# THERMOPHILIC LIGNIN DEGRADING ENZYMES FROM ACTINOMYCETES FOR BIOTECHNOLOGICAL APPLICATIONS

THESIS

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of MASTER OF SCIENCE of Rhodes University

By

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# ABSTRACT

Phenolic residues which accumulate in the environment as a result of agro-industrial practices has resulted in the need to find and use Eco-Friendly techniques, rather than the traditional methods of burning or burying this kind of waste. Bioremediation and bioconversion are attractive alternatives using whole cell or enzyme-based systems. The aims of this project were to isolate and uses thermophilic Actinomycetes, which produce thermo-tolerant oxidoreductase enzymes, which can be used to bioconvert a model industrial phenolic waste commonly genersated in the wine-making industry of South Africa. Current research in bioconversion and bioremediation focuses on mesophilic microbes in that their enzymes can catalyse reactions at higher temperatures without affecting its activity and lower contamination levels.

Three novel Actinomycete isolates were isolated (RU-A01, RU-A03 and RU-A06) from a compost site and characterized using a combination of conventional identification techniques and 16S rDNA methodology to identify the three isolates. All three isolates belong to the *Streptomyces* clade. In addition, five known Actinomycetes were selected from an internation culture collection and also screened for oxidoreductase activity in comparision to the three novel isolates. Although the five isolates were selected based on their ability to produce oxidoreductase enzymes, unexpectedly, no activity was detected.

Screening assays for peroxidase, polyphenol oxidase and laccase on RU-A01, RU-A03 and RU-A06, showed that all three isolated produced peroxidases and peroxidases but no laccase. Substrate specificity studies revealed that the most suitable substrates to determine peroxidase and polyphenol oxidase activity on these isolates were catechol for polyphenol oxidase, 2,4-dichlorophenol for peroxidases and veratryl alcohol for lignin peroxidases. Previous studies have indicated that peroxidases and polyphenol oxidases are produced in Actinomycetes during the primary stage of growth. This was the case with RU-A01, RU-A03 and RU-A06.

Growth rates were higher that other Actinomycetes, with maximum biomass being reached at 36 hours for the isolates RU-A01 and RU-A06 and 48 hours for isolate RU-A03. pH studies showed that the three isolates were adaptable and could grow over a broad pH range. Catabolism studies of phenolic model compounds showed that the three isolates were capable of catabolizing the model phenolic compounds within a period of 24 hours. Further studies were carried out to determine the effect of these microbes and their enzymes in whole cell and enzyme-based systems on a model phenolic waste, graoe waste consisting of compressed grape skins, pips and stalks. Whole cell studies showed that the isolates were capable of bioconverting the waste at a maximum concentration of 30% grape waste (vol:vol). Peroxidase and polyphenol oxidase activity increased indicating induction of these enzymes in the presence of phenolic compounds, with a maximum increase of upto 15.9 fold increase in extracellular lignin peroxidase activity in RU-A01. HPLC and phenolic determination assays indicated that bioconversion of the phenolic grape waste had occurred in the presence of the three isolates.

Attempts were made to isolate and identify a peroxidase or phenol oxidase gene from one the isolates. As bacteria, Actinomycetes are amendable to gene manipulation making them suitable candidates for methods such as site directed evolution in comparison to fungi. Two clones were selected for sequencing based on positive activity results when assayed for peroxidase activity. However the resultant sequences did not identify a functional gene sequence. Southern Blotting was then carried out to determine the nature of the peroxidase gene. Previous studies have been focused on the catalase-peroxidase gene (*CatC* gene) found Actinomycetes and other bacteria. A probe was developed from the *CatC* gene. No hybridization occurred with any of the enzyme restricted DNA from the three isolates. The implications of these results are that the peroxidase gene/s in the three isolates are in fact lignin peroxidase in nature,

This project has the potential in the bioconversion of phenolic wastes and is the first description of the use of thermophilic Actinomycetes in the bioconversion of an industrial phenolic waste.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| HPLC | high performance liquid chromatography |
| KDa | kilo Dalton |
| L-DOPA | L-3,4-dihydroxyphenylalanine |
| PEG | polyethylene glycol |
| PPO | polyphenol oxidase |
| UV | ultraviolet |
| Bp | base pair |
| Kbp | Kilo basepair |
| CTAB | Cetyl dimethyl ethylammonium bromide |
| DNA | Deoxy Ribo Nuckeic acid |
| Hrs | Hours |
| IPTG | Isopropyl-ß-D-Galactoside |
| Min | minutes |
| Nm | namometers |
| OD | Optical density |
| PCR | Polymerase chain reaction |
| Sec | seconds |
| X-Gal | 5-Bromo-4-chloro-3-Indoyl-ß-D-Galactoside |
| RU-A01 | Rhodes University- Actinomycete 01 |
| RU-A03 | Rhodes University- Actinomycete 03 |
| RU-A06 | Rhodes University- Actinomycete 06 |
| 4HR | 4-hexylresorcinol |
| MW | Molecular weight |
| EM | Electron microscopy |
| Mpa | Megapascals |
| BSA | Bovine serum albumin |
| *E.coli* | *Escherichia coli* |
| CSPD | Chemiluminescent substrate for alkaline phosphata |

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This project has the potential in the bioconversion of phenolic wastes and is the first description of the use of thermophilic Actinomycetes in the bioconversion of an industrial phenolic waste.

# CHAPTER 1

# INTRODUCTION

This thesis reports on a study of novel thermophilic Actinomycetes with the ability to produce peroxidases and phenol oxidases. Numerous studies have been conducted on the production of these enzymes by members of the Actinomycetes group, but these have been predominately mesophilic strains. Furthermore, the focus has been on the presence of these enzymes and little research has been done to demonstrate their ability to bioconvert or bioremediate common industrial effluents, which contain phenolic, or lignin derived material.

## 1.1 ACTINOMYCETES BIOLOGY

Actinomycetes are Gram-positive soil microorganisms, which differentiate into substrate mycelium, aerial mycelium and spores, and produce a broad range of different metabolites such as extracellular enzymes, enzyme inhibitors, pigments and antibiotics. Actinomycetes are one of the few classes of bacteria capable of producing oxidoreductase enzymes, such as peroxides and polyphenol oxidases (Adhi *et al.*, 1989; Antai and Crawford., 1981; Ball *et al.*, 1989; Burke *et a.l*, 1998; Mliki and Zimmermann., 1992; Antonopoulos *et al.*, 2001). Actinomycetes provides one of the focuses for the advancement of prokaryote genetics as they are considered to be valuable commercial organisms, particularly due to some of the antibiotic and lignin degrading species within the Taxon. Among all the genera in Actinomycetes, *Streptomyces* are represented in nature by the largest number of known species (Waksman, 1967). This is morphologically one of the more complex genera of soil bacteria. *Streptomyces* are heterotrophic bacteria, which undergo a complex cycle of morphological differentiation. This life cycle begins with the nutritive phase of colonial development whereby the vegetative mycelium develops, followed by the formation of aerial mycelium, which is usually thicker than the substrate mycelium (Waksman, 1967). Eventually spores develop from the aerial mycelium. Spores are suited for dispersal into the environment and are morphologically resistant to environmental elements such as drying but are not heat resistant like endospores (Hopwood, 1987).

There has been a significant increase in the knowledge of the genetics, molecular biology and physiology of the Actinomycetes. This interest has stemmed from two main stimuli, namely the development of more efficient systems and strategies for the cloning of genetic elements in *Streptomyces,* and the increasing importance of the commercial niche that these microorganisms occupy. In addition to the existence of *in-vivo* genetic analysis via plasmid mediated conjugation, and protoplast fusion, *in vitro* techniques have allowed for a more ordered genetic analysis of the production of commercially important metabolites e.g. antibiotics and a wide variety of enzymes (Hopwood *et al.*, 1985). The capacity of Actinomycetes to produce industrially important metabolites may have been a result of selective pressure due to competition with fungi and other bacteria (Charter, 1984; cited in Hopwood., 1987) within their environment. The complete genomic sequencing of *Streptomyces coelicolor* by collaboration between Professor Sir D Hopwood and the Sanger Institute is the accumulation, at present of these advances (www.sangerinstitute.com).

Actinomycetes occur in a wide range of environments in which they have the ability to grow on most naturally occurring substrates. Some form parasitic or symbiotic associations. Actinomycetes are saprophytes in soil, water, composts and other habitats from which many isolates have been obtained for scientific study or commercial exploitation (Ball *et al.*, 1989).

Some form of lignocellulosic degradation activity has been detected/identified in a large number of Actinomycetes strains from a range of genera (Crawford.,1974; Ball *et al.*, 1989). During the preparation of wheat straw compost from mushroom cultivation, Actinomycetes numbers increase dramatically and the population is dominated by *Thermomonosporas,* a group of thermotolerant Actinomycetes whose ability to degrade lignin is well documented (McCarthy *et al.*, 1988).

### 1.1.1 Industrial and potential use of the Actinomycetes

Efforts of a number of researchers have promoted the establishment of a collection of microorganisms able to degrade phenolic residues and pollutants (De Schrijver and De Mot, 1999; Mliki and Zimmermann, 1992; Muller *et al.*, 1996; Ramachandra *et al.*, 1987, Rozgaj, 1994, Tinnus and Pieper, 1999). Systems may involve whole cells of algae, fungi or bacteria, with bacteria being the organisms of choice as their pollutant end products have been better studied. Easy culturing of bacteria and faster growth rates has made bacteria popular for genetic manipulation. This would apply to the Actinomycetes, as the genetic aspects of Actinomycetes and in particular *Streptomyces,* have been well studied (Hopwood, 1987). Fungal systems do have the unique advantage that their metabolism of complex compounds is based on the secretion of extracellular enzymes into complex solid matrices. In contrast, fungi are not as amenable to genetic manipulations.

Actinomycetes are adapted to growth on solid substrates, with their primary carbon source being insoluble, polymeric compounds (McCarthy and Williamson, 1992) and hence, synthesizing a range of extra- and intracellular enzymes is necessary, including the oxidoreductase enzymes. Previous studies on *Streptomyces* have shown their ability to degrade lignin (Trigo and Ball, 1994), but white rot fungi such as *Phanerochaete chrysosporium* and *Trametes vesicolor* have been considered the primary lignocellulose degraders (Kurek *et al.*, 1997; Nutsubidze *et al.*, 1998; Crestini *et al.*, 1998 and Kauffman *et al.*, 1999). Large populations of lignocellulose utilizing Actinomycetes develop in compost, particularly those prepared for the cultivation of mushrooms, due to the alkaline conditions resulting from ammonification, which is ideal for this group of bacteria (McCarthy and Williamson, 1992).

Actinomycetes isolated from soil, compost and related substrates show primary biodegradative activity, secreting a range of extra- and intra- cellular enzymes exhibiting the capacity to degrade recalcitrant molecules. With the isolation of thermophillic Actinomycetes that have the capacity to produce oxidoreductase enzymes, heterologous expression studies of the protein(s) in a chosen expression host can be carried out, followed by sequencing studies to determine the genes encoding these enzymes. These studies contribute towards the development of bacteria genetically designed for bioremediation purposes. With the bioinformatic data generated from these studies, enzyme expression and gene position can be elucidated. Researchers are hence adequately equipped for subsequent DNA manipulation techniques to improve the enzyme(s) i.e. improvements in substrate specificity, thermostability etc, which all contribute towards the making of "designer" microorganisms. Being bacteria, Actinomycetes have advantages over fungi in terms of gene technology techniques and hence would be ideal candidates for the task of bioremediation and bioconversion of phenolic residues.

## 1.2 OXIDOREDUCTASE ENZYMES

Two groups of coupling enzymes for the oxidative catalysis and coupling of phenols and aromatic amines have been examined here. These are the peroxidases and phenol oxidase enzyme systems.

### 1.2.1 Peroxidases

Peroxidases (EC 1.11.1.7) are haem-containing enzymes that use hydrogen peroxide ($H_2O_2$) as the electron acceptor to catalyse a number of oxidative reactions and hydroxylations, utilizing both an oxidizing substrate and a reducing substrate. The oxidizing substrate is usually a peroxide or peroxy acid (Robinson, 1991). Typically peroxidases can use hydrogen peroxide, methyl-hydrogen peroxide and ethyl-hydrogen peroxide (Kermasha and Metche, 1988) as oxidizing agents in peroxidatic reactions, but most haem peroxidases follow the reaction mechanism below (Equations 1, 2 and 3) using hydrogen peroxide as the oxidizing agent.

**Equation 1**

$$\textbf{Peroxidase (Fe}^{3+}\textbf{)Porphyrin} + \textbf{H}_2\textbf{O}_2 \rightarrow \textbf{Peroxidase (Fe}^{4+}\textbf{=O)Porphyrin}^{+} + \textbf{H}_2\textbf{O}$$

**Equation 2**
**Peroxidase ($Fe^{4+}$=O) Porphyrin$^{+}$ + AH →Peroxidase ($Fe^{4+}$=O) Porphyrin + A•**

**Equation 3**
**Peroxidase ($Fe^{4+}$=O) Porphyrin + AH →Peroxidase ($Fe^{3+}$) Porphyrin + A• + $H_2O$**

**Figure 1.1** **Peroxidase reaction mechanism (Colonna *et al.*, 1999)**

### 1.2.1.1 Reaction mechanisms

The reaction mechanism of peroxidase (summarized in Figure 1.1) is a multi-step reaction which starts with the enzyme reacting with one equivalent of hydrogen peroxide and substrate (AH) to give compound I, a porphrin $\pi$-cation radical containing $Fe^{IV}$ (Equation 1). This results in a distinctive absorption spectrum due to the haem prosthetic group exhibiting transient changes on mixing with hydrogen peroxide. This is a two-electron oxidation/reduction reaction where hydrogen peroxide is reduced to water and the enzyme is oxidized. One oxidizing equivalent resides on iron, giving the oxoferryl ($Fe^{IV}$ =O) intermediate (Colonna *et al.*, 1999).

Compound I oxidizes an organic substrate to give a substrate radical (·AH). Compound I undergoes a second one- electron oxidation reaction yielding compound II, which contains an oxoferryl center coordinated to a normal (dianionic) porphyrin ligand (Equation 2). Finally, compound II, is reduced back to the native ferric state with concomitant one electron substrate oxidation. The overall charge on the resting state form and compound I is +1, while compound II is neutral (Equation 3; Piontek *et al.*, 2001).

#### 1.2.1.2 Peroxidase sources

Peroxidases can be found in bacteria, fungi, plants and animals. They have molecular weights ranging from 35 000 to 100 000 and approximately 25% of this is carbohydrate (O'Brien, 2000). In terms of sequence similarity and structural divergence, fungal, plant and bacterial peroxidases are viewed as members of a superfamily consisting of three major classes:

**Class I**, These are intracellular peroxidases, and includes: yeast cytochrome *c* peroxidase (CCP), a soluble protein found in the mitochondrial electron transport chain, where it probably protects against toxic peroxides; ascorbate peroxidase (AP), the main enzyme responsible for hydrogen peroxide removal in chloroplasts and cytosol of higher plants; and bacterial catalase-peroxidases, exhibiting both peroxidase and catalase activities. It is thought that catalase-peroxidase provides protection to cells under oxidative stress (Robinson *et al.*, 1991).

**Class II** consists of secretory fungal peroxidases: ligninases, or lignin peroxidases (LiP's), and manganese-dependent peroxidases (MnPs). These are monomeric glycoproteins involved in the degradation of lignin. In manganese peroxidase (MnP), $Mn^{2+}$ serves as the reducing substrate (Robinson *et al.*, 1991).

**Class III** consists of the secretory plant peroxidases, which have multiple tissue- specific functions: e.g., removal of hydrogen peroxide from chloroplasts and cytosol; oxidation of toxic compounds; biosynthesis of the cell wall; defense responses towards wounding; indole-3-acetic acid (IAA) catabolism; ethylene biosynthesis. Class III peroxidases are also monomeric glycoproteins. Class III includes the classic horseradish peroxidase isoenzyme (HRPC) and peanut peroxidase (PNP) (Paulos *et al.*, 1993).

#### 1.2.1.3 Substrates and Reactivity of peroxidases

Peroxidases are able to catalyze the oxidation of a large variety of substrates i.e phenol, aromatic amines and other compounds such as alkyl peroxides and aromatic peracids

(Sjoblad and Bollag, 1981), using hydrogen peroxide. Several other peroxidases also exist that contain either metal ions or a flavin prosthetic group (Table 1.1). Haem prosthetic type peroxidases are the most commonly found and are used as peroxidases in industrial applications. The specific biological function, the reduction potential of the iron and the nature of the substrates, which can be oxidised, are strongly determined largely by the structural features of the protein matrix around the prosthetic group (Table 1.1).

In microbial peroxidases such as lignin peroxidase and manganese peroxidases, the substrates include both phenolic and non-phenolic aromatic compounds; the phenolic substrates are oxidized to yield products similar to those of classical peroxidases such as HRP, while the oxidation of non-phenolic methoxybenzenes is unique to lignin peroxidases. The oxidation of these substrates to yield aryl cation radicals can result in:

- Demethoxylation (Fig 1.2 and Fig 1.3)
- $C\alpha - C_\beta$ cleavage of lignin model compounds (Figure 1.4)
- Benzylic alcohol oxidation (Figure 1.4)
- Hydroxylation of aromatic rings and side chains (Fig 1.2 and Fig 1.5)

The substrate range is very broad, with the reactivity being largely determined by redox potentials; lignin peroxidase can catalyze the oxidation of substrates with a reduction potential greater than 1.3 volts. The enzyme has been shown to oxidize lignin monomers, dimers and trimers as well as polycyclic aromatic compounds such as benzopyrene (Haemmerli *et al.*, 1986). The radicals (compound I and II) cause side chain fragmentation (C-C cleavage) leading to the breakdown of the lignin polymer (Evans and Fuchs, 1988). They can also catalyze lipid peroxidative pathways, which are capable of oxidising substrates such lignin-model dimers and polycyclic aromatic hydrocarbons.

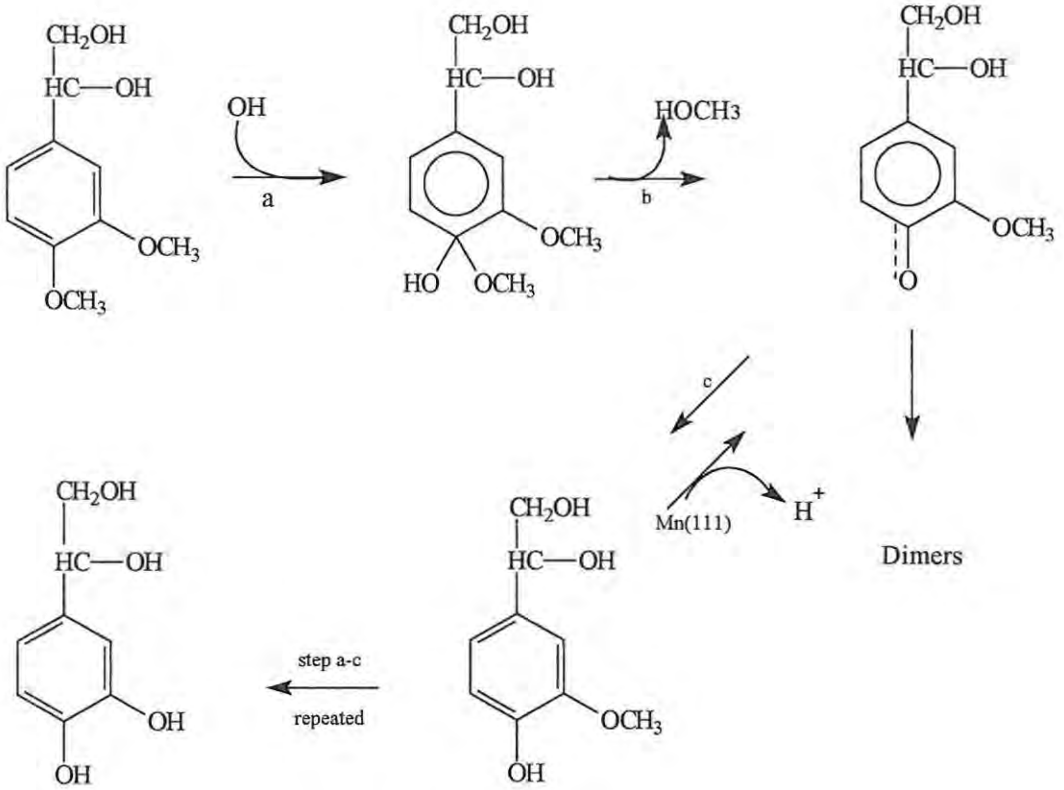


**Figure 1.2** **Suggested reaction generated by peroxidase, for replacement of methoxy and hydroxyl groups on the model compound Veratryl glycol (Hildèn et *al.*, 2000). Step a: a hydroxyl radical is attached and the free electron is delocalized on the ring; step b: a methanol leaves the VG; step c: a proton and an electron are donated (probably by an $FADH_2$ group). Steps a-c can then be repeated to generate another demethoxylation. In addition to this further demethoxylation, the peroxidase system can possibly generate dimers after step c.**

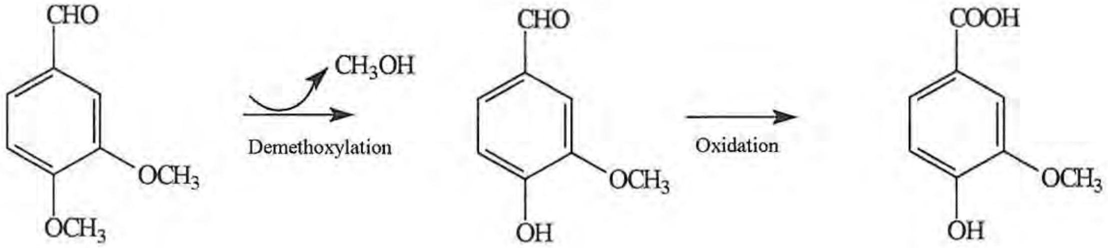


**Figure 1.3** **Demethoxylation pathway of veratric aldehyde (Lopretti *et al.*, 1998).**

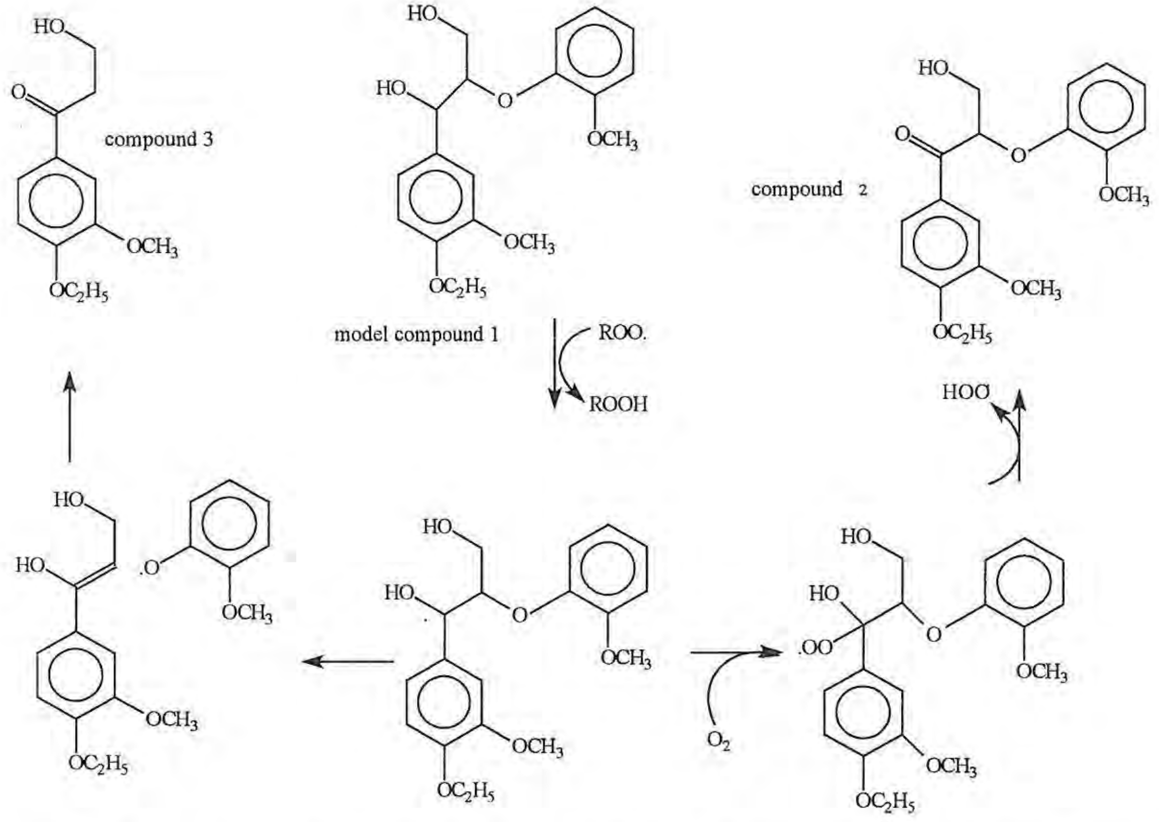


**Figure 1.4** **Proposed pathway for the oxidation of model 1 to compounds 2 and 3 by peroxyl radicals (peroxidase) (Kapich *et al.*, 1999)**

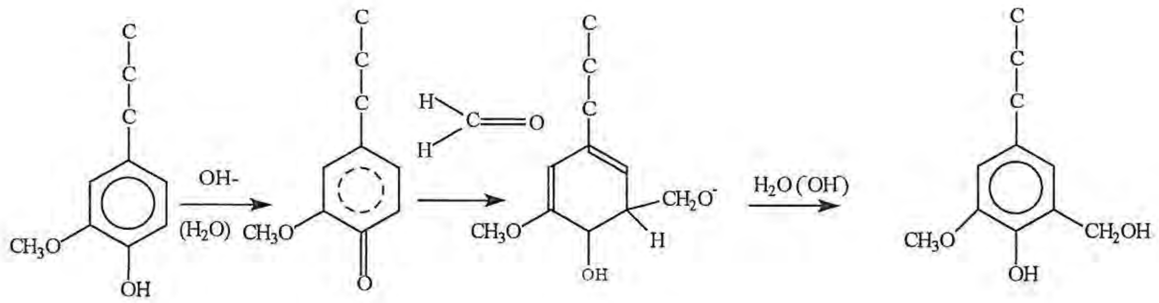


**Figure 1.5** **Hydroxymethylation of the phenolic ring of a lignin unit (guaiacyl type; Gonçalves and Benar, 2001) catalysed by a peroxidase.**

**Table 1.1** **Classification of peroxidases showing substrates and methods for peroxidase activity determination. Adapted from Robinson, (1991)**

| PROSTHETIC GROUP | COMMON NAME | SOURCE | POTENTIAL SUBSTRATES |
|---|---|---|---|
| Haem | peroxidases | | Test substrates including guaiacol and *o*-dinisidine |
| | Ascorbic acid peroxidase | Plants | Ascorbic acid |
| | Cytochrome C peroxidase | plants | Cytochrome C |
| | Lactoperoxidase | Animals | Thiocyanate |
| | Lignin peroxidase | Plants and fungal cell walls | Lignin precursors including ferulic and cinnamic acids; guaiacol and *o*-dinisidine |
| | Myeloperoxidase | Animals | Thiols |
| | Eosinophil peroxidase | Animals | Thiols |
| | Thyroid peroxidase | Animals | Thiols |
| Selenium | Glutathione peroxidase | Plants | Reduced glutathione |
| Vanadium | Bromoperoxidase | *Ascophyllum nodosum* | Halogens<br>2.4 DCP |
| Mangenese | Mn-haem peroxidase | *Phanerochaete chrysosporium* | Lignin<br>ABTS |
| Flavin | Flavoperoxidase | Micro-organisms | ----- |

### 1.2.1.4 Applications of Peroxidase.

Horseradish peroxidase is by far the best-studied and characterized peroxidase and has been accordingly exploited in several diagnostic applications. Alberti and Klibanov (1981) first proposed the use of the horseradish peroxidase for the removal of phenols from aqueous wastewaters. In 1984 Adler *et al.* used peroxidases to remove various phenols, aromatic amines, hydrocarbons and polychlorinated biphenyls (PCB's) from effluent. This process was successful and showed 85% -90% removal efficiency using 25 units per liter of peroxidase and 2mM hydrogen peroxide. A disadvantage of such a reaction was the use of stoichiometric amounts of costly hydrogen peroxide as oxidant.

Low temperature bleaching capabilities of peroxidases, e.g. horseradish peroxidase have been demonstrated in the paper industry and lignin peroxidases found in fungi and some bacteria have also been shown to depolymerize lignin model dimers, four different synthetic lignins and the high molecular weight chloro-lignins produced during the first alkaline stage of chlorine-based pulp bleaching (Antonopoulos *et al.*, 2001; Kauffman *et al.*, 1999).

Other peroxidases such as chloroperoxidases commonly found in *Streptomyces* species are unusually versatile; they catalyze not only the reactions typical of peroxidases but also those of catalases and monooxygenases, and they are also almost unique in catalyzing halogenation reactions in the presence of halide ions and hydrogen peroxide (Colonna *et al.*, 1999). The lack of stereospecificity in the halogenation reactions catalysed by chloroperoxidase is in agreement with the participation of an 'enzyme-free' halogenating species. Figure 1.6 summarizes the reactions catalysed by peroxidases.

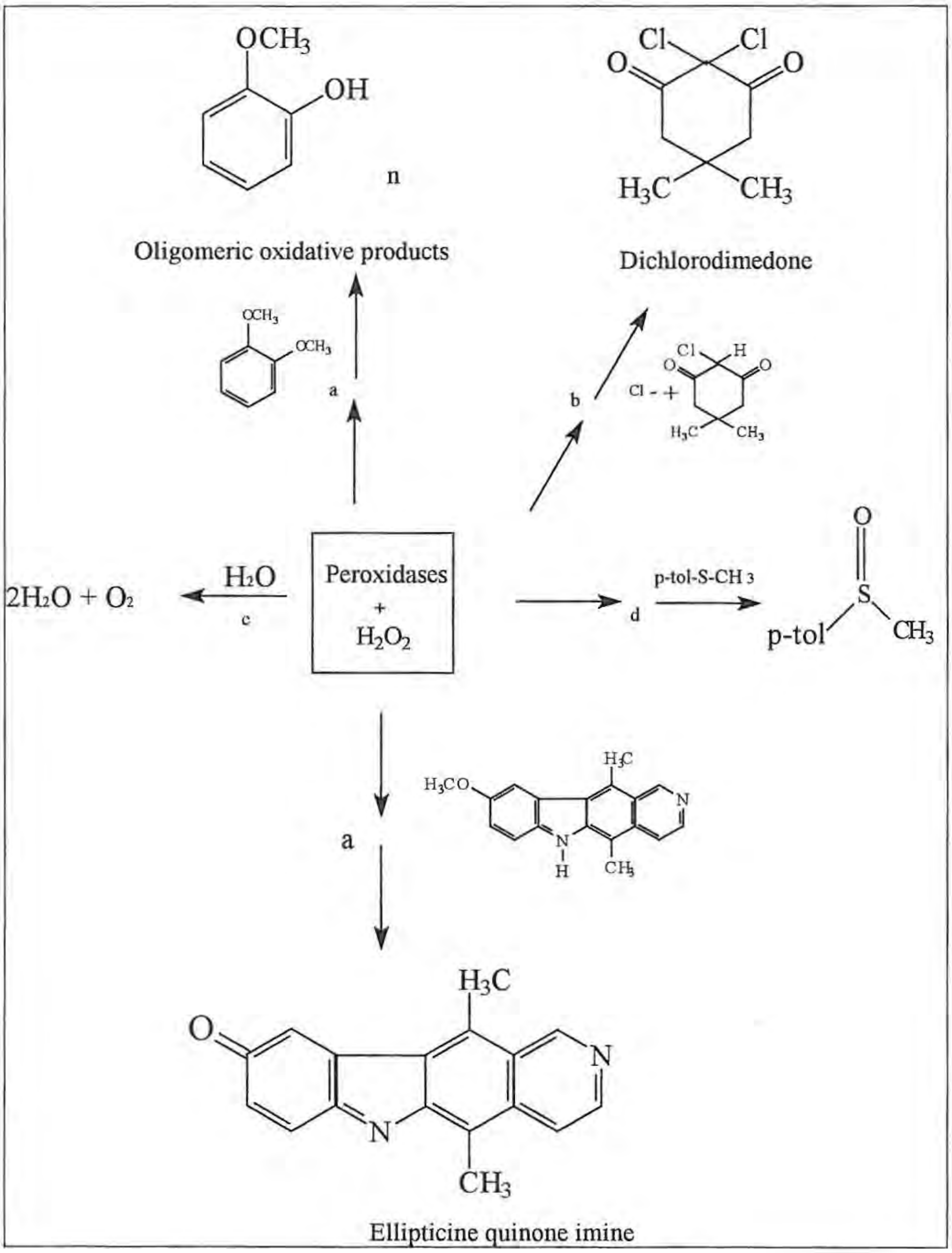


**Figure 1.6** **Examples of reactions catalysed by peroxidases: (a) oxidation; (b) halogenation (c) $H_2O_2$ dismutation; (d) oxygen transfer reactions (Colonna *et al.*, 1999).**

## 1.2.2 Phenol oxidases

### 1.2.2.1 Laccases

Laccases (p-diphenol: dioxygen oxidoreductase; EC 1.10.3.2) belong to the group of phenoloxidases, which are commonly found in many plants and secreted by numerous fungi and bacteria. They are multinuclear blue copper-containing oxidases, which catalyze the four-electron reduction of oxygen to water. The enzymatic activity is due to four copper (II) ions, which are arranged in the three different sites, each characterized by unique spectroscopic peculiarities. Laccases are widespread among plants and fungi and some bacteria and whose action is related to several physiological processes, and especially lignin metabolism (Bonomo *et al.*, 2001).

Laccases exploit the full oxidizing power of oxygen to perform the one-electron oxidation of a large number of organic compounds with a phenolic functional group (Figure 1.7), as well as inorganic compounds.

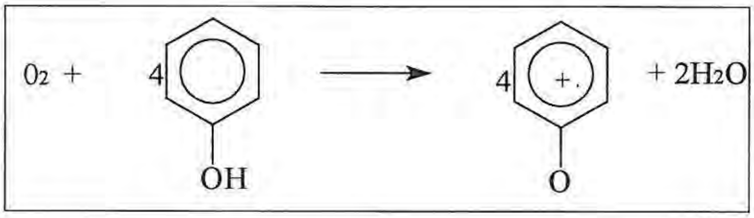


**Figure 1. 7** **Reaction example of the oxidizing power of oxygen on a phenolic compound (Roy-Arcand and Archibald, 1991).**

#### 1.2.2.1.1 Role of Laccases in nature

In nature, laccases have various functions, which include participation in lignin biosynthesis, plant pathogenicity, degradation of plant cell walls, insect sclerolization, bacterial melanization and melanin production in humans. Chemically all the functions of laccases are related to oxidation of a range of aromatic substances but the net effect of such oxidations can be very different and may work in opposite directions (Li *et al.*, 1999). Plant laccases are known to oxidize monolignols to form polymeric lignin, whereas microbial laccases degrade and depolymerize lignins.

### 1.2.2.1.2 Reaction mechanisms of Laccase

This group of phenol oxidases uses oxygen as an electron acceptor to remove hydrogen from the phenolic hydroxyl group. This reaction leads to the formation of free radicals, which can be demonstrated with most lignin compounds, and these can undergo rearrangements leading to alkyl-aryl cleavage of side chains and aromatic rings. The range of substrates oxidized by laccase depends on the type of laccase and the substrate specificity of laccases can be explained by their physiological functions.

The classical action of laccases is oxidation (Figure 1.8) of substrate by transferring electrons to oxygen in one-electron steps, resulting in polymerization of phenols and /or the formation of quinones (Majcherczyk *et al.*, 1998). Demethylation of phenolic and non-phenolic lignin substructures has been shown to happen, as well as the oxidation of phenolic lignin model compounds by Cα – Cβ cleavage of the side chain and thus the enhancing role in lignin degradation. The action of laccase on dimeric lignin model compounds in the presence of mediators such as ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid], phenol red, violuric acid and 1-Hydroxybenzotriazole expands the substrate spectrum of laccases to various benzoic acid derivatives (Li *et al.*, 1999). Mediators such as ABTS together with laccase have been shown to increase demethylation of pulps during bleaching processing in the pulp and paper industry (Monteiro and De Carvalho, 1989). Typical reaction patterns catalysed by microbial laccases are summarized in Table 1.2.

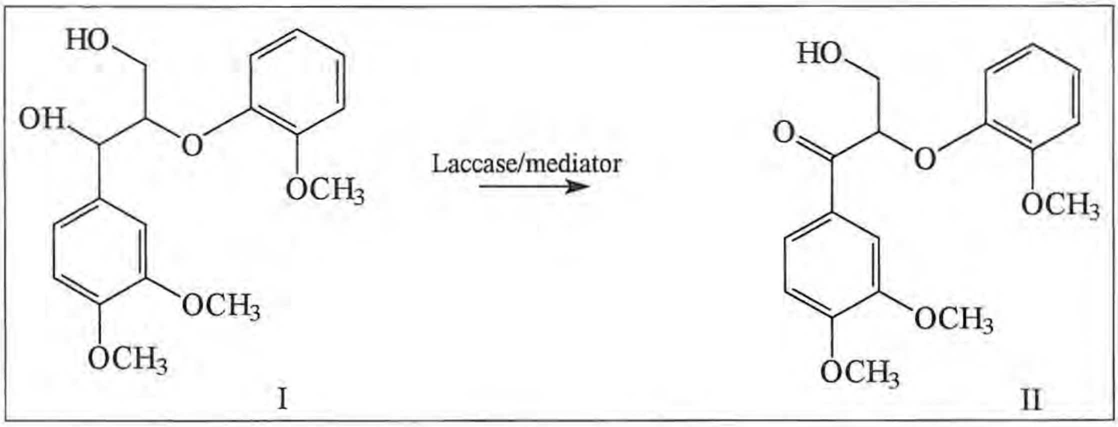


**Figure 1.8** **Oxidation of dimer I using laccase plus mediator (Li *et al.*, 1999).**

**Table 1. 2** **Reaction patterns catalyzed by microbial laccases (H.-D Youn *et al.*, 1995)**

| COMPOUND | REACTION PATTERN | MAJOR PRODUCTS |
|---|---|---|
| acetosyringone | Single electron transfer | Acetosyringone phenoxy radical |
| Vanillic acid | Polymerization | 2-meoxy-6-(2-meoxy-4-carboxyphenyl)-1,4-benzoquione |
| 1-(3,5-Demethoxy-4-hydroxyphenyl)-2-(3,5-dimethoxy-4-ethoxyphenyl) propane-1,3-diol; β-1 lignin model compound | Depolymerization | |
| | a. Cα oxidation | 1-(3,5-Dimeoxy-4-hydroxyphenyl)-2-(3,5-dimethooxy-4-ethoxyphenyl)-3-hydroxypropanone |
| | b. Alkyl- aryl cleavage | 2,6-Dimeoxy-*p*-hydroquinone and 1-(3,5-dimethoxy-4-ethoxyphenyl)-3-hydroxypropanal |
| | c. Cα – Cβ cleavage | Syringaldehyde and 1-(3,5-dimethoxy-4-ethoxyphenyl)-2-hydroxyethanone |
| 1-(3,5-Dimethoxy-4-hydroxyphenyl)-2-(2-methoxyphenyl)-1,3-dihydroxypropane;β-*O*-4 lignin model compound | Depolymerization | |
| | a. Cα oxidation | 1-(3,5-Dimethoxy-4-hydroxyphenyl)-2-(2-methoxyphenyl)-1-oxo-3-hydroypropane. |
| | b. alkyl-aryl cleavage | 2-(2-Methoxyphenyl)-3-hydroxypropanl and 2,6-dimethoxybenzoquinone |

| | | |
|---|---|---|
| | c. Cα – Cβ cleavage<br>Ring cleavage | Guaiacol and syringic acid<br>2,4-Di (*tert*-butyl)-4-(methoxycarbonylmethyl)-2-buten-4-olide |
| 4,6-Di (*tert*-butyl) guaiacol; aromatic ring compound 1-(3,4-Dimethoxyphenyl)-2-phenoxy-ethane-1,2-diol; non-phenolic β-1ligin model compound | Cα – Cβ cleavage (+ABTS) | Veratraldehyde and benzaldehyde |

### 1.2.2.2 Polyphenol oxidases

In contrast to peroxidases, polyphenol oxidases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) utilize oxygen as an oxidant. These enzymes are ubiquitous in nature and are involved in the biosynthesis of a wide range of natural products including lignins, tannins, melanins and alkaloids as well as polymerization of naturally occurring phenols resulting in the formation of humic acid and humus in soil by oxidative coupling (Trigo and Ball., 1994). 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 from the hydroxyl group. Polymerization of phenolic compounds by polyphenol oxidase has been reported and could possibly be related to lignin biosynthesis (Guerra *et al.*, 2000). Phenolic polymerization capacity in oxidative enzymes has been used in wastewater treatment, since the precipitation of phenolic compounds results in less toxic wastewaters (Geurra *et al.*, 2000).

Burton (1994) described polyphenol oxidase as a copper containing monoxygenase which catalyses two reactions:

1) The *ortho*-hydroxylation of phenols to catechols (cresolase activity)
2) The subsequent oxidation of catechols to *ortho*-quinones (catecholase activity).

Both reactions utilize molecular oxygen (Fig 1.9). The quinones are readily condensed to produce relatively insoluble high molecular weight brown polymers known as melanins, Fig 1.10 (Burton, 1994 and Rodriguez-Loperz *et al.*, 2001). *Ortho*-quinones are unstable, reactive species that may polymerize and oxidize other substrates or undergo nucleophillic attack by various substances including water, proteins or polyphenols.

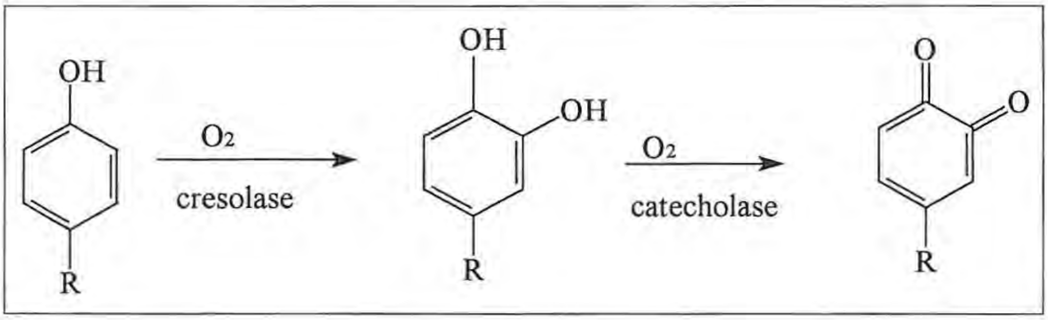


**Figure 1.9** **Reactions catalysed by polyphenol oxidase (Burton, 1994)**

The *o*-hydroxylation activity of polyphenol oxidase distinguishes it from other phenol oxidizing enzymes such as laccase and peroxidases (Sanchez-Ferrer *et al*, 1995). Polyphenol oxidase is an unusual monooxygenase in that it does not require a reducing cofactor such as NADH (Aitken *et al*, 1994). A wide variety of *o*-dihydroxy phenols, such a caffeic acid derivatives can also act as good substrates for polyphenol oxidase (Cheynior and Mountounet, 1992). Polyphenol oxidase oxidation of monophenols and *o*-diphenols has been extensively studied even though some of these substrates are not natural compounds (Jimenez *et al.*, 1999).

*In vitro* studies have suggested that polyphenol oxidase participates in the degradation of natural phenols with more complex structures such as anthocyanins and flavanols (Finger, 1994). These compounds are not directly oxidized by the enzymes but by the quinones formed by polyphenol oxidase from catechol and catechin (Jiménez *et al.*, 1999).

#### 1.2.2.2.1 Sources of polyphenol oxidase

Polyphenol oxidase is widely distributed throughout the phylogenetic scale from bacteria, including Actinomyces, fungi, plants and animals (including invertebrates and mammals). In some instances polyphenol oxidases are undetectable due to the presence of endogenous inhibitors or because they are in a latent form (Robb, 1984; Sanchez-Ferrer *et al.*, 1995). The role polyphenol oxidase plays varies from melanization in animals and browning in plants to cuticle formation in invertebrates (Cabones *et al.*, 1988; Ros *et al.*, 1994). It is evident that there are large differences in the properties, and compartmentalization of polyphenol oxidase across the phylogenetic scale (Longa *et al.*, 1996). Substrate specificity also differs with the sources of the enzymes (Burton, 1994) and appears to become narrower as one moves from single to more complex organisms (Robb 1984).

#### 1.2.2.2.2 Plant polyphenol oxidase

The most studied polyphenol oxidases in fruit are the enzymes from grapes and apples, due to their contribution to the quality of wine and juice. Polyphenol oxidase also contributes to the enzymic browning by oxidation of endogenous phenolic compounds released due to cutting, bruising or cellular damage.

#### 1.2.2.2.3 Microbial polyphenol oxidases

Common microbial sources of polyphenol oxidases are from the Actinomycetes group (this study) and fungi such as *Neurospora crassa* (Luke and Burton, 2001). The most studied microorganisms are *Streptomyces glaucescens* and *Neurospora crassa* (Burton, 1994). *Streptomyces glaucescens* produces the smallest reported polyphenol oxidase at 30.9kDa and shares 24% similarity on a DNA level to that from *Neurospora crassa*. *Neurospora crassa* polyphenol oxidase is a 46kDa monomer, which is formed upon proteolytic processing of the 75-kDa precursor (Van Gelder *et al.*, 1997).

Polyphenol oxidase exists in multiple and interconvertible forms. In some sources polyphenol oxidase has been shown to exist in a latent state, requiring activation by proteolysis, fatty acids, denaturants and detergents (Angleton and Flurkey 1984). In plants the predicted molecular weight of the pre-forms are 68-73 kDa; these are later processed to 58-68 kDa mature proteins. Proteolysis of the mature protein yields monomeric active forms of the 40-45 kDa (Martinez and Whitaker, 1995 and Van Gelder., 1997).

### 1.2.2.2.4 Structure and mechanisms of Polyphenol oxidase

The amino acid composition and sequences for *Neurospora crassa* and *Streptomyces glaucescens* have been published (Lerch *et al.*, 1982). The anti-ferromagnetically coupled dinuclear copper active site of polyphenol oxidase is close structurally to that of hemocyanin (Robb, 1984; Longa 1996) and exists in either the bicupric or bicuprous states with the oxytyrosinase capable of binding dioxygen to form a dioxygen di-copper II complex, which are stable at room temperature. 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.

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 to produce a high molecular weight mixed polymers, which readily precipitate (Figure 1.10).

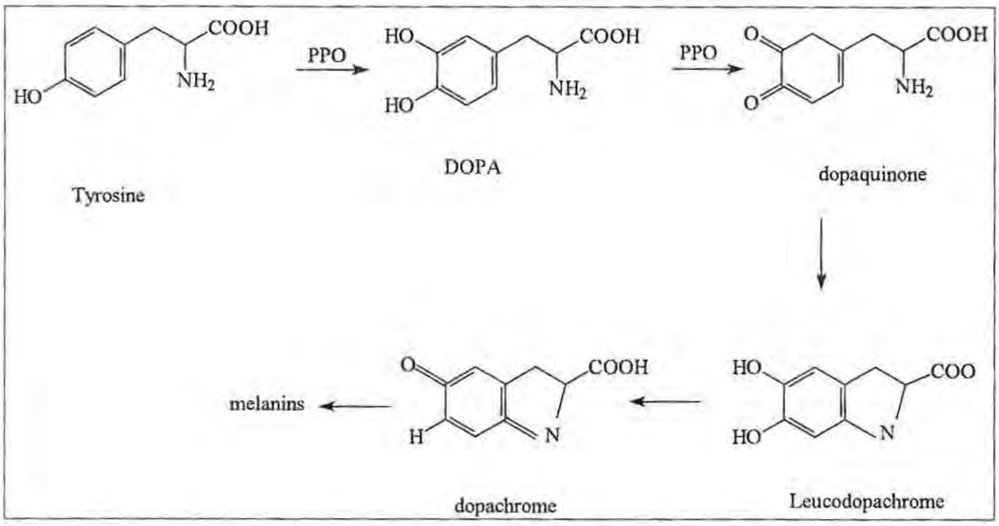


**Figure 1.10** **Schematic diagram of the general pathway for the formation of melanin using polyphenol oxidase (Neptuno *et al.*, 2001)**

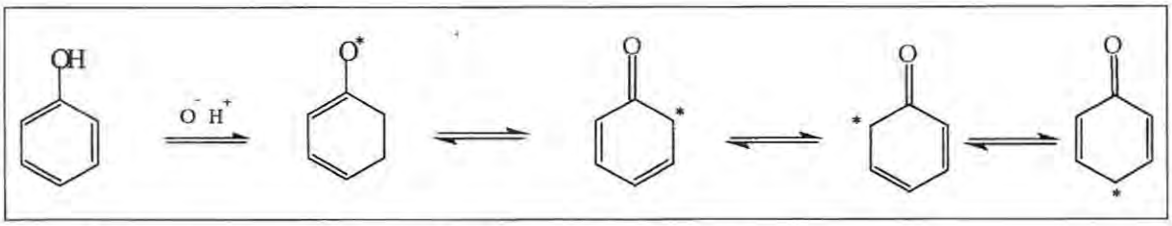


**Figure 1.11** **Formation of aryloxy radicals from phenol**

## 1.3 INDUSTRIAL POLLUTION: AN OVERVIEW

In recent decades there has been a substantial increase in the manufacture of synthetic chemicals such as solvents, plasticizers, insecticides, herbicides and fungicides. Many are classified as potentially harmful to humans (WRI report, 1987) and as we move into the next millenium, the importance of environmental health issues is becoming paramount. Humans have always had an impact on their environment, increasing with the advent of the Industrial Revolution and continuing throughout the 19th century (Goehl, 2000). Release of toxic chemicals into the environment due to man's agricultural and industrial activities creates novel ecotoxicological problems as a result of their persistence and recalcitrance to degradation (Galli, 1990). The public, typically, has an unsympathetic attitude towards companies causing adverse environmental impacts, which in turn influences potential markets (Murray and Claassen, 1999). Researchers have therefore seen a need for remediation and to explore eco-friendly methods of disposing of industrial waste using the processes of bioremediation and biotransformation (Faulds *et al.*, 1997).

Some of the main causes of industrial pollution include waste-management practices that involve methods of disposal rather than treatment. At present, the legislation is designed to ameliorate the adverse effects of past pollution and to elicit immediate action to minimize the impact when industrial accidents producing new pollutants occur. The traditional "dilute, bury or burn" practice is no longer tolerated. To enable industrial companies to operate within the law, the technology for pollution control must be made available, and recently, research into"eco-friendly" methods of remediating industrial waste in the form of bioremediation has increased in response to the increasing demand for bioremediation technologies. The challenge to biotechnology is to generate efficient, cost effective and environmentally safe bioremediation methods to replace existing technologies such as incineration. They must also provide unique solutions for the remediation of contaminated sites (Aitken, 1993). As a result of this, scientific researchers have formed partnerships with industries to look at ways of remediating industrial waste.

During the 1980's, there were expectations that modern biotechnology could be applied to biological cleanup. Such procedures were viewed as technically feasible, but the necessary research and biotechnological innovations were blocked by a decade of interim regulation by the United States of America Environmental Protection Agency (USA EPA) under the toxic substances control act (TSCA; Miller, 1997). These regulations were aimed at limiting the use of the then new genetic engineering techniques by putting in place disincentives to research and development. As a result of this, the application of recombinant DNA techniques in both industrial research and development and pre-commercial research was discouraged. This led to continued reliance on dated techniques for bioremediation, and ongoing environmental damage (Miller, 1997). With the increase of industrial pollution, it is difficult to ignore the new DNA recombination techniques and their potential for use in effluent clean-up, especially now that industry and research are moving away from conventional methods such as incineration, irradiation and solvent extraction, and looking towards bioremediation in the form of whole cell systems and enzyme-based catalysis.

## 1.4 BIODEGRADATION AND BIOREMEDIATION OF PHENOLICS.

Biological processes have long been in use for the treatment of wastes such as sewage. However, their use for treating hazardous refractory chemical wastes is a more recent development, due to our increasing knowledge of the pathways of biodegradation together with the isolation and utilization of newly identified microbes (Shannon and Unterman, 1993). The process of bioremediation technology involves detoxification and mineralisation of the pollutant to biomass, carbon dioxide and water. This makes it an attractive environmentally sound and potentially cost effective alternative to conventional methods. The general acceptance of bioremediation as a treatment technology requires that it demonstrates of its effectiveness, reliability, predictability and robustness in addition to showing that it has advantages over the conventional treatment technologies. Thus, it becomes important to understand the effectiveness, robustness and reliability of biological process involving industrial effluents and their ability to compete with proven physiochemical technologies.

### 1.4.1 Use of Whole cell systems for Bioremediation.

Exploiting microorganisms for remediation of waste is a promising alternative to conventional methods such as irradiation and chemical oxidation with the objective being to stimulate the growth of indigenous or introduced microorganisms.
Although microorganisms efficiently degrade many environment pollutants, others persist and constitute a severe health hazard (Timmis and Pieper, 1999). One important factor limiting the bioremediation of industrial effluents is the slow rate of degradation, which limits the practicality of using bacteria and fungi in remediation (Johri *et al.*, 1999).

Another problem pertaining to bacterial and fungal bioremediation is bioavailabity. Because bacteria and fungi are generally adapted to act on soluble substances in an aqueous medium and many organo-pollutants especially aromatics, are poorly soluble in water, this limits bioavailability to the bacteria. This has led to interest in the use of fungi, which can function under such conditions, because their extracellular enzymes catalyze the initial oxidation reactions. Another disadvantage of bacterial systems is an inability to degrade low levels of organo-pollutants where the level of the pollutant is too low to induce the biosynthesis of the enzymes required for degradation.

### 1.4.2 Characteristics of industrial enzymes and the criteria for successful applications of enzyme based systems.

An enzyme is evaluated according to its activity, which describes how much of a given substrate (S) is converted in a given time and under defined conditions into a corresponding product (P). For maximum enzyme activity, enzyme and substrate must have constant and unimpaired contact. Conversion rates are reduced when the enzyme is denatured due to an increase in temperature, resulting in inactivation (Uhlig, 1998). This is one area of concern in terms of feasibility of such processes in industry. As exothermic reactions, such as that which occur during the biocatalysis, may result in the thermal denaturation of the enzyme.

Every enzyme has an optimal activity, which is affected by pH and temperature. The media it is suspended in also affects catalytic activity. High enzyme stability is thus of great economic value and importance.

### 1.4.3 Gene technology for Bioremediation

Although a wide range of microorganisms has been discovered that are able to degrade highly stable toxic pollutants, many pollutants still persist in the environment. Recent advances in the field of molecular genetics have provided some solutions to these problems, making it possible to extend the range of substrates that an organism can utilize. It is even possible to endow an organism with the ability to degrade a predetermined range of pollutants. Advances in molecular genetics have opened new avenues to move toward the goal of genetically engineered microorganisms functioning as designed biocatalysts with desirable biodegradation pathways and enzymes from different organisms brought together in a single host to perform specific detoxification. Such catabolic genes have been cloned and characterized for pollutants such as organochlorines, polychlorinated biphenyls, chlorobenzoates, and napthalene (Johri *et al.*, 1999).

After almost 30 years in which gene technology has become an important part of modern biotechnology, genetically modified microorganisms still hold great promise for environmental applications. Nonetheless, some may have unintended adverse affects (Lenski, 1993). Unlike bacteria grown in the laboratory, genetically modified organisms (GMO's) destined for deliberate release as agents of bioremediation, must be able to express their phenotype under the control of external signals present in the environment (de Lorenzo, 1994).

### 1.4.4 Biological agents for bioremediation of plant phenolics

The concept of bioremediation of phenolic residues is that the phenolic residues in wastewaters are utilized as a carbon energy source, which supports a microbial community in the remediation process. Conventional biological systems include fluidized beds, trickling filters, packed beds, biological lagoons, oxidation ditches, rotational biological filters and activated sludge processes. Such systems are susceptible to shock loading, need long residence times for effective treatment and there are often inadequate conversion of materials that are biologically very stable, for example halogenated hydrocarbons. The low maintenance and operating costs of such systems are an advantage, and they are most useful when used in conjunction with other dephenolization methods or when treating low to medium concentrations of phenolic compounds (Verstraete and Top, 1992).

A specific but limited number of bacterial isolates have been used widely for the treatment of phenolic industrial residues. These include the *Psuedomonas* sp. (Wang and Loh, 1999; Chang *et al.*, 1993), Actinomycetes (Antonopoulos *et al.*, 2001); *Mycobacterium paratortuitum* E3 to degrade lower gaseous alkenes and produce stereo-specific epoxides (Weber *et al.*, 1990) and *Desulfomonile tiedjei* DCB 1 for dehalogenation of pentachlorophenol (Moh and Kennedy, 1992). De Schrijver and De Mot (1999) suggested that rather than single isolates, a consortium of bacteria maybe required for complete degradation.

## 1.5 PHENOLIC INDUSTRIAL RESIDUES

Many hazardous chemicals are synthesized from aromatic and aliphatic hydrocarbons such as phenolic compounds, which include phenols, and other organic compounds containing one or more hydroxyl groups attached to an aromatic ring (Fig 1.12).

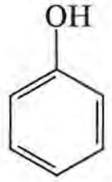


**Figure 1.12** **Aromatic ring structure of Phenol**

Phenol itself is highly soluble in water and is almost tasteless in pure solution. It is readily reactive with other chemicals and is rapidly absorbed through the gastrointestinal tract. The EPA's legal limit on phenol concentration in treated drinking water is 1 μg/ liter (USA EPA, 1980). A body of water contaminated with phenolic compounds may become rapidly depleted of oxygen due to a high biological oxygen demand (2.4 mg of oxygen per mg phenol) resulting in further pollution problems. The removal of aromatic phenolic compounds from aqueous effluents presents a serious problem for industries generating considerable quantities of these aromatic compounds.

The term 'phenolic compounds' embraces a wide range of substances, which possess an aromatic ring bearing a hydroxyl substitute. These include their functional derivatives and they are among the major classes of pollutants of concern in the US. They are listed priority pollutants regulated under the Clean Water Act (Patterson 1985, cited in Aitken, 1993). Several existing biological, chemical or physical treatment processes can be used to remove phenols from contaminated aqueous mixtures (Aitken, 1993). However it is often difficult to maximize treatment performance while minimizing cost with most of these technologies. Biological treatment systems can be cost effective but may not consistently remove specific chemicals to a significantly low level consistently (Grady 1990, cited in Aitken, 1993). For phenols and monochlorophenols, microbial degradation is the primary biotransformation process. Degradation rates decrease in water due to lower surface area and lower water temperature. Phenols can also polymerize and form molecules that are resistant to biological attack, but this also reduces the movement of these chemicals into the aquatic ecosystem.

Among the natural phenolic compounds of which several hundreds are known, the flavonoids and their relatives form the largest group, but phenol quinones, lignins and xanthones and other groups exist in considerable numbers.

### 1.5.1 Methods of remediating phenolic residues.

Conventional methods of remediating industrial residues have been in use for some time. These techniques include: -

- Solvent extraction e.g. the phenosoluan process,
- Irradiation,
- Enzymatic aerobic dephenolization,
- Adsorption on activated carbon,
- Chemical oxidation,
- Coagulation-flocculation,
- Ozonation
- Membrane processes e.g. reverse osmosis ultrafiltration (Klen and Lee, 1978).
- Ion exchange chromatography, which has been used to concentrate and separate polyphenols from high molecular weight humus materials in natural water.

### 1.5.2 Plant polyphenol residues

In the biosphere, aromatic compounds of biological origins are derivatives of the secondary metabolism of plants, biological and chemical cleavage of lignin and bioconversion of aromatic amino acids (Ruttiman *et al.*, 1998). A variety of aromatic compounds and phenols are produced by oil and petroleum refineries, coke plants, organic chemical plants, resins and plastics factories, olive oil production, wine fermentation etc. Plant derived polyphenolic compounds are produced by processes such as wine manufacture, the paper and pulp industry and the olive mill processing. Due to the large quantities of these phenolic residues produced annually, these compounds can be toxic or have undesirable effects when released into the environment. Bioremediation of these substances can be achieved via enzyme based and/or whole cell based systems (Nicell *et al.*, 1992).

The fate of these compounds is of interest due to their toxicity to all forms of life and particularly because they are a threat to human health. Phenolic compounds also present a particular problem to the agro-industry due to the presence of a high proportion of carbohydrate and phenols in their waste resulting in the formation of phenolic moieties, ester linked to polysaccaharides and forming structural cross-linking bridges between various polymers, restricting complete hydrolysis of these polymers (Faulds *et al.*, 1997). As a result these pollutants persist in the environment due to their resistance to microbial attack.

Plant polyphenolic compounds have been well studied and elucidated in different plants. Plant polyphenols includes phenolic acids, flavonols, flavanonols, proanthocyanidins and anthocyanins, which are responsible for many properties of fruit, juices and fermented beverages. All of these native phenolic compounds are more or less reactive and depending on their relative amounts, after enzymatic or chemical reaction give rise to a variety of products. The polyphenols are found in all parts of the plant at various concentrations (Table 1.3). These polyphenols pose a problem in waste produced during processes such as wine making. The resultant waste includes stems, skins, pips etc, which contain a relatively high concentration of polyphenols, especially phenolic acids, flavanols and flavanonolos such as astilbin, and lignin-derived compounds.

Tannins are also present and consist of polymeric proanthocyanidins. The relative concentrations of phenolic compounds detected in wines, grapes and grapewaste are depicted in Table 1.3 (Bonilla *et al.*, 1998), and molecular structures of some of the phenolic compounds in Figure 1.13. Table 1.4 gives concentration figures in mg/Kg of the different phenolic compounds in grape stems, seeds and skins from the Merlot grape variety. The flavonoids (flavones, isoflavones, flavonols, flavonones) are derived from a wide variety of compounds involved in plant photosynthesis (Schuler, 1990) with the best known being quercetin. Condensation of caffeic acid and phloroglucinol results in the formation of quercetin (Pratt and Hudson, 1990). The phenolic acids including gallic, coumaric, ferulic, vanillic and caffeic are the precursors to the flavonoids.

**Table 1.3** **Relative concentrations of polyphenolics in various plant tissue (Pratt *et al.*, 1990).**

| TISSUE | RELATIVE CONCENTRATIONS |
|---|---|
| Fruit | cinnamic acids>catechins=anthocyanins>flavonols |
| Leaf | flavonols=cinnamic acid>catechins=anthocyanins |
| Wood | catechin=anthocyanins>flavonols>cinnamic acid |
| Bark | As wood but in greater concentrations |

**Table 1. 4** **Concentration of the different phenolic compounds present in grape stems, seeds and skins of Merlot (Souquet *et al.*, 2000).**

| COMPOUND | MG/KG |
|---|---|
| Caftaric | 40 |
| Coutaric | 4.5 |
| Quercetin-3-glucuromide | 200 |
| Quercetin-3-gluside | 18 |
| Kaempferol-3-glucoside | Traces |
| Myricetin-3-glucoside | Traces |
| Myricetin-3-glucuconide | Traces |
| Catechin | 60 |
| Epicatechin | Traces |
| Astilbin | 35 |
| engeletin | Traces |

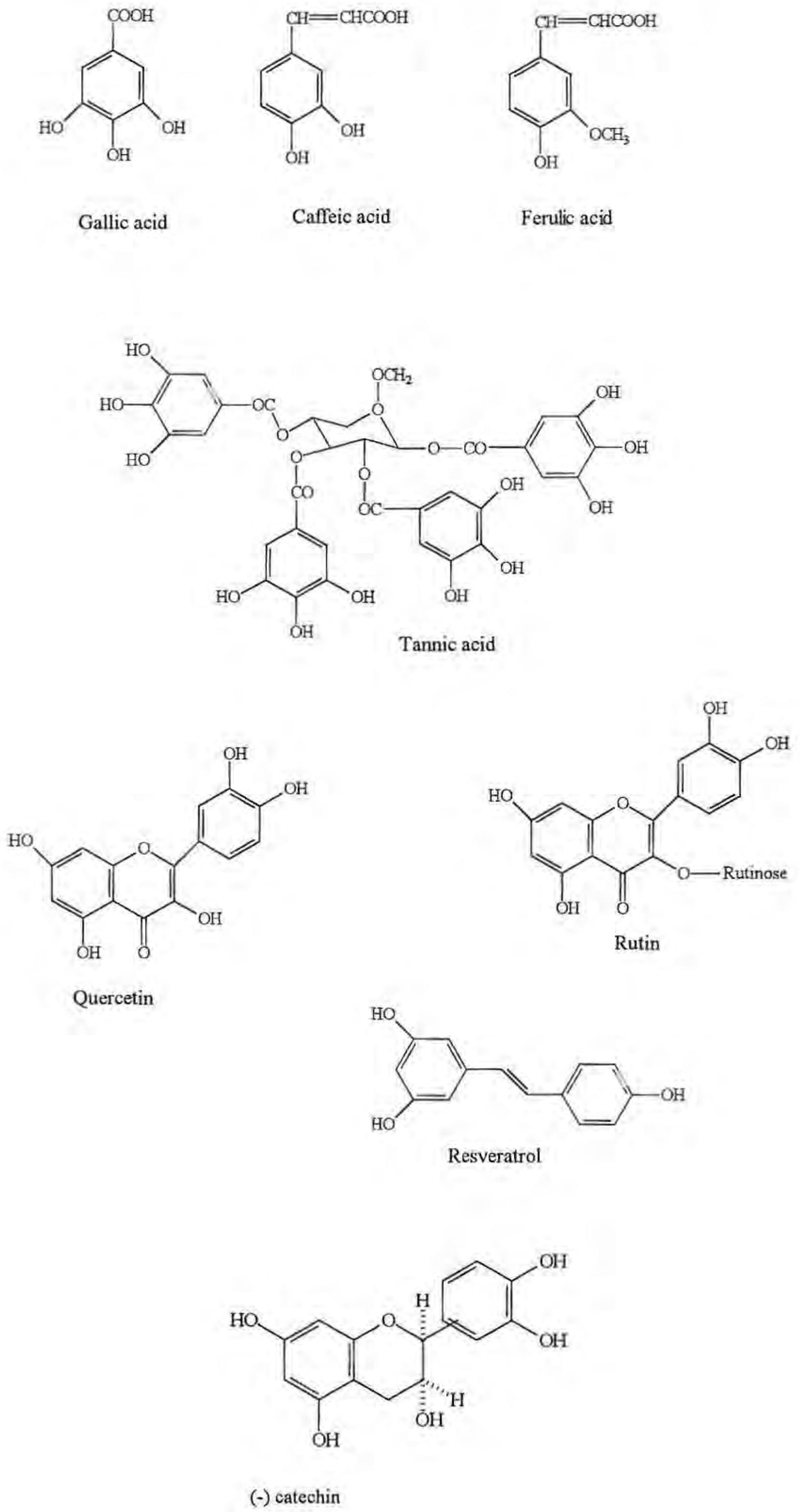


**Figure 1.13** **Structures of phenolic compounds found in grapes (Bonilla *et al.*, 1988; Cheeseman and Wilson., 1972; Gómez-Plaza *et al.*; 1999).**

## 1.6 THE APPLICATIONS OF PHENOL OXIDASES AND PEROXIDASES FOR THE TREATMENT OF PHENOLIC RESIDUES.

Oxidoreductases are known to catalyse biological oxidation/reduction reactions. The notion of developing practical biocatalytic applications using oxidoreductase enzymes is a useful and attractive goal. Examples of processes where oxidoreductase enzymes have, or could have, an immediate use and economic impact are:

- Pulp and paper bleaching
- Waste water treatment
- Soil remediation
- On-site waste destruction
- Bread dough conditioner; and
- Medical diagnostics

**Table 1.5** **Oxidoreductase enzymes with potential applicability in waste treatment (Aitken, 1993)**

| ENZYMEAND SOURCE | POLLUTANTS OR WASTE STREAM | REFERENCES |
|---|---|---|
| Laccase<br>*Trametes versicolor* and *Coriolus polyporus* | Halo-phenols, naphthols<br>Azo dye decolourisation | Bollag, 1988; Majchercyk, 1998, Ullah, 2000 |
| Peroxidases, chloroperoxidases<br>*Caldariomyces fumago* | Aromatic amines<br>Methylphenols,chlorophenols, phenol | Ricotta, 1996 |
| Lactoperoxidase<br>Lignin peroxidase<br>*Phanerochaete chrysosporium* | Dyes<br>Chlorophenols, polycyclic aromatic hydrocarbons | Yee and Wood, 1997 |
| Manganese peroxidase | Bleach plant effluent<br>meoxythphenols | Saski *et al.*, 2001<br>Tekere *et al.*, 2001 |
| Horseradish peroxidase<br>Sorghum peroxidase | Aromatic amines, bromophenols<br>Bleach plant effluent | Wagner and Nicell, 2001<br>Ximenes, 2001 |
| Polyphenol oxidase<br>*Agaricus bisporus* | Halo-phenols,<br>Methylphenols | Okeke *et al.*, 1997<br>Boshoff, 2001 |

In each of these areas listed in Table 1.5, these enzymes have advantages over the techniques currently in use and offer significant improvements over traditional chemical processes such as irradiation and solvent extraction by:

1. Minimizing the use of toxic chemicals
2. Lowering capital costs
3. Yielding cleaner products
4. Increasing cost efficiencies and
5. Improving production of unique chemical products.

While many of the waste treatment processes mentioned in this chapter are effective and useful, disadvantages include high cost, incomplete purification and formation of hazardous by-products and applicability over a limited range of concentrations. Like all enzymes, efficient enzyme production is dependant on pH, temperature and substrate moisture content (Rajarathnam and Bano, 1989).

The advantages of enzyme-based systems as opposed to whole cell systems may include the following for any one-enzyme system: -

- Rapid reaction rates with well characterized end products,
- Well-defined system with simpler process control,
- Operation over high and low concentration ranges of contaminants,
- Operation over wide pH and salinity ranges, operation over wide temperature ranges, action on compounds toxic to microbes.

The activity of the enzyme must, however, be well characterized in terms of the fate of toxic compounds before application can be considered (Aitken *et al.*, 1989).

The main disadvantage of enzyme-based systems is that complete degradation may only be achieved if multiple enzyme reactions are employed, and thus several enzymes and co-factors are required. Another limitation is the limited catalytic lifetime of enzymes, which affects the economic feasibility.

Enzyme treatment is by no means a new method of waste treatment. As early as 1980, 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. Peroxidases were first used for the efficient dissimilation of phenolic wastes in 1980 (Alberti and Klibanov, 1981). Parathion hydrolase is an enzyme effective at hydrolyzing organophosphates and is produced by a variety of cultures such as *Pseudomonas* spp., *Flavobacterium* sp. and a recombinant *Streptomyces*. (Smith *et al.*, 1992).

## 1.7 RESEARCH OBJECTIVES

The overall objective of this study was the development of an oxidase enzyme system capable of bioconverting grape waste polyphenolics from the wine-making industry in the Western Cape in South Africa.

The main aims of this study are divided into three distinct phases (Figure 1.14).

**Phase 1: The isolation of thermophilic Actinomycetes which have the ability to degrade phenolic compounds or which produce oxidoreductase enzymes, (primarily phenol oxidase and peroxidase) from an environment conducive for such Actinomycetes.** Our specific aim was to isolate Actinomycetes of a thermophilic nature, based on the fact that little information was available on thermophilic bacteria capable of degrading environmental contaminants. This was surprising, given the biotechnological importance of thermophilic bacteria as sources of thermostable enzymes and other products of industrial interest. Higher temperatures in the treatment of contaminated wastes have the advantage of increasing the solubility of the substrates. The quest to improve enzymes for bioremediation through screening for thermo-tolerant microorganisms/enzymes is one of the important objectives of this area of biotechnology (Harayama, 1998). Known thermophillic Actinomycetes will be screened for oxidoreductase activity.

**Phase 2: Biochemical profiling, secondary screening and demonstration of the ability of isolated thermophiles to bioconvert phenolic compounds using a common source of phenolic waste generated in industry.** The phenolic source was grape waste, which is a source of polyphenolics generated from the wine manufacturing process. Phenolic content from this type of waste is generally high and various extraction techniques are required to extract these polyphenolics. Thus, both whole cells and cell free crude extracts were to be used to determine whether the isolates were capable of bioconverting the polyphenolics, as the simpler products would be carbon sources that the isolates can utilize in whole cell systems, while the enzymes would be expectd to convert the polyphenolics to simpler or polymerised compounds. Analytical methods, which include HPLC, total phenolic determinations, and UV scanning, would be used to determine if any bioconversion has occured. Secondary screening would involve the use of model phenolic compounds found in phenolic-based effluents, incorporated into growth media and acting as the sole carbon source in the growth media, with monitoring of the decrease in concentration of these compounds.

**Phase 3: Isolation of the genes responsible for the enzymes detected through activities in Phase 1 and 2**. Methods used would include gene cloning, and Southern blotting by developing a probe for an oxidoreductase gene based on a previously studied *Streptomyces* strain.

## Research Outline

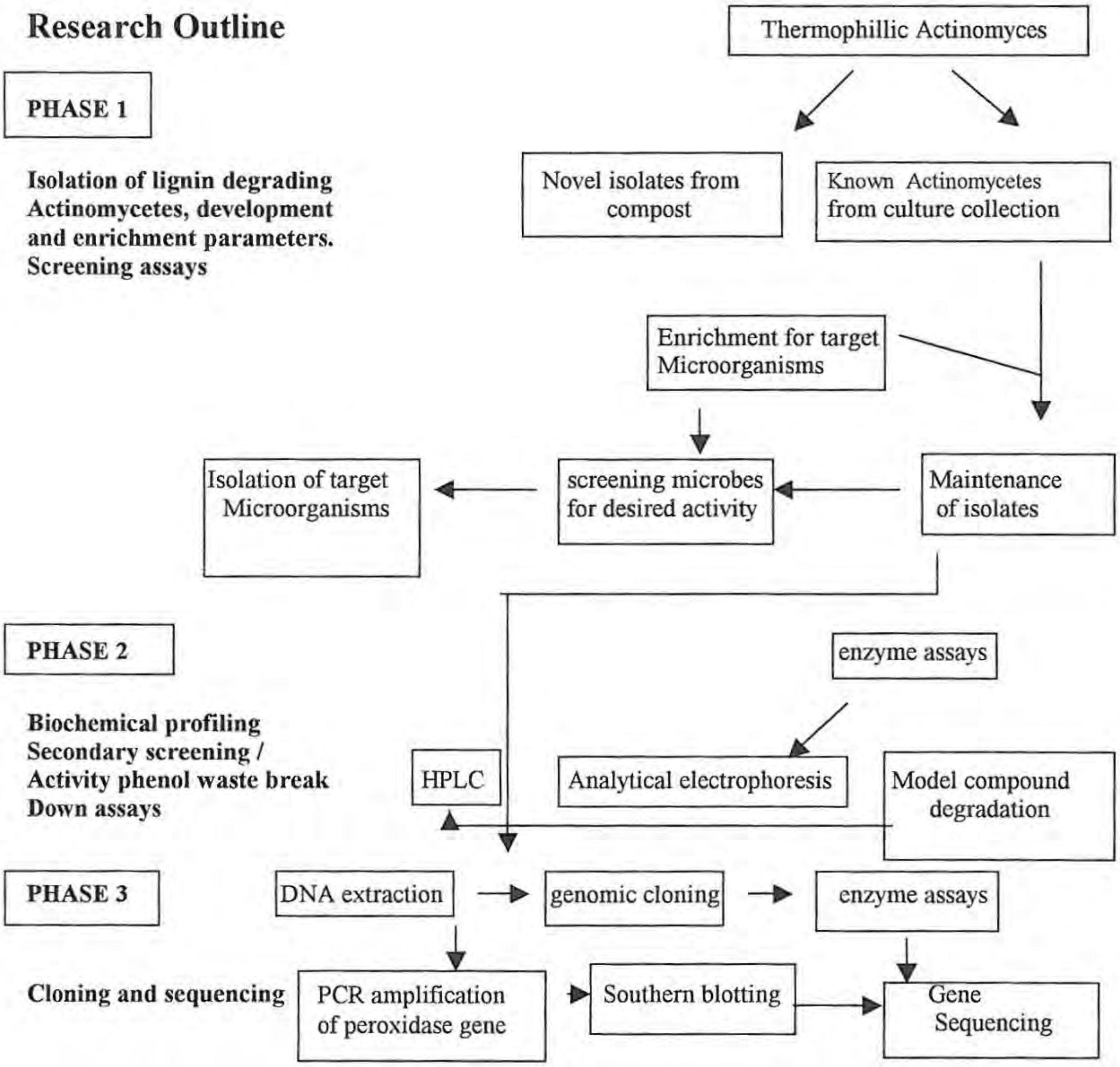


**Figure 1.14** **Research Outline of project**

# CHAPTER 2
# MATERIALS AND METHODS

## 2. 1 CULTURE CONDITIONS

### 2.1.1 Isolation of Actinomycetes.

#### 2.1.1.1 Materials

For the growth media, the following chemicals were purchased from Biolab chemicals, Merck (SA): - Yeast extract powder, Malt extract powder, calcium carbonate, sodium phosphate and potassium phosphate from Saarchem, Merck (SA). Thermophilic Actinomycete strains were ordered from DSMZ, Germany.

#### 2.1.1.2 Soil sampling

Thermophillic Actinomycetes were isolated from soil samples obtained from mushroom composting sites located in Grahamstown. The protocol carried out was as follows: - 1g samples of soil were placed into 20 mls of sterile saline in 100 ml flasks and left shaking at 150 r.p.m for 1 hour before serially diluting (1 in 100 series dilution) in sterile saline and plating out aliquots of $10\mu l$ each on M65 agar plates. The plates were inverted and incubated 50°C overnight.

#### 2.1.1.3 Subculturing of thermophilic Actinomycetes from primary plates

After a 24-hour incubation period, single Actinomycetes colonies were selected and streaked out on to fresh M65 agar plates and incubated overnight at 50○C. If there was poor or no growth, incubation was continued for a further 1-2 days at 50 °C. Single Actinomycetes colonies were then picked from the primary plates with a wire loop and streaked on fresh M65 agar plates.

The DMSZ thermophilic Actinomycetes strains (*Thermoactinomyces dichotomus* 43308, *Thermactinomyces thalpophilus* 43353, *Thermoactinomyces sacchari* 43356, *Thermobifida alba* 43795 and *Thermobifia fusca* 43793) and the three isolated thermophilic species (RU-A01, RU-A03 and RU-A06) were routinely maintained on *Streptomyces* media M65 agar (Appendix A). The thermophiles were sub-cultured monthly using a four-way streak method. The petri dishes with organisms fully grown were sealed with Para film and stored at $4^0C$.

Sporulation agar (Appendix A.14) was found to result in confluent growth of the above strains within 2-3 days incubation at $50^0C$ with the thermophiles growing confluently in 24 hrs at $50^0C$. This medium was used for providing cells for various tests and not for maintenance of cultures. M3 medium was used to prepare agar slants, which were inoculated with spores from the strains and incubated at room temperature for a week.

## 2.2 LONG-TERM STORAGE

### 2.2.1 Materials

The glycerol used was purchased from Unilab, Saarchem (Pty) Ltd. A Heraeus benchtop microfuge was used for centrifugation of the whole cells.

### 2.2.2 Storage media of thermophillic Actinomycetes

Actinomycetes spores were harvested from liquid culture media (0.6% yeast media, Appendix A) by centrifugation at 13 000rpm for 10 minutes. The supernatants were discarded and the pellets resuspended in 20% sterile glycerol and aliquoted into 1-1.5 ml volumes into vials suitable for deep-freezing at -20°C.

## 2.3 PREPARATION OF ACINOMYCETES FOR EM WORK.

Sections of confluent growth on growth agar were excised and placed in cold-buffered fixative (gluteraldehyde) in labelled vials and allowed to fix overnight. The fixative was decanted and replaced with cold phosphate buffer and washed for 10 to 15 minutes. This stage was repeated twice. The phosphate buffer was then decanted and replaced with 30% ethanol and allowed to dehydrate for 5-10 minutes. This step was repeated with each of the ethanol series concentrations (30%, 50%, 70%, 80% and 100%), with two changes of 100% ethanol. The specimens were then transferred to the critical point drying baskets, covered in 100%ethanol and placed into the critical point drying (CPD) chamber filled with liquid carbon dioxide. The specimens were then handed in and prepared further for electron microscopy work.

## 2.4 IDENTIFICATION OF THE THERMOPHILLIC ISOLATES

### 2.4.1 Materials

Qiagen DNEasy DNA extraction Kit was purchased from Southern Cross biotechnology (Pty Ltd), SA. PCR Kits containing *Taq* polymerase, $MgCl_2$-free PCR buffer, $MgCl_2$ buffer and dNTPs were purchased from Gibco. DNA molecular weight markers were purchased from Roche Biochemicals. Low melt agarose was purchased from Whitehead Scientific. PCR primers were ordered and synthesized from the Department of Molecular and Cell Biology, University of Cape Town. PCR reactions were carried out on a PCR sprint thermocycler.

### 2.4.2 DNA extraction

DNA was extracted from the isolates using the QIANeasy DNA extraction kit from Qiagen according to the manufacturer's instructions. DNA was quantified using a Genequant quantifier. DNA was stored at 4° C for short-term storage and at -20°C for long-term storage.

### 2.4.3 16S rDNA PCR

Actinomycetes primers were selected according to literature for amplification of the 16s rDNA genes designed to be specific to Actinomycetes (Table 2.1).

**Table 2. 1** **Primers used for i6s rDNA analysis.**

| Primer | 16s rDNA target (positions) | Sequence (5`-3`) |
|---|---|---|
| F9 | Actinomycetes | GATTTGATCCTGGCTCAG |
| 154IR | Actinomycetes | AAGGAGGTGATCCAGCC |

The PCR program used was the one according to Heuer *et al*, 1997 (Table 2.2).

The following PCR conditions were found to be ideal and were carried out in the HYBAID Ominigene. The "hot start" procedure was used where the *Taq* polymerase was added after the initial denaturation step, allowing the temperature to decrease until it reached $80^0$C where it was held to allow the addition of *Taq* DNA polymerase.

### 2.4.3.1 Optimization conditions for Polymerase Chain Reaction (PCR)

The components for all 16S rDNA amplification protocols and Southern blotting probes are shown in Table 2.4 and were used to obtain the respective final concentration. Master mixes were prepared for multiple reactions to reduce reagent loss and to enable accurate pipetting especially since small volumes were used.

**Table 2. 2** **PCR conditions used in 16S rDNA amplification**

| CONDITIONS | TEMPRATURE | DURATION (min) |
|---|---|---|
| Initial denaturation | 94°C | 5 |
| Denaturation | 80°C | Until *Taq* is added |
| Annealing | 63°C | 1 |
| Extension | 72°C | 2 |
| Final extension | 72°C | 10 |

The "hot start" procedure was used where the *Taq* polymerase was added after the initial denaturation step, allowing the temperature to decrease until it reached $80^0$C where it was held to allow the addition of *Taq* DNA polymerase.

**Table 2. 3** **Reagents and final concentrations used in all PCR reactions.**

| Reagent | Final Concentration |
|---|---|
| Buffer 10x | 1x |
| dNTP 10mM | 0.2mM each |
| $MgCl_2$ | 1.5 - 2.0mM |
| Forward and Reverse primers | 0.5$\mu$M |
| Template DNA | 10ng |
| *Taq* DNA polymerase | 2.5 Units |

The PCR reaction volume used was 25$\mu$l. The following PCR conditions were found to be ideal and were carried out in the HYBAID Ominigene PCR machine.

**Table 2. 4** **Volumes of reagents used for PCR reaction**

| REAGENTS USED | VOLUME ($\mu$l) |
|---|---|
| Sterile $dH_20$ | 15$\mu$l |
| Buffer x10 | 2.5$\mu$l |
| dNTPs | 1.0$\mu$l |
| $MgCl_2$ | 0.7$\mu$l |
| Forward Primer | 1.0$\mu$l |
| Reverse Primer | 1.0$\mu$l |
| 4% Acetamide | 2.0$\mu$l |
| Taq polymerase | 0.5$\mu$l |
| DNA template | 1.0$\mu$l |

15$\mu$l of sterile mineral oil was used to overlay the reaction mixture in order to avoid evaporation of the PCR mixtures. The PCR reaction volumes used was 25$\mu$l.

## 2.5 GROWTH CONDITIONS AND OPTIMUM PH STUDIES.

### 2.5.1.1 Growth studies

RU-A01, RU-A03 and RU-A06 were grown in 0.6% yeast buffered medium. Optical density readings were taken at regular intervals (4 hours) at wavelength 600nm. Intracellular protein was also determined. Whole cells were disrupted by French pressing (see section 2.5.2.3). Protein content was determined by using the Bradford protein determination assay (see section 2.5.1.2).

### 2.5.1.2 pH studies

For the pH studies 0.6% buffered yeast growth medium was used. pH was adjusted using HCl or NaOH. The pH's studied ranged from pH 6.0 to 9.0. Cultures were grown at 50 °C at 175 r.p.m until lag phase. Optical density readings were taken at wavelength 600nm.

### 2.5.2 Liquid growth media experiments

### 2.5.2.1 Medium and culture conditions

M65 broth buffered to pH 8.0 was the liquid medium of choice for the growth of the thermophilic Actinomycetes except for DNA extraction purposes due to the interference of the calcium carbonate in the lysis procedure. For the purpose of DNA extraction the liquid growth media of choice was M3 broth with 20% sucrose incorporated into the media to soften the outer wall for easier lysis attack of the lysozyme. 100 ml flasks containing 20ml growth medium were inoculated with spores from 2- to 3- week old agar slants and cultures were incubated at 50 °C and agitated at 200 r.p.m. $100\mu l$ samples were then inoculated into 1-litre flasks containing 200ml of growth medium. For the purposes of harvesting cells for crude extracts, spores were inoculated into 0.6% yeast buffered liquid medium.

#### 2.5.2.2 Metabolism of low molecular weight lignin-related compounds by Actinomyces isolates RU-A01, RU-A03 and RU-A06.

10mM concentrations of caffeic, *p*-coumaric, syringic acid, ferulic acid, vanillic acid and protocatechuic acid were used as sole carbon sources in minimal liquid media (200mL). 1 mL samples were taken at regular intervals and analysed by HPLC to monitor degradation of the compounds and possible products. Standard curve graphs were prepared from each model compound to convert peak areas into concentrations (Appendix J).

#### 2.5.2.3 French Pressing

Whole cells in sodium phosphate buffer (0.1M, pH 7.0) were French pressed at 15 MPa for two minutes after which the liquid was allowed to drip through into a beaker on ice at the rate of 1 drop per second.

## 2.6 ENZYME ASSAYS

### 2.6.1 Materials

For the assaying of peroxidase, *o*-dianisidine, hydrogen peroxide, 2,4 DCP, 4-aminoantipyrine and guaiacol were purchased from Sigma Chemical Co (USA). Veratryl alcohol was purchased from Aldrich. For the assaying of phenoloxidases, L-3,4-Dihydroxyphenyl alanine was purchased from Sigma Chemical Co (USA). ABTS and

Bovine serum albumin (BSA) was purchased from Boerhinger Manneheim, GBH (Germany) and catechol was purchased from Unilab, Saarchem (Pty) Ltd.

### 2.6.2 Enzyme activity

All kinetic activity was calculated using the enzyme activity equation (Appendix F.2) and using the various extinction coefficients for each substrate (Appendix F.1).

### 2.6.2.1 Peroxidase activity

Peroxidase activity was assayed with 2,4 DCP, guiaicol and *o*-dianisidine as substrates.

(a) 2,4 DCP as substrate.

The reaction mixture (total volume 1.0mL) contained 200$\mu$l of 100mM potassium phosphate buffer (pH 7.0); 200$\mu$l of 16 mM 4-aminoantipyrine; 200$\mu$l of 25mM 2,4-DCP; 200$\mu$l of culture supernatant or sonicated cells. The reaction was initiated with the addition of hydrogen peroxide (200$\mu$l of 50mM) and the reaction was monitored at 50 °C for 1 min at a wavelength of 510nm (Ramachandra *et al.*, 1988). Assays were conducted in duplicate.

(b) Guaiacol as substrate

The reaction mixture (total volume of 1.0mL) contained 0.2mL of crude enzyme extract incubated with 0.8 mL of guiaicol (40mM guiaicol in 0.1M sodium phosphate buffer, pH 5.6) to which 0.01mL 4mM hydrogen peroxidase was added. The reaction was monitored at a wavelength of 465nm for 240 seconds. Assays were conducted in duplicate.

(c) *o*-dianisidine as substrate

Peroxidase assays using 0-dianisidine as substrate was adapted from Vágújfalvi and Petz-Stifter (1982). 0.05mL pf substrate, 50mM o-dianisidine, was added to 6mL sodium phosphate buffer, pH6, containing 0.01M Hydrogen peroxide. The peroxidatic reaction was initiated by adding 0.1mL of enzyme solution to 2.9mL of the substrate mixture in a cuvette and monitored at 460nm. Assays were conducted in duplicate. Peroxidase activity was expressed in micromols of product formed per minute per mL of enzyme.

Equations and extinction coefficients for the calculations of the enzymes are given in Appendix F.

#### 2.6.2.2 Lignin peroxidase activity

LiP activity was determined according to Tien and Kirk (1983) using a final veratryl alcohol concentration of 10mM. 0.4ml distilled water was added to 0.3 ml sodium tartrate buffer (pH 2.5, 0.25mM), 0.1ml veratryl alcohol and 0.1 ml crude enzyme extract. The reaction was initiated by the addition of 6$\mu$l of hydrogen peroxide (10mM). Reaction was determined at absorbance 310 nm for 5 minutes.

### 2.6.3 Phenol oxidase activity

#### 2.6.3.1 Polyphenol oxidase

(a) L-DOPA as substrate

Polyphenol oxidase activity was measured using the Dopachrome assay (Burton *et al*, 1993). A fresh solution of L-DOPA (10mM) in 50mM of phosphate buffer, pH 6 was made up and 0.1 ml enzyme solution was added to 3 ml of the DOPA solution in a cuvette. The change in absorbance over 180 seconds was measured spectrophotometrically at 475 nm.

(b) Catechol as substrate.

0.1mL of crude enzyme extract was added to 0.1mL of copper sulphate (1mM) and 0.8mL of catechol (6mM) in a cuvette. The change in absorbance was over 180 seconds at wavelength 475nm.

### 2.6.4 Laccase

·ABTS as substrate.

0.8mL sodium acetate buffer (0.1M, pH 5.0) was added to 0.1mL of ABTS (5mM) and 0.06mL of crude enzyme extract. The change in absorbance was monitored for 180 seconds at wavelength 420nm.

## 2.7 PROTEIN DETERMINATION

Intra- and extracellular protein concentrations were determined by the method of Bradford (1976). Culture pellets were washed twice in sodium phosphate buffer (0.1M, pH 7.0) and then re-suspended in phosphate buffer, sonicated on ice at maximum amplitude for 2 cycles of 10 seconds each. The mass was then centrifuged for 3 minutes at 5000 r.p.m and the supernatant was aliqoted into 1.5 mL. Eppendorf tubes. Extracellular protein was collected by centrifugating growth medium to pellet the whole cells, collecting the supernatant. Total concentrations of supernatant and biomass protein were estimated from a calibration curve constructed using bovine serum albumin (BSA). Growth was measured as mg of intracellular protein per ml of culture supernatant.

## 2.8 CONCENTRATION AND PRESERVATION OF CRUDE ENZYME.

### 2.8.1 Materials

PEG 6000 was purchased from Merck (SA). Snakeskin dialysis tubing was purchased from Pierce (USA). Ammonium sulphate was purchased from Saarchem, Merck (SA).

### 2.8.2 Dialysis

Extracellular and intracellular crude proteins were collected and dialyzed against 20% PEG 6000 for 12 hours. Snakeskin dialysis tubing (molecular weight cutoff point 3 500 daltons) was used to remove small molecular weight compounds and to concentrate volumes by up to 10 fold. The resultant protein was analyzed on native PAGE gels and kinetic enzyme activities were conducted.

### 2.8.3 Ammonium sulphate precipitation studies

Ammonium sulphate cuts, from 30%, 55%, 85% and 100% were carried out by dissolving the relevant amount of ammonium sulphate in the supernatant to give the desired percentage of ammonium sulphate saturation, with gentle stirring at 4°C. the mixture was left to stand on ice for an hour and a half and centrifuged (20 000 x g, 20 minutes, 0°C). The supernatant was dialyzed against several changes of milli Q water, to remove salts and then dialyzed to concentrate down to 3mL quantities using 20% PEG. The supernatant was then assayed for peroxidase, lignin peroxidase and polyphenol oxidase activities. The fractionated procedure was repeated at the next ammonium sulphate level and the precipitate was harvested, dialyzed and assayed as before. The resultant extracts were subjected to PAGE (section 2.9).

### 2.8.4 Lyophilisation

Extracellular and intracellular protein was subjected to freeze drying in order to stabilize the protein and to preserve activity. Samples were frozen in round bottom flasks in liquid nitrogen, ensuring that a thin layer of the liquid formed in the interior surface of the flask. The flask was then placed onto the freeze dryer until all water was removed from the sample. The resultant powder was collected and stored in an airtight bottle and stored at -20°C.

## 2.9 GEL ELECTROPHORESIS.

### 2.9.1 Materials

The following chemicals were used for Polyacrylamide gel electrophoresis (PAGE). Ammonium persulphate, glycerol, methanol, glacial acetic acid, HCI and mercaptoethanol were from Unilab Saarchem (Pty) Ltd. Coomassie brillant blue R250 (CBB), *Tris*- (hydroxyethyl) aminomethane) and bromophenol blue were purchased from Sigma Chemical Co (USA). PAGE was carried out according to Laemmli (1970). Acrylamide was purchased from BDH chemicals, tetramethylethylene diamine (TEMED) was purchased from Merck NT lab supplies (Pty) Ltd and *N,N*-methylethylene-bis-acrylamide was from Boerhinger Manneheim. The SDS was from

Reidel-de Häen. The tall might small electrophoresis unit was set up according to the Hoefer Scientific Instruments manual (catalog No. SE280).

### 2.9.2 Activity gels

Gels containing no SDS were prepared with 4% stacking and 10% resolving gel concentrations and were run for approximately 3 hours at 100V. Enzyme samples (30ul) were loaded with tracking dye and electrophoresed using bath buffer (Appendix E). Peroxidase bands were detected by incubating the gels in 10mM o-dianisidine in the presence of 1.6 mM hydrogen peroxide for 30 minutes. Polyphenol oxidase was detected by incubating for 30 minutes in a 10mM solution of L-DOPA dissolved in 0.1M phosphate buffer.

### 2.9.2 Protein gels

PAGE gels containing SDS were prepared with 4% stacking and 10% resolving gel concentrations and were run for approximately 3 hours at 100v (Appendix E). Enzyme samples (30ul) were denatured by boiling in eppendorf tubes for 5 minutes in 15ul of dissociation buffer (Appendix E). 30ul of the denatured protein was loaded into the denaturing gel and electrophoresed in 1 X bath buffer (Appendix E). Protein marker solution containing proteins with known molecular weight (5ul) was applied to the gel and electrophoresed along with the enzyme samples in order to determine the molecular weight size of the proteins. Protein bands were detected by incubating the gel in Coomassie stain overnight, on a rocker. The stain was then discarded and destain was then added and replaced every 30 minutes until excess stain was removed and protein bands could be visualized.

## 2.10 EXTRACTION AND QUANTIFICATION OF PHENOLICS FROM GRAPEWASTE.

### 2.10.1 Materials

Grape waste in the form of compressed skins, pips and grape stalks were supplied from the Western Cape, South Africa. The organic solvents comprising of methanol, ethanol, ethyl acetate, acetone and 2-meoxyethanol were purchased from Saarchem. The drying agent sodium sulphate was purchased from Sigma Chemical Co (USA).

### 2.10.2 Extraction

Grape skins, pips and stalks were thawed and blended in a Warring blender with 0.1M potassium phosphate buffer (pH 7.0) to slurry. The slurry was then passed through cheesecloth to collect all the water-soluble phenols. 5g of the solid residue were then measured out and 30ml of methanol, 95% ethanol, ethyl acetate, acetone or 2- methoxy ethanol were added, and the mixture was allowed to stand for two hours in a fume hood. The flasks were shaken on a regular basis. The mixture was then filtered to remove any solid matter. The organic fractions were then were then dried using anhydrous sodium sulphate ($Na_2SO_4$) for 30 minutes, which was later filtered off through Whatman No. 4 filter paper. The filtrate was concentrated to dryness using a rotary evaporator and re-dissolved in methanol:water (3:2) mixture. Phenol concentrations were then determined using the Folin-Ciocalteau Phenol determination assay for all extracts including the water-soluble fraction (Section 2.10.2.3). Phenol concentrations were calculated based on the Folin-Ciocalteau standard curve (Appenix J).

### 2.10.2.3 Total phenolic determination.

The total phenolic assay procedure was adapted from the method described by Box *et al* (1983). This assay depends on the quantification of the reduction of phosphomolybdic and phosphotungstic acid to yield a heteropoly blue colour in alkaline solution and comparing this intensity of blue colour to that of different amounts of standard phenolic compounds. 1 mL of each sample was placed in a 100ml volumetric flask. 16ml of $dH_20$ was added to each flask and mixed well. 2.5 mL of Folin-Ciocalteau reagent was added and mixed thoroughly for 30 seconds. 15 mL of sodium carbonate, at a concentration of 100g/L was added, mixed and brought to the 100mL mark with distilled water. The solution was allowed to stand at room temperature for 1 hour. The control included all the reagents except for the unknown phenol sample, which was replaced with distilled water. Absorbance readings were taken at 765nm against a blank. Total phenol concentration was calculated against the standard curve extrapolated from the standards. Gallic acid was used as the standard and the standard curve is shown in Appendix J. The absorbance was read at 725nm using a Shimadzu UV-160A Spectrophotometer, against a reagent blank, and expressed as a concentration in mg/L.

### 2.10.3 Ethyl acetate extraction of phenolics

#### 2.10.3.1 Acidic fraction of grape waste

The second step of extraction was adapted from the method used by Garcia and co workers (2000), using ethyl acetate. 50mls of acetone extracted grape waste was acidified to pH 2 with HCl and extracted three times with double volumes of ethyl acetate (v/2v) at ambient temperature. The mixture was placed in separating funnel and mixed adequately. The solvent mixture was allowed to settle overnight. The organic fractions were dried using anhydrous $Na_2SO_4$ for 30 minutes and was then filtered through Whatman No 4 filter paper. The filtrate was then concentrated to dryness using a rotary evaporator and re-dissolved in 50mls methanol:water (6:4). A control was used in which was grape waste, not adjusted to pH 2 prior to extraction.

#### 2.10.3.2 Extraction of neutral phenolic fraction from grape waste

Extraction of phenolics using the method employed by Salagöity-Auguste *et al.* (1984) was adapted as follows: 100 mL distilled water was added to 100 mL red wine and concentrated to 100 mL by means of rotary evaporator at 35°C, which results in the evaporation of the alcohol that could interfere with the extraction by organic solvent, without destroying the phenolic compounds (Salagöity-Auguste *et al.*, 1984). The extract was then adjusted to pH 7 with 2 M NaOH and extracted three times with 100 mL ethyl acetate. The wine and solvent were mixed thoroughly with the use of a magnetic stirrer for 5 minutes followed by centrifugation for 10 minutes at 10 000 rpm. The organic upper phase was decanted into a separating funnel after each extraction. The three fractions were pooled together and evaporated to dryness using a rotary evaporator and the residue was re-dissolved in 10 mL distilled water. The aqueous phase containing the ionised acidic phenolics was adjusted to pH 2 with 2 M HCl. The un-ionised phenolics were extracted three times with ethyl acetate under the same conditions as the neutral phenolics. The phenolic compounds were then concentrated 10-fold.

### 2.10.4 Whole cell studies of phenolic grape waste using isolates RU-A01, RU-A03 and RU-A06

Red grape waste was added to 0.6 % yeast buffered medium (Appendix A) broth and agar at different concentrations ranging from 0.1% through to 50%. Enzyme activities and phenolic residue degradation were determined as described below. UV spectrum scans between wavelengths 220nm to 800 nm to determine peak areas were taken before and after inoculation, as well as phenol determination assays using the Folin- ciocalteau total phenol determination assay (See section 2.10.2.3).

## 2.11 ENZYME STUDIES FOR THE TREATMENT OF GRAPE WASTE PHENOLICS BY THERMOPHILLIC ISOLATE CRUDE ENZYMES.

### 2.11.1 Cell-free extract treatment for the breakdown of grape waste phenolics

2.5mL samples of red and green grape waste were aliquoted into sterile Bijou bottles. 2.5 mL of crude extract formed by the French pressing whole cells (See section 2.5.1.4) or extracellular extract formed by the removal of cells by centrifugation and use of the supernatant was added to these mixtures and 2.5mLs 0.1M phosphate buffer to the controls. These samples were closed and placed in an incubator set at 50°C, shaking at

175 r.p.m for 12 hours. The samples were then prepared for HPLC analysis and for total phenol determination by solvent extraction with ethyl acetate. The aqueous layer was extracted, and the organic extract was evaporated using a nitrogen stream. The extracts were then dissolved in water and methanol (3:2) in preparation for HPLC and total phenol determination.

### 2.10.4 Chromatographic analysis of the *in vitro* reaction of crude extract with red and green grape waste.

The disappearance of the phenolic compounds were monitored by HPLC (LaChrom, Merck, Germany) with UV detection (L-7400) using reverse-phase 5$\mu$m $C_{18}$ Waters (250mm x 4.6mm) column with a mobile phase of water/acetonitrile (80:20), containing 0.1% acetic acid, at a flow rate of 0.7ml/min. Peaks were detected at 280nm and analysed using HPLC system manager, (Merck, Hitachi) model D7000 data software.

## 2.11 HPLC METHOD DEVELOPMENT FOR THE ANALYSIS OF PHENOLIC COMPOUNDS FROM RED AND GREEN GRAPE WASTE.

### 2.11.1 Materials

Ascorbic acid, caffeic acid, (+) catechin, cinnamic acid, coumaric acid, (-) epicatechin, eugenol, ferulic acid, gallic acid, syringic acid, tannic acid and vanillic acid were all obtained from Sigma Chemical Company. Procyandins are not commercially available. The HPLC system used was LaChrom (Merck, Germany) with UV detection (L-7400) using reverse-phase 5$\mu$m $C_{18}$ Waters column.

### 2.11.2 Chromatographical conditions

Variations in solvent ratio of acetonitrile to water were investigated to obtain optimal separation of phenolic compounds as shown in Table 2.1. Acetic acid was dissolved in double distilled water. Samples were run at two different flow rates to achieve maximum separation (1ml/min and 0.7ml/min), with detection at a wavelength of 280nm at room temperature.

**Table 2. 5 Chromatographical conditions investigated in the HPLC separation and identification of grape waste phenolics using an isocratic system**

| METHOD | SOLVENT A | SOLVENT B | FLOW RATE | DURATION |
|---|---|---|---|---|
| | %Acetonitrile | % Acetic acid (0.1%)in milli Q water | ML/min | Min |
| 1 | 60 | 40 | 1ml/min | 45 |
| 2 | 60 | 40 | 0.7ml/min | 45 |
| 3 | 40 | 60 | 1ml/min | 45 |
| 4 | 40 | 60 | 0.7ml/min | 45 |
| 5 | 20 | 80 | 1ml/min | 45 |
| 6 | 20 | 80 | 0.7ml/min | 45 |

### 2.11.3 Identification of phenolics

The identification of the phenolic compounds in grape waste was achieved by comparing the retention times of the standard compounds under the same conditions used for the grape waste extracts.

### 2.11.4 Standard compounds

The standards were all dissolved in water except for eugenol, which was dissolved in methanol-water.

## 2.12 GENOMIC DNA EXTRACTION

### 2.12.1 Materials

Restriction endonucleases and molecular weight marker 3 were purchased from Roche Biochemical's (listed in Appendix G). Gel purification kits were purchased from, and high pure plasmid purification kits were purchased from. Cloning vectors pSE280 and pSE380 and TOP10F' *E.coli* cells were purchased from Invitrogen (Netherlands). Sybr green was purchased from. Bromophenol blue used in the tracking dye was purchased from. Triton X 100 was purchased from, sucrose, ampicillin, LB broth, boric acid, EDTA, Unicam spectrophotometer. PAGE and agarose gels were recorded using a Kodak Digital system 120 camera, using software.

### 2.12.2 Quantification of DNA

DNA used in this report was extracted using the Qiagen DNeasy Tissue extraction kit. The protocol used was carried out following the instructions provided in the manufacturer's handbook. Spectrophotometric quantification was carried out using a Unicam spectrophotometer by diluting DNA to $10^{-3}$ in TE buffer. Spectrophotometric readings (absorbance's) were converted to concentrations using the equation below.
1 Optical Density (OD) = 50μg/ml. Spectrophotometric measurements should generally lie between 0.1 and 1.0 to ensure reliability.

### 2.12.3 Restriction endonuclease digestion of DNA

#### 2.12.3.1 Restriction endonuclease digestion of genomic DNA

1 $\mu$l of DNA (concentration of 5 μg) was added into a final volume of 11$\mu$l of water, containing 2$\mu$l of the recommended digestion buffer (see table 2.1) and 1 $\mu$l of the restriction enzyme (5-10units/$\mu$l). DNA digestion was carried out at 37°C for 3 hours or overnight, followed by heating at 65° for 10 minutes to inactivate the enzyme.

#### 2.12.3.2 Preparation of digested genomic DNA for electrophoresis

To test whether the digestion was complete, 8$\mu$l of the digestion mix was added to 4$\mu$l loading buffer and 1$\mu$l of Sybr green (Appendix C.5) and 10$\mu$l was loaded onto 1% agarose (1X TBE, pH 8.3) (Appendix B.15). The DNA was electrophoretically fractionated at 80V. The DNA was visualised under an ultraviolet light. An even smear indicated complete digestion.

#### 2.12.3.3 Gel extraction and purification

The desired bands were excised and purified using Separations and Qiagen gel purification kits according to the manufacturer's instructions.

### 2.12.4 Plasmid preparations

#### 2.12.4.1 Culture of *Escherichia coli* (*E.coli*)

*E.coli* cells containing plasmids pSE280 and pSE380 were placed onto LB Amp (50μg/ml) plates, and incubated at 37°C overnight. Plasmid pSE280 and pSE380 were first transferred into competent *E.coli* cells by transformation before being placed onto LB Amp plates. Single colonies were picked from respective plates with a disposable loop and inoculated into 5mls of LB containing 50μg/ml ampicillin, and incubated at 37°C by shaking for not longer than 16 hours.

#### 2.12.4.2 Mini preps

##### 2.12.4.2.1 Plasmid DNA extraction (CTAB method)

Plasmid extraction using the CTAB method was carried. 1.5 ml of bacterial cells was pelleted by centrifugation for 5 minutes at 13000g. The supernatant was discarded and the pellet was resuspended in 200μl STET. 5 μl of lysozyme was added and incubated at room temperature for 5 mins. The Eppendorfs were boiled for 45 seconds and then centrifuged at 13000g for 15 mins. The supernatant was then poured into sterile Eppendorfs and the pellets discarded. 8μl of CTAB was added and centrifuged at 13000g for 5 mins. The pellet was resuspended in 300μl NaCl and 750μl absolute ethanol and centrifuged at 13000g for 10 mins. The supernatant was discarded and the pellet washed with 800μl 70% ethanol. The pellet dried under a vacuum for 30mins-1 hour. When almost dry, the pellets were resuspended in 20μl TE buffer.

The plasmid DNA was screened as follows: -

A 1% agarose gel was cast. 1 μl of sybr green was added to 5μl of plasmid DNA andand allowed to react at room temperature for 15 minutes. 3.5μl loading buffer was added and electrophoreses at 80v. The bands were visualised under UV light.

#### 2.12.4.2.2 Restriction endonuclease digestion of plasmid DNA

Plasmid DNA derived from mini-plasmid preparations (Section 2.12.4.2.1). Two microliters of plasmid DNA was incubated in a final volume of 11$\mu$l of water with 2$\mu$l of the recommended digestion buffer, containing approximately 1$\mu$l (5-10 units/$\mu$l) of the respective restriction endonuclease. Digestion was allowed to proceed at 37°C for 1-3 hours, followed by heating at 65°C for 10 minutes to inactivate the enzyme.

#### 2.12.4.2.3 Preparation of digested plamid DNA for electrophoresis

10 microliters of the digestion mixture was aliquoted, with 1$\mu$l syber green and 4 $\mu$l of loading buffer from which 15 $\mu$l was loaded on to 1% agarose gel and electrophoretically fractionated as described above.

### 2.12.5 Ligation of DNA fragments into vectors

#### 2.12.5.1 Linearisation of vector

The vector DNA, pSE280 and pSE380 were linerised by *BamH1, Bgl11* and *Sau3A* restriction endonuclease at the multiple cloning sites. (See Table G1 for conditions of digestions.

#### 2.12.5.2 Ligation of foreign DNA into linerised vectors

Reactions were mixed by pipetting several times in 0.5$\mu$l eppendorf tubes. These were then incubated at 4°C overnight to maximise the number of transformants obtained.

**Table 2. 6 Ligation reactions volumes between vectors, DNA and reagents.**

| LIGATIONS | Standard Reaction | Positive control | Background control |
|---|---|---|---|
| 2 x Rapid ligation buffer | 2.5$\mu$l | 2.5$\mu$l | 2.5$\mu$l |
| pSE vector | 0.5$\mu$l | 0.5$\mu$l | 0.5$\mu$l |
| DNA | 1.5$\mu$l | - | - |
| Control insert DNA | 0.5$\mu$l | 0.5$\mu$l | 0.5$\mu$l |
| T4 DNA ligase | - | 0.5$\mu$l | 0.5$\mu$l |
| dH$_2$O to 5$\mu$l final vol | - | 0.5$\mu$l | 1.5$\mu$l |
| Total volume | 5.0$\mu$l | 5.0$\mu$l | 5.0$\mu$l |

### 2.12.6 Preparation of *E.coli* competent cells.

5mls of LB broth was added in sterile test tubes. Each single colony was inoculated into a test tube and incubated at 37°C overnight. In 500 ml flasks containing 100ml LB broth, the following was added.

Over night cultures were inoculated as follows:

| | |
|---|---|
| Flask 1 | 1.5 ml over night culture |
| Flask 2 | 1.0ml over night culture |
| Flask 3 | 0.7ml |
| Flask 4 | 0.3ml |

Flasks were incubated on a shaker at 37°C for 2 hours. $OD_{600}$ absorbance readings were taken of Flask 2 after 2 hours. OD readings were taken until $OD_{600}$ readings were within the range of 0.8 (normally obtained after 2.5-2.75 hours). The flasks were placed on ice for 10 mins and then contents were transferred into pre-autoclaved centrifuge bottles. The cells were centrifuged at 5000rpm for 10 mins at 4°C. The supernatant was decanted and the pellet resuspended in 50 mL RF1 solution and incubated on ice for 20 minutes. The suspension was centrifuged using the same conditions in previous steps. The supernatant was decanted and each pellet was resuspended in 4 mL RF2.The flask contents were pooled together and 50 μl were aliquoted into sterile and pre-cooled eppendorfs and stored at -80°C.

### 2.12.7 Transformation Procedure

The vial(s) containing the ligation reaction(s) were centrifuged briefly and placed on ice. One 50μl vial of competent *E.coli* TOP10F’ cells was thawed on ice for each ligation/transformation. To transform, the following was used: 1 to 5μl of a ligation reaction DNA was directly added to the competent cells and mixed by tapping gently.

The vials were incubated on ice for 30 minutes, and then were incubated for exactly 30 seconds in the 42°C water bath without mixing or shaking. The vials were removed from the 42°C bath and quickly placed on ice. 250μl of pre-warmed SOC medium was added to each vial, which was then placed in a microfuge rack on its side and secured with tape. The vials were shaken at 37°C for 1 hour at 225 rpm in a rotary shaker-incubator. 10 to 50$\mu$l from each transformation vial were spread on separate, labelled LB agar plates. Two different volumes from each eppendorf were plated out to ensure at least one plate had well spaced colonies. For small volumes, 20$\mu$l of SOC was added to the ligation mixture prior to plating to allow for even spreading. Plates were inverted and incubated at 37°C overnight.

### 2.12.8 Protein expression

#### 2.12.8.1 Pilot expression

For each strain 2mL of SOB or LB containing 50$\mu$g/mL ampicillin was inoculated with a single recombinant *E.coli* colony and incubated overnight at 37°C, shaking at 225-250 rpm. This was followed by 10 ml of SOB or LB containing 50$\mu$g/mL ampicillin inoculated with 0.2 mL of over night culture, grown at 37°C with vigorous shaking to an $OD_{600}$= 0.6 (mid log phase). 1 mL aliquot of cells was removed, centrifuged at maximum speed for 30 seconds and the supernatant aspirated. The pellet was frozen at -20°C until further use. This was the zero time point sample. IPTG was added to the final concentration of 1mM (10 $\mu$l of a 1M IPTG stock to 10 mLs) and cells were grown at 37°C shaking. 1mL samples were taken every hour for 5 h (or more) and treated by centrifugation at maximum speed for 30 s and the supernatant aspirated. The tubes were labelled to correspond to the number of hours post-induction.

#### 2.12.8.2 Scale up expression

2 mL of SOB or LB containing 50$\mu$g/mL ampicillin was inoculated with a single recombinant *E.coli* colony and grown overnight at 37°C shaking at 225-250 rpm. 10 mL of SOB or LB containing 50$\mu$g/mL ampicillin was inoculated with 0.2 mL of overnight culture and grown at 37°C with vigorous shaking to an $OD_{600}$= 0.6 (mid log phase). IPTG was added to the final concentration of 1mM (50 $\mu$l of a 1M IPTG stock to 50 mL and grow at 37°C shaking until the optimal time point was reached, based on maximum

protein expression. Cells were harvested by centrifugation at 3000 r.p.m for 10 minutes at 4°C. The cell pellet was stored at -80°C for future use.

#### 2.12.8.3 Analysis of time point samples

Time point samples were collected, spun down to pellet the cells and each of the pellets were resuspended in 100µl of 1X SDS-PAGE sample buffer (Appendix E), boiled for 5 min and centrifuged using a top-bench microcentrifuge. Each sample was analyzed by loading 15µl into the wells of the SDS-PAGE gel and electrophoresed at 80V for 2.5 - 5hrs.

After electrophoresis, the top stacking gel was cut off and the plug removed leaving the resolving gel which was then stained with Coommasie blue (Appendix D.3) for 3h and destained overnight with half strength destain (Appendix D.5). The positive control was TOP10F` cells containing the vector pSE280 or pSE380, was included in the analysis of the samples to confirm that the growth and induction had been successful. The negative control was TOP10F` cells without any vector was used to distinguish recombinant proteins from background proteins. The bands on the gels were recorded using Kodak Digital system 120 camera.

## 2.13 SOUTHERN BLOTTING

### 2.13.1 Materials

DNA was extracted using the QIAneasy DNA tissue extraction Kit and purified using NuceloSpin extract 2 in 1 PCR and agarose purification kit purchased from Separations. Primers used for the amplification of the *CatC* gene were ordered and synthesized from the University of Cape Town. Probe labelling and detection was carried out using The DIG-Prime DNA labelling and Detection Starter Kit II from Roche Molecular Biochemicals. Blotting was carried out on nitrocellulose membranes purchased from Amersham.

### 2.13.2 Agarose Gel Electrophoresis and Southern Transfer

10μl of the digested sample containing sybr green was loaded onto a 25 x 20 cm 1% agarose gel and electrophoretically fractionated at 80 V for 5 hours in 1 X TBE, or until the dye front reached approximately 2 cm from the end of the gel. The DNA was visualised under ultraviolet light and the gel was photographed. The DNA was denatured by incubating the agarose gel in depurination solution (0.25M HCL, Appendix K) for 10 minutes with gentle shaking. The gel was washed in distilled water and then immediately soaked in denaturation solution (Appendix K) for 20-30 minutes with gentle shaking. The gel was washed again with distilled water and then immediately soaked in neutralisation solution (Appendix K) for 2x15 minutes with gentle shaking. The solution was discarded and allowed to stand in 2xSSC (Appendix K).

### 2.13.3 Transfer

The denatured DNA was Southern transferred by means of capillary action onto a nylon membrane. Each membrane was marked at the corners to facilitate alignment of sequential lanes. 3x3MM Whatman filter was cut to size1 cm bigger than the gel and 3 others were cut to the same size of the gel. The larger filter papers were soaked in 20 x SSC. A piece of Clingfilm was placed onto the bench, and soaked filter paper was layered out on top of the Clingfilm. The gel was placed on top of the filter papers, and the Nylon membrane of the same size placed onto the gel. The Clingfilm edges were folded around the membrane so that it just covered the edges of the gel. The 3 smaller pieces of filter paper were placed on the top of the nylon. About 5 cm of paper towels was placed on top of the Whatman paper and a weight was placed on top and left overnight. The apparatus was disassembled the following day and the nylon membrane was allowed to dry at between room temperature and 80°C depending on time. The DNA transferred to the membrane was fixed to the membrane by UV crosslinking at 0.35 J/cm$^2$, and stored in a Whatmann envelope sealed in a bag and stored at 4°C until use.

### 2.13.4 Labelling of DNA probes using the chemiluminescence method

The *cat C* gene was labelled and detected according to the manufacturer's instructions (Roche Biochemicals). Briefly, the nylon membrane was pre hybridised for 30 minutes with DIG Easy Hyb. 200-250 ng of probe was made up to 15µl of distilled water. The DNA was denatured by heating for 3 minutes at 100°C. followed immediately by cooling on ice for 5 minutes.

### 2.13.5 Hybridisation conditions for DIG High Prime DNA Labelling and detection starter Kit II.

The hybridisation buffer for the DIG kit was prepared according to the manufacturer's instructions. The nylon membrane was pre-hybridised at 38°C for 30 minutes in a hybridisation oven. The denatured DIG-labelled DNA probe was added to pre heated DIG Easy Hyb. The pre- hyb was removed and the probe/hybridisation mixture (about 25 ng/ml) was added to the membrane and incubated overnight with gentle agitation and 38°C (3.5ml/100cm$^2$).

After hybridisation the membrane was washed twice in SCC, 0.1% SDS (Appendix K.7) at 15°C under constant agitation for 5 minutes, followed by washing twice in 0.5% x SSC, 0.1% SDS (Appendix K.8; pre-warmed to wash temperature) at 65°C under constant agitation.

### 2.13.6 Immunological detection

After stringency washes, the membrane was rinsed briefly in washing buffer (Appendix K.3) and placed in blocking solution for 30 minutes, followed by antibody solution for 30 minutes. The membrane was then washed twice in washing buffer (Appendix K.3) and then equilibrated with detection buffer for 2-5minutes. The membrane was then placed onto Clingfilm and 1mL of chemiluminescent substrate for alkaline phosphatase (CSPD) was added and distributed without air bubbles evenly over the surface of the membrane and incubated for 5 minutes at 25°C. excess liquid was removed and the damp membrane was incubated for 10 minutes at 37°C to enhance luminescent reaction. The membrane was exposed X-ray film for 15-25 minutes at room temperature depending on the intensity of the signal required.

# CHAPTER 3
## RESULTS AND DISCUSSION

### 3.1 ISOLATION OF THERMOPHILLIC OXIDOREDUCTASE PRODUCING ACTINOMYCETES

The advantages of bioremediation practices have been discussed in detail in chapter 1 and hence the use of whole cells or their enzymes in the treatment of phenolic residues proves to be an attractive option to conventional methods such as solvent extraction enzymes and irradiation. The current focus of bioremediation and the use of microbes for this procedure has been primarily on mesophilic microorganisms, however there is an increase in interest in the use of thermophilic microorganisms. The main objective in this study was to isolate thermophillic/thermo-tolerant *Actinomycetes* because of their potential usefulness of these species in bioremediation/ biotransformation purposes. Before considering thermophilic microorganisms, a brief discussion of what actually qualifies as "thermophilic" is warranted. Brock (1986) defined thermophilic microorganisms as those that grow at temperatures between 50-60°C. From a bioremediation point of view, common terminology generally includes any process operating at temperatures from 45°C.

Although significant interest in the use of thermophilic microorganisms has developed only recently, the use of thermophilic microorganisms and their processes have been in operation for decades. Composting, commonly used to treat most organic solids such as garden refuse, sewage sludge etc represents a thermophilic waste treatment technology that has been used for centuries. Recently various applications of thermophilic microorganisms and their enzymes have been suggested, these include decolorization of industrial effluents, biopulping, and biobleaching in paper industries, biological desulfurization of coal and petroleum, production of aromatics from lignin, production of improved cattle feed and removal of chlorinated organic compounds from wastewaters.

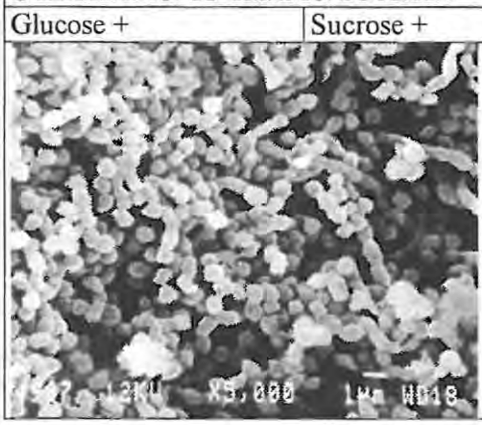
The genera of thermophilic actinomycetes isolated from compost include *Nocardia, Streptomyces, Thermoactinomycetes* and *Micromonospora* (Strom, 1985). *Actinomycetes* have been isolated from soil, mud, marine sediments, sand and compost (Cross, 1968). In their natural habitat, they are able to degrade cellulose and solubilize lignin and are known to tolerate higher temperatures and pHs in comparison to fungi. An ideal environment to isolate thermophilic Actinomycetes was at a composting site. Actinomycetes are known to be involved in the process of composting due to their ability to breakdown lignocelluose degradation during peak heating in the composting process.

Among the factors which contribute towards the development of microbial populations in compost include oxygen and nutrient availability, the temperature increase which rises to between 50 and 80°C resulting in a transition from mesophilic to thermophilic microbial communities which also results in a decrease in the microbial diversity (Blanc *et al.*; 1999). For isolation purposes in this study, it was hence crucial to select soil samples from specific regions of the composting site. Samples were hence taken from the compost core where temperatures are known to be highest.

Classical enrichment procedures select for bacterial strains with the highest growth rates under specified high nutrient and environmental conditions, which are often not representative of *in situ* soil communities. This technique helps increase microbial densities of the desired microorganisms. In this study, solid and liquid media specific for lab scale growth and enrichment of Actinomycetes was used to select for Actinomycetes. Incubation temperature selection was also crucial in terms of selecting for thermophilic Actinomycetes. All samples were incubated at 50°C although optimal temperature was later determined for the three isolates (Table 3.1, 3.2 and 3.3). Growth media used in this study was M65 agar and broth, consisting of malt, yeast, glucose and calcium carbonate. This method of isolation of microbes from natural environments is viewed as a traditional technique in cultivating microbes and subsequent characterization of pure isolates. This approach however does have its limitations, including insufficient information on cultivation requirements of most novel bacteria and the inability to recover starving dormant and viable but non-culturable cells. Using microscopic techniques, Torsvik *et al.*, 1996 demonstrated that while the majority of bacteria in soil samples are actively respiring, only about 1% of the total number of cells could be cultured on laboratory media. In addition, less that 1% of bacterial species have been isolated and characterized using enrichment techniques (McCaig *et al.*, 2001). However this technique in combination with molecular based analysis proves to be a useful method of identification.

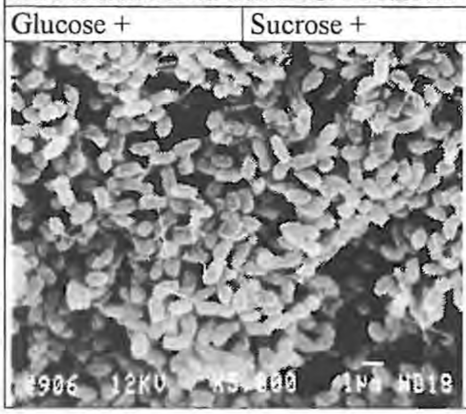
1g soil samples were serially diluted and 100μl of each sample was plated out in duplicate onto M65 agar, an *Actinomycetes* growth media consisting of malt, yeast and calcium carbonate. Plates were incubated at 50°C for a period of 24 –72 hours. *Actinomycetes* were isolated from the plates based on their phenotypic features, which differentiate *Actinomycetes* from other bacteria and/or pigment production commonly found in this group. Three isolates were isolated, sub-cultured and grown at 50°C. These isolates were then labeled RU-A01 (Figure 3.1), RU-A03 (Figure 3.2) and RU-A06 (Figure 3.3). Spore suspensions and hyphal fragments of the isolates were maintained in 20% (vol/vol) glycerol at -20°C. Isolates were routinely cultured on M65 plates and phosphate buffered suspensions were used to inoculate shake flasks containing M65 broth or a 0.6% buffered yeast medium. The three isolates were characterized and summarized in Tables 3.1, 3.2 and 3.3.

**Table 3. 1** **Biological characteristics of thermophilic isolate RU-A01**

| | | | |
|---|---|---|---|
| **MORPHOLOGICAL CHARACTERISTICS** | | | |
| Colour of aerial mycelium | Greyish | | |
| Spore shape | Coccus | | |
| Spore surface | Smooth | | |
| **CULTURABLE CHARACTERISTICS ON VARIOUS MEDIA** | | | |
| **M65 agar** | | **M65 broth** | |
| Colour of aerial mycelium | Grey | Colour | Purple/black |
| Reverse side | Purple/black, distinctive | Spores or mycelia | Mycelia |
| Soluble pigment | Brown | Brown | |
| **0.6% yeast buffered growth** | | **0.6% yeast buffered broth** | |
| Aerial mycelium | Grey | Colour | Purple/black |
| Reverse side | Purple/black | Spores or mycelia | Mycelia |
| Soluble pigment | Brown | Brown | |
| **PHYSIOLOGICAL CHARACTERISTICS** | | | |
| Melanin formation | | Positive | |
| Growth temperature | | 40˚C- 50˚C, optimum 47˚C | |
| Phenolic acid utilization | | Positive | |
| **UTILIZATION OF CARBON SOURCES** | | | |
| Glucose + | Sucrose + | Fructose + | Dextrose + |
|  | | | |

Maximum growth was achieved within 30-36 hours for RU-A01 and RU-A06 and 48 hours for RU-A03. A distinct purple pigment was produced by RU-A01 after an incubation time of 12 hours. This is common in some *Streptomyces* species and is often attributed to the production of melanin (Table 3.1 and 3.3) or the pigmented antibiotic actinohordin.

**Table 3. 2 Biological characteristics of thermophilic isolate RU-A03**

| | | | |
|---|---|---|---|
| MORPHOLOGICAL CHARACTERISTICS | | | |
| Colour of aerial mycelium | Greyish/brown | | |
| Spore shape | Oblong | | |
| Spore surface | Smooth | | |
| CULTURABLE CHARACTERISTICS ON VARIOUS MEDIA | | | |
| **M65 agar** | | **M65 broth** | |
| Colour of aerial mycelium | Greyish/brown | Colour | Greyish/brown |
| Reverse side | Not distinctive | Spores or mycelia | Spores |
| Soluble pigment | None | None | |
| **0.6% yeast buffered agar** | | **0.6% yeast buffered broth** | |
| Colour of Aerial mycelium | Greyish/brown | Brown | |
| Reverse side | Brown | Spores or mycelia | Spores |
| Soluble pigment | None | None | |
| PHYSIOLOGICAL CHARACTERISTICS | | | |
| Melanin formation | | Negative | |
| Growth temperature | | 40°C - 50°C, optimum 45°C | |
| Phenolic acid utilization | | Positive | |
| UTILIZATION OF CARBON SOURCES | | | |
| Glucose + | Sucrose + | Fructose + | Dextrose + |
|  | | | |

Five recognized *Actinomycetes* species, 3 belonging to the *Thermactinomyces* genus and 2 from the *Thermobifida* genus (Table 3.4) were selected, from an international culture collection and assayed for peroxidase and polyphenol oxidase activity. Growth was within 5 to 8 days on M65 agar and broth, which was comparatively slow compared to the compost isolates. The selection of these thermophiles was supported by the probability that these thermophiles could produce oxidoreductase enzymes that would exhibit thermostable properties of relevance to biotechnological applications.

**Table 3. 3** **Biological characteristics of thermophilic isolate RU-A06**

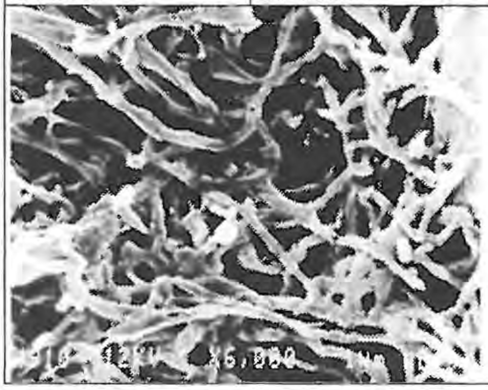
| | | | |
|---|---|---|---|
| MORPHOLOGICAL CHARACTERISTICS | | | |
| Colour of aerial mycelium | Grey | | |
| Spore shape | Rods | | |
| Spore surface | Smooth | | |
| CULTURABLE CHARACTERISTICS ON VARIOUS MEDIA | | | |
| **M65 agar** | | **M65 broth** | |
| Colour of aerial mycelium | Greyish/brown | Colour | Greyish/brown |
| Reverse side | Brown | Spores or mycelia | Mycelia |
| Soluble pigment | Brown | Brown | |
| **0.6% yeast buffered agar** | | **0.6% yeast buffered broth** | |
| Aerial mycelium | Greyish/brown | Brown | |
| Reverse side | Brown | Spores or Mycelia | Mycelia |
| Soluble pigment | None | None | |
| PHYSIOLOGICAL CHARACTERISTICS | | | |
| Melanin formation | Positive | | |
| Growth temperature | 40°C - 50°C, optimum 50°C | | |
| Phenolic acid utilization | Positive | | |
| UTILIZATION OF CARBON SOURCES | | | |
| Glucose + | Sucrose + | Fructose + | Dextrose + |
|  | | | |

**Table 3. 4** **Known Actinomycetes species strains and DSM number used in the study**

| Species description | DSMZ Number |
|---|---|
| *Thermactinomyces dichotomus* | 43308 |
| *Thermactinomyces thalpophilus* | 43353 |
| *Thermactinomyces sacchari* | 43356 |
| *Thermobifida fusca* | 43793 |
| *Thermobifida alba* | 43795 |

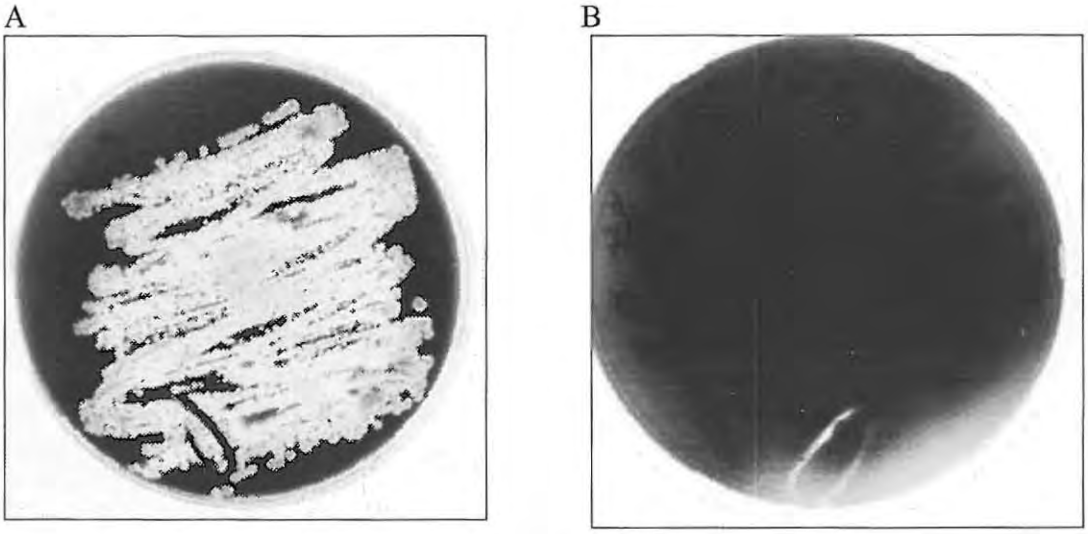


**Figure 3. 1** (A) Thermophilic isolate RU-A01 isolated from compost. (B) Reverse side pigment

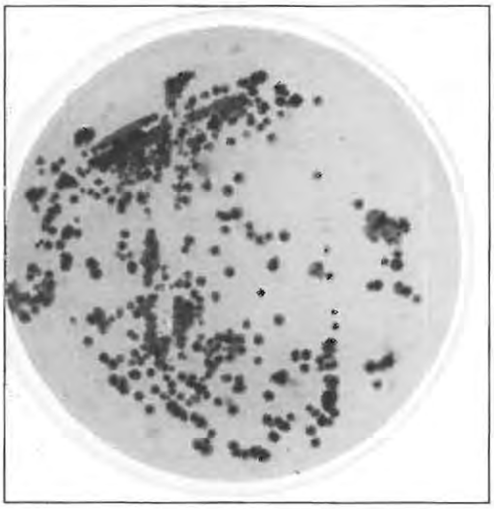


**Figure 3. 2** Thermophilic isolate RU-A03 isolated from compost grown on M65 agar, incubated at 50°C for 48 hours

**Figure 3. 3** **Thermophilic isolate RU-A06 isolated from compost grown on M65 agar, incubated at 50°C for 24 hours**

Light and electron microscopic techniques were utilized to determine any morphological differences between RU-A01, RU-A03 and RU-A06. Results were tabulated under morphological characteristics in Table 3.1, 3.2 and 3.3. The shape of the spores differed between the isolates with RU-A01 being of a coccoid shape, while RU-A03 were more oblong and RU-A06 were rod shaped.

By using the conventional transfer technique, an enrichment culture of bacteria able to grow on *Actinomycetes* growth media was easily obtained. Reduction of the culture complexity by the serial dilution plate technique and further purification resulted in the isolation of 3 *Actinomycete* isolates, RU-A01, RU-A03 and RU-A06.

## 3.2 IDENTIFICATION OF THE ISOLATES

Classification of novel isolates through taxonomic methods such as numerical taxonomy, fatty acid analysis, ribosomal protein analysis have been useful but more powerful tools of investigating phylogeny of microorganisms are available. Advances in molecular biology, in particular in development of 16S rRNA based methodologies enable cultivation-independent or dependant analysis of bacterial isolates for specific biotechnological applications. The most common approach involves PCR amplification and sequence analysis of 16S rRNA genes and has been used to investigate and identify bacteria. For this study a combination of traditional isolation methodologies in the form of enrichment techniques and physiological characterization, and 16S rRNA techniques were applied. Actinomycetes are a well-studied group, in particular the *Streptomyces* group. Actinomycetes are relatively easy to identify based on their phenotype, the extensive literature on laboratory culturing techniques, growth media and conditions of the group. Hence it was likely that using these techniques would result in the isolation of thermophilic Actinomycetes. 16S rRNA analysis was necessary to identify the isolates to determine whether these isolates were previously unidentified *Actinomycetes* species.

DNA was extracted from the isolates and Actinomycetes, oligonucleotide primers were selected and used for 16S rDNA amplification analysis. The resultant amplified DNA (Figure 3.4) was extracted purified and sequenced. The sequences were then compared and a phylogenetic tree was constructed (Figure 3.8). According to the sequence alignment comparisons, all three isolates were previously unidentified. The three isolates do however all fall within the *Streptomyces* clade, with isolate RU-A06 at the periphery of this clade (Figure 3.8). The phylogenetic tree was constructed using Clustalax software available at www.ebi.ac.uk. The e neighbour joining method with correction for multiple substitutions was used.

The primers utilized in the amplification of the 16S rDNA genes were designed to be specific for Actinomycetes (Table 3.5). Primer set F9 and 1541R cover a region of the 16s rRNA that is well represented in the Genbank. The region is not particularly variable, i.e. it is not an intergenic region but containing signature sequences for Actinomycetes.

**Table 3. 5 Primers used in 16 rDNA PCR experiments**

| Primer | 16S rDNA target (positions) | Sequence (5'-3') |
|---|---|---|
| F9 | *Actinomycetes* | GATTTGATCCTGGCTCAG |
| 1541R | *Actinomycetes* | AAGGAGGTGATCCAGCC |

The PCR program used was the one according to Heuer *et al.*, 1997.

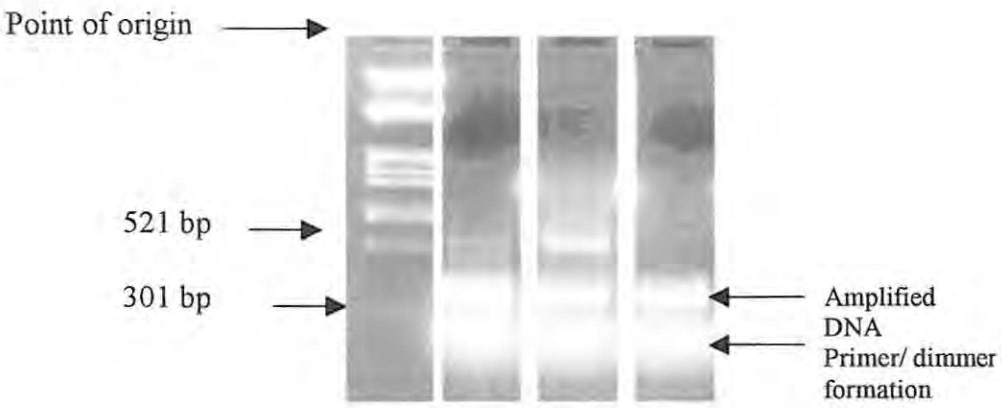


**Figure 3. 4** **16s rDNA amplification of the three thermophilic DNA using primers F9 and 1541R.**

## RU-A01 16ST7

```
1    TTAGTGGCGA ACGGGTGAGT AACACGTGGG CAATCTGCCC TGCACTCTGG
     GACAAGCCCT GGAAACGGGG TCTAATACCG GATA-CGACA C--AGGGAGG

101  CATCTCCTCT G-TGTGGAAA G-CTCCGGC- --GGTGCAGG ATGAGCCCGC
     GGCCTATCAG CTTGTTGGTG AGGTAACGGC TCACCAAGGC GA-CGA-CGG

201  GTAGCCGGCC TGAGAGGGCG ACCGGCCACA CTGGGACTGA GACACGGCCC
     AGACTCCTAC GGGAGGCAGC AGTGG-GGAA TATTGCACAA TGGGCGCAAG

301  CCTGATGCAG CGACGCCGCG TGAGGGATGA CGGCCTTCGG GTTGTAAACC
     TCTTTCAGCA G-GGAAGAA
```

**Figure 3. 5** **Nucleotide sequence for RU-A01 using primers F9 and 1541R**

## RU-A03 16S

```
1    TTAGTGGCGA ACGGGTGAGT AACACGTGGG CAATCTGCCC TGCACTCTGG
     GACAAGCCCT GAAAACGGGG TCTAATACCG TATA-CGACA C--ATGGAGG

101  CATCTNCTGT G-TGNGGAAA G-CTNCNGC- --GGTGCATG ATGAGCCCGC
     GGTCTATCAT CTTGTTGGNG AGGTAACGGN TCACCANGGC GA-CNA-CGG

201  GTAGNCGGCC TGAGAGGGCT ACCNGCCACA CTGGGACTGA GACACGGCCC
     AGACTCCTAC NGGAGGCAGC AGTGGTGGAA TATTGTANAA TGGNCGCGCG

301  CCTGATGCAN CGACGCCGCN NGAGGGATGA CGGCCTTTNG GTTGTNAACC
     TCTTTCATCA NTGGAAGAA
```

**Figure 3. 6** **Nucleotide sequence of RU-A03 using primers F9 and 1541R**

**RU-A06 16S 3F`T7**

```
1     TTAGCGGCGG ACGGGTGAGT AACACGTGGG CAACCTGCCT GTAAGACTGG
      GATAACTCCG GGAAACCGGA GCTAATACCG GATA-ACATT TTCTCTTGCA

101   TAAGAGAAAA TTGAAAGATG GTTTCGGCTA TCACTTACAG ATGGGCCCGC
      GGTGCATTAG CTAGTTGGTG AGGTAACGGC TCACCAAGGC AA-CGA-TGC

201   ATAGCCGACC TGAGAGGGTG ATCGGCCACA CTGGGACTGA GACACGGCCC
      AGACTCCTAC GGGAGGCAGC AGTAG-GGAA TCTTCCGCAA TGGACGAAAG

301   TCTGACGGAG CAACGCCGCG TGAGTGATGA AGGCTTTCGG GTCGTAAAAC
      TCTGTTGTTA G-GGAAGAA
```

**Figure 3. 7 Nucleotide sequence for RU-A06 using primers F9 and 1541R**

3 isolates were isolated and were identified according to the GenBank database as novel thermophilic *Streptomyces* species. A combination of both enrichment cultivation and the use of 16S rRNA analysis proved to be a successful in isolating the 3 isolates. The fact that the 3 isolates are novel is exciting and increasing the prospects in biotechnology research. Optimum growth and pH studies of the isolates were necessary as part of the evaluation process of these novel isolates.

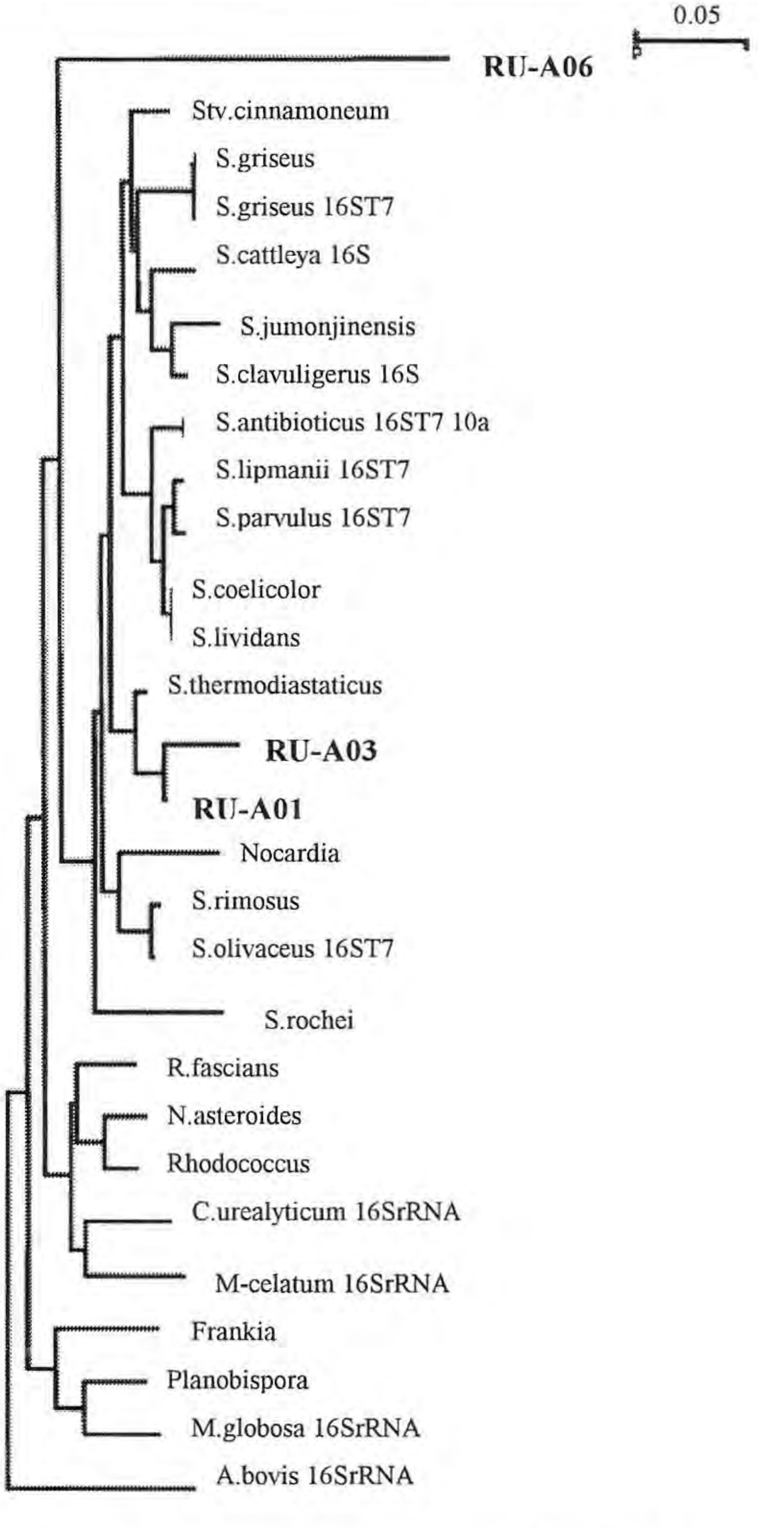


**Figure 3. 8** **Phylogenetic tree showing relationship of sequence from the thermophilic isolates in reference to other gram-positive bacteria. Analysis is based on a 300 base pair amplified sequence of aligned 16S rDNA data using primers F9 and 1541R.**

## 3.3 OPTIMUM GROWTH AND INITIAL PH CONDITION STUDIES OF THERMOPHILIC

Numerous papers have been published relating to the growth of Actinomycetes for the preparation of valuable proteins such has enzymes and antibiotics. The choice of a good medium is important to the success of the experimental program. Actinomycetes in general grow well in simple media. They are known to grow faster in media containing hydrozylates of protein, ground seed meals or the by-products of the cornstarch industry i.e. cornstreep liquor (Perlman, 1969). The presence of nitrogen based compounds results in faster cell production, more rapid utilization of carbohydrates and usually higher levels of enzymes in the cells (Perlman, 1969). Chemically defined media present distinct advantages from certain types of biochemical studies. The concentration of any one component or several components can be varied to determine its specific effect on cell growth and enzyme levels. These considerations allow the redesigning of a medium to obtain the greatest possible yield of product. Actinomycetes can be grown on the surface of liquid or on semisolid media in the form of agar, or in aerated flasks or vessels.

pH studies were carried out to correlate pH to biomass production and hence determine optimal growth at a specific pH. In this study the growth media used was both semi-solid agar and liquid broth that was aerated by rotation. Previous studies have shown that the production of oxidoreductase enzymes is optimal in an initially neutral to slightly alkaline medium. Actinomycetes are known to grow optimally at a slightly alkaline pH (pH 7.5-8.5; McCarthy and Williams, 1992). An investigation into the effects of pH on biomass growth was conducted within the pH range of 6.0 to 9.0. No definite trends could be derived from the data (Figures 3.9,3.10 and 3.11) but maximum final $OD_{600}$ readings (Table 3.6) showed that RU-A03 and RU-A06 grew best at pH 8.0 and isolate RU-A01 showed highest final $OD_{600}$ reading when grown at pH 6.5.

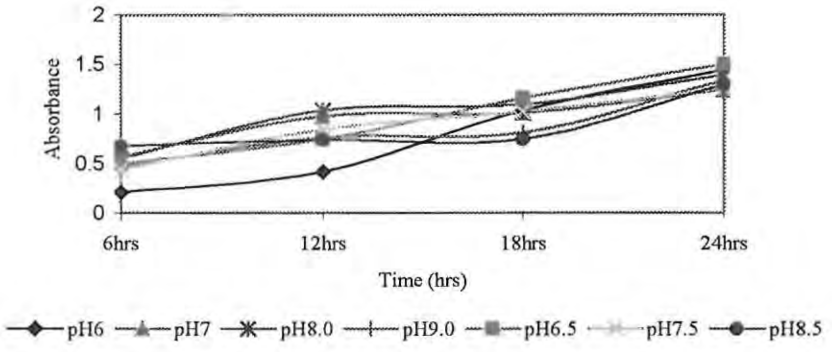


**Figure 3. 9** The effect of initial pH on growth of biomass for the isolate RU-A01 grown in 0.6% buffered yeast medium.

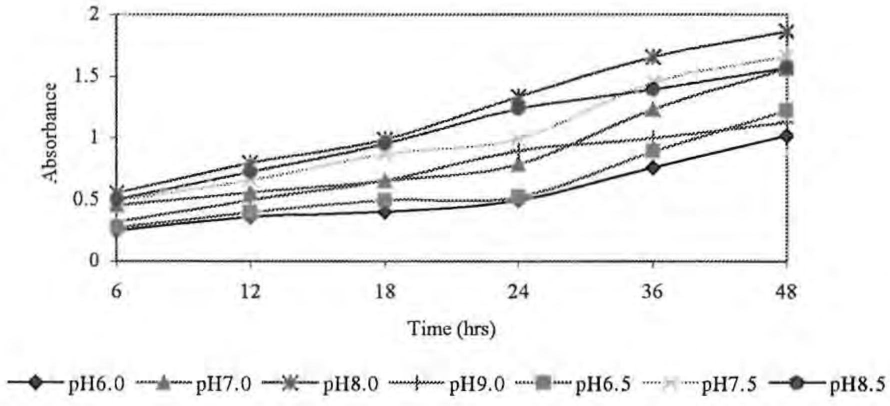


**Figure 3. 10** The effect of initial pH on growth of biomass for the isolate RU-A03 grown in 0.6% buffered yeast medium.

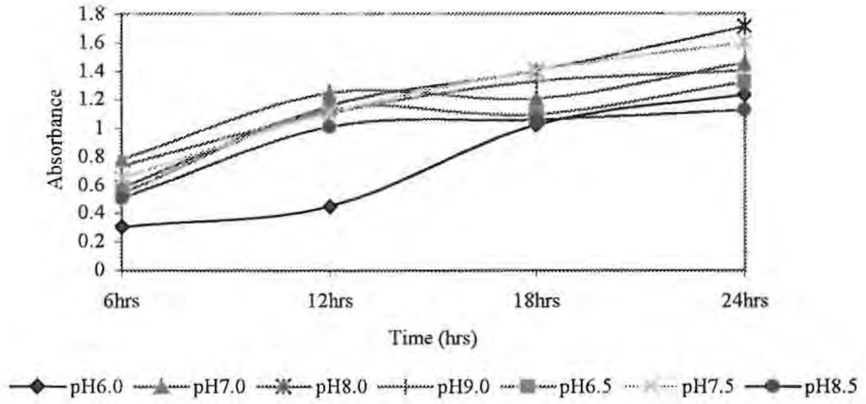


**Figure 3. 11** **The effect of initial pH on growth of biomass for the isolate RU-A06 grown in 0.6% buffered yeast medium.**

**Table 3. 6** **Final $OD_{600}$ readings of the three isolates at different pHs**

| | **pH** | | | | | | |
|---|---|---|---|---|---|---|---|
| **Isolate** | **6.0** | **6.5** | **7.0** | **7.5** | **8.0** | **8.5** | **9.0** |
| RU-A01 | 1.442 | **1.5** | 1.244 | 1.253 | 1.389 | 1.297 | 1.340 |
| RU-A03 | 1.023 | 1.225 | 1.566 | 1.662 | **1.867** | 1.568 | 1.132 |
| RU-A06 | 1.234 | 1.321 | 1.456 | 1.601 | **1.712** | 1.125 | 1.403 |

Optimum growth studies of the thermophilic *Streptomyces* isolates RU-A01, RU-A03 and RU-A06 were carried out. Although the growth medium of choice was M65 due to the rapid growth of the isolates in this media, the insoluble calcium carbonate interfered with optical density analysis. 0.6% buffered yeast medium was used instead for this set of experiments. Glass beads were incorporated into the growth medium to prevent the formation of large mycelium structures, which tend to adhere together preventing accurate optical density. A combination of high rotation speeds of 175rpm and above and the glass beads results in the formation of very fine spores enabling one to take optical density readings Maximum biomass production was measured within 36 hours for isolates RU-A01 and RU-A06 (Figures 3.12 and 3.14) at 50°C and within 48 hours for isolate RU-A03 (Figure 3.13). The decrease in growth rates and biomass after 48 hours would probably be due to the depletion of nutrients in the growth media. These growth rates are significantly faster than previously observed for mesophilic

*Streptomyces* species that take approximately 5-8 days to reach maximum growth in the laboratory using the same growth media and growth conditions (Mhlanga, 1998). Protein measurements were taken using the Bradford's protein determination assay (Bradford, 1976). As expected, biomass was directly proportional to protein concentration (Figures 3.13, 3.14 and 3.15).

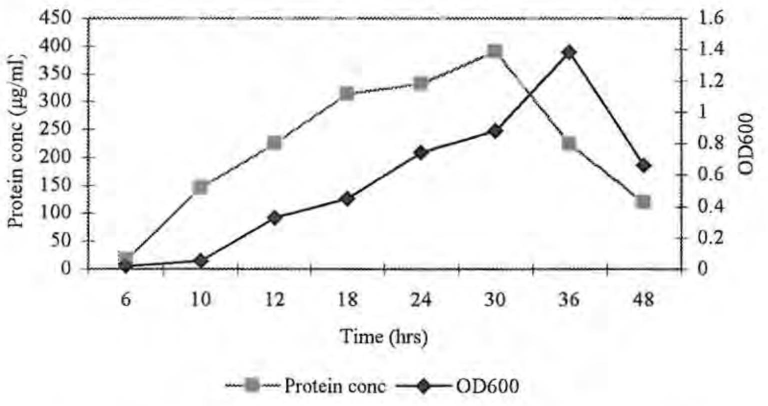


**Figure 3. 12** **$OD_{600}$ absorbance readings and protein concentrations of *Streptomyces* isolate RU-A01 grown in 0.6% buffered yeast growth medium over time**

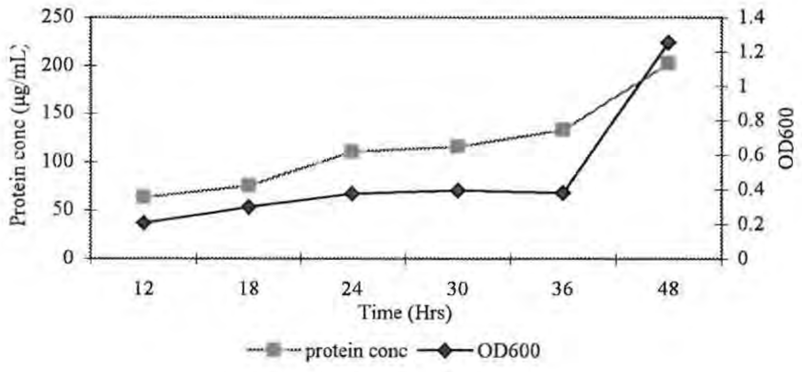


**Figure 3. 13** **$OD_{600}$ absorbance readings and protein concentrations of *Streptomyces* isolate RU-A03 grown in 0.6% buffered yeast growth medium over time.**

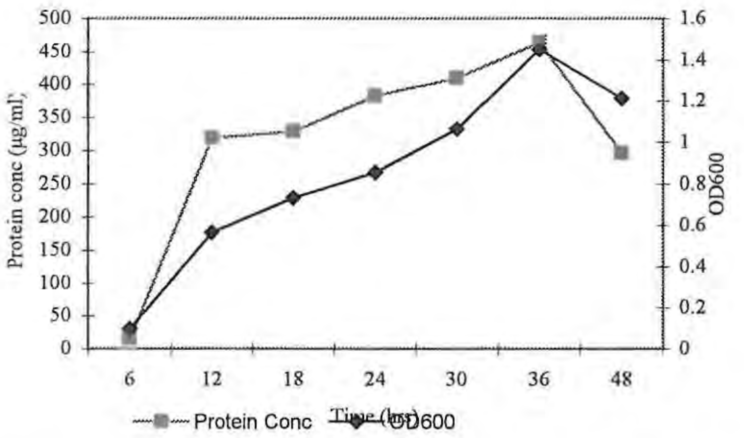


**Figure 3. 14** **$OD_{600}$ absorbance readings and protein concentrations of *Streptomyces* isolate RU-A06 grown in 0.6% buffered yeast growth medium over time.**

With optimal biomass growth occurring at pH 8.0 for isolates RU-A03 and RU-A06 and pH 6.5 for RU-A01 and maximum biomass/protein production occurring in the first 48 hours of growth, the next step involved the screening for oxidoreductase enzymes of these isolates as well as to determine at which stage maximum enzyme production occurs. Biomass growth was still high at lower pH's proving that these isolates are adaptable to grow at lower pHs.

## 3.4 SCREENING OF OXIDOREDUCTASE ENZYMES IN RU-A01, RU-A03 AND RU-A06.

The degradation of phenolic residues requires the concerted action of enzymes varying in substrate specificity. Researchers see it to be beneficial to screen a variety of microorganisms for their ability to produce oxidoreductase enzymes under a range of environmental conditions. In addition to screening established culture collections strains, new isolates can yield enzymes that are able to also degrade phenolic residues.

Peroxidase, polyphenol oxidase and laccases assays were used to determine the presence of these enzymes in the isolates. In addition, optimum enzyme activity was determined during the growth of these isolates in liquid cultures as well as protein determination of the isolates using the Bradfords protein determination assay (Bradfords, 1976). Unless otherwise noted, the spectrophotometric assays were performed at 50°C.

### 3.4.1 Substrate specificity of oxidoreductase enzymes isolated in RU-A01, RU-A03 and RU-A06

Several substrates were examined as potential substrates for peroxidase, laccase and polyphenol oxidase activity based on literature reviewed for this study. The aim was to determine substrate specificity for the oxidoreductase enzymes in RU-A01, RU-A03 and RU-A06 as well as enzyme activity and specific activity for comparative analysis of the various substrates used. Methods of determining enzyme activity include flourimetric analysis (e.g. flourographic detection of radioactive melanin), HPLC analysis, chemiluminescence and chronometric analysis.

The objective of this section was to evaluate the reliability and applicability of a range of available spectrophotometric assay procedures for the determination of Actinomycetes peroxidase and phenol oxidase activity. A total of 7 conventional peroxidase and phenol oxidase assay procedures were applied to intracellular and extracellular preparations from these isolates.

#### 3.4.1.1 Phenol oxidases.

In phenol oxidases, the copper-binding domains constitute the catalytic active site in enzymatic multi-copper oxidases i.e. Polyphenol oxidases and laccases (Solano *et al.*, 2001). Polyphenol oxidases and laccases are known to oxidise *o*- and *p*-quinols, aminophenols, pheylenediamines; anilines and benzenthiols, always couple to the reduction of $O_2$ and $H_2O$. The specificity to phenolic substrates varies with the group but simple diphenols e.g. hydroquinone and catechol are good substrates for most phenol oxidases. In terms of suitable substrates for standard spectrophotometer assays, diphenols are viewed as having low molar adsorption coefficients and the instability of the quinoic products hence limiting their use (Solanos *et al.*, 2001). Synthetic substrates with higher molar adsorption coefficients of the oxidized products have been used in a number of published studies such as *o*-dianisidine, ABTS and syringaldezine (Nganwa, 2000). However the oxidized products are not stable and reaction mixtures usually require a rather acidic pH which in this study needs to be noted due to the general optimum growth conditions at neutral to alkaline pHs. Methoxy substituted phenols such as guaiacol and L-DOPA have also been used to follow the enzymatic activity of phenol oxidases (Russell, 1999 and Boshoff, 2001).

Simpler and quicker methods have been developed in the form of PAGE activity gels, which enables the researcher to determine the number of isozymes present in the sample, which are commonly found in Actinomycetes species, and spectrophotometric assays, which quantifies the enzymes concerned and normally takes a few minutes to run. Based on the advantages of spectrophotometric assays, spectrophotometric assays were used to determine activity and substrate specificity of the oxidoreductatse enzymes in the three isolates.

Catechol, L-DOPA and ABTS were used to determine phenol oxidase activity. Catechol gave the highest activity as a substrate whereas L-DOPA gave the lowest activity (Table 3.7). In pervious studies, L-DOPA has been the substrate of choice when determining activity of tryrosinases and polyphenol oxidases in fungi such as the mushroom *Agarigus bisporus,* and *Neurospora crassa* (Russell, 1999; Boshoff, 2001 and Luke, 2001). However relatively low enzyme activity was displayed by RU-A01, RU-A03 and RU-A06 to L-DOPA. Catechol was hence used as a substrate to determine polyphenol oxidase activity. Catechol is light sensitive and darkens when exposed to light hence it was important to use freshly prepared catechol with each assay. ABTS was used to determine laccase activity.

### 3.4.1.2 Peroxidase.

The production of peroxidases, in particular extracellular peroxidases by Actinomycetes has been described (Mercer *et al.*, 1996, Ramachandra *et al.*, 1988, Winter *et al.*, 1991). Actinomycetes are a potentially rich source of peroxidases for introduction into a market that is substantial and almost totally dominated by Horseradish peroxidase

(HRP), which is a well characterised, highly reactive plant peroxidase (Dawson, 1998, Guerra *et al.*, 2000).

Techniques to determine peroxidase activity are based on the use of chromogenic substrates, particularly guaiacol and pyrogallol. Other substrates include phenol-containing compounds such as 2,7-diaminoflourene (DAF), *o*-dianisine, ABTS, ferulic acid and catchol. 2,4 Dichlorophenol is a common substrate used to detect peroxidases in the *Streptomyces* group (Mercer *et al.*, 1996, Ramachandra *et al.*, 1987, 1988). Normally analysis of substrate specificity of novel peroxidases is usually carried out under the optimal conditions determined for horseradish peroxidase (HRP). However the optimal conditions for catalysis by different peroxidases are not identical and hence react differently to different substrates. Hydrogen peroxide also affects enzyme determination. The role of hydrogen peroxide is an oxidative substrate for peroxidases and produces compound I from a resting form of peroxidase (Equation 1).

**Equation 1** $E + H_2O_2 \rightarrow \text{compound } 1 + H_2O_2$

Hydrogen peroxide is important to allow for enzyme kinetic analysis. Optimal concentrations of hydrogen peroxide and hydrogen donors as well as the efficiency of the two depend strongly on the chemical nature of the substrates.

The data in Tables 3.7 and 3.8 show that the peroxide-dependant oxidation of 2,4-DCP is the assay procedure that gives highest activity values. This substrate has been widely used to determine peroxidase activity amongst Actinomycetes (Manguson and Crawford, 1992; Mliki and Zimmermann, 1992; Ramachandra, 1987; Ramachandra and Crawford, 1988; Antonopoulos *et al.*, 2001). This assay is not specific however and can be termed as a general peroxidase assay as it does not appear to distinguish between lignin peroxidase and non-heam based peroxidases as shown by the specific activity differences between 2,4-DCP and veratryl alcohol as substrates (Table 3.8).

Previous assumptions that positive peroxidase activities in Actinomycetes are due to lignin peroxidase lead to the use of a specific substrate for lignin peroxidase activity determination. The peroxide-dependant oxidation of veratryl alcohol has been frequently used as the procedure for lignin peroxidase determination in white-rot fungi, after Tien and Kirk (1984). When applied in this study, activity was detected in all three isolates but was most pronounced in RU-A01 in the extracellular protein fraction (Table 3.7 and 3.8), giving a specific activity of 65 umols/mg/min. Previous studies have shown that few of the peroxidases produced by *Actinomycetes* species are due to lignin peroxidases except for *Streptomyces viridosporus* and *Streptomyces albus*, hence these results are of significant findings. Most peroxidases produced by *Actinomycetes* species are often non-heam peroxidases, in particular the intracellular peroxidases and are commonly belong to the haloperoxidases and catalase-peroxidase groups.

In general is it thought that extracellular peroxidases would be expected to have improved stability over their intracellular counterparts, particularly those from thermophiles and thus have potential for applications in biotechnology. In addition it is thought that the role of intracellular peroxidases are involved in cellular processes and that extracellular peroxidases are involved in the degradation of complex organic compounds in the *Actinomycetes* environment.

The present results involving guaiacol as a peroxidase substrate corroborate with previous findings (Mhlanga, 1998), which indicate that guaiacol was found to be unsuitable for the determination of *Actinomycetes* peroxidases. This was also found to be true by Mercer and co-workers (1996) who found guaiacol inadequate for the assessment of peroxidases in S*treptomyces* species.

No activity was detected from the intra- and extracellular crude samples of RU-A01, RU-A03 and RU-A06 towards *o*-dianisidine. This made *o*-dianisidine most unsuitable for quantitative determination of peroxidases in RU-A01, RU-A03 and RU-A06, however in a previous study conducted by Mercer *et al.*, 1996, *o*-dianisidine gave the second highest peroxidase activity out of 10 substrates used to determine peroxidase activity in *Streptomyces thermoviolaceus* and *Streptomyces* strain EC22. Specific activities however were not quoted in that study.

For polyphenol oxidases, L-3, 4-dihydroxyphenylalanine (L-DOPA) displayed relatively low reactivity towards intra-and extracellular proteins of isolates RU-AO1, RU-A03 and RU-A06 (Table 3.7). Previous studies using L-DOPA has found the reproducibility of L-DOPA for the determination of polyphenol oxidases relatively poor due to its extreme light sensitivity as well as its interference due to nonspecific oxidation of the substrate (Winter *et al.*, 1991). Catechol proved to be a better substrate for polyphenol oxidase determination as it was more stable than L-DOPA and the results were reproducible and less affected by light exposure. However, the results in Table 3.8 indicate the presence of a higher proportion of peroxide-dependant enzymes i.e. peroxidases than polyphenol oxidases.

No laccase was detected using 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as substrate. According to literature very few bacteria produce laccases and screening of laccase producing microbes has been focused mainly on white-rot fungi such as *Trametes versicolor, Trametes pubescens, Rhus vernicifera* and *Neurospora crassa* (S.G Burton, personal communication). To date there are no Actinomycetes that are known to produce laccases.

Peroxidases and phenol oxidases are often termed as ubiquitous and two main factors limit their exploitation.

1). The levels of enzyme production and
2.). The instability of activity under conditions such as high temperatures and pH, conditions generally encountered in industrial processes.

The evaluation of these Actinomycetes isolates has been aimed at addressing these factors hence the isolation of thermophilic Actinomycetes and also based on the fact that Actinomycetes thrive better at neutral to alkaline pH's. These two factors would hence have an advantage in terms of applicability in industrial processes.

**Table 3. 7** **Peroxidase and phenol oxidase reactivity towards different substrate as determined by enzyme activity from the isolates RU-A01, RU-A03 and RU-A06.**

| SUBSTRATE | ENZYME | ACTIVITY mols/mL/min | | | | | |
|---|---|---|---|---|---|---|---|
| | | RU-A01 | | RU-A03 | | RU-A06 | |
| | | Intracellular | Extracellular | Intracellular | Extracellular | Intracellular | Extracellular |
| 2,4 DCP + $H_2O_2$ | Peroxidase | 2.8000 | 3.5000 | 1.3600 | 0.9700 | 6.3000 | 4.6000 |
| Guaiacol + $H_2O_2$ | Peroxidase | 0.0000 | 0.0000 | 0.0014 | 0.0000 | 0.0000 | 0.0014 |
| o-dianisidine + $H_2O_2$ | Peroxidase | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| Veratryl alcohol + $H_2O_2$ | Lignin peroxidase | 0.4200 | 6.5000 | 0.4200 | 0.0000 | 1.2000 | 0.6000 |
| Catechol | Polyphenol oxidase | 0.6500 | 0.1300 | 0.8000 | 0.6000 | 0.3700 | 0.2000 |
| L-DOPA | Polyphenol oxidase | 0.0010 | 0.0050 | 0.0020 | 0.0000 | 0.0180 | 0.0120 |
| ABTS | Laccase | 0.0000 | 0.0000 | 0.0000 | 0.00000 | 0.0000 | 0.0000 |

**Table 3. 8** **Specific activity of intracellular, and extracellular protein from RU-A01, RU-A03 and RU-A06 using 7 different substrates to determine peroxidase and phenol oxidase activity.**

| SUBSTRATE | ENZYME | SPECIFIC ACTIVITY mols/min/mg | | | | | |
|---|---|---|---|---|---|---|---|
| | | RU-A01 | | RU-A03 | | RU-A06 | |
| | | intracelluar | Extracellular | intracelluar | Extracellular | intracelluar | Extracellular |
| 2,4 DCP + $H_2O_2$ | Peroxidase | 23.300 | 35.000 | 6.8000 | 9.700 | 21.000 | 46.000 |
| Guaiacol + $H_2O_2$ | Peroxidase | 0.005 | 0.0000 | 0.0070 | 0.0000 | 0.0020 | 0.014 |
| o-dianisidine + $H_2O_2$ | Peroxidase | 0.000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| Veratryl alcohol + $H_2O_2$ | Lignin peroxidase | 3.5000 | 65.0000 | 2.1000 | 0.0000 | 4.0000 | 6.0000 |
| Catechol | Polyphenol oxidase | 5.4000 | 1.3000 | 4.0000 | 6.0000 | 1.2000 | 2.0000 |
| L-DOPA | Polyphenol oxidase | 0.0050 | 0.0500 | 0.0100 | 0.0000 | 0.0600 | 0.1200 |
| ABTS | laccase | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.000 |

The specificity towards different substrates depends on several factors. Among them the suitable reduction potential of the active species with respect to those of the substrates, which in turn is determined by covalent and electrostatic factors. Structural properties of the protein are also relevant in defining the specificity towards different substrates.

## 3.5 Comparability between mesophillic and thermophillic Actinomycetes.

When thermophilic organisms were first considered for use in biotechnology, certain advantages were expected such as high reaction rates and insensitivity of processes to contamination. There has been an increasing interest in the possibility of deriving a wide variety of by-products from thermophiles. In addition to their high thermostability they also promise to have greater tolerance to organic solvents and a longer 'shelf life'. The possibility of recovering volatile products directly from a culture provides the opportunity to develop simplified bioprocesses (Sonnleitner and Fiecther, 1983), in comparison to that of mesophilic microbes.

The term 'thermophilic' is rather ill defined. Table 3.9 gives a brief outline of the minimal, optimal and maximal growth temperature ranges that mesophilic, thermo tolerant and thermophilic microbes can grow. Many microbes that can be cultivated at temperatures a little over 37°C, and are often named 'thermophilic' because they are often used for the production of thermostable enzymes. Many thermophilic organisms are known today, among then bacteria, algae and fungi.

**Table 3. 9** **Current nomenclature of 'thermophily' (Sonnleitner and Fiecther, 1983)**

| TECHNICAL TERM | GROWTH TEMPERATURE (°C) | | |
|---|---|---|---|
| | **Minimal** | **Optimal** | **Maximal** |
| Mesophilic | <20 | 20-30 | 40 |
| Thermo-tolerant | 15-25 | <50 | 50 |
| Thermophilic | 30-45 | 50-65 | 60-75 |
| Extremely thermophilic | >40 | >65 | >75 |

Based on this current nomenclature on the different definitions, RU-A01, RU-A03 and RU-A06 all lie at present under the term thermophilic, however growth studies done in this study did not exceed 50°C. Thermophiles are expected to be and /or to produce qualitatively new biocatalysts. Since they can grow at higher temperatures their cellular constituents such as enzymes, nucleic acids and lipids must function at these same high temperatures.

At present the most promising thermophilic biocatalysts are thermophilic enzymes because of their enhanced stability but also because they are more resistant to denaturing agents and more tolerant to higher solute concentrations. Whether these features are linked to thermo-tolarance is still not clear. The fact remains that researchers have a better chance of finding more thermostable enzymes in thermophiles or thermotolerant microbes. Besides the rapid growth rates displayed by RU-A01, RU-A-3 and RU-A06 at 50°C in comparison to other documented Actinomycetes with the majority of them being mesophilic, a comparison of peroxidase and polyphenol oxidase activities was necessary. The advantages of using thermophilic microbes for industrial processes have been discussed in this study and the advantages appear to outweigh the use of mesophilic microbes due to the high temperatures arising from exothermic reactions in bioremediation and enzyme catalysis, hence making it necessary to use microbes and enzymes that are thermotolerant in bioprocessing industry. However researchers cannot use thermophiles simply based on these factors, enzyme quantity and quality needs to be considered and compared with their mesophilic counterparts.

Peroxidase and polyphenol oxidase activity was compared between the thermophilic *Streptomyces* isolates and of mesophilic *Streptomyces* from a previous study (Mhlanga, 1998). Polyphenol oxidase activity between the two groups was comparable and peroxidase activity was significantly higher for the thermophiles than for the mesophiles (Table 3.10). RU-A01, RU-A03 and RU-A06 also have an advantage over the mesophilic *Streptomyces* strains in that they produce lignin peroxidases also found amongst some of the white rot fungi and known to play a role in lignin solubilization (Ball *et al.*, 1989). Peroxidase and polyphenol oxidase extracts from the thermophilic isolates reacted the same as the peroxidases and polyphenol oxidases from the mesophilic *Streptomyces* sp towards the various substrates.

**Table 3. 10** **Comparisons between mesophillic and thermophillic *streptomyces* polyphenol oxidase and peroxidase activity**[1]

| | **ACTIVITY (μmols/mL/min)** | | |
|---|---|---|---|
| **Species** | **Polyphenol oxidase**[2] | **Lignin peroxidase** | **Peroxidase**[3] |
| *Streptomyces antibioticus* | 0.69 | 0 | 0.51 |
| *Streptomyces hygroscopicus* | 0.85 | 0 | 0.62 |
| *Streptomyces* sp. strain 7 | 1.32 | 0 | 0.79 |
| RU-A01 | 0.60 | 6.5 | 3.50 |
| RU-A03 | 0.80 | 0.42 | 1.36 |
| RU-A06 | 0.37 | 1.2 | 5.28 |

[1] Optimum activity values were selected for each species
[2] Catechol was used as substrate for polyphenol oxidase activity
[3] 2,4 DCP was used as substrate for peroxidase activity.

At present the next step would be to do thermodynamic/kinetic studies of these enzymes from the three isolates to determine which category they actually fall under, their optimal reaction temperatures and stability lifespan (see future work, chapter 4).

## 3.6 ENZYME ACTIVITY AND SPECIFIC ENZYME ACTIVITY IN CORRELATION TO MICROBIAL CELL GROWTH IN RU-A01, RU-A03 AND RU-A06.

In *Streptomyces* and other genera of Actinomycetes, lignin degradation occurs during primary growth phase or log phase and the production of oxidoreductase activity is presumed to be a result of primary metabolic activity (McCarthy, 1987). Previous studies have shown that *Streptomyces viridosporus* peroxidase is associated with the growth phase. This is in contrast to *Phanerochaetae chrysosporium* and other white-rot fungi, in which both lignin degradation and lignin peroxidase are secondary metabolic events, which follow nutrient depletion.

Hernandez-Perez and co-workers (1999) found that lignin degrading enzymes produced by *Streptomyces viridosporus* were produced during the primary stage of growth, reaching a peak during this stage of growth and remaining stable and constant during the stationary phase of growth. These are valuable arguments for the use of Actinomycetes in a whole cell phenolic degradation strategy. Further studies also indicated that the use of growth media seems to be suitable for biomass growth applied to oxidoreductase enzyme production.

The aim of this work was to determine if the three isolates comply with the general view that oxidoreductase enzymes are produced at the primary stage of growth.

Enzyme activity in correlation to biomass growth studies were carried out on RU-A01, RU-A03 and RU-A06 to determine whether these novel isolates followed the general pattern of oxidoreductase enzyme production amongst the Actinomycetes.

Intra-and extracellular protein samples were harvested at 4 hourly intervals. Protein determinations using the Bradford protein determination assay (Bradford, 1976) was carried out for each sample as well as enzyme activities using catechol as substrate for polyphenol oxidase, 2,4 DCP as substrate for peroxidase, and veratryl alcohol as substrate for lignin peroxidase. Specific activities were also calculated at different harvest times.

Intracellular activity was determined by French pressing and spinning down the debris by centrifugation, the resultant supernatant was termed as intracellular crude protein extract. Culture supernatant was produced by growing the isolates in a buffered 0.6% yeast medium. Under these conditions, sufficient activity was produced to enable its detection by 2,4 DCP and catechol without first concentrating the supernatant samples.

### 3.6.1 Enzyme activity and specific enzyme activity in correlation to microbial cell growth in RU-A01.

#### 3.6.1.1 Polyphenol oxidase activity

Intracellular polyphenol oxidase activity in RU-A01 generally correlated with cell biomass growth, increasing proportionally to biomass growth up to time 30 hours of biomass growth. Activity peaked at time 36 hours of biomass growth and remained relatively constant at 0.62 μmols/mL/min at time 48 hours (Figure 3.15). Protein concentration peaked at time 30 hours (0.406 mg/ml) and then declined thereafter reaching a protein concentration of 0.296 mg/ml at time 48 hours. Specific polyphenol oxidase activity (Table 3.12) peaked at time 30 hours at 4.5 μmols/mg/min and then declined at time 36 hours to 2.3 μmols/mg/min and then peaked again at time 48 hours at 5.3 μmols/mg/min. Extracellular polyphenol oxidase activity first appeared at time 36 hours with an activity of 0.52 µmols/mL/min (Table 3.11) and a specific activity of 7.4 µmols /mg/min. Specific extracellular activity increased at time 48 hours to 12.0 µmols /mg/min (Table 3.12). Specific activity results show that polyphenol oxidase activity was highest in the extracellular protein samples than the intracellular protein samples. High extracellular specific activity may be due to either an increase in the quantity of the specific isozyme or/and an increase in the number of extracellular isozymes.

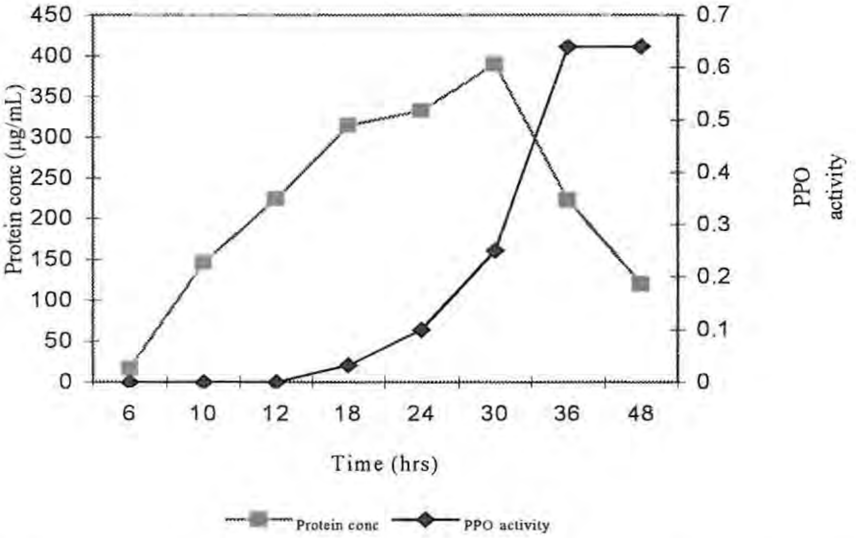


**Figure 3. 15** **Production of intracellular polyphenol oxidase activity versus intracellular protein concentration by RU-A01. The cultures were grown in 250ml flasks containing 100ml medium. After inoculation, cultures were incubated shaking (150 r.p.m) at 50°C.**

### 3.6.1.2 Peroxidase activity of RU-A01

Intracellular peroxidase activity was produced steadily and directly proportional to protein concentration up to time 30 hours (Figure 3.16). Protein concentration then declined at time 36 hours (0.120 mg/ml), however peroxidase activity peaked at this time (2.9 µmols/mL/min). Peroxidase activity then declined at time 48 hours to 2 µmols /mL/min). Extracellular activity peaked at 48 hours (1.2 µmols/mL/min). No trends could be followed in terms of specific intracellular peroxidase activity for the first 30 hours of biomass growth. At time 36 hours however peroxidase specific activity increased significantly from 0.64 µmols /mg/min at time 30 hours to 12.8 µmols

/mg/min at time 36 hours and then peaking at time 15.2 μmols/mg/min at time 48 hours (Table 3.12). Specific extracellular peroxidase activity first appeared at time 36 hours (19.3 μmols/mg/min) and then declined to 11.0 μmols/mg/min at time 48 hours.

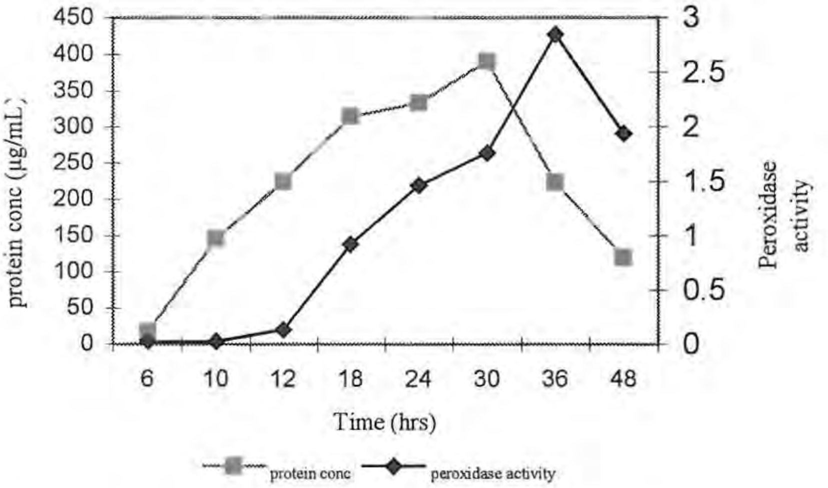


**Figure 3. 16** **Production of intracellular peroxidase activity by RU-A01 using 2,4 DCP as substrate versus intracellular protein concentration. The cultures were grown in 250ml flasks with 100mls medium, shaking at 150 r.p.m at 50°C. Cells were harvested and French pressed.**

### 3.6.1.2.3 Lignin peroxidase

Veratryl alcohol was used as a substrate for lignin peroxidase enzyme activity. Intracellular lignin peroxidase activity peaked at time 36 hours (Figure 3.17) and then sharply declined thereafter. There was no distinct pattern of specific lignin peroxidase activity between 6-30 hours. There was sharp increase however in activity from time 30-36 hours, peaking at time 48 hours at 15.2 μmols/mg/min. Extracellular activity peaked at time 36 hours (0.59μmols/mL/min). Extracellular specific activity first appeared at time 36 hours at a peak activity of 21.9 μmols/mg/min and declining sharply at 48 hours to 5.0 μmols/mg/min of lignin peroxidase (Table 3.12). Like specific peroxidase activity, extracellular lignin peroxidase specific activity was highest in the extracellular protein samples then the intracellular protein samples.

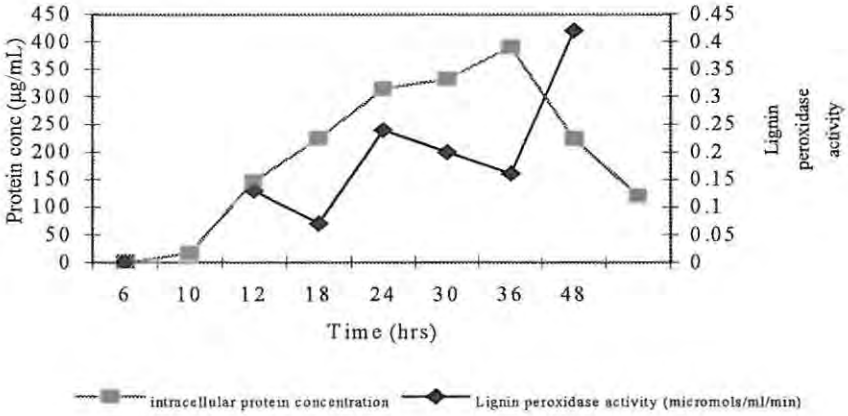


**Figure 3. 17** **Production of intracellular lignin peroxidase activity by RU-A01 versus intracellular protein concentration. The cultures were grown in 250ml flaks with 100mls medium, shaking at 150 r.p.m at 50°C. Cells were harvested and French pressed.**

## 3.6.2 Enzyme activity and specific enzyme activity in correlation to microbial cell growth in RU-A03.

### 3.6.2.1 Polyphenol oxidase activity

In terms of correlation between biomass growth and polyphenol oxidase activity, there was a distinct and direct correlation to polyphenol activity to biomass growth, with polyphenol oxidase activity peaking at time 48 hours at an activity 0.8 µmols /mL/min (Figure 3.18). Extracellular activity peaked at time 48 hours (0.6µmols/mL/min) Table 3.11). Polyphenol oxidase extracelluar specific activity first appeared at time 48 hours with a specific activity of 12.98 µmols /mg/min, which was the highest, recorded polyphenol oxidase activity recorded for isolate RU-A03 (Table 3.13).

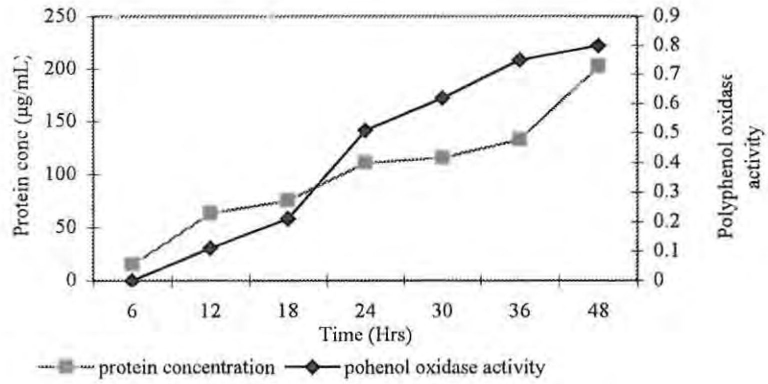


**Figure 3. 18** **Production of intracellular polyphenol oxidase activity versus intracellular protein concentration by RU-A03. The cultures were grown in 250ml flasks containing 100ml medium. After inoculation, cultures were incubated shaking (150 r.p.m) at 50°C**

### 3.6.2.2 Peroxidase activity of RU-A03

Intracellular peroxidase activity increased gradually with time peaking at time 48 hours (5.1 µmols /mL/min; Figure 3.19). Extracellular activity was first detected at time 48 hours (0.97µmols/mL/min). In terms of specific activity (Table 3.13), intracellular peroxidase activity increased and leveled off between time 18 and 30 hours and then peaked at time 36 hours at a concentration of 38.6 µmols/mg/min and the declining at 48 hours to a concentration of 26.2 µmols /mg/min. Extracellular peroxidase specific activity was lower to the intracellular peak activity at 20.9 µmols/mg/min at time 48 hours.

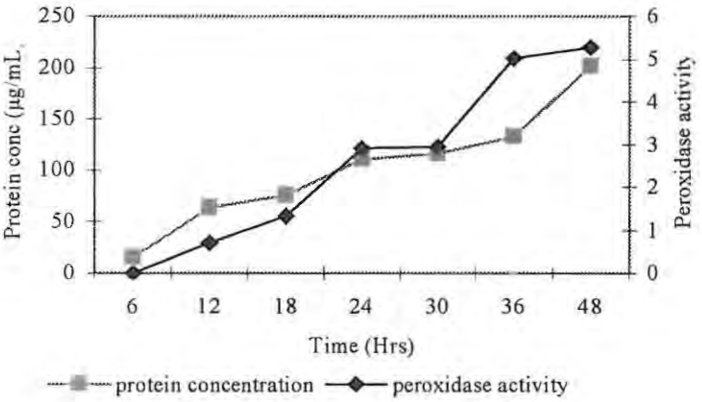


**Figure 3. 19** **Production of intracellular peroxidase activity by RU-A03 using 2,4 DCP as substrate versus intracellular protein concentration. The cultures were grown in 250ml flaks with 100mls medium, shaking at 150 r.p.m at 50°C. Cells were harvested and French pressed.**

### 3.6.2.3 Lignin peroxidase

Like peroxidase activity, specific activity (Table 3.13) levelled off between time 24 hours and 48 hours, with peak intracellular specific activity at time 24 hours (3.9µmols /mg/min). Extracellular activity was detected at 48 hours (0.59µmols/mL/min) Extracelluar activity peaked at time 48 hours (0.59 µmols/mL/min; Table 3.11). Extracellular specific lignin peroxidase activity was highest at a concentration of 9.1µmols/mg/min. There was also a direct correlation of lignin peroxidase activity to protein concentration (Figure 3.20).

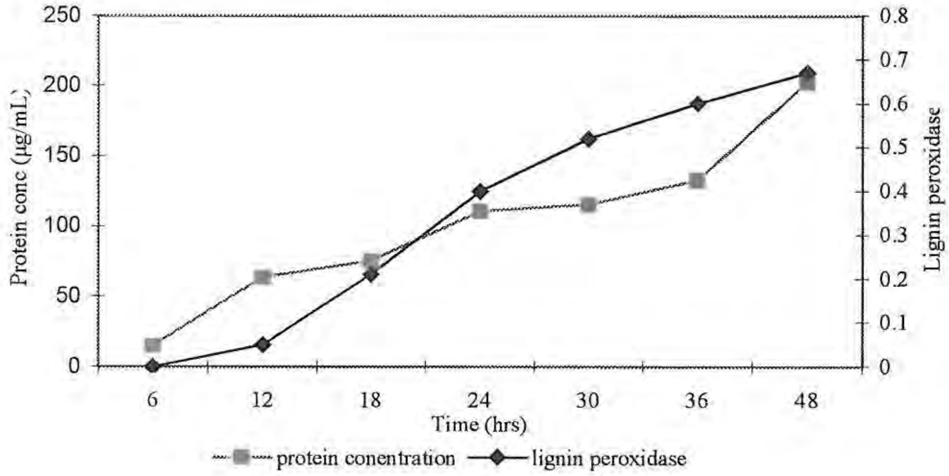


**Figure 3. 20** **Production of intracellular lignin peroxidase activity by RU-A03 versus intracellular protein concentration. The cultures were grown in 250ml flasks with 100mls medium, shaking at 150 r.p.m at 50°C. Cells were harvested and French pressed.**

## 3.6.3 Enzyme activity and specific enzyme activity in correlation to microbial cell growth in RU-A06.

### 3.6.3.1 Polyphenol oxidase

Polyphenol oxidase activity peaked at time 18 hours where it remained stable at that activity at time 24 hours, declining at time 30 hours and then increasing again at time 36 hours and then declined at time 48 hours (0.25 µmols /mL/min, Figure 3.21). Extracellular activity peaked at 48 hours (0.98 µmols/mL/min; Table 3.11). In terms of specific activity (Table 3.14), polyphenol oxidase specific activity showed no distinct pattern. Specific activity peaked at time 18 hours (1.1µmols /mg/min). Extracellular specific polyphenol oxidase activity was higher in comparison to intracellular samples, with a peak activity of 70.2 µmols /mg/min at time 36 hours (Table 3.14).

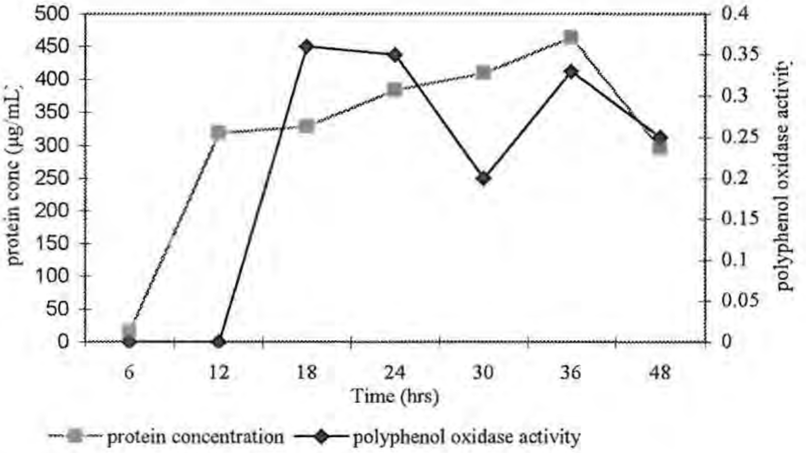


**Figure 3. 21** **Production of intracellular polyphenol oxidase activity in RU-A06 versus protein concentration over time.**

### 3.6.3.2 Peroxidase activity

Intracellular peroxidase activity peaked at time 36 hours (6.1 µmols /mL/min, Figure 3.22) and decreased at time 48 hours. In terms of specific activities, 15.9 µmols/mg/min was observed at time 12 hours, declined to 13.1 µmols /mg/min at time 18 hours, a further decline to 5.3 µmols /mg/min at time 24 hours, increasing gradually at time 30 hours and peaking at 18.8 µmols /mg/min at time 48 hours. The highest activity detected was at 36 hours from the extracellular protein samples at 26.4 µmols/mg/min, declining to 10.3 µmols /mg/min at time 48 hours. Extracellular peroxidase activity first appeared at time 36 hours (1.7 µmols/mL/min; Table 3.11).

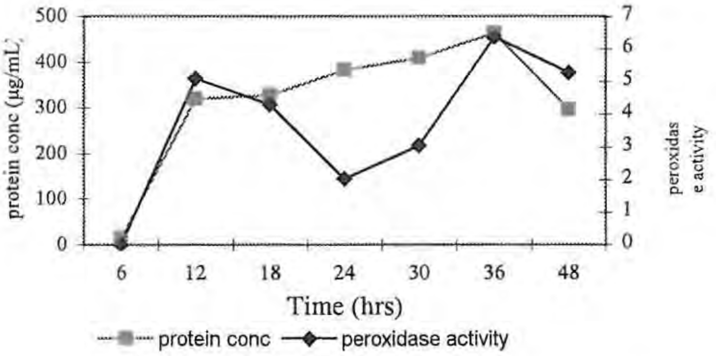


Figure 3. 22 **Production of intracellular peroxidase activity by RU-A06 using 2,4 DCP as substrate versus intracellular protein concentration. The cultures were grown in 250ml flaks with 100mls medium, shaking at 150 r.p.m at 50°C. Cells were harvested and French pressed.**

### 3.6.3.3 Lignin peroxidase

Lignin peroxidase activity peaked at time 24 hours whereby it then declined after that time (Figure 3.23). No activity at all was detected at time 48 hours. Intracellular specific activity peaked at time 24 hours at 3.0 µmols /mg/min. Extracellular activity was detected at time 48hours (0.59µmols/mL/min). No extracellular activity was detected at time 36 hours however the highest specific activity was detected at time 48 hours at a concentration of 24.4 µmols /mg/min (Table 3.14)

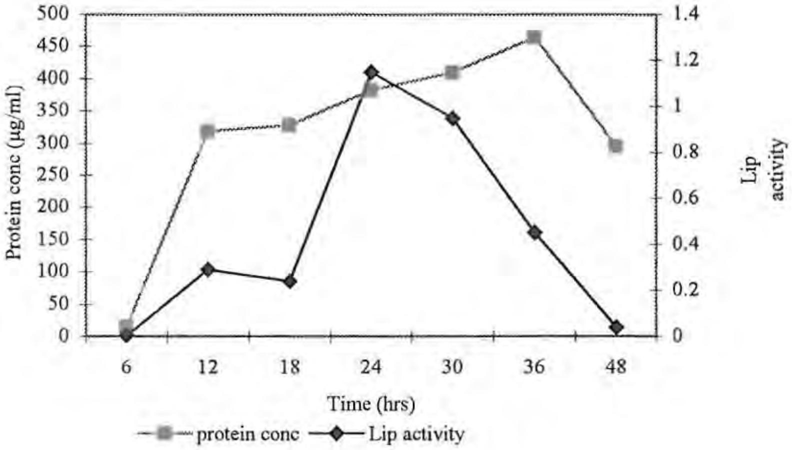


Figure 3. 23 **Production of intracellular lignin peroxidase activity by RU-A06 versus intracellular protein concentration. The cultures were grown in 250ml flaks with 100mls medium, shaking at 150 r.p.m at 50°C. Cells were harvested and French pressed.**

**Table 3. 11** **Extracellular lignin peroxidase, polyphenol oxidase and peroxidase activity of the 3 isolates after 36 and 48 hours of biomass growth.**

| THERMOPHILE AND TIME POINT SAMPLE | EXTRACELLULAR ENZYME ACTIVITY µmol/min/mL | | |
|---|---|---|---|
| | **Lignin peroxidase** | **Polyphenol oxidase** | **Peroxidase** |
| RU-A01 36 hrs | 0.59 | 0.52 | 0.2 |
| RU-A01 48 hrs | 0.5 | 1.1 | 1.2 |
| RU-A03 36 hrs | 0 | 0 | 0 |
| RU-A03 48 hrs | 0.42 | 0.6 | 0.97 |
| RU-A06 36 hrs | 0 | 0.64 | 1.7 |
| RU-A06 48 hrs | 0.59 | 0.98 | 4.6 |

In the course of screening the known Actinomycetes species for peroxidase and polyphenol oxidase activity, no activity was displayed by any of the five isolates. The use of these thermophiles was hence discontinued and subsequent studies were focused on the compost *Streptomyces* isolates. The five Actinomycetes species selected from an international culture collection were selected based on their ability to produce oxidoreductase enzymes. Their lack of activity during this study could be due to problems with the particular strains used in this study.

**Table 3. 12** **Specific activity of RU-A01 in relation to time and biomass growth**

| TIME (HRS) | SPECIFIC ACTIVITY (µmols/mg/min) | | |
|---|---|---|---|
| | **Lignin peroxidase** | **Peroxidase** | **Polyphenol oxidase** |
| **Intracellular enzyme** | | | |
| 6 | 0 | 1.76 | 0 |
| 12 | 0.58 | 0.62 | 0 |
| 18 | 0.22 | 2.92 | 0.101 |
| 24 | 0.72 | 4.38 | 0.3 |
| 30 | 0.5 | 0.64 | 4.5 |
| 36 | 0.7 | 12.8 | 2.8 |
| 48 | 2.3 | 15.2 | 5.3 |
| **Extracellular enzyme** | | | |
| 36 | 21.9 | 19.3 | 7.4 |
| 48 | 5.0 | 11.0 | 12.0 |

**Table 3. 13** **Specific activity of RU-A03 in relation to time and biomass growth**

| TIME (HRS) | SPECIFIC ACTIVITY (μmols/mg/min) | | |
|---|---|---|---|
| | Lignin peroxidase | Peroxidase | Polyphenol oxidase |
| **Intracellular enzyme** | | | |
| 6 | 0 | 0 | 0 |
| 12 | 0.78 | 14.1 | 2.3 |
| 18 | 2.8 | 22.6 | 0.31 |
| 24 | 3.9 | 28.8 | 4.9 |
| 30 | 3.4 | 28.4 | 5.7 |
| 36 | 3.4 | 38.6 | 5.6 |
| 48 | 3.1 | 26.2 | 4.0 |
| **Extracellular enzyme** | | | |
| 36 | 0 | 0 | 0 |
| 48 | 4.5 | 6.5 | 10.5 |

**Table 3. 14** **Specific activity of RU-A06 in relation to time and biomass growth**

| TIME (HRS) | SPECIFIC ACTIVITY (μmols/mg/min) | | |
|---|---|---|---|
| | Lignin peroxidase | Peroxidase | Polyphenol oxidase |
| **Intracellular** | | | |
| 6 | 0.2 | 0 | 0 |
| 12 | 0.91 | 15.99 | 0 |
| 18 | 0.73 | 13.1 | 1.1 |
| 24 | 3.0 | 5.3 | 0.78 |
| 30 | 2.3 | 7.5 | 0.49 |
| 36 | 0.97 | 13.7 | 0.7 |
| 48 | 0.14 | 18.8 | 0 |
| **Extracellular** | | | |
| 36 | 0 | 26.4 | 70.2 |
| 48 | 24.4 | 10.3 | 48.6 |

It has been proposed that lignin peroxidase is defined by the ability to oxidize nonphenolic substrates such as veratryl alcohol (Gold *et al*, 1993). Previous studies using Actinomycetes and their enzymes as agents of phenolic degradation has led to inconclusive results. This study however demonstrates the ability to bioconvert phenolic residues in wastes. Ramachandra *et al* (1988) and Winter *et al* (1999) reported that peroxidase activity though common amongst Actinomycetes, conceded that only a few were active against the substrate 2,4-DCP. This study reports that reactivity of peroxidases isolated from the 3 isolates are reactive to both 2,4-DCP and veratryl alcohol.

The production of extracellular peroxidases is a common feature amongst Actinomycetes but few of these Actinomycetes are actually thermophilic. Without further concentration of the supernatants, peroxidase and polyphenol oxidase was detected using 2,4-DCP, veratryl alcohol and catechol as substrates. The incubation time required for maximal peroxidase and polyphenol oxidase activity to be obtained was 36/48 hours for RU-A01 and RU-A03 and 48 hours for RU-A06, this period falls within the range frequently reported for Actinomycetes (48h to 72 h), including *Streptomyces thermoviolaceus* (Iqbal *et al.*, 1994), *Thermomonospora fusca* BD25 (Rob *et al.*, 1996, Tuncer *et al.*, 1999 and Zerbini *et al.*, 1999), *Streptomyces avermitilis* UAH30 (Rob *et al.*, 1997) and *Streptomyces viridosporus* T7A (Zerbini *et al.*, 1999).

The assessment of the activity of peroxidase against the chlorophenol substrate 2,4-DCP showed that the enzyme was able to oxidize successfully 2,4-DCP. The catalysis for the reaction proceeded with the formation of a pink/red colour due to the coupling with the colour-forming reagent 4-aminoantipyrine. The oxidation would be in accordance to Spiker *et al* (1992), that the oxidation of phenol containing one or no carbons in the para- position results in coupling with the 4-aminoantipyrine to form a pink/red colour.

More staining substrates for peroxidases and polyphenol oxidases should be investigated.

### 3.7 Enzyme activity comparisons between freeze dried extracts, extracellular enzyme and French pressed extracts from thermophilic isolates RU-A01, RU-A03 and RU-A06.

One of the major requirements for the use of enzymes in bioprocesses is that they remain stable when stored. Enzymes are temperature sensitive and need to be kept at temperatures of about -20°C until required. If enzymes are unstable, they cannot be useful for processes as they denature and are rendered inactive. Lyophilization is a freeze-drying process whereby water is removed leaving a concentrated powder that is stable even at temperatures above -20°C. By removing water, the activity of the enzyme/protein is preserved.

Attempts were made to preserve and concentrate the enzymes produced by RU-A01, RU-A03 and RU-A06 through the process of lyophilization. The initial attempt yielded no enzyme activity. Protease inhibitor as well as BSA to stabilize the crude proteins was added in the next batch. Though enzyme activity was detected, the activity was however significantly lower than extracellular and French pressed crude enzymes. It appears that at some stage of freeze-drying the enzymes become destabilized and hence lost activity (Tables 3.15, 3.16 and 3.17). There is no mention of this affect on enzymes from Actinomyces in literature and this area would need to be pursued further.

**Table 3. 15** **Comparative peroxidase activity of isolates RU-A01, RU-A03 and RU-A06 using 2,4 DCP as substrate. Activity expressed as μmols/min/mL.**

| | ACTIVITY (μmols/mL/min) | | |
|---|---|---|---|
| **Thermophiles** | **Extracellular** | **French pressed** | **Freeze dried** |
| RU-A01 | 3.5 | 1.9 | 0.2 |
| RU-A03 | 0.97 | 1.36 | 0 |
| RU-A06 | 4.6 | 5.28 | 0.2 |

**Table 3. 16** **Comparative lignin peroxidase activity of isolates RU-A01, RU-A03 and RU-A06 using veratryl alcohol as substrate. Activity expressed as μmols/min/mL.**

| | ACTIVITY (μmols/mL/min) | | |
|---|---|---|---|
| **Thermophiles** | **Extracellular** | **French pressed** | **Freeze dried** |
| RU-A01 | 6.5 | 0.96 | 3.0 |
| RU-A03 | 0.42 | 0.67 | 0 |
| RU-A06 | 0.60 | 11.5 | 1.04 |

**Table 3. 17** **Comparative polyphenol oxidase activity of isolates RU-A01, RU-A03 and RU-A06 using catechol as substrate. Activity expressed as μmols/min/mL.**

| | ACTIVITY (μmols/mL/min) | | |
|---|---|---|---|
| **Thermophiles** | **Extracellular** | **French pressed** | **Freeze dried** |
| RU-A01 | 0.13 | 0.6 | 0.05 |
| RU-A03 | 0.6 | 0.8 | 0.052 |
| RU-A06 | 0.2 | 0.37 | 0.041 |

Further studies on this area need to be addressed in order to determine what causes loss of activity during the stage of freeze-drying. At this point samples were cryo-preservation and stored at -20°C.

## 3.8 ENZYME FRACTIONATION AND PARTIAL PURIFICATION USING AMMONIUM SULPHATE AND DIALYSIS TECHNIQUES.

McLellan and Robinson (1981) suggested that peroxidases could be extracted from the pellet using high molarity salts. Salts have the ability to trap water and expose the hydrophobic areas on the surface of the enzyme molecules. The hydrophobic interaction between enzyme molecules results in aggregation, and consequently, in their precipitation. Salting out depends on hydrophobic interaction, alteration of pH or ionic strength, polarity and temperature (Ballesteros *et al.*, 1994). Another method of concentrating protein is through dialysis against a high molecular weight compound such as PEG. PEG dialysis works by drawing out molecules of a certain molecular weight, which in turn reduces protein volume, and concentrating the sample. Ammonium sulphate fractionation and PEG dialysis were used to determine individual enzyme activity and to also compare the results between the two methods.

The highest lignin peroxidase activity was detected at 35 % saturation of the extracellular protein fraction, with a specific activity of 49 μmols/min/mg, which was the highest specific lignin peroxidase activity in RU-A01 and fold purification of 9.8 (Table 3.18). For peroxidase activity using 2,4 DCP as substrate in RU-A01, the highest activity was detected in the 20% PEG dialysis intracellular fraction (25 μmols/mg/min) and a fold purification of 10.8 (Table 3.19). 156 μmols/min/mg of polyphenol oxidase was the highest amount detected in RU-A01. This activity came from 35% saturation pellet in the extracellular protein fraction with a fold purification of 13 (Table 3.19). Lignin peroxidase specific activity for isolate RU-A03 was highest at 163 μmols/min/mg. This activity was observed in the 20% PEG dialysis intracellular sample. The second highest specific activity was in the 20% PEG dialysis of extracellular protein sample, with a specific activity of 53 μmols/min/mg. Again the dialysis samples gave the highest peroxidase specific activity in both intra- and extracellular protein fractions (Table 3.22) for RU-A03. PEG dialysis of intracellular protein gave the highest specific polyphenol oxidase activity (151 µmols/min/mg.) with a fold purification of 75.5 (Table 3.23). PEG dialysed intracellular protein fraction gave the highest lignin peroxidase activity at 21 µmols/min/mg and a fold purification of 7 for RU-A06. 203 µmol/min/mg was detected in the 55% saturation pellet of the extracellular peroxidase protein fraction of RU-A06. This was the recorded highest specific activity, with a 4.2 fold purification. 182 µmols/min/mg closely followed by 151 µmols/min/mg were the highest polyphenol oxidase activities observed in the 55% and 85% saturation pellets of the extracellular protein fractions for RU-A06.

**Table 3. 18** **Comparison of extra-and intracellular partial purification and fractionation using ammonium sulphate and 20% PEG dialysis techniques of lignin peroxidase in isolate RU-A01.**

| **Fraction** | **Protein** µg/mL | **Activity** µmols/mL/min | **Specific activity** µmols/mg/min | **Total activity** µmols/mL | **Total protein** mg | **Yield (%)** | **Fold purification** |
|---|---|---|---|---|---|---|---|
| Extracellular protein | | | | | | | |
| Cell free extract | 99.6 | 0.5 | 5.0 | 100 | 19920 | 100 | 1 |
| 35% pellet | 36.5 | 1.8 | 49 | 9 | 182.5 | 3.6 | 9.8 |
| 55% pellet | 159 | 5.9 | 37 | 29.5 | 795 | 11.8 | 7.4 |
| 85% pellet | 518 | 17.4 | 34 | 87 | 2590 | 34.8 | 6.8 |
| 20% PEG dialysis | 115 | 1.3 | 11.3 | 13 | 1150 | 2.6 | 2.3 |
| Intracellular protein | | | | | | | |
| Cell free extract | 450 | 8.7 | 19.3 | 870 | 45000 | 100 | 1 |
| 35% pellet | 494.2 | 16.4 | 1.3 | 164 | 4942 | 1.88 | 0.56 |
| 55% pellet | 414.2 | 5.75 | 2.6 | 57.5 | 4142 | 0.7 | 1.1 |
| 85% pellet | 180 | 2.9 | 2.7 | 29 | 1800 | 0.3 | 1.2 |
| 20% PEG dialysis | 306.9 | 4.5 | 25 | 45 | 3069 | 0.5 | 10.8 |

**Table 3. 19** **Comparison of extra-and intracellular partial purification and fractionation using ammonium sulphate and 20% PEG dialysis techniques of peroxidase in isolate RU-A01.**

| **Fraction** | **Protein** µg/mL | **Activity** µmols/mL/min | **Specific activity** µmols/mg/min | **Total activity** µmols/mL | **Total protein** mg | **Yield (%)** | **Fold purification** |
|---|---|---|---|---|---|---|---|
| Extracellular protein | | | | | | | |
| Cell free extract | 99.6 | 1.2 | 11.0 | 240 | 19920 | 100 | 1 |
| 35% pellet | 36.5 | 0 | 0 | 0 | 182.5 | 0 | 0 |
| 55% pellet | 159 | 0 | 0 | 0 | 795 | 0 | 0 |
| 85% pellet | 518 | 0.56 | 1.1 | 2.8 | 2590 | 05 | 0.1 |
| 20% PEG dialysis | 115 | 0.25 | 2.2 | 2.5 | 1150 | 0.2 | 0.2 |
| Intracellular protein | | | | | | | |
| Cell free extract | 450 | 8.7 | 14 | 870 | 45000 | 100 | 1 |
| 35% pellet | 494.2 | 6.6 | 1.3 | 66 | 4942 | 0.8 | 0.06 |
| 55% pellet | 414.2 | 1.1 | 2.6 | 11 | 4142 | 0.1 | 0.1 |
| 85% pellet | 180 | 0.5 | 2.7 | 5 | 1800 | 0.05 | 0.13 |
| 20% PEG dialysis | 306.9 | 7.6 | 25 | 76 | 3069 | 0.8 | 1.3 |

**Table 3. 20** **Comparison of extra-and intracellular partial purification and fractionation using ammonium sulphate and 20% PEG dialysis techniques of polypehnol oxidase in isolate RU-A01.**

| **Fraction** | **Protein** µg/mL | **Activity** µmols/mL/min | **Specific activity** µmols/mg/min | **Total activity** µmols/mL | **Total protein** mg | **Yield (%)** | **Fold purification** |
|---|---|---|---|---|---|---|---|
| Extracellular protein | | | | | | | |
| Cell free extract | 99.6 | 1.1 | 12.0 | 220 | 19920 | 100 | 1 |
| 35% pellet | 36.5 | 5.7 | 156 | 28.5 | 182.5 | 5.2 | 13 |
| 55% pellet | 159 | 8.2 | 51 | 41 | 795 | 7.5 | 4.25 |
| 85% pellet | 518 | 5.4 | 10 | 27 | 2590 | 4.9 | 0.8 |
| 20% PEG dialysis | 115 | 7.7 | 70 | 77 | 1150 | 7 | 5.8 |
| Intracellular protein | | | | | | | |
| Cell free extract | 450 | 1.1 | 2.4 | 110 | 45000 | 100 | 1 |
| 35% pellet | 494.2 | 1.7 | 34 | 17 | 4942 | 0.6 | 14.2 |
| 55% pellet | 414.2 | 11.9 | 28 | 119 | 4142 | 10.8 | 11.7 |
| 85% pellet | 180 | 7.6 | 42 | 76 | 1800 | 6.9 | 17.5 |
| 20% PEG dialysis | 306.9 | 5.5 | 18 | 55 | 3069 | 5 | 7.5 |

**Table 3. 21** **Comparison of extra-and intracellular partial purification and fractionation using ammonium sulphate and 20% PEG dialysis techniques of lignin peroxidase in isolate RU-A03.**

| **Fraction** | **Protein** µg/mL | **Activity** µmols/mL/min | **Specific activity** µmols/mg/min | **Total activity** µmols/mL | **Total protein** mg | **Yield (%)** | **Fold purification** |
|---|---|---|---|---|---|---|---|
| Extracellular protein | | | | | | | |
| Cell free extract | 46.2 | 0.42 | 9.1 | 84 | 9240 | 100 | 1 |
| 35% pellet | 48.5 | 0 | 0 | 0 | 242.5 | 0 | 0 |
| 55% pellet | 12.3 | 0 | 0 | 0 | 61.5 | 0 | 0 |
| 85% pellet | 108.1 | 1.63 | 15 | 8.15 | 540.5 | 3.8 | 1.6 |
| 20% PEG dialysis | 35.4 | 1.9 | 53 | 19 | 354 | 1.95 | 5.8 |
| Intracellular protein | | | | | | | |
| Cell free extract | 448 | 0.97 | 20.9 | 194 | 4480 | 100 | 1 |
| 35% pellet | 373.8 | 12.2 | 32 | 122 | 3738 | 12.5 | 1.5 |
| 55% pellet | 370 | 6.2 | 17 | 62 | 3700 | 6.4 | 0.8 |
| 85% pellet | 226.5 | 12.1 | 53 | 121 | 2265 | 12.5 | 2.5 |
| 20% PEG dialysis | 31.2 | 5.1 | 163 | 51 | 312 | 5.3 | 7.8 |

**Table 3. 22** **Comparison of extra-and intracellular partial purification and fractionation using ammonium sulphate and 20% PEG dialysis techniques of peroxidase in isolate RU-A03.**

| **Fraction** | **Protein** µg/mL | **Activity** µmols/mL/min | **Specific activity** µmols/mg/min | **Total activity** µmols/mL | **Total protein** mg | **Yield (%)** | **Fold purification** |
|---|---|---|---|---|---|---|---|
| Extracellular protein | | | | | | | |
| Cell free extract | 46.2 | 0.97 | 20.9 | 194 | 9240 | 100 | 1 |
| 35% pellet | 48.5 | 1.4 | 28 | 7 | 242.5 | 1.4 | 1.3 |
| 55% pellet | 12.3 | 0.5 | 40 | 2.5 | 61.5 | 0.5 | 1.9 |
| 85% pellet | 108.1 | 11.4 | 105 | 57 | 540.5 | 11.6 | 5.0 |
| 20% PEG dialysis | 35.4 | 8.8 | 248 | 88 | 354 | 9.1 | 11.9 |
| Intracellular protein | | | | | | | |
| Cell free extract | 448 | 5.2 | 11.6 | 520 | 4480 | 100 | 1 |
| 35% pellet | 373.8 | 8 | 21 | 80 | 3738 | 1.5 | 1.8 |
| 55% pellet | 370 | 5 | 13 | 50 | 3700 | 1 | 1.1 |
| 85% pellet | 226.5 | 10.2 | 45 | 102 | 2265 | 2 | 3.9 |
| 20% PEG dialysis | 31.2 | 6.9 | 221 | 69 | 312 | 1.3 | 19.1 |

**Table 3. 23** **Comparison of extra-and intracellular partial purification and fractionation using ammonium sulphate and 20% PEG dialysis techniques of polyphenol oxidase in isolate RU-A03.**

| **Fraction** | **Protein** µg/mL | **Activity** µmols/mL/min | **Specific activity** µmols/mg/min | **Total activity** µmols/mL | **Total protein** mg | **Yield (%)** | **Fold purification** |
|---|---|---|---|---|---|---|---|
| Extracellular protein | | | | | | | |
| Cell free extract | 46.2 | 0.6 | 12.98 | 120 | 9240 | 100 | 1 |
| 35% pellet | 48.5 | 0 | 0 | 7 | 242.5 | 0 | 0 |
| 55% pellet | 12.3 | 0 | 0 | 2.5 | 61.5 | 0 | 0 |
| 85% pellet | 108.1 | 2.9 | 27 | 57 | 540.5 | 4.8 | 2.1 |
| 20% PEG dialysis | 35.4 | 0 | 0 | 0 | 354 | 0 | 0 |
| Intracellular protein | | | | | | | |
| Cell free extract | 448 | 0.8 | 2 | 80 | 4480 | 100 | 1 |
| 35% pellet | 373.8 | 4.6 | 12 | 80 | 3738 | 100 | 6 |
| 55% pellet | 370 | 6.4 | 17 | 50 | 3700 | 5.8 | 8.5 |
| 85% pellet | 226.5 | 10.0 | 33 | 102 | 2265 | 12.5 | 16.5 |
| 20% PEG dialysis | 31.2 | 4.9 | 151 | 69 | 312 | 6.1 | 75.5 |

**Table 3. 24** **Comparison of extra-and intracellular partial purification and fractionation using ammonium sulphate and 20% PEG dialysis techniques of lignin peroxidase in isolate RU-A06.**

| **Fraction** | **Protein** µg/mL | **Activity** µmols/mL/min | **Specific activity** µmols/mg/min | **Total activity** µmols/mL | **Total protein** mg | **Yield (%)** | **Fold purification** |
|---|---|---|---|---|---|---|---|
| Extracellular protein | | | | | | | |
| Cell free extract | 94.6 | 0.59 | 6.2 | 118 | 18920 | 100 | 1 |
| 35% pellet | 23.8 | 0 | 0 | 0 | 119 | 0 | 0 |
| 55% pellet | 38.8 | 0.4 | 10 | 2 | 194 | 0.7 | 1.6 |
| 85% pellet | 81.9 | 0.1 | 1.2 | 0.5 | 409.5 | 0.2 | 0.2 |
| 20% PEG dialysis | 141.5 | 2.6 | 18 | 26 | 1415 | 4.4 | 2.9 |
| Intracellular protein | | | | | | | |
| Cell free extract | 450 | 1.35 | 3 | 135 | 45000 | 100 | 1 |
| 35% pellet | 222.7 | 2.5 | 11 | 25 | 2227 | 1.85 | 3.7 |
| 55% pellet | 602.3 | 8.6 | 14 | 86 | 6023 | 6.4 | 4.7 |
| 85% pellet | 350.8 | 0.3 | 0.8 | 3 | 3508 | 0.2 | 0.3 |
| 20% PEG dialysis | 496.2 | 10.6 | 21 | 106 | 4962 | 7.9 | 7 |

**Table 3. 25** **Comparison of extra-and intracellular partial purification and fractionation using ammonium sulphate and 20% PEG dialysis techniques of peroxidase in isolate RU-A06.**

| **Fraction** | **Protein** µg/mL | **Activity** µmols/mL/min | **Specific activity** µmols/mg/min | **Total activity** µmols/mL | **Total protein** mg | **Yield (%)** | **Fold purification** |
|---|---|---|---|---|---|---|---|
| Extracellular protein | | | | | | | |
| Cell free extract | 94.6 | 4.6 | 48.6 | 920 | 18920 | 100 | 1 |
| 35% pellet | 23.8 | 0.5 | 20 | 2.5 | 119 | 0.1 | 0.4 |
| 55% pellet | 38.8 | 7.9 | 203 | 39.5 | 194 | 1.7 | 4.2 |
| 85% pellet | 81.9 | 7.6 | 92 | 38 | 409.5 | 1.6 | 1.8 |
| 20% PEG dialysis | 141.5 | 10.7 | 75 | 107 | 1415 | 2.3 | 1.5 |
| Intracellular protein | | | | | | | |
| Cell free extract | 450 | 5.99 | 13.3 | 599 | 45000 | 100 | 1 |
| 35% pellet | 222.7 | 4.2 | 18 | 42 | 2227 | 0.7 | 1.4 |
| 55% pellet | 602.3 | 2.1 | 3.4 | 21 | 6023 | 0.4 | 0.3 |
| 85% pellet | 350.8 | 1.6 | 0.4 | 16 | 3508 | 0.3 | 0.03 |
| 20% PEG dialysis | 496.2 | 13.9 | 28 | 139 | 4962 | 2.3 | 2.1 |

**Table 3. 26** **Comparison of extra-and intracellular partial purification and fractionation using ammonium sulphate and 20% PEG dialysis techniques of polyphenol oxidase in isolate RU-A06.**

| **Fraction** | **Protein** µg/mL | **Activity** µmols/mL/min | **Specific activity** µmols/mg/min | **Total activity** µmols/mL | **Total protein** mg | **Yield (%)** | **Fold purification** |
|---|---|---|---|---|---|---|---|
| Extracellular protein | | | | | | | |
| Cell free extract | 94.6 | 0.98 | 10.3 | 196 | 18920 | 100 | 1 |
| 35% pellet | 23.8 | 0.9 | 37 | 4.5 | 119 | 0.9 | 3.6 |
| 55% pellet | 38.8 | 7.1 | 182 | 35.5 | 194 | 7.2 | 17.7 |
| 85% pellet | 81.9 | 12.4 | 151 | 62 | 409.5 | 12.6 | 14.7 |
| 20% PEG dialysis | 141.5 | 7.4 | 52 | 74 | 1415 | 7.5 | 5.0 |
| Intracellular protein | | | | | | | |
| Cell free extract | 450 | 0.35 | 0.78 | 35 | 45000 | 100 | 1 |
| 35% pellet | 222.7 | 3.1 | 13 | 31 | 2227 | 8.9 | 16.7 |
| 55% pellet | 602.3 | 11.3 | 18 | 113 | 6023 | 32.3 | 23.1 |
| 85% pellet | 350.8 | 7 | 19 | 70 | 3508 | 20 | 24.4 |
| 20% PEG dialysis | 496.2 | 5.2 | 10 | 52 | 4962 | 14.9 | 12.8 |

Other methods of purification and fractionation could be used to effectively separate the 2 enzymes, namely gel filtration, ion exchange chromatography and ultrafiltration. The % yields were generally low and so looking into other fractionation and purification techniques would be an option.

### 3.9 Metabolism of low molecular weight lignin-related compounds.

Phenylpropanoid compounds found in soil generally originate from the breakdown of phenylalanine, tyrosine, lignin and other aromatic plant constituents. Several bacteria and fungi have been shown to metabolize representative phenylpropanoid compounds such as cinnamic acid, ferulic acid and *p*-coumaric acid. A few Actinomycetes are known to metabolize these compounds. Previous studies showed that *Streptomyces nigrifaciens* hydroxylates *p*-coumaric acid to caffeic acid (Nambudiri *et al.*, 1969), which catabolizes through protocatechuic acid. Another *Streptomyces* strain was able to convert cinnamic acid to cinnamamide (Bezanson *et al.*, 1970). Pathways have been reported by which Actinomycetes catabolizes model compounds such as cinnamic, *p*-coumaric and ferulic acids (Sutherland *et al.*, 1983).

Model compound research has mostly been focused on white-rot fungi, which have been viewed as probably the most efficient phenolic degraders. It is believed that's it's a radical reaction catalysed by a number of enzymes including polyphenol oxidases amd peroxidases, as well as xylanses and endoglucanses (Ramachandra, 1987; Georis, 1999). Bacteria including Actinomycetes can decompose phenolic compounds and probably can also further degrade low molecular weight bioconversion components produced from industrial phenolic bioremediation. Dimeric model compounds containing ß- ß, ß –5, ß –1 at 5-5 linkages have been shown to be decomposed by Actinomycetes species and *Pseudomonas* species (Ruttimann *et al.*, 1987).

Lignin structures consist of polypropane polymers consisting of coumaryl, guaiacyl and syringyl moieties linked together by numerous linkages, but primarily by the β-aryl ether bonds. Bioconversion of this recalcitrant polymer by *Streptomyces* is by an oxidative process in which the ether bonds are cleaved and the phenylpropane side chains are modified or removed. At this instance, some low molecular weight aromatic intermediates are released from lignin as a result of these reactions. A principle result of aryl ether bond cleavage is the substantial depolymerization of lignin (Crawford and Crawford, 1980). Based on this reaction mechanism performed in nature by

Actinomycetes, one could hypothesize that the same can occur with phenolic residues, resulting in mineralization and/or solubilization.

Microbial lignolysis continues to be the subject of intensive research and its applications can be extended beyond lignin degradation but also to phenolic residues that accumulate due to argo-industrial processes. The use of model compounds do have disadvantages in that the model compounds are of low molecular weight and unlike lignin and larger phenolic residues, they can be taken up and metabolised intracelluarly, making it difficult to determine whether degradation products observed really reflect true activity of the oxidoreductase enzymes. This has been a problem in terms of investigations aimed at finding extracellular oxidoreductase activity in bacteria. Ideally, phenolic model compounds should be macromolecular to facilitate. These model compounds would ideally be simpler structures than those of natural polymers. Unfortunately most of these compounds are not commercially available and hence have to be synthesized in the laboratory, which is a time consuming process. Studies that have followed this route of protocol have tagged the compounds concerned with high molecular weight compounds such as PEG which due to its size prevent the compound from entering the bacterial cell hence resulting in extracellular breakdown of the compound concerned. Such a process enables the researcher to follow the mechanism of bioconversion by the bacteria and the resultant products can be analysed by methods such as HPLC.

For this kind of research, the use of mono-phenolic acids were sufficient to detect whether the bacteria used in this study were capable of utilizing these model compounds irrespective of whether it was an extracellular or intracellular process. Metabolism of model phenolic compounds is not a true reflection of oxidoreductase activity but still serves as a good reference point in terms of the ability of these isolates to degrade such compounds and hence their potential in the bioconversion of phenolic residues.

Resting cell reactions were carried out in minimal buffered medium without a carbon source, which was replaced by the model compound. Initial concentrations of the model compound was10mM.

Phenolic acids are widely distributed in the plant kingdom and are present in grape waste hence the use of these acids as model compounds in the whole cell studies. Model compounds breakdown was determined by HPLC over time and concentrations were determined by the extrapolation of standard curves for each compound (Appendix H). The controls were minimal growth media with model compounds without any live biomass subjected to the same conditions as the flasks with minimal media, model compounds and live biomass. Based on the HPLC results the control flasks showed very little bioconversion of the model compounds. The three thermophiles were shown to degrade certain phenolic compounds, some of which were seen to decrease by 95% within 24 hours.

### 3.9.1 Ferulic acid metabolism

There was a gradual decline of ferulic acid over time for both RU-A01 and RU-A03 (Figure 3.24). RU-A06 was unable to utilize ferulic acid as the sole carbon source. Sutherland and co-workers (1983) found that in ferulate-grown cultures, ferulate, vanillin, vanillate and protocathechuate were detected, as well as acetate. These results suggested that the mechanism of ferulic acid metabolism of *streptomyces* species involves the removal of the 2 side-chain carbons as acetates and presumably other two substrates producing corresponding aldehydes, which in turn are oxidized to the corresponding substituted benzoic acid, probably by an oxygen-requiring oxidase (Crawford *et al.*, 1982; Figure 3.25).

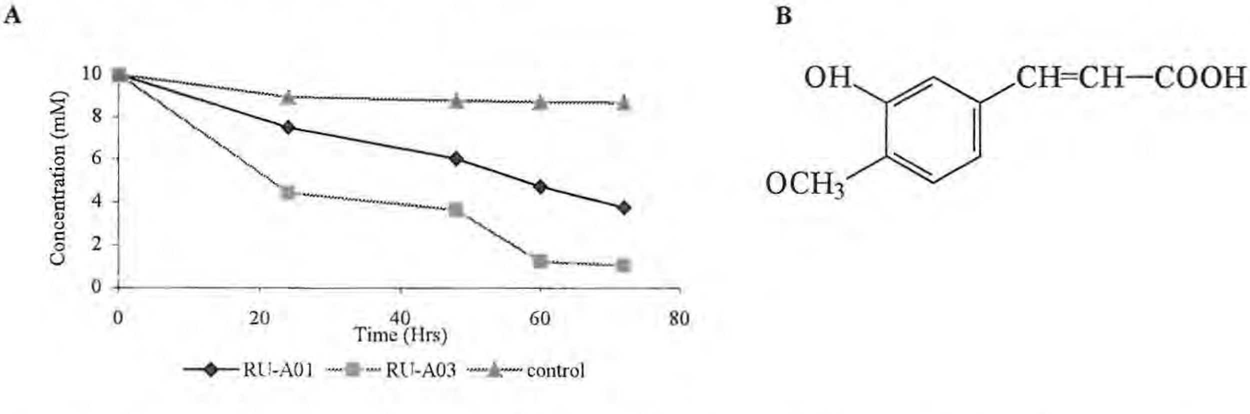


**Figure 3. 24** **(A) Ferulic acid catabolism by RU-A01 and RU-A03. The cultures were grown in 250 ml flasks contaning 50mls buffered minimal medium, with 10mM ferulic acid as the sole carbon source at 50°C. (B) Structural formula of ferulic acid.**

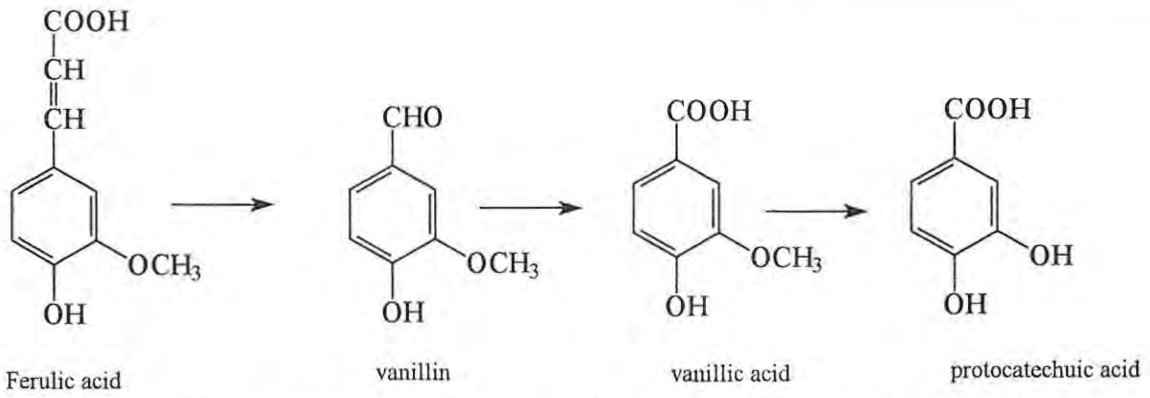


**Figure 3. 25** **Proposed catabolism of ferulic acid by Actinomycetes (Sutherland *et al.*, 1983).**

### 3.9.2 Vanillic acid metabolism

RU-A01 and RU-A06 were shown to catabolize vanillic acid significantly from an initial concentration of 10mM to below 1mM for both isolates (Figure 3.26). Previous studies have indicated that Actinomyces convert vanillin to guaiacol by decarboxylation and demethylate guaiacol to catechol (Pometto *et al.*, 1981; Figure 3.27). Demethylation is a common mechanism employed by peroxidases. *Streptomyces viridosporus* has been shown to convert vanillin into vanillic acid (Pometto *et al.*, 1983), and also further to catechol (Figure 3.27).

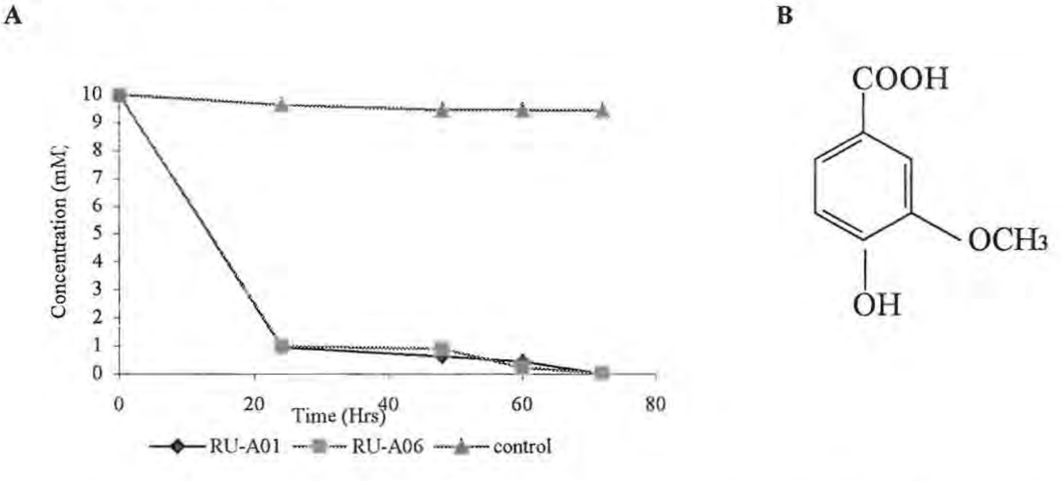


**Figure 3. 26** **(A) Vanillic acid catabolism by RU-A01 and RU-A06. The cultures were grown in 250ml flasks containing 50mls of buffered minimal medium with 10mM vanillic acid as the sole carbon source at 50°C. (B) structural formula of vanillic acid.**

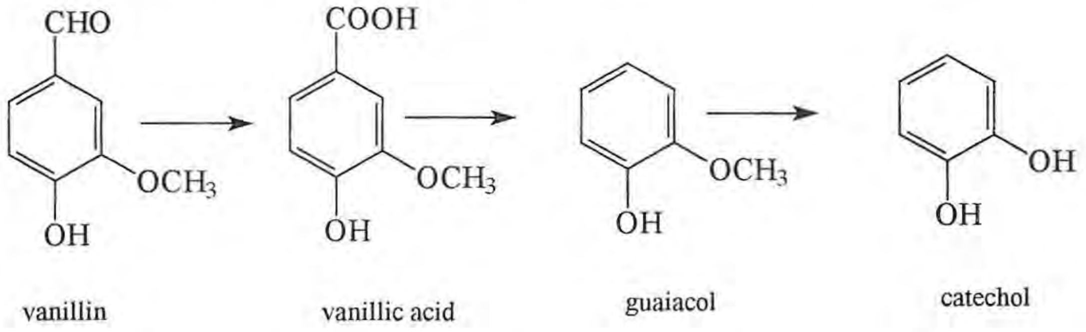


**Figure 3. 27** **Proposed vanillin catabolism by Actinomycetes (Sutherland *et al.*, 1983).**

There was a sharp decline in vanillic acid within the first 20 hours of incubation (Figure 3.26). At this point the concentration of vanillic acid was down to less than 1.5 mM for both RU-A01 and RU-A06.

### 3.9.3 Protocatechuic acid metabolism

There was a sharp decline in protocatechuic acid in the minimal media after 20 hours incubation for both RU-A01 and RU-A03 (Figure 3.28). RU-A03 depleted protocatechuic acid completely and RU-A01 depleted protocatechuic acid to less than 1mM after 60 hours. Protocathechuic acid is usually the end product of model compound metabolism as displayed in Figure 3.30 and is the product of oxygen-required oxidases such as phenol oxidases.

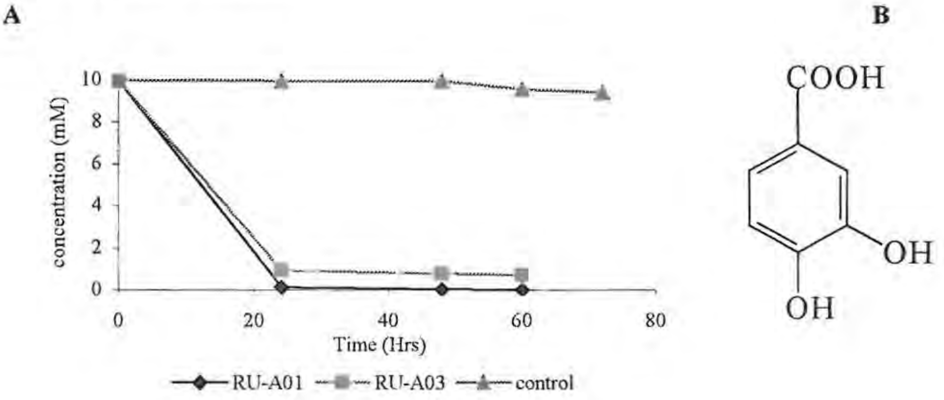


**Figure 3. 28** **(A) Protocatechuic acid catabolism by RU-A01 and RU-A03. The cultures were grown in 250ml flasks containing 50mls of buffered minimal medium with 10mM protocatechuic acid as the sole carbon source at 50°C. (B) Structural formula of protocatechuic acid.**

### 3.9.4 *P*-coumaric acid metabolism

Like ferulic acid, there was a gradual decline in *p*-coumaric acid with RU-A03 depleting *p*-coumaric acid within 60 hours, however there was slight increase in free *p*-coumaric acid at time 72 hours possibly due to adsorption of the acid to the spores and mycelia (Figure 3.29). The proposed metabolism pathway of *p*-coumaric acid by *Streptomyces* is shown in Figure 3.30. Again the action of oxygen dependant oxidases has been proposed to be responsible for the catabolism.

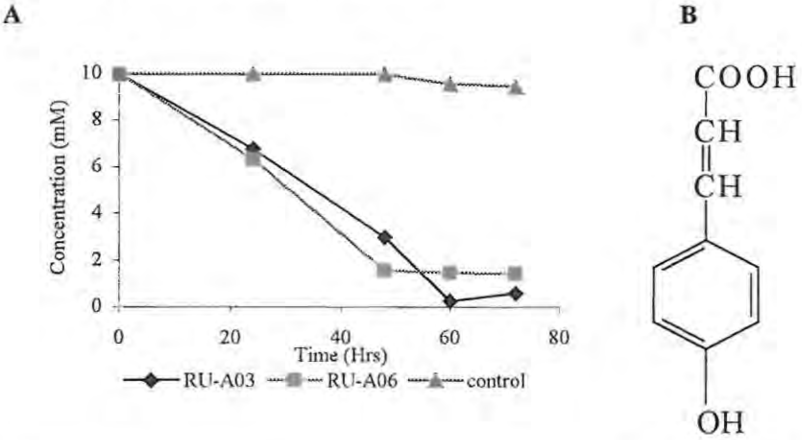


**Figure 3. 29** **(A) *p*-coumaric acid catabolism by RU-A03 and RU-A06. The cultures were grown in 250ml flasks containing 50mls of buffered minimal medium with 10mM *p*-coumaric acid as the sole carbon source at 50°C. (B) Structural formula of *p*-coumaric acid.**

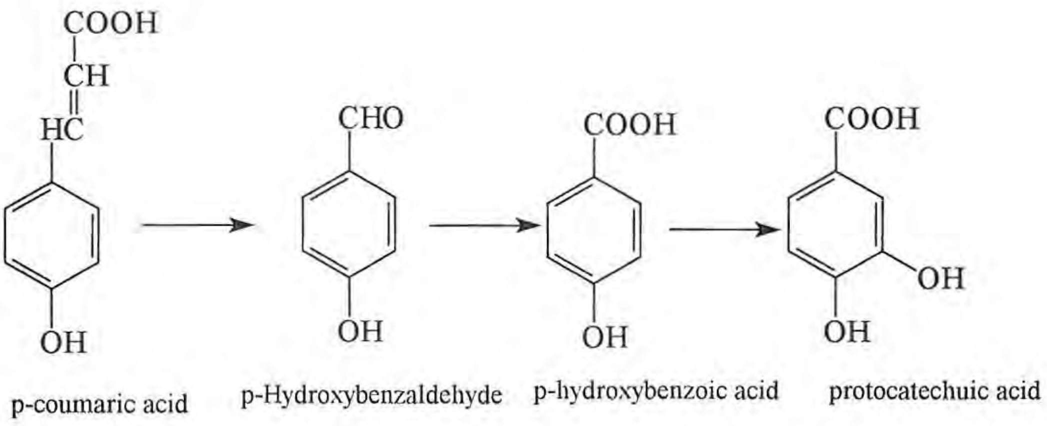


**Figure 3. 30** **Proposed catabolism of *p*-coumaric acid by Actinomycetes (Sutherland *et al.*, 1983).**

### 3.9.5 Syringic acid metabolism

All three isolates were capable of catabolizing syringic acid significantly within 24 hours of incubation with RU-A01 and RU-A06 depleting it completely by time 60 hours (Figure 3.31).

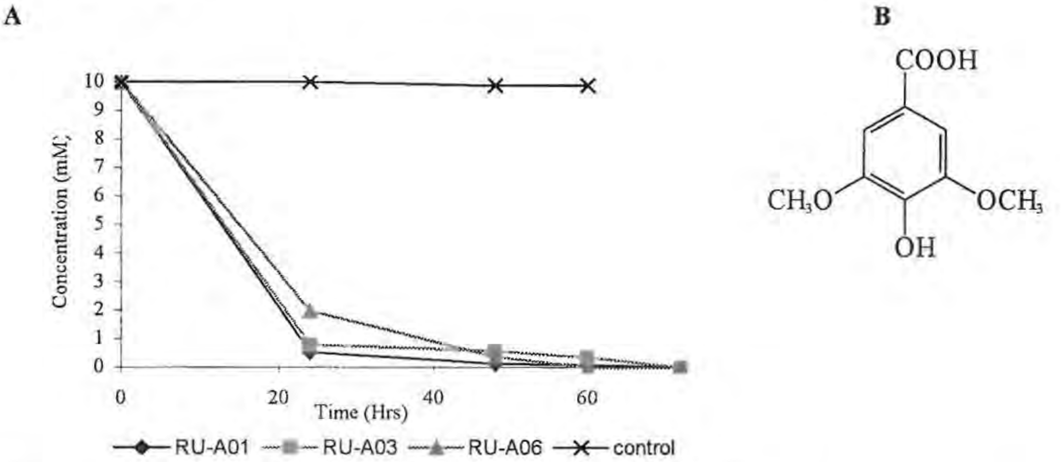


**Figure 3. 31** **(A) Syringic acid catabolism by RU-A01 and RU-A03. The cultures were grown in 250ml flasks containing 50mls of buffered minimal medium with 10mM syringic acid as the sole carbon source at 50°C. (B) structural formula of syringic acid**

Lignin-related aromatic acids, which contain phenylpropane ($C_6$ –$C_3$) type structures (such as ferulic acid and related compounds), are abundant molecules that play important functions in plant cells. The catabolism of these compounds is an important aspect for the mineralization of plant wastes as they are released during the breakdown of lignin. Caffeic, vanillic, ferulic, protocatechuic, syringic and p-coumaric acids are all low molecular weight lignin-model like compounds, which are also found in the lignin degradation pathway. Although bioconversion of lignin model compounds by microorganisms may not reflect true lignolytic capacity, this approach has proved successful in studies with filamentous fungi, in determining the capacity of microbial phenolic bioremediation.

The use of minimal growth media in this study was to determine if in a period of carbon starvation, the isolates would utilize and bioconvert the more complex carbon source represented by the model phenolic compounds used in this study. Catabolism of model phenolic compounds would normally occur via the cleavage of the phenolic ring via the *ortho-* or *meta*-pathways. Despite the vast array of aromatic compounds in natural environments, the bioconversion of different phenolic compounds usually proceeds through a limited number of metabolic pathways. Most of these phenolic model compounds are first converted to one of several di- or tri-hydroxylated substrates, such as catechol or protocathechuate, whose phenolic ring can be enzymatically cleaved (Buchan *et al.*, 2000).

The ability of RU-A01, RU-A03 and RU-A06 to catabolise model phenolic compounds found in phenolic residues and lignin indicate the probability of these thermophilic isolates to bioconvert phenolic residues in industrial effluents. No intermediate products however were detected which suggests that the reaction mechanisms when allowed to continue may result in the loss of intermediate products such as guaiacol (Figure 3.28) which maybe further catabolized by the isolates. The use of enzyme inhibitors such as α, α-dipyridyl which blocks oxidase activity in cultures grown in model compounds would prevent the reactions from going further resulting in the accumulation of bioconversion products (Sutherland *et al.*, 1983).

The ability of the 3 isolates to catabolize these model compounds suggests that these thermophilic *Streptomyces* maybe involved in the decomposition or bioconversion of phenylpropanoid compounds, which are phenolic-based compounds in their natural environment. Various explanations on the mechanisms by which bacteria catabolize model phenolic compounds have been offered (Saiqa *et al.*, 1998; Sutherland *et al.*, 1983). A number of thermophilic bacteria have been reported to utilize phenolic mono- and di-phenolic compounds, namely thermophilic bacilli (Saiqa *et al.*, 1998). No work to date has been done to demonstrate the ability of thermophilic Actinomycetes to bioconvert phenolic model compounds.

The possible biotechnological applications of these isolates and their constituent enzymes were investigated further under the hypothesis that the demonstrated ability of the three isolates to catabolize model phenolic compounds would therefore mean that they would be able to bioconvert a model phenolic waste. From the results presented in this section, it is possible to make some assessment of the feasibility of using the thermophilic Actinomycetes isolates in whole-cell and /or enzyme-based systems. If this hypothesis is proved, the potential to use these isolates in bioreactor systems, for the removal of phenolic wastes or treatment of contaminated waste streams is inherent.

## 3.10 A PRELIMINARY INVESTIGATION INTO THE LAB-SCALE APPLICATION OF ENZYMES FROM RU-A01, RU-A03 AND RU-A06 ON AN INDUSTRIAL PHENOLIC WASTE.

Agro-industrial plant waste material generated in the wine industry is particularly high. At present most of this waste goes towards animal feed, but academic and industrial researchers are putting more and more effort into the bioconversion and/or biodegradation of this waste into high-value compounds (Personal communication, S. G Burton, 2001). A high proportion of this waste material is phenolic and carbohydrate in nature. Phenolic moieties, ester-linked to polysaccharides forming structural cross-linking bridges between various polymers. These polymers often restrict complete hydrolysis of the plant material.

Current methods of removing phenolics from the agro-industry include microbial bioconversion and degradation, adsorption onto activated carbon, chemical oxidation and incineration. The choice of system depends mainly on economic and other factors.

After the discovery of oxidoreductase enzymes in bacteria and fungi, it was proposed that the non-specific activity of these enzymes and the associated microbes would be candidates for bioconversion and bioremediation. In 1983 Kirk (1983) wrote, "Waste treatment might well become the first directed use of an oxidoreductase system". Oxidoreductase enzymes are suitable for the elimination of phenolic residues, lignin and other phenolic –related compounds generated in industry.

### 3.10.1 Whole cell studies of RU-A01, RU-A03 and RU-A06 on grape phenolic waste

The potential of the 3 thermophilic *Streptomyces* isolates to produce peroxidases and polyphenol oxidases during its growth phase and the stability of this activity during the stationery phase of growth would be 2 valuable arguments for a phenolic bioconversion strategy. The number of compounds known to be bioconverted by lignolytic microbes continues to increase with ongoing research. According to the generally accepted theory, the initial attack of phenolic residues is made by extracellular enzymes. The enzymes perform a one-electron oxidation, thereby generating cation radicals of the contaminations. The cation radicals may undergo spontaneous chemical reactions such as C-C cleavage or hydroxylation resulting in more hydrophilic products such as acid precipitable polymeric lignin (APPL) commonly produced during the bioconversion of lignin by *Streptomyces* species (Ramachandra *et al.*, 1987 and 1988). These products can then be taken up by the cells and co-metabolized in the presence of a proper carbon source to carbon dioxide (Hammel *et al.*, 1991). This process is a complex process of oxidation, reduction, methylation and hydroxylation.

Whole cell experiments of RU-A01 and RU-A03 and RU-A06 were conducted on plates and in liquid culture to determine the efficiency of peroxidase and polyphenol oxidases on grape waste and the ability of the isolates to grow in media containing grape waste.

### 3.10.2 Growth determination studies of isolates on agar plates containing red grape waste.

Growth was observed on all agar plates of various concentrations of red grape waste (10-50%) (Figure 3.32). As the concentration of the grape waste increased, the growth rate decreased slightly. These were initial experiments to determine the adaptability of the isolates on this kind of carbon source. These plates were then stored and used as inoculums in liquid media containing grape waste.

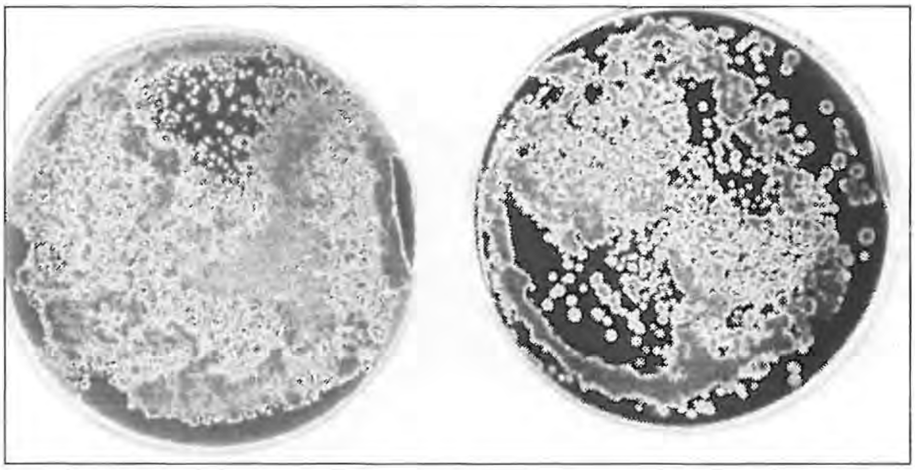


**Figure 3. 32** **Growth of RU-A03 and RU-A01 on M65 agar with 20% red grape waste incorporated into media.**

### 3.10.3 Whole cell determination of phenolic breakdown in liquid cultures supplemented with red grape waste.

RU-A01, RU-A03 and RU-A06 were inoculated into flasks containing 10, 20, and 30% (vol:vol) red grape waste and 0.1% yeast buffered minimal media. The flasks were allowed to incubate for 14 days and analyzed at time 7d and 14d. UV scans were carried out on the grape waste and peaks were detected within the wavelength region of 250 – 300nm, which is typical for phenolics compounds. UV scans were then carried out after incubation time 7d and 14d. A control sample was also run containing the grape waste without live biomass and a UV scan was taken of that.

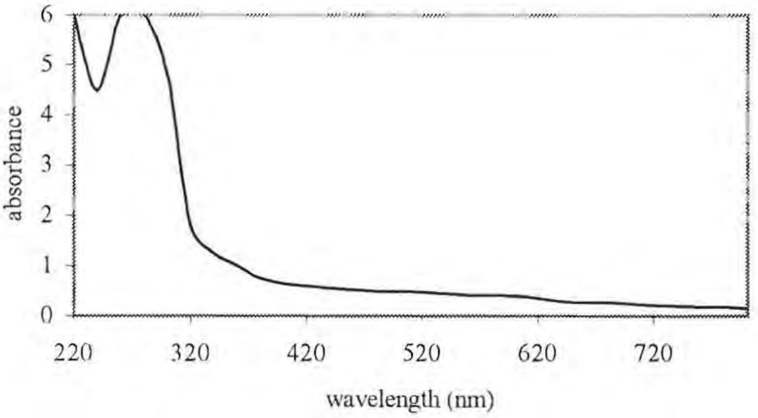


**Figure 3. 33** **UV spectrum range from 220-800nm of a 30% red grape waste sample in growth media. The red grape waste was extracted using the protocol in Figure 3.39. Peak ranges from 250 to 300 nm, which is typical for phenolic liquids.**

### 3.10.3.1 Whole cell determination of phenolic breakdown in liquid cultures supplemented with red grape waste by RU-A01.

After 3 days incubation time, a purple/blue pigment was produced and remained throughout the experimental time frame. UV scans showed a decrease in the major peak giving an indication of phenolic breakdown after a period of 7 days (Figure 3.34). Further incubation time until day 14 showed negligible phenolic breakdown, which could possibly indicate catabolite repression or the accumulation in the spent growth media of toxic intermediate products, which affected further breakdown of the phenolics in solution.

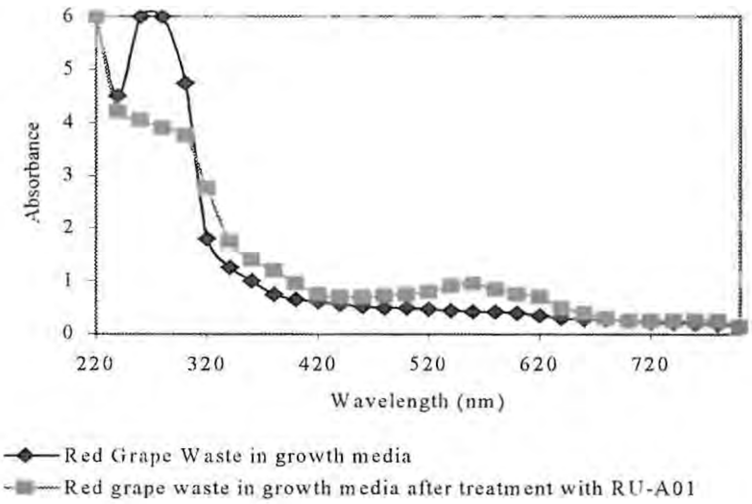


**Figure 3. 34** **UV spectrum of 30% red grape waste in growth media before and after treatment with the *Streptomyces* isolate RU-A01. There is a significant decline in absorbance around the area of 250 to 300 nm, indicating a breakdown in phenolics.**

Intermediate products in phenolic effluents have been known to occur which are toxic to the biomass resulting in the discontinuation of further phenolic breakdown. Ethyl acetate was used to extract phenolics from the medium to give a clearer colour indication of phenolic residue breakdown (Figure 3.37), a distinct colour change was observed indicating some form of reaction was occurring on the grape waste phenolics. Phenolic determination assays were conducted and these assays showed a significant decrease in phenol content after whole cell bioremediation studies with isolate RU-A01 (31.7 mg/L; Table 3.27).

### 3.10.3.2 Whole cell determination of phenolic breakdown in liquid cultures supplemented with red grape waste by RU-A03

Decolorisation of the growth media began to occur after 7 days of incubation. UV scans showed a significant decrease in the main peak at wavelength 250nm and a shift in peak to wavelength 300nm indicating the production of a by-product, which could possibly be phenolic. Further incubation up to day 14 showed complete disappearance of the phenolic peak (Figure 3.35), as well as a decrease in phenolic content (Table 3.27).

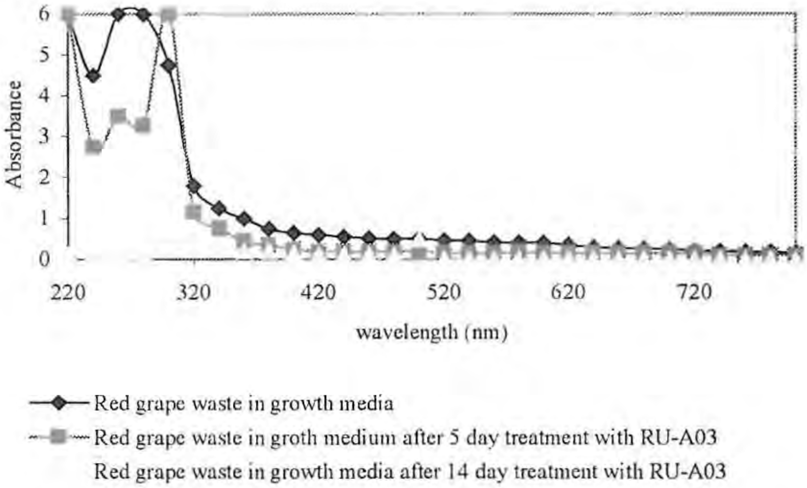


**Figure 3. 35** **UV spectrum of 30% red grape waste before and after treatment with RU-A03 over a period of 7days and 14 days. At day 7, there was a shift in absorbance peak to about 380nm. By day 14-absorbance reading were below 1 throughout the spectrum.**

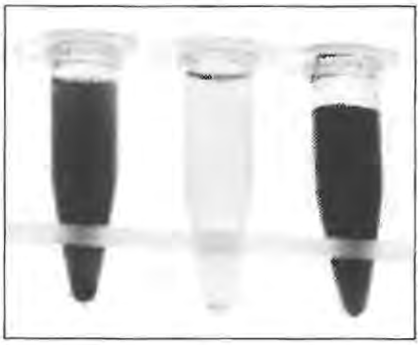
Colour changes of the growth medium were observed changing from a brick red colour to a pale orange colour with RU-A03. (Figure 3.36). Ethyl acetate was used to extract the phenolics in the organic phase showed a definite change in colour between the control and the flask cultures, which contained RU-A03 whole cells at day 7. At day 14 the organic extract was completely clear (Figure 3.37.).

### 3.10.3.3 Whole cell reactions to determine of phenolic breakdown in liquid cultures supplemented with red grape waste by RU-A06.

RU-A06 gave the least decrease and effect on the red grape waste with a phenol content of 108.3mg/L. This does not imply that RU-A06 was unable to effectively bioconvert the grape waste but could imply that the grape waste could have been bioconverted into smaller phenolic subunits such a di- and monophenolic acids, and other phenolic intermediates which are detected by the Folin-Ciocalteau reagent.

**Table 3. 27** **Final phenol concentration values of phenol content before and after whole cell treatments of red grape waste over. Cultures were incubated in 250 ml flask containing 50mls of growth medium with 30% red grape waste. Samples were taken on day 14.**

| THERMOPHILE | PHENOL CONCENTRATION (MG/L) |
|---|---|
| Control | 259.6 |
| Red grape waste | 1099 |
| 30% red grape waste in growth media | 279.2 |
| RU-A01 | 31.7 |
| RU-A03 | 59.2 |
| RU-A06 | 108.3 |



**A** **B** **C**

**Figure 3. 36** **Colour changes after whole cell treatment with RU-A01 and RU-A03. A, Red grape waste in growth media; B, Red grape waste in growth media after whole cell treatment with RU-A03; C, Red grape was in growth media after treatment with RU-A01.**

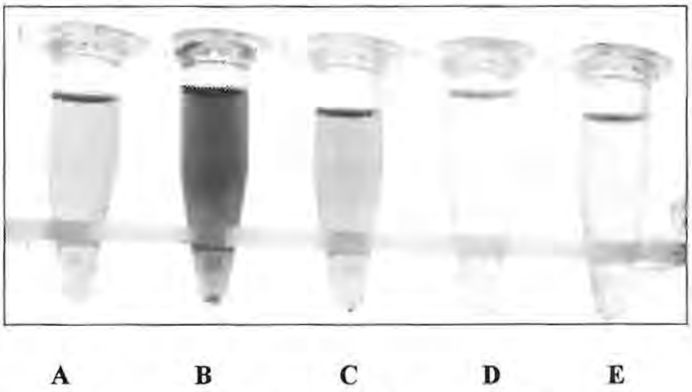


**Figure 3. 37** **Solvent extracted colour changes before and after whole cell treatment. A- red grape waste extracted from growth media; B, Red grape waste; C extracted phenolics after treatment with RU-A01; D, extracted phenolics after treatment with RU-A03 after 7 days; E, extracted phenolics after treatment with RU-A03 after 14 days.**

A change in absorbance at wavelength within the phenol range before and after whole cell treatment (Figures 3.33; 3.34; 3.35) indicated polymerisation of the phenolics in grape waste which would be the action of peroxidase on the waste and/or the biodegradation, mineralisation and decolourisation of the grape waste (Figure 3.36) which could be the action of polyphenol oxidases. These colour changes are due to the oxidation of phenolic substrates. Further reaction results in the polymerisation leading to high molecular weight dark pigments, which are insoluble in water, resulting in the precipitation of these high molecular weight compounds out of solution. The decolourisation observed after the whole cell treatment with RU-A03 (Figure 3.36 and 3.37) could be the result of depolymerisation of the polymeric phenols and /or due to the precipitation of most of the phenolics out of solution, resulting in decolourisation. Such reactions have been observed with the use of oxidoreductase enzymes on kraft pulp.

Previous studies have shown a drop in enzyme activity partially attributed to the adsorption process whereby the enzymes adsorb to the pellets in whole cell systems. A more controlled system is hence necessary to accurately monitor the effect of enzymes on wastes and would be best accomplished in *in vitro* studies in the form of cell-free enzyme-based systems, to accurately assay the effect of the enzymes activity on the phenolic residues. In conclusion to this section, the three isolates, RU-A01, RU-A03 and RU-A06 have demonstrated the ability to bioconvert red grape waste.

#### 3.10.3.4 Enzyme induction using grape waste of RU-A01, RU-A03 and RU-A06.

Enzyme activity was determined during whole cell reactions in red grape waste. It is common for microbes to increase enzyme production in the presence of phenolicss, which are seen as nutritionally unavailable. In some cases the production of oxidoreductase enzymes are not produced constitutively but are only produced when induced. In other cases, oxidoreductase enzymes are produced at lower levels during periods of abundant nutrient availability, with enzyme activity production increasing when low levels of an inducer are introduced into the media, which would the situation with the three thermophiles. High concentrations of the inducer however may result in catabolite repression whereby the enzymes are no longer produced due to the high levels of substrate. This was observed at concentrations above 30% vol:vol of red grape waste, with levels of less than 5% of normal enzyme activity being detected. Peak enzyme activity was detected at 30% vol:vol grape waste in culture flasks with fold increases as high as 13.1 for lignin peroxidase in RU-A01 and 15.9 fold increase for polyphenol oxidase activity (Table 3.28). There was a greater increase in enzyme activity in the extracellular fraction, which could mean that the phenolics in the red grape waste were too large to enter through the cell membrane and hence the initial stage of phenolic bioconversion occurred extracellularly, as the phenolics were broken down to smaller subunits, they could then be taken up into the cell and bioconverted further.

**Table 3. 28** **The effect of enzyme production on isolate RU-A01 in the presence of 30% red grape waste in flask cultures.**

| | Activity µmols/mL/min | | Fold increase | | Specific activity µmols/mg/min | |
|---|---|---|---|---|---|---|
| | Intracellular | Extracellular | Intracellular | Extracellular | Intracellular | Extracellular |
| Lignin peroxidase | 0.957 | 6.5 | 3.5 | 13.1 | 7.9 | 65.3 |
| Polyphenol oxidase | 10.1 | 11.3 | 15.9 | 9.8 | 84.2 | 117.1 |
| Peroxidase | 3.8 | 3.5 | 2.1 | 3.2 | 31.7 | 35.1 |
| **Enzyme activity in the absence of grape waste** | | | | | | |
| Lignin peroxidase | 0.42 | 6.5 | 1 | 1 | 2.3 | 4.98 |
| Polyphenol oxidase | 0.65 | 0.13 | 1 | 1 | 5.2 | 11.9 |
| Peroxidase | 2.8 | 3.5 | 1 | 1 | 15.1 | 10.9 |

There was a significant increase in enzyme activity for isolate RU-A03 in the presence of red grape phenolics (Table 3.29). Initial enzyme activity studies showed that RU-A03 displayed the least enzyme activity, however in the presence of red grape waste, enzyme levels increased significantly which would mean that RU-A03 needs to be induced in order for it to produce appreciatable oxidoreductase enzyme levels. Other Actinomycetes species do respond to induction, whereby enzyme levels increase significantly which corroborates with the theory that in their natural environment, Actinomycetes will produce oxidoreductase enzymes only during periods of low nutrient availability (McCarthy, 1987).

**Table 3. 29 The effect of enzyme production on isolate RU-A03 in the presence of 30% red grape waste in flask cultures**

| | Activity µmols/mL/min | | Fold increase | | Specific activity µmols/mg/min | |
|---|---|---|---|---|---|---|
| | Intracellular | Extracellular | Intracellular | Extracellular | Intracellular | Extracellular |
| Lignin peroxidase | 1.96 | 4.21 | 3.0 | 10.0 | 9.8 | 46.8 |
| Polyphenol oxidase | 7.63 | 11.51 | 6.3 | 19.2 | 38.15 | 127.9 |
| Peroxidase | 5.3 | 4.2 | 0.85 | 4.3 | 26.5 | 46.7 |
| **Enzyme activity in the absence of grape waste** | | | | | | |
| Lignin peroxidase | 0.42 | 0 | 1 | 1 | 3.3 | 4.68 |
| Polyphenol oxidase | 0.8 | 0.6 | 1 | 1 | 1.98 | 6.66 |
| Peroxidase | 0.37 | 0.2 | 1 | 1 | 31.2 | 10.86 |

Enzyme activity did increase in RU-A06 however not has much as the other 2 isolates. Extracellular activity did increase the most, which would again tally with the theory that the initial stage of red grape waste phenolic enzymatic breakdown occurs extracellularly. Extracellular lignin peroxidase activity did however decrease which would suggest that the red grape waste phenolics, at that concentration had a negative effect on the enzyme production mechanism (catabolite repression; Table 3.30).

**Table 3. 30** **The effect of enzyme production on isolate RU-A06 in the presence of 30% red grape waste in flask cultures.**

| | **Activity** µmols/mL/min | | **Fold increase** | | **Specific activity** µmols/mg/min | |
|---|---|---|---|---|---|---|
| | **Intracellular** | **Extracellular** | **Intracellular** | **Extracellular** | **Intracellular** | **Extracellular** |
| Lignin peroxidase | 0.8 | 0.6 | 0.67 | 0.26 | 2 | 6.4 |
| Polyphenol oxidase | 0.5 | 7.1 | 1.25 | 7.30 | 0.2 | 75 |
| Peroxidase | 7 | 1.7 | 1.13 | 1.75 | 17.5 | 18.1 |
| **Enzyme activity in the absence of grape waste** | | | | | | |
| Lignin peroxidase | 1.2 | 0.8 | 1 | 1 | 2.98 | 24.6 |
| Polyphenol oxidase | 0.37 | 0.2 | 1 | 1 | 0.16 | 10.27 |
| Peroxidase | 2.8 | 3.5 | 1 | 1 | 10 | 10.34 |

## 3.11 EXTRACTION OF GRAPE WASTE PHENOLICS

The phenolic content in grape waste is high. Many methods have been employed in the extraction of grape phenolics and the recovery of phenols from plant tissue have been performed using various solvents such as ethanol (Kanner *et al.*, 1994), methanol, ethyl ether and ethyl acetate (Salagoity-Auguste *et al.*, 1984; Bonnila *et al.*, 1998, Gomez-Plaza et al., 1999). As the grape waste comes in solid form consisting of compressed skins and pips, a method was devised to first increase surface area of the grape waste mass and then extract the phenolics. The method used in this study (summarized in Figure 3.38) involved the homogenization of the grape waste in a buffered solution, removal of solid particles, and the use of several different organic solvents for solvent extraction.

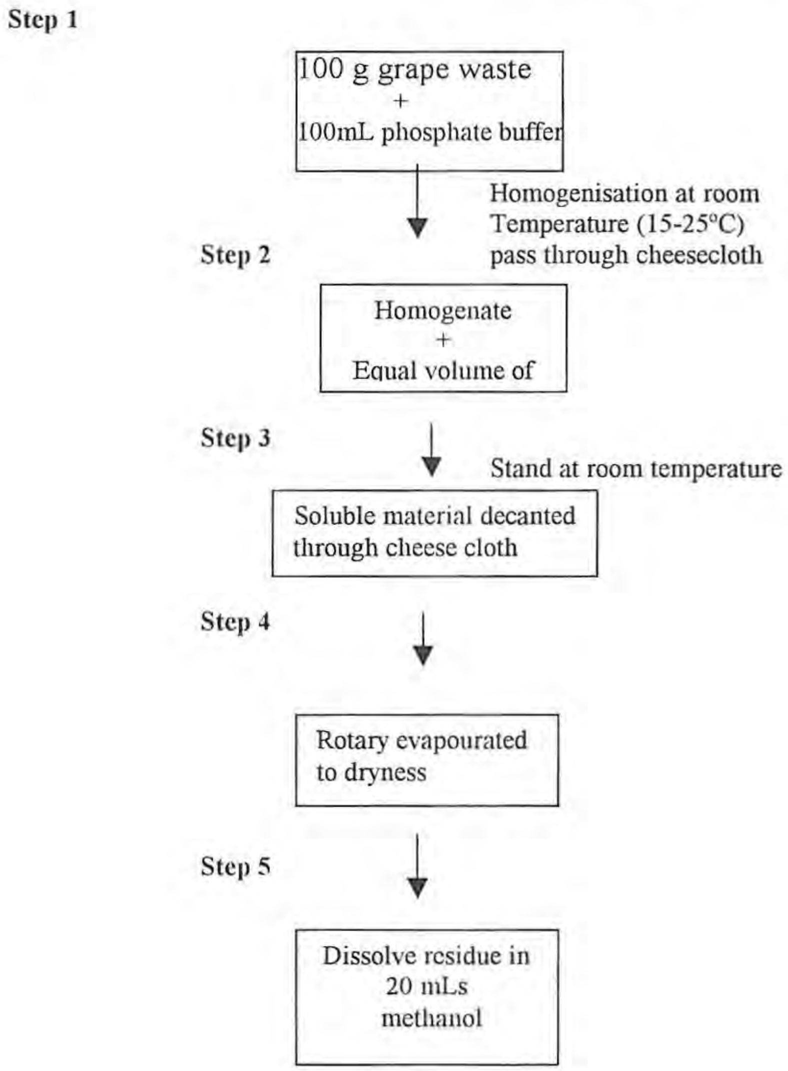


**Figure 3. 38** Summarized flow chart of the extraction protocol used for the extraction of grape waste phenolics in the study.

### 3.11.1 Phenol Determination of grape waste

The determination of total phenolic content is based on the reaction of Folin-Ciocaltaeu phenol reagent (Folin and Ciocaltaeu, 1927) and the aromatic ring of the phenols. According to Bray *et al.*, 1950 Folin-Ciocaltaeu is the most useful colorimetric method since it is a very sensitive and widely applicable method for determining the phenol content whilst other techniques such as Gibbs, using 2,6-dibromoquininone-4-cholorimide, are used for many phenols but are more troublesome to apply. Mohler and Jacobs (1957) and Chesseman and Wilson (1972) reviewed various colorimetric methods from phenols and concluded that methods such as the indophenol method, involving *p*-amino-*N*, *N*-dimethylaniline, and Gibbs method were unsuitable due to a combination of negative reactions with certain phenolics (often the *p*-substituted phenols), wide variations in the wavelength maximum absorbance to be used for quantification, and long complex procedures. The Folin-Ciocalteau reagent is employed to determine a wide range of phenolic substances due to its sensitivity, provided non-phenolic reducing compounds are absent. This reagent was developed for protein determination and therefore to quantify total phenolic content, the use of ethyl acetate is important in order to remove any protein present, allowing for the true value of phenolic content.

The nature of the chemical reaction (involved in the reduction of phosphomolybdic and phosphotungstic acid in the Folin-Ciocalteau phenol reagent) and the composition of the resultant heteropoly blue are unknown (Box, 1983). Colour is not given with the Folin-Ciocalteau reagent by all phenols. In general, the reactions commonly used for the detection of phenols are not specified for particular phenols but may serve as a guide to narrowing down identification. With colour reactions, shades of colour are difficult to describe precisely and final interpretation should be by direct comparison with a standard (Bray *et al.*, 1950).

Since the advantages of using the Folin-Ciocalteau phenol reagent clearly out weighed the advantages of the other reagents, Folin-Ciocalteau phenol reagent was the reagent of choice in this study.

Acetone, ethanol, methanol, ethyl acetate, 2-methoxy-ethanol and water were the solvents used to extract phenols from red and green grape waste. The efficiency of these solvents to extract phenols was determined by using the Folin-Ciocalteau phenol reagent to determine phenolic content.

**Table 3. 31 Phenolic content in Red and Green grape using different extraction solvents***

| **Solvent** | **Acetone** | **Ethanol** | **Methanol** | **Ethyl acetate** | **2-meoxy-ethanol** | **Water** |
|---|---|---|---|---|---|---|
| **Red grape waste (g/l)** | 366.7 | 39.2 | 43.3 | 15.2 | 111.1 | 46.9 |
| **Green grape waste (g/l)** | 253.54 | 73.5 | 50.6 | 115.6 | 115.2 | 67.9 |

Acetone proved to be the most effective solvent to extract the phenols based on the phenolic assays conducted on organic samples (Table 3.31). Acetone and the other solvents do not however exclusively remove phenols from the grape waste but also other compounds such as sugars, proteins, pigments etc. Therefore it was necessary to remove these sugars, proteins, pigments etc by adding ethyl acetate to collect the phenolics in the organic layer leaving the other compounds in the aqueous layer. In this study, ethyl acetate was the most useful solvent due to its relatively easy removal, the formation of a biphasic system with acetone, and separation of sugars, fiber and protein present in the stalks, seeds and grape skins.

The organic phase was then evaporated and the residue dissolved in methanol after which phenolic content was determined (Tables 3.32 and 3.33). The control fractions were used for all subsequent studies.

**Table 3. 32** **Total phenolic content of acidic, neutral and control fractions of red grape waste.**

| Red grape fraction | Phenolic content (g/kg) |
|---|---|
| Neutral | 5.108± 0.025 |
| Acidic | 6.408± 0.067 |
| Control | 5.054± 0.098 |

**Table 3. 33** **Total phenolic content of acidic, neutral and control fractions of green grape waste**

| Green grape waste | Phenolic content (g/kg) |
|---|---|
| Neutral | 3.513± 0.103 |
| Acidic | 3.930± 0.062 |
| Control | 3.553± 0.082 |

The grape waste residues were collected and dissolved in methanol and stored at -20°C until needed.

## 3.12 HPLC ANALYSIS OF GRAPE WASTE PHENOLICS

High performance Liquid chromatography (HPLC) is a popular method of analysis since it is not limited by the volatility or stability of a compound. HPLC has many applications, which include separation, identification, purification and quantification of various compounds. Separation occurs when certain compounds have different migration rates given in a particular column and mobile phase. The degree of separation is determined by the choice of stationary phase and mobile phase. Many HPLC methods have been developed with the use of different columns, mobile phases and run times for the separation of grape phenolics.

### 3.12.1 Chromatographic conditions

The chromatographic method used for the separation and analysis of grape waste phenolics was an isocratic system consisting of acetonitrile, milli Q water and glacial acetic acid (20:20:01). The most common wavelength used for the analysis of most plant phenols is 280nm.

### 3.12.2 HPLC spiking experiments

Standard phenolic model compounds found in grapes were used in spiking experiments. The retention times of individual phenolic acids are listed in Table 3.34. Spiking the retention times under the same analytical conditions with the corresponding authentic phenolic acid standards was also performed to further ascertain the identity of each of the suspected phenolic compounds.

### 3.12.3 Retention times of model compounds

The retention times of the model compounds used in the spiking experiments were determined (Table 3.34). Once that was determined an overlay method provided by the HPLC program enabled us to overlay several chromatograms over each other. In this case those of the actual waste and the chromatogram of the grape waste with the spiked compound were compared. Corresponding peaks would imply a positive match between the spiked sample and the original sample.

**Table 3. 34** **Retention times of model compounds using an isocratic HPLC method with a mobile phase of acetonitrile, milli Q water and glacial acetic acid (20:20:0.1).**

| COMPOUND | RETENTION TIME (MINUTES) |
|---|---|
| Ferulic acid | 30.71 |
| Gallic acid | 6.07 |
| Coumaric acid | 25.55 |
| Syringic acid | 24.87 |
| (+) catechin | 16.17 |
| Catechol | 10.82 |
| Vanillic acid | 20.39 |
| Vanillin | 22.58 |
| Cinnamic acid | 30.98 |
| Epicatechin | 14.65 |
| Protocatechuic acid | 8.21 |
| Caffeic acid | 14.73 |

### 3.12.4 HPLC separation of grape waste phenolics

HPLC allows for the separation of phenolic compounds resulting in a distinct phenolic fingerprint or profile that is unique and differs from any other phenolic sample. HPLC phenolic fingerprints even differ from one extraction to another of the same compound, in this case the HPLC phenolic fingerprints differed with each extraction of red and green grape waste depending on exposure time to acetone, extraction time with ethyl acetate and the dominant mass in the grape waste sample, i.e. skins, pips or stalks.

Figure 3.39 shows a typical chromatogram of an extract of red grape waste treated according to the summarized method in Figure 3.38 recorded at 280nm. 24 different peaks appear in the chromatogram. Some of them could be assigned to different phenolic compounds (Table 3.35) on the basis of their retention times as compared with those of the standards isolated in grapes. The relative amounts (%) and the retention times (minutes) have been noted and tabulated. Figure 3.41 shows a typical HPLC profile chromatogram of green grape waste recorded at 280nm. The phenolic HPLC separation fingerprint of green grape waste differs from that observed for the HPLC separation fingerprint of red grape waste. Protocatechuic acid and epicatechin/catechin were both identified in both red and green grape was residues, but in varying relative amounts. The majority of peaks isolated remained unidentified. These peaks could possibly be tannins, polymerized polyphenols, flavonols, anthocyanins and other glycosylated phenolics.

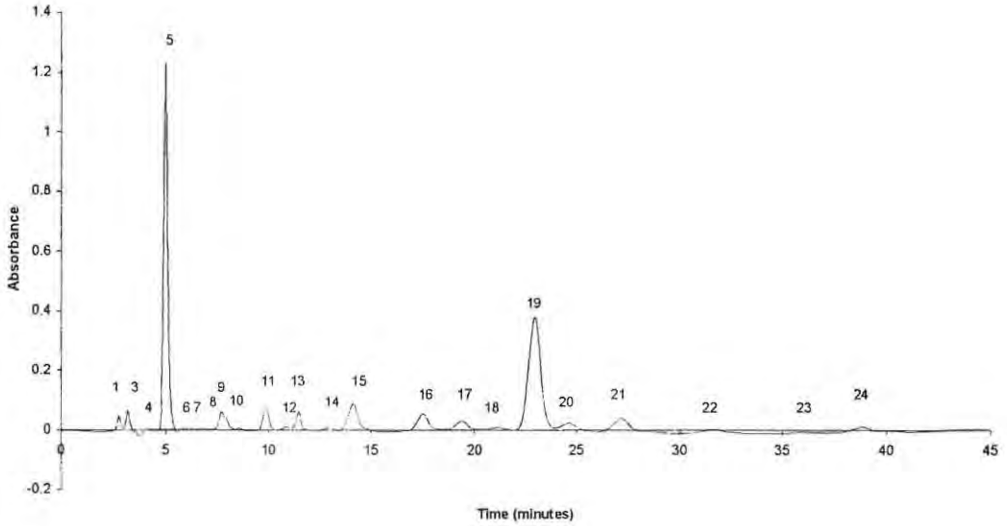


**Figure 3. 39** **HPLC profile of red grape extract extracted by the process of acetone, followed by ethyl acetate, which was evaporated to dryness. The resultant film was dissolved in methanol and run on HPLC using mobile phase acetonitrile, milli Q water and acetic acid (20:20:0.1)**

**Table 3. 35** **Compound, retention times and relative concentrations in red grape waste based on HPLC profile.**

| PEAK | COMPOUND | RETENTION TIME | RELATIVE CONCENTRATION (%) |
|---|---|---|---|
| 1 | Unknown | 2.28 | 0.057 |
| 2 | Unknown | 2.75 | 1.517 |
| 3 | Unknown | 3.17 | 2.399 |
| 4 | Unknown | 4.09 | 0.765 |
| 5 | Unknown | 5.00 | 32.743 |
| 6 | Gallic acid | 6.18 | 0.123 |
| 7 | Unknown | 6.68 | 0.04 |
| 8 | Unknown | 7.15 | 0.132 |
| 9 | Protocatechuic acid | 7.7 | 2.906 |
| 10 | Unknown | 8.57 | 0.121 |
| 11 | Unknown | 9.88 | 3.097 |
| 12 | Catechol | 10.86 | 0.570 |
| 13 | Unknown | 11.48 | 2.019 |
| 14 | Unknown | 13.01 | 0.577 |
| 15 | Caffeic acid | 14.13 | 5.753 |
| 16 | Catechin/epicatechin | 17.52 | 3.990 |
| 17 | Vanillic acid | 19.42 | 2.055 |
| 18 | Unknown | 21.17 | 0.475 |
| 19 | Vanillin | 22.97 | 30.874 |
| 20 | Syringic acid/coumaric acid | 24.64 | 1.604 |
| 21 | Unknown | 27.18 | 3.807 |
| 22 | Ferulic acid | 31.63 | 1.889 |
| 23 | Unknown | 35.95 | 0.279 |
| 24 | Unknown | 38.88 | 1.583 |
| 25 | Unknown | 40.27 | 0.053 |
| 26 | Unknown | 44.19 | 0.574 |

Gallic, protacatechuic, caffeic, vanillic, syringic, coumaric and ferulic acids were detected in red grape waste through spiking experiments, as well as vanillin and catechin/epicatechin. The remaining peaks remained unknown but they would most likely be tannins, glycosylated and polymerized phenolic acids and anthocyanins.

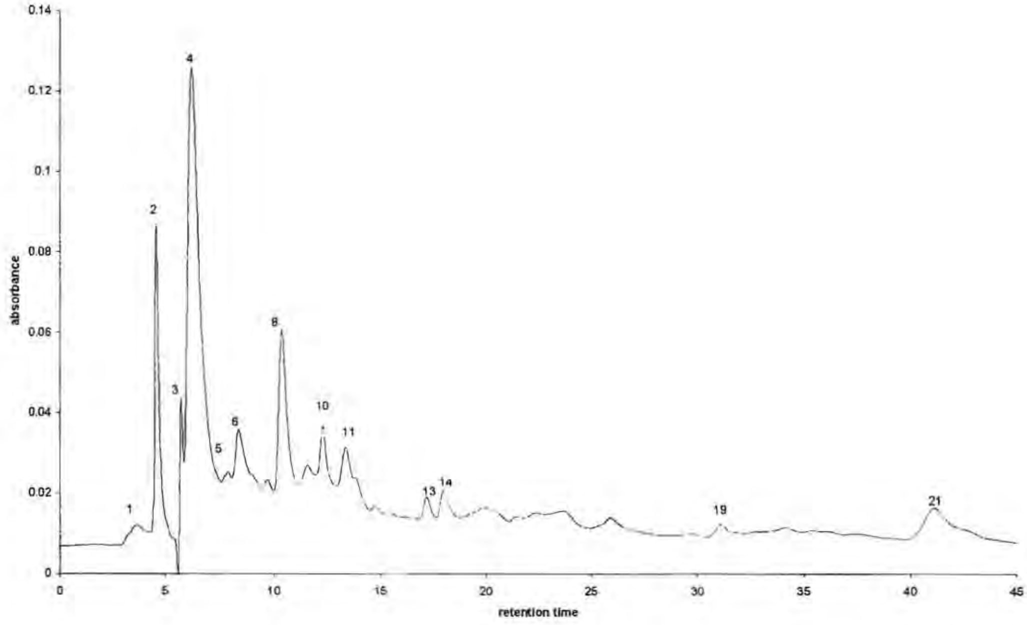


**Figure 3. 40** **HPLC profile of green grape waste extract extracted by the process of acetone, followed by ethyl acetate, which was evaporated to dryness. The resultant film was dissolved in methanol and run on HPLC using mobile phase acetonitrile, milli Q water and acetic acid (20:20:0.1).**

A total of 21 peaks were detected by HPLC during the separation of green grape waste. Gallic, protacatechuic, vanillic, coumaric and cinnamic acids were identified in green grape waste, as well as catechin/epicatechin (Figure 3.40). As can be seen the HPLC profile or fingerprint does differ between red and green grape waste, making HPLC a good method in not only separating compounds in a sample but also creating unique fingerprints that helps one to differentiate between different samples.

**Table 3. 36** **Compound, retention time and relative concentrations of peaks separated by HPLC of green grape waste, based on Figure 3.41.**

| PEAK | COMPOUND | RETENTION TIME | RELATIVE CONCENTRATION % |
|---|---|---|---|
| 1 | Unknown | 3.68 | 0.995 |
| 2 | Unknown | 4.56 | 11.034 |
| 3 | Unknown | 5.72 | 4.508 |
| 4 | Gallic acid | 6.19 | 45.854 |
| 5 | Unknown | 7.89 | 0.147 |
| 6 | Protocatechuic acid | 8.36 | 2.506 |
| 7 | Unknown | 9.71 | 0.293 |
| 8 | Catechol | 10.37 | 10.938 |
| 9 | Unknown | 11.57 | 2.847 |
| 10 | Unknown | 12.28 | 5.485 |
| 11 | Catechin/epicatechin | 13.35 | 6.303 |
| 12 | Unknown | 14.74 | 0.156 |
| 13 | Unknown | 17.17 | 1.090 |
| 14 | Vanillic acid | 17.95 | 1.419 |
| 15 | Unknown | 19.94 | 0.513 |
| 16 | Unknown | 21.48 | 0.113 |
| 17 | Coumaric acid | 23.67 | 0.999 |
| 18 | Unknown | 25.92 | 0.323 |
| 19 | Cinnamic acid | 31.11 | 0.751 |
| 20 | Unknown | 34.16 | 0.284 |
| 21 | Unknown | 41.15 | 3.423 |

## 3.13 ACTINOMYCETES CRUDE OXIDOREDUCTASE ENZYME TREATMENT OF RED AND GREEN GRAPE WASTE.

### 3.13.1 Actinomycetes crude oxidoreductase enzyme treatment of red and green grape waste without hydrogen peroxidase.

There is a growing recognition that enzymes can be used in many remediation processes to target specific pollutants for treatment. In this direction, recent biotechnological advances have allowed the production of cheaper and more readily available enzymes through better isolation procedures. The potential advantages of the enzymatic treatment as compared with conventional treatments include: the enzymes application to recalcitrant materials, operation at high and low contaminant concentrations over wide pH, temperature and salinity ranges, needs of biomass acclimatization and the easy control process amongst others (Duran and Esposito, 2000). The most recent research in this area includes the development of enzyme processes for the treatment of phenolic effluents.

The use of oxidoreductase enzymes to remove phenolic and aromatic amines from phenolic effluents has attracted much attention since the initial work of Klibanov *et al* (1980). Treatment of the phenolic residues was accomplished through the oxidation of substrates under the influence of the biocatalyst. The free radical reaction products spontaneously react to form polymeric products of reduced solubility, which can be separated from solution by precipitation or sedimentation (Ikehata and Nicell, 2000). Polyphenol oxidase and peroxidase reactions on phenolic substrates have been characterized and their mode of action described.

In this study the peroxidases and polyphenol oxidases isolated from the three thermophilic Actinomycetes isolates were used to determine their effectiveness on bioconverting liquid grape waste, which has a high phenolic content.

Peroxidase and polyphenol oxidase enzymes from RU-A01, RU-A03 and RU-A06 were grown in flask cultures to a point of maximum enzyme production, the extra- and intracellular crude enzymes were harvested and used to react with the grape waste with the objective of determining the effect of these enzymes on the grape waste phenolic samples. To determine the effects of peroxidase and polyphenol oxidases from the isolates, equal volumes of grape waste to crude intracellular extract were mixed together and incubated for 12 hours at 50°C. After incubation, the samples were then solvent extracted using ethyl acetate to remove proteins, the organic phase evaporated and the residue dissolved in methanol. Total phenol assays were carried out on the dissolved residue.

Crude extracts of the intracellular and extracellular protein from RU-A01, RU-A03 and RU-A06 were added to red and green grape waste extract and incubated at 50°C for 12 hours. For the intracellular studies, experiments were also carried out to compare the effectiveness of phenolic residue removal between fresh intracellular crude extract and intracellular crude extract that had been stored previously at -20°C. Tables 3.39 and 3.40 show that fresh intracellular crude has higher phenolic removal properties than the stored extracts. The samples were harvested and centrifuged briefly to remove any precipitate and the supernatant was subjected to HPLC analysis to trace the disappearance or appearance of peaks. A decrease in peak areas for some of the phenolic compounds and complete removal of some compounds occurred under the conditions stated above at 50°C. Total phenol determination assays were conducted.

High temperatures are known to have an effect on the degradation of phenols and this was evident in the control sample. Prior to incubation, the phenol content and HPLC profile of the control differed to the control after incubation. This may have been due to two factors: firstly the loss of phenols during the recovery process using ethyl acetate. Secondly, the effect of heat on the degradation of plant phenols. At 50°C, it is possible that the heat had an effect on the grape phenolics. Results from the analysis of total phenolics and HPLC profiles indicated that each thermophile reduced the phenolic content in both red and green grape waste at varying degrees (Table 3.37 and 3.38). A distinct peak was seen in most of the experiments after treatment with the crude extracts. This peak had a retention time of 7.46 minutes, which is yet to be identified.

**Table 3. 37** **Total phenol determination of cell free crude extracts reactions on red grape waste**

| RED GRAPE | PHENOLIC CONCENTRATION (G/KG) | |
|---|---|---|
| **Isolate** | **Intracellular crude** | **Extracellular crude** |
| Control | 1.325 | 1.962 |
| RU-A01 | 0.138 | 1.579 |
| RU-A03 | 0.075 | 0.967 |
| RU-A06 | 0.342 | 1.517 |

**Table 3. 38** **Total phenol concentration of cell free extracts reactions on green grape waste**

| GREEN GRAPE | PHENOLIC CONCENTRATION (G/KG) | |
|---|---|---|
| **Isolate** | **Intracellular crude** | **Extracellular crude** |
| Control | 1.720 | 1.720 |
| RU-A01 | 0.175 | 0.529 |
| RU-A03 | 0.175 | 0.617 |
| RU-A06 | 0.358 | 0.612 |

Phenol determination assays indicate that the intracellular crude extract bioconverted grape waste phenolics more than the extracellular crude extracts (Table 3.37 and 3.38). Based on the HPLC chromatograms however there is no significant difference hence leading to the premise that the extracellular enzymes probably reduced larger phenolic polymers found in grape waste into smaller units, which can be detected by the phenol determination assay using Foli-Ciocalteau phenol reagent. Extracellular enzymes may hence be most useful in producing valuable bioconversion products. Precipitation of the phenolic grape waste was observed and this was centrifuged and removed prior to HPLC analysis. Previous studies using peroxidases and polyphenol oxidases from Actinomyces have mainly focused on the effect of these enzymes on lignin structures so in terms of using oxidoreductase enzymes from Actinomycetes on phenolic wastes, this is a novel approach towards determining the applicability of these enzymes beyond lignin structures and focusing on industrial phenolic residues.

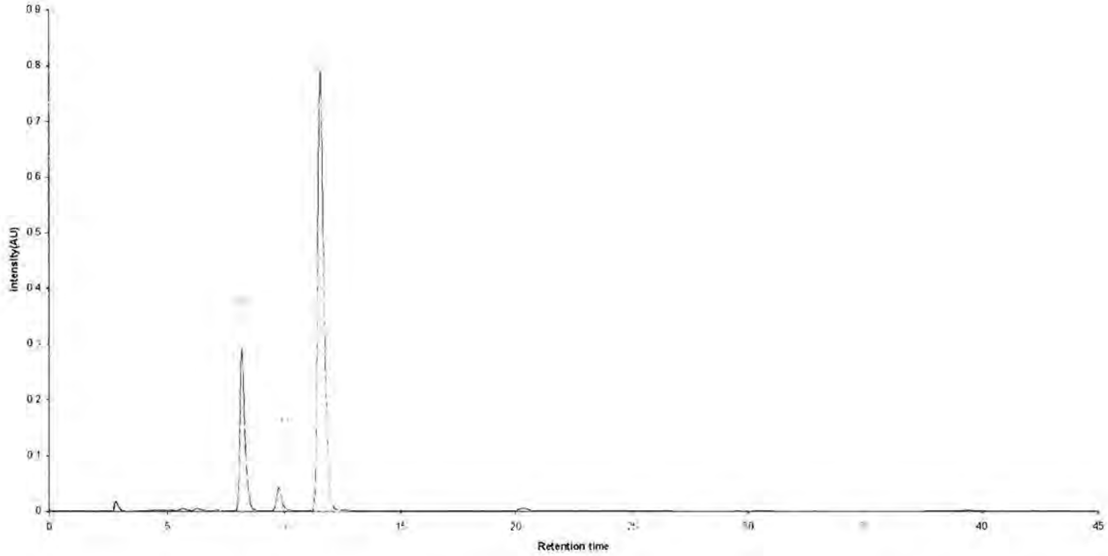


**Figure 3. 41** **HPLC profile of red grape waste after 12-hour treatment with intracellular crude extract from *Streptomyces* thermophile RU-A06. The sample was then solvent extracted with ethyl acetate to remove proteins and run on HPLC using Acetonitrile, water and acetic acid (20:20:0.1) as mobile phase at a flow rate of 1ml/min.**

Phenolic removal varied between isolate, however there were common peaks that were removed by all crude extracts (Table 3.39). Greater phenolic removal was detected using fresh intracellular crude extracts. Protocatechuic acid levels increased significantly after treatment with fresh intracellular crude from RU-A01. In whole cell systems the production of protocatechuic acid is the result of the bioconversion of ferulic or *p*-coumaric acids in Actinomyces. Ferulic acid was removed by fresh intracellular extracts, which could possibly have been bioconverted to protocatechuic acid (Sutherland *et al*, 1983). Catechol concentrations doubled after treatment with all crude extracts. In whole cell bioconversion studies, catechol is the end product of vanillin catabolism or the result of that specific metabolic bioconversion pathway.

Oxidoreductase enzymes from different microbes have varying substrate preferences hence the reason why some phenolic compounds were bioconverted more than others in this section would be due to the preference of the enzyme towards certain substrates. As previously discussed, substrate preference depends largely on optimal conditions for catalysis by the enzyme, reduction potential of the active species with respect to those of the substrates, as well as covalent and electrostatic factors. It can be assumed that the highly reactive substrates in the grape waste were bioconverted first or the most. Substrates that were not as reactive towards the enzymes were then only partially bioconverted due to a lesser substrate preference by the enzyme.

**Table 3. 39** **Compounds, retention times and relative concentrations (%) of peaks separated by HPLC of red grape waste before and after treatment with (1) stored crude intracellular cell free crude extracts stored at -20°C for a period exceeding 7 days, and (2) fresh crude intracellular extract. Equal volumes of crude extracts to grape waste were incubated shaking at 150 r.p.m at 50°C**

| Peaks | Retention Time | RELATIVE CONCENTRATION (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Control | RU-A01 | | RU-A03 | | RU-A06 | |
| | | | 1 | 2 | 1 | 2 | 1 | 2 |
| 1 | 2.24 | 3.610 | 0.766 | 0 | 0 | 0.889 | 0 | 0 |
| 2 | 2.94 | 12.689 | 14.103 | 6.147 | 10.273 | 0.826 | 1.117 | 1.285 |
| 3 | 4.43 | 11.457 | 7.015 | 0 | 0 | 0 | 0 | 0 |
| 4 | 4.7 | 1.118 | 0 | 0 | 0 | 0 | 0.258 | 0 |
| 5 | 5.12 | 0.399 | 0.412 | 0 | 0 | 0 | 0 | 0 |
| 6 | 5.6 | 5.036 | 1.789 | 0 | 0 | 0 | 0.162 | 0 |
| 7 | 5.8 | 19.593 | 3.998 | 0 | 0.456 | 4.111 | 0 | 0 |
| 8 | 6.47 | 9.907 | 2.685 | 0 | 0.529 | 0 | 0.194 | 0 |
| Protocatechuic acid | 7.29 | 2.959 | 5.210 | 72.013 | 0.115 | 0 | 0.021 | 1.725 |
| 10 | 8.62 | 3.299 | 52.582 | 21.840 | 81.920 | 93.170 | 21.234 | 89.43 |
| Catechol | 10.15 | 2.571 | 1.173 | 0 | 0 | 0 | 3.321 | 0.439 |
| 12 | 12.07 | 1.551 | 0.744 | 0 | 0.211 | 0 | 72.985 | 5.306 |
| 13 | 20.82 | 14.999 | 4.369 | 0 | 3.254 | 0 | 0.604 | 0 |
| Ferulic acid | 31.89 | 1.905 | 1.064 | 0 | 0.252 | 0 | 0 | 0 |
| 15 | 39.24 | 3.922 | 1.439 | 0 | 0.480 | 0 | 0.105 | 0 |
| 16 | 41.08 | 4.985 | 1.537 | 0 | 0.406 | 0 | 0 | 0 |

For green grape extract treatment, gallic and coumaric acids were removed to an extent after treatment with the extracts of all 3 isolates (Table 3.40). Peak 8 increased significantly after treatment with the extracts of all 3 isolates. The compound could possible be a polymerized phenolic compound, which would be the product of the activity of peroxidases and polyphenol oxidases.

Fresh intracellular crude extract was more efficient in removing grape waste phenolics as seen in Tables 3.39 and 3.40. Preservation of crude extract activity is an important issue and needs to be addressed in future work. Earlier work showed that freeze drying compromises activity,and in some cases inactivated the enzymes.

**Table 3. 40** **Compounds, retention times and relative concentrations (%) of peaks separated by HPLC of green grape waste before and after treatment with (1) stored crude intracellular cell free crude extracts stored at -20°C for a period exceeding 7 days, and (2) fresh crude intracellular extract. Equal volumes of crude extracts to grape waste were incubated shaking at 150 r.p.m at 50°C**

| | | RELATIVE CONCENTRATION (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Compound** | **Retention time** | Control | **RU-A01** | | **RU-A03** | | **RU-A06** | |
| | | | 1 | 2 | 1 | 2 | 1 | 2 |
| 1 | 2.22 | 7.863 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 2.93 | 0.784 | 3.665 | 33.043 | 1.819 | 7.186 | 5.809 | 4.261 |
| 3 | 3.61 | 52.645 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 4.65 | 5.282 | 0 | 4.940 | 0 | 1.225 | 0.636 | 0.566 |
| 5 | 5.35 | 9.976 | 37.238 | 0 | 13.427 | 0 | 0.242 | 0.247 |
| Gallic acid | 6.42 | 6.826 | 7.090 | 0 | 1.303 | 0.170 | 0.099 | 0.046 |
| 7 | 7.03 | 0.476 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | 8.57 | 2.299 | 44.046 | 62.016 | 80.785 | 90.763 | 92.419 | 93.533 |
| 9 | 10.25 | 0.720 | 1.763 | 0 | 0.855 | 0.128 | 0 | 0 |
| 10 | 12.05 | 1.508 | 1.905 | 0 | 0.529 | 0 | 0 | 0 |
| 11 | 14.05 | 1.206 | 0 | 0 | 0 | 0 | 0 | 0 |
| Caffeic acid | 16.87 | 3.906 | 0 | 0 | 0 | 0 | 0 | 0.154 |
| 13 | 18.24 | 2.782 | 4.293 | 0 | 1.282 | 0.407 | 0 | 1.086 |
| Coumaric acid | 26.89 | 3.726 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 3. 41** **Relative peak concentrations of phenols treated with crude extracellular extracts in red grape waste.**

| | | RELATIVE CONCENTRATION (%) | | | |
|---|---|---|---|---|---|
| **Peaks** | Retention times | Control | **RU-A01** | RU-A03 | **RU-A06** |
| 1 | 1.16 | 0 | 2.597 | 100 | 0 |
| 2 | 3.16 | 28.985 | 7.189 | 0 | 10.011 |
| 3 | 4.27 | 42.143 | 0 | 0 | 0 |
| Gallic acid | 6.11 | 0 | 18.325 | 0 | 16.316 |
| Protocatechuic acid | 7.25 | 17.827 | 0 | 0 | 0 |
| Catechol | 10.20 | 2.692 | 2.480 | 0 | 1.848 |
| Caffeic acid | 14.41 | 0.541 | 0 | 0 | 0 |
| Catechin/epicatechin | 16.05 | 0 | 0 | 0 | 0 |
| 9 | 20.08 | 0 | 6.082 | 0 | 6.153 |
| Vanillin | 22.13 | 0.472 | 0.775 | 0 | 0.981 |
| 11 | 23.67 | 0 | 1.842 | 0 | 2.327 |
| 12 | 27.63 | 5.094 | 0 | 0 | 0 |
| 13 | 29.43 | 0 | 2.938 | 0 | 0 |
| 14 | 34.32 | 0 | 55.608 | 0 | 58.539 |
| 15 | 41.07 | 2.246 | 0 | 0 | 0 |

Gallic acid was an end product produced after treatment of red grape waste by RU-A01 and RU-A06 extracellular crude extract. The relative concentration of vanillin increased after the treatment by extracellular crude enzyme extracts from RU-A01 and RU-A06, which is a by-product of ferulic acid catabolism. Unidentified peak 14 had a significantly high concentration after treatment with RU-A01 and RU-A06. Only one product was formed after the treatment with extracellular crude of RU-A03 at a retention time of 1.16 minutes.

**Table 3. 42** **Peak concentrations before and after treatment with extracellular crude extract on green grape waste.**

| | | RELATIVE CONCENTRATION (%) | | | |
|---|---|---|---|---|---|
| **Peaks** | **Retention time** | **Control** | **RU-A01** | **RU-A03** | **RU-A06** |
| 1 | 3.15 | 0 | 29.537 | 12.724 | 23.110 |
| 2 | 4.50 | 5.957 | 0 | 0 | 0 |
| 3 | 5.38 | 8.202 | 38.687 | 74.015 | 52.023 |
| Protocatechuic acid | 8.45 | 12.450 | 0 | 0 | 0 |
| Catechol | 10.25 | 0 | 10.909 | 0 | 0 |
| 6 | 13.81 | 31.666 | 0 | 0 | 0 |
| 7 | 15.93 | 0 | 0 | 2.740 | 6.107 |
| 8 | 18.25 | 2.267 | 0 | 0 | 0 |
| Vanillic acid | 20.23 | 0 | 20.867 | 10.521 | 18.760 |
| Cinnamic acid/ferulic acid | 29.05 | 19.402 | 0 | 0 | 0 |
| 11 | 35.28 | 20.055 | 0 | 0 | 0 |

Protocatechuic acid was completely removed after treatment of the green grape waste by the 3 isolates. This correlates with whole cell studies using protocatechuic acid where it was catabolized by the isolates (Section 3.9). A catechol peak was detected after treatment with extracellular crude extract from RU-A01, which is usually the end product of the bioconversion of vanillin in some Actinomycetes (Sutherland *et al.*, 1983). The appearance of vanillic acid after treatment with the 3 isolates may also be the result of vanillin catabolism as vanillic acid is often a by-product of vanillin catabolism (Sutherland *et al.*, 1983). The peak that represents cinnamic acid or ferulic acid was completely removed by all three isolates.

In comparison to the mode of action by intra-and extracellular crude extracts, there are subtle differences in terms of peak removal preferences. Based on phenolic determination assays after treatment with extracellular crude extracts, phenolic removal is not as high as that of intracellular crude extracts which could mean that phenolic compounds in the grape waste are being converted to other phenolic compounds and possibly depolymerization of large compounds into smaller phenolic units.

### 3.13.2 Studies with crude grape extract and hydrogen peroxide using crude enzyme extracts from RU-A01, RU-A03 and RU-A06

In whole cell systems where peroxidases are used, the microbes have the ability to generate its own supply of hydrogen peroxides for the normal function of the peroxidases. In cell free enzyme systems, the hydrogen peroxide needs to be added in the reaction in order for the peroxidases to function. The concentration and quantity of the hydrogen peroxidase is crucial for optimal activity of the peroxidase. High concentrations of hydrogen peroxidase will inactivate the enzyme and low concentrations will decrease peroxidase activity. The mode of action of peroxidases includes depolymerization, polymerization, demethylation and oxidation. A 50mM concentration of hydrogen peroxodase was used according to previous studies on this area (Antonopoulous *et al.*, 2001).

**Table 3. 43** **Compounds, retention times and relative concentrations (%) of peaks separated by HPLC of red grape waste before and after treatment with crude intracellular extracts from RU-A01, RU-A03 and RU-A06. Equal volumes of crude extracts to grape waste and 50mM hydrogen peroxide were incubated shaking at 150 r.p.m at 50°C.**

| | | RELATIVE CONCENTRATION (%) | | | |
|---|---|---|---|---|---|
| **Peaks** | **Retention time** | **Control** | **RU-A01** | **RU-A03** | **RU-A06** |
| 1 | 2.82 | 12.635 | 9.012 | 1.401 | 0.990 |
| 2 | 4.27 | 11.688 | 6.434 | 0.611 | 0.592 |
| 3 | 4.95 | 26.008 | 0 | 1.313 | 1.007 |
| 4 | 5.42 | 25.805 | 0 | 0.842 | 0.451 |
| 5 | 6.48 | 6.885 | 3.145 | 0.150 | 0 |
| 6 | 7.46 | 3.390 | 81.409 | 93.517 | 96.176 |
| 7 | 33.99 | 13.588 | 0 | 0 | 0 |

**Table 3. 44** **Compounds, retention times and relative concentrations (%) of peaks separated by HPLC of green grape waste before and after treatment with crude intracellular extracts from RU-A01, RU-A03 and RU-A06. Equal volumes of crude extracts to grape waste and 50mM hydrogen peroxide were incubated shaking at 150 r.p.m at 50°C.**

| | | RELATIVE CONCENTRATION (%) | | | |
|---|---|---|---|---|---|
| Peaks | Retention time | Control | RU-A01 | RU-A03 | RU-A06 |
| 1 | 2.81 | 12.525 | 1.800 | 1.171 | 1.400 |
| 2 | 4.21 | 23.501 | 2.028 | 1.115 | 1.675 |
| 3 | 4.86 | 2.912 | 0.436 | 0 | 0.270 |
| 4 | 5.70 | 3.857 | 0.276 | 0 | 0 |
| 5 | 6.29 | 0 | 55.140 | 1.344 | 1.680 |
| 6 | 7.59 | 8.893 | 36.908 | 95.697 | 94.518 |
| 7 | 8.29 | 4.809 | 0 | 0 | 0 |
| 8 | 11.91 | 10.866 | 0 | 0 | 0 |
| 9 | 15.35 | 8.936 | 2.828 | 0.267 | 0.457 |
| 10 | 17.95 | 10.751 | 0.583 | 0 | 0 |
| 11 | 21.42 | 7.411 | 0 | 0 | 0 |
| 12 | 27.40 | 5.538 | 0 | 0 | 0 |

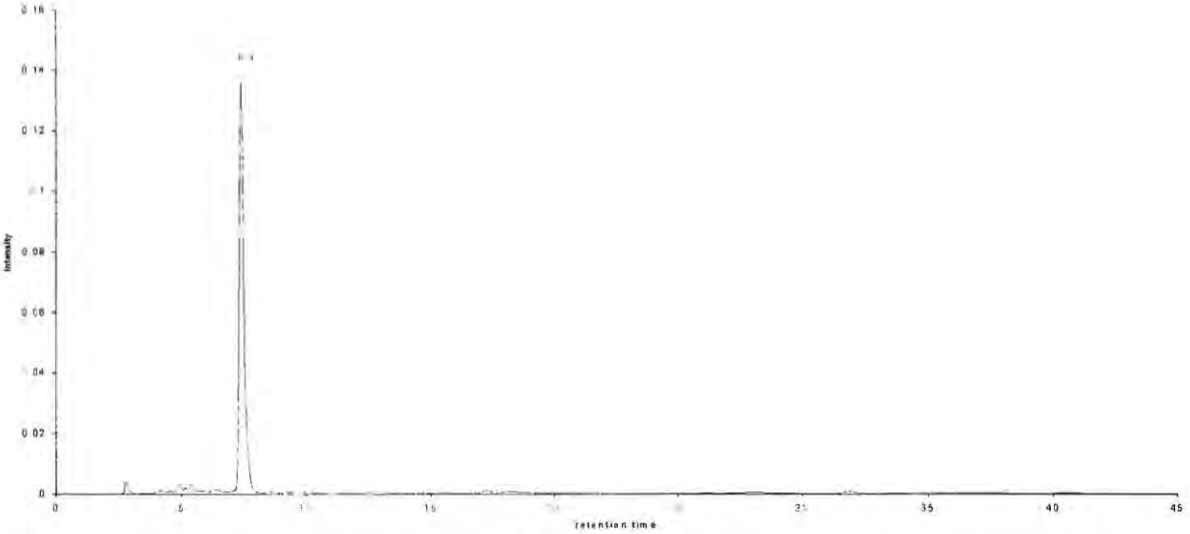


**Figure 3. 42** **Green grape waste after treatment with intracellular protein extract from RU-A03 and equal volume of 50mM hydrogen peroxide.**

The basic HPLC fingerprint was similar for all intra-and extracellular treatments with the three thermophiles was similar making it difficult to determine which enzyme; peroxidase or polyphenol oxidase was responsible for the reduction in total phenols and peaks (Figures 3. 42). There was a significant decrease in phenolics and again the prominent peak at time 7.46 minutes (Table 3.43 and 3.44) was present in all samples after treatment with crude extracts from the 3 thermophilic isolates, with all compounds eluting out before time 9 minutes. At this point, based on the results, its difficult to accurately deduce which enzyme, peroxidase or polyphenol oxidase is responsible or dominant in terms of phenolic removal in grape waste.

Irrespective of the natural role and function of these enzymes, the high levels of production, specific activity and particularly the robustness of these enzymes suggest potential applications. They exhibited high thermostability, being able to biconvert phenolic residues in grape waste at 50°C, which in comparison to the maximum temperature stability limit in fungi at 30-37°C, these bacteria and their enzymes prove to have immense potential in biconversion processes. Enzyme activity speed doubles for every 10°C increase, hence this would suggest that under optimal conditions bioconversion rates would increase 2/3 fold higher than that of in fungi which operate at a lower optimal temperature. In addition, high temperatures increase accessibility of the reaction by- and end products and decreases contamination levels.

A few Actinomycetes species have been isolated and their enzymes characterized. Based on their results, researchers have reached the conclusions that the use of these microbes and their enzymes in bioremediation and bioconversion processes would be viable processes, however they have not actually demonstrated the ability of Actinomycetes species on bioremediating or bioconverting a model phenolic waste. Previous studies of peroxidases and polyphenol oxidases in Actinomycetes have focused on the ability of these bacteria to bioconvert single compounds commonly found in targeted phenolic-based effluents (Spiker *et al.*, 1992, Antonopoulos et al., 2001). No work to date has been carried out on demonstrating the effect of these enzymes in whole cell or enzyme-based systems on the actual phenolic waste. In this study, it has been demonstrated that the three isolates, RU-A01, RU-A03 and RU-A06 can bioconvert a phenolic waste resulting in the reduction of the phenolic content in the waste as well as complete removal of some of the phenolic compounds in the phenolic waste. This study hence reports the first demonstration of the application of thermophilic peroxidases and polyphenol oxidases from thermophilic Actinomycetes on a phenolic waste.

The search for and identification of by-and end products would be required, possibly by the fractionation of the extracts and the possible application of nuclear magnetic resonance (NMR) and liquid chromatography-mass spectrometry (LC-MS). These techniques would be needed to identify the chemical structures of all products to further elucidate the bioconversion. The formation of the major peak in most of the cell-free reactions would need to be identified. These results confirmed the hypothesis that the ability of the isolates to catabolize the model phenolic compounds would suggest that their ability to bioconvert a phenolic waste would be inherent which in this study was grape waste generated in the wine-making industry in South Africa.

## 3.14 Molecular cloning and isolation of a polyphenol oxidase or peroxidase gene in the thermophilic *Streptomyces* isolates.

Gene cloning is a useful technique in biotechnology, resulting in the production of protein of interest in high yield. In addition to the three techniques available to researchers which include screening of microbes from soil and similar environments to find new high yielding isolates; medium and process parameters optimisation to increase yield in known cultures and also mutagenesis and selection of the new overproducing mutant, gene cloning offers a useful option. The process of gene cloning enables researchers to clone a gene of an already known biocatalyst and over-express it or modify it for the desired characteristics as well as to study its primary structure.

In cases when the genes in a particular group of microbes have not been isolated, researchers can use cloning techniques in order to isolate the genes responsible for the production of a particular biocatalyst by means of screening clones for a particular activity. In order to detect the desired protein, heterologous genes must be expressed at a relatively high level for it to be detected at the screening stage. Thus the most important parameter is the fractional abundance of the protein at the time the culture is harvested. Induction of the heterologous gene at some later stage of growth to obtain a high fractional synthetic rate for the protein is commonly done. One can arrange an expression module that yields a reasonable fractional synthetic rate throughout the growth period and harvest the culture at the highest possible cell concentration. *E.coli* can be grown to very high cell densities, thus one can obtain high fractional synthetic rate for any heterologous protein in *E.coli*.

The purpose of this section was for two main purposes:

1). Originally, the numerous oxidoreductase isozymes often detected from Actinomycetes were thought to be a result of posttranslational variability of the same gene product, but in terms of fungi, several research groups have been able to isolate and characterize several genes of the same enzyme. The aim was to determine if this was the case with the thermophilic *Streptomyces* in the three novel isolates.

2.) Previous genetic studies concerning oxidoreductase enzymes have been conducted on *Streptomyces* species and their genes isolated and characterized, namely lignin peroxidases however their sequence data has not been published (Thomas and Crawford, 1998). The aim was to screen for the genes, study and compare is genomic structure to other commonly producing microbes and also use it for further gene techniques such as gene shuffling.

In previous studies on Actinomycetes, in particular *Streptomyces* species, various expression vectors and strains have been used, most commonly *Streptomyces* expression strain *Streptomyces lividans* TK64 and expression plasmid pIJ702. This vector has been used successfully in expressing Actinomycetes proteins. In this particular case however, this expression vector was found to be undesirable due to the fact that *Streptomyces lividans* TK64 is known to produce its own peroxidase and polyphenol oxidase (Mhlanga, 1998, Crawford, 1990,), which would complicate the interpretation of data. Other expression hosts include *Pichia pastoris*, which has been used to express *Streptomyces* lignin peroxidases, xylanases and endoglucanases with some success. *E.coli* expression vectors have also been used to express *Streptomyces* proteins with a great degree of success (Tarr, 2000). Faster growth rates, high production rates of heterologous proteins and well studied expression mechanisms of *E,coli* expression vectors led to the use of this system in an attempt to isolate and identify a peroxidase or polyphenol oxidase gene from any one of the three thermophilic isolates.

The PCR primers, bacterial strains and plasmids used in this study are listed in Table 3.45 and Figure 3.47. The cloning procedure in isolating either a polyphenol oxidase or a peroxidase gene from any one of the thermophilic *Streptomyces* isolates is summarized in the flow diagram (Figure 3.44).

**Table 3. 45** **PCR primers, bacterial strains and plasmids used in this study**

| **Primers** | **Sequences** | **Source** |
|---|---|---|
| Forward *Cat C* gene | 5`CCCAGGTCATCTACCGGGGC3` | Hahn *et al.*, 2000 |
| Reverse *Cat C* gene | 5`CCCGGGCGGGGCGGGGTGG3` | Hahn *et al.*, 2000 |
| **Description and/or relevant genotype** | | |
| **Bacterial strains** | | |
| *E.coli* Top10F` | F`{lac$^{a}$Tn10(Tet$^{R}$)}mcrA-(mrr-hsdRMS-mcrBC)Φ80lacZ-M15-lacX74 deoR recA1 araD139 -(ara-leu)7697 galU galK rpsL endA1 nupG. | Invitrogen |
| **Plasmids** | | |
| pSE280 | pKK233-2 derivative | Invitrogen |
| pSE380 | PTrc99A derivative | Invitrogen |

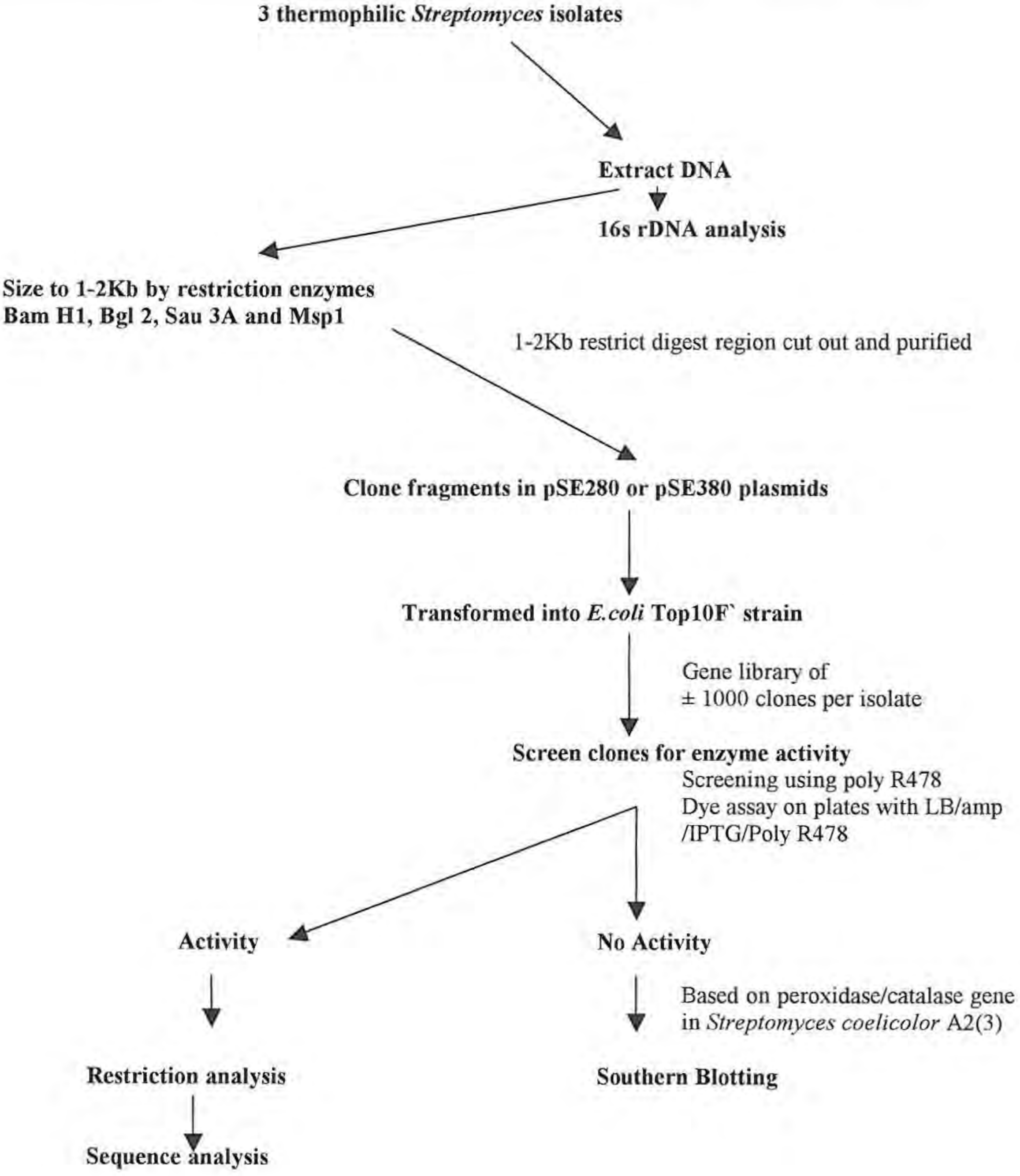


**Figure 3. 43** **Flow diagram of summarized methods used in the isolation of a peroxidase or polyphenol oxidase genes from the three thermophilic *streptomyces* isolates.**

Total DNA was extracted using the QiaNeasy DNA extraction kit (Figure 3.44) and quantified. The DNA extracts were stored at 4°C until required.

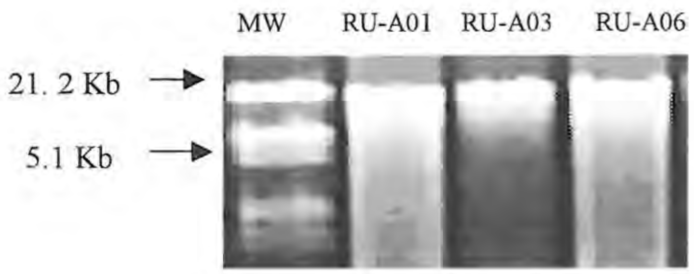


**Figure 3. 44** **DNA extracted from RU-A01, RU-A03 and RU-A06 using the Qiagen DNeasy extraction kit.**

The DNA was fragmented using restriction enzymes according to compatible ligation sites within the superlinker region of the vectors (Table 3.46). The restriction enzymes used on the *Streptomyces* DNA isolates were *BamHI*, *Bgl II*, *Sau 3A* and *Msp I*. Single, double and triple digests were carried out using *BamH I*, *Bgl II* and *Sau 3A* (Figure 3.45) which are known to cut *Streptomyces* DNA frequently resulting in small DNA fragments while *Msp I* cuts less frequently generating larger DNA fragments. Double and triple digests were carried out to increase variety in the fragments hence increasing the size of the genomic library. DNA ladders were formed when the restriction enzymes *BamHI*, *BglII* and *Sau3A* were used (Figure 3.45). DNA in the 1-2 Kilo base pair region was extracted, gel purified and ligated to vector pSE280, which had been linearised with a compatible restriction enzyme prior to ligation. Ligation was carried out according to the ligation kit manufacturer's instructions. The ligated DNA was then transformed into *E.coli* Top10F` (Table 3.45) and plated out onto Luria Broth agar plates with ampicillin, IPTG and Poly R478 dye.

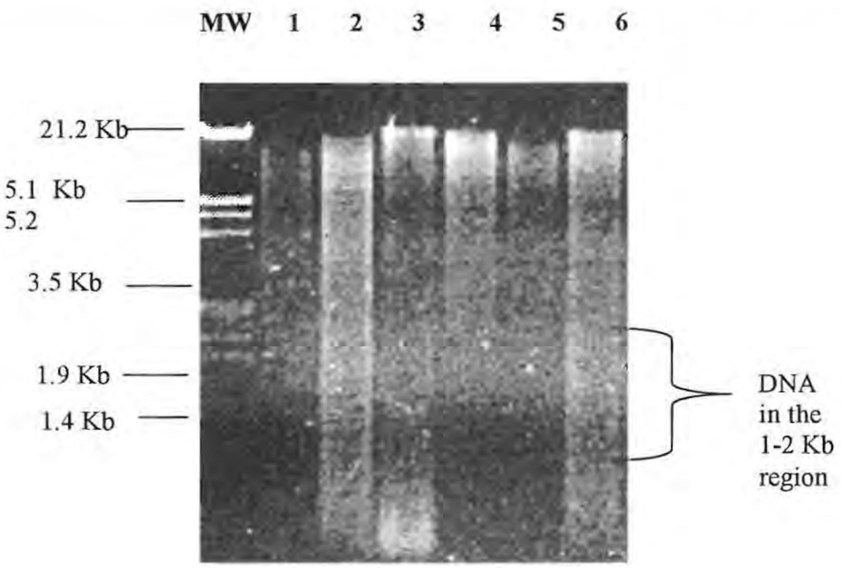


**Figure 3. 45** **Thermophilic *Streptomyces* DNA fragmented by restricion enzymes Lanes 1,2 and 3, RU-A01, RU-A03 and RU-A06 fragmented with *BamHI*; Lanes 4,5 and 6, RU-A01, RU-A03 and RU-A06 fragmented with *Bgl II*.**

**Table 3. 46** **Superlinker enzyme positions in vectors (a) pSE280 and (b) pSE380**

| A SUPERLINKER | | | | B SUPERLINKER | | | |
|---|---|---|---|---|---|---|---|
| **Enzyme position** | | | | **Enzyme position** | | | |
| Nco I | 275 | Kpn I | 460 | Nco I | 275 | Xba I | 450 |
| BstE II | 283 | Hpa I | 468 | Dra III | 294 | Kpn I | 460 |
| Dra III | 294 | Nae I | 478 | Bsm I | 304 | Nae I | 478 |
| PflM I | 294 | SnaB I | 484 | BstB I | 306 | SnaB I | 484 |
| Bsm I | 304 | Stu I | 507 | EcoR I | 308 | Stu I | 507 |
| BstB I | 306 | Avr II | 508 | BspM I | 326 | Avr II | 508 |
| EcoR I | 308 | Nsi I | 518 | Sal I | 332 | Eag I | 524 |
| Sal I | 332 | Eag I | 524 | Aat II | 333 | Not I | 524 |
| Aat II | 333 | Not I | 524 | Bsu36 I | 338 | Pst I | 535 |
| Bsu36 I | 338 | Pst I | 535 | Xma I | 353 | Nru I | 562 |
| Xma I | 353 | **Nar I**[b] | 540 | Sma I | 355 | Sac II | 569 |
| Sma I | 355 | Mlu I | 551 | Eco0109 I | 357 | Xho I | 584 |
| Apa I | 360 | Nru I | 562 | **BamH I**[a] | 369 | Sac I | 585 |
| **BamHI**[a] | 369 | Sac II | 569 | **Bgl II**[a] | 396 | Spe I | 594 |
| BcI I | 383 | Xho I | 584 | Eco47 III | 420 | Sfi I | 607 |
| **Bgl II**[a] | 396 | Sac I | 585 | Nhe I | 438 | Afl II | 619 |
| BssH II | 402 | Spe I | 594 | | | HindIII | 622 |
| EcoR V | 414 | Sfi I | 607 | | | | |
| Nhe I | 438 | Afl II | 619 | | | | |
| Sph I | 438 | Hind III | 622 | | | | |
| Xba I | 450 | | | | | | |

[a] Enzyme position sites compatible with *BamHI, BglII* and *Sau 3A* restriction sites on *Streptomyces* DNA

[b] Enzyme position sites compatible with *Msp I* restriction sites on *Streptomyces* DNA

**Figure 3. 46** **Plasmids used in cloning of DNA from the *streptomyces* isolates (A) pSE280 plasmid, (B) pSE380 and both plasmid run on an 1% agarose gel.**

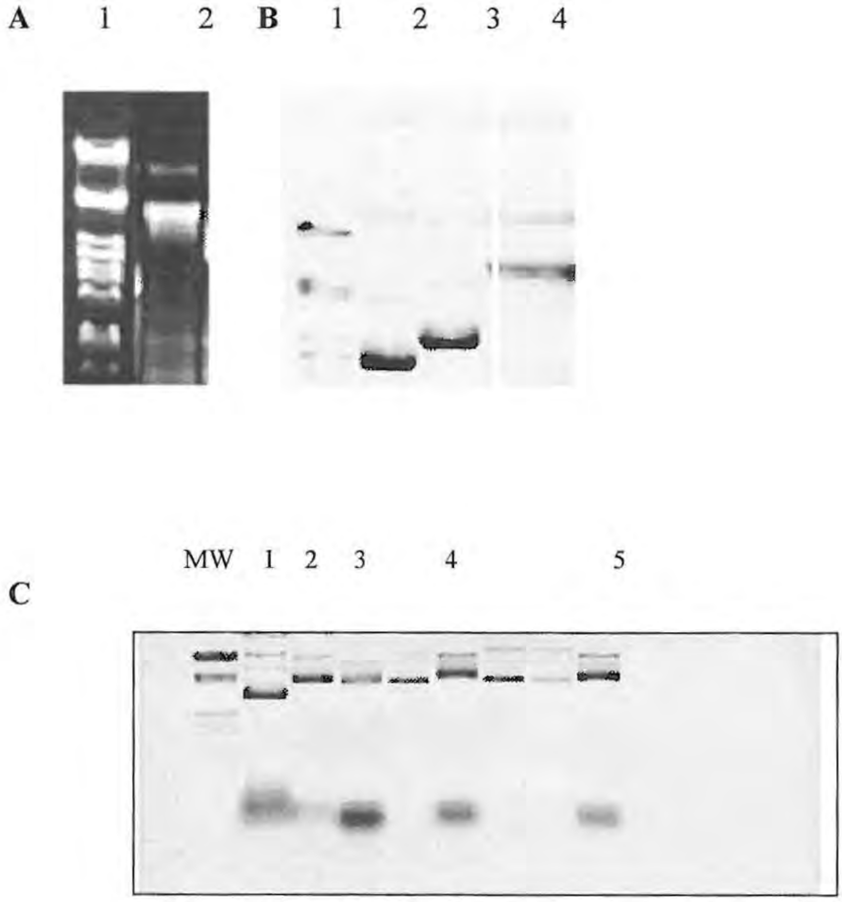


**Figure 3. 47** **Clones isolated in the study and vectors. A. Lane 2, RU/A03/3; B. Lane 2, pSE280; Lane 3, pSE380; Lane 4, RU-A03/19; C, Lane 2 RU-A06/27; Lane 3, RU-A03/5; Lane 4, RU-A06/8; Lane 5, RU-A01/20.**

### 3.14.1 Screening clones for activity.

In order to isolate clones which have the desired activity, its important to develop a foolproof screening assay. In some cases this appears to be the bottleneck of such research considering the large number of clones needed to assay for activity. Poly R478, a dye that has been used to detect the presence of oxidoreductase activity in fungi and bacteria (Gold and Glenn, 1988) was incorporated into the growth media after transformation. Other screening possibilities included the use of bromophenol blue with an aromatic acid incorporated into the media, a colour change from blue to green would indicated the utilization of the aromatic acid due to the presence of an oxidoreductase enzyme. The disadvantages of the latter method outweighed those of the former method but both methods do have their loopholes. In the presence of oxidoreductase enzymes, poly R478, a red dye is decolorized. When localized on agar, the resultant decolourisation resulting in a halo, which is not permanent and after a period of time, the red dye in its immediate vicinity diffuses through into the halo. The degree of decolourisation and halo intensity is directly related to the amount and concentration of the enzyme produced.

The volume of transformed clones was initially reduced from 100 μl to 50 μl to ensure that the clones were well spaced out and hence to clearly determine which clones gave halos. The degree of clearing was very low and hence difficult to determine if the clone was producing any oxidoreductase enzymes. Clones were therefore selected if there appeared to be even remote signs of clearing. Based on that, 7 clones were isolated (Table 3.47 and Figure 3.47) and assayed further for activity.

**Table 3. 47** **Clone, restriction inserts and size of inserts of the 7 clones selected on screening plates with the dye Poly R478 based on their ability to decolourise the dye**

| CLONE | RESTRICTION INSERTS | | SIZE OF INSERTS |
|---|---|---|---|
| **Isolate origin of insert/number** | **DNA** | **Vector** | |
| RU-A03/5 | BgI II | BamH I | 1.3 Kb |
| RU-A06/8 | BgI II | BamH I | 1.2 Kb |
| RU-A03/19 | Msp I | Nar I | 1.7 Kb |
| RU-A06/27 | BamH I/BgI II/Sau 3A | BgI II | 1.4 Kb |
| RU-A03/26 | BamH I/BgI II | BgI II | 1.1 Kb |
| RU-A03/3 | BgI II | BamH I | 1.1 Kb |
| RU-A01/20 | BamH I/BgI II/Sau 3A | BamH I | 1.0 Kb |

### 3.14.2 Enzyme activity of clones

The clones were inoculated into 10mls of Luria broth and ampicillin. They were grown up to mid- log phase and then induced with IPTG. Samples were taken every hour for 6 hours and sonicated to collect the crude extract and then assaying for peroxidase, lignin peroxidase and polyphenol oxidase activities. Controls were also assayed for activity, the negative control being *E.coli* Top10F` plus vector. The negative comtrol gave positive kinetic enzyme activity for polyphenol oxidase and peroxidase (Figure 3.47, 3.48 and 3.49), hence complicating the interpretation of the data. This activity was viewed however as background activity. It was then necessary to bring into account the activity of the positive control. Peroxidase activity using 2,4 DCP as substrate was observed over time with 2 clones (RU-A03/19 and RU-A03/26) and of the negative control (Figure 3.48). This can be seen as an increase in activity over time peaking at time 6 hours. For the 2 clones, there was a gradual increase in activity for the first 2 hours and then a sharp increase from time 2 hours up to and peaking at time 6 hours. Although there was a general increase in activity for the control, the increase was gradual throughout the 6-hour period, unlike the activity of the clones.

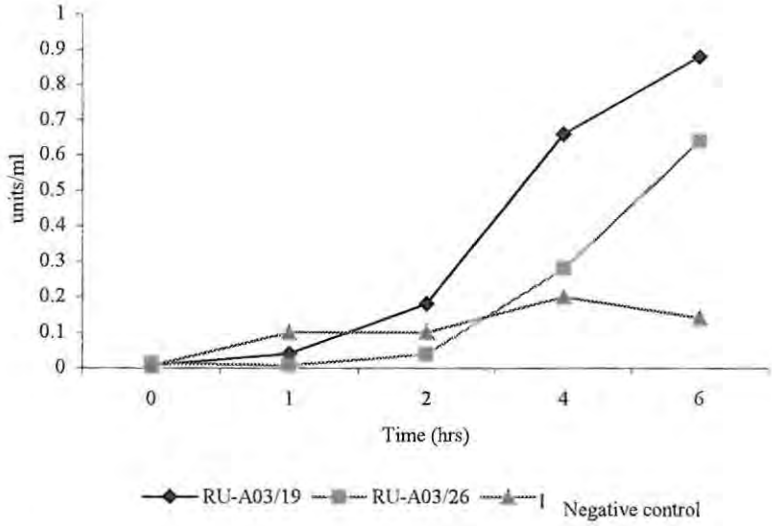


**Figure 3. 48** **Production of peroxidase over time after induction with IPTG by 2 selected clones and a control (plasmid without insert). Peroxidase activity was seen to increase with time peaking at time 6 hours.**

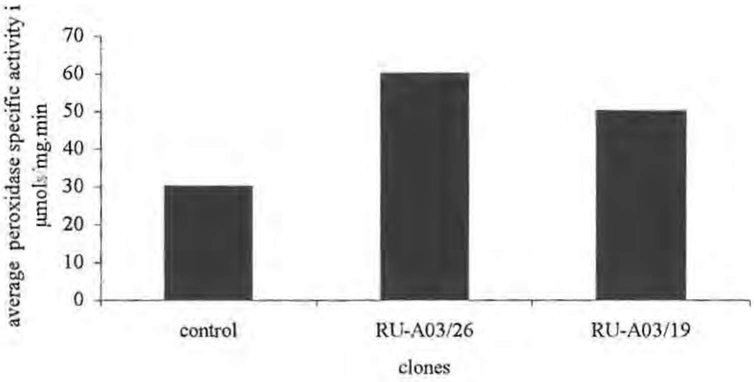


**Figure 3. 49** Specific peroxidase activity using 2,4 DCP as substrate of clones RU-A03/19 and RU-A03/26 and the control.

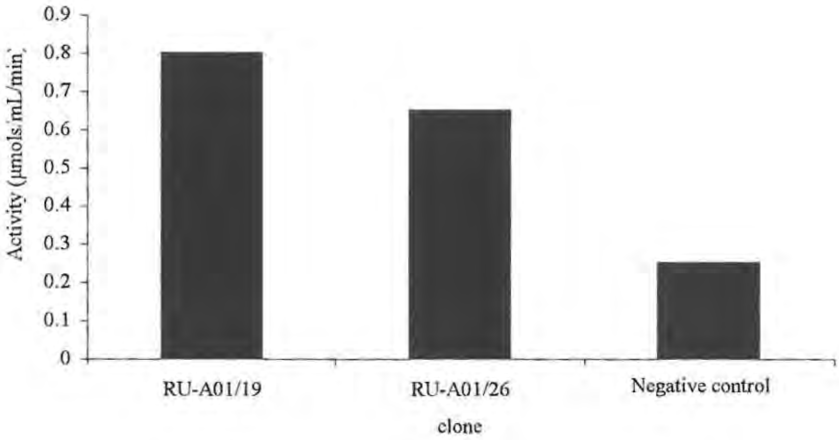


**Figure 3. 50** Peroxidase activity of RU-A03/19, RU-A03/26 and control. Substrate used for assay was 2,4 DCP.

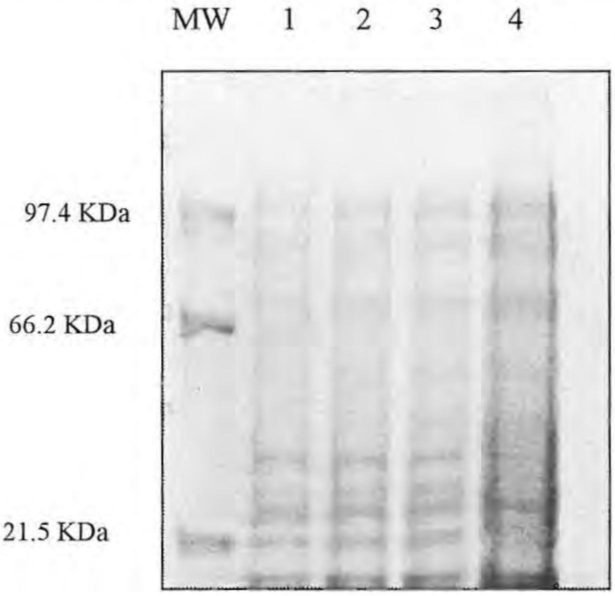


**Figure 3. 51** **SDS-PAGE gel time point samples of clone RU-A03/26 lane 1, time 2 hours; lane 2, time 3 hours; lane 3, time 4 hours; Lane 4, time 5 hours.**

SDS-PAGE gels were then run to determine the presence of a band/s and comparisons between positive clones, the negative control and the control (Figure 3.51).

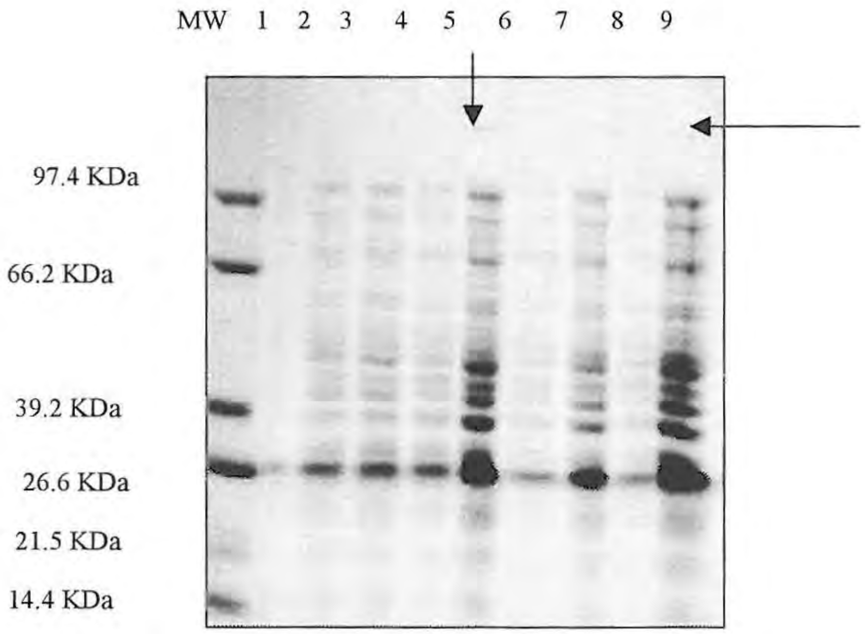


**Figure 3. 52** **SDS-Page gel of non-induced and induced clones and controls. IPTG was the inducer. Lane 1, -ve control; 2, + control uninduced; 3, + control induced; 4,RU-A03/19 uninduced; 5 RU-A03/19 induced; 6, RU-A03/3 uninduced; 7, RU-A03/3 induced; 8, RU-A03/26 uninduced; 9, RU-A03/26 induced.**

The results obtained were inconclusive as the activities of the clones were overshadowed by the activity of the positive control. The SDS-PAGE gels run gave similar banding patterns to that of the positive control however there was no difference in the intensity of banding patterns between the non-induced and induced fractions of the positive control. A faint band was detected in the induced fractions of 2 clones (RU-A03/19 and RU-A03 /26; highlighted by the arrows in Figure 3.52). Based on the results in Figure 3.51 and enzyme kinetics the two clones were selected and sent for sequencing. The primers used were universal primers M13. However the resultant sequences did not identify a functional gene sequence.

The size of the inserts chosen in this study in comparison to DNA fragment sizes used in isolating oxidoreductase enzymes in fungi is considerably smaller. However fungal DNA comparises of introns, which are regions of DNA that do not code for anything. Bacterial DNA do not have introns. In addition the simplest peroxidase or polyphenol isolated in Actinomycetes consisted of approximately 450 amino acids, which would be approximately 1350 bp.

The prospects of isolating a gene encoding for a thermophilic peroxidase or polyphenol oxidase has exciting prospects, namely to manipulate the enzymes in terms of immobilization and gene manipulation. At this point, one other option was available, Southern blotting using the peroxidase *Cat C* gene sequence from *Streptomyces coelicolor* A3(2) as a probe.

A final attempt was carried out to try and isolate and identify the peroxidase genes in the isolates by Southern blotting with a probe of a published peroxidase gene sequence in *Streptomyces coelicolor* A3(2).

### 3.15 Southern Blotting analysis

Southern Blotting is a chromatographic technique used for isolating and identifying specific fragments of DNA as the fragments formed as a result of DNA cleavage by restriction enzymes. This is a common method used in molecular biology techniques and in this case the use of Southern blotting was to determine if the peroxidase gene in *Streptomyces coelicolor* was of similar structure to the peroxidase genes in the three thermophilic isolates, RU-A01, RU-A03 and RU-A06. Peroxidase assays used to determine the peroxidase in *Streptomyces coelicolor* include *o*-dianisidine and 2,4 DCP as substrates. Veratryl alcohol and other lignin peroxidase substrates have not been used on this particular peroxidase due to its lack of reactivity towards these substrates.

Due to the inconclusive results obtained the next approach was to use Southern blotting to try and determine the nature of the gene. At present lignin peroxidase, or polyphenol oxidase genes have not been published in current literature. The *cat C* gene, which encodes for a peroxidase gene in *Streptomyces coelicolor* A3(2) (Hahn *et al.*, 2000) as well as non-heam peroxidases in *Streptomyces* species have however been isolated and sequenced (Pfeiffer *et al.*, 1992; Pelletier *et al.*, 1994 and Facey *et al.*, 1996). The *catC* gene in *Streptomyces coelicolor* A3(2) encodes a protein of 740 amino acids with a molecular mass of 80,860 Daltons and is situated downstream to the *fur A* gene. The predicted amino acid sequence is highly homologous to all known bacterial catalase-peroxidases (Hahn *et al.*, 2000). The aim was to develop a probe based on the *cat C* gene in *Streptomyces coelicolor* A3 (2) and perform blots on the three thermophillic *Streptomyces* isolates.

DNA was extracted from S.coelicolor A3(2) and analysed on 0.8 % agarose.The primers used for PCR amplification of the *cat C* gene in S*treptomyces coelicolor* are listed in Table 3.45. Amplification was performed by use of 30 cycles of 1 minute denaturation at 94°C, 1 minute at 63°C for primer annealing, and 2 minutes at 72°C for primer extensions followed by a final step at 72°C for 7 minutes and cooling at 4°C. Products were first analysed by electrophoresis on 1% agarose gels (Figure 3.53). The main PCR product was a 1.0 Kb fragment with smaller non- specific bands of less than 500 base pairs in size.

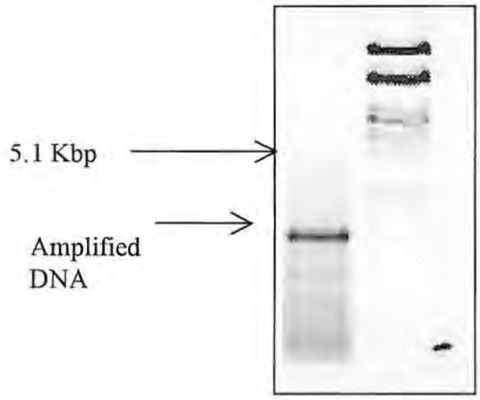


**Figure 3. 53** **1.0 Kbp PCR product of *S.coelicolor* using *cat C* gene primers.**

The band was excised and gel purified and quantified in preparation for labeling according to the DIG-prime labeling kit. DNA was extracted from RU-A01, RU-A03 and RU-A06 and then restrict digested using restriction enzymes *BamH I, Bgl II* and *Msp I* and transferred to a nylon membrane.

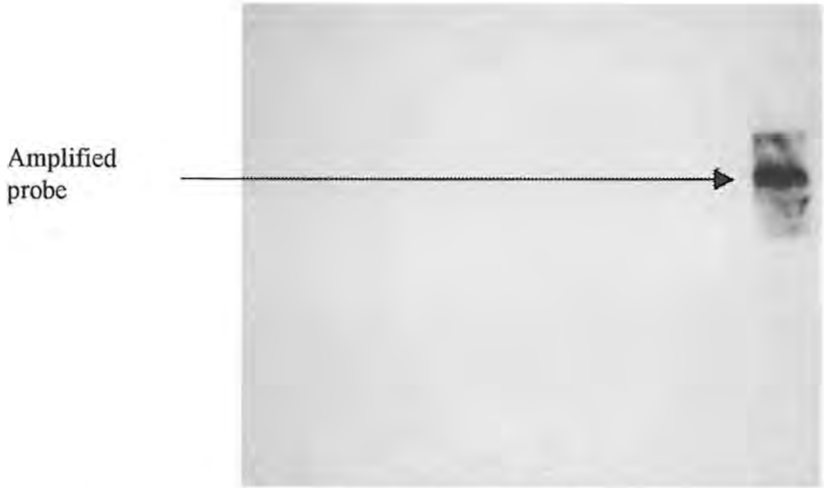


**Figure 3. 54** **Southern Blot of restricted digested DNA of isolates RU-A01, RU-A03 and RU-A06 using restriction enzymes *BamHI, Bgl II* and *MSP I*, plus PCR product of probe. The positive band is that of the probe.**

No hybridisation was observed to any of the restricted DNA fragments from RU-A01, RU-A03 and RU-A06. The only positive signal came from the probe hybridising to itself

(Figure 3.54). There is hence no evidence of the *CatC* gene, which encodes for a peroxidase gene found in some *Streptomyces* species in any of the 3 isolates. The results or lack of results in this Southern blotting experiment could imply that the peroxidase gene/s present in the three thermophilic isolates is in fact a lignin peroxidase gene or a peroxidase gene that does not belong to the same class as the *CatC* type peroxidases. The difference between the *CatC* type peroxidase and a lignin peroxidase is that they belong to different classes. *CatC* type peroxidases belong to the Class I peroxidases while lignin peroxidases belong to the class II peroxidases (see Chapter 1p.6). With the peroxidase reactivity towards veratryl alcohol, which is a substrate for lignin peroxidase, the nature of the peroxidases isolated in these isolates would be lignin peroxidase.

# CHAPTER 4

# CONCLUSION AND FUTURE WORK

## 4.1. CONCLUSION

This work has been the first step of a research project that has focused on the isolation of novel actinomycetes, which are of a thermophilic nature, producing thermostable oxidoreductase enzymes for biotechnological purposes such as the removal, and/or bioconversion of phenolic residues found in the agro-industrial sector of South Africa.

Three thermophilic Actionmycetes isolates, RU-A01, RU-A03 and RU-A06 were isolated from compost. The source was selected due to the relatively high temperatures that arise during the process of composting at a composting site, hence the likely chance of isolating thermophiles. With the advances in molecular biology techniques, screening for new microorganisms in environments such as soil in combination with older techniques such as soil sampling, and enrichment techniques, enhances the prospects of isolating microbes that may be of value to researchers. In this study, a combination of enrichment and lab culturing techniques together with PCR methodologies to isolate 16S rDNA and subsequent sequencing led to the identification of three novel thermophilic Actinomycetes which belong to the *Streptomyces* genera. The discovery of these three thermophilic *Streptomyces* isolates using basic enrichment techniques in combination with 16S rDNA analysis did not require a large or complex screening method. Samples taken from a single compost site resulted in the isolation of these three novel isolates. This hence proves that the potential of finding other novel Actinomycetes with biotechnological applications such as the three isolated in this study remains untouched in comparison to fungi that have been extensively studied and described. Other environments can be investigated in the search for more novel Actinomycetes, namely marine areas, hotsprings and phenolic effluent dumps.

The three isolates displayed relatively high growth rates in comparison to other *Streptomyces* species, as well as the ability to produce peroxidase and polyphenol oxidase enzymes, which may have arisen due to the fact that the primary carbon sources in soil environments are insoluble and polymeric, necessitating the secretion of a range of extracellular enzymes, which would include peroxidases and polyphenol oxidases. RU-A01 and RU-A03 had the abilty to produce lignin peroxidase, an enzyme thought to be produced only by some white rot fungi. A range of different peroxidase and phenol oxidase substrates were used to determine the substrate specificity. Ideal substrates included 2,4-Dichlorophenol, and veratryl alcohol for peroxidases of which the former has been used extensively in assaying peroxidases in Actinomycetes. Catechol was the substrate of choice for polyphenol oxidases, which has been viewed to be more stable than L-DOPA, a substrate commonly used to detect polyphenol oxidase activity in the brown mushroom *Agaricus bisporus* and other polyphenol oxidase producing fungi. Laccase was not detected in any of the isolates and has not been isolated in any Actinomycetes to date.

A lot of focus has been placed on the white rot fungi and their biodegradative enzymes, however, Actinomycetes do have advantages over fungi in that their growth rates are significantly faster, maximum growth rates for the three isolates was reached at 48h incubation time; although Actinomycetes are known to prefer neutral to alkaline growth pHs, they are adaptable to lower pHs and in this study, RU-A03 and RU-A01 removed grape waste in a whole cell system of an initial pH of 3.5. Most fungi are mesophilic, as are the enzymes produced by these mesophiles, making them intolerant to increased temperature changes that occur during exothermic enzyme catalyzed reactions.

Studies have shown that oxidoreductase enzymes in Actinomycetes are produced during the primary stage of growth, with activity remaining stable during the lag phase of growth, making this potential whole cell system ideal for phenolic bioremediation systems. This is ideal because even during the lag phase of growth, whole cell bioremediation can continue, as the enzyme remains stable during this growth phase. In this study it was observed that peroxidase and poyphenol oxidase was produced in the primary stage of growth, peaking during the later phase of primary growth, extracellular activity was only detected after 36 hours of growth which would be the later stage of log phase, specific extracellular activity however peaked at 36 hours and then declined for most enzymes thereafter. At this point enzyme activity may be made to remain constant in the presence of an inducer or the addition of an inducer at this point. Induction studies showed that these isolates respond to and increase enzyme activity in the presence of a phenolic inducer. It was also observed that extracellular specific activity was highest for both polyphenol oxidase and peroxidase.

Resting cell studies of the catabolism of low molecular weight model phenolic compounds were conducted to determine whether these isolates were capable of metabolizing these phenolic subunits. Catabolism of model phenolic compounds has been used as a method to determine whether the microbes have the ability to degrade phenolic residues and compounds. Vanillin, syringic acid, ferulic acid, *p*-coumaric acid and protacatechuic acid were catabolised by the isolates at varying degrees within 48 hours of incubation. No bioconversion products were detected however previous studies have shown the bioconversion of these phenolic acids with the aid in some cases of enzyme inhibitors such as $\alpha,\alpha$-dipyridyl.

A model waste was used to determine the efficiency of these isolates in removing phenolic residues. The winemaking industry in the Western Cape in South Africa is relatively large with a large percent of the grape waste, which consists of compressed skins, pips and stalks going towards animal feed. A large proportion of the waste however remains and accumulates. Several solvents and methods of extracting the phenolics in this grape waste were explored. Acetone and ethyl acetate proved to be the best solvents in extracting grape phenolics. The extracts were subsequently used in whole cell and enzyme extract studies. Red and green grape wastes were separated and analyzed by HPLC, with each one displaying a unique HPLC phenolic fingerprint. A number of model phenolic compounds were run; retention times determined and subsequent spiking experiments were carried out to determine the presence of these compounds in the grape waste. Ferulic acid, vanillic acid, coumaric acid, protocatechuic acid and gallic acid were identified in red and green grape waste. The remaining unidentified peaks are likely to be tannins, anthocyanins, glycosylated and polymerized phenolic acids.

Whole cell studies were then conducted to determine if the isolates were capable of degrading red grape waste in liquid media. Phenolic residues are often acidic and Actinomycetes grow optimally at neutral to alkaline pH, however they are adaptable at lower pHs making them ideal microbes for remediation purposes. UV scans and phenol determination assays indicated that the isolates were capable of degrading the grape phenols, with RU-A03 being the most efficient isolate. Enzyme activities were also determined, showing a significant increase in enzyme activity, particularly extracelluar activity when cells were exposed to a concentration of 30% red grape waste. Higher concentrations resulted in a decline in enzyme activity and in some cases no activity at all, which could have been a result of catabolite repression.

One of the disadvantages of using whole cell systems to determine bioremediation capabilities is that researchers need to take into account the degree of adsorption of the phenolics onto the cell biomass, hence a true reflection of enzyme capacity on the phenolic residues would be to use enzyme extracts. Crude enzyme extracts were used in this study to determine the effects of the enzymes on red and green grape waste. HPLC was the method of choice to separate the phenolics in grape waste and to observe changes before and after treatment with enzyme extracts. Phenolics were significantly removed by all isolates, both in the presence of hydrogen peroxide and also in its absence, making it difficult to determine which enzyme (peroxidase or polyphenol oxidase) was responsible for the phenolic removal. Precipitation did occur in both sets of experiments, which may have been the result of polymerization of the phenolics, a common mechanism of both polyphenol oxidase and peroxidases. It would therefore be feasible to consider using these enzymes in the biotransformation of phenolics, however further extensive studies would need to be carried out.

Isolation of the genes for peroxidase and polyphenol oxidase in the 3 isolates proved to be challenging, as the expression vector used and the expression host (*E.coli*) produced it own polyphenol oxidase in the negative controls making interpretation of the data difficult. The aim of this section of the study was to answer a number of questions; to compare gene sequences of that of other producing microbes, as well as to allow for the use of genetic engineering techniques such as gene shuffling increasing enzyme substrates, increase enzyme production and enhance thermostability. 3 clones, were selected and sequenced, however their resultant sequences were of no significance. Due to time constraints that area of the study could not be explored further. Southern blotting was carried out using a probe of the *Cat C* gene from *Streptomyces coelicolour*, which encodes for a peroxidase gene. No hybridizaton occured for any of the isolates indicating that the type of peroxidase found in the isolates differ from that of *Streptomyces coelicolor* 2(A) 3 and is likely to be a lignin peroxidase gene.

In conclusion, the work presented in this study is the first description of the application of novel thermophilic Actinomycetes that produce both peroxidases and polyphenol oxidases and their application on an industrial phenolic waste. Both whole-cell and cell-free enzyme-based reactions demonstrated the bioconversion of grape waste with reactions occurring at 50°C.

## 4.2 FUTURE WORK

Several aspects of this project can be investigated in great depth. Two such aspects of this research project can be explored further:

Biochemical aspects of the enzymes produced and the molecular biology aspect. Biochemical profiling of these enzymes can be carried out to determine the enzymes reactivity to other industrial model wastes. At present several other industrial wastes are being investigated (Personal communication, S. G. Burton), namely pulp and paper effluents, cresylic effluents and olive waste waters. Other bacteria and some fungi are being investigated in their ability to bioconvert these wastes and it would be an option to investigates the ability of these three novel isolates on these phenolic-based effluents and to compare their effectiveness against other microbes. The effects of these isolates on these effluents would give a clearer picture on their true capacity in terms of applications in bioconversion and bioremediation. Processes. In addition, optimal pH and temperature ranges, buffer systems as well as solvent tolerance studies can be carried out to determine the robustness of these enzymes.

In terms of the molecular aspects, isolation of the lignin peroxidase gene would still be feasible due to the immense potential in the utilization of genetic engineering techniques such as gene shuffling to produce a robust form of the enzyme. Other expression vectors may need to be looked into in particular yeast expression vectors, which have been used recently with some success in expressing *Srteptomyces* enzymes such as xylanases.

# APPENDICES

## APPENDIX A:

## Culture media

### Solid and liquid media

Actinomyces were cultured on solid and liquid media using M3, M65 agar and broth and 0.6% yeast buffered liquid media in this study. All media was autoclaved for 20 minutes. The following compositions are in grams per liter:

#### A.1 M3 Agar

| | |
|---|---|
| Malt extract | 10g |
| Yeast extract | 4g |
| Purified agar | 20g |

#### A.2 M3 Broth

| | |
|---|---|
| Malt extract | 24g |
| Yeast extract | 5g |

#### A.3 M65 agar

| | |
|---|---|
| Glucose | 4g |
| Yeast extract | 4g |
| Malt extract | 10g |
| Calcium carbonate | 2g |

The pH was adjusted to 7.2 with 1M potassium hydroxide before the addition of 20g purified agar.

#### A.4 M65 Broth

| | |
|---|---|
| Glucose | 4g |
| Yeast extract | 4g |
| Malt extract | 10g |
| Calcium carbonate | 2g |

The pH was adjusted to 7.0 using potassium hydroxide (1M).

*E.coli* was cultured and maintain on solid and liquid media using LB agar and broth and Soc media in this study. The media was made up according to the manufacturers' instructions.

### A.5 Luria-agar (LB agar) for plates

The following components were added successively with stirring at room temperature:

| | |
|---|---|
| Tryptone | 10g |
| Yeast | 5g |
| Sodium chloride | 10g |
| Agar Agar | 15g |
| Distilled water | 1000mLs |

The pH was adjusted to 7.0 with sodium hydroxide before the addition of agar

### A.6 Luria-Bertani Medium (LB Broth)

The following components were added successively with stirring at room temperature:

| | |
|---|---|
| Tryptone | 10g |
| Yeast | 5g |
| Sodium chloride | 10g |
| Distilled water | 1000mLs |

The pH was adjusted to 7.0 with 1M sodium hydroxide

### A.7 Soc medium

The following was made up with with 97 mLs de-ionised water and autoclaved at 121 °C for 15 minutes

| | |
|---|---|
| Bactotryptone | 2.0g |
| Yeast extract | 0.5g |
| 1M Sodium chloride | 1ml |
| 1M potassium chloride | 250$\mu$l |

After the solution was autoclaved and cooled to room temperature, the following was added:

2M MgCl 1ml filter sterilized

2M glucose 1ml filters sterilized

2M MgCl stock and 2M glucose each was added to a final concentration of 20mM. The volume was brought to 100 mL with deionised water (sterile) and filter sterilized through a 0.2 mm filter unit.

## A.8 Minimal media

In 615 mLs distilled water, the following components were added with continuous stirring:

| | |
|---|---|
| 10 X M9 salts | 100 mls |
| 40% glucose | 25mls |
| Trace elements | 10 mLs |
| 1M magnesium chloride | 200$\mu$l |
| 1M calcium chloride | 200$\mu$l |

## A.9 M9 salts

To 500 mLs of distilled water, the following was added:

| | |
|---|---|
| $Na_2HPO_4$ | 30g |
| $KH_2PO_4$ | 15g |
| NaCL | 2.5g |

## A.10 Trace elements

To 990 mLs of distilled water, dissolve successively with stirring at room temperature

| | |
|---|---|
| Boric acid | 0.05 |
| $MnSO_4.2H_2O$ | 0.04 |
| $(NH_4)_6MO_2O_{24}$ | 0.02 |
| KI | 0.01 |
| $CuSO_4$ | 0.004 |
| $ddH_2O$ | 990 mLs |

0.02g $FeCl_2$ was added to 10 mLs $dH_2O$ and then added to the above solution

## A.11 SOB medium

| | |
|---|---|
| Tryptone | 2% |
| Yeast | 0.5% |
| NaCl | 0.05% |
| KCl | 2.5mM |
| $MgCl_2$ | 10mM |

20g tryptone, 5g yeast and 0.5 g NaCl was dissolved in 950 mls d $H_2O$. A 250mM KCl solution was made and added to the solution above. The pH of the solution was adjusted to 7.5 with 5M NaOH and distilled water was added to make a final volume of 1 liter. The solution was autoclaved, cooled to about 55 °C to which 10 ml sterile 1M $MgCl_2$ was added. 50$\mu$g/ml of ampicillin was added and stored at ± 4 °C for no longer than 1-2 weeks.

### A.12 Yeast buffered minimal medium

The following components were added to make a buffered media: -

0.1M phosphate buffer

| | |
|---|---|
| $KH_2PO_4$ | 13.61g |
| $MgSO_4$ | 0.2g |
| NaCl | 0.2g |
| $CaCl_2$ | 0.05g |
| Yeast | 0.1% |
| $dH_2O$ | 1000 mLs |

To reach pHs 6.0, 6.4, 6.8, 7.2, 7.6 and 8.0, 1M NaOH was used.

### A.13 Yeast Growth Media

The following components were added to 1000mLs of distilled water:

| | |
|---|---|
| Yeast extract | 0.6% |
| $Na_2HPO_4$ | 5.3g |
| $KH_2PO_4$ | 1.98g |
| $MgSO_4.7H_2O$ | 0.2g |
| NaCl | 0.2g |
| $CaCl.2H_2O$ | 0.05g |
| Trace elements | 1 ml |

# APPENDIX B

## Buffers and Solutions

The following solutions and buffers were used for DNA and plasmid extraction protocols. All solutions and buffers were autoclaved at 121°C for 15 minutes unless otherwise stated.

### B.1 Chloroform : Isoamyl alcohol

24 : 1
The above solution was not autoclaved

### B.2 CTAB solution (100ml)

The following componetes were added to 90mLs distilled water

| | |
|---|---|
| CTAB | 1g |
| NaCl | 4.38g |
| Tris-base | 0.61g |
| EDTA | 0.29g |

pH was then adjusted to 8.0 and the volume made to 100mLs

### B.3 CTAB/NaCl solution

| | |
|---|---|
| NaCl | 4.1g |
| CTAB | 10g |

Distilled $H_20$ to 100mL

### B.4 EDTA 0.5M (pH 8.0)

| | |
|---|---|
| EDTA.$2H_2O$ | 186.1g |
| Distilled $H_2O$ | 800mL |

The pH was adjusted to 8.0 with NaOH pellets and volume adjusted to 1000mL

### B.5 Ethidium Bromide (10mg/mL)

1.0g of ethidium bromide were added to 100mL of distilled water. The solution was wrapped in aluminium foil and stored in the dark at 4°C. the solution was not autoclaved.

### B.6 Gel loading buffer (6x)

To 10 mLs of distilled water the following components were added:

| | | |
|---|---|---|
| Bromophenol blue | | 0.025g |
| Xylene cyanol | | 0.025g |
| Sucrose | | 4.0g |
| Distilled $H_2O$ | to | 10mL |

The mixture was stirred thoroughly to allow the dye to mix properly and stored at 4 °C

### B.7 Lysozyme solution

50mg of lysozyme powder was added to 1000 µl Tris-HCl 0.1M (pH 8.0) and mixed thouroughly using a 1mL pipette. The resultant solution was stores at -20 °C.

### B.8 Sodium chloride (5M)

292.2g of soudium chloride was added to 1000mL distilled water stirring continuosly until dissolved.

### B.9 Phenol / Chloroform / Isoamyl alcohol

25 : 24 : 1

| | |
|---|---|
| Phenol | 500.0g |
| Chloroform | 500mL |
| Isoamyl alcohol | 25mL |
| TE buffer | 525mL |

Solution was stirred to emulsify and phases were allowed to separate. The top phase was removed and TE buffer solution addition was repeated. When phases had separated the top phase was removed leaving 1 cm of the aqueous phase, which was stored at 4°C in a dark bottle.

### B.10 Proteinase K

20mg of Proteinnase K was addd to 1mL distilled water. 100µl aliquots were prepared and stored at -20$^0$C.

### B.11 RNase A

20mg of Rnase A was added to 1mL of distilled water. The solution was heated to 100$^0$C for 10 minutes, cooled and aliquots of 100µl were stored at -20$^0$C.

### B.12 SDS (10%)

100g of electrophoresis grade SDS was added to 900 mL of distilled water. The solution was heated to 68$^0$C to assist dissolution and the pH was adjusted to 7.2 using concentrated HCl, followed by adjustment of the volume to 1000mL.

### B.13 Sodium acetate (3M)

40.81g of sodium acetate was added to 80mL distilled water. The pH was adjusted to 5.2 with glacial acetic acid before making up the volume to 100ml

### B.14 TAE 50x (250mL)

The following components were combined:

| | |
|---|---|
| Tris base | 60.5g |
| Glacial acetic acid | 14.3mL |
| EDTA 0.5M (pH 8.0) | 25mL |

The volume was adjusted to 250mL before autoclaving

### B.15 TBE 5X

The following components were combined:

| | |
|---|---|
| Tris base | 108g |
| Boric acid | 55g |
| Distilled $H_2O$ | 900mL |
| EDTA 0.5M pH 8.0 | 40mL |

The volume was then adjusted to 1000mL with distilled water.

### B.16 TE buffer

| pH 7.4 | pH 7.6 | pH 8.0 |
|---|---|---|
| Tris-Cl (pH 7.4) | Tris-Cl (pH 7.6) | Tris-Cl (pH 8.0) |
| EDTA (pH 8.0) | EDTA (pH 8.0)<br>1mM | EDTA (pH 8.0) |

The volume was adjusted to 1000mLs before autoclaving

### B.17 Electrophoresis buffer

The electrophoresis buffers were made up according to Sambrook *et al* (198) and has the following composition (in grams per litre)

| | **Working solution** | **Concentrated solution** |
|---|---|---|
| **50X Tris-acetate buffer (Tris-acetate)** | 1 x 0.04 M Tris-acetate<br>0.01 M EDTA | 50x: 242g Tris base<br>57.1 mL glacial acetic acid<br>100mL 0.5M EDTA |
| **5 x Tris-Borate-EDTA buffer (TBE)** | 0.001M EDTA | 0.5 x 0.045M Tris borate<br>5x: 54g Tris base<br>27.5g boric acid<br>20 ml 0.5M EDTA |

## APPENDIX C

### PCR Reagents

### C.1 Acetamide (4%)

2.0g acetaminde was added 50mL distilled water and stores at 4°C

### C.2 Agarose 1%

1.0g of agarose was added to 1x concentration TAE buffer and 3.0 μl ethidium bromide. The mixture was microwaved until the solution had fully melted and allowed to cool before pouring into a casting tray.

### C.3 Deoxyribonucleotides (dNTPs) 100mM Stock

16 µl of sterile water was added to the following to make a 100mM stock solution

| | |
|---|---|
| dATP | 1.0µl |
| dCTP | 1.0µl |
| dGTP | 1.0µl |
| dTTP | 1.0µl |

The above solution was diluted for PCR reactions to obtain 100µM

### C.5 Primers

Primers were diluted to obtain a concentration of 0.8 µM using sterile water.

### Oligonucletides

Oligonucleotides for PCR and sequencing were purchased from the University of Cape Town's Department of Molecular Biology and are listed in Table C.1

**Table C.1 Oligonucleotides**

| Name | Sequence | Application |
|---|---|---|
| 9F | 5‘GATTTGATCCTGGCTCAG | Primer pairs used to PCR amplify 16s rDNA analysis |
| 1541R | 5‘AAGGAGGTGATCCAGCC | |
| F | 5‘CACAGGAAACAGACCATGGC | Primer pairs for sequencing of clones |
| R | 5‘GACGTACGCGTATCGATGGC | |
| F | 5‘CCGAGGTCATCTACCGGGGC | Cat C gene in *S.coelicolor.* Probe for Southern blotting |
| R | 5‘CCCGGGCGGGGCGGGGTGG | |

Theoretical $T_m = 4(G+C) + 2(A+T)$

### C.6 Sybr green (1000x)

1.0µl of sybr green was added to 1.0mL DMSO. 10 µl was aliquoted into sterile eppendorfs and stored at -20°C.

### C.7 *Taq* polymerase (500U/µl)

*Taq* polymerase was diluted to obtain 5U/µl for all the PCR reactions that were carried out.

# APPENDIX D

**Transformation and Expression**
The following reagents were made for transformation and expression purposes in *E.coli.*

## D.1 CTAB 5%

5.0g Cetyl trimethyl ammonium bromide was added to 100mL of distileed water and stored at room temperature.

## D.2 Glucose 2M (10mL)

3.6g of glucose was added to 10mL sterile milli Q water and stored at room temperature.

## D.3 IPTG stock solution (0.1M; 50mL)

| | |
|---|---|
| IPTG | 1.2g |

A final volume with 50mL distilled water was made up and filter-sterilized and stored at 4°C

## D.4 $Mg^{2+}$ stock (2M; 100mL)

The following components were added to 100mL of distilled water and filter sterilized.

| | |
|---|---|
| $MgCl_2.6H_2O$ | 20.33g |
| $MgSO_4.7H_2O$ | 24.65g |

## D.5 $RF_1$ solution

The following components were added to 1000mL of distilled water

| | |
|---|---|
| KCl (100mM) | 7.456g |
| $MnCl_2$ (50mM) | 9.8955g |
| $CH_3COOK$ (30mM) | 2.974g |
| $CaCl_2$ | 1.47g |
| Glycerol (15% m/v) | 150g |

The pH was adjusted to pH to 5.8 and filter-sterilized.

## D.6 $RF_2$ solution (1000mL)

The following components were added to 1000mL of distilled water

| | |
|---|---|
| MOPS (10mM) | 2.093g |
| KCl | 0.7456g |

| | |
|---|---|
| $CaCl_2$ | 11.025g |
| Glycerol (15%m/v) | 150g |

The pH solution was adjusted to 6.8.

### D.7 SOC medium

| | |
|---|---|
| Tryptone | 2.0g |
| Yeast extract | 0.5g |
| NaCl (1M) | 1.0mL |
| KCl (1M) | 0.25mL |
| $Mg^{2+}$ (2M) | 1.0mL |
| Glucose (2M) | 1.0mL |

The tryptone, yeast extract, NaCl and KCl was added to 97ml distilled water, stirred to dissolve and autoclaved. Addition of Mg and glucose followed and thereafter the complete medium was filtered through a 0.2μm filter unit.

### D.8 STET buffer

The following compoments were added to 100mLs distilled water:

Sucrose (8% w/v)
Triton X-100 (0.1% v/v)
EDTA (50mM)
Tris-HCl (50mM, pH 8.0)

### D.9 X-Gal (2ml)

100mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was added to 2mL N,N' - dimethyl-formamide. The X-Gal was dissolved, covered in aluminum foil and stored at -20$^0$C.

### D.10 Antibiotic stock solutions

Ampicillin

Ampicillin was dissloved in $H_20$ at a concentration of 50mg/ml and stored at -20°C.

# APPENDIX E

## Gels for electrophoresis

### Polyacrylamide gels

### E.1 30% Acrylamide

The following reagents were added tp 60mL of distilled water:

| | |
|---|---|
| acrylamide | 29g |
| Bis acrylamide | 1g |

The solution was heated to 37°C to dissolve chemicals and the volume adjusted to 100mLs with distilled water. The solution was sterilized by filtration and the pH was maintained at 7.0 or less.

### E.2 10% Ammonium persulphate

1g of ammonium persulphate was added to 10mL distilled water.

### E.3 Acrylamide buffers and solutions.

**Plug**

| | |
|---|---|
| Acrylamide stock solution | 3mLs |
| 10% APS | 80$\mu$l |
| TEMED | 40$\mu$l |

| **E.4 Resolving gel** | 7.5% | 10% |
|---|---|---|
| Acrylamide stock | 10mL | 13.35mL |
| Tris/Hcl pH 8.8 | 15mL | 15mL |
| Distilled water | 12.3mL | 9.25mL |
| 10% APS | 0.3mL | 0.3mL |
| TEMED | 60$\mu$l | 60$\mu$l |

| **E.5** **Stacking gel** | 4% | 3% |
|---|---|---|
| Acrylamide Stock | 2.0mL | 1.5mL |
| 1M Tris/hcl pH 6.8 | 1.9mL | 1.9mL |
| Distilled water | 9.25mL | 9.75mL |
| 10% APS | 0.1mL | 0.1mL |
| TEMED | 60$\mu$l | 60$\mu$l |

### E.8 Bath buffer stock

| | |
|---|---|
| Tris | 30.3g |
| Glycine | 144.1g |
| SDS | 10.0g |
| Distilled water | 1000mL |

Dilute 1 in 10 for use.

### E.9 Staining Solution (Coomassie)

| | |
|---|---|
| Methanol | 45mL |
| Acetic Acid | 10mL |
| Coomassie Brilliant Blue | 0.2g |
| Distilled water | 45mL |

### E.10 Destaining Solution

| | |
|---|---|
| Methanol | 450mL |
| Glacial acetic acid | 70mL |
| Distilled water | 400mL |
| Glycerol | 100mL |

### E.11 Dissociation buffer

| | |
|---|---|
| SDS | 5g |
| Mercaptoethanol | 5mL |
| Glycerol | 7.5mL |
| bromophenol blue (0.2%) | 2.5mL |

| | |
|---|---|
| 1M tris pH 6.8 | 6.3mL |
| Distilled water | 28.7mL |

# APPENDIX F

## Extinction coefficients and enzyme activity Formulae

### F.1 Extinction coefficients

The extinction coefficients for enzyme substrates are as follows:

### F.1.1 Peroxidase activity

| | | |
|---|---|---|
| F.1.1.1 | 2,4 DCP | $1.36 \times 10^4\ M^{-1}\ cm^{-1}$ |
| F.1.1.2 | Veratryl alcohol | $9\ 300\ \ M^{-1}\ cm^{-1}$ |
| F.1.1.3 | Guaiacol | $6\ 390\ M^{-1}\ cm^{-1}$ |

### F.1.2 Polyphenol oxidase activity

| | | |
|---|---|---|
| F.1.2.1 | Catechol | $1417\ M^{-1}\ cm^{-1}$ |
| F.1.2.2 | L-DOPA | $3\ 600\ M^{-1}\ cm^{-1}$ |

### F.1.3 Laccase

| | | |
|---|---|---|
| F.1.3.1 | ABTS | $3.4 \times 10^4\ M^{-1}\ cm^{-1}$ |

### F.2 Enzyme activity calculation

Although the enzymatic activity in all samples were determined by a spectrophotometer, the activities were calculated as follows:

$$\text{Activity} = \frac{\frac{\Delta A}{t} \text{ x } 1/x \text{ x } y \text{ x dilution factor x } 10^{-3}}{\Sigma \text{ x } 10^{-6}}$$

$\Delta A/t$ = dA/Min

Where:
X = enzyme extract volume

Y = total volume of reaction mixture

$\Sigma$ = extinction coefficient

**F3: Formulae**

Total protein = protein (mg) x total volume of solution (mL)

Total activity = Activity ($\mu$mol.min.ml$^{-1}$) x total volume of solution (mL)

$$\text{Specific activity} = \frac{\text{Total activity (U/mL)}}{\text{Total protein (mg)}}$$

$$\%\ \text{Yield} = \frac{\text{activity in particular solution}}{\text{activity of crude extract}}$$

$$\text{Fold purification} = \frac{\text{Specific activity in particular solution}}{\text{Specific activity in crude extract}}$$

## APPENDIX G

### Enzymes for DNA work

### G.1 Restriction enzymes for DNA work

Restriction endonucleases were purchased from Roche Biochemicals (Table G.1).

**TABLE G.1 Restriction endonucleases, buffers and reaction temperatures**

| Endonuclease | Manufacturer | Buffer | Temperature |
|---|---|---|---|
| *BamH1* | Roche molecular | B | 37°C |
| *Bgl11* | Roche molecular | B | 37°C |
| *Msp1* | Roche molecular | L | 37°C |
| *Nar1* | Roche molecular | A | 37°C |
| *EcoR1* | Roche molecular | H | 37°C |
| *Sau3A* | Roche molecular | A | 37°C |

# APPENDIX H

## Enzyme Assay Reagents

The following reagents were made up for enzyme assays and stored at 4°C.

### H.1 Peroxidases

#### H.1.2 2,4 DCP

A 5mM solution of 2,4 dichlorophenol (2,4 DCP) was made up with distilled water (1000mL).

#### H.1.3 4-aminoantipyrine

A 1mM solution of 4-aminoantipyrine was made up with distilled water (1000mL).

**H.1.4** 0.1M sodium phosphate buffer

The following components were added to 500mLs of distilled water:

Add 1.74g $K_2HP0_4$ and 1.3g $KH_2PO_4$ to dH2O.

The resultant pH of the solution was adjusted to pH 7.0

**H.1.5** 0.01M hydrogen peroxide

1.8875ml made up to 1000mL in dH20

#### H.1.6 Guaiacol

2.9.1.6 10mM guiaicol solution was made up in 1000mLs of distilled water

H.1.7 0.01M hydrogen peroxide

### H.2 Veratryl alcohol

H.2.1 0.25M Sodium tartrate buffer (pH 2.5)

H.2.2 10mM veratryl alcohol

H.2.3 0.01M hydrogen peroxide

### H.3 Polyphenol oxidase

H.3.1 catechol

A 6mM catechol solution was made up in 1000mL of distilled water and stored in a dark bottle.

H.3.2 1mM copper sulphate

A 1mM solution of copper sulphate was made in 1000mLs distilled water.

H.3.3 L-DOPA soutlion for spectrophotometer assays

A 1mM L-DOAP solution was made up in 1000mLs of distilled water and stored in a dark bottle.

H.3.4 L-DOPA solution for non-denaturing PAGE activity gels

A 10mM solution of L-DOPA was made up in 1000mLs of distilled water and stored in a dark bottle at 4°

## APPENDIX I

## HPLC reagents

### I.1 Mobile phase reagents

Acetonitrile, glacial acetic acid, and methanol solvents suitable for gradient analysis were purchased from Merck (HiPerSolv for HPLC). Mobile phases were de-gased using 0.45μm millipore filter.

### I.2 Lignin aromatic model compounds

The following model phenolic compounds listed in table I.1 were used for HPLC analysis and grape waste determination assays.

Table I.1 Chemical formulae and molecular structures of model compounds used in the study

| Compound | Chemical formula | Compound | Chemical formula |
|---|---|---|---|
| Ferulic acid<br>4-Hydroxy-3-methoxycinnamic acid | $C_{10}H_{10}O_4$ | Eugenol<br>2-methoxy-4-[2-propenyl]phenol) | $C_{10}H_{12}O_2$ |
| Vanillic acid<br>4-Hydroxy-3-methoxybenzoic acid | $C_8H_8O_4$ | Vanillin<br>4-hydroxy-3-methoxybenzaldehyde | $C_8H_8O_3$ |
| Syringic acid<br>3, 5-Dimethoxy-4-hydroxybenzoic acid;<br>4-hydroxy-3,5-dimethoxy-benzoic acid | $C_9H_{10}O_5$ | Quercetin<br>3,3',4',5,6-pentahydroxyflavone | $C_{15}H_{10}O_7.2H_2O$ |
| Protocatechuic acid<br>3,4-Dihydroxybenzoic acid | $C_7H_6O_4$ | Gallic acid<br>3,4,5-trihydroxybenzoic acid | $C_7H_6O_5$ |
| *p*-coumaric acid<br>4-Hydroxycinnamic acid | $CH_9H_8O_3$ | Caffeic acid<br>Trans-3,4 Dihydroxycinnamic acid | $(HO)_2C_6H_3CH{=}CHCO_2H$ |
| Catechin | $C_{15}H_{15}O_6$ | Tannic acid | Gallic acid polymer |

# APPENDIX J

## Standard Calibration Curves

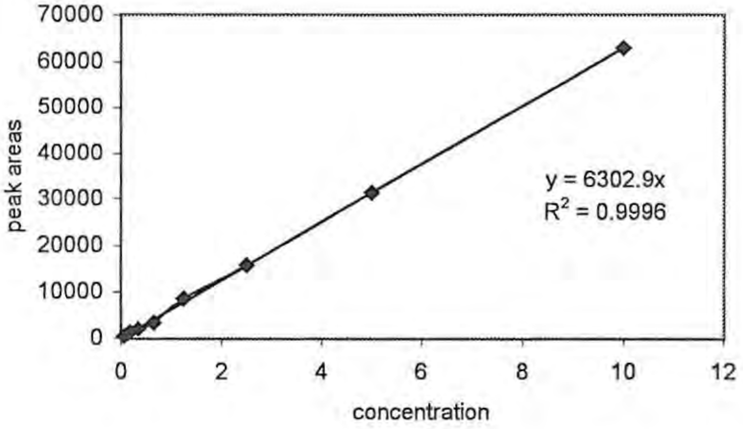


**Figure J. 1** Standard curve for vanillic acid

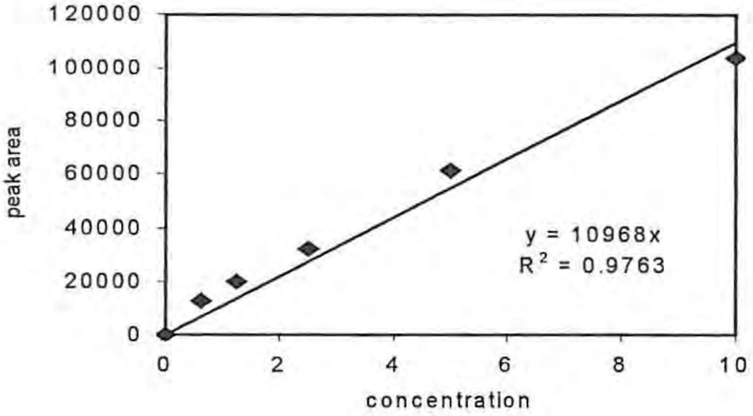


**Figure J. 2** Standard curve for Ferulic acid

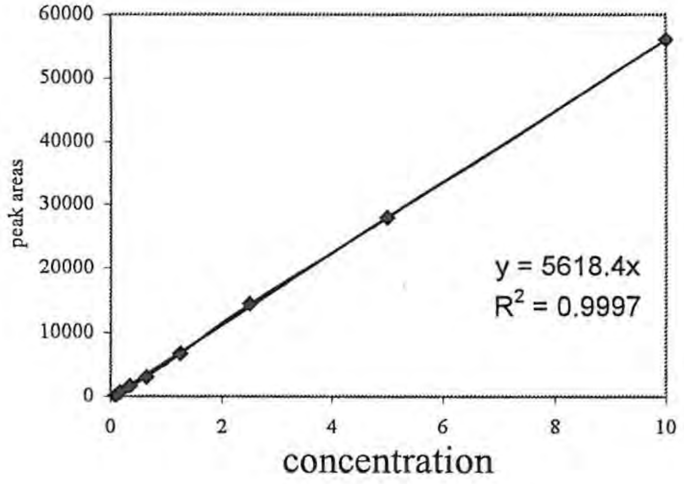


**Figure J. 3** **Standard curve for protocatechuic acid**

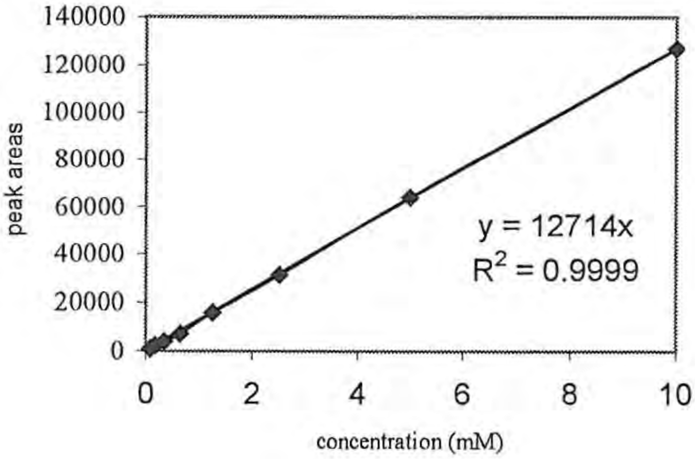


**Figure J. 4** **Standard curve for *p*-coumaric acid**

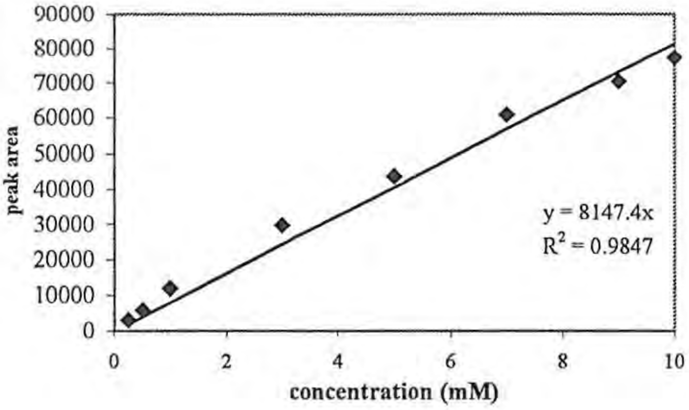


**Figure J. 5** **Standard curve for syringic acid**

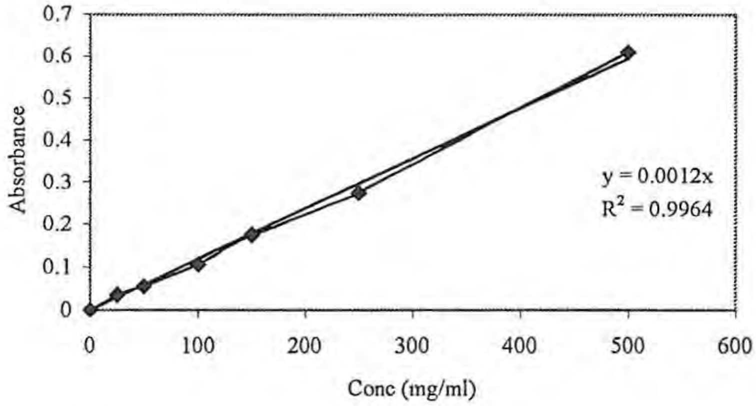


**Figure J. 6** **Standard Folin-Ciocalteaus total phenol determination assay curve using gallic acid as standard compound**

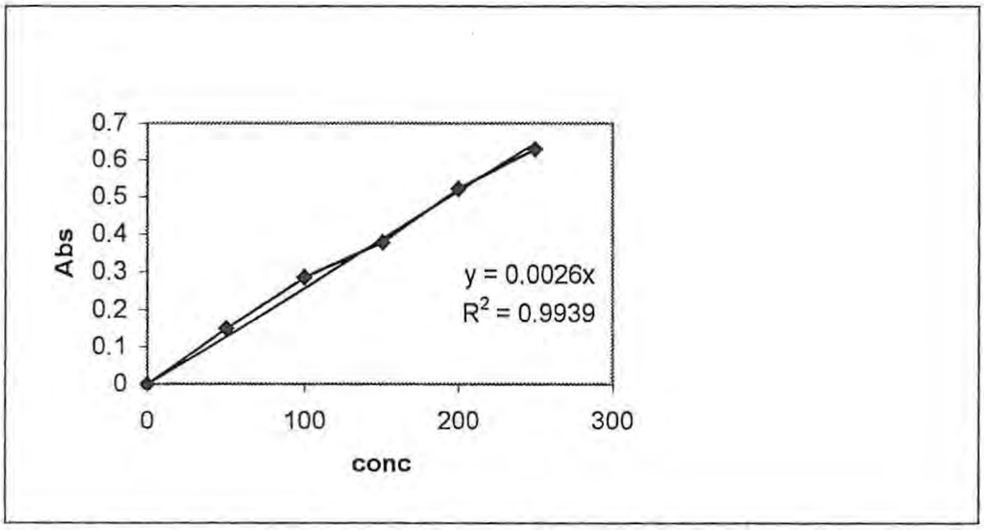


**Figure J. 7** **Standard Bradfords protein curve**

## APPENDIX K

### Southern blotting Dig solutions

The following solutions and reagents were prepared for the use of the Southern Blotting DIG-labelling Kit by Roche.

### K.1 20 x SSC

The following components were added to 800mLs of distilled water:

| | |
|---|---|
| Sodium chloride | 175.3 g |
| Sodium citrate | 88.2 g |

The pH was adjusted to pH 7.0 and made up to 1L

### K.2 Maleic Acid buffer (1L)

The following components were added to 1000mL of distilled water:

| | |
|---|---|
| Maleic acid buffer | 11.607g |
| Sodium chloride | 8.766 g |

The pH was adjusted to pH to 7.5 using HCL (conc).

**K.3 Washing buffer** (1L)

| | |
|---|---|
| Maleic acid buffer | 997 ml |
| Tween 20 | 3 ml |

**K.4 Detection buffer** (1L)

| | |
|---|---|
| Tris | 12.11 g |
| NaCl | 5.844 g |

pH with HCL to 9.5

**K.5 Stripping solution** (500 ml)

| | |
|---|---|
| NaOH | 8.401 g |
| SDS | 0.5g |
| Distilled water | 500 ml |

**K.6 Blocking solution** (100 ml)

| | |
|---|---|
| Maleic acid buffer | 90 ml |
| Blocking solution stock | 10 ml |

**K.7 2 x SSC, 0.1% SDS** (500ml)

| | |
|---|---|
| 20 x SSC | 50 ml |
| Distilled water | 450 ml |
| 10% SDS | 0.5 ml |

**K.8 0.5 x SSC, 0.1% SDS** (500ml)

| | |
|---|---|
| 20x SSC | 12.5 ml |
| Distilled water | 487.5 ml |
| 10%SDS | 0.5 ml |

**K.9 2 x SSC (1L)**

| | |
|---|---|
| 20 x SSC | 100ml |
| Distilled water | 900 ml |

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