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EXPLOITATION OF THE POTENTIAL OF A NOVEL BACTERIAL PEROXIDASE FOR THE DEVELOPMENT OF A NEW BIOCATALYTIC PROCESS

By

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DECLARATION

I, AMOS MUSENGI, declare that the contents of this dissertation/thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

14 February 2014

Signed

Date

ABSTRACT

Peroxidases are ubiquitous catalysts that oxidise a wide variety of organic and inorganic compounds employing peroxide as the electron acceptor. They are an important class of oxidative enzymes which are found in nature, where they perform diverse physiological functions. Apart from the white rot fungi, actinomycetes are the only other known source of extracellular peroxidases. In this study, the production of extracellular peroxidase in wild type actinomycete strains was investigated, for the purpose of large-scale production and finding suitable applications.

The adjustment of environmental parameters (medium components, pH, temperature and inducers) to optimise extracellular peroxidase production in five different strains was carried out. Five *Streptomyces* strains isolated from various natural habitats were initially selected for optimisation of their peroxidase production. *Streptomyces* sp. strain BSII#1 and *Streptomyces* sp. strain GSIII#1 exhibited the highest peroxidase activities (1.30±0.04 U ml^{-1} and 0.757±0.01 U ml^{-1}, respectively) in a complex production medium at 37°C and pH 8.0 in both cases. Maximum enzyme production for *Streptomyces* strain BSII#1 was obtained in the presence of 0.1 mM veratryl alcohol or pyrogallol, while 0.1 mM guaiacol induced the highest peroxidase production in *Streptomyces* sp. strain GSIII#1.

As the highest peroxidase producer, *Streptomyces* sp. strain BSII#1 was selected for further studies. The strain was first characterised by a polyphasic approach, and was shown to belong to the genus *Streptomyces* using various chemotaxonomic, genotypic and phenotypic tests. Production of peroxidase was scaled up to larger volumes in different bioreactor formats. The airlift configuration was optimal for peroxidase production, with *Streptomyces* sp. strain BSII#1 achieving maximum production (4.76±0.46 U ml^{-1}) in the 3 l culture volume within 60 hrs of incubation.

A protocol for the purification of the peroxidase was developed, which involved sequential steps of acid and acetone precipitation, as well as ultrafiltration. A purification factor of at least 46-fold was achieved using this method and the protein was further analysed by LC-MS. The protein was shown to be a 46 kDa protein, and further biochemical characterisation showed that the peroxidase had a narrower spectrum of substrates as compared to reports on other peroxidases derived from actinomycetes. With 2,4-dichlorophenol as the substrate, the K_m and V_{max} for this enzyme were 0.893 mM and 1.081 µmol min^{-1}, respectively. The purified peroxidase was also capable of catalysing coupling reactions between several phenolic monomer pairs.

Overall, the peroxidase from *Streptomyces* sp. strain BSII#1 could feasibly be produced in larger scales and there remains further room to investigate other potential applications for this enzyme.

ACKNOWLEDGEMENTS

DEDICATION

Molly Musengi

Gone too soon, beloved sister, wonderful friend

Your life has been short, way too short, but was an example well lived.

Rest in eternal peace.

Love you always

LIST OF PUBLICATIONS

The following is a list of the publication and conference presentations resulting from the work presented in this thesis.

Publication

Musengi, A., Khan, N., Le Roes-Hill, M., Pletschke, B.I., and Burton, S.G., 2013. Increasing the scale of peroxidase production by *Streptomyces* sp. strain BSII#1. *Journal of Applied Microbiology,* in press; doi:10.1111/jam.12380.

Conference Papers

1. *CPUT research day, November 2011, Cape Town Campus.* Production of peroxidase from selected actinomycete strains. A Musengi, N Khan, M Le Roes-Hill, B Pletschke, D Cowan, S Burton (poster presentation).

2. *16th International Symposium on the Biology of Actinomycetes, December 2011 (Puerto Vallerta, Mexico).* In search of novel peroxidases from Actinobacteria. N Khan, A Musengi, K Durrell, M Le Roes-Hill, D Cowan, S Burton (poster presentation).

3. *South African Society of Biochemistry and Molecular Biology, Federation of African Societies of Biochemistry and Molecular Biology, January 2012 (Drakensberg, South Africa).*Production of peroxidase from selected actinobacterial strains. A Musengi, N Khan, M Le Roes-Hill, BI Pletschke, DA Cowan, SG Burton (poster presentation).

4. *Joint conference on Science and Technology for development in Africa, Cape Town, June 2012.* Production of peroxidase from selected actinomycete strains. A Musengi, N Khan, M Le Roes-Hill, BI Pletschke, DA Cowan and SG Burton (oral presentation).

5. *CPUT research day, November 2012, Cape Town Campus.* Purification of peroxidase from a novel actinobacterial strain. A Musengi, N Khan, M Le Roes-Hill, B Pletschke, D Cowan, SG Burton (Third prize for Best Research Poster presentation).

6. *South African Society of Microbiology, November 2013, Bela-Bela Hot Springs, Limpopo Province.* Increasing the scale of peroxidase production by *Streptomyces* sp. strain BSII#1. A Musengi, N Khan, M Le Roes-Hill, B Pletschke, SG Burton.

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GLOSSARY

Terms/Acronyms/Abbreviations	Definition/Explanation
2,4-DCP	2,4-dichlorophenol
CCP	Cytochrome *c* peroxidase
CPO	*Caldariomyces fumago* peroxidase
DNA	Deoxynucleic acid
DTT	Dithiothreitol
FAD	Flavin adenine nucleotide
FMN	Flavin mono nucleotide
HRP	Horseradish peroxidase
LC-MS	Liquid chromatography-Mass spectrometry
LiP	Lignin Peroxidase
MnP	Manganese-dependent peroxidase
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydrogen
PEG	Polyethylene glycol
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

CHAPTER ONE

INTRODUCTION

1.1. Background

Biocatalysis entails the use of enzymes in biodegradative or biosynthetic reactions. It is a core element of Biotechnology, which the European Federation of Biotechnology has defined as "the integration of natural sciences and engineering sciences in order to achieve the application of organisms, cells, parts thereof and molecular analogues for products and services" (Nagel *et al.*, 1992). In general, enzyme technology is an attractive alternative to the conventional petrochemical-based chemical industry because it is considered to be more ecologically friendly. Enzyme technologies utilise renewable feedstocks; this aligns with the global paradigm shift to a bio-economy strategy (OECD, 2009). Enzymes also operate at mild or ambient reaction conditions, hence impose less energy demands as compared to conventional chemical-based catalysis, and produce non-toxic products (Tufvesson *et al.*, 2010). Enzymes are also versatile and efficient catalysts which catalyse a very wide range of reactions (Burton and Le Roes-Hill, 2008) and have impressive turnover numbers (orders of thousands to millions of molecules converted to products per second, depending on the actual enzyme). Biocatalysis has also become an important technology for the production of fine chemicals and pharmaceutical compounds (van Beilen and Li, 2002; van Beilen *et al.* 2003).

In general terms, oxidative enzymes catalyse oxidation reactions. Oxidative enzymes are highly sought after enzymes, particularly because they can catalyse some reactions that are difficult to carry out using conventional chemical catalysis (personal communication, Marilize Le Roes-Hill). Due to their inherent stereo- and regio-selectivity, and efficiency, oxidative enzymes have attracted attention as potential biocatalysts for various biotechnological processes (Sariaslani, 1989). Peroxidases are a class of oxidative enzymes that utilise hydrogen peroxide to catalyse oxidative reactions (Conesa *et al.*, 2002). They have been shown to have several applications that include use in diagnostics (Krell, 1991; Hailu *et al.*, 2010), treatment of wastewaters for removal of toxic contaminants (Nicell *et al.*, 1995; Dalai and Gupta, 2007), fine chemicals production (Mantha *et al.*, 2002; Casella *et al.*, 2010) and industrial applications such as Kraft pulp bleaching (Harazono *et al.*, 1996; Moreira *et al.*, 2001). Fungal and plant peroxidases have been well-studied, while relatively less work has been done to attempt to harness the catalytic potential of bacterial peroxidase enzymes in industrial processes. Most of the investigations have been based on the plant-derived horseradish peroxidase (HRP) and the family of lignin peroxidases (LiP) from the white rot fungus, *Phanerochaete chrysosporium*, which generally have served as model peroxidases.

HRP is widely used in diagnostic kits and for antibody labelling applications. These have generally taken the form of biosensors for the detection of compounds such as hydrogen peroxide, organic hydroperoxides, glucose, alcohols, glutamate and choline (Hamid and Rehman, 2009). Peroxidases have been demonstrated to be useful for the decolourisation of dyes (Bhunia *et al.*, 2001) and removal of phenolic compounds from industrial wastewaters (Dalai and Gupta, 2007).In spite of high turnover numbers, the two model peroxidases, HRP and LiP, have limited applicability because of restrictions arising from inherent properties of the enzymes. For example, the optimum pH for *P. chrysosporium* LiP activity has been reported as pH 3.0 (Oyadomari *et al.*, 2002). This implies that this peroxidase cannot be used to catalyse a reaction requiring an alkaline environment, as occurs typically with textile and paper and pulp mill effluents. The process parameters for the industrial biotransformation of substrates into important bioactive compounds may not necessarily conform to the requirements of these enzymes, thus facilitating the need to find other biocatalysts that can meet the needs of the particular transformation process.

In light of these limitations it is imperative that ways to overcome these hurdles be investigated and new systems for biocatalysis employing new enzymes with potentially refined features be developed. Some of the approaches could include:

(i)   Screening of new habitats for microbial species that may have enhanced enzyme activity. The marine environment, for example, has been described as a source of chemical diversity for drug discovery (Ward and Bora, 2006) because many bioactive substances are isolated from marine organisms.

(ii)   Investigating the properties of peroxidases from newly-discovered micro-organisms, with a view towards enzyme stabilisation and optimisation for industrial biocatalysis. The latter may include investigations into whole-cell and enzyme immobilisation. Extremozymes (enzymes isolated from extremophiles) are potentially very attractive for industrial applications.

(iii)   Manipulation of the enzyme's characteristics to render conformance to requirements of the biocatalytic process. The advent of designer biocatalysts, produced by informed selection and mutation through recombinant DNA technology, enables production of process-compatible enzymes (Burton *et al.*, 2002).

Peroxidases have many potential industrial applications, including their use for production of bioactive substances. In spite of their potential, peroxidases have been underutilised because there are limited sources for feasible production and they are relatively more expensive as compared to other oxidative enzymes that are available on the market. It is

against this background that this study proposes to carry out investigations into potential peroxidase-producing micro-organisms, to assess their suitability for producing the enzyme in a more cost-effective manner and to show the potential of the enzyme to be utilised in selected biocatalysis reactions.

1.2. Motivation

The investigation of actinomycetes as a potentially significant source of peroxidases for possible industrial applications is an area that has not been extensively investigated. Members in this group of organisms, particularly *Streptomyces* spp. have been shown to be producers of peroxidase, but activity has been generally low (typically less than 1 U ml^{-1} in culture medium). Because bacteria may be cultured on defined medium and their growth and enzyme production regulated, actinomycete-based peroxidase production may be more cost-effective than producing the enzyme from other non-prokaryotic sources.

In comparison to fungal-based peroxidase studies, there are relatively fewer reports available on the characteristics of bacterial peroxidases. Therefore it is likely that bacterial peroxidases may offer, as yet, undiscovered novel characteristics that could be exploited in innovative biotechnological applications.

1.3. Hypothesis

Actinomycete-derived peroxidases can be feasibly produced in useful quantities and utilised in biocatalysis for the production of industrially-relevant chemicals.

1.4. Objectives

The broad aims of this work may be listed in four categories, and these were to:

(i)   To optimise peroxidase production in five peroxidase-producing streptomycetes and select the two highest enzyme-yielding strains for characterisation.

(ii)   To scale-up production in a laboratory size bioreactor.

(iii)   To purify and characterise the peroxidase from the highest yielding strain.

(iv)   To investigate the feasibility of using the peroxidase in a biocatalytic application.

1.5. Methodology

To achieve the objectives of this study, the methodology outlined in Figure 1.1 was followed. An initial screening step from five peroxidase-producing strains to two enabled the elimination of lower yielding strains and selection of the two highest yielding strains. These were then characterised via a polyphasic approach. After scaling up production of the enzyme from the highest peroxidase yielding strain, there was sufficient enzyme to perform

purification and biochemical characterisation of the peroxidase. The potential of the partially-purified peroxidase to catalyse coupling reactions between organic molecules investigated.
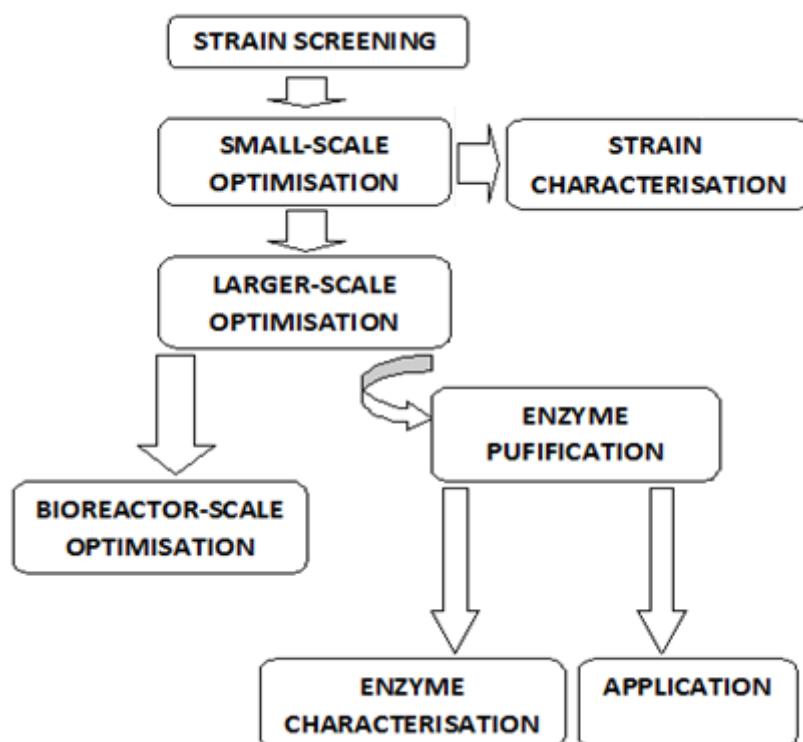
Figure 1.1: Summary outline of study methodology

1.6.   Organisation of thesis

This thesis is divided into seven chapters. This chapter (Chapter 1) provides an overview of the whole thesis and background information to the study. Chapter 2 is a review of literature on peroxidases, while Chapter 3 describes the initial screening work carried out in streamlining the investigations from an initial set of five peroxidase-producing actinomycete strains to two. Chapter 4 gives a description of the highest yielding strain giving its phylogenetic, phenotypic and partial chemotaxonomic characterisation. Chapter 5 covers the optimisation of peroxidase production by the strain identified as the highest peroxidase-yielder (*Streptomyces* sp. strain BSII#1), from a 10 ml culture volume to 6 l bioreactor capacity. Chapter 6 describes the purification and biochemical characterisation of the peroxidase from *Streptomyces* sp. strain BSII#1 and its application in coupling reactions between aromatic monomers. Chapter 7 summarises the conclusions drawn from this study and offers recommendations for future studies.

CHAPTER TWO

LITERATURE REVIEW

2.1.   Oxidative Enzymes

Oxidative enzymes (also known as oxidoreductases or redox enzymes) are enzymes capable of catalysing electron transfer or hydrogen transfer, resulting in the oxidation of the substrate (Burton and Le Roes-Hill, 2008). They catalyse the exchange of electrons or redox equivalents between donor and acceptor molecules, in reactions involving electron transfer, proton abstraction, hydrogen extraction, hydride transfer, oxygen insertion, or other key steps (Xu, 2005). They have been extensively studied as they are key components of all respiratory and energy-transducing processes (Munro *et al.,* 2000). Oxidoreductases are an emerging class of biotechnologically relevant enzymes because of the vast range of reactions they catalyse and their useful properties for application.

Aside from hydrolases, oxidative enzymes represent the most applied enzyme class in industry (Blank *et al.*, 2010). These enzymes have activity towards a wide range of substrates and display both regio- and stereo-specificity in their reactions (Van Beilen and Li, 2002; Burton, 2003; Nolan and O'Connor, 2008). Unresolved problems associated with chemical catalysts, such as lack of control and predictability of the product structures, have made the use of oxidative enzymes an attractive alternative for biocatalytic applications (Burton, 2003). In addition, oxidative enzymes are capable of facilitating reactions with chemically resistant starting compounds because of their generally high redox potentials (Burton, 2003). This makes them useful in environmental bioremediation programmes where they are used to oxidise toxic organic pollutants to less harmful derivatives.

2.1.1.   Classification of Oxidising Enzymes

Depending on what may be convenient, oxidative enzymes are classified in different ways. The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NCIUBMB) classifies oxidative enzymes in different Enzyme Commission (E.C.) sub-categories depending on the nature of the electron acceptor. Alternatively, Xu (2005) classified oxidoreductases using four main designations based on their signature catalysis and/or cofactor dependence:

2.1.1.1.   Oxygenases/Hydroxylases

These are enzymes that catalyse the incorporation of molecular oxygen into a substrate. They facilitate the regio-, stereo- and chemo-selective introduction of oxygen in a number of organic molecules (van Beilen *et al.,* 2003). They are widely distributed in nature and play

crucial roles in the metabolic conversion of both synthetic and degradative reactions of various natural compounds such as amino acids, lipids, hormones, vitamins, as well as synthetic drugs (Hayaishi, 2013). Oxygenases are also important because they enable the selective oxy-functionalisation of organic substrates in organic synthesis reactions (Pazmiño *et al.*, 2010). Two main sub-categories of oxygenases exist:

a. Monooxygenases

Monooxygenases (EC 1.13.x.x and EC1.14.x.x) are enzymes that catalyse the insertion of a single oxygen atom from dioxygen into an organic substrate (Pazmiño *et al.*, 2010). The cytochrome P450-dependent systems are perhaps the best characterised monooxygenases (Burton and le Roes-Hill, 2008) Monooxygenases generally employ nicotinamide adenine dinucleotide hydrogen (NADH) as a cofactor (Burton, 2003).

b. Dioxygenases

Dioxygenases require four electrons and generate two water molecules (Burton and le Roes-Hill, 2008) for the incorporation of both oxygen atoms into a given substrate**.**

2.1.1.2. *Oxidases*

Oxidases are enzymes that use oxygen as their electron acceptor (Burton, 2003). Oxidases receive electrons from organic substrates via an intermediate electron carrier to form oxygen-containing derivatives such as hydrogen peroxide or superoxide, which in turn react with further reducing substrates (Burton, 2003). As a result, the reactions can be non-specific or can produce several different products (Burton, 2003). Examples of enzymes in this category include xanthine oxidase, monoamine oxidase and cytochrome *c* oxidase.

a. Peroxidases

Peroxidases are enzymes that are characterised by achieving the oxidation of organic and inorganic substrates using peroxide as the electron acceptor. Hydrogen peroxide is the most commonly used oxidant, but other peroxides, including alkyl peroxides may be used as well. The reaction catalysed by peroxidases is generally of the form:

$$H_2O_2 + 2AH \implies 2H_2O + 2A \qquad \textbf{[Equation 1]}$$
electron donor \qquad\qquad oxidised electron donor

b. Dehydrogenases/reductases

These are enzymes that oxidise substrates by the removal of two protons and two electrons from their substrates, generally using nicotinamide adenine dinucleotide (NAD) or flavin cofactors and are typically highly specific for their substrates (Burton and Le Roes-Hill,

2008). Flavin cofactors include flavin adenine dinucleotide (FAD) and flavin mono nucleotide (FMN). Dehydrogenases are distinguished from the other classes of oxidising enzymes by virtue of effecting oxidation without direct participation of oxygen.

This study sought to investigate the potential of an actinomycete-derived peroxidase with a view towards high level production of the enzyme and utilisation in an economically relevant industrial process. The remainder of this chapter will therefore focus on peroxidases.

2.2. Peroxidases

Peroxidases are ubiquitous oxidoreductases that reduce hydrogen peroxide to water with the concomitant one- and/or two-electron oxidation of a variety of both organic (AH_2) and inorganic (e.g., halides, X_2) substrates according to Equations 2 and 3 (Bernroitner *et al.*, 2009):

$$H_2O_2 + 2AH2 \implies 2H_2O + 2 \cdot AH \qquad \textbf{[Equation 2]}$$
$$H_2O_2 + X^- \implies 2H_2O + HOX \qquad \textbf{[Equation 3]}$$

In Equation 2, there is reduction of peroxide by means of two one-electron donors (AH), which can be aromatic molecules, aliphatic molecules, small inorganic anions or metal cations; whereas the two-electron donors (X), in Equation 3 may be halides (Bernroitner *et al.*, 2009). Free radicals ($\cdot$AH) (as generated in the Equation 2) may subsequently dimerise, while the corresponding oxidation product in Equation 3 (HOX) usually tends to be a hypohalous acid or hypothiocyanate (Bernroitner *et al.*, 2009).

Peroxidases occur widely in nature, where they perform a variety of physiological functions. The peroxidase-catalase superfamily, for example is widespread among bacteria, fungi and plants (Zamocky and Obinger, 2010). Several fungal species are involved in carbon cycling via lignin degradation (Christian *et al.*, 2005; Zamocky and Obinger, 2010), and secreted peroxidases from algae and plants are included during the various forms of secondary metabolism processes (Zamocky and Obinger, 2010). In addition to the common fungal strains identified to degrade lignin and natural compounds for nutrient cycling, actinomycetes have also been identified as peroxidase-producing microorganisms (Mercer *et al.*, 1996; Svistunenko *et al.*, 1999).

2.2.1. <u>Classification, Occurrence and Function of Peroxidases</u>

Based on the type of electron donor, the NCIUBMB classifies peroxidases biochemically into 17 categories, under the Enzyme Commission (E.C.) numbering system (different peroxidases are designated E.C.1.11.1.x, donor: hydrogen-peroxide oxidoreductase).

Examples of peroxidases include NADH peroxidase (E.C. 1.11.1.1), cytochrome *c* peroxidase (E.C. 1.11.1.5), glutathione peroxidase (E.C. 1.11.1.9), lignin peroxidase (E.C. 1.11.1.14) and versatile peroxidase (E.C.1.11.1.16). Different peroxidases fall within the E.C. numbers from EC 1.11.1.1 to EC 1.11.1.16 with the exception of EC 1.11.1.4 which has been removed (Passardi *et al.*, 2007). Some enzymes with peroxidase as well as another second enzyme function are classified as peroxidases; these include linoleate diol synthase (EC 1.13.11.44), prostaglandin endoperoxide synthase (EC 1.14.99.1), NAD(P)H oxidase (EC 1.6.3.1) and 4-carboxymuconolactone decarboxylase (EC 4.1.1.44) (Le Roes-Hill, *et al* 2011). Peroxidasins, peroxinectins, other non-animal peroxidases, DyP-type peroxidases, hybrid ascorbate-cytochrome *c* peroxidases and other Class II peroxidases are classified in EC 1.11.1.7 (Koua *et al.,* 2008).

Alternatively, peroxidases may also be classified as haem-peroxidases, vanadium peroxidases or non-metal peroxidases, depending on the nature of the active centre. Vanadium peroxidases contain a vanadate ion at their active site and are most commonly found in marine environments (Valderrama *et al.*, 2002). The non-metal peroxidases are of bacterial origin, and are active only in the presence of acetate or propionate buffer (de Velde *et al.*, 2001).

From a phylogenetic point of view, peroxidases may be classified under two broad categories: haem peroxidases and non-haem peroxidases (Figure 2.1).
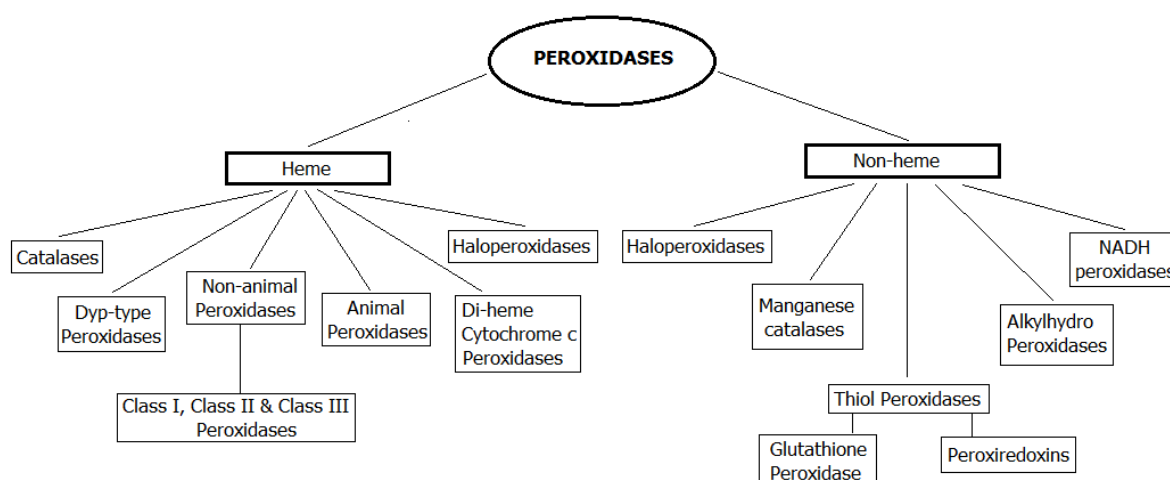
Figure 2.1: Classification of peroxidases according to phylogenetic relationships (adapted from Koua *et al.*, 2008).

2.2.1.1.   Haem Peroxidases

Haem peroxidases are enzymes that contain iron (III) protoporphyrin IX (ferriprotoporphyrin IX) as a prosthetic group (Hamid and Rehman, 2009) and include horseradish peroxidase

(derived from *Amoracia rusticana* roots), lactoperoxidase (from bovine milk) and lignin peroxidase (from the white rot fungus, *Phanerochaete chrysosporium*). The majority of all peroxidases contain the haem moiety. In a 2010 study of at least 6800 known peroxidase sequences found on Peroxibase (a database that contains identified peroxidase-encoding sequences), at least 73% of the sequences coded for haem peroxidases (Zamocky and Obinger, 2010).

From a phylogenetic point of classification, the haem peroxidases may be sub-divided into two main superfamilies (peroxidase-cyclooxygenase, peroxidase-catalase) and three families (di-haem, DyP-type and haloperoxidase).

a. The Peroxidase-Cyclooxygenase superfamily (or 'animal' haem peroxidases)

The Peroxidase-cyclooxygenase superfamily are a large group of metalloenzymes that exhibit both peroxidase and cyclooxygenase activities. Members in this superfamily are haem-containing oxidoreductases and are involved in the innate immune systems of many kinds of organisms, from prokaryotes to complex mammals. Well-researched mammalian peroxidases such as thyroid peroxidase (TPO), lactoperoxidase (LPO) and myeloperoxidase belong to the peroxidase-cyclooxygenase superfamily.

Bacterial members include primordial peroxidases, peroxicins and peroxidockerins. Cyanobacteria, as well as proteobacteria, have been shown to contain open reading frames of primordial peroxidases (Zamocky and Obinger, 2010). Peroxicins are multi-domain peroxidases that possess a short N-terminal peroxidase motif in addition to the normal length peroxidase domain as well as C-terminal haemolysin-like calcium-binding repeats which may be involved in defence mechanisms against competitor bacteria (Zamocky and Obinger, 2010). Peroxidockerins are multi-domain proteins of unknown physiological role composed of a transmembrane domain, dockerin type I repeats and a catalytic peroxidase domain (Bernroitner *et al.*, 2009).

Analogues of bacterial, fungal and animal cyclooxygenases occur in nature. Peroxinectins function as haemoperoxidase and cell adhesion factor involved in invertebrate immune reaction (Vizzini *et al.*, 2013) and are mainly found among various arthropod and nematode species.

The peroxidasins, which consist of a peroxidase moiety fused with an immunoglobulin domain, occur among invertebrates as well as vertebrates including mammals (Zamocky and Obinger, 2010). The peroxidasin has been described as a novel protein structure with

potential to participate in several significant biological processes including removal and destruction of cells that have undergone programmed cell death, targeted consolidation of extracellular matrix by covalent cross-link formation and protection of the organism against non-self-immunity reactions (Nelson *et al.*, 1994).

b. The Peroxide-Catalase superfamily (or 'non-animal' haem peroxidase)

The Peroxidase-catalase superfamily includes the most well studied members including HRP, ascorbate peroxidase and yeast cytochrome c peroxidase. It was originally referred to as the superfamily of plant, fungal and bacterial haem peroxidases (Zamocky and Obinger, 2010). Three sub-classes, namely Class I, Class II and Class III are identified within this superfamily:

i. Class I peroxidases

Class I peroxidases are intracellular enzymes, and include cytochrome *c* peroxidases and ascorbate peroxidases (Jones *et al.*, 1998). They are not glycosylated and do not have signal peptides, calcium ions, or disulphide bridges. Three E.C. numbers define this class: EC 1.11.1.5 (ferrocytochrome-*c*: hydrogen-peroxide oxidoreductase), EC 1.11.1.6 (hydrogen-peroxide: hydrogen-peroxide oxidoreductase, more commonly known as catalase peroxidase), and EC 1.11.1.11 (l-ascorbate: hydrogen-peroxide oxidoreductase) (Passardi *et al.*, 2007). Zamocky and Obinger (2010) further identified two sub-categories within the Class I group of peroxidases, which are the catalase-peroxidases as well as ascorbate peroxidase, cytochrome *c* peroxidase and their putative hybrid types.

- **Catalase-Peroxidases (KatGs)**: Catalase-peroxidases are mainly found in prokaryotes but also occur in dome fungi (Ascomycetes) and typically exhibit dual activity in acting as both a catalase and a peroxidase (Passardi *et al.*, 2007). KatGs exhibit a peroxidase activity similar to that of conventional peroxidases (EC 1.11.1.7, hydrogen peroxide, donor oxidoreductase; Bernroitner *et al.*, 2009).

- **Ascorbate Peroxidase, Cytochrome *c* Peroxidase and their putative hybrid types:** Ascorbate peroxidases utilise ascorbate as its specific electron donor to reduce hydrogen peroxide to water with the concomitant generation of mono dehydroascorbate and functions to prevent the accumulation of toxic levels of hydrogen peroxide in photosynthetic organisms (Shigeoka *et al.*, 2002). The known ascorbate peroxidases are classified as cytosolic, peroxisomal or chloroplastic peroxidases depending on their location in the cell (Passardi *et al.*, 2007).

- **Cytochrome *c* peroxidases (CcP):** (Ferrocytochrome *c*: hydrogen peroxide oxidoreductase, EC 1.11.1.5) catalyse the oxidation of ferrocytochrome *c* by hydrogen peroxide (Erman and Vitello, 1980) and play a major role in scavenging hydrogen peroxide generated during aerobic respiration from the oxidation of cytochrome *c* in the mitochondrial intermembrane space (Passardi *et al.*, 2007).

ii. Class II peroxidases

Class II peroxidases include fungal enzymes that are secreted and include lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP). Class II peroxidases have special biotechnological relevance as they can oxidise compounds with high redox potential (e.g. lignin) and have been utilised in environmental remediation programmes for the removal of organic pollutants (Nicell *et al.*, 1993) and in Kraft pulp bleaching operations (Sasaki *et al.*, 2001). Class II peroxidases are mainly derived from fungi and consist mainly of two classes: LiP (EC 1.11.1.14) and MnP (EC 1.11.1.13). Class II peroxidases differ from Class I peroxidases in that they are glycosylated and contain calcium ions, disulphide bridges and a peptide signal directing the protein to the endoplasmic reticulum for secretion (Passardi *et al.*, 2007).

The versatile peroxidases (no defined EC number exists) encompass multifunctional peroxidases that share the catalytic properties of LiP and MnP (Passardi *et al.*, 2007). This class of enzymes has been shown to oxidise Mn^{2+} directly (as MnP does), as well as high redox potential methoxybenzenes (a property of LiP), and phenolic aromatic substrates (similar to HRP), in addition to other substrates that cannot be directly oxidised by other peroxidases (Ruiz-Dueñas *et al.*, 2007).

iii. Class III peroxidases

Class III peroxidases comprise the plant peroxidases (secreted) including the classical HRP and peanut peroxidases (Jones *et al.*, 1998). Class III peroxidases are involved in cell elongation, cell wall construction and differentiation as well as in the defence against various plant pathogens (Zamocky and Obinger, 2010).

c. Di-haem peroxidase family

The di-haem peroxidase family is characterised by having two haem groups in one protein moiety and its predominant members are various bacterial and a few archaeal members (Zamocky and Obinger, 2010). Di-haem peroxidases are periplasmic proteins that provide protection from oxidative stress.

d.   The DyP-type peroxidases

The DyP-type peroxidases are a distinct group of peroxidases that may be bi-functional enzymes with hydrolase or oxygenase activity, in addition to typical peroxidase activities (Sugano, 2009). DyP-type peroxidases are not related in primary sequence, structure, and reaction characteristics to peroxidases belonging to the plant and animal peroxidase superfamilies (van Bloois *et al.*, 2010). The DyP-type peroxidases exhibit greater activity towards anthraquinone dyes than towards azo dyes, and different degradation spectra with phenolic compounds such as 2,6-dimethoxyphenol, guaiacol and veratryl alcohol (VA) (Sugano, 2009). The DyP-type peroxidases were first discovered in basidiomycetous fungi but differ structurally from other Class II fungal peroxidases and have been classified as a separate group of peroxidases. Subsequently some bacterial homologs have been discovered (van Bloois *et al.*, 2010). The natural roles of the DyP-type peroxidases remain largely unknown. Their strong ability to decolourise dyes makes them potentially significant for biotechnological application, in the removal of dyes from textile effluents, for instance.

e.   Haloperoxidases

Halogenating enzymes belong to three main groups (Murphy, 2003):

- (i)   Non-selective haloperoxidases and perhydrolases (originally regarded as metal-free haloperoxidases; Picard *et al.*, 1997);
- (ii) methyl transferases, which transfer a methyl group from *S*-adenosylmethionine to a halide (Wuosmaa and Hager, 1990); and
- (iii) selective flavin-dependent halogenases.

Two sub-groups are distinguishable within the haloperoxidases: haem and non-haem haloperoxidases. Haloperoxidases contain either vanadium (V) or ferric haem (ferri-protoporphyrin IX) in the active site and generate, as perhydrolases, hypohalous acid but through the direct hydrogen peroxide-dependent oxidation of halides (Murphy, 2003). Perhydrolases take effect via primarily formed peracids, which in turn oxidise halide ions (X^-) into hypohalous acid (XOH) acting as a free diffusible halogenating agent (Van Peé and Patallo, 2006).

Among the halogenating haem peroxidases, *Caldariomyces fumago* peroxidase (generally referred to as CPO) is particularly interesting because it bears a cysteine residue ligated to the proximal side of the haem iron (haem-thiolate protein; Sundaramoorthy *et al.*, 1995) whereas most other haem peroxidases have a histidine at this position (Van Rantwijk and Sheldon, 2000). In consequence, CPO does not only halogenate organic substrates susceptible to electrophilic attack but possesses oxygenase properties as well and catalyses

a multitude of biotechnologically important oxygen transfer reactions (Hofrichter and Ullrich, 2006).

2.2.1.2.   *Non-Haem Peroxidases*

Non-haem peroxidases form five independent families that do not appear to be linked evolutionarily (Koua *et al.*, 2008). The thiol peroxidase family, which is the largest, contains two subfamilies: glutathione peroxidases and peroxiredoxins. Alkylhydroperoxidase, non-haem haloperoxidase, manganese catalase and NADH peroxidase are the remaining four non-haem peroxidase families. These families are not discussed further as they are not within the scope of the objectives of this work.

2.2.2. Structure of Haem Peroxidases

The haem peroxidases are the most, well-characterised type of peroxidases and much is known about the molecular structure and function of this diverse group. The active site of all haem peroxidases are strikingly similar (Gumiero *et al.*, 2010) and during catalysis uses a similar mechanism involving the formation of an intermediate, referred to as Compound I. Most of the information regarding the molecular structure of haem peroxidases comes from the crystal structures of yeast cytochrome *c* peroxidase (CCP), LiP and HRP, which may be regarded as representatives of Class I, II and III peroxidases, respectively. A comparison of the three proteins reveals that they share a similar topology, with identical regions of secondary and tertiary structure (Ruiz-Dueñas and Martinez, 2010). This may point to their evolutionary relatedness despite great differences in individual peroxidase amino acid sequences within the superfamily (Ruiz-Dueñas and Martinez, 2010).

The first haem peroxidase structure to be resolved was that of yeast CCP (Poulos *et al.*, 1980; Figure 2.2). In general, Class I peroxidases contain no disulphide bridges, carbohydrate, structural Ca^{2+} or endoplasmic reticulum signal sequence (Welinder, 1992). This is in contrast to the fungal and plant peroxidases that are secreted, which are routed via the endoplasmic reticulum. The structure of yeast CCP peroxidase reveals that the haem group is enclosed within a crevice formed by two protein domains. His-175 coordinates to the haem iron and defines the proximal haem pocket while the side-chain of Trp-191 is parallel to, and in Van der Waals contact with the imidazole ring of His-175 and the porphyrin ring (Erman and Vitello, 2002). The carboxylate group of Asp-235 is hydrogen bonded to the side chains of both His-175 and Trp-191, thereby introducing acidity in the active site. Arg-48, Trp-51, and His-52 define the distal haem pocket (Erman and Vitello, 2002).
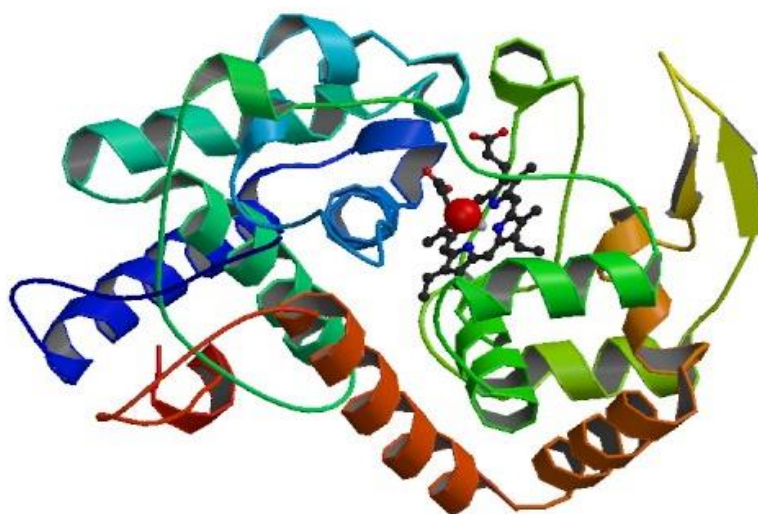
Figure 2.2: Crystal structure of yeast CCP. Protein chains are coloured from the N-terminal to the C-terminal using a rainbow (spectral) colour gradient. Source: RSCB Protein databank (http://www.rcsb.org/pdb/explore.do?structureId=1ebe)

The greatest insights into the molecular physiology of the haem peroxidases have come from studies on the most well-researched haem peroxidase, HRP. The most important structural elements of HRP include a Fe^{3+} protoporphyrin IX (b type haem) with a histidine (His170) as fifth ligand to the Fe^{3+} as well as an extended hydrogen bonding network, four disulphide bonds and two hepta-coordinated Ca^{2+} ions (one at the distal and one at the proximal side of the haem; Figure 2.3(a)) (Szigeti *et al.*, 2008). The proximal and distal Ca^{2+} act as structural stabilisers of the haem cavity thereby maintaining the positions of the conserved histidines (Gajhede *et al.*, 1997). The Ca^{2+} ions are held in place by a network of hydrogen bonds (Gajhede *et al.*, 1997). The proximal and distal histidines are involved in the modulation of the peroxidase redox potential and in the acid–base activation of hydrogen peroxide at the beginning of the catalytic reaction, respectively (Gajhede *et al.*, 1997; Verdin *et al.*, 2006). The haem is sandwiched between the proximal and distal domains in a channel that is about 5 Å wide at the mouth and reaches the haem edge at a minimum depth of about 10 Å (Coates *et al.*, 1998). This channel is believed to allow for transport of substrate peroxides into and out of the active site. Figure 2.3(b) shows the arrangement of these groups in space.
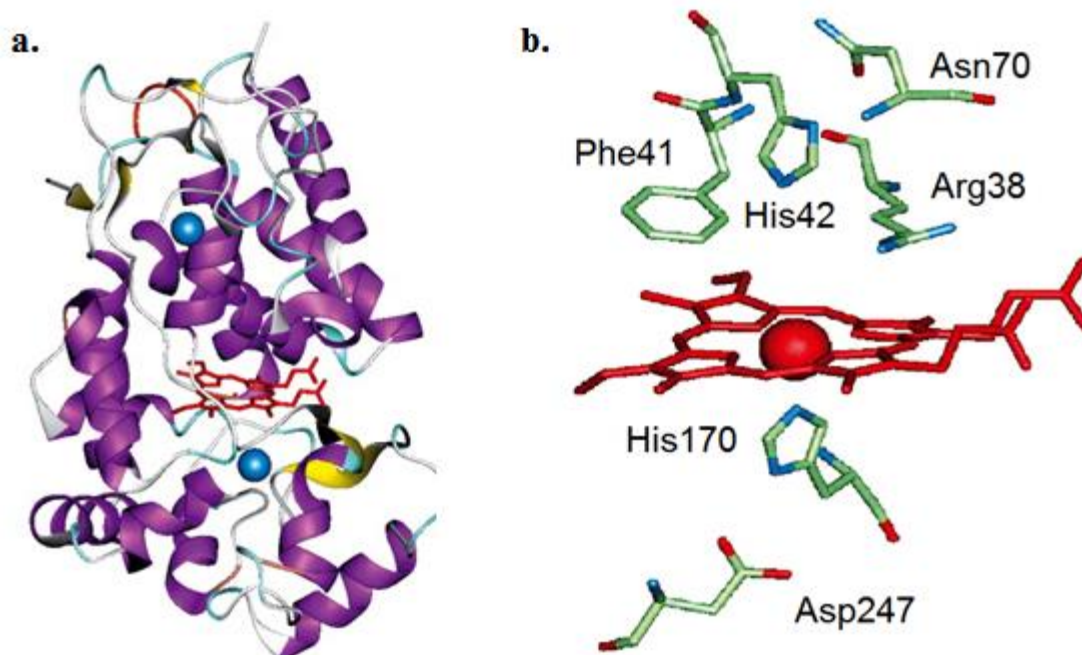
Figure 2.3: (a) The three-dimensional structure of HRP isoenzyme C as depicted by X-ray crystallography. The blue spheres represent the Ca²⁺ ions, while the red plane is the haem group. Alpha-helical and β-sheet regions of the enzyme are shown in purple and yellow respectively. (b) Key amino acid residues in the haem-binding region of HRP. The haem group and haem iron atom are shown in red, the remaining residues in atom colours (Veitch, 2004). Reproduced with permission from Elsevier Ltd.

The proximal histidine residue (His170) of the enzyme links to the haem moiety via a coordinate bond between the histidine side-chain atom and the haem iron atom (Veitch, 2004). Small molecules bind to the haem iron atom at the second distal axial coordination site and binding may be stabilised by hydrogen bond interactions with the distal haem pocket amino acid side-chains of Arg38 (the distal arginine) and His42 (the distal histidine) (Figure 2.3; Veitch, 2004).

2.2.3. Peroxidase mechanism of catalysis

The spectroscopic properties of HRP are representative of those of all peroxidases in which the haem iron is coordinated to the nitrogen of a histidine residue (de Montellano, 1987). The classic HRP peroxidase cycle therefore describes the mechanism of catalysis of most haem peroxidases (with some exceptions and variations) that are found in nature (Figure 2.4).
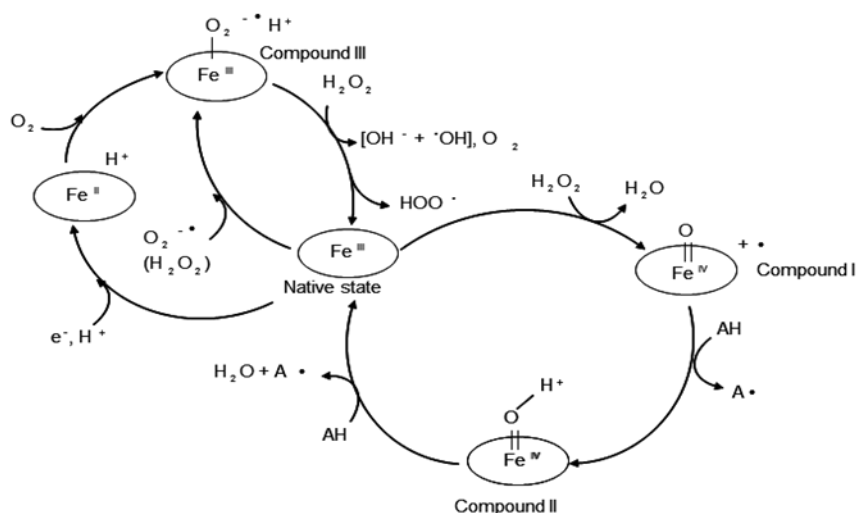
Figure 2.4: General catalytic cycle of haem-peroxidases (reproduced with permission from Le Roes-Hill *et al.*, 2011).

Peroxidases react non-selectively via free-radical mechanisms, using hydrogen peroxide as the electron acceptor (Burton, 2003). The complete peroxidatic mechanism is a cyclic process and involves two key intermediate forms of the enzyme, namely Compound I and Compound II (Figure 2.4). Oxidation of the native ferric haemoprotein by hydrogen peroxide yields Compound I, with the concomitant reduction of hydrogen peroxide to water. Compound I is an iron (IV)-oxo species carrying a porphyrin or protein radical and is formed by the two-electron oxidation of the enzyme by the peroxide molecule (De Riso *et al.*, 2003). Compound I then oxidises the reduced substrate (AH) to generate a substrate radical (A$^\bullet$) and is itself reduced to Compound II (Figure 2.4) which contains an oxoferryl centre coordinated to a porphyrin (Torres *et al.*, 2003).

Compound II reacts with a second molecule of reduced substrate (AH) and is thereby reduced to the ferric (native) form of the enzyme with the release of a water molecule (Torres *et al.*, 2003). The free radicals generated in these processes can form dimers or react with other compounds depending on the conditions of the reaction (Di Cerbo *et al.*, 2001). In the presence of excess hydrogen peroxide, Compound II is converted to a highly reactive peroxyiron (III)-porphyrin free radical, called Compound III (Torres *et al.*, 2003). Compound III is a key intermediate leading to the irreversible inactivation of the enzyme; this process is referred to as suicide-inactivation (Valderrama, 2010).

2.2.4. <u>Reactions catalysed by Peroxidases</u>

Peroxidases can catalyse a wide variety of oxidation reactions and a wide array of biologically active compounds may be formed they have great potential for use in the fine chemicals, agrochemical and pharmaceutical industries. Colonna *et al.* (1999) grouped the different types of reactions catalysed by peroxidases into four general categories:

2.2.4.1. *Oxidative Dehydrogenation*

$$2\ AH + H_2O_2 \quad \Longrightarrow \quad 2\ A^{\bullet} + 2\ H_2O_2 \qquad \textbf{[Equation 4]}$$
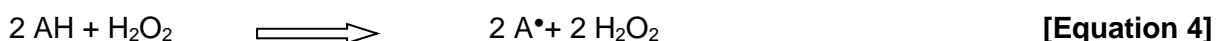
Peroxidases catalyse the one-electron oxidation of sufficiently oxidisable substrates but generally do not catalyse oxygen transfer reactions in which the ferryl oxygen is transferred to the substrate (de Montellano, 1987). Oxidative dehydrogenation is the classical reaction catalysed by peroxidases in general. Oxidative dehydrogenation involves one-electron transfer processes between an oxo-iron (IV) porphyrin-based π-free radical or an oxo-iron (IV) porphyrin and any of a variety of organic and inorganic substrates, with a peroxide (hydrogen peroxide, organic hydroperoxides, peracids) or inorganic oxides (such as periodate and chlorite), as electron donors (Valderrama *et al.*, 2002). Considering the peroxidase-catalysed oxidative dehydrogenation reaction of phenol (Figure 2.5), for example: radical coupling between two phenoxy radicals can occur through an *ortho–ortho* process, giving rise to an *o,o'*-biphenyl adduct, or through an *ortho–para* process, forming the Pummerer's ketone, which is a pharmacophoric synthon (Figure 2.5; Casella *et al.*, 2010).
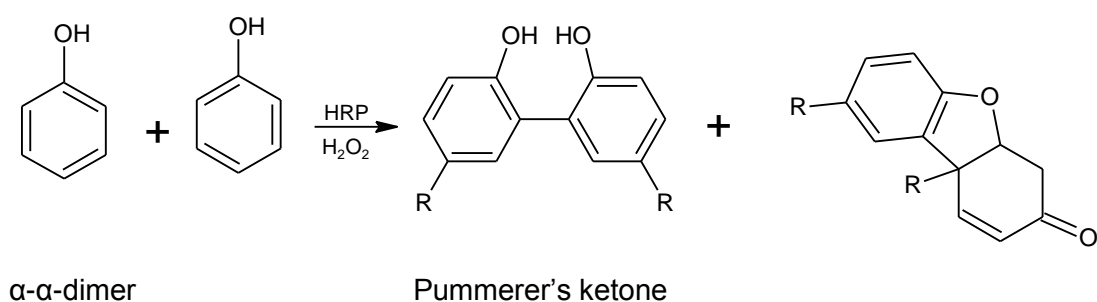
Figure 2.5: The peroxidase-catalysed oxidative dehydrogenation reaction of phenol. Adapted from Casella *et al.* (2010).

2.2.4.2. Oxidative Halogenations

$$AH + H_2O_2 + H^{(+)} + X^{(-)} \implies AX + 2\,H_2O \qquad \textbf{[Equation 5]}$$
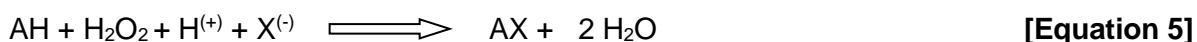
CPO catalyses the conversion of alkenes in the presence of halide ions and hydrogen peroxide to give α,β halohydrins (Colonna *et al.*, 1999). CPO also catalyses the halogenation of a range of aromatic compounds, including anilines, phenols and heterocycles (Colonna *et al.*, 1999). The molecular architecture of CPO makes it unique among the regular haem peroxidases in that it also has oxygenase-like capabilities (Sundaramoorthy *et al.*, 1995). The vanadium and non-haem peroxidases also facilitate oxidative halogenations.

2.2.4.3. Hydrogen Peroxide Dismutation

$$H_2O_2 \implies 2\,H2O + O2 \qquad \textbf{[Equation 6]}$$

Some peroxidases, such as CPO, and to a lesser extent HRP, have this catalase-type of activity, but since this reaction has limited synthetic applications and is not industrially useful, it should be minimised (Colonna *et al.,* 1999).

2.2.4.4. Oxygen Transfer Reaction

$$AH + H_2O_2 \implies AOH + H2O \qquad \textbf{[Equation 7]}$$
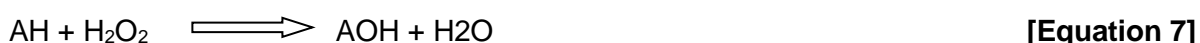
Oxygen transfer oxidations include hetero-atom oxidation (*S*-oxidation and *N*-oxidation), epoxidation, and C-H bond oxidation (Valderrama *et al.*, 2002). These types of reaction allow for the selective oxidation of organic compounds under mild conditions. Due to its versatility, CPO remains the enzyme of choice for the majority of these conversions.

Figure 2.6 depicts the different types of peroxidase-catalysed reactions with examples.
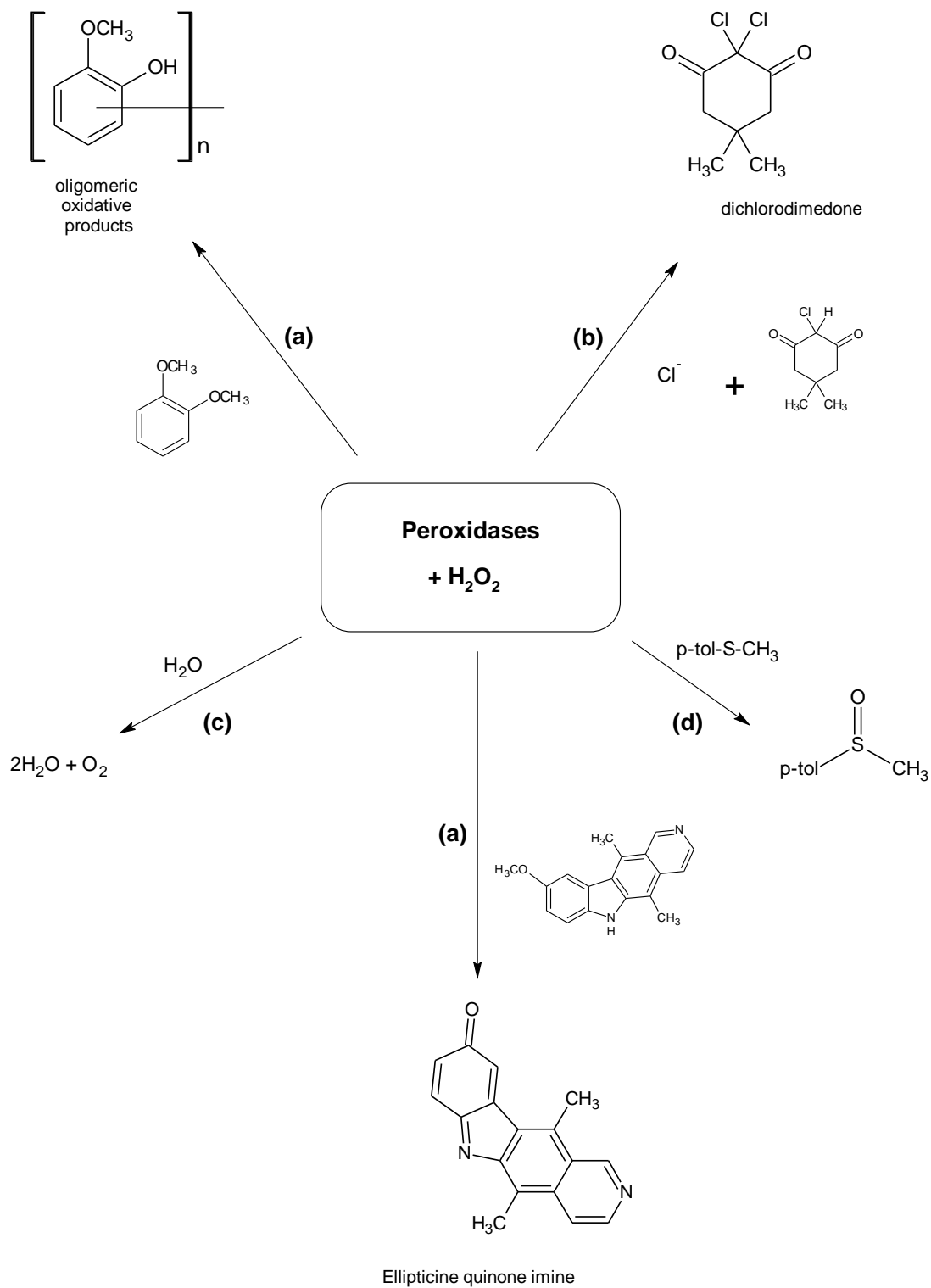
Figure 2.6: Examples of reactions catalysed by peroxidases: (a) oxidative dehydrogenation; (b) oxidative halogenation; (c) hydrogen peroxide dismutation; (d) oxygen transfer reactions (Adapted from Colonna _et al._, 1999).

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2.2.5. Biochemical Properties of Peroxidases

The underlying feature of peroxidases is their ability to catalyse oxidation reactions using peroxide as the electron acceptor. Most peroxidases suffer from the 'suicide inactivation' phenomenon, as described in Section 2.2.3. This section presents a survey of the general biochemical and some biocatalytic characteristics of peroxidases, using selected representatives from plant, fungal and bacterial groups. For the bacteria, the properties of extracellular peroxidases from actinomycetes are presented, as exemplified by the well-studied LiP of *Streptomyces viridosporus* T7A and the relatively less well characterised DyP-type peroxidase class.

2.2.5.1. Biochemical Properties of HRP

HRP is a monomeric haem-containing plant enzyme (44 kDa) which has found enormous biotechnological applications because of its unusually high stability in aqueous solution (Chattopadhyay and Mazumdar, 2000). At least fifteen HRP isozymes have been isolated from horse radish (Veitch, 2004). While the different peroxidases catalyse the same reaction, the individual isozymes differ markedly in their physicochemical and kinetic properties, which in turn explain the apparent discrepancies in peroxidase literature (Shannon *et al.*, 1966).

HRP C is the predominant isoform that has received the most attention. Relative to the other HRP isozymes, HRP C exhibits good stability at 37°C and high activity at neutral pH (Veitch, 2004). Different studies have shown that the thermostability of HRP is affected by several factors including Ca^{2+} concentration, glycosylation, temperature, pH and buffer strength (Haifeng *et al.*, 2008). In the case of haem peroxidases in general, pH is critical for haem stability and activity, as it has a bearing on the ionic form of the enzyme active site residues and thus on the binding of substrates to the haem group.

Studies on the HRP-catalysed oxidation of phenol on magnetic beads showed that the optimum pH for free and immobilised HRP was pH 7.0 (Bayramoglu and Arıca, 2008). The optimum temperature range for the oxidation of phenols with immobilised HRP was between 25 and 35°C and the immobilised HRP was found to be more resistant to temperature inactivation than the free form.

The following compounds are inhibitors of horseradish peroxidase: sodium azide, cyanide, L–cysteine, dichromate, ethylenethiourea, hydroxylamine, sulphide, vanadate, *p*–aminobenzoic acid, Cd^{+2}, Co^{+2}, Cu^{+2}, Fe^{+3}, Mn^{+2}, Ni^{+2}, Pb^+ (Sigma-Aldrich, 2013). Typical reducing substrates that are oxidised by HRP C and other HRP isozymes include aromatic phenols, phenolic acids, indoles, amines and sulphonates (Veitch, 2004).

2.2.5.2.   Biochemical Properties of Fungal LiP and MnP

The white rot fungus, *P. chrysosporium* secretes into its medium two classes of extracellular peroxidases involved in lignin degradation (LiP and MnP). LiP consists of a mixture of different isozymes which are glycoproteins of 38–46 kDa size, with pI values of 3.2–4.0 (Wong, 2009). The multiplicity of LiP isozymes has been described in different *P. chrysosporium* strains and in other white rot fungi. The LiP isozymes differ in their physical characteristics, substrate specificity and stability (Rothschild *et al.*, 1997).

LiP has the unique ability to catalyse oxidative cleavage of C–C bonds and ether (C–O–C) bonds in non-phenolic aromatic substrates of high redox potential (up to 1.4V) (Valli *et al.*, 1990; Wong, 2009). LiP oxidation of aromatic substrates generates aryl cation radicals which are decomposed spontaneously by various pathways (Gold *et al.*, 1989). Compared with other lignin-degrading enzymes, LiP is relatively non-specific towards its substrates and oxidises phenolic aromatic substrates, different non-phenolic lignin model compounds as well as a range of organic compounds (Wong, 2009). Examples of LiP substrates include single-ring, aromatic substrates such as guaiacol, vanillic acid, and syringic acid, and non-phenolic VA, and dimethylphenylenediamine. LiP catalyses the oxidation of phenolic compounds preferentially at a much faster rate compared to non-phenolic substrates (Harvey and Palmer, 1990).

Tien and Kirk (1983) indicated the inhibition of LiP by concentrations of hydrogen peroxide greater than 5 mM. Other inhibitors include sodium azide (Tuisel *et al.*, 1991), ethylenediamine tetraacetic acid (EDTA) (Chang and Bumpus, 2001) and chloride ions (Cai and Tien, 1991).

The MnP from *P. chrysosporium* is an acidic glycoprotein with a pI near 4.5 and a MW of 46kDa (Wong, 2009). It exists in several related isozymes forms.   MnP oxidises Mn(II) to Mn(III), which facilitates the degradation of phenolic compounds including simple phenols, amines, dyes, as well as phenolic lignin substructures and dimers (Wong, 2009). Alternatively, Mn (III) oxidises a second mediator for the breakdown of non-phenolic compounds.

The pH optimum for LiP and MnP is low for both enzymes: pH 3 (Tien and Kirk, 1984; Conesa *et al.*, 2002) and 4.7 (Glenn and Gold, 1985), respectively. Couto *et al.* (2005) reported the optimal pH and temperature for MnP as 4.5 and 32°C, respectively.

2.2.5.3.   Biochemical Properties of LiP of Streptomyces viridosporus T7A

The LiP from *S. viridosporus* T7A depolymerises lignin while degrading cellulose (Crawford, 1978) and produces a modified water-soluble, acid-perceptible polymeric lignin (APPL) as a major lignin degradation product (Crawford *et al.*, 1983; Ramachandra *et al.*, 1988). The predominant P-3 isoform of this enzyme was designated AliP-P3 and its molecular mass has been estimated to be 17.7kDa (Ramachandra *et al.*, 1988). In the presence, but not the absence, of hydrogen peroxide, AliP-P3 was shown to catalyse C_α-C_β, bond cleavage in the side chains of a β-0-4 model compound in a manner similar to that seen with the lignin peroxidase of *P. chrysosporium* (Ramachandra *et al.*, 1988).

S. viridosporus T7A LiP was shown to be stable in the pH range 3.0 to 8.2 in temperatures below 40°C (Lodha *et al.*, 1991; Gottschalk *et al.*, 2008). Sensitivity toward temperature was observed upon the enzyme incubation at 50°C for 30 min at pH 5.5 or 7.0, which resulted in 25% and 38% activity loss, respectively (Lodha *et al.*, 1991; Gottschalk *et al.*, 2008).

AliP-P3 showed activity against 2,4-dichlorophenol (2,4-DCP), homoprotocatechuic acid, caffeic acid, and N,N,N',N'-tetramethylphenylenediamine (TEMED) but was inhibited by potassium cyanide and sodium azide (Ramachandra *et al.*, 1988).

2.2.5.4.   Biochemical Properties of Fungal DyP-type peroxidases

The DyP-type peroxidases are of special biotechnological significance due to their ability to degrade a wide range of important dyes (Kim and Shoda, 1999). The typical characteristic of the DyP-type peroxidases is their ability to oxidise the high-redox potential anthraquinone-type dyes which are hardly oxidised by other peroxidases. The dye-decolourising peroxidase, isolated from *Thanatephorus cucumeris* Dec 1 (designated, DyP), contains a haem group and catalyses peroxidase reactions, but shows no primary or tertiary structural homology to the other known peroxidases (Kim and Shoda, 1999; Sugano *et al.*, 2006). DyP is a glycoprotein which is glycosylated with *N*-acetyl glucosamine and mannose (17%). DyP has a molecular mass of 60 kDa and a pI of 3.8 (Kim and Shoda, 1999). The optimal temperature for DyP activity was 30°C, and DyP activity was stable even after incubation at 50°C for 11rs. The enzyme exhibits anthraquinone dye decolourisation activity which is relatively rare compared to azo dye decolourisation activity (Kim and Shoda 1999). The wide range of substrate specificity shown by the *T. cucumeris* Dec 1 strain itself, which includes azo and anthraquinone dyes, is thought to indicate the involvement of multiple enzymes from this strain (Sugano *et al.*, 2006). Also, the ability of *T. cucumeris* Dec 1 to completely decolourise Reactive blue 5 *in vivo*, but not *in vitro*, means that dye degradation *in vivo* is likely to involve other enzymes in addition to the peroxidase.

Liers *et al.* (2010) demonstrated the production of two haem peroxidase forms (AjP I and AjP II) with DyP-like characteristics by the jelly fungus *Auricularia auricula-judae.* Both AjP I and AjP II catalysed not only the conversion of typical peroxidase substrates such as 2,6-dimethoxyphenol and 2,2′-azino-bis(3-ethylthiazoline-6-sulphonate) but also the decolourisation of the high-redox potential dyes Reactive Blue 5 and Reactive Black 5 (Liers *et al.*, 2010). The N-terminal amino acid sequence of AjP matched well with sequences of fungal dye-decolourising peroxidases. Interestingly, both AjPs oxidised nonphenolic lignin model compounds (VA; adlerol, a nonphenolic β-O-4 lignin model dimer) at low pH (maximum activity at pH 1.4), which indicates a certain ligninolytic activity of dye-decolourising peroxidases.

2.2.5.5.   Biochemical Properties of Bacterial DyP-type peroxidases

Escherichia coli YcdB is one of the well-characterised bacterial DyP-type peroxidase and it has been shown that YcdB is a haemoprotein (Sturm *et al.*, 2006). It exhibits modest peroxidase activity with guaiacol as a substrate and is most active under acidic conditions (Sturm *et al.*, 2006). Van Bloois *et al.* (2010) characterised a novel DyP-type peroxidase (*Tfu*DyP) from *Thermobifida fusca* and showed that the monomeric, robust enzyme contained non-covalently bound haem as cofactor and had an optimum pH of 3.8. *Tfu*DyP was shown to oxidise several dyes to differing extents (including Reactive Blue 19, Reactive Blue 4 and Reactive Black 5) as well as the general peroxidase substrates guaiacol, 2,6-dimethoxyphenol, VA and *o*-phenylenediamine (Van Bloois *et al.*, 2010). Ahmad *et al.* (2011) showed that DypB, a recombinant DyP-type peroxidase from *Rhodococcus jostii* RHA1, was capable of oxidising both polymeric lignin and a lignin model compound, and appeared to possess both Mn(II) and lignin oxidation sites.

2.2.6. Applications of Peroxidases

A wide range of applications for peroxidases is possible due to their ability to non-specifically oxidise different organic and inorganic substrates. HRP and LiP have been the main peroxidases that have been exploited commercially, but new sources of peroxidase such as soybean (Hailu *et al.*, 2010) and turnip (Agostini *et al.*, 2002) have received attention and have been suggested for possible commercial exploitation. Peroxidases have found the greatest world market in diagnostic kits as well as in poly-enzyme-based biosensors and as components of Enzyme Linked Immunosorbent Assay (ELISA) detection systems.

2.2.6.1.   Biosensors, Analytical Devices and Diagnostic Kits

Biosensors are analytical devices that combine a biological component with a suitable transducer, which converts the biological signal into an electrical signal. Biosensors are

increasingly becoming important in many sectors such as medicine, food production, pharmaceuticals as well as fundamental research. Due to its many favourable biochemical characteristics that include stability, good sensitivity in the range of analyte detection, availability in pure form, and the ability to oxidise numerous chromogenic substrates HRP is one of the most widely used enzymes in analytics (Azevedo *et al.*, 2003). HRP is generally incorporated into biosensor systems, where its function is to catalyse the formation of an easily detectable coloured product from the analyte. Peroxidase-based electrodes are employed in analytical systems to detect hydrogen peroxide and other organic hydroperoxides (Jia *et al.*, 2002). When co-immobilised with another hydrogen peroxide-producing oxidase, these biosensors may be exploited for determination of glucose, alcohols, glutamate, choline, lactate, uric acid, pyruvate and amino acids (Ruzgas *et al.*, 1996; Azevedo *et al.*, 2003). Glucose and cholesterol sensors containing HRP are widely used for the detection and during management of diabetes mellitus and heart conditions, respectively.

Peroxidases are particularly suited for the preparation of enzyme-conjugated antibodies because of their ability to form chromogenic products at low substrate concentrations (Krell, 1991). Universal covalent conjugates of proteins, antibodies and other molecules with HRP are useful and versatile tools for ultra-sensitive detection in immunoassays, nucleic acid detection, and histo- and cyto-chemical applications (Azevedo *et al.*, 2003). HRP has also been incorporated in medical kits for the detection of compounds indicative of different conditions such as bladder and prostate cancer risks (8-hydroxydeoxyguanosine in urine) (Chiou *et al.*, 2003); cystic fibrosis (cystic fibrosis delta F508 mutation in blood) (Hopfer *et al.*,1995) as well as tumours (human tumour necrosis factor). HRP-based ELISA test assays have been developed for screening monoclonal antibodies against mycotoxins (Hamid and Rehman, 2009). This approach has several advantages over traditional chromatography-based detection methods including simplicity, low cost, reliability, low requirements for technical skills and simple equipment.

Non HRP-based kits have also been devised. A diagnostic test kit for the detection of uric acid was developed based on peroxidase from hairy root tip cultures of turnip (Agostini *et al.*, 2002). The capability of actinomycete-derived peroxidases to function as useful chromogen-generating agents for application in biosensors and related systems has largely not been investigated.

2.2.6.2.   *Bioremediation of Phenolic and Related Compounds*
Aromatic compounds including phenols and aromatic amines, arising from different industries (coal mining, textiles manufacture, petroleum refinery, paint, leather, paper and pulp milling) result in the release of toxic compounds which are dangerous to plant and animal life if

released directly into the environment. Enzymatic approaches for removal of these pollutants have proven useful because enzymes are highly efficient and can degrade toxic compounds in both dilute and concentrated solutions.

HRP has been shown to be capable of polymerising phenols and aromatic amines, resulting in the precipitation of the polymers out of solution. The majority of the degradation products formed are insoluble polymers, which are less harmful and can be removed by coagulation and precipitation followed by filtration or sedimentation (Azevedo *et al.*, 2003). The ability of HRP to induce formation of mixed polymers between certain difficult-to-remove contaminants and the easily-removable contaminants makes the enzyme potentially very useful for industrial wastewater clean-up programs. For example, polychlorinated biphenyls can easily be co-precipitated with phenols in the presence of HRP (Klibanov, 1983). Different approaches that have been shown to improve the efficiency of HRP-catalysed removal of toxic compounds from wastewaters including immobilisation of the HRP (Liu *et al.*, 2011), addition of enzyme protecting compounds such as polyethylene glycol (Nicell *et al.*, 1995; Cooper and Nicell, 1996) or Ni^{2+} ions (Nazari *et al.*, 2007) and adjustment of bioreactor configuration to optimise enzyme performance (Nicell *et al.*, 1993).

LiP and MnP from *P. chrysosporium* and other white rot fungi are high redox potential enzymes capable of catalysing the oxidation of phenol and related compounds, in a manner analogous to HRP-based catalysis of the same compounds. LiP has been shown to oxidise a number of polycyclic aromatic and phenolic compounds (Aitken *et al.*, 1989). In addition, LiP catalyses the oxidation of many other recalcitrant pollutants, including chlorinated aromatic compounds, dichlorodiphenyltrichloroethane (DDT) and other pesticides, dyes, munitions, cyanides, azides and cross-linked acrylic polymers (Higson, 1991; Barr and Aust, 1994; Sutherland *et al.*, 1997; Conesa *et al.*, 2002).

While the potential for peroxidases has been shown, the bioremediation of contaminated wastewaters using peroxidases has not been applied on an industrial commercial scale. There are constraints that have limited the use of HRP for the removal of organic contaminants from wastewaters. These include the inactivation of the enzyme by its co-substrate (hydrogen peroxide) and by the phenoxy radicals that are formed during catalysis. Due to inactivation a considerable amount of starting enzyme is required for the process to be economically viable. In addition, in certain cases trace amounts of soluble, low molecular weight products can be produced which are more toxic than the original compound (Wagner and Nicell, 2002).

Peroxidases from other non-traditional sources such as soybean, turnip roots, and bitter gourd, have been proposed as alternatives to HRP (Torres-Duarte and Vazquez-Duhalt, 2010). Immobilised turnip peroxidase in the presence of polyethylene glycol (PEG) efficiently catalysed phenol removal from a real wastewater effluent and covalent chemical modification with PEG and immobilisation of the modified turnip peroxidase showed improved characteristics including an enhanced protein conformational and thermal stability, higher solvent tolerance, and a slight increase in catalytic efficiency (Quintanilla-Guerrero *et al.*, 2008). These improvements allowed for an extended re-use of the peroxidase preparation in the transformation of a highly concentrated phenol solution.

2.2.6.3.   Application in Decolourisation of Synthetic Dyes

Some dyes from the photography, textile and printing industries, such as azo dyes, are recalcitrant, toxic compounds that should be detoxified prior to release into the environment. Enzyme-facilitated breakdown of these compounds is the preferred approach over currently used methods such as chemical oxidation, reverse osmosis and adsorption. This is mainly because the use of enzymes is generally considered to pose less threat to the environment. HRP was shown to be effective for the degradation and precipitation of the azo dyes Remazol Blue and Cimacron Red (Bhunia *et al.*, 2001). Similarly fungal peroxidases have been shown to possess similar capabilities. Asgher *et al.* (2006) reported that *P. chrysosporium* and *Trametes versicolor* could effectively decolourise the textile dyes Remazol Brilliant Yellow 3GL, Procion Blue PX-5R and Cibacron Blue P-3RGR in shake flask cultures. The authors attributed decolourisation of the dyes to the activity of the consortium of oxidative enzymes including LiP, MnP, laccase and Mn-independent versatile peroxidase (VP) produced by the white rot fungi.

Triphenylmethane dyes are a group of dyes that are widely used for many applications such as dying nylon and cotton fabrics in the textiles industry, for colouring plastics, gasoline, varnish, oil and waxes, as well as for imparting colour in medical and biological stains. They are generally considered as xenobiotic compounds and they are very recalcitrant to biodegradation (Shedbalkar *et al.*, 2008). Yatome *et al.* (1991) reported one of the earliest positive decolourisation results of triphenylmethane dyes via the use of actinomycetes. They showed that '*Nocardia corallina*' (*Gordonia rubripertincta*) and *Nocardia globerula* could completely decolourise crystal violet in 24 hrs. They also showed that '*N. corallina*' had the capacity to decolourise (albeit to a lesser extent than crystal violet) other dyes such as Methyl Violet, Ethyl violet, Basic Fuschin and Victoria Blue. However, the underlying mechanism of decolourisation was not shown to be specifically due to peroxidase activity in these studies.

Some actinomycetes (e.g. *T. fusca*) and fungi (e.g. *Thanatephorus cucumeris*) secrete the so-called DyP-type peroxidases. This type of peroxidase is remarkable in the ability to oxidise anthraquinone dyes which are a particularly inert group of dyes. Sugano (2009) suggested that the versatility of DyP-type peroxidases may be useful for the treatment of wastewater contaminated with recalcitrant xenobiotics.

2.2.6.4. Application in the Paper and Pulp Industry

Biopulping entails the use of ligninolytic microorganisms or their enzymes to degrade wood components to produce a soft pulp which can be further processed to produce paper products. Considerable research has investigated the lignin degradation mechanisms of fungi with the aim of degrading the lignocellulose contaminants in industrial wastewaters from the pulp and paper milling industries. Effluent from these industries pollutes water, air and land. The biological colour removal process uses bacteria, fungi and algae to degrade the polymeric lignin-derived chromophoric material (Murugesan, 2003). Laccase (EC 1.10.3.2, benzenediol: oxygen oxidoreductase), LiP, MnP, VP, and hydrogen peroxide-forming enzymes such as glyoxal oxidase (GLOX) and aryl alcohol oxidase (AAO, EC 1.1.3.7) are all enzymes that play a part in the natural degradation of lignin. They are produced by lignin-degrading white-rot fungi and litter-decomposing fungi in different combinations (Hatakka, 1994; Hatakka, 2001) and have been shown to effectively treat paper mill effluent.

Antonopoulos and co-workers (2001) showed the suitability of extracellular peroxidase produced by *Streptomyces albus* ATCC 3005 for application in the bleaching of Kraft pulps. Advantages to the process included: (*i*) short retention times within which significant kappa number reductions occurred, (*ii*) high peroxidase activity that could be applied directly in alkaline Kraft pulps, (*iii*) stability of the peroxidase with high hydrogen peroxide concentrations, and (*iv*) no addition of costly mediators was required. Niladevi and Prema (2005) reported a newly-isolated mangrove actinomycete, *Streptomyces psammoticus* (strain NJP49), capable of producing all three major ligninolytic enzymes (LiP, laccase and MnP). These studies show that bacterial enzymes may also have potential for use in decolourisation of paper mill effluent.

2.2.6.5. Application of peroxidases in organic and polymer synthesis

Peroxidases of non-bacterial origin have been utilised extensively for the production of useful novel compounds and materials. Regarded as the most versatile peroxidase, CPO catalyses a wide range of reactions including the non-specific chlorination, bromination, and iodation of a variety of electrophilic organic substrates (Hofrichter and Ullrich, 2006). In the absence of halides, CPO resembles cytochrome P450s and epoxidises and hydroxylates activated

substrates such as organic sulphides and olefins (Hofrichter and Ullrich, 2006). Some of these reactions have a promising biotechnological potential because they lead to the regio- and enantio-selective oxygenation of substrate molecules and, hence, to pro-chiral or chiral products (Littlechild, 1999). The direct oxidation of olefins catalysed by CPO, in the presence of hydrogen peroxide, is an important means for the production of chiral epoxides, which are important for the preparation of important compounds in the fine chemicals industry (Casella *et al.*, 2010).

The production of conducting polymers is of currently of interest because of their wide range of applications, including uses in anticorrosive protection, optical displays and light-emitting diodes (Raitman *et al.*, 2002). HRP-catalysed polymerisation of aniline has been used to create polyaniline, a versatile electrically-conducting polymer with good environmental stability (Hamid and Rehman, 2009). By a similar mechanism, MnP derived from *Bjerkandera adusta* has been used to prepare polyacrylamide, and features of the resultant compound showed that it could be used as a thermoplastic resin (Iwahara *et al.*, 2000).

2.2.6.6.   Other applications
Potentially cheaper sources of peroxidases (relative to HRP) could be used for some bulk-use, non-analytical applications. Compounds such as phenols, indoles, volatile fatty acids, ammonia, hydrogen sulphide and mercaptans that are found in manure could be removed by application of peroxidases, thereby improving animal health and livestock growth rates in animal husbandry programs (Ye *et al.*, 2009). In contrast to fungal and plant peroxidases, bacterial peroxidases have not been widely used in these kinds of applications.

2.2.6.7.   Production of antioxidant compounds
The use of oxidative enzymes for the production of novel, bioactive compounds with antioxidant properties has been demonstrated using laccase from a *Micromonospora* sp. (Goodwin, 2010) and from *Trametes pubescens* (Adelakun *et al.*, 2012). In the latter investigation, the laccase from *T. pubescens* was used to catalyse the coupling of ferulic acid monomers a monophasic or biphasic system, for the production of dimeric compounds with increased antioxidant capacity.

The potential for peroxidases to catalyse oxidation of phenolic compounds to produce similar products has not been demonstrated and may be an area well-worth investigating. An objective of this work was to demonstrate this capacity by a peroxidase derived from an actinomycete.

2.2.7. Factors limiting the use of peroxidases

One of the important requirements for a successful biocatalytic process is the availability of the enzyme in adequately large amounts (Burton *et al.*, 2002). A significant factor limiting the use of peroxidases is their relatively high isolation and purification cost (Ferrer *et al.*, 1991) in comparison to other commercially available oxidases. Aside from the white rot fungi, actinomycetes are the only other known source of extracellular peroxidases. While ubiquitous in other living systems, peroxidases occur as intracellular enzymes and are secreted in small amounts. The production of extracellular enzymes is advantageous because it simplifies the downstream purification and recovery of the enzyme from the growth medium. Inexpensive enzyme sources should be identified if peroxidases are to find greater biocatalytic applications on an industrial scale. Furthermore, large-scale production of enzymes by actinomycetes has been shown to be possible, e.g. nitrile hydratase production by *Rhodococcus* spp. Actinomycetes, therefore, offer an attractive advantage to fungi as peroxidase producers, as they can be easily cultured in defined media and peroxidase production appropriately optimised.

Often, peroxidases are characterised by rapid deactivation by hydrogen peroxide or other organic hydroperoxides (Torres *et al.*, 2003). This phenomenon is referred to as the 'suicide inactivation' of haem peroxidases. Hydrogen peroxide is the 'suicide' substrate which converts Compound II to a highly reactive peroxyiron(III)-porphyrin free radical, called Compound III (Figure 2.4). A second hydrogen peroxide molecule reacts with this free radical, producing a hydroxyl radical which attacks the tetrapyrrol structure of the haem group leading to irreversible inactivation of the enzyme (Torres *et al.*, 2003). Polymeric substances that are formed in this free-radical catalysed reaction also attach to the enzyme and tend to inhibit its activity (Wu *et al.*, 1993). However, Torres and co-workers (2003) suggested that deactivation of the enzyme by hydrogen peroxide should be studied for each application as the process is influenced by the composition of the reaction media. For example, the presence of organic substances, such as PEG (Cheng *et al.*, 2006), or of Ni^{2+} ions (Louie and Meade, 1999) in the medium, is able to protect the biocatalyst from deactivation. De Velde and co-workers (2001) improved the operational stability of CPO using two methods: feed-on-demand addition of hydrogen peroxide and *in situ* generation of hydrogen peroxide. It is clear, however, that the increased number of process operations also increases the costs involved. Elevated temperatures also severely reduce the activity of peroxidases (Hamid and Rehman, 2009), and thermostable enzymes derived from novel strains or from engineered microorganisms could offer a solution to this problem. The fact that the peroxidase from *S. albus* was tolerant to higher concentrations of hydrogen peroxide (Antonopoulos *et al.*, 2001) makes it a useful alternative to the traditionally-employed HRP and other peroxidases that are more susceptible to suicide inactivation for Kraft pulp bleaching operations.

Overall, peroxidases are useful for a number of industrial applications, from wastewater remediation to fine chemicals production. A number of factors limit the use of peroxidases, particularly finding alternative inexpensive sources of the enzyme. Therefore the search for novel sources of peroxidase with new, improved or different properties is necessary.

2.3.   Actinomycetes

The name 'actinomycete' is derived from the Greek words 'aktis-' (ray) and 'mykes' (fungus), a term which was coined for this group of microorganisms with reference to their fungus-like, filamentous morphology (Figure 2.7).
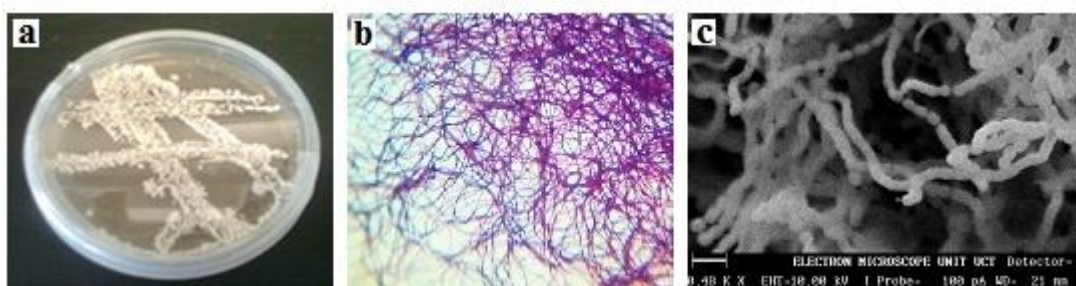
Figure 2.7: Morphological characteristics of *Streptomyces* sp. strain GSIII#1. (a) Typical growth features on a solid agar plate; (b) Gram stained micrograph at X1000 magnification and (c) an electron micrograph showing growing spores in chains.

Actinomycetes comprise a large and diverse group of largely filamentous, aerobic bacteria, many of which are ecologically important and are exploited commercially for the production of natural products such as antibiotics and enzymes (Edwards, 1993). They are Gram positive bacteria characterised by high G+C content DNA (55-75%; Dhananjeyan *et al.*, 2010) and typically have soil as their natural habitat, although marine (and other environmental) forms are being increasingly described. They are ubiquitous microorganisms that can be found in both man-made and natural environments and are predominantly aerobic, heterotrophic, saprophytic and non-motile (Kulkarni, 2003).

It is a well-known attribute of the actinomycetes to produce inhibitory compounds that repress the growth of other bacteria (Waksman *et al.*, 2010). Their abundance in soils and other ecological niches such as marine waters and mangrove swamps, coupled with the wide range of diversity among the actinomycetes means they harbour great capacity and potential for the production of a wide range of important, bioactive compounds. Actinomycetes are well-known producers of many biological compounds of importance to man, notably antibiotics such as streptomycin, actinomycin, streptothrycin, mycetin and micromonosporin. In addition to antibiotics, actinomycetes are an important source of other bioactive

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metabolites that include enzymes, enzyme inhibitors, antitumour agents and nutraceuticals. Actinomycetes have been described as virtually unlimited sources of novel compounds (Subramani and Aalbersg, 2012). The notably higher relative contributions made by actinomycetes to the total number of bioactive compounds discovered is shown in Table 2.1. Since most bioactive compounds from actinomycetes are commercially valuable compounds, this group of microorganisms continues to be screened routinely for the production of novel compounds (Prakash *et al.*, 2013).

Table 2.1: Approximate numbers of bioactive microbial metabolites that have been described since 1940 to 2010 (adapted from Berdy, 2012).

Periods	1940-1974	%	1975-2000	%	2001-2010	%	Total
Micro-organism							
Actinobacteria	3 400	62	7200	42	3 100	28.5	13 700
Streptomyces spp.	2 900		5100		2 400		10 400
Other actinobacteria	500		2100		700		3 300
All microscopic bacteria	800	15	2300	13	1 100	10	4 200
Myxobacteriales	25		400		210		635
Cyanobacteria	10		30		1 250		1 290
Fungi	1 300	23	7700	45	6 600	61	15 600
Microscopic fungi	950		5400		4 900		11 250
Basidiomycetes	300		1800		1 500		3 600
Other fungi	20		200		160		380
Total /total per year	5 500/180		17000/690		10 800/ 1 100		33 500

Berdy (2012) reviewed the distribution of known bioactive natural compounds according to their sources and historical use. The study showed that *Streptomyces* spp. and filamentous fungi were the most notable producers of bioactive metabolites (with respect to numbers, versatility and diversity).

The order *Actinomycetales* is one of ten orders within the phylum *Actinobacteria*; the other nine orders being *Acidimicrobiales, Bifidobacteriales*, *Coriobacteriales, Euzebyales, Gaiellales, Nitriliruptorales, Rubrobacterales, Solirubrobacterales,* and *Thermoleophilales* (http://bacterio.net/-classifordersclasses.html#Actinobacteria). The order *Actinomycetales* comprises a very large group of diverse microorganisms whose characteristics have been exploited in the pharmaceutical industry (drug and antibiotic production), in bioprocesses (for the production of enzymes, including peroxidases, laccases and other oxidative enzymes) and in environmental remediation (degradation of textile dyes from industrial effluents) (Arenskötter *et al.*, 2004; Mane *et al.*, 2008; Bagewadi *et al.*, 2011; Le Roes-Hill *et al.*, 2011).

Actinomycetes are primarily terrigenous microorganisms and are intimately involved in soil ecology (Kirby, 2006). Filamentous bacteria of the genus *Streptomyces* are involved in carbon cycling in the natural ecological environment. They (together with other non-*Streptomyces* actinomycetes), have been shown to produce ligninolytic enzymes and can mineralise up to 15% of labelled lignins (Ramachandra *et al.*, 1988; Adhi *et al.*, 1989; Ball *et al.*, 1989; Tuncer and Ball, 2002; Hatakka, 2001). Typically, *Streptomyces* spp. solubilise part of lignin, and the end product is water-soluble acid-perceptible polymeric lignin (Crawford *et al.*, 1983; Hatakka, 2001).

Some members of the order *Actinomycetales* are medically important, as they are human pathogens. *Corynebacterium, Nocardia* and *Mycobacterium* spp. are responsible for major types of human infections such as actinomycosis, nocardiosis and tuberculosis (Kulkarni, 2003). The air-borne spores of some pathogenic strains can result in the manifestation of diseases such as 'farmer's lung' and 'hypersensitive pneumonitis' (Kulkarni, 2003). In agriculture, some actinomycete species are also responsible for disease manifestation, for example *Streptomyces scabies* (later re-classified as *Streptomyces scabiei*) causes potato scab disease in potato. In water treatment plants, incidental establishment and overgrowth of actinomycetes causes problems such as scumming and compromise the efficiency of the treatment process (Kulkarni, 2003).

2.3.1. Actinomycetes as a Source of Peroxidases

The interest in actinomycetes in the present study is due to their ability to secrete extracellular peroxidases. The actinomycetes have been described as a potentially rich source of peroxidases for introduction into a market that is substantial and almost totally dominated by HRP (Mercer *et al.*, 1996).

Actinomycetes produce peroxidases and other oxidative enzymes which are key factors for the degradation of organic matter and the recycling of nutrients (Mason *et al.*, 2001). The production and purification of peroxidases from actinomycetes is advantageous for two important reasons. Firstly, actinomycetes secrete peroxidases into their extracellular environment (Mason *et al.,* 2001). This implies that the downstream purification and recovery of the enzyme may be simplified. In contrast, the recovery of peroxidase from horseradish roots for example, is a lengthy and complex procedure involving multiple precipitation, fractionation, chromatography and filtration steps, with the whole process taking 5-8 weeks, depending on the grade of the enzyme preparation (Barnard, 2012). In addition, only a few grams of the highest grade peroxidase are produced from 1 tonne of roots (Barnard, 2012). Secondly, because actinomycete-based peroxidases, unlike their fungal counterparts, are

largely unexplored, they could potentially offer, as yet, undiscovered properties that could be exploited in novel biotechnological applications.

Studies by different workers demonstrate the potential of actinomycetes as a noteworthy source of peroxidase with potentially novel applications. The following section is a brief overview of the properties of some of the actinomycete-derived peroxidases that have been reported, and the suggested biotechnological applications for them:

a. Iqbal *et al.* (1994) studied the characteristics of peroxidases obtained from a thermophilic actinomycete, *Streptomyces thermoviolaceus.* The microorganism produced two predominant isoforms (P-3 and P-5) which were shown to be peroxidase haem proteins that catalysed the oxidation of substrates only in the presence of hydrogen peroxide. The P-3 isoform was stable at 50°C for more than 24 hrs and had a half-life of 70 min at 70°C. Both isoforms were stable after incubation at 60°C in the presence of sodium dodecyl sulphate (SDS). Additionally, the P-3 isoform showed an unusually high hydrogen peroxide requirement for optimal activity, making it a potentially good alternative over HRP and other peroxidases that suffer from inactivation by hydrogen peroxide. The authors suggested potential applications similar to those employing HRP (Iqbal *et al.,* 1994).

b. The peroxidase from *T. fusca* BD25 has been described as a novel non-haem peroxidase showing activity over the temperature range between 50 and 80°C (50% maximum activity retained) and pH 6.0 – 8.0 and a high stability against denaturing agents (e.g. 5% (w/v) SDS and 8 M urea (Svistunenko *et al.,* 1999). *T. fusca* BD25 secretes at least four non-haem peroxidase isoforms. The P-1 isoform is capable of cleaving simple and complex aromatic compounds. Rob *et al.* (1997) demonstrated that the pre-treatment of straw and paper pulp with *T. fusca* BD25 prior to subsequent treatment with pure xylanases and cellulases increased the yield of released sugars by a magnitude of three times. The thermostability of *T. fusca* peroxidase and its ability to depolymerise lignin could make it potentially useful for the biobleaching of pulp (Tuncer and Ball, 2002). Van Bloois *et al.* (2010) characterised an extracellular haem-containing peroxidase from *T. fusca*, which not only was capable of decolourising dyes, but also catalysed enantioselective sulfoxidations. The properties of the bacterial DyP-type peroxidases have been presented in Section 2.2.5.5.

c. Antonopoulos and co-workers (2001) studied the suitability of an extracellular peroxidase produced by *S. albus* ATCC 3005 for application in the bleaching of Kraft pulps. The enzyme obtained maximal activity at pH 9.9, while exhibiting a

broad pH optimum (activity >90%) between pH 8.1 and 10.4. This property makes peroxidase from *S. albus* ATCC 3005 suitable for direct application on alkaline Kraft pulp. In addition to stability under alkaline conditions, the enzyme remained active (>64.6% activity) after 1 hr in the presence of 100 mM hydrogen peroxide, making its performance superior to that of fungal peroxidases. Fungal peroxidases are typically inactivated by hydrogen peroxide concentrations greater than 0.1 mM (Glenn and Gold, 1985; Paice *et al.*, 1995).

In conclusion, actinomycetes represent a biotechnological resource that potentially can be exploited further for producing useful bioactive compounds and for carrying out industrial transformations. The following chapter describes the screening and optimisation of selected actinomycete strains for peroxidase production.

CHAPTER THREE

INITIAL SCREENING EXPERIMENTS

3.1.   Introduction

Enzyme-based catalysis is an attractive approach over conventional petrochemical-based catalysis due to several reasons, including catalysis at physiological conditions, generation of non-toxic wastes and high yields of production. At least 50% of the enzymes used in industry are derived from bacteria. The increasing demand for robust, high turnover, economical and easily available biocatalysts has resulted in a renewed surge in the search for novel sources of enzymes, enzymes with novel properties or the improvement of existing enzymes by engineering at gene and protein level (Prakash *et al.*, 2013). Because the substrates used in modern industrial processes are generally artificial compounds, enzymes known to catalyse suitable reactions for such processes are unknown. Hence, continuous screening for novel enzymes capable of catalysing new reactions is needed (Ogawa and Shimizu, 1999).

The identification of appropriate enzyme candidates depends on the use of efficient and sensitive screening strategies and the greatest possible input of diverse candidate genes and organisms for screening purposes (Lee *et al.*, 2010). The traditional approach of obtaining novel enzymes by cultivation and subsequent screening of pure strains of organisms remains a standard and powerful approach (Ogawa and Shimizu, 1999). The traditional screening approach enables the rapid detection and elimination of poor-performing candidates and allows identification of the best performing ones for further studies such as scale-up and characterisation. Industrial enzymes usually come from two kinds of sources: 'common' environments (e.g. soil or seawater) and 'extreme' environments (e.g. hot springs, Antarctic ice and alkaline lakes) (Lee *et al.*, 2010), yielding a variety of enzymes that catalyse reactions under normal or extreme conditions. The advent of designer biocatalysts, produced by informed selection and mutation through recombinant DNA technology, enables production of process-compatible enzymes from these microorganisms (Burton *et al.*, 2002). Enzyme engineering, for example with the aim to enable activity in non-aqueous media, and protein engineering to provide the enzyme with altered structures, functions and activities, can improve catalytic efficiency (Singh, 2010).

Screening a large number of microorganisms with a well-considered screening system based on microbial diversity and versatility is one of the most efficient and successful means of finding new enzymes (Lee *et al.*, 2010). Traditional screening methods involve application of enrichment and isolation strategies, and provide information about the original functions of the enzyme in its natural environment. However, because more than 99% of environmental

microbes cannot be cultured under current laboratory conditions, metagenomic approaches have been used successfully in recent years to unlock this potential and obtain novel microbial products from uncultured microorganisms (Singh, 2010).

Screening collections or isolates for potentially high enzyme-producing strains/microorganisms is a quick way to determine which microbes to select for further analysis and optimisation of production. In a study that involved the screening of forty actinomycete strains for extracellular peroxidase production using different spectrophotometric analyses, Mercer *et al.* (1996) found that this trait was relatively common, but quantitatively variable. The assays included various substrates: 2,4-dichlorophenol (2,4-DCP), *o*-dianisidine, L-3,4-dihydroxyphenylalanine (L-DOPA), guaiacol, phenol red, and vanillyl acetone, amongst others. The study showed that some substrates were not suitable for peroxidase screening purposes due to interference by degradative compounds in some of the assays, and poor reproducibility. It was concluded that the 2,4-DCP assay was the most appropriate test for extracellular peroxidase activity determination for actinomycetes as it showed good reproducibility, and higher activity determination.

The realisation that fungal and bacterial peroxidases have a wide range of potential biotechnological applications has caused an increased interest in identifying new species that produce beneficial peroxidase activity (Orth *et al.*, 1993; Mercer *et al.*, 1996; Tuncer *et al.*, 1999). Among the actinomycetes studied, the majority of studies report on peroxidase production by different *Streptomyces* spp., but non-*Streptomyces* based reports are also available. A number of studies report on the effects of different environmental parameters (pH, temperature, inclusion of various inducers) on the production of peroxidase. Various studies have shown that extracellular peroxidase production is dependent upon medium components including carbon and nitrogen sources and their concentrations. Tuncer *et al.* (2009) showed that maximum peroxidase production by *Streptomyces* sp. F6616 was obtained when the microorganism was grown in basal medium containing either oat spelt (C:N ratio 4.6:1) or ball-milled straw (C:N ratio 6.8:1). Gottschalk *et al.* (1999) showed that the incorporation of 0.5% corn oil (C:N ratio 16:9) in the medium for the production of LiP by *S. viridosporus* T7A resulted in higher levels of LiP activity compared to the medium without the oil. The resultant LiP was reportedly more stable either in the presence or absence of glucose. Different studies have investigated the induction of LiP production in actinomycetes by incorporation of these compounds in culture media (Ramachandra *et al.*, 1988; Adhi *et al.*, 1989; Lodha *et al.*, 1991; Pasti *et al.*, 1991; Zerbini *et al.*, 1999). However, direct induction of peroxidase solely by the presence of these substrates has not been conclusively proven, owing to the heterogeneity of the reported results (Zerbini *et al.*, 1999). Pasti *et al.* (1991) showed that the presence or absence of lignocellulose in the culture media did not

significantly influence the level or the time of appearance of extracellular peroxidase activity of either *Streptomyces chromofuscus* A2 or *S. viridosporus* T7A. This is in contrast to reports on positive induction of peroxidase by *T. fusca* BD25 (Tuncer *et al.*, 1999) in the presence of oat spelt xylan and by *S. viridosporus* T7A in the presence of straw (Tuncer *et al.*, 2009).

Cultivation pH, temperature and length of incubation affect the extent of growth of microorganisms in general, and it is expected that these factors should have an effect on the production of peroxidase by actinomycetes. Tuncer *et al.* (1999) reported the optimum pH and temperature for the production of extracellular lignocellulose degrading enzymes (including peroxidase) by the thermophile *T. fusca* BD25 to occur at pH 7.0-8.0 and 50°C. Similar results were reported for *Streptomyces badius* (Adhi *et al.*, 1989). In general, the majority of peroxidase production reports by actinomycetes have employed neutral to alkaline pH conditions (Iqbal *et al.*, 1994; Rob *et al.*, 1997, Tuncer *et al.*, 2009; Fodil *et al.*, 2012).

The Biocatalysis and Technical Biology (BTB) Research Group has a large actinomycete culture collection with strains isolated from diverse environments from several parts of the world. This includes strains that originate from places such as the Antarctic Dry Valleys, thermal hot springs in Zambia, and alkaline lakes in Ethiopia, Namibian desert soils and geothermal locations in New Zealand. Other strains have been isolated from organisms including termite guts, sea sponges and sea squirts. As part of a larger 'Oxidase Project', the BTB Research Group has carried out an intensive screening program to identify notable producers of oxidative enzymes (laccases, tyrosinases and peroxidases) for application in lignocellulose degradation, detoxification of organic pollutants and synthesis of novel biomolecules with useful properties. The microorganisms that were used in this study originated from the BTB collection and have been previously shown to be producers of peroxidase. The aim of the work presented in this chapter was to screen a range of five pre-selected actinomycete strains for optimal peroxidase production under varied conditions of pH, temperature and a set of potential peroxidase inducers. The highest-yielding strains were selected for further studies.

3.2.   Materials and Methods

All chemicals and solvents used were of analytical grade and purchased from Merck-Millipore (South Africa) and Sigma-Aldrich (South Africa).

3.2.1. Microorganisms and Culture Maintenance

A previous multi-faceted screening programme identified five streptomycetes isolated from different environments (Table 3.1) capable of producing extracellular peroxidases.

Table 3.1: Selected actinomycete strains used in this study

Strain name	Source
Streptomyces sp. strain BSII#1	Bwanda Hot Springs, Zambia
Streptomyces sp. strain GSIII#3	Gwisho Hot Springs, Zambia
Streptomyces sp. strain MS56	Mindolo stream (Copper belt region), Zambia
Streptomyces sp. strain MID27	Douglas coal mine, Middelburg, South Africa
Streptomyces sp. strain MV32	Termite hindgut (*Amitermes hastatus*)

Spore/ hyphae suspensions (500 µl) were used to inoculate 10 ml modified phenoxazinone medium (in 50 ml Erlenmeyer flasks) and flasks were incubated at 30°C for two days, shaking, at 160 rpm on an orbital shaker. The optimal peroxidase production medium (initial pH 8.0) which was modified from phenoxazinone production medium (Graf *et al.*, 2007) contained (per litre): 10 g glycerol, 10 g glucose, 10 g soy flour, 5 g yeast extract, 5 g casein hydrolysate, 4 g calcium carbonate and 1 ml trace salts solution (per litre: 1 g $FeSO_4.7H_2O$; 0.9 g $ZnSO_4$; 0.2 g $MnSO_4.7H_2O$).

The stock cultures were prepared and stored in 20% (v/v) glycerol at -20°C and routinely sub-cultured every 4-6 weeks. For routine inoculations the strains were first grown on starch-casein-nitrate/yeast extract (SCN/YE) agar (pH adjusted to 8.0 before autoclaving, and containing per litre: 10 g starch; 0.3 g casein; 2 g KNO_3; 0.05 g $MgSO_4.7H_2O$; 0.3 g $CaCO_3$; 0.01 g $FeSO_4.7H_2O$; 0.01 g yeast extract; 20 g agar and 1 ml trace salts solution) (same composition as trace salts solution used for preparation of the modified phenoxazinone production medium) for at least 5 days at 30°C. Spore/hyphae suspensions prepared in 5 ml sterile distilled water were used for further inoculations.

3.2.2. Peroxidase Production and Crude Extracts

Spore/hyphae suspensions (500 µl) were used to inoculate 10 ml modified phenoxazinone medium and flasks were incubated at 30°C for two days. This served as a pre-culture for subsequent inoculation in all the experimental flasks. Purity of pre-cultures was assessed by performing the Gram stain and viewing under the light microscope to confirm the expected actinomycete Gram positive, filamentous morphology prior to inoculation. Samples from the growing cultures were centrifuged at 10 000 g for 5 min at room temperature and the decanted supernatant served as the crude enzyme preparation.

3.2.3. Peroxidase Assay

Peroxidase activity was measured using the 2,4-DCP assay (ε = 14 800 $M^{-1}cm^{-1}$; Antonopoulos *et al*., 2001). The reaction mix contained equal volumes of 100 mM potassium phosphate buffer (pH 7.0), 16 mM 4-aminoantipyrine, 50 mM hydrogen peroxide, 25 mM 2,4-DCP and the enzyme. The reaction was initiated by the addition of hydrogen peroxide and the increase in A_{510} monitored for 5 min on an Anthos Zenyth 1100 microtiter plate reader. One unit (U) of peroxidase activity was defined as the amount of enzyme required for the oxidation of 1 µmol of substrate (2,4-DCP) in one min.

In all cases, a 0.002 mg ml^{-1} horseradish peroxidase solution prepared in water was used as the positive control, while water was substituted for the enzyme in the negative control. Further experimental negative controls in the form of un-inoculated medium containing all the medium components for that experiment were always used.

3.2.4. Optimisation Studies

Small-scale optimisation experiments were carried out in 10 ml culture volume in 50 ml Erlenmeyer flasks. Sterile medium was inoculated with 500 µl of two-day old pre-cultures for each of the five strains (Table 3.1).

3.2.4.1. Optimal Temperature

Peroxidase production by the five different *Streptomyces* strains was assessed during growth at ambient temperature (25±2°C, 30°C, 37°C and 45°C in modified phenoxazinone production medium (pH 7.0).

3.2.4.2. Optimal pH

The effect of pH on peroxidase production by each of the five strains in modified phenoxazinone production medium was assessed over the pH range 5.0-10.0 at the noted optimal temperature of peroxidase production.

3.2.4.3. Effect of Phenolic Aromatic Compounds

Each of the five strains was cultured in modified phenoxazinone production medium at their respective optimum temperature and pH and monitored for peroxidase production in the presence of each of the following chemical inducers: veratric acid, anisaldehyde, VA, pyrogallol or guaiacol (each inducer at 0.1 mM, 0.5 mM and 1 mM concentration). The effect of indulin (0.01%, 0.05% and 0.1% w/v) on the production of peroxidase was also assessed. Induction of peroxidase by *Streptomyces* sp. strain MS56 and *Streptomyces* sp. strain MV32

by phenolic aromatic compounds was not performed because peroxidase production appeared to be quite erratic and non-reproducible for both strains.

3.2.4.4.   *Effect of Natural Lignocellulosic Compounds*

The number of strains tested was narrowed down to the two highest peroxidase-yielding strains. The effect of different natural lignocellulosic materials on peroxidase production by the two strains was assessed. These materials included banana peel, orange peel, wheat bran, sugarcane bagasse and Birchwood shavings. The materials were added to the modified phenoxazinone medium at 1% (w/v) concentrations (unless specified otherwise).

3.2.5. Statistical Analyses

The data is represented as mean±SEM. Statistical analyses were conducted using IBM® SPSS® Statistics, Version 21. Depending on the experiment design, univariate analysis of variance or one-way ANOVAs were used to determine the significance of results. This was followed by the Bonferroni post hoc test for pairwise comparisons of factors.

3.3.   Results

3.3.1. Optimisation Studies

3.3.1.1.   *Temperature optimization*

Figure 3.1 summarises the production of peroxidase by *Streptomyces* sp. strain BSII#1. At each of the different temperatures tested, maximum peroxidase production typically occurred on either day 4 or day 5 (Figure 3.1). Maximum peroxidase production (0.48 ± 0.06 U ml^{-1}) occurred when *Streptomyces* sp. strain BSII#1 was cultured at 37°C (Figure 3.1). No observable growth of *Streptomyces* sp. strain BSII#1 or any of the other four strains occurred at 45°C. On the fourth day of incubation, peroxidase activity was significantly different between all temperatures tested ($p<0.05$) with maximum peroxidase production at 37°C. . In this, and in subsequent experiments, the 2,4-DCP assay was used to determine peroxidase activity, as detailed in Section 3.2.3.
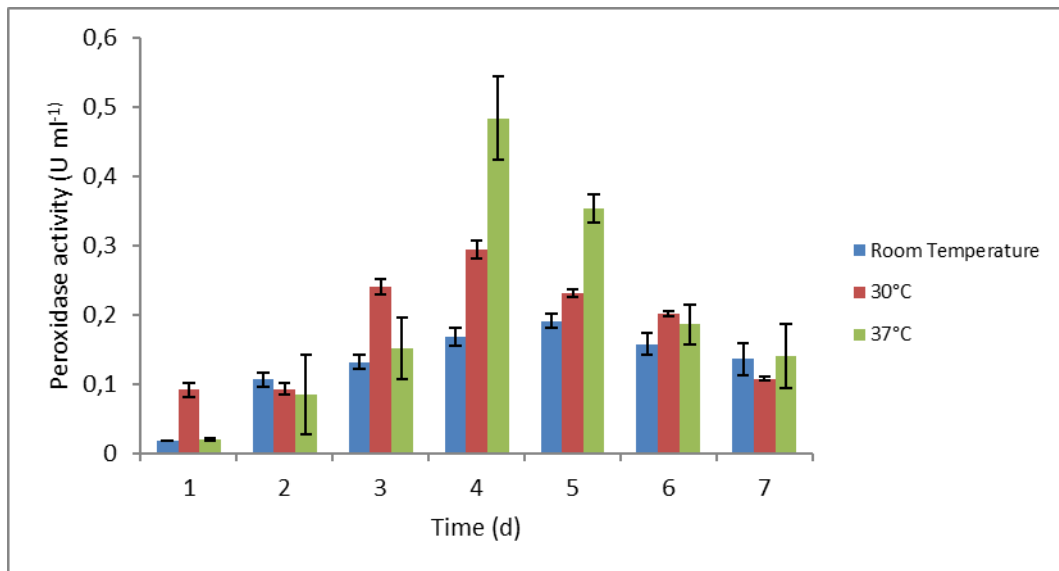
Figure 3.1: Peroxidase production by *Streptomyces* sp. strain BSII#1 when incubated at ambient temperature (25±2°C), 30°C and 37°C over a period of 7 days, shaking at 160 rpm (mean±SEM; n=2).

Figure 3.2 shows the production of peroxidase by *Streptomyces* sp. strain MS56 at 30°C and 37°C. Peroxidase activity was not detected when *Streptomyces* sp. strain MS56 was cultured at ambient temperature. Maximum peroxidase production occurred on the fourth day of incubation (0.38±0.01 U ml^{-1}) at 30°C.
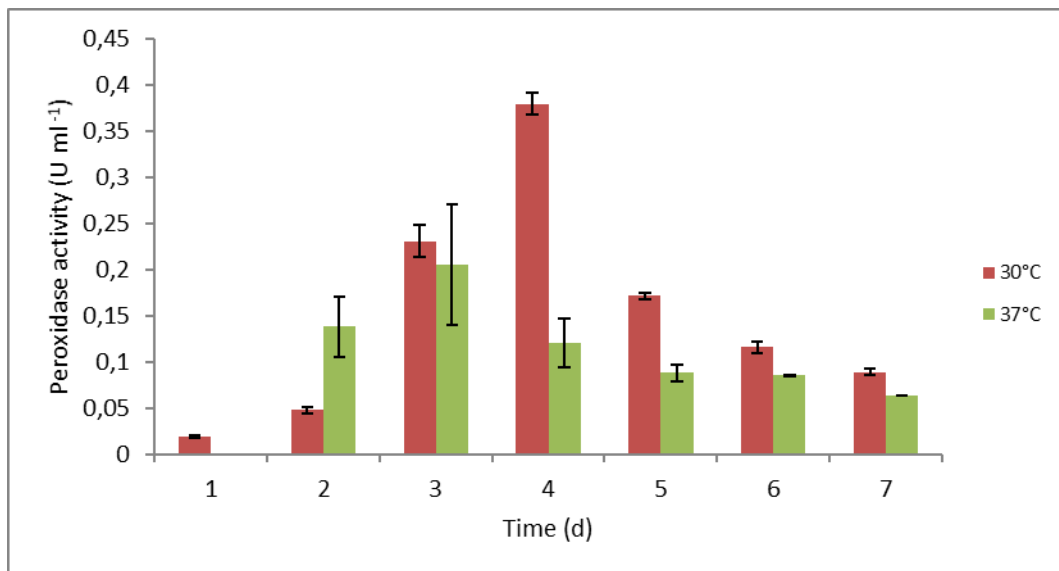
Figure 3.2: Peroxidase production by *Streptomyces* sp. strain MS56 when incubated at 30°C and 37°C over a period of 7 days, shaking at 160 rpm (mean±SEM; n=2).

Figure 3.3 shows the production of peroxidase by *Streptomyces* sp. strain MID27. Maximum activity was detected on the fourth day of incubation (0.29±0.01 U ml^{-1}) at 37°C. However, the enzyme production profile was erratic when the microorganism was cultured at ambient temperature and at 30°C, with unexpected decreases then increases in activity. Furthermore,

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on day 4, there was no significant difference in peroxidase production at 30°C and 37°C (p>0.05).
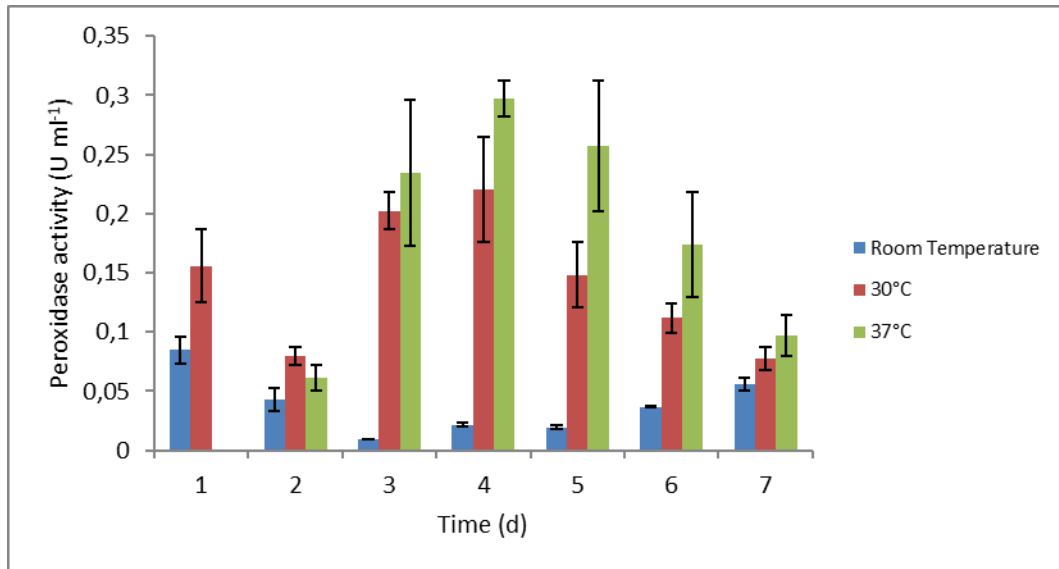
Figure 3.3: Peroxidase production by *Streptomyces* sp. strain MID27 when incubated at 30°C, 37°C and ambient temperature (25±2°C) over a period of 7 days, shaking at 160 rpm (mean±SEM; n=2).

Figure 3.4 shows the temperature optimisation profile for *Streptomyces* sp. strain MV32. Maximum peroxidase production was achieved on the fifth day of incubation at ambient temperature (0.35±0.009 U ml^{-1}). When cultured at 30°C, maximum production occurred on day 6 (0.29±0.007 U ml^{-1}). However, this was not significantly different from production at ambient temperature on day 5 (p<0.05). Culturing *Streptomyces* sp. strain MV32 at 37°C resulted in the lowest enzyme activities throughout the entire culture period.
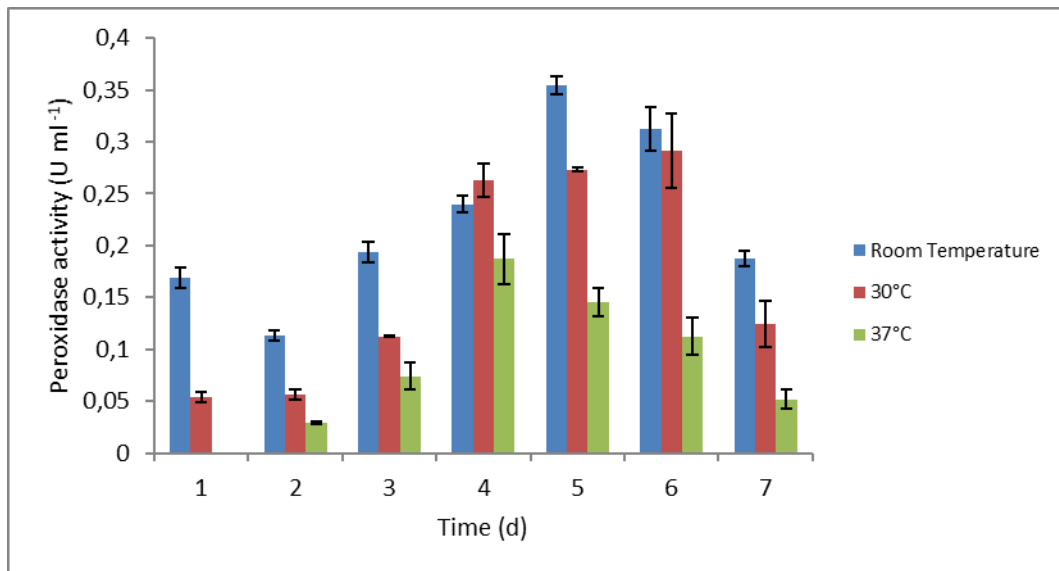
Figure 3.4: Peroxidase production by *Streptomyces* sp. strain MV32 when incubated at 30°C, 37°C and ambient temperature (25±2°C) over a period of 7 days, shaking at 160 rpm (mean±SEM; n=2).

Figure 3.5 summarises the production of peroxidase by *Streptomyces* sp. strain GSIII#1. The highest peroxidase activity (0.46±0.05 U ml^{-1}) was detected on the third day of incubation at 37°C. This result was significantly higher than production at the other temperatures tested (p<0.05).
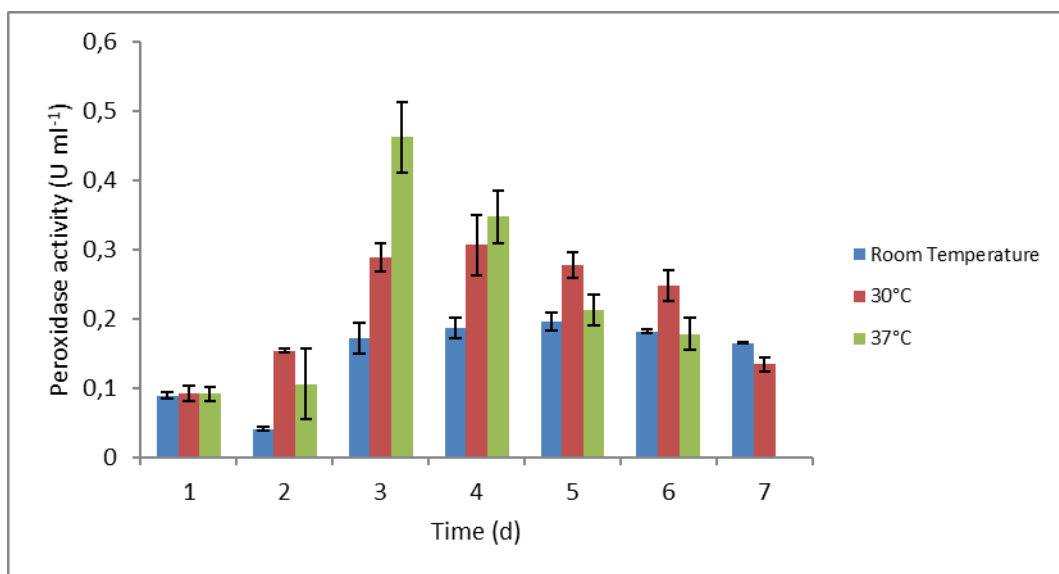
Figure 3.5: Peroxidase production by *Streptomyces* sp. strain GSIII#1 when incubated at 30°C, 37°C and ambient temperature (25±2°C) over a period of 7 days, shaking at 160 rpm (mean±SEM; n=2).

All five strains produced peroxidase maximally within the mesophilic temperature range. The two strains, *Streptomyces* sp. strain BSII#1 and *Streptomyces* sp. strain GSIII#1 both had maximum peroxidase activity when cultured at 37°C (0.48±0.06 U ml^{-1} and 0.46±0.05 U ml^{-1}

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respectively). Incubation at 30°C resulted in the greatest peroxidase activity for *Streptomyces* sp. strain MS56 (0.38±0.01 U ml⁻¹). While maximum production occurred at 37°C on day 3 for *Streptomyces* sp. strain MID27 (0.29±0.01 U ml⁻¹), there was no significant difference in production between 30°C and 37°C ($p > 0.05$). Maximum peroxidase production for *Streptomyces* sp. strain MV32 was achieved at ambient temperature (25±2°C; 0.35±0.009 U ml⁻¹). None of the five strains were capable of growth at 45°C.

3.3.1.2.   pH optimization

Peroxidase production was assayed for each strain in modified phenoxazinone medium at a pH range of pH 5.0 to 10.0 at the optimal temperature of production. Figure 3.6 shows the pH optimisation profile for *Streptomyces* sp. strain BSII#1. The highest production of peroxidase (0.59±0.007 U ml⁻¹) occurred on the fifth day of incubation with the medium having an initial pH of 8.0. There was no significant difference in the production of enzyme within the range of pH 6.0 to pH 9.5 on the fifth day of incubation ($p > 0.05$). The most extreme pH values tested (pH 5.0 and pH 10.0) resulted in significantly lower peroxidase production (0.15±0.02 U ml⁻¹ and 0.09±0.02 U ml⁻¹, respectively; $p < 0.05$).
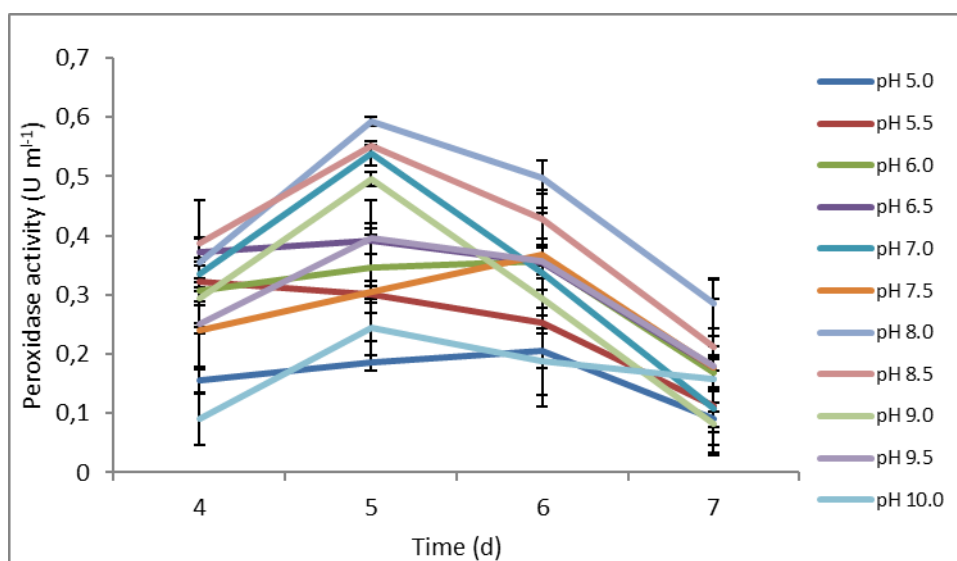
Figure 3.6: Effect of pH on peroxidase production by *Streptomyces* sp. strain BSII#1; 37°C; 160 rpm, 7 days of incubation (mean±SEM; n=2).

The pH optimisation profile of *Streptomyces* sp. strain MS56 is shown in Figure 3.7. The highest peroxidase activity (0.18±0.003 U ml⁻¹) was achieved with modified phenoxazinone medium with an initial pH of 8.5; however, this was less than half the maximum activity obtained during the temperature optimisation (0.38±0.01 U ml⁻¹; Figure 3.2). The highest activity previously achieved with the pH 7 medium during the temperature optimisation stage (Figure 3.2) was not improved, but instead decreased significantly ($p < 0.05$) to 0.098±0.003

U ml^{-1} (day 4, pH 7; Figure 3.7) after the attempt to optimise peroxidase production via pH adjustment.
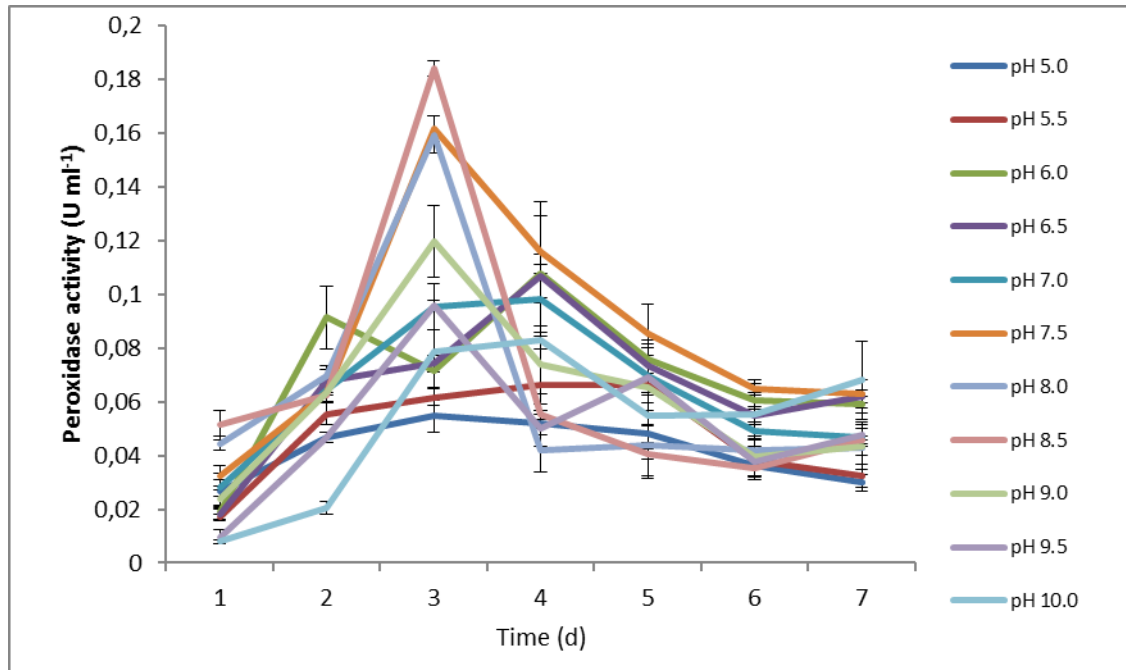
Figure 3.7: Effect of pH on peroxidase production by *Streptomyces* sp. strain MS56; 30°C; 160 rpm, 7 days of incubation (mean±SEM; n=3).

Figure 3.8 shows the pH optimisation profile for peroxidase production by *Streptomyces* sp. strain MID27. The individual pH profiles showed that the production of peroxidase did not follow a normal distribution pattern and was very variable between replicates; peroxidase activity appeared to be erratic and non-reproducible. In addition, pH optimisation did not result in improved activity, but resulted in similar level of maximum activity: 0.31±0.004 U ml^{-1} compared to activity detected under non-optimised conditions (0.29±0.01 U ml^{-1}; Figure 3.3).
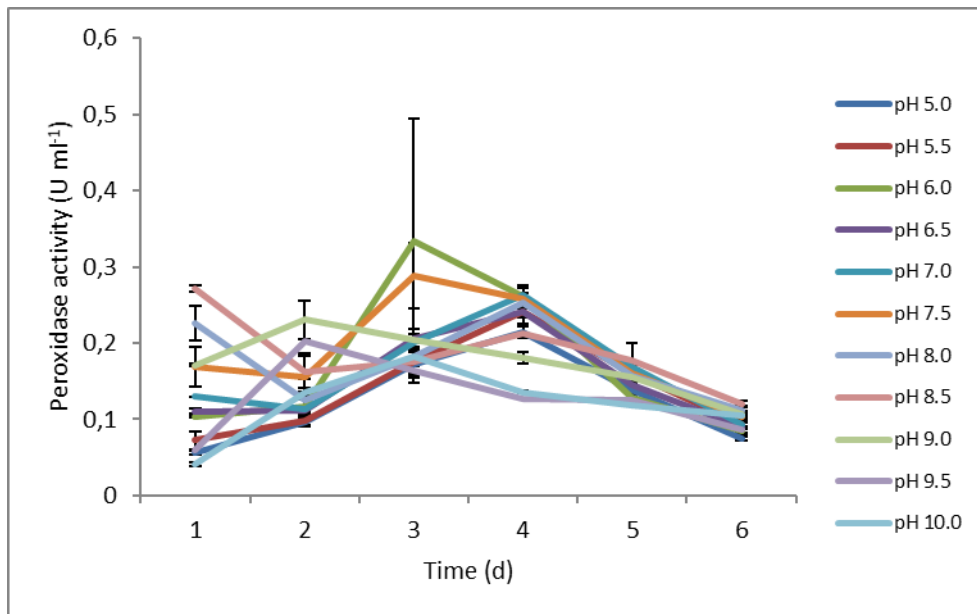
Figure 3.8: Effect of pH on peroxidase production by *Streptomyces* sp. strain MID27; 30°C; 160 rpm, 7 days of incubation (mean±SEM; n=2).

The pH optimisation profile for *Streptomyces* sp. MV32 is summarised in Figure 3.9. The pattern of peroxidase production appeared to be erratic when considering each individual pH profile over the time period. The highest peroxidase production (0.34±0.01 U ml^{-1}) was observed at pH 5.5 on the seventh day of incubation. There was a major decrease in peroxidase production between the ninth and the tenth day at all the pH points assayed.
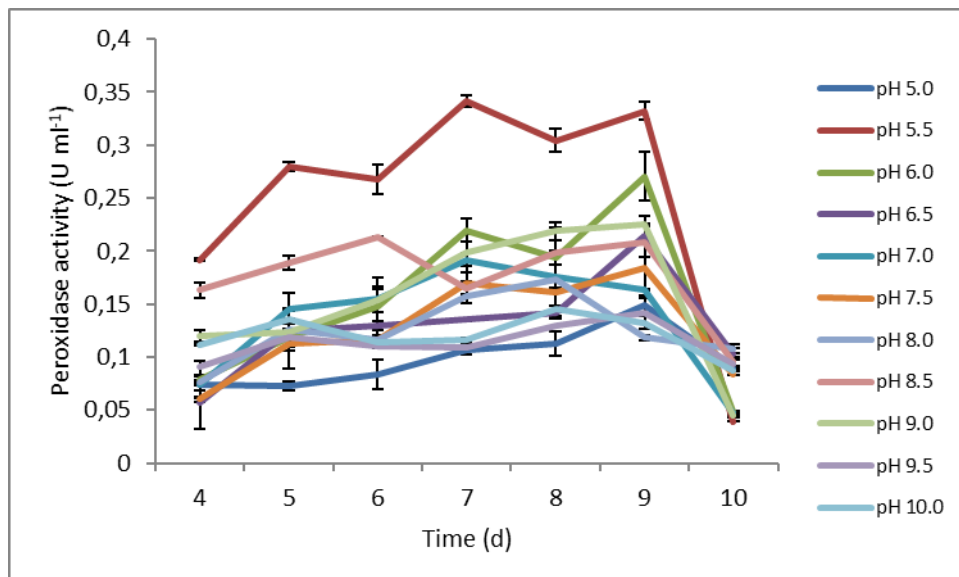
Figure 3.9: Effect of pH on peroxidase production by *Streptomyces* sp. strain MV32; ambient temperature (25±2°C); 160 rpm, 10 days of incubation (mean±SEM; n=2).

Figure 3.10 summarises the pH optimisation profile for *Streptomyces* sp. strain GSIII#1. Maximum peroxidase production was determined when the strain was cultured in modified phenoxazinone medium with an initial pH of 8.0 on the fifth day of incubation. There was no significant difference in production in medium with an initial pH in the range of pH 7.5 to pH 9.
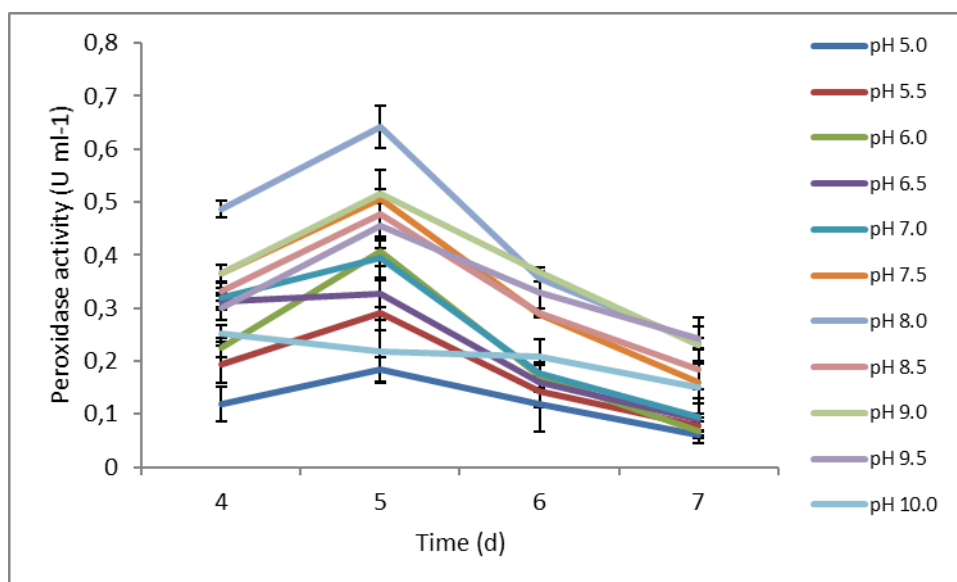
Figure 3.10: Effect of pH on peroxidase production by *Streptomyces* sp. strain GSIII#1; 37°C; 160 rpm, 7 days of incubation (mean±SEM; n=2).

pH optimisation was successful for the two highest-yielding strains, *Streptomyces* spp. strain BSII#1 and *Streptomyces* sp. strain GSIII#1, where peroxidase production improved with adjustment of the medium pH to 8.0 (Figure 3.6 and Figure 3.10). Maximum peroxidase production occurred on the fifth day of incubation in both cases. Peroxidase activity could not be improved with a pH optimisation step for the remaining three strains, with peroxidase activities either remaining similar or decreasing with pH adjustment. *Streptomyces* sp. strain MS56 was the only strain that produced maximum peroxidase at an acidic pH with optimal production observed at pH 5.5 (Figure 3.7).

At this point, it was decided that further optimisation would be carried out with two highest-yielding strains thus far: *Streptomyces* sp. strain BSII#1 and *Streptomyces* sp. strain GSIII#1.

3.3.1.3.   Effect of Phenolic Aromatic Compounds
The effect of five different lignin-based model compounds on the production of peroxidase by *Streptomyces* sp. strain BSII#1 and *Streptomyces* sp. strain GSIII#1 was determined. The inclusion of either 0.1 mM VA or 0.1 mM pyrogallol in the growth medium resulted in similar, significant increases in peroxidase production by *Streptomyces* sp. strain BSII#1 on day 5

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(1.30 ± 0.04 U ml^{-1} and 1.27 ± 0.06 U ml^{-1}, respectively, $p < 0.01$) compared to the uninduced control culture (0.84 ± 0.06 U ml^{-1}) (Figure 3.11). Guaiacol and anisaldehyde yielded significantly lower peroxidase activities than the uninduced control on the fifth day of incubation ($p < 0.01$), while veratric acid did not significantly affect production ($p > 0.05$) (Figure 3.11).
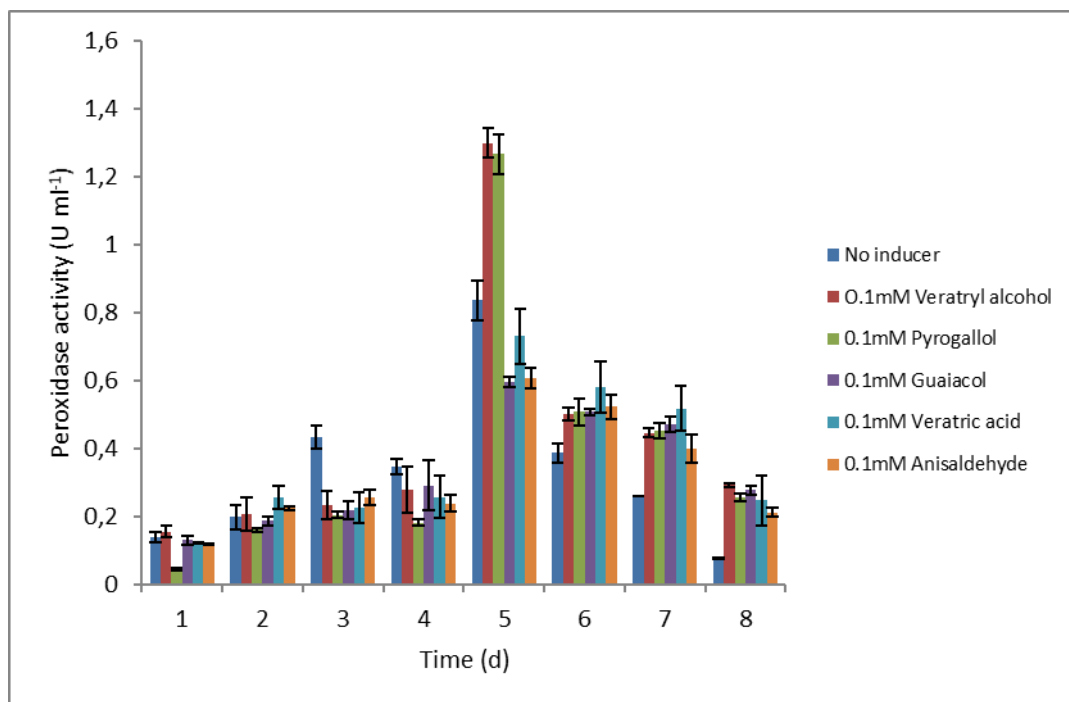
Figure 3.11: Effect of selected phenolic aromatic compounds (0.1 mM) on peroxidase production by *Streptomyces* sp. strain BSII#1 in modified phenoxazinone medium, pH 8.0; 37°C; 160 rpm, 8 days of incubation (mean±SEM; n=3).

Increasing the concentration of the inducers from 0.1 mM to 0.5 mM did not improve peroxidase production by *Streptomyces* sp. strain BSII#1 (Figure 3.12) and a further increase in inducer concentration to 1 mM resulted in inhibition of the growth of the microorganism. One-way ANOVA followed by Bonferroni post hoc tests for each treatment on the day of maximum production showed that there was significantly greater peroxidase production in the uninduced control compared to all the inducer treatments ($p < 0.05$). That is, at 0.5 mM concentration, all the phenolic aromatic compounds tested inhibited peroxidase production by *Streptomyces* sp. strain BSII#1.
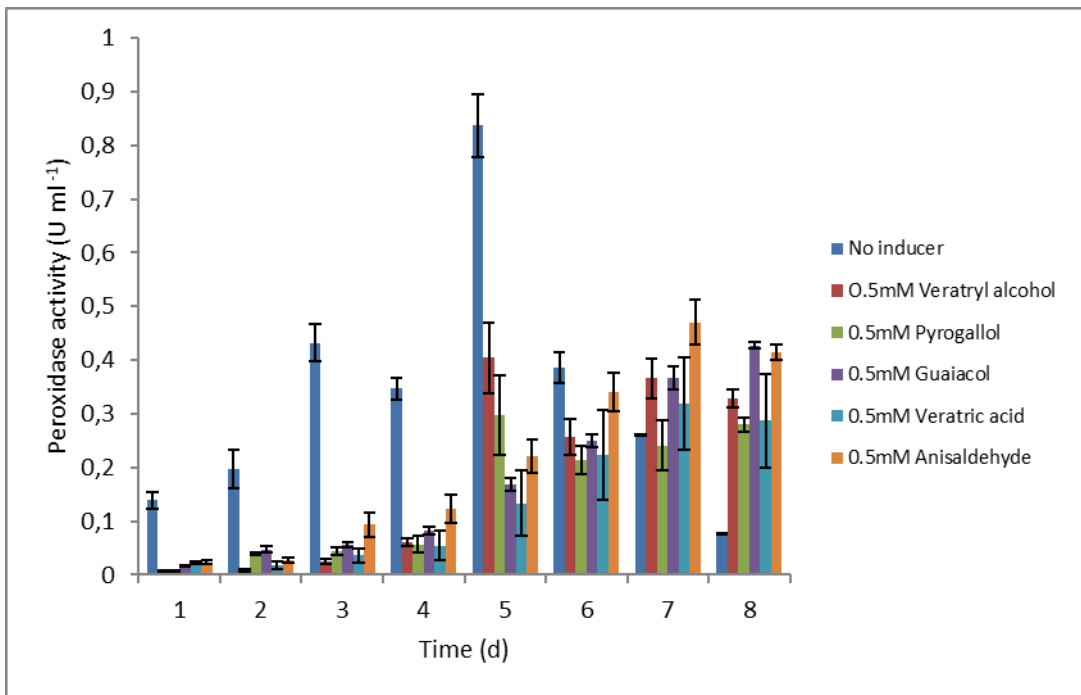
Figure 3.12: Effect of selected phenolic aromatic compounds (0.5 mM) on peroxidase production by *Streptomyces* sp. strain BSII#1 in modified phenoxazinone medium, pH 8.0; 37°C; 160 rpm, 8 days of incubation (mean±SEM; n=3).

The effect of the phenolic aromatic compound inducers at 0.1 mM concentration on peroxidase production by *Streptomyces* sp. strain GSIII#1 (Figure 3.13) was tested. Significant improvements in peroxidase production by *Streptomyces* sp. strain GSIII#1 occurred in the presence of each inducer tested on day 5 ($p < 0.05$).
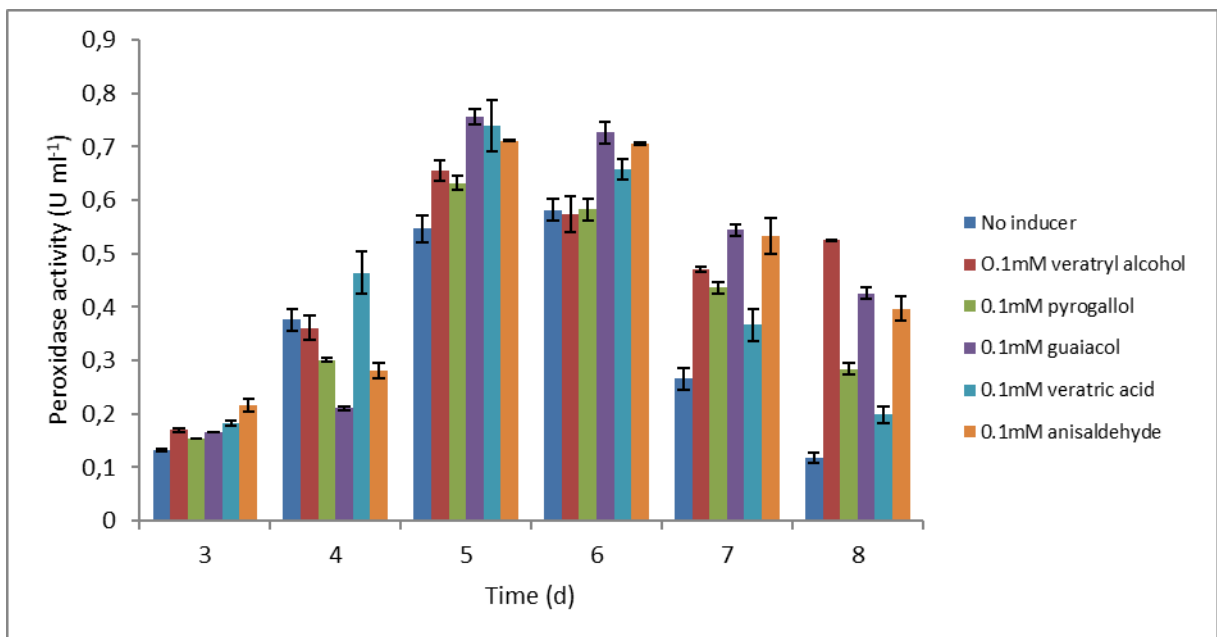
Figure 3.13: Effect of selected phenolic aromatic compounds (0.1 mM) on peroxidase production by *Streptomyces* sp. strain GSIII#1; in modified phenoxazinone medium, pH 8.0; 37°C; 160 rpm, 8 days of incubation (mean±SEM; n=2).

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Maximum increase in peroxidase production occurred in the presence of 0.1 mM guaiacol on days 5 and 6 of incubation (38.4% and 25.0% improvement over the uninduced control, respectively; Figure 3.13). However, maximum peroxidase production by *Streptomyces* sp. strain GSIII#1 in the presence of 0.1 mM guaiacol (0.757 ± 0.01 U ml^{-1}) remained lower than production by *Streptomyces* sp. strain BSII#1 in the presence of 0.1 mM VA (1.30 ± 0.04 U ml^{-1}).

3.3.1.4.   Effect of Indulin

Low concentrations of indulin were tested as a potential inducer of peroxidase production by *Streptomyces* sp. strains BSII#1 and GSIII#1. However, at all concentrations tested (0.01-0.1%, w/v) there was no significant difference in peroxidase production ($p > 0.05$) compared to the uninduced control on the day of maximum production (day 4; Figure 3.14). Similar results were obtained for *Streptomyces* sp. strain GSIII#1 (Figure 3.15). Although there appears to be some induction of peroxidase production by indulin (0.01 and 0.05%) between day 5 and day 7, maximum production in the presence of indulin on these days still remained lower than the uninduced control on day 4 (Figure 3.14).
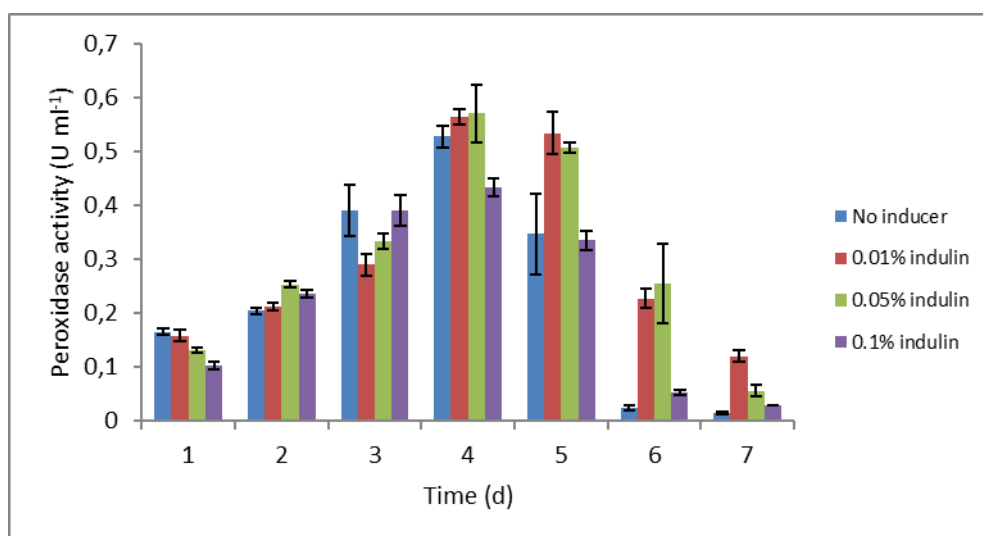
Figure 3.14: Effect of indulin on peroxidase production by *Streptomyces* sp. strain BSII#1 in modified phenoxazinone medium, pH 8.0; 37°C; 160 rpm, 7 days of incubation (mean±SEM; n=3).

Figure 3.15 shows the effect of indulin on peroxidase production by *Streptomyces* sp. strain GSIII#1. Maximum peroxidase production was noted with 0.05% (w/v) indulin on the third day of incubation (1.07 ± 0.09 U ml^{-1}); however, production between all concentrations of indulin and the uninduced control on the day of maximum production was not significant ($p > 0.05$; day 3, Figure 3.15).
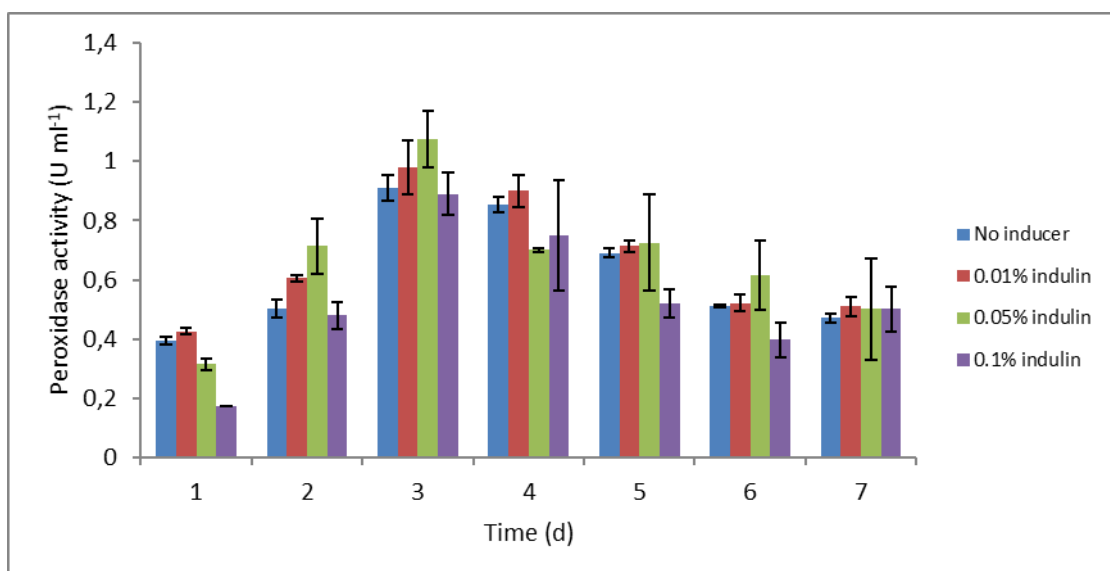
Figure 3.15: Effect of indulin on peroxidase production by *Streptomyces* sp. strain GSIII#1 in modified phenoxazinone medium, pH 8.0; 37°C; 160 rpm, 7 days of incubation (mean±SEM; n=2).

3.3.1.5. Effect of Natural Lignocellulosic Substrates

The following natural lignocellulosic substrates were tested for their ability to induce peroxidase production by *Streptomyces* sp. strain BSII#1 and *Streptomyces* sp. strain GSIII#1: orange peel, banana peel, wheat bran and Birchwood shavings (all at 1% w/v). With the exception of wheat bran, all the other natural lignocellulosic materials tested showed no significant induction ($p > 0.05$) of peroxidase production in *Streptomyces* sp. strain BSII#1 when compared to the control on the day of maximum peroxidase production (days 4 and 5; Figure 3.16).
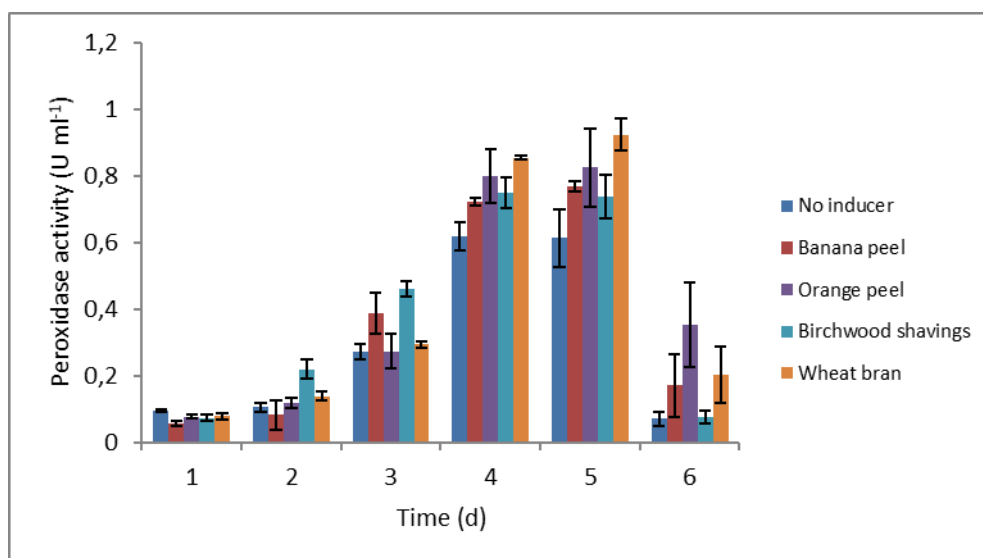
Figure 3.16: Effect of different natural lignocellulosic substrates (1% w/v) on peroxidase production by *Streptomyces* sp. strain BSII#1 in modified phenoxazinone medium, pH 8.0; 37°C; 160 rpm, 6 days of incubation (mean±SEM; n=3).

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When comparing the day of maximum peroxidase production (day 4 for the control without wheat bran and day 6 for all concentrations of wheat bran tested), there was only a significant improvement in peroxidase production in the presence of 1.5% (w/v) wheat bran ($p < 0.05$; Figure 3.17). There was no significant difference between maximum peroxidase production in the control and in medium containing 0.5% or 1% wheat bran ($p > 0.05$). Maximum peroxidase production in the presence of 1.5% (w/v) wheat bran was 0.94 ± 0.1 U ml^{-1}; although this was an improvement over the control, it took longer for production to reach peak levels (Figure 3.17).
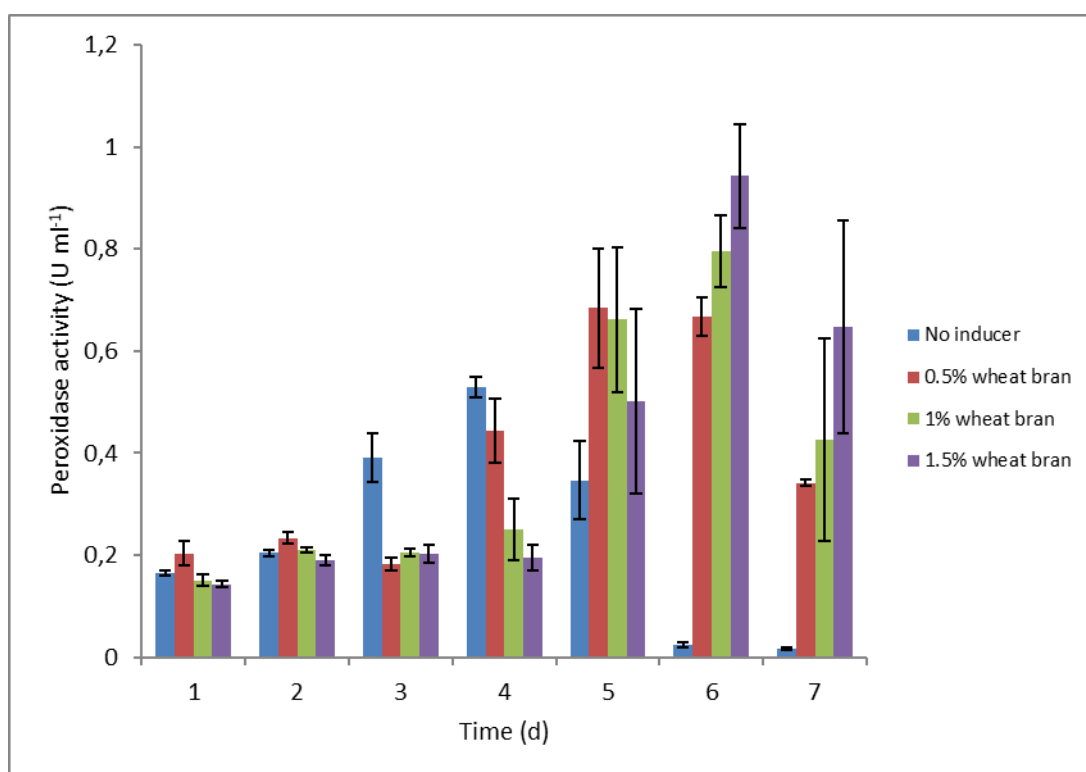
Figure 3.17: Effect of wheat bran on peroxidase production by *Streptomyces* sp. strain BSII#1 in modified phenoxazinone medium, pH 8.0; 37°C; 160 rpm, 7 days of incubation (mean±SEM; n=3).

There was an apparent increase in peroxidase production by *Streptomyces* sp. strain GSIII#1 on days 3 and 4 in the presence of 1% (w/v) Birchwood shavings compared to the control (Figure 3.18). However, one-way ANOVA analysis followed by Bonferroni post-hoc test for multiple comparisons showed that there was no significant difference between this result and the uninduced control ($p > 0.05$). There was also no significant induction of peroxidase production by 1% wheat bran, orange peels or banana peels throughout the entire 7-day incubation period ($p > 0.05$).
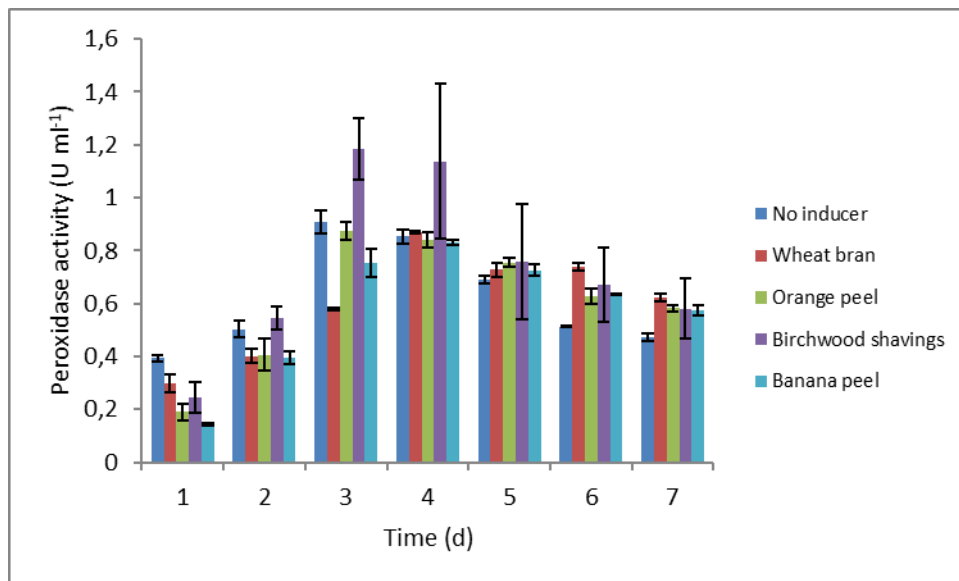
Figure 3.18: Effect of different natural lignocellulosic substrates (1% w/v) on peroxidase production by *Streptomyces* sp. strain GSIII#1 in modified phenoxazinone medium, pH 8.0; 37°C; 160 rpm, 7 days of incubation (mean±SEM; n=2).

An overall summary of the small-scale optimisation tests is presented in Table 3.2. Once optimal temperatures and initial pH conditions were determined, *Streptomyces* spp. strains BSII#1 and GSIII#1 were identified as the highest-yielding peroxidase producers and were the focus of subsequent studies.

Table 3.2: Summary of maximum peroxidase production by five *Streptomyces* spp. during optimisation of various conditions (temperature, pH, lignin-based model compounds and ligninocellulose-containing natural substrates.

Strain	Temperature optimisation	pH optimisation	Phenolic aromatic compound (inducer)	Lignocellulosics natural substrates
Streptomyces sp. strain BSII#1	0.48±0.06 U ml^{-1} (37°C)	0.59±0.007 U ml^{-1} (pH 8.0)	1.30±0.04 U ml^{-1} (0.1 mM VA) 1.27±0.06 U ml^{-1} (0.1mM pyrogallol)	0.94±0.1 U ml^{-1} (1.5% wheat bran)
Streptomyces sp. strain MS56	0.38±0.01 U ml^{-1} (30°C)	0.18±0.003 U ml^{-1} (pH 8.5)	ND	ND
Streptomyces sp. strain MID27	0.29±0.01 U ml^{-1} (30°C)	0.31±0.004 U ml^{-1} (pH 8.0)	ND	ND
Streptomyces sp. strain MV32	0.35±0.009 U ml^{-1} (ambient temperature 25±2°C)	0.34±0.01 U ml^{-1} (pH 5.5)	ND	ND
Streptomyces sp. strain GSIII#1	0.46±0.05 U ml^{-1} (37°C)	0.64±0.03 U ml^{-1} (pH 8.0)	0.757±0.01 U ml^{-1} (0.1 mM guaiacol)	1.18±0.12 U ml^{-1} (1% Birchwood shavings)

3.4. Discussion

3.4.1. Temperature and pH optimization

Peroxidase production is a growth-associated process in actinomycetes (Tuncer *et al.*, 1999; Ramachandra *et al.,* 1988; Niladevi and Prema, 2008). This means that factors that affect the growth of the microorganism in culture, including temperature and pH of incubation, as well as medium components have a bearing on peroxidase production. Typically, actinomycetes are terrigenous microorganisms and grow well within the mesophilic temperature range (McCarthy, 1987). *Streptomyces* sp. strain BSII#1 and *Streptomyces* sp. strain GSIII#1 were isolated from a hot spring and optimal peroxidase production occurred at 37°C (Table 3.2). *Streptomyces* sp. strain MID27 and *Streptomyces* sp. strain MS56 were isolated from a coal mine and a water stream environment, respectively, and optimally produced peroxidase at 30°C. Despite the source, all these strains optimally produced peroxidases in the mesophilic temperature range. The growth of all five *Streptomyces* strains, and hence their peroxidase production, was inhibited at 45°C.

S*treptomyces* sp. strain MV32 had maximum peroxidase production at ambient temperature and acidic pH. This rather anomalous finding among the other actinomycetes may be related to the isolation source (termite gut). The pH in the termite hindgut ranges from about neutrality (in the main hindgut chamber) to slightly acidic (in the colon and rectum) (Bignell and Eggleton, 1995). The pattern of peroxidase production from *Streptomyces* sp. strain MV32 was unstable and good reproducibility of results was not possible. The peroxidase from *Streptomyces* sp. strain MV32 is therefore not a good candidate for biocatalytic application, over and above the lower levels of enzyme production.

The pH optimisation step was successful for *Streptomyces* sp. strain BSII#1 and *Streptomyces* sp. strain GSIII#1 as adjustment in pH resulted in significantly better peroxidase production (Figures 3.6 and 3.10). Maximum peroxidase production occurred at alkaline pH (pH 8.0) for three of the five strains studied, and for most strains, production was only significantly hindered when the initial medium pH was at the extremes (pH 5 and pH 10). Optimal peroxidase production at alkaline pH is in conformance with other reports on actinomycete-based peroxidase production (Tuncer *et al.,* 1999).

At this point in the optimisation, it was determined that *Streptomyces* sp. strain BSII#1 and *Streptomyces* sp. strain GSIII#1 were the best candidates for further study as they produced the highest peroxidase activity of the strains tested.

3.4.2. Effect of Phenolic aromatic Compounds

Identifying inexpensive means of producing an enzyme for industrial use is an essential prerequisite for improving the cost-effectiveness of the biotechnological application. The use of inexpensive growth substrates, such as agri-industrial residues, enables the simultaneous removal of unwanted materials from the environment and value addition. As such, a variety of agri-industrial residues were tested to induce peroxidase production in the highest enzyme-yielding strains. Actinomycetes are recognised lignin-degrading microorganisms and because their primary substrate in soil is plant biomass (Godden *et al.*, 1992) it was thought that lignocellulosic agri-industrial residues could induce peroxidase production by the actinomycetes used in this study. The selected phenolic aromatic compounds tested (VA, pyrogallol, veratric acid, guaiacol and anisaldehyde) were chosen on the same basis and have been shown to be effective laccase inducers in *S. psammoticus* (Niladevi and Prema, 2008).

Induction of ligninolytic peroxidase production in the presence of veratryl alcohol (VA) has been reported for several white rot fungi including *Phanerochaete chrysosporium*, *Bjerkandera* spp. and *Phlebia radiata* (e.g. Niku-Paavola *et al.,* 1990; Mester *et al.,* 1995 and

Couto *et al.,* 1999, respectively), but has not been shown for an actinomycete. In this study we report the production of relatively high amounts of peroxidase activity detected in the crude extract of *Streptomyces* sp. strain BSII#1 in the presence of 0.1 mM VA or 0.1 mM pyrogallol (1.30 ± 0.04 U ml^{-1} and 1.27 ± 0.06 U ml^{-1}, respectively; Table 3.2) as compared to most reports on peroxidase production by actinomycetes; the majority of reports on actinomycete-based peroxidase production indicate enzyme activities that are generally less than 1 U ml^{-1} (Iqbal *et al.,* 1994; Bon *et al.*, 1999; Macedo *et al.,*1999; Tuncer *et al.,* 1999; Zerbini *et al.,* 1999; Antonopoulos *et al.,* 2001; Tuncer *et al.,* 2009). This result indicated that *Streptomyces* sp. strain BSII#1 is a potentially good source of peroxidase and warranted further investigation.

Pyrogallol was an equally effective inducer for peroxidase production by *Streptomyces* sp. strain BSII#1 in 10 ml culture volumes. However, because there is very limited information on pyrogallol-based induction available, more attention was paid in this study to VA, for which evidence for induction of other oxidative enzymes in non-actinomycetes is available (Saraiva *et al.*, 2012; Barbosa *et al.*, 1996). Similarly, *Streptomyces* sp. strain GSIII#1, in the presence of 0.1 mM guaiacol, showed a lower degree of induction for peroxidase production. These results indicate that different *Streptomyces* strains respond differently to the presence of low concentrations chemical inducers. Their growth is inevitably inhibited, in the presence of higher inducer concentrations (1 mM).

Sensitivity to VA-induced toxicity has been reported in *Botryosphaeria* sp*.,* a fungal species, at concentrations greater than 30 mM; however, the fungus could still grow on 100 mM VA (Dekker *et al.*, 2001). Growth of the *Streptomyces* strains in this study was completely inhibited in the presence on 1 mM VA. This indicates that the *Streptomyces* strains are much more sensitive to VA.

3.4.3. Effect of Natural Lignocellulosic Compounds

Of the natural lignocellulosic substrates tested, wheat bran appeared to be the most promising candidate for peroxidase induction in *Streptomyces* sp. strain BSII#1, with peroxidase activity reaching 0.94 ± 0.1 U ml^{-1} on day 6 of cultivation (Figure 3.17). Wheat bran is an important and readily-available by-product of the cereal industry, which makes it quite attractive as a raw material feedstock for peroxidase production. While the addition of 1.5% (w/v) wheat bran increased peroxidase production (78.4% improvement), it also increased the time required for maximum peroxidase production from 4 days (without wheat bran) to 6 days (Figure 3.17) with *Streptomyces* sp. strain BSII#1.

Birchwood shavings proved to be the best inducer for peroxidase production by *Streptomyces* sp. strain GSIII#1 (30.1% improvement in peroxidase production over the control). On the fifth day of incubation *Streptomyces* sp. strain GSIII#1 gave 1.18 ± 0.12 U ml^{-1} in the presence of 1% (w/v) Birchwood shavings (Figure 3.18). It is possible that the shavings provide a high surface area solid substratum onto which the bacteria could adsorb and proliferate, thereby resulting in improved peroxidase production.

The finding that that there was no appreciable induction of peroxidase production in the presence of indulin for both *Streptomyces* sp. strain BSII#1 and *Streptomyces* sp. strain GSIII#1 lends further support to previous reports that indulin is not a good growth substrate and therefore not a good inducer for peroxidase production in actinomycetes (Giroux *et al.,* 1988; Godden *et al.,* 1992).

3.5. Conclusion

VA and pyrogallol (0.1 mM) are effective inducers of peroxidase production in *Streptomyces* sp. strain BSII#1, and guaiacol also induced enzyme production by *Streptomyces* sp. strain GSIII#1. Of the natural lignocellulosic substrates tested, 1.5% (w/v) wheat bran showed the greatest positive induction effect on peroxidase production by *Streptomyces* sp. strain BSII#1. Birchwood shavings were the most effective natural inducer for *Streptomyces* sp. strain GSIII#1.

In the following chapter, the phenotypic, selected chemotaxonomic and genotypic characteristics of *Streptomyces* sp. strain BSII#1 are described to determine their relation to known peroxidase-producing actinomycete species.

CHAPTER FOUR

SPECIES DESCRIPTION

4.1.   Introduction

Microbial taxonomy is the science that deals with the classification, identification and nomenclature of microorganisms (Prakash *et al.*, 2007). Early microbial taxonomy relied heavily on the phenotypic characteristics of microorganisms such as colour, shape, size, staining properties and the assimilation of carbon sources (Woese, 1987; Clarridge, 2004; Prakash *et al.*, 2007; Labeda *et al.*, 2012). These features have proven insufficient for the complete description of microbial species due to the great diversity among prokaryotes, especially when considering their biochemical and physiological characteristics. In order for the biotechnological potential of a microorganism to be fully exploited, its identity as well as biochemical and metabolic capabilities should be known. Methods are constantly being developed to identify microorganisms unambiguously, with greater precision and reliability.

The polyphasic approach to microbial identification and characterisation refers to the integration of genotypic, chemotypic and phenotypic information of a microbe in order to reliably group microorganisms (Colwell, 1979). It is an approach that distinguishes bacteria based on morphological and biochemical characteristics, supplemented with information from molecular techniques to collectively determine the taxonomic position of a microorganism (Prakash *et al.*, 2007). The polyphasic approach is favourable as it offers a more complete identification of a microorganism, is potentially capable of resolving differences among the strains of a species and it enables the identification of the natural evolutionary relationships between microorganisms (Prakash *et al.*, 2007). Figure 4.1 depicts an outline of the procedure for taxonomical identification of a microorganism based on the polyphasic approach. The first step is the isolation of the microorganism from its natural environment followed by genotypic characterisation using DNA profiling and 16S rRNA gene sequencing. A Basic Local Alignment Search Tool (BLAST) can be used to identify the microorganism's most closely-related phylogenetic neighbours. DNA-DNA hybridisation with related strains gives an indication of the novelty of the isolate under investigation. Further characterisation, such as chemotaxonomic and phenotypic analyses, provides valuable information for full taxonomic characterisation of the strain.
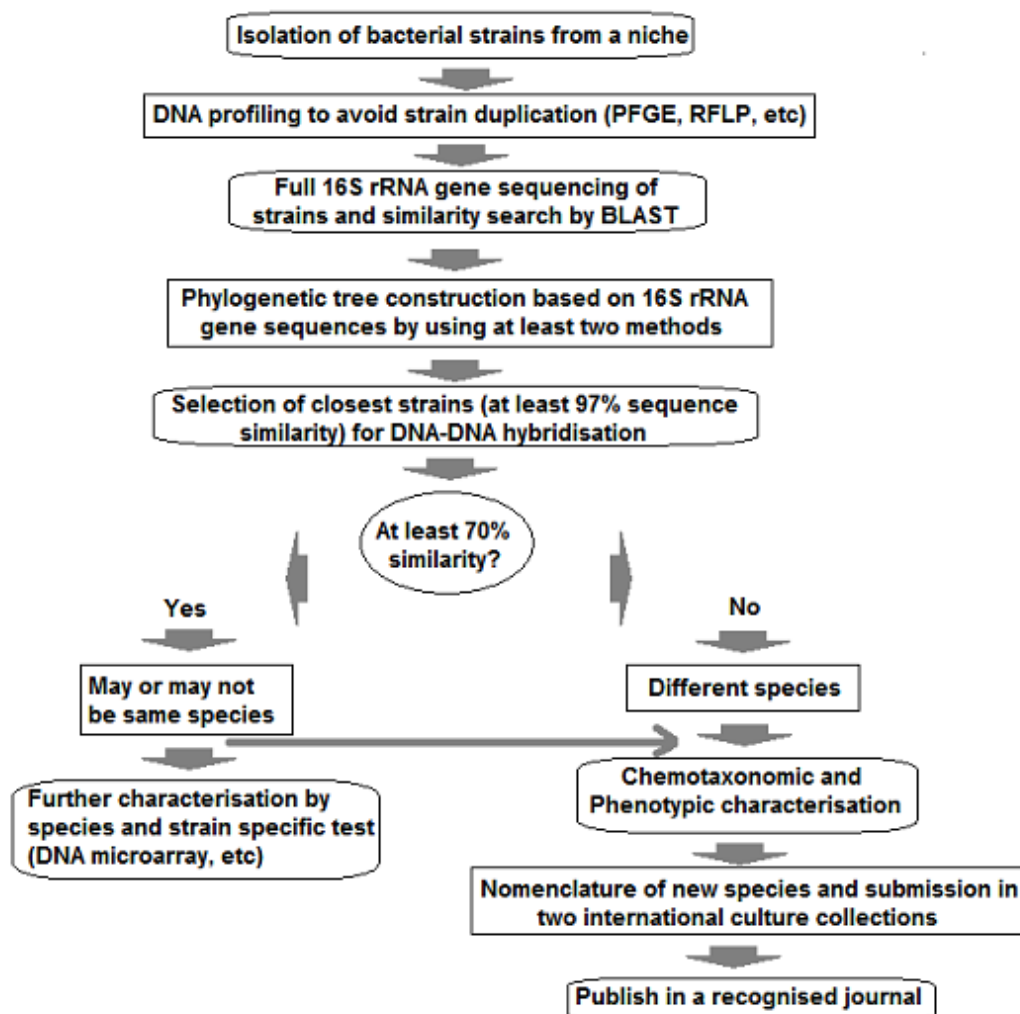
Figure 4.1: Steps in the taxonomical characterisation of a newly identified strain followed by its deposition in culture collection centres and publication as a novel species. Adapted from Prakash *et al.* (2007).

The family *Streptomycetaceae* was proposed by Waksman and Henrici (1943). It contains the genera *Streptomyces*, *Streptacidiphilus* and *Kitasatospora*. *Streptomyces* is the type genus and members of this genus are notable producers of various antimicrobial agents and bioactive compounds. Members of the genus *Streptomyces* represent an important biotechnological resource (Le Roes-Hill *et al.*, 2011). Some of the common features of the genus *Streptomyces* are summarised in Table 4.1.

Table 4.1: General characteristics of members of the genus *Streptomyces* as determined through a polyphasic approach (Adapted from Kampfer, 2006).

Characteristic	Brief description
Phenotypic	Gram-positive, aerobic bacteria whose mycelia rarely fragment. Colonies growing on solid plates take on a variety of different forms (lichenoid, leathery, butyrous, powdery, velvety) and may produce coloured pigments. Non-motile spores are formed on aerial mycelia and take on different surface topologies (smooth, hairy, rugose, spiny, and warty). Typically mesophilic organisms, but thermophiles also present. Non-fastidious and utilize a wide range of carbon and nitrogen sources.
Genotypic	G+C content of 69 - 78 mol%.
Chemotaxonomic features	Cell wall peptidoglycan contains major amounts of LL-diaminopimelic acid; lack mycolic acids. Contain predominantly saturated, iso- and anteiso-fatty acids. Possess either hexa- or octahydrogenated menaquinones.

In a previous study, 16S rRNA gene sequence analyses (F2 and R2 regions only) showed that both the peroxidase-producing strains in this study, BSII#1 and GSIII#2 are members of the genus *Streptomyces*. A polyphasic approach was used to describe the genotypic, phenotypic and chemotaxonomic features of the highest yielding peroxidase-producing strain, *Streptomyces* sp. strain BSII#1, to determine its status as a potentially novel species and its relation to known peroxidase producers within the genus *Streptomyces*. Additional 16S rRNA gene sequence analysis of *Streptomyces* sp. strain GSIII#2 was also performed to determine its relation to strain BSII#1 and other members of the genus *Streptomyces*.

4.2.   Materials and Methods

All media components and chemicals used in this study were obtained from Merck-Millipore SA or Sigma-Aldrich. The preparation of standard media used for the characterisation as well as the respective, detailed methods for the phenotypic characterisation, are given in Appendix I.

4.2.1. Phenotypic Characteristics

A spore suspension was prepared in 5 ml sterile distilled water and 20 µl of the spore suspension was streaked onto the agar plate surface using the standard streaking technique as described by Shirling and Gottlieb (1966). This inoculation technique was used for all the tests described below with the exception of nitrate and H_2S production (stab-inoculation of the culture into the agar) and testing for the ability to utilise selected sole carbon and sole

nitrogen sources (see below for details). For the hydrolysis and degradation tests, the spore suspension was streaked in single lines to allow the observation of zones of clearing. All plates were incubated at 30°C unless otherwise indicated.

4.2.1.1.   Growth on International Streptomyces Project (ISP), Bennett's and Starch-Casein-Nitrate (SCN) Media

The morphological characteristics of *Streptomyces* sp. strain BSII#1 cultured on various ISP media, Bennett's medium and SCN medium were noted.

4.2.1.2.   Analyses of Strain Morphology: Light Microscopy and Scanning Electron Microscopy

The standard Gram stain technique was performed on *Streptomyces* sp. strain BSII#1 prior to viewing under a light microscope. In addition, morphological features of the strain grown on an ISP4 agar plate were captured under a UoP UB203i digital light microscope. Electron micrographs of a 14d old *Streptomyces* sp. strain BSII#1 culture on ISP4 medium were also taken using cryo-scanning electron microscopy (Leica Stereoscan S440 Scanning Electron Microscope, University of Cape Town).

4.2.1.3.   Hydrolysis Tests

The ability of *Streptomyces* sp. strain BSII#1 to hydrolyse specific components in different media was tested. These components included pectin, egg yolk and Tween 80 (Sierra medium).

4.2.1.4.   Nitrate and H_2S production Tests

400 µl 0.8% sulphanilic acid in 5 M acetic acid: 0.05% α-naphthylamine in 5 M acetic acid (1:1, v/v) was added to a stab-inoculated slant culture grown in the presence of potassium nitrate to detect nitrate reductase activity. H_2S production was tested by including a lead acetate strip in the tube during the growth of a stab-inoculated slant culture over 14 days.

4.2.1.5.   Degradation Activity Tests

The ability of *Streptomyces* sp. strain BSII#1 to degrade various substrates incorporated into Bennett's agar medium was tested. These substrates were nitrogenous bases (adenine, guanine, hypoxanthine and xanthine) and various natural polymers (cellulose, elastin, gelatine, starch, L-tyrosine and xylan). Degradation of allantoin, arbutin, casein, hippurate and urea was also tested.

4.2.1.6.　Growth in the Presence of Inhibitory Compounds

The ability of *Streptomyces* sp. strain BSII#1 to grow in the presence of growth inhibitors and under inhibitory temperature (37°C) and pH (pH 4.3) conditions was examined. Growth was tested in the presence of the following chemical inhibitors: 0.0001% (w/v) crystal violet; 0.1% (w/v) phenol; 0.01 and 0.02% (w/v) NaN_3; 4, 7, 10 and 13% (w/v) NaCl.

4.2.1.7.　Sole Carbon and Sole Nitrogen Source Utilisation

The ability of *Streptomyces* sp. strain BSII#1 to grow on basal mineral salts agar supplemented with different carbon or nitrogen sources was ascertained. The strain was cultured in 10 ml ISP1 liquid media at 30°C, shaking at 160 rpm on an orbital shaker until the formation of cell mass was observed. Cell mass was collected by centrifugation at 10 000 g for 5 min and the supernatant discarded. The cell mass was washed twice with sterile distilled water (centrifugation at 10 000 g for 5 min for each washing step) and finally resuspended in sterile distilled water. The washed cells were used for the inoculation of the sole carbon or sole nitrogen source agar plates. Cells were washed to ensure that no residual carbon or nitrogen sources from the liquid growth medium would interfere with determining the strain's ability to utilise specific carbon or nitrogen sources.

4.2.2.　Chemotaxonomic Tests
4.2.2.1.　Cell Wall Chemotype and Whole Cell Sugar Pattern

A modified method of Hasegawa *et al.* (1983) was used to determine the dominant di-aminopimelic acid (DAP) and whole cell sugar pattern for *Streptomyces* sp. strain BSII#1. For DAP analysis, 1 ml 6 M HCl was added to 10 mg dried cell mass in a pyrex test tube. For the sugar analysis, 1 ml 0.25 M HCl was added to 100 mg dried cell mass in a pyrex test tube. The tubes prepared for the DAP and sugar analyses were covered and autoclaved using standard conditions (20 minutes at 121°C).

After cooling, the resultant hydrolysates and appropriate standards were spotted onto a cellulose thin layer chromatography (TLC) plate (Merck). For the DAP analysis, 3 µl of 0.1% (w/v in sterile water) DAP standard, 4 µl of 0.1% (w/v in sterile water) glycine and 2 µl of the DAP hydrolysate were applied to the TLC plates. For the sugar analysis, 1 µl of a 1% (w/v in sterile water) of glucose, mannose and ribose solution, 1 µl of a 1% (w/v in sterile water) of galactose, arabinose and xylose solution and 2 µl of the sugar hydrolysate were applied to the TLC plates. The following solvent systems were used for TLC development: methanol-distilled water-6 M HCl-pyridine (80:26:4:10, v/v/v/v) for DAP analysis and n-butanol-distilled water-pyridine-toluene (10:6:6:1, v/v/v/v) for sugar analysis.

For visualisation of the DAP and sugars, the following reagents were applied to the dried TLC plates: The DAP TLC plate was sprayed with 0.1% (w/v) ninhydrin prepared in acetone. The plate was allowed to dry and was heated at 110°C for 5 minutes. For the analysis of the sugars, a spray reagent consisting of the following was used: 2 ml aniline, 3.3 g pthalic acid and 100 ml water-saturated n-butanol. After the plate has been sprayed, it was allowed to air-dry, and was heated at 100°C for 2-5 minutes.

4.2.2.2. Predominant Menaquinones and Phospholipids

The extraction of menaquinones and phospholipids were performed using the method described in Minnikin *et al.* (1984). Briefly, 50 mg of dry cell mass was mixed with methanol-0.3% (w/v) aqueous sodium chloride (100:10, v/v) (2 ml) and petroleum ether (2 ml) for 15 min on a carousel (20 rpm). The upper layer was transferred to a small vial and petroleum ether (1 ml) was added to the cell mass mix. After mixing (carousel, 15 min, 20 rpm), the upper layer was removed. The upper layers collected were combined and evaporated with nitrogen gas (< 37°C) to obtain the isoprenoid quinone extract. The lower layer was heated in a boiling water bath (5 min), cooled at 37°C (5 min) and chloroform-methanol-water (90:100:30, v/v/v) (2.3 ml) added. The solution was mixed (60 min), centrifuged at 10 000 g for 5 min, and the supernatant transferred to a glass tube. Chloroform-methanol-water (50:100:40, v/v/v) (0.75 ml) was added to the lower layer and mixed for 30 min. The sample was centrifuged as before and the supernatant combined with the above (this step was repeated twice). Chloroform (1.3 ml) and 0.3% (w/v) aqueous sodium chloride (1.3 ml) was added to the combined supernatants, mixed thoroughly, centrifuged as before, and the upper layer removed. The lower layer was concentrated by evaporation under a gentle stream of nitrogen gas (< 37°) to give the polar lipid (phospholipid) extract.

For the quinone analysis, the isoprenoid quinine extracts were resuspended in petroleum ether and applied as 4 cm bands on 10 x 10 cm silica gel 60 F254 TLC sheets (Merck). Vitamin K1 was applied as a positive control. The plates were developed in petroleum ether-acetone (95:5, v/v) and the quinones visualized by UV (254 nm). The bands were cut out and the silica scraped off. The quinones were extracted from the silica by shaking for 15 min in 1 ml diethyl ether; this was repeated twice. The solvent was removed from the silica (centrifuged at 10 000 g for 5 min) and evaporated off under nitrogen gas. The identities of the dominant menaquinones were determined by mass spectrometry using a Brucker ESI Q-TOF mass spectrometer (Bruker Daltonik GmbH, Germany) and high performance liquid chromatography-time of flight mass spectrometer (LC-MS).

Phospholipid extracts were dissolved in chloroform-methanol (2:1, v/v) (60 µl) and 10 µl was spotted on the corner of a 10 X 10 cm silica gel 60 F254 TLC plate and subjected to two-

dimensional TLC analysis. Chloroform-methanol-water (65:25:4, v/v/v) was used for the first dimension; and chloroform-acetic acid-methanol-water (40:7.5:6:2, v/v/v/v) for the second dimension. Identification of the predominant polar lipids was achieved by using different detection reagents which is described in Appendix II.

4.2.3. Genotypic Tests

4.2.3.1. DNA Extraction and 16S rRNA Gene Sequence Analysis

Streptomyces spp. strains BSII#1 and GSIII#2 were inoculated into 10 ml liquid broth (ISP2) and incubated at 30°C with shaking on an orbital shaker (160 rpm) until sufficient cell mass was produced. The cells were harvested by centrifugation at 10 000 g for 5 min. The supernatant was removed and cell harvesting was repeated until approximately 200 µl of cell mass was collected. DNA was isolated according to Wang *et al.* (1996), with the exception that 25 mg ml^{-1} lysozyme was used instead of 5 mg ml^{-1} in the lysozyme buffer, and the incubation time for the RNase A step was extended from 30 min (at 37°C) to 24 h (at 22°C).

The 16S rRNA gene was amplified by the polymerase chain reaction (PCR) using the universal bacterial 16S rRNA gene primers F1 and R5 (Cook and Meyers, 2003). The internal primers, F3 and R4 were used in a second round of amplification to generate overlapping sequences for confirmation of the 16S rRNA gene sequence. Recombinant Taq DNA Polymerase (Fermentas) was used together with 25 mM MgCl$_2$ (KAPA Biosystems, South Africa). The PCR products were analysed by electrophoresis on 1% (w/v) agarose gels containing ethidium bromide (0.8 µg ml^{-1} final concentration). The PCR cycling conditions were as follows: 95°C for 5 min; 30 cycles of 95°C for 30 s, 48-56°C for 1 min and 72°C for 1 min; and 72°C for 5 min. Amplicons were purified using the MSB Spin PCRapace® PCR purification kit (Invitek). The concentration of the purified DNA was determined using a Nanodrop™ spectrophotometer (model ND-1000) and submitted for sequencing to Inqaba Biotech (Pretoria, South Africa).

Sequencing results were analysed using Chromas LITE Version 2.01 (Technelysium Pty Ltd). The edited 16S rRNA gene sequence fragments (F1, F2, F4, R5, R4, R2 and R1) were assembled using DNAMAN Version 4.13 (Lynnon Biosoft). The 1490 bp consensus 16S rRNA gene sequence of strain BSII#1 was submitted for BLASTN analysis (Altschul *et al.*, 1990) to determine which sequences in the database were most similar. The same analysis was performed for the 1297bp consensus 16S rRNA gene sequence obtained for strain GSIII#2. The closest described phylogenetic neighbours were identified using the EzTaxon-e database (Kim *et al.*, 2012). Sequences of the closest phylogenetic neighbours were downloaded (only validly published species) and were used in the generation of a Neighbour-joining tree (Saitou and Nei, 1987) using the program MEGA 5.1 (Molecular Evolutionary

Genetics Analysis; Tamura *et al.*, 2011). The 16S rRNA gene sequences of known peroxidase producers were also included in the phylogenetic tree.

To determine the degree of similarity between the 16S rRNA gene sequence of strain BSII#1 and strain GSIII#2, a sequence alignment was performed using the program DNAMAN Version 4.13 (Lynnon Biosoft), using the default settings of the software program.

4.2.3.2. Mol (%) G+C Determination

Streptomyces sp. strain BSII#1 was cultured in 200 ml ISP2 medium, pH 7.2. Total genomic DNA was isolated using the method proposed by Marmur (1961). For a complete method description, see Appendix III. The DNA was redissolved in 0.1% Saline-Sodium Citrate (SSC) buffer and used in the analysis. The mol (%) G+C content was determined using the method described by Mandel and Marmur (1968). Working solutions of DNA were diluted to a final concentration of 50 μg ml^{-1} in 0.1% SSC.

Thermal melting was determined using a Lamba 25 UV/Vis temperature-controlled spectrophotometer (Perkin-Elmer) by measuring the absorbance at 260nm (A_{260nm}) at the following temperatures:

1) 50°C
2) 60°C
3) 60°C (after holding at temperature for 2 minutes)
4) 70°C
5) 80°C
6) 81-90°C (absorbance measured at increments of 1°C)
7) 90°C (measured four times, each after holding at temperature for 2 minutes)

The melting curve was constructed by plotting the A_{260nm} as a function of temperature. To determine the melting temperature (T_m), the observed mid-point of the thermal transition curves was measured graphically. These T_m values (0.1% SSC) were corrected to correspond to T_m values in 1% SSC via the relationship described by Mandel and Marmur (1968) using the following equation:

$$T_m (1\% \text{ SSC}) = T_m (0.1\% \text{ SSC}) + 15.4°C \qquad \textbf{[Equation 8]}$$

The mol%G+C content of the DNA was determined using the empirical relationship between G+C content in DNA and the observed T_m for DNA in 1% SSC, as described by Marmur and Doty (1962):

GC% = 2.44 (T_m – 69.3)   **[Equation 9]**

4.3. Results

4.3.1. <u>Phenotypic Characteristics</u>

4.3.1.1.   Growth on ISP, Bennett's and Starch-Casein-Nitrate (SCN) Media

The growth characteristics exhibited by *Streptomyces* sp. strain BSII#1 when grown on different ISP media, Bennett's medium and SCN medium, are summarised in Table 4.2. The strain was able to grow on all the media tested, but only sporulated on selected media. The production of diffusible pigments was only noted on SCN agar.

Table 4.2: Characteristics of *Streptomyces* sp. strain BSII#1 growing on ISP, Bennett's and SCN media

Medium	*Streptomyces* sp. strain BSII#1
ISP1	Pale yellow, smooth colonies with off-white aerial mycelium
ISP2	Yellow, smooth growth streak (substrate mycelium); off-white to yellowish aerial mycelium
ISP3	Faint yellow colonies; no aerial mycelium
Bennett's medium	No diffusible pigments noted. Colonies appeared pale yellow in colour and there was little evidence of spore formation.
SCN medium	Red to red-brown pigments observed on underside of plates. Colonies grew as loose leathery colonies yellow to dark green in colour.
ISP4	Straight, white filaments; white to off-white aerial mycelium; Colonies appeared off-white to rust-grey in colour and formed aerial mycelium, which is white in colour.
ISP5	No colour change of agar block observed with either HCl or NaOH in the test to assess the sensitivity of the pigment colour to pH. Colonies were off-white to dull-grey in colour, and no pigments were observed.
ISP6	No melanin production was noted; faint yellow growth streak; no aerial mycelium
ISP7	No melanin production was noted; deep yellow growth streak; no aerial mycelium. Growth of colonies was sparse.

4.3.1.2.   Examination of strain BSII#1: Light Microscopy and Scanning Electron Microscopy

Streptomyces sp. strain BSII#1 was viewed under the light microscope after performing the standard Gram's staining technique. The microorganism is a Gram positive, filamentous bacterium. In addition, examination of the growth on ISP4 using a light microscope (x400

magnification) showed that the mycelial filaments were straight and unbranched (Figure 4.2a). This was confirmed when the strain morphology was examined using SEM (Figure 4.2b).
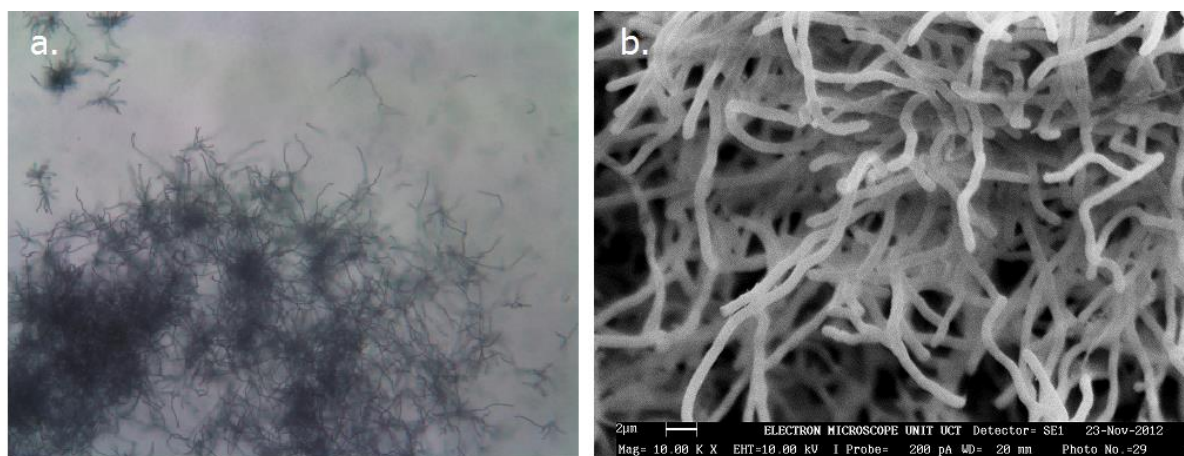
Figure 4.2: (a) Photograph of *Streptomyces* sp. strain BSII#1 grown on ISP4 medium (x400 magnification; UoP UB203i digital microscope). The aerial mass appears as straight, unbranched hyphae. (b) *Streptomyces* sp. strain BSII#1 as viewed with a Leica Stereoscan S440 SEM after two weeks of growth on ISP4 medium at 30°C. The aerial mass appears as smooth, straight, unbranched hyphae.

4.3.1.3.   Hydrolysis Tests

Streptomyces sp. strain BSII#1 was able to hydrolyse either pectin or Tween 80 (Sierra medium) (Figure 4.3a and 4.3b). A turbid region around the colony growth was observed on Sierra medium indicating positive degradation of Tween 80.
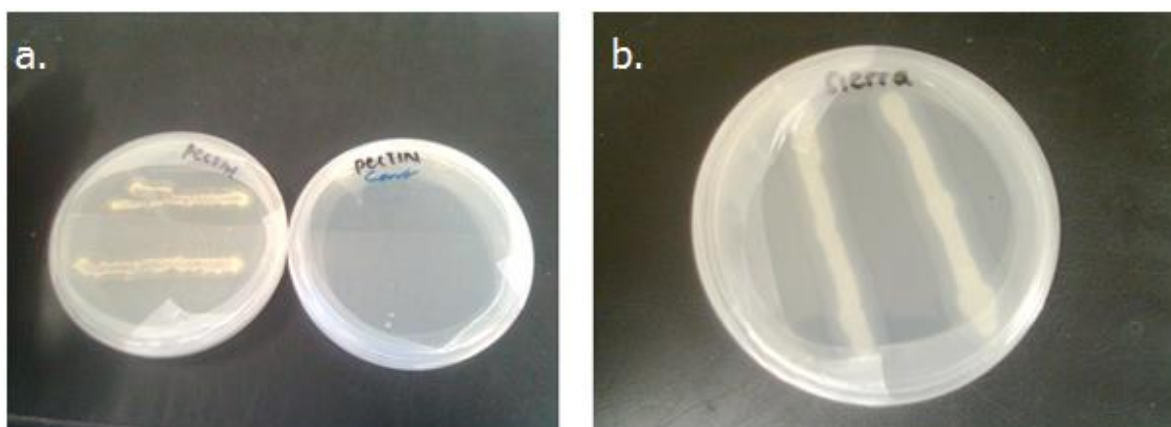
Figure 4.3: (a) Growth of *Streptomyces* sp. strain BSII#1 on pectin-containing Bennett's medium (left) versus an uninoculated control (right); (b) Growth of *Streptomyces* sp. strain BSII#1 on Sierra medium (contains Tween 80). The slight turbidity around the growth streak is evident. Both pictures were taken after 6 days of incubation at 28°C.

Proteolytic clearing around the growth streak of strain BSII#1 was observed on egg yolk agar. In addition, a pearly sheen along the edge of the culture was also observed, indicating

that the strain exhibits lipolytic activity (Figure 4.4). No deposit along the growth streak indicative of lecithinase activity was observed.
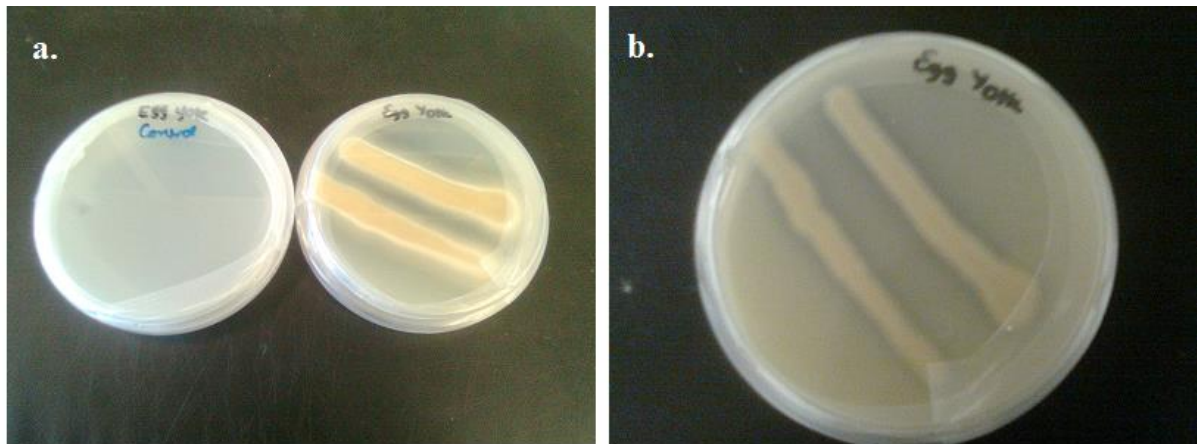
Figure 4.4: (a) Hydrolysis of egg yolk by *Streptomyces* sp. strain BSII#1. The uninoculated control is shown on the left while the experimental plate is on the right hand side; (b) Close-up view showing egg yolk hydrolysis by *Streptomyces* sp. strain BSII#1. The zone of clearing around the growth streak is evident. Both pictures were taken after 6 days of incubation at 28°C.

4.3.1.4.   *Nitrate and H$_2$S production Tests*

Streptomyces sp. strain BSII#1 tested positive for nitrate reduction: the solution of sulphanilic acid and α-naphthylamine turned pink when added to the culture, indicating the presence of nitrite (due to the reduction of nitrate). Furthermore, lead acetate paper strips were blackened upon incubation in tubes stab-inoculated with cultures of *Streptomyces* sp. strain BSII#1, indicating the production of H$_2$S (Figure 4.5).
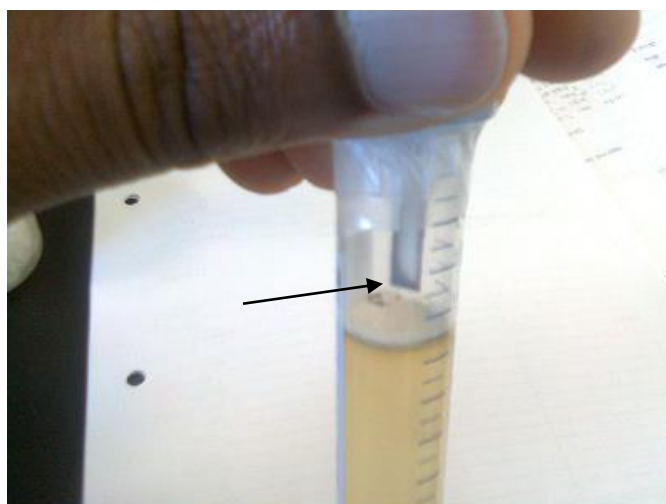
Figure 4.5: Blackening of lead acetate paper (arrow) after 14 days' incubation at 28°C in an agar tube stab-inoculated with *Streptomyces* sp. strain BSII#1 indicates the production of H$_2$S.

4.3.1.5.   Degradation Activity Tests

The ability of *Streptomyces* sp. strain BSII#1 to degrade various compounds is summarised in Table 4.3. Strain BSII#1 was able to degrade all the substrates tested in this study.

Table 4.3: Summary of degradation activity tests on various substrates by *Streptomyces* sp. strain BSII#1 (+ denotes positive activity; - denotes no degradation activity).

Substrate	Results
Adenine	+
Guanine	+
Hypoxanthine	+
Xanthine	+
Carboxymethyl cellulose	+
Gelatine	+
Starch	+
L-Tyrosine	+
Xylan	+
Casein	+
Urea and Allantoin	+
Hippurate	+
Arbutin	+

4.3.1.6.   Growth in the Presence of Inhibitory Compounds

The growth of *Streptomyces* sp. strain BSII#1 on Bennett's agar plates supplemented with various inhibitory compounds was assessed. The results are summarised in Table 4.4. In addition, the ability of the strain to grow at 37°C and at pH 4.3 is also presented in Table 4.4. Growth in the presence of crystal violet was only observed after a 14 d incubation period. The strain was unable to grow in the presence of phenol, sodium azide and 10% NaCl. Good growth was observed when the strain was cultured at 37°C and at pH 4.3.

Table 4.4: Growth of *Streptomyces* sp. strain BSII#1 in the presence of inhibitory compounds and factors (+ denotes growth; - denotes no growth).

Inhibitor (%, w/v)	*Streptomyces* sp. strain BSII#1	
	Day 7	Day 14
0.0001% crystal violet	-	+
0.1% phenol	-	-
0.02% sodium azide	-	-
4% NaCl	+	+
7% NaCl	+	+
10% NaCl	-	-
37°C	+	+
pH 4.3	+	+

4.3.1.7.   Carbon and Nitrogen Source Utilisation

In general, *Streptomyces* sp. strain BSII#1 was able to grow on most of the carbon sources and all of the nitrogen sources that were tested in this study (Table 4.5).

Table 4.5: Growth of *Streptomyces* sp. strain BSII#1 on sole carbon and nitrogen sources (+ denotes growth; - denotes no growth).

Carbon source	Observation	Nitrogen source	Observation
Growth on sole carbon source		Growth on sole nitrogen source	
Control (without carbon source)	-	Control (without nitrogen source)	-
Adonitol	+		
L-arabinose	+	L-arginine	+
D-cellobiose	+	L-cysteine	+
D-fructose	+	L-methionine	+
D-galactose	+	L-phenylalanine	+
D-glucose	+	Potassium nitrate	+
D-lactose	+	L-serine	+
D-mannitol	+	L-threonine	+
D-mannose	+	L-valine	+
D-melibiose	+		
Raffinose	+		
L-rhamnose	-		
Ribose	+		
Sodium citrate	+		
Sucrose	+		
D-xylose	+		

4.3.2. Chemotaxonomic Tests

4.3.2.1.   Cell Wall Chemotype and Whole Cell Sugar Pattern

DAP and whole cell sugar analyses were performed on cell hydrolysates prepared for *Streptomyces* sp. strain BSII#1. Even though the separation of the samples was poor, the presence of LL-DAP was confirmed (Figure 4.6a). No distinctive sugar pattern was observed for strain BSII#1, but galactose and traces of arabinose were visualised on the TLC plate (Figure 4.6b).
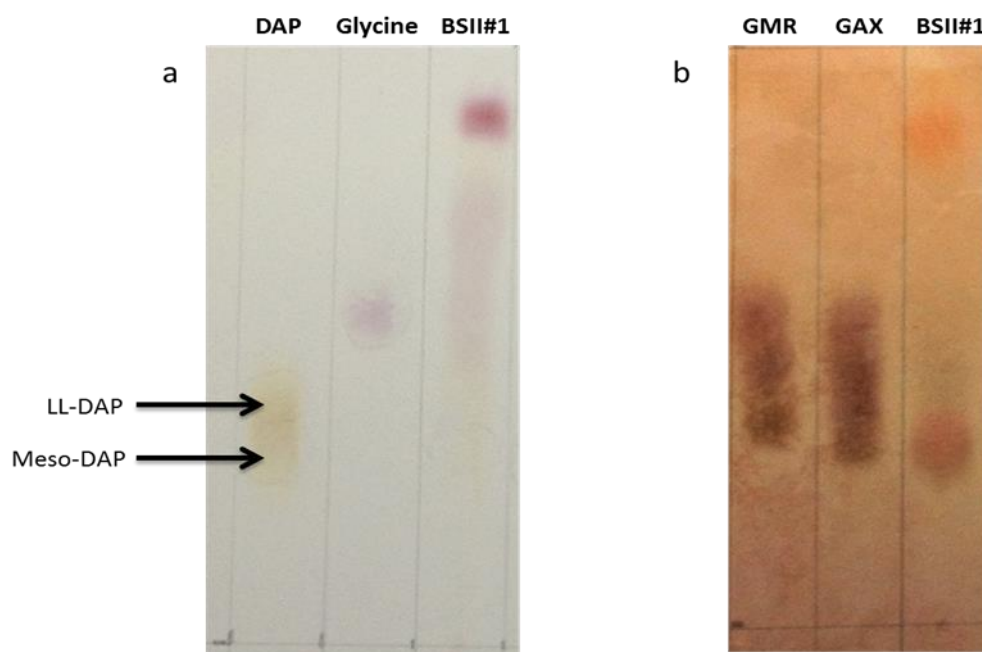
Figure 4.6: (a) DAP analysis for the determination of dominant di-aminopimelic acid in the whole cell hydrolysate of *Streptomyces* sp. strain BSII#1; (b) Whole cell sugar pattern for *Streptomyces* sp. strain BSII#1. DAP = di-aminopimelic acid standard; GMR = glucose, mannose and ribose; GAX = galactose, arabinose and xylose.

4.3.2.2.   Predominant Menaquinones and Phospholipids

Menaquinone extracts were analysed in positive mode using a Bruker LC-MS. MS spectra were analysed for the presence of known molecular weights of menaquinones reported in actinomycetes. Two peaks were identified as potentially representing menaquinones: 791 and 789 (Figure 4.7a and b). These molecular weights correspond to MK-9(H_6) and MK-9(H_4), respectively. Both were detected at very low concentrations.
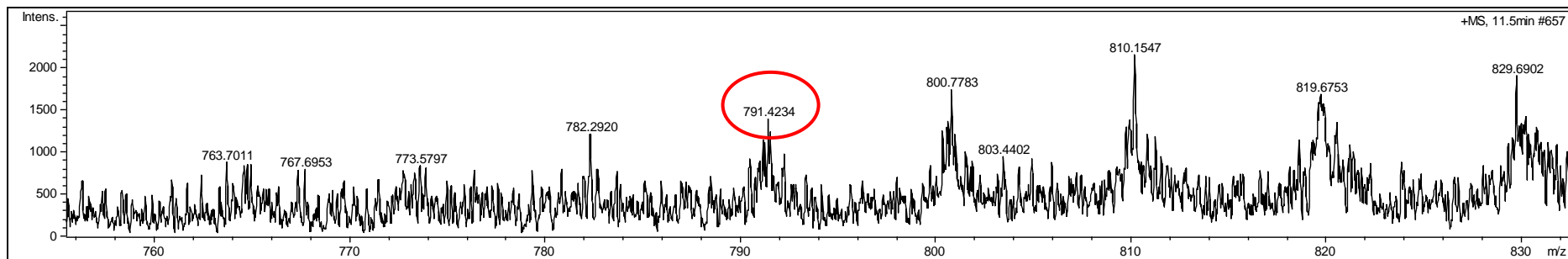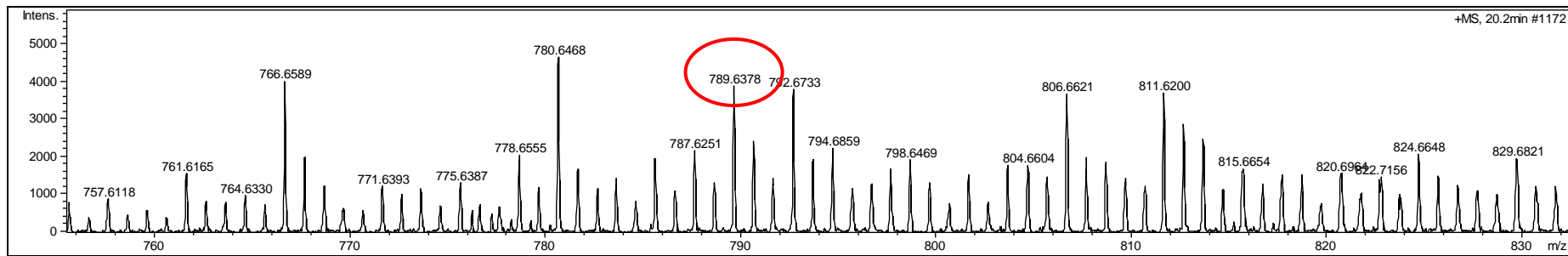
Figure 4.7: LC-MS analysis (positive mode) of menaquinone extracts (a) a peak observed at 791.4234 and (b) a peak observed at 789.6378 corresponds to known menaquinone masses.

Phospholipid content was analysed using two dimensional TLC. Phospholipids were visualised through the use of different reagents (Figure 4.8). The glycolipids, phosphatidyl glycerol (PG) and diphosphatidyl glycerol (DPG) were visualised with the periodate Schiff's reagent (Figure 4.8c) and α-naphthol (Figure 4.8a). Phosphatidyl inositol mannosides (PIMs) were visible as blue spots on the TLC plate stained with periodate Schiff's reagent (Figure 4.8c), but no phosphatidyl inositol (PI) was detected (should appear as yellow spots). Two pink spots were faintly visible on the TLC plate stained with ninhydrin (Figure 4.8d), indicating the presence of either phosphatidyl ethanolamine (PE) or phosphatidyl serine (PS). Two, and a possible third, phosphate esters were also detected (Figure 4.8b).
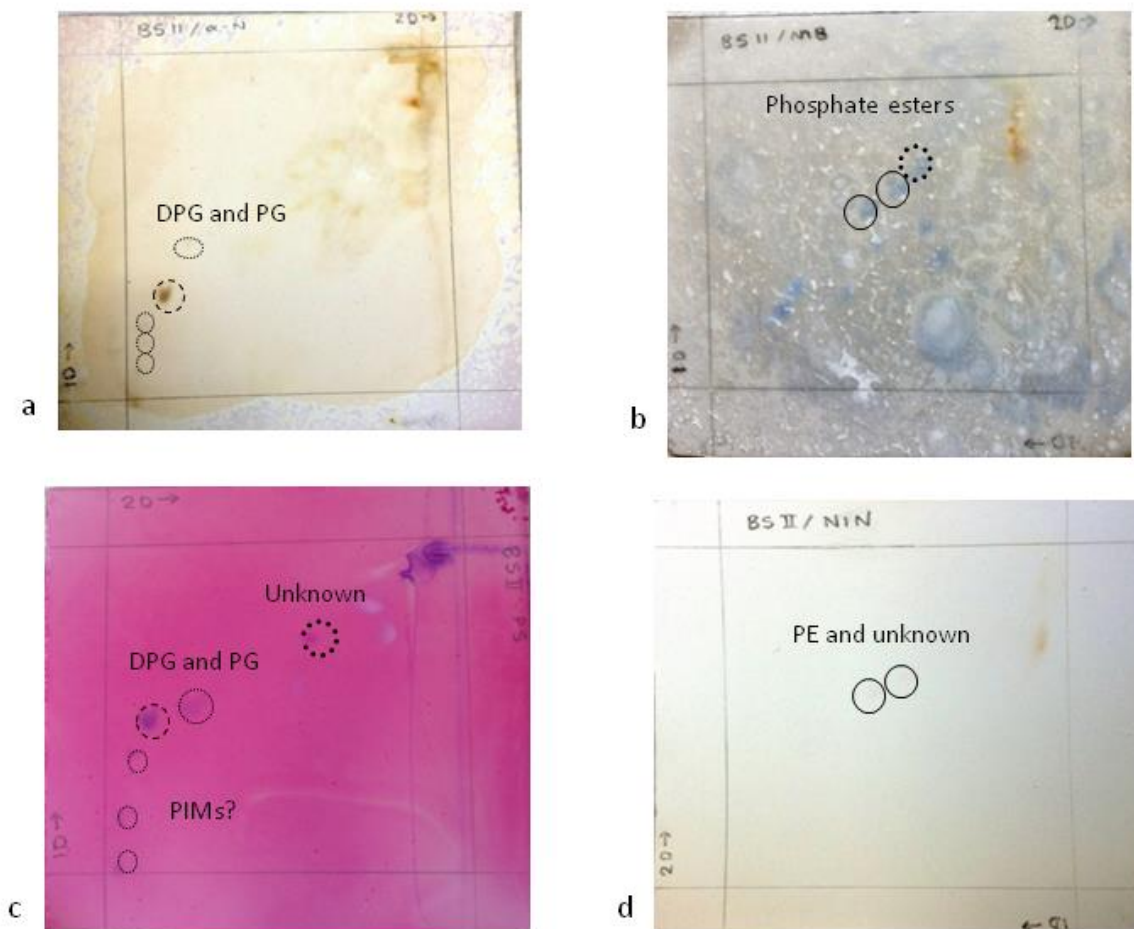
Figure 4.8: Two dimensional TLC analysis of phospholipid extracts from *Streptomyces* sp. strain BSII#1. (a) TLC plate stained with α-naphthol; (b) TLC plate stained with molybdenum blue reagent; (c) TLC plates stained with periodate Schiff's reagent; and (d) TLC plate stained with ninhydrin. DPG = diphosphatidyl glycerol; PG = phosphatidyl glycerol; PIMs = phosphatidyl inositol mannosides; PE = phosphatidyl ethanolamine.

4.3.3. Genotypic Tests

4.3.3.1. DNA Extraction and 16s rRNA Gene Sequence Analysis

A 1490 bp consensus 16S rRNA gene sequence was obtained for *Streptomyces* sp. strain BSII#1. BLAST analysis and identification by EzTaxon-e revealed that the strain BSII#1 16S rRNA gene sequence showed >99% sequence similarity to six streptomycete strains: *Streptomyces albidoflavus* DSM 40455[T] (99.73%), *Streptomyces somaliensis* NBRC 12916[T] (99.66%), *Streptomyces hydrogenans* NBRC 13475[T] (99.66%), *Streptomyces violascens* ISP 5183[T] (99.65%), *Streptomyces daghestanicus* NRRL B-5418[T] (99.45%) and *Streptomyces koyangensis* VK-A60[T] (99.26%). The same results were obtained when the re-identify function of EzTaxon-e was used. The 1297 bp sequence obtained for *Streptomyces* sp. strain GSIII#2 gave the same results as for strain BSII#1 and a sequence alignment between the 16S rRNA gene sequence of strain BSII#1 and GSIII#2, showed 100% similarity (Figure 4.9), indicating that these strains are most probably the same species.

```
Fast alignment of DNA sequences BSII#1 (1490bp) and GSIII#2 (1297bp)

Ktuple=2 Gap penalty=7

Upper line: BSII#1, from 12 to 1307
Lower line: GSIII#2, from 1 to 1296

BSII#1: GSIII#2 identity= 100.00 %( 1296/1296) gap=0.00 %( 0/1296)

12    TGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCGCTT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1     TGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCGCTT

72    TCGGGCGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
61    TCGGGCGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTG

132   GGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACCGTCTGCCGCATGGTGGATGG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
121   GGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACCGTCTGCCGCATGGTGGATGG

192   TGTAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGTG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
181   TGTAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGTG

252   GCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
241   GCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGA

312   GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
301   GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGC
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 372  CTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 361  CTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAG

 432  GGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 421  GGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGC

 492  CGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 481  CGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGG

 552  CGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 541  CGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGG

 612  GCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGAT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 601  GCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGAT

 672  ATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 661  ATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCG

 732  AAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 721  AAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCA

 792  CTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 781  CTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCC

 852  TGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 841  TGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGC

 912  GGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 901  GGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCG

 972  GAAACGTCTGGAGACAGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 961  GAAACGTCTGGAGACAGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGT

1032 CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGT
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1021 CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGT

1092 TGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGG
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1081 TGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGG

1152 AAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACA
```

```
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1141 AAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACA


1212 ATGGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTC
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1201 ATGGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTC


1272 AGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAG
          ||||||||||||||||||||||||||||||||||
1261 AGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAG
```

Figure 4.9: Sequence alignment of the 16S rRNA gene sequence from *Streptomyces* sp. strain BSII#1 and *Streptomyces* sp. strain GSIII#2.

A phylogenetic analysis of the 16S rRNA gene sequence of *Streptomyces* sp. strain BSII#1, its closest phylogenetic neighbours and known peroxidase-producing streptomycete strains showed that even though strain BSII#1 showed a high sequence similarity (>99%) to various streptomycete strains, it branched on a separate clade (Figure 4.10).
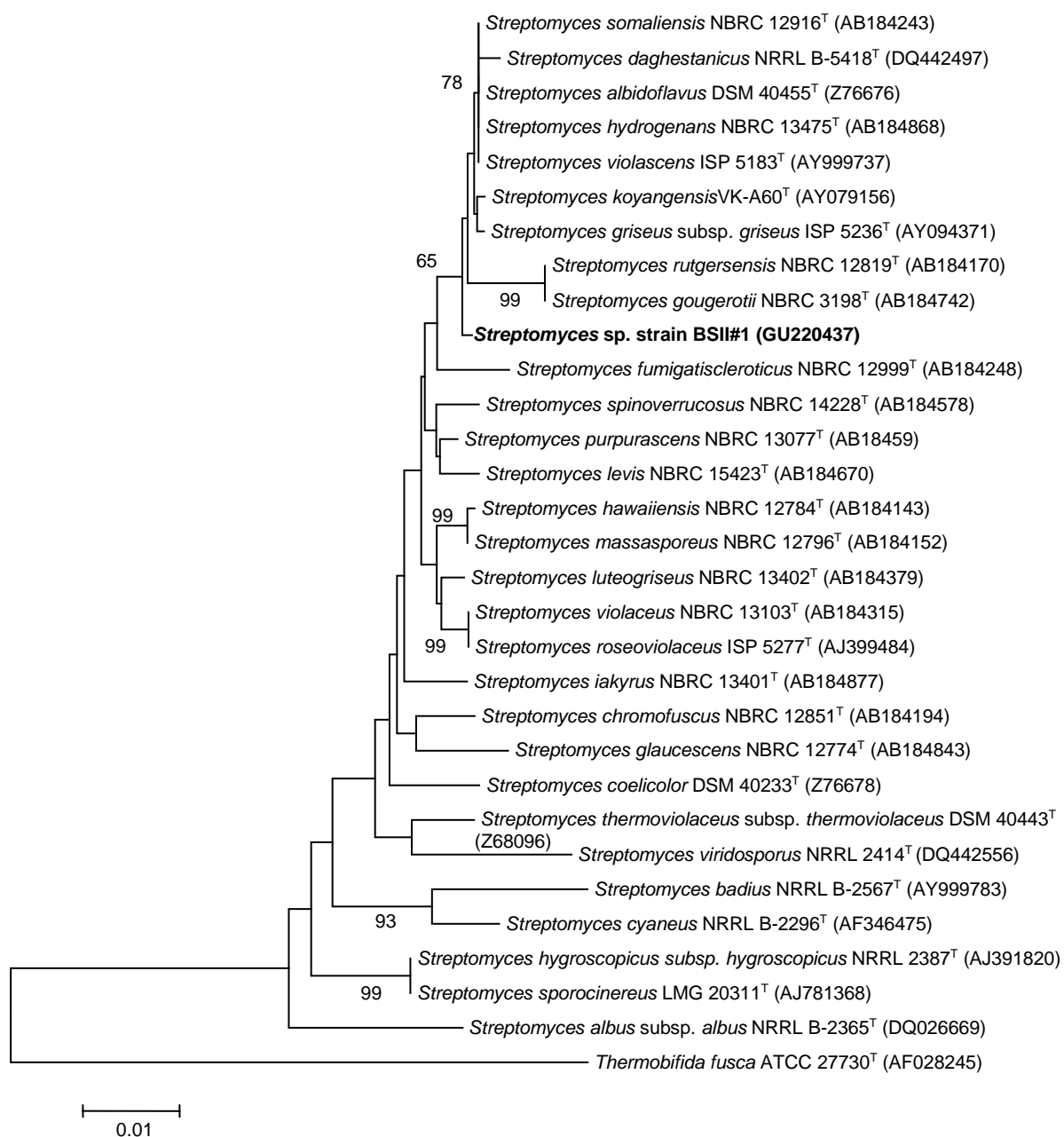
Figure 4.10: Neighbour-joining tree of the 16S rRNA gene sequences of *Streptomyces* sp. strain BSII#1, its phylogenetic neighbours as determined from EzTaxon-e analysis, and selected known peroxidase producers, which includes the out-group used in this tree (*T. fusca*). The tree was constructed using the longest common length of all the sequences (1421 bp). GenBank accession numbers are provided in parenthesis. The error bar represents 10 nucleotide substitutions per 1 000 nucleotides and percentage bootstrap values of 1 000 replications are shown at each node (only values above 50% are shown) (Please note: The GenBank data for the *Streptomyces* sp. strain BSII#1 sequence still needs to be updated; the accession number refers to the shorter, 791bp sequence previously submitted).

4.3.3.2.   Mol (%) G+C Determination

Thermal melting curves for the genomic DNA extracted from *Streptomyces* sp. strain BSII#1 were constructed in triplicate (Figure 4.11; Appendix III for all replicates and calculations).
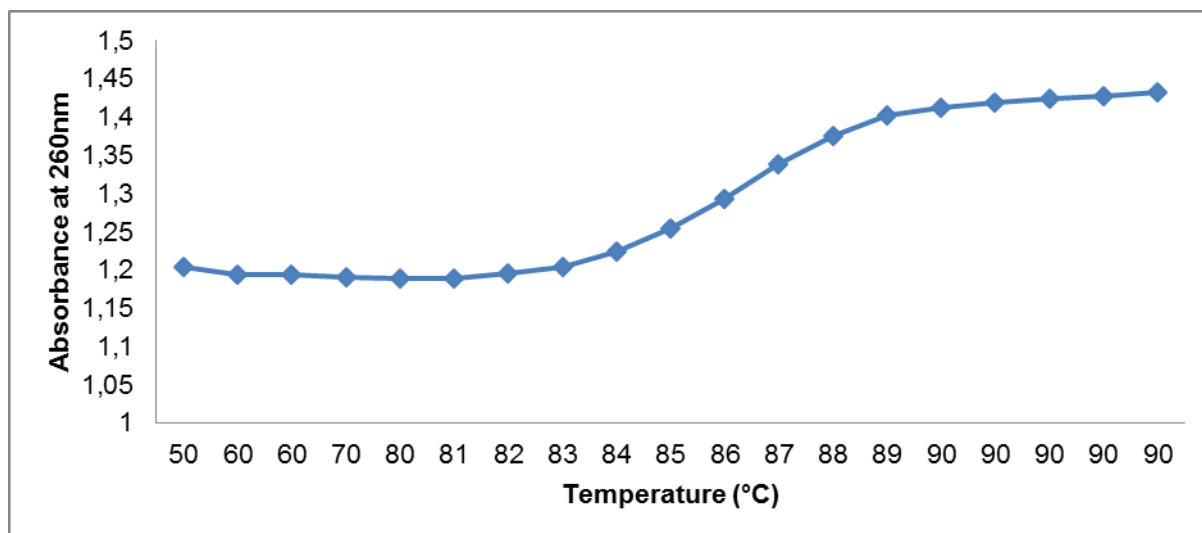
Figure 4.11: An example of a replicate thermal melting curve of genomic DNA isolated from *Streptomyces* sp. strain BSII#1.

The observed T_m (in 0.1% SSC) and corrected (to 1% SSC) T_m values are presented in Table 4.6. The equation (Equation 8) applied for the calculation of mol% G+C content determined that the mol% G+C for the *Streptomyces* sp. strain BSII#1 genome was 77.92±0.85% (mean ±SEM; n=3).

Table 4.6: Observed and Corrected T_m values used for the calculation of the mol% G+C

	Observed T_m (°C)	Corrected T_m (°C)
Replicate 1	86.4	101.8
Replicate 2	85.9	101.3
Replicate 3	85.6	100.6

4.4.   Discussion

Streptomyces sp. strain BSII#1 was isolated from sediment from the Bwanda hot springs, Lochinvar National Park, Zambia. Through the use of a polyphasic approach, it could be concluded that the isolate shows characteristics typical of the genus *Streptomyces* and its assignment to this genus is supported by not only genotypic information, but also phenotypic and chemotaxonomic information. Aerial mycelia developed into smooth, rounded spores that are formed in linear chains. *Streptomyces* sp. strain BSII#1 did not produce any pigments on ISP 5 or melanoid pigments on either ISP 6 or ISP 7 medium, but a deep red to

red-brown pigment was readily produced on SCN medium. The red-brown pigment was also produced in liquid modified phenoxazinone production medium (Section 3.2.1). On ISP4 medium, the aerial spore mass colour changed from an initial off-white colour to rust grey as the colonies aged.

16S rRNA gene sequence analysis showed that *Streptomyces* sp. strain BSII#1 is most closely related to *S. albidoflavus*. A comparison of phenotypic characteristics (Table 4.7) revealed that the two strains are very similar. A phylogenetic tree of the 16S rRNA gene sequences of strain BSII#1 and the top hits from EzTaxon-e, showed that strain BSII#1 branches on a separate clade. The top five hits (as indicated in Section 4.3.3.1) is part of a taxonomic group called the *S. somaliensis* group. According to EzTaxon-e, strains within this group cannot be distinguished from each other on the basis of 16S rRNA gene sequence alone. In order to determine the true taxonomic status of strain BSII#1, DNA-DNA hybridisation needs to be performed and will form part of future studies.

Table 4.7: A comparison of the phenotypic characteristics of *S. albidoflavus* and strain BSII#1. Information on *S. albidoflavus* was taken from Shirling and Gottlieb (1969).

Characteristic	*S. albidoflavus*	*Streptomyces* sp. strain BSII#1
Spore chain morphology	Rectiflexibiles	Rectiflexibiles
Spore surface morphology	Smooth	Smooth
Aerial mass colour	White or grey colour series (ISP2); no sporulation on other ISP media	White or grey colour series (ISP4); Off-white to yellow colour series on ISP2; no sporulation on other ISP media
Diffusible pigments	No distinctive pigments	Red to red-brown pigment on SCN agar
Melanoid pigments	No production on ISP6 or ISP7	No production on ISP6 or ISP7
Carbon source utilisation	D-glucose L-arabinose D-xylose D-mannitol D-fructose	D-glucose L-arabinose D-xylose D-mannitol D-fructose Raffinose
Not utilised as sole carbon source	Myo-inositol Rhamnose Raffinose	Rhamnose

Chemotaxonomic test results supported the genotypic information for grouping strain BSII#1 in the genus *Streptomyces*: LL-DAP as the dominant di-aminopimelic acid and no discernible

sugar pattern. MK-9(H$_6$) and MK-9(H$_4$) were detected in the menaquinone extract, but was only present in low quantities. In future work, these extractions will be repeated on a larger scale and concentrated to confirm the presence of these menaquinones, especially the presence of MK-9(H$_4$), which is not typically found in streptomycetes [typically, MK-9(H$_6$) and MK-9(H$_8$) are the dominant menaquinones found in streptomycetes].

The phospholipid extract contained DPG and PG, visualised as deep blue-purple spots on the silica TLC plate stained with α-naphthol, with PIMs being visualised as blue spots. No yellow spots were observed on the α-naphthol silica TLC plate, indicating that PI was not present or was too low in concentration for detection. Two pink spots (faint) was observed on the plate stained with ninhydrin, indicating the presence of either PE or PS. Streptomycetes typically have DPG, PG, PI, PIMs and PE, indicating that one of the pink spots could be PE and the other an unknown amino-containing phospholipid. The phospholipid pattern needs to be confirmed and the fatty acid profile determined in order to complete the chemotaxonomic description of strain BSII#1. The mol% G+C for strain BSII#1, 77.92±0.85%, lies within the reported range for streptomycetes.

4.5. Conclusion

The overall descriptions of *Streptomyces* sp. strain BSII#1 in this chapter confirm its identity as a *bona fide* member of the *Streptomyces* genus. Additional tests still need to be performed to verify the potential novelty of the strain.

CHAPTER FIVE

SCALE-UP OF PEROXIDASE PRODUCTION

5.1. Introduction

One of the major limiting factors for the greater application of oxidative enzymes is their unavailability due to limited supplies. This is particularly true of peroxidases which are expensive enzymes compared to other oxidases available on the market (Ferrer *et al.*, 1991; Torres *et al.*, 2003). Often, peroxidase production in wild-type strains is too low for the feasible production of sufficient enzyme for use in industrial or biotechnological applications. Therefore it becomes important to develop industrial strains from nature's toolset for the enzyme to be further exploited. The most-widely applied peroxidase is HRP. However, the recovery of peroxidase from horseradish roots is a lengthy and complex procedure with generally low yields (Barnard, 2012). Microbial production of peroxidase has been dominated by white rot fungi due to their relatively high extracellular and inducible production by wild-type strains. The white rot fungi including *P. chrysosporium*, *Bjerkandera* sp. and *Coprinus* sp. have been well studied for their production of extracellular peroxidase enzymes (Conesa *et al.*, 2000; Ikehata, 2004). Actinomycetes are the only other known extracellular peroxidase producers, with most producers belonging to the genus *Streptomyces*. However, production in actinomycetes is generally lower than in white rot fungi and the majority of peroxidase production reports have focused on white rot fungi.

Enhancement of peroxidase production has been reported in different fungal strains under optimised environment and culture conditions or after genetic manipulation. Among other factors, the composition of the production medium has been shown to be important for increased peroxidase production. The synthesis of ligninolytic enzymes in *P. chrysosporium,* for example, is known to occur in response to carbon, nitrogen or sulphur limitation (Hamman *et al.*, 1997; Urek and Pazarlioglu, 2007); this fact that has been exploited to produce large quantities of the enzyme in bioreactors. Manganese and nitrogen reportedly have strong regulatory effects on the ligninolytic machinery of this organism (Hamman *et al.*, 1999). Urek and Pazarlioglu (2007) showed that supplementing *P. chrysosporium* cultures with 0.05% (v/v) Tween 80 and 174 µM Mn^{2+} doubled the MnP production. Using full factorial design (FFD) with aeration and agitation as the two main parameters, Alam *et al.* (2009) optimised LiP production by *P. chrysosporium.* The highest peroxidase activity achieved (0.74 U ml^{-1}) occurred after five days of fermentation in 1.5 l culture volume. Genetic engineering has also been used to improve peroxidase production. An engineered *Pleurotus ostreatus* strain showed more than 30-fold overproduction of versatile peroxidase compared to the non-engineered wild-type strain (Tsukihara *et al.*, 2006).

Streptomycetes are important industrial microorganisms due to their ability to produce a large number of secondary metabolites, including enzymes with different industrial applications (Macedo *et al.*, 1999). The potential of streptomycetes has not been fully unravelled or exploited. Despite numerous whole-genome studies confirming the presence of genes or gene clusters with huge biosynthetic potential (often revealed as cryptic pathways), the nutritional requirements and physiology of most of the groups screened and the key elements involved in the regulation of their secondary metabolite production are still largely unknown (Genilloud *et al.*, 2011). Although the *Streptomyces* genus is the largest genus in the *Actinobacteria* with over 600 validly published species (Parte, 2013), there is still potential for the discovery of novel, useful metabolites and the mechanisms for their production.

Reports based on microfermentations of various actinomycete (Genilloud *et al.*, 2011) and fungal strains (Bills *et al.*, 2008) under a range of conditions show that effective manipulation of media and culture conditions can promote the biosynthetic potential of microorganisms. The extensive multiplicity of possible media composition/environmental factor combination simply that there is a possibility for discovering actinomycete strains (either novel or known) that are capable of producing peroxidases at levels that make industrial use feasible.

Studies have shown that peroxidase production in actinomycetes is dependent on different factors (Tuncer *et al.*, 2009). These include the producing microorganism, composition of the medium (including presence of inducers, enhancers and activators), the pH of the medium, temperature and aeration (Rob *et al.*, 1997; Tuncer *et al.*, 1999; Tuncer *et al.*, 2009). Tuncer *et al.* (2009) conducted optimisation studies of extracellular lignocellulolytic enzymes that included peroxidase by *Thermomonospora fusca* BD25 in shake flasks as well as in an automated bioreactor (1 l culture volume). They found that the highest production of extracellular peroxidase (12 mU ml^{-1}), occurred in a medium containing either oat spelt xylan or ball milled straw (Tuncer *et al.*, 2009).

The large-scale production of extracellular peroxidases by actinomycetes is a subject that has received less attention than production by fungi. Currently, there is no information available on commercial or industrial-scale peroxidase production by actinomycetes. The majority of studies have centred on production in flask culture with culture volumes ranging between 20 and 100 ml (Iqbal *et al.*, 1994; Rob *et al.*, 1997; Bon *et al.*, 1999; Macedo *et al.*, 1999; Tuncer *et al.*, 1999; Zerbini *et al.*, 1999; Antonopoulos *et al.*, 2001). Laboratory-scale bioreactors have only been reported for peroxidase production by actinomycetes in a few instances: 0.5 l culture volume (Adhi *et al.*, 1989) and1 l culture volume (Tuncer *et al.*, 2009).

The study by Adhi *et al.* (1989) showed that maximum extracellular peroxidase production (about 0.12 U ml^{-1}) by *S. viridosporus* T7A in agitated, submerged culture occurred early in the stationary phase, but as the production of peroxidase was not the main objective of their experiments, they did not present further details. In their study on production of extracellular peroxidase by *Streptomyces* sp. F6616, Tuncer *et al.* (2009) attributed the better peroxidase production to the presence of inducers (xylan and wheat straw) in the medium, rather than optimised fermentation conditions.

The results discussed in Chapter 3 established that *Streptomyces* sp. strain BSII#1 was the most significant peroxidase-producing strain of the five strains tested in 10 ml culture volume: 1.30±0.04 U ml^{-1} in the presence of 0.1 mM VA; 1.27±0.06 U ml^{-1} in the presence of 0.1 mM pyrogallol and up to 0.94±0.1 U ml^{-1} in the presence of 1.5% (w/v) wheat bran. Based on these results, *Streptomyces* sp. strain BSII#1 was selected for further scale-up of the peroxidase production. The aim in this part of the study was to incrementally increase the scale of peroxidase production by *Streptomyces* sp. strain BSII#1 in a simple, cost-effective system to produce yields at least similar to those achieved under optimised conditions on a small scale.

5.2. Materials and Methods

All chemicals and solvents used were of analytical grade and purchased from Merck-Millipore (South Africa) and Sigma-Aldrich (South Africa).

5.2.1. Microorganism and Culture Maintenance

Stock cultures of *Streptomyces* sp. strain BSII#1 were prepared, handled and stored as described previously (Section 3.2.1).

5.2.2. Pre-cultures

Spore/hyphae suspensions (500 μl) of *Streptomyces* sp. strain BSII#1 were used to inoculate 10 ml modified phenoxazinone medium (initial pH 8.0) and incubated at 30°C for two days, shaking at 160 rpm (Section 3.2.2). This served as a pre-culture for subsequent inoculation in all the experimental flasks. The contents of the pre-culture flasks were pooled together for all but the stirred tank bioreactor experiment and served as the inoculum for the scale-up studies.

5.2.3. Peroxidase Assay

Peroxidase activity was measured using the 2,4-DCP assay (Antonopoulos *et al.*, 2001) as detailed in Section 3.2.3. Samples from the growing cultures were centrifuged at 10 000 g for

5 min at room temperature and the decanted supernatant served as the crude extracellular extract for peroxidase determinations.

5.2.4. Incremental Scale-Up

5.2.4.1.   100 ml Erlenmeyer and Baffled Flask Cultures

The effects of aeration and varying the inoculum size was assessed in 100 ml culture volumes in 500 ml Erlenmeyer and baffled flasks (Figure 5.1). The inoculum sizes that were investigated were 5%, 10%, 15% and 20% (v/v). Flasks were incubated at the previously established optimum temperature for peroxidase production (37°C), shaking at 160 rpm.
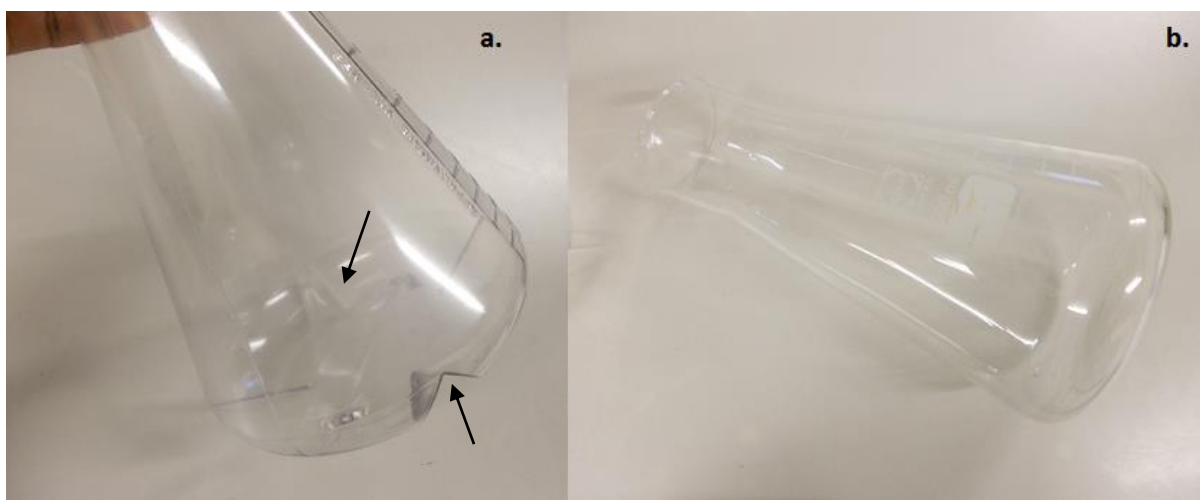
Figure 5.1: Comparison between (a) baffled flask, which has indentations (the 'baffles' indicated by the arrowheads) in its walls to increase turbulent flow and (b) flat-bottomed Erlenmeyer flask.

5.2.4.2.   400 ml Baffled Flasks

Peroxidase production was assessed in 400 ml culture volumes (10% v/v inoculum) in 2 l baffled flasks. Flasks were incubated at 37°C with shaking at 160 rpm.

5.2.4.3.   Bubble Bioreactors

Two-day old pre-cultures were used to inoculate 350 ml modified phenoxazinone production medium (10% (v/v) inoculum) in 1 l bubble bioreactor vessels. Figure 5.2 shows the principal components of the bubble bioreactor. Air input was facilitated by the use of fish tank air pumps set at maximum feed rate (Regent air pump; Model 9500; aeration capacity range 0.3-1.4 l min^{-1}). Antifoam 204 (Sigma) was added as necessary at 1 µl of antifoam per 1 ml of culture. The pH was neither monitored nor controlled over the fermentation period.
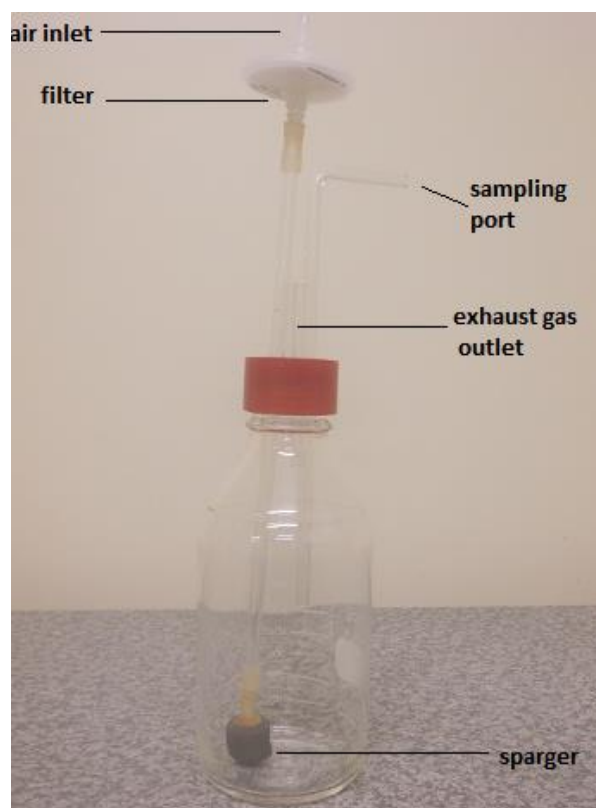
Figure 5.2: Principal components of the bubble bioreactor used, showing the air inlet, air filter, sampling port, exhaust gas outlet and sparger.

5.2.4.4.   Airlift Bioreactors

Two-day old pre-cultures served as inoculum to seed 3 l modified phenoxazinone production medium (pH 8.0; 10% (v/v) inoculum) in an airlift bioreactor. Figure 5.3 is a photograph of the airlift bioreactor used. Air input was facilitated via the use of an air compressor, and the aeration rate was gradually increased from 2 l hr^{-1} to 18 l hr^{-1} as fermentation proceeded to ensure adequate mixing and aeration of the culture. Peroxidase activity was assessed at 12 hr intervals. Antifoam 204 (Sigma) was added as necessary at 1 µl of antifoam per 1 ml of culture. The pH was neither monitored nor controlled over the fermentation period.

Figure 5.3: Photograph showing *Streptomyces* sp. strain BSII growing in an airlift reactor. On the right (in the rear) is a flow meter device for measuring air flow rate as it was pumped into the reactor.

5.2.4.5.   Stirred Tank Bioreactors

Peroxidase production by *Streptomyces* sp. strain BSII#1 was assessed in two types of stirred tank bioreactor: a parallel Sixfors reactor (300 ml culture volume) and a Sartorius Biostat B fermentor (6 l culture volume).

a. Parallel Sixfors Bioreactor

Two-day old pre-cultures served as inoculum to seed 300ml modified phenoxazinone production medium (pH 8.0; 10% v/v inoculum) in six parallel Sixfors fermentors (Figure 5.4). The system consisted of six fermentation units with individual controls for aeration rate, stirring speed, pH and temperature control. Three six-flat-blade impellers (Rushton turbines) were mounted on the rotor shaft of each fermentation unit. The aeration rate was controlled by a gas flow regulator; sterile air was distributed in the fermentor via a sparger device. The stirrer speed was independent of the aeration parameters. pH and temperature during fermentation were monitored and controlled: pH was adjusted as appropriate by automatic addition of base or alkali, and temperature was controlled using a heating/cooling jacket.

Foaming within each fermentation vessel was monitored by an antifoam probe, which triggered the automatic dispensation of antifoam when activated.

Figure 5.4: Photograph of the parallel Sixfors bioreactor system used. The system comprises six independent fermentation units that can be independently controlled for pH, temperature, stirrer speed and antifoam activity.

b. Sartorius Biostat B Fermentor

Figure 5.5 shows the components of the Sartorius Biostat B fermentor used in this study. The fermentor had four equidistant baffles, two Rushton turbines along the rotor shaft and was equipped with control and monitoring systems for aeration, temperature, pH and foaming. An electric jacket wrapped around the vessel, together with circulating water for cooling, maintained the temperature within set limits. The pre-culture was prepared as follows: spore/hyphae suspensions (15 ml) of *Streptomyces* sp. strain BSII#1 prepared in water were used to inoculate two 1 l baffled flasks each containing 300 ml modified phenoxazinone medium (initial pH 8.0) and incubated at 30°C for two days with shaking at 160 rpm.

Figure 5.5: Photograph showing a Sartorius Biostat B fermentor. On the left is the stirred tank fermentor and its associated attachments, and on the right is the control unit.

Two-day old pre-cultures served as inoculum to seed 6 l modified phenoxazinone production medium (10% v/v inoculum) in the Sartorius Biostat B fermentor. In the first experiment the fermentor was set to maintain a partial pressure of oxygen of 20% (pO_2) and the stirrer speed limits varied between 200 rpm and 500 rpm. In the second experiment the fermentor was set to maintain a pO_2 of 10% and the stirrer speed limits varied between 200 rpm and 365 rpm. In both experiments the pH was controlled at pH 7.0 and the temperature maintained at 30°C. Manual adjustment of the stirrer speed was effected when the pO_2 drifted from the set values.

5.2.5. Statistical Analyses

The data is presented as mean±SEM. Statistical analyses were conducted using IBM® SPSS® Statistics, Version 21. Depending on the experiment design, univariate analysis of variance or one-way ANOVAs were used to determine the significance of results. This was followed by the Bonferroni post hoc test for pairwise comparisons of factors.

5.3. Results

The aim of this section of the work was to scale-up production of peroxidase by *Streptomyces* sp. strain BSII#1. Preliminary investigations during scale-up to 100 ml culture volume (in baffled flasks) indicated that the inclusion of VA in the medium did not result in appreciable induction of peroxidase as it had done in 10 ml culture volume (Figure 5.6). In fact, production on this scale in the absence of the inducer was significantly higher on the day of maximum production ($p < 0.05$, day 4; Figure 5.6).
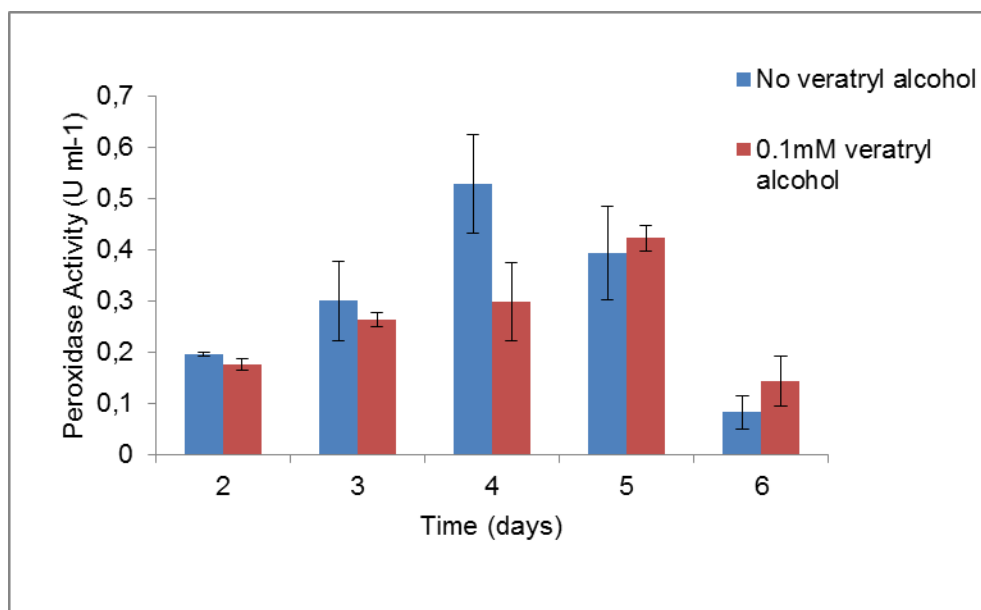
Figure 5.6: Peroxidase production by *Streptomyces* sp. strain BSII#1 in flat-bottomed Erlenmeyer flasks with 100 ml culture volume in modified phenoxazinone production medium (pH 8.0) with or without 0.1 mM VA (37°C, 160 rpm) (mean±SEM; n=2).

Similarly, the inclusion of wheat bran did not result in improved peroxidase production. This was because, during agitation, the bran particles tended to adhere onto the walls of the flask above the level of the liquid. In line with the objective to design the simplest but most effective bioprocess for peroxidase production at larger scale, it was decided to exclude inducers from further scale-up investigations and to focus on other parameters to determine if a sub-optimal larger scale production could generate peroxidase yields at least comparable to the optimised, induced small-scale production.

5.3.1. 100 ml Culture Volume

Upon scale-up from 10 ml to 100 ml culture volume in flat-bottomed Erlenmeyer flasks (no inducers), there was a significant decrease ($p < 0.05$) in maximum peroxidase production. Production decreased from 0.84 ± 0.06 U ml^{-1} in 10 ml culture volume to 0.068 ± 0.004 U ml^{-1} in 100 ml culture volumes (i.e. almost 90% decrease in maximum production) under the same culture conditions (Figure 5.7). These results indicated that aeration may be the limiting

factor for peroxidase production in the larger volume since all other factors were kept constant.
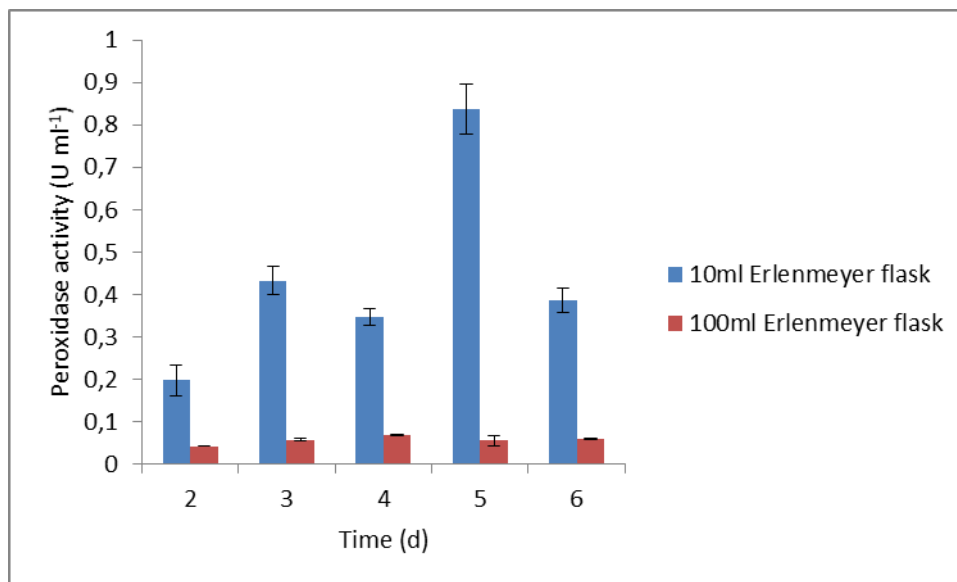
Figure 5.7:Peroxidase production by *Streptomyces* sp. strain BSII#1in flat-bottomed Erlenmeyer flasks with 10 ml and 100 ml culture volume (modified phenoxazinone production medium (pH 8.0), 37°C, 160 rpm) (mean±SEM; n=2).

To determine if increased aeration could improve production in 100ml culture volumes, baffled flasks were employed. The effect of inoculum size on peroxidase production was concurrently assessed. In this experiment, the physical conditions for maximum peroxidase production previously established for *Streptomyces* sp. strain BSII#1 in 10 ml culture volumes were maintained. The results showed that, in comparison to Erlenmeyer flasks, maximum production was significantly increased ($p<0.05$) in 100 ml culture volume when baffled flasks were used (Figure 5.8). This trend applied for all inoculum sizes tested; while the highest peroxidase activity (0.53 ± 0.1 U ml^{-1}) was observed using 10% (v/v) inoculum, there was no significant difference in production using 5, 10 or 15% (v/v) inoculum.
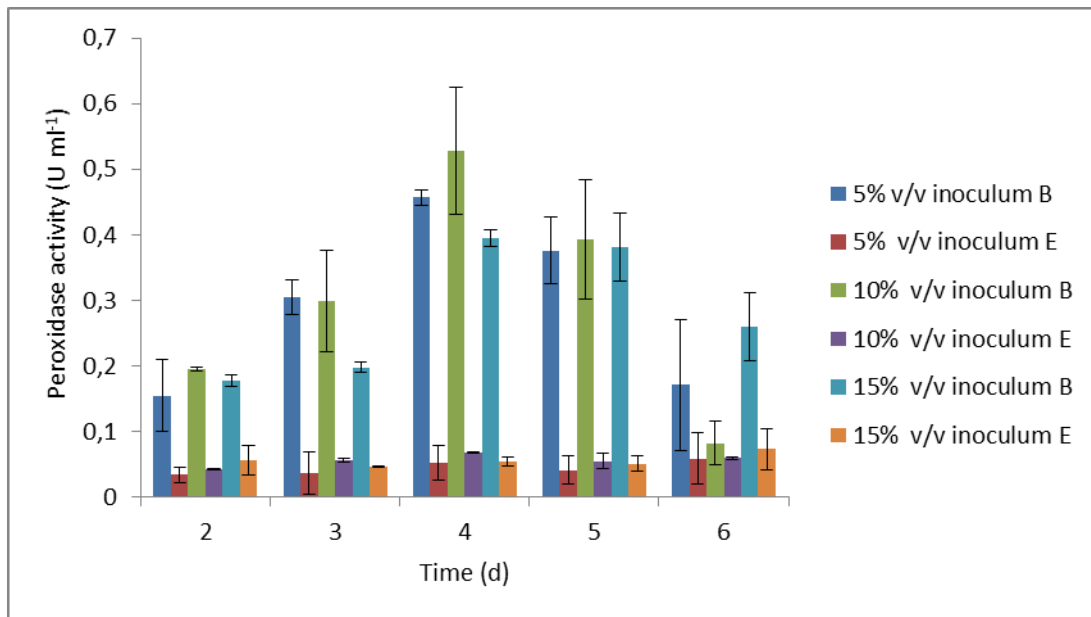
Figure 5.8: Effect of inoculum size and flask type (E=Erlenmeyer flask; B= baffled flask) on peroxidase production by *Streptomyces* sp. strain BSII#1 in 100 ml culture volume, pH 8.0, 37°C, 160 rpm (mean±SEM; n=2).

5.3.2. 380 ml to 400 ml Culture Volume

As increased aeration using baffled flasks significantly improved peroxidase production by *Streptomyces* sp. strain BSII#1in 100 ml culture volumes, the next step in the incremental scale-up to 400 ml culture volumes was conducted in baffled flasks and bubble bioreactors.

Scale-up of peroxidase production to 400 ml culture volume in baffled flasks resulted in both a decrease in the maximum peroxidase production (0.26 ± 0.08 U ml^{-1}) and an increase in the retention time required for maximum peroxidase production (6 days compared to 4 days; Figure 5.9) compared to 100ml culture volumes in baffled flasks (Figure 5.8).
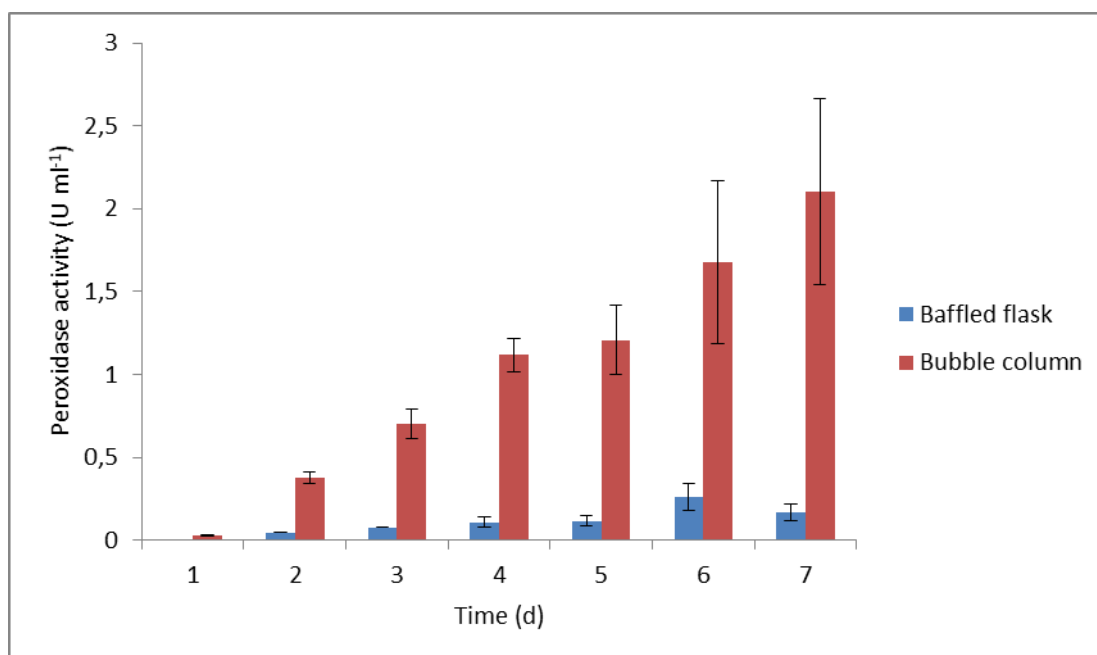
Figure 5.9: Peroxidase production by *Streptomyces* sp. strain BSII#1in modified phenoxazinone production medium (initial pH 8.0) in baffled flasks (400 ml culture volume; pH 8.0, 37°C, 160 rpm) and in bubble bioreactors (380 ml culture volume; pH 8.0, 25±2°C) (mean±SEM; n=2).

As aeration appeared to improve or restore maximum peroxidase production by *Streptomyces* sp. strain BSII#1 on scale-up, a bubble bioreactor (using fish tank pumps for aeration) was used to increase the aeration efficiency in 380 ml culture volumes. Production in the bubble bioreactors was conducted at room temperature rather than at the previously established production optimum of 37°C as this was more practical in our laboratory. The results showed that aeration through bubbling was an effective method of enhancing peroxidase production in 380 ml culture volumes (up to 2.10±0.56 U ml^{-1} on day 7; Figure 5.9). The yields of peroxidase that were achieved under sub-optimal conditions (ambient temperature, no inducers) using bubble bioreactors was much higher than those from small-scale optimised flask cultures (1.30±0.04 U ml^{-1}, Table 3.2).

While the peroxidase concentration continued to increase (Figure 5.9), it was not feasible to allow the process to continue longer than 7 days as the culture medium became thick and viscous with excessive biomass. This made the downstream handling difficult and resulted in a decrease in the volume of crude peroxidase that could be harvested after centrifugation.

5.3.3. 3 l Culture Volume

A further scale increase to 3 l culture volume was achieved using an airlift bioreactor format. On this scale, with improved aeration rates due to the use of compressed air, the retention time for high peroxidase production was dramatically shortened (1.66±0.05 U ml^{-1} within 36 hrs; Figure 5.10). Furthermore, the peroxidase production continued to increase up to

4.76±0.46 U ml^{-1} after 60 h fermentation. At this point, cultures were halted as the biomass growth was excessive (50 mg dry weight per ml culture) and any further retention in the reactor resulted in difficulty in downstream processing and reduced yields in terms of the crude extract volume that could be extracted after centrifugation.
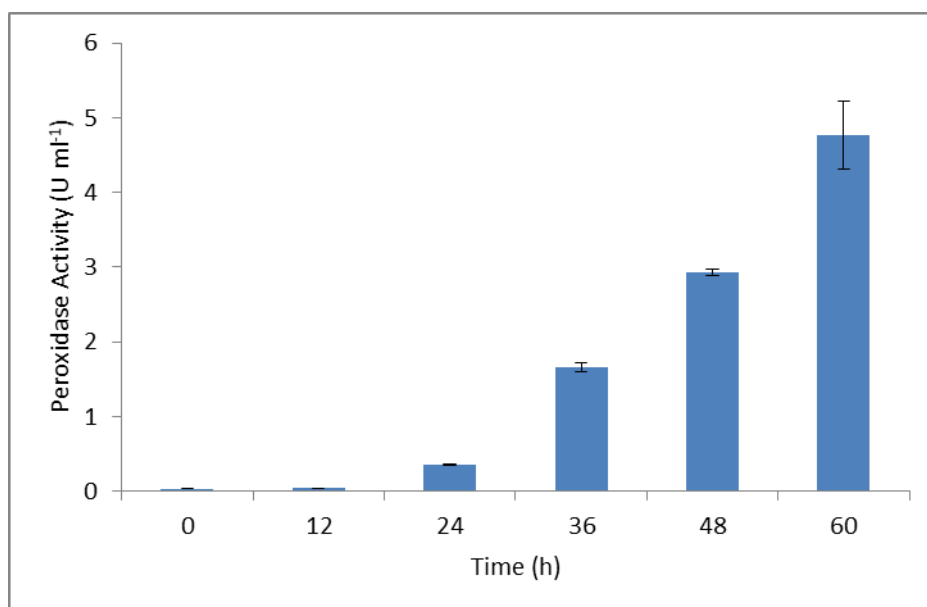
Figure 5.10: Peroxidase production by *Streptomyces* sp. strain BSII#1 in modified phenoxazinone production medium (initial pH 8.0) in 3 l culture volume in an airlift bioreactor, pH 8.0, 25±2°C (mean±SEM; n=2).

5.3.4. Stirred Tank Bioreactors

5.3.4.1. *Parallel Sixfors Reactors*

There was rapid uptake of oxygen during the first day of fermentation and it became necessary to gradually increase the stirrer speed from the initial 300 rpm in each fermentor unit. At the end of the first day of incubation, it was decided to set the stirrer speed at 1200 rpm in all six fermentors to ensure adequate aeration.

Figure 5.11 shows peroxidase production in the parallel Sixfors fermentors under the different conditions described. Maximum peroxidase production (0.288 U ml^{-1}) was achieved in the fermentor set at 37°C and without any pH maintenance or control.
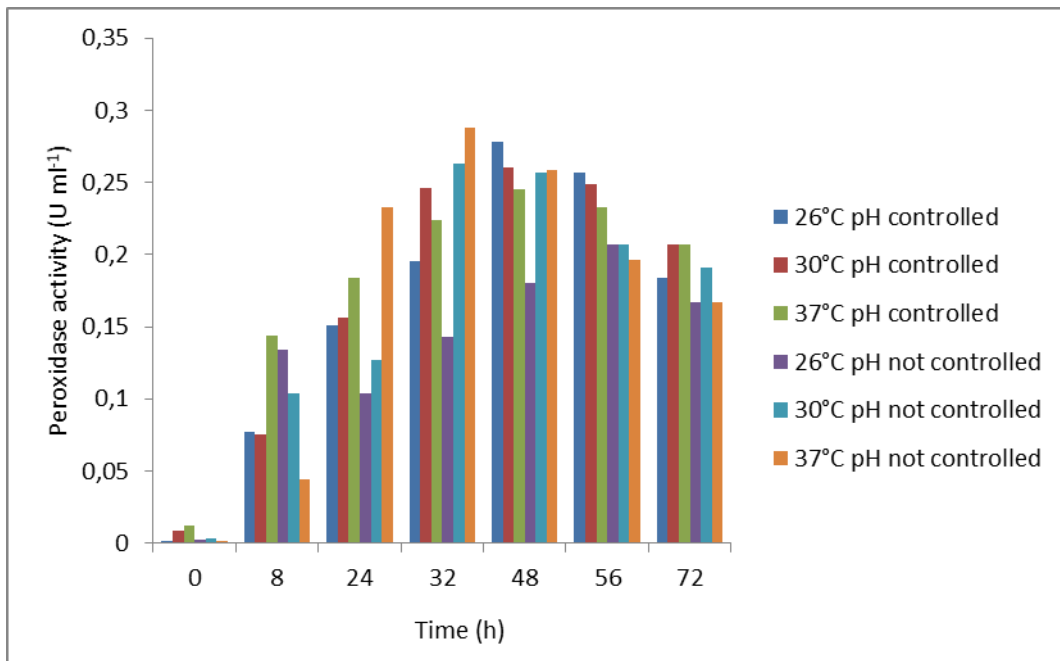
Figure 5.11: Peroxidase production by *Streptomyces* sp. strain BSII#1 in modified phenoxazinone production medium at different temperature and with or without pH control (pH maintained at pH 8.0 in the pH-controlled reactors) in 300 ml culture volume in parallel Sixfors fermentors (mean±SEM; n=2).

pH monitoring in the parallel Sixfors fermenters showed that the pH of modified phenoxazinone production medium dropped from pH 8.0 (before autoclaving) to pH 7.0 (after autoclaving) (Figure 5.12). Furthermore, pH was maintained around pH 7.0 during the fermentation without any outside control (Figure 5.12a). Overall, maximum peroxidase production reached in all cases tested in the stirred tank reactors was low, ranging from 0.180 to 0.288 U ml^{-1} maximum peroxidase activity (32-48 hrs), before decreasing (Figure 5.11).
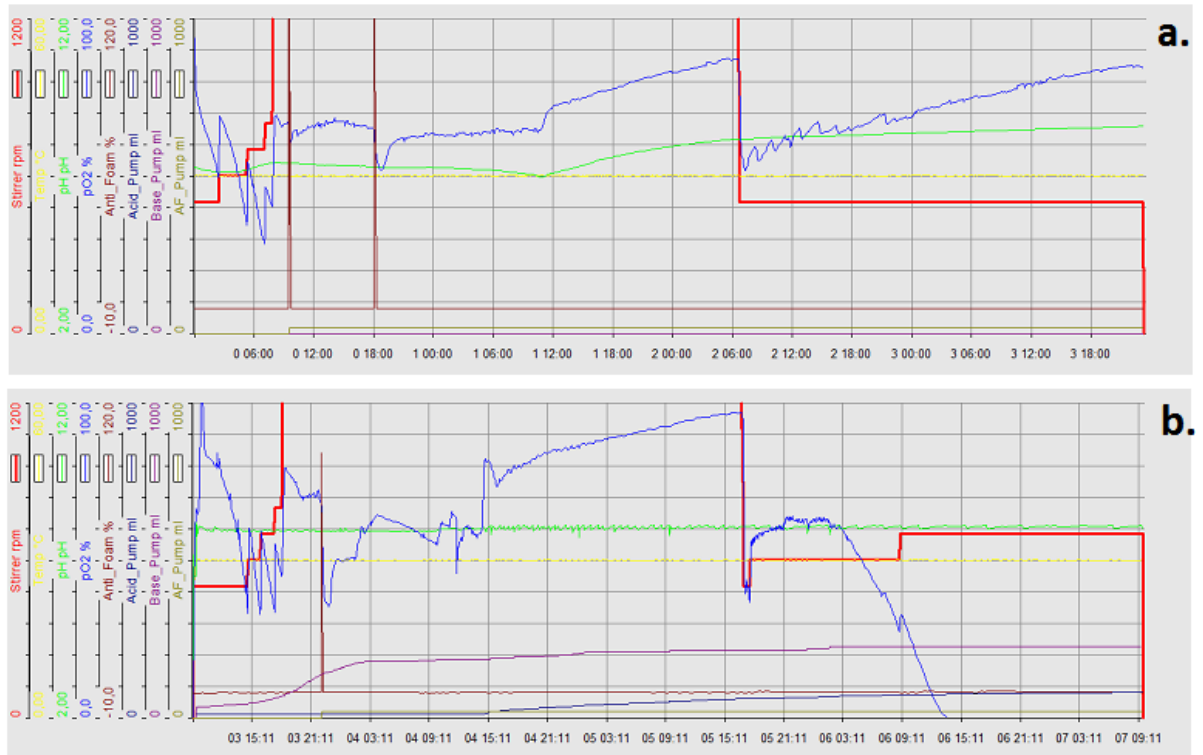
Figure 5.12: Examples of the fermentation profiles for two parallel Sixfors fermentor units set at 30°C. In (a) the pH was not controlled throughout the fermentation and it can be seen that the starting pH was about 7.0 In (b) pH was set to be controlled at pH 8.0 and there is an immediate initial adjustment to correct the starting pH of 7.0.

5.3.4.2.   Sartorius Biostat B Fermentor

Figure 5.13 shows the profile of pO_2 and stirrer speed in the Sartorius bioreactor, where the pO_2 was maintained at 20%. The stirrer speed was manually adjusted (between 200 rpm to 500 rpm) in an attempt to maintain pO_2 at 20%. The stirrer speed was increased from 400 rpm to 500 rpm after 28 hrs to increase pO_2 to 20%. After 40 hrs, there was a rapid increase in pO_2 and the stirrer speed was decreased to 350 rpm. The stirrer speed was continually adjusted to compensate for fluctuations in pO_2.
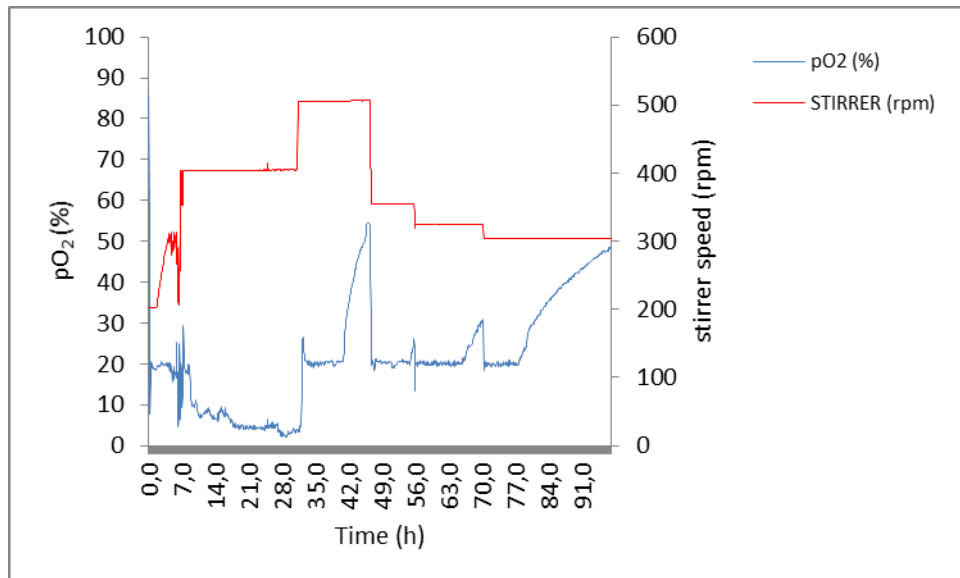
Figure 5.13: Profile of pO$_2$ and stirrer speed during fermentation of *Streptomyces* sp. strain BSII#1 in modified phenoxazinone production medium (6 l culture volume, pH 7.0, 30°C). The stirrer speed was manually adjusted to maintain pO$_2$ at 20%.

Figure 5.14 shows the profile of pO$_2$ and stirrer speed in the Sartorius bioreactor where the pO$_2$ was maintained at 10%.
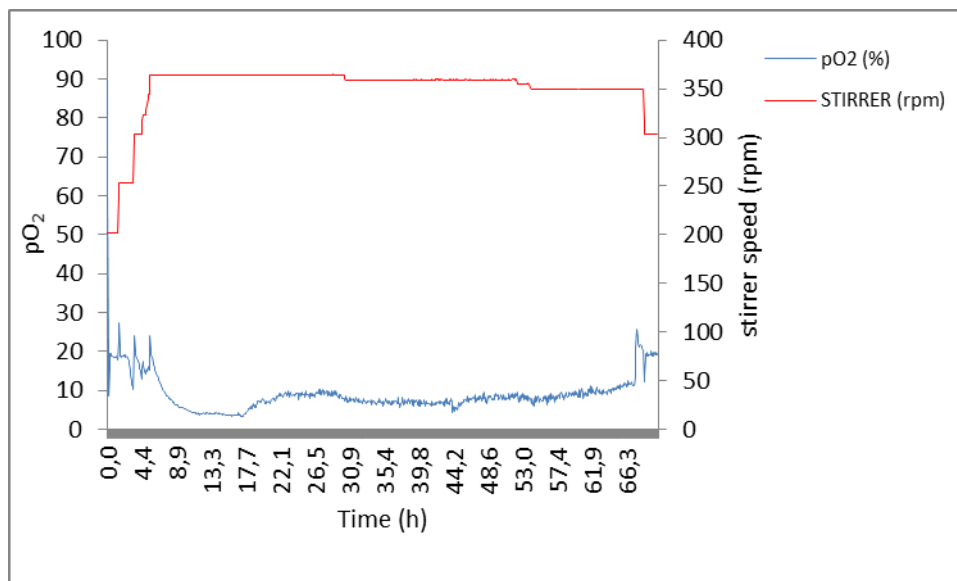
Figure 5.14: Profile of pO$_2$ and stirrer speed during fermentation of *Streptomyces* sp. strain BSII#1 in modified phenoxazinone production medium (6 l culture volume, pH 7.0, 30°C). The stirrer speed was manually adjusted to maintain pO$_2$ at 10%.

Manual adjustments of the stirrer speed between 200-500 rpm were necessary to correct and maintain the pO$_2$ at 20% (Figure 5.13). Maintaining pO$_2$ at 8-10% was achieved by making adjustments in the stirrer speed within the 200-350 rpm range (Figure 5.14). There was less fluctuation in the actual pO$_2$ levels observed at 10% pO$_2$ (Figure 5.14) as compared to 20%

(Figure 5.13). Furthermore, after 72 hrs of incubation, growth was visually observed to have proceeded to a greater extent compared to when the pO_2 was maintained at 20%.

Peroxidase production was still steadily increasing after 72 hrs of incubation when the pO_2 was maintained at about 10% and the stirrer speed was mostly at 350 rpm (Figure 5.15). When pO_2 was higher (20%) and stirrer speed was between 300-500 rpm, peroxidase production peaked between at 48 hrs to 72 hrs before starting to decrease (Figure 5.15). Visual inspection of the fermentors at 72 hrs showed that biomass in the fermentor set at 10% pO_2 was markedly greater than in the one set at 20% pO_2. Maximum production occurred when pO_2 was maintained at a lower saturation level (8-10%) and, hence, stirrer speed was lower.
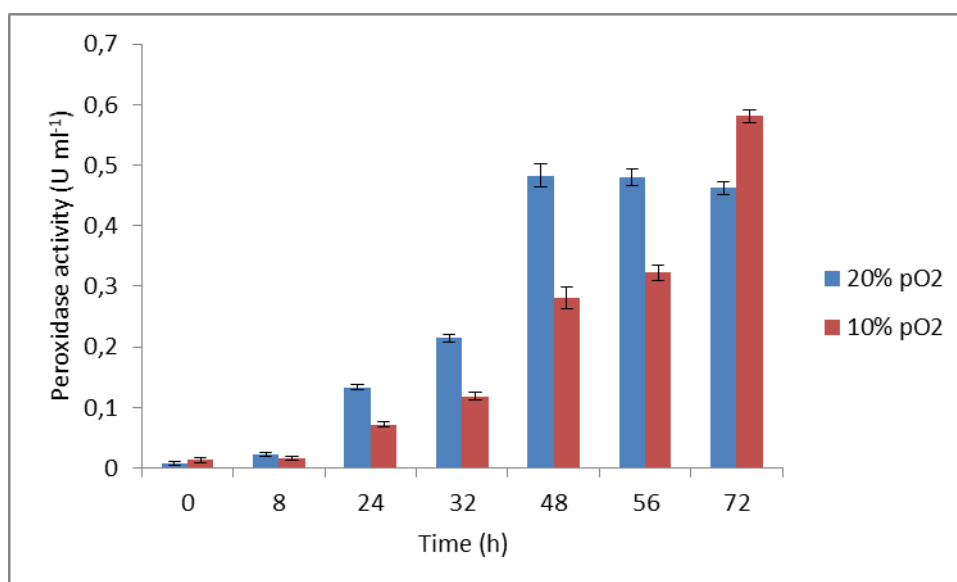
Figure 5.15: Peroxidase production by *Streptomyces* sp. strain BSII#1 in 6 l culture volume in a Sartorius stirred tank bioreactor, pH 7.0, 30°C and pO_2 maintained at either 10% or 20%.

Table 5.1 summarises the results of the incremental scale-up of peroxidase production by *Streptomyces* sp. strain BSII#1 by listing the highest peroxidase activities recorded for each bioreactor format and the culture volume tested in each case.

Table 5.1: Summary of maximum peroxidase activities obtained from *Streptomyces* sp. strain BSII#1 fermentation with the different fermentation formats

Bioreactor configuration	Culture volume (l)	Maximum peroxidase activity (U ml^{-1})
Erlenmeyer flask	0.1	0.068±0.004
Baffled flask	0.1	0.53±0.1
Baffled flask	0.4	0.26±0.08
Bubble bioreactor	0.4	2.10±0.56
Airlift bioreactor	3.0	4.76±0.46
Parallel Sixfors stirred tank bioreactor	0.3	0.288±0.01
Stirred tank bioreactor: Sartorius (20% pO$_2$) Sartorius (10% pO$_2$)	 6.0 6.0	 0.48 0.58

5.4.   Discussion

In general peroxidase production by actinomycetes is low and typically less than 1 U ml^{-1} of enzyme activity is reported (Iqbal *et al.,* 1994; Bon *et al.,* 1999; Tuncer *et al.,* 1999; Macedo *et al.,* 1999; Zerbini *et al.,* 1999; Antonopoulos *et al.,* 2001; Tuncer *et al.,* 2009). The results of this work indicate that, under appropriate conditions of production, *Streptomyces* sp. strain BSII#1 is capable of producing significantly higher levels of peroxidase activity.

5.4.1. 10 ml Erlenmeyer and 100 ml Erlenmeyer Flask Cultures

The comparison of peroxidase production in 50 ml Erlenmeyer flasks versus 500 ml Erlenmeyer flasks indicated that, while all production parameters (except the culture volume) between the two sets of experiments were kept constant, there was almost 90% decrease in the peroxidase production during scale-up from 10 ml to 100 ml culture volume. The greater surface area to volume ratio in the smaller vessel (10 ml culture volume) means that processes such as oxygen transfer and gas exchange between the medium and the surrounding air occurs more rapidly and efficiently. Therefore the 10 ml culture medium was well-aerated and growth of the microorganism was promoted, as observed by the culture becoming more viscous within two days of inoculation. In the 100ml cultures in 500 ml flasks, the surface area to volume ratio was lower, which limits mass oxygen transfer between the medium and the environment. The culture remained much less viscous in 100 ml culture volume; better growth resulted in greater peroxidase production in 10 ml culture volume as compared to 100 ml volume.

5.4.2. 100 ml Erlenmeyer and Baffled Flask Cultures

Since peroxidase production decreased with a linear scale-up of culture volume and flask size, it was hypothesized that aeration was the limiting factor. To test this hypothesis, peroxidase production was assessed between flasks with similar dimensions but different aeration efficiency (i.e. flat-bottomed Erlenmeyer flasks and baffled flasks).

Comparison of peroxidase production in 100 ml culture volumes between baffled and Erlenmeyer flasks clearly showed better production in the baffled flasks (Figure 5.8). There was almost 90% lower peroxidase production in Erlenmeyer flasks (0.068±0.004 U ml^{-1}) as compared to the same volume in baffled flasks (0.53±0.1 U ml^{-1}). Baffled flasks have grooves ('baffles') either in the side wall or at the base of the vessel which helps to create turbulent flow as the medium is agitated on an orbital shaker. The increased turbulence promotes greater aeration efficiency, as compared to Erlenmeyer flasks where laminar flow of medium prevails. This was also supported by evidence from visual inspection of the flasks which pointed to improved growth of the microorganism occurring in the baffled flasks (greater viscosity of the culture), as compared to cultures in the Erlenmeyer flasks which remained thin and watery after 6 days' incubation.

5.4.3. 400 ml Culture Volume

Increasing the scale of production from 100 ml to 400 ml in 500 ml and 2 l baffled flasks (respectively) resulted in an almost50% decrease in the maximum peroxidase production (Table 5.1). Comparison of the 100 ml and 400 ml culture volumes in baffled flasks (Figure 5.8 and Figure 5.9) shaking at the same speed (culture volume was 20% of flask volume in both cases), showed better enzyme production in the smaller vessel where the surface area to volume ratio, hence aeration, was greater. The extent of growth observed in the 400 ml culture volumes was also reduced compared to that typically achieved with 10 ml and 100 ml culture volumes. The poorer growth and related decrease in peroxidase production as the culture volume was increased was also attributed to oxygen transfer limitations, resulting in reduced peroxidase production.

5.4.4. Bubble Bioreactors

The use of a bubble bioreactor to improve the aeration efficiency resulted in relatively high production of peroxidase (2.10±0.56 U ml^{-1}; Figure 5.9) as compared to the highest activity recorded for the baffled flasks (0.53±0.1 U ml^{-1}) as well as reported in literature (0.9 U ml^{-1}; Iqbal *et al.*, 1994). Oxygen Transfer Rate (OTR) is a frequently employed parameter for quantifying the physiological state of an aerobic culture (Suresh *et al.*, 2011). Shake flask culture systems depend on agitation of the vessel (and turbulent flow due to baffles) for

oxygen transfer into the growth medium. Bubble bioreactors, on the other hand, introduce oxygen directly into the growth medium. Thus, it is possible to enhance the aeration capacity of a bubble bioreactor using spargers with appropriate pore sizes and sufficient oxygen flow rates for efficient mixing. Improved aeration facilitated by direct introduction of air into the medium resulted in improved growth of the aerobic *Streptomyces* sp. strain BSII#1 and increased peroxidase production.

5.4.5. Airlift Bioreactors

Enzyme production was highest in the airlift bioreactor (4.76 ± 0.46 U ml^{-1}; Figure 5.10) where aeration and mixing was most efficient. The design of the airlift bioreactor, which incorporates a central draft tube, enabled more efficient mixing and oxygenation of the culture in the medium. As the airlift bioreactor system does not rely on shaking or agitation effected by baffles or impellers, minimal shearing occurs. The gentler mixing and improved aeration enabled faster growth of *Streptomyces* sp. strain BSII#1, and greater enzyme production over a shorter time period: 4.76 ± 0.46 U ml^{-1} was recorded after 60 hrs of incubation in the airlift reactor, whereas 2.10 ± 0.56 U ml^{-1} was achieved after 7 days of incubation in the bubble bioreactor.

5.4.6. Stirred Tank Bioreactors

5.4.6.1. Parallel Sixfors Bioreactors

Maximum peroxidase production in the parallel Sixfors bioreactors was relatively low (0.288 ± 0.01 U ml^{-1}) compared to other reactor formats (Table 5.1). In an attempt to ensure adequate aeration of the medium, the cultures in this fermentation system were exposed to high rates of stirring (1200 rpm). Each fermentation unit was equipped with three Rushton turbines mounted on the rotor shaft. Rushton turbines are flat blade impellers that generate a high shear environment that is useful for thorough mixing of fluids.

Streptomyces sp. strain BSII#1 is a filamentous bacterium and the exposure to the high shear environment led to the disruption of the filamentous form of the microorganism, thereby disrupting its normal growth pattern. This was evidenced by the failure of the microorganism to form its characteristic turbid mass in culture. Instead, the culture medium remained thin and watery, which was accompanied by low peroxidase production.

5.4.6.2. Sartorius Biostat B Fermentor

Two experiments were carried out on the Sartorius Biostat B fermentor. In the first run, the fermentor was set to maintain a pO$_2$ of 20%, which is considered adequate for the growth of most bacteria (Syldatk, C., personal communication). However, as the microorganism proliferated, there was a greater demand on oxygen supply. To compensate for the oxygen

uptake, the stirrer speed on the bioreactor had to be manually adjusted from time to time. Stirrer speeds between 300 and 500 rpm were used. The resultant shearing stress created led to disruption of the filamentous nature of *Streptomyces* sp. strain BSII#1. The ensuing sub-optimal growth of the microorganism was reflected in the lower uptake of oxygen from the medium, which manifested as spikes in the pO_2 profile at 30.5, 42.6, 67 and 79.1h (Figure 5.13).

In the second set of Sartorius Biostat B fermentor experiments, the $pO_2$was maintained at 10%. Rotor speeds required to maintain this pO_2were lower (between 300 and 360 rpm) than for maintaining pO_2 at 20%. This meant that *Streptomyces* sp. strain BSII#1 was subjected to a lower shearing stress environment allowing for better growth of the filamentous biomass. This was reflected in the more constant pO_2 profile, indicating steadier uptake of O_2 and growth. Peroxidase production appeared to be increase more steadily in the lower shear system (10% pO_2; Figure 5.15). Maximum peroxidase production in this system was slightly higher (0.58 U ml^{-1}) than in the system where the $pO_2$was maintained at 20% (0.48 U ml^{-1}).

In summary, the greatest shearing forces were experienced in the Parallel Sixfors fermentor, where the stirrer speed was set at 1200 rpm (three Rushton turbines) in a 300 ml culture volume. Growth of *Streptomyces* sp. strain BSII#1 was poor in this system and peroxidase production was low. The least shearing forces were experienced in the 6 l Sartorius reactor with two Rushton turbines and lower stirrer speeds between 300 and 360 rpm. Growth of *Streptomyces* sp. strain BSII#1 was much improved under lower shear stress and peroxidase production was more stable.

Overall, while it was possible to achieve considerable peroxidase (0.58 U ml^{-1}) production using the stirred tank bioreactor in a large culture volume (6 l), this type of reactor is not the best format for cultivating filamentous microorganisms such as *Streptomyces* sp. strain BSII#1.In general, the low-shear environment associated with bubble and airlift reactors compared to stirred tank reactors enables the successful cultivation of shear-sensitive and filamentous organisms (Garcia-Ochoa and Gomez, 2009).

5.5.   Conclusions and Recommendations

As with most bioprocesses, a linear scale-up of peroxidase production could not be achieved and adjustment of different factors had to be effected. An incremental scale-up was successful in identifying the key factor for restoring and enhancing peroxidase production by *Streptomyces* sp. strain BSII#1 on a larger scale without the use of inducers: aeration. The highest peroxidase production by *Streptomyces* sp. strain BSII#1 (4.76±0.46 U ml^{-1} in 3 l culture volume) was achieved via the use of an airlift reactor. Since peroxidase production is

a growth-associated process in actinomycetes (Ramachandra *et al.,* 1988; Tuncer *et al.,* 1999; Niladevi and Prema, 2008), reactor formats that favoured good growth of the filamentous *Streptomyces* sp. strain BSII#1 resulted in higher peroxidase production. While aeration was shown to be a key factor in improving growth and peroxidase production, the mode of aeration is also important. Efficient aeration delivered in a low shear environment with gentle mixing (airlift reactor format) was shown to result in better and faster enzyme production. Growth of *Streptomyces* sp. strain BSII#1 (and hence production of peroxidase) was shown to be sensitive to the shearing forces experienced in a stirred tank bioreactor.

The enhanced biomass growth associated with increased peroxidase production may be problematic on larger scales in batch culture, as increased biomass growth and viscosity of the culture reduces the crude extracellular yields that could be obtained after centrifugation. However, a continuous process may be useful to minimise this problem and should be investigated. Furthermore there may be viable uses for the biomass generated such as single cell protein for feeding livestock, or as composting material. Valuable bioactive compounds such as antibiotics could also be potentially extracted.

In the next chapter, the purification and characterisation of the peroxidase from *Streptomyces* sp. strain BSII#1 is considered. Its potential for use in coupling reactions is also considered.

CHAPTER SIX

PURIFICATION, CHARACTERISATION AND BIOCATALYTIC APPLICATION OF THE PEROXIDASE FROM *Streptomyces* sp. strain BSII#1

6.1. Introduction

From a historical perspective, the peroxidases that are derived from actinomycetes have received considerably less attention compared to their plant and fungal counterparts (Gottschalk *et al.*, 2008; van Bloois *et al.*, 2010; Fodil *et al.*, 2011; Fodil *et al.*, 2012). This is reflected in the limited amount of information that is available on purification and characterisation studies of peroxidases originating from actinomycetes. The major reason for this difference has been attributed to the relatively low stability and poor catalytic activity under industrial conditions, of the majority of known actinomycete peroxidases (Fodil *et al.*, 2011). A second limitation has been the typically lower levels of peroxidase secretion by actinomycetes (generally less than 1 U ml^{-1}; Iqbal *et al.,*1994; Bon *et al.,*1999; Tuncer *et al.,*1999; Macedo *et al.,*1999; Zerbini *et al.,*1999; Antonopoulos *et al.,* 2001; Tuncer *et al.,*2009) compared to fungal peroxidase secretion levels. Indeed, the difficulty in detecting extracellular peroxidases in culture supernatant without a preliminary concentration step has been noted in some reports (Rob *et al.*, 1997; Tuncer *et al.,* 1999; Tuncer *et al.,* 2009).

From the reports that are available, the purification of peroxidases from actinomycetes has generally employed common methods such as centrifugation and filtration (Fodil *et al.*, 2011) to recover the crude enzyme from the culture medium. Other methods that have been used for further concentration and purification of peroxidase from actinomycetes include ammonium sulphate precipitation, ultrafiltration, dialysis and different forms of column chromatography (Mliki and Zimmerman, 1992; Tuncer *et al.*, 2009). The specific activity in the purified fraction of actinomycete peroxidases has generally been lower than that of purified plant peroxidases and typically less than 10 U mg^{-1} protein (Iqbal *et al.*, 1994; Fodil *et al.*, 2011; Fodil *et al.*, 2012). However, Mliki and Zimmerman (1992) reported much higher activity in the purified fraction from *Streptomyces cyaneus* (247 U mg^{-1} protein). In contrast, specific activity of purified plant peroxidases is typically in the order of thousands of U mg^{-1} protein (Cai *et al.*, 2012). Aside from low initial concentrations, it is also difficult to achieve homogenous actinomycete peroxidase preparations as multiple purification steps do not completely remove other proteins and the peroxidase is rapidly inactivated after several chromatography steps (Mliki and Zimmerman, 1992). These challenges may also help to explain the limited information that is currently available on actinomycete peroxidase purification and characterisation.

A summary of key biochemical characteristics of some actinomycete peroxidases is given in Table 6.1. Actinomycete peroxidases generally have their optimum pH within the neutral to alkaline range and this is generally reflected in Table 6.1 (with exceptions).

Table 6.1: Summary of key biochemical properties of selected actinomycete peroxidases

Organism	Enzyme description	Optimum pH	Thermostability characteristics	Reference
Streptomyces sp. strain AM2	Extracellular monomeric proteins HaP1 and HaP2 (40.3 and 25.2 kDa respectively); catalyse biodegradation of humic acids	6.0 (HaP1) 7.5 (HaP2)	Stable at 55°C for more than 24 hrs	Fodil *et al.* (2011)
Streptomyces cyaneus	Bifunctional, intracellular haem protein with catalase and peroxidase activities	5.0 (peroxidase) 8.0 (catalase)	Not specified	Mliki and Zimmerman (1992)
Streptomyces avermitilis UAH30	Non-haem peroxidase capable of removing colour from paper mill effluent	6.5-8.5	Stable at 50°C for 1 hr	Rob *et al.* (1997)
Streptomyces thermoviolaceus	Extracellular haem proteins (P3-82kDa; P5-60kDa)	6.5-7.0	P3 stable at 50°C for more than 24 hrs	Iqbal *et al.* (1994)
Streptomyces sp. strain F6616	Extracellular haem protein	9.0-10	Stable at 50°C for over 2 hrs	Tuncer *et al.* (2009)
Streptomyces sp. strain AH4	Extracellular thermostable humic acid peroxidase (HaP3) (60.2Da)	5.0	Stable at 60-90°C for 4 hrs	Fodil *et al.* (2012)

This chapter describes the purification of peroxidase from growing cultures of *Streptomyces* sp. strain BSII#1, and subsequent characterisation of the enzyme. The last part of the chapter reports the use of this peroxidase for oxidative coupling of phenolic monomers.

6.2.   Materials and Methods

6.2.1. Preparation of Crude Enzyme Extract

Spore/hyphae suspensions (500 μl) in water of *Streptomyces* sp. strain BSII#1 were used to inoculate 10 ml modified phenoxazinone medium and flasks were incubated at 30°C for two days. This served as a pre-culture for subsequent inoculation in all the experimental flasks. Inoculation of sterile modified phenoxazinone medium in different bioreactor configurations (10 ml and 100 ml culture volumes in conical and baffled flasks; 380 ml and 500 ml culture volumes in bubble column reactors and 3 l culture volume in airlift reactors) was carried out using 10% (v/v) inoculum (See Chapter 5).

Growing cultures were centrifuged at 10 000 g for 5 min at room temperature. The decanted supernatant was passed through coffee filters and the filtrate served as the crude enzyme fraction.

6.2.2. Assays and Statistical Analyses

Peroxidase activity was monitored throughout using the 2,4-DCP assay (see Section 3.2.3). Protein concentrations were measured using the Bradford's assay. Commercial Bradford's reagent (Sigma) was used and the assays were performed in microtitre format according to the manufacturer instructions. Standard curves of known protein concentrations [0-100 mg ml^{-1} bovine serum albumin (BSA)] were constructed every time the assay was used. Univariate analysis of variance (Univariate ANOVA) followed by Bonferroni post hoc tests were used to compare results and test for significance (IBM® SPSS® Statistics, Version 21). Only p-values ≤ 0.05 were considered significant.

6.2.3. Purification of Peroxidase

The steps in purification of the peroxidase from *Streptomyces* sp. strain BSII#1 were carried out at temperatures not exceeding 4°C unless otherwise specified. Different strategies were investigated for the recovery of peroxidase from crude fractions:

6.2.3.1.   Acid and Acetone Precipitation followed by Ultrafiltration

The pH of the crude enzyme was adjusted from an initial value of pH 6.8 to pH 2.3 by addition of concentrated HCl. The acid treated crude enzyme was incubated at ambient temperature (25±2°C) for 15 mins before centrifugation at 10 000 rpm for 10 mins. The pellet was resuspended in sufficient 100 mM Tris-HCl (pH 8.0) buffer for complete dissolution of the pellet ('acid fraction'). Ten volumes of ice-cold acetone was added to the acid fraction and the mixture was incubated at -20°C for at least 12 hrs before centrifugation at 10 000 rpm for 10 mins. The resultant supernatant was discarded and the pellet was resuspended in

sufficient 100 mM Tris-HCl (pH 8.0) buffer to enable complete dissolution ('acetone fraction'). The acetone fraction was filtered through Amicon® Ultracel®-30K centrifugal filters (30 000 MWCO; 7 500 g for 30 mins). The resultant filtrate was designated the 'ultrafiltrate fraction'.

6.2.3.2. Other Approaches

Different approaches, used alone and in varied combinations, were tested for effectiveness in the purification of peroxidase from *Streptomyces* sp. strain BSII#1.

a. Ammonium Sulphate Precipitation

Fractions of the crude enzyme were subjected to precipitation using a range of ammonium sulphate saturation (from 10% to 80%) by addition of appropriate amounts of the salt. Precipitates were collected by centrifugation (10 000 rpm for 10 min) and resuspended in 100 mM potassium phosphate buffer (pH 8.0).

Peroxidase activity, via the 2,4-DCP assay, and protein estimation via the Bradford method were determined for both the pellet and supernatant fractions. More ammonium sulphate (as appropriate) was slowly added to the supernatant fraction collected after 50% ammonium sulphate precipitation to increase the saturation levels to 70% and to 80%. After centrifugation (10 000 rpm for 10 min), the pellet fractions were resuspended in 100 mM potassium phosphate buffer (pH 8.0).

b. Acetone Precipitation

Sufficient volumes of ice-cold acetone were added to crude extract samples to achieve a range of final acetone concentrations (from 10 to 80% v/v). Samples were incubated at -20°C overnight to allow precipitation of the proteins. Pellets were collected after centrifugation (10 000 rpm for 10 min) and resuspended in 100 mM potassium phosphate buffer (pH 7.0).

c. Dialysis

Resuspended pellets (from ammonium sulphate precipitation) were placed in dialysis tubing (10 000 MWCO) and dialysed overnight at 4°C against excess 10 mM potassium phosphate (pH 7.0) buffer solution.

d. Size Exclusion Chromatography (SEC)

Enzyme samples (1-2 ml) were applied to Sephadex G-25 columns (5 ml bed volume) and eluted with 10 mM potassium phosphate buffer (pH 7.0). 1 ml fractions each were collected and the fractions giving the highest peroxidase activity were pooled for subsequent analyses.

e.   Polyethylene glycol (PEG) concentration

Partially purified enzyme fractions in dialysis tubing (10 000 MWCO) were placed in a beaker with crystalline PEG 80 000 at 4°C to allow osmosis of water out of the sample in order to concentrate the enzyme fraction.

f. Polyvinyl polypyrrolidone (PVPP) and Amberlite® XAD-4

Partially purified enzyme fractions were added to either PVPP or Amberlite®XAD-4 and incubated at 4°C for 12 hrs to allow for adsorption of melanin from the sample. PVPP or Amberlite® XAD-4 were removed from the sample by centrifugation (10 000 rpm for 5 min).

g.   Ion Exchange Chromatography (IEC)

Enzyme samples (1-2 ml) were applied to DEAE Sephadex A50 columns (5 ml bed volume). The columns were washed with 10 mM potassium phosphate buffer (pH 7.0) and elution was achieved with 0 to 1 M NaCl (added in step-wise increments). 1 ml fractions each were collected and the fractions with the highest peroxidase activity were pooled for subsequent analyses.

h.   Acetone-trichloroacetic acid (TCA) precipitation

Varying concentrations of acetone and TCA were added to crude enzyme samples. Proteins were allowed to precipitate at -20°C overnight and collected by centrifugation (10 000 rpm for 5 min). The pellets were resuspended in 100 mM potassium phosphate buffer (pH 8.0).

6.2.4. Storage of Purified Peroxidases

Aliquots of purified peroxidase (acid and acetone precipitation followed by ultrafiltration) were mixed with different volumes of glycerol to achieve final glycerol concentrations of between 2.5% and 25% (v/v) in 1 ml volumes in Eppendorf tubes. The tubes were divided into three sets which were incubated at ambient temperature (25±2°C), 4°C and at -20°C. Residual peroxidase activity for each glycerol concentration at each storage temperature was determined on a weekly basis for a period of six weeks.

6.2.5. Characterisation of Purified Peroxidases

All characterisation studies were conducted using peroxidase purified by acid and acetone precipitation followed by ultrafiltration.

6.2.5.1.   SDS-PAGE Analysis

20 µl samples were mixed with 5 µl 5x sample loading buffer (3.1 ml 1 M Tris-HCl [pH 8.0], 5 ml glycerol, 0.5 ml bromophenol Blue, 1.4 ml water and 5 mM dithiothreitol [DTT] or 5 mM).

Alternatively 20 µl samples were mixed with 4 µl 6x sample loading buffer (375 mM Tris-HCl pH 6.8, 6% SDS, 48% glycerol, 9% 2-Mercaptoethanol, and 0.03% bromophenol blue). The samples were boiled for 15 mins and allowed to cool. Gels and running buffers were prepared as per the manufacturer instructions for the Biorad Protean Mini-Gel system (Biorad). Samples (and PageRuler prestained protein ladder [ThermoScientific]) were loaded in lanes on a 12% resolving gel and run at 180 V until the dye front had reached the bottom of the gel. The gel was stained with PageBlue™ Coomassie stain (Fermentas). Staining was allowed to proceed on a rocking shaker overnight and excess stain was removed by washing the gel with distilled water for at least ten minutes.

6.2.5.2.   Optimum pH and Substrate Range

The substrate range of the purified peroxidase was determined (at 25±2°C) using the following substrates, each at a final concentration of 1 mM and at pH 3, 5, 7 and 9: 2,4-DCP, 2,6-dimethoxyphenol (2,6-DMP), guaiacol, resorcinol, syringaldazine, phenol, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), L-3,4-dihydroxyphenylalanine (L-DOPA), 4-*tert*-butylcatechol (4-TBC), 3-hydroxyanthranilic acid (3-HAA), 4-methylcatechol, caffeic acid, catechol, *o*-aminophenol, pyrogallol, *p*-cresol and L-tyrosine. The optimum pH for the oxidation of 2,4-DCP was determined within the pH range 3 to 10 in increments of 0.5 pH units.

6.2.5.3.   Optimum Temperature

The oxidation of 2,4-DCP by the purified peroxidase at different temperatures (from 5°C to 80°C at 5°C intervals) was monitored at 510 nm using a UV/VIS Lambda 25 spectrophotometer with a PTP-6+6 Peltier system for temperature control (PerkinElmer). The peroxidase was suspended in 100 mM Tris-HCl and in 100 mM potassium phosphate buffer, and the optimum temperature in both systems was evaluated using the 2,4-DCP assay (pH 8.0, see Section 3.2.3).

6.2.5.4.   Thermostability

The purified enzyme samples were incubated in a Corning LSE™ digital dry bath at temperatures between 30 and 80°C (10°C increments) for 10, 20, 30 and 60 min and immediately cooled on ice. The control sample was incubated on ice throughout the duration of the experiments and had no further treatments applied. The temperature stability of the peroxidase in 100 mM Tris-HCl and in 100 mM potassium phosphate buffer was evaluated. Once cool, residual peroxidase activity was measured at ambient temperature (25±2°C) using the 2,4-DCP assay (see Section 3.2.3).

6.2.5.5. pH stability

The purified peroxidase was mixed with equal amounts of each buffer (100 mM concentration) and incubated at 4°C for 24 hrs. Sodium acetate buffer (pH 3.0-5.5), potassium phosphate buffer (pH 6.0 – 7.5) and Tris-HCl buffer (pH 8.0 – 10.0) were used and all were applied at 0.5 pH unit increments. Residual peroxidase activity was measured using the 2,4-DCP assay (25±2°C, see Section 3.2.3).

6.2.5.6. Effects of Inhibitors, Reducing Agents and Metal Ions

The effect of the following compounds (0 to 50 mM) on the ability of the peroxidase to oxidise 2,4-DCP was assessed: NaCl, L-cysteine, SDS, *p*-arbutin, sodium metabisulphite, EDTA, L-ascorbic acid, hydrogen peroxide and sodium azide. The effect of the following metal ions was also determined: Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} (each at 0.1, 0.5, 1, 2 and 5 mM concentrations). The enzyme sample was incubated in the presence of the compound or metal ion tested for 1 hr before assaying for peroxidase activity (25±2°C, pH 8.0, see Section 3.2.3) and compared to the control enzyme activity where no inhibitor or metal ions were added.

6.2.5.7. Effects of Organic Solvents on Enzyme Activity

The effect of the following organic solvents on the ability of the peroxidase to oxidise 2,4-DCP was assessed: ethanol, methanol, acetone, acetonitrile, dimethylsulfoxide (DMSO) and 2-propanol. The organic solvent was added to the 2,4-DCP assay mix (at 10, 20, 30 and 40% v/v concentration) prior to running the assay (25±2°C, pH 8.0, see Section 3.2.3). The reference control did not have the organic solvent component.

6.2.5.8. Enzyme Kinetics

The kinetic parameters V_{max}, K_m and K_{cat} were determined for the oxidation of 2,4-DCP. The enzyme concentration was constant while the concentration of 2,4-DCP was varied. Assays were performed at ambient temperature (25±2°C) and pH 8.0 (see Section 3.2.3).

6.2.5.9. Spectral Characteristics

A complete wavelength scan (200 nm to 700 nm) of the purified peroxidase from *Streptomyces* sp. strain BSII#1 was acquired using a PerkinElmer UV/VIS Lambda 25 spectrophotometer. In addition, LC-MS analysis of the purified peroxidase was conducted using a Dionex outlet 3000 HPLC coupled to the micrOTOF-Q11 (Bruker).

6.2.5.10. Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS was performed on a Dionex HPLC system (Dionex Softron, Germering, Germany) equipped with a binary solvent manager and autosampler coupled to a Brucker ESI Q-TOF mass spectrometer (Bruker Daltonik GmbH, Germany). The products were separated by reversed phase chromatography on a Waters Sunfire C18 column 5 µm; 4.6 × 150 mm (Dublin Ireland) using gradient elution at a flow rate of 0.5 ml min^{-1}, an injection volume of 10 µl and an oven temperature of 40°C. The gradient was set up as follows: 98% A to 40% A (60 min); 40% A to 98% A (60 - 60.1 min); 98% A (60.1- 80 min); solvent A - 0.1% formic acid in water, solvent B - acetonitrile. MS spectra were acquired in positive mode using the full scan mode with dual spray for reference mass solution. Electrospray voltage was set to - 3500 V. Dry gas flow was set to 8 l min^{-1} with a temperature of 200°C and nebulizer gas pressure was set to 17.5 psi.

6.2.6. <u>Coupling Reactions</u>

The potential of the partially purified peroxidase for catalysis of oxidative coupling between phenolic monomers was evaluated. Reactions consisted of the enzyme (final concentration of 1 U ml^{-1}), two phenolic monomer substrates (final concentration 2 mM each), hydrogen peroxide (final concentration 0.37 mM) and 100 mM ammonium bicarbonate buffer (pH 8.0) in a total reaction volume of 650 µl. The substrates tested consisted of combinations of catechin, catechol or guaiacol with trans-cinnamic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, cinnamyl alcohol, syringaldazine or with each other (Table 6.2). The reactions were initiated upon addition of hydrogen peroxide and the tubes were incubated at 30°C for 10 mins. The reaction was terminated by the addition of 650 µl ice-cold methanol (99.8%). The tubes were incubated on ice for 30 min before centrifugation at 4°C for 10 min. The supernatant was collected from each reaction tube and stored at 4°C in glass vials prior to analysis by LC-MS.

Table 6.2 Phenolic monomer combinations used for coupling reactions with peroxidase from *Streptomyces* sp. strain BSII#1

	Substrate 1	Substrate 2
Group 1	catechin	trans-cinnamic acid
	catechin	chlorogenic acid
	catechin	*p*-coumaric acid
	catechin	ferulic acid
	catechin	cinnamyl alcohol
	catechin	guaiacol
	catechin	syringaldazine
	catechin	catechol
	catechin	caffeic acid
Group 2	catechol	trans-cinnamic acid
	catechol	chlorogenic acid
	catechol	*p*-coumaric acid
	catechol	ferulic acid
	catechol	cinnamyl alcohol
	catechol	guaiacol
	catechol	syringaldazine
	catechol	catechol
	catechol	caffeic acid
Group 3	guaiacol	trans-cinnamic acid
	guaiacol	chlorogenic acid
	guaiacol	*p*-coumaric acid
	guaiacol	ferulic acid
	guaiacol	cinnamyl alcohol
	guaiacol	syringaldazine
	guaiacol	caffeic acid

6.2.6.1.   LC-MS Analysis of coupling products

LC-MS was performed on a Dionex HPLC system (Dionex Softron, Germering, Germany) equipped with a binary solvent manager and autosampler coupled to a Bruker ESI Q-TOF mass spectrometer (Bruker Daltonik GmbH, Germany). The products were separated by reversed phase chromatography on a Thermo Fischer Scientific C18 column 5 µm; 4.6 × 150 mm (Bellefonte, USA) using gradient elution at a flow rate of 0.8 ml min^{-1}, using an injection volume of 10 µl and an oven temperature of 30°C. The gradient was set up as follows: 98% A to 0% A (20 min); 0% A to 98% A (20- 21 min); 98% A (21-25 min); solvent A - 0.1% formic acid, solvent B -acetonitrile. MS spectra were acquired in negative mode using

the full scan mode with dual spray for reference mass solution. Electrospray voltage was set to +3500 V. Dry gas flow was set to 9 l min^{-1} with a temperature of 300°C and nebulizer gas pressure was set to 35 psi.

6.3.   Results

6.3.1. Purification of Peroxidase

6.3.1.1.   Acid and Acetone Precipitation followed by Ultrafiltration

Table 6.3 shows the purification profile for the peroxidase from *Streptomyces* sp. strain BSII#1 after the crude extract was subjected to acid (HCl) and acetone precipitation followed by ultrafiltration through a 30 000 MWCO Amicon® centrifugal filter.

Table 6.3: Purification of peroxidase from *Streptomyces* sp. strain BSII#1 by acid and acetone precipitation followed by ultrafiltration

	Volume (ml)	Protein Conc (mg ml^{-1})	Total Protein (mg)	Sample Activity (U ml^{-1})	Total Activity (U)	Sp. Activity (U mg^{-1})	Yield (%)	Purification Fold
Crude Extract	280.00	0.76	213.00	0.76	213.87	1.00	100.00	1.00
Acid fraction	26.50	3.70	97.93	7.26	192.38	1.96	89.95	1.96
Acetone fraction	21.50	3.31	71.21	8.10	174.07	2.44	81.39	2.43
Ultrafiltration: residue	11.80	3.65	43.12	9.44	11.14	2.58	52.09	2.57
Ultrafiltration: filtrate	3.60	0.03	0.09	1.22	4.40	46.51	2.06	46.32

Changes in the colour of the enzyme fractions were observed at different stages of the purification. While the crude enzyme was yellow/yellow-brown in colour, the acid fraction appeared to be red to red-brown, and the ultrafiltrate fraction was pink (Figure 6.1).
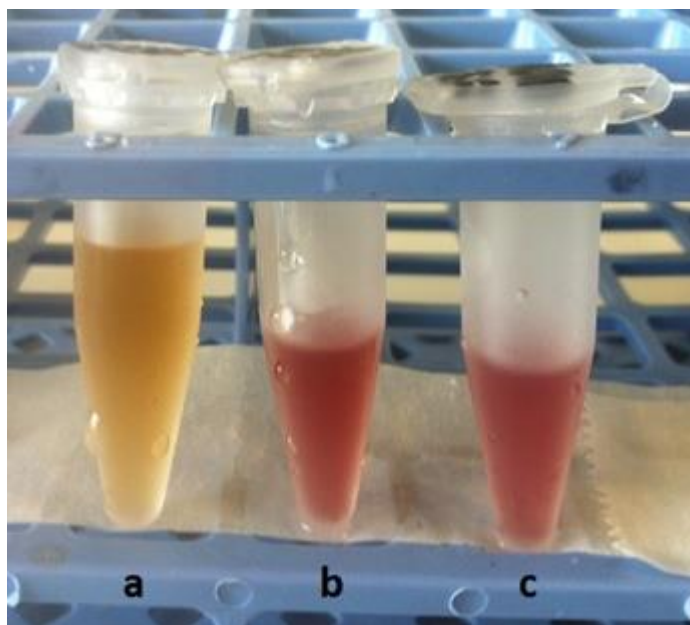
Figure 6.1: Colour changes in peroxidase fractions during the course of purification: (a) crude enzyme (1.0 U mg^{-1}), (b) acetone fraction (2.4 U mg^{-1}) and (c) ultrafiltrate fraction (46.5 U mg^{-1}).

6.3.1.2.   Other Approaches

There was an immediate loss in the peroxidase activity after each treatment step with little to no improvement in the purification fold (Table 6.4). Dialysis for desalting prior to SEC resulted in a complete loss of activity (results not shown). Attempts to concentrate the peroxidase using PEG were not successful (Table 6.4). The specific activity of acetone-TCA treated fractions decreased by 75% relative to the crude enzyme (Table 6.4). Even greater activity loss (>90%) occurred after PVPP or Amberlite® XAD-4 treatments (Table 6.4). A large decrease (>95%) in the activity of the purified fractions relative to the crude was consistently observed (from 1.65 U ml^{-1} in the crude, to 0.043 U ml^{-1} in the fraction with the highest activity) when the peroxidase was loaded directly onto a DEAE Sephadex A50 column. These treatments did not result in acceptable purification or yields (summarised in Table 6.5) and were not investigated any further.

Table 6.4 Other approaches tested for the purification of the peroxidase from *Streptomyces* sp. strain BSII#1.

Method	Specific Activity in Purified Fraction (U ml^{-1})	Yield (%)	Purification Fold
Ammonium Sulphate	0.39	13.38	0.76
Dialysis	0.79	3.42	1.54
SEC (Sephadex G-25)	0.50	8.99	1.98
PEG	0.14	1.75	0.28
PVPP	0.08	0.36	0.37
Amberlite®XAD-4	0.60	1.33	0.41
IEC (Sephadex A50)	0.045	0.52	2.17
Acetone-TCA	0.016	24.14	0.24

6.3.2. Storage of Peroxidase

The crude peroxidase extract from *Streptomyces* sp. strain BSII#1 was typically a yellow or yellow-brown liquid. Peroxidase activity (determined using the 2,4-DCP assay; see Section 3.2.3) gradually decreased to zero within several weeks upon storage at 4°C.

6.3.2.1. Stability of Purified Peroxidase in Storage

The stability of purified peroxidase in different glycerol concentrations (up to 25% v/v glycerol) at three different storage temperatures was assessed over a period of six weeks (Table 6.5). Purification of the peroxidase resulted in improved storage stability (85% activity retained after 6 weeks of storage at -20°C).

Table 6.5: Residual activity of purified peroxidase from *Streptomyces* sp. strain BSII#1 when stored over a six-week period in different concentrations of glycerol (v/v). Activity was assessed by the 2,4-DCP assay.

Storage Temperature	Optimal glycerol concentration (% v/v)	Residual activity (6 weeks)
Ambient (25±°2C)	0	22.3±0.9%
4°C	0	63.8±1.2%
-20°C	7.5-10	85.4-85.8%

6.3.3. Characterisation of Purified Peroxidase

6.3.3.1. SDS-PAGE Analysis

The estimation of the molecular mass of the purified peroxidase from *Streptomyces* sp. strain BSII#1 was done by SDS-PAGE analysis (Figure 6.2). Individual bands stained more distinctly when the samples were boiled in loading buffer containing β-mercaptoethanol rather than DTT (Figure 6.2). After acid and acetone precipitation, there were several faint bands visible and only two distinct bands (approx. 24.4 and 26.6 kDa; Lanes 5 and 7, Figure 6.2). There were no distinct bands visible in the crude extract where protein concentrations were relatively low (0.76 mg ml^{-1}, Table 6.3). The relatively harsh purification treatments used resulted in samples streaking on the gel, indicating protein degradation (Lanes 4 to 7, Figure 6.2). The ultrafiltrate fraction could not be visualised by SDS-PAGE, most probably due to the very low protein concentration (0.03 mg ml^{-1}, Table 6.3).
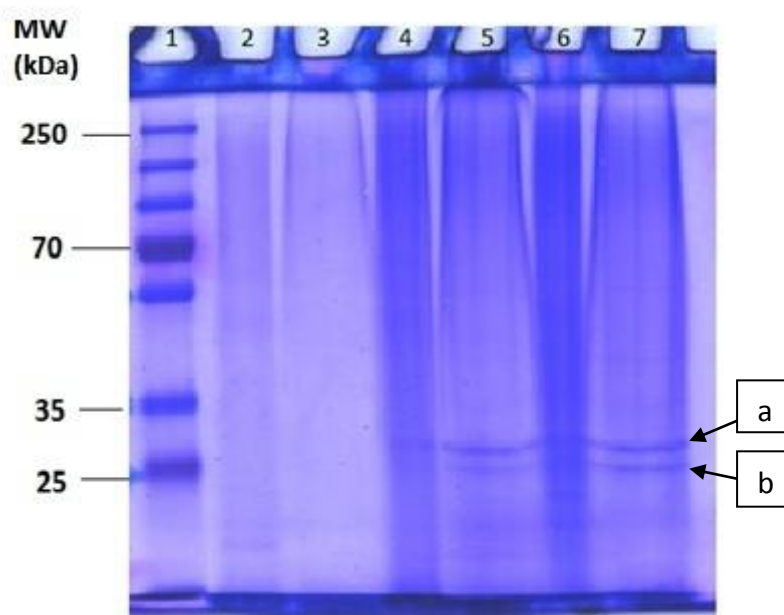
Figure 6.2: SDS-PAGE with samples from each purification step for the peroxidase from *Streptomyces* sp. strain BSII#1. Lane 1, protein size marker (10 to 250 kDa). Lanes 2 and 3, crude enzyme boiled in DTT-containing and β-mercaptoethanol-containing loading buffer, respectively. Lanes 4 and 5, acid fraction boiled in DTT-containing loading buffer and β-mercaptoethanol-containing loading buffer, respectively. Lanes 6 and 7, the acetone fraction boiled in DTT-containing and β-mercaptoethanol-containing loading buffer, respectively. The arrows point to the most prominent protein bands with sizes estimated at (a) 26.6 kDa and (b) 24.4 kDa (b).

6.3.3.2.   Optimum pH and Substrate Range

The maximum 2,4-DCP oxidation occurred at pH 8.0 (Figure 6.3). There was no significant difference in the oxidation rate from pH 5.0 to pH 8.5 ($p > 0.05$), with the exception of the anomalous reaction rate at pH 6.0.
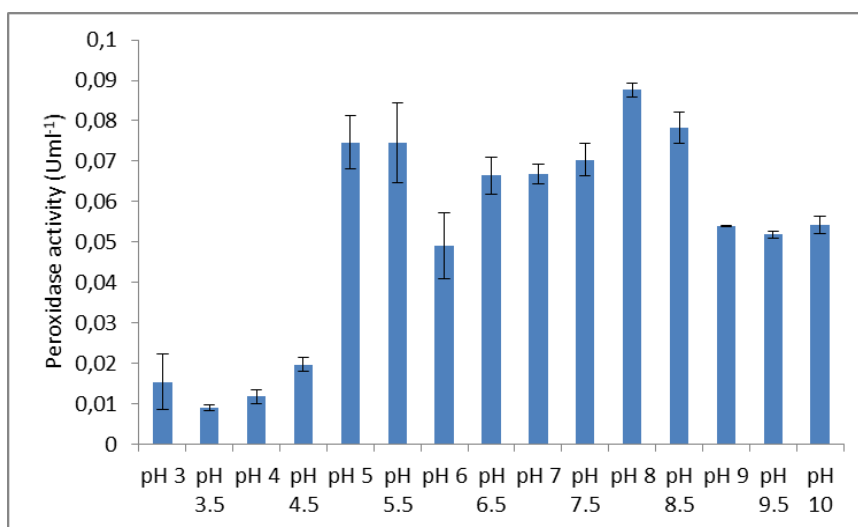
Figure 6.3: Optimum pH profile for the peroxidase from *Streptomyces* sp. strain BSII#1 with 2,4-DCP as the substrate. The enzyme was incubated on ice for 1 hour in the presence of 100 mM of the appropriate buffer before the assay was initiated.

Apart from 2,4-DCP whose activity was tested over a greater pH range, the peroxidase from *Streptomyces* sp. strain BSII#1 was capable of oxidising only one other substrate of the sixteen different substrates tested at pH values 3, 5, 7 and 9 (guaiacol; Table 6.6). Typical peroxidase substrates like ABTS and pyrogallol were not oxidised, making the enzyme potentially an atypical peroxidase.

Table 6.6 Optimum pH of oxidation and activity determined for the purified peroxidase

Substrate	Optimal pH for oxidation	Activity (U ml^{-1})
2,4- DCP	pH 8.0	0.087±0.001
Guaiacol	pH 5.0	0.052±0.0004

6.3.3.3.   Optimum temperature

The optimum temperature for peroxidase activity was tested using two buffers: Tris-HCl (pH 8.0) and potassium phosphate buffer (pH 8.0) (Figure 6.4a and Figure 6.4b, respectively). In both buffering systems, the activity was relatively unstable at temperatures higher 35°C resulting in large discrepancies in activities between replicates at the same temperature. Due to the large variance between replicates, it was not possible to statistically determine an accurate optimal temperature for activity. However, it was apparent that the enzyme was active over a wide temperature range (from 5°C to 85°C). While the most stable activity (in terms of reproducibility) occurred below 40°C, maximum activity occurred between 40° and 60°C.
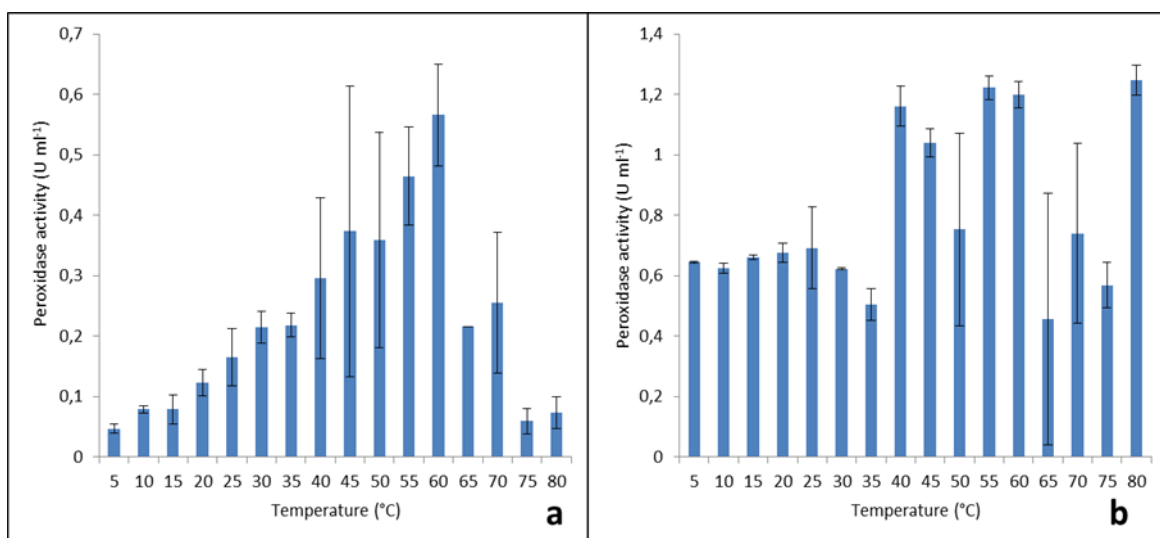
Figure 6.4: Temperature profile of activity for the peroxidase from *Streptomyces* sp. strain BSII#1 using 2,4-DCP. The enzyme was suspended in(a) 100 mm Tris-HCl buffer (pH 8.0)and(b) 100 mM potassium phosphate buffer (pH 8.0) (mean±SEM; n=2).

6.3.3.4.   Thermostability

Figure 6.5 shows the residual activity of the peroxidase from *Streptomyces* sp. strain BSII#1 after incubation in a range of temperatures for different time intervals. The peroxidase was suspended in two buffering systems for the thermostability test: 100 mM Tris-HCl buffer (pH 8.0) (Figure 6.5a) and 100 mM potassium phosphate buffer (pH 8.0) (Figure 6.5b). The control consisted of the peroxidase incubated on ice throughout the duration of the experiments.
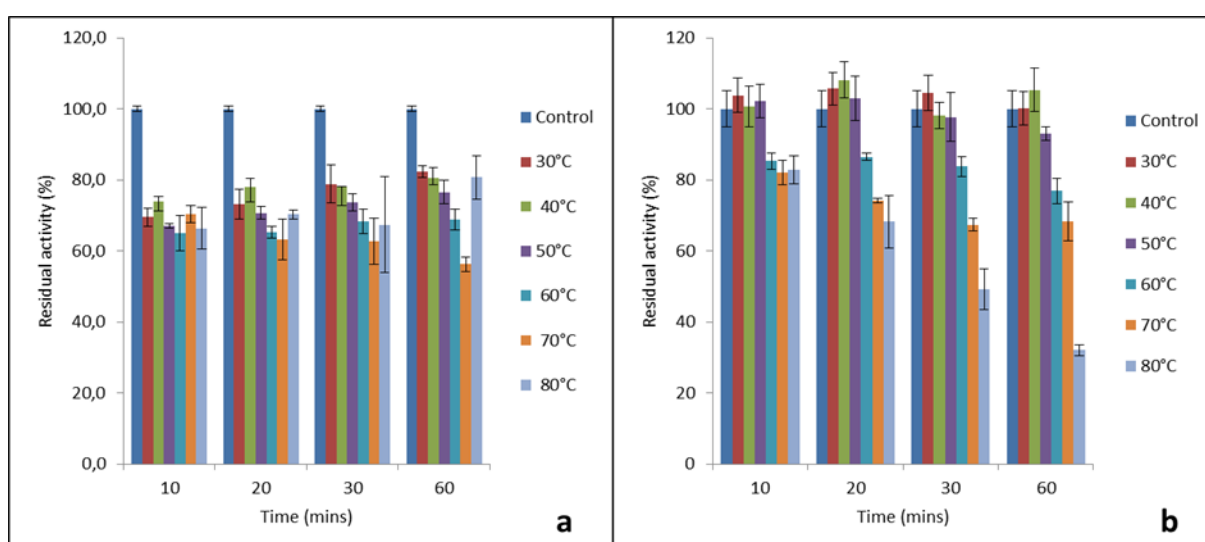
Figure 6.5 The residual activity of the peroxidase from *Streptomyces* sp. strain BSII#1 in (a) 100 mM Tris-HCl buffer (pH 8.0) and (b) 100 mM Potassium phosphate buffer (pH 8.0) after incubating in a range of temperatures for different time periods (mean±SEM; n=3). The control was kept on ice for the duration of the experiment. Activity was determined using the standard 2,4-DCP assay (at ambient temperature) after all the tubes had cooled to 25±2°C.

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When the peroxidase was suspended in 100 mM Tris-HCl buffer, there was a significant loss of activity over all the temperature tested after 10 minutes ($p<0.05$; Figure 6.6a). However, peroxidase activity stabilised after this initial loss. After the initial loss of activity, there was no further significant loss of activity ($p>0.05$) when the enzyme was incubated between 30°C and 80°C for up to 60 min.

In contrast, when the enzyme was suspended in 100 mM potassium phosphate buffer, there was no significant difference in activity between the control and all incubation temperatures after 10 mins ($p>0.05$; Figure 6.6b). After 20 and 30 min of incubation, there was no significant difference in activity between the control and samples incubated at 30, 40, 50 and 60°C ($p>0.05$), indicating that the enzyme was not affected by holding at these temperatures for up to 30 min. However there was a significant difference between the control and the tubes held at 70 and 80°C ($p<0.05$) when the enzyme was incubated for these durations (20 and 30 mins). After 60 mins of incubation, there was no significant difference between the control and enzyme incubations at 30, 40 and 50°C; however, there was a significant difference between the control and the higher incubation temperatures (i.e. 60, 70 and 80°C). There was no significant change in activity over the 60 min duration between 30 and 70°C. The only significant decrease in activity with time occurred with incubation at 80°C.

6.3.3.5.   pH stability

The residual activity of the enzyme was determined after a 24-hour incubation period in different buffers (pH 3.0 to pH 10.0; Figure 6.6). Peroxidase activity appeared to be most stable between pH 5 and 10 with maximum residual activity upon incubation at pH 9.0, where 81% activity was retained after 24 hrs. There was total loss of activity after the peroxidase was incubated at pH 3.0, 3.5 and 4.0, and >90% loss of activity after incubation at pH 4.5.
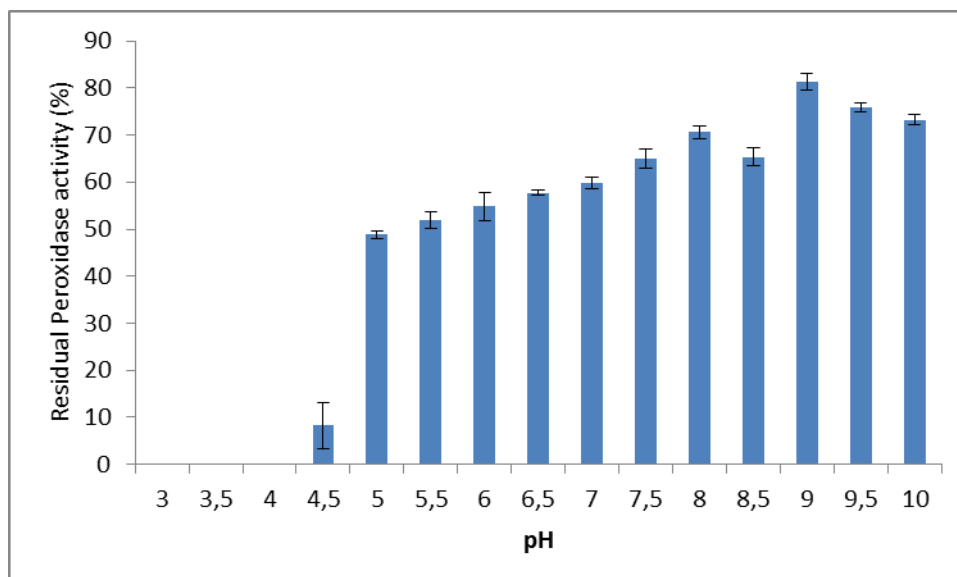
Figure 6.6 Residual peroxidase activities after incubation of the purified enzyme in different buffers at 4°C over 24 hrs. Activity was determined using the standard 2,4-DCP assay.

6.3.3.6.   Effects of Inhibitors, Reducing Agents and Metal Ions

The effect of different concentrations of inhibitors on peroxidase activity was determined after incubating the enzyme with the inhibitor for one hour at ambient temperature (25±2°C). The minimum concentration of each inhibitor that resulted in a significant inhibitory effect on peroxidase activity and the level of the resultant inhibition are presented in Table 6.7. Different compounds had different effects on the activity of the enzyme but the peroxidase was most sensitive to hydrogen peroxide, where complete inhibition ('suicide' inhibition) was observed at 0.05 mM concentration. Moreover, significant inhibition (42%) was noted at the lowest concentration tested (0.01 mM; Table 6.7). EDTA, SDS and NaCl did not have any significant effects on peroxidase activity at all concentrations tested (0.01 mM to 50 mM) under conditions of the experiment.

Table 6.7 Effect of different inhibitors on purified peroxidase of *Streptomyces* sp. strain BSII#1.

Inhibitor	Lowest inhibitor concentration resulting in significant inhibition (% inhibition)	Complete inhibition at:
H_2O_2	0.01 mM (42.0%)	0.05 mM
cysteine	0.02 mM (33.9%)	0.1 mM
Sodium ascorbate	0.05 mM (39.5%)	0.5 mM
Sodium metabisulphite	0.5 mM (93.5%)	1.0 mM
p-arbutin	1 mM (24.2 %)	Max (66.4%) at 50 mM
Sodium azide	5 mM (26.6%)	Maximum (68.4%) at 50 mM

The effects of different metal ions on peroxidase activity are indicated in Figure 6.7. Copper interfered with the assay as false positive 'peroxidase' activity was detected in the controls that contained copper ions but not the enzyme. After correction of peroxidase activity in the controls, there still was significant induction of peroxidase with copper at all concentrations tested except at 0.1 mM concentration. Inclusion of cobalt or iron resulted in significant inhibitory effects at all concentrations tested, while magnesium and zinc had no effect on peroxidase. Calcium only showed a significant inhibitory effect at 0.1 mM concentration. Manganese showed significant inhibitory effects at all concentrations tested except at 5 mM concentration.
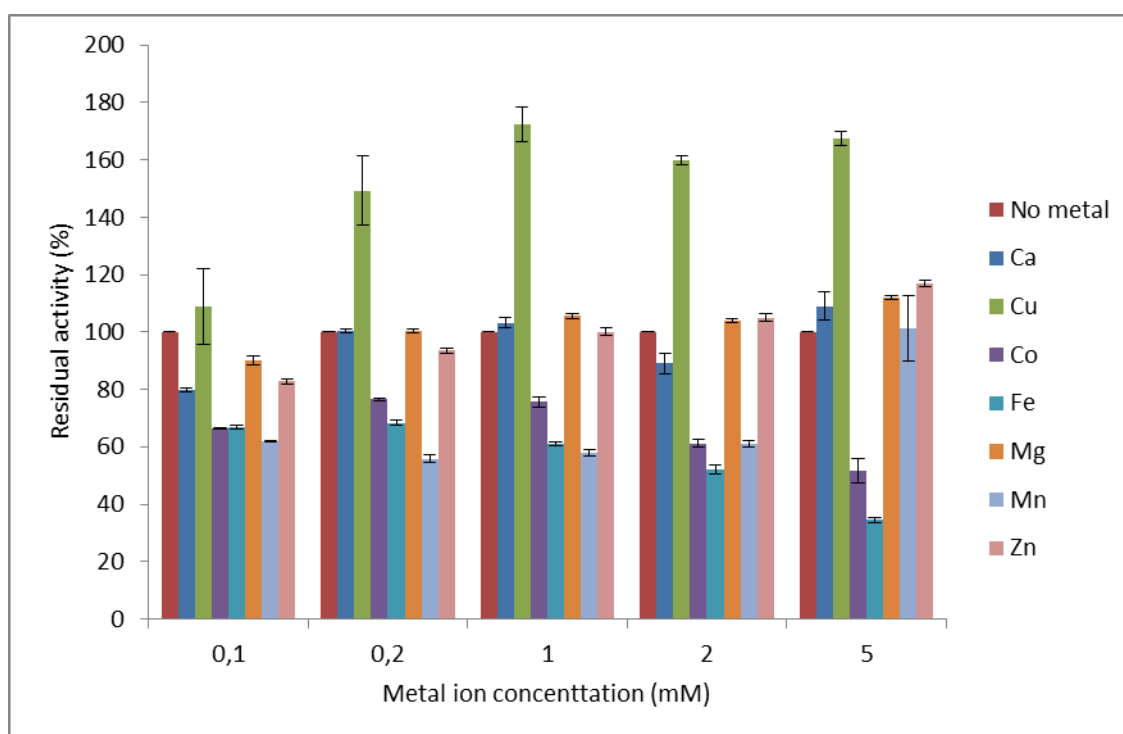
Figure 6.7: Effect of different metal ion concentrations on the residual activity of peroxidase from *Streptomyces* sp. strain BSII#1.

6.3.3.7.   Effect of Organic Solvents

The effect of different organic solvents on the oxidation of 2,4-DCP by peroxidase was assessed at 10, 20, 30 and 40% (v/v) concentration (Figure 6.8). At all concentrations of each solvent tested, there was a significant decrease in activity relative to the control.
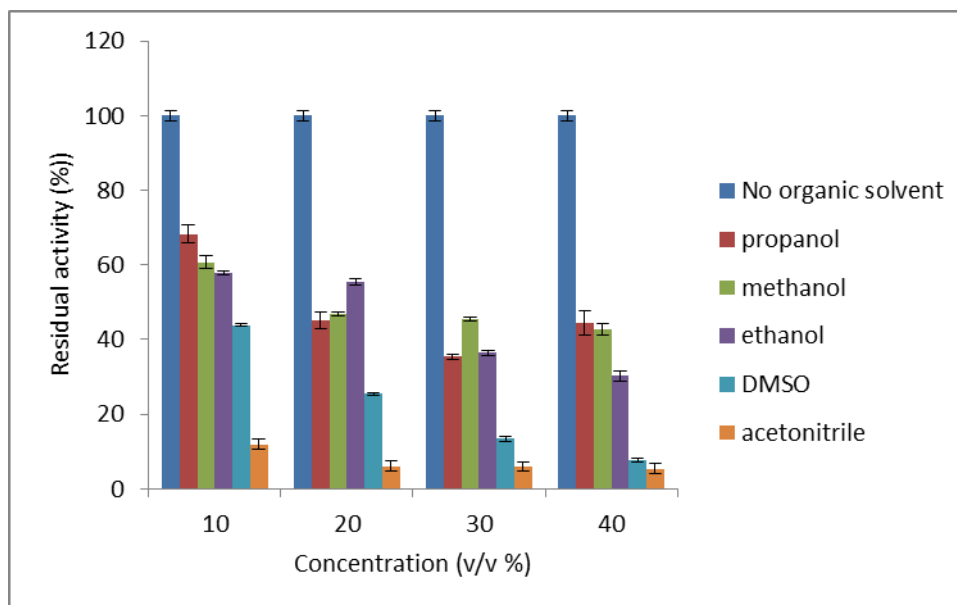
Figure 6.8 Residual activities after incubating the peroxidase from *Streptomyces* sp. strain BSII#1 with different concentrations of organic solvent.

6.3.3.8.   Enzyme Kinetics

Figure 6.9 shows the relationship between 2,4-DCP concentration and the oxidation reaction rate (velocity) at pH 8.0 using the purified peroxidase from *Streptomyces* sp. strain BSII#1. As the substrate concentration is increased above 4 mM concentration, the enzyme becomes saturated with the reaction velocity reaching the V_{max}. Therefore, this peroxidase displays typical Michaelis-Menten kinetics.

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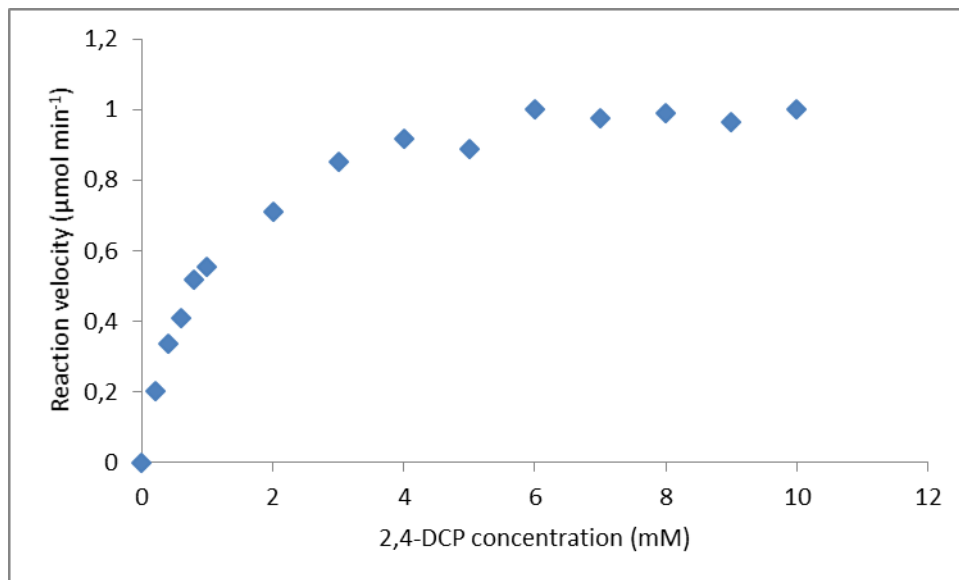
Figure 6.9 Relationship between reaction velocity and 2,4-DCP concentration catalysed by the purified peroxidase from *Streptomyces* sp. strain BSII#1 at pH 8.0, 25±2°C.

Derivative plots of the reaction velocity and substrate concentration were used to determine the kinetic parameters of the enzyme. Figure 6.10 shows the Hanes-Woolf plot, from which the K_m and the V_{max} were calculated as 0.918 mM and 1.094 µmol min^{-1}, respectively.
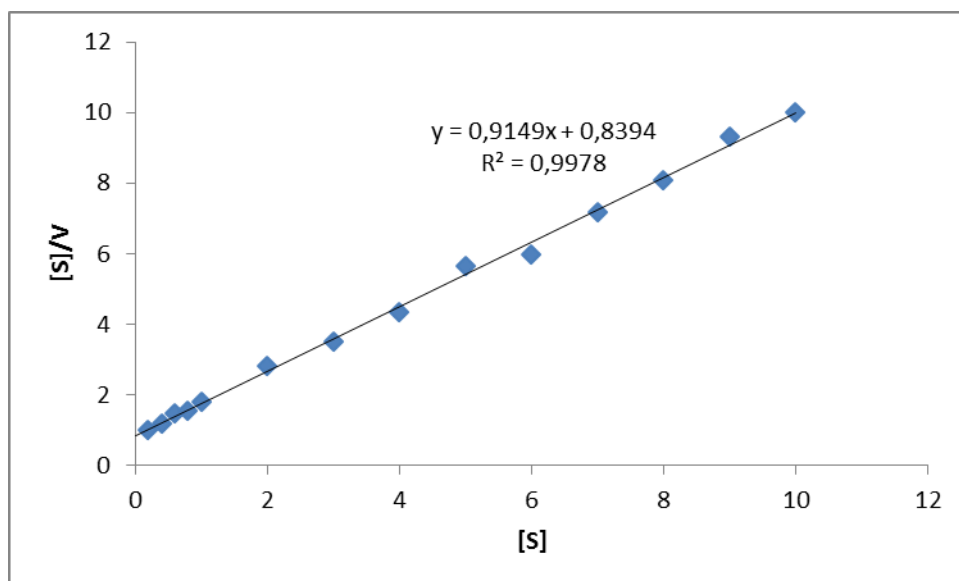
Figure 6.10 Hanes-Woolf plot for 2,4-DCP oxidation by purified peroxidase from *Streptomyces* sp. strain BSII#1. [S] and V are, respectively, the 2,4-DCP substrate concentration (mM) and the reaction velocity (µmol min^{-1}).

The Lineweaver-Burke (double reciprocal) plot is shown in Figure 6.11. From Figure 6.11, the maximum rate of reaction (V_{max}) was calculated as 1.068 µmol min^{-1}, while the K_m was 0.868 mM.
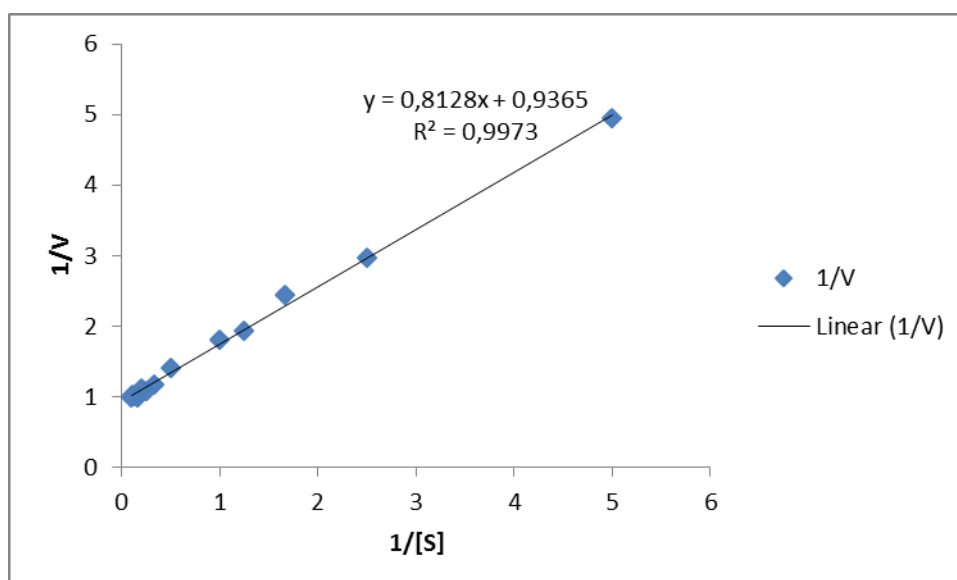
Figure 6.11 Lineweaver-Burke plot for 2,4-DCP oxidation by purified peroxidase from _Streptomyces_ sp. strain BSII#1. [S] and V are, respectively, the 2,4-DCP substrate concentration (mM) and the reaction velocity (µmol min^{-1}).

Table 6.8 below summarises the calculated kinetic parameters for the purified peroxidase from _Streptomyces_ sp. strain BSII#1 with the substrate 2,4-DCP at pH 8.0, 25±2°C. The calculated values for K_m and V_{max} from the Eadie-Hofstee plot differed from those provided by the Hanes-Woolf and the Lineweaver-Burke plots, were hence not used in the final calculations (Table 6.8).

Table 6.8: Kinetic parameters calculated for the peroxidase from _Streptomyces_ sp. strain BSII#1

PLOT	K_m (mM)	V_{max} (µmol min^{-1})
Hanes-Woolf	0.918	1.094
Lineweaver-Burke	0.868	1.068
Overall	0.893	1.081

The k_{cat} for the purified peroxidase (considering MW of 46 kDa according to LC-MS analysis, see Section 6.3.3.9) was calculated as 3.315 x 10^4 min^{-1} (or 552 s^{-1}).

6.3.3.9.   Spectral Characteristics

Figure 6.12 is a complete wavelength scan of the purified peroxidase from *Streptomyces* sp. strain BSII#1. The peak at approximately 400 nm corresponds to the Soret band. This suggests that the peroxidase contains a haem moiety in its structure and is a haem peroxidase.
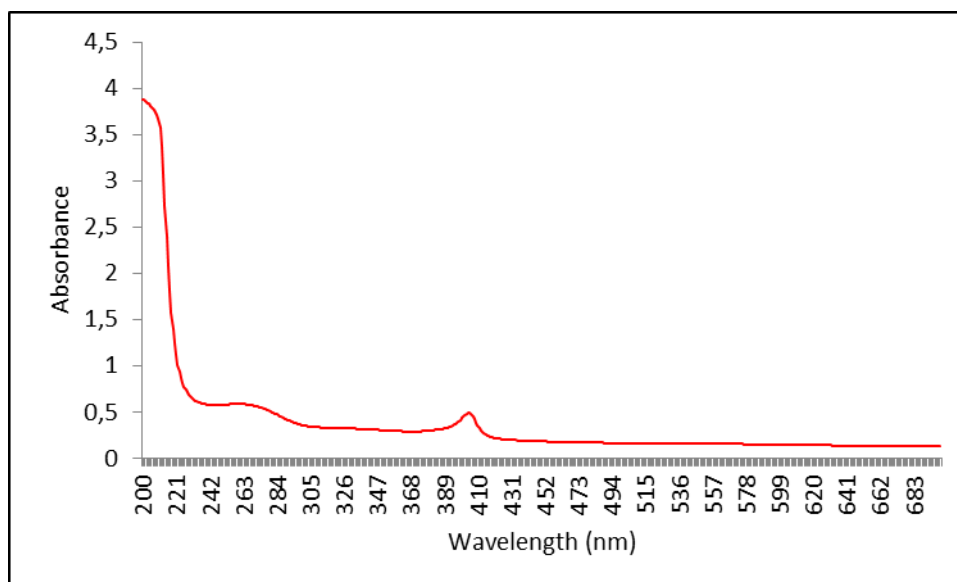
Figure 6.12 UV/VIS scan of the purified peroxidase, with a peak corresponding to the Soret band at approximately 400 nm.

Since SDS-PAGE analysis was inconclusive in determining the protein size, the purified protein was analysed by LC-MS. Figure 6.13 (a) shows the high performance liquid chromatography (HPLC) chromatogram. The single large peak (at 48 min) with only two minor peaks (32 min and 65 min) in the chromatogram suggests that the enzyme was almost completely purified. The 48 min peak (presumed to be the peroxidase of interest) was analysed by mass spectrometry. Mass spectra of the 48 min peak showed that the size of the protein was 46 kDa. However, since the protein was not purified to homogeneity, further investigations and confirmation by further analysis methods are required to verify these presumptions.
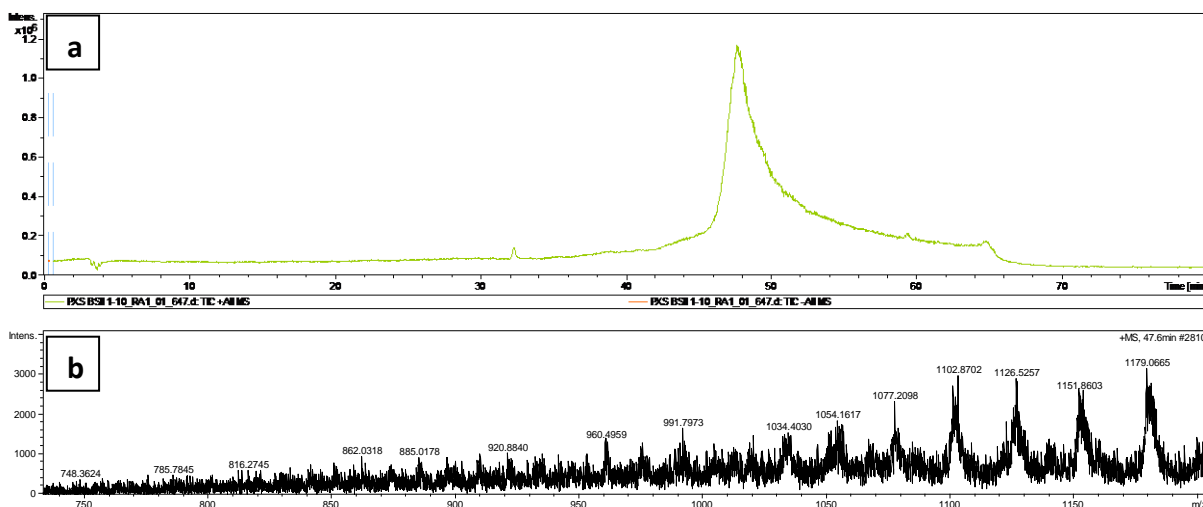
Figure 6.13 LC-MS analysis of the partially-purified peroxidase from *Streptomyces* sp. strain BSII#1. (a) HPLC chromatogram with major peak at 48 min (presumed to be the peroxidase). (b) Mass spectra of the protein (calculated size 46 kDa).

6.3.4. Coupling Reactions

The ability of the purified peroxidase from *Streptomyces* sp. strain BSII#1 to catalyse the coupling of phenolic monomers was assessed. The phenolic monomer pairs that resulted in a coupling reaction product are shown in Table 6.9. The majority of coupling reactions occurred between catechin and a second substrate. Figure 6.14 shows two coupling products that were formed in the reaction between catechin and guaiacol.

Table 6.9 Combinations of phenolic monomers that were coupled using the peroxidase from *Streptomyces* sp. strain BSII#1

Substrate 1	Substrate 2
guaiacol	chlorogenic acid
guaiacol	syringaldazine
guaiacol	caffeic acid
catechol	*p*-coumaric acid
catechol	ferulic acid
catechin	syringaldazine
catechin	*p*-coumaric acid
catechin	guaiacol
catechin	cinnamyl alcohol
catechin	caffeic acid
catechin	catechol

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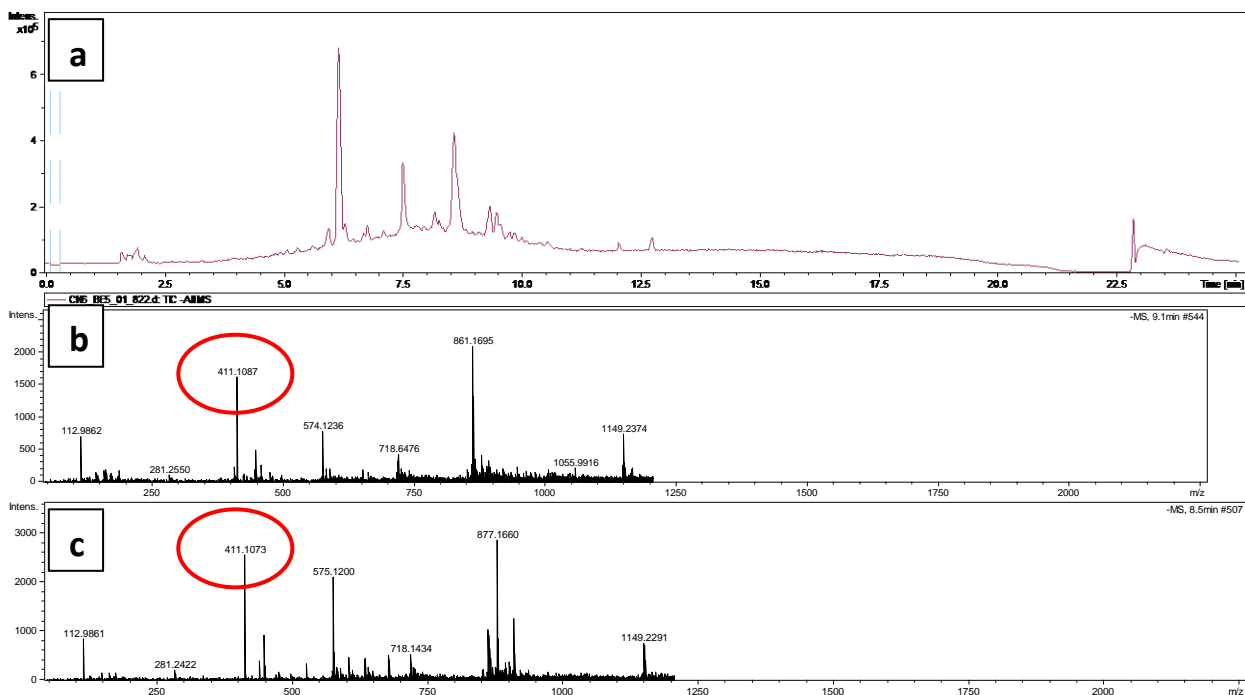
Figure 6.14 (a) LC-MS analysis of the coupling product formed between catechin and guaiacol. Total ion chromatogram showing (b) first coupling product (*m/z*= 411.1073) (c) second coupling product (*m/z*=411.1087)

6.4. Discussion

6.4.1. Production and Peroxidase and Preparation of Crude Enzyme

Peroxidase from *Streptomyces* sp. strain BSII#1 was produced and the crude extracts prepared as described in Chapters 3 and 5.

6.4.2. Purification of Peroxidase

6.4.2.1. Acid and Acetone Precipitation followed by Ultrafiltration

The first step used for the successful purification of the peroxidase (Section 6.2.3.1) was an acidification step via HCl addition. A red to reddish-brown precipitate was formed at this stage and acidification resulted in doubling the purity index with only a 10% drop in the yield (Table 6.3). Although acidification has not been used before to precipitate peroxidases from actinomycetes at any stage during the purification procedure, this study showed that it was an effective initial step for removing other unwanted proteins. The ultrafiltration step proved to be effective, resulting in nearly 20 times improvement in the purity of the acetone fraction. The red colour in the ultrafiltrate fraction could be indicative of the association of the enzyme with a chromogenic cofactor (van Bloois *et al.*, 2010; Fodil *et al.*, 2012) and most likely the haem component.

The advantage associated with the protocol used in this study is that it is readily amenable for application on a large-scale. Although it must be stated that the greatest drawback is the

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large volume of acetone required; this may make the process costly. For applications that may require crude enzyme, but with high activity (such as the removal of toxic chemicals from the environment or removal of dyes from waste streams), the acidification step alone may suffice as it concentrates the peroxidase very well with minimal activity loss (Table 6.3).

6.4.2.2.   Other Approaches

Attempts to purify the peroxidase using other traditional purification approaches had limited success. Ammonium sulphate precipitation resulted in a large loss of activity (13.3% yield; Table 6.4), and activity was further lost with different desalting techniques (dialysis and SEC). Dialysis against phosphate buffer resulted in a purification factor of only 1.5, and there was a large decrease in the yield (3.4% yield; Table 6.4). SEC performed before the dialysis step resulted in no net improvement in the purification fold (1.98; Table 6.4).

Peroxidase purification based on chromatography techniques resulted in significant loss of activity (typically over 90%) from the initial value; while there was an overall purification in some instances (for example with the DEAE Sephadex A50 column (IEC), the final activity in the purified fraction was too low for any envisaged applications. The purification on DEAE Sephadex A50 column showed purity indices between 2 and 5 in certain fractions. However, in addition to the low activities (less than 0.045 U ml^{-1}), yields were also very low (less than 1%) indicating the inefficiency of this technique for the enzyme of interest.

The peroxidase adsorbed onto Amberlite® XAD-4 and PVPP and did not elute under conditions tested. There were also high losses of activity after PEG concentration and acetone-TCA precipitation (Table 6.4).Purification of the enzyme fraction was not possible by these means. Overall, acidification and acetone precipitation followed by ultrafiltration was considered the best option for purification of the peroxidase from *Streptomyces* sp. strain BSII#1.

6.4.3. Storage of Peroxidase

The crude peroxidase extract was typically a yellow or yellow-brown colour. The peroxidase activity of the crude enzyme gradually decreased to zero within several days upon storage at 4°C. However, storage of the purified peroxidase in 10% glycerol solution at -20°C preserved over 85% of the activity after 6 weeks of storage (Table 6.5). This means that the enzyme can be stored with good retention of enzyme activity under these conditions. It is likely that freeze-drying the peroxidase may further improve stability in storage over longer time periods due to the drastic reduction in the water activity and should be investigated in future. Overall, the removal of contaminating proteins through a purification process improved the storage characteristics of the enzyme.

6.4.4. Characterisation of Purified Peroxidase

6.4.4.1. SDS-PAGE Analysis

The use of the denaturant β-mercaptoethanol rather than DTT resulted in the visualisation of more distinct protein bands after staining. There was streaking of the proteins across the gel indicating degraded proteins; this was attributed to the extreme pH that was used during purification. The 26.6 kDa and 24.4 kDa bands were the predominant bands identified and were presumed (due to the increased specific activity as purification proceeded), but not shown, to be peroxidase bands. No bands stained with the substrate 2,4-DCP in either native or SDS-PAGE gel analysis. In addition, the two bands could represent different monomeric units of the peroxidase. Further analysis, as by protein sequencing, will be required to validate this information.

6.4.4.2. Optimum pH and Substrate Range

Maximum 2,4-DCP oxidation occurred at pH 8.0 (Figure 6.3) but the optimal pH range was from pH 5.0 to pH 8.0 since statistical analysis showed no significant differences in activities within this range (with the exception of pH 6.0, which was taken to be an anomaly). Other studies on the pH for activity of actinomycete peroxidases indicate that the optimal pH is usually in the alkaline range (Table 6.1).

Of the substrates tested, the peroxidase was capable of oxidising only guaiacol and 2,4-DCP. This finding differs from the majority of actinomycete peroxidases whose substrate ranges have generally been reported to be quite broad. For example, the peroxidase from *Streptomyces albus* ATCC 3005 showed activity against L-DOPA, 2,4-DCP and other chlorophenols including 4-chlorophenol, 2,6-dichlorophenol, 2,4,5-trichlorophenol and 2,4,6-trichlorophenol (Antonopoulos *et al.*, 2001).

The very narrow range of substrates oxidised by the peroxidase from *Streptomyces* sp. strain BSII#1 may indicate a specialised function performed by the peroxidase in the natural environment and/or that this is an atypical peroxidase; however, further investigation is required in order to draw firm conclusions.

6.4.4.3. Optimum Temperature

Two buffer systems were used in the investigation of the optimum temperature for peroxidase activity: Tris-HCl (pH 8.0) and potassium phosphate buffer (pH 8.0) (Figure 6.4a and Figure 6.4b, respectively). In both buffering systems, there was large variance between replicates at higher temperatures, particularly between 40 and 60°C. The variance could be due to instability of the enzyme or of the reaction components. Since the peroxidase

appeared to be stable at temperatures above 40°C (Figure 6.4a), the variability was most probably due to non-specific reactions between assay components at the elevated temperatures.

The concept of 'optimum temperature of enzymes' has been challenged as one of dubious validity and limited value because the apparent optimum temperature arises from an unknown mix of thermal stability and temperature coefficient, and is dependent upon assay duration (Daniel *et al.*, 2001). It has been described as a non-intrinsic parameter whose dependence on a variety of other factors may not make it a very useful reference point. In this study, the instability of the assay mix at elevated temperatures illustrated this point.

6.4.4.4.   Thermostability

Statistical analyses of the results presented in Figure 6.5a indicated that while there was a similar significant decrease in activity (20-30%) of the enzyme suspended in Tris-HCl (pH 8.0) after 10 min incubation at all temperatures tested ($p < 0.05$), there was no further loss of activity with up to 60 min incubation at temperatures between 30 and 80°C ($p > 0.05$). The enzyme suspended in potassium phosphate buffer (pH 8.0) showed no significant change in activity after incubation at temperatures between 30 and 50°C for up to 60 min, although there was an initial significant drop in Tris-HCl which however stabilised subsequently. There were significant differences between activity in the control and activity after incubation at 60, 70 and 80°C for 60 min. The analyses indicate that the enzyme is fairly stable at high temperatures and that the suspension buffer plays a role in the thermostability.

The inference that can be drawn from these results is that for higher temperature applications (greater than 50°C), suspending the peroxidase in Tris–HCl buffer (pH 8.0) may be preferable over suspending in potassium phosphate buffer (pH 8.0) as this enhanced the thermostability of this enzyme.

6.4.4.5.   pH stability

The residual activity of the enzyme was determined after a 24-hour incubation period in different buffers (pH 3.0 to pH 10.0). The peroxidase appeared to be most stable between pH 5 and 10 with maximum residual activity upon incubation at pH 9.0, where 81% activity was retained after 24 hrs. There was total loss of activity after the peroxidase was incubated at pH 3.0, 3.5 and 4.0 and >90% loss of activity after incubation at pH 4.5. These results are in agreement with the general finding that actinomycete peroxidases tend to be more stable at alkaline pH conditions (Tuncer *et al.*, 2009).

6.4.4.6.   Effects of Inhibitors, Reducing Agents and Metal Ions

The peroxidase from *Streptomyces* sp. strain BSII#1 showed greatest sensitivity to the presence of hydrogen peroxide. Significant inhibition occurred at the lowest concentration tested (Table 6.7). Hydrogen peroxide is a cofactor for peroxidases, but it inhibits the enzyme at higher concentrations (suicide inactivation phenomenon, Section 2.2.7). Under the experimental conditions, the purified peroxidase was exposed to a range of hydrogen peroxide concentrations for 60 mins. The duration of this exposure is most likely the reason for significant inhibition even at very low concentrations. Evidence for this is that the activity of the enzyme has been routinely assayed at final concentrations of hydrogen peroxide of 10 mM (1×10^3 times fold the minimum inhibitory concentration) without inhibitory effects being observed. In future, this could mean that continuous rather than batch addition of hydrogen peroxide would be preferable for long-running reaction processes.

Sodium metabisulphite, L-cysteine, sodium ascorbate and *p*-arbutin can all act as reducing agents and may be grouped as preservatives with antioxidant properties. These reagents were expected to react with groups in the protein structure and result in either activation (due to protection of catalytic groups) or inhibition (due to blockage of active sites) of peroxidase activity. All four compounds resulted in inhibition of peroxidase activity, with complete inhibition occurring after 60 min of incubation with 0.1 mM cysteine. The other compounds showed lower degrees of inhibition compared to cysteine, which could be explained in terms of its strong reduction potential. Inhibition studies using these compounds with actinomycete peroxidases have not been reported, therefore comparisons for validation cannot be made.

From the absorption spectrum that showed the characteristic peak of the Soret band, it was thought that the peroxidase of interest was a haem peroxidase (Figure 6.12). Most haem peroxidases are readily inactivated by sodium azide (Ramachandra *et al.*, 1988; Rob *et al.*, 1997; Mliki and Zimmerman, 1992; Jeong-Ho *et al.*, 2002; Fodil *et al.*, 2011; Fodil *et al.*, 2012); however, the enzyme of interest in this study showed considerable resistance against inhibition by sodium azide, with no significant loss of activity in the presence of up to 2 mM sodium azide and retaining 32% activity after incubation with 50 mM sodium azide for 60 min. In a similar experiment performed under similar conditions, Fodil *et al.* (2012) showed that the peroxidase from *Streptomyces* sp. strain AH4 retained 40% of its activity after incubation with 5 mM sodium azide for 60 min. Thus the peroxidase from *Streptomyces* sp. strain BSII#1 retained almost the same level of activity but in ten-fold greater concentration of sodium azide. Jeong-Ho *et al.* (2002) showed 90% inhibition of the peroxidase from *Streptomyces* sp. AD001 after 30 mins exposure to 1 mM sodium azide at 4°C. These results further indicate the atypical properties of the peroxidase from *Streptomyces* sp. strain BSII#1.

Some metal ions have been shown to pose similar effects on the peroxidases from different actinomycetes. Fodil *et al.* (2011) and Fodil *et al.* (2012) reported on the inhibition of the peroxidases from *Streptomyces* sp. strain AM2 and *Streptomyces* sp. strain AH4, respectively. The authors reported that both peroxidase' activity were inhibited to different extents by the presence of 5 mM zinc ions, but induced in the presence of 5 mM calcium, manganese or copper ions. In this study, most of the metal ions tested showed an inhibitory action on the activity of the peroxidase (Figure 6.7), except for copper, which enhanced activity. Similarly, Fodil *et al.* (2012) showed induction of peroxidase activity by copper ions, where they reported a residual activity of 111% after 60 min incubation in the presence of 5 mM $CuCl_2$. In contrast, however, zinc, calcium and manganese ions had no significant effect on activity of the peroxidase from *Streptomyces* sp. strain BSII#1. Therefore, the effect of a single type of metal ion on peroxidase activity does not seem to be typical for all actinomycete-derived peroxidases and could be dependent on the source organism. Alternatively, the atypical characteristics of the peroxidase of interest extend to the effect of metal ions.

The ability of a peroxidase to exhibit high activity in the presence of metal ions is considered a useful attribute when considering its potential for industrial application (Fodil *et al.*, 2011). The potential of the peroxidase from *Streptomyces* sp. strain BSII#1for use in applications that require metal ion tolerance is therefore dependent on the identity and concentration of the particular metal ion.

6.4.4.7.   Effect of Organic Solvents

A few natural enzymes which are stable in the presence of organic solvents have been discovered but the majority of natural enzymes are easily denatured and inactivated in the presence of organic solvents (Ogino and Ishikawa, 2001). In this regard, the peroxidase of interest is quite typical: the presence of all of the organic solvents tested resulted in an inhibitory effect, even at low concentrations (10%) (Figure 6.8). The simple alcohols showed the least inhibition of peroxidase activity, while the presence of acetonitrile resulted in the greatest inhibition at all the concentrations tested (Figure 6.8). The exposure of enzymes to organic solvents affects the three-dimensional conformation of the enzyme at the active site. Optimisation of the stability of catalytic groups in an organic solvent system leads to enhanced activity of the enzyme, while destabilisation of this stability results in an inhibitory effect on the enzyme. Enzymes are affected in different ways by different organic solvents and optimisation of their stability in organic solvents improves catalytic performance. This peroxidase would require some protein modification in order to be used in an application that required activity in an organic solvent medium. Equivalent data for comparing K_{cat} values

among the actinomycete peroxidases was quite limited, but however the K_{cat} value for *Streptomyces* sp. strain BSII#1 (3.358×10^4 min^{-1}) was slightly lower than that of *P. chrysosporium* (3.52×10^4 min^{-1}; Ryu *et al.*, 2008) indicating comparable catalytic efficiency.

6.4.4.8.   Enzyme Kinetics

Comparison of the kinetic properties of the peroxidase under current study with that of other actinomycete peroxidases reported (Table 6.10) indicates that the peroxidase from *Streptomyces* sp. strain BSII shares a similar affinity for 2,4-DCP with HRP; but a higher affinity for 2,4-DCP (lower K_m) compared to other *Streptomyces* spp. reported (with the exception of ALiP-P3). The peroxidase from the fungus *P. chrysosporium,* has the highest affinity for the substrate. The peroxidase from *Streptomyces* sp. strain BSII also shows much higher reaction rates (V_{max} greater than 10^4 orders higher) than the peroxidase from *Streptomyces viridosporus* T7A and *Streptomyces* sp. AD 001.

Table 6.10 Comparison of the kinetic constants of the peroxidase from *Streptomyces* sp. BSII#1 with other actinomycetes, HRP and *P. chrysosporium*

	K_m	V_{max}	Reference
Streptomyces sp. BSII#1	0.893 mM	1.081 µmol min^{-1} (or 5.765×10^6 nmol/mg protein.min)	This study
Streptomyces avermitilis UAH30	1.45 mM	Not determined	Rob *et al.* (1997)
Streptomyces sp. F6616	1.52 mM	Not determined	Tuncer *et al.* (2009)
ALiP-P3 (*Streptomyces viridosporus*T7A)	0.372 mM	465.8 nmol/mg protein.min	Yee and Wood (1997)
Streptomyces sp. AD 001	1.7 mM	529.7 nmol/mg protein.min	Jeong-Ho *et al.* (2002)
HRP	0.84 mM	Not determined	Laurenti *et al.* (2003)
P. chrysosporium	0.79 µM	Not determined	Ryu *et al.*, 2008

The low Km (high affinity; comparable to HRP) and the high V_{max} with the substituted phenol substrate indicates the potential this enzyme has for industrial application since efficiency is an important consideration in developing biocatalytic processes.

6.4.4.9.   Spectral Characteristics

The absorption maximum at 400 nm is characteristic of the haem peroxidases and the characteristic peak (Soret band) at this wavelength is attributed to the presence of a haem

moiety within the enzyme structure. The peroxidase from *Streptomyces* sp. strain BSII#1 is believed to be a haem peroxidase (Figure 6.12).

The single major peak in the HPLC chromatogram revealed that the purification was effective in isolating one major product (Figure 6.13). Analysis of this product peak (presumed to be the peroxidase of interest) with LC-MS revealed that it was a protein of 46 kDa, but confirmation with another method is required to validate the initial finding.

6.4.5. Coupling Reactions

The ability of the peroxidase from *Streptomyces* sp. BSII#1 to catalyse coupling reactions between pairs of phenolic monomers was tested. The experiment was an initial survey step to consider the potential of the peroxidase for use in biocatalytic reactions. The results indicated that the peroxidase was able to catalyse coupling of a number of different pairs of phenolic monomers (Table 6.9) resulting in the formation of dimers. The most prominent coupling peaks were formed between catechin and guaiacol (Figure 6.14), and also between catechin and catechol. The remainder of the coupling peaks (Appendix IV) were quite small, indicating that only small yields of coupling product were formed in each case.

Several factors affect the successful coupling of phenolic monomers, as catalysed by an enzyme. One of these factors is steric hindrance (Garcia-Ubasart *et al.*, 2013). Catechol and guaiacol are small molecules with a small number of substituents (two). This reduces steric hindrance and increases chances of coupling. Analysis of the structures of the monomers in Table 6.9 shows that the majority of coupling reactions took place between catechin and a second substrate. This is probably due to the fact that upon oxidation catechin does not readily polymerise, which increases the chances of catechin radicals coupling with other molecules.

Environmental factors within the system (including temperature, pH, medium components, enzyme inhibitors and cofactors) also influence coupling reactions. The coupling reactions were performed in an aqueous ammonium/bicarbonate buffer solution and the enzyme was freely suspended in solution. Optimisation of these factors may significantly increase the yield of the coupling product. Optimisation may take the form of using an appropriate organic solvent for catalysis rather than in an aqueous buffer system. However, this was not an immediate goal of the current study.

6.5.   Conclusions

A protocol for the successful purification of the peroxidase from *Streptomyces* sp. strain BSII#1 involving acidification, acetone fractionation and ultrafiltration steps, was developed. The purified peroxidase size was 46 kDa and is believed to be a haem peroxidase. The enzyme is active over a wide range of pH and temperature. It was capable of catalysing coupling reactions between several phenolic monomer pairs and displayed characteristics atypical of those described for other actinomycete peroxidases.

CHAPTER SEVEN
CONCLUSIONS AND RECOMMENDATIONS

7.1.   Conclusions

Peroxidases are an important class of oxidative enzymes whose potential has not been widely applied in synthetic or biodegradative processes, mainly due to the cost of these applications. Relative to other oxidative enzymes available on the commercial market, peroxidases are relatively expensive and their sources are limited. Actinomycetes are a well-known source of extracellular peroxidases, but have not been thoroughly investigated as a source that can supply this enzyme in sufficient quantities for cost-effective field applications. Furthermore, the properties of peroxidases from actinomycetes have not been as extensively examined as their plant and fungal counterparts.

The production of peroxidases from selected actinomycetes was investigated in this study and found that actinomycetes differ in their capacity to secrete extracellular peroxidase. They also differ in their response to optimisation of environmental conditions for increased peroxidase production. *Streptomyces* sp. strain MV32 and *Streptomyces* sp. strain MS56 were found to be unstable producers of peroxidase and did not respond positively to optimisation. *Streptomyces* sp. strain MID27 consistently yielded relatively low levels of peroxidase. While *Streptomyces* sp. strain GSIII could be induced to produce higher levels of peroxidase, *Streptomyces* sp. strain BSII#1 showed the best induction response with veratryl alcohol, pyrogallol or 1.5% w/v wheat bran added to the medium in small scale culture volumes (10ml). The optimal temperature and pH for peroxidase production at small scale (10 ml culture volume) was 37°C and pH 8.0, respectively, for both *Streptomyces* sp. strain BSII#1 and *Streptomyces* sp. strain GSIII.

Streptomyces sp. strain BSII#1 was chosen for further investigation. This organism exhibited the expected basic genotypic, phenotypic and chemotaxonomic traits characteristic of members of the *Streptomyces* genus. Its closest relative in phylogenetic terms was found to be *Streptomyces albidoflavus.*

Aeration was identified as the key factor necessary to significantly improve peroxidase yield at higher scales of production in the absence of inducers. The use of baffled flasks showed significantly higher (up to ten times) peroxidase production than flat-bottomed Erlenmeyer flasks. The use of aerated bioreactors resulted in further improvement in the production of peroxidase. Peroxidase production was up to four-fold great in bubble bioreactors compared to baffled flasks. However, the best peroxidase production was achieved in an airlift

bioreactor (4.76±0.46 U ml^{-1}) which, to the author's knowledge, is the highest reported peroxidase production by an actinomycete.

In addition, the mode of aeration has an effect on peroxidase production. Peroxidase production by actinomycetes is growth-associated (Tuncer *et al.*, 2009). Stirred tank bioreactors are associated with high shear forces and are not conducive to the growth of filamentous organisms. The high shear forces in the stirred tank bioreactors tested hindered the growth of the filamentous *Streptomyces* sp. strain BSII#1 and resulted in poorer peroxidase production.

A new purification regime was demonstrated to be useful for the purification of the peroxidase from *Streptomyces* sp. strain BSII#1. The protocol involves the following sequential steps: acidification, acetone precipitation and ultrafiltration. The protocol is simple and amenable for purification of larger volumes of peroxidase.

The purified peroxidase was a 46 kDa haem protein that showed atypical features from other actinomycete peroxidases that have been described. Unlike typical peroxidases, the purified peroxidase had a narrower spectrum of target substrates, but showed considerable resistance to inhibition by sodium azide. Characterisation of the peroxidase from *Streptomyces* sp. strain BSII#1 showed that it had a narrower substrate range than other actinomycete peroxidases and could oxidise only 2,4-DCP and guaiacol. The purified peroxidase had an optimum pH between pH 5.0 and 8.0 and was most stable between pH 5.0 and 10.0. The optimum temperature could not be accurately determined due to the large variance within readings, particularly between 40°C and 60°C, which was thought to be due to instability of the 2,4-DCP assay components at higher temperature. The thermostability results indicated that the enzyme is fairly stable at high temperatures (up to 80°C) and that the suspension buffer plays a role in the thermostability of the enzyme. It was inactivated by the metal ions and organic solvents that were tested and was inhibited by low (0.01 mM) concentrations of hydrogen peroxide. The peroxidase was shown to be capable of catalysing coupling reactions between several phenolic monomer substrates.

7.2. Recommendations

This study has demonstrated the feasibility of peroxidase production from an actinomycete (*Streptomyces* sp. strain BSII#1) on a larger scale. The peroxidase was purified and its successful application in coupling of phenolic monomers demonstrated. Recommendations for future work include:

- Further field applications in biocatalysis and environmental bioremediation.

- Further examination of the characteristics of this atypical enzyme, e.g. expanding the range of substrates and environmental parameters tested.
- DNA-DNA hybridisation of the genome from *Streptomyces* sp. strain BSII#1 with its closest known relatives in order to determine whether this is a new species.

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Appendix I- Standard Characterisation media

International *Streptomyces* Project (ISP) Media
* NOTE: Always incubate plates with an uninoculated control plate.

ISP Medium Number 1 (Tryptone-Yeast extract Broth)
Tryptone   5.0 g
Yeast extract   3.0 g
H_2O to: 1 litre
Adjust pH to 7.0. Autoclave at 15 psi for 15-20 minutes.

ISP Medium Number 2 (Yeast Extract -Malt Extract Agar)
Yeast extract   4.0 g
Malt extract   10.0 g
Glucose   4.0 g
H_2O to   1 litre
Adjust pH to 7.3. *Add 20 g agar. Autoclave at 15 psi for 15-20 minutes.

ISP Medium Number 4 (Inorganic salts-starch agar)
Solution I:
10.0 g of soluble starch (BDH potato starch). Make a paste of the starch using a small amount of water and then adjust the volume to 500 ml.

Solution II:
K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	1.0 g
NaCl	1.0 g
$(NH_4)_2SO_4$	2.0 g
$CaCO_3$	2.0 g
*Trace salts solution	1.0 ml
H_2O to:	500 ml

The pH of Solution II should be between 7.0 and 7.4. Do not adjust the pH if it is within this range. Mix the starch suspension and the salts solution. Add 20.0 g of agar. Autoclave at 15 psi for 15-20 minutes. Cool, pour plates.

* Trace salts solution (Filter sterilize)
$FeSO_4.7H_2O$	0.1 g
$MnCl_2.4H_2O$	0.1 g
$ZnSO_4.7H_2O$	0.1 g
H_2O to:	100 ml

METHOD: Streak the actinomycete isolates onto ISP #4 plates and incubate at 28°C for 14 days.
Use these plate cultures for the determination of the colour of the spore mass (aerial mycelium), spore-chain morphology (by examination of the colonies at $100 \times$ magnification under the light microscope), and the colour of the substrate mycelium.

ISP MEDIUM No.5

L-Asparagine monohydrate (Merck)   1.0 g
Glycerol   10.0 g

K$_2$HPO$_4$	1.0 g
*Trace salts solution	1.0 ml
H$_2$O to:	1 litre

* trace salts solution as for ISP#4.

Adjust pH to 7.0 with HCl. Add 20.0 g agar. Autoclave at 15 psi for 15-20 minutes. Cool, pour plates.

METHOD:
Streak the actinomycete isolates onto ISP #5 plates and incubate at 28°C for 14 days.

ISP MEDIUM No. 6 (*Ammonium Ferric Citrate)*

Peptone	15.0 g
Proteose peptone	5.0 g
#Ferric ammonium citrate	0.5 g
K$_2$HPO$_4$	1.0 g
Na$_2$S$_2$O$_3$	0.08 g
Yeast extract	1.0 g
H$_2$O to:	1 litre
Agar	15.0 g

Adjust pH to 7.0. Autoclave at 15 psi for 15-20 minutes. Cool, pour plates.

METHOD:
Streak the actinomycete isolates onto ISP #6 plates (ONE bug per plate) and incubate at 28°C for 4 days.

ISP MEDIUM No. 7

Glycerol	15.0 g
L-Tyrosine	0.5 g
L-Asparagine monohydrate (Merck)	1.0 g
K$_2$HPO$_4$	0.5 g
MgSO$_4$.7H$_2$O	0.5 g
NaCl	0.5 g
FeSO$_4$.7H$_2$O	0.01 g
*trace salts solution	1.0 ml
H$_2$O to:	1 litre

* trace salts solution as for ISP#4.

Adjust pH to 7.2 with HCl. Add 20.0 g agar. Autoclave at 15 psi for 15-20 minutes. Cool, pour plates.

METHOD:
Streak your chosen actinomycete isolates onto ISP #7 plates and incubate at 28°C for 4 days.

MEDIA TO TEST FOR ENZYME ACTIVITY

PECTIN HYDROLYSIS MEDIUM

Solution A:

KH_2PO_4	4.0 g
$Na_2HPO_4.2H_2O$	7.52 g
H_2O to:	200 ml

Adjust pH to 7.0 with NaOH.

Solution B:

Pectin (from citrus fruit rind)	5 g
H_2O	200 ml

Solution C:

$(NH_4)_2SO_4$	2.0 g
Yeast extract	1.0 g
$MgSO_4.7H_2O$	2.0 g
$FeSO_4.7H_2O$	0.1 ml of a 1% solution
$CaCl_2$	0.1 ml of a 1% solution
H_2O to:	600 ml

Add 10.0 g agar. Do not adjust the pH. Autoclave the three components separately at 15 psi for 15-20 minutes. Cool, combine A, B and C and mix. Pour plates.

METHOD:
Streak the actinomycete isolates onto pectin plates and incubate at 28°C for 6 days. Flood the plates with 1% CTAB solution and leave for 30-40 minutes to allow the unhydrolysed pectin to precipitate. Look for zones of clearing around the growth streaks.

EGG-YOLK MEDIUM

Bacteriological peptone	2.0 g
Yeast extract	1.0 g
NaCl	2.0 g
H_2O to:	178 ml

No pH adjustment required.

Add to a 250-ml blue-top bottle containing 2.4 g agar. Autoclave at 15 psi for 15-20 minutes. Cool to 60°C and add: 2 ml sterile 10% glucose and 20 ml 50% egg-yolk emulsion*

Mix well (swirl, do *not* shake). Pour 8 plates.

* Egg-yolk emulsion supplied by Biolab Diagnostics Ltd (catalogue number: BX14).

METHOD:
Streak the actinomycete isolates onto the egg-yolk plates in two parallel streaks and incubate at 28°C for 6 days. Look for zones of proteolytic clearing around the growth streaks at 2 days. Looks for lipolysis and lecithinase activity at 2, 4 and 6 days.

MEDIUM FOR THE H$_2$S TEST AND THE NITRATE REDUCTION TEST
(NOTE: the same medium is used for *both* of these tests)

Nutrient broth (Biolab Diagnostics)	make up according to manufacturer's instructions
KNO$_3$	1.0 g
Agar (Biolab Diagnostics)	3.0 g
H$_2$O to	500 ml

(This medium consists of normal nutrient broth supplemented with 0.2% KNO$_3$ and 0.6% agar).

Heat in the microwave to melt the agar. Dispense into universals in 10-ml volumes. Autoclave. Allow the agar to set with the universals upright (i.e. these are *not* slants).

SOLUTIONS FOR THE NITRATE REDUCTASE TEST
Solution A
Dissolve 0.08 g sulphanilic acid in 10 ml 5M acetic acid.

NOTE: Sulphanilic acid dissolves very slowly – leave overnight to dissolve.
Solution B
Dissolve 0.05 g α–naphthylamine (also called 1-naphthylamine) in 10 ml 5M acetic acid.

5 M acetic acid
Pipette 14.3 ml of glacial acetic acid into a measuring cylinder and adjust the volume to 50 ml. This will give 50 ml of 5M acetic acid.

LEAD ACETATE PAPERS FOR THE H$_2$S TEST

Mix 0.5 g lead acetate [(CH$_3$COO)$_2$Pb] with 10 ml H$_2$O to make a saturated solution (5%). Heat for 30-50 seconds in the microwave (on full power) to make the solution hot.

Cut Whatman No. 1 filter paper into about 50 strips (each one measuring *about* 1 $\times$ 6 cm). Soak the strips of filter paper in the hot lead acetate solution (it is easiest to do this in a Petri dish). Remove the wet filter-paper strips one by one with a pair of forceps, allow excess liquid to drip off, and transfer to a 100-ml blue-top bottle (divide the strips between *two* such blue-top bottles). *Try and keep the strips as straight as possible by allowing them to stick to the inside walls of the blue-top bottles.*

Let the lead acetate strips dry for 1-2 hours at 60°C *with the lids of the bottles off*. Replace lids of bottles and autoclave the bottles. After autoclaving, loosen the lids and leave the bottles at 37°C overnight so that the lead acetate strips can dry out again. Fasten lids and keep the sterile lead acetate papers at room temperature until it is time to inoculate the test medium.

NOTE: Lead acetate filter papers are stable at room temperature for over a year.

METHOD:
Stab inoculate your chosen actinomycete isolates into the agar in the bottles. Insert a lead acetate paper strip into the tube so that when the lid is screwed on, it suspends the paper strip *above* the agar (the paper must not touch the agar or the sides of the bottle). Incubate at 28°C for 14 days. Look for blackening of the paper strip at 7 days and 14 days.

At 14 days remove the lead acetate strip. Mix equal volumes of Nitrite Detection Solution A and Solution B and add 400 µl of the mixture to each culture bottle. If the solution turns dark red or pink, there is nitrite present in the medium (and therefore the bug has reduced the nitrate to nitrite).

MEDIA FOR TESTING DEGRADATION ACTIVITY

TEST SUBSTRATES AND AMOUNTS (per 100 ml Bennett medium):

Adenine	0.5 g
Guanine	0.05g
Hypoxanthine	0.4 g
Xanthine	0.4 g
Cellulose	0.3 g
Elastin	0.3 g
Gelatine	0.4 g
Starch	1.0 g
L-Tyrosine	0.5 g
Xylan	0.4 g

Bennett Medium:

Glycerol	10.0 g
Casitone (Difco)	2.0 g
Yeast extract	1.0 g
"Lab Lemco" Powder (Oxoid)	1.0 g
H_2O to:	1 litre

Adjust pH to 7.0.

Pour 100-ml volumes of the Bennett Medium into 250-ml blue-top bottles containing 2 g agar plus the appropriate amount of cellulose, elastin, gelatine, starch, tyrosine or xylan.

For the adenine, guanine, hypoxanthine and xanthine plates:
Add 90 ml Bennett Medium to 2 g agar in a 250-ml bottle.

Place the appropriate amount of adenine, guanine, hypoxanthine and xanthine in universals and add 10 ml H_2O to each universal. NOTE: these nitrogenous bases will *not* dissolve.

Autoclave separately (15 psi for 15-20 minutes).

After autoclaving, allow the agar to cool to 60°C, then add the 10-ml volume of autoclaved adenine, guanine, hypoxanthine or xanthine. Pour plates.

METHOD:
Streak the actinomycete isolates onto the plates. Incubate all plates at 28 C.
The gelatine and starch plates are incubated for 7 days.

All other plates are incubated for 21 days. Look for zones of clearing around the growth streaks at 7d, 14d and 21d.

At 7 days, flood the starch plates with Gram's iodine solution and look for zones of starch hydrolysis around the growth streaks.

At 7 days, flood the gelatine plates with a saturated solution of $(NH_4)_2SO_4$ and leave for 15-30 minutes to allow unhydrolysed gelatine to precipitate. Look for zones of clearing around the growth streaks.

MEDIUM FOR TESTING CASEIN DEGRADATION

Mix 1 g of skim-milk powder with 10 ml H_2O.

Autoclave for 10 min at 15 psi (note the shorter autoclaving time).

Add 90 ml of Bennett Medium, prepared as for the degradation-activity media, to a 250-ml blue-top bottle containing 2 g agar.

Autoclave the Bennett Agar at 15 psi for 15-20 minutes. Cool, add the autoclaved skim-milk solution to the agar, mix, and pour plates.

METHOD:
Streak the actinomycete isolates onto the casein plates. Incubate at 28°C for 21 days. Look for zones of clearing around the growth streaks at 7d, 14d and 21d.

SIERRA'S MEDIUM (Tween 80 degradation)

Peptone (Difco)	10.0 g
NaCl	5.0 g
$CaCl_2.2H_2O$	0.114 g
H_2O to:	900 ml

Adjust pH to 7.4. Add 15.0 g agar.

10% Tween 80.
Mix 10 ml Tween 80 with 90 ml of H_2O.

Autoclave the agar medium and the Tween 80 separately at 15 psi for 15-20 minutes. Cool, add the 10% Tween 80 solution to the agar and mix thoroughly. Pour plates.

METHOD:
Streak the actinomycete isolates onto the Tween 80 plates. Incubate the plates at 28°C for 14 days. Look for zones of clearing around the growth streaks at 7d and 14d.

MEDIUM FOR TESTING UREA AND ALLANTOIN DEGRADATION

KH_2PO_4	3.0 g
$Na_2HPO_4.2H_2O$	3.57 g
Yeast extract	0.3 g
1% Phenol Red solution	240 µl
H_2O to:	300 ml

Adjust pH to 6.7.

For urea plates:
Add 300 ml basal medium to a 500-ml blue-top bottle containing 3.6 g agar.

Autoclave for 15-20 minutes at 15 psi. Cool to 60°C, add 40 ml of a 15% urea solution (filter sterilised), mix and pour plates.

For allantoin plates:
Add 300 ml basal medium to a 500-ml blue-top bottle containing 3.6 g agar AND 1.0 g allantoin. Autoclave for 15-20 minutes at 15 psi. Cool to 60°C and pour plates.

METHOD:
Streak the actinomycete isolates onto the urea and allantoin plates. Incubate at 28°C for 21 days. Look for a change in the colour of the medium at 7d, 14d and 21d. If urea or allantoin is degraded (i.e. ammonia is released) the medium will change colour from orange to a vibey, shocking pink. If the urease activity is weak the colour change will be less dramatic.

NOTE: Always incubate with an uninoculated control plate.

MEDIUM FOR TESTING THE HYDROLYSIS OF HIPPURATE

Tryptone	2 g
"Lab Lemco" Powder (Oxoid)	0.6 g
Yeast extract	0.2 g
$Na_2HPO_4.2H_2O$	1.254 g
Hippuric acid, sodium salt	2.0 g
H_2O to:	200 ml

Adjust the pH to 7.0. Add 2.4 g agar. Autoclave for 15-20 minutes at 15 psi. Cool to 60°C, add 2 ml 10% glucose solution, mix and pour plates.

METHOD:
Streak the actinomycete isolates onto the hippurate plates. Incubate at 28°C for 21 days. Flood the plates with 50% H_2SO_4 and leave for a few minutes. The appearance of crystals (of sodium benzoate) indicates that hydrolysis of hippurate has occurred.

MEDIUM FOR TESTING THE HYDROLYSIS OF AESCULIN AND ARBUTIN

Yeast extract	0.6 g
Ferric Ammonium Citrate	0.1 g
Aesculin *or* arbutin	0.2 g
H_2O to:	200 ml

Adjust pH to 7.0.

NB: Prepare a third medium, as above, *but omitting aesculin and arbutin* (these plates will serve as colour-control plates).

Add medium to a 500-ml blue-top bottle containing 2 g agar. Autoclave for 15-20 minutes at 15 psi.

METHOD:
Streak the actinomycete isolates onto the aesculin, arbutin and control plates. Incubate at 28°C for 18 days. Examine plates for a change in colour to dark brown at 7, 14 and 21 days. It is *very important* that the colour change occurs only on the aesculin and/or arbutin plates and not on the control plates.

MEDIUM FOR TESTING GROWTH IN THE PRESENCE OF INHIBITORY COMPOUNDS

INHIBITORY COMPOUNDS AND TEST CONCENTRATIONS:
Crystal violet (0.0001%).
Phenol (0.1%).
2-Phenylethanol (0.1 and 0.3%).
Potassium tellurite (0.001 and 0.01%)
NaN_3 (sodium azide) (0.01 and 0.02%).
NaCl (4, 7, 10, 13%).
Thallous acetate (0.001 and 0.01%).

Bennett Medium:
Glucose	10.0 g
Casitone (Difco)	2.0 g
Yeast extract	1.0 g
"Lab Lemco" Powder (Oxoid)	1.0 g
H_2O to:	1 liter

Adjust pH to 7.0.

Add 99 ml Bennett Medium (98 ml for the 0.02% NaN_3 plates) to a 250-ml blue-top bottle containing 2 g agar. Autoclave for 15-20 minutes at 15 psi.

To the sterile agar add:
1 ml filter-sterilised 0.01% crystal violet solution; or

1 ml filter-sterilised 0.1% potassium tellurite; or
1 ml filter-sterilised 1% potassium tellurite; or

1 ml filter-sterilised 0.1% thallous acetate; or
1 ml filter-sterilised 1% thallous acetate; or

1 ml filter-sterilised 1% NaN_3; or
2 ml filter-sterilised 1% NaN_3.

For the phenol and 2-phenylethanol plates:
Add 90 ml Bennett Medium (70 ml for the 0.3% 2-phenylethanol) to a 250-ml blue-top bottle containing 2 g agar. Autoclave for 15-20 minutes at 15 psi.

To the sterile agar add:
10 ml filter-sterilised 1% phenol solution; or

10 ml filter-sterilised 2-phenylethanol solution; or
30 ml filter-sterilised 2-phenylethanol solution.

For the NaCl plates:
Weigh out 4, 7, 10 or 13 g NaCl into a 200-ml beaker.
Add 80 ml of Bennett Medium and dissolve the NaCl.
Pour the solution into a 100-ml measuring cylinder.
Adjust the volume to 100 ml using Bennett Medium.

Pour the solution into a 250-ml blue-top bottle containing 2 g agar. Autoclave at 15 psi for 15-20 minutes. Cool, pour plates.

Streak the actinomycete isolates onto the inhibitory-compounds plates. Incubate at 28°C for 14 days. Record the presence or absence of growth on the plates at 7 days and 14 days.

MEDIUM FOR TESTING GROWTH AT 37°C AND AT pH 4.3

Prepare Bennett Medium as for growth in the presence of inhibitory compounds, but *do not* add any of the inhibitors.

Add 100 ml of Bennett Medium to 2 g agar in a 250-ml blue-top bottle. Autoclave at 15 psi for 15-20 minutes. Cool, pour plates.

Use these plates for testing growth at 37°c.

To a second volume of 100 ml of Bennett medium, add concentrated HCl and adjust the pH to 4.3. Add the solution to 2 g agar in a 250-ml bottle. Autoclave at 15 psi for 15-20 minutes. Cool, pour plates.

Use these plates for testing growth at pH 4.3.

METHOD:
Streak the actinomycete isolates onto the Bennett Agar and pH 4.3 Bennett Agar plates. Incubate at 37 (28°C for pH 4.3 plates) for 14 days. Record the presence or absence of growth on the plates at 7 days and 14 days.

INOCULATIONS

General inoculum
For the inoculation of the media, you need a spore suspension of the actinomycete. Scrape several (2-4) loopfuls of spores from a plate on which the bug has sporulated profusely and transfer them to a sterile universal containing 3 ml sterile water. Shake *very* vigorously to disperse the spores. This constitutes the general inoculum. Use 15 µl of this spore suspension to inoculate each test agar plate. Inoculate by streaking, not spreading.

NOTE: *Streptomyces* spores are hydrophobic, so need to be dispersed well before being used as an inoculum.

Seal the inoculated plates in plastic bags and incubate at 28°C. The plates for testing growth at 37°C are incubated at 37°C. Check the plates after a day or two to make sure there is no fungal contamination.

Appendix II: Reagents for Chemotaxonomic tests

Periodate Schiff's reagent
This reagent is used for the detection of carbohydrates. The plate is first sprayed with a 1% periodate solution and allowed to dry. This is followed by spraying with Schiff's reagent (dissolve 0.5 g of fuchsin and 9 g of sodium metabisulphite in 500 ml water and add 10 ml HCl – filter through activated charcoal).

Glycolipids, including phosphatidyl inositol mannosides, show up blue, whereas phosphatidyl inositol presents a yellow colour. The appearance of the yellow colour only occurs after 40 minutes. Phosphatidyl glycerol shows up as a deep purple colour.

Molybdenum blue reagent
This reagent is used for the detection of phosphate esters. Preparation of the reagent is as follows: 40.11 g molybdenum trioxide is dissolved in 1 litre of 25 M sulphuric acid by constantly stirring and heating until totally dissolved. The reagent is diluted 1:2 before use. The solution is yellow-green and can be kept for several months.

α-Naphthol
This spray is used for the visualization of glycolipids, including PIMs. The reagent is a mixture of 10.5 ml of 15% (w/v) α-naphthol ethanol solution, 6.5 ml concentrated sulphuric acid, 40.5 ml ethanol and 4 ml water. After spraying, the plate is heated at 100°C for 5 minutes. Lipids containing carbohydrates show up as blue spots.

Ninhydrin
Phospholipids with free amino groups react positive with ninhydrin (phosphatidyl ethanolamine and phosphatidyl serine). The 0.1% solution of ninhydrin in water-saturated n-butanol is sprayed on the plate and the plate heated at 120°C for 10 minutes.

For the visualization of all lipids extracted, a plate can be sprayed with 50% sulphuric acid solution and charred at 150°C for 5 minutes.

Appendix III: Mol% G+C determination: Solutions and method

Buffers and solutions:
Cell suspension buffer: For 100 ml – 1 ml of 1 M Tris-HCl (pH 8.0); 0.2 ml of 0.5 M EDTA; 12 g sucrose; water to 100 ml.
Lysing solution: (2x) For 100 ml – 10 ml of 1 M Tris-HCl (pH 8.0); 4 ml of 0.5 M EDTA; 6 ml of 5 M NaCl; 8 ml of 25% w/v SDS; 2 ml β-mercaptoethanol and 500 µl of 20 mg/ml proteinase K.
Other solutions required: 5 M $NaClO_4$ (700 g/l); isopropanol; 75% ethanol; 3 M sodium acetate; TE buffer; chloroform:isoamyl (24:1); phenol:chloroform (2:1); 10 µl RNase (10 mg/ml) in 90 µl TE buffer.

For 200 – 600 ml cultures

1. Harvest cells by centrifugation at 7500 rpm for 10 minutes. Decant the SNF and allow to drain. Resuspend cells in 20 ml suspension buffer.
2. Add dry lysozyme (8 mg/ml) and incubate at 37°C. Start doing the lysis test from 15 minutes onwards.
3. Add an equal volume of the 2X lysing solution and a volume of 5 M $NaClO_4$ equal to ¼ of the combined volume. This mixture forms a precipitate – heat to 50°C to get back into solution. Mix with a swirling action to get to a uniform mixture before all the cells lyse. Lysis is evident by the turbid solution becoming translucent and very viscous. Incubate the lysate O/N at 50 - 60°C to degrade cellular proteins.
4. Add 15 ml phenol:chloroform, shake by hand to form a uniform emulsion and then shake on a wrist action shaker for 20 minutes. Transfer the mixture to a 50 ml centrifuge tube and centrifuge at 12 000 rpm for 10 minutes at room temperature. Meanwhile rinse the flask and let it drain. Slowly decant as much of the upper aqueous phase back into the flask as you can. Do not allow any of the organic phase to go over. The remainder of the aqueous phase can be collected by using an inverted 2 – 5 ml glass pipette. Repeat the extraction procedure twice more. Extraction of proteins is complete if there is little or no white precipitate at the interface after centrifugation.
5. Carefully decant the aqueous phase from the last phenol:chloroform extraction into a suitable flask (125 ml). Taring the flask and then weighing the solution is a good way of estimating volume (1 g = 1 ml). Add 0.6 volumes of isopropanol and swirl to mix. DNA and RNA will precipitate and form a loose clot. Hold the clot back with a Pasteur pipette and pour off the lysate-isopropanol solution. Add 25 ml 76% ethanol and allow to stand for 10 minutes. Decant the ethanol and repeat the wash. Decant the ethanol and press the clot of nucleic acid with a Pasteur pipette to get rid of most of the ethanol. Allow the precipitate to dry at 37°C for 15 minutes.
6. Dissolve the precipitate in 20 ml TE buffer O/N. Add 0.25 ml RNase mix and incubate at 37°C for 1 hour. Extract the solution once with 5 ml chloroform:isoamyl solution, centrifuge and save the aqueous layer.
7. Add 0.1 volumes of 3 M sodium acetate, mix, overlay with 2 volumes of 95% Ethanol, and collect DNA by spooling onto a Pasteur pipette.
8. Dissolve the DNA in 3 – 5 ml TE buffer. For G+C determination, the DNA can be redissolved in a 1% SSC solution and diluted according to type of DNA (DNA with low G+C is submitted in 1% SSC, whereas DNA with high G+C is submitted for analysis in 0.1% SSC).

1% SSC: For 500 ml – 4.38 g NaCl and 0.22 g trisodium citrate are dissolved in 500ml distilled water and autoclaved

Replicate thermal melting curves and %mol G+C for *Streptomyces* sp. strain BSII#1
- Replicate 1:
Replicate 1 thermal melting curve.
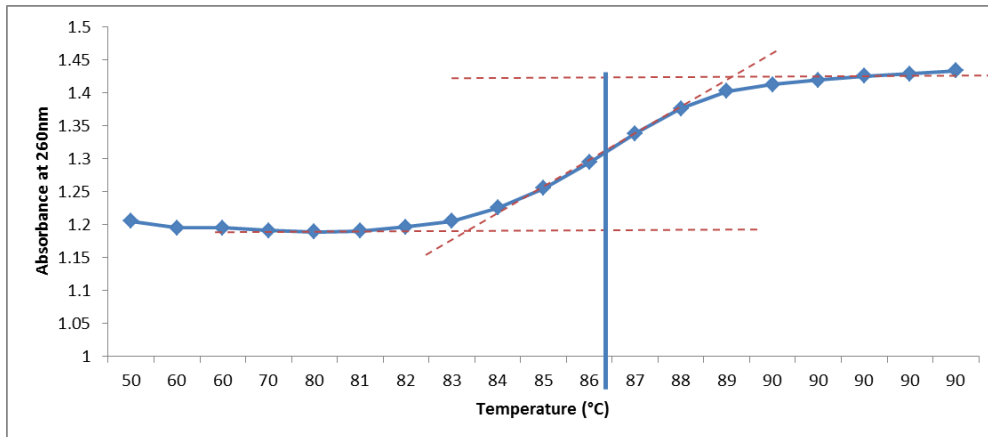
Measured midpoint T_m (0.1% SSC):86.4°C
Calculate Corrected T_m (1% SSC):
T_m (1% SSC) = T_m (0.1% SSC) + 15.4°C
= 86.4°C + 15.4°C
= 101.8°C
Calculate mol% G+C:
Mol% G+C = 2.44 (T_m − 69.3)
= 2.44 (101.8 − 69.3)
= **79.3 %**

- Replicate 2:

Replicate 2 thermal melting curve.
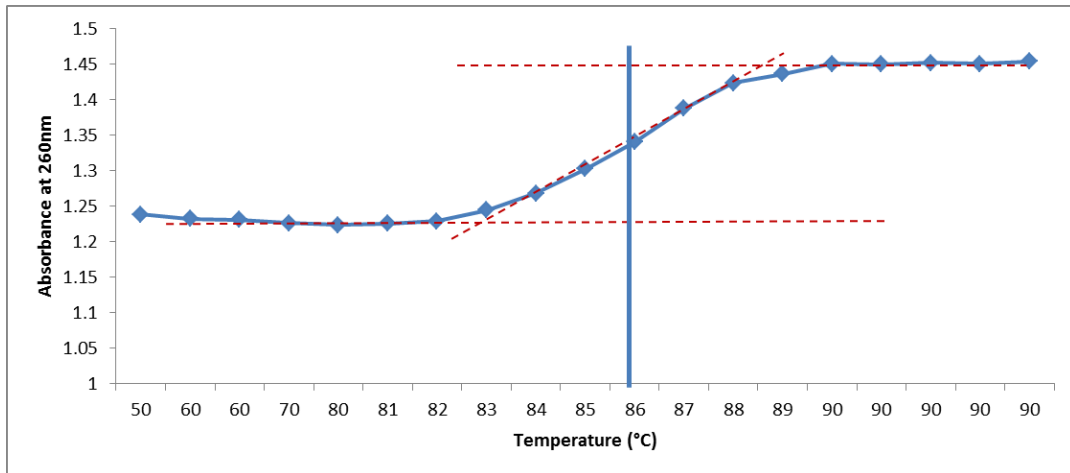
*Measured midpoint T_m (0.1% SSC):*85.9°C
Calculate Corrected T_m (1% SSC):
T_m (1% SSC) = T_m (0.1% SSC) + 15.4°C
= 85.9°C + 15.4°C
= 101.3°C
Calculate mol% G+C:
Mol% G+C = 2.44 (T_m – 69.3)
= 2.44 (101.3 – 69.3)
= **78.08 %**

- Replicate 3:

Replicate 3 thermal melting curve.
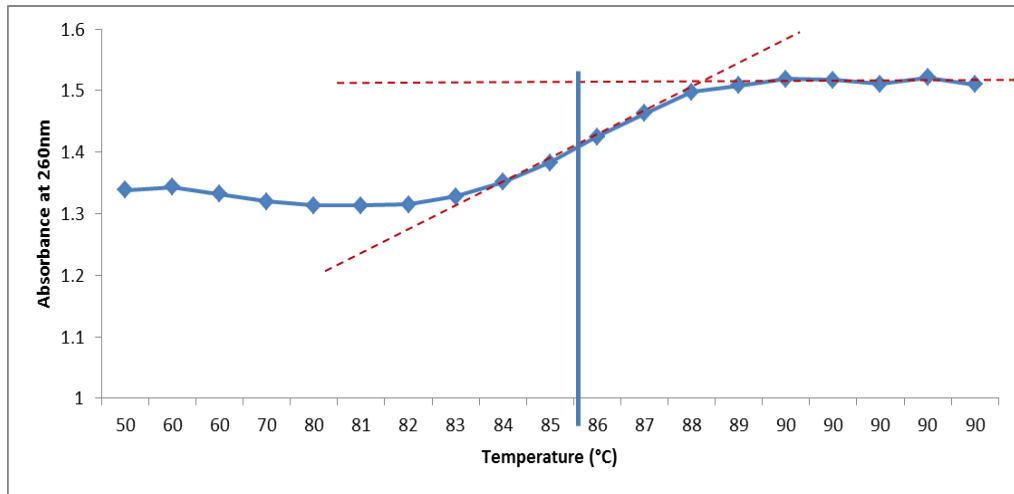
*Measured midpoint T_m (0.1% SSC):*85.6°C
Calculate Corrected T_m (1% SSC):
T_m (1% SSC) = T_m (0.1% SSC) + 15.4°C
= 85.6°C + 15.4°C
= 100.6°C
Calculate mol% G+C:
Mol% G+C = 2.44 (T_m − 69.3)
= 2.44 (100.6 − 69.3)
= **76.37 %**
Mol%G+C:

	%
Replicate 1	79.3
Replicate 2	78.08
Replicate 3	76.37
Average %	77.92
SEM	0.85

Appendix IV: Coupling reaction products
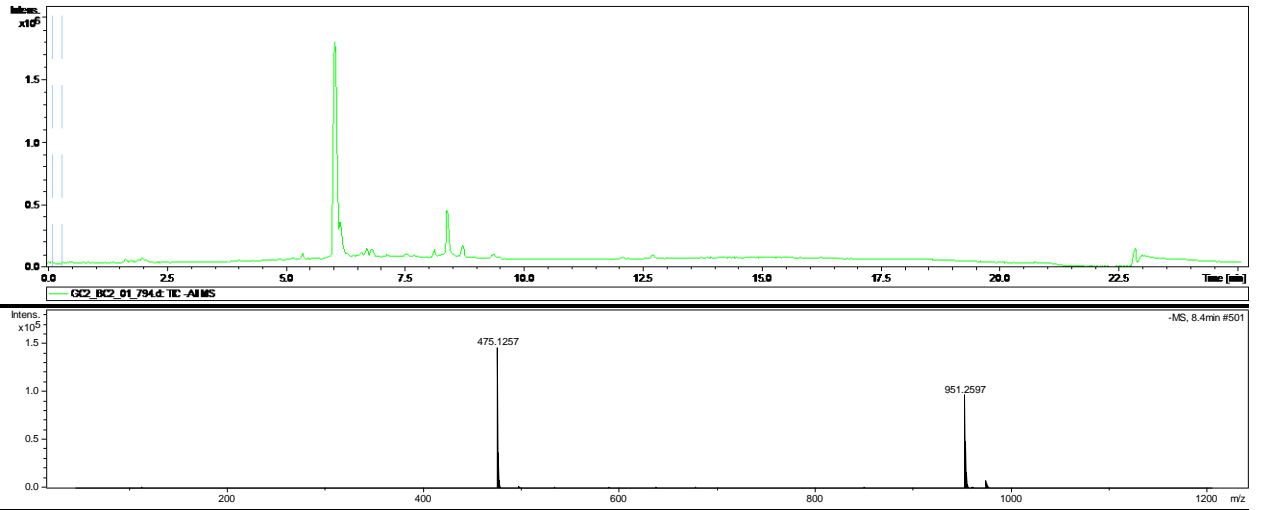
Guaicol+chlorogenic acid
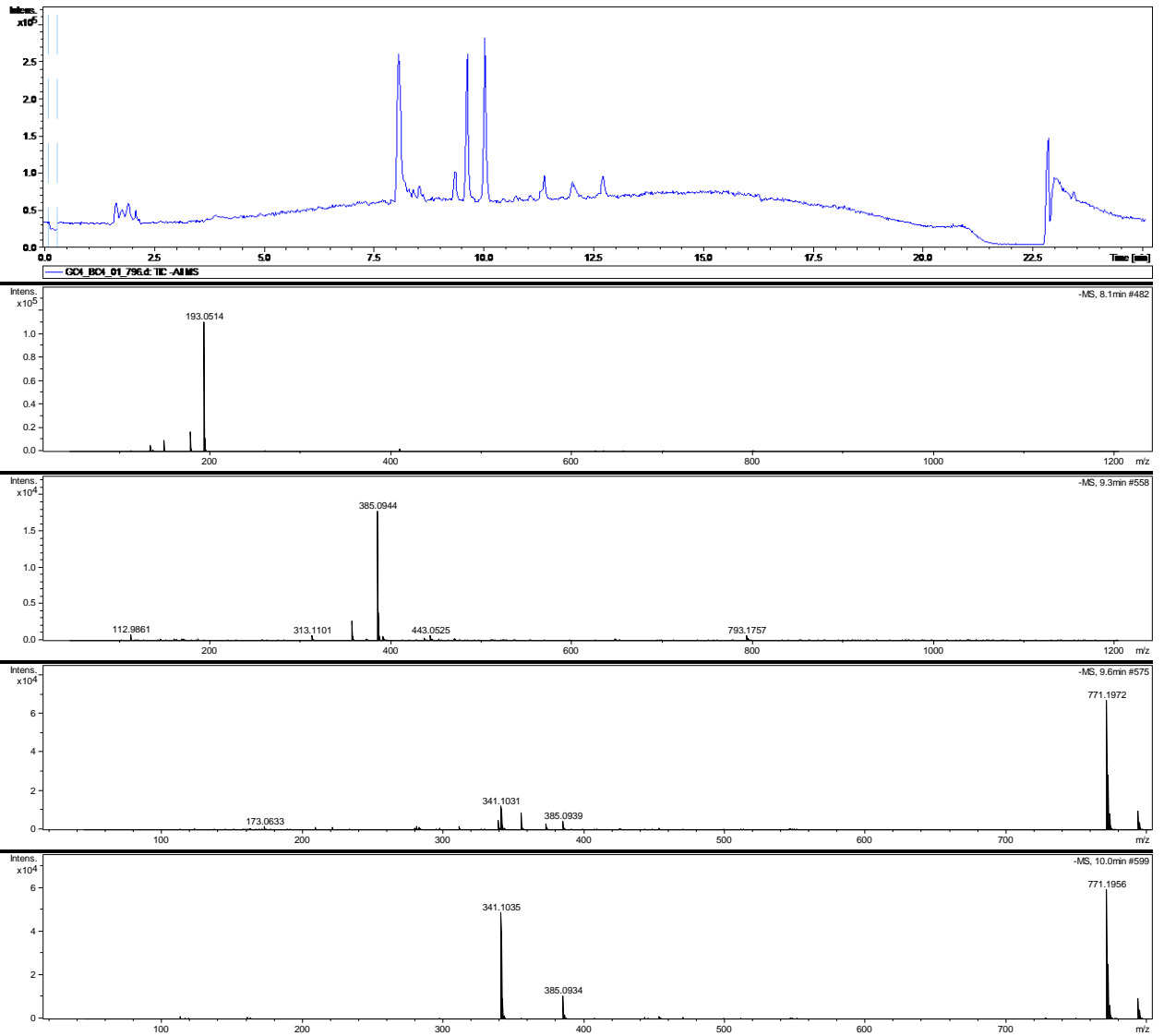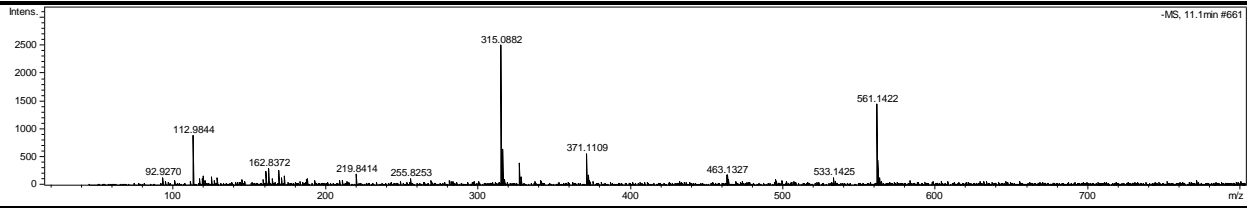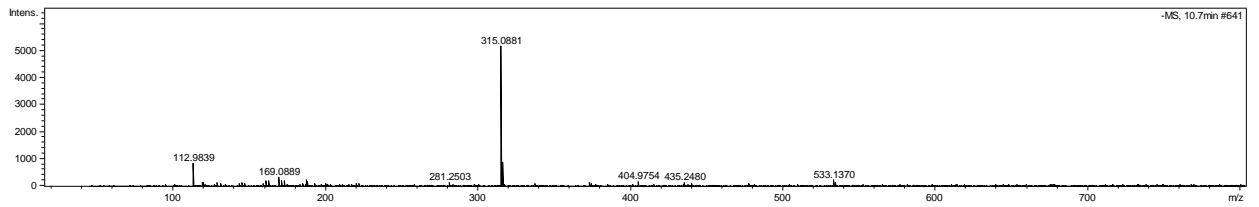
Guaiacol+ferulic acid

Guaiacol+syringaldazine

Guaiacol+caffeic acid

171
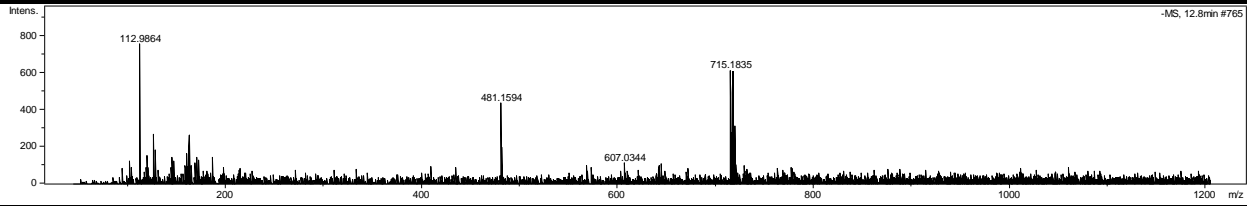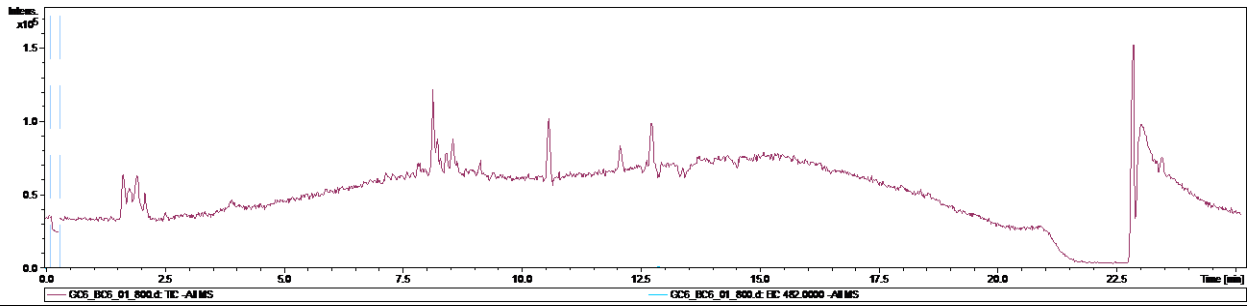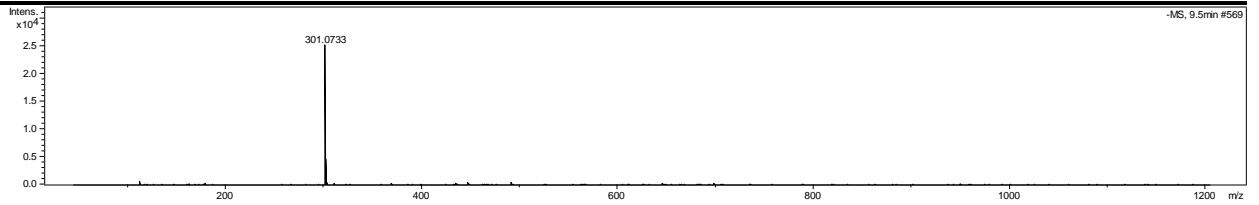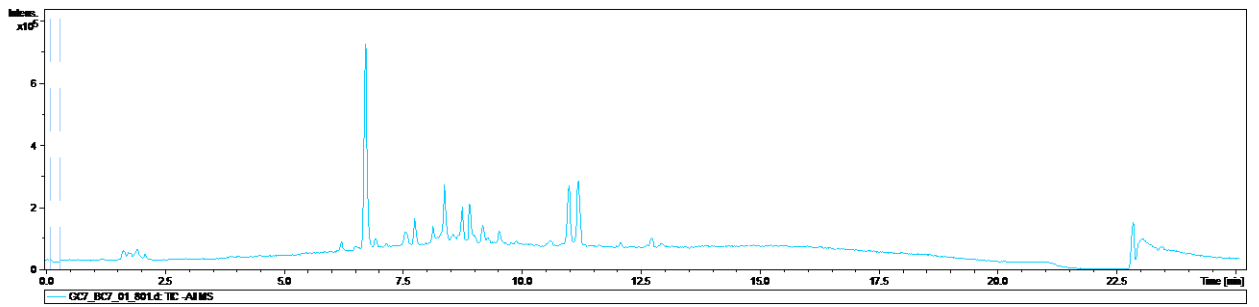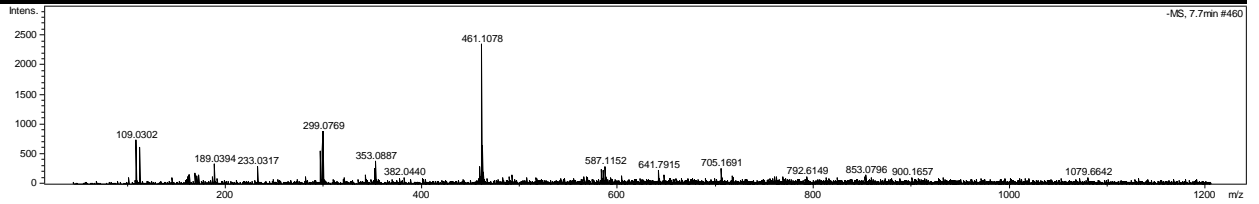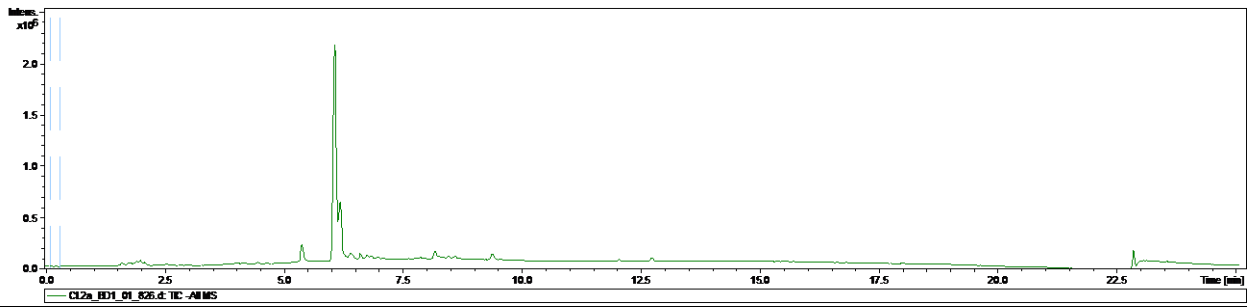
Catechol+cholorogenic acid
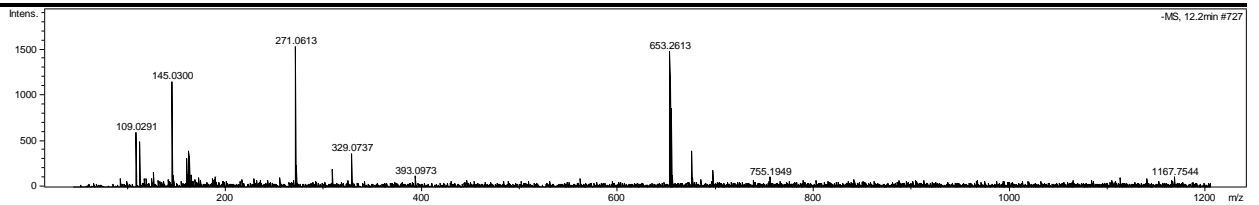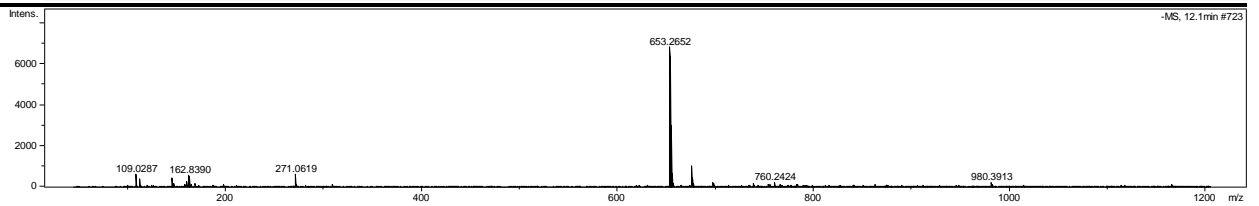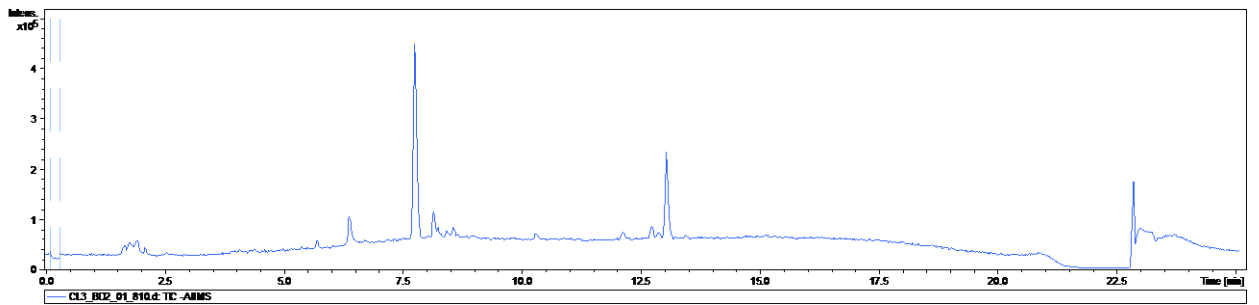
Catechol+coumaric acid

Catechol+ferulic acid

Catechin+chlorogenic acid

Catechin +coumaric acid

-MS, 9.5min #566

861.1716

421.1289

Catechin+cinnamyl alcohol

-MS, 8.5min #507

877.1660

411.1073

575.1200

112.9861

281.2422

718.1434

1149.2291

-MS, 9.1min #544

861.1695

411.1087

574.1236

112.9862

281.2550

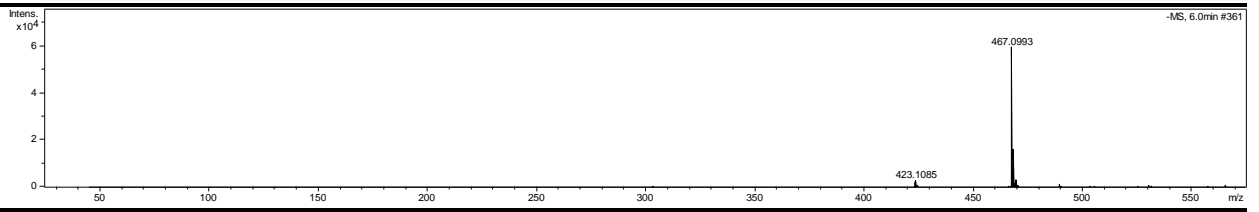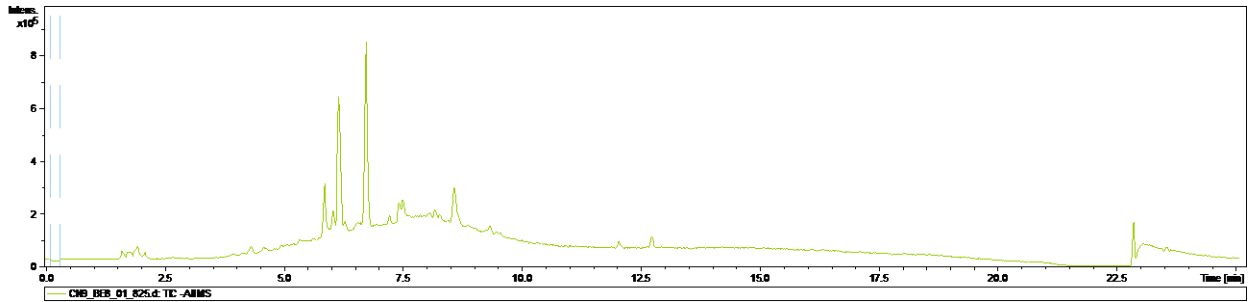718.6476

1055.9916

1149.2374

Catechin+guaiacol

174

Catechin+syringaldazine

Catechin+catechol

Catechin +caffeic acid