IMBM Institute for Microbial Biotechnology and Metagenomics

UNIVERSITY of the WESTERN CAPE

Characterisation of a lignocellulosic degrading *Bacillus* strain isolated from thermophilic compost

By

Matshaya Munaka

Submitted in partial fulfillment of the requirement for the degree of *Magister Scientiae* (M.Sc) in the Department of Biotechnology,
University of the Western Cape

Supervisor: Prof D.A. Cowan

Co-supervisors: Dr R. Bauer, Prof M. Tuffin and Dr B. Kirby

November 2011

Declaration

I hereby declare that **Characterisation of a lignocellulosic degrading *Bacillus* strain isolated from thermophilic compost** is my own original work and that I have accurately reported and acknowledged all sources, and that this document has not previously, in its entirety or in part been submitted at any university for the purpose of obtaining an academic qualification.

Matshaya Munaka

November 2011

.....

Abstract

The negative environmental impact of fossil fuels and growing concerns about petroleum supplies has driven the search for alternative, renewable transportation fuels. An 'ideal' fuel replacement would be a biofuel produced from lignocellulosic biomass. Unfortunately, the presence of lignin in plant cell walls impedes the breakdown of cell wall polysaccharides into simple sugars and the subsequent conversion of these sugars into useable fuels. One of the most common fates of lignin in nature is to be metabolized by lignin peroxidases (LiPs), predominantly of microbial origin. This study aims to isolate and characterise microorganism(s) involved in the degradation of lignocellulose. Thermophilic bacteria were isolated from straw-based compost and screened for lignin peroxidase activity. One isolate, CP11, showed significant lignin peroxidase activity and based on 16S rRNA gene sequence analysis, the isolate was found to be most closely related to *Bacillus thermoamylovorans*. Morphological, physiological and biochemical characterisation was conducted to determine whether the isolate was a novel species. Morphologically, CP11 was characterised as an endospore-forming, Gram positive rod. In addition, the isolate was found to be a facultative anaerobe, catalase positive and capable of utilising a range of carbon sources including glucose, sucrose and arabinose. Isolate CP11 was moderately thermotolerant and grew between 37°C and 55°C, with an optimum growth temperature of 45°C. Based on its phenotypic characteristics CP11 could be clearly distinguished from its closest phylogenetic neighbours. Preliminary characterisation of the lignin peroxidase was conducted using crude enzyme extract and Azure B dye as the substrate. Activity was detected in the supernatant only and a growth curve was constructed to determine the growth phase of lignin peroxidase production. In order to identify the gene encoding the lignin peroxidase a small insert library was constructed and screened for ligninase activity using Azure B as the substrate.

Acknowledgements

I would like to extend my heartfelt gratitude to:

- God (mighty counsellor), for HIS eternal love and keeping me. Great is thy faithfulness.
- My supervisor Professor Don Cowan for allowing me the opportunity of this research project.
- Professor Marla Tuffin, Dr Rolene Bauer and Dr Ana Casanueva for their leadership and guidance throughout the project.
- Dr Bronwyn Kirby for her patience, effort and knowledge. I could never wish for a better supervisor.
- Dr Heide Goodman for her caring attitude.
- My friendly and cheerful research group, for being a blessing each day with invaluable support and assistance. FACT, IMBM rocks!
- NRF for providing me with a personal funding.
- TIA and LEAF for funding my project.

This thesis is dedicated to my family for their prayers, love, support with no measures and encouragement throughout the study. And also to those who believe in the richness of learning.

List of figures

Chapter 2

Figure 2.2.1 A simplified overview of the conversion of lignocellulose into biofuels process.....8

Figure 2.3.1 Schematic representation of the structure of lignocelluloses.....10

Figure 2.4.1.1 The H_2O_2 dependant lignin degradation mechanism by lignin peroxidases....16

Figure 2.4.2.1 The H_2O_2 dependent manganese oxidation mechanism by manganese peroxidases.....19

Chapter 4

Figure 4.1.1 Isolates from the mushroom compost at different temperatures grown on TSB agar plates……43

Figure 4.2.1 Isolates from thermophilic compost were screened for peroxidase activity on alkali lignin TSA plates with 4-amino-antipyrine as a substrate.....44

Figure 4.2.2 Growth of isolate CP11 on TSA containing Azure B dye.....45

Figure 4.3.1.1 Gram stain morphology of isolate CP11.....46

Figure4.3.1.2 Endospores produced by isolate CP11 stained green with malachite green.....47

Figure 4.3.2.1 Isolate CP11 induced metabolic reactions in API microtubes.....48

Figure 4.3.2.2 Results from the catalase test of isolate CP11.....50

Figure 4.3.3.1 Agarose gel (0.8%) showing total genomic DNA of isolate CP11.....53

Figure 4.3.3.2 Agarose gel (0.8%) showing PCR amplification of bacterial 16S rRNA gene using primers F1 and R5.....54

Figure 4.3.3.3 Agarose gel (1%) showing Amplified rDNA Restriction Analysis (ARDRA) patterns of colonies isolated from pGEM-T-Easy cloning.....55

Figure 4.3.3.4 A neighbour joining phylogenetic tree based on the aligned 16S rRNA gene sequences of isolate CP11 and the closest genera.....57

Figure 4.4.1.1 Growth curve of CP11 isolate with enzyme activity from the supernatant and the cell free extract.....58

Figure 4.4.2.1 Relative activity of the lignin peroxidase, determined by the Azure B assay, after dialysis of the cell free extract and supernatant.....60

Figure 4.5.1.1 Analysis of randomly selected Superbac 1 clones showing no insert DNA with a control of an uncut insert background.....62

Figure 4.5.2.1 Agarose gel (1%) containing samples of genomic DNA digests performed with increasing time using 0.1U *Sau*3A1 restriction endonuclease.....63

Figure 4.5.2.2 Restriction analysis of pJET 1.2/ isolate CP11 constructs. Fragments were created by a 5min *Not*I digestion.....64

Figure 4.5.2.3 The recombinant clones showing growth on TSA containing Azure B dye....65

List of tables

Chapter 2

Table 2.1. A general overview of the enzymes involved in lignin decomposition.....13

Chapter 3

Table 3.1. Oligonucleotide PCR primers used in this study.....31

Table 3.2. Designed primers for full gene length using primer walking analysis used in this study.....39

Chapter 4

Table 4.1. The API reference table showing the characteristic reactions of isolate CP11.....49

Table 4.2. Physiological characteristics of isolate CP11 in comparison to two other *Bacillus* strains.....51

Table 4.3. BLAST sequence analysis of the 16S rRNA gene sequences for isolate CP11 showing its closest relatives.....56

List of Abbreviations

BLAST	Basic Local Alignment Search Tool
Bp	Base pair
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
°C	Degree Celcius
EC	European Commission number
EDTA	Ethylene diamine tetraacetic acid
EtBr	Ethidium bromide
EtOH	Ethanol
g	Gram
H_2O	Water
IPTG	Isopropyl β-D-thiogalactosidase
kDa	Kilo Dalton
Kb	Kilobase
LBA	Luria Bertani Agar
M	Molar
Min	Minute
mM	Millimolar
µg	Microgram
µl	Microlitre
ml	Millilitre
MW	Molecular weight
NaCl	Sodium Chloride
NaOAc	Sodium Acetate

NCBI	National Center for Biotechnology Institute
ng	Nanogram
nm	Nanometer
OD	Optical density
Ω	Ohm
PCR	Polymerase chain reaction
Redox	Reduction-oxidation
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
S	Second(s)
sp.	Species
TAE	Tris acetate EDTA
TE	Tris EDTA
Tris-HCl	Tris (hydroxymethyl) methylamine hydrochloride
U	Units
Vol	Volume
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Table of contents

Declaration.....**ii**

Abstract.....**iii**

Acknowledgements.....**iv**

Dedications.....**v**

List if figures.....**vi**

List of tables.....**vii**

List of abbreviations.....**viii**

Chapter 1: General introduction and project aims.....**1**

1.1General introduction.....1

1.2 Project aim and objectives.....3

Chapter 2: Literature review.....**4**

2.1 Introduction.....4

2.2 Biofuels.....6

2.3 Lignocellulose.....9

2.3.1.1 Cellulose.....10

2.3.1.2 Hemicellulose.....11

2.3.1.3 Lignin.....12

2.4 Ligniniolytic enzymes.....13

2.4.1 Lignin peroxidases.....14

2.4.2 Manganese peroxidases.....18

2.4.3 Laccases.....20

2.5 Thermophilic lignin degrading enzymes.....21

Chapter 3: Materials and methods.....23

3.1 General laboratory chemicals and reagents.....23

3.2 Isolation f bacteria from thermophilic compost.....23

3.3 Characterisation of lignin peroxidase.....24

3.3.1 Screening for general peroxidase.....24

3.3.2 Screening for lignin peroxidase activity.....24

3.4 Phenotypic characterisation of the isolate.....25

3.4.1 Physiological characterisation of the isolate.....25

3.4.2 Growth curve.....27

3.4.3 Crude protein analysis.....28

3.5 Phylogenetic characterisation of the isolate.....29

3.5.1 DNA extractions.....29

3.5.2 DNA amplification by the 16S rRNA gene.....30

3.5.3 Drop dialysis.....32

3.5.4 Preparation of elecrocompetent cells.....33

3.5.5 Transformation.....33

3.5.6 Colony PCR amplification.....34

3.5.7 Amplified rDNA restriction analysis (ARDRA).....35

3.5.8 Sequencing.....35

3.5.9 Phylogenetic analysis.....35

3.6 Identification of the gene encoding lignin peroxidase.....36

3.6.1 DNA size fractionation and purification.....36

3.6.2 Cloning high molecular weight DNA.....37

3.6.3 Small insert library construction.....38

Chapter 4: Results and Discussion.....41

4.1 Isolation of bacteria from thermophilic compost.....42

4.2 Screening for general peroxidases.....44

4.3 Characterisation of isolate CP11.....46

4.3.1 Morphological characterisation.....46

4.3.2 Physiological characterisation.....48

4.3.3 Phylogenetic analysis of isolate CP11.....53

4.4 Lignin peroxidase activity screening.....58

4.4.1 Growth parameters.....58

4.4.2 Crude protein analysis.....60

4.5 Molecular analysis.....61

4.5.1 Superbac library construction.....61

4.5.2 Small insert library.....63

Chapter 5: Concluding remarks.....67

References.....69

UNIVERSITY of the
WESTERN CAPE

Chapter 1 General introduction and project aims

1.1 Introduction

The high demand for fossil fuels by the transportation sector, coupled to growing concerns over global climate change has driven the search for alternative, environmentally friendly fuel sources, of which bioethanol is regarded as an ideal candidate. First generation biofuels are produced by fermenting sugars from plants, whereby the glucose from the carbohydrate part of the plant is converted into alcohol (Nigam and Singh, 2011). The major disadvantage of this strategy is that economically it may be more viable for industries to channel food grade plant material to the production of fuel which may negatively affect the food supply, particularly in developing countries (Nigam and Singh, 2011).

Second generation biofuels are produced from the fermentation of biomass (non-edible feed stocks), thereby limiting the food versus fuel competition associated with first generation fuels and preventing the conversion of food into fuel. An added advantage is that second generation biofuels are renewable and carbon-neutral. Production processes utilise microbial enzymes to convert the cellulose and hemicellulose components to sugars prior to fermentation to produce ethanol. The main obstacle in the production of biofuels from biomass is that cellulose and hemicellulose fractions must first be separated from the lignin fraction, as lignin may impede enzymatic degradation (Kleinert *et al*., 2009).

In a biological system, microorganisms, such as bacteria and fungi, produce ligninases (lignin peroxidase (LiP), manganese peroxidase and laccase) which degrade lignin through oxidation (Dashtban *et al*., 2010). The initiation step during the production of biofuels from cellulose is the depolymerisation of lignin which is catalysed by lignin peroxidases (Perez *et al*., 2002). Similar to other lignocellulose degraders, the advantage of using bacterial LiPs is that these

organisms can be readily modified to produce large amounts of enzyme and can utilise a range of growth substrates (DeAngelis *et al*., 2011).

Thermophilic bacteria are a source of a wide variety of industrial enzymes. Compared to mesophiles, thermophiles have higher metabolic rates at elevated temperatures, have a greater tolerance to organic solvents generated during the production of biofuels and reduce the risk of contamination (Turner *et al*., 2007). The present study reports the identification and characterisation of a bacterial LiP producing strain isolated from a thermophilic environment.

1.2 Project aims and objectives

Aim

This study aims to isolate and fully characterise a novel bacterial strain isolated from thermophilic straw-based compost. The strain was selected based on its ability to produce lignin peroxidase, a class of enzymes involved in the biodegradation of lignocellulose.

The objectives of the study were:

- To isolate lignin degrading bacteria from a thermophilic environmental source.
- To screen isolates for lignin peroxidase activity.
- To conduct full morphological and physiological characterisation of lignin degrading isolate(s).
- To construct a small insert DNA library to identify the lignin peroxidase encoding gene.
- To sequence and clone the lignin peroxidase encoding gene.

Chapter 2: Biofuels and lignin degrading enzymes

2.1 Introduction

Earth is being transformed by humans. These changes include the depletion of the ozone layer, tropical deforestation, acid deposition and increased atmospheric concentrations of gases that trap heat, which leads to global warming. Many of these human activities far exceed the natural processes that serve as "messengers" of the planet's environmental state (Silver and DeFries, 1990). Certain gases are described as being 'trace gases', including, water vapour, carbon dioxide (CO_2), methane, tropospheric ozone and nitrous oxide. These gases trap heat, which accumulates close to the earth's surface initiating the greenhouse effect. This ultimately results in a rise in temperature, adjustment of precipitation patterns and unforeseeable global climate change (Silver and DeFries, 1990).

After water vapour, CO_2 is the most plentiful greenhouse gas. While it can occur naturally, it is also produced in great quantities during the combustion of fossil fuels, particularly coal. When fuel is burned carbon is oxidised into carbon dioxide. Since the industrial revolution, when coal was first introduced as the primary energy source, atmospheric CO_2 concentrations have increased by 25%, with an annual increase of 0.4% (Jahi, 2009). In addition, CO_2 is released when forests are cleared and the trees are burned or allowed to decay (Silver and DeFries, 1990). For decades scientists have been monitoring the accumulation of CO_2 in the atmosphere and they have proven that this accumulation is linked to an increase in the earth's surface temperature.

Similarly, methane (CH_4) plays a major role in maintaining the greenhouse effect. Methane originates from natural processes including emissions from paddy rice fields, anaerobic decomposition in natural wetlands, livestock production systems and the anaerobic decomposition of organic waste in landfills. Human activities such as biomass burning (including forest fires, charcoal combustion and firewood burning) as well as the exploration for and transport of fossil fuels also tends to play a major role in global methane emissions (Heilig, 1994).

2.2 Biofuels

Due to over-consumption by humans, valuable economic resources, such as fossil fuels, are becoming scarce. Increased awareness of the environmental impact of fossil fuels, escalating fuel prices, reliance on energy imports and declining availability has sparked an interest in biofuels. Biofuels can either be liquid or gaseous, and are produced from biomass. Biofuels have the potential to supply transportation energy in a carbon-neutral way (Von Blottnitz and Curran, 2006), and have a number of potential applications such as transportation, generating electricity and lubrication (Furfari, 2008). Biofuels encompass a broad range of nonpetroleum based fuels, which includes everything from fuels made from agricultural products, to fuels made from used vegetable oil (Barnes-Davies, 2009).

Bioethanol is the most widely used liquid biofuel (Demirbas, 2006), and is produced from the fermentation of sugars, starches and cellulosic biomass. Most commercial production of bioethanol is from either cane or beet sugar, as starches and cellulosic biomass usually requires expensive pre-treatment. Bioethanol is a renewable energy fuel source and it is used in the manufacturing of cosmetics and pharmaceuticals, and for the production of alcoholic beverages (Kumar *et al*., 2007).

There are numerous reasons why biofuels have suddenly become attractive alternatives to fossil fuels. Biofuels are considered environmentally friendly. They have lower sulphur content and are a renewable resource. With the projected increase in fossil fuel prices, biofuels carry a significant economic potential (Demirbas, 2006).

South Africa is an emerging economy. The South African biofuels industry mainly relies on the production of bioethanol from maize and sugarcane, although a small volume of bioethanol is also manufactured from molasses which is produced as a by-product by the

sugar industry. However, most of the bioethanol currently produced in South Africa is not used as fuel but as potable alcohol, in paints and inks and by the pharmaceutical industry. Notwithstanding, between the years 2000 and 2004 almost 200 000 tons of bioethanol was exported, highlighting South Africa's significance as a bioethanol exporter (Cartwright, 2007).

Biomass resources used in the production of biofuels include natural and man-made derived materials such as woody and herbaceous plant species, wood wastes, bagasse, agricultural and industrial residues, waste paper, municipal solid waste, sawdust, biosolids, grass, waste from food processing, animal waste, and aquatic plants and algae (Yaman, 2004).

Several processes can be used to degrade biomass, such as hydrolysis (chemical, acidic or thermal) (Knauf and Moniruzzaman, 2004), pyrolysis (Di Blasi, 1995) and microbial degradation. Ideally, the microorganisms utilised in biofuel's production should have a broad substrate utilisation range, have a good tolerance to inhibitors and display a high metabolic flux (Alper and Stephanopoulos, 2009).

Microorganisms release constituent sugar monomers from biomass via a process called saccharification, and these monomers can be used in secondary fermentation to produce high volumes of biofuels at a reduced price (Edwards, 1990). Wang and Chen (2009) described the basic process for the conversion of lignocellulose to biofuels in four steps. Firstly, delignification liberates cellulose and hemicellulose from lignin entanglement by nonspecific physical and chemical methods. A wide spectrum of inhibitory by-products such as weak acids, furan derivatives and phenolic compounds are also generated. During the second step, the pre-treated suspension is exposed to microbial enzymes which hydrolyse the cellulosic

and hemicellulosic components into simple carbohydrate products, primarily six and five carbon sugars. During the third step, the sugars are fermented by specific microorganisms into various products, primarily ethanol. The final step involves the separation of these products from the reaction components - including the substrate, microorganisms and culture broth.

The conversion of lignocellulosic material into biofuels involves several processes, whereby the chemical energy stored in plant cell walls is liberated. Plant cell walls are made-up of sugar chains and polymers that encircle individual plant cells. The plant biomass is harvested, crudely cut and the cell walls are thermally denatured (Rubin, 2008). As illustrated in Figure 2.2.1, there is a clear carbon cycle: carbon dioxide is incorporated into plant biomass, which is subsequently used to produce ethanol releasing carbon dioxide back into the atmosphere.

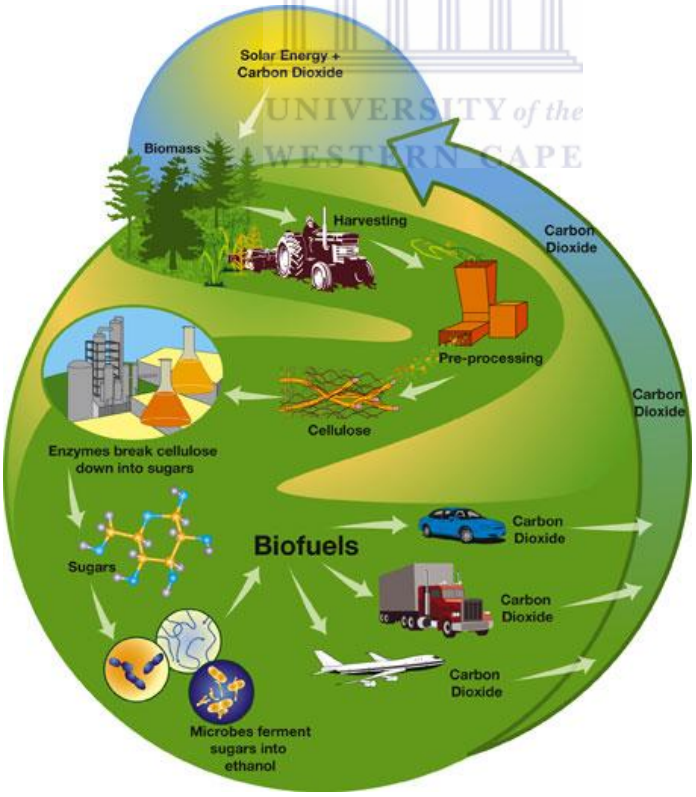

Figure 2.2.1 A simplified overview of the conversion of lignocellulose into biofuels process. (Taken from www.biorefinery.ws/images/bio_f1_b.jpg&imgrefurl 01/04/10).

Ethanol and other biofuels produced from lignocellulosic biomass represent a renewable, more carbon-balanced alternative to fossil fuels and corn- or sugarcane-derived ethanol. Unfortunately, the presence of lignin in plant cell walls impedes the breakdown of cell wall polysaccharides into simple sugars and the subsequent conversion of these sugars into useable fuel. The physical removal of lignin from plant tissue before saccharification is therefore necessary. The current approaches used for lignin removal from biomass are not feasible as they are energy-intensive, making the large scale production of cellulosic biofuels unrealistic. In nature, one of the most common fates of plant cell wall lignin is to be hydrolysed by lignin peroxidases (LiPs), manganese peroxidases (MnPs) and closely-related enzymes from white rot basidiomycetes (Hamid and Rehmad, 2009).

2.3 Lignocellulose

Lignocellulose is regarded as an important structural component of woody and non-woody plants, and represents a significant source of organic, renewable matter (Howard *et al.*, 2003). As the main component of cellulosic biomass, it is mainly comprised of cellulose, hemicelluloses and lignin (Fig. 2) (Edwards, 1990). The two main challenges to using lignocellulosic biomass as a feedstock for biofuels production are that it is recalcitrant to biodegradation, and contains both pentose and hexose sugars (Chen *et al.*, 2009).

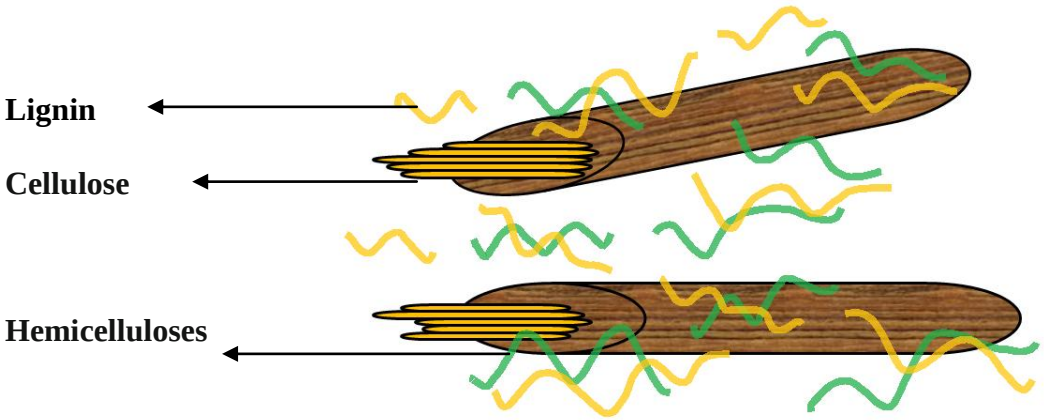

Figure 2.3.1 Schematic representation of the structure of lignocellulose. The hemicellulose and cellulose microfibrils are crosslinked with the lignin acting as a 'glue' that binds adjacent cells.

2.3.1.1 Cellulose

Cellulose is a linear polysaccharide which consists of cellobiose units that are connected by ß-(1→4)-glycosidic bonds. The glycosyl units form fibrils that are stabilised by strong intermolecular hydrogen bonds between the hydroxyl groups of adjacent molecules (Bon and Ferrara, 1996). As it constitutes the main component of the plant cell wall, cellulose is the most abundant biomass resource on Earth and represents over 50% of wood mass. Cellulose is an inexhaustible, renewable raw material, and possesses excellent mechanical and thermal properties such as thermal stability, flexibility and conductivity (Nishino *et al.*, 2004).

Cellulose can be utilized as a feedstock for biofuel production. During the initial stage of the process the cellulosic chains are broken down to produce glucose that is subsequently utilised during the fermentation by fungi or bacteria (Alzate and Torooj, 2006). Certain microbial enzymes have been implicated in the enzymatic hydrolysis of cellulose. ß-1-4 endoglucanases (EC 3.2.1.4.) attack the low crystalline regions of the cellulose fibres creating free chain ends,

which are subsequently hydrolysed by ß-1-4 exoglucanases (cellobiohydrolase, EC 3.2.1.91.) which cleave the molecule by removing the cellobiose units from the free chain ends. In the final step, ß-glucosidase (cellobiase, EC 3.2.1.21.) hydrolyses the cellobiose units and glucose is produced as an end-product (Mussatto *et al*., 2008).

2.3.1.2 Hemicellulose

Hemicellulose is regarded as the second most common polysaccharides in nature and represents about 20–35% of lignocellulosic biomass, of which xylans are the most abundant (Lee *et al.*, 2005). Hemicellulose bioconversion has recently received increased attention due to its practical applications in agro-industrial processes, including the production of fuels and chemicals, the delignification of paper pulp, enhancing the digestibility of animal feedstock, the clarification of juices, and improving the consistency of beer (Saha, 2003).

Hemicelluloses are derived mainly from chains of pentose sugars, which act as a cement holding the cellulose micelles and fibres together. Hemicelluloses are largely soluble in alkali and are more easily hydrolysed compared to lignin. Structurally, hemicelluloses are similar to plant gums and compared to cellulose, occur in shorter molecule chains (Demirbas, 2006).

Several microbial species degrade hemicellulose, namely *Clostridium thermocellum*, *Erwinia chysantheni* (Antoni *et al*., 2007), *Streptomyces reticuli* (Wang and Chen, 2009) and *Zymomonas mobilis*. Numerous hemicellulases, such as xylanases and xylosidases, have been isolated from saprophytic microorganisms (Mielenz, 2001).

2.3.1.3 Lignin

Lignin is an integral cell wall constituent that provides the plant with strength (Baharuddin *et al.*, 2010). Lignin is composed of complex polymers consisting of phenylpropane units interconnected by a variety of carbon-carbon bonds and ether linkages. In nature, lignin physically encapsulates cellulose (Ramachandra *et al.*, 1988) and is involved in water transportation in plants. Lignin is resistant to microbial degradation and forms a barrier against microbial destruction by protecting the readily assimilable polysaccharides (Hofrichter, 2002). Lignin degradation results in the release of lignin-rich, water soluble fragments called acid precipitable polymeric lignin (Kuhad *et al.*, 2007).

Early work on lignin degradation focused on fungal systems. Recently, attention has shifted towards bacterial genera as a potential source of lignocellulose degrading enzymes. In particular, thermophilic actinobacteria have received special recognition for their role in the breakdown of plant matter, as well as in the composting process. However, despite this interest, the actinobacterial enzymes characterised thus far are not as efficient as those produced by white rot fungi (Edwards, 1990). Some of the most active lignin degraders among the actinobacteria are *Streptomyces* and *Thermomonospora* species (Kuhad *et al.*, 2007). These organisms have been found to possess a range of ligninolytic enzymes such as lignin peroxidases, manganese peroxidases and laccases, as well as hydrogen producing oxidases (Jeffries, 1994).

2.4 Ligninolytic enzymes

There is increasing scientific interest in enzymes involved in the mineralisation and depolymerisation of lignin. Ligninases include a range of enzymes that have the capability to degrade numerous lignin model compounds, and lignin peroxidases (LiPs), manganese peroxidases (MnPs) and laccases are three families of enzymes which have been implicated in the biological degradation of lignin (Jeffries, 1994). The various classes of lignin degrading enzymes can be characterised by their ability to cleave at different sites of the lignin molecule. Table 2.1. shows ligninolytic enzymes and the reactions catalysed during lignin biodegradation.

Table 2.1. A general overview of the enzymes involved in lignin decomposition.

Enzyme activity	Molecular mass (kDa)	Mediator (substrate)	Main effect (Reaction)
Lignin peroxidase	**43-45**	**H_2O_2**	**Oxidation of the aromatic ring into a cationic radical.**
Manganese peroxidase	**38-63**	**H_2O_2**	**Oxidation of Mn (II) to Mn (III); the chelated Mn (III) oxidises phenolic compounds to phenoxyl radicals.**
Laccase	**50-100**	**ABTS**	**Oxidation of phenoxyl radicals by phenols.**

(Adapted from Hatakka *et al.*, 2001).

Apart from lignin biodegradation, ligninolytic enzymes have the ability to mineralise xenobiotic compounds such as polycyclic hydrocarbons, chlorinated phenols, polychlorinated biphenyls, dioxin, alkylhalides and nitrotoluenes. Additionally, the decolourisation of polymeric dyes in liquid culture has been demonstrated to be related to the lignin degradation system, with the dye serving as the substrate (Ryu *et al.*, 2003).

Peroxidases (E.C. 1.11.1.x) are a diverse group of enzymes with molecular weights ranging from 30,000 to 150,000 Da (Hamid and Rehmad, 2009). Peroxidases are found in most living organisms including bacteria, plants and animals, and their ubiquitous distribution highlights their importance in biological systems (Passardi *et al*., 2004). Peroxidases have the ability to catalyse various substrate oxidations, provided that hydrogen peroxide (H_2O_2) is present. Most of the peroxidases characterized thus far contain a heme group in their tertiary structure (Rob *et al.*, 1995).

2.4.1 Lignin Peroxidases

Lignin peroxidases (EC 1.11.1.14) play a central role in the biodegradation of plant cell wall lignin by microorganisms, the most extensively studied of which is the white rot fungi *Phanerochaete chrysosporium* (Tuor *et al.*, 1995). The *P. chrysosporium* peroxidase is a unique heme peroxidase with a higher redox potential, lower pH optima and broader substrate specificity than any other microbial peroxidase characterised. Like other peroxidases, this peroxidase is capable of oxidizing certain non-phenolic aromatic substrates including substituted aromatic ethers and thioethers (Ward *et al.*, 2003).

Although much of the literature focuses on fungal lignin peroxidase, several bacterial lignin peroxidases have been characterised, including enzymes from *Streptomyces vidiosporus*, *Streptomyces lividans* and *Thermonospora mesophilia* (Bugg *et al.*, 2010). In addition, lignin peroxidases have also been isolated from *Bacillus* and *Pseudomonas* species (Dawker *et al.*, 2009). The enzymes produced by *Bacillus* species have the ability to decolourise various textile dyes and are involved in the asymmetric cleavage of dyes, such as anthraquionine, triphenylmethane and azo. Azo dyes such as Congo red, orange II and Azure B are organic compounds which have vivid colours (Deng *et al.*, 2008).

Traditionally, lignin peroxidases were characterised using chromatographic techniques whereby the lignin peroxidase polymers were radio-labelled and the catalysis was monitored by the generation of different soluble products (Hammel *et al*., 1993). Currently, quantitative methods have been developed, including spectrophotometric assays, whereby the oxidation of a substrate is monitored by a change in absorbance, such as the Azure B assay which is based on the oxidation of micromolar concentrations of the Azure B dye by lignin peroxidase (Archibald, 1992).

Lignin peroxidases and iso-enzymes range in molecular weight from 38 to 43 kDa (Farrel *et al.*, 1989). Iso-enzymes differ from lignin peroxidases in terms of stability, substrate specificity and physical characteristics including molecular weight, iso-electric points and N-terminal sequences (Rothschild *et al.*, 1996). Both lignin peroxidase and iso-enzymes possess a single porphyrin IX ring (per mol of an enzyme) as a prosthetic group. In addition, they contain cysteine residues that form disulfide bridges. Calcium-binding sites maintain the topology of the active sites and carbohydrate chains mainly play a role in protecting the C-

terminal peptide from proteolysis (Wong, 2009). Lignin peroxidases are highly liable and due to over-oxidation may be inactivated when exposed to excess H_2O_2 (Kadish *et al.*, 2003).

The enzymology of bacterial lignin peroxidase degradation is not well understood, although it has emerged that bacteria use the same enzymatic mechanisms as fungi. In comparison to fungi, bacteria degrade lignin inefficiently, and studies have shown that bacteria utilise the low molecular weight portion of lignin and cleave the intermonomeric linkages of the lignin polymer (Raj *et* al., 2007).

The most studied mechanism of lignin degradation is through the utilisation of extracellular lignin peroxidases, (Bugg *et al*., 2010) and H_2O_2-dependant oxidation by lignin peroxidases has been investigated for different model compounds. The sequence of the reaction can be simplified to the following equations, whereby H_2O_2 (which is the natural electron acceptor) is an oxidising substrate for ferric enzymes.

Native peroxidase (Fe^{3+}) + H_2O_2 → Compound- I + H_2O

Compound- I + AH_2 → Compound- II + $AH^{\bullet}$

Compound- II + AH_2 → Native peroxidase (Fe^{3+}) + AH*+ H_2O

Figure 2.4.1.1 The H_2O_2 dependant lignin degradation mechanism by lignin peroxidases (Adapted from Li *et al.*, 2009).

Where:

Fe^{3+} is the oxyferryl,

Compound I is the lignin peroxidase carrying both oxidising equivalents of H_2O_2,

AH_2 is the first electron donor,

$AH^{\bullet}$ is the cation radical,

Compound II is the lignin peroxidase carrying one oxidising equivalent.

This mechanism involves the two-electron oxidation of the ferriheme (Fe^{3+}) prosthetic group from the native peroxidase by H_2O_2, generating the intermediate compound-I which consists of oxyferryl iron (Fe^{4+} - O) and a porphyrin π cation radical. In the subsequent reaction the first electron donor AH_2 reduces compound-I, leading to the formation of compound II. In the final step compound-II accepts an additional electron from AH_2, thereby returning the enzyme to its native state as ferriperoxidase (Li *et al*., 2009).

The substrate specificity of lignin peroxidases is broad. The most widely used synthetic substrates are 3, 4-dimethoxybenzyl alcohol (also known as veratryl alcohol), 3, 4-dimethoxybenzyl glycerol, 3, 4, 5- trimethoxybenzyl alcohol and 3, 4, 5-trimethoxybenzyl glycerol. Amongst these substrates veratryl alcohol is the 'best' substrate discovered, as it serves as a mediator and enzyme protector during lignin peroxidase catalysed oxidation (Dengbo *et al*., 1994).

2.4.2 Manganese Peroxidases

Manganese peroxidases (EC 1.11.1.13) (MnPs) are glycoproteins with a molecular weight ranging between 38-62.5 kDa. Manganese peroxidises are produced by several white rot fungal species and in nature they degrade lignin from wood. MnPs exist as a series of isozymes containing one iron protoporphyrin IX prosthetic group (Wariishi *et al.*, 1989). Due to their broad substrate specificities, MnPs have the ability to degrade a wide range of synthetic dyes and a variety of pollutants including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). MnPs have been applied in bioprocesses such as bioremediation, bio-pulping, bio-bleaching and bio-ethanol production (Fujihara *et al.*, 2010).

MnPs catalyse the H_2O_2- and manganese-dependent oxidation of a variety of phenols, amines and organic dyes. Electronic absorption and resonance Raman spectral evidence indicates that the heme environment of MnPs have features similar to plant peroxidases. The mechanism of MnPs (Figure 2.4.2.1) involves the native enzyme reacting with H_2O_2 forming compound I (MnI) which oxidises compound II (Mn II, a metal naturally present in wood) into compound III (Mn III). Mn III acts as a reducing agent during the subsequent oxidation of monomeric phenols and phenolic lignin dimers via the formation of a phenoxy radical (Wariishi *et al.*, 1989).

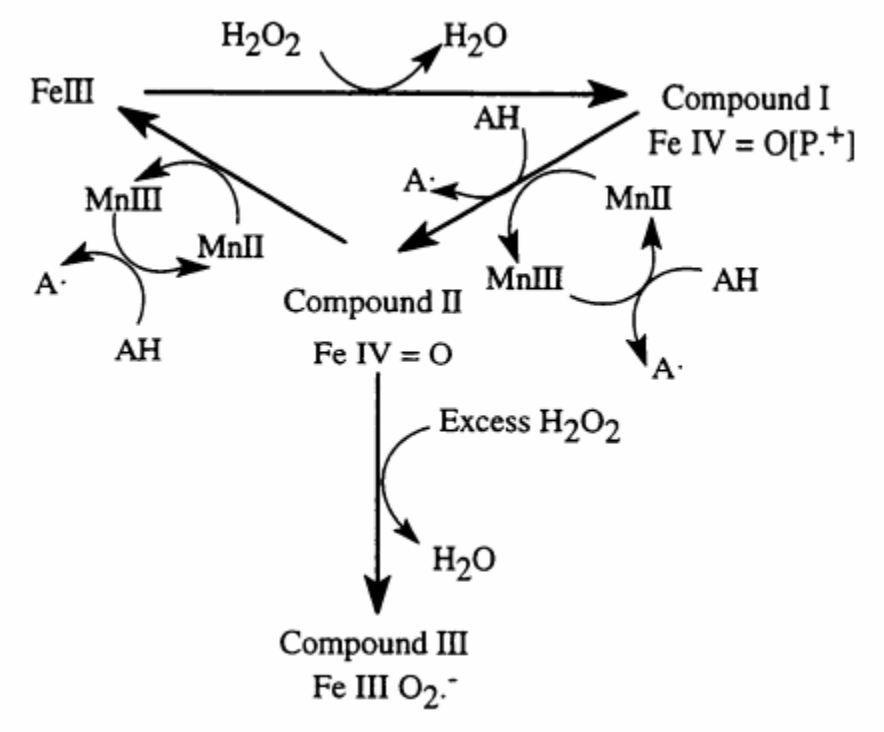

Figure 2.4.2.1 The H_2O_2 dependent manganese oxidation mechanism by manganese peroxidases (adapted from Castillo, 1997).

Mn III behaves as a low molecular weight mediator that diffuses to remote regions of the lignin molecule and initiates the oxidation process. The oxidation of Mn II to Mn III is dependent on the presence of chelating agents such as lactate, succinate and malonate (Duhalt and Ramirez, 2004).

2.4.3 Laccases

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are enzymes that catalyse the reduction of oxygen to water, followed by the oxidation of phenolic compounds through coupling electrons with a wide range of aromatic compounds. Many laccases have been identified from plant and fungal species based on homology searches of protein and bacterial genome databases. Microbial laccases are believed to be involved in various cellular activities such as conidial pigmentation and development, lignin formation, lignin degradation and pathogenicity (Endo *et al.*, 2003).

The molecular mass of a laccase monomer ranges from 50 to 100 kDa. An important feature of a laccase monomer is a covalently linked carbohydrate moiety (10–45%), which contributes to the high stability of the enzyme. Most laccases have acidic pH optima (Claus, 2004) and are generally thermostable enzymes (Arias *et al*., 2003).

Laccases have several roles in bacterial systems, including melanin production, spore coat resistance and morphogenesis. To date, laccase activity has been reported in only a few bacterial species, including *Azospirillum lipoferum*, *Bacillus subtilis*, *Marinomonas mediterranea* and *Streptomyces griseus* (Madhavi and Lele, 2009). Laccases have potential applications in various biotechnological applications such as bio-bleaching, increasing the strength of cellulose fibres, textile dye finishes and bioremediation. Laccases are considered to be one of the most promising enzymes for future industrial applications and are active in the presence of chemical mediators, such as 2, 2 -azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) (Arias *et al.*, 2003).

Laccases oxidise phenol and polyphenols via electron abstraction, resulting in radicals that are subsequently polymerised. As oxidation may be limited by phenolic subunits, bulk delignification requires the breaking of many bonds. In the presence of the appropriate substrates, laccases are capable of oxidising both phenolic and non-phenolic lignin structure (Bourbonnais and Paice, 1990).

2.5 Thermophilic lignin degrading organisms

Microbial growth is regulated by temperature and microorganisms can be classified based on their temperature growth range into one of three broad groups namely psychrophiles, mesophiles and thermophiles (Kristjansson, 1992). Thermophiles are defined as microorganisms with an optimal growth temperature above 60°C. Most thermophiles cannot grow below 45°C (Blumer-Schuette *et al*., 2008). Thermophiles have been isolated from a number of marine and terrestrial geothermally-heated habitats including shallow terrestrial hot springs, deep sea hydrothermal vent systems and sediment from volcanic islands (Bitton, 2002).

Much interest has been expressed in using enzymes produced by thermophilic organisms for the generation of bioethanol. An advantage of the use of thermophilic enzymes include the possibility to distil off the ethanol continuously as ethanol evaporates at the growth temperature of the producing strain thereby making the downstream processing steps more economical, since the removal of cells from the fermentation broth to recover the ethanol is not required (Edwards, 1990). During the hydrolysis of lignocellulosic materials, thermophilic enzymes have been shown to display high specific activity, stability and increased flexibility (Olsson and Ahring, 2007).

At elevated temperatures thermophiles produce thermostable proteins that are highly resistant to denaturation and proteolysis. Thermophiles produce specialised proteins known as chaperonins that refold and restore the protein to its native form, helping to maintain functionality after denaturation. Thermophiles' cell membranes possess saturated fatty acids which create an internal hydrophobic environment within a cell and maintain cell rigidity at elevated temperatures. Thermophilic proteins are highly functional at high temperatures due to increased intermolecular and intramolecular interactions such as electrostatic, disulphide bridges and hydrophobic bonds (Haki and Rakshit, 2003).

In nature, many thermophilic fungi display cellulolytic or/and lignolytic activities. The most extensively studied lignin biodegrading taxa are the *Basidiomycota* (white rot fungi) and the *Ascomycota*, which both produce oxidative enzymes. Although previous research has concentrated on using fungal treatments for lignin degradation, it is becoming increasingly important to focus research on the isolation and identification of ligninolytic bacteria. Studies have shown that lignin degrading bacteria have a wider tolerance for temperature, pH and oxygen limitation compared to fungi (Abd-Elsalam and El-Hanafy, 2009). Bacterial species have immense environmental adaptability and biochemical versatility, which makes them stable during the seemingly "extreme" conditions (high substrate concentrations, high concentrations of organic solvents and diverse substrate range) imposed in the laboratory (Li *et al.*, 2009).

Chapter 3 Materials and methods

3.1 General Laboratory chemicals and reagents

Chemicals were supplied by Merck Chemicals and Laboratory Supplies (Darmstadt, Germany), Sigma-Aldrich Chemical Company (Deissenhofen, Germany), and Kimix Chemical and Laboratory Supplies (South Africa). Unless stated, all chemicals were of analytical grade. Oxoid Ltd (Cambridge, UK) and New England Biolabs (UK) supplied culture media. DNA size markers and all DNA modifying enzymes (polymerases and restriction endonucleases) were purchased from Fermentas Life Sciences Ltd (South Africa). Oligonucleotide primers for polymerase chain reaction (PCR) were synthesised by Whitehead Scientific (South Africa).

3.2 Isolation of bacteria from thermophilic compost

Microorganisms were isolated from straw-based compost collected from Medallion Mushroom farm in Stellenbosch (Western Cape Province, South Africa; November 2009). The temperature of the compost at the time of collection was recorded using a temperature probe.

To isolate microorganisms, 25 g of compost was added to 100 ml 0.8 % NaCl (isotonic solution, pH 7.0) in a 500 ml Erlenmeyer flask. The compost slurry was incubated for 2 hours at 37°C with shaking at 150 rpm. After incubation, the sample was serially diluted (10^{-1} to 10^{-10}) with 0.8 % NaCl. Aliquots (100 µl) of each dilution were spread-plated on TSA (17 g Bacto-tryptone, 3 g Bacto-soytone, 2.5 g dextrose, 5 g NaCl, 2.5 g hydrogen dipotassium phosphate and 15 g bacteriological agar) supplemented with cycloheximide at

50 µg/ml. Five plates were prepared per dilution and incubated at either 28°C, 45°C, 55°C, 68°C or 72°C for 7 days.

Bacterial colonies were picked from the isolation plates using a sterile toothpick and streaked onto new TSA plates (without antibiotics). The plates were incubated at 45°C overnight. The bacteria were purified by repeated re-streaking onto fresh TSA plates until a pure culture was obtained. Isolates were stored as 15% glycerol stocks at -80°C. Glycerol stocks were prepared by adding 300 µl of 50% glycerol to 700 µl of broth culture.

3.3 Characterisation of the lignin peroxidase

3.3.1 Screening for general peroxidase activity

Isolates were screened on TSA containing 2.5 mM 4-Aminoantipyrine (AAP) with 0.17 M phenol as an indicator (Worthington, 1988). The isolates were grown at 45°C for 10 days. Bacteria able to produce peroxidase would be able to grow on the media, while peroxidase negative strains would fail to grow.

3.3.2 Screening for lignin peroxidase activity

Isolates were screened for lignin peroxidase activity on modified TSA. Standard TSA (10 ml) supplemented with 0.025% alkali lignin was poured into an empty Petri dish. Once solidified, the medium was overlaid with 10 ml TSA containing 0.025% Azure B dye. The isolates were streaked onto the plates and incubated at 45°C for 10 days. Lignin peroxidase positive bacteria would oxidise the blue dye and positive colonies would appear yellowish-green in colour.

3.4 Phenotypic characterisation of the isolate

3.4.1 Physiological characterisation of the isolate

API strips were used for the preliminary characterisation of the isolate. A single colony was grown in 5 ml TSB media pH 7.0 with 0.025% alkaline lignin and incubated overnight at 45°C, while shaking. The overnight culture was diluted 1:100 in a final volume of 100 ml TSB alkali lignin medium and the cells were grown at 45°C for 3-4 hours with shaking until an OD_{600} of 1. Twenty millilitres (20 ml) of the culture was centrifuged at 8000 rpm for 10 min and the cell pellet was resuspended in 10 ml containing 0.85% (w/v) NaCl solution. The API strip was inoculated with 200 µl of the resuspended cells and incubated overnight at 37°C.

Gram staining (Gram, 1884) was performed according to standard methods and the bacteria were viewed under a BA 2000 series Biological Light Microscope (Chongquing Optical and Electrical Instrument Company, China) equipped with a 100 × oil immersion lens. Colony morphology was examined on cultures grown on TSA for 5 days at 45°C. Motility of the isolate was observed under a microscope using the hanging drop method (Ventosa *et al*., 1982; Queseda *et al*., 1984).

The pH and temperature growth range was determined on TSA. The pH range for growth was tested at pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0. The temperature range for growth was determined at 28°C, 37°C, 45°C, 55°C, 68°C and 72°C (Dussault, 1955). A result was considered positive if there was visible growth after 7 days incubation, except for the plates incubated at 28°C where growth was recorded after 14 days.

Catalase activity was tested by growing culture in 5 ml TSB medium for 18 hours. Three to four drops of 3% (w/v) H_2O_2 was added to the test tube which was covered with foil. The

evolution of bubbles was considered a positive result and would indicate the breakdown of H_2O_2, with the subsequent release of oxygen (Ventosa *et al*., 1982).

The ability of the isolate to grow anaerobically was evaluated on TSA incubated at 45°C for 16 hours in jars with the GasPak Anaerobic system [BBL Microbiology Systems, Cockeys-ville, Md.].

Gelatine hydrolysis (Frazier, 1926) was tested on TSA containing 1% (w/v) gelatine. Plates were incubated at 45°C for 16 hours and gelatinase activity was detected by flooding the plates with a saturated $(NH_4)_2SO_4$ solution, which would precipitate any unhydrolysed gelatine remaining in the agar. A positive result would be observed as a zone of clearing around the colony.

Casein hydrolysis was determined on TSA containing 1.3% (w/v) skim milk powder. The plates were streaked with the isolate and incubated at 45°C for 16 hours. Zones of clearing around the growth streak would indicate a positive result. Starch hydrolysis was determined by streaking the isolate on TSA containing 1% potato starch, followed by incubation at 45°C for 16 hours. The presence of starch was detected by flooding the plate with Gram's iodine which formed a purple-blue complex with the starch (Cowan *et al*., 1974). Lipase activity (Sierra, 1957) was detected on Sierra's fat agar incubated at 45°C for 7 days. The plate was flooded with a saturated $CuSO_4$ solution and left to stand at room temperature for 10 min. Activity was detected by examining the greenish-blue fat globules under and around the bacterial colonies.

Acid fast staining test was examined by spreading a loopful of bacterial culture onto a clean slide. Once heat fixed, the cells were covered with Carbolfuschin and placed over a boiling water bath for 5 min. The cells were decolourised by rinsing with acid alcohol for

15-20 sec. The cells were counter-stained with methylene blue for 30 sec, rinsed with tap water and blotted dry with tissue paper. The cells were observed under a microscope using a 100 × oil immersion lens (Sheehan and Hrapchak, 1980).

An endospore stain was performed according to standard methods (Dussault, 1955). A loopful of bacterial culture was spread on a glass slide, heat fixed and flooded with malachite green and heated for 5 min over a boiling water bath. The smear was washed gently with tap water. The cells were counter-stained with Safranin for 30 sec. The slide was washed with tap water and blotted dry with tissue paper. The slide was observed under a microscope using a 100 × oil immersion lens.

3.4.2 Growth curve

The isolate was grown overnight in 5 ml TSB supplemented with 0.025% alkali lignin (pH 7.0) at 45°C, with shaking. The overnight culture was transferred to a 500 ml Erlenmeyer flask containing 100 ml of TSB alkali lignin medium pH 7.0 and grown for 2 days at 45°C, with shaking. The 100 ml culture was used to inoculate 250 ml TSB alkali lignin medium in a 1000 ml Erlenmeyer flask and the cultures were incubated at 50°C with shaking. Growth was monitored by measuring the optical density (OD) at 600 nm every 12 hours.

3.4.3 Crude Protein analysis

Crude cell extracts were prepared from a 250 ml liquid culture supplemented with 0.025% alkali lignin pH 7.0, grown at 45°C for 7 days. Cell mass was harvested by centrifugation at 10 000 x *g* for 10 minutes at 4°C. The cells were lysed by sonication in 5 ml 100 mM

potassium phosphate buffer pH 7.0 using a Sonoplus HD-070 sonicator (Bandelin, Germany). Sonication was performed as follows: 4× cycles bursts for 2 minutes, 60% max amplitude, with 30 seconds incubations in an ice bath between cycles. The cell lysates were centrifuged at 10 000 × *g* for 30 minutes at 4°C.

Ammonium sulphate was added separately to the crude supernatant and the lysed cellular extract to 50% saturation and incubated at 4°C for 1 hour while shaking. Precipitated protein was collected by centrifugation at 6000 × *g* for 10 minutes at 4°C. The particulate matter of both the supernatant and lysed cellular extract was removed by filtering through a 0.22 µM sterile syringe filter, retained and resuspended in pH 7.0 potassium phosphate buffer.

Following ammonium sulphate precipitation the resuspended protein of both the crude supernatant and filtered cell-free extract were desalted by dialysis. To hydrate the dialysis cassette, it was pre-incubated in 100 mM potassium buffer, pH 7.0 for 5 minutes. The protein extract was injected into the cassette using a 21 gauge needle. The protein was dialysed against 100 mM phosphate buffer pH 7.0 for 24 hours, changing the buffer every 12 hours. The dialysed protein was removed using a syringe and assayed for lignin peroxidase activity.

The dialysed protein extract was used to screen for lignin peroxidase activity using the Azure B assay (Archibald, 1992). The reaction mixtures contained 150 mM citrate buffer pH 3.5 and 32 µM Azure B. After the reaction mixtures were prepared, 300 µl culture supernatant was added to each reaction and incubated at 25°C for 3 - 4 minutes. The reaction was initiated by the addition of 100 µM hydrogen peroxide and the volume was

made up to a final volume of 0.9 ml with distilled water. Changes in absorbance were monitored spectrophotometrically at 651 nm for 5 - 7 minutes. A positive reaction would result in a decreased OD in the 0.4 – 1.0 range over time. A control reaction contained culture supernatant and the hydrogen peroxide was substituted with distilled water.

3.5 Phylogenetic characterisation of the isolate

3.5.1 DNA extractions

Genomic DNA extraction was performed according to the protocol of Zhou *et al*. (1996). A 50 ml overnight culture was centrifuged at 8000 × *g* for 10 minutes. The supernatant was discarded and the pellet was resuspended in 10 ml of extraction buffer (25 mM Tris-HCl pH 8.0, 50 mM glucose, 10 mM EDTA, 25 mg/ml lysozyme). Proteinase K (100 µl/ml) was added to the sample and the tube was incubated horizontally at 37°C for 30 min while shaking at 150 rpm. SDS was added to a final concentration of 1% and the sample was incubated at 65°C for 2 hours, with gentle inversion every 20 min.

An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the sample and centrifuged at 16 000 × *g* for 10 minutes. The aqueous layer was removed and transferred into a new tube. An equal volume of chloroform was added to the supernatant and the sample was centrifuged as before. Chloroform extractions were repeated until the aqueous phase was clear. The DNA was precipitated with 0.6 vol of isopropanol.

The DNA was collected by centrifugation at 16 000 × *g* for 30 min, and the pellet was washed with 70% ethanol, air-dried and re-suspended in 50 µL of 1 × TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0). The concentration of genomic DNA was

determined using a Nanodrop 1000 (Thermo Scientific) and the integrity of the DNA was evaluated on a 0.8% agarose gel containing 0.5 µg/ml of ethidium bromide. Prior to loading on the gel the samples were mixed with standard loading dye (60% [v/v] glycerol, 0.25% [w/v] bromophenol blue). A λ*Pst*I molecular weight marker was included on all gels. DNA was visualized using an AlphaImager HP (Alpha Innotech) gel imaging system. DNA was stored in TE buffer at -20°C for long term and 4°C for short term storage.

3.5.2 DNA amplification of the 16S rRNA gene

PCR was performed in 20 µl reaction volumes containing 1 × Dream*Taq* buffer containing $MgCl_2$, 2U Dream*Taq* polymerase, 0.2 mM of each dNTP, 0.5 µM of each primer (Table 3.1) and 2 µl of template DNA. *E.coli* DH5α chromosomal DNA was included as a positive control. PCR reactions were performed in a Thermocycler Gene Amp®2700 (Applied Biosystems, USA). The PCR programme included an initial denaturation step at 96°C for 2 min, followed by 35 cycles of denaturing at 96°C for 45 sec, annealing at 56°C for 30 sec and extension at 72°C for 2 min, followed by a final extension of 72°C for 5 min (Cook and Meyers, 2003).

Table 3.1. Oligonucleotide PCR primers used in this study

Primer	Sequence (5' – 3')[a]	Application	Reference
F1	AGAGTTTGATCITGGCTCAG	Amplification of the full length 16S rRNA gene	Weisberg *et al.*, 1991
F3	GCCAGCAGCCGCGGTAATAC	Bacterial 16S rRNA gene internal sequencing primer	Lalloo *et al.*, 2007
F5	GCATGGITGTCGTCAGCTCGTG	Bacterial 16S rRNA gene internal sequencing primer	Lalloo *et al.*, 2007
R1	GTATTACCGCGGCTGCTGCTGGCAC	Bacterial 16S rRNA gene internal sequencing primer	Lalloo *et al.*, 2007
R3	CACGAGCTGACGACAICCATGC	Bacterial 16S rRNA gene internal sequencing primer	Lalloo *et al.*, 2007
R5	ACGGITACCTTGTTACGACTT	Amplification of the full length 16S rRNA gene	Weisberg *et al.*, 1991
M13F	CCCAGTCACGACGTTGTAAAACG	Universal vector primer – colony PCR	Jin *et al.*, 2007
M13R	AGCGGATAACAATTTCACACAGG	Universal vector primer – colony PCR	Jin *et al.*, 2007

a – I, Inosine.

The PCR products were analysed by agarose gel electrophoresis on 0.8% (w/v) Tris-Acetate EDTA agarose gels [TAE; 0.2% (w/v) Tris-base, 0.5% (v/v) glacial acetic acid, 1% (v/v) 5M EDTA, pH 9.0], containing 5 µg/ml ethidium bromide (Sambrook and Russel, 2001). The DNA was visualised on an Alphaimager 2000 imaging system.

The amplified 16S rRNA gene was purified using the GFX^{TM} PCR DNA and Gel Band Purification kit (GE Healthcare, United Kingdom) according to the manufacturer's instructions. Purified 16S rDNA amplicons were cloned into the pGEM-T-Easy vector (Promega, United Kingdom) according to the manufacturer's instructions. The ligation reaction (final reaction volume of 10 µl) included 50 ng pGEM-T-Easy vector, 150 ng insert DNA, 2 × ligation buffer and 1U of T4 ligase enzyme. The ligation mixture was incubated at 4°C for 16 hours. Ligated plasmids were transformed into electrocompetent *E.coli* Genehog cells (Invitrogen, USA).

3.5.3 Drop dialysis

Drop dialysis was used to desalt the ligation reactions prior to transformation. A small volume (approximately 2 ml) of sterile, deionised water was placed in a Petri dish and a 0.22 µM Type GSWP nitrocellulose filter (Millipore) was gently lowered onto the water so that it floated shiny side up. Once the filter was completely hydrated, 3 µl of the ligation reaction was applied to the filter. The sample was allowed to dialyse for 10 minutes undisturbed at room temperature and 2 µl was used per transformation (Silhavy *et al*., 1984).

3.5.4 Preparation of electrocompetent cells

Electrocompetent GeneHog *E.coli* and EPI-300 cells were prepared as outlined in Sambrook and Russel (2001). Glassware was thoroughly washed with 70% EtOH, rinsed and autoclaved prior to use. Competent cells was kept on ice throughout the protocol, dispensed into pre-chilled 0.5 ml Eppendorf tubes and stored at -80°C (Inoue *et al.,* 1990). To test the electro-competency, the competent cells were transformed with 100 ng of Superbac vector DNA.

3.5.5 Transformation

Electrocompetent *E.coli* GeneHog cells (section 3.5.4) were transformed by adding 2 µl of the drop-dialysed DNA ligation mixture to 50 µl of freshly thawed competent cells in a sterile electroporation cuvette (0.1 mm gap junction, BioRad, USA). The cells were incubated on ice for 10 min prior to transformation. Electroporation was performed using a Gene Pulser (Biorad, USA) with standard conditions for *E. coli*: 1.8kV, 25µF, 200Ω. After the addition of 950 µl ice-cold Super Optimal Broth with catabolite repression (SOC) (Hanahan, 1985), the transformed *E. coli* cells were allowed to recover for 1 hour in a 37°C water bath. An aliquot of the transformed cells (100 µl) was spread-plated on LB agar (LBA) plates supplemented with 100 µg/ml ampicillin, 20 µg/ml IPTG and 30 µg/ml X-Gal, and incubated at 37°C overnight, covered in foil to exclude light. *E.coli* GeneHog cells harbouring putative recombinants pGEM-T constructs (white colonies) were picked onto LBA plates supplemented with 100 µg/ml ampicillin and incubated overnight at 37°C. Plasmid DNA was extracted from the overnight broth cultures using the PureYield Plasmid Miniprep kit (Promega, USA) according to the manufacturer's instructions.

Isolated plasmid DNA was quantitated spectrophotometrically using a Nanodrop spectrophotometer. Putative recombinant clones were analysed by restriction digestion to confirm the correct sized fragment had been cloned.

3.5.6 Colony PCR amplification

DNA templates for sequencing were generated by PCR using universal M13 forward and M13 reverse (Table 1) primers, which bind on either side of the multiple cloning site. A sterile toothpick was stabbed into a colony and used to transfer a small amount of cell mass to a microfuge tube containing 5 µl of sterile water. The sample was vortexed vigorously and used as the template DNA for PCR. Colony PCR was performed on 20 randomly selected clones. The reaction mix contained 1× NEBlab buffer, 0.2 mM of each dNTPs, 0.5 µM of each universal primer and *Taq* polymerase (prepared in the lab). The PCR programme was an initial denaturation period at 95°C for 5 min (extended to ensure bacterial cells were completely lysed), followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 2 min. An aliquot of the PCR product was examined by agarose gel electrophoresis to check whether the correct sized fragment had been generated.

3.5.7Amplified rDNA Restriction Analysis (ARDRA)

Amplified ribosomal DNA restriction analysis (ARDRA) (Martin-Laurent *et al*., 2001; Sambrook *et al*., 2001) of the 1.5 kb 16S rRNA gene PCR amplicon was performed using two 4 bp cutter restriction endonuclease, *Alu*I and *Hae*III [Fermentas]. The 20 µl mixture contained 100 ng (2 µl) PCR amplified DNA, 0.1 U restriction endonuclease and 1× restriction endonuclease buffer. The digestions were incubated overnight at 37°C and the reaction was terminated by the addition of 5 µl of 6 × DNA loading dye. Banding patterns were visualised by separation on 1% (w/v) agarose gels.

3.5.8 Sequencing

Sequencing reactions were carried out by the DNA sequencing facility at the Department of Molecular and Cell Biology, University of Cape Town. Sequencing was performed using primers F1, F3, F5, R1, R3 and R5 in order to obtain the full length sequence of both DNA strands.

3.5.9 Phylogenetic Analysis

Sequences were analysed using the software packages BioEdit Version 7.0 (Hall, 1999), Chromas (Andersen *et al*., 1992) and DNAMAN Version 4.13 (Lynnon Biosoft). The GenBank database was used for the analysis of DNA sequences and homology searches. The Basic Local Alignment Search Tool (BLAST*n*) programme (www.ncbi.nlm.nih.gov/) was used to determine the sequence similarity to known sequences in the GenBank database. Sequences were aligned using ClustalW multiple alignments feature in BioEdit

sequence Alignment Editor. Phylogenetic analyses were conducted using MEGA version 4 (Tamura *et al*., 2007) and a Neighbour-joining tree (Saitou and Nei, 1987) was constructed.

3.6 Identification of the gene encoding lignin peroxidase

3.6.1 DNA size fractionation and purification

A square (4 × 1 cm) was cut out of a 0.7% agarose gel using a sterile scalpel and the hole was filled with 0.7% low melting point (LMP) agarose. High molecular weight (HMW) genomic DNA was loaded on the gel alongside a λ-*Hind*III ladder and fosmid control DNA (Epicentre). Electrophoresis was carried out at 30 V for 18 hours. The section of gel containing the two marker lanes was cut from the gel and stained in TAE –EtBr buffer (EtBr 5 mg/ml, 0.5 × TAE) for 30 minutes. The markers were viewed at 302 nm UV and the 23 kb *Hind*III band was marked using a sterile pipette tip. The gel was re-assembled and the slice of gel containing the desired size DNA was excised from the LMP agarose and transferred to a pre-weighed 1.5 ml Eppendorf tube. DNA was recovered from the gel slice by agarase digestion according to the manufacturer's instructions (Fermentas). DNA was precipitated with 1/10 vol 3 M NaOAc pH 5.2 and 2.5 vol of 99% ethanol. The sample was placed at -20°C for 30 minutes and the precipitated DNA was collected by centrifugation at 13 000 × *g* for 10 minutes. The supernatant was removed and the pellet was washed with 70% ice-cold EtOH. After air-drying the pellet, the DNA was re-suspended in 50 µl TE buffer (pH 8.0) and the DNA concentration was quantified by fluorimetry.

3.6.2 Cloning high molecular weight DNA

DNA was end-repaired to generate 5'-phosphorylated blunt-ended DNA fragments using an End Repair kit (Epicentre). The following reagents were combined on ice to a total volume of 50 µl; end-repair buffer, dNTP mix containing 2.5 mM each of dATP, dCTP, dGTP, dTTP, 10 mM ATP, 2 µl End- repair enzyme mix (including T4 DNA Polymerase and T4 Polynucleotide Kinase) and 20 µg insert DNA. The end-repair reaction was incubated at room temperature for 45 min and the enzymes were inactivated by incubation at 70°C for 15 min. The DNA was precipitated using 0.1 vol 3 M NaOAc (pH 7.0) and 2.5 volumes of ice-cold absolute ethanol. After incubation at -20°C for 30 min, the sample was centrifuged at 13 000 × *g* for 20 min and the pellet was resuspended in 10 µl of TE buffer (pH 8.0). The DNA was quantified using a Qubit™ system (Invitrogen, Oregon, USA). Each ligation reaction contained 7 µl of insert DNA at a minimum concentration of 250 ng.

Superbac1 cloning vector was isolated from a 200 ml overnight LB culture containing 34 µg/ml chloramphenicol and incubated at 37°C with shaking. Plasmid DNA was isolated using a Midi-prep kit (Qiagen) and digested with *Hind*III. The digested vector was blunt ended using 1U of the Klenow restriction enzyme, followed by dephosphorylation and quantification using a Qubit. Each ligation reaction contained vector DNA at a minimum concentration of 50 ng.

The following reagents were added to a total volume of 20 µl in the order given in the manual, with gentle mixing after each addition: 2 × Fast-Link ligation buffer, 1 µl 10 mM ATP, 3 µl Superbac vector (80 ng), 9 µl concentrated DNA insert, and 1U Fast-Link DNA ligase. The reaction was incubated at room temperature for 1 hour, transferred to 70°C for 15 min to inactivate the ligase and stored at -20°C. Competent EPI-300 cells were transformed by electroporation with 10 µl of the ligation reaction. SOC broth (1 ml) was

immediately added and the cells were allowed to recover for 1 hour at 37°C prior to plating onto LB containing 34 µg/ml chloramphenicol. In order to determine the average insert sizes, BACs were extracted from six transformants using a Midi-prep kit (Qiagen), followed by digestion with 0.5 U *Not*I restriction endonuclease.

3.6.3 Small insert library construction

Genomic DNA was partially digested with *Sau*3A1 and separated by agarose gel electrophoresis. DNA fragments in the size range 5-10 kb were excised from the gel, purified using the QIAquick Gel Extraction kit (Qiagen, USA) and ligated into a ready to use positive selection cloning pJET 1.2/blunt vector (Fermentas). The ligation reactions were incubated overnight at 4°C and transformed into electrocompetent *E.coli* Genehogs cells. After electroporation the cells were plated on LB agar containing 100 µg/ml ampicillin and incubated overnight. The recombinant clones were clearly identified as white colonies on the agar plate.

To estimate the average insert size of the transformed clones, a few transformants were randomly selected from the agar plate and were inoculated into 5 ml of LB containing 100 µg/ml ampicillin. Cultures were incubated at 37°C overnight with shaking. The plasmid DNA was isolated using a standard Mini-prep kit (Qiagen). The DNA inserts were excised from the vector using *Not*I restriction enzyme and analysed on 1% agarose gel. Gels were visualised using the Alphaimage system. For maintenance, the colonies were inoculated into 100 µl LB containing 100 µg/ml ampicillin in a 96 well-plate. The plates was incubated overnight at 37°C with shaking and kept at 4°C for later analysis.

Library screening was based on plate assays. Positive transformants were screened for ligninase (lignin peroxidase and laccase) activity by plating on media containing either Azure B or ABTS as a substrate, as described in section 2.3.2. For laccase activity, the medium contained 1 mM ABTS and 10 µM $CuSO_4$, the pH of the LBA was adjusted to pH's 5.0; 7.0 and 9.0, and the clones were incubated at 37°C overnight. Laccase positive clones would appear dark green/purple in colour, while negative clones would remain whitish in colour.

The genomic libraries were maintained as a cell suspension at -80°C in 15% (v/v) glycerol (Sambrook *et al*., 1989). Inserts from randomly selected clones were sequenced by primer walking. Sequencing primers were designed using DNAMAN Version 4.13 (Lynnon Biosoft), synthesised at Inqaba Biotech (South Africa) and sequenced at the University of Stellenbosch sequencing facility using an ABI PRISM automated DNA sequencer.

Table 3.2. Primers designed in this study for primer walking analysis to obtain the full length gene.

Primer	**Sequence (5' – 3')[a]**
MNAKA 31F	**ACATGTCAAAGCAAGCGC**
MNAKA 31R	**GCTAAAGATGCTGCAGAG**
MNAKA 70F	**TTAGTACGAGAGGACCGG**
MNAKA 70R	**GCTGGTGTACCAGTTGTC**

Plasmid DNA was extracted from two LiP positive clones (CPM31 and CPM70) using the Mini-prep kit (Qiagen). End sequences were obtained by sequencing with M13F and M13R primers. Sequencing primers were designed from the end sequences (Table 3.2). PCR amplicons were generated using the plasmid DNA as the template. The 20 µl reaction volume contained 50 ng (2 µl) purified plasmid DNA, 1 × Dream*Taq* buffer containing $MgCl_2$, 2U Dream*Taq* polymerase, 0.2 mM of each dNTPs, and 0.5 µM of each primer. Amplicons were analysed by electrophoresis in a 0.8% agarose gel at 302 nm UV. DNA fragments were excised from the gel and purified using the GFX kit. The purified DNA was ligated into a pJET 1.2/blunt vector and transformations were performed as described in section 3.6.3.

The selected recombinant clones were analysed by colony PCR (Fermentas) performed according to the standard method. The PCR programme included an initial denaturation step at 96°C for 3 min, followed by 25 cycles of denaturing at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for a minute, followed by final extension at 72°C for 5 min. The presence of the PCR product was analysed on a 0.8% agarose gel purified, using the GFX kit and the purified DNA was sequenced at the University of Stellenbosch.

Chapter 4 Results and discussion

The composting process involves the decomposition of organic material by microorganisms into carbon dioxide, biomass and thermal energy. The organic matter is made up of components such as cellulose, proteins, lipids and lignin (Toumela *et al*., 2000), and the degrading organic matter provides food for the colonising microorganisms. Microbial taxa most frequently isolated from compost include *Bacillus* species (Strom, 1985), members of the order *Actinomycetales* (Beefa *et al*., 1996) and *Thermus* species (Mc Caig *et al*., 2001), as well as fungi, predominantly *Aspergillus* species (Song *et al*., 2001). Despite the elevated temperatures inside the compost heap, microorganisms are able to survive and the microbial populations vary over time as the temperature fluctuates. Compost serves as an ideal habitat for microbial colonization as it is nutrient rich, moist, provides carbon- and nitrogen sources, and heat (Yang *et al*., 2007). This project describes the characterisation of a novel lignin peroxidase producing *Bacillus* strain isolated from thermophilic straw-based compost.

4.1 Isolation of bacteria from thermophilic compost

Compost samples were collected from Medallion Mushroom farm, outside Stellenbosch, South Africa. The temperature of the compost heap was 71°C at the time of sampling. Compost was incubated in an isotonic solution prior to dilution and plating to dislodge the microorganisms adhered to compost particles. The isotonic solution served to protect microorganisms against cell lysis. The microorganisms present within the compost were isolated on TSA supplemented with cycloheximide to suppress fungal growth. Cycloheximide is a protein synthesis inhibitor that acts on the 60S subunit of the eukaryotic ribosome (Obring *et al*., 1971). Tryptic casein soy agar (TSA) was selected as the isolation medium as it is nutrient rich and supports the growth of a wide variety of non-fastidious, heterotrophic microorganisms (MacFaddin, 1985). Bacterial growth was observed within a few days incubation at all temperatures tested, with increase in temperature (Figure 4.1.1 X axis) and dilution factor (Y axis). Interestingly, based on morphology neither fungi nor actinobacteria were isolated.

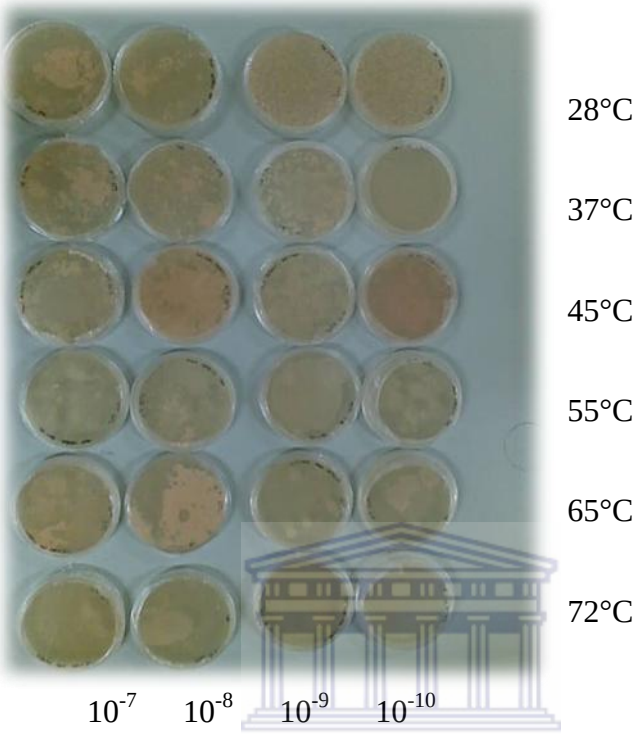

Figure 4.1.1 Isolates from the straw-based compost at different temperatures grown on TSB agar plates. Compost samples were diluted up to 10^{-10} in an isotonic solution prior to plating.

As bacterial numbers were high, the 10^{-10} (with an average of 1.28×10^{8} CFU/ml) dilution was used for isolation as the bacterial colonies were well distributed at this dilution. A range of isolation temperatures (28°C to 72°C) was included to favour the growth of both mesophilic and thermophilic bacteria. Bacterial growth was observed within a few days of incubation at all temperatures tested (Fig. 4.1.1) with most of the isolated bacteria appearing to favour the more moderately thermophilic temperatures (37°C and 45°C), while limited bacterial growth occurred at temperatures above 55°C. Bacterial selection

was based on morphology. In total, 29 pure isolates with different morphologies were selected from the isolation plates incubated at 45°C.

4.2 Screening for general peroxidases

All 29 compost isolates were screened by the general peroxidase plate assay using amino-antipyrine as the substrate (Figure 3.2.1). Of these, 21 tested positive for general peroxidase activity with the phenol- 4 amino-antipyrine assay. Peroxidase positive isolates were subsequently tested for lignin peroxidase activity using the Azure B assay (Figure 3.2.2). Isolate CP11was the only isolate positive for lignin peroxidase activity.

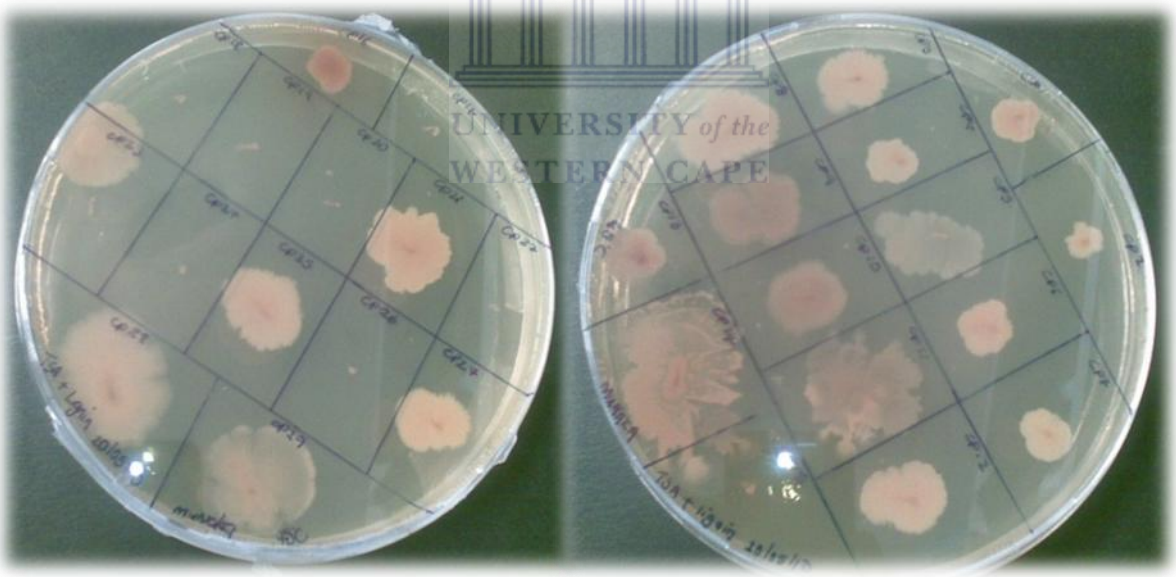

Figure 4.2.1 Isolates from thermophilic compost were screened for peroxidase activity on alkali lignin TSA plates with 4-amino-antipyrine as a substrate. Bacterial growth indicates peroxidase activity, while no growth indicates an inability to produce peroxidases.

While several substrates can be used to screen for peroxidases, Azure B assay is specific for lignin peroxidase activity (Archibald, 1992), where a change in colour from dark blue to yellowish green signifies the bio-oxidation of Azure B dye by a microorganism.

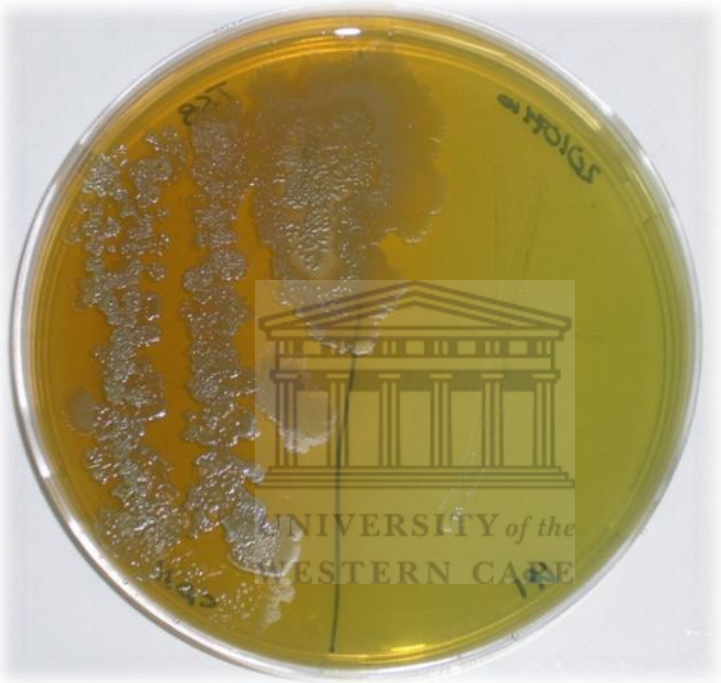

Figure 4.2.2 Growth of isolate CP11 on TSA containing Azure B dye (colony on the left) resulted in a colour change from blue to yellowish green indicating the oxidation of the Azure B dye by the isolate. The negative control (right) shows no colour change.

4.3 Characterisation of isolate CP11

4.3.1 Morphological characterisation

Morphological characterisation was determined by Gram staining (Figure 4.3.1.1). Isolate CP11 was a Gram positive bacilli with rounded ends, occurring singly, in pairs or in short chains (3-5 cells).

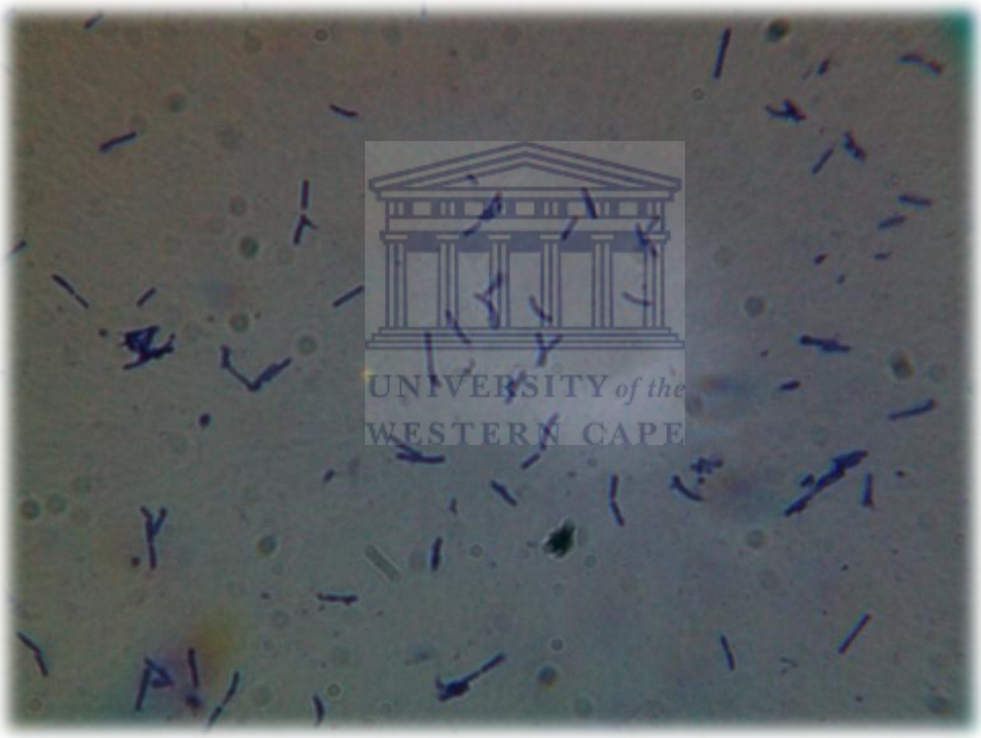

Figure 4.3.1.1 Gram morphology of isolate CP11 as seen under the microscope (magnification 100× with oil immersion).

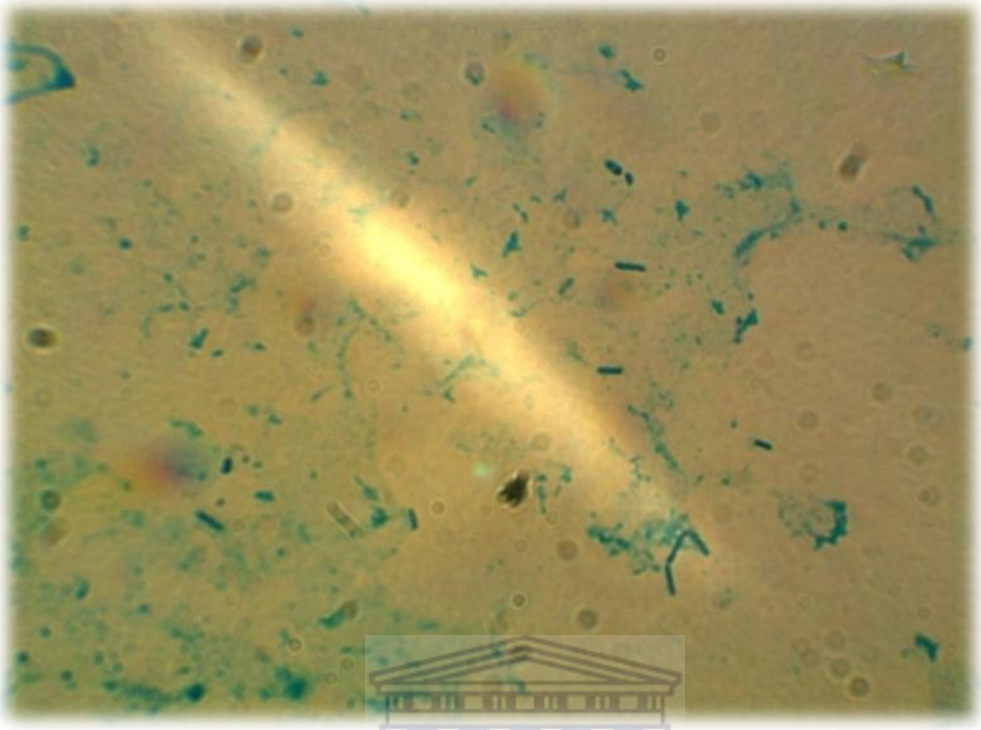

Figure4.3.1.2 Endospores produced by isolate CP11 stained green with malachite green as seen under a light microscope (magnification 100× with oil immersion).

Isolate CP11 produced endospores visible as thick, short, green bacilli when stained with malachite green (Figure 4.3.1.2). While resistant to Gram staining, endospores stain green with malachite green, while the vegetative tissues appears as pinkish-red cells as they take up the Safranin counterstain. Endospores are mostly formed at times of nutritional stress, allowing the organism to persist in the environment until conditions become favourable as endospores provide resistance to harsh environmental conditions such as heat, acid and salt. Several *Bacillus* and *Clostridium* species are known to form endospores (Nicholson *et al*., 2000).

4.3.2 Physiological characterisation

API (Analytical Profile Index) tests

Analytical Profile Index (API) is a standard system for identifying bacteria and consists of strips, each with 20 microtubes containing dehydrated substrates which are inoculated with a bacterial cell suspension. Active metabolism results in a pH change which is visualised by a colour change (Figure 4.3.2.1). The results obtained for isolate CP11 are presented in Table 4.1.

Figure 4.3.2.1 Isolate CP11 induced metabolic reactions in API microtubes after overnight incubation at 37°C.

Table 4.1. Physiological characterisation of isolate CP11 using the API strips.

TEST	SUBSTRATE	REACTION TESTED	NEGATIVE RESULT	POSITIVE RESULT
ONPS	ONPG	Beta galactosidase		X
ADH	Arginine	Arginine dihydrolase		X
LCD	Lysine	Lysine decarboxylase		X
ODC	Ornithine	Ornithine decarboxylase		X
CIT	Citrate	Citrate utilisation	X	
H2S	Na thiosulfate	H2S production	X	
URE	Urea	Urea hydrolysis	X	
TDA	Tryptophan	Deaminase	X	
IND	Tryptophan	Indole production	X	
OVP	Na pyruvate	Acetoin production	X	
GEL	Charcoal gelatine	gelatinase		X
GLU	Glucose	Fermentation/oxidation		X
MAN	Mannitol	Fermentation/oxidation		X
INO	Inositol	Fermentation/oxidation		X
SOR	Sorbitol	Fermentation/oxidation		X
RHA	Rhamnose	Fermentation/oxidation		X
SAC	Sucrose	Fermentation/oxidation		X
MEL	Melibiose	Fermentation/oxidation		X
AMY	Amygdalin	Fermentation/oxidation		X
ARA	Arabinose	Fermentation/oxidation		X

Isolate CP11 tested positive for carbohydrate utilisation including glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygladin and arabinose. These substrates were used oxidatively as carbon and energy sources. Urea was not hydrolysed. Isolate CP11 utilised arginine, lysine and ornithine as a sole nitrogen source. Indole, acetoin and deaminase tests were negative. Hydrogen sulphide was not produced. Isolate CP11 was found to be catalase positive (Figure 4.3.2.2).

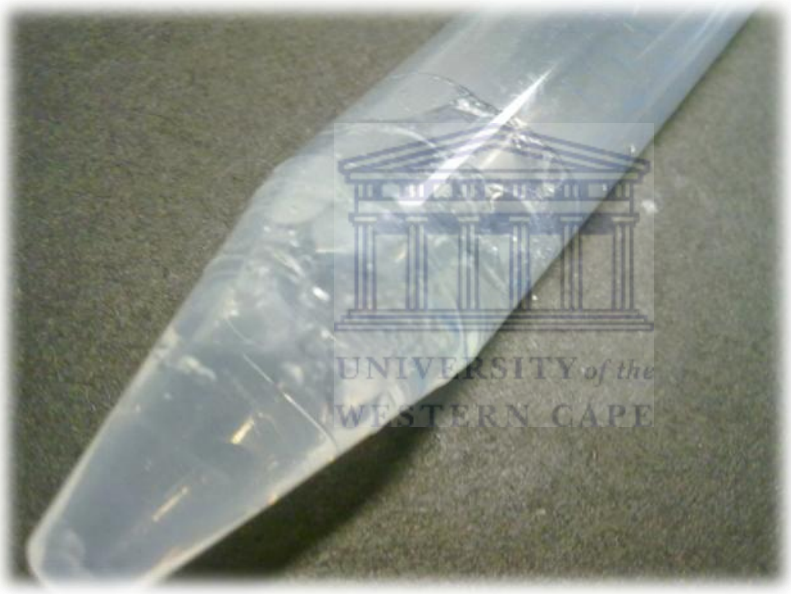

Figure 4.3.2.2 Results from the catalase test. Bubbles indicate the release of oxygen and are a result of the interaction between the isolate and hydrogen peroxide.

The production of catalase is found ubiquitously in Gram positive bacteria, since the enzyme neutralises the bactericidal effects of H_2O_2. As a result, the bacterium is able to repair and escape oxidative damage caused by free radicals.

Table 4.2. Physiological characteristics of isolate CP11 in comparison to three other *Bacillus* strains.

Characteristics	1	2°	3*	4 #
Temperature optimum	45°C	50°C	45°C	55°C
pH optimum	7	6.5-7.5	7	8
Gram staining	+	+	+	+
Cell morphology	Rod	Rod	Rod	Rod
Colony pigmentation	White	White	White	White
Acid fast staining	-	ND	-	ND
Endospore staining	+	ND	+	+
Motility	-	+	+	+
Anaerobic growth	+	+	+	-
Gelatine hydrolysis	-	+	+	ND
Casein hydrolysis	No growth	+	+	+
Starch hydrolysis	-	+	+	-
Lipase activity	+	-	-	-
Catalase activity	+	+	+	+

Taxa are indicated as: 1, Isolate CP11; 2, *Bacillus thermoamylovorans*; 3, *Bacillus subtilis* and 4, *Bacillus alveayuensis*. Characteristics are scored as: +, positive; -, negative; ND not determined. Data was obtained from: ° Combert-Blanc *et al* (1995), *Todar (2011) and #Bae *et al.,* (2005).

Morphological and physiological characteristics of CP11 are shown in Table 4.2. These are consistent with the already published characteristics for members of the genus *Bacillus*. The characteristics of the isolate were compared with *Bacillus thermoamylovorans* (Combert-Blanc *et al*., 1995), *Bacillus subtilis* (Todar, 2011) and *Bacillus alveayuensis* (Bae *et al.*, 2005). The isolate can be differentiated from *Bacillus thermoamylovorans* by its inability to hydrolyse starch, gelatine and casein. In comparison with the other three strains, the colonies of the isolate were white, circular and the edges were entire after several days of incubation.

Isolate CP11 was able to grow under both aerobic and anaerobic conditions, probably due to a relatively high catalase activity. Growth was apparent between of 37°C and 55°C, with optimal growth at 45°C. The pH range supporting growth stretched from pH 5.0 to pH 11, with optimal growth at pH 7.0. These growth parameters are similar to those reported for other *Bacillus* species. Phenotypic differences between isolate CP11 and *Bacillus subtilis* and *Bacillus alveayuensis*, include the fact that CP11 is non-motile, and lacked gelatinolytic and caseinolytic activity.

4.3.3 Phylogenetic analysis of isolate CP11

The isolate's genomic DNA (Figure 4.3.3.1) was successfully extracted from an overnight culture using the method described by Zhou *et al.* (1996). High molecular weight DNA greater than 14.0 kb was purified from the agarose gel and the concentration determined by fluorimetry. The 16S rRNA gene was amplified using universal eubacterial primers F1 and R5 (Figure 4.3.3.2). A single amplicon of the correct size (1400 bp) was obtained. The PCR products were cloned into pGEM-T-Easy vector. Recombinant clones with the correct insert size were identified using colony PCR with M13 primers (Jin *et al*., 2007) (Table 4.1).

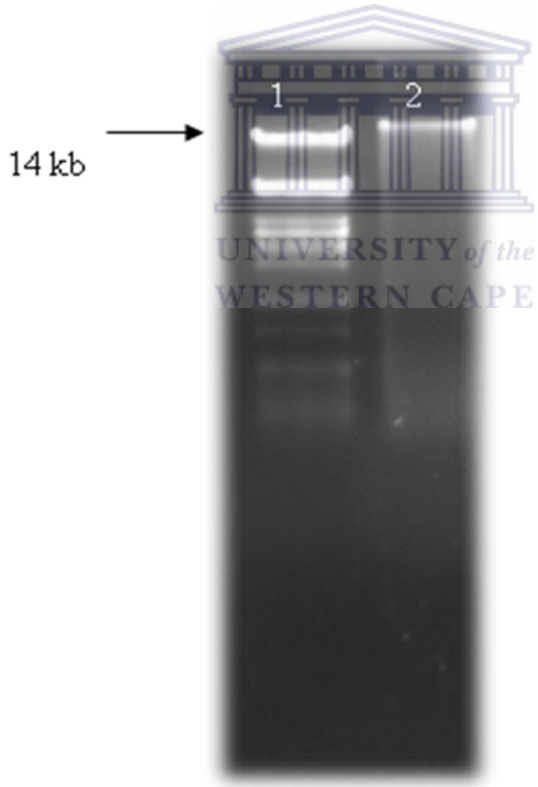

Figure 4.3.3.1 Agarose gel (0.8%) showing total genomic DNA of isolate CP11. Lane 1- DNA molecular weight marker, λ*Pst*1 digest DNA. Lane 2- Isolate CP11 genomic DNA.

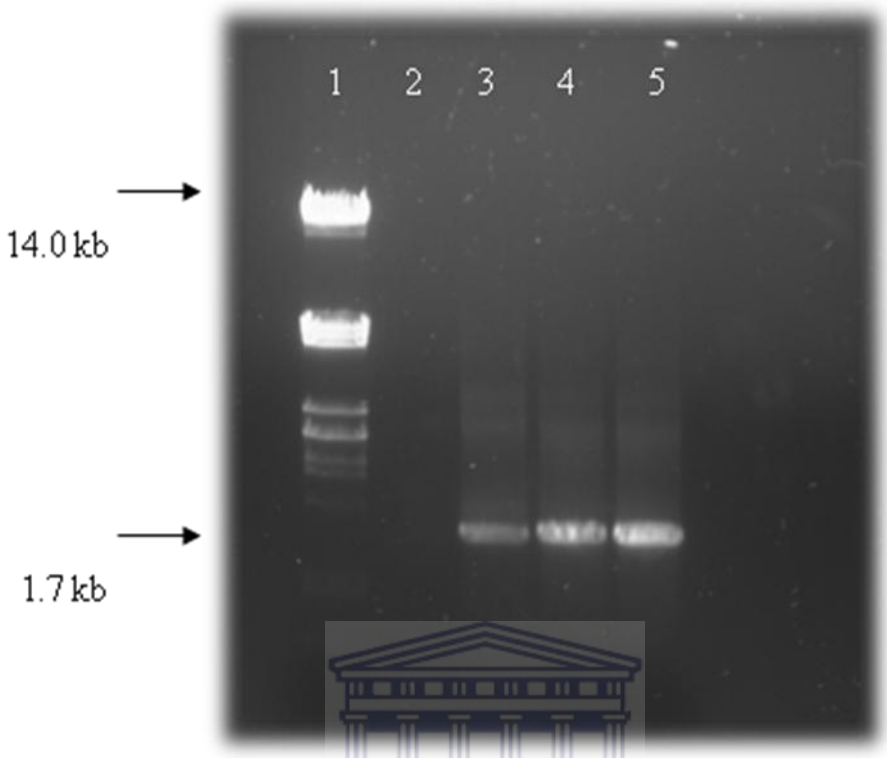

Figure 4.3.3.2 Agarose gel (0.8%) showing PCR amplification of bacterial 16S rRNA gene using primers F1 and R5. Lane 1- DNA molecular weight marker, λ*Pst*1 digest DNA. Lane 2- negative control. Lane 3- 16S rDNA amplicon from *E.coli* genomic DNA. Lane 4 and 5- 16S rDNA amplicons from isolate CP11.

To ensure a single 16S rDNA amplicon had been cloned, Amplified rDNA Restriction Analysis (ARDRA) was performed. The M13 colony PCR amplicons were subjected to ARDRA using *Alu*I and *BsuR*I restriction enzymes (Figure 4.3.3.3).

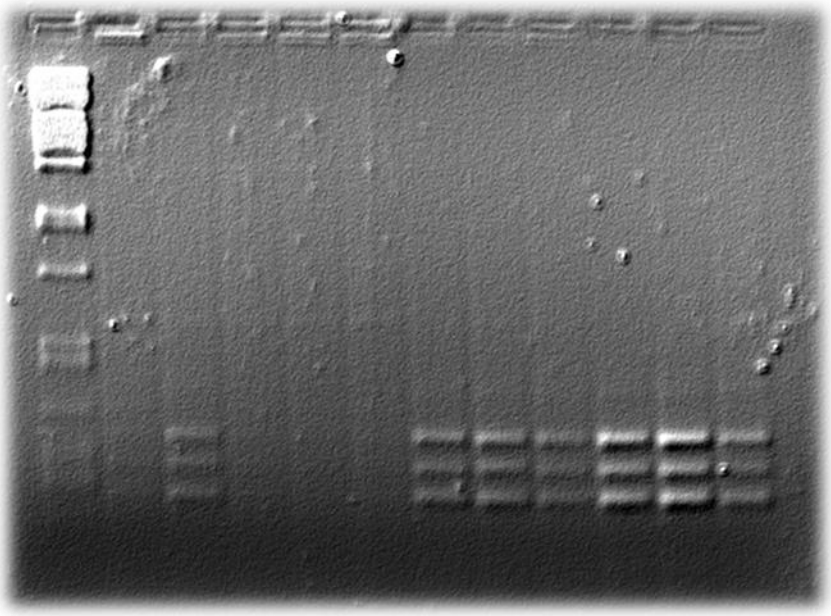

Figure 4.3.3.3 Agarose gel (1%) showing Amplified rDNA Restriction Analysis (ARDRA) patterns of colonies isolated from pGEM-T-Easy cloning.

The 16S rRNA gene sequence for isolate CP11 was compared to existing sequences in the NCBI nucleotide database using BLAST (BLAST*n*). Based on sequence analysis, strain CP11 was assigned to the genus *Bacillus*. CP11 was found to be most closely related to *Bacillus thermoamylovorans* with 93% sequence similarity (Table 4.3). *B. thermoamylovorans* was first isolated from a palm wine sample in Senegal (Combert-blanc *et al*., 1995). It was described as a non-spore forming, moderately thermophilic, facultatively anaerobic, Gram positive bacterium. This amylolytic bacterium is heat resistant, supporting the finding that isolate CP11 is a thermophilic microorganism. As the standard level of 16S rRNA gene sequence similarity to define novel bacterial species is 97% sequence similarity (Wayne *et al.,* 1987), isolate CP11 can be recognised as a unique genomic species. This hypothesis is supported by the fact that CP11's phenotypic characteristics clearly distinguish it from *B. thermoamylovorans*.

Table 4.3. 16S rRNA gene sequence similarities obtained by BLAST analysis for isolate CP11 showing its closest relatives.

SEQUENCE LENGTH (bp)	CLOSEST RELATIVE	% IDENTITY	ACCESSION NUMBER
1475	**Uncultured compost bacterium**	**96**	**AB034711**
1485	***Bacillus thermoamylovorans***	**93**	**AB360823**
1440	***Bacillus species***	**93**	**AJ586361**
1473	***Bacillus circulans***	**92**	**Y13065**
1093	***Geobacillus pallidus***	**90**	**HQ324908**
1433	***Proteobacterium***	**89**	**EU250939**
1432	***Bacillus kaustophilus***	**88**	**X60618**
1523	***Bacillus thermoglucosidasius***	**85**	**AB021197**
1435	***Bacillus amylolyticus***	**82**	**X60606**

Using 16S rRNA gene primers, an almost complete sequence consisting of 1442 bp was determined. A neighbour joining phylogenetic tree (Figure 4.3.3.4) was constructed using MEGA version 4. Bootstrap values provided a measure of the reliability of phylogenetic analysis.

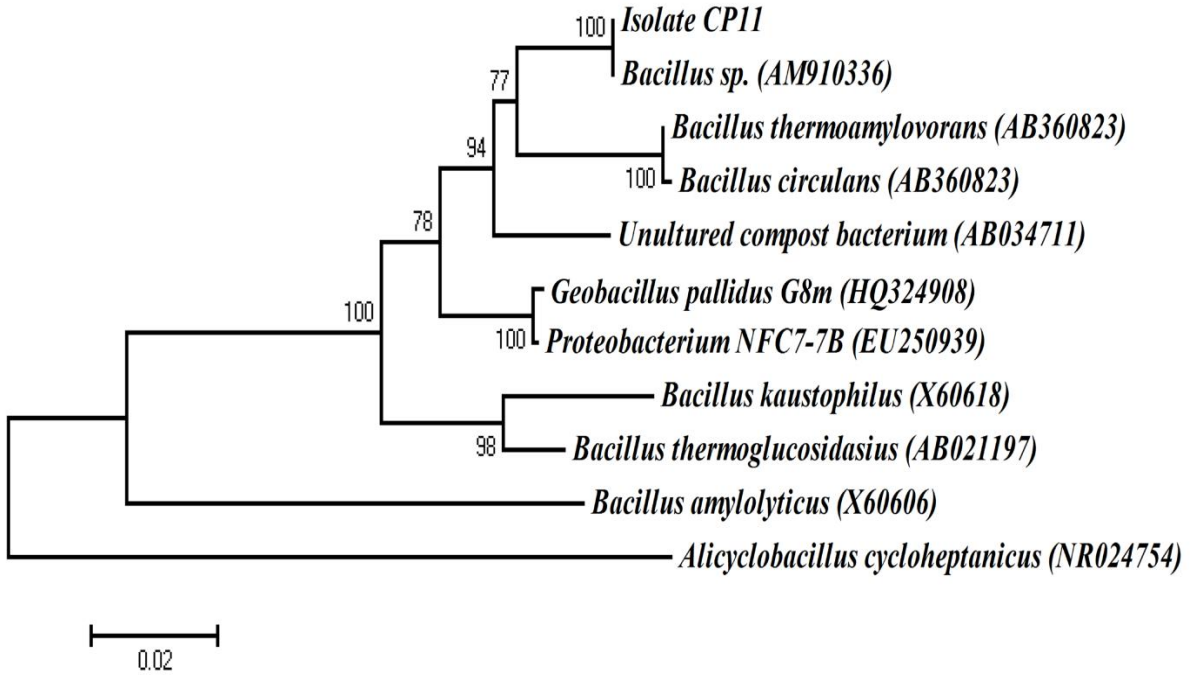

Figure 4.3.3.4 A neighbour joining phylogenetic tree based on the aligned 16S rRNA gene sequences of isolate CP11 and its nearest phylogenetic neighbours. Bootstrap values (expressed as percentages of 1000 replications) are shown at the nodes. *Alicyclobacillus cycloheptanicus* was used as the outgroup. The scale bar of the tree represents a 0.2% difference in nucleotide sequences.

A nearly complete 16S rRNA gene sequence (1442bp) was obtained for isolate CP11. Comparison of the 16S rRNA gene sequence to related members of the family *Bacillaceae* revealed that isolate CP11 was most closely related to a cluster comprising of *Bacillus* species, with *Bacillus thermoamylovorans* (AB360823) (Figure 4.3.3.4) being its closest phylogenetic neighbour, followed by *Bacillus circulans*. These three strains formed a monophyletic clade in the phylogenetic tree, constructed with the neighbour joining algorithm.

4.4 Lignin Peroxidase activity screening

4.4.1 Growth parameters

A growth curve was generated at 45°C for isolate CP11 over 96 hours, taking OD readings at 12 hourly intervals (Figure 4.4.1.1). Samples were assayed for ligninase activity at each sampling point as it was essential to determine at what stage in the growth cycle enzyme production occurred. Ligninase activity was detected in the supernatant (blue line) and not in the cell free extracts (yellow line).

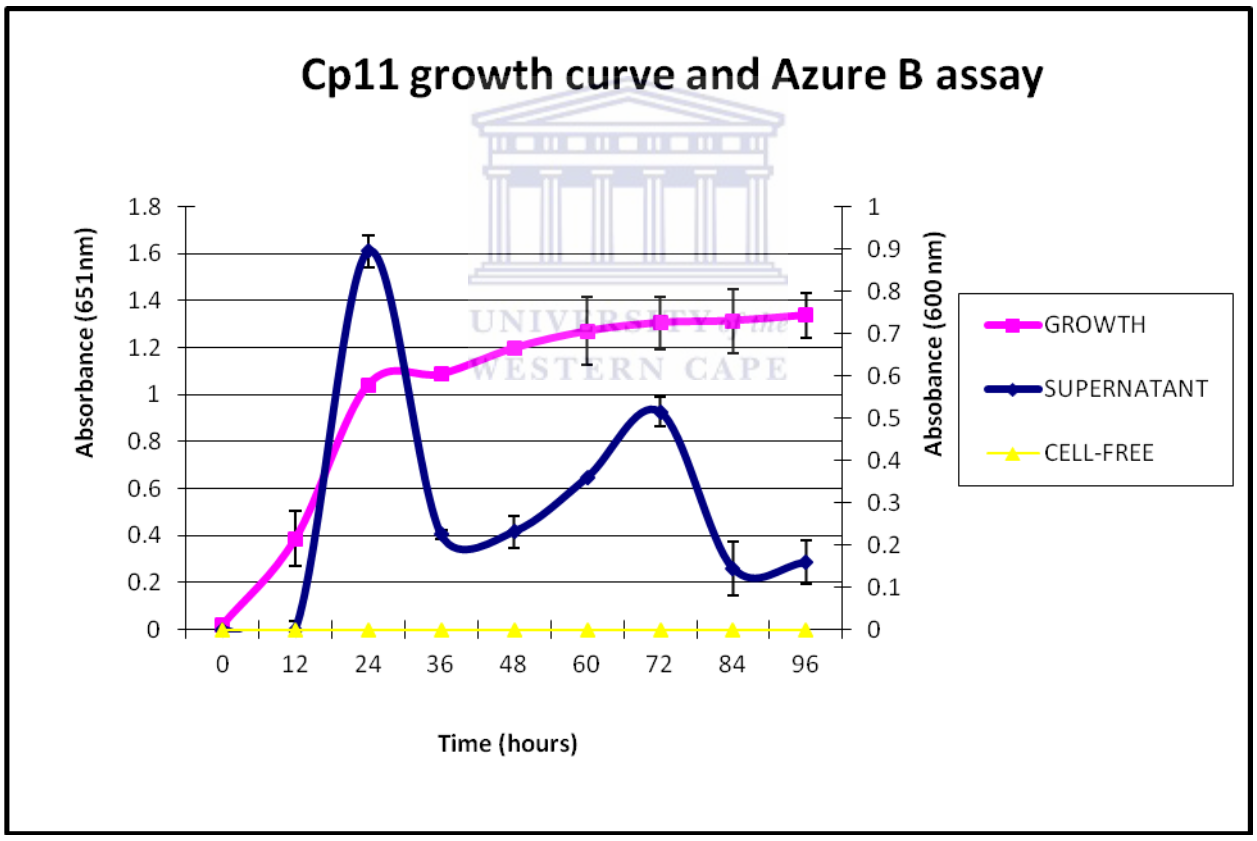

Figure 4.4.1.1 Growth curve of CP11 isolate (pink line), with enzyme activity from the supernatant (blue line) and the cell free extract (yellow line).

Enzyme production in the supernatant increased during log phase and peaked after 18-24 hours of incubation. A second activity peak formed during stationary phase, peaking after 72 hours. This could indicate the presence of more than one lignin peroxidase or the production of the same enzyme at different stages of growth.

The colourimetric assay used for the determination of lignin activity was the Azure B assay. Compared to utilisation of substrates such as L-DOPA and DCP, the assay is relatively economical and free from interference caused by UV-absorbing materials such as lignins, phenolics and quinonic compounds. The disappearance of the substrate is measured as a decrease in OD which is caused by a hyperchromic shift of the visible absorbance peak of the substrate (Archibald, 1992).

Considering its complex structure, lignin is likely degraded by numerous enzymes that cleave at different parts of the lignin structure. Compared to fungal lignin degrading enzymes, the enzymology of bacterial lignin degradation is poorly understood. Nonetheless, several reports suggest that bacteria use similar types of extracellular lignin degrading enzymes as fungi (Bugg *et al*., 2010).

4.4.2 Crude protein analysis

Isolate CP11's ligninase activity was detected using Azure B as a substrate. The proteins were precipitated using ammonium sulphate from both the culture supernatant and cellular extract. After precipitation, salts were removed by dialysis using a dialysing cassette. Following dialysis of the supernatant and cell free extract, preliminary crude enzyme assays were performed using the Azure B liquid assay. However, no lignin peroxidase activity could be detected in the dialysed cell free extract or the supernatant (Section 3.4.3).

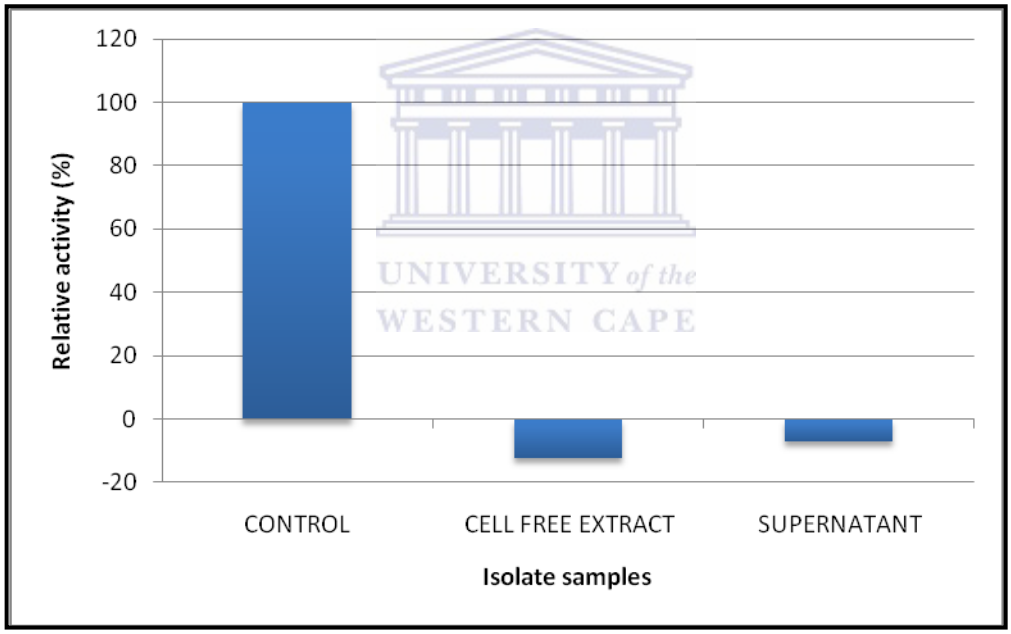

Figure 4.4.2.1 Relative activity of the lignin peroxidase, determined by the Azure B assay, after dialysis of the cell free extract and supernatant. Undialysed supernatant was used as a control sample.

No enzyme activity was observed for either the dialysed cell free extract or the supernatant. Azure B was not oxidised when the enzyme preparation or H_2O_2 was omitted from the assay. One possible explanation for this finding (no activity) is that the isolate is producing H_2O_2 which is required for enzyme activity. While most of the H_2O_2 produced is likely to be broken down by the catalase, small amounts of H_2O_2 may still be present in the crude extracts. The H_2O_2 would be removed during dialysis which may account for the observed loss of activity. Therefore, to exclude the possibility that the lack of activity was due to the absence of H_2O_2, the assay was repeated including H_2O_2, and both extracts again tested negative. Another explanation is that the observed loss of activity is due to enzyme instability or denaturation of the protein in the dialysis buffer leading to enzyme inactivation (Have *et al*., 1998).

4.5 Molecular analysis

4.5.1 Superbac library construction

Super bacterial artificial chromosome (BAC) is a modified pBeloBAC1, and it is one of the vectors used for large insert library construction. BACs have been used for large scale gene discovery, and annotation of gene function and regulation (Farrar and Donnison, 2007) as BAC vectors stably take up large pieces of insert DNA ranging from 50 kb to over a 100 kb.

One of the research goals of this study was to clone the gene responsible for the observed lignin degrading activity seen for isolate CP11. High molecular weight DNA was extracted and digested with *Hind*III. Fragments were cloned into the *Hind*III site of the Superbac1 vector. The BAC library was constructed using 5:1, 1:1 and 1:5 ratios of vector to insert DNA, and 5 µl of the ligation mixture yielded between 200 and 2400 recombinant clones.

Several parameters were evaluated for the quality of the BAC library, including the insert size (Figure 4.5.1.1), integrity of the library and the transformation efficiency.

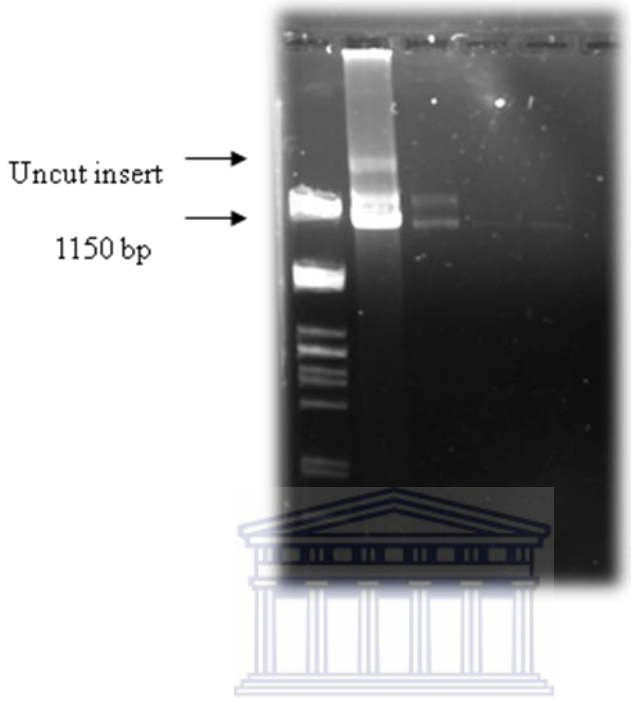

Figure 4.5.1.1 Analysis of randomly selected Superbac 1 clones showing no insert DNA with a control (lane 2) of an uncut insert background. DNA of the clones was released by *Not*I digestion and run on a 1% agarose gel.

During the analysis of randomly selected Superbac1 clones, plasmids were extracted using a Qiagen Midi kit with the protocol that has been adapted for very low copy plasmid extraction. The clones were evaluated using *Not*I restriction enzyme digestion and analysed by gel electrophoresis (Figure 3.5.1.1). However, analysis of the agarose gels showed that all clones did not include insert DNA. Possible explanations for this finding may be that the initial modifications including blunting and dephosphorylation may have been unsuccessful or the ligation itself might have failed. Additionally, as the Superbac is a low copy number vector, it was difficult to isolate sufficient vector DNA, even though larger cultures were used for the

extractions. Therefore, in order to circumvent these challenges it was decided to construct a small insert library in an *E. coli* host.

4.5.2 Small insert library

Genomic DNA was partially digested with *Sau*3A1 restriction endonuclease and fragments were size fractionated on agarose gel. Fragments in the range 5-10 kb were excised from the gel, purified and cloned into pJET 1.2 blunt vector (Figure 3.7.2.1).

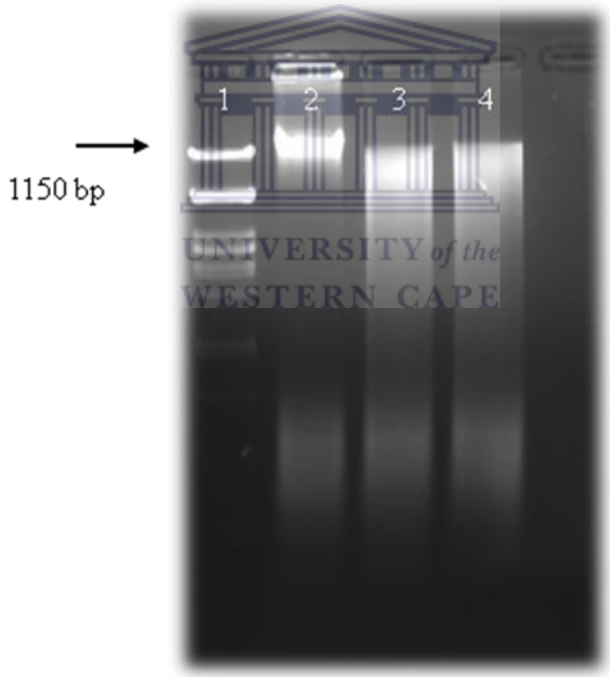

Figure 4.5.2.1 Agarose gel (1%) containing samples of genomic DNA digests performed with increasing time using 0.1U *Sau*3A1 restriction endonuclease. Lane 1- DNA molecular weight marker, λPst digest DNA. Lane 2- undigested genomic DNA. Lane 3 and 4- Genomic DNA (5µg) was digested for 18 min and 22 min, respectively.

To determine the average insert size several recombinant pJET 1.2 clones were randomly selected and the plasmid DNA was digested with *Not*I restriction endonuclease. Insert sizes were found to range from 1700 bp to 3000 bp (Figure 4.5.2.2).

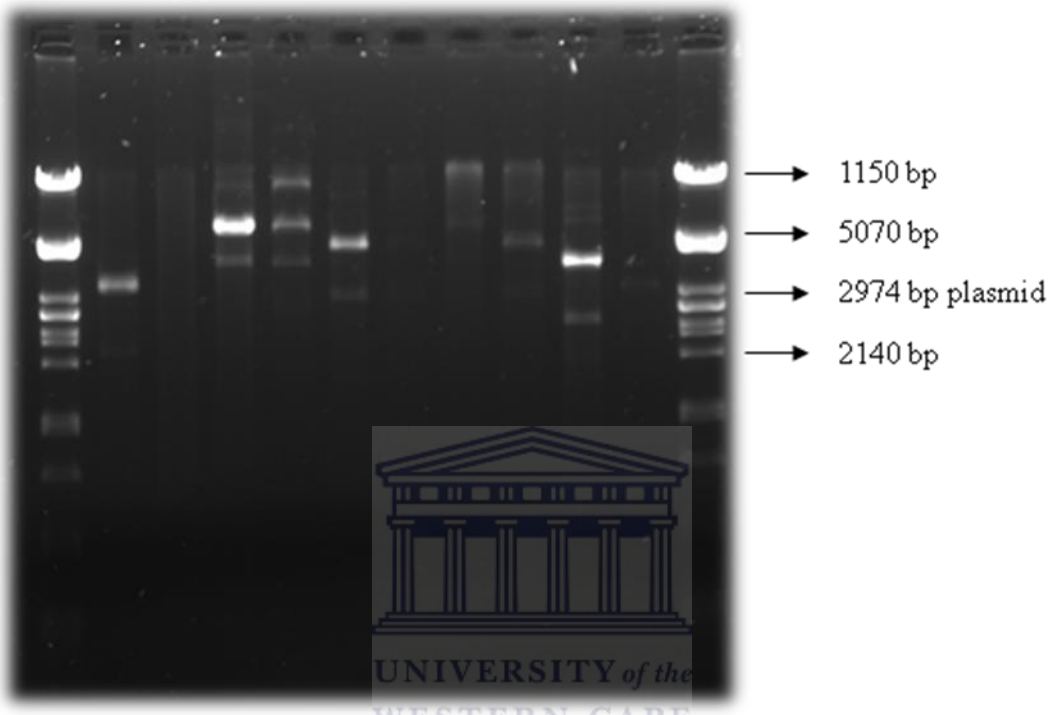

Figure 4.5.2.2 Restriction analysis of pJET 1.2/ isolate CP11 constructs. Plasmid DNA was restricted with *Not*I for a 5 min. The 2974 bp plasmid backbone is present. λ*Pst* marker digest DNA was used as a molecular marker.

The small insert library consisted of 147 recombinant clones with an average insert size of 2.3 kb. Therefore, it was estimated that less than 340 kb of genomic DNA had been cloned, accounting for less than 10% of a *Bacillus* genome (Ye *et al*., 2007). The clones were assayed for ligninase activity using Azure B and ABTS as substrates to screen for lignin peroxidases and laccases, respectively. A positive result is indicated by a change in medium colour due to the oxidation of the substrates by the clones (Figure 4.5.2.3). Of the 147 recombinant clones

tested, 15 exhibited significant activity on Azure B, while none of the clones oxidised the ABTS.

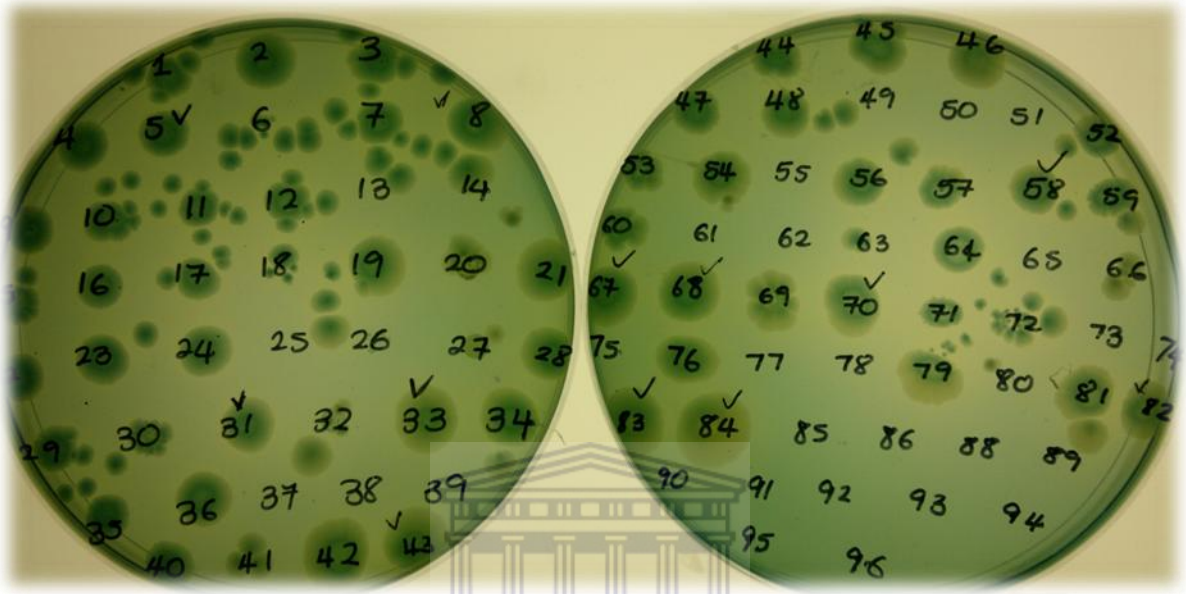

Figure 4.5.2.3 the recombinant clones showing growth on TSA containing Azure B dye. Some of the clones resulted in a colour change from blue to yellowish green indicating the oxidation of the Azure B dye. The negative recombinant clones show no colour change.

Two lignin peroxidase clones were selected and end sequences were obtained with M13F and M13R primers. These sequences were used to design sequencing primers in order to sequence the full length insert by primer walking (Section 3.6.3). Primer walking allows one to sequence/PCR amplify a region of DNA without having prior knowledge of sequence beyond the targeted priming site. However, the procedure has low sensitivity and efficiency. Sequencing was performed on the original plasmids using primers designed in this study. However, all the sequences obtained were found to be mixed products. A possible explanation for this finding is that the primers were non-specific and bound to several sites on

the plasmid. Given that the library was so small and that after repeated attempts to sequence the gene of interest failed, it was decided to stop working on this library. Future work would therefore involve constructing a larger small insert library in order to sequence and clone the ligninase gene.

Chaper 5 Concluding remarks

Microorganisms play a vital role in the composting process and thermophilic compost heaps have been identified as a source of potentially novel thermophilic/thermotolerant organisms. Due to the recalcitrant nature of plant cell wall polymers, the biofuels industry requires robust, thermostable enzymes for the conversion of biomass into fuel (Mizuguchi *et al*., 2006). In this study, thermophilic bacteria were isolated from straw based compost sampled at a mushroom farm. All isolates were screened for general peroxidase activity using the 4-Aminoantipyrine plate assay. Amongst the 21 peroxidase positive isolates, only one isolate (CP11) subsequently tested positive for the production of lignin peroxidase on Azure Blue dye.

Phylogenetic analysis of the 16S rRNA gene revealed that isolate CP11 belonged to the genus *Bacillus*. The closest relative of isolate CP11 was *Bacillus thermoamylovorans* with a sequence similarity of 93%. While the physiological characteristics of isolate CP11 were similar to those reported for *Bacillus thermoamylovorans* (Combert-Blanc *et al*., 1995), isolate CP11 was able to form endospores, was motile and displayed lipase activity. In addition, CP11 was unable to hydrolyse gelatine, casein and starch. These phenotypic differences, combined with the low level of 16S rRNA gene sequence similarity suggest that strain CP11 be recognised as a unique genomic species. Studies have shown that the diversity of thermotolerant *Bacillus* species in compost heaps is fairly high at temperatures of up to 55°C, and decreases dramatically at temperatures higher than 60°C. The ability to produce endospores (as displayed by isolate CP11) may allow bacilli to withstand the higher temperatures within the compost heap (Combert-Blanc *et al*., 1995).

Preliminary protein characterisation of the crude enzyme extract was performed using Azure B as a substrate and in the presence of hydrogen peroxide. Lignin peroxidase activity was detected in the supernatant only and not in the cell free extract. However, activity was lost after ammonium sulphate precipitation and dialysis, possibly due to enzyme instability or incorrect folding of the enzyme in the dialysis buffer which leads to enzyme deactivation.

High molecular weight genomic DNA (above 14 kb in size) was extracted from isolate CP11 and a small insert library was constructed to identify the gene encoding the lignin peroxidase. The library was screened to identify the recombinant clones conferring lignin peroxidase activity on medium containing Azure B as a substrate. Fifteen clones were identified and two of these (CPM31 and CPM70) were selected for further study. End sequences were obtained for these two clones, however attempts to sequence the insert DNA by primer walking failed. Future studies would include the identification of the gene(s) responsible for lignin peroxidase activity and characterisation of the cloned gene product.

References

Abd-Elsam, H.E. and El-Hanafy, A.A. (2009). Lignin biodegradation with ligninolytic strain and comparison of *Bacillus subtilis* and *Bacillus sp*. isolated from Egyptian soil. *Agriculture and Environmental Sciences*. **5**: 39-44

Alper, H. and Stephanopolous, G. (2009). Engineering for biofuels: exploiting innate microbial capacity or importing biosythentic potential. *Nature Reviews*. **71**: 715-723

Alzate, C.A. and Toroo, J. (2006). Energy consumption analysis of intergrated flow sheets for production of ethanol from lignocellulosic biomass. *Journal of Energy*. **31**: 2447-2459

Anderson, J.A., Huprikar, S.S., Kochian, L.V., Lucas, W.J. and Gaber, R.F. (1992). Functional expression of a probable *Arabidop**sis** thaliana* potassium channel in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences*. **89**: 3736-3740

Antoni, D., Zverlov, V.V. and Schwarz, W.H. (2007). Biofuels from microbes. *Applied Microbiology and Biotechnology*. **73**: 23-35

Archibald, F.S. (1992). A new assay for lignin type peroxidase employing the dye Azure B. *Applied and Environmental Microbiology*. **58**: 3110-3116

Arias, M.E., Arenas, M., Rodriguez M., Soliveri, J., Ball, A.S. and Hernandez, M. (2003). Kraft pulp biobleaching and mediated oxidation of a nonphenolic Substrate by laccase from *Streptomyces cyaneus* CECT 3335. *Applied and Environmental microbiology*. **69**: 1952-1958

Baharuddin, A.S., Razak, M.N., Hock, L.S., Ahmad, M.N., Abdul-Aziz, S., Rahman, N.A., Shah, U.K., Hassan, M.A., Sakai, K., and Shirai, Y. (2010). Isolation and characterization of thermophilic cellulose-producing bacteria from empty fruit bunch-palm oil mill effluent compost. *American Journal of Applied Sciences*. **7**: 56-62

Barnes-Davies, R. (2009). 50 ways to help save the earth. *Westminster John Knox Press*. USA. **255**: 16-44

Beefa, T., Blanc, M., Lyon, P., Vogt, G., Marchiani, M., Fischer, J.L. and Aragno, M. (1996). Isolation of *Thermus* strains from hot composts (60 to 80°C). *Applied and Environmental Microbiology*. **62**: 1723–1727

Bitton, G. (2002). Encyclopaedia of Microbiology. Volume 3. John Wiley and Sons Publishers. New York. 3527 pp

Blottnitz, V.A. and Curran, M.A. (2006). A review of assessments conducted on bioethanol as a transportation fuel from a net energy, green house gas, and environmental life cycle perspective. *Cleaner Production*. **15**: 607-619

Blumer-Schuette, S. E., Kataeva, I., Westpheling, J., Adams, M. W. and Kelly, R. M. (2008). Extremely thermophilic microorganisms for biomass conversion: status and prospects. *Current Opinion in Biotechnolgy*. **19:** 210-217

Bon, E.P.S. and Ferrara, M.A. (1996). Bioethanol production via enzymatic hydrolysis of cellulose biomass. Food and agriculture organisation of the United Nations. Brazil. pp 1-11

Bourbonnais, R. and Paice, M.G. (1990). Oxidation of non-phenolic substrates: an expanded role for laccase in lignin biodegradation. *Federation of European Microbiology societies*. **267**: 99-102

Bugg, T.D.H., Ahmad, M., Hardiman, E.M. and Singh, R. (2010). The emerging role for bacteria in lignin degradation and bio-product formation. *Current Opinion in Biotechnology*. **22**: 1-7

Cartwright, A. (2007). Biofuels trade and sustainable development: An analysis of South African bioethanol. *The International Institute for Environment and Development*. pp 1-6

Chen, J., Zhang, W., Tan, L., Wang, Y. and He, G. (2009). Optimization of metablolic pathways for bioconversion of lignocellulose to ethanol through genetic engineering. *Biotechnology Advances*. **27**: 593-598

Claus, H. (2004). Laccases: structure, reactions, distribution. *Micron*. **35**: 93-96

Combet-Blanc, Y., Ollivier, B., Streicher, C., Patel, B. K. C., Dwivedi, P. P., Pot, B., Prensier, G. and Garcia, J.L. (1995). *Bacillus thermoamylovorans* sp. nov., a moderately thermophilic and amylolytic bacterium. *International journal of Systematic and Evolutionary Bacteriology*. **45**: 9–16

Cook, A.E. and Meyers, P.R. (2003) Rapid identification of filamentous actinomycetes to the genus level using genus specific 16S rRNA gene restriction fragment patterns. *International Journal of Systematic and Evolutionary Bacteriology*. **53**:1907-1915

Cowan, S.T. Steel, K.J. (1974). Manual for the identification of medical bacteria. Cambridge University Press. 146 pp

Dawkar, V.V., Jadhav, U.U., Telke, A.A. and Govihawar, S.P. (2009). Peroxidase from *Bacillus* sp. VUS and its role in the decolorization of textile dyes. *Biotechnology and Bioprocess Engineering*. **14**: 361-368

Dashtban, M., Schraft, H., Syed, T.A. and Qin, W. (2010). Fungal biodegradation and enzymatic modification of lignin. *International Journal of Biochemistry and Molecular Biology*. **1**: 36-50

DeAngelis, K.M., Allgaler, M., Chavarria, Y., Fortney, J.L., Hugenholtz, P., Simmons, B., Sublette, K., Silver, W.L. and Hazen, T.C. (2011). Characterization of trapped lignin-degrading microbes I tropical forest soil. *Applied and Environmental Microbiology*. **6**: 1-9

Deng, D., Guo, J., Zeng, G. and Sun, G. (2008). Decolorization of anthraquinone, triphenylmethane and azo dyes by a new isolated *Bacillus cereus* strain DC11. *International Journal of Biodeterioration and Biodegradation*. **62**: 263-269

Dengbo, M.A., Gao, P., Wang, Z., Hattori, T. and Shimada, M. (1994). Lignin peroxidase- catalyzed oxidation of monomeric lignin model substrates. *Wood Science*. **81**: 1-4

Dermibas, A. (2006). Progress and recent trends in biofuels. *Progress in Energy and Combustion Science*. **33**: 1-18

Di Blasi, C. (1996). Heat, momentum and mass transportation through a shrinking biomass particle exposed to thermal radiation. *Chemical Engineering Science*. **51**:1121-1132

Duhalt, R. V. and Ramirez, R. Q. (2004). Petroleum Biotechnology: developments perspectives. 1st edition. Library of Congress. Netherlands. 635 pp

Dussault, H.P. (1955). An improved technique for staining red halophilic bacteria. *Bacteriology*. **70**: 484-485

Edwards, C. (1990). Microbiology of extreme environment. Alden press. Oxford University. pp 21-23

Endo, K., Hayashi, Y., Hibi, T., Hosono, K., Beppu, T. and Ueda, K. (2003). Enzymological characterization of EpoA, a laccase-like phenol oxidase produced by *Streptomyces griseus*. *Biochemistry*. **133**: 671-677

Farrar, K. and Donnison, I.S (2007). Construction and screening of BAC libraries made from *Brachypodium* genomic DNA. *Nature Protocols*. **2**: 1661-1674

Farell, R. L., Murtagh, K. E., Tien, M., Mozuch, M. D. and Kirk, T. K. (1989). Physical and enzymatic properties of lignin peroxidase isoenzymes from *Phanerochaete chrysosporium*. *Enzyme and Microbial Biotechnology*. **11**: 322-328

Frazier, W. C. (1926). A method for the detection of changes in gelatin due to bacteria. *Infectious Diseases*. **39**:302-309

Fujihara, S., Hafashita, M., Sakurai, A. and Sakakibara, M. (2010). Production of Manganese peroxidase by white rot fungi from potato-processing waste water: role of amino acids on biosynthesis. *African Journal of Biotechnology*. **9**: 725-731

Furfari, A. (2008). Biofuels: Illusion or reality?.The European Experience. Editions TECHNIP. Paris. 137 pp

Gold, M. H., Wariishi, H. and Valli, K. (1989). Extracellular peroxidases involved in lignin degradation by the white rot basidiomycete *Phanerochaete chrysosporium*. In Biocatalysis in Agricultural Biotechnology ed. *American Chemical Society*. pp 127-140

Gram, H.C. (1884). The differential staining of *schizomycetes* in tissue sections and in dred preparations. *Pioneers in Medical Laboratory Science*. **2**: 185-189

Haki, G.D. and Rakshit, S.K. (2003). Development in industrially important thermostable enzymes: A review. *Bioresources Technology*. **89**: 17-34

Hall, T.A. (1999). Bioedit: A user friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic acids Symposium Series. pp 95-98

Hamid, M. and Rehmad, K. (2009). Potential applications of peroxidases. *Food Chemistry*. **115**: 1177-1186

Hammel, K.E., Jensen, K.A., Mozuch, M.D., Landucci, L.L., Tien, M. and Pease, E.A. (1993). Ligninolysis by a purified Lignin peroxidase. *Biological Chemistry*. **268**: 12274-81

Hanahan, D. (1985). Techniques for transformation of *E.coli*. In DNA Cloning. Glover D.M edition. IRL Press. Oxford. Vol. 1. pp 109-135

Hatakka, A., Steinbuchel, A. and Hofrichter, M. (2001). Biopolymers: Biodegradation of lignin. Volume 1.Wiley- VHC Publishers. Germany. pp 143-145

Have, R., Hartmans, S., Teunissen, P.J.M. and Field, J.A. (1998). Purification and characterisation of two lignin peroxidase isozymes produced by *Bjerkandera sp*. Strain BOS55. *Federation of European Microbiology Societies*. **422**: 391-394

Heilig, G.K. (1994). The greenhouse gas methane (CH_4) sources and sinks the impact of population growth, possible interventions. *Population and Environment*. **16**: 109-137

Hofrichter, M. (2002). Review: lignin conversion by manganese peroxidase. *Enzyme and Microbial Technology*. **30**: 454-466

Howard, R.L., Abotsi, E., van Rensburg J.E.L. and Howard, S. (2003). Lignocellulose biotechnology: Issues of bioconversion and enzyme production. *African Journal of Biotechnology*. **2**:602-619

http://www.biorefinery.ws/images/bio_f1_b.jpg&imgrefurl 01/04/10

Inoue, H., Nojima, H. and Okayama, H. (1990). High efficiency transformation of *Escherichia coli* with plasmids. *Gene*. **96**:23-28

Jahi, J.M. (2009). Environmental quality and human security: the need for an integrated environmental management system. *European Journal of Social Sciences*. **9**: 1-10

Jeffries, T.W. (1994). Biodegradation of lignin and hemicelluloses. Kluwer Academic Publishers. Netherlands. pp 233–277

Jin, Q., Dong, H.,Peng, Y., Chen, B., Shao, J., Deng, Y., Dai, C., Fang, Y., Lou, Y.,Li, Y. and Li, D. (2007). Application of cDNA array for studying the gene expression profile of mature appressoria of *Magnaporthe grisea. Zhejiang University Science*. **8**: 88–97

Kadish, K.M., Smith, K.M. and Guilard, R. (2003). The porphyrin handbook. Academic Press. **4**:189-218

Kirk, J.L., Beaudette, L.A., Hart, M., Moutoglis, P., Klironomos, J.N., Lee, H. and Trevors, J.T. (2004). Methods of studying soil microbial diversity. *Microbiological Methods*. **58:** 169-188

Kleinert, M., Gasson, J.R. and Barth, J. (2009). Optimizing solvolysis conditions for intergrated depolymerisation and hydrodeoxygenation of lignin to produce liquid biofuel. *Analytical and Applied Pyrolysis*. **85**: 108-117

Knauf, M. and Moniruzzamun, M. (2004). Lignocellulosic biomass processing: A perspective. *International Sugar Journal*. **106**: 1-4

Kristjansson, J.K. (1992). Thermophilic bacteria. CRC press. USA. 228 pp

Kuhad, R.C. and Singh, A. (2007). Lignocellulose biotechnology: Future prospects. International Publishing House. New Dehli. 390 pp

Kumar, A.C., Ravinder, R., Venkateswar, R.L., Pogaku, R. and Lakshmi, N.M. (2007). Industrial enzymes in bio-industrial sector development: An Indian perspective. *Commercial Biotechnology*. **13**: 283-291

Lalloo, R., Ramchuran, S., Ramduth, D., Go¨ rgens, J. and Gardiner, N. (2007). Isolation and selection of *Bacillus* spp. as potential biological agents for enhancement of water quality in culture of ornamental fish. *Applied Microbiology*. **103**: 1471-1479

Lee, C., Wong, D., and Robertson, G. (2005). Cloning and characterization of the xylan gene from *Lentinula edodes*. *Proteins*. **24**: 21-26

Li, J., Yuan, H. and Yang, J. (2009). Bacteria and lignin degradation. *Frontiers in Biology*. **4**: 29-38

MacFaddin, J.F. (1985). Media for the isolation – cultivation – maintenance of medical bacteria. Vol 1. Williams and Wilkins Publishers. Baltimore. 620 pp

Madhavi, V. and Lele, S.S. (2009). Laccase: properties and applications. *Bioresources*. **4**: 1694-1717

McCaig, A.E. Grayston S.J., Prosser, J.I. and Glover, L.A. (2001). Impact of cultivation on characterisation of species composition of soil bacterial communities. *Federation of European Microbiology Societies*. **35**: 37–48

Martin-Laurent, F., Philippot, L., Hallet, S., Chaussod, R., Germon, J.C., Soulas, G. and Catroux, G. (2001). DNA extraction from soils: old bias for new microbial diversity analysis methods. *Applied and Environmental Microbiology.* **67**: 2354–2359

Mielenz, J.R. (2001). Ethanol production from biomass: technology and commercialization status. *Current Opinions in Microbiology*. **4**: 324-329

Mizuguchi, K., sele, M. and Cubellis, M.V. (2006). Environmental specific substitution tables for thermophilic proteins. *Bioorganic Medicinal Chemistry*. **8**: 1-15

Mussatto, S.I., Fernandez, M. and Milagres, A.M.F. (2008). Effect of hemicelluloses and lignin on enzymatic hydrolysis of cellulose from brewer's spent grain. *Enzyme and Microbial Technology*. **41**: 124-129

Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., and Setlow, P. (2000). Resistance of *bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiology and Molecular biology* . **63**: 548-572

Nigam, S.P. and Singh, A. (2011). Production of liquid biofuels from renewable resources. *Progress in Energy and Combustion Science*. **37**: 52-68

Nishino, T., Matsuda I. and Hirao K. (2004). All-cellulose composite. *Macromolecules*. **37**: 7683–7687.

Olsson, L., and Ahring, B. K. (2007). *Biofuels*: Advances in Biochemical Engineering/Biotechnology. Library of Congress. New York. pp 124-136

Obring, T.G., Culp, W.J., McKeehan, W.L. and Hardesty, B. (1971). The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. *Biology and Chemistry*. **246**: 174-181

Passardi, F., Longet, D., Penel, C. and Dunand, C. (2004). The class III peroxidase multigenic family in rice and its evolution in land plants. *Phytochemistry*. **65**: 1879-1893

Perez, J., Munoz-Dorado, J., Rubia, T. and Martinez, J. (2002). Biodegradation and biological treatments of cellulose, hemicelluloses and lignin: An overview. *International Journal of Microbiology*. **5**: 53-63

Quesada, E., Ventosa, A., Rodriguez-Valera, F., Megias, T. and Ramos-Cormenzana, A. (1983). Numerical taxonomy of moderately halophilic Gram-negative bacteria from hypersaline soils. *General Microbiology*. **129**: 2649-2657

Raj, A., Reddy, M.M.K., Chandra, R., Purohit, H.J. and Kapley, A. (2007). Biodegradation of kraft-lignin by *Bacillus* sp. Isolated from sludge of pulp and paper mill. *Biodegradation*. **18**: 738-792

Ramachandra, M., Crawford, D.L., and Herte, L.G. (1988). Characterization of extracellular lignin peroxidase of the lignocellulytuc actinomycete *streptomyces viridosporus*. *Applied and Environmental Microbiology*. **54**: 3057-3063

Rob, A., Hernandez, M., Ball. A.S., Tuncer, M., Arias, M.E. and Wilson, M.T. (1995). Production and partial characterization of extracellular peroxidises produced by *streptomyces avermitilis* UAH30. *Applied Biochemistry and Biotechnology*. **62**: 159-174

Rothschild, N., Hadar, Y. and Dosoretz, C.G. (1996). Lignin peroxidase isozymes from *Phanerochaete chrysosporium* can be enzymatically dephosphorylated. *Applied and Environmental Microbiology*. **63**: 857–861

Ryu, W.Y., Jang, M.Y. and Cho, M.H. (2003). The selective visualization of lignin peoxidase, manganese peroxidase and laccase, produced by white rot fungi on solid media. *Biotechnology and Bioprocess Engineering*. **8**: 130-134.

Rubin, E.M. (2008). Genomics of cellulosic biofuels. *Nature Reviews*. **454**: 841-845

Saha, B.C. (2003). Hemicellulose bioconversion. *Industrial Microbiology and Biotechnology*. **30**: 279-291

Saitoun, N and Nei, M. (1987). The neighbour-joining method: A new method for reconstructing Phylogenetic trees. *Molecular Biology and Evolution*. **4**: 406-425

Sambrook, J., Fritsch, E.F and Maniatis, T. (1989). Molecular cloning: A laboratory manual. 2nd edition. Cold Spring Harbor Laboratory press. UK. pp 1-74

Sambrook, J., Maccallum, P. and Russel, D. (2001). Molecular cloning: A laboratory manual, 3rd edition. Cold Springs Harbour Press. UK. 2344 pp

Sheehan, D. and Hrapchak, B. (1980). Theory and practice of Histotechnology. 2nd edition. Battelle Press, Ohio. pp 235-237

Sierra, G. (1957). A simple method for the detection of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. *Microbiology and Serology*. **23**: 15-22

Silver, S.C. and Defries, R.S. (1990). One earth one future: Our changing Global environment. National Academy of Sciences. USA. 196 pp

Silhavy, T., Berman, M. and Enquist, L. (1984). Experiments with Gene Fusions. Cold Spring Harbor, N.Y. Press

Song, J., Weon, H. Y., Yoon, S. H., Park, D.S., Go S. J. and Suh, T. W. (2001). Phylogenetic diversity of thermophilic actinomycetes and *Thermoactinomyces* spp. isolated from mushroom composts in Korea based on 16S rRNA gene sequence analysis. *Federation of the Societies of Biochemistry and Molecular Biology*. **202**: 97–102

Starr, C., Evers, A.C. and Starr, L. (2010). Biology: Concepts and applications. Library of congress. USA. 222 pp

Strom, P. F. (1985). Identification of thermophillic bacteria in solid-waste composting. *Applied and Environmental Microbiology*. **50:** 906-913

Tamura, K., Dudley, j., Nei, M. and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*. **24**: 1596-1599.

Todar, K. (2011). Todar's online textbook of bacteriology. (Http: www.Textbook of bacteriology.net)

Toumela, M., Vikman, M., Hatakka, A. and Itavaara, M. (2000). Biodegradation of lignin in a compost environment: a review. *Bioresource Technology*. **72**: 169-183

Tuor, U., Winterhalter, K. and Fietchter, A. (1995). Enzymes of white rot fungi involved in lignin degradation and ecological determinants for wood decay. *Biotechnology*. **41**: 1-17

Ventosa, A., Quesada, E., Rodriguez-Valera, F., Ruiz-Berraquero, F. and Ramos-Comenzana, A. (1982). Numerical taxonomy of moderately halophilic Gram-negative rods. *General Microbiology*. **128**: 1959-1968

Wang, Z., and Chen, S. (2009). Potential of biofilm-based biofuel production. *Applied Microbiology and Biotechnology*. **83**: 1-18

Ward, G., Hadar, Y., Bilkis, I. And Dosoretz, C.G. (2003). Mechanistic features of lignin peroxidase-catalysed oxidation of substituted phenols and 1,2-Dimethoxyarens. *Biological Chemistry*. **276**: 39726-39734

Wariishi, H., Valli, K., Renganathan, V., and Gold, M.H. (1989). Thiol mediated oxidated of non-phenolic lignin model compounds by manganese peroxidase of *Phanerochaete chrysosporium*. *Biological Chemistry*. **264**: 14185-14191

Wayne, L.G., Brenner, D.J., Colwell, R.R., et al. (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *International Journal of Systematic Bacteriology* **37**:463-464

Weisburg, W. G., Barns, S. M., Pelletier, D. A. and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Bacteriology*. **173:** 697-703

Wong, D.W.S. (2009). Structure and action mechanism of lignolytic enzymes. *Applied Biochemistry and Biotechnology*. **157**: 174-209

Worthington, C.C. (1988). The Worthington Manual. Worthington Biochemical Corporation. Canada. pp 254-257

Yang, Z.H., Xiao, Y., Zeng, G.M., Xu, Z.Y. and Liu, Y.S. (2007). Comparison of methods for total community DNA extraction and purification from compost. *Applied Microbiology and Biotechnology*. 7**4**: 918–925

Yaman, S. (2004). Pyrolysis of biomass to produce fuels and chemical feedstocks. *Energy Conversion and Management*. **45**: 651-671

Ye, M.H., Wen, J., Cao, H.H., Li, H.B., Chen, J.L., Zhao, G.P., and Zheng, M.Q. (2007). Construction of genomic DNA library of Beijing fatty chick in phage EMBL3 and screen of chick heart type fatty acid binding protein gene. *International Journal of Poultry Science*. **6**: 599-603

Zhou, J., Bruns, M.A. and Tiedje, J.M. (1996). DNA recovery from soils of diverse composition. *Applied Environmental Microbiology*. **62**: 16-322

UNIVERSITY of the
WESTERN CAPE