



Institute for Microbial
Biotechnology and
Metagenomics



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WESTERN CAPE

Laccases from actinomycetes for lignocellulose degradation

by

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A thesis submitted in partial fulfilment of the requirements for the degree of
Magister Scientiae (M.Sc.) in the Department of Biotechnology,

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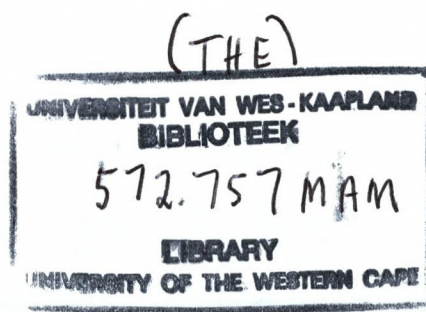
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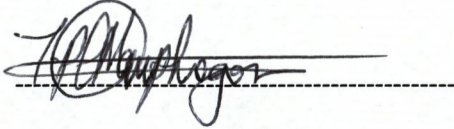


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Declaration

I declare that "Laccases from actinomycetes for lignocellulose degradation" is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.



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Abstract

Lignocellulose has a complex structure composed mainly of lignin, hemicellulose and cellulose. Several enzymes are needed for the degradation of lignocellulose into simple sugars. Actinomycetes are known to produce laccases which are able to degrade lignin. Laccase activities were detected in actinomycete strains MS26 isolated from soil collected from the Zambian Copperbelt and DFNR17 isolated from soil collected from a New Zealand farm. Morphological studies showed that the strains produced extensively branched substrate mycelia and aerial hyphae. Micromorphological characteristics were consistent with the assignment of these strains to the genus *Streptomyces*. Isolates were found to be mesophiles, with growth occurring in a temperature range of 16 and 45°C. Optimal growth occurred at temperatures between 30 and 37°C. Analysis of the 16S rRNA gene sequences of the strains showed that strain MS26 had the highest sequence similarity (99%) to *Streptomyces atrovirens* strain NRRL B-16357 and *Streptomyces viridodiataticus* strain IFO 13106. Strain DFNR17 had the highest 16S rRNA gene sequence similarity (99%) to *Streptomyces althioticus* strain KCTC 9752. The strains shared several physiological and biochemical characteristics with their closest neighbours which, along with 16S rRNA gene sequences analysis, confirmed that the strains were members of the genus *Streptomyces*. Attempts to identify the laccase genes from these isolates by screening a fosmid library failed. Subsequently isolates were screened by PCR using laccase-like copper oxidase degenerate primers designed from several *Streptomyces* strains. A 300 bp amplicon was obtained from both isolates. Phylogenetic analysis was performed and both amplicons from strains MS26 and DFNR17 had the highest similarities with the copper oxidase gene from *Streptomyces griseoflavus* strain Tu4000. Therefore it is probable that the laccase activity observed for these strains is due to the activity of copper oxidase gene products.

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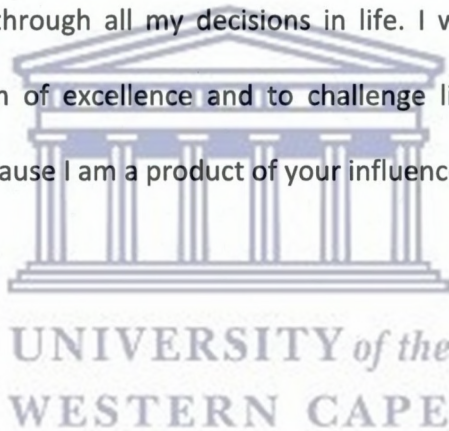
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Dedication

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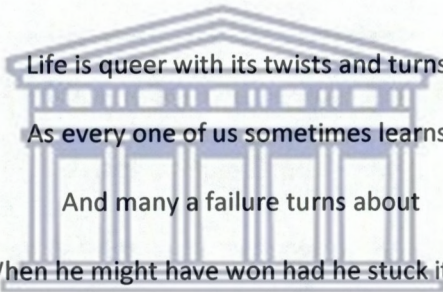
List of Abbreviations

ABTS	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
bp	Base pair
BLAST	Basic local alignment sequencing tool
BSA	Bovine serum albumin
CaCO₃	Calcium carbonate
CaCl₂	Calcium chloride
cfu	Colony forming units
CTAB	Cetyl trimethyl ammonium bromide
dATP	Deoxy-adenine 5'-triphosphate
dCTP	Deoxy-cytidine 5'-triphosphate
ddH₂O	Deionized distilled water
dGTP	Deoxy-guanosine 5'-triphosphate
DMP	2,6-dimethoxyphenol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxy-thymidine 5'-triphosphate
°C	Degrees celsius
EC	European commission
EDTA	Ethylenediaminetetra-acetate
FeSO₄.7H₂O	Ferrous sulfate heptahydrate
g	Gram
"g"	Gravitational force
HBT	1-hydroxybenzotriazole
H₂O₂	Hydrogen peroxide
ISP	International <i>Streptomyces</i> project
KCl	Potassium chloride
K₂HPO₄	Dipotassium phosphate
L	Litre
LB	Luria Bertani medium
LB-amp	Luria Bertani medium containing ampicillin
M	Molar
mg	Milligram
MgCl₂	Magnesium chloride
MgSO₄	Magnesium sulphate
MgSO₄.7H₂O	Magnesium sulfate heptahydrate
MnCl₂.4H₂O	Manganese chloride tetrahydrate
MnCl₂	Manganese chloride
min	Minute(s)

ml	Millilitre
mm	Millimetre
mM	Millimolar
μF	Microfarad
μg	Microgram
μl	Microlitre
μM	Micromolar
NaOH	Sodium hydroxide
NaH₂PO₄	Sodium dihydrogen orthophosphate
Na₂HPO₄·2H₂O	Disodium phosphate dehydrate
Na₂S₂O₃	Sodium hyposulfite
ng	Nanogram
nm	Nanometre
OD	Optical density
PCR	Polymerase chain reaction
rpm	Revolutions per minute
rRNA	Ribosomal nucleic acid
s	Seconds
SLAC	Small laccase
TAE	Tris acetic acid EDTA
TE	Tris EDTA
TEMPO	2,2',6,6'-tetramethylpiperidine- <i>N</i> -oxyl
Tris	Tris-hydroxymethyl-aminomethane
UV	Ultraviolet
VLA	Violuric acid
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase
ZnSO₄ · 7H₂O	Zinc sulphate heptahydrate

DON'T QUIT

When things go wrong,
As they sometimes will,
When the road you're trudging seems all uphill,
When the funds are low and the debts are high,
And you want to smile, but you have to sigh,
When care is pressing you down a bit
Rest if you must, but don't you quit.



Life is queer with its twists and turns,
As every one of us sometimes learns,
And many a failure turns about
When he might have won had he stuck it out.
Don't give up though the pace seems slow
You may succeed with another blow.

Success is failure turned inside out
The silver tint of the clouds of doubt,
And you never can tell how close you are,
It may be near when it seems so far;
So stick to the fight when you're hardest hit
It's when things seem worst that you mustn't quit.

(author.....Unknown)

Chapter 1: Literature review

1.1 Lignocellulose

Lignocellulose is the main structural component of both woody and non-woody plants and represents a major source of renewable organic matter (Howard *et al.*, 2003). Lignocellulose consists of lignin, hemicellulose and cellulose (Malherbe and Cloete, 2002) and as the building block of all plants is ubiquitous to all regions on earth. The ratio of lignin:hemicellulose:cellulose has a profound effect on the tertiary structure of lignocellulose. Table 1.1 shows the distinctive compositions of the three components in different lignocellulosic materials (Howard *et al.*, 2003). Cellulose and hemicellulose are composed of sugars which can be used in various biotechnological applications including biofuel production.

In processes involving the degradation of lignocellulose the use of weak acids to degrade lignin can result in a less effective hydrolysis of cellulose while the use of strong acids requires the use of expensive apparatus due to the extremely corrosive nature of the process (Howard *et al.*, 2003). Enzymatic hydrolysis of lignocellulose could be a suitable alternative for biotechnological applications. The complex mechanism by which lignocellulose is degraded enzymatically in nature is yet to be fully understood, but significant advances have been made in gaining insight into the microorganisms and the lignocellulolytic enzymes involved in the process.

Table 1.1: Percentage of cellulose, hemicellulose and lignin present in the lignocellulose of common agricultural residues and wastes (Howard *et al.*, 2003).

Lignocellulosic material	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood	40-45	24-40	18-25
Softwood	45-50	25-35	25-35
Nutshells	25-30	25-30	30-40
Corn cobs	45	35	15
Papers	85-99	0	0-15
Wheat straw	30	50	15
Rice straw	32.1	24	18
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seeds hair	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste paper from pulp	60-70	10-20	5-10
Primary wastewater solids	8-15	N/A	24-29
Fresh bagasse	33.4	30	18.9
Swine waste	6	28	NA
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0

1.1.1 Cellulose

Cellulose is the main constituent of plant cell walls and about 50% of wood is comprised of cellulose (Lynd *et al.*, 1999). Structurally cellulose is closely associated with hemicellulose and lignin (Figure 1.1) and the isolation of cellulose requires intensive chemical treatments. Cellulose consists of D-glucopyranose monomer units bound by β -1-4-glycosidic linkages. The successive glucose residues are rotated by 180°C relative to each other forming cellobiose dimer units and thus the repeating unit of the cellulose chain is cellobiose. The average degree of polymerization of plant cellulose varies between 700 and 1500 glucose units, depending on the source (Fengel and Wenger, 1983; Lynd *et al.*, 1999).

Cellulose contains hydroxyl groups (OH^-) which serve as the functional group on both ends of the cellulose chain (O'Sullivan, 1997). These OH^- groups are able to interact with each other or with O^- , N^- , and S^- groups, forming hydrogen bonds and making the surface of cellulose largely hydrophilic. The cellulose chains are packed together to form highly crystalline microfibrils in which the individual cellulose chains are bound together by hydrogen bonds. An individual cellulose crystal contains tens of glucan chains in a parallel orientation. Crystal polymorphs identified for cellulose are designated as $\text{I}\alpha$, $\text{I}\beta$, II_1 , III_{11} , IV_1 , and IV_{11} , with the first two polymorphs appearing as the most abundant crystal forms (Atalla and Van der Hart, 1984). Several reviews have surveyed the structure of cellulose and it is still the subject of intense study (Hon, 1994; O'Sullivan, 1997; Kadla and Gilbert, 2000).

1.1.2 Hemicellulose

Hemicelluloses are mainly classified according to the type of sugar residue in the backbone. Classes include xylans, mannans, galactans and glucans, with xylans and mannans being the most abundant types of hemicellulose (Jeffries, 1990). Hemicellulose is chemically cross-linked with polysaccharides, proteins and/or lignin. Xylans appear to be the major interface between lignin and other carbohydrates. In woody trees the average degree of polymerization of hemicelluloses varies between 70 and 200 depending on the species (Fengel and Wenger, 1983; Vincent, 1999; Mosier *et al.*, 2005).

The hemicellulose component in hardwoods and plants is mainly xylan (15-30%), whereas softwood hemicelluloses consist of galactoglucomannans (15-20%) and xylans (7-10%). Hardwood xylans are composed of β -D-xylopyranosyl units, which contain 4-O-methyl- α -D-glucuronic acid and acetyl side groups. The 4-O-methyl- α -D-glucuronic acid is linked to the xylan backbone by O-(1 \rightarrow 2) glycosidic bonds and the acetic acid side groups

are esterified at the carbon 2 and hydroxyl group. Softwood xylans are arabino-4-O-methylglucuronoxylans, are non-acetylated and the xylan backbone is substituted at carbon 2 and 3 with 4-O-methyl- α -D-glucuronic acid and α -L-arabinofuranosyl residues (Mosier *et al.*, 2005).

1.1.3 Lignin

Lignin is defined as a rigid material embedded in the cellulose matrix of vascular plant cell walls and plays a significant structural role in supporting terrestrial plant species (Chabannes *et al.*, 2001; Jones *et al.*, 2001). Lignin is typically found between plant cells but can also be found inside the cells, and it binds cellulose fibres together. The highest concentration of this recalcitrant polymer is found in the middle lamella where it acts as a cement between the wood fibres (Figure 1.1). It is also found in layers in the cell wall where it forms an amorphous matrix with hemicelluloses in which the cellulose fibrils are embedded and protected against biodegradation (Figure 1.1). The function of lignin is to control the transport of liquid in the living plant, partly by reinforcing the cell walls and keeping them from collapsing, and partly by regulating the flow of liquid. The increased rigidity conferred by lignin enables trees to grow tall and compete for sunshine (Boerjan *et al.*, 2003).

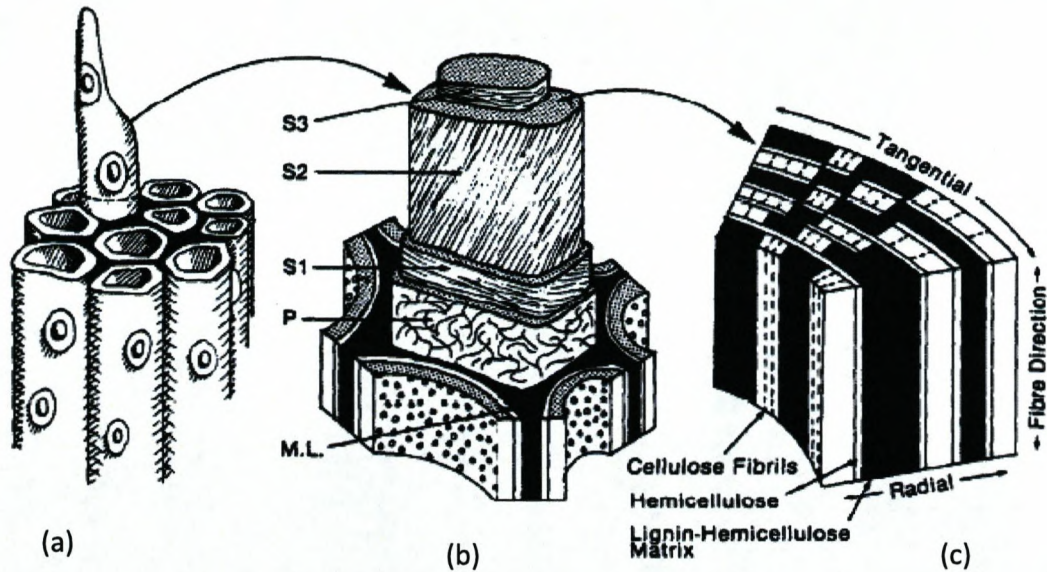


Figure 1.1: Structural design of woody tissues: (a) collection of adjacent wood cells, (b) cross sectioning showing the distinct cell wall layers, (c) part of the secondary wall showing the connection of hemicellulose and lignin to the cellulose fibrils. Key: P, primary cell wall layers; S1, S2 and S3, secondary cell wall layers; ML, middle lamella. (Kirk and Shimida, 1985).

Lignin is very resistant to degradation because of its high molecular weight and the presence of biologically stable linkages. It is a complex polymer in which the building blocks are phenolic compounds (Howard *et al.*, 2003). It contains three different aromatic alcohol units: coniferyl alcohol, p-coumaryl alcohol and sinapyl alcohol. Lignin macromolecules typically comprise of phenylpropanoid units linked to each other by various ether and carbon-carbon bonds. Lignin from woody plants contains small amounts of incomplete or modified monolignols, while other monomers are important in non-woody plants (Ralph *et al.*, 2001). The complexity of lignin is the main reason for its recalcitrance. Due to the variety of molecules making up the lignin macromolecule the activities of a consortium of enzymes are needed to degrade lignin (Adler, 1977).

1.2 Biofuels

Due to the high demand for energy and the limited amount of fossil fuel alternatives, sustainable energy sources such as bioethanol are required (Goldemberg, 2007). An added advantage of using ethanol as a source of fuel is the decrease in carbon dioxide emissions associated with its use (Hill *et al.*, 2006). Lignocellulosic biomass can be used as a resource for the production of biofuels such as bioethanol, whereby glucose generated from the degradation of cellulose can be fermented to produce ethanol (Delgenes *et al.*, 1996). Likewise pentoses from the degradation of xylan can be fermented to ethanol (Hahn-Hägerdal *et al.*, 1994).

Lignocellulose, as previously mentioned (section 1.2), is the major structural component of plant material. Compared to other fuel sources lignocellulose is cheap, abundant and is a renewable energy source as it can be obtained from agricultural waste materials (Belkacemi *et al.*, 2002; Hill *et al.*, 2006), municipal solid waste (Li *et al.*, 2007; Chester and Martin, 2009) as well as waste from forestry and the pulp and paper industry (Lynd *et al.*, 1991; Goldemberg, 2007). Presently cornstarch is used for the production of ethanol but its cultivation requires a large amount of agricultural land that is normally used for food production (Hill *et al.*, 2006). Therefore, the use of lignocellulose wastes for bioethanol production is a more practical solution than the use of corn crops as the former does not involve the use of valuable land resources for biomass production (Hill *et al.*, 2006).

Currently bioethanol production costs are high compared to production costs of fossil fuels and bioethanol has not replaced fossil fuel as the main energy source (Goldemberg, 2007). The use of lignocellulosic biomass for bioethanol production is at present not economical because the enzymes and chemicals needed for its bioconversion

are expensive (Zheng *et al.*, 2009). For lignocelluloses to be a cost effective sustainable alternative resource for bioethanol production, lignin degrading enzymes are required to degrade the lignin component of plant matter. This would allow cellulases to access the cellulose, which would subsequently be degraded into its constituent sugars. For this process to be economically viable enzymes are needed to break down the different components of lignocellulosics into simple sugars that can then be fermented to bioethanol.

1.3 Lignin degrading enzymes

As mentioned previously, a large number of enzymes are implicated in the degradation of lignin. Table 1.2 shows the various types of ligninolytic enzymes and their specific substrates within the lignin molecule. This review focuses on the three most characterised enzymes, lignin peroxidases, manganese peroxidases and laccases.

Table 1.2: Enzymes involved in the degradation of lignin and their main reaction (Hatakka, 1994).

Enzyme Activity	Cofactor or Substrate, "Mediator"	Main Effect or Reaction
Lignin peroxidase	H ₂ O ₂ , veratry alcohol	Aromatic ring oxidized to cation radical
Manganese peroxidase	H ₂ O ₂ , Mn, organic acids as chelator, thiols, unsaturated lipids	Mn (II) oxidized to Mn(III); chelated Mn(III) oxidizes phenolic compounds to phenoxyl radicals; other reactions in the presence of additional compounds
Laccase	O ₂ ; mediators e.g. hydroxybenzotriazole or ABTS	Glyoxal oxidized to glyoxylic acid; H ₂ O ₂ production
Glyoxal oxidase	Glyoxal, methyl glyoxal	Aromatic alcohols oxidized to aldehydes; H ₂ O ₂ production
Aryl alcohol oxidase	Aromatic alcohols (anisyl, veratryl alcohol)	O ₂ reduced to H ₂ O ₂

1.3.1 Lignin peroxidase

Lignin peroxidase (LiP; EC 1.11.1.14) is one of the many enzymes that is able to degrade lignin. The first characterised LiP, discovered in 1983, was isolated from *Phanerochaete chrysosporium* (Glenn *et al.*, 1983; Tien and Kirk, 1983). Lignin peroxidases are produced by many wood degrading fungi as a family of isoenzymes (Kirk and Farrell, 1987). Recent research has also identified them in bacteria such as *Streptomyces viridosporus* (Macedo *et al.*, 1999).

LiPs are heme proteins which are roughly 37,000 Daltons in size (Tien *et al.*, 1986). They are related to the plant peroxidases in structure and mechanism and use hydrogen peroxide and organic peroxides to oxidize a range of substrates. Substrates for LiP may be either phenolic or non-phenolic aromatic compounds. LiPs are characterised by their ability to oxidize high redox-potential, non-phenolic methoxybenzene aromatic compounds such as veratryl (3,4-dimethoxybenzyl) alcohol and methoxybenzene (Gerini *et al.*, 2003). The oxidation of these substrates to form aryl cation radicals can result in demethoxylation, C_a-C_β cleavage of lignin model compounds, benzyl alcohol oxidation, and the hydroxylation of aromatic rings and side chains.

The substrate range of LiPs is very broad and reactivity is determined by the redox potential. LiPs can catalyze the oxidation of substrates with a reduction potential greater than 1.3 volts (d'Acunzo *et al.*, 2003). LiPs oxidize lignin monomers, dimers and trimers, as well as polycyclic aromatic compounds such as benzopyrene. The nonspecific nature of lignin peroxidase activity has lead to investigations into their possible use in diverse applications including the fields of chemical synthesis, biodegradation of toxic chemicals, pulp and paper processing and the textile industry.

1.3.2 Manganese Peroxidase (MnP)

Manganese peroxidases (MnP; EC1.11.1.13) are heme containing enzymes and were first isolated from the extracellular medium of lignolytic cultures of *Phanerochaete chrysosporium*, a white rot fungus (Hirai *et al.*, 1994). It is considered to be a key enzyme in lignolysis by white rot fungi and has become one of the most important enzymes in the delignification of Kraft pulps (Hirai *et al.*, 1994) where it increases the brightness of hardwood Kraft pulp.

MnPs release methanol from methoxyl groups on rings with free phenolic hydroxyls (Hao *et al.*, 2010). MnPs partially oxidize the lignin in the pulp but do not degrade it into soluble fragments. For this reason they are only used at an early stage in the degradation of lignin.

1.3.3 Laccase

Laccases (LAC; EC 1.10.3.2) were first described by Yoshida in 1883 when he extracted the enzyme from the exudates of the Japanese lacquer tree, *Rhus vernicifera* (Thurston, 1994). The extracts were only confirmed to be fungal enzymes in 1896 by Bertrand and Laborde (Thurston, 1994). Laccases belong to the small group of large blue copper-containing proteins and/or blue copper oxidases (Gavnholt and Larsen, 2002). Plant ascorbate and the mammalian plasma protein ceruloplasmin are other enzymes in this group (Thurston, 1994; Xu, 1996; Ducros *et al.*, 1998).

Laccases can be either mono- or multimeric copper-containing oxidases, and catalyze the oxidation of a large range of substrates with the concomitant reduction of molecular oxygen into two molecules of water (Ducros *et al.*, 1998). The ability of laccases to oxidize phenolic compounds and to reduce molecular oxygen to water has led to widespread

interest in these enzymes. The oxidation of phenolic compounds within lignin which allows for delignification and the oxidation of organic compounds in waste waters are some of the many useful applications of these enzymes (O'Malley *et al.*, 1993; Thurston, 1994; Xu, 1996; Jolivalt *et al.*, 1999).

The applications of laccases range from effluent decolourisation, detoxification and pulp bleaching to the removal of phenolics from wine, as well as dye transfer blocking functions in detergents and washing powders (Elshafei *et al.*, 2012). The introduction of laccase mediator systems, which oxidize non-phenolic compounds that could not previously be reduced, has led to the expansion of biotechnological applications of these enzymes (Brijwani *et al.*, 2010).

It has been reported that the inclusion of a mediator such as ABTS (2,2'-azonobis(3-ethylbenzthiazoline-6-sulfonate), HBT (1-hydroxybenzotriazole), VLA (violuric acid) and TEMPO (2,2',6,6'-tetramethylpiperidine-*N*-oxyl) can extend the substrate range of laccases to non-phenolic subunits of lignin (Fabbrini *et al.*, 2002; Hernández *et al.*, 2006).

1.3.3.1 Location and physiological role of laccases

Of all the large blue copper-containing proteins, laccases are the most widely distributed enzymes found in higher plants, fungi (Leontievsky *et al.*, 1997) and bacteria (Diamantidis *et al.*, 2000). Laccases have been isolated from many plants including the sycamore (Bligny and Douce, 1983), peach (Lehman *et al.* 1974) poplar (Ranocha *et al.*, 1999) and tobacco (De Marco and Roubelakis-Angelakis, 1997). In the plant xylem laccases play an important role in oxidizing monolignols in the early stages of lignification (De Marco and Roubelakis-Angelakis, 1997). *Rhus vernicifera* laccase has been extensively studied, especially with regard to its spectroscopic properties (Malmström *et al.*, 1970; Woolery *et al.*

al., 1984). *R. vernicifera* laccase has been widely used in investigations of the general reaction mechanism of laccases (Lee *et al.*, 2002; Battistuzzi *et al.*, 2003; Johnson *et al.*, 2003).

The majority of laccases have been characterized from fungi, in particular from the white-rot basidiomycetes that are capable of degrading lignin (Bao *et al.*, 1993). Recognized laccase-producing fungi include *Agaricus bisporus* (Wood, 1980), *Botrytis cinerea* (Marbach *et al.*, 1984), *Phlebia radiata* (Niku-Paavola *et al.*, 1988) and *Pleurotus ostreatus* (Sannia *et al.*, 1986), *Pycnoporus cinnabarinus* (Eggert *et al.*, 1996b) and *Trametes versicolor* (Rogalski *et al.*, 1991). Fungal laccases have diverse physiological roles. Laccases produced by *Trametes versicolor* and *Pycnoporus cinnabarinus* participate in lignin biodegradation, where they mostly oxidize the phenolic subunits of lignin (Bourbonnais and Paice, 1990; Thurston, 1994; Eggert *et al.*, 1996a; Eggert *et al.*, 1996b; Hatakka, 2001). Laccases are the main virulence factors in plant pathogenic fungi. The grapevine grey mould *Botrytis cinerea* produces a laccase that is essential for pathogenesis where it is hypothesised that the laccase is involved in the detoxification of the toxic defence metabolites produced by the plant (Bar-Nun *et al.*, 1988).

Laccases have also been shown to be important for pathogenesis in the chestnut blight fungus *Cryphonectria parasitica* (Rigling and van Alfen, 1991; Choi *et al.*, 1992; Mayer and Staples, 2002) and in the human pathogen *Cryptococcus neoformans* (Williamson, 1994). In *Aspergillus nidulans* laccase activity is related to pigment production, and deletion of the laccase I gene (gene *yA*) abolishes the green colour of conidial spores (Aramayo and Timberlake, 1993; Adams *et al.*, 1998). Laccases have also been proposed to participate in fungal morphogenesis in *Armillaria* spp. (Worrall *et al.*, 1986), *Lentinus edodes* (Leatham and Stahmann, 1981) and *Volvariella volvacea* (Chen *et al.*, 2004).

Only a few bacterial laccases have been described. The first bacterial laccase was detected in *Azospirillum lipoferum*, a plant root-associated bacterium (Givaudan *et al.*, 1993), where it was found to be involved in the formation of melanin (Faure *et al.*, 1994). Subsequently, an atypical laccase consisting of six putative copper-binding sites was discovered from *Marinomonas mediterranea* but no functional role has been assigned to this enzyme (Solano *et al.*, 1997; Sanchez- Amat *et al.*, 2001). *Bacillus subtilis* produces a thermostable *CotA* laccase, which participates in pigment production in the endospore coat (Martins *et al.*, 2002).

After fungi, actinomycetes are believed to be the second most prolific producers of laccases. Purification and characterisation of laccases from actinomycetes, especially different *Streptomyces* species, has been reported. The laccase-like phenol oxidase from *Streptomyces griseus* has been reported to have a highly unique homotrimer structure (Endo *et al.*, 2003), while the small laccase (SLAC) from *Streptomyces coelicolor* has been described as a dimer, lacking the second domain (Machczynski *et al.*, 2004). Laccase from *Streptomyces lavendulae* is thermotolerant and is stable at 70 °C (Suzuki *et al.*, 2003). The laccase from *Streptomyces cyaneus* was the first described enzyme capable of oxidizing non-phenolic compounds in the presence of mediators (Arias *et al.*, 2003).

1.3.3.2 Occurrence of laccases in bacteria

There is a large amount of information on the widespread occurrence of laccases in prokaryotes (Table 1.3), yet until 2007 only three bacterial laccases had been purified and characterised (Sharma *et al.*, 2007). *Azospirillum lipoferum* laccase (Givaudan *et al.*, 1993), the first reported bacterial laccase, is a multimeric enzyme containing a catalytic subunit and two large chains. This enzyme functions in the pigmentation of the cell, and is involved in the consumption of plant phenolic compounds (Faure *et al.*, 1994; Faure *et al.*, 1995) and electron transport (Alexandre *et al.*, 1999). The most studied bacterial laccase is *CotA*, an endospore coat component of *Bacillus subtilis* (Martins *et al.*, 2002). It plays a role in brown spore pigment biosynthesis, and is thought to produce melanin which protects the spore coat against hydrogen peroxide and UV light (Driks, 2004). An unusual multi-potent polyphenol oxidase (PPO) has been reported from *Marinomonas mediterranea*, a marine melanogenic bacterium (Solano *et al.*, 1997). A PPO is a laccase capable of oxidizing substrates of both laccases and tyrosinases. *EpoA* from *Streptomyces griseus* is a homotrimer of 114 kDa. The enzyme has moderately narrow substrate specificity and can oxidize well known model laccase substrates such as guaiacol and syringaldazine, albeit ineffectively (Endo *et al.*, 2002). *EpoA* has subsequently been cloned and expressed as a recombinant protein in *E. coli* (Endo *et al.*, 2003). The wildtype enzyme is thought to play an important role in morphogenesis in *Streptomyces* species. The *CopA* protein from *Xanthomonas campestris* (Mellano and Cooksey, 1998) and the *PcoA* protein from *Escherichia coli* (Brown *et al.*, 1995) are structurally homologous to multi-copper oxidases with regard to canonical copper binding sites. They have laccase-like activity and play an important role in copper resistance in bacteria.

Table 1.3: Known bacterial laccases/ laccase-like proteins

Species	Potential function	References
<i>Aquifex aeolicus (suffl)</i>	Cell division	Deckert et al. (1998)
<i>Azospirillum lipoferum</i>	Pigmentation, oxidation of phenolic compounds, electron transport	Givaudan et al. (1993)
<i>Bacillus sphaericus</i>	Sporulation, pigmentation	Claus and Filip (1997)
<i>Bacillus subtilis (cota)</i>	Pigmentation of spores, UV and H ₂ O ₂ resistance	Hullo et al. (2001)
<i>Bacillus halodurans C-125 (lbh 2082)</i>	Cu ²⁺ resistance	Ruijsennars and Hartmans (2004)
<i>Escherichia coli (yack)</i>	Cu ²⁺ efflux, oxidation of phenolate-siderophores ferroxidase activity	Roberts et al. (2002)
<i>Marinomonas mediterranea (ppoA)</i>	Pigmentation	Sanchez-Amat et al. (2001)
<i>γ-Proteobacterium JB</i>	Oxidation of toxic compounds	Bains et al. (2003)
<i>Pseudomonas maltophilia</i>	Nucleoside oxidase activity	Isono and Hoshino (1989)
<i>Pseudomonas putida GB-1 (cuma)</i>	Mn ²⁺ oxidation	Brouwers et al. (1999)
<i>Pseudomonas syringae pv. tomato (copA)</i>	Cu ²⁺ resistance	Cha and Cooksey (1991)
<i>Streptomyces antibioticus</i>	Phenoxazinone synthesis	Freeman et al. (1993)
<i>Streptomyces coelicolor</i>	Oxidation of phenolic compounds	Machczynski et al. (2004)
<i>Streptomyces cyaneus</i>	Oxidation of non-phenolic compounds	Arias et al. (2003)
<i>Streptomyces griseus (epoA)</i>	Pigmentation, morphogenesis	Endo et al. (2002)
<i>Xanthomonas campestris(copA)</i>	Cu resistance	Lee et al. (1994)

1.3.3.3 Defining laccases according to substrate specificity

Laccases and tyrosinases overlap in the range of substrates they are capable of degrading, and as such it is not easy to define laccases according to their substrate specificity. Tyrosinases possess both catecholase and cresolase activities. One difference between laccases and tyrosinases is that laccases have the ability to oxidise syringaldazine while tyrosinases cannot (Thurston, 1994; Eggert *et al.*, 1996a). An enzyme exhibiting both tyrosinase and laccase activities has been identified from *Alteromonas* sp. MMB1 (Sanchez-Amat and Solano, 1997). Polyphenol oxidases (PPO) are copper containing proteins with the general attribute that they are capable of oxidising aromatic compounds using molecular oxygen as an electron acceptor (Mayer, 1987). The classification of PPOs is based on substrate specificity (Walker and McCallion, 1980; Mayer, 1987).

Laccases can utilise a range of substrates. While hydroquinones, catechols and ABTS are good laccase substrates and are routinely used in the laboratory, guaiacol and 2,6-dimethoxyphenol (DMP) have been found to be better substrates (Thurston, 1994). Thus, laccases oxidise polyphenols, diamines, methoxy-substituted phenols and many other compounds (Thurston, 1994). The laccase-catalysed reactions frequently lead to polymerisation through oxidative coupling, from C-O and C-C coupling of phenolic substrates and from N-N and C-N coupling of aromatic amines (Hublik and Schinner, 2000). *Rhizoctonia praticola* laccase is able to catalyse the coupling of two differently halogenated phenols, 2,4-dichlorophenol and 4-bromo-2-chlorophenol. The laccase catalysed reaction leads to the construction of three dimers with asymmetric shapes (Bollag *et al.*, 1979).

1.3.3.4 Structure and catalytic mechanism of laccases

The overall structure of laccases comprises of three cupredoxin-like domains A, B and C, which are equal in size (Figure 1.3) (Ducros *et al.*, 1998; Bertrand *et al.*, 2002; Piontek *et al.*, 2002; Enguita *et al.*, 2003). All three domains are significant for the catalytic activity of laccases: the substrate-binding site is located in a cleft between domains B and C. A mononuclear copper centre is located in domain C and a trinuclear copper center is located at the interface between domains A and C (Solomon *et al.*, 1996).

The mononuclear copper centre contains one type-1 (T1) copper atom that is triangularly coordinated to two histidines and a cysteine. The coordination bond between T1 and SCys is covalent. The trinuclear cluster contains one type-2 (T2) copper atom and a pair of type-3 (T3) copper atoms (Messerschmidt, 1997). The T2 copper and the T3 copper atoms are coordinated by two and by six conserved histidines, respectively (Bertrand *et al.*, 2002; Piontek *et al.*, 2002). The T3 copper pair is antiferromagnetically coupled by a bridging hydroxide, which makes the T3 coppers EPR-silent (Solomon *et al.*, 1996).

The catalytic cycle of laccases involves the formation of a fully reduced laccase in which all four coppers are in a reduced state (Shin *et al.*, 1996; Solomon *et al.*, 1996; Lee *et al.*, 2002). Molecular oxygen then oxidizes the fully reduced laccase, via a peroxy intermediate, and is reduced to water (Shin *et al.*, 1996; Solomon *et al.*, 1996).



Figure 1.2: Three-dimensional structure of *M. albomyces* laccase. Domains A, B, and C are colored red, green and blue, respectively. The four copper atoms are shown as yellow balls and carbohydrates as grey sticks (Hakulinen *et al.*, 2002).

1.3.3.5 Applications of the laccase enzyme

Due to their wide reaction capabilities as well as their broad substrate specificity laccases have a great biotechnological potential (Sharma *et al.*, 2007). Promising applications include textile-dye bleaching (Kierulff, 1997), pulp bleaching (Palonen and Viikari, 2004), food improvement (Minussi *et al.*, 2002), bioremediation of soils and water (Li *et al.*, 1999; Wasenberg *et al.*, 2003), polymer synthesis (Marzoorati *et al.*, 2005) and the development of biosensors and biofuel cells (Trudeau *et al.*, 1997). Some of the potential applications are outlined in Table 1.4.

Table 1.4: Diverse fields of potential industrial applications of laccase

Potential industrial application	References
Pulp delignification	Kunamneni <i>et al.</i> (2007)
Bioremediation	Riva (2006)
Ethanol production	Senthilguru <i>et al.</i> (2011)
Biosensors	Ncanana <i>et al.</i> (2007)
Wine clarification	Rosana <i>et al.</i> (2002)
Detergent manufacturing	Sharma and Kuhad (2008)
Transformation of antibiotics and steroids	Ncanana <i>et al.</i> (2007)
Herbicide degradation	Mougin <i>et al.</i> (2002)

1.4 Actinomycetes

The actinomycetes are Gram positive bacteria, characterised by the high G+C (>55%) content of their genomic DNA. The term “actinomycete” was derived from Greek ‘aktis’ (a ray) and ‘mykes’ (fungus), and was given to this group of organisms based on their morphology upon initial examination. They were originally thought to be an intermediate group between bacteria and fungi, but are now accepted to be prokaryotic organisms (Hemashenpagam, 2011).

Most actinomycetes are saprophytic free living bacteria found widely dispersed in soil and water, as well as colonizing plants (Benizri *et al.*, 2001). They are recognized as one of the major groups of bacteria in soil and population size and composition has been shown to vary with soil type. Actinomycetes participate in the turnover of soil components, particularly in the transformation of organic compounds (Benizri *et al.*, 2001).

Actinomycetes are important producers of antibiotics, making three quarters of all known bacterially derived natural products (Kieser *et al.*, 2000). *Streptomyces*, a genus belonging to the actinomycetes, is particularly prolific and produces around 80% of the antibiotics characterised, with the second most prolific genus *Micromonospora* producing less than one-tenth as many as the genus *Streptomyces* (Kieser *et al.*, 2000).

1.4.1 The genus *Streptomyces*

Streptomycetes are Gram-positive, aerobic bacteria, which produce widespread branching vegetative mycelia and aerial mycelium chains. Both the vegetative and aerial mycelia can be pigmented (Rattanaporn *et al.*, 2010). They form lichenoid, leathery colonies on agar plates (Panchagnula, 2011). Their genomic DNA has a G+C content of about 69-78%. L- diaminopimelic acid is the characteristic compound present in the cell wall peptidoglycan of streptomycetes. Streptomycetes are able to make effective use of a variety of organic compounds as a sole carbon source, as well as complex biological materials such as cellulose and lignin (Lynd *et al.*, 2002). Streptomycetes produce many secondary metabolites such as antibiotics and other bioactive compounds (Kieser *et al.*, 2000).

1.4.2 *Streptomyces* Classification

The number of known *Streptomyces* species is continuously increasing. There are over 600 validly published species – as of September 2002 there were 650 species recorded in the DSMZ collection of microorganisms and cell cultures. *Streptomyces* is the largest genus in the order *Actinomycetales* within the class *Actinobacteria* (Stackebrandt *et al.*, 1997).

The classification of *Streptomyces* species was initially based on morphological and biochemical characterisation, and subsequently, on physiological tests (Kämpfer *et al.*, 1991;

Goodfellow *et al.*, 1992). Other methods used to classify streptomycetes include protein profiling (Ochi, 1995; Taguchi *et al.*, 1997), phage typing (Korn-Wendish and Schneider, 1992) and serological methods (Ridell *et al.*, 1986). With the development of molecular biology the application of methods such as 16S rRNA gene sequence analysis (Gladek *et al.*, 1985; Stackebrandt *et al.*, 1992; Kim *et al.*, 1996; Takeuchi *et al.*, 1996; Hain *et al.*, 1997; Kataoka *et al.*, 1997) and DNA-DNA reassociation (Labeleda, 1992; Kim *et al.*, 1999) have been used to confirm phenotypic classifications.

1.4.3 Identification of novel *Streptomyces* species

The classical methods for identifying *Streptomyces* species, based on characteristics like spore chain and spore morphology, pigmentation, physiological abilities, and resistance to antibiotics, were laid out by the International *Streptomyces* Project (Shirling and Gottlieb 1966; Williams *et al.*, 1983).

Since the advent of molecular biology, DNA based molecular methods have been used for species delineation and the identification of *Streptomyces* species. DNA-DNA reassociation has proved to be suitable for the study of relationships between closely related taxa, such as species. Strains belonging to the same species will have a greater than 70% DNA-DNA relatedness (Stackebrandt and Goebel, 1994). While DNA-DNA reassociation can be useful in the characterisation of *Streptomyces* species, genome instability requires that this method should be used in correlation with other tests (Anderson and Wellington, 2001).

Sequence analysis of the genes coding for the ribosomal subunits (16S, 23S and 5S rRNA), in particular the 16S rRNA gene, has become an important tool in bacterial identification since it provides information about the phylogenetic placement of species

(Brown *et al.*, 2007). While the overall DNA sequences of the ribosomal genes are highly conserved, the genes also contain variable regions, which can be useful for species discrimination (Stackebrandt and Goebel 1994, Rosselló-Mora and Amann, 2001).

1.4.4 Isolation of *Streptomyces*

The selective isolation of *Streptomyces* species can be achieved using selective nutrient sources in the cultivation media (Atalan *et al.*, 2000). Streptomycetes are capable of utilizing many biopolymers and are able to grow on an inorganic nitrogen source such as nitrate. Media containing starch as the carbon source and nitrate, casein or arginine as the nitrogen source have been shown to be the most useful growth media for their selective isolation (Brandelli *et al.*, 2010). Antifungal agents such as cycloheximide, nystatin and pimaricin can be added to the isolation media to suppress fungal growth.

Mesophilic streptomycetes are normally cultivated at temperatures from 22 to 37°C, while thermophilic species are cultured between 40 and 55°C (Rebollido *et al.*, 2008). As the majority of streptomycetes are neutrophilic, isolation media are typically at a neutral pH (pH 7.2-7.6). When acidophilic strains are to be isolated, the pH of the medium can be adjusted to 4.5 and for alkalophilic strains to pH 10-11. However, some species may show adaptation to a wide pH range (Suutari *et al.*, 2000).

1.4.5 Secondary metabolite production by *Streptomyces*

Secondary metabolites are compounds produced by an organism but are not necessary for the growth or other vital processes in the cell (Vining, 1990). They are mostly produced by microbial genera inhabiting soil and undergoing morphological differentiation (Vining, 1990). More than 23 000 secondary metabolites are known of which 42% are produced by actinobacteria, 16% by other bacteria and 42% by fungi (Lazzarini *et al.*, 2000).

Out of the 10 000 recognized antibiotics, 55% are produced by *Streptomyces* species, making streptomycetes the most effective producers of secondary metabolites (Demain, 1999; Lazzarini *et al.*, 2000). The bioactive compounds produced by streptomycetes have a wide spectrum of biological activities; e.g. antibacterial (streptomycin, tetracycline, chloramphenicol), antiparasitic (ivermectin), antitumor (actinomycin, mitomycin C, anthracyclines), antiviral (tunicamycin), immunosuppressive (rapamycin), diabetogenic (bafilomycin, streptomycin) and enzyme inhibitory (clavulanic acid). Secondary metabolites have comparable structures, similar to spore pigments and are synthesized by the same kinds of mechanisms (Metsä-Ketelä *et al.*, 1999; Nakano *et al.*, 2000). Table 1.5 lists some of the secondary metabolites.

Genes coding for the proteins that play a role in the synthesis of secondary metabolites are often clustered (Pissowotzki *et al.*, 1991). These clusters incorporate genes for biosynthesis, determinants for regulation and self-resistance. Sometimes these genes are situated in plasmids and horizontal transfer of genes coding for secondary metabolites can take place in the soil (Egan *et al.*, 1998; Ōmura *et al.*, 2001).

Availability of nutrients affects the production of secondary metabolites. Antibiotic production is often improved by the presence of a non preferred carbon source or by phosphate starvation in fermentation experiments (McDowall *et al.*, 1999). Production of secondary metabolites could also be influenced by the availability of nitrogen sources (Aharonowitz, 1980).

The regulation of sporulation appears to be linked to regulation mechanisms for the production of secondary metabolites (Horinouchi and Bappu, 1992). In *Streptomyces griseus* the A-factor, a hormone-like regulatory factor, induces these processes. Streptomycetes possess a complex regulatory apparatus as illustrated by the fact that 12% of the proteins

coded by the genes of *S. coelicolor* are predicted to have regulatory functions (Bentley *et al.*, 2002).



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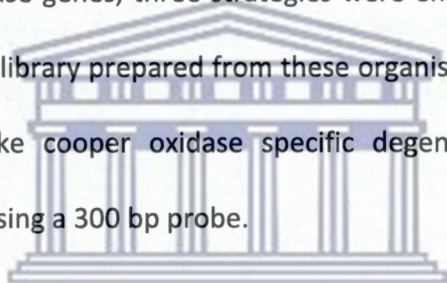
Table 1.5: Secondary metabolites produced by *Streptomyces*

Compound	Biological activity	Species	Reference
Streptozotocin	Diabetogenic	<i>S. achromogenes</i>	Herr <i>et al.</i> (1967)
Streptomycin	Antimicrobial	<i>S. griseus</i>	Egan <i>et al.</i> (1998)
Bafilomycin C	ATPase inhibitor of micro-organisms, plant and animal cells	<i>S. griseus</i> ,	Frändberg <i>et al.</i> (2000)
Mitomycin C	Antitumor, binds to double-stranded DNA	<i>S. lavendulae</i>	Mao <i>et al.</i> (1999)
Hygromycin	Antimicrobial, immunosuppressive	<i>S. hygrosopicus</i>	Uyeda <i>et al.</i> (2001)
Lincomycin	Antibacterial, inhibitor of protein biosynthesis	<i>S. lincolnensis</i>	Peschke <i>et al.</i> (1995)
Chloramphenicol	Antimicrobial, inhibitor of protein biosynthesis	<i>S. venezuelae</i>	Bewick <i>et al.</i> (1976)
Valinomycin	Ionophor, toxic for pro- and eukaryotes	<i>S. griseus</i>	Anderson <i>et al.</i> (1998)
Anthracyclines	Antitumor	<i>S. galileus</i>	Fujii and Ebizuka (1997)
Avermectin	Antiparasitic	<i>S. avermitilis</i>	Burg <i>et al.</i> (1979)
Tetracycline	Antimicrobial	<i>S. rimosus</i>	Hansen <i>et al.</i> (2001)
Rapamycin	Immunosuppressive, antifungal	<i>S. hygrosopicus</i>	Vezina <i>et al.</i> (1975)

1.5 Research Objectives

The presented research had two core objectives. The first was the full polyphasic classification of two actinobacterial isolates. Strain MS26 was isolated from a soil sample collected from the Zambian Copperbelt region and isolate DFNR17 from a soil sample collected from a New Zealand farm. Identification of these strains was based on cultural, morphology, physiology and biochemical characteristics, as well as 16S rRNA gene analysis.

The second objective was to screen strains MS26 and DFNR17 for novel laccases using degenerate primers designed from several *Streptomyces* multicopper oxidases. For the isolation of the full laccase genes, three strategies were employed: functional/activity-based screening of a fosmid library prepared from these organisms, PCR-based screening of the library using laccase-like copper oxidase specific degenerate primers and colony hybridization of the library using a 300 bp probe.



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Chapter 2: Materials and methods

2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 2.1. Stock cultures were maintained at -80 °C as cell suspensions in 25% (v/v) glycerol (Sambrook *et al.*, 1989).

Table 2.1: Bacterial strains and plasmids used in this study

Name	Genotype/ relevant feature(s)	Reference/supplier
Strains:		
BM4	Mesophilic actinobacterial isolate	Le Roes-Hill, unpublished
HMC13	Mesophilic actinobacterial isolate	Le Roes-Hill, unpublished
7H1	Mesophilic actinobacterial isolate	Le Roes-Hill, unpublished
MS26	Mesophilic actinobacterial isolate	Le Roes-Hill, unpublished
DFRN17	Mesophilic actinobacterial isolate	Le Roes-Hill, unpublished
#18	Mesophilic actinobacterial isolate	Le Roes-Hill, unpublished
ORS`#3	Thermophilic actinobacterial isolate	Le Roes-Hill, unpublished
<i>E. coli</i> (DH5α)	<i>recA endA1 hsdR17 supE4 gyrA96 relA1</i> <i>Δ(lacZYA-argF)U169 (φ80dlacZΔM15)</i>	Promega
<i>E. coli</i> (GeneHog®)	F- <i>mcrA D(mrr-hsdRMS-mrcBC)</i> <i>f80lacZDM15 DlacX74 deoR recA1 endA1</i> <i>araD139 D(araleu) 7697 galU galK l rpsL</i> <i>nupG</i>	Invitrogen
Plasmids		
pGEM®-T-Easy	Size 3015 bp, T7 promoter, SP6 promoter, <i>Amp^r</i> , <i>lac</i> operator, <i>LacZ</i> start codon, phage <i>f1</i> region, pUC M13 priming sites, 3' – T overhangs	Promega

2.2 Media and growth conditions

Luria-Bertani (LB) broth

This medium was routinely used to grow bacterial strains.

Constituent	L ⁻¹
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
dH ₂ O up to	1000 ml

The medium was sterilized by autoclaving. LB agar (LBA) medium contained 1.5 % (w/v) agar. For laccase assays LBA was prepared using 0.05 M sodium acetate buffer (pH 5.0), 0.05 M potassium phosphate buffer (pH 7.0) and 0.05 M Tris-HCl buffer (pH 9.0). When necessary, the appropriate antibiotic was added after autoclaving.

SOB agar

This medium was used to culture the starter inoculum for the preparation of electrocompetent *E. coli* cells.

Constituent	L ⁻¹
Tryptone	20.0 g
Yeast extract	0.50 g
NaCl	0.50 g
250 mM KCl	10.0 ml
Agar	15.0 g
dH ₂ O up to	1000ml

The pH was adjusted to 7.0 with 10 M NaOH before autoclaving. After autoclaving, the broth was cooled to ~55°C and the following filter sterilized solutions were added aseptically: 5 ml of MgCl₂ (final concentration 2 M) and 20 ml glucose (1 M).

SOC Medium

SOC medium was used for the recovery of newly transformed *E.coli* cells.

Constituent	L⁻¹
Tryptone	20.0 g
Yeast extract	5.0 g
NaCl	0.5 g
250 mM KCl	10.0 ml

The pH was adjusted to 7 before autoclaving; the medium was cooled to ~ 55°C and the following were filter sterilized solutions and added aseptically, 5 ml of 2 M MgCl₂ and 20 ml of 1 M glucose. The medium was made up to 1 L.

2xYT medium

2xYT media was used to culture *E. coli* cells for the preparation of electrocompetent cells.

Constituent	L⁻¹
Tryptone	16 g
Yeast extract	10 g
NaCl	5 g

The pH was adjusted to 7.0 with 10 M NaOH and the final volume was adjusted to 1 L with H₂O. The medium was sterilized by autoclaving and stored at room temperature.

Bennett's medium

This medium was used for determining the degradation activity of actinomycete strains.

Constituent	L⁻¹
Glycerol	10.0 g
Casitone (Difco)	2.0 g
Yeast extract	1.0 g
Beef extract (Oxoid)	1.0.g

The pH was adjusted to 7.0 with 10 M NaOH and the final volume was adjusted to 1 L with dH₂O. The medium was sterilized by autoclaving, and allowed to cool to ~ 55°C before use.

YEME Medium

This medium was used for routine maintenance of the actinomycete isolates.

Constituent	L⁻¹
Yeast extract	4.0 g
Glucose	4.0 g
Malt extract	10.0 g
Agar	20.0 g

The pH was adjusted to 7.2 with 10 M NaOH and the final volume was adjusted to 1 L with dH₂O. The medium was sterilized by autoclaving and allowed to cool to ~ 55°C before use.

ISP Medium No.4

This medium was used for determination of the colour of the spore mass (aerial mycelium), the spore-chain morphology and the colour of the substrate mycelium.

Solution 1

Constituent	500 ml
Soluble starch (BDH potato starch)	10.0 g
dH ₂ O up to	500 ml

Solution 2

Constituent	500 ml ⁻¹
K ₂ HPO ₄	1.0 g
MgSO ₄ ·7H ₂ O	1.0 g
NaCl	1.0 g
(NH ₄) ₂ SO ₄	2.0 g
CaCO ₃	2.0 g
Trace salts solution	1.0 ml
dH ₂ O up to	500 ml



The pH of solution 2 was adjusted to 7.0 with 10 M NaOH and the volume to 500 ml with dH₂O. The two solutions were mixed together and 20 g agar was added. The medium was sterilized by autoclaving and allowed to cool to ~ 55°C and poured into petri dishes. Pure colonies of the isolate were streaked on the solidified medium and the plates were incubated at 28°C for 14 days.

Trace salts solution

Constituent	100 ml ⁻¹
FeSO ₄ ·7H ₂ O	0.1 g
MnCl ₂ ·4H ₂ O	0.1 g
ZnSO ₄ ·7H ₂ O	0.1 g

Made up to 100 ml and filter sterilized.

ISP Medium No.5

This medium was used for determination of the colour of the spore mass (aerial mycelium), the spore-chain morphology and the colour of the substrate mycelium.

Constituent	L ⁻¹
L-Asparagine monohydrate	1.0 g
Glycerol	10.0 g
K ₂ HPO ₄	1.0 g
Trace salts solution (see ISP4)	1.0 ml
dH ₂ O up to	1000 ml

The pH of the medium was adjusted to 7.0 with 0.1 M HCl and the volume to 1000 ml with dH₂O and 20 g agar was added. The medium was sterilized by autoclaving and allowed to cool to ~ 55°C and poured into petri dishes. Pure colonies of the isolate were streaked on the solidified medium and the plates were incubated at 28°C for 14 days.

ISP Medium No.6

This medium was used for determination of the colour of the spore mass (aerial mycelium), the spore-chain morphology and the colour of the substrate mycelium.

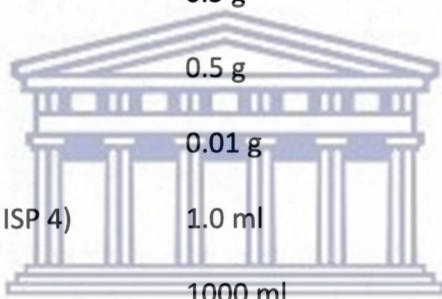
Constituent	L ⁻¹
Peptone	15.0 g
Proteose peptone	5.0 g
Ferric ammonium citrate	0.5 g
K ₂ HPO ₄	1.0 g
Na ₂ S ₂ O ₃	0.08 g
Yeast extract	1.0 g
dH ₂ O up to	1000 ml

The pH of the medium was adjusted to 7.0 with 10 M NaOH and the volume to 1L with dH₂O. 15.0 g agar was added. The medium was sterilized by autoclaving and allowed to cool to ~ 55°C and poured into petri dishes. Pure colonies of the isolate were streaked on the solidified medium and the plates were incubated at 28°C for 4 days.

ISP Medium No.7

This medium was used for determination of the colour of the spore mass (aerial mycelium), the spore-chain morphology and the colour of the substrate mycelium.

Constituent	L ⁻¹
Glycerol	15.0 g
L-Tyrosine	0.5 g
L-Asparagine monohydrate	1.0 g
K ₂ HPO ₄	0.5 g
MgSO ₄ .7H ₂ O	0.5 g
NaCl	0.5 g
FeSO ₄ .7H ₂ O	0.01 g
Trace salts solution (see as in ISP 4)	1.0 ml
dH ₂ O up to	1000 ml



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The pH of the medium was adjusted to 7.2 with 0.1 M HCl and the volume to 1L with dH₂O. 20.0 g of agar was added. The medium was sterilized by autoclaving and allowed to cool to ~ 55°C and poured into the petri dishes. Pure colonies of the isolate were streaked on the solidified medium and the plates were incubated at 28°C for 4 days.

R2A

This medium was used for isolating actinomycetes.

Constituent	L ⁻¹
Yeast extract	1.0 g
Peptone	1.0 g
Casamino acids	1.0 g
Glucose	1.0 g
Starch	1.0 g
Sodium tartrate	0.6 g
K ₂ HPO ₄	0.6 g
MgSO ₄ ·7H ₂ O	0.1 g
dH ₂ O	1000 ml

The pH was adjusted to 7.0 with 10 M NaOH and the volume to 1 L with H₂O. The medium was sterilized by autoclaving and allowed to cool to ~ 55°C before use.

2.3 General recombinant DNA procedures

E. coli plasmid DNA was prepared by the alkaline lysis method of Ish-Horowicz and Burke (1981) (Section 2.6.3.1) or using the QIAprep[®] Spin Miniprep Kit (Qiagen). All DNA modifications and manipulations were performed according to standard procedures (Sambrook *et al.*, 1989).

2.3.1 Agarose gel electrophoresis

Genomic DNA and PCR products were separated in 1 % and 1.5 % (w/v) agarose gels prepared in 0.5x TAE buffer respectively (Sambrook *et al.*, 1989). Samples were prepared by adding 10µl 6x loading buffer (20 % (v/v) glycerol and 5 mg/ml bromophenol blue). Electrophoresis was performed in 0.5x TAE buffer at 100 V. Ethidium bromide solution (0.5 µg/ml) was added to molten agarose before the gels were cast. DNA bands were sized according to their migration in the gel as compared to a DNA molecular weight marker (e.g., λ DNA cut with *Pst*I restriction enzyme). Gels were visualized via ultraviolet (UV) light illumination at a peak wavelength of 302 nm and photographed with a digital imaging system (Alphamager 2000, Alpha innotech, San Leandro, CA).

2.3.2 DNA quantification

For routine quantification DNA concentrations were determined using a Nanodrop ND-1000. DNA was resuspended in double distilled water overnight at 4°C. For more accurate quantification DNA concentrations were measured using the Qubit™ DNA assay kit according to the standard procedures.

2.3.3 Gel extraction and DNA purification

DNA fragments were briefly visualized under UV illumination at a peak wavelength of 302 nm and excised from agarose gels using a sterile scalpel blade. A GFX PCR DNA and gel band purification kit (GE Healthcare Life Sciences) was used to purify the DNA from the gel slices according to the manufacturer's instructions. The DNA was eluted in 10mM Tris-buffered double distilled sterile water at pH 8.0.

2.4 Genomic DNA extraction

Genomic DNA extraction from isolates was carried out according to the method described by Wang *et al.* (1996) with modifications. Cells were harvested by centrifugation at 10 000 rcf for 2 minutes until a pellet was obtained. Cells were resuspended in 500 µl of lysis buffer (25 mM Tris-HCl pH 8, 50 mM glucose, 10 mM EDTA and 25 mg/ml lysozyme) and incubated at 37°C overnight. SDS was added to a final concentration of 1 % and the tubes were mixed by inversion and incubated at 65°C for 30 minutes. An equal volume of phenol was added to the samples and the tubes were mixed ten times by inversion and centrifuged at 10 000 rcf for 1 minute. The upper aqueous phase was transferred to a new microcentrifuge tube, an equal volume of chloroform:isoamyl alcohol (24:1) was added and the tubes were mixed gently. Cells were centrifuged as before and the upper aqueous phase was transferred to a new tube. DNA was precipitated with 1 volume of ice-cold isopropanol. The tubes were centrifuged at 10 000 rcf for 5 minutes and the supernatant was discarded. The DNA pellet was air-dried and the DNA was resuspended in 50 µl of 1 X TE buffer and stored at 4°C.

2.5 PCR amplification

PCR amplifications were performed in 0.2 ml thin walled tubes using an Eppendorf Mastercycler gradient thermocycler equipped with a heated lid. Primer sets employed in this study are listed in Table 2.2. A standard 50 µl PCR reaction contained approximately 100 ng chromosomal DNA template, 0.5µM of each primer, 200 µM of each dNTP (dATP, dTTP, dCTP, dGTP), 1XPCR buffer (100 mM Tris-HCl pH 8.8, 10mM KCl, 10mM (NH₄)₂SO₄, 0.1 % (w/v) Triton X-100, 2 mM MgCl₂) and 1 U/µl *Taq* polymerase. The reaction mixture was

made up to 50 μ l with sterile ddH₂O. The PCR products were electrophoresed on a gel to confirm the correct fragment had been amplified.

2.5.1 M13 Colony PCR

M13 colony PCR was used to verify the presence of the correct sized insert cloned into pGEM[®]-T Easy. A sterile toothpick was used to transfer a small amount of cell mass from white colonies growing on LBA Amp plates containing Xgal (0.5 mM) and IPTG (80 μ g/ml) into 20 μ l TE buffer. The mixture was vortexed and 2 μ l was used as the template for PCR. Amplification was performed in an automated thermal cycler (Thermo Hybaid) with the cycling parameters detailed in Table 2.2. The products were purified using the QIAprep[®] Spin Miniprep Kit (QIAGEN) and sequenced by the DNA sequencing facility at the Department of Molecular and Cell Biology, University of Cape Town.

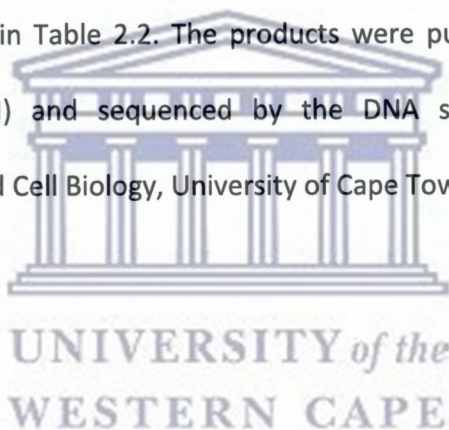


Table 2.2: Primers used in this study for PCR amplification of genes

Primer set	Sequence (5' to 3')	Amplification cycles	Specificity	Reference
16S-F1	AGAGTTTGATCITGGCTCAG	[94°C for 5min 30 x (94°C for 30s, 52°C for 30s, 72°C for 1:5 min) 72°C for 7min] [#]	Bacterial universal 16S rRNA	Weisberg <i>et al.</i> (1991)
16S-R5	ACGGITACCTTGTTACGACTT			
16S-F3	GCCAGCAGCCGGGTAATAC	[94°C for 5min 30 x (94°C for 30s, 52°C for 30s, 72°C for 1:5 min) 72°C for 7min] [#]	Bacterial universal 16S rRNA	Weisberg <i>et al.</i> (1991)
16S-R3	CACGAGCTGACGACAICCATGC			
M13F	GTAAAACGACGGCCAGT	[94°C for 3mins 35 x (94°C for 30s, 55°C for 30s, 72°C for 30s) 72°C for 5min] [#]	pGEM [®] T-Easy	Yanisch-Perron <i>et al.</i> (1985)
M13R	ATTACCGGGCTGTGG			
SCuOxF	CSRTC GTCTTCAACGAYATG	[94°C for 3mins 30 x (94°C for 30s, 56°C for 30s, 72°C for 30s) 72°C for 7min] [#]	<i>Streptomyces</i> laccase like gene	This study
SCuOxR	GCASRTGGCAGTGGTACAT			

* IMBM lab *Taq* used

Phusion *Taq* polymerase used

2.5.2 *Streptomyces* laccase-like copper oxidase primer design

Streptomyces laccase-like copper oxidases primers (SCuOxF/R, Table 2.2) amplifying a 300 bp product were designed by Dr Ana Casanueva, Institute for Microbial Biotechnology and Metagenomics, University of the Western Cape. Table 2.3 lists the sequences used to design the primers.

Table 2.3: Laccase-like copper oxidase sequences used for alignments to design primers

Species	NCBI Accession no.	Number of Amino acids	Assigned Function
<i>S. coelicolor</i>	CAB45586	343 aa	SLAC
<i>S. griseus</i>	BAB64332	348 aa	EpoA
<i>S. ipomoeae</i>	ABH10611	335 aa	SilA
<i>S. clavuligerus</i>	ZP_03185908	335 aa	copper oxidase
<i>S. pristinaespiralis</i>	ZP_06908025	330 aa	copper oxidase

2.6 Cloning of PCR products

Ligations were carried out using a p-GEM[®]-T Easy kit (Promega) according to the manufacturer's instructions. Ligations were carried out in 10 µl volumes containing 5 µl of rapid ligation buffer, 1 µl of pGEM-T[®] Easy vector, 3 µl of PCR product and 1 µl ligase.

2.6.1 Preparation of *E. coli* competent cells

Competent *E. coli* DH5 α cells were prepared and transformed as described by Inoue *et al.* (1990). A single colony of a freshly streaked *E. coli* DH5 α culture was inoculated into 20 ml SOC medium and cultured for 8 hours at 37°C with agitation at 250 rpm. A 2 ml aliquot of the overnight culture was inoculated into 250 ml sterile SOC medium and incubated at 18°C with shaking to mid-exponential phase (OD₆₀₀ of 0.4 to 0.55). The cells were pelleted in polypropylene tubes by centrifugation at 4,000 x *g* for 10 min at 4°C in a J2-21M rotor (Beckman-USA). The supernatant was decanted and the pellet was washed twice with transformation buffer. The cells were resuspended gently in 2 ml of ice-cold Inoue transformation buffer (Inoue *et al.*, 1990) to which 150 μ l DMSO had been added. Following incubation on ice for 15 minutes, 50 μ l of cells were aliquoted into 0.5 ml microcentrifuge tubes, frozen immediately using liquid nitrogen and stored at -70°C until needed.

2.6.2 Electroporation of *E. coli*

The electrocompetent Gene Hog® *E. coli* cells were transformed as follows. A 50 μ l aliquot of electrocompetent cells was removed from -80°C storage and allowed to thaw on ice. Once thawed, 2 μ l of the ligation mixture (section 2.6) was added and the cells were gently mixed. The mixture was incubated on ice for ~ 1 min then transferred to a pre-chilled 0.1 cm sterile electroporation cuvette (Bio-Rad). Electroporation was performed under the following conditions: 1.8 kV, 25 μ F, 200 Ω in a BioRad Gene Pulser machine. Immediately following electroporation 1 ml of SOB broth was added to the cuvette and the cells were transferred to a 15 ml Falcon tube and incubated at 37°C for 1 hour with agitation at 250 rpm. Following recovery 100 μ l of the cells were plated onto LB-agar plates supplemented with ampicillin (100 μ g/ml), IPTG (20 μ g/ml) and X-Gal (30 μ g/ml). Recombinant

transformants were selected by blue/white colour selection based on insertional inactivation of the *lacZ* gene for transformations done using pGEM T-Easy™.

2.6.3 Small scale plasmid purification

2.6.3.1 Alkaline lysis method

Single colonies were selected from the agar plates and inoculated into 5 ml LB broth supplemented with the appropriate antibiotic(s). The culture was incubated overnight at 37°C with agitation at 250 rpm. Plasmid DNA was isolated from the overnight cultures by the alkaline lysis method (Sambrook and Russell, 2001), with slight modifications. A 2 ml aliquot of the overnight culture was transferred into 2 ml tubes and the cells were harvested by centrifugation at 10,000 x *g* for 1 minute at room temperature. The supernatant was discarded and the pellet was resuspended in 200 µl of solution 1 (50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA pH 8.0). 200 µl of solution 2 (1% [w/v] SDS and 0.2 M NaOH) was added to the mixture and the tube was mixed by inversion and incubated for 5 minutes on ice. Following the addition of 300 µl 7.5 M ammonium acetate (pH 7.6) the tubes were incubated on ice for 10 minutes and centrifuged at 13,000 x *g* for 15 minutes at room temperature. The supernatant was transferred to a new tube, 500 µl of chloroform: isoamyl alcohol (24:1) was added and the samples were centrifuged at 13,000 x *g* for 10 minutes at 4°C. The supernatant was transferred to a new tube and the plasmid DNA was precipitated by the addition of an equal volume of isopropanol. The tubes were incubated at -80°C for 15 min and centrifuged at 13,000x *g* for 10 mins at 4°C. The pellet was dried and dissolved in TE containing RNase A to a final concentration of 20 µg/ml.

2.6.3.2 Plasmid minipreps using a kit

Plasmid DNA for DNA sequencing was extracted using the Zymo miniprep kit according to the manufacturer's instructions (Zymo Research, USA).

2.7 Sequencing

Sequencing reactions were performed using M13F and M13R oligonucleotide primers (Table 2.2) for constructs in the pGEM T Easy™ vector. Sequencing of the gel-purified DNA fragments of the bacterial 16S rRNA gene PCR amplification reactions was carried out with primers 16S-F1/16S-R5 and 16S-F3/16S-R3 (Table 2.2). Sequencing was performed using the Hitachi 3130xl DNA Analyzer (Applied Biosystems) using the Big Dye Terminator v3.1 system.

2.8 Phylogenetic analysis

The chromatograms of the DNA sequences were edited using Chromas software before alignment using BioEdit (Tamura *et al.*, 2007). Unrooted phylogenetic trees were constructed using the neighbourhood joining method (Saitou and Nei, 1987) in *MEGA 4* (Tamura *et al.*, 2007). The robustness of the tree topology was evaluated by bootstrap analysis based on 1000 resamplings (Felsenstein, 1985). The amplified DNA sequences were identified through homology searches using BLAST against the NCBI non-redundant database.

2.9 Biochemical testing and physiological characterisation

2.9.1 Degradation of tyrosine

100 ml of Bennett's medium (as prepared in section 2.2) containing 2 g of agar was mixed with 0.5 g of tyrosine and autoclaved for 15-20 minutes at 121°C at 15psi. This was cooled to 55°C, poured into plates and allowed to solidify. Pure colonies of MS26 and DFNR17 isolates were streaked on the agar and incubated at 28°C for 3 weeks and observed for zones of hydrolysis adjacent to the growth streak.

2.9.2 Degradation of gelatin

100 ml of Bennett's medium (as prepared in section 2.2) containing 2 g of agar was mixed with 0.4 g of gelatin, autoclaved and poured into plates. Pure colonies of the isolate were streaked on the agar and incubated at 28°C for one week. After incubation the plates were flooded with (NH₄)₂SO₄ and observed for zones of hydrolysis along the growth streak.

2.9.3 Degradation of starch

100 ml of Bennett's medium (as prepared in section 2.2) containing 2 g of agar was mixed with 1.0 g of starch, autoclaved and poured into plates. Pure colonies of the isolate were streaked on the agar and incubated at 28°C for one week. After incubation the plates were flooded with Gram's iodine and observed for zones of hydrolysis along the growth streak.

2.9.4 Degradation of xylan

100 ml of Bennett's medium (as prepared in section 2.2) containing 2 g of agar was mixed with 0.4 g of xylan and autoclaved and poured into plates. Pure colonies of the isolate

were streaked on the agar and incubated at 28°C for 3 weeks and observed for zones of hydrolysis.

2.9.5 Degradation of nitrogenous bases

Adenine and guanine agar plates were prepared by mixing 0.5 g of adenine and 0.05 g of guanine separately in 10 ml of distilled water and autoclaved. Bennett's medium (90 ml) containing 2 g of agar (section 2.2) was also prepared. The sterile nitrogenous base suspensions were added to the molten agar and plates were poured. Pure colonies of MS26 and DFNR17 isolates were streaked onto the agar plates and incubated at 28°C for 3 weeks and observed for zones of hydrolysis.

2.9.6 Degradation of hypoxanthine and xanthine

Hypoxanthine and xanthine agar was prepared as follows. 0.4 g of hypoxanthine or 0.4 g of xanthine was dissolved in 10 ml of distilled water and autoclaved. Bennett's medium (90 ml) (section 2.2) containing 2 g of agar was also prepared. The sterile hypoxanthine or xanthine suspension was added to the molten agar and plates were poured. Pure colonies of MS26 and DFNR17 isolates were streaked onto the agar plates and incubated at 28°C for 3 weeks. The plates were observed for zones of hydrolysis.

2.9.7 Degradation of casein

Casein agar plates were prepared by mixing 1 g skim milk powder with 10 ml distilled water. The solution was autoclaved for 10 minutes at 121 psi. Bennett's medium (90 ml) containing 2 g agar was prepared. The mixtures were combined and the agar was poured into plates. Isolates were streaked onto the agar and incubated at 28°C for 3 weeks and observed for zones of hydrolysis.

2.9.8 Hydrolysis of pectin

Pectin agar was prepared by dissolving 4.0 g KH_2PO_4 and 7.25 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in 200 ml distilled water and the pH was adjusted to pH 7.0 with NaOH. 5 g pectin was dissolved in 200 ml of distilled water. Basal medium contained 10 g agar, 2.0 g $(\text{NH}_4)_2\text{SO}_4$, 1.0 g yeast extract, 2.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 ml 1 % solution $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.1 ml 1 % solution of CaCl_2 . The medium was made up to a final volume of 600 ml with distilled water. The three solutions were sterilized separately. The solutions were added to the molten agar and plates were poured. Isolates were streaked onto the agar plates and incubated at 28°C for 6 days. After incubation the plates were flooded with 1 % CTAB solution and left for 30-40 minutes to allow unhydrolysed pectin to precipitate.

2.9.9 Lecithinase activity

Egg-yolk agar was prepared by dissolving 2.4 g of agar, 2.0 g peptone, 1.0 g yeast extract and 2.0 g NaCl in distilled water. The final volume was adjusted to 178 ml. 2 ml of sterile glucose (10 %) and 2 ml of egg-yolk (50 %) emulsion were added to the tempered molten agar. The mixtures were combined and the plates were poured. Pure colonies of isolates were streaked onto the agar and incubated at 28°C for 6 days. Plates were observed for lecithinase activity.

2.9.10 Degradation of Tween 80

Sierra agar was prepared by dissolving 10.0 g peptone, 5.0 g NaCl and 0.114 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in distilled water and the final volume was adjusted to 900 ml. The pH was adjusted to 7.4 and 15.0 g of agar was added. 10 ml Tween 80 was mixed with 90 ml of distilled water and autoclaved. The two mixtures were combined and plates were poured.

Pure colonies of isolates were streaked onto the agar and incubated at 28°C for 2 weeks and observed for droplet formation.

2.9.11 Hydrolysis of aesculin and arbutin

Aesculin and arbutin agar plates were prepared by dissolving 0.6 g yeast extract, 0.1 g ferric ammonium citrate and 0.2 g of either aesculin or arbutin in distilled water to a final volume of 200 ml. The pH of the solutions was adjusted to 7.0 and 2 g of agar was added. A third solution was prepared as above omitting the substrates (aesculin and arbutin) to serve as a colour-control plate. The isolates were streaked onto the plates and incubated at 28°C for 3 weeks. Plates were observed for a colour change from light brown to dark brown.

2.9.12 Inhibition by NaCl

NaCl agar was prepared by dissolving 4.0 g, 7.0 g, 10.0 g and 13.0 g NaCl separately in 80 ml of Bennett's medium as prepared in section 2.2. The final volume was adjusted to 100 ml with Bennett's medium. Each solution was mixed with 2.0 g agar and sterilized by autoclaving. The isolates were streaked onto the plates and incubated at 28°C for 2 weeks. Plates were observed for the presence or absence of growth.

2.9.13 Antibiotic susceptibility

Antibiotic susceptibility agar was prepared by dissolving 2.0 g agar in 100.0 ml Bennett's medium (section 2.2) and the solution was sterilized by autoclaving. Working stocks of the antibiotics (Table 2.4) were prepared in water, filter sterilised and added to a final concentration as indicated in Table 2.4. A plate which did not contain any antibiotics was included as a control. Plates were incubated at 28°C and observed for the presence of growth on a daily basis for 7 days.

Table 2.4: Antibiotics used for susceptibility testing of actinomycetes

ANTIBIOTICS	FINAL CONCENTRATION
Ampicillin	100 µg/ml
Chloramphenicol	12.5 µg/ml
Gentamycin	100 µg/ml
Kanamycin	50 µg/ml
Lincomycin	100 µg/ml
Penicillin G	10 I.U./ml
Streptomycin	100 µg/ml

2.9.14 Growth temperature

The growth temperature range of the isolates was determined on YEME agar (Section 2.2). The plates were incubated at 16°C, 30°C, 37°C, 45°C, 55°C, 60°C and 68°C for 2 weeks and observed for colony growth.

2.10 Microscopy

Approximately 10 µl of a culture was placed on a sterile microscope slide and covered with a coverslip. The cells were observed with a light microscope using a 100X oil-immersion objective (Axioplan 2, Zeiss).

2.11 Construction of the fosmid library

Fosmid library MD# was prepared from isolates MS26, DFNR17 and #18 using the Copy Control Fosmid Library Production Kit (EPICENTRE) according to the manufacturer's instructions by Dr Ana Casanueva, Institute for Microbial Biotechnology and Metagenomics, University of the Western Cape. The fosmid library was infected into *E. coli* EPI300 cells.

2.11.1 Activity-based screening of the fosmid library

The fosmid DNA library was screened for laccase activity by adapting the assay based on the chromogenic oxidative coupling reaction between ABTS/guaiacol and CuSO_4 to a solid agar assay. Laccase activity was screened on LBA supplemented with 12.5 $\mu\text{g}/\text{ml}$ chloramphenicol, 0.01 %arabinose, 1mM CuSO_4 and either 1 mM ABTS or 0.012 % guaiacol. Media with both substrates were prepared at pH 5, 7 and 9.

2.12 Southern hybridization and colony hybridization

Genomic DNA from the actinomycete strains MS26 and DFNR17 and plasmid DNA (for probe preparation) were digested to completion with the appropriate restriction endonucleases. The DNA was size fractionated by electrophoresis on an 0.8% agarose gel in TRIS-acetate-EDTA buffer and transferred to a Hybond N⁺ nylon membrane (Amersham Pharmacia Biotech) according to the manufacturer's instructions. DNA probes were purified from a 0.8% agarose gel and random prime labelled with digoxigenin-11-dUTP (DIG) using the Digoxigenin Labelling and Detection Kit (Roche Diagnostics) according to the manufacturer's instructions. DNA hybridization with the DIG-labelled probe was performed overnight at 42°C using DIG-EASY hybridization buffer. Signals were detected using chemiluminescent detection with CDP-Star[®] (Roche Diagnostics).

Chapter 3: Characterisation of laccase producing actinomycete strains

3.1 Introduction

In biological terms, soil is a complex system. Many biological processes occur in the first few inches below the surface (Riesenfeld *et al.*, 2004). The ever-expanding field of phylogenetics is revealing the immense diversity of microbial life within this ecosystem (Riesenfeld *et al.*, 2004). Bacteria and fungi are in the greatest abundance in soils due to their nutritional versatility and fast doubling time. It is estimated that one gram of soil can be inhabited by up to 1 billion microorganisms belonging to thousands of different species (Fredrickson *et al.*, 2004). The complex web of biological interactions in soil serves a multitude of purposes such as decomposition, nutrient recycling and shuffling, toxin sequestration and disease suppression, many of which have been exploited by researchers for valuable purposes in society (Roselló-Mora and Amann, 2001; Riesenfeld *et al.*, 2004).

Actinomycetes are widely dispersed in soil and play an important role in break down and mineralization cycles by producing extracellular enzymes such as chitinases, cellulases, laccases and peroxidises, and by participating in the turnover of soil components, most importantly in the transformation of organic compounds (Bhattarai *et al.*, 2007). Streptomycetes are saprophytic bacteria and take part in important environmental processes including the decomposition of organic matter, especially complex mixtures of polymers such as chitin, hemicellulose, keratin, lignocellulose, pectin, starch and even some man-made compounds that may reach soil as contaminants. They also participate in biodegradation by recycling nutrients associated with recalcitrant polymers (McCarthy, 1987; McCarthy and Williams, 1992; Crawford, 1993).

Actinomycetes are also important in the rhizosphere, where they play an important role in influencing plant growth and protecting plants roots against invasion by pathogenic fungi (Goodfellow and O'Donnell, 1993; Loqman *et al.*, 2009). The possible role of actinomycetes as biological control agents of soil-born root diseases in crop plants has been investigated, mostly in greenhouse experiments, and several *Streptomyces* species have been shown to protect different plant species against soil borne fungal pathogens (Crawford *et al.*, 1993). Some genera have also been shown to produce herbicidal and insecticidal compounds (Crawford *et al.*, 1993; Hoagland *et al.*, 2007). Similarly, members of the actinobacterial genus *Frankia* can fix nitrogen. *Frankia* have a broad host range and have been shown to form root nodule symbioses with more than 200 species of flowering plants (Mincer *et al.*, 2002).

Actinomycetes are of great interest to industry because of their ability to produce important secondary metabolites. Secondary metabolites are organic compounds that are not directly involved in the normal growth and development of the producing organisms (Martín *et al.*, 2005). Microbial secondary metabolites have biotechnological applications as antibiotics, pigments, toxins, enzymes and antitumor agents. It is estimated that approximately 7000 of the bioactive compounds reported in literature are actinobacterial secondary metabolites, with the genus *Streptomyces* being the major antibiotic-producing genus, accounting for approximately 80% of the actinobacterial-derived natural products (Kieser *et al.*, 2000; Jensen *et al.*, 2005). Although thousands of antibiotics have been described, these are thought to represent only a minor fraction of the repertoire of bioactive compounds that members of the genus *Streptomyces* are able to produce (Watve *et al.*, 2001). Apart from antibiotics, actinomycetes also have the ability to synthesize other economically important compounds such as vitamins, immunomodulators and enzymes

which are widely used in industry as biocatalysts (Watve *et al.*, 2001). The discovery of gentamicin (an aminocyclitol containing aminoglycoside antibiotic complex that inhibits bacterial protein biosynthesis by binding to the 30S subunit of the ribosome) produced by *Micromonospora purpurea* and *Micromonospora echinospora* greatly stimulated interest in screening non-streptomycete actinobacterial genera for novel antibiotics (Cundliffe and Demain, 2010). Several *Actinomadura* and *Amycolatopsis* species have been found to produce vancomycin-type glycopeptides. A number of macrolide-type antibiotics are produced by *Micromonospora* and *Saccharopolyspora* species, whilst *Actinomadura* species have been found to produce macrolactam and naphthacene-quinone antibiotics (Moncheva *et al.*, 2002).

Identification of actinobacteria involves assigning newly cultured/discovered organisms to a particular rank in a previously published classification system (Goodfellow and O'Donnell, 1993; Janssen, 2006). Physiological, morphological and biological properties are usually employed to identify actinobacterial species. Common methods and characteristics are discussed below.

Morphology forms the basis of traditional actinobacterial taxonomy (Ventura *et al.*, 2007). Some of the morphological characteristics considered in actinobacterial taxonomy include the size, shape and colour of colonies on specific media, the Gram stain reaction, acid-fastness and the production of diffusible pigments. Other morphological features that are taxonomically important include the colour of the mycelium and the morphology of the sporangium, as well as surface arrangement of the spores (Ventura *et al.*, 2007; Wiese *et al.*, 2008). Actinobacteria display a wide range of morphologies including cocci (e.g. *Micrococcus*) or rod-cocci (e.g. *Arthrobacter*), fragmentation hyphal forms (e.g. *Nocardia*) and permanent and highly differentiated branched mycelium (e.g. *Streptomyces*).

Physiological characteristics such as nutritional requirements (e.g. sole carbon and nitrogen sources), fermentation products and growth conditions (oxygen, temperature and inhibitory products) are also important when classifying actinobacteria (Bryant and Frigaard, 2006).

Chemotaxonomy is the study of the intermittent distribution of chemical macromolecules such as amino acids, lipids, polysaccharides and related polymers, proteins and isoprenoid quinones amongst members of different taxa and the use of such information for classification and identification (Goodfellow and O'Donnell, 1989; Schleifer, 2009). Chemotaxonomic analysis of macromolecules such as amino acids, isoprenoid quinones (e.g. menaquinones and ubiquinomes), lipids (lipopolysaccharides and fatty acids including mycolic acids and polar lipids), polysaccharides and related polymers (e.g. methanochondrium and wall sugars) and proteins (e.g. bacteriochlorophylls, whole organism protein patterns and enzymes) provide valuable data for the classification of actinobacteria (Ward and Goodfellow, 2004).

Chemotaxonomy also involves the grouping of organisms according to the chemistry of the cell wall constituents, membranes and quinones (Zaitlin and Watson, 2006). The composition of the cell wall varies greatly amongst the different groups of actinobacteria. For taxonomical purposes, the isomer of diaminopimelic acid (DAP) present in the cell wall is one of the key properties of Gram positive bacteria. The 2,6-DAP form is widely distributed in cell walls and has three optical isomers (Sasaki *et al.*, 1998). Bacteria generally contain either the LL - form or the *meso* – form, mostly located in the peptidoglycan. Four cell wall types can be distinguished according to three major features of the peptidoglycan composition and structure: i) the amino acid present in the tetrapeptide side chain 3 ii) the presence of glycine in the interpeptide bridges iii) the peptidoglycan sugar content (Lechevalier and Lechevalier, 1970; Hermoso, 2007).

Although chemotaxonomy is considered useful in actinobacterial taxonomy, it is not always reliable as several genera may exhibit similar chemical properties. For example, members of the genera *Actinomadura*, *Microbispora*, *Microtetraspora* and *Nonomura*, cannot be distinguished from each other using chemotaxonomy as they exhibit highly similar chemotaxonomic characteristics (Wang *et al.*, 1999). In addition, several of the techniques used in chemotaxonomy are cumbersome and time consuming. Growth conditions, including media composition, may affect the results obtained making it difficult to generate reproducible data (Gevers *et al.*, 2005). Due to these disadvantages bacterial taxonomy is no longer solely based on phenotypic properties. Molecular-based methods are currently used in prokaryote systematics, because the end product of this approach highlights natural relationships between prokaryotes as encoded by their DNA sequences (Head *et al.*, 1998; Gevers *et al.*, 2006; Alam *et al.*, 2010; Jensen, 2010). A major advantage of molecular-based systematics over chemotaxonomic approaches is that the acquisition of sequence data is independent of cultivation conditions (Head *et al.*, 1998).

The molecular phylogenetic approach is useful in determining relatedness at levels ranging from kingdom to species. The comparison of DNA nucleotide sequences between two strains provides a rapid and accurate method for establishing relatedness. Techniques for carrying out the comparisons include DNA-DNA hybridization (whole genome comparison) and PCR- based gene sequence analysis (comparison of single/several gene sequences). The analysis of DNA for bacterial taxonomy focuses on analysis of the 16S rRNA gene. Ribosomal RNAs are essential elements in protein synthesis and are therefore present in all living organisms (Priest and Austin, 1995). Additional factors that make these molecules ideal for the analysis of evolutionary relationships are i) lateral/horizontal transfer of rRNAs between different organisms is extremely rare ii) longer rRNAs (16S, 18S

and 23S) contain distinct regions which are highly conserved, moderately variable or highly variable. The highly variable regions are used in taxonomy while the conserved regions provide priming sites for PCR amplification (Letowski *et al.*, 2004; Gentry *et al.*, 2006).

The 16S rRNA genes of many phylogenetic groups have characteristic oligonucleotide signatures, which are sequences that occur in most or all members of a particular phylogenetic group (Woese *et al.*, 1985). These oligonucleotide signatures can be used to design primers which are genus- or species-specific (Park *et al.*, 2000).

The 16S rRNA gene can be analyzed by a number of methods which include amplified ribosomal DNA restriction analysis (ARDRA), restriction fragment length polymorphisms (RFLP), amplified random length polymorphisms (AFLP) and rep-DNA (Gürtler and Mayall, 2001; Cook and Meyers, 2003). An advantage of some of these PCR-based methods is that the amplified DNA can either be sequenced directly or cloned into a phage or plasmid vector prior to sequencing. Sequences are compared by aligning the corresponding nucleotide sites, the comparison of sequence positions providing an estimation of the relatedness of the organisms (Priest and Austin, 1995). Analysis of the 16S rRNA gene offers a rapid alternative to the time-consuming classical methods of identification such as chemotaxonomy (Alfaresi and Elkosh, 2006). Based on 16S rRNA gene sequence analysis streptomycetes are classified as belonging to the family *Streptomycetaceae*, order *Actinomycetales*, suborder *Streptomycineae*, phylum *Actinobacteria*, (Stackebrandt *et al.*, 1997).

An exponentially growing number of bacterial 16S rRNA gene sequences are available in public databases. There are however drawbacks to employing the 16S rRNA gene for phylogenetic studies including (i) in many bacterial genomes the gene is present in multiple copies (Acinas *et al.*, 2004) (ii) in actinobacterial phylogenetics, analysis of the 16S

rRNA gene alone has been shown to be insufficient to distinguish between closely related species, notably species within certain *Streptomyces* clades (Liu *et al.*, 2005; Guo *et al.*, 2008).

DNA-DNA hybridization (DDH) is one method that provides better resolution when defining species and strains (Stakebrandt and Goebel, 1994) and is generally necessary in order to define a novel bacterial species. DDH measures the degree of similarity between the genomes of the different species and is therefore useful for delineating novel species, and for the definitive assignment of a strain with ambiguous phenotypic properties to the correct taxonomic group (Garrity and Holt, 2001; Stackebrandt *et al.*, 2002). However, DDH has several disadvantages including the high cost of the required pairwise cross-hybridizations and the requirement for isotopic or fluorescent dye labeling. In addition, the method is labour-intensive and results are often not reproducible between laboratories. The establishment of a central database is difficult as results between laboratories are not comparable (Vandamme *et al.*, 1996; Cho and Tiedje, 2001; Coenye *et al.*, 2005; Gevers *et al.*, 2005).

Although DDH cannot be replaced in species delineation, multilocus sequence analysis (MLSA), which examine the sequences of several conserved housekeeping genes distributed over at least 100 kb of the genome, has been proposed as a more accessible and reproducible tool for assessing the phylogeny and taxonomy of prokaryotes (Brett *et al.*, 1998; Maiden *et al.*, 1998; Godoy *et al.*, 2003; Cooper and Feil, 2004). MLSA is a procedure which yields reproducible results and which characterizes bacterial isolates using the sequence of internal fragments of (usually) four housekeeping genes (Stepkowski *et al.*, 2003; Stepkowski *et al.*, 2005; Vinuesa *et al.*, 2005b). A short 450-500 bp internal fragment of each gene is used. Small fragments are used in MLSA as a full length sequence can be

obtained with a single Sanger sequencing reaction (Maiden *et al.*, 1998). MLSA is a useful tool in general actinobacterial taxonomy and the approach has been applied in the study of taxonomic relationships in a number of genera such as *Streptomyces* (Guo *et al.*, 2008; Mignard and Flandrois, 2008) and *Ensifer* (Naser *et al.*, 2006; Martens *et al.*, 2007).

Housekeeping genes that are present as a single copy in a bacterial genome can be used in MLSA. An ideal candidate housekeeping gene should typically be a gene that is constitutively expressed, is required for the maintenance of basic cellular function and is found in all members of a taxonomic group. Some of the house keeping genes that have been used in actinobacterial taxonomy include *atpD*, *gyrB*, *recB*, *rpoB* and *trpB* (Naser *et al.*, 2006; Martens *et al.*, 2007; Guo *et al.*, 2008; Mignard and Flandrois, 2008; Young *et al.*, 2008).

In the present study Strain MS26 was isolated from a soil sample collected from the Zambian Copperbelt region and isolate DFNR17 from a soil sample collected from a New Zealand farm. Both strains were isolated by Dr Marilize Le Roes-Hill (Biocatalysis and Technical Biology Research Group, Cape Peninsula University of Technology). Identification of these strains was based on cultural, morphological, physiological and biochemical characteristics, as well as on 16S rRNA gene analysis.

3.2 Isolation of laccase producing actinomycete strains

Actinomycete strain MS26 was isolated from a soil sample collected from the Mindolo stream in the Zambian Copperbelt region. The sample was air-dried for 5 days. Following serial dilution, aliquots were plated onto YEME and modified R2A agar, both supplemented with nalidixic acid (10 µg/ml) and cycloheximide (100 µg/ml). Plates were incubated at 30°C for two weeks. Strain MS26 was isolated on YEME agar and maintained on this medium.

Strain DFNR17 was isolated from a soil sample collected from a New Zealand farm. A standard serial dilution was prepared and plated as described above. Plates were incubated at 25°C for two weeks. Strain DFNR17 was isolated on R2A agar, but was subsequently maintained on YEME agar.

Both strains were isolated by Dr Marilize Le Roes-Hill (Biocatalysis and Technical Biology Research Group, Cape Peninsula University of Technology). The two isolates were identified as actinomycetes based on colony morphology. Strains MS26 and DFNR17 were screened for the presence of a number of industrially relevant enzymatic activities. Both strains were shown to possess tyrosinase, laccase and peroxidase activities. The aim of the present study was to further characterise the isolates to the genus level and to perform phenotypic characterisation.

3.3 Identification of actinomycete strains MS26 and DFNR17 based on 16S rRNA gene sequence analysis

3.3.1 Extraction of genomic DNA from isolates MS26 and DFNR17

Genomic DNA was extracted from isolates MS26 and DFNR17 using the method described by Wang *et al.* (1996) as described in Section 2.4. The extraction yielded high molecular weight DNA from both isolates (Fig. 3.1).



Figure 3.1: Agarose gel electrophoresis of genomic DNA from isolates MS26 and DFNR17. Lanes 1-4: quadruplicate extractions from isolate DFNR17. Lanes 5-8: quadruplicate extractions from isolate MS26. Lane 9: molecular weight marker (λ Pst).

The $A_{260/280nm}$ ratios for the extracted DNA samples varied from 1 to 1.8, showing a suitable purity for use in downstream applications. The extractions were conducted independently and in quadruplicate for each isolate. In spite of evidence of substantial DNA shearing, sufficient DNA was extracted, with DFNR17 yielding between 970 – 3170 ng/ μ l DNA from a 50 ml culture, and extractions from isolate MS26 yielding 958 – 3169 ng/ μ l DNA. Both isolates yielded similar amounts of DNA per volume cultured. The DNA from the quadruplicate extractions was pooled and used as a template for polymerase chain reaction (PCR) amplification.

3.3.2 Amplification of the 16S rRNA gene

In order to identify isolates DFNR17 and MS26 the 16S rRNA gene was amplified by PCR (section 2.5). The universal bacterial primer set F1 and R5 (Table 2.2) was used to directly amplify the bacterial 16S rRNA gene from the genomic DNA resulting in an approximately 1,5 kb DNA fragment (Fig. 3.2).

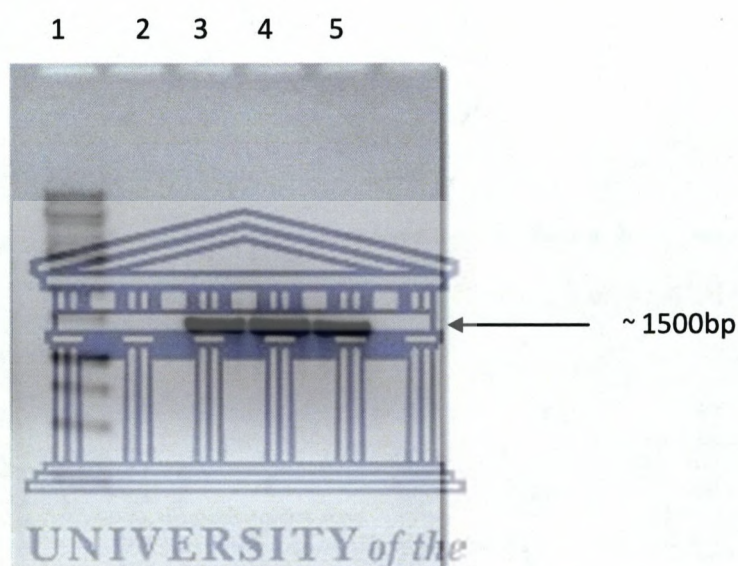


Figure 3.2: Agarose gel electrophoresis of the 1500 bp amplicons from strains DFNR17 and MS26 amplified using the universal 16S rRNA gene bacterial primers F1/R5. Lane 1: *Pst*I digested lambda DNA, lane 2: negative control, lane 3:DFNR17, lane 4: MS26, lane 5: positive control (*Streptomyces* strain ORS`#3).

PCR amplicons were cloned into the p-GEM[®]-T Easy vector (section 2.6). The recombinant clones were verified as containing DNA of the correct insert size by colony PCR using the primers M13F and M13R (section 2.5.1). Randomly selected recombinant clones containing amplicons from the two isolates gave a fragment of approximately 1700 bp, representing the size of the 16S rRNA gene amplification product which was approximately 1,5 kb, plus approximately 200 bp of the vector sequence (Figure 3.3). Plasmids from clones

with the correct insert size were extracted and sequenced using the M13F and M13R primers.

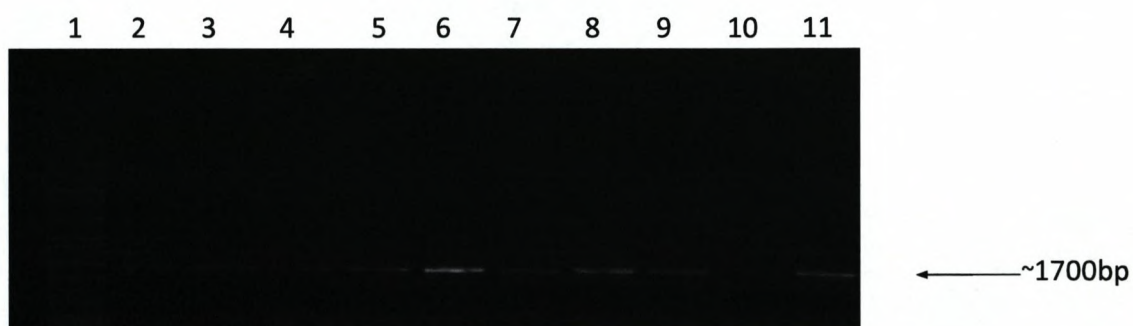


Figure 3.3: M13 PCR amplification of representative clones containing the actinobacterial 16S rRNA gene. Lane 1: *Pst*I digested λ DNA, Lane 2-6: 1700 bp amplicons generated from the DFRN17 colonies, Lanes 7-11: 1700 bp amplicons generated from MS26 colonies.

3.3.3 Sequence analysis of the 16S rRNA gene sequences and phylogenetic analysis

Based on homology searches using BLAST against the NCBI non-redundant database (<http://www.ncbi.nlm.nih.gov/>) the 16S rRNA gene sequences from both isolates were found to have highest sequence similarities to *Streptomyces* species. The closest validly published matches were retrieved and aligned against the sequences obtained in this study. Strain MS26 shared the highest 16S rRNA gene sequence similarity to *Streptomyces viridodiastaticus* IFO 13106 (99.0 %) and *Streptomyces atrovirens* NRRL B-16357 (99.0 %), while DFRN17 shared the highest 16S rRNA gene sequence similarity to *Streptomyces althoticus* KCTC 9752 (99.0 %). Phylogenetic analysis of the 16S rRNA gene confirmed that strains MS26 and DFRN17 clustered with other *Streptomyces* species (Figure 3.4).

The *S. atrovirens* strain NRRL B-16357, *S. althoticus* KCTC 9752 and *S. viridodiastaticus* strain IFO 13106 were characterized as part of International *Streptomyces*

Project (Zarantonello *et al.*, 2002; Remsing *et al.*, 2003). The three strains were isolated from soils. *S. althioticus* KCTC 9752 was originally characterised by Yamaguchi *et al.* (1957) as the producer of althiomycin, a peptide antibiotic which is active against Gram positive bacteria. The strain also possesses anticoccidial and antiherpes activity (Zarantonello *et al.*, 2002). *S. atrovirens* strain NRRL B-16357 was characterised as the producer of mithramycin, an antineoplastic antibiotic which binds to DNA and prevents cells from making RNA and proteins (Remsing *et al.*, 2003). *S. viridodiastaticus* strain IFO 13106 was characterised as the producer of bioxalomycin α_2 , a bactericidal compound that inhibits DNA synthesis. This antimicrobial agent has activity against Gram positive and Gram negative bacteria and demonstrates potent activity against methicillin-resistant *Staphylococcus aureus* (Herberich *et al.*, 2001).



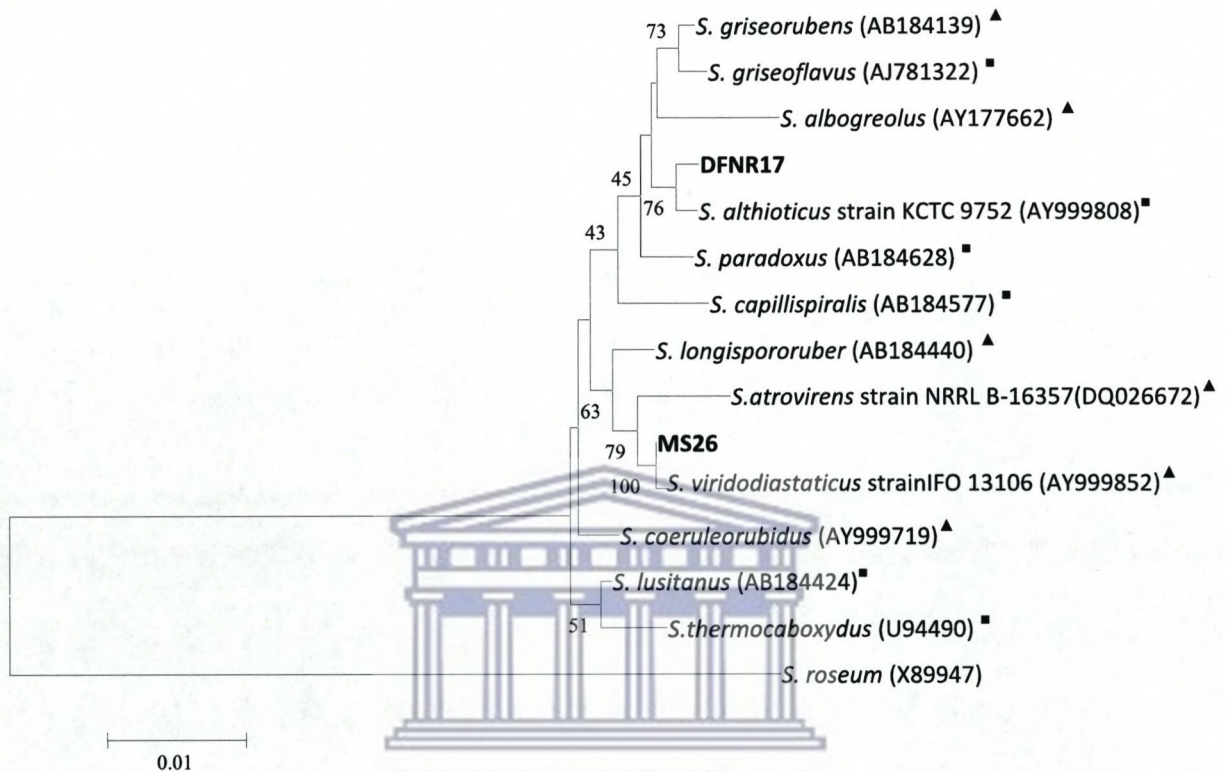


Figure 3.4: Phylogenetic tree showing the position of strains MS26 and DFNR17 and other *Streptomyces* species based on the 16S rRNA gene sequence analysis. The phylogenetic tree was constructed using the neighbour-joining algorithm. The tree is based on 1000 resampled datasets and numbers at nodes indicate the percent level of bootstrap support (only values greater than 40% are shown). The bar represents 0.01 nt substitution per nt. *Streptosporangium roseum* was set as the out-group. Sequences obtained in this study are in bold uppercase letters. GenBank sequences identified from BLAST analysis against DFNR17 are designated with ■ while sequences obtained from BLAST analysis against MS26 are designated with a triangle ▲.

3.4 Physiological characterisation of actinomycete strains MS26 and DFNR17

3.4.1 Morphological characteristics of strains MS26 and DFNR17 strains

Strains MS26 and DFNR17 grew on ISP-4 agar and displayed typical streptomycete characteristics. Extensively branched substrate mycelia and aerial hyphae which differentiated into long, straight spore chains were formed. Both isolates formed small balls when grown in liquid media. Gram stain analysis of these strains showed that they were Gram positive and filamentous (Figure 3.5).



Figure 3.5: Light microscopy (X50) of Gram stained (A) strain MS26 and (B) strain DFNR17.

3.4.2 The biochemical and physiological characteristics of strains MS26 and DFNR17

The biochemical and physiological characteristics of strains MS26, DFNR17 and closely related strains are summarized in Tables 3.1, 3.2a and 3.2b. Both strains MS26 and DFNR17 were capable of growing in the temperature range 16 to 45°C with neither strain showing growth at temperatures of 55°C or higher. Optimal growth was evident at temperatures between 30 and 37°C suggesting that both strains are mesophiles. Growth occurred in the presence of 4 and 7 % NaCl, with no growth occurring in the presence of NaCl concentrations of 10 % or greater. Both strains grew on ISP-4 medium, but were unable to grow on ISP-5, ISP-6 and ISP-7 media. Therefore standard characteristics normally determined on these media (production of diffusible pigments and melanin) could not be determined.

Both isolates DFNR17 and MS26 strongly degraded aesculin, arbutin, pectin, hypoxanthine, tyrosine, xanthine, xylan, starch, casein and Tween 80, but could not degrade egg-yolk. In addition adenine, gelatin and guanine were not hydrolysed. Growth occurred in the presence of penicillin G (10 I.U./ml), lincomycin (100 µg/ml) and ampicillin (100 µg/ml), but both strains were inhibited by streptomycin (100 µg/ml), kanamycin (50 µg/ml), gentamicin (100 µg/ml) and chloramphenicol (12.5 µg/ml).

Table 3.1: Comparison of the biochemical and physiological characteristics of strain DFNR17 and *S. althioticus* strain KCTC 9752

Characteristics	DFNR17	<i>S. althioticus</i> strain KCTC 9752	Characteristics	DFNR17	<i>S. althioticus</i> strain KCTC 9752	
Degradation activity						
Tyrosine	+	+	Hydrolysis activity			
Gelatin	-	+		Egg-yolk (lecithin)	-	-
Starch	+	+		Pectin	+	+
Xylan	+	+		Aesculine	+	+
Adenine	-	+		Arbutin	+	+
Guanine	-	+		Microscopic characteristics		
Hypoxanthine	+	+		Gram reaction	+	+
Xanthine	+	-		Cell morphology	BF	BF
Casein	+	-		Type of spores chains	LCS	LCS
Siera's/Tween 80	+	+++		Temperature growth range		
Resistance to antibiotics ($\mu\text{g ml}^{-1}$)						
Ampicillin (100)	+	+	16°C	+	++	
Chloramphenicol (12.5)	-	-	30°C	+++	+++	
Gentamicin (100)	-	-	37°C	+++	+++	
Kanamycin (50)	-	-	45°C	+	++	
Lincomycin (100)	+	-	55°C	-	+	
Penicillin (10i.u/ml)	+	+	60°C	-	-	
Streptomycin (100)	-	-	68°C	-	-	
Effect of NaCl concentration						
4 %	+	+	Growth on ISP medium			
7 %	+	+	ISP 4	++	++	
10 %	-	+	ISP 5	-	++	
13 %	-	-	ISP 6	-	++	
			ISP 7	-	++	

+=positive, slight growth; ++ =strong growth; +++ = excellent growth; - = negative, no growth; BF = branched filaments and LCS = long chain spores (Rattanaporn *et al.*, 2010; Ningthoujam *et al.*, 2011 and Houssam *et al.*, 2011).

Table 3.2(a): Comparison of the biochemical and physiological characteristics of strain MS26 and *S. atrovirens* NRRL B-16357

Characteristics	MS26	<i>S. atrovirens</i> strain NRRL B-16357	Characteristics	MS26	<i>S. atrovirens</i> strain NRRL B-16357	
Degradation activity						
Tyrosine	+	+	Hydrolysis activity	-	-	
Gelatin	-	+		Egg-yolk (lecithin)	+	+
Starch	+	+		Pectin	+	+
Xylan	+	+		Aesculine	+	+
Adenine	-	+		Arbutin	+	+
Guanine	-	+	Microscopic characteristics			
Hypoxanthine	+	+	Gram reaction	+	+	
Xanthine	+	-	Cell morphology	BF	BF	
Casein	+	+	Type of spores chains	LCS	LCS	
Siera's/Tween 80	+	+++	Temperature growth range			
Resistance to antibiotics ($\mu\text{g ml}^{-1}$)						
Ampicillin (100)	+	+	16°C	+	++	
Chloramphenicol (12.5)	-	-	30°C	+++	+++	
Gentamicin (100)	-	-	37°C	+++	+++	
Kanamycin (50)	-	-	45°C	+	+++	
Lincomycin (100)	+	-	55°C	-	+	
Penicillin (10 i.u./ml)	+	+	60°C	-	-	
Streptomycin (100)	-	-	68°C	-	-	
Effect of NaCl concentration						
4 %	+	+	Growth on ISP medium			
7 %	+	+	ISP 4	++	++	
10 %	-	+	ISP 5	-	++	
13 %	-	-	ISP 6	-	++	
			ISP 7	-	++	

+=positive, slight growth; ++ =strong growth; +++ =excellent growth; - = negative, no growth; **BF** = branched filaments and **LCS** = long chain spores (Rattanaporn *et al.*, 2010; Ningthoujam *et al.*, 2011 and Houssam *et al.*, 2011).

Table 3.2(b): Comparison of the biochemical and physiological characteristics of strain MS26 and *S. viridodiastaticus* strain IFO 13106

Characteristics	MS26	<i>S. viridodiastaticus</i> strain IFO 13106	Characteristics	MS26	<i>S. viridodiastaticus</i> strain IFO 13106	
Degradation activity						
Tyrosine	+	+	Hydrolysis activity			
Gelatin	-	+		Egg-yolk (lecithin)	-	-
Starch	+	+		Pectin	+	+
Xylan	+	+		Aesculine	+	+
Adenine	-	+		Arbutin	+	+
Guanine	-	+	Microscopic characteristics			
Hypoxanthin	+	+	Gram reaction	+	+	
Xanthine	+	-	Cell morphology	BF	BF	
Casein	+	+	Type of spores chains	LCS	LCS	
Siera`s/Tween 80	+	+++	Temperature growth range			
Resistance to antibiotics ($\mu\text{g ml}^{-1}$)						
Ampicillin (100)	+	+	16°C	+	++	
Chloramphenicol (12.5)	-	-	30°C	+++	+++	
Gentamicin (100)	-	-	37°C	+++	+++	
Kanamycin (50)	-	-	45°C	+	++	
Lincomycin (100)	+	-	55°C	-	+	
Penicillin (10i.u/ml)	+	+	60°C	-	-	
Streptomycin (100)	-	-	68°C	-	-	
Effect of NaCl concentration						
4 %	+	+	Growth on ISP medium			
7 %	+	+	ISP 4	++	++	
10 %	-	+	ISP 5	-	++	
13 %	-	-	ISP 6	-	++	
			ISP 7	-	++	

+ = positive, slight growth; ++ = strong growth; +++ = excellent growth; - = negative, no growth; BF = branched filaments and LCS = long chain spores (Rattanaporn *et al.*, 2010; Ningthoujam *et al.*, 2011 and Houssam *et al.*, 2011).

3.5 Discussion.

In this study, genomic DNA was obtained using the method described by Wang *et al.* (1996). Chemical lysis methods have been shown to be most effective for lysing the cell walls of actinomycetes (McGuire *et al.*, 1984). Both isolates MS26 and DFNR17 yielded high concentrations of DNA per volume cultured with yields of between 970.0 and 3170.3 ng/ μ l DNA being recorded. The purity of the DNA preparations was high, as indicated by the $A_{260/280\text{nm}}$ ratios which ranged between 1 and 1.8, showing an acceptable level of purity for downstream applications.

According to the *ad hoc* Committee on Reconciliation of Approaches to Bacterial Systematics, members of a bacterial species should share a 16S rRNA gene sequence similarity of at least 97 % and at this level DNA-DNA hybridization should be conducted (Wayne *et al.*, 1987). However, more recent findings showing that a 16S rRNA gene sequence similarity range above 98.7–99 % should be mandatory for testing the genomic uniqueness of a novel isolate (Stackebrandt and Goebel, 1994; Stach *et al.*, 2003; Stackebrandt and Ebers 2006). In this study, both isolates showed 16S rRNA gene sequence identities of 99 % to known streptomycetes.

Phylogenetic analysis showed that the isolates had a high similarity to a number of previously cultured *Streptomyces* species. Strain MS26 is positioned with *S. viridodiastaticus* IFO 13106 and *S. atrovirens* NRRL B-16357, while strain DFNR17 is most closely related to *S. althioticus* KCTC 9752. Based on BLAST analysis it is proposed that MS26 and DFNR17 are members of a validly described species since they shared 99 % 16S rRNA gene sequence similarity to other validly published members of the genus *Streptomyces*.

While the isolates grew relatively well on ISP-4 medium, no growth was observed on ISP-5, ISP-6, and ISP-7 media (Tables 3.1, 3.2a and 3.2b). Therefore morphological

characterisation of the strains could only be performed on ISP-4 agar. This is an interesting finding as most streptomycetes including the closest relatives are able to grow well on ISP-4, ISP-5, ISP-6 and ISP-7 plates (Rattanaporn *et al.*, 2010). As such the morphological characteristics reported in this study for strains MS26 and DFNR17 were determined on YEME solid agar.

As expected for members of the genus *Streptomyces* neither strain produced sporangia or flagellated spores (Tables 3.1, 3.2a and 3.2b). Both strains were Gram positive and formed extensively branched grey substrate mycelia with grey aerial hyphae which differentiated into long, straight spore chains (Figure 3.5). Strains MS26 and DFNR17 differed from their closest relatives in that *S. atrovirens* strain NRRL B-16357 and *S. viridodiastaticus* strain IFO 13106 produce golden-yellow substrate mycelia with grey aerial mycelium which differentiate into long, straight mature spore chains while *S. althioticus* strain KCTC 9752 produce brown beige substrate mycelia with grey aerial mycelium which differentiated into long, straight mature spore chains (Zarantonello *et al.*, 2002).

In the laboratory the ability of an isolate to grow is dependent on factors including the available nutrients and the physical growth conditions. In this study the isolates were cultured on various media and optimal growth was observed on YEME. This medium was used to determine the growth temperature profile. The temperature growth range for both isolates was 16°C - 45°C and they shared optimum and maximum growth temperatures of 30°C - 37°C and 45°C, respectively (Tables 3.1, 3.2a and 3.2b). Based on this temperature growth range the isolates could be defined as being mesophilic streptomycetes. *S. althioticus* strain KCTC 9752, *S. atrovirens* strain NRRL B-16357 and *S. viridodiastaticus* strain IFO 13106 did however show slight growth at 55°C (Tables 3.1, 3.2a and 3.2b). This anomaly might be due to calibration issues with the incubator used for this study.

Strains MS26 and DFNR17 grew on salt concentrations of 4 % and 7 % and their nearest relatives are able to grow on salt concentrations of 4 %, 7 % and 10 % (Tables 3.1, 3.2a and 3.2b). Based on these growth responses the strains MS26 and DFNR17 could be defined as mild halophiles whereas their nearest relatives which grow in the presence of 10 % NaCl are moderate halophiles (Gattinger *et al.*, 2002).

Many streptomycetes produce antibiotics such as the aminoglycosides namely kanamycin, gentamicin and streptomycin, chloramphenicol and many other antibiotics that alter the integrity of the bacterial cell membranes of prokaryotic cells and inhibit the synthesis of protein (Siegenthaler *et al.*, 1986; Begg and Barclay, 1995; Davies and Wright, 1997). Antibiotic producing strains must be able to protect themselves from their own antibiotics. The mechanisms involved in the antibiotic resistance are permeability changes in the bacterial cell wall which restricts antimicrobial access to target sites, active efflux of the antibiotic from the microbial cell, enzymatic modification of the antibiotic, degradation of the antimicrobial agent, acquisition of alternative metabolic pathways to those inhibited by the drug and modification of antibiotic targets and overproduction of the target enzyme (Spratt, 1994; McDermott *et al.*, 2003; Magnet and Blanchard, 2005; Wright, 2005).

In this study strains MS26 and DFNR17 and their nearest relatives were inhibited by the aminoglycoside antibiotics kanamycin, gentamicin and streptomycin, as well as by chloramphenicol (Tables 3.1, 3.2a and 3.2b). Therefore, it is probable that the isolates lack the mechanisms that confer resistance to aminoglycoside-type and chloramphenicol antibiotics. Lincomycin inhibits protein synthesis in susceptible bacteria by binding to the 50S subunits of bacterial ribosomes and preventing peptide bond formation upon transcription (Tenson *et al.*, 2002). Both isolates were resistant to lincomycin, whereas their

nearest relatives lack the mechanisms that confer resistance to lincomycin (Tables 3.1, 3.2a and 3.2b).

Most actinomycetes, especially *Streptomyces*, produce a diverse mixture of hydrolytic enzymes that permit the utilization of organic compounds such as starch, cellulose and hemicelluloses (Kumar *et al.*, 2011; Saenna *et al.*, 2011). The data presented in Tables 3.1 and 3.2a and 3.2b shows that both isolates display many characteristics in common with their closest relatives. There are however some differences that exist between the biochemical and physiological characteristics of strains MS26 and DFNR17 and their closest relatives. Both isolates are unable to degrade adenine, gelatin and guanine while their closest relatives *S. althioticus* strain KCTC 9752, *S. atrovirens* strain NRRL B-16357 and *S. viridodiastaticus* strain IFO 13106 are able to produce the enzymes required to hydrolyse the substrates. Both strains and their nearest relatives are unable to hydrolyse egg-yolk. This could mean that they both lack lecithinase, an enzyme required to degrade lecithin to insoluble diglycerides (Thaler *et al.*, 1998).

From comparison of the biochemical and physiological characteristics of the strains in this study and the closest validly published strains (Tables 3.1, 3.2a and 3.2b) it is concluded that strains MS26 and DFNR17 are members of a validly published *Streptomyces* species and share a large number of biochemical and physiological characteristics with the type strains of these species.

Chapter 4: Identification of *Streptomyces* laccase genes

4.1 Introduction

The versatility and increasing importance of laccases in the biotechnology industry is demonstrated by their various uses. Laccases have important biotechnological applications in the chemical industry and are used in the production of agrichemicals, paints, pharmaceuticals, photographic developers, stains, natural aromatic flavours, synthetic dyes, and in the synthesis of complex natural products and cosmetics. In addition, laccases degrade polycyclic aromatic hydrocarbons which exhibit cytotoxic, mutagenic and carcinogenic properties and are a serious risk to human health (Cripps *et al.*, 1990; Lesage-Meessen *et al.*, 1996; Anastasi *et al.*, 2009).

To date, research has focused exclusively on fungal laccases. This is not surprising considering the high yield and ease of purification of fungal laccases as the majority of the enzyme is excreted into the growth medium (Thurston, 1994). The biotechnological importance of this group of enzymes has led to a drastic increase in demand.

Laccase activity has primarily been demonstrated in wood-degrading fungi where it plays a role in lignin degradation (Thurston, 1994). Laccases have also been isolated from bacteria such as the *Streptomyces griseus* *epoA* laccase which plays a role in pigmentation and morphogenesis (Endo *et al.*, 2002). There is an interest in identifying and isolating novel bacterial laccase genes (Kramer *et al.*, 2001; Claus, 2003; Claus, 2004; Dittmer *et al.*, 2004). Due to the advances in molecular biology technology it is now possible to access much of the available bacterial diversity and thereby investigate the occurrence of laccase genes in bacteria.

A classical approach to characterising novel genes involves identifying and purifying the target protein, obtaining amino acid sequences from peptides generated by proteolytic digestion and reverse translation of the peptides. The derived DNA sequence, which may be ambiguous due to the degeneracy of the genetic code, can then be employed for the construction of probes to screen a gene library (Laging *et al.*, 2001). Cloning the gene of interest is the crucial first step in the functional analysis of a gene, e.g. as a means to get hold of the protein by overexpression mutants.

The ligation of restriction-digested or blunt-ended genomic DNA fragments into vectors and subsequent transformation into a library host strain can be performed using a variety of different strategies, depending on the type of library required. Points to consider include the subsequent screening strategies planned and whether these include sequence-based or function-based approaches (Aakvik *et al.*, 2009).

Construction of small-insert libraries (average insert size of < 10 kb) in a standard vector (e.g. pSK⁺ and pUC) employing *Escherichia coli* as the host strain are usually chosen due to the fact that they are a lot easier to establish (Henne *et al.*, 1999). However, small insert libraries do not allow the detection of large gene clusters or operons. To circumvent this limitation large insert libraries (40-300 kb) are employed. In these systems specialized vectors such as cosmids, fosmids and BACs, which are able to maintain the integrity of large inserts are used (Rondon *et al.*, 2000; Lee *et al.*, 2004). Large insert libraries are more informative, allowing access to neighbouring genes or *cis*-elements required for the effective expression of target genes, which can easily be missed in small insert libraries. Large insert libraries can provide insight into the evolutionary origin of the functional gene (Streit and Schmitz, 2004). Another advantage of large insert libraries is the high level of sequence coverage, which might allow for the reconstruction of whole novel genomes

(Venter *et al.*, 2004). Due to the size of the inserts expression screening using large insert libraries is usually entirely reliant on native promoters. The only drawback of functional screening methods is that expression of the target gene is reliant on the host cell recognizing the heterologous transcription signals.


The choice of strategy for library construction becomes more complicated when performing function-based screening. To be able to detect novel activities in genomic libraries, the vector borne heterologous gene(s) of interest need to be successfully expressed and this requires several criteria to be fulfilled (Gabor *et al.*, 2004). Firstly, the chosen insert size must be large enough to include the entire gene or cluster of genes of interest. Secondly, a promoter and an appropriately located ribosome binding site that is compatible with the expression machinery of the host are necessary (Ermolaeva *et al.*, 2000). These *cis*-acting factors can either be provided by the cloning vector used, or be internal signals within the cloned DNA fragment (Gold *et al.*, 1981; Staden, 1983).

In addition, several *trans*-factors need to be provided by the host cell such as the proper transcription factors, inducers, precursors, chaperones, cofactors, post-translationally acting factors, and secretion mechanisms (Streit and Schmitz, 2004). Other possible factors include codon usage and the potential toxicity of the heterologous product to the host cell. One way to partially overcome these complex obstacles is to use vectors that can be transferred to and maintained in a variety of different hosts. This provides the possibility to screen the libraries in hosts that are considered likely to express the types of genes that are searched for. Favourable qualities of such vectors include high transfer efficiencies and a broad range of hosts in which they can replicate (Gold *et al.*, 1981; Staden, 1983; Ermolaeva *et al.*, 2000).

A range of different methods have been applied for functional screening based on the metabolic activities of genomic library-containing-clones. As the frequency of genomic clones that express a given trait may be low, the screening method should preferably be either highly sensitive or carried out in a high throughput manner (multi-well plate format) (Aakvik *et al.*, 2009). As sequence information is not required, this is the only strategy that bears the potential to identify entirely novel genes and/or gene classes encoding both known and novel functions (Handelsman, 2004; Riesenfeld *et al.*, 2004; Daniel, 2005; Ferrer *et al.*, 2009). Three different function-driven approaches have been used to recover novel biomolecules: phenotypical detection of the desired activity (Liaw *et al.*, 2010); heterologous complementation of host strains or mutants (Riesenfeld *et al.*, 2004; Simon *et al.*, 2009; Chen *et al.*, 2010) and induced gene expression (Uchiyama *et al.*, 2005; Uchiyama and Miyazaki, 2010).

In most cases, phenotypical detection employs chemical dyes and insoluble or chromophore-bearing derivatives of enzyme substrates incorporated into the growth medium where they register the specific metabolic capabilities of individual clones (Ferrer *et al.*, 2009). An example of such an activity-driven screen targeted genes encoding bacterial β -D-glucuronidases which are part of the human intestinal microbiome (Gloux *et al.*, 2010). Another example was the identification of novel glycosyl hydrolases in *E. coli* clones harbouring fosmid libraries derived from cellulose-depleting microbial communities of a fresh cast of earthworms. The libraries were screened for enzymes able to hydrolyze p-nitrophenyl- β -D-glucopyranoside and p-nitrophenyl- α -L-arabinopyranoside. Two of the recovered glycosyl hydrolases had no sequence similarity to any known glycosyl hydrolases and represented two novel families of β -galactosidases/ α -arabinopyranosidases (Beloqui *et al.*, 2010).

A different category of function-driven screens is based on heterologous complementation of host strains with mutants which require the targeted genes for growth under selective conditions. This technique allows the rapid screening of complex genomic libraries comprising millions of clones. Since almost no false positives are detected, this approach is highly selective for the targeted genes of interest (Simon *et al.*, 2009). Recent example of screens employing heterologous complementation includes the identification of genes encoding lysine racemases (Chen *et al.*, 2010), antibiotic resistance (Riesenfeld *et al.*, 2004; Deneff *et al.*, 2009), enzymes involved in poly-3-hydroxybutyrate metabolism (Wang *et al.*, 2006), DNA polymerases (Simon *et al.*, 2009) and Na⁺/H⁺ antiporters (Majerník *et al.*, 2001).



The third type of activity-driven screen, termed substrate-induced gene expression screening (SIGEX) is a high-throughput screening approach which employs an operon trap *gfp*-expression vector in combination with fluorescence-activated cell sorting. The screen is based on the fact that catabolic-gene expression is induced mainly by specific substrates and is often controlled by regulatory elements located close to catabolic genes (Uchiyama *et al.*, 2005). Subsequently, positive clones are identified by fluorescent microscopy (Williamson *et al.*, 2005).

Functional searches for novel genes in genomic libraries have often been performed using highly sophisticated picking and pipetting robots. Often several hundred thousand clones must be analyzed to detect less than ten active clones in a single screen (Henne *et al.*, 1999; Henne *et al.*, 2000; Majernik *et al.*, 2001). This is mainly owing to the lack of efficient transcription of the genomic-derived genes in the host strain. The drawbacks of the function-driven method include reliance of the method on expression of the genes in a

foreign host and proper protein folding to yield functional gene products (Gabor *et al.*, 2004).

Sequence-based screening - approaches are used to identify genes within a library on the basis of sequence homology. This approach includes the use of PCR-based or hybridization-based techniques for the identification of target genes with primers or probes, respectively, designed from conserved regions of known genes or protein families (Aakvik *et al.*, 2009). This strategy has led to the successful identification of genes encoding novel enzymes, such as dimethylsulfoniopropionate-degrading enzymes (Varaljay *et al.*, 2010), dioxygenases (Morimoto and Fujii, 2009; Sul *et al.*, 2009, Zaprasis *et al.*, 2010), nitrite reductases (Bartossek *et al.*, 2010), [Fe-Fe]-hydrogenases (Schmidt *et al.*, 2010), [NiFe] hydrogenases (Maróti *et al.*, 2009), hydrazine oxidoreductases (Li *et al.*, 2010), chitinases (Hjort *et al.*, 2010) and glycerol dehydratases (Knietsch *et al.*, 2003).

For example the genes encoding homologs of copper-dependent nitrite reductases (NirK) in ammonia-oxidizing archaea were identified using a PCR-based approach. Based on deduced amino acid sequences of NirK proteins from bacteria and two archaeal homologs, different sets of degenerated primers for the amplification of *nirK*-related genes from archaea were designed and used for amplification (Bartossek *et al.*, 2010).

In order to gain comprehensive insights into the available sequence space of the genes of interest, PCR-based screening approaches have been combined with large-scale pyrosequencing of amplicons. This sequence information can subsequently be used to design probes which are suitable to recover full-length versions of the target genes. This approach was used by Iwai *et al.* (2010) who applied the method to recover genes encoding aromatic dioxygenases from polychlorinated-biphenyl-contaminated samples.

In the present study a fosmid library was constructed by Dr Ana Casanueva from the Institute for Microbial Biotechnology and Metagenomics, University of the Western Cape. Genomic DNA was derived from three actinomycete genomes and the large insert library was produced using the CopyControl™ Fosmid Library Production Kit (Epicentre). This library had total size of 6MB. The library was screened for bacterial laccase activity.



4.2 PCR amplification of an internal fragment of the laccase gene from MS26 and DFNR17 strains using the SCuOxF/R primer combination

To demonstrate the presence of laccase genes, the genomic DNA from strains MS26, DFNR17, #18, BAM4, HMC13 and 7H1 was screened by PCR using the laccase-like cooper oxidase specific degenerate primers SCuOxF and SCuOxR under the conditions described in Table 2.2. The strains are described in Table 4.1.

Table 4.1: Description of strains used to demonstrate the presence of laccase genes

Strain	Source	Growth Medium	Growth Temperature	Genus	Enzymes produced
#18	Termite gut	YEME	30°C	Streptomyces	Laccase
BAM4	Soil	YEME	30°C	Streptomyces	Laccase
HMC13	Soil	YEME	30°C	Streptomyces	Laccase
7H1	Soil	YEME	30°C	Streptomyces	Laccase
DFNR17*	Soil	YEME	30°C	Streptomyces	Laccase
MS26*	Soil	YEME	30°C	Streptomyces	Laccase

Strain #18 from the gut of the termite *Amitermus hastatus*; strain BAM4 from garden soil collected in Stellenbosch, South Africa; strains 7H1 and HMC13 from soil collected from the Swartberg Nature Reserve, South Africa. Strains DFNR17 and MS26 are described on Section 3.2. Strains were sourced from the IMBM culture collection, University of the Western Cape.

A fragment of the expected size of 300 bp was amplified from MS26 and DFNR17 genomic DNA (Figure 4.1). The amplicons were cloned into the pGEM®-T Easy vector as described (Section 2.6). As the degenerate primers are likely to detect a number of different laccase genes, 11 white colonies were picked from each plate after blue-white selection. The colonies were directly screened by PCR using the vector primers M13 forward and M13 reverse (Table 2.2). Randomly selected clones yielded a fragment of approximately 500 bp,

representing the size of the 300 bp laccase gene fragment plus approximately 200 bp of the vector sequence. Plasmid DNA from the clones with the correct insert size was sequenced.

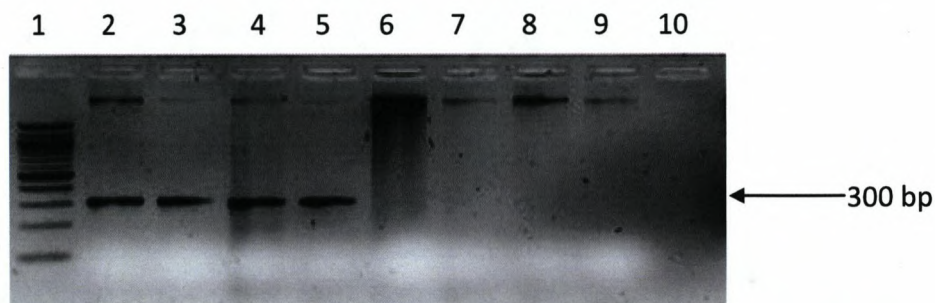


Figure 4.1: Agarose gel electrophoresis of the 300 bp PCR products amplicons from genomic DNA of strains MS26 and DFNR17 and other actinomycete isolates with laccase activity using the SCuOx/F/R primer combination. Lane 1: *Pst*I digested λ DNA, lanes 2 and 3: MS26, lane 4 and 5: DFNR17, lane 6: #18, lane 7: BAM4, lane 8: HMC13, lane 9: 7H1 and lane 10: negative control.

4.3 Southern hybridization

To elucidate the genomic organisation of the laccase genes, the 300 bp SCuOx/F/R laccase gene PCR products from MS26 and DFNR17 were labelled as specific probes for Southern hybridization. Analysis of the Southern autoradiographs suggested that a laccase gene was present in both isolates (Figure 4.2).

For isolate MS26, a positive signal was detected on a 10 kb fragment generated by digesting the genomic DNA with *Pst*I, while a positive signal was detected on a 7.5 kb *Xho*I fragment. For DFNR17, a single band was detected on a 7.5 kb fragment of genomic DNA digested with *Pst*I, while three signals were observed for the genomic DNA digest with *Xho*I on 3.1 kb, 3kb and 0.3 kb fragments. The detection of three fragments containing part of the gene of interest could be an indication that the DFNR17 genomic DNA was only partially digested with *Xho*I or the isolate contains multiple genes with close sequence identity. However, following restriction enzyme sequence analysis of the 300 bp fragments

for both MS26 and DFNR17, it was discovered that the fragments did not contain *Pst*I or *Xho*I sites.

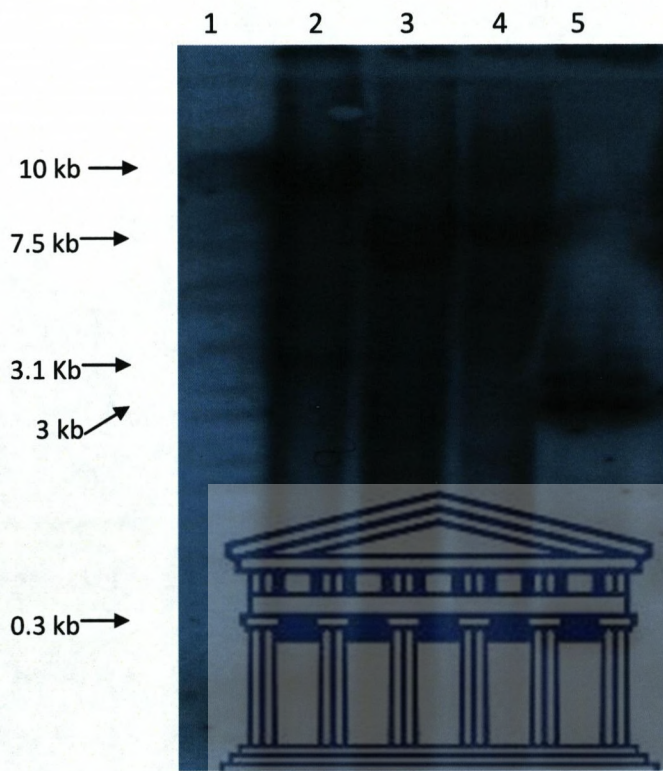


Figure 4.2: Southern hybridization with the laccase PCR product as the probe. Shown is a Southern blot of the *Pst*I and *Xho*I digested genomic DNA of MS26 and DFNR17 isolates probed with the labelled laccase gene 300 bp SCuOxFR PCR fragment. Lane 1: *Pst*I digested λ DNA, lane 2: MS26 genomic DNA digested with *Pst*I, lane 3: MS26 genomic DNA digested with *Xho*I, lane 4: DFNR17 genomic DNA digested with *Pst*I, lane 5: DFNR17 genomic DNA digested with *Xho*I.

4.4 Characterisation of the partial laccase gene sequences

The nucleotide sequences were translated into amino acid sequences *in silico* and compared by BLAST analysis against the NCBI protein database (<http://www.ncbi.nlm.nih.gov/>). Based on BLAST analysis of the translated DNA sequences for isolates MS26 and DFNR17, the predicted amino acid sequences had high homology to copper oxidase genes, multicopper oxidase type 2 genes and laccase genes from several *Streptomyces* species (Table 4.2), with the percentage identity at the amino acid level ranging from 70 % to 83 %. The closest matches were retrieved from the GenBank database and aligned against the amino acids sequences obtained in this study.

Sequence homology is inferred when the alignment generated between a sequence of interest and in the queried database exceeds a specific alignment score, S (Hofmann, 2000). The biological significance of the alignment is quantified by a statistical E-value which represents the number of different alignments with scores equivalent to or better than S that are likely to occur in the database search simply by chance (Hofmann, 2000). In this context, low E-values are considered more biologically significant than larger E-values. Based on the low E-values obtained for the alignments in this study (Table 4.1), the BLAST results were considered to be statistically significant.

A multiple alignment (Figure 4.3) was generated from clones MS26, DFNR17 and the laccase protein sequences originally used to design primers SCuOxFR (Table 2.4) using Multalin (Corpet, 1988). To investigate the phylogenetic relationship between the MS26 and DFNR17 gene fragments, the alignments were subjected to cluster analysis (Section 2.8) and a phylogenetic tree was constructed (Figure 4.4). Phylogenetic analysis of the *Streptomyces* species laccase genes confirmed that the highest identities existed between bacterial copper oxidase genes from *S. griseoflavus* Tu4000 and the two isolates (MS26 and DFNR17).

Table 4.2 BLAST analysis of the partial laccase gene fragments amplified from strains MS26 and DFNRR17

Species	BLAST results				
	NCBI Accession no.	Sequence (no AA)	Function	E-value	% Identity
<i>Streptomyces griseoflavus</i> Tu4000	ZP07309495	328	Copper oxidase	6e-55	83
<i>Streptomyces</i> sp. C1	AEP17492	141	Laccase	1e-51	79
<i>Streptomyces ghanaensis</i> ATCC 14672	ZP06575241	349	SilA	2e-54	83
<i>Streptomyces viridochromogenes</i> DSM 40736	ZP07307823	325	Copper oxidase	2e-50	79
<i>Streptomyces albus</i> J1074	ZP06594703	347	SilA	3e-49	76
<i>Streptomyces flavogriseus</i> ATCC 33331	ADW02154	329	Multi copper Oxidase type 2	3e-52	81
<i>Streptomyces hygroscopicus</i> ATCC 53653	ZP07292585	344	Putative Copper oxidase	2e-47	77
<i>Streptomyces coelicolor</i> 3(2)	CAB45586	343	Putative Copper oxidase	5e-53	83
<i>Streptomyces griseus</i>	BAB64332	348	EpoA	1e-41	70
<i>Streptomyces ipomoeae</i>	ABH10611	335	SilA	1e-51	81
<i>Streptomyces clavuligerus</i> ATCC 27064	ZP06774671	355	Copper oxidase	3e-47	74
<i>Streptomyces sviceus</i> ATCC 29083	ZP06921179	325	Copper oxidase	2e-53	83
<i>Streptomyces pristinaespiralis</i> ATCC 25486	ZP06908025	338	Copper oxidase	3e-46	77
<i>Streptomyces</i> sp. SPB74	ZP06822512	331	Copper oxidase	1e-45	77
<i>Streptomyces</i> sp. SirexAA-E	YP004806206	333	Multi copper oxidase type 2	6e-52	81
<i>Streptomyces coelicolor</i> A3(2)	NP630785	343	Copper oxidase	5e-53	83
<i>Streptomyces</i> sp. SPB78	ZP07275255	349	Copper oxidase	1e-46	78
<i>Streptomyces violaceusniger</i> Tu 4113	YP004813620	334	Multi copper oxidase type 2	2e-46	76
<i>Streptomyces scabiei</i> 87.22	YP003487081	355	Copper oxidase	1e-50	79
<i>Streptomyces roseosporus</i> NRRL 11379	ZP04712722	355	Copper oxidase	1e-48	77

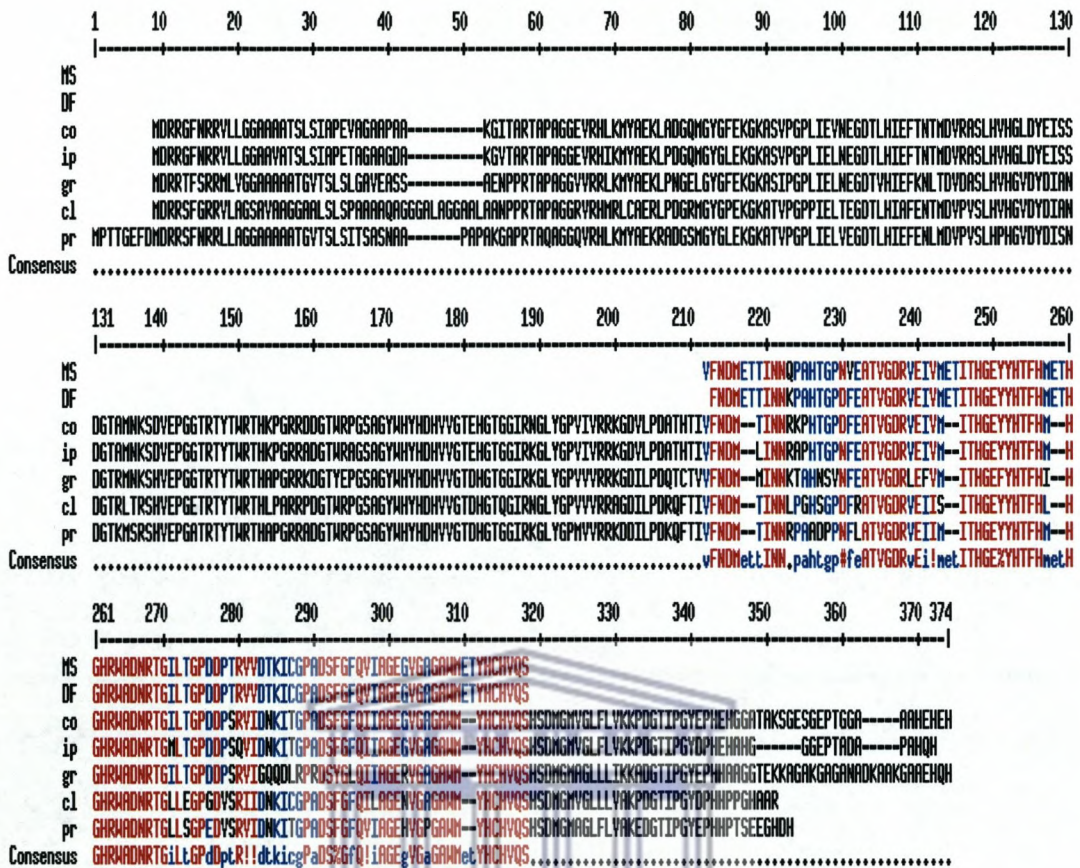


Figure 4.3: Amino acid alignment of the deduced MS26 and DFNR17 amino acid sequences with those of other *Streptomyces* laccase-like sequences used in the primer design. The sequences are as follows: MS (MS26); DF (DFNR17); co (*Streptomyces coelicolor*); ip (*Streptomyces ipomoeae*); gr (*Streptomyces griseus*); cl (*Streptomyces clavuligerus*) and pr (*Streptomyces pristinaespiralis*). Similar amino acids showing a significant degree of conservation are highlighted in red and identical amino acids are highlighted in blue. The highly conserved amino acids residues consisting of histidine-rich motifs have been identified as playing a significant central role in binding copper ions (Solano *et al.*, 2001).

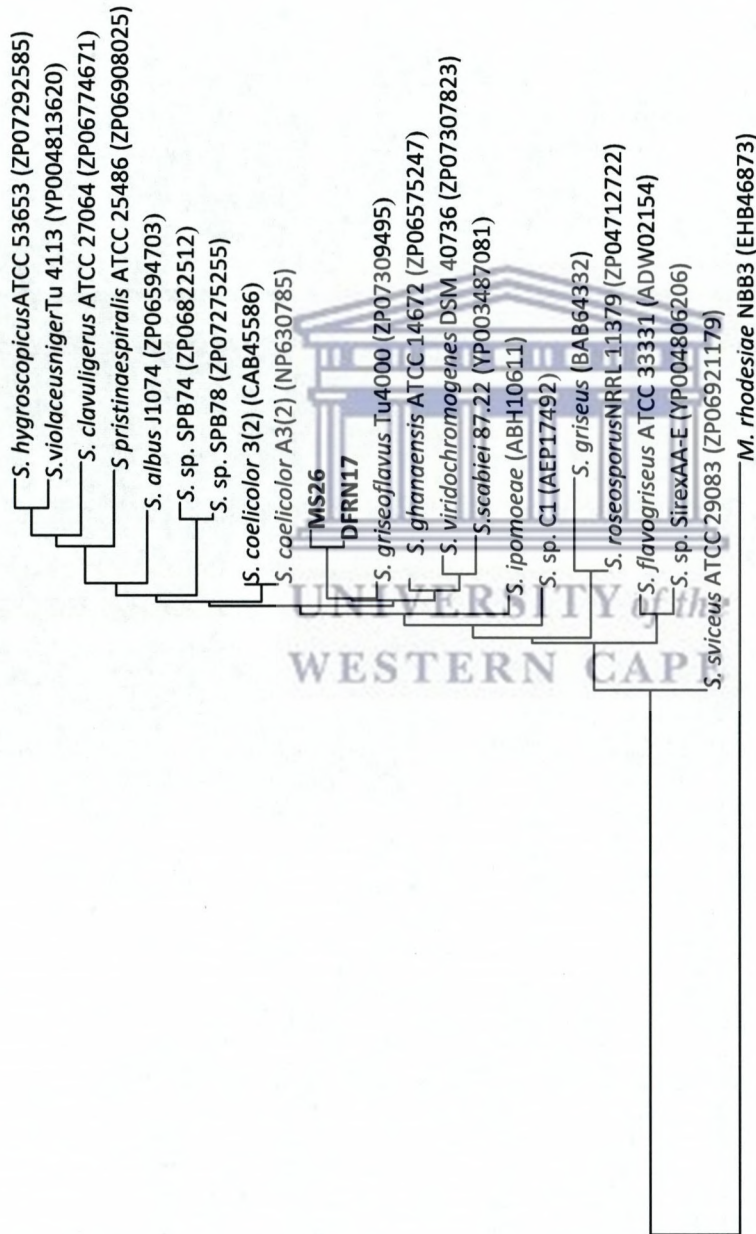


Figure 4.4: The phylogenetic relationship between copper oxidase gene fragments generated from isolates MS26 and DFRN17 and representative members of the multicopper oxidase type 2, copper oxidase and laccase genes. The phylogenetic tree was based on neighbour joining analysis of 1000 resampled datasets. The bar represents 0.2 nt substitution per nt. *Mycobacterium rhodesiae* NBB3 was set as the outgroup.

4.5 Activity-based screening of the fosmid library

A fosmid library, designated MD#, was constructed from the genomic DNA extracted from three mesophilic actinobacterial strains, MS26, DFNR17 and #18, with proven laccase activities. The fosmid library was constructed in *E. coli* (Section 2.11). Based on restriction analysis this library had an average fosmid insert size of 40 kb and contained approximately 1500 clones. Functional screening for laccase activity was performed using a chromogenic oxidative coupling reaction between the substrate 2,2'-azino-di(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and CuSO_4 (Li *et al.*, 1999). The ABTS assay involves the oxidation of ABTS to an intensely-coloured nitrogen-centred radical cation, $\text{ABTS}^{•+}$ (Ferrer *et al.*, 2009). Laccase activity is detectable as an intense green-blue coloured product (Li *et al.*, 1999). The screening plates were prepared at three different pHs to increase the probability of identifying functionally active genes (Section 2.11.1). The screening plates were monitored for the presence of laccase activity for up to 5 days, however, no positive clones were identified. *Coriolus versicolor* laccase served as a positive control and was found to be most active at pH 5.0 (data not shown).

4.6 Colony hybridization screening of the fosmid libraries

Colony hybridization was performed on MD# fosmid library clones using a mixed probe from both MS26 and DFNR17 strains which was generated by PCR with the SCuOxF and SCuOxR primers. A number of putative positive clones were detected, however, following reprobing and PCR analysis, these were shown to be false positives.

4.7 PCR-based screening of the fosmid library

In order to determine whether the failure to detect laccase activity on indicator plates was due to the absence of expressible laccase genes in the library, PCR analysis of the library was performed to screen for laccase genes in the library. A library was subjected to alkaline lysis (Section 2.6.3.1) to isolate the fosmid DNA for PCR analysis. PCR was performed using *Streptomyces* laccase-like copper oxidase specific primers SCuOxF and SCuOxR (Section 2.5.2 and Table 2.2). No PCR amplicons were detected following PCR, which suggests that bacterial laccase genes were not present in the fosmid library.

4.8 Discussion

There has been increasing interest in identifying and isolating bacterial laccase genes. The presence of laccase activity has been shown in a number of diverse bacterial taxa including *Azospirillum lipoferum* (Givaudan *et al.*, 1993), *Marinomonas mediterranea* (Sanchez-Amat *et al.*, 2001), *Escherichia coli* (Grass and Rensing, 2001) and *Bacillus halodurans* (Ruijsenaars and Hartmans, 2004). The widespread presence of laccases in bacteria has also been suggested based on sequencing bacterial genomes (Alexandre and Zhulin, 2000). Due to the advances in molecular biology where genomes are sequenced with steadily improving techniques it is now feasible to access much of the extant bacterial diversity and therefore investigate the occurrence of laccase genes in bacteria.

The PCR screening assay for bacterial laccase genes has several useful applications. Apart from the obvious detection of laccase genes in individual bacterial isolates, it could also be used to determine laccase gene distribution in the genome. To identify the presence of laccase genes and to verify the effectiveness and specificity of the primer set, laccase positive actinomycetes strains MS26, DFNR17, #18, BAM4, HMC13, 7H1 were used as model

organisms for the PCR detection of the laccase gene. A single 300 bp amplicon was specifically generated for strains MS26 and DFNR17, whereas no amplicon was generated for strains #18, BAM4, HMC13 and 7H1.

Southern hybridization can be used to confirm the identity of PCR amplification products from cultured isolates or from environmental DNA (Bej *et al.*, 1991; Erb and Wagner-Döbler, 1993) and to ensure that amplified products are in fact the gene of interest, as opposed to non-specific products. Such confirmation is especially important in the analysis of amplification products from environmental DNA where there is a greater likelihood of false positive PCR amplification clones. In this study the amplification of a partial laccase gene fragment from MS26 and DFNR17 genomic DNA by PCR was confirmed using Southern hybridization of *Pst*I and *Xho*I-digested genomic DNA from MS26 and DFNR17.

Based on BLAST analysis of the PCR generated sequences the amplicons were shown to be similar to a number of *Streptomyces* genes including copper oxidase, multi-copper oxidases, laccase and putative copper oxidase gene fragments. As no other amplicons were obtained from the MS26 and DFNR17 genomic DNA, the primers were considered copper oxidase specific. Phylogenetically both MS26 and DFNR17 were found to be most related to the *S. griseoflavus* strain Tu4000 with a percentage identity at the amino acid level of 83 % (Table 4.1). Therefore, it is probable that the laccase activity observed for these strains is due to a copper binding atom on the active site.

Construction and subsequent screening of the expression libraries for the presence of a desired enzyme activity has become a useful tool for the discovery of novel biocatalysts (Henne *et al.*, 1999; Henne *et al.*, 2000; Lorenz and Schleper, 2002). The collective genomes of microbes inhabiting an ecosystem are referred to as the metagenome (Handelsman *et al.*,

1998), and are considered to be an excellent source of novel and potentially economically valuable genes (Cowan, 2000). Various technologies have been developed to target specific genes within environmental samples. One of the earliest and still prevalent approaches is activity based screening of libraries.

In the present study a fosmid library was successfully constructed, with an average genome insert size of 25-40 kb. Based on an average insert size of 40 kb, the library represented a maximum genome coverage of 6 MB and contained approximately 1500 clones, the number being lower than those generated in other studies (Henne *et al.*, 2000; Ranjan *et al.*, 2005).

No laccase activity was detected following activity-based screening, which could be attributed to a number of factors. Although the average insert size (40 kb) of the library was more than adequate to represent full-length genes, there may have been a low nucleotide coverage i.e. the fraction of DNA captured during library construction was not large enough to include a functional laccase gene. Statistically, libraries of 10^5 clones need to be screened to ensure a positive hit assuming an average insert size of 3 kb (Gabor *et al.*, 2004). The fosmid library screened in this study contained only 1500 clones ($<10^5$), which may account for no positive clones being detected. Gabor and co-workers (2004) describe a binomial distribution describing the number of clones (Np) necessary to detect a cloned target gene at least once with the probability P :

$$Np = \frac{\ln(1 - p)}{\ln\left(1 - \frac{I - X}{G.c.z}\right)}$$

where l is the average insert size, X the size of the gene of interest, G the average genome size present in the sample, z the number of genomes assuming even distribution and c a constant equal to 1 when expression of the interest is independent of the vector (Gabor *et al.* 2004).

The above expression assumes that all genomes present are represented equally. This assumption, however, underestimates the impact made by individual genomes to the total DNA. Two statistical models were proposed by Gans and co-workers (2005) to more accurately estimate of the number of clones necessary for effective screening. The first assumed equal distribution of genomes which indicated lower genome abundance while the second assumed uneven distribution of genomes which indicated higher genome abundance compared to the even distribution figures. If the calculated genome estimates are true for uneven distribution, the value of z according to Gabor *et al.* (2004) may be much larger, allowing for an even higher value for Np .

Assuming the following for the fosmid library used in this study: average insert size (l) = 3 kb; average gene size (X) = 1.5 kb; average size of a *Streptomyces* genome (G) = 8 Mb; $c = 1$; $z = 2000$; with a probability P of 0.99 of achieving a positive hit during expression screening, the number of clones needed within the fosmid library would be 5×10^7 clones. This theoretical figure is much larger than the constructed fosmid library. Even if the figure of 2000 genomes is overestimated tenfold, the number of theoretical clones required for a 0.99 probability of obtaining a positive hit still far exceeds the current size of the library.

In this study *E. coli*, a Gram-negative bacterium, was used as the host for the construction of the fosmid library. The disadvantage of using *E. coli* as a host is that expression of the gene and the gene product, in this case laccase, is limited and dependent on the host containing the cellular components required to express the gene or secrete a

functional gene product (Lam *et al.*, 1999; Gabor *et al.*, 2004; Kashima and Udaka, 2004; and Nakashima *et al.*, 2005).

The problems involved with the selection of the heterologous host that must be considered when expressing a foreign gene include poor transcription, translation, and excretion of the product (Gabor *et al.*, 2004). Also, in several cases the desired protein is not folded correctly because essential chaperones are not present in the host strain to produce functional proteins (Ferrer *et al.*, 2003; Ferrer *et al.*, 2004; Gabor *et al.*, 2004). In addition, cofactors which are essential for the functional expression of the protein may not be present (Gabor *et al.*, 2004). Lastly, a different codon usage could result in poor protein expression and low activities (Sharp and Li, 1987). Codon usage can result in a bias, especially where organisms preferably use certain codons to code for an amino acid instead of using other synonymous codons provided by the host (Sharp and Li, 1987; Grote *et al.*, 2005).

To verify whether the absence of laccase activity was due to the absence of laccase genes in the library, PCR analysis was performed on fosmid DNA extracted from the library. Using laccase-like copper oxidase specific primers in PCR analysis of the native isolates enabled the detection of a variety of laccase genes. Following PCR analysis of the fosmid library no signals were obtained in any of the PCR reactions and it was concluded that no laccase genes were cloned from the native strains.

The host that is most commonly used for protein expression is *E. coli* (Handelsman *et al.*, 1998). The reasons for this are that batch production, separation, and downstream processing methods used in the production of valuable products are already well-studied for *E. coli* (Daniel, 2004). *Streptomyces* and *Pseudomonas* strains have been used as a host to express prokaryotic genes (Courtois *et al.*, 2003; Martinez *et al.*, 2004; Ono *et al.*, 2007). The

advantages of using *Streptomyces* or other actinomycetes as heterologous hosts are that they possess a greater number of complex promoters (Strohl, 1992), they can post-transcriptionally modify products that *E. coli* cannot (Gabor *et al.*, 2004), they can express high G+C DNA content genes (Muto and Osawa, 1987) and actinomycetes are known to produce an array of metabolites so there is a greater chance that the biosynthetic machinery is present to express and produce these products. However the use of these hosts is technically difficult and requires intricate optimization procedures to ensure the expression of the genes of interest (Wilkinson *et al.*, 2002).

A possible explanation for the number of putative positive clones detected by colony hybridization which subsequently proved to be negative after reprobing and PCR analysis are that the false positives result from high background hybridization caused by inadequate removal of cellular debris (Hu and Wu, 2000). Alternatively the signals may result from the hybridization of the probe to the *E. coli* copper oxidase gene *CueO*.



Chapter 5: General discussion, conclusion and future work

5.1 General discussion and conclusion

The choice of DNA extraction methods, particularly the effective dissociation of cells from debris and efficient cell lysis, is crucial for the recovery of representative community of DNA (Heuer *et al.*, 2001). DNA extraction methods do not equally lyse all cells. Most microorganisms that form vegetative cells, spores and hyphae, especially actinomycetes, are difficult to lyse. In this study, high molecular weight DNA was obtained using the method described by Wang *et al.* (1996). This method was found to be more effective for lysing actinomycetes cell walls and the isolates yielded good quality, high molecular weight DNA.

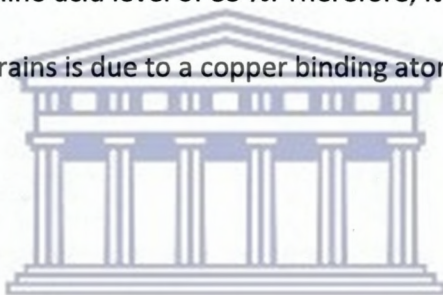
Members of a bacterial species are at least 97% identical in 16S rRNA gene sequence (Wayne *et al.*, 1987). Currently a 16S rRNA gene sequence similarity range above 98.7–99% (Stach *et al.*, 2003; Stackebrandt and Ebers, 2006) is mandatory for testing the genomic uniqueness of a novel isolate. This overturns the old value of 97%. In this study, DNA from both samples was PCR amplified using F1/ R5 primers. Samples from both strain produced the required amplicons which were successfully cloned into a p-GEM[®]-T Easy vector. The F1/R5 primers could amplify 16S rRNA gene sequences from both isolates and their sequence showed 16S rRNA sequence gene identities of 99% with known *Streptomyces* species, with MS26 sharing the highest 16S rRNA gene sequence similarity to *S. viridodiastaticus* IFO 13106 (99.0 %) and *S. atrovirens* NRRL B-16357 (99.0 %), and DFNR17 sharing the highest 16S rRNA gene sequence similarity with *S. althioticus* KCTC 9752 (99.0 %) (Figure 3.4). Based on the high level of sequence similarity it is probable the both isolates are members of validly described species. This finding was supported by conducting full phenotypic characterisation on the isolates. To confirm whether the strains belonged to

already described taxa full polyphasic classifications of strains MS26 and DFNR17 were conducted. Both isolates display many characteristics in common with their closest relatives (Tables 3.1, 3.2a and 3.2b). There are however some differences that exist between the biochemical and physiological characteristics of strains MS26 and DFNR17 and their closest relatives. Both isolates are unable to degrade adenine, gelatin and guanine while their closest relatives *S. althioticus* strain KCTC 9752, *S. atrovirens* strain NRRL B-16357 and *S. viridodiataticus* strain IFO 13106 are able to degrade adenine, gelatin and guanine i.e they are able to produce the enzymes required to hydrolyse the substrates. Both isolates were resistant to lincomycin, whereas their nearest relatives lack the mechanisms that confer resistance to lincomycin. However these characteristics neither validate that the isolates have been previously described nor that they are novel isolates.

In order to identify the laccase genes a fosmid library, designated MD#, was constructed from the genomic DNA extracted from three mesophilic actinobacterial strains, MS26, DFNR17 and #18, with proven laccase activities. The library was screened for clones exhibiting laccase activity on solid medium using a chromogenic oxidative coupling reaction between the substrate 2,2'-azino-di(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and CuSO_4 . The MD# library consisted of approximately 1500 clones. Activity screening was unsuccessful as no laccase positive clones were identified

Colony hybridisation was attempted to detect the laccase gene in the MD# library, using the 300bp SCuOxR PCR product probe from MS26 and DFNR17. Unfortunately, the screening was unsuccessful as no laccase positive clone was detected. This could be due to hybridisation of the probe with the *E. coli* copper oxidase gene *CueO*. Few *Streptomyces* sequences coding for laccase genes are available. As a consequence designing the primers

necessary for the identification of laccase genes from the isolates MS26 and DFNR17 hinder the PCR based approach and a traditional activity based approach was useful. A single 300 bp amplicon was specifically generated from strains MS26 and DFNR17. These PCR generated sequences were shown to be similar to a number of *Streptomyces* genes including copper oxidase, multi-copper oxidases, laccase and putative copper oxidase gene fragments. As no other amplicons were obtained from the MS26 and DFNR17 genomic DNA the primers were considered copper oxidase specific. Phylogenetically both strains MS26 and DFNR17 were found to be most related to the *S. griseoflavus* strain Tu4000 with a percentage identity at the amino acid level of 83 %. Therefore, it is probable that the laccase activity observed for these strains is due to a copper binding atom on the active site.



5.2 Future work

Isolates MS26 and DFNR17 have been partially characterised phenotypically. Future work should include characterisation of carbon and nitrogen source utilisation. Additional physiological characterisation including, scanning electron microscopy should be performed to determine the spore chain morphology.

As attempts to screen a fosmid library failed, there are several possible methods which can be used to obtain the functional laccase gene from these isolates. The first is to sequence the fosmid library employing new 454 sequencing technology. A disadvantage of this approach is that there is a possibility that the fosmid library constructed in this study did not contain the laccase gene. Secondly, a large library could be constructed. The probability of finding a positive clone would be greatly increased if at least 5×10^7 clones were screened.

Another opinion would be to generate a separate small insert library from each strain (ideally consisting of at least $\geq 10^7$ clones) from partially digested genomic DNA and to screen each library separately for laccase activity. If clones with laccase activity are detected the gene of interest can be identified by sub-cloning and primer walking. Once a portion of the new insert sequence is known, it can then be used to design a new primer to read further sequence of the insert. This process is repeated until the whole insert is sequenced.

Once a full length gene is obtained it can be cloned into an expression vector and the extracellular laccase expressed by the positive clone could be purified for proteomic analysis. The physical and biochemical properties of the enzyme such as pH stability, optimum temperature and enzyme kinetics would be determined.



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