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,

University of the Western Cape

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May 2012

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 viridodiastaticus* 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 cooper 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.

Acknowledgements

The success of this work can be attributed to the assistance, guidance, and prayers of so many people who are hereby gratefully acknowledged. Firstly, I would like to give the Almighty God all the glory, honour and adoration for giving me the strength and wisdom through the Holy Spirit to complete this work.

To my supervisors, Prof. Don Cowan and Prof. Marla Tuffin, thank you for granting me the opportunity to learn high class science (molecular biology). I am sincerely grateful for your belief in me, your patience, support and encouragement and for allowing me to learn to the level I am today.

To my co-supervisor Dr. A Casanueva, I know it was not an easy road but it was worth it. You made it endurable throughout. I am grateful to you for everything, for assisting with all the molecular techniques, critically reading my research and finally for being patient with me, without which this thesis would not have been possible.

To Dr. H Goodman for making me always feel at home, your support assistance, day to day running of the lab, and reading of my thesis throughout is highly appreciated.

To Mrs. Ruth Coetzee thank you for always making me feel at home

I gratefully acknowledge the National Research Foundation (NRF) of South Africa for funding my research programme. The support of my colleagues in IMBM, University of the Western Cape, including Thulani, William, Claude, Layla-Lucinda, Freedom , Rhulani, William Bopda, Munaka , Rudzani , Timna, Dean, Dr Kambulu, Victor, Justice Baruti, Dr Mulaudzi Takalani and Stephen Mailu is gratefully acknowledged. The leadership, role and cooperation from

our post doctoral fellows Dr. Mark Paul Taylor, Dr. Inonge Mulako, Dr. Bronywn Kirby, Dr. Francesca Stomeo and Dr. Rob Huddy is also acknowledged.

I am deeply grateful to Dr Bronwyn Kirby for your contribution towards helping me putting my thoughts down on paper in a scientific manner; your critical reading of my thesis throughout is highly appreciated.

I am also appreciative of everyone who has helped teach me some of the techniques that I have learned over the course of my M.Sc.: Mr William Mavengere, Mr Lonnie van Zyl and Dr Bronwyn Kirby.

Thank you to Dr Marilise le Roux-Hill from the Cape Peninsula University of Technology for providing strains.

I gratefully acknowledge Dr. Samuel Kojo Kwofie and the Division for Postgraduate Studies for academic support.

Thank you to Makhadzi Mutshinya Constance Netshidzati for all the encouragement

To my family: The Netshilema and Rasivhaga families: thank you for being a wonderful family to me. I really appreciate everything you did and have done for me, for giving me support, advice, and strength when I needed it the most. I am very grateful to all of you for supporting me emotionally, spiritually, financially, and for all the long distances calls. Finally, for giving me a shoulder to cry on whenever I needed it**,** you are one in a million. The road we travelled together was not easy but it was worth every step. Through good times and the worst times, you have been there with me. I wouldn't have made it this far without you.

Mrs Maemu Shiela Rasivhaga: The pain I felt due to your untimely death was, and still is, unbearable. “It won’t be so bad, after a while”. So say these friends of mine. But they have never lost a mother who is so caring, supporting and loving. The pain will never go away yet it softens some, with time you are gone. It hurts to say I’ll never be “just fine” anytime soon. However goodbyes are not forever, goodbyes are not the end. They simply mean I will miss you until we meet again. You are always in my thoughts, and I know that you will be watching over me from heaven. May your soul rest in peace.

To Mrs AvhatakaIi Netshilema: I am sanctified for being blessed with a mother like you who has always supported me through all my decisions in life. I would like to thank you for positioning me on the path of excellence and to challenge life when necessary. I have become who I am today because I am a product of your influence.

Dedication

To Mrs Avhatakali Netshilema and Mrs Maemu Shiela Rasivhaga, your belief in me has allowed me to reach this point. Your constant encouragement and sustenance has enabled me to accomplish my dream. I love you always.

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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
$CaCO_3$	Calcium carbonate
$CaCl_2$	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
$FeSO_4.7H_2O$	Ferrous sulfate heptahydrate
g	Gram
"g"	Gravitational force
HBT	1-hydroxybenzotriazole
H_2O_2	Hydrogen peroxide
ISP	International *Streptomyces* project
KCl	Potassium chloride
K_2HPO_4	Dipotassium phosphate
L	Litre
LB	Luria Bertani medium
LB-amp	Luria Bertani medium containing ampicillin
M	Molar
mg	Milligram
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
$MnCl_2.4H_2O$	Manganese chloride tetrahydrate
$MnCl_2$	Manganese chloride
min	Minute(s)

ml	Millilitre
mm	Millimetre
mM	Millimolar
µF	Microfarad
µg	Microgram
µl	Microlitre
µM	Micromolar
NaOH	Sodium hydroxide
NaH_2PO_4	Sodium dihydrogen orthophosphate
$Na_2HPO_4.2H_2O$	Disodium phosphate dehydrate
$Na_2S_2O_3$	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-*N*-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
$ZnSO_4 \cdot 7H_2O$	Zinc sulphate hepthahydrate

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.

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 Iα, Iβ, II_I, III_{II}, IV_I, and IV_{II}, 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→2) glycosidic bonds and the acetic acid side groups

are esterified at the carbon 2 and hydroxyl group. Softwood xylans are arabino-4-O-methyglucuronoxylans, 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).

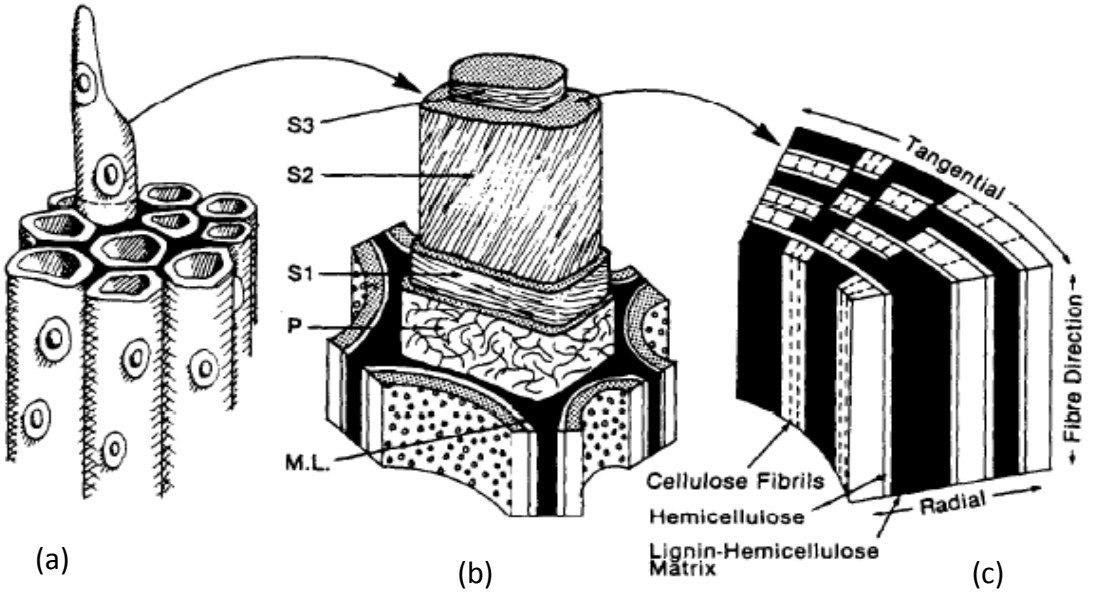

Figure 1.1: Structural design of woody tissues: (a) collection of adjacent wood cells, (b) cross sectioning showing the distinct cell wall layers, (c) part of the secondary wall showing the connection of hemicellulose and lignin to the cellulose fibrils. Key: P, primary cell wall layers; S1, S2 and S3, 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 lignocellose 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_2O_2, veratry alcohol	Aromatic ring oxidized to cation radical
Manganese peroxidase	H_2O_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. hydroxybenzatriazole or ABTS	Glyoxal oxidized to glyoxylic acid; H_2O_2 production
Glyoxal oxidase	Glyoxal, methyl glyoxal	Aromatic alcohols oxidized to aldehydes; H_2O_2 production
Aryl alcohol oxidase	Aromatic alcohols (anisyl, veratryl alcohol)	O_2 reduced to H_2O_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-$C_ß$ 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 concomittant 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 lead 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 lead 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., 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. (Worral *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 campesteris* (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
Aquifex aeolicus (sufI)	Cell division	Deckert *et al.* (1998)
Azospirillum lipoferum	Pigmentation, oxidation of phenolic compounds, electron transport	Givaudan *et al.* (1993)
Bacillus sphaericus	Sporulation, pigmentation	Claus and Filip (1997)
Bacillus subtilis (cotA)	Pigmentation of spores, UV and H_2O_2 resistance	Hullo *et al.* (2001)
Bacillus halodurans C-*125 (lbh 2082)*	Cu^{2+} resistance	Ruijsennars and Hartmans (2004)
*Escherichia coli (yac*K*)*	Cu^{2+} efflux, oxidation of phenolate-siderophores ferrooxidase activity	Roberts *et al*. (2002)
*Marinomonas mediterranea (ppo*A*)*	Pigmentation	Sanchez-Amat *et al.* (2001)
y-Proteobacterium JB	Oxidation of toxic compounds	Bains *et al.* (2003)
Pseudomonas maltophila	Nucleoside oxidase activity	Isono and Hoshino (1989)
Pseudomonas putida GB-1 *(cum*A*)*	Mn^{2+} oxidation	Brouwers *et al.* (1999)
*Pseudomonassyringae pv.tomato (cop*A*)*	Cu^{2+} resistance	Cha and Cooksey (1991)
Streptomyces antibioticus	Phenoxazinone synthesis	Freeman *et al.* (1993)
Streptomyces coelicolor	Oxidation of phenolic compounds	Machczynski *et al.* (2004)
Streptomyces cyaneus	Oxidation of non-phenolic compounds	Arias *et al.* (2003)
*Streptomyces griseus (epo*A*)*	Pigmentation, morphogenesis	Endo *et al.* (2002)
*Xanthomonas campesteris(cop*A*)*	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 practicola* 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).

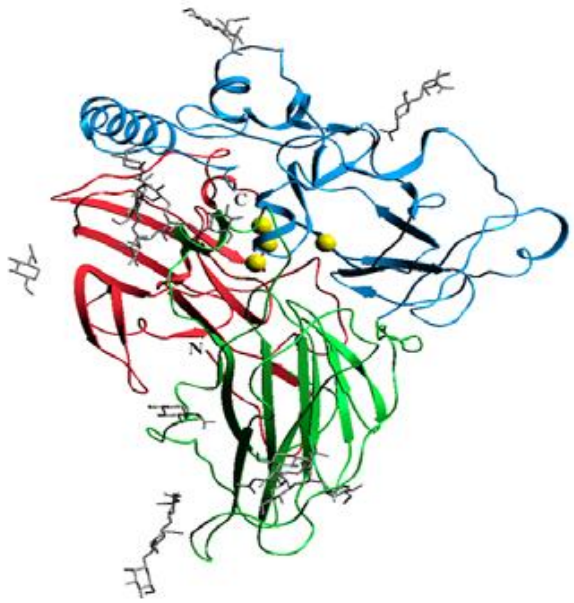

Figure 1.2: Three-dimensional structure of *M. albomyces* laccase. Domains A, B, and C are colored red, green and blue, respectively. The four copper atoms are shown as yellow balls and carbohydrates as grey sticks (Hakulinen *et al.,* 2002).

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 *et al.* (2007)
Bioremediation	Riva (2006)
Ethanol production	Senthilguru *et al.* (2011)
Biosensors	Ncanana *et al.* (2007)
Wine clarification	Rosana *et al.* (2002)
Detergent manufacturing	Sharma and Kuhad (2008)
Transformation of antibiotics and steroids	Ncanana *et al.* (2007)
Herbicide degradation	Mougin *et al.* (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 (Labeda, 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 (avermectin), 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; Ômura *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).

Table 1.5: Secondary metabolites produced by *Streptomyces*

Compound	Biological activity	Species	Reference
Streptozotocin	Diabetogenic	*S. achromogenes*	Herr *et al.* (1967)
Streptomycin	Antimicrobial	*S. griseus*	Egan *et al.* (1998)
Bafilomycin	ATPase inhibitor ofmicro-organisms, plant and animal cells	*S. griseus,*	Frändberg *et al.* (2000)
Mitomycin C	Antitumor, binds to double-stranded DNA	*S.lavendulae*	Mao *et al.* (1999)
Hygromycin	Antimicrobial, immunosuppressive	*S. hygroscopicus*	Uyeda *et al.* (2001)
Lincomycin	Antibacterial, inhibitor of protein biosynthesis	*S. lincolnensis*	Peschke *et al.* (1995)
Chloramphenicol	Antimicrobial, inhibitor of protein biosynthesis	*S. venezuelae*	Bewick *et al.* (1976)
Valinomycin	Ionophor, toxic for pro-and eukaryotes	*S. griseus*	Anderson *et al.* (1998)
Anthracyclines	Antitumor	*S. galileus*	Fujii and Ebizuka (1997)
Avermectin	Antiparasitic	*S. avermitilis*	Burg *et al.* (1979)
Tetracycline	Antimicrobial	*S.rimosus*	Hansen *et al.* (2001)
Rapamycin	Immunosuppressive, antifungal	*S.hygroscopicus*	Vezina *et al.* (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 *Streptomycete* 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 cooper oxidase specific degenerate primers and colony hybridization of the library using a 300 bp probe.

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 ^{o}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
***E.coli* (DH5α)**	*recA endA1 hsd*R17 *sup*E4 *gyr*A96 *rel*A1 Δ(*lac*ZYA-*arg*F)U169 (ϕ80d*lac*ZΔM15)	Promega
***E.coli* (GeneHog®)**	F- mcrA D(mrr-hsdRMS-mrcBC) f80lacZDM15 DlacX74 deoR recA1 endA1 araD139 D(araleu) 7697 galU galK l rpsL nupG	Invitrogen
Plasmids		
pGEM®-T-Easy	Size 3015 bp, T7 promoter, SP6 promoter, Amp^{r}, *lac* operator, *Lac*Z start codon, phage f1region, 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_2O 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_2O up to	1000ml

The pH was adjusted to 7.0 with 10 M NaOH before autoclaving. After autoclaving, the broth was cooled to ~55^{o}C and the following filter sterilized solutions were added aseptically: 5 ml of $MgCl_2$ (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^{o}C and the following were filter sterilized solutions and added aseptically, 5 ml of 2 M $MgCl_2$ 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 H_2O. 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 dH_2O. The medium was sterilized by autoclaving, and allowed to cool to ~ 55^{o}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 dH_2O. The medium was sterilized by autoclaving and allowed to cool to ~ 55^{o}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_2O up to	500 ml

Solution 2

Constituent	**500 ml^{-1}**
K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	1.0 g
NaCl	1.0 g
$(NH_4)_2SO_4$	2.0 g
$CaCO_3$	2.0 g
Trace salts solution	1.0 ml
dH_2O 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 dH_2O. The two solutions were mixed together and 20 g agar was added. The medium was sterilized by autoclaving and allowed to cool to ~ 55^{o}C and poured into petri dishes. Pure colonies of the isolate were streaked on the solidified medium and the plates were incubated at 28^{o}C for 14 days.

Trace salts solution

Constituent	**100 ml^{-1}**
$FeSO_4.7H_2O$	0.1 g
$MnCl_2.4H_2O$	0.1 g
$ZnSO_4 \cdot 7H_20$	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_2HPO_4	1.0 g
Trace salts solution (see ISP4)	1.0 ml
dH_2O 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 dH_2O and 20 g agar was added. The medium was sterilized by autoclaving and allowed to cool to ~ 55^{o}C and poured into petri dishes. Pure colonies of the isolate were streaked on the solidified medium and the plates were incubated at 28^{o}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_2HPO_4	1.0 g
$Na_2S_2O_3$	0.08 g
Yeast extract	1.0 g
dH_2O up to	1000 ml

The pH of the medium was adjusted to 7.0 with 10 M NaOH and the volume to 1L with dH_2O. 15.0 g agar was added. The medium was sterilized by autoclaving and allowed to cool to ~ 55^{o}C and poured into petri dishes. Pure colonies of the isolate were streaked on the solidified medium and the plates were incubated at 28^{o}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_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.5 g
NaCl	0.5 g
$FeSO_4.7H_2O$	0.01 g
Trace salts solution (see as in ISP 4)	1.0 ml
dH_2O up to	1000 ml

The pH of the medium was adjusted to 7.2 with 0.1 M HCl and the volume to 1L with dH_2O. 20.0 g of agar was added. The medium was sterilized by autoclaving and allowed to cool to ~ 55^{o}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^{o}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_2HPO_4	0.6 g
$MgSO_4.7H_2O$	0.1 g
dH_2O	1000 ml

The pH was adjusted to 7.0 with 10 M NaOH and the volume to 1 L with H_2O. The medium was sterilized by autoclaving and allowed to cool to ~ 55^{o}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 (Alphalmager 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 $(NH_4)_2SO_4$, 0.1 % (w/v) Triton X-100, 2 mM $MgCl_2$) and 1 U/µl *Taq* polymerase. The reaction mixture was

made up to 50 µl with sterile ddH_2O. 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 µg/ml) into 20 µl TE buffer. The mixture was vortexed and 2 µl 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 *et al*. (1991)
16S-R5	ACGGITACCTTGTTACGACTT			
16S-F3	GCCAGCAGCCGCGGTAATAC	[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 *et al*. (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 *et al.* (1985)
M13R	ATTACCGCGGCTGCTGG			
SCuOxF	CSRTCGTCTTCAACGAYATG	[94°C for 3mins 30 x (94°C for 30s, 56°C for 30s, 72°C for 30s) 72°C for 7min][#]	*Streptomyces* 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
S. coelicolor	CAB45586	343 aa	SLAC
S. griseus	BAB64332	348 aa	EpoA
S. ipomoeae	ABH10611	335 aa	SilA
S. clavuligerus	ZP_03185908	335 aa	copper oxidase
S. pristinaespiralis	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* DH5α cells were prepared and transformed as described by Inoue *et al*. (1990). A single colony of a freshly streaked *E. coli* DH5α 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 (OD_{600} 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 µl DMSO had been added. Following incubation on ice for 15 minutes, 50 µl 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 µl aliquot of electrocompetent cells was removed from -80°C storage and allowed to thaw on ice. Once thawed, 2µl 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 µF, 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 µl of the cells were plated onto LB-agar plates supplemented with ampicillin (100 µg/ml), IPTG (20 µg/ml) and X-Gal (30 µg/ml). Recombinant

transformants were selected by blue/white colour selection based on insertional inactivation of the *lac*Z 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^{o}C at 15psi. This was cooled to 55^{o}C, poured into plates and allowed to solidify. Pure colonies of MS26 and DFNR17 isolates were streaked on the agar and incubated at 28^{o}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^{o}C for one week. After incubation the plates were flooded with $(NH_4)_2SO_4$ 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^{o}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 waterand 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 hypoxantine 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 $Na_2HPO_4.2H_2O$ 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 $(NH_4)_2SO_4$, 1.0 g yeast extract, 2.0 g $MgSO_4.7H_2O$, 0.l ml 1 % solution $FeSO_4.7H_2O$ 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^{o}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^{o}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 $CaCl_2.2H_2O$ 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 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 ily 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
Gentamacin	100 µg/ml
Kanamycin	50 µg/ml
Lincomycin	100 µg/ml
Penicillin G	10 l.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^{o}C, 30^{o}C, 37^{o}C, 45^{o}C, 55^{o}C, 60^{o}C and 68^{o}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 12.5µg/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 aminocylitol 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 *Saccharoplyspora* species, whilst *Actinomadura* species have been found to produce macrolactam and napthacene-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 characteristics 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 sequence 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).

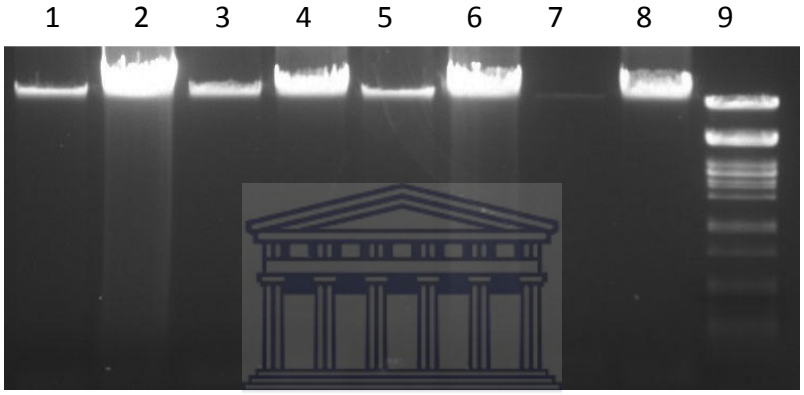

Figure 3.1: Agarose gel electrophoresis of genomic DNA from isolates MS26 and DFNR17. Lanes 1-4: quadruplicate extractions from isolate DFRN17. Lanes 5-8: quadruplicate extractions from isolate MS26. Lane 9: molecular weight marker (λ*Pst*).

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/µl DNA from a 50 ml culture, and extractions from isolate MS26 yielding 958 – 3169 ng/µl 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).

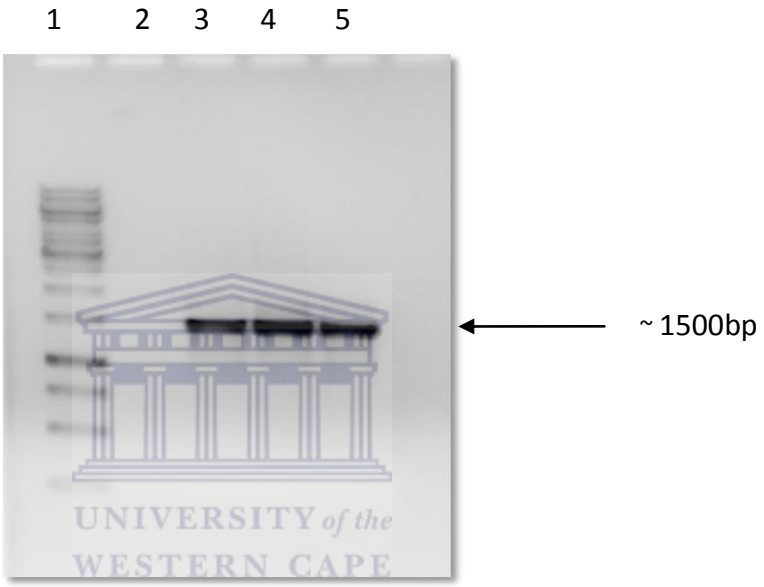

Figure 3.2: Agarose gel electrophoresis of the 1500 bp amplicons from strains DFNR17 and MS26 amplified using the universal 16S rRNA gene bacterial primers F1/R5. Lane 1: *Pst*I digested lambda DNA, lane 2: negative control, lane 3:DFNR17, lane 4: MS26, lane 5: positive control (*Streptomyces* strain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

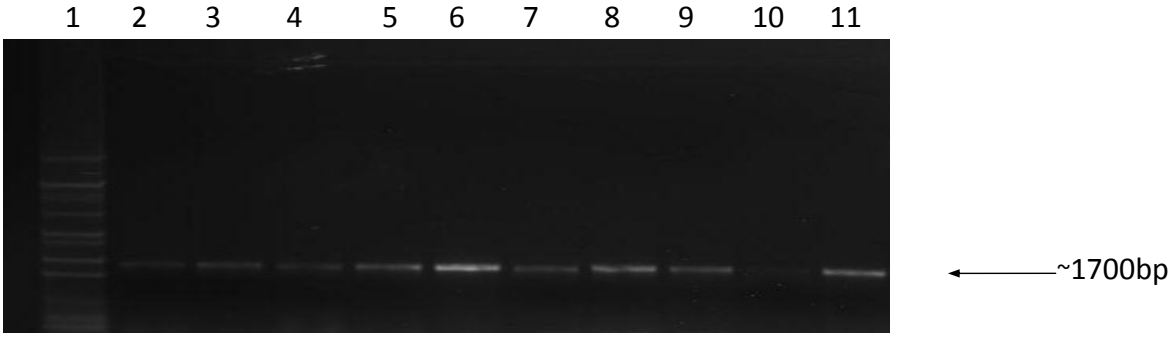

Figure 3.3: M13 PCR amplification of representative clones containing the actinobacterial 16S rRNA gene. Lane 1: *Pst*I digested λ DNA, Lane 2-6: 1700 bp amplicons generated from the DFRN17 colonies, Lanes 7-11: 1700 bp amplicons generated from MS26 colonies.

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 DFNR17 shared the highest 16S rRNA gene sequence similarity to *Streptomyces althioticus* KCTC 9752 (99.0 %). Phylogenetic analysis of the 16S rRNA gene confirmed that strains MS26 and DFNR17 clustered with other *Streptomyces* species (Figure 3.4).

The *S. atrovirens* strain NRRL B-16357, *S. althioticus* 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).

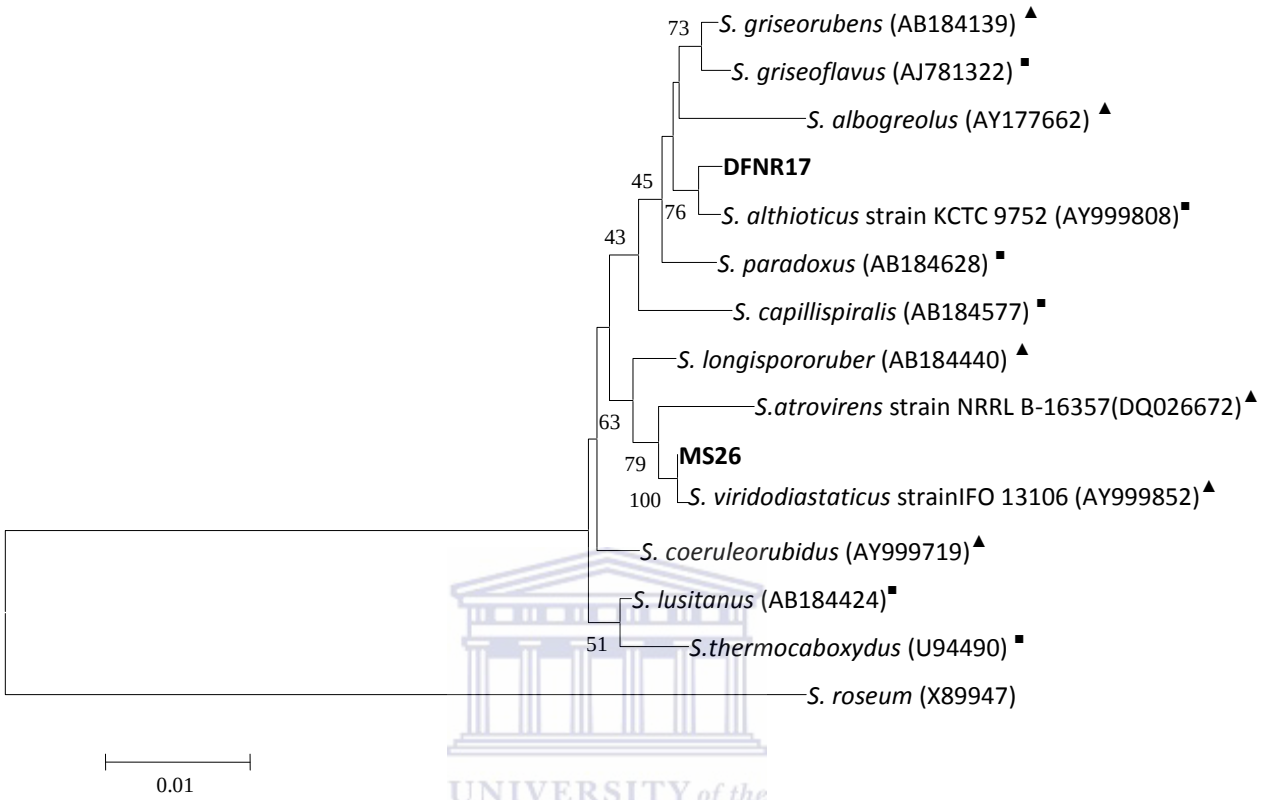

Figure 3.4: Phylogenetic tree showing the position of strains MS26 and DFNR17 and other *Streptomyces* species based on the 16S rRNA gene sequence analysis. The phylogenetic tree was constructed using the neighbour-joining algorithm. The tree is based on 1000 resampled datasets and numbers at nodes indicate 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 DFRN17 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).

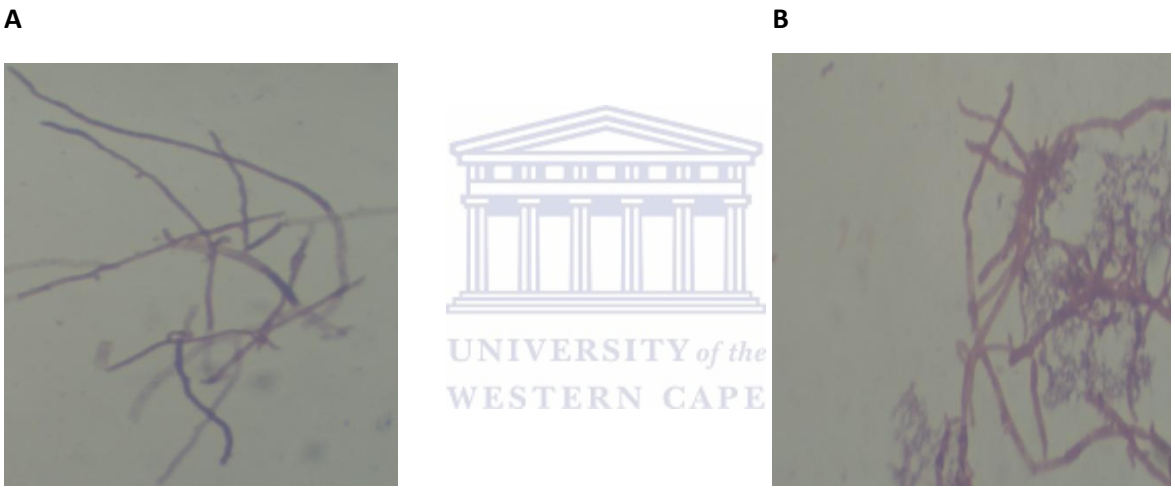

Figure 3.5: Light microscopy (X50) of Gram stained (A) strain MS26 and (B) strain DFNR17.

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^{o}C with neither strain showing growth at temperatures of 55^{o}C or higher. Optimal growth was evident at temperatures between 30 and 37^{o}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 l.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**	*S. althioticus* strain KCTC 9752	**Characteristics**	**DFRN17**	*S. althioticus* strain KCTC 9752
Degradation activity			**Hydrolysis activity**		
Tyrosine	**+**	**+**			
Gelatin	**-**	**+**	Egg-yolk (lecithin)	**-**	**-**
Starch	**+**	**+**	Pectin	**+**	**+**
Xylan	**+**	**+**	Aesculine	**+**	**+**
Adenine	**-**	**+**	Arbutin	**+**	**+**
Guanine	**-**	**+**	**Microscopic characteristics**		
Hypoxanthine	**+**	**+**			
Xanthine	**+**	**-**	Gram reaction	**+**	**+**
Casein	**+**	**-**	Cell morphology	**BF**	**BF**
Siera`s/Tween 80	**+**	**+++**	Type of spores chains	**LCS**	**LCS**
Resistance to antibiotics (µg ml^{-1})			**Temperature growth range**		
Ampicillin (100)	**+**	**+**	16^{o}C	**+**	**++**
Chloramphenicol (12.5)	**-**	**-**	30^{o}C	**+++**	**+++**
Gentamicin (100)	**-**	**-**	37^{o}C	**+++**	**+++**
Kanamycin (50)	**-**	**-**	45^{o}C	**+**	**++**
Lincomycin (100)	**+**	**-**	55^{o}C	**-**	**+**
Penicillin (10i.u/ml)	**+**	**+**	60^{o}C	**-**	**-**
Streptomycin (100)	**-**	**-**	68^{o}C	**-**	**-**
Effect of NaCl concentration			**Growth on ISP medium**		
4 %	**+**	**+**	ISP 4	**++**	**++**
7 %	**+**	**+**	ISP 5	**-**	**++**
10 %	**-**	**+**	ISP 6	**-**	**++**
13 %	**-**	**-**	ISP 7	**-**	**++**

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

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

+=postive, slight growth; **++** =strong growth; **+++** = excellent grwoth; **-** = 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/µl DNA being recorded. The purity of the DNA preparations was high, as indicated by the $A_{260/280nm}$ 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^{o}C - 45^{o}C and they shared optimum and maximum growth temperatures of 30^{o}C - 37^{o}C and 45^{o}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^{o}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 DFRN17 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; Denef *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, DFRN17, #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 saurced 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.

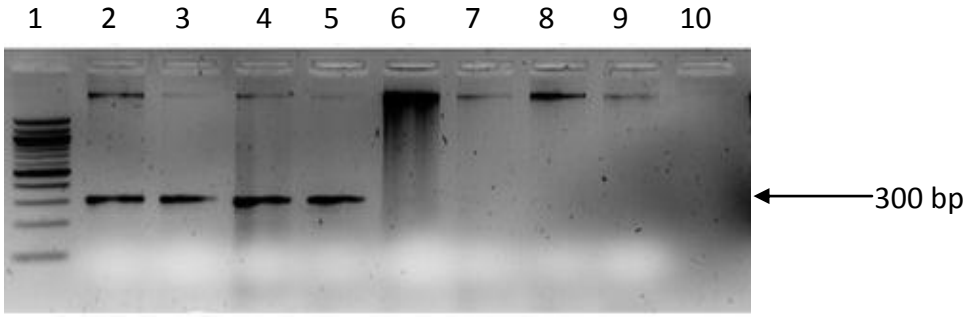

Figure 4.1: Agarose gel electrophoresis of the 300 bpPCR products amplicons from genomic DNA of strains MS26 and DFNR17 and other actinomycete isolates with laccase activity using the SCuOxF/R primer combination. Lane 1: *Pst*I digested λ DNA, lanes 2 and 3: MS26, lane 4 and 5: DFRN17, lane 6: #18, lane 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 SCuOxF/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 *PstI,* while three signals were observed for the genomic DNA digest with *XhoI* 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 *XhoI* 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 *PstI* or *XhoI* sites.

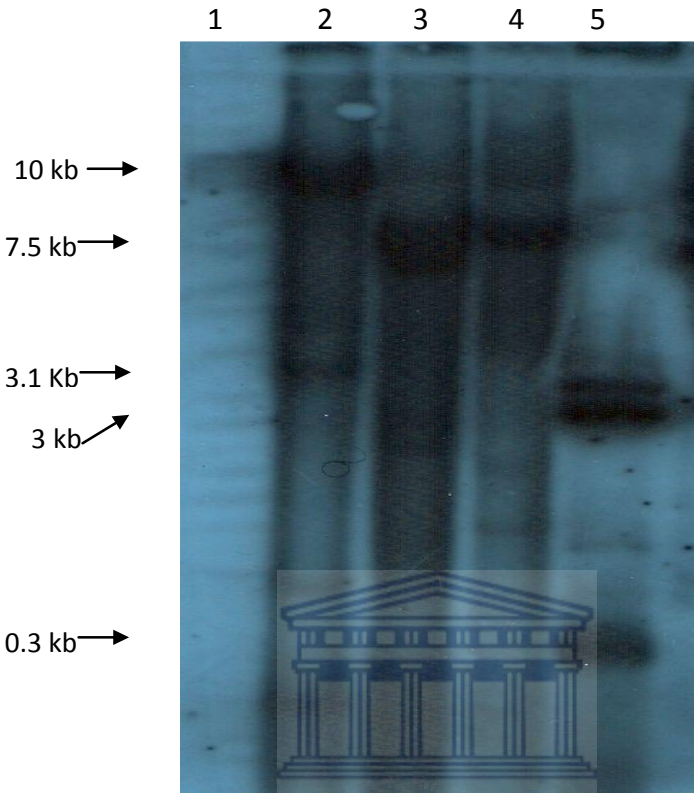

Figure 4.2: Southern hybridization with the laccase PCR product as the probe. Shown is a Southern blot of the *Pst*I and *Xho*I digested genomic DNA of MS26 and DFNR17 isolates probed with the labelled laccase gene 300 bp SCuOxFR PCR fragment. Lane 1: *Pst*I digested λ DNA, lane 2: MS26 genomic DNA digested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

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

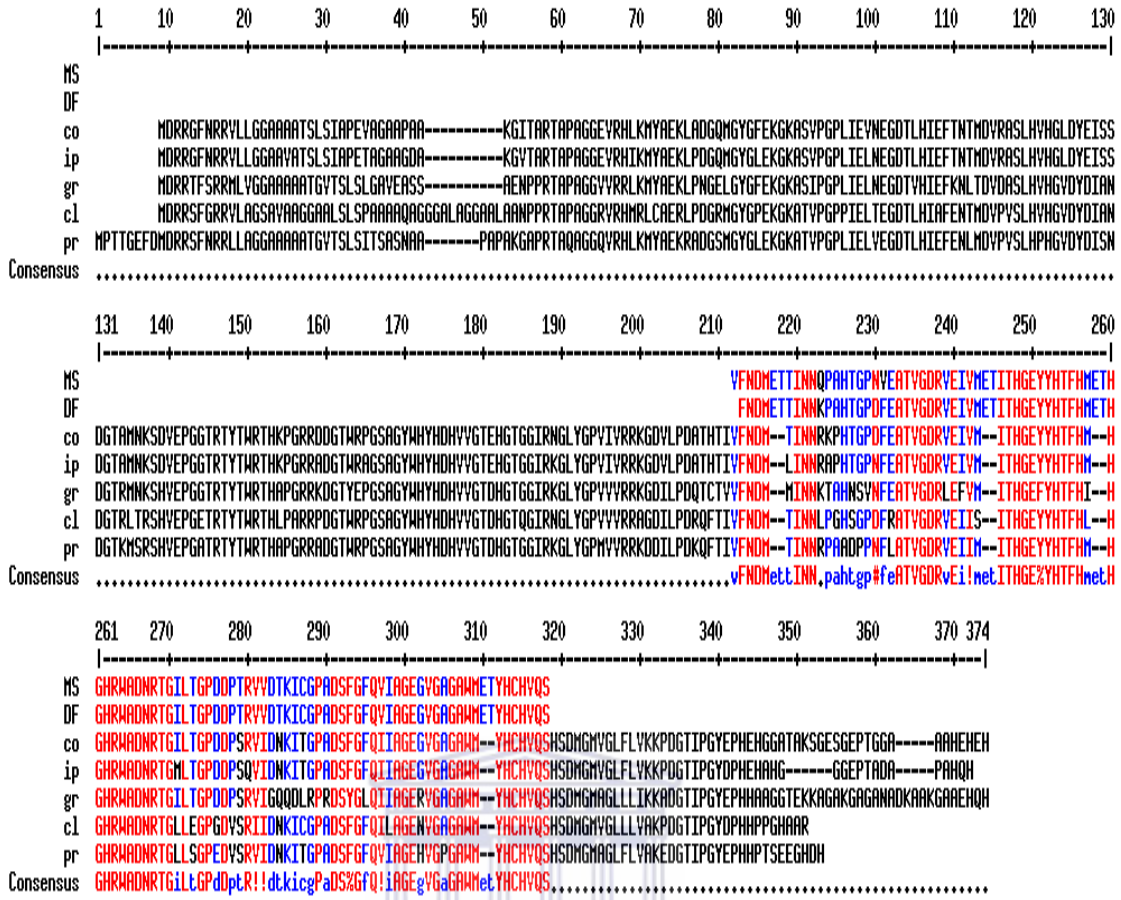

Figure 4.3: Amino acid alignment of the deduced MS26 and DFNR17 amino acid sequences with those of other *Streptomyces* laccase-like sequences used in the primer design.The sequences are as follows: MS (MS26); DF (DFNR17); co (*Streptomyces coelicolor*); ip (*Streptomyces ipomoeae*); gr (*Streptomyces 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).

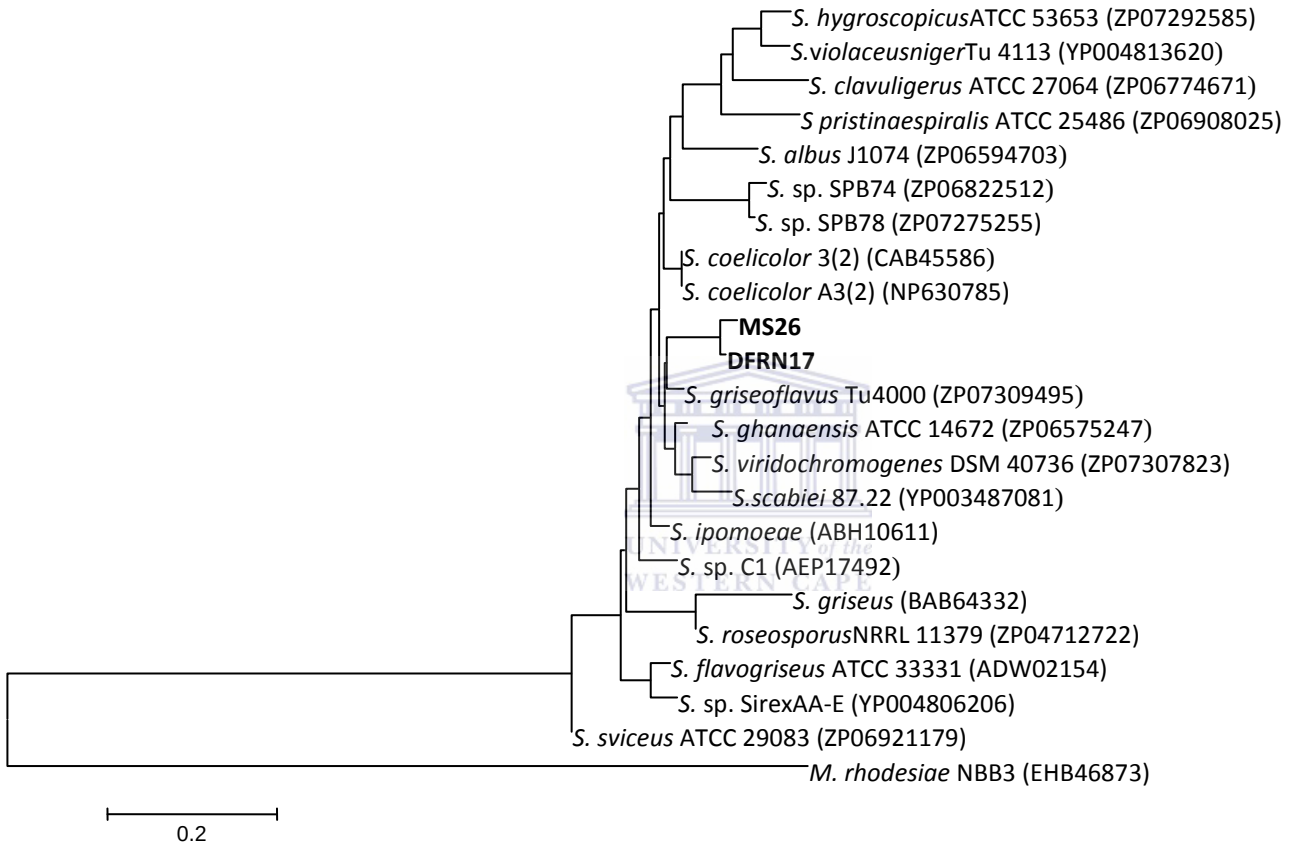

Figure 4.4: The phylogenetic relationship between copper oxidase gene fragments generated from isolates MS26 and DFNR17 and representative members of the multicopper oxidase type 2, copper oxidase and laccase genes. The phylogenetic tree was based on neighbour joining analysis of 1000 resampled datasets. 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, $ABTS^{\bullet+}$ (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 cooper 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* (Ruijssenaars 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 *PstI* and *XhoI*-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 10^7 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 (N*p*) necessary to detect a cloned target gene at least once with the probability P:

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

where *I* 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 N*p.*

Assuming the following for the fosmid library used in this study: average insert size (I) = 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 cooper 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). *Streptomyce*s 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. viridodiastaticus* 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 SCuOxF/R PCR product probe from MS26 and DFRN17. 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 ≥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.

Reference List

Aakvik, T., Degnes, K.F., Dahlsrud, R., Schmidt, F., Dam, R., Yu, L., Volker, U., Ellingsen, T.E. and Valla, S. (2009). A plasmid RK2-based broad-host-range cloning vector useful for transfer of metagenomic libraries to a variety of hosts. *FEMS Microbiology Letters*. **296:** p.149-58.

Acinas, S.G., Marcelino, L.A., Klepac-Ceraj, V. and Polz, M.F. (2004b). Divergence and redundancy of 16S rRNA sequences in genomes with multiple *rrn* operons. *Journal of Bacteriology.* **186:** p.2629-2635.

Adams, T.H., Wieser, J.K. and Yu, J.H. (1998). Asexual sporulation in *Aspergillus nidulans*. *Microbiology and Molecular Biology Reviews*. **62:** p.35-54.

Adler, E. (1977). Lignin chemistry - past, present and future. *Wood Science and Technology.* **11:** p.169-218.

Aharonowitz, Y. (1980). Nitrogen metabolite regulation of antibiotic biosynthesis. *Annual Review of Microbiology.* **34:** p.209-233.

Alam, M. T., Merlo, M. E., Takano, E. and Breitling, R. (2010). Genome-based phylogenetic analysis of *Streptomyces* and its relatives. *Molecular Phylogenetics and Evolution.* **54:** p.763-772.

Alexandre, G., Bally, R., Taylor, B.L. and Zhulin, I.B. (1999). Loss of cytochrome oxidase activity and acquisition of resistance to quinone analogs in a laccase-positive variant of *Azospirillum lipoferum*. *Journal of Bacteriology.* **181:** p.6730-6738.

Alexandre, G. and Zhulin, I.B. (2000). Laccases are widespread in bacteria. *Trends in Biotechnology.* **18:** p.41-42.

Alfaresi, M. and Elkosh, A. (2006). Rapid identification of clinically relevant *Nocardia* species using real time PCR with SYBR green and melting curve analysis. *Journal of Medical Microbiology.* **55:** p.1711-1715.

Anastasi, A., Coppola, T., Prigione, V. and Varese, G. (2009). Pyrene degradation and detoxification in soil by a consortium of basidiomycetes isolated from compost: role of laccases and peroxidases. *Journal of Hazardous Materials.* **165**p.1229-1233.

Anderson, A.S. and Wellington, E.M.H. (2001). The taxonomy of *Streptomyces* and related genera. *International Journal of Systematics and Evolutionary Microbiology.* **51:** p.797-814.

Andersson, M.A., Mikkola, R., Kroppenstedt, R.M., Rainey F.A., Peltola, J. and Helin, J. (1998). The mitochondrial toxin produced by *Streptomyces griseus* strains isolated from an indoor environment is valinomycin. *Applied and Environmental Microbiology.* **64:** p.4767-4773.

Aramayo, R. and Timberlake, W.E. (1993). The *Aspergillus nidulans yA* gene is regulated by *abaA*. *EMBO Journal*. **12:** p.2039-2048.

Arias, M.E., Arenas, M., Rodríguez, J., Soliveri, J., Ball, A.S. and Hernández, M. (2003). Kraft pulp biobleaching and mediated oxidation of a nonphenolic substrate by laccase from *Streptomyces cyaneus* CECT 3335. *Applied and Environmental Microbiology.* **69:** p.1953-1958.

Atalan, E., Manfio,G.P., Ward, A.C., Kroppenstedt, R.M. and Goodfellow, M. (2000). Biosystematic studies on novel streptomycetes from soil**.** *Antonie van Leeuwenhoek.* **77:** p.337-353.

Atalla, R.H. and Van der Hart, D.L. (1984). Native cellulose: a composite of two distinct crystalline forms. *Science.* **223**: p.283-285.

Bains, J., Capalash, N. and Sharma, P. (2003). Laccase from a nonmelanogenic, alkalotolerant c-proteobacterium JB isolated from industrial waste water drained soil. *Biotechnology Letters.* **25:** p.1155-1159.

Bao, W., O'Malley, D.M., Whetten, R. and Sederoff, R.R. (1993). A laccase associated with lignification in Loblolly pine xylem. *Science.* **260:** p.672-674.

Bar-Nun, N., Tal-Lev, A., Harel, E. and Mayer, A.M. (1988). Repression of laccase formation in *Botrytis cinerea* and its possible relation to phytopathogenicity. *Phytochemistry*. **27:** p.2505-2509.

Bartossek, R., Lanzen, G.W.A., Klenk, H.P. and Schleper, C. (2010). Homologues of nitrite reductases in ammonia-oxidizing archaea: diversity and genomic context. *Environmental Microbiology.* **12:** p.1075-1088.

Battistuzzi, G., Di Rocco, G., Leonardi, A. and Sola, M. (2003). H NMR of native and azide-inhibited laccase from *Rhus vernicifera*. *Journal of Inorganic Biochemistry.* 2003. **96:** p.503-506.

Begg, E.J. and Barclay, M.L. (1995). Aminoglycosides – 50 years on. *British Journal of Clinical Pharmacology.* **39:** p.597-603.

Bej, A.K., DiCesare, J.L., Haff, L. and Atlas, R.M. (1991). Detection of *Escherichia* coli and *Shigella* spp in water by using the polymerase chain reaction and gene probes for *uid. Applied and Environmental Microbiology.* **57**: p.1013-1017.

Belkacemi, K., Turcotte, G. and Savoie, P. (2002). Aqueous/steam-fractionated agricultural residues as substrates for ethanol production. *Industrial and Engineering Chemistry Research.* **41:** p.173-179.

Beloqui, A., Nechitaylo, T.Y., López-Cortés, N., Ghazi, A., Guazzaroni, M., Polaina, J., Strittmatter, A.W., Reva, O., Waliczek, A., Yakimov, M.M., Golyshina, O.V., Ferrer, M. and Golyshin, P.N. (2010). Diversity of glycosyl hydrolases from cellulose-depleting communities enriched from casts of two earthworm species. *Applied and Environmental Microbiology.* **76:** p.5934-5946.

Benizri, E., Baudoin E. and Guckert, A. (2001). Root colonization by inoculated plant growth-promoting rhizobacteria. *Biocontrol Science and Technology*. **11:** p.557-574.

Bentley, S.D., Chater, K.F., Cerdeño-Tárraga, A.M., Challis, G.L., Thomson, N.R. and James, K.D. (2002). Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature.* **417:** p.141-147.

Bertrand, T., Jolivalt, C., Briozzo, P., Caminade, E., Joly, N., Madzak, C. and Mougin, C. (2002). Crystal structure of a four-copper laccase complexed with an arylamine: insights into substrate recognition and correlation with kinetics. *Biochemistry.* **41:** p.7325-7333.

Bewick, M.W, Williams S.T and Veltkamp, C. (1976). Growth and ultrastructure of *Streptomyces venezuelae* during chloramphenicol production. *Microbios.* **16:** p.191-199.

Bhattarai, K., Tiwari, K.B. and Agrawal, V.P. (2007). Enhanced antibacterial activity of sodium azide treated mutant *Streptomyces* strain. *Journal of Nepal Medical Association.* **8:** p.67-78.

Bligny, R. and Douce, R. (1983). Excretion of laccase by sycamore (*Acer pseudoplatanus* L.) cells. Purification and properties of the enzyme. *Biochemical Journal.* **209:** p.489-496.

Boerjan, W., Ralph, J. and Baucher, M. (2003). Lignin biosynthesis. *Plant Journal.* **54:** p.519-546.

Bollag, J.M., Liu, S.Y. and Minard, R.D. (1979). Asymmetric diphenol formation by a fungal laccase. *Applied and Environmental Microbiology.* **38:** p.90-92.

Brandelli, A., Daroit, D.J. and Riffel, A. (2010). Biochemical features of microbial keratinases and their production and applications. *Applied Microbiology and Biotechnology.* **85:** p.1735-1750.

Brett, P.J., Deshazer, D. and Woods, D.E. (1998). *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. *International Journal of Systematic Bacteriology.* **48:** p.317-320.Fungal laccases: production, function, and applications in food processing. *Enzyme Research.* **2010:** p. 1-10.

Brouwers, G.J., de Vrind, J.P.M., Corstjens, P.L.A.M., Cornelis, P., Baysse, C. and de Vrind-deJong, E.W. (1999). *CumA*, a gene encoding a multicopper oxidase, is involved in Mn - oxidation in *Pseudomonas putida* GB-1. *Applied and Environmental Microbiology.* **65:** p.1762-1768.

Brown, D.R., Whitcomb, R.F. and Bradbury, J.M. (2007). Revised minimal standards for description of new species of the class *Mollicutes* (division *Tenericutes*). *International Journal of Systematics and Evolutionary Microbiology.* **57:** p.2703-2719.

Brown, N.L., Barrett, S.R., Camakaris, J., Lee, B.T. and Rouch, D.A. (1995). Molecular gene and transport analysis of the copper resistance determinant (*pco*) from *Escherichia coli* plasmid pRJ 1004. *Molecular Microbiology*. **17:** p.1153-1166.

Bryant, D.A. and Frigaard, N.U. 2006. "Prokaryotic photosynthesis and phototrophy illuminated". *Trends in Microbiology.* **14 :** p.488-496.

Burg, R.W., Miller, B.M., Baker, E.E., Birnbaum, J., Currie, S.A. and Hartman, R. (1979). Avermectins, new family of potent anthelmintic agents: producing organism and fermentation. *Antimicrobial Agents and Chemotherapy.* **15:** p.361-367.

Cha, J. and Cooksey, D.A. (1991). Copper resistance in *Pseudomonas syringae* by periplasmic and outer membrane proteins. *Proceedings of the National Academy of Sciences of the United States of America.* **88:** p.8915-8919.

Chabannes, M., Ruel, K., Yoshinaga, A., Chabbert, B., Jauneau, A., Joseleau, J. and Boudet, A. (2001). *In situ* analysis of lignins in transgenic tobacco reveals a differential impact of individual transformations on the spatial patterns of lignin deposition at the cellular and subcellular levels. The *Plant Journal.* **28:** p.271-282.

Chen, I.C., Thiruvengadam, V., Lin, W.D., Chang, H.H. and Hsu, W.H. (2010). Lysine racemase: a novel non-antibiotic selectable marker for plant transformation. *Plant Molecular Biology.* **72**:p.153-169.

Chen, S., Ge, W. and Buswell, J.A. (2004). Biochemical and molecular characterization of a laccase from the edible straw mushroom, *Volvariella volvacea*. *European Journal of Biochemistry*. **271:** p.318-328.

Chester, M. and Martin, E. (2009). Cellulosic ethanol from municipal solid waste: a case study of the economic, energy, and greenhouse gas impacts in California. *Environmental Science and Technology.* **43:** p.5183-5189.

Cho, C.J. and Tiedje, M.J. (2001). Bacterial species determination from DNA-DNA hybridization by using genome fragments and DNA microarrays. *Applied and Environmental Microbiology.* **67:** p.3677-3682.

Choi, G.H., Larson, T.G. and Nuss, D.L. (1992). Molecular analysis of the laccase gene from the chestnut blight fungus and selective suppression of its expression in an isogenic hypovirulent strain. *Molecular Plant-Microbe Interactions*. **5:** p.119-128.

Claus, H. and Filip, Z. (1997). The evidence of a laccase-like activity in a *Bacillus sphaericus* strain. *Microbiology Research.* **152:** p.209-215.

Claus, H. (2003). Laccases and their occurrence in prokaryotes. *Archives of Microbiology*. **179:** p.145-150.

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

Coenye, T., Gevers, D., Van de Peer, Y., Vandamme, P. and Swings, J. (2005). Towards a prokaryotic genomic taxonomy. *Federation of European Materials Societies Microbiology Reviews.* **29:** p.147–167.

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

Cooper, J.E. and Feil, E.J. (2004). Multilocus sequence typing – what is resolved? *Trends in Microbiology.* **12:** p.373-377.

Corpet, F. (1988). Mutiple sequence alignment with hierarchical clustering. *Nucleic Acids Research.* **16 (22):** p.10881-10890.

Courtois, S., Cappellano, C. M., August, P. R., Martinez, A., Kolvek, S. J., Hopke, J., Osburne, M. S., Nalin, R., Guérineau, M., Jeannin, P., Simonet, P., Pernodet, J. L., Ball, M., Francou, F. X., Normand, P. and Helynck, G. (2003). Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products. *Applied and Environmental Microbiology.* **69:** p.49-55.

Cowan, D.A. (2000). Microbial genomes – the untapped resource. *Trends in Biotechnology.* **18:** p.14-16.

Crawford, D.L., Lynch, J.M., Whipps, J.M. and Ousley, M.A. (1993). Isolation and characterization of actinomycete antagonists of a fungal root pathogen. *Applied and Environmental Microbiology.* **59:** p.3899-3905.

Cripps, C., Bumpus, J.A. and Aust, S.D. (1990). Biodegradation of azo and heterocyclic dyes by *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology.* **56**: p.1114-1118.

Cundliffe, E. and Demain, A.L. (2010). Avoidance of suicide in antibiotic-producing microbes. *Journal of Industrial Microbiology and Biotechnology.* **37:** p.643-672.

Daniel, R. (2004). The soil metagenome--a rich resource for the discovery of novel natural products. *Current Opinions in Biotechnology.* **15**: p.199-204.

Daniel, R. (2005). The metagenomics of soil. *Nature Reviews Microbiology.* **3:** p.470-478.

Davies, J. and Wright, G.D. (1997). Bacterial resistance to aminoglycoside antibiotics. *Trends in Microbiology.* **5:** p.234-240.

Deckert, G., Warren, P.V., Gaasterland, T., Young, W.G., Lenox, A.L., Graham, D.E., Overbeek, R., Snead, M.A., Keller, M., Aujay, M., Huber, R., Feldman, R.A., Short, J.M., Olson, G.J. and Swanson, R.V. (1998). The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. *Nature.* **393:** p.353-358.

Delgenes, J.P., Moletta, R. and Navarro, J.M. (1996). Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*, and *Candida shehatae*. *Enzyme and Microbial Technolology*. **19:** p.220-225.

Demain, A.L. (1999). Pharmaceutically active secondary metabolites of microorganisms. *Applied Microbiology and Biotechnology.* **52:** p.455-463.

De Marco, A. and Roubelakis-Angelakis, K.A. (1997). Laccase activity could contribute to cell-wall reconstitution in regenerating protoplasts. *Phytochemistry.* **46:** p.421-425.

Denef, V.J., VerBerkmoes, N.C., Shah, M.B., Abraham, P., Lefsrud, M., Hettich, R.L. and Banfield, J.F. (2009). Proteomics-inferred genome typing (PIGT) demonstrates inter-

population recombination as a strategy for environmental adaptation. *Environmental Microbiology.* **11**:p.313-325.

Diamantidis, G., Effosse, A., Potier, P. and Bally, R. (2000). Purification and characterisation of the first bacterial laccase in rhizospheric bacteria, *Azospirillum lipoferum*. *Soil Biology and Biochemistry.* **32:** p.919-927.

Dittmer, N.T., Suderman, R.J., Jiang, H., Zhu, Y.C., Gorman, M.J., Kramer, K.J. and Kanost, M.R. (2004). Characterization of cDNAs encoding putative laccase-like multicopper oxidases and developmental expression in the tobacco hornworm, *Manduca sexta*, and the malaria mosquito, *Anopheles gambiae*. *Insect Biochemistry and Molecular Biology.* **34**: p.29-41.

Driks, A. (2004). The *Bacillus subtilis* spore coat. *Phytopathology.* **94:** p.1249-1251.

Ducros, V., Brzozowski, A.M., Wilson, K.S., Brown, S.H., Ostergaard, P., Schneider, P., Yaver, D.S., Pedersen, A.H. and Davies, G.J. (1998). Crystal structure of the type-2 copper depleted laccase from *Coprinus cinereus* at 2.2 Å resolution. *Nature Structural and Molecular Biology.* **5:** p.310-316.

D'Acunzo, F., Baiocco, P. and Galli, C. (2003). A study of the oxidation of ethers with the enzyme laccase under mediation by two N–OH–type compounds. *New Journal of Chemistry*. **27:** p. 329-332.

Egan, S., Wiener, P., Kallifidas, D. and Wellington, E.M.H. (1998). Transfer of streptomycin biosynthesis gene clusters within streptomycetes isolated from soil. *Applied and Environmental Microbiology.* **64:** p.5061-5063.

Eggert, C., Temp, U., Dean, J.F.D. and Eriksson, K.E.L. (1996a). A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS Letters*. **391:** p.144-148.

Eggert, C., Temp, U. and Eriksson, K.E.L. (1996b). The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. *Applied and Environmental Microbiology.* **62:** p.1151-1158.

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

Endo, K., Hosono, K., Beppu, T. and Ueda, K. (2002). A novel extracytoplasmic phenol oxidase of *Streptomyces*: it's possible involvement in the onset of morphogenesis. *Microbiology.* **148:** p.1767-1776.

Enguita, F.J., Martins, L.O., Henriques, A.O. and Carrondo, M.A. (2003). Crystal structure of a bacterial endospore coat component. A laccase with enhanced thermostability properties. *Journal of Biological Chemistry.* **278:** p.19416-19425.

Elshafei, A.M., Hassan, M.M., Haroun, B.M., Elsayed, M.A. and Othman, A.M. (2012). Optimization of laccase production from *Penicillium martensii* NRC 345. *Advances in Life Sciences*. **2:** p.31-37.

Erb R.W. and Wagner-Döbler, I. (1993). Detection of polychlorinated biphenyl degradation genes in polluted sediments by direct DNA extraction and polymerase chain reaction. *Applied and Environmental Microbiology.* **59:** p.4065-4073.

Ermolaeva, M.D., Khalak, H.G., White, O., Smith, H.O. and Salzberg, S.L. (2000). Prediction of transcription terminators in bacterial genomes. *Journal of Molecular Biology.* **301:** p.27-33.

Fabbrini, M., Galli, C. and Gentili, P. (2002). Radical or electron-transfer mechanism of oxidation with some laccase/mediator systems. *Journal of Molecular Catalysis B: Enzymatic*. **18:** p.169-171.

Faure, D., Bouillant, M.L. and Bally, R. (1994). Isolation of *Azospirillum lipoferum* 4T Tn5 mutants affected in melanization and laccase activity. *Applied and Environmental Microbiology.* **60:** p.3413-3415.

Faure, D., Bouillant, M. and Bally, R. (1995). Comparative study of substrates and inhibitors of *Azospirillum lipoferum* and *Pyricularia oryzae* laccases. *Applied and Environmental Microbiology*. **61:** p.1144-1146.

Fengel, D. and Wenger, G. (1983). Wood: chemistry, ultrastructure and reactions. Berlin: Walter de Gruyter and Co. p.613.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution.* **39:** p.783-791.

Ferrer, M., Beloqui, A., Timmis, K.N. and Golyshin. P.N. (2009). Metagenomics for mining new genetic resources of microbial communities. *Journal of Molecular Microbiolgy and Biotechnology.* **16:** p.109-123.

Ferrer, M., Chernikova, T.N., Timmis, K.N. and Golyshin, P.N. (2004). Expression of a temperature-sensitive esterase in a novel chaperone-based *Escherichia coli* strain. *Applied and Environmental Microbiology.* **70:** p.4499-504.

Ferrer, M., Chernikova, T.N., Yakimov, M.M., Golyshin, P.N. and Timmis, K.N. (2003). Chaperonins govern growth of *Escherichia coli* at low temperatures. *Nature Biotechnology.* **21:** p.1266-1267.

Frändberg, E., Petersson, C., Lundgren, L.N. and Schnurer, J. (2000). *Streptomyces halstedii* K122 produces the antifungal compounds bafilomycin B1 and C1. *Canadian Journal of Microbiology.* **46:** p.753-758.

Fredrickson, J.K., Zachara, J.M. and Balkwill, D.L. (2004). Geomicrobiology of high-level nuclear waste-contaminated vadose sediments at the Hanford site, Washington state. *Applied and Environmental Microbiology.* **70:** p.4230-4241.

Freeman, J.C., Nayar, P.G., Begley, T.P. and Villafranca, J.J. (1993). Stoichiometry and spectroscopic identity of copper centers in phenoxazonine synthase: a new addition for the blue copper oxidase family. *Biochemistry.* **32:** p.4826-4830.

Fujii, I. and Ebizuka, Y. (1997). Anthracycline biosynthesis in *Streptomyces galileus. Chemical Reviews*. **97:** p.2511-2523.

Gabor, E.M., Alkema W.B.L. and Janssen D.B. (2004). Quantifying the accessibility of the metagenome by random expression cloning techniques. *Environmental Microbiology.* **6:** p.879-886.

Gans, J., Wolinsky, M. and Dunbar, J. (2005). Computational improvements reveal great bacterial diversity and high metal toxicity in soil. *Science.* **309:** p.1387-1390.

Garrity, G.M. and Holt, J.G. (2001). The road map to the manual. In Bergey's Manual of Systematic Bacteriology, 2nd edn. Boone, D. R. Castenholz R. W. and Garrity, G. M. (eds). New York. **1:** p.119-166.

Gattinger, A., Schloter, M. and Munch, J.C. (2002). Phospholipid etherlipid and phospholipid fatty acid fingerprints in selected euryarchaeotal monocultures for taxonomic profiling. *FEMS Microbiology Letters.* **213:** p.133-139.

Gavnholt, B. and Larsen, K. (2002). Molecular biology of plant laccases in relation to lignin formation. *Physiologia Plantarum.* **116:** p.273-280.

Gentry, T.J., Wickham, G.S., Schadt, C.W., He, Z. and Zhou, J. (2006). Microarray applications in microbial ecology research. *Microbial Ecology.* **52:** p.159-175.

Gerini, M.F., Roccatano, D., Baciocchi, E. and Di Nola, A. (2003). Molecular dynamic simulations of lignin peroxidase in solution. *Biophysical Journal.* **84:** p. 3883-3893.

Gevers, D., Cohan, F. M., Lawrence, J. G., Sprat, B. G., Coeyne, T., Feil, E. J., Stackebrandt, E., Van de Peer, Y., Vandamme, P., Thompson, F. L. and Swings, J. (2005). Opinion: re-evaluating prokaryotic species. *Nature Reviews Microbiology.* **3:** p.733-739.

Gevers, D., Dawyndt, P., Vandamme, P., Willems, A., Vancanneyt, M., Swings, J. and De Vos, P. (2006). Stepping stones towards a new prokaryotic taxonomy. *Philosophical Transactions of the Royal Society B.* **361:** p.1911-1916.

Givaudan, A., Effose, A., Faure, D., Potier, P., Bouillant, M.L. and Bally, R. (1993). Polyphenol oxidase in *Azospirillum lipoferum* isolated from rice rhizosphere: evidence for laccase activity in non motile strains of *Azospirillum lipoferum*. *Federation of European Materials Societies Letters.* **108***:* p.205-210.

Glenn, J.K., Morgan, M.A., Mayfield, M.B., Kuwahara, M. and Gold, M.H. (1983). An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white-rot basidiomycete *Phanerochaete chrysosporium*. *Biochemical and Biophysical Research Communications.* **114:** p.1077-1083.

Gladek, A., Mordarski, M., Goodfellow, M. and Williams, S.T. (1985). Ribosomal ribonucleic acid similarities in the classification of *Streptomyces*. *Federation of European Materials Societies letters.* **26**: p.175-180.

Gloux, K., Berteau, O.E.l., Oumami, H., Béguet, F., Leclerc, M. and Doré. J. (2010). Microbes and Health Sackler Colloquium: a metagenomic β-glucuronidase uncovers a core adaptive function of the human intestinal microbiome. *Proceedings of the National Academy of Science of the United States of America.* **108:** p.4539-4546.

Godoy, D., Randle, G., Simpson, A.J., Aanensen, D.M., Pitt, T.L., Kinoshita, R. and Spratt, B.G. (2003). Multilocus sequence typing and evolutionary relationships among the causative agents of melioidosis and glanders, *Burkholderia pseudomallei* and *Burkholderia mallei*. *Journal of Clinical Microbiology.* **41:** p.2068-2079.

Gold, L., Pribnow, D., Schneider, T., Shinedling, S., Singer, B. and Stormo, G. (1981). Translational initiation in prokaryotes. *Annual Review of Microbiology.* **35:** p.365-403.

Goldemberg, J. (2007). Ethanol for a sustainable energy future. *Science.* **315**: p.808-810.

Goodfellow, M., Ferguson, E.V. and Sanglier, J.J. (1992). Numerical classification and identification of *Streptomyces* species – a review. *Gene.* **115:** p.225-233.

Goodfellow, M. and O'Donnell, A.G. (1989). Search and discovery of industrially-significant actinomycetes. In *Microbial Products: New Approach*. Baumberg, S., Hunter, I.S. and Rhodes. P.M. (eds.), Cambridge, UK: Cambridge University Press. p.343-383.

Goodfellow, M. and O'Donnell A.G. (1993). Roots of bacterial systematics, *In Handbook of new bacterial systematics*. M. Goodfellow and A.G. O'Donnell (ed.), Academic Press, London. p.3-54.

Grass, G., and Rensing, C. (2001). Genes involved in copper homeostasis in *Escherichia coli*. *Journal of Bacteriology.* **183:** p.2145-2147.

Grote, A., Hiller, K., Scheer, M., Münch, R., Nörtemann, B., Hempel, D. C. and Jahn, D. (2005). JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. *Nucleic Acids Researsch.* **33:** p.526-531.

Guo, Y., Zheng, W., Rong, X. and Huang, Y. (2008). A multilocus phylogeny of the *Streptomyces griseus* 16S rRNA gene clade: use of multilocus sequence analysis for streptomycete systematics. *International Journal of Systematics and Evolutionary Microbiology.* **58:** p.149-159.

Gürtler, V. and Mayall, B.C. (2001). Genomic approaches to typing, taxonomy and evolution of bacterial isolates. *International Journal of Systematic and Evolutionary Bacteriology.* **51:** p.3-16.

Hahn-Hägerdal, B., Jeppsson, H., Olsson, L. and Mohagheghi, A. (1994). An interlaboratory comparison of the performance of ethanol-producing micro-organisms in a xylose-rich acid hydrolysate. *Applied Microbiology and Biotechnology.* **41:** p.62-72.

Hain, T., Ward-Rainey, N., Kroppenstedt, R.M., Stackebrandt, E. and Rainey, F.A. (1997). Discrimination of *Streptomyces albidoflavus* strains based on the size and number of 16S-23S ribosomal DNA intergenic spacers. *International Journal of Systematic Bacteriology.* **47:** p.202-206.

Hakulinen, N., Kiiskinen, L., Kruus, K., Saloheimo, M., Paananen, A., Koivula, A. and Rouvinen, J. (2002). Crystal structure of a laccase from *Melanocarpus albomyces* with an intact trinuclear copper site. *Nature Structural Biology.* **9:** p.601-605.

Handelsman, J. (2004). Metagenomics: application of genomics to uncultured microorganisms. *Microbiology and Molecular Biology Reviews.* **68**:p.669-68.

Handelsman, J., Rondon, M. R., Brady, S. F., Clardy, J. and Goodman, R.M. (1998). Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. *Chemistry and Biology.* **5:** p.245-249.

Hansen, L.H, Ferrari B., Sørensen, A.H, Veal D. and Sørensen S. (2001). Detection of oxytetracycline production by *Streptomyces rimosus* in soil microcosms by combining whole cell biosensors and flow cytometry. *Applied and Environmental Microbiology.* **67:** p.239-244.

Hatakka A. (1994). Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. *Federation of European Materials Societies Microbiology Letters.* **13:** p.125-135.

Hatakka, A., (2001). Biodegradation of lignin. In: *Biopolymers. Lignin, humic substances and coal*. Hofrichter, M. and Steinbüchel, A. (eds). Wiley-VCH, Weinheim, Germany. **1:** p.129-180.

Head, I.M., Saunders, J.R. and Pickup, R.W. (1998). Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microbial Ecology.* **35:** p.1-21.

Hemashenpagam, N. (2011). Purification of secondary metabolites from soil actinomycetes. *Indian Journal of Medical Research.* **3:** p.148-156.

Henne, A., Daniel, R., Schmitz, R.A. and Gottschalk, G. (1999). Construction of environmental DNA libraries in *Escherichia coli* and screening for the presence of genes conferring utilization of 4-hydroxybutyrate. *Applied and Environmental Microbiology.* **65:** p.3901-3907.

Henne, A., Schmitz, R.A., Bömeke, M., Gottschalk, G. and Daniel. R. (2000). Screening of environmental DNA libraries for the presence of genes conferring lipolytic activity on *Escherichia coli. Applied and Environmental Microbiology.* **66:** p.3113-3116.

Herberich, B., Kinugawa, M., Vazquez, A. and Williams, R.M. (2001). Sequential Staudinger/Picket-Spengler cyclization strategy for the construction of tetrahydroisoquinolines of the bioxalomycin and ecteinascidin family of alkaloids. *Tetrahedron Lett*ers*.* **42:** p.543-546.

Hermoso, J. A., García, J. L. and García, P. (2007). Taking aim on bacterial pathogens: from phage therapy to enzybiotics*. Current Opinions in Microbiology.* **5:** 461-472.

Hernández Fernaud, A. Carnicero, F. Perestelo, M. Hernández Cutuli, E. Arias and Falcón, M.A. (2006). Upgrading of an industrial lignin by using laccase produced by *Fusarium proliferatum* and different laccase-mediator systems. *Enzyme and Microbial Technology.* **38**: p.40-48.

Herr, R.R., Jahnke, H.K. and Argoudelis, A.D. (1967). The structure of streptozotocin. *Journal of American Chemical Society.* **89:** p.4808-4809.

Heuer, H., Wieland, G., Schonfeld, J., Schnwalder, A., Gomes, N.C.M. and Smalla, K. (2001). Bacterial community profiling using DGGE or TGGE analysis. In: Environmental Molecular Microbiology: Protocols and Applications (ed. P.A. Rochelle). Horizon Scientific Press, Wymondham, UK. p.177-190.

Hill, J., Nelson, E., Tilman, D., Polasky, S. and Tiffany, D. (2006). Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *Proceedings of the National Academy of Science of the United State of America.* **103:** p.11206-11210.

Hirai, H., Kondo, R. and Sakai, K. (1994). Screening of lignin-degrading fungi and their ligninolytic enzyme activities during biological bleaching of kraft pulp. *Mokuzai Gakkaishi.* **40:** p.980-986.

Hjort, K., Bergström, M., Adesina, M.F., Jansson, J.K., Smalla, K. and Sjöling, S. (2010). Chitinase genes revealed and compared in bacterial isolates, DNA extracts and a metagenomic library from a phytopathogen suppressive soil. *FEMS Microbiology Ecology*. **71:** p.197-207.

Hoagland, R.E., Boyette, C.D. and Weaver, M.A. (2007). Bioherbicides: research and risks.*Toxin Reviews*. **26:** p.313-342.

Hofmann, K. (2000). Sensitive protein comparisons with profiles and hidden Markov models. *Briefings in Bioinformatics*. **1:** p.167-178.

Hon, D.N.S. (1994). "Cellulose: A random walk along its historical path," *Cellulose*. **1:** p.1-25.

Horinouchi, S. and Beppu, T. (1992). Regulation of secondary metabolism and cell differentiation in *Streptomyces*: A-factor as a microbial hormone and the AfsR protein as a component of a two-component regulatory system. *Gene.* **115:** p.167-172.

Houssam, M.A, El-Sayed, A.S., El-Desoukey, M.A., Mona Hassan, M. and Manal El-Gazar, M. (2011). Taxonomic Study, phylogenetic characterization and factors affecting on the biosynthesis antimicrobial agents produced by *streptomyces lydicus*. *Academia Arena.* **3:** p.72-84.

Howard, R.L., Abotsi, E., Jansen Van Rensburg, E.L. and Howard, S. (2003). Lignocellulose biotechnology. *African Journal of Biotechnology*. **2:** p.602-619.

Hu, T.L. and Wu, S.C. (2000). Comparism of colony lift with direct spotting methods of blot preparation on the effect of colony hybridisation in the detection of environmental organisms. *Journal of Microbiology, Immunonlogy and Infection.* **33**: p.123-126.

Hublik, G. and Schinner, F. (2000). Characterisation and immobilisation of the laccase from *Pleurotus ostreatus* and its use for the continuous elimination of phenolic pollutants. *Enzyme and Microbial Technology.* **27:** p.330-336.

Hullo, M.F., Moszer, I., Danchin, A. and Martin-Verstraete, I. (2001). *CotA* of *Bacillus subtilis* is a copper-dependent laccase. *Journal of Bacteriology.* **183:** p.5426-5430.

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

Ish-Horowicz, D. and Burke, J.F. (1981). Rapid and efficient cosmid cloning. *Nucleic Acids Research.* **9:** p.2989-2998.

Isono, Y. and Hoshino M. (1989). Laccase-like activity of nucleoside oxidase in the presence of nucleosides. *Agriculture and Biological Chemistry.* **53**: p.2197-2203.

Iwai, S., Chai, B., Cole, J.R., Hashsham, S.A. and Tiedje, J.M. (2010). Gene-targeted-metagenomics reveals extensive diversity of aromatic dioxygenase genes in the environment. *Journal of the International Society for Microbial Ecology.* **4:** p.279-285.

Janssen, P.h. (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA Genes. *Applied and Environmental Microbiology.* **72:** p.1719-1728.

Jeffries, T.W. (1990). Biodegradation of lignin-carbohydrate complexes. *Biodegradation.* **1:** p.808-812.

Jensen, P.R. (2010). Linking species concepts to natural product discovery in the post-genomic era. *Journal of Industrial Microbiology and Biotechnology.* **37:** p.219-224.

Jensen, P.R., Mincer, T.J., Williams, P.G. and Fenical, W. (2005). Marine actinomycete diversity and natural product discovery. *Antonie van Leeuwenhoek.* **87**: p.43-48.

Johnson, D.L., Thompson, J.L., Brinkmann, S.M., Schuller, K.A. and Martin, L.L. (2003). Electrochemical characterization of purified *Rhus vernicifera* laccase: voltametric evidence for a sequential four-electron transfer. *Biochemistry.* **42:** p.10229-10237.

Jolivalt, C., Raynal, A., Caminade, E., Kokel, B., Le Goffic, F. and Mougin, C. (1999). Transformation of N',N'-dimethyl-N=(hydroxyphenyl) ureas by laccase from the white rot fungus *Trametes versicolor*. *Applied Microbiology and Biotechnology.* **51:** 676-681.

Jones, L., Ennos, A.R. and Turner, S.R. (2001). Cloning and characterization of irregular xylem4 (irx4): a severely lignin-deficient mutant of *Arabidopsis*. *Plant Journal.* **26:** p.205-216.

Kadla, J.F. and Gilbert, R.D. (2000). Cellullose structure: a review. *Cellulose Chemistry and Technology.* **34:** p. 197-216.

Kämpfer, P., Kroppenstedt, R.M. and Dott, W. (1991). A numerical classification of the genera *Streptomyces* and *Streptoverticillium* using miniaturized physiological tests. *Journal of General Microbiology.* **137:** p.1831-1891.

Kashima, Y. and Udaka, S. (2004). High-level production of hyperthermophilic cellulose in the *Bacillus brevis* expression and secretion system. *Bioscience, Biotechnology and Biochemistry.* **68**: p.235-237.

Kataoka, M., Ueda, K., Kudo, T., Seki, T. and Yoshida, T. (1997). Application of the variable region in 16S rDNA to create an index for rapid species identifiaction in the genus *Streptomyces*. *FEMS Microbiology Letters.* **151:** p.249-255.

Kierulff, J.V. (1997). Denim bleaching. *Textile Horizons.* **17:** p.33-36.

Kieser, T., Buttner, M.J., Charter, K.F. and Hopwood, D.A. (2000). Practical *Streptomyces* Genetics, The John Innes Foundation, Norwich, England. **1:** p.1-42.

Kim, B., Sahin, N., Minnikin, D.E., Zakrzewska-Czerwinska, J., Mordarski, M. and Goodfellow, M. (1999). Classification of thermophilic streptomycetes, including the

description of *Streptomyces thermoalkalitolerans* sp. nov. *International Journal of Systematic Bacteriology.* **49:** p.7-17.

Kim, D., Chun, J., Sahin, N., Hah, Y.C. and Goodfellow, M. (1996). Analysis of thermophilic clades within the genus *Streptomyces* by 16S ribosomal DNA sequence comparisons. *International Journal of Systematic Bacteriology.* **46:** p.581-587.

Kirk T.K. and Farell R.L. (1987). Enzymatic "combustion": the microbial degradation of lignin. *Annual Review of Microbiology.* **41:** p.465-505.

Kirk, T.K. and Shimida, M. (1985). Lignin biodegradation: the microorganisms involved, and the physiology and the biochemistry of degradation by white-rot fungi. In: *Biosynthesis and biodegradation of wood components.* Higuchi, T. (ed.), Academic Press Ltd., London. p. 579-605.

Knietsch, A., Bowien, S., Whited, G., Gottschalk, G. and Daniel. R. (2003). Identification and characterization of coenzyme B_{12}-dependent glycerol dehydratase- and diol dehydratase-encoding genes from metagenomic DNA libraries derived from enrichment cultures. *Applied and Environmental Microbiology.* **69:** p.3048-3060.

Korn-Wendish, F. and Schneider, J. (1992). Phage-typing - a useful tool in actinomycete systematics. *Gene.* **115:** p.243-247.

Kramer, K.J.; Kanost, M.R., Hopkins, T.L., Jiang, H.; Zhu, Y.C., Xu, R., Kerwin, J.L. and Turecek, F. (2001). Oxidative conjugation of catechols with proteins in insect skeletal systems. *Tetrahedron letters.* **57:** p.385-392.

Kumar, K.S., Haritha, R., Mohan, Y.S.Y.V.J. and Ramana, **T.** (2011). Screening of marine actinobacteria for antimicrobial compounds. *Research Journal of Microbiology.* **6:** p.385-393. a versatile enzyme for biotechnological applications. *in Communicating Current Research and Educational Topics and Trends in Applied Microbiology*. Mendez-Vilas, A. (ed.), Formex, Badajoz., Spain. **1**: p.233–245.

Labeda, D.P. (1992). DNA-DNA hybridisation in the systematics of *Streptomyces*. *Gene.* **115:** p.249-253.

Laging, M., Fartmann. B and Kramer.W. (2001). Isolation of segments of homologous genes with only one conserved amino acid region via PCR. *Nucleic Acids Research.* **29.** p.1-6.

Lam, K.H.E., Chow, K.C. and Wong, W.K.R. (1999). Construction of an effiecint *Bacillus subtilis* system for extracellular production of heterologous proteins. *Journal of Biotechnology.* **63:** p.167-177.

Lazzarini, A., Cavaletti, L., Toppo, G. and Marinelli, F. (2000). Rare genera of actinomycetes as potential producers of new antibiotics. *Antonie van Leeuwenhoek.* **78:** p.399-405.

Leatham, G.F. and Stahmann, M.A. (1981). Studies on the laccase of *Lentinus edodes*: specificity, localization and association with the development of fruiting bodies. *Journal of General Microbiology.* **125**: p.147-157.

Lechevalier, M.P. and Lechevalier, H.A. (1970). Chemical composition as a criterion in the classification of aerobic actinomycetes. *International Journal of Systematic Bacteriology.* **20**: p.435-443.

Lee, S.K., George, S.D., Antholine, W.E., Hedman, B., Hodgson, K.O. and Solomon, E.I. (2002). Nature of the intermediate formed in the reduction of O_2 to H_2O at the trinuclear

copper cluster active site in native laccase. *Journal of American Chemical Society.* **124:** p.6180-6193.

Lee, S.W., Won, K., Lim, H.K., Kim, J.C., Choi. G.J. and Cho, K.Y. (2004). Screening for novel lipolytic enzymes from uncultured soil microorganisms. *Applied and Environmental Microbiology.* **65:** p.720-726.

Lee, Y., Hendson, M., Panopoulos, N.J. and Schroth, M.N. (1994). Molecular cloning, chromosomal mapping, and sequence analysis of copper resistance genes from *Xanthomonas campestris* pv. *juglandis*: homology with small copper proteins and multicopper oxidases. *Journal of Bacteriology.* **176:** p.173-188.

Lehman, E., Harel, E. and Mayer, A.M. (1974). Copper content and other characteristics of purified peach laccase. *Phytochemistry.* **13:** p.1713-1717.

Leontievsky, A., Myasoedova, N., Pozdnyakova, N. and Golovleva, L. (1997). 'Yellow' laccase of *Panus tigrinus* oxidises non-phenolic substrates without electron-transfer mediators. *FEBS Letters*. **413:** p.446-448.

Lesage-Meessen, L., Delattre, M., Haon, M., Thibault J.F., Ceccaldi, B.C., Brunerie, P.and Asther, M. (1996). A two-step bioconversion process for vanillin production from ferulic acid combining *Aspergillus niger* and *Pycnoporus cinnabarinus*. *Journal of Biotechnology.* **50**: p.107-113.

Letowski, J., Brousseau, R. and Masson, L. (2004). Designing better probes: effect of probe size, mismatch position and number on hybridization in DNA oligonucleotide microarrays. *Journal of Microbiological Methods.* **57:** p.269-78.

Li, A., Antizar-Ladislao, B. and Khraisheh, M. (2007). Bioconversion of municipal solid waste to glucose for bio-ethanol production. *Bioprocess and Biosystem Engineering.* **30:** p.189-196.

Li, K., Xu, F. and Eriksson, K.H.L. (1999). Comparison of fungal laccases and redox mediators in oxidation of a nonphenolic lignin model compound. *Applied and Environmental Microbiology.* **65**: p.2654-2660.

Li, M., Hong, Y., Klotz, M.G. and Gu, J.D. (2010). A comparison of primer sets for detecting 16S rRNA and hydrazine oxidoreductase genes of anaerobic ammonium-oxidizing bacteria in marine sediments. . *Applied and Environmental Microbiology.* **86:** p.781-790.

Liaw, R.B., Cheng, M.P., Wu, M.C. and Lee. C.Y. (2010). Use of metagenomic approaches to isolate lipolytic genes from activated sludge. *Bioresource Technology.* **101:** p.8323-8329.

Liu, J., Wang, E.T. and Chen, W.X. (2005). Diverse rhizobia associated with woody legumes *Wisteria sinensis*, *Cercis racemosa* and *Amorpha fruticosa* grown in the temperate zone of China. *Systematic and Applied Microbiology.* **28:** p.465-477.

Loqman, S., Barka, E.A., Cle´ment C. and Ouhdouch, Y. (2009). Antagonistic actinomycetes from Moroccan soil to control the grapevine gray mold. *World Journal of Microbiology and Biotechnology.* **25:** p.81-91.

Lorenz, P. and Schleper, C. (2002). Metagenome—a challenging source of enzyme discovery. *Journal of Molecular Catalysis B.* **19:** p.13-19.

Lynd, L. R., Wyman, C.E. and Gerngross, T.U. (1999). Biocommodity engineering. *Biotechnology Progress.* **15**: p.777-793.

Lynd, L.R., Weimer, P.J., van Zyl, W.H. and Pretorius, I.S. (2002). Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews.* **66:** p.506-577.

Macedo, J.M.B., Gottschalk, L.M.F and Bon, E.P.S. (1999). Lignin peroxidase and protease production by *Streptomyces viridosporus* T7A in the presence of calcium carbonate. Nutritional and regulatory carbon sources. *Applied Biochemistry and Biotechnology*. **79:** p.735-744.

Machczynski, M. C., Vijgenboom, E., Samyn, B. and Canters, G.W. (2004). Characterization of SLAC: a small laccase from *Streptomyces coelicolor* with unprecedented activity. *Protein Science.* **13:** p.2388-2397.

Magnet S. and Blanchard J. S. (2005). Molecular insights into aminoglycoside action and resistance. *Chemical Reviews.* **105:** p.477-497.

Maiden, M.C.J., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J. and Zurth, K. (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Science of the United States of America*. **95:** p.3140-3145.

Majerník, A., Gottschalk, G. and Daniel. R. (2001). Screening of environmental DNA libraries for the presence of genes conferring $Na^+(Li^+)/H^+$ antiporter activity on *Escherichia coli*: characterization of the recovered genes and the corresponding gene products. *Journal of Bacteriology.* **183:** p.6645-6653.

Malherbe, S. and Cloete, T.E. (2002). Lignocellulose biodegradation: fundamentals and applications. *Reviews in Environmental Science and Biotechnology.* **1:** p.105-114.

Malmström, B.G., Reinhammar, B. and Vanngård, T. (1970). The state of copper in stellacyanin and laccase from the lacquer tree *Rhus vernicifera*. *Biochimica et Biophysica Acta.* **205:** p.48-57.

Mao, Y., Varoglu, M. and Sherman, D.H. (1999). Molecular characterisation and analysis of the biosynthetic gene cluster for the antitumor antibiotic mitomycin C from *Streptomyces lavendulae* NRRL 2564. *Chemistry and Biology.* **6:** p.251-263.

Marbach, I., Harel, E. and Mayer, A.M. (1984). Molecular properties of extracellular *Botrytis cinerea* laccase. *Phytochemistry.* **23:** p.2713-2717.

Maróti, G., Tong, Y., Yooseph, S., Baden-Tillson, H., Smith, H.O., Kovács, K.L., Frazier, M., Venter, J.C. and Xu, Q. (2009). Discovery of [NiFe] hydrogenase genes in metagenomic DNA: cloning and heterologous expression in *Thiocapsa roseopersicina. Applied and Environmental Microbiology.* **75:** p.5821-5830.

Martens, M., Delaere, M., Coopman, R., De Vos, P., Gillis, M. and Willems, A. (2007). Multilocus sequence analysis of *Ensifer* and related taxa. *International Journal of Systematic and Evolutionary Microbiology.* **5:** p.489-503.

Martín, J.F., Casqueiro, J. and Liras, P. (2005). Secretion systems for secondary metabolites: how producer cells send out messages of intercellular communication. *Current Opinions in Microbiology.* **8**: p.282-293.

Martinez, A., Kolvek, S.J., Yip, C.L.T., Hopke, J., Brown, K.A., MacNeil, I.A. and Osburne, M.S. (2004). Genetically modified bacterial strains and novel bacterial artificial chromosome shuttle vectors for constructing environmental libraries and detecting heterologous natural products in multiple expression hosts. *Applied and Environmental Microbiology.* **70:** p.2452-2463.

Martins, L.O., Soares, C.M., Pereira, M.M., Teixeira, M., Costa, T., Jones, G.H. and Henriques, A.O. (2002). Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. *Journal of Biological Chemistry*. **277:** p.18849-18859.

Marzoorati, M., Danieli, B., Haltrich, D. and Riva, S. (2005). Selective laccase-mediated oxidation of sugars derivatives. *Green Chemistry.* **7:** p.310-315.

Mayer, A.M. (1987). Polyphenoloxidases in plants: recent progress. *Phytochemistry*. **26:** p.11-20.

Mayer, A.M. and Staples, R.C. (2002). Laccase: new functions for an old enzyme. *Phytochemistry.* **60:** p.551-565.

McCarthy, A.J. (1987). Lignocellulose degrading actinomycetes. *FEMS Microbiology Reviews*. **46:** p.145-163.

McCarthy, A.J. and Williams, S.T. (1992). Actinomycetes as agents of biodegradation in the environment—a review. *Gene*. **115:** p.189-192.

McDermott, P.F., Walker, R.D. and White, D.G. (2003). Antimicrobials: modes of action and mechanisms of resistance. *International Journal of Toxicology.* **22: p.**135-143.

McDowall, K.J., Thamchaipenet, A. and Hunter, I.S. (1999). Phosphate control of oxytetracycline production by *Streptomyces rimosus* is at the level of transcription from promoters overlapped by tandem repeats similar to those of the DNA-binding sites of the OmpR family. *Journal of Bacteriology.* **181:** p.3025-3032.

McGuire, R.M., Jones, J.M., Means, E.G., Izaguirre, G. and Reston, A.E. (1984). Controlling attached blue-green algae with copper sulfate. *Research Technology.* p.60-65.

Mellano, M.A. and Cooksey, D.A. (1988). Nucleotide sequence and organization of copper resistance genes from *Pseudomonas syringae* pv.tomato. *Journal of Bacteriology.* **170:** p.2879-2883.

Messerschmidt, A., Ladenstein, R., Huber, R., Bolognesi, M., Avigliano, L., Petruzzelli, R., Rossi, A. and Finazzi-Agro, A. (1997). Refined crystal structure of ascorbate oxidase at 1.9 Å resolution. *Journal of Molecular Biology*. **224:** p.179-205.

Metsä-Ketelä, M., Salo, V., Halo, L., Hautala, A., Hakala, J., Mäntsälä, P. and Ylihonko, K. (1999). An efficient approach for screening minimal PKS genes from *Streptomyces*. *FEMS Microbiology Letters*. **180:** p.1-6.

Mignard, S. and Flandrois, J.P. (2008). A seven-gene, multilocus, genus-wide approach to the phylogeny of mycobacteria using supertrees. *International Journal of Systemic and Evolutionary Microbiology.* **58:** p.1432-1441.

Mincer, T.J., Jensen, P.R., Kauffman, C.A. and Fenical, W. (2002). Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments. *Applied and Environmental Microbiology*. **68:** p.5005-5011.

Minussi, R.C., Pastore, G.M. and Durán, N. (2002). Potential applications of laccase in the food industry. *Trends in Food Science and Technology*. **13:** p.205-216.

Moncheva, P., Tishkov, S., Dimitrova, N., Chipera, V., Nikolova, S.A. and Bogatzevska, N. (2002). Characteristics of soil actinomycetes from Antarctica. *Journal of Culture Collections.* **3**: p.3-14.

Morimoto, S., and Fujii, T. (2009). A new approach to retrieve full lengths of functional genes from soil by PCR-DGGE and metagenome walking*. Applied Microbiology and Biotechnology.* **83: p**.389-396.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M. and Ladisch. **M.** (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology.* **96:** p.673-686.

Mougin C., Kollmann A. and Jolivalt, C. (2002). Enhanced production of laccase in the fungus *Trametes versicolor* by the addition of xenobiotics. *Biotechnology Letters.* **24:** p.139-142.

Muto, A. and Osawa, S. (1987). The guanine and cytosine content of genomic DNA and bacterial evolution. *Proceedings of the National Academy of Science of the United States of America.* **84:** p.166-169.

Nakano, T., Miyake, K., Ikeda, M., Mizukami, T. and Katsumata, R. (2000). Mechanism of the incidental production of a melanin-like pigment during 6-demethylchlortetracycline production in *Streptomyces aureofaciens*. *Applied and Environmental Microbiology.* **66:** p.1400-1404.

Nakashima, N., Mitani, Y. and Tamura T. (2005). Actinomycetes as host cells for production of recombinant proteins. *Microbial Cell Factories.* **4: p.**7.

Naser, S.M., Vancanneyt, M., Hoste, B., Snauwaert, C., Vandemeulebroecke, K. and Swings, J. (2006). Reclassification of *Enterococcus flavescens* (Pompei *et al.,* 1992) as a later synonym of *Enterococcus casseliflavus* (Vaughan *et al.,* 1979) and *Enterococcus saccharominimus* (Vancanneyt *et al.,* 2004) as a later synonym of *Enterococcus italicus* (Fortina *et al.,* 2004). *International Journal of Systematic and Evolutionary Microbiology.* **56:** p.413-416.

Ncanana, S., Baratto, L., Roncaglia, L., Riva, S..and Burton, S.G. (2007). Laccase mediated oxidation of totarol. *Advanced Synthesis and Catalysis*. **349:** p.1507-1513.

Niku-Paavola, M.L., Karhunen, E., Salola, P. and Raunio, V. (1988). Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. *Biochemical Journal.* **254:** p.877-884.

Ningthoujam, D.S., Sanasam, S. and Nimaichand, S. (2011). Studies on BioactiveActinomycetes in a Niche Biotope, Nambul River in Manipur, India. *Journal of Biochemical and Microbiological Technology*. **5:** p.221-225.

Ochi, K. (1995). A taxonomic study of the genus *Streptomyces* by analysis of ribosomal protein AT-L30. *International Journal of Systematic Bacteriology.,* **45:** p.507-514.

O'Malley, D.M., Whetten, R., Bao, W., Chen, C.L. and Sederoff, R.R. (1993). The role of laccase in lignification. *Plant Journal.* **4:** p.751-757.

Ômura, S, Ikeda, H., Ishikawa, J., Hanamoto, A., Takahashi, C. and Shinose, M. (2001). Genome sequence of an industrial microorganism *Streptomyces avermitilis*: Deducing the

ability of producing secondary metabolites. *Proceedings of the National Academy of Science of the United States of America.* **98:** p.12215-12220.

Ono, A., Miyazaki, R., Sota, M., Ohtsubo, Y., Nagata, Y. and Tsuda, M. (2007). Isolation and characterization of naphthalene-catabolic genes and plasmids from oil-contaminated soil by using two cultivation-independent approaches. *Applied Microbiology and Biotechnology.* **74:** p.501-510.

O'Sullivan, A.C., (1997). Cellulose: the structure slowly unravels. *Cellulose.* **4:** p.173-207.

Palonen, H. and Viikari, L. (2004). Role of oxidative enzymatic treatments on enzymatic hydrolysis of softwood. *Biotechnology and Bioengineering.* **86:** p.550-557.

Panchagnula, B. (2011). Screening of marine sediments from Bay of Bengal near Pudimadaka Coast of Andhra Pradesh for isolation of lipolytic actinobacteria and characterization of the most potent isolates. *International Journal of Biology.* **3:** p. 33-43.

Park, H., Jang, H., Kim, C., Chung, B., Chang, C. L., Park, S.K. and Song, S. (2000). Detection and Identification of Mycobacteria by Amplification of the Internal Transcribed Spacer Regions with Genus- and Species-Specific PCR Primers. *Journal of Clinical Microbiology.* **38:** p.4080-4085.

Peschke, U., Schmidt, H., Zhang, N.Z. and Piepersberg, W. (1995). Molecular characterisation of the lincomycin-production gene-cluster of *Streptomyces lincolnensis*. *Molecular Microbiology.* **16:** p.1137-1156.

Piontek, K., Antorini, M. and Choinowski., T. (2002). Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-Å resolution containing a full complement of coppers. *Journal of Biological Chemistry.* **277:** p.37663-37669.

Pissowotzki, K., Mansouri, K. and Piepersberg, W. (1991). Genetics of streptomycin production in *Streptomyces griseus*: molecular structure and putative function of genes *strELMB2N*. *Molecular and General Genetics.* **231:** p.113-123.

Priest, F. and Austin, B. (1995). Modern bacterial taxonomy, Chapman and Hall, UK, p.74-79

Ralph, J., Lapierre, C., Marita, J.M., Kim, H., Lu, F., Hatfield, R.D., Ralph, S., Chapple, C., Franke, R., Hemm, N.R., van Doorsselaere, J., Sederoff, R.R., O'Malley, D.M., Scott, J.T., MacKay, J.J., Yahiaoui, N., Boudet, A.M., Pean, M., Pilate, G., Jouanin, L. and Boerjan, W. (2001). Elucidation of new structures in lignins of CAD- and COMT- deficient plants by NMR. *Phytochemistry.* **57:** p.993-1003.

Ranjan, R., Grover, A., Kapardar, R.K. and Sharma, R. (2005). Isolation of novel lipolytic genes from uncultured bacteria of pond water. *Biochemical and Biophysical Research Communications*. **335:** p.57-65.

Ranocha, P., McDougall, G., Hawkins, S., Sterjiades, R., Borderies, G., Stewart, D., Cabanes-Macheteau, M., Boudet, A.M. and Goffner, D. (1999). Biochemical characterisation, molecular cloning and expression of laccases – a divergent gene family – in poplar. *European Journal of Biochemistry*. **259:** p.485-495.

Rattanaporn, S., Kanpicha, J., Morakot, S., Shinji, T. and Wasu, P. (2010). Taxonomic characterization of *Streptomyces* strain CH54-4 isolated from mangrove sediment. *Annual Review of Microbiology.* **60:** p.299-305.

Rebollido, R., Martínez J., Aguilera, Y., Melchor, K., Koerner, I. Stegmann. R. (2008). Microbial populations during composting process of organic fraction of municipal solid waste. *Applied Ecology and Environmental Research.* **6:** p.61-67.

Remsing, L.L., González, A.M., Nur-e-Alam, M., Fernández-Lozano, M.J., Braña, A.F., Rix, U., Oliveira, M.A., Méndez, C., Salas, J.A. and Rohr, J. (2003). Mithramycin SK, a novel antitumor drug with improved therapeutic index, mithramycin SA, and demycarosyl-mithramycin SK: three new products generated in the mithramycin producer *Streptomyces argillaceus* through combinatorial biosynthesis. *Journal of American Chemical Society.* **125 :** p.5745-5753.

Ridell M, Wallerström G, Williams S.T. (1986). Immunodiffusion analysis of phenetically defined strains of *Streptomyces*, *Streptoverticillium* and *Nocardiopsis. Systematic and Applied Microbiology* **8**: 24-27.

Riesenfeld, C.S., Goodman, R.M. and Handelsman. J. (2004). Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. *Environmental Microbiology.* **6:** p.981-989.

Rigling, D. and van Alfen, N.K. (1991). Regulation of laccase biosynthesis in the plant-pathogenic fungus *Cryphonectria parasitica* by double-stranded RNA. *Journal of Bacteriology.* **173**: p.8000-8003.

Riva, S. (2006). Laccases: blue enzymes for green chemistry. *Trends in Biotechnology*. **24:** p. 219–226.

Roberts, S.A., Weichsel, A., Grass, G., Thakali, K., Hazzard, J.T., Tollin, G., Rensing, C. and Montfort, W.R. (2002). Crystal structure and electron transfer kinetics of *CueO*, a

multicopper oxidase required for copper homeostasis in *Escherichia coli*. *Proceedings of the National Academy of Science of the United States of America.* **99:** p.2766-2771.

Rogalski, J., Lundell, T., Leonowicz, A. and Hatakka, A. (1991). Production of laccase, lignin peroxidase and manganese-dependent peroxidase by various strains of *Trametes versicolor* depending on culture conditions. *Polish Journal of Microbiology.* **40:** p.221-234.

Rondon, M. R., August, P. R., Bettermann, A. D., Brady, S. F., Grossman, T. H., Liles, M. R., Loiacono, K. A., Lynch, B. A., MacNeil, I. A., Minor, C., Tiong, C. L., Gilman, M., Osburne, M. S., Clardy, J., Handelsman, J. and Goodman, R. M. (2000). Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Applied and Environmental Microbiology.* **66**: p.2541-2547.

Rosana, C., Minussi, Y., Pastore, G.M. and Durany, N. (2002).Potential applications of laccase in the food industry. *Trends in Food Science and Technology*. **13:** p.205-216.

Rosselló-Mora, R. and Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiology Reviews.* **25:** p.39-67.

Ruijssenaars, H.J. and Hartmans, S. (2004). A cloned *Bacillus halodurans* multicopper oxidase exhibiting alkaline laccase activity. *Applied Microbiology and Biotechnology.* **65:** p.177-182.

Saenna, P., Gilbreath, T., Onpan, N. and Panbangred,W. (2011). Actinomycetes community from starch factory wastewater. *Research Journal of Microbiology.* **6:** p.534-542.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution.* **4**: p.406-425.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sambrook, J. and Russell, D.W. (2001). Molecular cloning: a laboratory manual. New York: CSH Laboratory Press.

Sanchez-Amat, A., Lucas-Elio, P., Fernandez, E., Garcia-Borron, J.C. and Solano, F. (2001). Molecular cloning and functional characterization of a unique multipotent polyphenol oxidase from *Marinomonas mediterranea*. *Biochemica et Biophysica Acta.* **1547:** p.104-116.

Sanchez-Amat, A. and Solano, F. (1997). A pluripotent polyphenol oxidase from the melanogenic marine *Alteromonas sp* shares catalytic capabilities of tyrosinases and laccases. *Biochemical and Biophysical Research.* **240:** p.787-792.

Sannia, G., Giardina, P., Luna, M., Rossi, M. and Buonocore, V. (1986). Laccase from *Pleurotus ostreatus*. *Biotechnology Letters.* **8:** p.797-800.

Sasaki, J., Chijimatsu, M. and Suzuki, K. (1998). Taxonomic significance of 2,4-diaminobutyric acid isomers in the cell wall peptidoglycan of actinomycetes and reclassification of *Clavibacter toxicus* as *Rathayibacter toxicus* comb. nov. *International Journal of Systematic Bacteriology.* **48**: p.403-410.

Schleifer, K. H. (2009). Classification of *Bacteria* and *Archaea*: past, present and future. *Systematic Applied Microbiology.* **32:** p.533-542.

Schmidt, O., Drake, H.L. and Horn, M.A. (2010). Hitherto unknown [Fe-Fe]-hydrogenase gene diversity in anaerobes and anoxic enrichments from a moderately acidic fen. *Applied and Environmental Microbiology.* **76:** p.2027-203.

Senthilguru, K., George, T.S, Vasanthi , N.S. and Kannan, K.P. (2011). Ethanol production from lignocellulosic waste. *World Journal of Science and Technology*. **1:** p.12-16.

Sharma, P., Goel, R. and Capalash, N. (2007). Bacterial laccases. *World Journal of Microbiology and Biotechnology.* **23:** p.823-832.

Sharma, K.K and Kuhad, R.C. (2008). Laccase enzyme revisited and function redefined. *Indian Journal of Microbiology*. **48:** 309–316.

Sharp, P.M. and Li, W.H. (1987). The codon adaptation index-a measure of directional synonymous codon usage bias, and its potential applications. *Nucleic Acids Research.* **15:** p.1281-1295.

Shirling, E.B. and Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *International Journal of Systematic and Evolutionary Microbiology.* **16**: p.313-340.

Shin, W., Sundaram, U.M., Cole, J.L., Zhang, H.H., Hedman, B., Hodgson, K.O. and Solomon, E.I. (1996). Chemical and spectroscopic definition of the peroxide level intermediate in the multicopper oxidases: relevance to the catalytic mechanism of dioxygen reduction to water. *Journal of American Chemical Society.* **118:** p.3202-3215.

Siegenthaler, W.E., Bonetti, A. and Luthy, R. (1986). Aminoglycoside antibiotics in infectious diseases. An overview. *American Journal of Medicine.* **80**: p.2-14.

Simon, C., Herath, J., Rockstroh, S. and Daniel. **R.** (2009). Rapid identification of genes encoding DNA polymerases by function-based screening of metagenomic libraries derived from glacial ice*. Applied and Environmental Microbiology.* **75:** p**.**2964-2968.

Solano, F., Garcia, E., Perez De Egea, E. and Sanchez-Amat, A. (1997). Isolation and characterization of strain MMB-1 (CECT 4803), a novel melanogenic marine bacterium. *Applied and Environmental Microbiology.* **63:** p.3499-3506.

Solano, F., Lucas-Elio, P., Lopez-Serrano, D., Fernandez, E. and Sanchez-Amat, A. (2001). Dimethoxyphenol oxidase activity of different microbial blue multicopperproteins. *FEMS Microbiology Letters*. **204:** p.175-181.

Solomon, E.I., Sundaram, U.M. and Machonkin, T.E. (1996). Multicopper oxidases and oxygenases. *Chemical Reviews.* **96:** p.2563-2606.

Spratt, B.G. (1994). Resistance to antibiotics mediated by target alterations. *Science.* **264:** p.388-393.

Stach, J.E., Maldonado, L.A., Masson, D.G., Ward, A.C., Goodfellow, M. and Bull, A.T. (2003). Statistical approaches for estimating actinobacterial diversity in marine sediments. *Applied and Environmental Microbiology.* **69:** p.6189-6200.

Stackebrandt, E. and Ebers J. (2006). Taxonomic parameters revisited: tarnished gold standards. *Microbiology Today.* **33**: p.152-155.

Stackebrandt, E. and Goebel B.M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology.* **44:** p.846-849.

Stackebrandt, E., Liesack W. and Witt, D. (1992). Ribosomal RNA and rDNA sequence analysis. *Gene.* **115:** p.255-260.

Stackebrandt, E., Rainey, F.A. and Ward-Rainey, N.L. (1997). Proposal for a new hierarchic classification system, *Actinobacteria classis nov. Systematic Bacteriology*. **47 :** p.479-491.

Stackebrandt. E., Frederiksen, W., Garrity, G.M., Grimont, P.A.D., Kämpfer, P., Maiden. M.C., Nesme, X., Rosselló-Mora, R. and Swings, J. (2002). Report of the *ad hoc* committee for the reevaluation of the species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology.* **52:** p.1043-1047.

Staden, R. (1983). Computer methods to locate signals in nucleic acid sequences. *Nucleic Acids Research.* **12:** p.509-519.

Stepkowski, T., Czaplínska, M., Miedzinska, K. and Moulin, L. (2003). The variable part of the *dnaK* gene as an alternative marker for phylogenetic studies of rhizobia and related alpha Proteobacteria. *Systematic and Applied Microbiology.* **26:** p.483-494.

Stepkowski, T., Moulin, L., Krzyzanska, A., McInnes, A., Law, I.J. and Howieson, J. (2005). European origin of *Bradyrhizobium* populations infecting lupins and serradella in soils of western Australia and South Africa. *Applied and Environmental Microbiology.* **71:** p.7041-7052.

Streit, W.R. and Schmitz, R.A. (2004). Metagenomics – the key to the uncultured microbes. *Current Opinions in Microbiology.* **7**: p.492-498.

Strohl, W. R. (1992). Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. *Nucleic Acids Research.* **20**: p.961-974.

Sul, W. J., Park, J., Quensen III, J.F., Rodrigues, J.L.M., Seliger, L., Tsoi, T.V., Zylstra, G.J. and Tiedje, J.M. (2009). DNA-stable isotope probing integrated with metagenomics for retrieval of biphenyl dioxygenase genes from polychlorinated biphenyl-contaminated river sediment. *Applied and Environmental Microbiology.* **75:** p.5501-5506.

Suutari, M., Lignell, U., Hirvonen, M.R. and Nevalainen, A. (2000). Growth pH ranges of *Streptomyces* spp. *American Society of Microbiology News.* **66:** p.588-589.

Suzuki, T., Endo, K., Ito, M., Tsujibo, H., Miyamoto, K. and Inamori, Y. (2003). A thermostable laccase from *Streptomyces lavendulae* REN-7: purification, characterization, nucleotide sequence, and expression. *Bioscience, Biotechnology and Biochemistry.* **67:** p.2167-2175.

Taguchi, S., Kojima, S., Terabe, M., Kumazawa, Y., Kohriyama, H., Suzuki, M., Miura K.I. and Momose, H. (1997). Molecular phylogenetic characterisation of *Streptomyces* protease inhibitor family. *Journal of Molecular Evolution*. **44:** p.542-551.

Takeuchi. T., Sawada, H., Tanaka, F. and Matsuda, I. (1996). Phylogenetic analysis of *Streptomyces* spp. causing potato scab based on 16S rRNA sequences. *International Journal of Systematic Bacteriology.* **46:** p.476-479.

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

Tenson, T. and Ehrenberg, M. (2002). Regulatory nascent peptides in the ribosomal tunnel. *Cell.* **108:** p.591-594.

Thaler, J.O., Duvic, B., Givaudan, A. and Boemare, N. (1998). Isolation and entomotoxic properties of the *Xenorhabdus nematophilus* F1 lecithinase. *Applied and Environmental Microbiology.* **64:** p.2367-2373.

Thurston, C.F. (1994). The structure and function of fungal laccases. *Microbiology.* **140**: p.19-26.

Tien, M. and Kirk, T.K. (1983). Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds. *Science.* **211:** p.661-663.

Tien M., Kirk T.K., Bull, C. and Fee J.A. (1986). Steady-state and transient state kinetic studies on the oxidation of 3,4-dimethoxybenzyl alcohol catalysed by the lignase of *Phanerocheate chrysosporium* Burds. *Journal of Biological Chemistry.* **261**: p.1687-1693.

Trudeau, F., Diagle, F. and Leech, D. (1997). Reagentless mediated laccase electrode for the detection of enzyme modulators. *Analytical Chemistry.* **69:** p.882-886.

Uchiyama, T., Abe, T., Ikemura, T. and Watanabe. K. (2005). Substrate-induced gene-expression screening of environmental metagenome libraries for isolation of catabolic genes. *Nature Biotechnology.* **23:**p.88-93.

Uchiyama, T. and Miyazaki. K. (2010). Product-induced gene expression (PIGEX): a product-responsive reporter assay for enzyme screening of metagenomic libraries. *Applied and Environmental Microbiology.* **76**: p.7029-7035.

Uyeda, M., Mizukami, M., Yokomizo, K. and Suzuki, K. (2001). Pentalenolactone I and hygromycin A, immunosuppressants produced by *Streptomyces filipinensis* and *Streptomyces hygroscopicus*. *Bioscience, Biotechnology and Biochemistry.* **65:** p.1252-1254.

Vandamme, P., Pot, B., Gillis, M., de Vos, P., Kersters, K. and Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiology Reviews.* **60:** p.407-438.

Varaljay, V.A., Howard, E.C., Sun, S. and Moran, M.A. (2010). Deep sequencing of a dimethylsulfoniopropionate-degrading gene (*dmdA*) by using PCR primer pairs designed on the basis of marine metagenomic data. *Applied Microbiology.* **76:** p.609-617.

Venter, J. C.,Remington, K.,Heidelberg, J.F., Halpern, A.L., Rusch,D., Eisen, J.A., Wu, D.,Paulsen, I., Nelson, K.E., Nelson, W., Fouts, D.E., Levy, S.,Knap, A.H., Lomas,M.W., Nealson, K., White, O., Peterson, J., Hoffman, J., Parsons, R.,Baden-Tillson,H., Pfannkoch, C., RogersY. and SmithH.O. (2004). Environmental genome shotgun sequencing of the Sargasso Sea. *Science.* **304:** p.66-74.

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G.F., Chater, K.F. and van Sinderen, D. (2007). Genomics of *Actinobacteria*: tracing the evolutionary history of an ancient phylum. *Microbiology and Molecular Biology Reviews.* **71:** p.495-548.

Vezina, C., Kudelski, A. and Sehgal, S.N. (1975). Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *Journal of Antibiotics.* **28:** p.721-726.

Vincent, J. F. (1999). From cellulose to cell. *Journal of Experimental Biology.* **202:** p.3263-3268.

Vining, L.C. (1990). Functions of secondary metabolites. *Annual Review of Microbiology.* **44:** p.395-427.

Vinuesa, P., Silva, C., Werner, D. and Martinez-Romero, E. (2005b). Population genetics and phylogenetic inference in bacterial molecular systematics: the roles of migration and recombination in *Bradyrhizobium* species cohesion and delineation. *Molecular Phylogenetics and Evolution.* **34:** p.29-54.

Walker, J.W.R. and McCallion, R.F. (1980). The selective inhibition of ortho and paradiphenol oxidases. *Phytochemistry*. **19:** p.373-373.

Wang, C., Meek, D.J., Panchal, P., Boruvka, N., Archibald, F.S., Driscoll B.T. and Charles, T.C. (2006). Isolation of poly-3-hydroxybutyrate metabolism genes from complex microbial communities by phenotypic complementation of bacterial mutants. *Applied and Environmental Microbiology.* **72:** p.384-391.

Wang, Y., Zhang, Z. and Kuen, J. (1996). A proposal to transfer *Microbiospora bispora* (Lechevalier 1967) to a new genus, *Thermobiospora* gen.nov., as *Thermobiospora bispora* comb.nov. *International Journal of Systematic Evolution.* **46:** p.933-938.

Wang, Y., Zhang, Z.S., Ruan, J.S., Wang, Y.M. and Ali, S.M. (1999). Investigation of actinomycete diversity in the tropical rainforests of Singapore. *Journal of Industrial Microbiology and Biotechnology.* **23:** p.178-187.

Wasenberg, D., Kryriakides, I. and Agathos, S.N. (2003). White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnology Advances*. **22 :** p.161-187.

Watve, M.G., Tickoo, R., Jog, M.M. and Bhole, B.D. (2001). How many antibiotics are produced by the genus *Streptomyces***?** *Archives of Microbiology.* **176**: p.386-390.

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

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

Wiese, J., Jiang, Y.,Tang, S., Thiel, V.,Schmaljohann, R., Xu, L., Jiang, C. and Imhoff, J.F. (2008). A new member of the family *Micromonosporaceae*, *Planosporangium flavigriseum* gen. nov., sp. nov. *International Journal of Systematic and Evolutionary Microbiology.* **58 (6):** p.1324-1331.

Williams, S.T., Goodfellow, M., Wellington, E.M.H., Vickers J.C., Alderson, G., Sneath, P.H.A. (1983). A probability matrix for identification of some streptomycetes. *Journal of General Microbiology.* **129**: p.1815-1830.

Williamson, L.L., Borlee, B.R., Schloss, P.D., Guan, C.I., Allen, H.K. and Handelsman, J. (2005). Intracellular screen to identify metagenomic clones that induce or inhibit a quorum-sensing biosensor. *Applied and Environmental Microbiology.* **71:** p.6335-6344.

Williamson, P.R. (1994). Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: identification as a laccase. *Journal of Bacteriology*. **176**: p.656-664.

Wilkinson, D.E., Jeanicke, T. and Cowan, D.A. (2002). Efficient molecular cloning of environmental DNA from geothermal sediments. *Biotechnology Letters.* **24:** p.155-161.

Woese, C.R., Stackebrandt, E., Macke, T.J. and Fox, G.E. (1985). A phylogenetic definition of the major eubacterial taxa. *Systematic and Applied Microbiology.* **6:** p.143-151.

Wood, D.A. (1980). Inactivation of extracellular laccase during fruiting of *Agaricus bisporus*. *Journal of General Microbiology.* **117:** p.339-345.

Woolery, G.L., Powers, L., Peisach, J. and Spiro, T.G. (1984). X-ray absorption study of *Rhus* laccase: evidence for a copper-copper interaction, which disappears on type 2 copper removal. *Biochemistry.* **23:** p.3428-3434.

Worral, J.J., Chet, I. and Hüttermann, A. (1986). Association of rhizomorph formation with laccase activity in *Armillaria* spp. *Journal of General Microbiology.* **132:** p.2527-2533.

Wright, G.D. (2005). Bacterial resistance to antibiotics: enzymatic degradation and modification. *Advanced Drug Delivery Reviews.* **57:** p.1451-1470.

Wu, H., Wu, C., He, Q., Liao, X. and Shi, B. (2010).Collagen fiber with surface-grafted polyphenol as a novel support for Pd(0) nanoparticles: Synthesis, characterization and catalytic application. *Materials Science and Engineering C.* **30:** p. 770-776.

Xu, F. (1996). Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. *Biochemistry*. **35:** p.7608-7614.

Yamaguchi, H., Nakayama, Y., Takeda, K., Tawara, K., Maeda, K., Takeuchi, T. and Umezawa, H. (1957). A new antibiotic, althiomycin. *Journal of Antibiotics.* **10:** p.195-200.

Yanisch-Person, C., Vieira, J. and Messing, J. (1985). Improved M13 phage cloning vectore and host strains: nucleotide sequencing of the M13mp18 and Puc9 vectors. *Gene.* **33:** p.103-119.

Young, J.M., Park, D.C., Shearman, H.M. and Fargier, E. (2008). A multilocus sequence analysis of the genus *Xanthomonas*. *Systematic and Applied Microbiology.* 31: p.366-377.

Zaitlin, B. and Watson, S.B. (2006). Actinomycetes in relation to taste and odour in drinking water: myths, tenets and truths. *Water Research Reviews.* p.1742-1753.

Zaprasis, A., Liu, Y.J., Liu, S.J., Drake, H.L. and Horn. M.A. (2010). *Abundance* of novel and diverse *tfdA*-like genes, encoding putative phenoxyalkanoic acid herbicide-degrading dioxygenases in soil. *Applied and Environmental Microbiology.* **76:** p.119-128.

Zarantonello, P., Leslie, C.P., Ferritto, R. and Kazmierski. W.M. (2002). Total synthesis and semi-synthetic approaches to analogues of antibacterial natural product althiomycin. *Bioorgganic and Medicinal Chemistry Letters.* **12:** p.561-565.

Zheng, Y., Pan, Z. and Zhang, R. (2009). Overview of biomass pretreatment for cellulosic ethanol production. *International Journal of Agricultural and Biological Engineering*. **2:** p 51-68.