Enrichment, isolation, and phylogenetic identification of cholesterol-degrading fungi

By

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DECLARATION

I, **MTHAKATHI NTSANE TREVOR** (South African ID number: [redacted]), hereby certify that the dissertation submitted by me for the degree MASTER OF HEALTH SCIENCES IN BIOMEDICAL TECHNOLOGY, is my own independent work; and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology (Free State). I hereby declare, that this research project has not been previously submitted before to any university or faculty for the attainment of any qualification. I further waive copyright of the dissertation in favour of the Central University of Technology (Free State).

MTHAKATHI NTSANE TREVOR **DATE**

DEDICATION

It would be disrespectful to forget those who came before me and paved this successful path for me. It is with deep spiritual respect that I dedicate my novel Masters study and pay tribute to the following noble individuals:

- My late mother Lieketseng Lydia Lengau. The woman who sacrificed a lot for me to be educated from the primary and secondary level. The woman who compromised too many times for me to attend university because she believed so much in me. She urged me to pursue my Masters because she believed I have a potential to be the best in what I do and encouraged me to serve mankind with my knowledge. She always stood by me and could not wait to see the successful completion of my Masters study. Unfortunately death caught up with her and she passed on in June 2016. May Her soul rest in peace!

- To all my Ancestors known and unknown. The ancient legends and pioneers who invented amongst other things mathematics, physics, engineering, astronomy, chemistry, sciences in general and medicine. It is their genius minds and divine inventions that started it all and motivates me to stay in alignment with their great discoveries and continue their greatness even in this modern era. It is an honour as their descendent to continue their legacy and follow in their great footsteps.

"Education develops the intellect; and the intellect distinguishes man from other creatures. It is education that enables man to harness nature and utilize her resources for the well-being and improvement of his life."

Haile Selassie

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LIST OF ABBREVIATIONS AND ACRONYMS

%	Percentage
≥	Greater or equal to
>	Greater than
±	Plus –minus
~	Approximately
-	Minus
°C	Degree Celsius
3D	Three-dimensional
α	Alpha
β	Beta
λ	Gamma
μ	Micro
*	Asterisk
A. simplex	*Arthrobacter simplex*
A. terreus	*Aspergillus terreus*
ABC transporter	ATP-binding cassette transporters
Acetyl coA	Acetyl coenzyme A
AcmA	Acetylmuramidase
AD	Androst-4-ene3,17 Dione
ADD	Androsta-1,4- diene-3,17-dione
ATP	Adenosine triphosphate

B	Boron
BBB	Blood-brain barrier
BLAST	Basic Local Alignment Search Tool
B. sterolicum	*Brevibacterium sterolicum*
bp	Base pairs
Ca^{2+}	Calcium ions
CC	Carbon-carbon
C	Carbon
CDC	Centre for Disease Control
ChOx	Cholesterol oxidase
CO_2	Carbon dioxide
C. testosterone	*Comamonas testosteroni*
C-terminal	Carboxyl terminal
CYP450	Cytochrome P450 monoxygenase
DHC	Dehydrocholesterol
DHEA	Dehydroepiandrosterone
3,4-DHSA	3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-trien-9,17-dione
4,9-DSHA	4,5,9,10-*diseco*-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid (4,9-DSHA)
DOHNAA	9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid
DNA	Deoxyribonucleic acid

e.g.	For example
ER	Endoplasmic Reticulum
et al.,	*Et alia* (and others)
F	Forward
FAD	Flavin adenine dinucleotide
FDA	Food Drug and Administration
Fe	Iron
FP	Forward primer
Fwd	Forward
g	Grams
g/l	Grams per litre
G	Gravity
GMC	Glucose-methanol-choline
H_2O	Water molecule
3-HSA	3-hydroxy-9,10-secoandrosta-1,3,5(10)-trien-9,17-dione
HSD	Hydroxysteroid dehydrogenase/isomerase
H	Hydrogen
hr	Hour
I	Iodine
ID	Identity
IFN	Interferon
ITS	Internal transcribed spacer

K	Potassium
L	Liter
LB	Luria Bertani
LSU	Large sub unit
M. smegmatis	*Mycobacterium smegmatis*
M. tuberculosis	*Mycobacterium tuberculosis*
m	Milli
M	Molar
mce	Mammalian cell entry
MEGA	Molecular Evolutionary Genome Analaysis
Mg	Magnesium
Min	Minutes
mL	Millilitre
mM	MIllimolar
Mn	Manganese
M	Molybdium
mRNA	Messenger ribonucleic acids
Na	Sodium
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
NO	Nitrogen oxide

NH_4	Ammonium
N-terminal	Amino terminal end
NTMSK	Ntsane Trevor Mthakathi Syed Khajamohiddin
O	Oxygen
OH	Hydroxyl group
P. pinophilum	*Penicillium pinophilum*
P	Phosphate
PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase chain reaction
PD	Potato dextrose
PDA	Potato dextrose agar
R. erythropolis	*Rhodococcus erythropolis*
PKA	Protein kinase A
PO	Phosphate
PRD	Prestige Research Day
R	Reverse
RP	Reverse primer
Rev	Reverse
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acids
RNA	Ribonucleic acid

rpm	Revolutions per minute
Rrna	Ribosomal ribonucleic acid
SO	Sulphate
sp.	Species
SDR	Short-chain dehydrogenase reductase
SSU	Small sub unit
T. longibrachiatum	*Trichoderma longibrachiatum*
T. pinophilum	*Talaromyces pinophilum*
t	Time
Tm	Melting temperature
tRNA	Tranfer ribonucleic acid
UV	Ultra violet
WHO	World Health Organization
Zn	Zinc

CONTENTS

	Page
DECLARATION	**II**
DEDICATION	**III**
ACKNOWLEDGEMENTS	**IV**
LIST OF ABBREVIATIONS AND ACRONYMS	**VII**
LIST OF FIGURES	**XVII**
LIST OF TABLES	**XIX**
CHAPTER 1: ABSTRACT	**1**
CHAPTER 2: INTRODUCTION AND LITERATURE REVIEW	
2.1. Introduction to cholesterol	**4**
2.2. Physiologic importance of cholesterol	**5**
2.2.1. Bile acids	**6**
2.2.2. Vitamin D3 (Cholecalciferol)	**7**
2.2.3. Steroid hormones	**8**
2.2.4. Other important functions of cholesterol	**9**
2.3. Microbial cholesterol metabolism	**10**
2.3.1. History and significance	**10**
2.3.2. Bacterial cholesterol degradation	**12**

2.3.2.1. Transformation of cholesterol into cholestanone 13

2.3.2.2. Side chain degradation 15

2.3.2.3. Central pathway 17

2.3.2.4. Lower catabolic pathway 19

2.3.3. Anoxic metabolism 19

2.4. Cholesterol uptake 20

2.5. Cholesterol and other steroids as environmental hazards and role in pathogenesis 22

2.6. Clinical and biotechnological applications of cholesterol and its degrading enzymes 23

2.6.1. Biosensors for cholesterol analysis in biological samples 23

2.6.2. Microbial enzymes for insecticides and fungicides 24

2.6.3. Probiotics 24

2.6.4. Production of pharmaceuticals 25

2.7. Cholesterol bioconversion by Eukaryotes 26

2.8. Motivation and objectives of the study 27

2.9. References 28

CHAPTER 3: ENRICHMENT AND ISOLATION OF CHOLESTEROL DEGRADING FUNGI

3.1. Introduction **52**

3.2. Materials and methods **54**

3.2.1. Soil sampling area **54**

3.2.2. Soil sample collection and preparation **56**

3.2.3. Culture media **56**

3.2.4. Isolation of fungi **56**

3.3. Results and discussion **57**

3.3.1. Isolation of cholesterol utilizing fungi **57**

3.3.2. The significance of fungal cholesterol utilization **62**

3.4. Conclusion **63**

3.5. References **64**

CHAPTER 4: PHYLOGENETIC IDENTIFICATION OF CHOLESTEROL UTILIZING FUNGI

4.1. Introduction **68**

4.2. Materials and methods **71**

4.2.1. Genomic DNA isolation **71**

4.2.2. PCR amplification and sub-cloning of ribosomal DNA subunits **71**

4.2.3. Sequence analysis **72**

4.2.4. Construction of phylogenetic trees 73

4.3. Results and discussion 73

4.3.1. PCR amplification of different rRNA genes and phylogenetic identification of fungal isolates 73

4.4. Conclusion 81

4.5. References 82

4.6. Appendix A 88

CHAPTER 5: CONCLUSION 91

RESEARCH OUTPUTS 93

Research articles 93

Conference attendance 97

Award 99

Media coverage 100

LIST OF FIGURES

		Page
Figure 2.1.	The chemical structure of cholesterol	**4**
Figure 2.2.	Cholesterol and catabolic products	**6**
Figure 2.3.	Cholesterol degradation pathway under aerobic conditions	**14**
Figure 2.4.	Proposed β-oxidation-like reactions for cholesterol side-chain degradation	**17**
Figure 3.1.	South African map indicating the sample collection sites in the Mpumalanga province	**55**
Figure 3.2.	The initial six sample flasks after a two week incubation period along with the control flask	**58**
Figure 3.3.	Growth of cholesterol utilizing microorganisms on LB-agar plates	**59**
Figure 3.4.	Four cholesterol utilizing fungi on Selective A medium	**60**
Figure 3.5.	Growth of fungal isolates on PDA	**61**
Figure 3.6.	Growth of fungal isolates on Selective A medium where cholesterol is supplemented as a sole carbon source	**61**
Figure 4.1.	Diagram of primer locations in the ribosomal cassette consisting of SSU, ITS1, 5.8S, ITS2 and LSU rDNA	**70**
Figure 4.2.	Aragose gel electrophoresis analysis of ribosomal DNA genes	**74**

Figure 4.3.	The phylogenetic tree of *A. terreus* strain NTMSK1	**76**
Figure 4.4.	The phylogenetic tree of *A. terreus* strain NTMSK2	**77**
Figure 4.5.	The phylogenetic tree of *T. longibrachiatum* strain NTMSK3	**78**
Figure 4.6.	The phylogenetic tree of *T. pinophilum* strain NTMSK4	**79**

LIST OF TABLES

		Page
Table 3.1.	Bacterial species known to degrade cholesterol and use it as the sole source of carbon	**53**
Table 4.1.	Oligonucleotide primer sequences for amplification of rDNA subunits	**72**
Table 4.2.	PCR parameters for amplification of rDNA subunits using KAPA HiFi DNA polymerase	**72**
Table 4.3.	Information regarding fungal isolates identification	**75**

CHAPTER 1

ABSTRACT

Cholesterol is one of the most abundant lipids playing crucial role in the physiology of humans. This precursor of vitamin D, steroid hormones and bile acids maintains structural integrity and the fluidity of the lipid bilayer and also possess a tremendous role in cellular signalling. Plants also synthesize close homologs of cholesterol collectively called phytosterols, which play a crucial role in plant physiology. The significance of cholesterol is not only in maintaining normal physiology in eukaryotic organisms as its main producers. For over 70 years, bacteria have been the only group of microorganisms known and demonstrated to degrade or biotransform or utilize cholesterol as the source of carbon and energy. The etiologic agent of tuberculosis, *Mycobacterium tuberculosis*, uses human cholesterol as the carbon and energy source to survive and cause the disease. Cholesterol degradation pathway and enzymes involved in the degradation is under immense study to use as novel drug targets.

The current study is the first of its kind in fungal lipid research, bringing a novel concept of cholesterol utilization by fungi. In this study, four fungal strains able to utilize cholesterol as the sole source of carbon and energy were isolated from South African soil. The fungal strains were identified using the three ribosomal subunits 5.8S rRNA, 18S rRNA and 28S rRNA gene sequences. Based on the ribosomal gene sequences and phylogenetic analysis, the fungal strains were identified. Two fungal strains were found to be the same and hence named as *Aspergillus terreus* strain NTMSK1 and NTMSK2. The two other strains were identified and named as *Trichoderma longibrachiatum* strain NTMSK3 and *Talaromyces pinophilum* strain NTMSK4. All isolated fungal species are human pathogens and *A. terreus* also causes diseases in other animals and plants.

The current study demonstrating that cholesterol serves as the carbon source to fungal pathogens opens a new field in fungal pathogenicity and studying of fungal infection mechanisms. Cholesterol and its homologs including phytosterols are ubiquitous in nature as much as cholesterol is an abundant lipid in the human body. Hence, unravelling the cholesterol degradation pathway in fungi will catalyse the development of novel fungal drug targets against animals (including humans) and plants pathogens.

Considering the novel concept of fungal utilization of cholesterol, further experiments are under progress (as part of PhD) that will enable this study to be published in high impact factor journal.

Microbiology is one of the broadest branches of biology. As a master's student, I took an initiation and obligation of supervising B Tech students as my contribution for inspiration and development of not only myself as a growing researcher, but of other potential researchers and scientist. Our Unit of Drug Discovery Research (UDDR) specialises among other fields in microbial P450s, therefore the outputs are the following papers, conference proceeding and awards:

Published:

1. **Mthakathi NT** (3rd author) (2016) Molecular evolutionary dynamics of cytochrome P450 monooxygenases across kingdoms: Special focus on mycobacterial P450s. Scientific Reports 6, Article number: 33099. Doi: 10.1038/srep33099. Impact factor: 5.2. http://www.nature.com/articles/srep33099?WT.feed_name=subjects_evolution.
2. **Mthakathi NT** (11th author) (2015) Diversity and evolution of cytochrome P450 monooxygenases in Oomycetes. Scientific Reports 5, Article number: 11572 Doi:10.1038/srep11572. Impact factor:5.2. http://www.nature.com/articles/srep11572.
3. **Mthakathi NT**, Kgosiemang IKR, Chen W, Mohlatsane ME, Mojahi RJ, J-H Yu, Mashele SS, Syed K (2015) Cytochrome P450 monooxygenase analysis in free-living and

symbiotic microalgae *Coccomyxa* sp. C-169 and *Chlorella* sp. NC64A. Algae 30(3): 233-239. Doi: http://dx.doi.org/10.4490/algae.2015.30.3.233. Impact factor 1.8. http://www.e-algae.org/journal/view.php?number=2765

Under review:

1. Qhanya LB, **Mthakathi NT**, Boucher CE, Mashele SS, Theron CW, Syed K (2016) Isolation and characterization of endocrine disruptor nonylphenol-using bacteria from South Africa. South African Journal of Science, Article number: SAJS-2016-0287 (Under review).

International conference:

1. **Mthakathi NT**, Chen W, Yu J-H, Mashele SS, Syed K (2016) Cytochrome P450 monooxygenase analysis in free-living and symbiotic microalgae *Coccomyxa* sp. C-169 and *Chlorella* sp. NC64A. The 13th International Symposium on Cytochrome P450 Biodiversity and Biotechnology, Vancouver, BC, Canada, 22-26 July 2016.

Academic Prestige Research Day (PRD)

1. **Mthakathi NT**, Qhanya LB, Theron CW, Mashele SS, Syed K (2016) Cholesterol utilizing fungi: a novel concept in fungal pathogenesis. Oral presentation. The Central University of Technology Prestige Research Day, Free State, 31 October 2016.
2. **Mthakathi NT,** Mashele SS and Syed K (2015) Significance of Cytochrome P450 monooxygenases in the physiology and biotechnological applications of free-living and symbiotic microalgae *Coccomyxa* sp. C-169 and *Chlorella* sp. NC64A. Oral presentation. The Central University of Technology Prestige Research Day, Free State, 29 October 2015.
3. CUT Prestige Research Day (PRD): **Best Masters' student presentation (2015)**, The Central University of Technology Prestige Research Day, Free State, 29 October 2015.

CHAPTER 2

INTRODUCTION AND LITERATURE REVIEW

2.1. Introduction to cholesterol

Cholesterol is a polycyclic steroid compound that is widely distributed in the biosphere (García *et al.*, 2012). This 27 carbon-atom compound is an amphiphilic lipid, structurally bearing 4 alicyclic rings and the side chain (Figure 2.1). The side chain is a flat and rigid carbon skeleton fused to four alicyclic rings (Figure 2.1) (García *et al.*, 2012). The molecular structure of cholesterol includes a tetracyclic ring skeleton, with a single hydroxyl group at carbon 3, side chain at carbon 17 (Urich, 1994; Vance and Vance, 2002). The rings are assembled in the *trans* configuration, making cholesterol very planar and rigid, except for the flexible isooctyl side chain (Nes, 1974; Yeagle, 1985). The three dimensional characteristic of cholesterol structure shows that the 3β-OH group, the two methyl groups and the side chain are all located on the same side of the ring skeleton (β-configuration) (Duax *et al.*, 1988; Urich, 1994; Bittman, 1997).

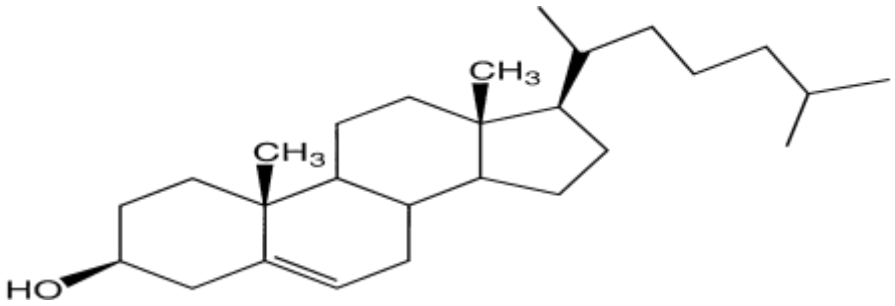

Figure 2. 1. The chemical structure of cholesterol (Taken from Ohvo-Rekila *et al.*, 2002).

The hydroxyl group in cholesterol is very important as it attributes amphiphilic character (Tanford, 1980; Bloch, 1983) and therefore orients the molecule in membranes (Ohvo-Rekila *et al.*, 2002). Furthermore, the hydroxyl group

can also mediate the hydrogen bonding of cholesterol with water and possibly with other lipid components of the cellular membranes (Bittman, 1997).

Although ubiquitous in nature, cholesterol is a recalcitrant molecule to biodegradation because of its low number of functional groups (one carbon-double bond and a single hydroxyl group), its low solubility in water ($3x10^{-8}$) and its complex spatial conformation constituted by four alicyclic rings and two quaternary carbon atoms (García *et al.*, 2012). The high rate of ubiquity and persistence of cholesterol and some derived compounds such as coprostanol, have been used as reference biomarkers for environmental pollution analysis (Veiga *et al.*, 2005) as a result of human activity (Gagné, *et al.*, 2006). Cholesterol, including its metabolites, possesses many functions in both prokaryotes and eukaryotes relating to biology, medicine and biotechnology. Cholesterol is an essential structural component of animal cell membranes (Staytor and Bloch, 1965) and also required for viability and cell proliferation (Dahl and Dahl, 1988; Yeagle, 1993).

It is naturally classified as a terpenoid. Terpenes are a large class of natural hydrocarbon secondary metabolites built up from five-carbon isoprene units linked together most commonly in a head-to-tail arrangement (Zhang *et al*., 2002). They can also be assembled through other chemical variations including oxidation, unsaturation, the presence of ring closures and functional groups, during which they'll now be referred to as terpenoids. Such chemical modifications yield new compounds with obviously altered structures, giving rise to terpene-like compounds.

2. 2. Physiologic importance of cholesterol

Depending on the physiological demand and biomolecule necessity, cholesterol can be catabolized for the biosynthesis of many physiologically important steroids. Steroids possess important biological functions in eukaryotic organisms (Merino *et*

al., 2013) such as membrane components (e.g., cholesterol and phytosterols), hormones (e.g., testosterone and estradiol), bile salts and detergents and vitamins (Wollam and Antebi, 2011). Figure 2.2 illustrates the chemical structure of cholesterol and some biologically important steroids derived from cholesterol. In the following sections the physiologic importance of each derivative is discussed.

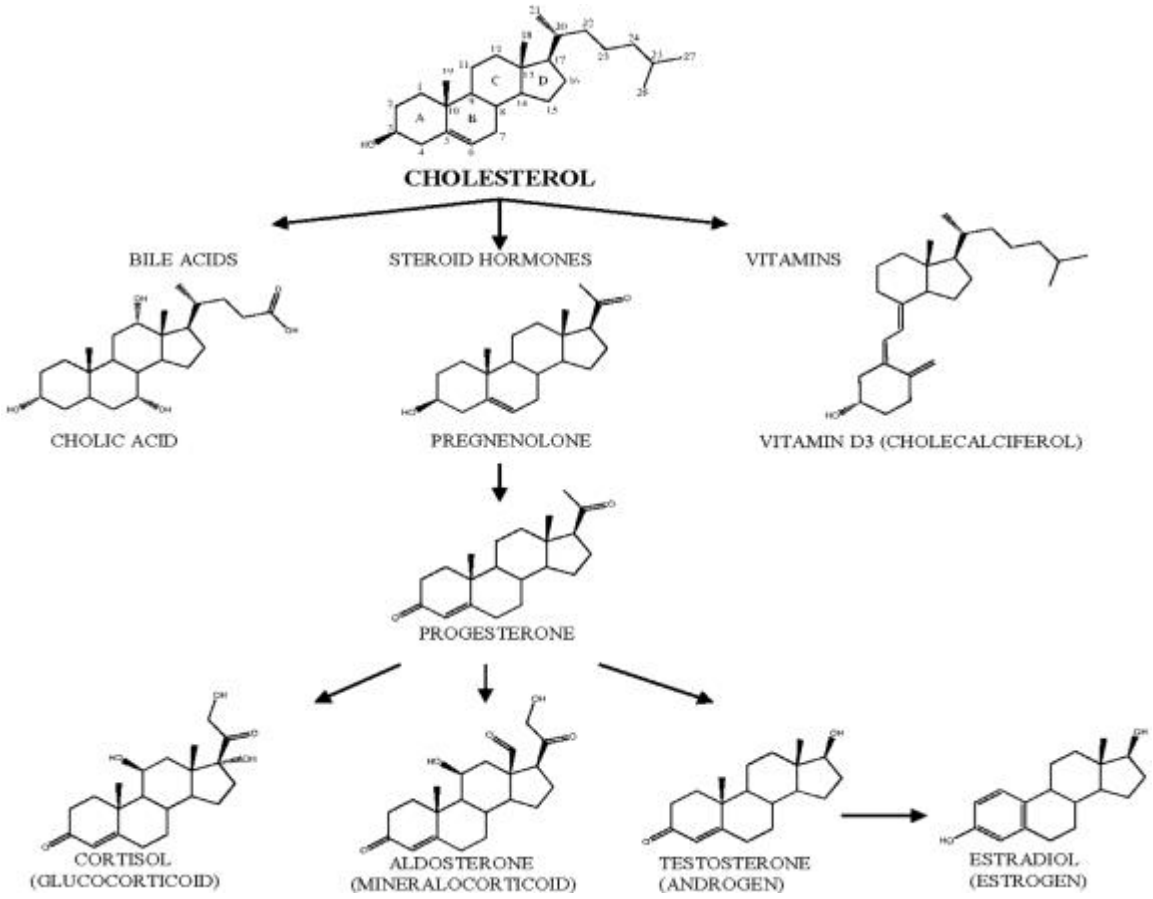

Figure 2.2. Cholesterol and catabolic products (Taken from García *et al.*, 2012)

2.2.1. Bile acids

Primary bile acids (cholate and chenodeoxycholate) are synthesized in the liver from cholesterol (Merino *et al*., 2013). The synthesis of bile acids requires 17 individual enzymes located in different organelles including the cytosol, endoplasmic reticulum (ER), mitochondria and perixosomes (Michael, 2016). The most abundant bile acids

found in bile are chenodeoxycholate (45%) and cholic acid (31%) and these are called primary bile acids (Michael, 2016). Later, these polycyclic compounds are conjugated either to taurine or glycine before they are released into the gallbladder (Björkhem and Eggertsen, 2001), from where they are then secreted into the intestinal lumen to facilitate the absorption of lipids from the dietary sources (Merino *et al*., 2013). In the intestinal lumen, primary bile acids are converted to secondary bile acids (e.g., deoxycholic acid and litholic acid) by microbial enzymes (Michael, 2016). Bile acids have also been shown to function as signaling molecules participating in the regulation of systemic endocrine functions (Wollam and Antebi, 2011). Also, waste products including bilirubin are eliminated from the body by secretion into bile acids and ultimately excreted in feces (Bowen, 2001). Additionally, the production of bile acids is one of the major mechanisms of excreting excess cholesterol (Michael, 2016).

2.2.2. Vitamin D3 (Cholecalciferol)

One of the major functions of cholesterol in the body is its function as a precursor of vitamin D (Masterjohn, 2006). Physiologically, the penetration of the skin by the UV-B spectrum converts 7-dehydrocholesterol to Vitamin D3 (Engelsen, 2010). 7-Dehydrocholesterol is a very close precursor of cholesterol, as the result, it's often called "cholesterol" or "a form of cholesterol" (Masterjohn, 2006). Cholecalciferol is hydroxylated in the liver; in the kidney, it's α-hydroxylated to produce the active form (1.25-dihydroxycholecalciferol) by the regulated enzyme α-1-hydroxylase (van der Wat, 2007). Vitamin D is known for its involvement and function in calcium metabolism and bone health; new roles are discovered, including its role in mental health, sugar regulation, the immune system and cancer prevention (Masterjohn, 2006).

2.2.3. Steroid hormones

Cholesterol also plays an important role in the synthesis of steroid hormones. The major synthetic pathway for the adrenal steroid hormones begins with cholesterol, and then goes to the adrenal products aldosterone, cortisol and dehydroepiandrosterone (DHEA) and then to the non-adrenal steroid hormones progesterone, testosterone and estradiol (Brandt, 2003). Steroid hormones, synthesized in tissue including the adrenal cortex, testes, ovary and some non-adrenal tissues (adipose tissue and brain) are all derived from cholesterol and they only differ in their ring structure and side chains (LaPolt, 2010). All of them are synthesized from the intermediate of cholesterol called pregnolone (Craigie *et al*., 2008; LaPolt, 2010). Although synthesized from the common intermediate and their structures almost alike, they exert different functions in the body.

Aldosterone exerts its functions in the kidneys, where it increases water and sodium reabsorption, while also increasing potassium excretion (Marieb and Hoehn, 2009), it is for the regulation of these two electrolytes or minerals that this hormone is also classified as the mineralocorticoid. Cortisol plays role in the maintenance of electrolyte balance, regulation of carbohydrates, lipids, protein metabolism, mediating allergic reactions and inflammatory response (http://dfard.weebly.com/uploads/1/0/5/3/10533150/ch24_cas.pdf). Because this hormone is mostly significant in glucose metabolism, it's also known as glucocorticoid. DHEA is biologically important in prepubertal growth and secondary sexual development (Berman, 2003). Testosterone is a potent anabolic hormone and is also responsible for the development of male secondary characteristics at puberty; estradiol stimulates the appearance of female sexual characteristics at puberty,

enhances the physiology of the female reproductive system such as stimulating the endometrial growth in the first half of the menstrual cycle and also in regulating normal vaginal epithelium (Berman, 2003). Progesterone stimulates the preparation of the endometrium, already primed by estradiol for implantation of the conceptus; it also maintains a uterine environment conducive for fetal growth (Berman, 2003).

2.2.4. Other important functions of cholesterol

For homeostasis, it's important that cholesterol amount be maintained at normal physiologic levels. Cholesterol existing in excess amounts in the circulation may accumulate in macrophages and vascular cells and ultimately cause atherosclerosis (Martin *et al.,* 1986), while excessive amounts in the bile may lead to cholelithiasis (Bennion and Grundy, 1978). Impaired biosynthesis during early embryogenesis may lead to organ defects, intrauterine or neonatal death (Tint *et al.,* 1994). Although most of cholesterol is synthesized in the liver, the most cholesterol rich organ is the brain. In this organ, cholesterol plays role as the component of the myelin sheath (Björkhem *et al.,* 1997). However, because of the difficulty of cholesterol to cross the blood-brain barrier (BBB), its homeostasis is regulated by a number of enzymes that convert it to 24-hydroxycholesterol, which can be transported easily through the BBB (Björkhem *et al.,* 1997).

Cholesterol has great physiologic role in plasma membranes. The most important function of cholesterol in the lipid bilayer is its ability to modulate physicochemical properties of cellular membranes (Yeagle, 1985 and 1988; Finegold, 1993). This steroid lipid is an abundant structural component of cell membranes (Staytor and Bloch, 1965). About 20% of membrane lipids is cholesterol (Marieb and Hoehn, 2009). A great order in the plasma membrane results in a more condensed

membrane with increased density of the phospholipids (Lund-Katz *et al*., 1988; Smaby *et al*., 1994; McIntosh, 1999). This increases the mechanical strength and decreases the permeability of the membrane (Yeagle, 1985; Needham and Nunn, 1990). As the result, the relatively high rates of both lateral and rotational diffusion of the lipid bilayer are maintained (Yeagle, 1988; Vist and Davis, 1990; Davis, 1993). Cholesterol wedges its plate-like hydrocarbon rings between the phospholipid tails, rendering stability to the membrane while increasing the mobility of other lipids (Marieb and Hoehn, 2009) and the fluidity of the membrane (Kusumi *et al*., 1983; Kusumi *et a*l., 1986). Additionally, it also raises hydrophobic barriers for polar molecules and increases rigidity barriers for non-polar molecules (Subczynski and Wisniewska, 2000). Moreover, cholesterol can also play role in cellular signaling by modulating the physical properties of the lipid bilayer, thereby affecting the activity of receptors and enzymes residing on it, or directly as a regulator of enzymes in the biosynthesis of cholesterol (Dahl and Dahl, 1988; Jackson *et al*., 1997; Edwards and Ericsson, 1998).

2.3. Microbial cholesterol metabolism

2.3.1. History and significance

The search for microorganisms capable of degrading cholesterol has been going on for over seven decades (García *et al*., 2012). From the early 20th century, it was observed that several species of *Mycobacterium* could use cholesterol as the sole source of carbon and energy (Söhngen, 1913; Tak, 1942). Following this discovery, it was reported that microorganisms belonging to the genus *Proactinomyces*, like *Rhodococcus erythropolis* (formely *Nocardia erythropolis*), have the ability to degrade cholesterol and a potential capacity for the synthesis of new steroid

derivatives (Turfitt, 1944; 1948). Moreover, some species of *Azotobacter* transform cholesterol into cholest-4-en-3-one (cholestenone) or 7-dehydrocholesterol, and have the ability to hydrolyze the side-chain of cholesterol generating methylheptanone (Horvath and Kramli, 1947). From then until recently, bacteria belonging to the genera *Nocardia*, *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Streptomyces*, *Microbacterium*, *Serratia*, *Achromobacter*, *Pseudomonas, Protaminobacter*, isolated from the soil, water and different waste materials, were reported to accomplish either partial or complete cholesterol degradation (García *et al*., 2012; Merino *et al*., 2013; Saranya *et al.,* 2014).

The genome sequence analysis and subsequent functional analysis of cholesterol degrading bacteria revealed involvement of different gene loci in cholesterol metabolism i.e., *igr* (side chain degradation), *hsa* (Central pathway) and *mce* (uptake and transport) (García *et al.*, 2012). Confirmation of the different gene locus in cholesterol degradation and uptake by bacteria was demonstrated by high density mutagenesis and deep sequencing analysis in *Mycobacterium tuberculosis* (Griffin *et al*., 2011). However, some bacteria do not contain some genes in their genomes, suggesting that although these bacteria could transform cholesterol, they may not be able to completely mineralize it (García *et al.*, 2012). Therefore, even today, it is still not known with enough precision how steroids like cholesterol are mineralized (Fernández de Las Heras *et al*., 2009), particularly by bacteria lacking cholesterol catabolism and transport genes. In spite of the large number of described bacteria able to use cholesterol as a sole source of carbon and energy, only the genomes of few of them have been completely sequenced (García *et al*., 2012) and available for public use. The significance of microbial conversion of cholesterol when compared to chemical reaction is that, microbial conversions of cholesterol are more

regio- and stereo-selective and have been used in the production of pharmaceutical products for a long time (Ahmad *et al*., 1992; Naghibi *et al*., 2002). Chemical approach is not effective because of its low conversion rate and economic disadvantage (Saranya *et al*., 2014). This is caused by low specificity of the reactions, resulting in considerable contamination by some undesired by-products (Naghibi *et al*., 2002). It's not only the disadvantage of the steroid chemical production that fuels and catalyzes the microbial cholesterol conversion; other uses including medicinal, clinical and biotechnological applications (García *et al*., 2012), as well as bioremediation purposes (Zhang *et al*., 2011) are key aspects of this approach.

2.3.2. Bacterial cholesterol degradation

Cholesterol catabolism by bacteria has not yet been fully elucidated in any of the bacterial strains having cholesterol degrading abilities, thus the metabolic pathways are suggested by merging biochemical and genetic studies done in different organisms (Martin, 1977; Schoemer and Martin, 1980; Owen *et al*., 1983; Kieslich, 1985; Sedlaczek and Smith, 1988; Szentimal, 1990; Yam *et al*., 2010; García *et al*., 2011; Griffin *et al*., 2011; Ouellet *et al*., 2011; García *et al*., 2012). Using a ^{14}C-radiolabelled cholesterol in experiments, it was observed that carbon from the steroid ring was converted to CO_2 and then used for the generation of energy through the citric acid cycle, while the side-chain is assimilated into the mycobacterial lipids (Cox *et al*., 1999). The catabolism pathway is divided into four major steps (Figures 2.3 and 2.4) (García *et al*., 2012).

2.3.2.1. Transformation of cholesterol into cholestanone

It is generally assumed that in the first reaction of the pathway, cholesterol is oxidized through two sequential reactions (Figure 2.3); first is the oxidation of cholesterol to cholest-5-en-3-one [2], secondly, this product is isomerized to cholest-4-en-3-one [3] (García *et al*., 2012). These reactions are catalyzed by cholesterol oxidases (ChOx), or 3*β*-hydroxysteroid dehydrogenase/isomerases (HSDs) (García *et al*., 2012). ChOx is classified as a monomeric flavoenzyme that catalyzes the oxidation of cholesterol to cholestenone compounds with a concomitant reduction of flavin adenine dinucleotide (FAD) (García *et al*., 2012). It exists in two different isoforms across different bacteria. That is, the ChOx from *Streptomyces* sp. SA-COO (Yue *et al*., 1999; Lario *et al*., 2003) and from *Brevibacterium sterolicum* (Vrielink *et al*., 1991; Li *et al*., 1993) have been classified as type I oxidases and belong to the glucose-methanol-choline oxidoreductase family of flavoenzymes where the FAD cofactor is non-covalently bound (García *et al*., 2012). The other form of ChOx, from *B. sterolicum* (Coulombe *et al*., 2001) has been classified as a type II oxidase belonging to the family of vanillyl-alcohol oxidase enzymes, which contain a covalently bound FAD molecule (Motteran *et al*., 2001). Both classes of ChOx share the same catalytic activity but show significant differences in their redox and kinetic properties. In general, they exhibit a broad range of steroid specificities and can oxidize a number of hydroxysterols, including sterols, steroid hormones and bile acids, but the presence of a 3*β*-hydroxyl group is an important requirement for activity (Pollegioni *et al*., 1999).

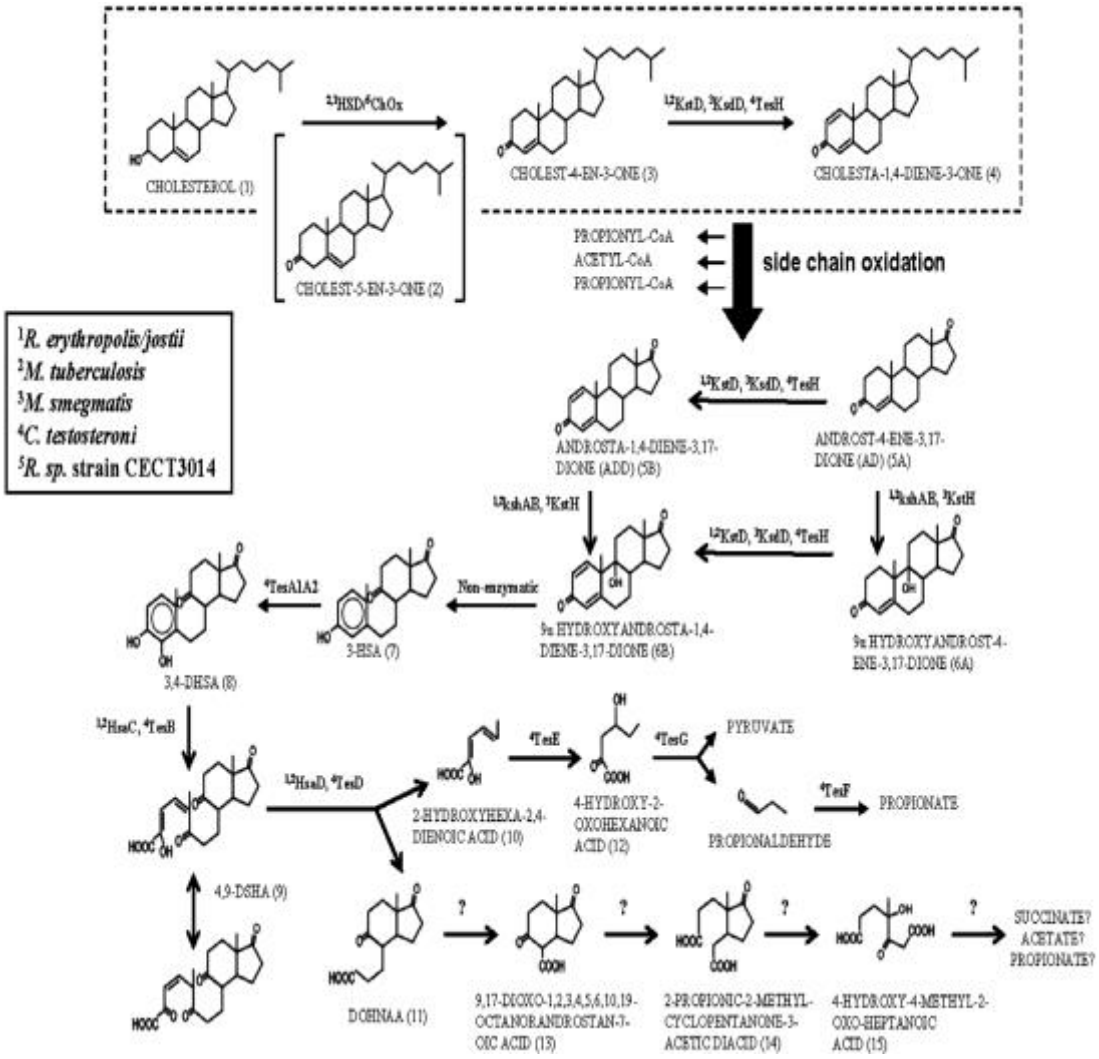

Figure 2.3. Cholesterol degradation pathway under aerobic conditions. Numbers in superscripts indicate the catabolic activity as demonstrated by bacteria listed on the left hand side of the diagram, while numbers in brackets are assigned to facilitate the compound identification in the text (Taken from García *et al*, 2012).

Overall, they demonstrate a wide range of steroid specificities and can oxidize a number of hydroxysterols, including sterols, steroid hormones and bile acids, but the presence of a 3*β*-hydroxyl group is an important requirement for activity (Pollegioni *et al*., 1999). ChOx are extracellular and occur as secreted and/or cell-surface-associated forms, depending on the microorganism and growth conditions (Kreit and Sampson, 2009). These enzymes also possess potential industrial and clinical

applications (Saranya *et al*., 2014). These applications include role in the quantification of serum and food cholesterol (Richmond, 1973; Yazdi *et al*., 2000); as precursors in the production of pharmaceutical steroids (Watanabe *et al*., 1989); in the degradation and lowering the dietary cholesterol (Kaunitz, 1978), thus providing a diagnostic kit for degrading cholesterol (Noma and Nakayama, 1976) and also as bio-insecticides (Purcell *et al*., 1993; García *et al*., 2012). These are some of the reason microbial ChOx have received much attention in recent years (Saranya *et al*., 2014).

2.3.2.2. Side chain degradation

It is generally assumed that conclusive of the first oxidative process in the catabolism of cholesterol (Figure 2.4), the long aliphatic chain of cholesterol is removed *via* a process similar to *β*-oxidation of fatty acids, leading to the formation of a steroid C22-oic intermediate with the concomitant release of two molecules of propionly-CoA and one acetyl-CoA (García *et al*., 2012). Study demonstrated that the CYP125 enzyme from *Rhodococcus jostii* RHA1 (*ro04679* coding gene) and *M. tuberculosis* (*Rv3545c* coding gene) initiates the side-chain degradation of cholesterol (Capyk *et al*., 2009a; Rosłoniec *et al*., 2009). The CYP125 from *M. tuberculosis* CDC1551 strain was proved to be involved not only in the hydroxylation of cholesterol but also in its further oxidation to cholest-4-en-3-one-26-oic acid (Ouellet *et al*., 2010), thus this P450 has more than one catalytic activities in the side chain degradation of cholesterol. Although this P450 protein is important in the degradation of the side chain, it's not the only P450 with such significance. CYP142 is able to replace the activity of CYP125 in *M. tuberculosis* H37Rv (Driscoll *et al*., 2010; Johnston *et al*., 2010), explaining why a CYP125 deletion mutant of this strain is still able to grow using cholesterol as a carbon source (García *et al*., 2012).

Following the formation of the carboxylic acid intermediate in the pathway,

cholesterol side-chain is shortened by a *β*-oxidation-like process initiated by an ATP-dependent sterol/steroid CoA ligase catalyzing the CoA activation of the C26 carboxylic acid intermediate (Sih, 1968; Sih *et al*., 1968). Subsequent cleavage of the side-chain to 17-ketosteroid takes place stepwise in three consecutive cycles (figure 2.4.), producing two molecules of propionyl-CoA and one molecule of acetyl-CoA (García, *et al*., 2012). A number of genes suggested to be involved in cholesterol side chain degradation and also related to *β*-oxidation have been recently identified in *M. tuberculosis* by transposon mutagenesis (Griffin *et al*., 2011) and some of them were found to be located inside the large cholesterol degradation gene cluster (*hsd4A, ltp2, fadE29, fadE28, fadA5, fadE30, fadE32, fadE33, fadE34 and hsd4B*), but others were found outside of the cluster (*fadE5, echA9*, *fadD36* and *fadE25*) (García *et al*., 2012). The oxidation of cholesterol together with the degradation of its lateral chain produces the central intermediate androst-4-ene3,17 dione (AD) [Figure. 2.3 (5A)], which is further metabolized by the central catabolic pathway (García *et al*., 2012).

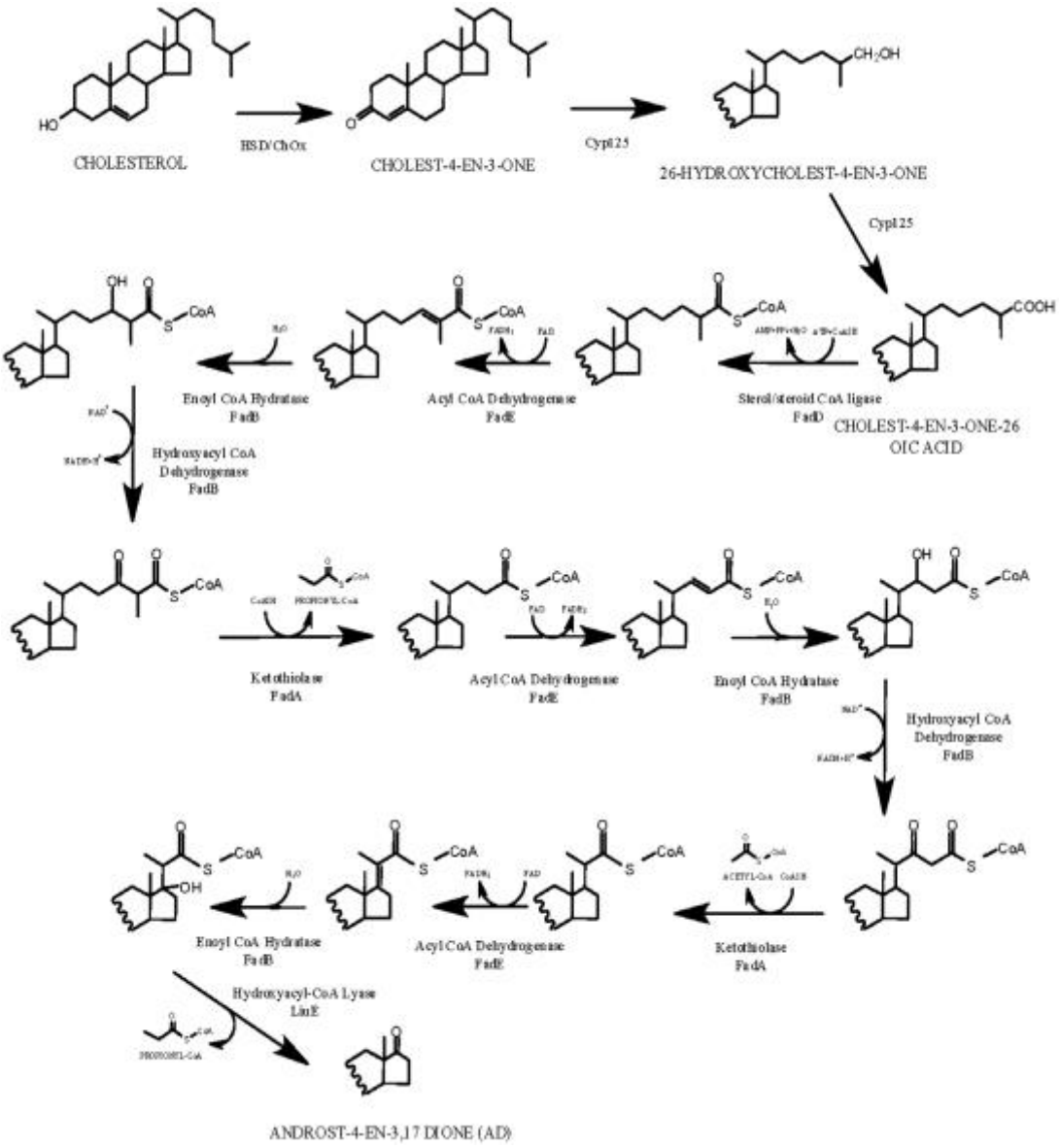

Figure 2.4. Proposed *β*-oxidation-like reactions for cholesterol side-chain degradation (Taken from García *et al.*, 2012).

2.3.2.3. Central pathway

Once the aliphatic chain has been oxidized, cholesterol catabolism appears to follow the common pathway described for C19 steroids (García *et al.*, 2012). Briefly, 3-ketosteroid-D^1-dehydrogenases, as KsdD in *Mycobacterium smegmatis* (Brzostek *et al.*, 2005), KstD in R*hodococcus erythropolis* (van der Geize *et al.*, 2000; 2001;

2002a) and *M. tuberculosis* (Knol *et al*., 2008) that are equivalent to TesH of *Comamonas testosteroni* (Horinouchi *et al*., 2003a) transform AD into androsta-1,4-diene-3,17-dione (ADD) [Figure 2.4 (5B)]. This is followed by a 9*α*-hydroxylation that is catalysed by KshAB in *R. erythropolis* (van der Geize *et al*., 2001; 2002b; 2008a), KshAB (Rv3526/Rv3571) in *M. tuberculosis* H37Rv (Capyk *et al*., 2009b), and KstH (KshA-like) in *M. smegmatis*) (Andor *et al*., 2006). The non-enzymatic transformation of the 9*α*-hydroxyandrosta-1,4-diene-3,17-dione [figure 2.4 (6B)] to 3-hydroxy-9,10- secoandrosta-1,3,5 (10)-trien-9,17-dione (3-HSA) [figure 2.4 (7)] then occur.

The next step in the pathway is the hydroxylation of 3-HSA by enzymes similar to the two-component oxygenase TesA1A2 from *C. testosteroni* (Horinouchi *et al*., 2004) to produce 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-trien-9,17-dione (3,4-DHSA) [figure 2.4 (8)] (García *et al*., 2012). The last catechol derivative is cleaved by a *meta* extradiol dioxygenase similar to TesB in *C. testosteroni* (Horinouchi *et al*., 2001), which was named HsaC in *R. jostii* RHA1 (van der Geize *et al*., 2007) or *M. tuberculosis* (Yam *et al*., 2009) yielding 4,5,9,10-*diseco*-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid (4,9-DSHA) [figure 2.4. (9)] (García *et al*., 2012). This compound is then hydrolyzed by enzymes similar to TesD from *C. testosteroni* (Horinouchi *et al*., 2003b) named HsaD in *M. tuberculosis* (Lack *et al*., 2008; 2010) or *R. jostii* RHA1 (van der Geize *et al*., 2007) yielding 2-hydroxyhexa-2,4- dienoic [Figure 2.3. (10)] and 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic (DOHNAA) acids [Figure 2.3 (11)] (García *et al*., 2012).

2.3.2.4. Lower catabolic pathway

This is the last of the four major steps in the catabolism of cholesterol. In the first reaction the 2-hydroxyhexa-2,4-dienoic acid is metabolized to 4-hydroxy-2-oxo-hexanoic acid by the TesE-like hydratase [Figure 2.3 (12)] that is consequently transformed by the catalytic activity of TesG-like aldolase into pyruvic acid and propionaldehyde; ultimately, this product is transformed into propionic acid by the action of TesF-like aldehyde dehydrogenase (Horinouchi *et al*., 2005). In the following step, DOHNAA is transformed to succinic acid and it is assumed that the first step in the degradation of this compound could be the elimination of a propionyl moiety, to produce 9,17-dioxo-1, 2,3,4,5,6,10,19-octanorandrostan-7-oic acid [Figure 2.3 (13)] through a typical *β*-oxidation (Kieslich, 1985). This reaction comprises two steps, first is the activation of DOHNAA by ATP and coenzyme A and in the second step, DOHNAA is activated by a reduction of DOHNAA-CoA by an NADPH-dependent dehydrogenase (Miclo and Germain, 1990). As can be seen from the diagram, the enzymes catalyzing these reactions have not been precisely identified nor characterized (García *et al*., 2012).

2.3.3. Anoxic catabolism

Bacteria can catabolize cholesterol under denitrifying conditions (Taylor *et al*., 1981). Under such conditions the double bond of cholesterol is reduced to yield coprostanol (Li *et al*., 1995; Harder and Probian, 1997). Many of the intestinal bacteria possessing fermentation abilities can catalyze the transformation of cholesterol to coprostonol (Groh *et al*., 1993; Freier *et al*., 1994), and the reductase responsible for this reaction has been characterized in *Eubacterium coprostanoligenes* (Li *et al*., 1995). Initially, the anoxic metabolism of cholesterol was mainly studied at the biochemical level in

the denitrifying *β*-proteobacterium, *Sterolibacterium denitrificans*, but now used as a model organism (Tarlera and Denner, 2003). Also important is the observation that this bacterium can aerobically catabolize testosterone (Chiang *et al*., 2010), which is one of the derivatives of cholesterol. Although the conditions are different, the first steps of the anoxic catabolism of cholesterol are similar to those described for the aerobic process and thus, first the hydroxyl group at C-3 is oxidized into the keto group leading to the production of cholest-5-en-3-one followed by a subsequent isomerization to yield cholestenone (Chiang *et al*., 2007). The enzyme catalyzing these reactions is a bifunctional enzyme AcmA that belongs to the short-chain dehydrogenase reductase (SDR) superfamily (Chiang *et al*., 2008a). In contrast with the aerobic pathway where cholestenone is hydroxylated by a monooxygenase at C-27 to produce the corresponding primary alcohol, the anaerobic hydroxylation of the cholesterol side-chain is oxygen-independent (Chiang *et al*., 2007).

2.4. Cholesterol uptake

It is evident from the previous sections that many bacteria do degrade cholesterol and use it as the carbon source. However, what is missing is how cholesterol is transported into the organism. Cholesterol uptake system studies demonstrated that cholesterol was transported by an inducible transport system in *Pseudomonas testosterone* (Watanabe and Po, 1974). These are generally termed the mammalian cell entry (mce) transport systems. The first discovered *mce* gene was found to promote the uptake of mycobacteria into nonphagocytic cells (Arruda *et al*., 1993). The genome of *M. tuberculosis* contains four *mce* operons (*mce1-4*) each including 9–13 genes with a similar arrangement encoding two transmembrane proteins with homology to the permease subunits of ABC transporters, along with several putative

secreted or cell-surface proteins (Pandey and Sassetti, 2008). Recent studies in this system reported that the mycobacterial gene cluster *mce4* encodes a cholesterol import system that enables *M. tuberculosis* to derive both carbon and energy from this component of host membranes (Pandey and Sassetti, 2008). The energy is generated by a common ATPase, MceG, belonging to the Mlk family (Joshi *et al*., 2006). Even though the MceG generates energy for the Mce4 proteins, the genome analysis demonstrates that he *mceG* gene is located far away from the four *mce* operons in *M. tuberculosis* (Mohn *et al*., 2008).

Exploration of genome from diverse *Actinobacteria*, including members *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Streptomyces* genera, demonstrates the presence of *mce* transporter operons involved in steroid uptake (Casali and Riley, 2007; García *et al*., 2011). Of four *mce* operons, the *mce4* locus is the only one presently associated with steroid or cholesterol metabolism in all annotated bacterial genomes (García *et al*., 2012), suggesting that other operons might have other functions unrelated to steroid uptake and catabolism. On the other hand, the Mce4 uptake system is also essential for growth of *R. jostii* RHA1 in cholesterol related compounds such as 5*α*-cholestanol, 5*α*-cholestanone and *β*-sitosterol (Mohn *et al*., 2008). Also, the Mce4 uptake system is also essential for growth of *R. jostii* RHA1 in cholesterol related compounds such as 5*α*-cholestanol, 5*α*-cholestanone and *β*-sitosterol (Mohn *et al*., 2008). However, it appears that only the side chains with at least eight carbons are recognized by mce4 steroid uptake system (Mohn *et al*., 2008; García *et al*., 2012).

2.5. Cholesterol and other steroids as environmental hazards and role in pathogenesis

Cholesterol and some related steroids constitute a new class of pollutants discharged into the environment as a result of human activity (Gagné *et al*., 2006). These pollutants are found in different environments. Steroid contamination studies performed in 1990 showed the presence of estrogens and other steroids in seawater, lakes and rivers in different areas (Shore *et al.,* 1993; Soto *et al*., 2004; Barel-Cohen *et al*., 2006). Significantly, steroidal estrogens were shown to be the main contributors to the estrogenic activity observed in aquatic systems contaminated with sewage treatment effluents (Shore *et al*., 1993). The presence and accumulation of these pollutants (mainly as a consequence of pharmaceutical industry spillages) may have significant eco-toxicological impacts, including lower fertility in mammals and reproductive anomalies in fish (Länge *et al.,* 2001; Colborn, 2004; Lange *et al*., 2008). Thus, these pollutants cause abnormalities and diseases not only in humans, but other animals as well.

Cholesterol catabolism by microorganisms is also implicated in the etiology of diseases, especially Tuberculosis. Study demonstrated that *M. tuberculosis* can grow using cholesterol as the sole carbon and energy source (Pandey and Sassetti, 2008). Therefore, cholesterol has recently been identified as an important lipid for mycobacterial infection (de Chastellier and Thilo, 2006; Ouellet *et al*., 2011). The relatively abundant cholesterol distributed in host cells is an important growth substrate for bacteria in different infection stages (García *et al*., 2012). *M. tuberculosis* growing in the humans appears to obtain the energy from host lipids rather than other nutrients such as carbohydrates (Dubnau *et al*., 2005). The Mce4

transport system is essential for cholesterol transport into bacterial cells and also plays an important role in pathogenesis because a mutant lacking this transporter fails to persist in the lungs of chronically infected mice and cannot grow in macrophages (Pandey and Sassetti, 2008). This means that the Mce4 transport system transports host cholesterol into the bacterial cell where cholesterol is degraded to produce energy for the bacteria to survive and cause diseases. Also, the ability of Mce4 proteins to bind cholesterol could act as a signal allowing the pathogen to interact with the host (Mohn *et al*., 2008).

2.6. Clinical and biotechnological applications of cholesterol and its degrading enzymes

2.6.1. Biosensors for cholesterol analysis in biological samples

Hypercholesterolemia is the major cause of cardiovascular diseases; it's for this reason that biosensors have been developed to clinically target and quantify cholesterol (Arya *et al*., 2008; Jubete *et al*., 2009; Solanki *et al*., 2011), meaning that cholesterol biosensors can measure the amount of cholesterol in the body. ChOx are used in enzymatic analysis of cholesterol (García *et al*., 2012), and most of them are from *Streptomyces hygroscopicus*, *B. sterolicum* and *Pseudomonas fluorescens* (Arya *et al*., 2008; Nien *et al*., 2009; Singh *et al*., 2009). In the blood, about 70% of cholesterol exists in an ester form, while 30% exists in free form, thus cholesterol esterases are also included in biosensors together with ChOxs (Arya *et al*., 2008; Shih *et al*., 2009).

2.6.2. Microbial enzymes for insecticides and fungicides

The catalytic role of ChOx allows them to be used as effective insecticides and fungicides. This came after the discovery of an effective ChOx protein that killed boll weevil (*Anthonomus grandis grandis Boheman*) larvae in *Streptomyces* culture filtrates (Purcell *et al.,* 1993). The purified enzyme was tested and found active against boll weevil larvae at a concentration comparable with the bioactivity of *Bacillus thuringiensis* proteins against other insect pests (García *et al*., 2012). The mechanism of this enzyme involves the lysis of the midgut epithelial cells of the larvae and also demonstrates insecticidal abilities against lepidopteran cotton insect pests, including tobacco budworm (*Heliothis virescens*), corn earworm (*Helicoverpa zea*) and pink bollworm (*Pectinophora gossypiella*) (Corbin *et al*., 2001). Study also reported that *Chromobacterium subtsugae* has insecticidal properties (Martin *et al*., 2007), probably because it produces ChOx enzyme (Doukyu *et al*., 2008).

2.6.3. Probiotics

Some probiotic bacteria are known to have cholesterol-lowering effects. Such bacteria include genera as *Lactobacillus, Lactococcus, Enterococcus*, *Streptococcus* or *Bifidobacterium* (Pereira and Gibson, 2002). Several mechanisms are proposed on how probiotics can lower cholesterol levels in the body. They include enzymatic deconjugation of bile acids by bile-salt hydrolases of probiotics (Lambert *et al*., 2008), assimilation of cholesterol (Pereira and Gibson, 2002), co-precipitation of cholesterol with deconjugated bile (Liong and Shah, 2006), cholesterol binding to cell walls of probiotics (Liong and Shah, 2005), incorporation of cholesterol into the cellular membranes of probiotics during growth (Lye *et al*., 2010a), production of short-chain fatty acids upon fermentation by probiotics in the presence of prebiotics

(de Preter *et al*., 2007) and conversion into coprostanol (Lye *et al*., 2010b). Many intestinal fermenting bacteria are known to catalyze the reduction of cholesterol to coprostanol (Groh *et al*., 1993; Freier *et al*., 1994), thus decreasing the amount of absorbed cholesterol and leading to a reduced concentration in the physiological cholesterol pool (Ooi and Liong, 2010). This perception is already applied in clinical therapy. This done by directly administering cholesterol reductase enzymes to humans to bioconvert cholesterol to coprostanol in the small intestines to reach a bloodstream cholesterol-lowering effect (Ooi and Liong, 2010). Of all the mechanism described above, the most optimum is the one with enzymatic deconjugation of bile acids. Following the enzymatic conjugation of bile acids and their subsequent excretion in feces, the response is the synthesis of new bile acids as a homeostatic response, resulting in a hypocholesterolemic effect of probiotics (Begley *et al*., 2006; Ooi and Liong, 2010).

2.6.4. Production of pharmaceuticals

One of the important roles of cholesterol degrading bacteria is the biotransformation/bioconversion of cholesterol into steroid hormones and drugs. This approach of producing pharmaceuticals is also known to be environmentally beneficial (García *et al*., 2012). Microbial bioconversion has been focused mainly in steroid hydroxylation, D^1-dehydrogenation and sterol side-chain cleavage that associated to chemical synthesis steps, have enhanced the large-scale production of both natural and modified steroid analogues (García *et al*., 2012). This application is optimum with use of whole bacterial cells instead of just bacterial enzymes, because the production costs are lower and time saving, as it is possible to perform multi-steps conversions with a single biocatalyst (Bortolini *et al*., 1997). Steroid compounds

manufactured in this manner possess anti-inflammatory, immunosuppressive, diuretic, anabolic and contraceptive activities and are also breast and prostate cancer and anti-diabetic agents (García *et al*., 2012). Some of them are neurosteroids because of their role as memory enhancers, inducers of endocrine response to stress, anxiolytic agents, anticonvulsants, antidepressives and neuroprotective effects (Fernandes *et al.*, 2003).

Some bacteria having the ability to degrade the side chain of cholesterol and thus used for their potential interest as biocatalysts for steroid synthesis include *R. coralline*, *Arthrobacter simplex, R. equi*, *Mycobaterium fortuitum*, *R. erythropolis, Mycobacterium neoaurum* or *Micrococcus roseus* (García *et al*., 2012). Additionally, *Mycobacterium* sp. NRRL B-3805 (Liu *et al*., 1994b; Liu and Lo, 1997) and *Lactobacillus bulgaricus* (Kumar *et al*., 2001) have been used to produce testosterone from cholesterol using a single strain. Cholesterol can also be converted to ADD by a two-step reaction via microbial transformation (Lee *et al*., 1993); firstly, cholesterol is oxidized to cholestanone by ChOx and secondly, cholestanone is transformed into ADD by *Mycobacterium* sp. (Mahato and Garai, 1997). Other microorganisms *Mycobacterium* sp. (Smith *et al*., 1993), *R. equi* (Ahmed and Johri, 1993), *Escherichia* (Panchishina, 1992), *Nocardia rubra* (Osipowicz *et al*., 1992), *A. simplex* (Liu *et al*., 1994a), *R. erythropolis* (Jadoun and Bar, 1993), *R. equi* (Myamato and Toyoda, 1994), *Agrobacterium* sp. M4 (Mahato and Garai, 1997; Yazdi *et al*., 2000) can transform cholesterol into cholestanone.

2.7. Cholesterol bioconversion by Eukaryotes

Even though eukaryotes such as plants and fungi are the main producers of cholesterol and other steroids, they are known to lack bioconversion pathways for recycling the carbon content of these compounds (Wang *et al*., 2013). Eukaryotic

organisms have not been demonstrated to exhibit any metabolic effects on cholesterol, that is, no study or any form of scientific experimentation has shown any microorganism other than bacteria to be able to either breakdown or transform cholesterol into any compound.

2.8. Motivation and objectives of the study

Research is gaining momentum on isolating microorganisms that can effectively bio-transform cholesterol. This aspect of cholesterol metabolism carries many potential medical and biotechnological applications as discussed on the previous section. One of the critical roles that have not been explored is the role of cholesterol in microbial pathogenesis and pathophysiology. This is an aspect of microbial lipid research that current researchers and scientists always overlook and never explore. It's one of the aspects this study aims to promote, shed light on and bring into attention and consideration regarding microbial disease-mechanism studies.

Few studies (Chimuka *et al*., 2012; Nekhavhambe *et al*., 2014) indicated that South African air, land and water are contaminated with many chemicals that can cause many detrimental physiologic conditions in humans and animals (Tijani *et al.,* 2016). Cholesterol and other related steroid compounds are among some of the contaminants in such environments. Additionally, it has been shown that microorganisms especially *M. tuberculosis* use cholesterol as the sole source of carbon and energy and for the etiology of Tuberculosis (Pandey and Sassetti, 2008; García *et al*., 2012). The objective of the study is to isolate, identify and characterize microorganisms that can effectively degrade and utilize cholesterol as the sole source of carbon from the South African soil.

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CHAPTER 3

ENRICHEMENT AND ISOLATION OF CHOLESTEROL DEGRADING FUNGI

3.1. Introduction

Microbiologists have been trying to isolate cholesterol degrading microorganisms for several decades now, with the first bacteria isolated in 1942 (Tak, 1942). The ubiquity and abundance of this compound renders it scientifically significant in biogeochemistry as its transformation has potential applications in biotechnology, pharmaceuticals and clinical research (Fernandes *et al*., 2003; Doukyu, 2009; Wang *et al*, 2013). All microorganisms demonstrated to have the ability to recycle the carbon contents of cholesterol are bacteria, as eukaryotic organisms are known to lack pathways to recycle its carbon contents (Wang *et al*., 2013). In spite of the large number of described bacteria able to use cholesterol as a sole source of carbon and energy, only the genomes of few of them have been completely sequenced (García *et al*., 2012) and available for public use (Table 3.1). Cholesterol degrading bacteria are isolated from different environments and sources such as the soil, waste waters and many other waste materials and they belong to different genera (García *et al.*, 2012; Merino *et al*., 2013; Saranya *et al.,* 2014). To date, eukaryotic microorganisms have not been described nor demonstrated to grow on cholesterol as the only source of carbon.

Table 3.1. Bacterial species known to degrade cholesterol and use it as the sole source of carbon (taken from García *et al.*, 2012).

Organisms[a]	**GenBank**	**Chromosome (nt)**	***Igr***[b]	***Hsa***[b]	***Mce***[b]
Gornadia heofelifaeecis	NZ_AEUD00000000	4 257 286	Yes*	Yes	Yes
Mycobacterium avium	NC_008595	5 475 491	Yes	Yes	Yes
Mycobacterium bovis	NC_008769	4 374 522	Yes	Yes	Yes
Mycobacterium smegmatis	NC_008596	6 988 209	Yes	Yes	Yes
Mycobacterium tuberculosis	NC_000962	4 411 532	Yes	Yes	Yes
Rhodococcus equi	NC_014659	5 043 170	Yes*	Yes	Yes
Rhodococcus erythropolis	NC_012490	6 516 310	Yes*	Yes	Yes
Rhodococcus jostii	NC_008268	7 804 765	Yes*	Yes	Yes
Streptomyces venezuelae	FR845719.1	8 226 158	Yes*	No	Yes
Streptomyces viridochromogenes	NZ_ACEZ00000000	8 548 109	Uncomplete*	No	Yes

[a] only one representative organism is included in the table.

[b] The presence of the *igr, hsa* and *mce* genes has been analysed.

The asterisk (*) means that the CYP125 encoding gene is not present in the locus.

Fungal species have been thoroughly studied and described to grow on many culture mediums utilizing many different carbon sources, yet no study has demonstrated the ability of fungi to utilize cholesterol as the sole source of carbon and energy. This study describes a novel concept in fungal lipid research, four fungal organisms utilizing cholesterol as the source of carbon and energy. This is a novel concept in fungi with undescribed, unlimited biotechnological, medical and biological impacts.

3.2. Materials and methods

3.2.1. Soil sampling area

Soil samples were collected from different locations in Mpumalanga province, South Africa (Figure 3.1). This province has quite a number of coal-fired power plants and this province is famous for its mountains and forest. Samples 1 and 2 were collected around Matla power station and Duvha power station where soil is covered with black layer of coal residues. Samples 3 and 5 were collected in a forest located next to Swadini Forever Resort where decomposition of dead trees is underway. Sample 4 is collected between R544 and R515 on the side of the road. Sample 6 is the soil sample collected from the mining deposit next to Kriel Coal mine.

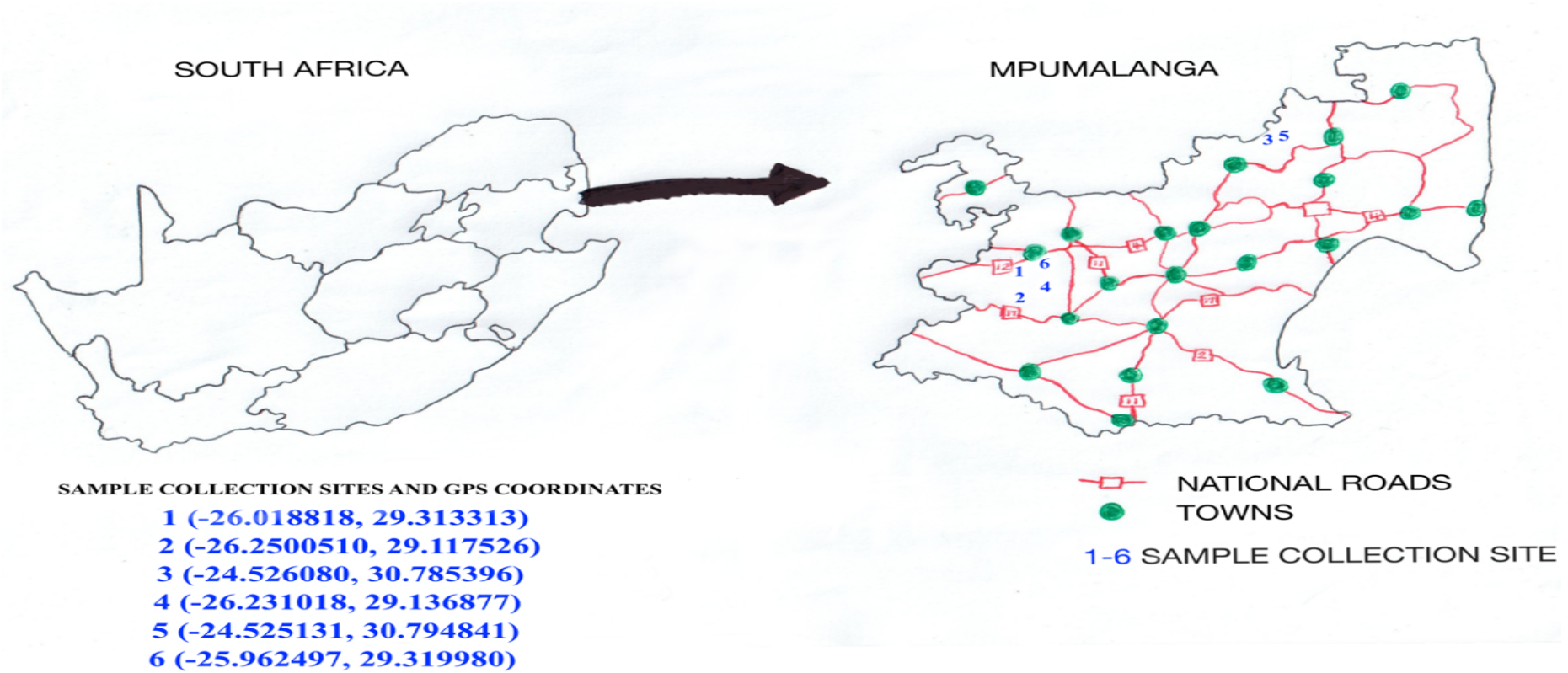

Figure 3.1. South African map indicating the sample collection sites in the Mpumalanga province. Only major towns and major national roads are included in the map. The GPS coordinates of sample collection (1 to 6) are given in the map.

3.2.2. Soil sample collection and preparation

Six soil samples were aseptically collected from the specified areas in sterile falcon tubes. 5 g of each sample was resuspended in 30 ml of DNase and RNase free distilled water by vortexing for 5 min, followed by incubation for one hour on a rotary shaker at room temperature. After incubation the soil was allowed to settle and the supernatants were collected for further analyses.

3.2.3. Culture medium

All chemicals and reagents used in this study were purchased form Sigma-Aldrich, unless otherwise stated. A modification of Selective A medium was used for isolation of microorganisms (Arima *et al*., 1969; Yadzi *et al*., 2000). The modified Selective A medium contained: 0.25 g/l K H_2PO_4; 17.00 g/l NH_4NO_3; 0.25 g/l $MgSO_4.7H_2O$; 0.005 g/l NaCl; 0.1 ml Tween-20; 0.001 g/l $FeSO_4.7H_2O$ and 2 mM cholesterol. The Selective A medium was further supplemented with 10 ml of trace element solution [0.4 mg/l $CuSO_4$; 1.0 mg/l KI; 4.0 mg/l $MnSO_4.H_2O$; 4.0 mg/l $ZnSO_4.7H_2O$; 5.0 mg/l H_3BO_3; 1.2 mg/l $Na_2MO_4.2H_2O$ and 2.0 mg/l $FeCl_3.6H_2O$] per litre of medium (Zeng *et al*., 2010).

3.2.4. Isolation of fungi

Aliquots (1 ml) of supernatants from soil samples were inoculated into 100 ml of modified Selective A medium, and incubated on a rotary shaker at 100 rpm and 37^{o}C. After two weeks of incubation, 1 ml of culture medium from incubating flasks was re-inoculated into 100 ml of fresh modified Selective A medium, followed by further incubation on a rotary shaker at 100 rpm and 37^{o}C. The enrichment was repeated until a pure culture was obtained. Culture aliquots (100 µl) were spread on modified Selective A medium agar plates or Potato Dextrose Agar (PDA) plates or LB plates for observation of fungal growth and morphology.

3.3. Results and discussion

3.3.1. Isolation of cholesterol utilizing fungi

The soil in the areas sampled in this study is contaminated with discharges from the coal-fired power stations and decomposing organic matter from decaying trees and dead forest animals. A recent study conducted in the area around coal-fired power stations revealed that these sites were contaminated with different polycyclic aromatic hydrocarbons (PAHs) (Okedeyi *et al.,* 2013). It can therefore be expected that the organisms growing in such sites have capabilities to degrade aromatic compounds including PAHs, as well as different compounds present in decomposing trees and dead forest animals. Considering that cholesterol is a polycyclic aromatic compound, microorganisms in the sites where soil samples were collected, could possibly have the capability to degrade this compound. In this study, enrichment of microorganisms providing cholesterol as the sole source of carbon was set up.

The culture medium were prepared as described elsewhere (Arima *et al.*, 1969; Yadzi *et al*., 2000) with cholesterol supplied as the only source of carbon and energy. After 14 days of incubation, the presence of microorganisms was noted by the turbidity of the culture medium and the growth was observed in five of the first culture flasks. The 6th sample showed no growth and the control flask was negative as expected, indicating that there was no contamination of the medium during the preparation. To confirm the microorganisms' growth in the medium, 100 µl of the culture was spread on freshly prepared LB agar plates. The plates were incubated overnight and the growth was observed after 18 hours. As can be observed from the plates (Figures 3.2 and 3.3), a heterogeneous growth of both bacteria and surprisingly, fungi was observed on a medium described for the isolation of cholesterol utilizing bacteria (Arima *et al*., 1969; Yadzi *et al*., 2000). After subsequent enrichments, a

homogenous fungal growth was observed on Selective A medium plates where cholesterol was supplemented as a carbon source (Figure 3.3).

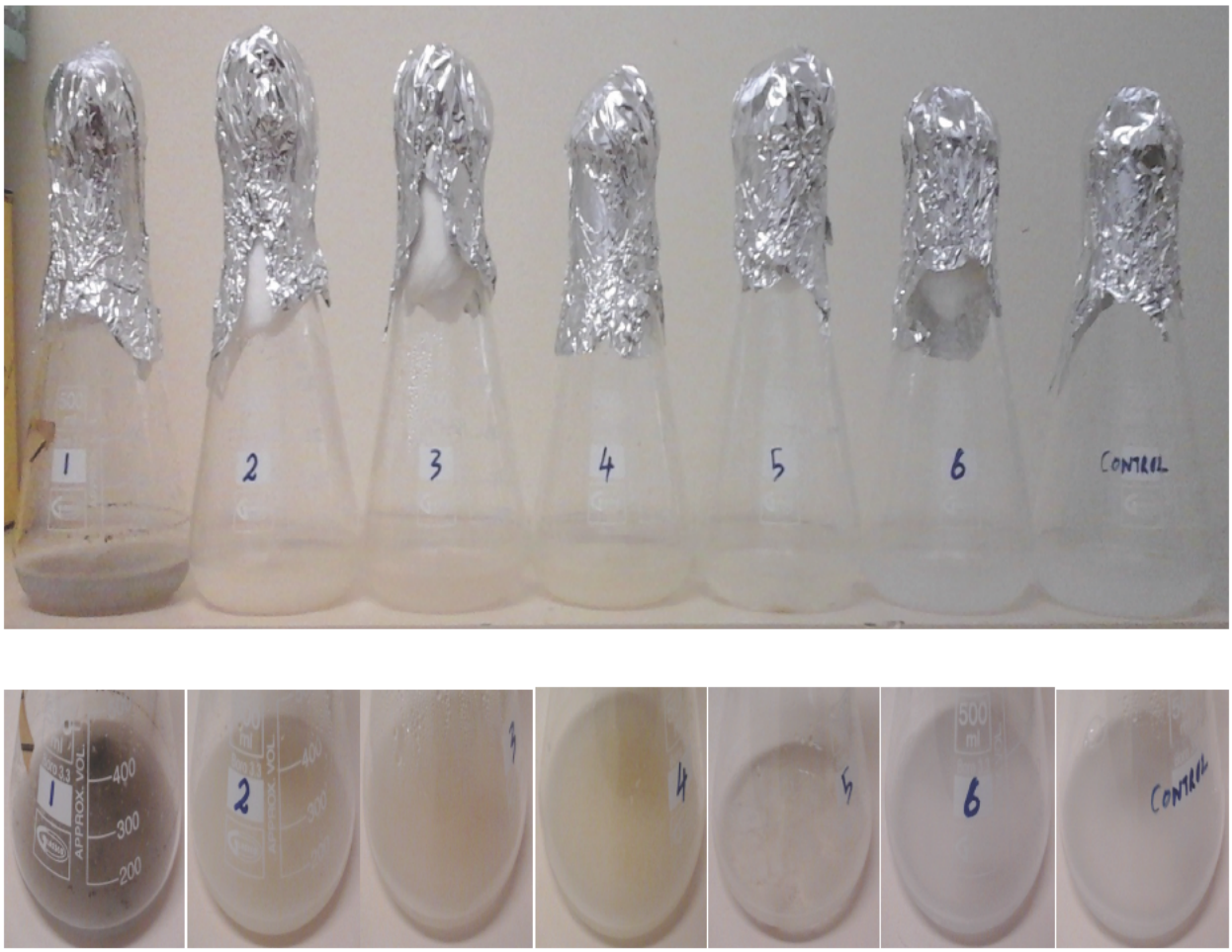

Figure 3.2. The initial six sample flasks after a two week incubation period along with the control flask.

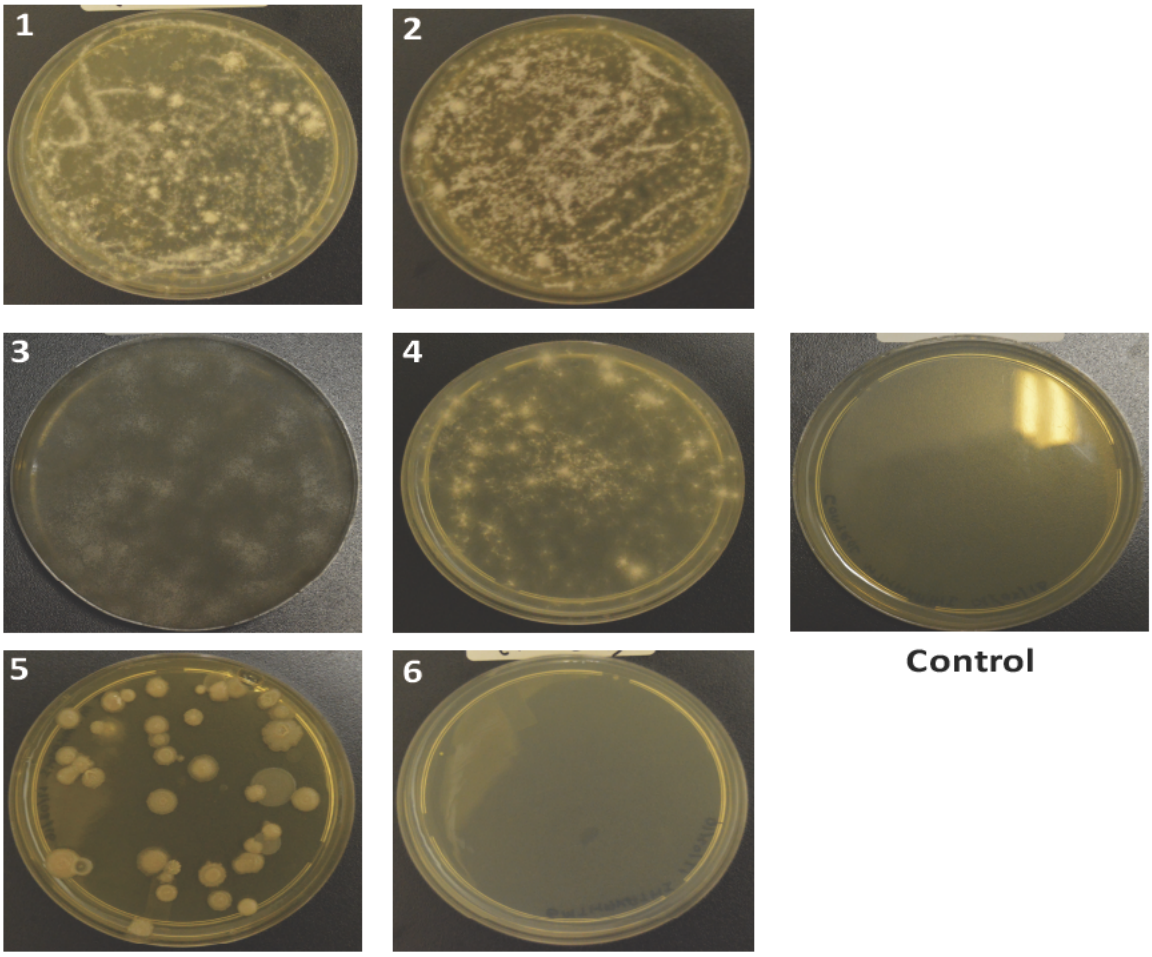

Figure 3.3. Growth of cholesterol utilizing microorganisms on LB-agar plates. 100 µl of 2 week old culture from Selective A medium was spread on the plates to see growth of microorganism. Fungal biomass is evident on the first 5 samples, with evidence of bacterial growth observable on close inspection of the agar plates. Sample number 6 is negative for cholesterol utilizing microorganisms. The control plate is negative as expected.

After obtaining homogenous fungal growth on plates, the fungi were subcultured by inoculating the agar block onto fresh Selective A medium plates where cholesterol was supplemented as the only carbon source. As suspected, fungal isolates flourished and produced a lot of homogeneous biomass (figure 3.4). Based on phenotypic observation

sample 5 isolate was found to be a mould. Due to this reason sample 5 was not taken for further study. However, sample 5 is stored for future studies.

To observe distinct fungal growth characteristics and morphological features of the four remaining fungal isolates, 100 µl of cultures were transferred from the liquid medium and inoculated on freshly prepared PDA plates and incubated for 5-6 days. Fungal isolates from samples 1 to 2 (Figure 3.5) demonstrated similar growth characteristics, while isolates 3 and 4 have unique growth characteristics. The isolates also exhibit the same growth characteristics when cultured on the Selective A medium for the same incubation period and conditions (Figure 3.6).

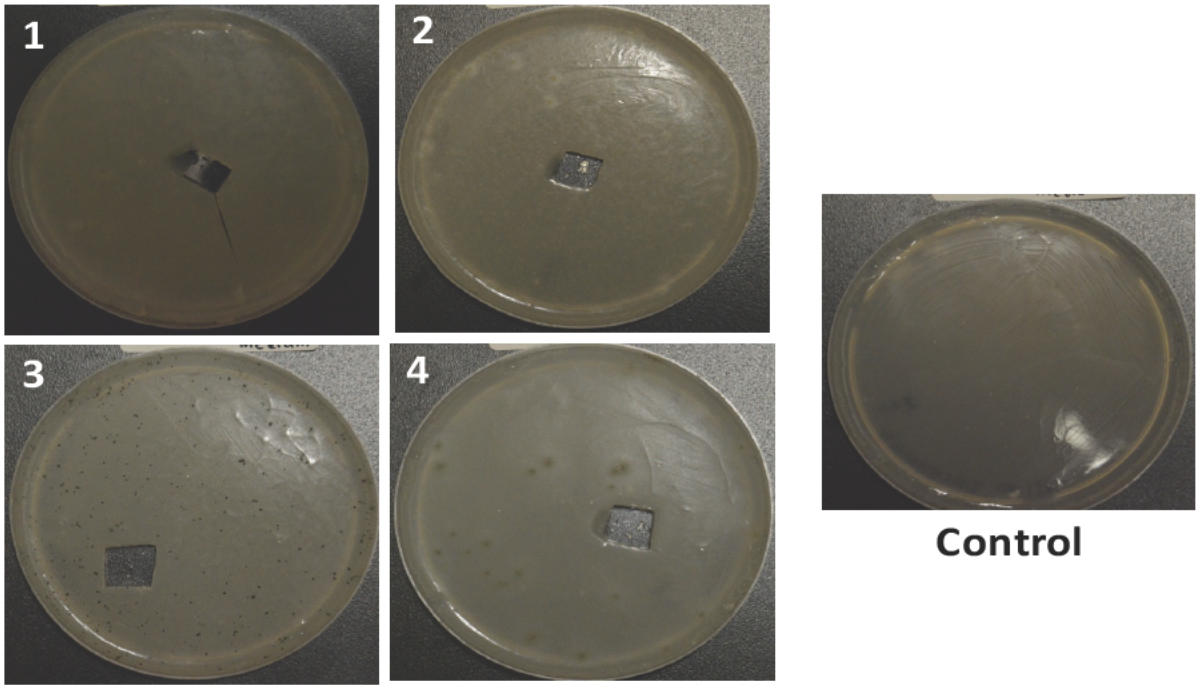

Figure 3.4. Four Cholesterol utilizing fungi on Selective A medium. Small blocks of cultures were cut from the plates and used to inoculate PD broth for further serial culturing and characterization.

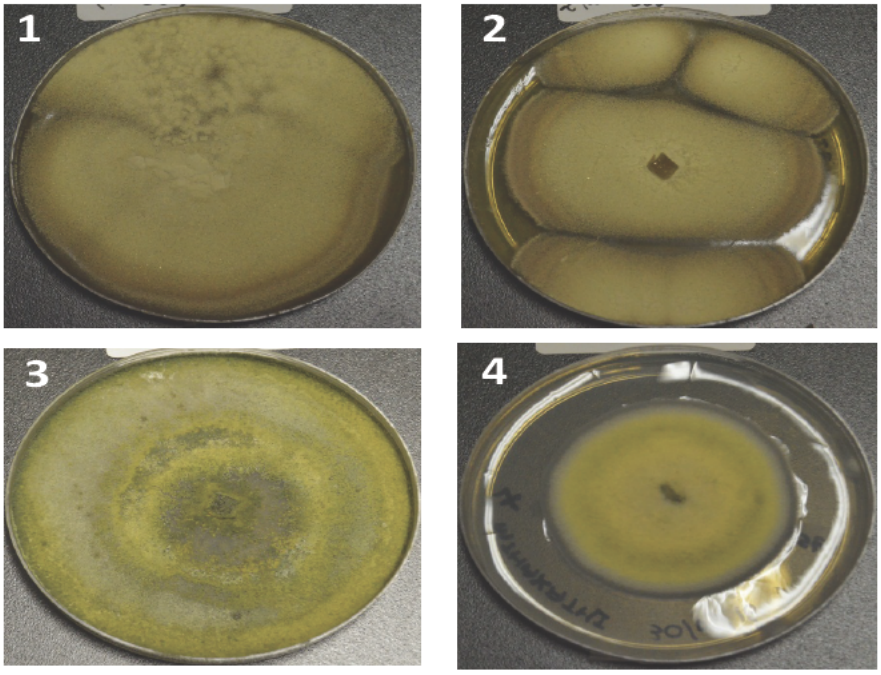

Figure 3.5. The growth of fungal isolates on PDA.

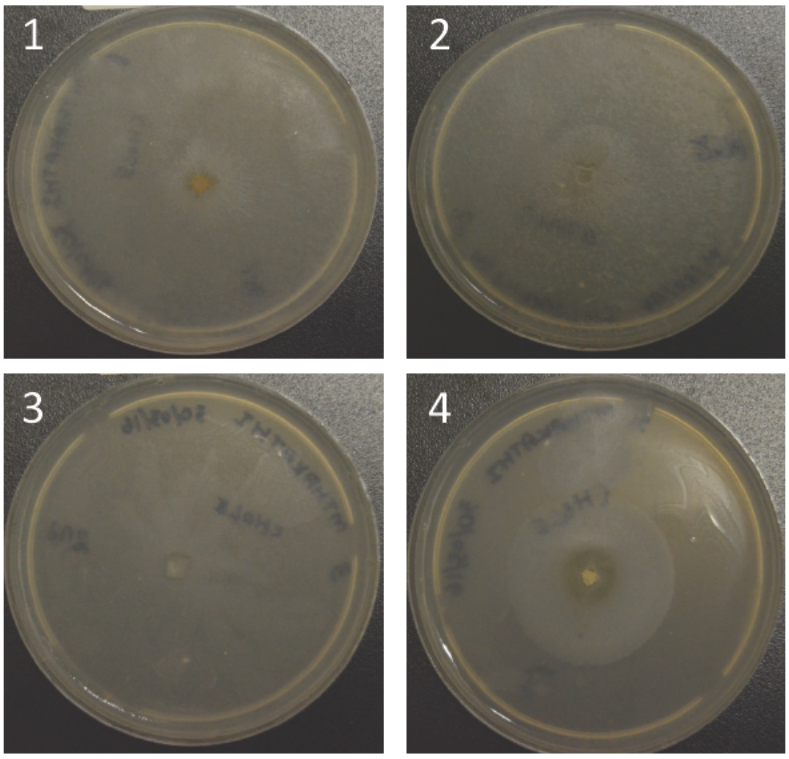

Figure 3.6. Growth of fungal isolates on Selective A medium where cholesterol is supplemented as a sole carbon source.

3.3.2. The significance of fungal cholesterol utilization

Many fungal species have been studied and characterized to grow on many carbon sources, however until this day, not one fungal specie has been shown neither documented anywhere to grow on cholesterol as the sole source of carbon. This study reports for the first time, fungal species utilizing cholesterol as the only source of carbon. This is a novel concept in fungi with undescribed, unlimited biotechnological, medical and biological impacts. This is because although fungi is one of the least explored biodiversity in our planet (Webster and Weber, 2007), fungi is a group of highly rich kingdom with about 120 000 identified (Hawksworth, 2001; Kirk *et al.,* 2001) and potentially 1.5-5.1 million undescribed species (Hawksworth, 2001; O'Brien *et al*., 2005; Schmit and Mueller, 2007; Kirk *et al*., 2008) in different habits such as aquatic systems (Kagami *et al.*, 2007; Kagami *et al*., 2007), forest and polar region soils (Allen, 1991; Smith and Read, 2008) and the phylloshere (Clay, 1988; Santamaría, and Bayman, 2005; Jumpponen and Jones, 2009). These organisms exert wide biological impacts such as being decomposers, symbionts of plants, viral hosts and parasites of various organisms (Toju *et al.,* 2012). They also carry human benefits in the fields of agriculture (Cardoso and Kuyper, 2006; Sawers *et al.,* 2008), pharmacy (Zjawiony, 2004), food industry and some environmental technologies (Mortimer, 2000; Wang and Chen, 2006). Still, the exploration of fungal diversity is crucial to provide invaluable resources for various fields of applied microbiology (Toju *et al*., 2012). Therefore, this novel concept of fungal cholesterol utilization will open a new window of possibilities and production of novel discoveries, including drug targets against pathogenic fungi. Many bacteria have been shown to degrade cholesterol and this study also shows that fungi can also degrade cholesterol, despite being known to be so recalcitrant to biodegradation.

3.4. Conclusion

The study demonstrates that bacteria are not the only group of microorganisms capable of degrading and utilizing cholesterol as the sole source of carbon and energy. However, it is no surprise that fungi isolated from the soil sample can degrade and utilize cholesterol as the carbon source, because fungi are found in different environments exerting a wide range of biologic impacts. Interestingly, the culture medium described to isolate cholesterol degrading bacteria, is also effective in isolating fungi with the same metabolic activity when supplemented with the trace elements. The fact that fungi can also utilize this steroid lipid as the source of carbon might change the perception of regarding cholesterol as the recalcitrant compound. As cholesterol is also present in the soil, it might be one of the overlooked carbon and energy sources driving the survival of fungal organisms in the soil. This also suggests that cholesterol, its analogs and phytosterols might also be the major fuel sources in fungal pathogenesis.

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CHAPTER 4

PHYLOGENETIC IDENTIFICATION OF CHOLESTRERROL UTILIZING FUNGI

4.1. Introduction

Fungi represent a class of microorganisms with great significance and biologic impacts in other domains of life. These range from agricultural applications (Cardoso and Kuyper, 2006; Sawers *et al.,* 2008) environmental technologies (Wang and Chen, 2006), them being pathogens with the potential of causing life-threatening diseases (Ponton *et al*., 2000) and to their abilities in performing critical ecosystem functions (Mosier *et al*., 2016). Their ability to synthesize primary and secondary metabolites is best utilized to manufacture active compounds such as antibiotics and many other drugs in the pharmaceutical industry (Lange, 2014; Sharma and Sharma, 2016). Fungal microorganisms are found everywhere, occupying every environment in the biosphere. Of all 1.5 million estimated fungal species, only about 300 species are known to cause diseases in humans and these pathogens are usually those that are common in the environment (CDC, 2014). According to an online article, fungal pathogens kill more people than malaria and tuberculosis worldwide and they also destroy about a third of all arable food crops (http://www.dailymail.co.uk/sciencetech/article-2912033/Could-fungus-big-killer-Experts-warn-fungal-diseases-cause-deaths-malaria-tuberculosis.html).

Considering there are quite a number of fungal species, it is essential to identify fungal species in order to understand or connect its characteristic to a particular condition or process etc. Accurate identification of fungal species by DNA sequencing is gaining momentum. Formerly, fungal species were identified by traditional methods comprising of morphological features (de Hoog *et al*., 2000) and carbohydrate assimilation and fermentation tests (Freydiere *et al.*, 2001). As the result of some endophytic fungi

demonstrating some intricacies in morphological features that could not allow identification to the specie level; some endophytic fungi producing a biased data by lacking spores (Jeewon *et al.,* 2013) and the fact that traditional methods are cumbersome and not suitable for non-specialized clinical microbiology laboratories, molecular testing strategies emerged.

Interestingly, molecular methodologies led to the discovery of an accurate fungal DNA barcoding method by means of the polymerase chain reaction. These studies led to the identification of ribosomal DNA (rDNA) sequence information (Pryce *et al*., 2003) by amplifying the internal transcribed spacer region (ITS) of fungal species. The ITS region has the highest successful identification for the broadest range of fungi (Schoch *et al*., 2012) and this region is also a variable sequence of great importance in distinguishing fungal species (Martin and Rygiewicz, 2005). This significant region is also employed in some fungal organisms to provide delimitation by a measure of the genetic distances (Del-Prado *et al*., 2010). Amplification of the ITS region coupled with phylogenetic analysis has enabled a reliable identification and classification of a number of unidentified fungal species (Jeewon *et al*., 2013) and over 90 000 fungal ITS region sequences have been deposited in public databases (Toju *et al.*, 2012).

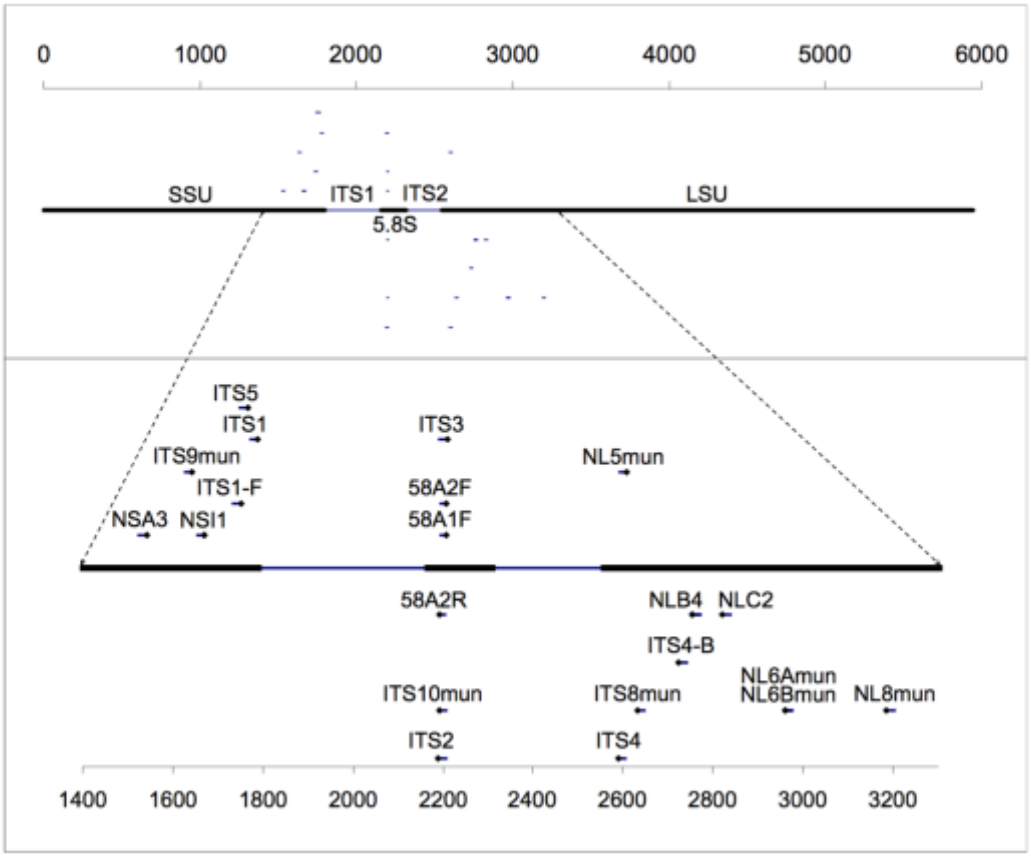

Figure 4.1. Diagram of primer locations in the ribosomal cassette consisting of SSU, ITS1, 5.8S, ITS2, and LSU rDNA. The diagram also shows the sub regions of the ITS1 and ITS2 along with appropriate primers, F (forward) and R (reverse). Taken from Martin and Rygiewicz, 2005).

The ITS region in fungal genomes has two main regions, ITS1 and ITS2, located between the 18S and 28S ribosomal subunits and which are separated by the 5.8S subunit (Chen *et al.*, 2000, 2001), which is located within the ITS region (Figure 4.1). In eukaryotes, the rRNA is transcribed by RNA polymerase as a cistron consisting of the 18S, 5.8S and the 28S rRNA, all three playing an important role in identification, classification and phylogenetic analyses. Though transcribed as a unit, the cistron is split by posttranscriptional processes to remove the two internal transcribed spacers from the 18S and the 28S subunit, thus, the two internal transcribed spacers and the 5.8S gene are the ITS (Schoch *et al.*, 2012).

The 18S nuclear ribosomal small subunit rRNA gene (SSU) is usually used in phylogenetic studies, while the 28S nuclear ribosomal large subunit gene (LSU) is rather used sometimes to discriminate species on its own or in combination with the ITS (Stackebrandt and Goebel, 1994; Schoch *et al.*, 2012). This chapter reports the identification of four fungal isolates by amplifying the ITS region in combination with the 18S and 28S subunits to accurately identify the fungal isolates to specie level.

4.2. Materials and methods

4.2.1. Genomic DNA isolation

Genomic DNA was isolated from fungal cultures using the ZR fungal/Bacterial DNA MiniPrep kit (Cat. No. D6005, ZymoResearch, South Africa,) following the manufacturer's protocol, and analysed using Agarose-gel electrophoresis.

4.2.2. PCR amplification and sub-cloning of ribosomal DNA subunits

Primers for amplification of the 28S, 18S and 5.8S subunits of rDNA were selected from Fungal Barcoding Database (http://www.fungalbarcoding.org/), and supplied by Integrated DNA Technologies (Table 4.1). The target regions were amplified accordingly using the KAPA HiFi HotStart PCR kit (KAPA Biosystems) according to manufacturer instructions and the parameters provided (Table 4.2). Generated amplicons were purified from agarose gels using the Wizard® SV Gel and PCR Clean-Up System (Promega). The 5.8S amplicons were phosphorylated using T4 polynucleotide kinase (Thermo Fischer Scientific) and sub cloned using the Zero Blunt® PCR cloning kit (Invitrogen). The recombinant plasmid was propagated using SIG10 5α chemically competent cells (Sigma-Aldrich), and isolated using the lysis by boiling method described by Sambrook and Green (2001).

Table 4.1. Oligonucleotide primer sequences for amplification of rDNA subunits. bp, is the base pairs; Tm, is the melting temperatures; FP, is forward primer; RP, is reverse primer.

Subunit	Primer name	Sequence (5'-3')	Length (bp)	Tm (°C)
28S	NS1-18FP	GTA GTC ATA TGC TTG TCT C	19	52.4
	NS1-18RP	CTT CCG TCA ATT CCT TTA AG	20	51.1
18S	LR0R-28FP	ACC CGC TGA ACT TAA GC	17	47.2
	LR0R-28RP	TCC TGA GGG AAA CTT CG	17	48.7
5.8S	ITS1-5.8FP	TCC GTA GGT GAA CCT GCG G	19	59.5
	ITS1-5.8RP	TCC TCC GCT TAT TGA TAT GC	20	52.1

Table 4.2. PCR parameters for amplification of rDNA subunits using KAPA HiFi DNA polymerase.

			Subunit				
			28S	**18S**	**5.8S**		
Temperature (°C)	95	98	60.5	56.1	63.5	72	72
Time	3 min	20 sec	15 sec			45 sec	1 min
Number of cycles	1	25					1

4.2.3. Sequence analysis

Samples were prepared for sequencing using the BigDye™ Terminator V3.1 Cycle Sequencing Kit (Catalog number: 4337455, Thermo Fischer Scientific, South Africa). The aforementioned primers were used for sequencing, except in the case of the sub cloned 5.8S regions, for which the M13 Forward and Reverse primers provided in the kit were used. The sequencing reactions were performed according to the parameters described by the manufacturer.

Sequencing reactions were purified using the EDTA-Ethanol method described by the manufacturer, and submitted for sequencing using a 3130xl Genetic Analyzer (Applied Biosystems) at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State. Consensus sequences were derived from the sequences obtained

from the forward and reverse primer reactions for each product, using Geneious® R9 9.1.2. software.

4.2.4. Construction of phylogenetic trees

Phylogenetic analysis of fungal isolates was carried out using the nucleotide sequences of the 5.8S, 18S and 28S ribosomal rDNA subunits. Nucleotide sequences were compared with sequences in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/BLAST.cgi), as well as the fungal barcoding database (http://www.fungalbarcoding.org/) (Schoch *et al*., 2012(. The sequences for the first hits were downloaded and subjected to evolutionary analysis. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura- Nei model)Tamura and Nei, 1993). Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016). An out-group rDNA sequence was also included in the phylogenetic tree.

4.3. Results and discussion

4.3.1. PCR amplification of different rRNA genes and phylogenetic identification of fungal isolates

In order to identify the fungal isolates, rRNA gene sequence-based phylogenetic analysis was carried out. The rRNA genes (5.8S, 18S, and 28S) of fungal isolates were PCR amplified using the primers listed in Table 4.1 using the protocol listed in Table 4.2. Analysis of the PCR amplified products on Agarose gel showed prominent DNA bands with different sizes as per rRNA gene (Figure 4.3). This indicates specific amplification of different rRNA genes. The amplified rRNA genes were gel purified and subjected to sequence analysis using the same primers used for their amplification. Sequence analysis was performed using both the forward and reverse primers, yielding a consensus sequence of 300-500 overlapping base

pairs between the sequences. The rRNA gene sequences generated in this study is provided in the Appendix.

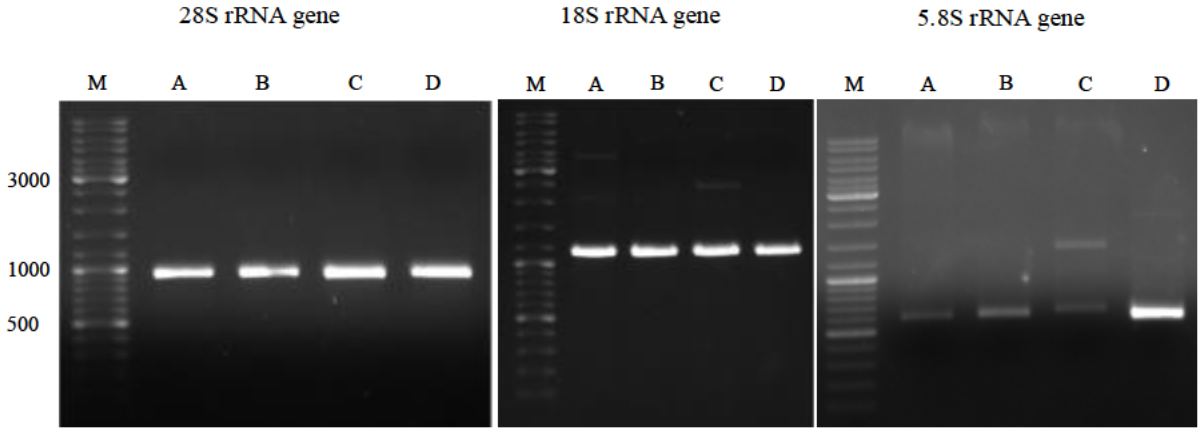

Figure 4.2. Agarose gel electrophoresis analysis of ribosomal DNA genes. PCR amplified products were run on 1% Agarose gel. Lane M, indicates DNA Ladder (O'GeneRuler DNA Ladder Mix 100-10000 bp, from ThermoFisher, Catalog number SM1173). Lanes A to D indicate the respective fungal species.

The size of rRNA gene sequence obtained for each of the isolate is presented in Table 4.3. 5.8S, 18S and 28S rRNA sequence of isolates 1 and 2 showed 100% identity to *Aspergillus terreus* strain (Table 4.3). Isolates 3 and 4 showed 100% identity for all three rRNA genes sequences with *Trichoderma longibrachiatum* and *Talaromyces pinophilum*. Phylogenetic analysis of isolates based on different rRNA gene sequences compared to the rRNA gene sequences of hit species, highlighted the differential alignment of fungal isolates with different species (Figures 4.3-4.6). Based on the phylogenetic alignment and percentage identity, the four fungal isolates were named as shown in Table 4.3. Furthermore, homology analysis (percent identity) of rRNA gene sequences between two *A. terreus* strains NTMSK1 and NTMSK2 revealed they were 100% identical.

Table 4.3. Information regarding fungal isolates identification.

Sample number	rRNA gene	rRNA gene sequence length in base pairs	NCBI Blast hit results			Name assigned to the bacterial isolate
			Fungi	Percent identity	Query cover percentage	
1	5.8S	538	*A. terreus* strain FJAT-31038	100	100	*A. terreus* strain NTMSK1
	18S	1003	*A. terreus* strain VV08	100	100	
	28S	871	*A. terreus* strain DAOM	100	100	
2	5.8S	605	*A. terreus* strain F9L	100	100	*A. terreus* strain NTMSK2
	18S	961	*A. terreus* strain VV08	99	100	
	28S	865	*A. terreus* strain KAML04	100	100	
3	5.8S	637	*T. longibrachiatum* strain T161	100	100	*T. longibrachiatum* strain NTMSK3
	18S	756	*Hypomyces chrysospermus* strain JADVS1	100	100	
	28S	698	*T. longibrachiatum* strain ATCC	100	100	
4	5.8S	579	*T. pinophilum* strain SGE75	100	100	*T. pinophilum* strain NTMSK4
	18S	1000	*Penicillium pinophilum* isolate MR-*2*	100	100	
	28S	886	*P. pinophilum* strain KUC1758	100	100	

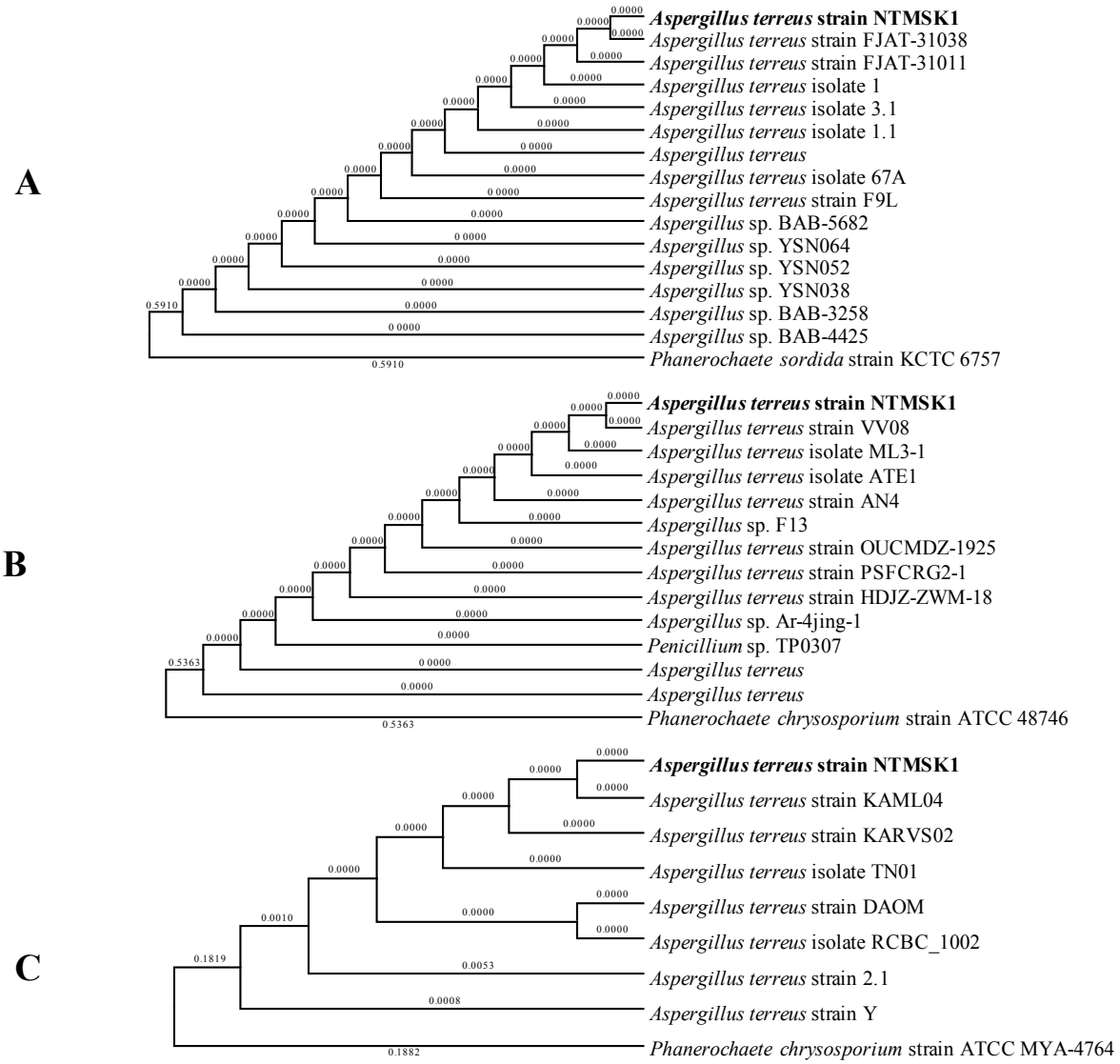

Figure 1.3. The phylogenetic tree of *A. terreus* strain NTMSK1. The phylogenetic analysis and representation of the ITS, SSU and LSU subunits with the close homologs of *A. terreus* strain NTMSK1 and the outgroup, *Phanerochaete* spp. A, B, C represent phylogenetic tree of the 5.8S, 18S and 28S rRNA gene sequence. Branch lengths are also shown in the tree. Fungal species isolated and named in this study are highlighted with bold font.

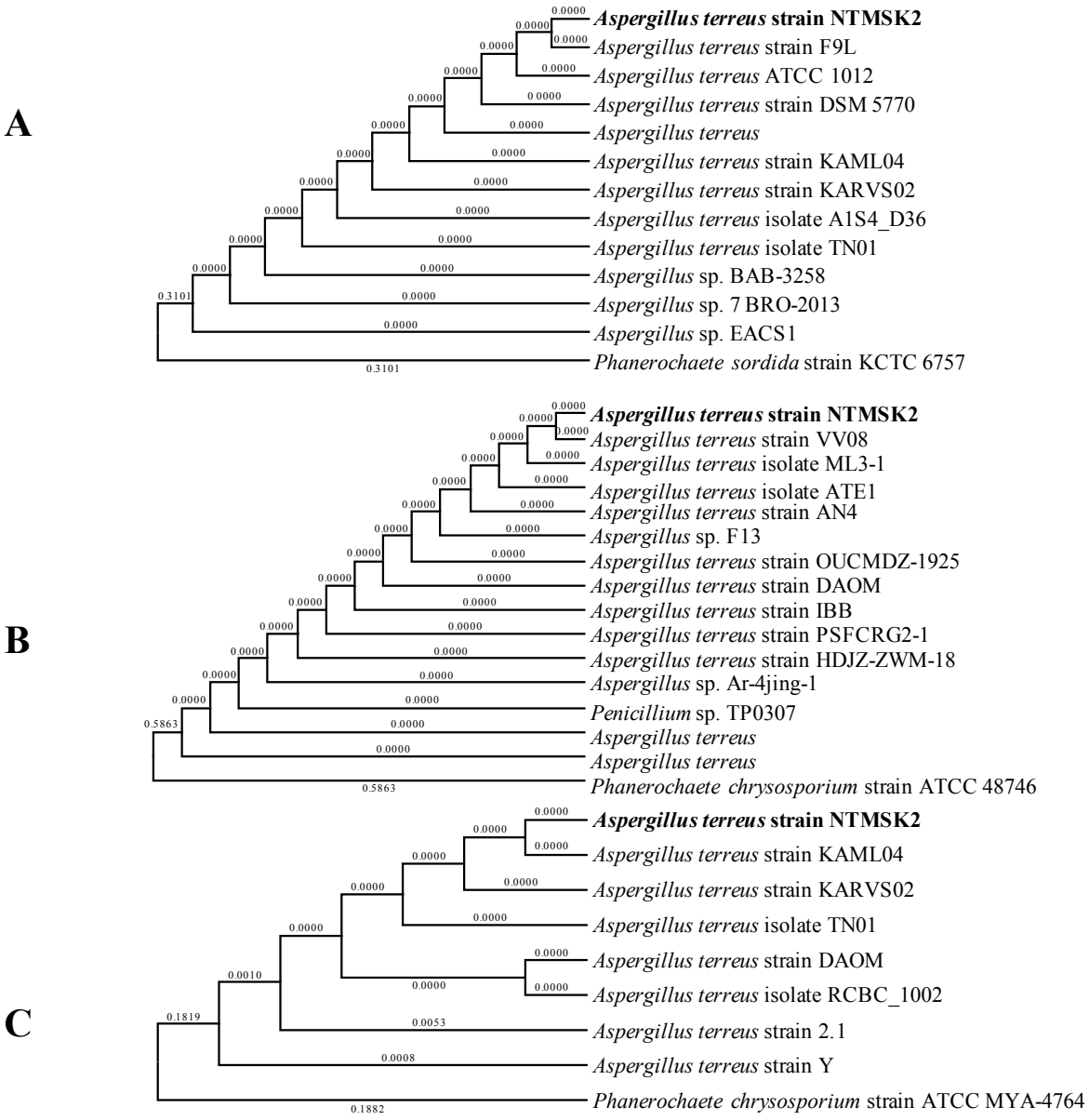

Figure 4.4. The phylogenetic tree of *A. terreus* strain NTMSK2. The phylogenetic analysis and representation of the ITS, SSU and LSU subunits with the close homologs of *A. terreus* strain NTMSK2 and the outgroup, *Phanerochaete* spp. A, B, C represent phylogenetic tree of the 5.8S, 18S and 28S rRNA gene sequence. Branch lengths are also shown in the tree. Fungal species isolated and named in this study are highlighted with bold font.

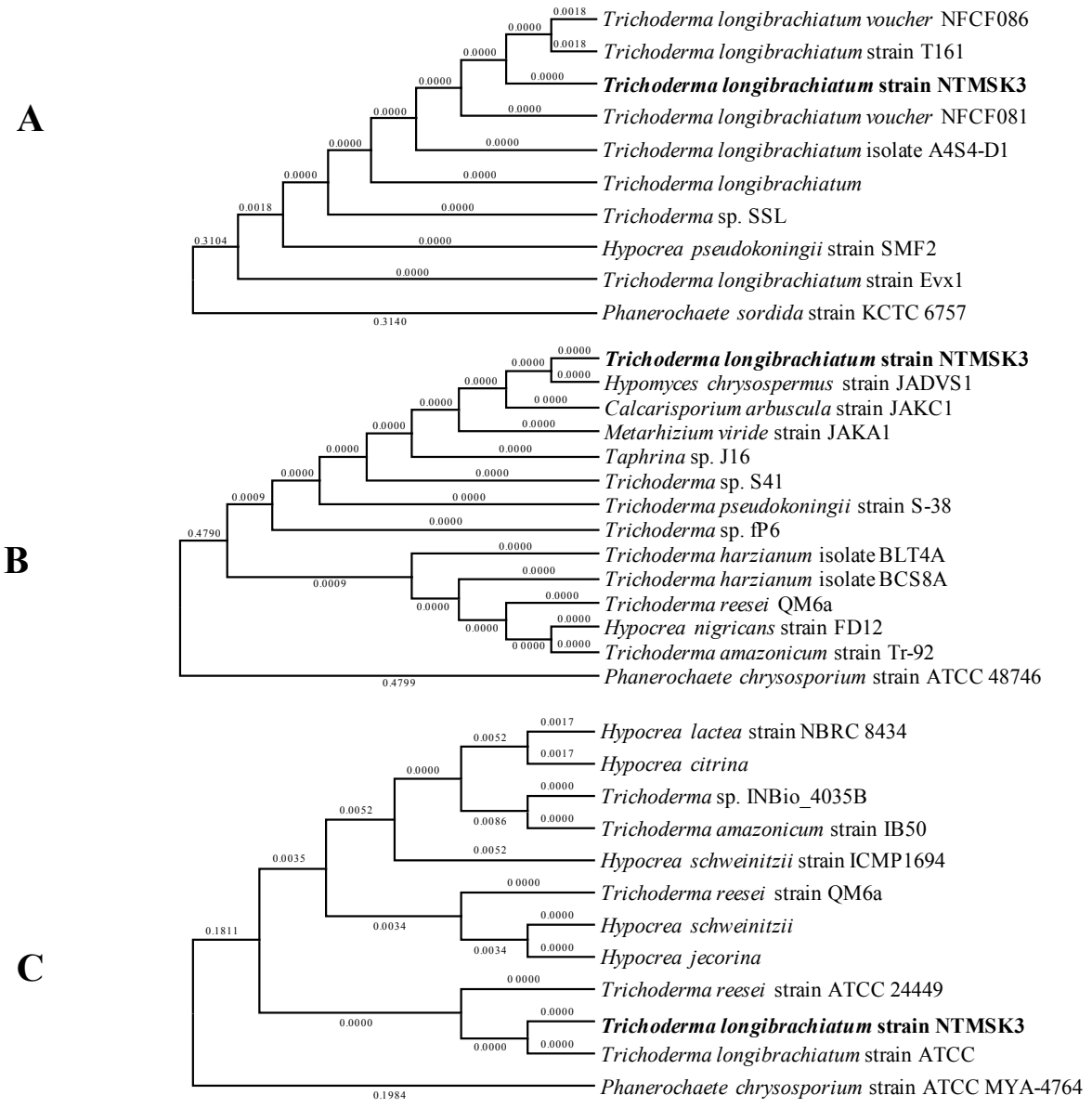

Figure 4.5. The phylogenetic tree of *T. longibrachiatum* strain NTMSK3. The phylogenetic analysis and representation of the ITS, SSU and LSU subunits with the close homologs of *T. longibraciutum* strain NTMSK3 and the outgroup, *Phanerochaete* spp. A, B, C represent phylogenetic tree of the 5.8S, 18S and 28S rRNA gene sequence. Branch lengths are also shown in the tree. Fungal species isolated and named in this study are highlighted with bold font.

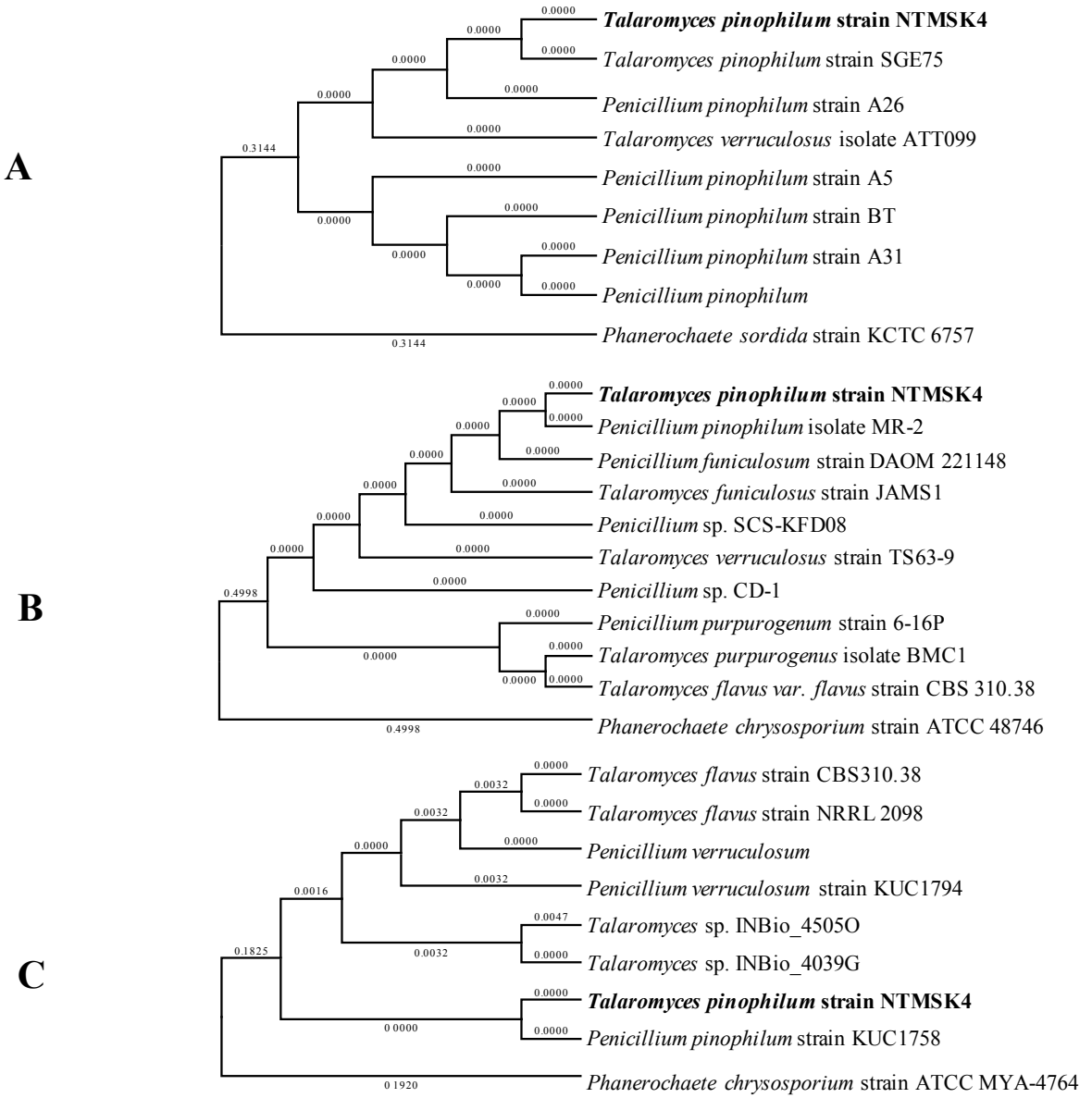

Figure 4.6. The phylogenetic tree of *T. pinophilum* strain NTMSK4. The phylogenetic analysis and representation of the ITS, SSU and LSU subunits with the close homologs of *T. pinophilum* NTMSK4 and the outgroup, *Phanerochaete* spp. A, B, C represent phylogenetic tree of the 5.8S, 18S and 28S rRNA gene sequence. Branch lengths are also shown in the tree. Fungal species isolated and named in this study are highlighted with bold font. *T. pinophilum* was originally called *P. pinophilum* (Ohte *et al*., 2011; Uchida *et al*., 2013) hence aligning with the *Penicillium* spp.

All the isolates in the study belong to Ascomycota phylum and are known common inhabitants of the soil. *T. longibrachiatum*, an emerging clinical pathogen, is a known soil inhabitant thriving as a toxic house mould that produces trilongins peptides (Mikkola *et al*., 2010) and is also important industrially for production of xylanase (Azin *et al*., 2007). *T. pinophilum* is another soil inhabitant known to degrade agricultural wastes and can produce *β*-glucosidase (El-Naggar *et al*., 2015). *A. terreus* is a fungus inhabiting the soil, decomposing vegetation and dust (Al-Doory and Domson, 1984). It is the most pathogenic and virulent fungi (Lass-Flörl *et al*., 2005) and known to be an emerging opportunistic pathogen (Steinbach *et al*., 2012) with a high mortality of invasive infections (Pastor and Guarro, 2014). This opportunistic pathogen is capable of causing both systemic and superficial infections in human beings (Mariana *et al*., 2013). It's not only an opportunistic human pathogen, but can also cause detrimental diseases in other animals and plants. These include mycotic abortions in cattle, sinusitis and osteomyelitis in dogs (Richard, 2013). In plants, it can affect the third-most important food crop potatoes, by causing the foliar blight of the potatoes (Louis *et al*., 2013). It can also disrupt the male reproductive cycle of *Arabidopsis thaliana*, ultimately posing a negative genetic diversity in the plant species (Shimada *et al.,* 2002). This fungus is also a prolific producer of many secondary metabolites with many clinical and industrial uses (Schimmel *et al*., 1998). The most significant results about these fungi are their pathogenicity towards humans, other animals and plants. It was demonstrated in the beginning of the 20th century that cholesterol, phytosterols and other structurally related compounds can be found on the soil (Schreiner and Shorey, 1911).

4.4. Conclusion

Correct identification of microbes is critical, as accurate identification is a cornerstone for subsequent biologic interpretation, manipulation and testing. In the current study, isolates were accurately identified to species level using three fungal subunits, 5.8S rDNA, 18S rDNA and 28S rDNA. All isolates were found to belong to Ascomycota phylum. Two isolates are *A. terreus* and remaining species are *T. longibrachiatum* and *T. pinophilum*. The study demonstrates that fungi can survive in the soil by utilizing cholesterol and possibly other structurally related compounds like phytosterols as carbon sources. Hence, it is likely that these pathogens also utilize *in vivo* cholesterol as the carbon source to survive in mammals and cause diseases.

4.5. References

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Appendix A

Fungal isolates ribosomal rRNA gene sequences

A. terreus strain NTMSK1:

28S rDNA sequence:

CAATAAGCGGAGGAAAAGAAACCCACCCGGGATTGCCTCAGTAACGGCGAGTGAAGCGGCAAGAGCTCAAATTTGAAAGCTGGCTCCTTCGGGG
TCCGCATTGTAATTTGCAGAGGATGCTTCGGGTGCAGCCCCCGTCTAAGTGCCCTGGAACGGGCCGTCATAGAGGGTGAGAATCCCGTATGGGG
CGGGGTGTCTGCGTCCGTGTGAAGCTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGTGGTAAATTTCATCTAAAGCTAAAT
ACTGGCCGGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGAAAAGAGAGTTAAACAGCACGTGAAATTGTTGAAAGG
GAAGCGCTTGCAACCAGACTCGCTCGCGGGGTTCAGCCGGGCTTCGGCCCGGTGTACTTCCCCGCGGGCGGGCCAGCGTCGGTTTGGGCGGCCG
GTCAAAGGCCTCCGGAATGTAGCGCCCTTCGGGGCGCCTTATAGCCGGGGGTGCAATGCGGCCAGCCTGGACCGAGGAACGCGCTTCGGCACGG
ACGCTGGCATAATGGTTGTAAACGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATCTACGCGAGTGTTCGGGTGTCAAACCCGTACGCGC
AGTGAAAGCGAACGGAGGTGGGAGCCCCCTCGCGGGGCGCACCATCGACCGATCCTGATGTCTTCGGATGGATTTGAGTACGAGCGTAGCTGTG
GGGACCCGAAAGATGGTGAACTATGCCTGAATAGGGCGAAGCCAGAGGAAACTCTGGTGGAGGCTCGCAGCGGTTCTGACGTGCAAATCGATCG
TCAAATTTGGGTATAGGGGCGAAAG

18S rDNA sequence:

GCGAATGGCTCATTAAATCAGTTATCGTTTATTTGATAGTACCTTACTACATGGATACCTGTGGTAATTCTAGAGCTAATACATGCTAAAAACC
CCGACTTCGGAAGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCTCCTTGGTGATTCATAATAACTTAACGAATCGCATGGCCTT
GCGCCGGCGATGGTTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAGTGGCCTACCATGGTGGCAACGGGTAACGGGGAATTAGG
GTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGT
GACAATAAATACTGATACGGGGCTCTTTCGGGTCTCGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAGGAACAATTGGAGGGCAAGTCT
GGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAACCTTGGGTCTGGCTGGCC
GGTCCGCCTCACCGCGAGTACTGGTCCGGCTGGACCTTTCCTTCTGGGGAATCCCATGGCCTTCACTGGCTGTGGGGGGAACCAGGACTTTTAC
TGTGAAAAAATTAGAGTGTTCAAAGCAGGCCTTTGCTCGAATACATTAGCATGGAATAATAGAATAGGACGTGCGGTTCTATTTTGTTGGTTTC
TAGGACCGCCGTAATGATTAATAGGGATAGTCGGGGGCGTCAGTATTCAGCTGTCAGAGGTGAAATTCTTGGATTTGCTGAAGACTAACTACTG
CGAAAGCATTCGCCAAGGATGTTTTCATTAATCAGGGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACT
ATGCCGACTAGGGATCGGGCGGTGTTTCTATGATGACCCGCTCGGCACCTTACGAGAAATCAA

5.8S rDNA sequence:

CAAGTTGCAAATAAATGCGTCGGCGGGCGCCGGCCGGGCCTACGGAGCGGAAGACGAAGCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCG
CTGCCTTTCGGGCCCGTCCCCCGGGAGCCGGGGGACGAGGGCCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCC
CCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTAGTTATCGCATTTCGCTGCGTTCT
TCATCGATGCCGGAACCAAGAGATCCATTGTTGAAAGTTTTAACTGATTGCAAAGAATCACACTCAGACTGCAAGCTTTCAGAACAGGGTTCAT
GTTGGGGTCTCCGGCGGGCACGGGCCCGGGGGCGAGTCGCCCCCCGGCGGCCAGCAACGCTGGCGGGCCCGCCGAAGCAACAAGGTACAATAGT
CACGGGTGGGAGGTTGGGCCATAAAGACCCGCACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGA

A. terreus strain NTMSK2:

28S rDNA sequence:

AACGGCGAGTGAAGCGGCAAGAGCTCAAATTTGAAAGCTGGCTCCTTCGGGGTCCGCATTGTAATTTGCAGAGGATGCTTCGGGTGCAGCCCCC
GTCTAAGTGCCCTGGAACGGGCCGTCATAGAGGGTGAGAATCCCGTATGGGGCGGGGTGTCTGCGTCCGTGTGAAGCTCCTTCGACGAGTCGAG
TTGTTTGGGAATGCAGCTCTAAATGGGTGGTAAATTTCATCTAAAGCTAAATACTGGCCGGAGACCGATAGCGCACAAGTAGAGTGATCGAAAG
ATGAAAAGCACTTTGAAAAGAGAGTTAAACAGCACGTGAAATTGTTGAAAGGGAAGCGCTTGCAACCAGACTCGCTCGCGGGGTTCAGCCGGGC
TTCGGCCCGGTGTACTTCCCCGCGGGCGGGCCAGCGTCGGTTTGGGCGGCCGGTCAAAGGCCTCCGGAATGTAGCGCCCTTCGGGGCGCCTTAT
AGCCGGGGGTGCAATGCGGCCAGCCTGGACCGAGGAACGCGCTTCGGCACGGACGCTGGCATAATGGTTGTAAACGACCCGTCTTGAAACACGG
ACCAAGGAGTCTAACATCTACGCGAGTGTTCGGGTGTCAAACCCGTACGCGCAGTGAAAGCGAACGGAGGTGGGAGCCCCCTCGCGGGGCGCAC
CATCGACCGATCCTGATGTCTTCGGATGGATTTGAGTACGAGCGTAGCTGTGGGGACCCGAAAGATGGTGAACTATGCCTGAATAGGGCGAAGC
CAGAGGAAACTCTGGTGGAGGCTCGCAGCGGTCTGACGTGCAAATCGATCGTCAAATTTGGGTATAGGGGCGAAAGACTAATCGAAACCATCTG
GTAGCTGGTTCCTGCCGAA

18S rDNA sequence:

ACTGCGAATGGCTCATTAAATCAGTTATCGTTTATTTGATAGTACCTTACTACATGGATACCTGTGGTAATTCTAGAGCTAATACATGCTAAAA
ACCCCGACTTCGGAAGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCTCCTTGGTGATTCATAATAACTTAACGAATCGCATGGC
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TCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAACCTTGGGTCTGGCTG
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TTCTAGGACCGCCGTAATGATTAATAGGGATAGTCGGGGGCGTCAGTATTCAGCTGTCAGAGGTGAAATTCTTGGATTTGCTGAAGACTAACTA
CTGCGAAAGCATTCGCCAAGGATGTTTTCATTAATCAGGGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAA
ACTATGCCGACTAGGGATCGG

5.8S rDNA sequence:

TCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGCGGGTCTTTATGGCCCAACCTCCCACCCGTGACTATTGTACCTTGTTGCTTCGGCGG
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TGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCT
GCCCTCAAGCCCGGCTTGTGTGTTGGGCCCTCGTCCCCCGGCTCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAG
CGTATGGGGCTTCGTCTTCCGCTCCGTAGGCCCGGCCGGCGCCCGCCGACGCATTTATTTGCAACTTGTTTTTTTCCAGGTTGACCTCGGATCA
GGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGA

T. longibrachiatum strain NTMSK3:

5.8S rDNA sequence:

TCCGTAGGTGAACCTGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCAATGTGAACGTTACCAATCTGTTGCCTCGGCGGGATTCTC
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CTCTGTTTTATTTTTGCTCTGAGCCTTTCTCGGCGACCCTAGCGGGCGTCTCGAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTG
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TATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGGTCGGCGTTGGGGATCGGCCCCTCACCGGGCCGCCCC
CGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCACCGGGAGCGCGGCGCGGCCACAGCCGTAAAACACCC
CAAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

18S rDNA sequence:

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TCGACCCCGGAGAAGGAGCCTGAGAAACGGCTACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGA
CAATAAATACTGATACAGGGCTCTTTTGGGTCTTGTAATCGGAATGAGTACAATTTAAATCCCTTAACGAGGAACAATTGGAGGGCAAGTCTGG
TGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGTGGTTAAAAAGCTCGTAGTTGAACCTTGGGCCTGGCTGGCCGG
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TTGAAAAAAATTAGAGTGCTCAAGGCAGGCCTATGCTCGAATACATTAGCATGGAATAATAGAATAGGACGTGTGGTTCTATTTTGTTGGTTTC
TAGG

28S rDNA sequence:

CCCCAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCCCTTACGGGTCCGAGTTGTAATTTGTAGAGGATGCTTTTGGCAAG
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GGGTTCTCCCCGGTGCACTTCGCCGCGTCCAGGCCAGCATCAGTTCGTCGCGGGGGAAAAAGGCTTCGGGAACGTGGCTCCCTCGGGAGTGTTA
TAGCCCGTTGCGTAATACCCTGCGGTGGACTGAGGACCGCGCATCTGCAAGGATGCTGGCGTAATGGTCACCAGCGACCCGTCTTGAAACACGG
ACCAAGGAGTCGTCTTCGTATGCGAGTGTTCGGGTGTCAAACCCCTACGCGTAATGAAAGTGAACGCAGGTGAGAGCTTCGGCGCATCATCGAC
CGATCCTGATGTTCTCGGATGGATTTGAGTAAGAGCATAC

T. pinophilum strain NTMSK4:

5.8S rDNA sequence:

TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTAACTCCTACCTGATCCGAGGTCAACCGTGGTAAAACATGGTGGTGACCAACCCCCGCAG
GTCCTTCCCGAGCGAGTGACAGAGCCCCATACGCTCGAGGACCAGACGGACGTCGCCGCTGCCTTTCGGGCAGGTCCCCGGGGGGACCACACCC
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ATTCACGGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCATTGTTGAAAGTTTTGAC
AATTTTCACAGTACTCAGACAGCCCATCTTCATCAGGGTTCACAGAGCGCTTCGGCGGGCGCGGGCCCGGAGACGTGCGTCCCCCGGCGACCAG
GTGGCCCCGGTGGGCCCGCCAAAGCAACAGGTGTATAGAGACAAGGGTGGGAGGTTGGGCCGCGAGGGCCCGCACTCGGTAATGATCCTTCCGC
AGGTTCACCTACGGA

18S rDNA sequence:

GCGAATGGCTCATTAAATCAGTTATCGTTTATTTGATAGTACCCTACTACATGGATACCTGTGGTAATTCTAGAGCTAATACATGCGCAAAACC
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CGGTCCGCCTCACCGCGAGTACTGGTCCGGATGGGCCTTTCTTTCTGGGGAATCCCATGGCCTTCACTGGCTGTGGCGGGGAACCAGGACTTTT
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TCTAGGACCGCCGTAATGATTAATAGGGATAGTCGGGGGCGTCAGTATTCAGCTGTCAGAGGTGAAATTCTTGGATTTGCTGAAGACTAACTAC
TGCGAAAGCATTCGCCAAGGATGTTTTCATTAATCAGGGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAA
CTATGCCGACTAGGGATCGGGCGGGGTTTCTATGATGACCCGCTCGGCACCTTACGAGAA

28S r

DNA sequence:

GATTAGTCTTTCGCCCCTATACCCAAATTTGACGATCGATTTGCACGTCAGNAACCGCTGCGAGCCTCCACCAGAGTTTCCTCTGGCTTCGCCC
TATTCAGGCATAGTTCACCATCTTTCGGGTCCCAACAGCTATGCTCTTACTCAAATCCATCCGAAGACATCAGGATCGGTCGATGGTGCGCCCC
GAGGGGCTCCCACCTCCGTTCGCTTTCACTGCGCGGACGGGTTTGACACCCGAACACTCGCATAGATGTTAGACTCCTTGGTCCGTGTTTCAAG
ACGGGCCGTTGACCACCATTACGCCAGCATCCTCGCCGAAGCGCGGGCCTCGGTCCAGGCTGGCTGTATGGCACCCCGGGCTATAAGGCACCCC
GAAGGGTGGCACATTCCCGGGGCCTTTCACCAGCCGCCCAAACCGATGCTGGCCCGCCCGGAGAGGAGTACACCGGCACACGTGCCGGCTGAAC
CCCCCCGGGCGAGTCTGGTGGACAACGCTTCCCTTTCAACAATTTCACGTGCTGTTTAACTCTCTTTTCAAAGTGCTTTTCATCTTTCGATCAC
TCTACTTGTGCGCTATCGGTCTCCGGCCAGTATTTAGCTTTAGATGAAATTTACCACCCGCTTAGAGCTGCATTCCCAAACAACTCGACTCGTC
GAAGGAGCTTCACACGGGCGCGGCCGCCCATCCCAGACGGGATTCTCACCCTCTATGACGGCCCGTTCCAGGGCACTTAGACGGGGACCGCACC
CGAAGCATCCTCTGCAAATTACAACTCGGACCCCAAAGGGGCCAGATTTCAAATTTGAGCTCTTGCCGCTTCACTCGCCGTTACTGAGGCAATC
CCGGTTGGTTTCTTTTCCTCCGCTTATTGATATGCTTAAG

CHAPTER 5

CONCLUSION

Cholesterol biotransformation studies have been performed with positive results for over seven decades. Many bacteria capable of bio-transforming and/or utilizing cholesterol as the carbon source were isolated from many sources in the environment. These studies over the years showed that bacterial bioconversion of cholesterol can be utilized for production of pharmaceutical compounds, fungicides, pesticides, clinical cholesterol biosensors and probiotics. It is now well-established that the etiologic agent of tuberculosis, *Mycobacterium tuberculosis*, uses human cholesterol as the carbon and energy source to survive and cause the disease. To date, bacteria are the only organism demonstrated and known to metabolize cholesterol.

The current study performed the isolation of cholesterol degrading microorganisms, like all other previous studies, but the results were far astonishing. Surprisingly, fungal organisms capable of using cholesterol as carbon source were isolated. The isolates were phylogenetically identified to specie level by using the three fungal ribosomal subunits, 5.8S rRNA, 18S rRNA and 28S rRNA. The isolated fungal strains are animal, plant and human pathogens. Fungi has never been shown to utilize cholesterol as their only source of carbon and energy. Demonstrating that fungi can grow utilizing cholesterol as the source of carbon and energy is novel in fungal lipid research.

Although fungal diseases are attributed to less than 5 % of known fungal organisms, they kill more people than tuberculosis and malaria globally. The high statistics can be blamed to patients' non-compliance with medication and lack of medical attention. However, scientists have reported the developing resistance of fungal pathogens to currently administered drugs, and the limitation and shortcoming of other fungal drugs. This dilemma has led scientists to suggest intense experimentation and more studies on the discovery of

novel drug targets. Studies also show that fungal pathogenicity destroys a third of arable food crops. This does not only affect the economy, but severely reduces and limits food supply that escalates the problem of malnutrition, starvation and infection from eating contaminated foods. All these factors ultimately lead to rise in fungal mortality rates.

The current study demonstrating that cholesterol serves as the carbon source to fungal pathogens opens a new field in fungal pathogenicity and studying of fungal infection mechanisms. Cholesterol and its homologs including phytosterols are ubiquitous in nature as much as cholesterol is an abundant lipid in the human body. Hence, unravelling the cholesterol degradation pathway in fungi will catalyse the development of novel fungal drug targets against animals (including humans) and plants pathogens.

The current study opens a new field in fungal pathogenesis. Bacteria as prokaryotic organisms were the only organisms known to degrade cholesterol before the dawn of the current study.

RESEARCH OUTPUTS

Research articles

SOUTH AFRICAN Journal of Science

Isolation and characterization of endocrine disruptor nonylphenol-using bacteria from South Africa

Journal:	*South African Journal of Science*
Manuscript ID	SAJS-2016-0287
Manuscript Type:	Research Article
Significance of the Work:	First report on endocrine disruptor nonylphenol-using bacteria from South Africa, Study will pave the way for further exploration of endocrine disruptors degrading microbes from South Africa, Six bacterial species capable of using nonylphenol as carbon source isolated, Phylogenetic analysis of isolates were carriedout using 16S rRNA gene sequence, HPLC analysis revealed degradation of Nonylphenol by all six bacterial isolates
Keywords:	Bacteria, Biodegradation, Endocrine disrupting chemicals, Nonylphenol, Phylogenetic analysis

Isolation and characterization of endocrine disruptor nonylphenol degrading bacteria from South Africa

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Running head: Nonylphenol-using bacteria from South Africa

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Molecular evolutionary dynamics of cytochrome P450 monooxygenases across kingdoms: Special focus on mycobacterial P450s

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Since the initial identification of cytochrome P450 monooxygenases (CYPs/P450s), great progress has been made in understanding their structure-function relationship, diversity and application in producing compounds beneficial to humans. However, the molecular evolution of P450s in terms of their dynamics both at protein and DNA levels and functional conservation across kingdoms still needs investigation. In this study, we analyzed 17 598 P450s belonging to 113 P450 families (bacteria —42; fungi —19; plant —28; animal —22; plant and animal —1 and common P450 family —1) and found highly conserved and rapidly evolving P450 families. Results suggested that bacterial P450s, particularly P450s belonging to mycobacteria, are highly conserved both at protein and DNA levels. Mycobacteria possess the highest P450 diversity percentage compared to other microbes and have a high coverage of P450s (≥1%) in their genomes, as found in fungi and plants. Phylogenetic and functional analyses revealed the functional conservation of P450s despite belonging to different biological kingdoms, suggesting the adherence of P450s to their innate function such as their involvement in either generation or oxidation of steroids and structurally related molecules, fatty acids and terpenoids. This study's results offer new understanding of the dynamic structural nature of P450s.

Cytochrome P450 monooxygenases, also known as CYPs/P450s, are heme-thiolate enzymes playing key roles in nature, particularly in the evolution of organisms, including the dawn of multicellular life[1]. P450s are well known for their capabilities for stereo- and regio-specific oxidation of substrates, which makes these enzymes essential in the primary and secondary metabolism of organisms. Since their identification five decades ago, quite a large

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Note

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Open Access

Cytochrome P450 monooxygenase analysis in free-living and symbiotic microalgae *Coccomyxa* sp. C-169 and *Chlorella* sp. NC64A

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Microalgae research is gaining momentum because of their potential biotechnological applications, including the generation of biofuels. Genome sequencing analysis of two model microalgal species, polar free-living *Coccomyxa* sp. C-169 and symbiotic *Chlorella* sp. NC64A, revealed insights into the factors responsible for their lifestyle and unravelled biotechnologically valuable proteins. However, genome sequence analysis under-explored cytochrome P450 monooxygenases (P450s), heme-thiolate proteins ubiquitously present in species belonging to different biological kingdoms. In this study we performed genome data-mining, annotation and comparative analysis of P450s in these two model algal species. Sixty-nine P450s were found in two algal species. *Coccomyxa* sp. showed 40 P450s and *Chlorella* sp. showed 29 P450s in their genome. Sixty-eight P450s (>100 amino acid in length) were grouped into 32 P450 families and 46 P450 subfamilies. Among the P450 families, 27 P450 families were novel and not found in other biological kingdoms. The new P450 families are CYP745-CYP747, CYP845-CYP863, and CYP904-CYP908. Five P450 families, CYP51, CYP97, CYP710, CYP745, and CYP746, were commonly found between two algal species and 16 and 11 P450 families were unique to *Coccomyxa* sp. and *Chlorella* sp. Synteny analysis and gene-structure analysis revealed P450 duplications in both species. Functional analysis based on homolog P450s suggested that CYP51 and CYP710 family members are involved in membrane ergosterol biosynthesis. CYP55 and CYP97 family members are involved in nitric oxide reduction and biosynthesis of carotenoids. This is the first report on comparative analysis of P450s in the microalgal species *Coccomyxa* sp. C-169 and *Chlorella* sp. NC64A.

Key Words: carotenoid biosynthesis; *Chlorella* sp. NC64A; *Coccomyxa* sp. C-169; cytochrome P450 monooxygenases; nitric oxide reductase; P450nor; symbiosis

INTRODUCTION

Green algae belonging to the phylum Chlorophyta consist of microorganisms adapted to diverse ecological niches. Green algae are photosynthetic in nature and it is estimated that more than one billion years ago terrestrial plants emerged from these organisms (Heckman et al. 2001). These organisms played a key role during the evo-

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Diversity and evolution of cytochrome P450 monooxygenases in Oomycetes

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Mopeli Marshal Sello[1], Norventia Jafta[1], David R Nelson[2], Wanping Chen[3], Jae-Hyuk Yu[4], Mohammad Parvez[1], Ipeleng Kopano Rosinah Kgosiemang[1], Richie Monyaki[1], Seiso Caiphus Raselemane[1], Lehlohonolo Benedict Qhanya[1], Ntsane Trevor Mthakathi[1], Samson Sitheni Mashele[1] & Khajamohiddin Syed[1]

Cytochrome P450 monooxygenases (P450s) are heme-thiolate proteins whose role as drug targets against pathogens, as well as in valuable chemical production and bioremediation, has been explored. In this study we performed comprehensive comparative analysis of P450s in 13 newly explored oomycete pathogens. Three hundred and fifty-six P450s were found in oomycetes. These P450s were grouped into 15 P450 families and 84 P450 subfamilies. Among these, nine P450 families and 31 P450 subfamilies were newly found in oomycetes. Research revealed that oomycetes belonging to different orders contain distinct P450 families and subfamilies in their genomes. Evolutionary analysis and sequence homology data revealed P450 family blooms in oomycetes. Tandem arrangement of a large number of P450s belonging to the same family indicated that P450 family blooming is possibly due to its members' duplications. A unique combination of amino acid patterns was observed at EXXR and CXG motifs for the P450 families CYP5014, CYP5015 and CYP5017. A novel P450 fusion protein (CYP5619 family) with an N-terminal P450 domain fused to a heme peroxidase/dioxygenase domain was discovered in *Saprolegnia declina*. Oomycete P450 patterns suggested host influence in shaping their P450 content. This manuscript serves as reference for future P450 annotations in newly explored oomycetes.

Ongoing genome sequencing momentum has resulted in genome sequencing of a large number of species from different biological kingdoms. Lower eukaryotic kingdoms occupy a special place among biological kingdoms because of the presence of a large number of species and their adaptation to diverse ecological niches. Genome sequencing of lower eukaryotes such as fungi revealed high diversity in their genomes compared to other biological kingdoms. For example, not only the presence of a large number of cytochrome P450 monooxygenases (P450s) was detected in many of their genomes, but also high diversity in terms of the number of P450 families[1].

P450s are heme-thiolate proteins and ubiquitously present in species across the biological kingdoms[2]. These proteins are well known to perform enzymatic reactions in a stereo- and regio-specific manner[3,4]. Because of this characteristic these enzymes become critical in organisms' primary and secondary metabolism, drug development, generation of human valuables and xenobiotic compound degradation[2,5]. Progress has been made in understanding P450s from lower eukaryotic organisms, such as their

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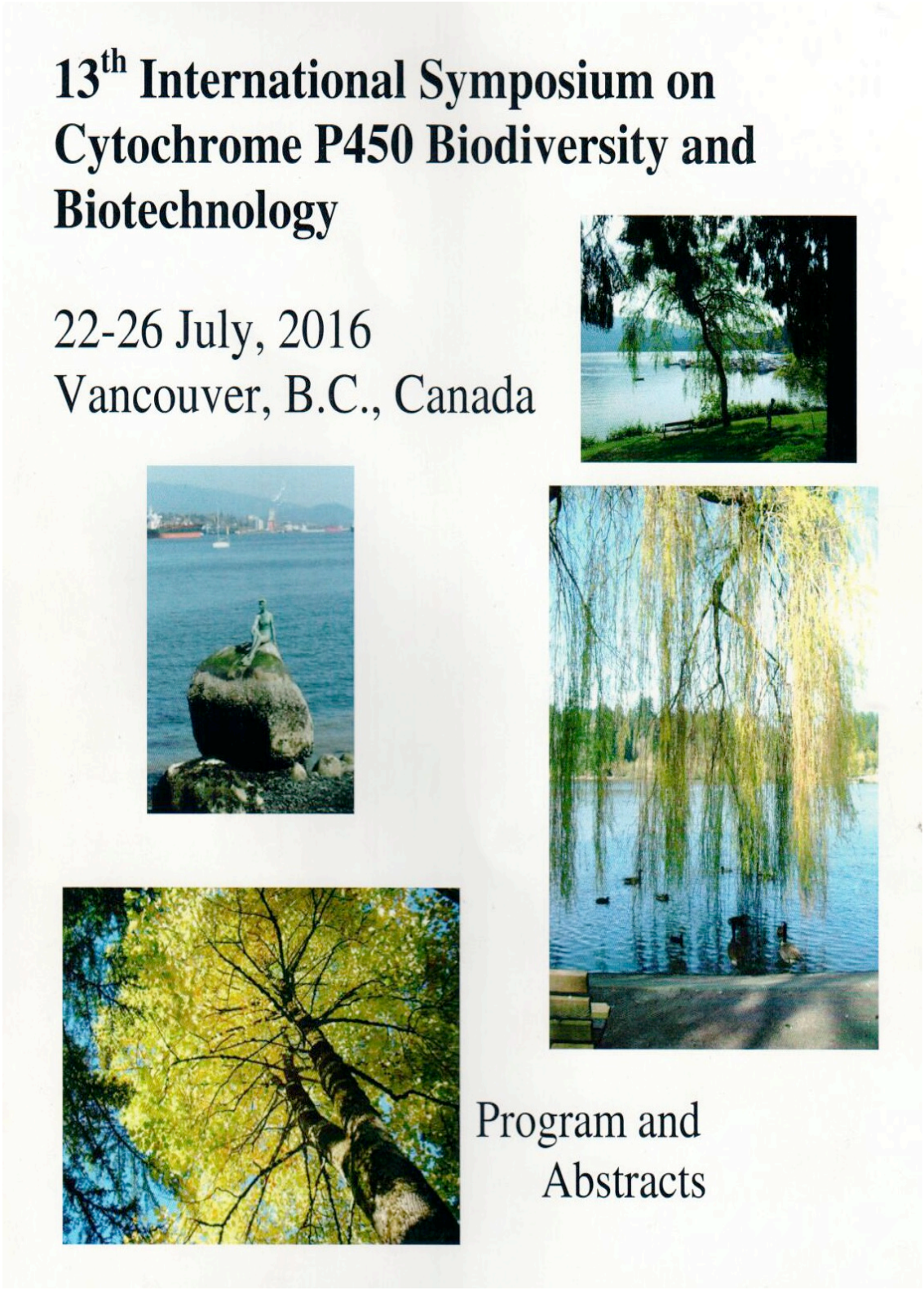
Conference Attendance



P 63-S1

Cytochrome P450 monooxygenase analysis in free-living and symbiotic microalgae *Coccomyxa* sp. C-169 and *Chlorella* sp. NC64A

Mthakathi, Ntsane Trevor;[1] Chen, Wanping;[2] Yu, Jae-Hyuk;[3] Mashele, Samson Sitheni;[1] Syed, Khajamohiddin[1]

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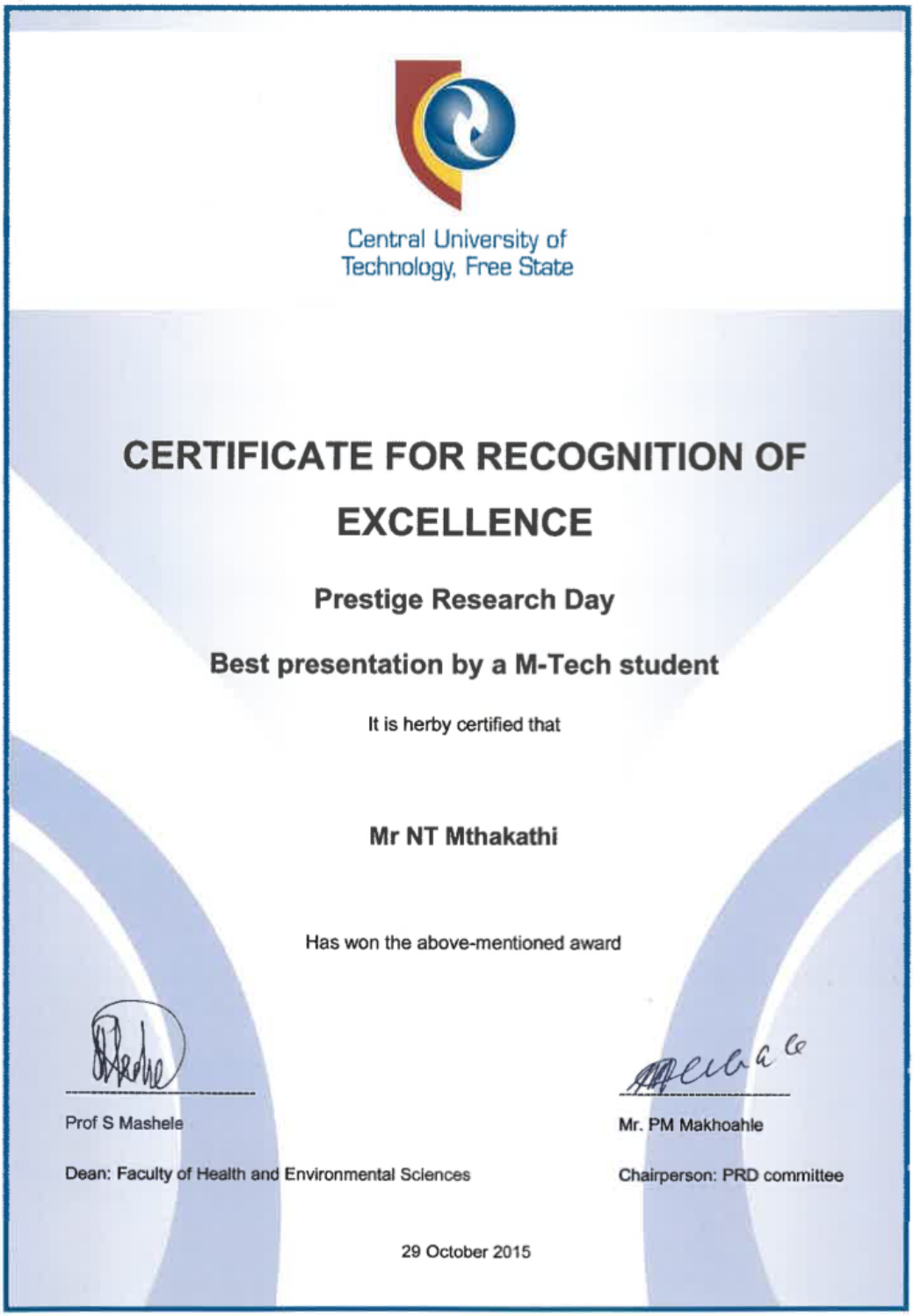
Microalgae research is gaining momentum because of their potential biotechnological applications, including the generation of biofuels. Genome sequencing analysis of two model microalgal species, polar free-living *Coccomyxa* sp. C-169 and symbiotic *Chlorella* sp. NC64A, revealed insights into the factors responsible for their lifestyle and unravelled biotechnologically valuable proteins. However, genome sequence analysis under-explored cytochrome P450 monooxygenases (P450s), heme-thiolate proteins ubiquitously present in species belonging to different biological kingdoms. In this study we performed genome data-mining, annotation and comparative analysis of P450s in these two model algal species. Sixty-nine P450s were found in two algal species. *Coccomyxa* sp. showed 40 P450s and *Chlorella* sp. showed 29 P450s in their genome. Sixty-eight P450s (>100 amino acid in length) were grouped into 32 P450 families and 46 P450 subfamilies. Among the P450 families, 27 P450 families were novel and not found in other biological kingdoms. Synteny analysis and gene-structure analysis revealed P450 duplications in both species. This is the first report on comparative analysis of P450s in the microalgal species *Coccomyxa* sp. C-169 and *Chlorella* sp. NC64A.

Award



Central University of Technology, Free State

CERTIFICATE FOR RECOGNITION OF EXCELLENCE

Prestige Research Day

Best presentation by a M-Tech student

It is herby certified that

Mr NT Mthakathi

Has won the above-mentioned award

Prof S Mashele

Dean: Faculty of Health and Environmental Sciences

Mr. PM Makhoahle

Chairperson: PRD committee

29 October 2015

Media coverage

pressreader

CUT's groundbreaking discovery

New discovery may help fight aquatic animal infection

The New Age (Free State) 17 Jun 2015 News

leading: Research team leader Dr Khajamohiddin Syed and faculty of environmental science acting dean professor Sam Mashele.

pioneers: The CUT science students who helped discover the drug that may help fight aquatic animal infection.

Acting dean of the faculty Prof Sam Mashele said some of the deadliest pathogens in the world were found in the fish farming industry.

He said the discovery was made by researchers from the unit for drug discovery in the institution.

"For many years researchers across the world have been trying to understand these micro- organisms in order to control the disease and develop novel drugs against these pathogens, and CUT researchers are leading the way in finding solutions that will bring an end to this socio-economic challenge facing aquatic farming," Mashele said.

He said microorganisms were widely known in the scientific world and they continued to wreak havoc on the aqua farming sector worldwide.

"They are considered the deadliest of pathogens, causing diminished production of aquatic food," Mashele said.

The team of researchers who discovered the drug were led by Dr Khajamohiddin Syed.

"We collaborated with highly acclaimed international scientists such as professors David Nelson from the University of Tennessee in the US, ae-Hyuk Yu from the University of Wisconsin-Madison and Dr Wanping Chen from Huazhong Agricultural

University in China," Mashele said.

"The university researchers are investigating solutions that would sustain the aquatic resources while helping to increase high production levels of aqua farming for commercial purposes, food security and poverty alleviation."

He said their work highlighted the important role aqua farming plays in promoting healthy living and in fighting poverty and hunger.

Syed said aqua farming was a big industry across the world and it involved the farming of fish, shrimps, prawns, squid and octopus and is considered by the UN as an important sector that can provide a livelihood for more than 60 million people in Africa and Asia.

"Consumption of these animals remains a vital source of protein and essential nutrients, especially for developing countries, where they constitute almost half of the total value of their traded communities," Syed said.

"The results of this study have been accepted for publication in the Nature Publication Group journal Scientific Reports, a prestigious multidisciplinary scientific international journal with an impact factor of 5.1."

dag 16 Junie 2015 **Volksblad**

Navorsers by SUT maak deurbraak

Caydene Davids

'n SPAN navorsers aan die Sentrale Universiteit vir Tegnologie (SUT) het 'n wegspringpunt vir 'n middel ontdek wat groot potensiaal toon om siektes, wat deur parasiete veroorsaak word, in seediere te bestry.

Navorsers van oor die wêreld heen het jare lank probeer om die swamagtige mikro-organismes te verstaan ten einde die siekte, wat onder meer in visse, garnale, skaalvisse en seekatte voorkom, te beheer en 'n middel daarteen te ontwikkel.

Maar meer as 50 jaar lank was dit tevergeefs – totdat die navorsers aan die SUT en wêrelderkende wetenskaplikes koppe byeengesit het.

Dan Maritz, woordvoerder, sê 'n hul span jong navorsers onder leiding van prof. Samson Sitheni Mashele en dr. Khajamohiddin Syed loop nou voor om oplossings te vind wat die maatskaplik-ekonomiese uitdagings wat die siektes vir veral die visserybedryf bied, te beëindig.

Syed sê miljoene seediere vrek jaarliks weens siektes wat deur parasiete veroorsaak word en dit het 'n regstreekse invloed op die visserybedryf.

Volgens hom is dit alombekend in die wetenskaplike wêreld dat die parasiete (*oomycete*) chaos in die visserybedryf wêreldwyd saai en die lewensduur van die diere aansienlik verkort.

Die navorsers het die *P450 monooxygenase*-proteïen in 13 patogeniese genome bestudeer en bevind dit kan as wegspringpunt gebruik word vir 'n middel om die swamagtige mikro-organismes te beveg.

Die navorsers ondersoek dit nou om oplossings te vind om seehulpbronne te beskerm.

Maritz sê hul (die navorsers se) werk beklemtoon die belangrikheid van die visserybedryf in die bevordering van gesonde lewe en die stryd teen armoede en honger.

Die uitkoms van die navorsing is reeds vir plasing in die *Nature Publication Group Journal* aanvaar.

■ Die wêreldbekende wetenskaplikes wat ook deel van die navorsingspan is, sluit in prof. David R. Nelson van die Universiteit van Tennessee, prof. Jae Hyuk Yu van die Universiteit van Wisconsin-Madison, en dr. Wanping Chen Huazhong van die Landbou-universiteit in China.

Die span navorsers aan die Sentrale Universiteit vir Tegnologie (SUT) wat die wegspringpunt vir 'n middel ontdek het om siektes wat deur parasiete veroorsaak word, in seediere te bestry. Hier is van links, voor: dr. Khajamohiddin Syed; middel: Lorato Mongale, dr. Ntsoaki Malebo, Poojah Jawallapersanei en Mohammad Parvez; agter: prof. Sitheni Mashele, Mopeli Sello, Seiso Raselemane, Lehlohonolo Qwanya, Mthakathi Ntsane, Thapelo Makhele, Euginiah Rampana en Richie Monyaki.
Foto: MLUNGISI LOUW