Biological Properties and Interactions of *Kalaharituber pfeilii*

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By

Krele Viwe (g15k7469)

https://orcid.org/0000-0002-1975-1707

Supervised by: Prof. Joanna F. Dames

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Abstract

Dessert truffles are seasonal macro fungi and have been identified in several parts of the world including South Africa. The first part of the present study dealt with the assessment of the biologically active compounds of the Kalahari truffles found in the Northern Cape of South Africa. Truffles extracts (methanol, ethanol, aqueous) were investigated for their antimicrobial properties towards Gram-positive and Gram-negative bacteria. The results demonstrated that the truffle extracts tested had no inhibitory effects against the bacterial isolates. The truffle mycelial growth was also noted to be ineffective against the selected bacteria. The bacteria tested in the present study showed some antagonistic effects against the fungus. Cultures of *K. pfeilii* were also screened for enzyme production including amylase, protease, cellulose, and laccase. Evaluation of the potential of *K. pfeilii* mycelia to produce these industrially and economically important enzymes demonstrated both amylase and protease activity. However, for laccase and cellulose, no activity was detected.

The second part of the present study aimed at optimizing biomass production by *K. pfeilii* in liquid culture media. FF Microplate containing 95 discreet carbon sources were employed to test for substrate utilization. Blanked readings above 0.1 were regarded as positive for utilization, and 4 substrates were selected as potential substrates and were included in liquid media. Media was evaluated for mycelial biomass production. Of the carbon sources tested sucrose proved to be the most suitable for supporting mycelial growth.

The third part of the current study included investigating the diversity of microbial communities colonizing the rhizosheath of *Stipagrostis ciliata* var. *capensis* (the host plant of *K. pfeilii*) and these were identified by means of next-generation sequencing using Illumina Miseq. Bioinformatics tools were utilized in analyzing the data. Actinobacteria were found to be the most dominant bacterial phylum, followed by unclassified bacteria, Proteobacteria, and Acidobacteria. The top 25 sequences were selected and clustered into bacterial OTUs (at 97% threshold) which were assigned into 1 phylum (Actinobacteria), 1 family (Geodermatophilaceae) and 23 genera. This phylum is well known for its secondary metabolites. *Streptomyces sp*. was the most frequently encountered genus. The results from this study necessitate further investigations with regards to the function and evolution of fungal-bacterial associations. Wheather these bacteria have a contribution towards the truffle development, it is still not confirmed.

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List of Abbreviations

AWCD	Average Well Color Development
µ	Miro
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
cm	centimeters
CMC	Carboxymethyl cellulose
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	dideoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
ECM	Ectomycorrhiza
ENDO	Endomycorrhiza
FNT	Fontana
g	gram
hr	hour
ITS	Internal Transcribed Spacer
KT	Kalahari truffle
l	litre
MHB	Mycorrhizal Help Bacteria
Min	minute

MMN	Modified Melin- Norkrans
NA	Nutrient agar
NB	Nutrient broth
NCBI	National Center of Biotechnology Information
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
pH	Potential hydrogen
s	second
TBE	Tris-Borate-EDTA

Dedication

This thesis is dedicated to my late mom Boniswa Sylvia Krele.

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Chapter one

1. Introduction

1.1 Truffles

Truffles are edible mushrooms that form their fruiting bodies below ground; they belong to the order Tuberales and the phylum Ascomycotina (Mello et al., 2006; Tang et al., 2007). Their distribution is among six Pezizalean families: Glaziellaceae, Discinaceae-Morchellaceae, Helvellaceae, Tuberaceae, Pezizaceae and Pyronemataceae (Hansen and Pfister, 2006). Generally, species of *Tuber* form an obligate mycorrhizal symbiosis with the roots of specific trees (Bofante and Genre, 2010). These include poplar, oak, pecan, and hazelnut. Truffles are descendants of the epigeous mushroom (Bonito et al., 2013). They are widely distributed in the world, more especially in the regions of the northern hemisphere. Mycologists have discovered more than 60 different forms of truffles around the world, most of which grow in different parts of Europe (Tang et al., 2007; Mello et al., 2006).

Truffles belong to the fungal phylum, Ascomycota. The Pezizales are predominantly a large group of ectomycorrhizal fungi having a symbiotic relationship with the roots of many plant species belonging to both Angiosperms and Gymnosperms. The truffle fungus forms a subterranean ascoma (Vita et al., 2015). Tuberaceae are well known for their hypogeous ascomata or forest truffles which are highly regarded, fetching handsome prices when in season. The ascomata of many species in this genus are aromatic, flavourful, and highly sort after for their gastronomical properties (Healy et al., 2016).

Truffles are usually difficult to find especially to an untrained eye and therefore require a specialist who possesses high skills to explore these underground fungi. Truffles have characteristic aromatic compounds, which help truffle hunters to be able to locate them quickly with the use of animals like pigs and dogs (Enshasy et al., 2013). These animals have a greater sensitivity towards these compounds hence their efficiency in locating them. These aromatic compounds are derived from low molecular weight carbon compounds and have a collective name of volatile organic compounds (VOCs). These compounds are unique for every species and play an important role in terms of their market, nutritional as well as medicinal value.

Murcia et al., (2002) and Kalač (2009) have reported these fungi to be good sources of proteins, amino acids, fiber, minerals, vitamins, terpenoids, sterols, flavour compounds, and

carbohydrates. Hence, traditionally these food delicacies are enjoyed either raw or cooked and have been used in traditional medicine.

Truffles (black and white) form part of the Italian and French economy, being regarded as the world's most expensive mushrooms. Depending on the species and season, one kilogram of truffles sells on the market for between 600-6000 Euros (Luard, 2006). They are also beneficial to terrestrial ecosystems and their host plants contributing to nutrient uptake. This has been attributed to their capacity to colonize the soil through mycorrhiza (Mello et al., 2006; Wang and Marcone, 2011). In general, truffles do not have a stipe or gills, and their vegetative mycelium grows underground. Upon ripening, they move from being soft and fragile and become firm, dense and woody (Hall et al., 2007). They are thus of interest for use in reforestation projects where they reduce the need for fertilizers (Harley and Smith 1983) and provide an additional crop.

Mycorrhizal symbiosis is based on resource exchange where fungi exchange mineral nutrients for carbon with their autotrophic partners (Vita et al., 2015). This symbiosis is vital for the completion of the fungal life cycle, and only if fungi form this symbiosis with the plant roots will they be able to establish an ectomycorrhizal association and eventually after a series of events form truffles (Splivallo et al., 2011).

1.1.1 Life cycle of truffles

The truffle life cycle begins with the proliferation of hyphae in the presence of roots in order to ensure contact. The symbiotic phase begins once the fungus attaches to the root of a host plant leading to the development of the association. Truffles form a symbiotic association with the roots of trees such as oak, willow, poplar, and hazel as well as some shrubs (Napoli et al., 2010; Benucci et al., 2018). The ectomycorrhizal hyphae then aggregate and form a fruiting body commonly known as a truffle through a meiotic reproduction process; the ascospores of the ascoma become disseminated at a later stage into the environment. This then leads to the formation of vegetative mycelia, which eventually leads the development of the extraradical phase, and the truffle life cycle is completed (Vita et al., 2015; Mello et al., 2005).

Truffle formation is affected by abiotic and biotic factors. The abiotic factors include, soil composition, climatic weather conditions such as rain, sunshine, and temperature whereas

biotic factors could include other soil fungi, yeasts, bacteria, mesofauna, and plant host. These parameters have the power to enhance or inhibit the ascoma formation (Ceruti et al., 2003). In general, truffle formation occurs rapidly when there is adequate rainfall and warm but not hot temperatures (Ceruti et al., 2003; El Enshasy et al., 2013).

1.1.2 Forest truffles

Humans have long been collecting truffles of various species and genera including those of the genus *Tuber* (Ascomycota) either for self-consumption or for commercial purposes to make a living (Benucci and Bonito, 2016). These fungi play a crucial role in promoting forest health aiding in plant nutrition through the symbiotic relationship they form with the plant roots (Egli, 2011; Benucci et al., 2013). *Tuber* species are mostly identified in different regions of Europe, Australia, New Zealand, North America, Asia, and in Africa (Berch and Bonito, 2016; Ferdman et al., 2005). *Tuber* species are divided into two main groups i.e. black and white *Tuber* sp. White truffles species encompasses *Tuber magnatum, T. maculatum, T. borchii, T. dryphilum, T. peberulum, T. oregonense* and *T. lastisporum,* while black truffles include *T. brumale, T. melanosporum, T. aestivum, T. indicum* and *T. himalayense* (Zhang et al., 2005; Patel et al., 2017)

The value of truffles in the market is based on the species, quality of the harvest, and place in which they are produced or harvested and sold (Álvarez-Lafuente et al., 2018). In Italy and some parts of Europe, the white truffle (*Tuber magnatum*) is the most expensive truffle species (Mello et al., 2010). White truffles are generally sold on the market at US $5000 per kg, however, desert truffles are more reasonably priced (Patel et al., 2017). Truffle prices differ in various geographical regions, the same species could be worth more; for example in the United States and in New Zealand. For example *Tuber melanosporum* is worth almost double, of its worth in Europe (Alvis 2001). In addition, truffles are utilized for various purposes, such as being incorporated in fermentation processes to make liquors, contribute towards the final tobacco scent, and is recognized as aphrodisiacs (Mandeel and Al-Laith, 2007, El Enshasy et al., 2013).

Mushrooms and truffles are economically important forestry products particularly in the Northern Hemisphere. Truffle fruiting bodies contain a mixture of volatile compounds, which

are emitted as they mature and these compounds aid in the communication of truffles with plants, animals (particularly trained dogs and pigs) and microorganisms (Splivallo et al., 2011). These compounds play an important role in the determination of the truffle economic value. *Tuber melanosporum* Vittad. ('Black truffle') and *Tuber magnatum* Pico ('White truffle') are by far the most appreciated forest truffle among the *Tuber* species and this is attributed to their intense aroma and taste, resulting from the many hundreds of volatile compounds they emit (Gioacchini et al., 2005).

T. melanosporum is characterized by a 'wet forest' smell, with a bit of a radish taste and a hint of hazelnut. *T. magnatum* has a musky, earthy, garlicky, cheesy smell with subtle methane overtones (Wang and Marcone, 2011). *T. melanosporum* occupies carbonated soil in impoverished mountain ecosystems within the Mediterranean continental climate hence it is of great ecological and agricultural value (Montecchi and Sarasini, 2000, and García-Montero et al., 2007).

1.1.3 Desert truffles

Dessert truffles are seasonal macro fungi and have been identified in several parts of Bahrain, Jordan, Syria, Iraq, Oman, Kuwait, Saudi Arabia, Morocco, Libya, Egypt, Algeria, South Africa, Botswana, Namibia, and Tunisia (El Enshasy et al., 2013; Mandeel and Al-Laith, 2009). Species of *Terfezia* and *Tirmania* are predominantly the most common desert truffles (El Enshasy et al., 2013).

Dessert truffles are edible ascoma, which form their fruiting bodies below ground (Kagan-Zur and Roth-Bejerano 2008). Taxonomically, these species belong to the Pezizaceae family (Pezizales, Ascomycota) with the *Terfezia* and *Tirmania* genera being the most predominant among them (Kagan-Zur & Roth-Bejerano 2008). The term desert truffle refers to their geographical distribution and the ability of these genera to grow under arid and semi-arid conditions (Enshasy et al., 2013). They form mycorrhizal associations with the roots of perennial *Helianthemum spp* plants (Cistaceae) and have the capacity to form either or both ecto or endo mycorrhiza (Bradai et al., 2015; Kagan-Zur and Roth- Bejerano 2008). Dessert truffles are found in Africa, Southern Europe, and in the Middle East as well as countries

around the Mediterranean (Bradai et al., 2014; Ferdman et al., 2005; Morte et al., 2009; Mandeel and Al-Laith, 2007; Enshasy et al., 2013).

Desert truffles have been found in South Africa and Botswana (Morte et al., 2008; Enshasy et al., 2013). Desert truffle formation depends on several factors in particular rainfall, these fungi generally must receive an annual rainfall ranging between 50-380 mm (Enshasy et al., 2013). In North Africa and the Middle East, good yields of truffles are obtained if an adequate and properly distributed rain fall has been received by no later than December, whereas in southern Europe it should not be later than October (Morte et al., 2008).

Desert inhabitants such as communities from the Middle East regions and North Africa find temporary occupation in the collection of desert truffles after harvesting they sell them to the local markets as well as for national and international trade (Feeney 2002; Kagan-Zur and Roth-Bejerano 2008, and Volpato et al., 2012).

Truffles are rich sources of diverse essential nutrients and chemical analysis revealed that dessert truffles consist up to 60% carbohydrates of the dry matter, 20-27% protein, 3-7.5% fat (both saturated and unsaturated fatty acids), 7-13% fiber, and 2-5% ascorbic acid (Kagan-Zur and Bejerano, 2008; Wang and Marcone, 2011; Al-Laith, 2010; Al-Laith, 2014, Hamza et al., 2010). Chemical profiling that was conducted on three desert truffle species from Iraq, the white desert truffle, *Tirmania nivea*, and black desert truffles *Tefezia claveryi* and *Tirmania pinoyi*, reported that the dry matter carbohydrate concentration ranged between 16.6 and 24.8%, their protein content ranged between 8.1-13.8%, phosphorus was from 9.7-25.5%, and ash content ranged between 4.9-5.9% (Hussain and Al-Ruqaie, 1999). Other studies have shown these truffles to be a source of essential minerals including Al, Zn, Cu, Mg, Ca, Na, K, Si, and Mn (Wang and Marcone, 2011) and to have a higher fat, and crude fiber content than other desert truffle species. Despite being different species all were found to contain essential sulphur containing amino acids (Sawaya et al., 1985).

Some Western Saharan tribes collect truffles for various purposes; either in preparation for an expedition or for immediate consumption. They prepare truffles in different ways sometimes they eat them boiled, roasted, and are consumed with butter or with camel hump fat. Being a delicacy, truffles are also used as a food enhancer and in some cases as emergency food in times of poverty (Volpato et al., 2012).

1.2 Mycorrhizal fungi

Soil contains a broad spectrum of microorganisms that continually interact with plants during their life cycle. These interactions can either be harmful or mutualistic (Sander, 2011). Among the beneficial microorganisms, the mycorrhizal association is the most abundant and is of great significance to plant ecology, plant growth, agriculture and forestry (Smith and Read, 2008). Mycorrhizal fungi form a symbiotic association with plant roots (Brundrett, 2002). The mycorrhizal fungus has coexisted with their host plants (dual soil-plant inhabitants) over many millions of years, which make them as ancient as the first land plants (Brundett, 2002). These mutualistic associations are believed to have led to the evolution of plant roots (Brundrett, 2002). Previous studies have proven that nearly all plants species form a symbiosis with soil fungi, with the exception of a few species that have evolved the capacity to exclude mycorrhizal associations due to their ability to form fine/cluster roots or produce compounds making them non-mycorrhizal (Behie and Bidochka 2014).

Mycorrhizal symbioses are ubiquitous in nature and are formed by over 90 % of plant species with a wide diversity of soil fungi. Mycorrhizal fungal association with plants is a two-way exchange process, whereby plants reallocate around 5-21% of their total photosynthetically fixed carbon to their symbiotic partners and in turn plants receive limiting nutrient resources from the fungus (Strullu-Derrien et al., 2014; Brundrett, 2002). The fungal mycelium is well adapted to a three-dimensional exploration of the soil environment and certain mycorrhiza have a weathering potential which may allow access to non-soluble mineral elements (Hoffland et al., 2004).

Uptake of immobile elements such as Phosphorus (P), Zinc (Zn) and Cupper (Cu); and mobile ions which include Sulfur(S), Calcium (C), Potassium (K), Iron (Fe), Magnesium (Mg), Manganese (Mn), Chlorine (Cl) , Bromine (Br), and Nitrogen (N) are enhanced in the presence of mycorrhizal fungi (Tinker, 1984). In addition, it has been reported that plants colonized by mycorrhizal fungi have enhanced water uptake and/or have the capacity to alter the plant's physiology to reduce the stress response in times of drought (Parke et al., 1983; Safir and Nelson, 1985). Furthermore, plants colonized by mycorrhizal fungi have shown increased absorptive surface area, disease resistance by activation of plant defence mechanisms, and increased tolerance to other soil stresses such as salinity and heavy metals toxicity (Bofante and Genre, 2010; Smith and Read, 2008).

Certain types of mycorrhizal fungi have the capacity to form mushrooms and truffles as part of their life cycle. Propagules of mycorrhizal fungi in the environment are in the form of spores and hyphae in root fragments, which then responds to the root exudates leading to the proliferation of hyphae and spore germination resulting in subsequent colonization of the plant roots by the fungus. Mycorrhizal colonization begins on the outside of the root tissue and proceeds internally. The physiological and morphological changes of both plant and the fungi due to root colonization of various hosts leads to the formation of either ecto-, ectendo-, or endomycorrhizal relationships (Linderman, 1988).

Ectomycorrhizal (ECM) fungi form mutualistic associations with most of the world's temperate trees, which control primary production and nutrient remineralization in terrestrial ecosystems (Moeller and Peay, 2016). They are an integral part of forest ecosystems interacting with temperate tree species such as pine (Pinaceae) and beech (Fagaceae) as well as tropical species such as members of the Dipterocarpaceae. ECM fungi play a vital role in seedling establishment in forests and contribute greatly to the health and growth of trees, shaping the present forests (Smith and Read. 2008).

Characteristic features of this type of mycorrhiza include the formation of a hyphal sheath or mantle of fungal tissue around feeder roots, a 'Hartig' net around root cells and the extraradical mycelium and rhizomorphs, which exploit the soil environment (Bücking et al., 2012).

ECM fungi have multiple lineages (high diversity) given the number of times both plants and fungi have evolved (Tedersoo and Smith, 2013). Nearly all ECM fungi originated from a diverse range of saprotrophic ancestors (Hibbett et al., 2000). However, certain ECM fungal species (e.g *Hebeloma* spp) are referred to as facultative ECM fungi; meaning under normal conditions they exist as ECM symbionts but can grow as saprotrophs when the need arises (Taylor and Alexander, 2005). There are at least 66 independent lineages of ECM fungi, predominantly in the phylum Basidiomycota, Ascomycota and to a lesser extent Zygomycota within the genus *Endogone* (Molina et al., 2004; Tedersoo et al., 2010). ECM symbiosis involves around 60 000 plant species and approximately 20 000- 25 000 fungal species (Rinaldi et al., 2008; Brundrett, 2009).

Most ECM fungi reproduce through meiosis and form fruiting bodies such as mushrooms and truffles unlike the endomycorrhizal (ENDO) fungi (Luoma et al., 2004; Tedersoo et.al. 2009). Endomycorrhizal associations are recognised by the presence of fungal structures inside the root cells forming either fine branches arbuscles or coils depending on the type of

endomycorrhizal fungus involved. Ectendomcorrhizal associations are intermediate between ECM and ENDO (Smith and Read, 2008; Turgeman et al., 2016). They are distributed among four ascomycetous fungal taxa namely *Wilcoxina* (which is subdivided into *W. mikolae* and *W. rehmii*); *Sphaerosporella brunnea; Phialophora finlandia* and *Chloridium paucisporum* (Trevor et al., 2001). In addition, these fungi are restricted to the conifer genera, *Pinus* and *Larix.* Ectendomycorrhizas also include arbutoid and monotropoid mycorrhizas (Smith and Read, 2008). These mycorrhizal associations are characterized by the thin layer of sheath (which is some cases is lacking), a 'Hartig' net and intracellular hyphae (Finlay, 2008). Some of these fungi are known to have the ability to hydrolize complex carbohydrates (Peterson et al., 2004). Furthermore, *Wilcoxina spp*. are known to produce the siderophore, ferricrocin, which is released to scavenge iron in cases where plants are exposed to heavy metals such as iron, protecting them from toxicity (Peterson et al., 2004).

1.2.1 *Kalaharituber pfeilii*

Kalaharituber pfeilii (Hennings) Trappe & Kagan-Zur was first misidentified as *Terfezia boudieri* Chatin or *Terfezia claveryi* Chatin and *Tirmania pinoyi* (Maire) then later identified as *Terfezia pfeilii* (Hennings) and finally in 2005 Ferdman and his colleagues renamed it as *Kalaharituber pfeilii* based on the genetic sequences of the ribosomal RNA subunit (Ferdman et al., 2005; Trappe et al., 2010). *Kalaharituber pfeilii* is an underground edible ascoma found in the Kalahari Desert of southern Africa (Ferdman et al., 2005). It was named after its original collector Count Pfeil. They are widely distributed in the Kalahari Desert which includes Botswana, Namibia and the adjacent areas of South Africa. An area is classified as desert if it receives an annual rainfall which is below 250 milliliters. The Kalahari does not perfectly fit this description as the northern parts of the Kalahari receive more rainfall and has dense vegetation including palm trees growing among the camel thorn bush and forests of deciduous trees used for timber. The southwestern Kalahari experiences very low precipitation, with few trees and short grasses. The central Kalahari receives more rain than the southwestern Kalahari and its plant coverage includes scattered tress, some shrubs and grasses. Kalahari soil is mostly sand and has been reported to be nutrient poor with low organic matter (Aswegen, 2017; Ntshakaza, 2013; Adeleke, 2007).

Kalaharituber pfeilii in Botswana have been observed in Ghanzi district and in Namibia they were found in Damaraland and finally in South Africa they were found in the Northern Cape (Ferdman et al., 2005). These truffles have been observed to form their fruiting bodies from April to June amongst the sand dunes. The local inhabitants (Khoisans) of the Kalahari Desert refer to these truffles as n'abba and they are also known as Kalahari truffles (Adeleke and Dames 2014). *K. pfeilii* has a solid gleba with white veins, have fertile pockets which turn from yellowish white to brown on exposure to air. They have a mild taste with a fungoid smell. The peridium is usually smooth with no hair, and has a yellowish to a dark brown colour with cracks or in some cases wrinkles (Trappe et al., 2010).

Kalaharituber pfeilii occurring in South Africa were previously studied for their host plant relations (Ntshakaza, 2014) and interactions with associated bacteria (Adeleke and Dames, 2014). According to Ntshakaza (2013), among the collected potential host plants only *Stipagrostis ciliata* var. *capensis* was confirmed to be the host plant of *K. pfeilii.* After the roots of these plants were stained, *Stipagrostis ciliata* var. *capensis* displayed an ectendomycorrhizal association and this was in line with previous findings by Adeleke (2007). These finding and the molecular identification of the fungi found in the rhizosheath of *Stipagrostis ciliata* var. *capensis* confirmed the association with *Stipagrostis ciliata* var. *capensis.* Other potential host plants such as *S. obtuse* and *Schmidtia kaliharinsis* were reported to have endomycorrhizal associations with morphological colonisation features of the arbuscular mycorrhizal fungi and the presence of vesicles and arbuscules was noted. Hence their exclusion as potential hosts, because in spite of truffle's ability to form endomycorrhizal associations, they have not been seen to produce intracellular arbuscules. Adeleke and Dames (2014), investigated the *in vitro* interaction between associated bacteria and *K. pfeilii.* The isolated bacteria were from the Actinobacteria, Firmicutes, and Proteobacteria. Some of these bacterial strains were observed to have mycorrhizal helper ability which stimulated mycelial growth.

1.3 Biological properties of truffles

Truffles provide a comprehensive coverage of health benefits; they have been used as health promoters, for disease prevention and to treat various diseases (Enshasy 2013). Literature has reported truffles to have immunosuppressing, antioxidant, anti-carcinogenic and anti-

inflammatory properties as well as antimicrobial activity (Al-Laith 2004; Villares et al., 2012; Janakat et al., 2004; Stanikunaite et al., 2009; Stanikunaite et al., 2007; Patel 2012)

Research on desert truffles has revealed that truffles have been used in Arabian countries for more than two millennia with no known complications (Al- Rahmah, 2001). Trachoma one of the first recorded eye diseases by the World Health Organisation (WHO) has been treated by boiled truffle water extracts in Arabian countries (Enshasy 2013).

1.3.1 Antimicrobial activity

Fungi have been highly recognized for their antimicrobial secondary metabolites since the early 20th century (El Enshasy et al., 2013). The antibacterial and antiviral activities of desert truffles were first studied in the 1980s by Al-Marzooky (1981) who investigated the *in vitro* biological activities of all aqueous polar and nonpolar extracts of *Terfezia claveryi*. These extracts exhibited good wide spectrum antimicrobial activities especially against the trachoma causing pathogen, *Chlamydia trachomatis*, stomach ulcers, and wounds. Desert truffles have a long history of utilization in traditional medicine for treating not only ophthalmic diseases but also as sexual stimulants (El Enshasy et al., 2013, Omer et al., 1994). Other research has shown water extracts of *T. claveryi* to have inhibitory effects against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Janakat et al., 2004; Janakat et al., 2005). The antimicrobial activity attributed to *T. claveryi* is based mainly on the production of small molecular weight peptide compounds (Janakat et al., 2004). *Terfezia* extracts have also been shown to have antiviral activities (Hussain and Al-Ruqaie, 1999). According to Mekawey (2015) *Terfezia boudieri* extracts showed some degree of inhibition against Herpes Simplex virus type-2 (HSV-2) and Vesicular Stomatitis virus (VSV). Aqueous extracts of *T. boudieri* had a 70% inhibition, followed by methanol extracts at 50%, chloroform extract at 33%, and finally acetone extract had a 32% inhibition against VSV. HSV-2 inhibition concentrations were as follows in the order above, 75%, and 46%, 49% and 35% respectively (Mekaway, 2015). Dib-Bellahouel and Fortas (2011) showed that ethyl acetate extract of *Tirmania pinoyi* exhibited potent antimicrobial activities against the Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*. These properties make truffles important candidates for complementary medicine and targets for drug discovery (Molitoris, 1994).

1.4 Interactions with soil microbes

1.4.1 Plant Growth-Promoting Bacteria

Mycorrhizal fungal associations with plant roots are continuously influenced by various microorganisms in the soil adjacent to the roots of the host plant and more especially by bacteria (Garbaye, 1994). It has been suggested at least that even if the bacteria is tightly or loosely associated with mycorrhizal fungi, they form a third component of the mycorrhizal interaction (Bofante and Anca, 2009, Garbaye, 1991). Bacterial populations associated with fungi may have a negative, neutral or positive effect on the fungal fitness (de Boer et al., 2005; Compant et al., 2010). Mycorrhizal fungi can also indirectly influence bacterial communities in the mycorrhizosphere by stimulating root growth as well as by altering root exudation patterns and the structure of the surrounding soil (Rilling, 2004; Johansson et al., 2004; Jones et al., 2004). Fungi can benefit from the relationship by obtaining a supply of energy from for example cyanobacteria. Unlike the green algae photobionts in most lichens, some cyanobacterial partners fix nitrogen and supply a portion of the yield to the fungus (Honegger, 1998; Honegger, 2001; Richardson 1999; Rai et al., 2000). Nitrogen transfer may also be important in the association of organotrophic bacteria with fungi. Plants derive various benefits from the plant growth-promoting bacteria (PGPB) apart from growth promotion; PGPB provide an increased protection against invading pathogens, induce production of plant growth hormones and help distribute soil nutrients that would otherwise be unavailable to the plant (Hu et al., 2016; Hu et al., 2017).

Certain bacteria such as *Pseudomonas fluorescence* have the capacity to stimulate the development of symbiotic fungi such as ectomycorrhizal fungi and Dupunnois and Garbaye (1991) were the first to observe this phenomenon. This phenomenon is referred to as mycorrhizal helper bacteria because it involves mycorrhizal fungi (Garbaye, 1994). Several follow-up studies have confirmed the stimulating effect of these bacteria on the mycorrhizal establishment, but the actual mechanisms are largely unknown (Frey-Klett et al., 1997; Brulé., et al., 2001).

Some helper bacterial species can influence both plants and their mycorrhizal symbionts this is called a multiple helper effect. The bacteria seem to be involved in the development of

mycorrhizal fruiting bodies suggesting that they do not only colonize the extraradical hyphae of the mycorrhizal fungi but the mycorrhizal roots and fruiting bodies as well (Bofante and Anca 2009). The bacterial interactions studied by Adeleke and Dames (2014) were strains isolated from the internal gleba of *K. pfeilii* indicating an association with the truffle fruiting bodies. Moreover, these bacteria complement the role of the external mycelium by mobilizing nutrients from complexed sources or through the production of volatile organic compounds that contribute to truffle aroma in association with other truffle-associated microbes (Bofante and Anca, 2009). Initiation of fruiting bodies of some mushrooms such as *Agaricus bisporus* is dependent on the presence of *Pseudomonas putida*, however, the exact mechanism is not well understood but it has been suggested that the mycelium produce self-inhibiting compounds, which are removed or neutralised by the bacteria (Rainey et al., 1990).

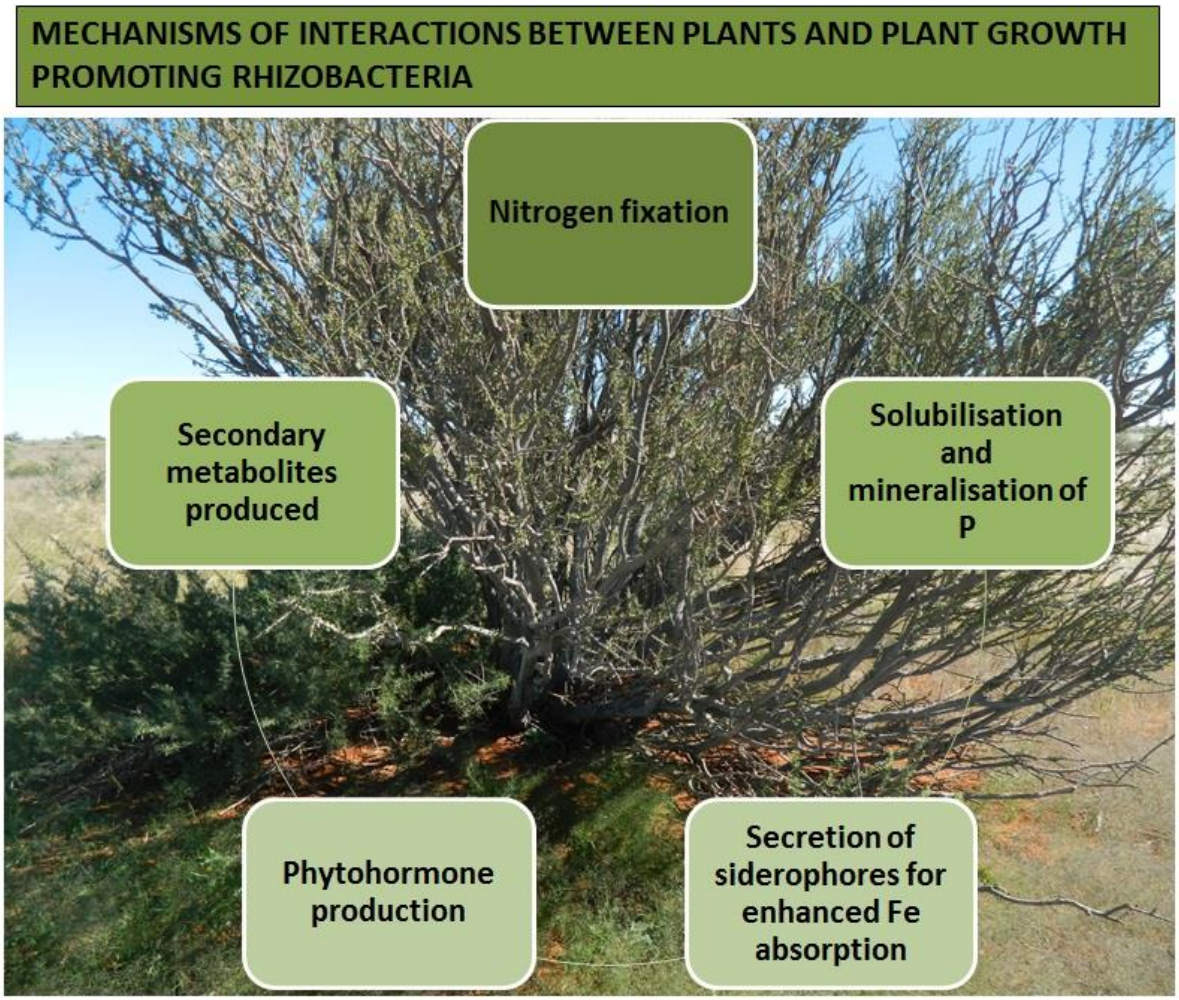

Figure 1.1: Interactions of host plant and the soil microbes.

1.4.2 Rhizosphere

The rhizosphere is described as the bulk of soil surrounding the plant roots, which is under the influence of plant root activity (e.g. exudates) (Basu et al., 2017). This environmental area hosts a large and diverse community of microorganisms including bacteria, fungi, actinomycetes, protozoa, and algae (Basu et al., 2017). However, this zone is highly dominated by bacteria of which less than 1% are culturable. A number of factors, but most of all the type of soil (Schoenborn et al., 2004; Basu et al., 2017) influences bacterial growth. The bacteria are recruited by the abundance of the nutrients made available through root exudates, which accounts for up to a 1/3 of the fixed carbon resulting in greater densities of bacteria in this region (Badri and Vivanco 2009; Basu et al., 2017).

This is a highly competitive region where plants compete with invading root systems of neighbouring plants for space, water, mineral nutrients, and some soil-borne organisms, including bacteria, fungi and insects feeding on the abundant source of organic material (Ryan and Delhaize, 2001; Bais et al., 2004). Each gram of rhizospheric soil may contain up to 10^6-10^9 bacteria, 10^4 protozoa, 10^1-10^2 nematodes, and 10^5-10^6 fungi (Watt et al., 2006; Hinsinger et al., 2009; Mendes et al., 2013).

Bacterial and fungal diversity increases soil quality by affecting soil aggregation and fertility. Both groups of microorganisms are important in nutrient cycling and in enhancing plant health through direct and indirect means. In addition, a healthy rhizospheric population can help plants deal with biotic and abiotic stresses such as pathogens, drought, and soil contamination (Kirk et al., 2004).

1.4.3 Rhizosheath

Rhizosheaths are a mass of sand grains entangled by a mesh of hair-like epidermal structures, mucilage, and other root exudates (Wullstein and, Pratt 1981). An adhesive agent called mucilage (Bergmann et al., 2009; George et al., 2014; Brown et al., 2017) tightly binds sand grains to the root. Root mucilage forms a gelatinous layer surrounding root tips and is a visible sign of organic carbon excretion from roots, which infiltrates the soil pores and eventually coats the soil particles (Jones et al., 2009; Ahmadi et al., 2017). This substance comprises of

polysaccharides, proteins and some phospholipids (Read et al., 2003). The mucilage released into the soil protects root meristems from toxic metals, enhances soil aggregation stability, promotes soil aeration and root growth, prevents soil erosion and maintains a continuous water flow towards the rhizoplane, hydrating the soil in close contact with the root (Albasmeh and Ghezzehei, 2014; Read et al., 2003).

Rhizosheaths are a result of modifications that occur in the rhizosphere in sheath forming grasses (Bailey and Scholes, 1997). These structures are defined, as sandy sheaths, if sand grains were held together simply by the presence of root hairs, but if the connective tissue was apparent they are referred to as rhizosheaths. Initially research was conducted on or using herbarium specimens, mucilage could not be easily detected from the specimens, this then resulted in the broadening of the definition of these sheaths to include all sandy structures (Bailey and Scholes, 1997).

Rhizosheath formation is quite complex but it is strongly correlated to root hair length, microbial mucilage production, mycorrhizal fungi, soil texture and free-living bacteria (Haling et al., 2010; Delhaize et al., 2012; Brown et al., 2017). Prize hypothesized in 1911 that rhizosheaths are mechanisms to better tolerate drought and protect roots under harsh conditions this has been supported by Benard and his colleagues in 2016 (Bernard et al., 2016). Rhizosheaths help plants cope with other abiotic stresses and help alleviate phosphorus, nitrogen and zinc deficiencies (Brown et al., 2012; Haling et al., 2013; Brown et al., 2017). They keep roots and soil in contact during drying, help maintain soil moisture adjacent to the roots thus providing favourable conditions for microorganisms inhabiting that zone and increasing their microbial activity (Drenovsky et al., 2004; Kuzyakov and Blagodatskaya, 2015; Ahmed et al., 2017; Brown et al., 2017). Hence, rhizosheaths are efficient in alleviating such stresses. The rhizosheath of grasses is more tightly bound to the root particularly under dry conditions (Watt et al., 1994; Ahmed et al., 2017) suggesting greater adaptation to arid soils (Ahmed et al., 2017).

1.5 Motivation, aims and objectives

Macrofungal structures such as mushrooms and truffles are sources of various types of essential nutrients and their nutritional and chemical profiles have been studied and reviewed by several authors (Wang and Marcone, 2011; Hamza et al., 2016). *Kalaharituber pfeilii* is a desert truffle that thrives in the Kalahari region of the Northern Cape, South Africa (Adeleke, 2007; Ntshakaza, 2013, Trappe et al., 2008). Analysis of these truffles has shown that they comprise of over 20% dry matter, approximately 23% of human digestive protein, 3-7.5% crude fibre, 60% of carbohydrates and ascorbic acid (Kagan-Zur & Roth-Bejerano, 2008). Linoleic acid is the primary fatty acid of the Kalahari truffle (67.2% of the total fatty acid). These fungi are of high nutritional value containing eight essential amino acids, four semi-essential amino acids and six non-essential amino acids (Ackerman et al., 1975). Furthermore, truffles represent a vast untapped potential of therapeutic compounds constituting antimicrobial, antioxidant, anti-inflammatory, immune- suppressor, anti-carcinogenic properties. To the best of our knowledge, the potential biological properties of *K. pfeilii* occurring in South Africa has not been explored and was the primary aim of this study. Biological properties of interest include the antimicrobial properties as well as the potential enzymes, which these fungi may produce. Additionally, aims were to: Optimise growth of *K. pfeilii* in liquid cultures and to assess bacterial diversity associated with the rhizosheath of the truffle host, *Stipagrostis ciliata* var. *capensis.* These aims were achieved in the following manner.

Aim 1: Assess biological properties of *Kalaharituber pfeilii*

In order to assess biological properties of the Kalahari truffle the following objectives were necessary.

1. Collection of truffles from the Northern Cape in order to establish cultures

2. Molecular verification of the fungal culture identity

3. Determination of antimicrobial properties

4. Screening for enzyme activity

Aim 2: Optimisation of fungal growth in liquid medium.

Liquid extracts are required for many bioassays therefore optimisation of *K. pfeilii* growth under these conditions was investigated. In order to achieve this, the following objectives were necessary.

5. Determination of substrate preference

6. Media optimization

Aim 3: Assessment of rhizosheath bacterial diversity

Previous research (Ntshakaza, 2013) has shown that *K. pfeilii* is associated with the rhizosheath of Bushmans grass (*Stipagrostis ciliata* var. *capensis).* Bacteria also inhabit this niche but little is known about their diversity.

7. DNA extraction and PCR amplification of the 16S region of rhizosheath material.

8. Assessment of bacterial diversity using Next Generation Illumina Sequencing and bioinformatics analysis.

Chapter 2

2. Methods

2.1 Sample collection

Fresh ascomata (sporocarps) of the *Kalaharituber pfeilii* (*K. pfeilii*) were collected from Karukul Research Institute Upington, Northern Cape in May 2015. Samples were placed in paper bags and kept in a cooler box for transportation. Root samples of the grass, *Stipagrostis ciliate* var. *capensis*, a host of *K. pfeilii* (Ntshakaza, 2013) were collected in May 2016. Root samples were cut into 3 cm pieces, placed into sterile microcentrifuge tubes, and were stored at -20°C until further processing. Roots for staining were placed in 50% ethanol.

2.2 Isolation and culturing of *Kalaharituber pfeilii*

The truffles were rinsed in running water to remove sand and then surface sterilized in 70% ethanol. The sporocarps were broken open to avoid contamination by bacteria residing on the outer surface. Explants were plated onto modified Fontana (FNT) media (Appendix A - Adeleke, 2007; Fontana, 1968). The pH of the medium was adjusted to 7.2 and plates were incubated at 32°C (Adeleke, 2007). Fungal cultures were sub-cultured at regular intervals to maintain growth. Once cultures were established, mycelial plugs were inoculated into FNT broth and incubated on a rotary shaker at 150 rpm in a 28°C controlled environment incubation room. The lower temperature was selected to avoid excessive evaporation of the liquid medium.

2.3 Molecular identification of cultures

2.3.1 DNA extraction

DNA extraction was performed using the Zymo Research Fungal/ Bacterial DNA MiniPrep™ Kit (Catalog No. D6005) as per manufacturer's instruction. Extractions were conducted on 7 *K. pfeilii* isolates, mycelia (approximately 200 mg) was scraped off from the media using a

sterilised scalpel and suspended in 200 µl of sterile distilled water in a microcentrifuge tube. This mixture was transferred into a ZR BashingBead™ Lysis tube followed by the addition of 750-µl lysis solution to break open the cell. Subsequently the tube was secured in a bead beater, which was processed at high speed for 5 min. The ZR BashingBead™ Lysis Tube was centrifuged in a microcentrifuge (Hangzhou Allsheng Instruments) at 10 000 x g for 1 min. Following centrifugation, 400 µl of the supernatant was transferred to a Zymo-Spin™ IV Spin filter in a collection tube and was centrifuged at 7 000 x g for 1 min. Fungal/Bacterial DNA binding buffer (1200 µl) was added to the filtrate obtained in the collection tube to enhance the binding of the DNA to the silica. Eight hundred microliters of the mixture were transferred to a Zymo-Spin™ IIC column in a new collection tube and was centrifuged at 10 000 x g for 1 min. The flow through was discarded from the collection tube and the step above was repeated to ensure proper binding of DNA to the column. DNA pre-wash buffer (200 µl) was added to the Zymo-Spin™ IIC column in a new collection tube and centrifuged at 10 000 x g for 1 min. DNA (500µl) wash buffer was added to the Zymo-Spin™ IIC column and centrifuged at 10 000 x g for 1 min. Thereafter, the Zymo-Spin™ IIC column was transferred into a clean 1.5 microcentrifuge tube and 50 µl of DNA elution buffer was added directly to the column matrix to elute the DNA and centrifugation was performed at 10 000 x g for 30 sec. The eluted DNA was used for Polymerase chain reaction (PCR) amplification.

2.3.2 Polymerase chain amplification

The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA) was amplified with the universal fungal primers ITS1 (5’ TCCGTAGGTGAACCTGCGG) and ITS4 (5’ TCCTCCGCTTATTGATATGC) (White et al., 1990). The reaction was carried out in a 50 µl reaction containing 2 µl of each primer, 16 µl of distilled water, 5 µl of DNA template and 25 µl of KAPA *Taq* ReadyMix (2X) DNA polymerase (Catalog No. KK1024). The 2X ReadyMix contained KAPA *Taq* DNA polymerase (1 U per 50 µl reaction), KAPA *Taq* buffer, dNTPs (2.2 mM of each dNTP at 1X), $MgCl_2$ (1.5 mM at 1X) and stabilizers. The PCR was performed using an Applied Biosystems 2720 Thermal Cycler, using the cycling conditions presented in Table 2. 1. The DNA obtained was eletrophoresed in 1% agarose gel stained with ethidium bromide with a concentration of 0.5 µg/mL and run in TBE buffer pH 8 at 80 volts

for 1 hr. The gel was viewed using Gel DocTM XR+ and ChemiDocTM XRS+ imaging system to check for the quality of the PCR products.

Table 2.1: PCR cycling parameters used for amplification of fungal DNA.

Steps	Temperature (°C)	Time (sec)	Cycles
Initial denaturation	95	120	X 1
Annealing	94	30	X 25
Denaturation	47	45	X 25
Elongation/extension	72	60	X 25
Final elongation	72	420	X 1

2.3.3 PCR product clean-up

The PCR product was purified using a Wizard® SV Gel and PCR Clean-up System kit (Catalog No. A9281) as instructed by the manufacturer. Equal volumes of the PCR product and membrane binding solution were added to a SV minicolumn inserted into a collection tube. This was incubated for 1 min at 25°C. Following incubation, the minicolumn assembly was centrifuged at 16 000 x g for 1 min. The flow through was discarded and the minicolumn was re-inserted into the collection tube. To the minicolumn, 700µl of membrane wash solution was added and the minicolumn assembly was centrifuged at 16 000 x g for 1 min. The flow through was discarded and the minicolumn was re-inserted into the collection tube. This step was repeated with 500 µl of membrane wash solution and centrifugation was performed at 16 000 x g for 5 min. To allow for evaporation of any residual ethanol, the collection tube was emptied and the minicolumn assembly was centrifuged open for 1 min at 16 000 x g. The minicolumn assembly was carefully transferred into a clean microcentrifuge and 50 µl of nuclease free water was added to the column to elute the DNA. This was allowed to stand at 25°C for 1 min. Following incubation, the minicolumn assembly was centrifuged at 16 000 x g for 1 min. The eluted DNA was eletrophoresed in 1% agarose gel stained with ethidium bromide with a concentration of 0.5 µg/mL and run in TBE buffer pH 8 at 80 volts for 1 hr. The gel was viewed using Gel DocTM XR+ and ChemiDocTM XRS+ imaging system to check for the

quality of the PCR products. The PCR products obtained were sent for Sanger sequencing (Sanger et al., 1977) to Inqaba Biotechnologies in Pretoria.

Sanger sequencing also known as chain termination sequencing is a polymerase-dependent synthesis method (Zhou and Li, 2015). Reagents required for sequencing include the DNA template, primers, DNA polymerase, deoxynucleotides (dNTPs) and the fluorescently labeled dideoxynucleotides (ddNTPs). Sanger sequencing is a DNA sequencing technique that produces DNA strands of various lengths owing to the random termination of the reaction by the incorporation of non-reversible synthesis terminator ddNTPs. The various segments are separated by size through electrophoresis and are arranged in the capillary tube from the shortest to the longest. Each ddNTP is labeled with a different color and the sequencing machine records the color of the terminator bases as a series of colored blocks revealing the DNA sequence of the template strand (Morozova and Marra, 2008).

The sequences were analyzed using FinchTV DNA sequencing analysis program and then submitted to the NCBI database (Zhang et al., 2000; Morgulis et al., 2008).

2.4 Biological properties

2.4.1 Determination of antimicrobial properties

Bacterial isolates were obtained from the Department of Biochemistry and Microbiology, Rhodes University, Grahamstown, South Africa. The four bacterial isolates used in this study were *Bacillus subtilis, Escherichia coli, Pseudomonas putida* and *Staphylococcus aureus.*

Bacterial isolates were discontinuously streaked onto Nutrient agar (NA) and were incubated overnight at 37°C to check for purity. A single colony was subsequently inoculated into Nutrient broth (NB) and incubated overnight at 37°C. All tests were conducted in triplicates.

2.4.1.1 Truffle extracts preparation

One hundred grams of fresh surface sterilized truffle was placed in a beaker with 250 ml of the extraction solvent (40% concentration). Water, ethanol, methanol, and dimethyl sulfoxide (DMSO) were used as extractants according to standard methods for mushroom/truffle extactions (Janakat et al., 2004; Janakat et al., 2005; Neggaz & Fortas, 2013). The truffles were homogenized using a blender. Two antimicrobial methods were employed namely disc diffusion and agar well diffusion methods (Bauer et al., 1966). Tests were performed using both NA and FNT agar media.

2.4.1.2 Determination of antimicrobial activity

Using the disc diffusion method, *K. pfeilii* extracts were filter sterilized using a syringe filter with pore size of 0.45-µm diameter into microcentrifuge tubes containing filter disc. The discs were left for 20 min to allow for proper absorption of the extracts. An aliquot of 100 µl of the bacterial cultures were spread plated onto the surface of NA agar plate and the plates were left to dry for 15 min at 25°C. Using sterile forceps, the filter disks were placed onto the agar surface and gently pressed down to ensure contact. The plates were incubated at 32°C for 24 h. A zone of inhibition around the disc indicated a positive reaction. The lower temperature was selected to prevent the disc from drying too quickly.

A subsequent antimicrobial activity screening of fungal isolates was evaluated on FNT agar plates. A plug of actively growing mycelium was transferred to new FNT agar plates and incubated at 32°C for 6 days. Bacterial isolates were streaked along the sides of the plates 2 cm away from the growing mycelium and incubated as above. The tests were considered positive if an inhibition zone was observable between the fungal colony and the bacterial growth.

Five equidistance agar wells were made in FNT media plates using a sterile 6 mm diameter corer (Fig 2.1). The middle well was inoculated with *K. pfeilii* plug and the four wells on the sides were inoculated with 70 µl of a bacterial broth culture. All the plates were incubated at 32°C development of a zone of inhibition represented a positive response.

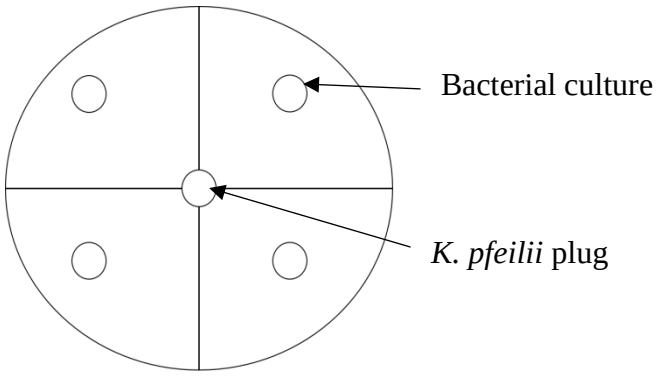

Figure 2.1: Agar well diffusion method for antimicrobial screening.

2.4.3 Screening for enzyme activity

Screening for *K. pfeilii* enzyme activity was conducted on both FNT agar plates and in FNT liquid culture since the FNT media is a chemically defined media and nutrient sources can be adjusted if required.

2.4.3.1 Cellulose utilization

One percent carboxymethylcellulose (CMC) sodium salt (Catalog No. Sigma C4888) was sterilized with FNT medium minus glucose, in an autoclave and allowed to cool down. The media was aseptically transferred to Petri dishes. A plug of *K. pfeilii* was inoculated onto the media and incubated at 32°C for 7 days. After incubation, the FNT + CMC agar plates were flooded with a freshly prepared 1% w/v Congo Red (Catalog No. Sigma C.I. 22120) solution for 15 min at 25°C, after which the stain was discarded. CMC degradation was positive if a yellow opaque zone occurred around the colony against a red background indicating un-degraded CMC (Pointing, 1999). Uninoculated FNT medium was used as controls.

Cellulose utilization by *K. pfeilii* was also tested in FNT broth without shaking. For cellulase production 1% CMC, (Catalog No. Sigma C4888) was added to liquid medium. After autoclaving, the media was allowed to cool down and was aseptically poured into sterile petri dishes. A plug of *K. pfeilii* was inoculated in the FNT + CMC liquid medium and was incubated for 14 days. The experiments was conducted in triplicate and controls were uninoculated. The

liquid cultures were filtered using a Buchner funnel under vacuum. Mycelium was collected on a dried 90 mm Whatman #1 filter paper. Fungal biomass was dried at 37°C for 72 hr and weighed. Weight was corrected for filter paper.

2.4.3.2 Starch utilization

Amylase screening was conducted according to the method of Behlet et al., (2006) and Rele (2004) using a FNT medium minus glucose amended with 1% soluble starch (Catalog No. Sigma 9004-32-4). A plug of *K. pfeilii* was inoculated onto the amended media and incubated at 32°C for 7 days. To test for starch hydrolysis, the plates were flooded with iodine solution. A clearance zone around the colony indicated a positive reaction for amylase activity. Uninoculated FNT medium was used as controls.

Screening for starch utilization was also conducted using FNT liquid media amended with 1% soluble starch (Catalog No. Sigma 9004-32-4). After autoclaving, the media was allowed to cool down, and it was aseptically poured into sterile petri dishes. A plug of *K. pfeilii* was inoculated in the FNT liquid medium and was incubated for 14 days. The experiment was conducted in triplicate. Mycelial was collected on a 90 mm Whatman #1 filter paper using a Buchner funnel under vaccum. Fungal biomass was determined as described above (section 2.4.3.1).

Laccase activity was assessed on FNT media minus glucose with 1% guaiacol as a sole carbon source (Catalog No. Sigma G5502). After autoclaving, the media was allowed to cool down and was aseptically poured into sterile petri dishes and was allowed to cool. A plug of *K. pfeilii* was inoculated onto the amended media and incubated at 32°C for 7 days. The production of an intense brown color around the fungal colony was considered a positive reaction for the presence of laccase activity (Jebapriya and Gnanadoss, 2013). Laccase production by *K. pfeilii* was investigated in FNT liquid media using the laccase indicator-guaiacol. The procedure was as described above (section 2.4.3.1) and fungal biomass was recorded.

2.4.3.4 Protein utilization

Screening for protease activity was conducted using FNT medium amended with low fat skim milk. The long-life milk was aseptically added to the cooled FNT media after autoclaving (1:1) and gently mixed before pouring into plates. The media was inoculated with a plug of *K. pfeilii* and incubated for 7 days. A zone of clearance around the colony indicated a positive reaction. Uninoculated plates were used as controls.

Screening for protein utilization was also conducted using FNT liquid medium amended with 1.6% of low fat skim milk. The milk was aseptically added to the cooled media after autoclaving. A plug of *K. pfeilii* was inoculated in the FNT liquid medium and was incubated for 14 days. The experiment was conducted in triplicate. Fungal biomass was determined as described above (section 2.4.3.1).

2.5 Determination of substrate preference using Biolog FF MicroPlate

K. pfeilii cultures, inoculated into a FNT liquid (250 ml) media, were incubated at 28°C on a rotary shaker at 150 rpm. After incubation, the liquid media was carefully discarded and the biomass was re-suspended in 100 ml sterile saline (0.2 %) and homogenized with an Ultraturrex, which was pre-sterilized in 70 % ethanol. A Biolog FF MicroPlate (Catalog No. 1006) containing 95 discrete carbon substrates was pre-warmed at 25°C for 30 min. Wells were inoculated with (150 µl) homogenized culture suspension. Assimilation and utilization of substrates was evaluated daily at 490 nm using a plate reader (Power WaveX Bio-Tek Instruments) for ten days. Blanked readings above 0.1 were regarded as positive for utilization (Rice and Currah, 2005).

2.6 Determination of substrate preference using selected carbon souces

The FNT modified media was optimized to produce high quality inoculum using four different carbon sources. The effect of carbon sources on the growth of *K. pfeilii* mycelium in culture was assessed using culture media containing glucose (control), maltose, xylose, sucrose and malic acid (Ceccaroli et al., 2001)**.** Each carbon source had three replicates. The cultures were incubated for three weeks at 25°C poured into petri-dishes without shaking. The liquid medium

was filtered using Buchner funnel under vacuum and mycelia was collected on a 90 mm Whatman #1 filter paper. Fungal biomass was dried at 37°C for 72 hr. and weighed. Weight was corrected for filter paper.

2.7 Optimization of mycelial biomass production

K. pfeilii was inoculated into five different liquid media to determine which was best to support fungal growth. The media used were modified MMN (Appendix A), PGY (Appendix A), PGY with no yeast, modified FNT and FNT media (Appendix A) as a control. Each media had three replicates. The inoculated media was incubated at 28°C for 3 weeks. The fungal biomass was determined as described above (section 2.6).

2.8 Rhizosheath diversity

2.8.1 Identification of mycorrhizal association found in roots.

Roots stored in 50% ethanol were used for staining. The ethanol was carefully discarded over the 125-µm sieve to prevent loss of roots. To clear roots, 5% KOH was added to each sample bottle and was allowed to stand overnight at room temperature. The KOH was discarded and the roots were rinsed with distilled water. The roots were bleached with a freshly prepared bleaching solution (H_2O_2) for 30 min. After the bleach, solution was discarded and roots were rinsed with distilled water. Roots were acidified by covering with 0.1 M HCl solution overnight. HCl was discarded and roots were stained with a lactoglycerol solution containing 0.05 % trypan blue. Roots were left overnight at room temperature. The staining solution was discarded followed by de-staining with lactoglycerol (Smith and Dickson, 1997).

The roots were mounted, in lactoglycerol, on microscope slides, covered with coverslips, and placed under a compound microscope (Leica CME) to identify the mycorrhizal structures present. Images were captured using a digital compound light microscope (Olympus BX50) with a camera (Olympus DP72) using analySIS software.

2.8.2 Assessment of bacterial diversity using Next Generation Illumina Sequencing and bioinformatics analysis

2.8.2.1 DNA extraction

Roots were placed in a sterile mortar and frozen with liquid nitrogen before crushing with a sterile pestle. The crushed root material was transferred to a Bashing Bead Lysis tube and DNA was extracted using the Zymo Research Plant/Seed DNA Miniprep Kit (Catalog no. D6005) as per manufacturer's instructions (method similar to that described in Section 2.3.1).

2.8.2.2 Polymerase chain amplification

Extracted DNA was first amplified with the universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG) and rP2 (ACGGCTACCTTGTTACGACTT) (Weisburg et al., 1991) in a 25 µl reaction mix containing 2.5 µl DNA template, 1 µl of each primer, 12.5 µl Kapa Hifi master mix (Catalog no. KK1024) and 8-µl of sterile distilled water. PCR amplification of 16S rDNA was performed using an Applied Biosystems 2720 Thermal Cycler using the cycling parameters presented in Table 2.4.

Table 2.2: PCR cycling parameters for bacterial DNA amplification.

Steps	**Temperature (°C)**	**Time (sec)**	**Cycles**
Initial denaturation	98	300	X 1
Denaturation	95	45	X 30
Annealing	52	45	X 30
Extension	72	60	X 30
Final extension	72	120	X 1

The PCR products were then electrophoresed on 1% agarose gel stained with 2µl of ethidium bromide (with a concentration of 5µg/ml) along with Pst DNA ladder (Catalog no. 29278810) and run in 1× TBE buffer at 100 volts for 1 hour. The gel was viewed using Gel Doc™ XR+ and ChemiDoc™ XRS+ imaging system to check for the quality of the PCR products. The PCR products were purified using a Wizard® SV gel and PCR clean-up System.

2.8.2.3 PCR product purification

The DNA fragment of interest was excised with a minimum volume of agarose using a clean sterile scalpel. The gel slice was transferred into the weighed microcentrifuge tube and the weight was recorded. The weight of the empty tube was subtracted from the total weight to obtain the weight of the gel slice. For every 10 mg of agarose gel slice, 10 µl of the membrane binding solution was added. The mixture was vortexed and incubated at 55°C until the gel slice was completely dissolved. The SV minicolumn was placed in a collection tube for each dissolved gel slice. The dissolved gel mixture was transferred to the SV minicolumn assembly, and incubated for 1 min at 25° C. The SV minicolumn assembly was centrifuged in a microcentrifuge at 16000 x g for 1 min. The SV minicolumn assembly was removed from the spin column assembly, and the liquid in the collection was discarded and the column was subsequently returned to the collection tube. The column was washed by adding 700 µl of membrane wash solution and centrifuged at 16 000 x g for 1 min. The collection tube was emptied and the SV minicolumn was placed back in the collection tube. This step was repeated with 500 µl of membrane wash solution and was centrifuged at 16 000 x g for 5 min. The SV minicolumn was carefully removed from the microcentrifuge and the collection tube was emptied and placed back into the SV minicolumn assembly. To allow evaporation of any residual ethanol, the column assembly was centrifuged for a minute with the tube lid open. The SV minicolumn was carefully transferred to a clean 1.5 microcentrifuge tube. Fifty microliters of nuclease-free water were added directly to the center of the column without touching the membrane with the pipette tip. This was allowed to stand at room temperature for one minute. Thereafter, the tube was centrifuged at 16 000 x g for 1 min. The SV minicolumn was discarded and the microcentrifuge tube containing the eluted DNA was then stored at -20 ° C.

The PCR product obtained was further amplified using Miseq primers in preparation for Illumina sequencing.

2.8.2.4 PCR using tagged primers

Amplification of bacterial 16S rRNA sequencing genes were amplified using Miseq primers F (5′-CAGCAGCCGCGGTAA-3′) and R (5′-GTAAGGTTCTTCGCGT-3) for paired–end microbial community and sequenced on the Illumina Miseq platform. The reaction was carried out in 25 µl reaction volume containing 5 µl DNA template, 0.75 µl Miseq Sa-forward primer, 0.75 Miseq Sa- reverse primer, 5 µl $MgCl_2$, 0.75 dNTPs, 0.5 µl BSA, 0.5 Kapa HiFi Hotstart Ready Mix (KK1006.), and 11.75 µl of nuclease free water (Table 2.5).

Table 2.3: PCR cycling parameters for the paired-end sequencing.

Steps	**Temperature (°C)**	**Time (s)**	**Cycles**
Initial denaturation	98	300	X 1
Denaturation	98	45	
Annealing	45	30	X 20
Elongation/extension	72	60	
Denaturation	98	45	
Annealing	50	30	X 20
Extension	72	60	
Final extension	72	120	X 1

The PCR products were electrophoresed in 1% agarose gel stained with 2-µl ethidium bromide (5 µm/mg) along with 1500 bp lambda DNA in TBE buffer for 1 hr. at 80 volts to determine the exact size of the DNA. A NanoDrop 2000 spectrophotometer (Thermal Scientific) was used to analyze the quantity of the extracted DNA. DNA absorbance was measured at 260 nm and nuclease free water was used as a blank. The PCR products were stored at -20°C for Illumina

Miseq sequencing. Illumina next generation sequencing was used for sequencing of 16S rDNA to characterize bacterial community in the rhizosheath. Sequencing was conducted at University of the Western Cape next generation sequencing facility.

2.8.2.5 Statistical and Bioinformatic analysis

Mothur version (v.1.38.1) was used for sequence processing. All sequences/bases with low quality scores and short sequences were removed using a Qscore of 20. The sequence data was trimmed to remove reads with ambiguous bases and ones that are too long. Contaminating sequences were removed. Sequencing data was aligned to Silva-compatible alignment database (Quast et al., 2013). Chimeras were identified and removed using UCHIME (Edgar et al., 2011). Sequences above 97% were clustered into OTUs.

Chapter 3

3.1 *Kalaharituber pfeilii* isolation

Fresh ascoma of the *K. pfeilii* were collected from Karakul Research Institute Upington, Northern Cape, South Africa (Figure 3.1A). Isolates were successfully established on FNT medium and growth of colonies was observed after 14 days (Figure 3.2.B).

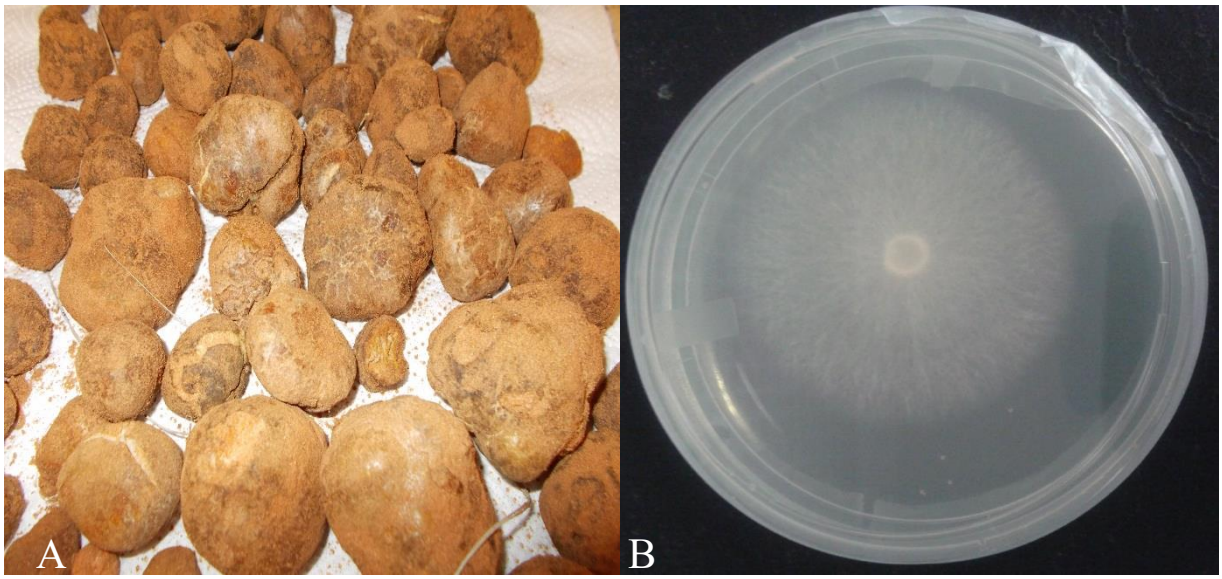

Figure 3.1: A) *Kalaharituber pfeilii* fresh ascoma and B) *K. pfeilii* mycelium growing on Fontana agar media.

3.2 Molecular identification of culture

Fungal DNA was successfully extracted from the 7 cultures of *K. pfeilii* (Figure3.2). This was followed by successful PCR amplification of the ITS region using the primer pair ITS 1 and ITS 4. A band size of 750 bp was observed on the gel (Figure 3.2). PCR products were sequenced.

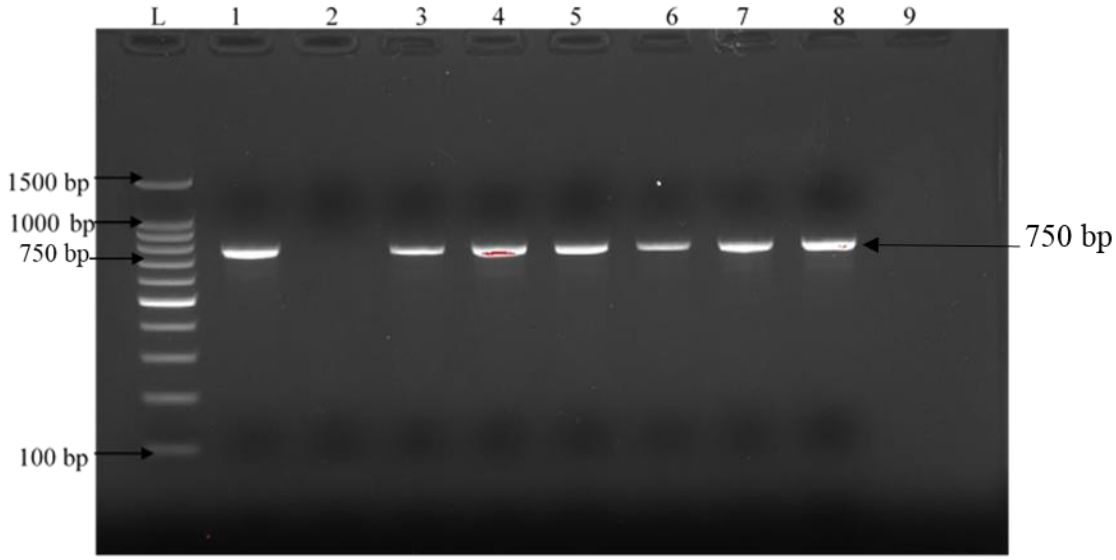

Figure 3.2: DNA was eletrophoresed in 1% agrose gel stained with 2 µl of ethidium bromide (at a concentration of 5mg/ml) and run in TBE buffer pH 8 at 80 volts for 1 hr. Lane L-100 bp ladder, lane 1 and 3-8 shows *K. pfeilii* PCR products from cultures. Lane 2 was broken therefore no sample was loaded.

Sequences obtained from Inqaba Biotechnologies were analysed and edited with FinchTV and were subsequently submitted to GenBank. Five isolates were confirmed to be *Kalaharituber pfeilii* (Table 3.1). Five sequences had a 99% similarity. KT2 had a short read. KT5 had a 95% similarity indicating a same genus (Liu et al., 2014). All subsequent analysis was conducted using KT1.

Table 3.1 Isolate identification from GenBank database identified using BLAST algorithm.

Samples	**GenBank Accession no.**	**Best database match**	**E-value**	**Query cover (%)**	**Identity (%)**
KT1	AF301422.1	*Kalaharituber pfeilii*	0.0	89	99
KT2	No significant similarity found				
KT3	AF301422.1	*K. pfeilii*	0.0	95	99
KT4	AF301422.1	*K. pfeilii*	0.0	96	99
KT5	AF301422.1	*Kalaharituber*	0.0	96	95
KT6	AF301422.1	*K. pfeilii*	0.0	96	99
KT7	AF301422.1	*K. pfeilii*	0.0	96	99

3.3 Biological properties of *K. pfeilii*

3.3.1 Determination of antimicrobial activity

Bacterial properties of *K. pfeilii* was tested against the four selected bacteria. No bacterial inhibition was recorded with any of the *K. pfeilii* extracts tested regardless of solvent used.

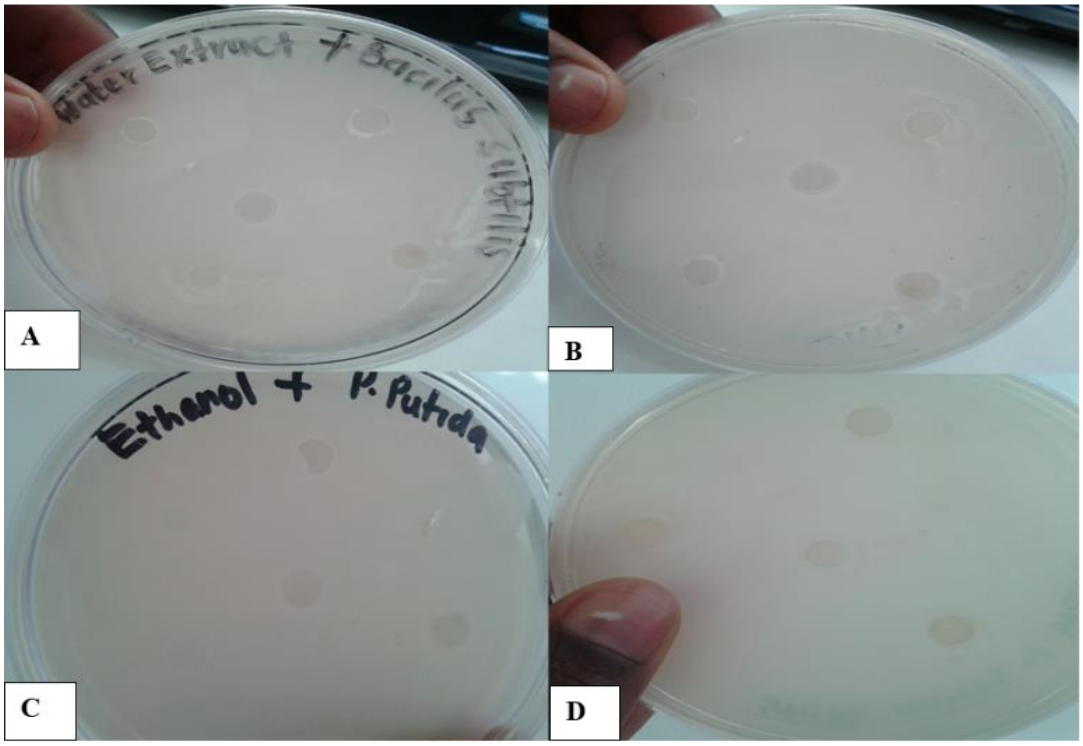

Figure 3.3: *Kalaharituber pfeilii* truffle extracts and effect on selected bacteria. A) Water extract against *Bacillus subtilis*, B) Methanol extract against *Escherichia coli*, C) Methanol extract against *Staphylococcus aureus* and D) Ethanol extract against *Pseudomonas putida*.

Microbial inhibition was also assessed using a dual inoculation quick screening technique (Figure 3.4).

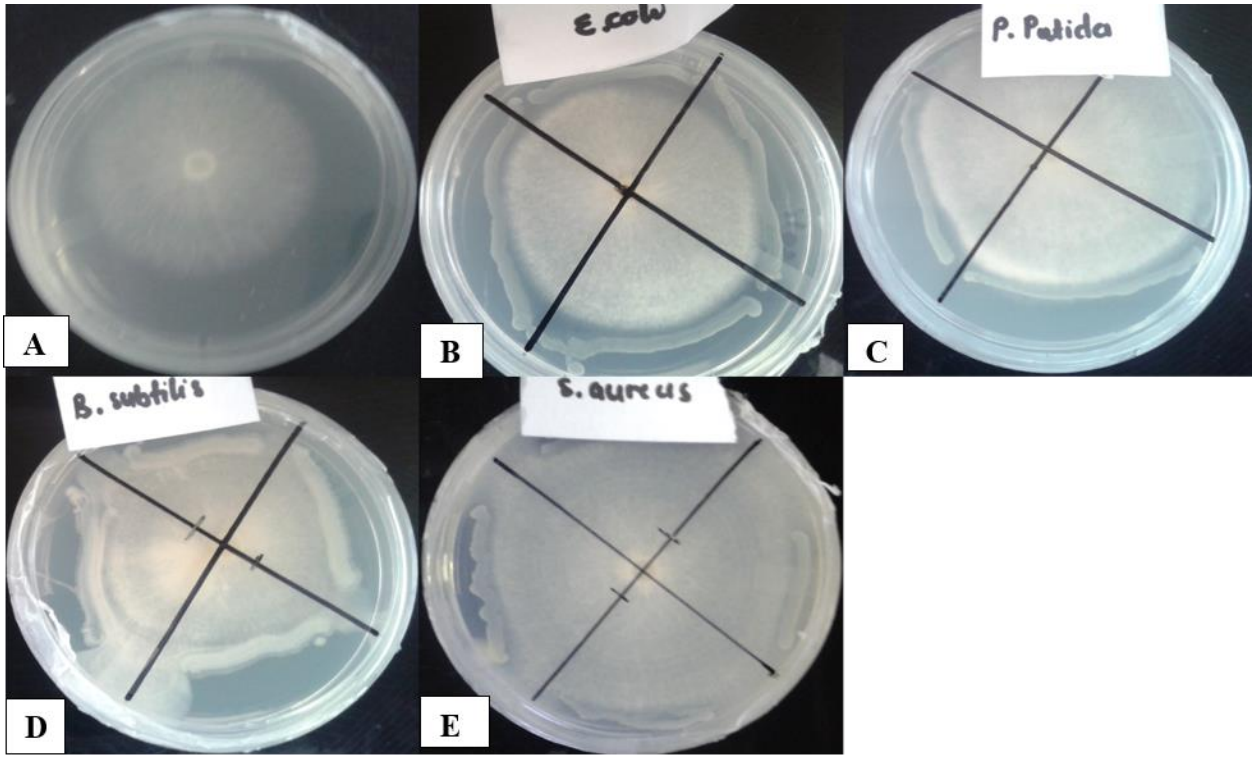

Figure 3.4: Dual culture assay with an actively growing *Kalaharituber pfeilii* culture in the center and bacterial isolate streaked along sides. A) Control, B) *Kalaharituber pfeilii* challenged by *Escherichia coli,* C) *Pseudomonas putida,* D) *Bacillus subtilis* and E) *Staphylococcus aureus.*

Some inhibition of fungal growth was noted when *K. pfeilii* grew with *E. coli* and *P. putida*. Lack of inhibition of the bacterial isolates was confirmed.

When fungal isolates were inoculated onto plates and challenged by the selected bacteria, fungal growth was inhibited around the wells containing the bacteria (Figure. 3.5). Indicating microbial activity against *Kalaharituber pfeilii*.

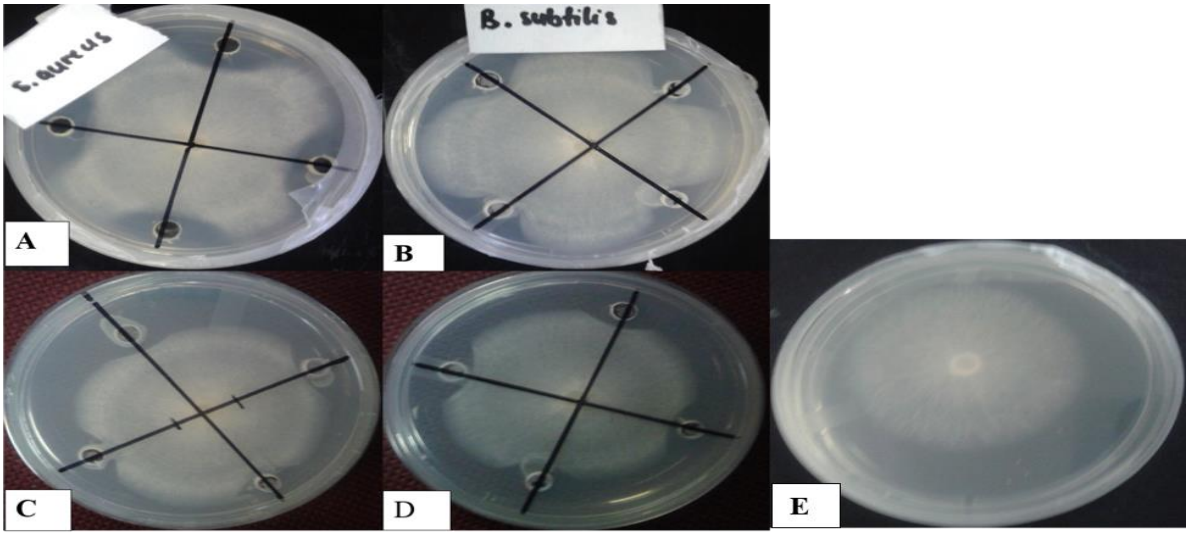

Figure 3.5: Microbial activity against *Kalaharituber pfeilii*. A) *Kalaharituber pfeilii* challenged by *Staphylococcus aureus*. B) *Bacillus subtilis*. C) *P. putida.* D) *Escherichia coli.* E) Control.

3.4 Screening for enzyme activity

Kalaharituber pfeilii was screened for enzyme activity including cellulase, protease, laccase and amylase. *Kalaharituber pfeilii* as shown in figure 3.6 has a capability to secrete enzymes such as amylase, protease but was unable to produce laccases and cellulose.

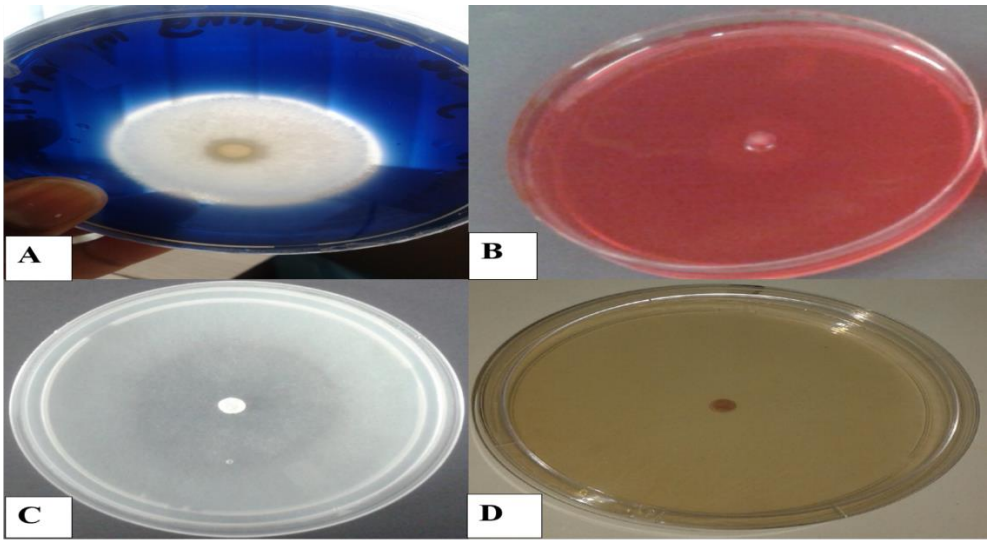

Figure 3.6: Enzyme screening of *Kalaharituber pfeilii* in solid media. A) Amylase activity. Zone of clearance indicating positive reaction. B) Cellulase activity. No zone of clearance indicating negative results. C) Protease activity. Zone of clearance indicating positive reaction. D) Laccase activity. No zone of clearance indicating negative reaction.

Fungal biomass was not recorded for liquid media amended with CMC and guaiacol indicating that *K. pfeilii* could not utilize these compounds for growth, confirming results obtained on solid media. Biomass was recorded in starch and protein amended FNT liquid medium (Fig 3.7).

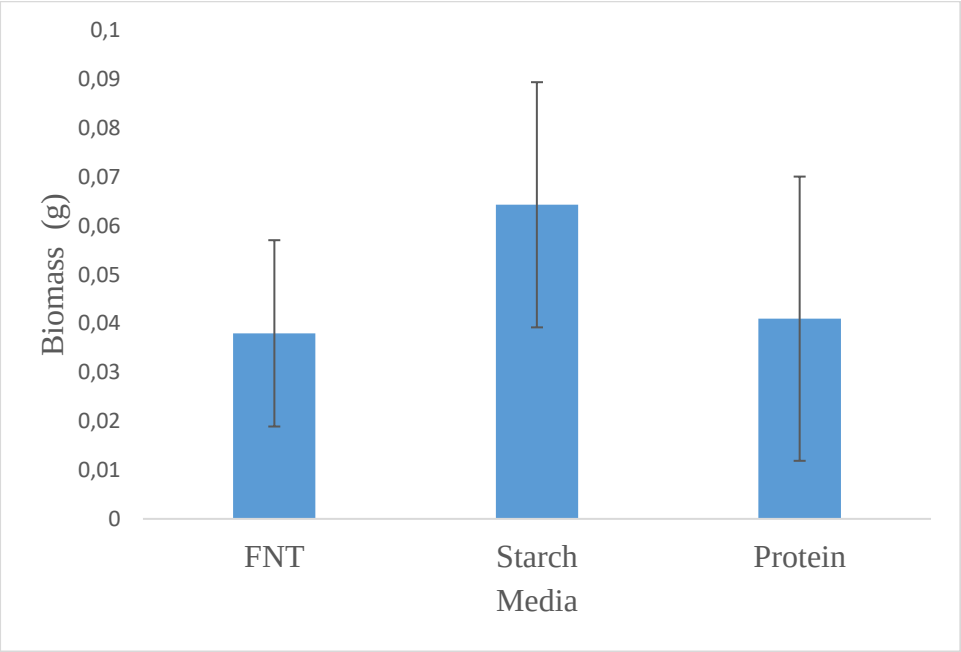

Figure 3.7: Utilization of starch and protein by *Kalaharituber pfeilii* in liquid media.

Fungal biomass was however not significantly different from that achieved in non-amended FNT control medium ($F_{(2,6)} = 1.016$; $p = 0.417$). Successful growth in the media containing starch and protein indicated ability to produce enzymes such as amylase and protease.

3.5 Determination of substrate preference using Biolog FF MicroPlate

The Biolog plates for filamentous fungi (FF) contains various substrates and the dye called iodonitrophenyltetrazolium redox dye which upon utilization turns purple (Atanasova and Druzhinina, 2010). Substrates which turned purple and had an absorbance of more than 0.1 (highlighted below), were regarded as positive for fungal utilization with an average well color development (AWCD) of 0.46 (Table 3.2). The substrates maltose, xylose, sucrose and malic acid were selected for further testing. These substrates were selected as they were most readily available.

Table 3.2: Carbon substrate utilization by *Kalaharituber pfeilii* using Biolog FF MicroPlate. Carbon sources utilized are in bold.

Cell no.	Substrate	O.D (490 nm)
A1	Water	0
A2	Tween 80	-0.15
A3	N-Acetyl-D-Galactosamine	-0.631
A4	N-Acetyl-D-Glucosamine	-0.204
A5	N-Acetyl-D-Mannosamine	-0.486
A6	Adonitol	-0.444
A7	Amygdalin	-0.247
A8	D-Arabinose	-0.224
A9	L-Arabinose	-0.134
A10	D-Arabitol	**0.387**
A11	Arbutin	**0.239**
A12	D-Cellubiose	-0.506
B1	α-Cyclodextrin	-0.108
B2	β-Cyclodextrin	-0.001
B3	Dextrin	**0.654**
B4	i-Erythritol	-0.383
B5	D-Fructose	0.044
B6	L-Fucose	-0.271
B7	D-Galactose	-0.48
B8	D-Galacturonic Acid	-0.208
B9	Gentiobiose	0.014
B10	D-Gluconic Acid	-0.413
B11	D-Glucosamine	0.043
B12	α-D-Glucose	-0.084
C1	Glucose-1- Phosphate	0.134
C2	Glucuronamide	-0.259
C3	D-Glucuronic Acid	-0.485
C4	Glycerol	-0.266
C5	Glycogen	**1.027**
C6	m-Inositol	-0.276

Cell no.	Substrate	O.D (490 nm)
C7	2-Keto-D-Gluconic Acid	-0.05
C8	α-D-Lactose	-0.069
C9	Lactulose	-0.133
C10	Maltitol	-0.088
C11	Maltose	**0.6**
C12	Maltotriose	**0.781**
D1	D-Mannitol	-0.124
D2	D-Mannose	-0.211
D3	D-Melezitose	0.035
D4	D-Melibiose	-0.632
D5	α-Methyl-DGalactoside	-0.395
D6	β-Methyl-DGalactoside	-0.449
D7	α-Methyl-DGlucoside	0.056
D8	β-Methyl-DGlucoside	-0.271
D9	Palatinose	-0.015
D10	D-Psicose	-0.195
D11	D-Raffinose	**0.308**
D12	L-Rhamnose	**0.171**
E1	D-Ribose	0.045
E2	Salicin	-0.175
E3	Sedoheptulosan	-0.327
E4	D-Sorbitol	-0.021
E5	L-Sorbose	**0.169**
E6	Stachyose	**0.142**
E7	Sucrose	**0.167**
E8	D-Tagatose	**0.146**
E9	D-Trehalose	0
E10	Turanose	0.042
E11	Xylitol	**0.203**
E12	D-Xylose	**0.171**
F1	γ-Amino-butyric Acid	0.02
F2	Bromosuccinic Acid	**0.175**

Cell no.	Substrate	O.D (490 nm)
F3	Fumaric Acid	-0.051
F4	β-Hydroxy-butyric Acid	-0.289
F5	γ-Hydroxy-butyric Acid	-0.214
F6	p-Hydroxyphenylacetic Acid	**0.173**
F7	α-Keto-glutaric Acid	-0.219
F8	D-Lactic Acid Methyl Ester	-0.245
F9	L-Lactic Acid	0.022
F10	D-Malic Acid	0.017
F11	L-Malic Acid	**0.434**
F12	Quinic Acid	0.047
G1	D-Saccharic Acid	-0.079
G2	Sebacic Acid	**1.183**
G3	Succinamic Acid	**2.634**
G4	Succinic Acid	-0.48
G5	Succinic Acid Mono-Methyl Ester	**0.137**
G6	N-Acetyl-LGlutamic Acid	**0.328**
G7	Alaninamide	-0.238
G8	L-Alanine	-0.084
G9	L-Alanyl-Glycine	-0.052
G10	L-Asparagine	**0.16**
G11	L-Aspartic Acid	**0.193**
G12	L-Glutamic Acid	-0.059
H1	Glycyl-L-Glutamic Acid	0.015
H2	L-Ornithine	-0.209
H3	L-Phenylalanine	-0.446
H4	L-Proline	-0.079
H5	L-Pyroglutamic Acid	-0.131
H6	L-Serine	-0.38
H7	L-Threonine	-0.357
H8	2-Amino Ethanol	-0.054
H9	Putrescine	-0.116
H10	Adenosine	-0.308

Cell no.	Substrate	O.D (490 nm)
H11	Uridine	**0.359**
H12	Adenosine-5'-Monophosphate	0.069

3.6 Determination of substrate preference using selected carbon sources

Kalaharituber pfeilii biomass grown in FNT medium amended with selected carbon substrates was evaluated. Medium containing sucrose as the sole carbon source (glucose being excluded from the FNT medium) and medium with the addition of sucrose and glucose (FNT-Sucrose) was significantly improved when compared to other carbon source combinations (Figure 3.8).

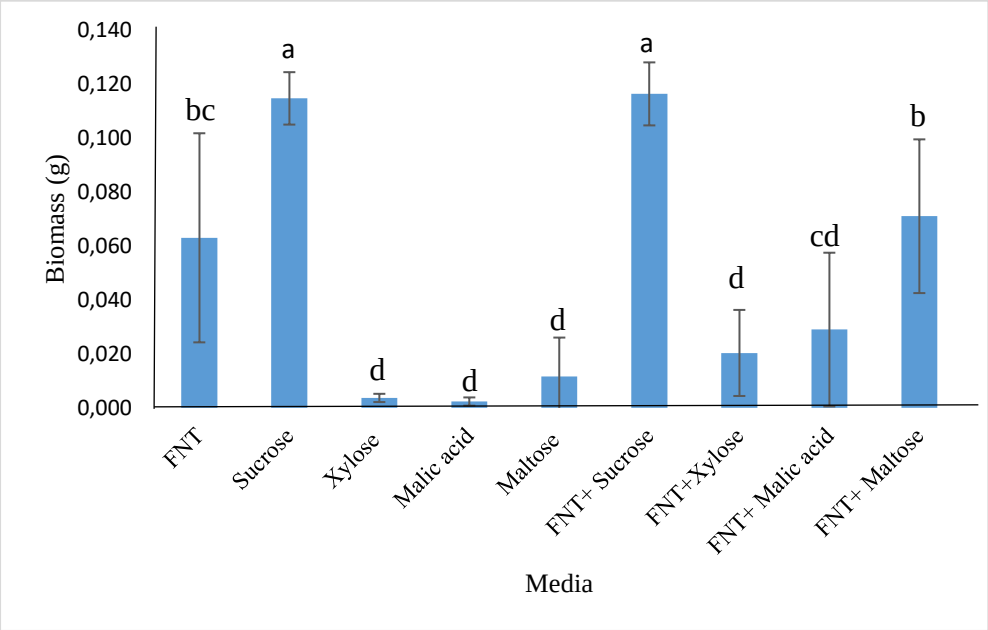

Figure 3.8: Effects of various carbon sources on mycelial biomass production by *Kalaharituber pfeilii* in liquid culture. Columns represent mean biomass ± standard deviations, n=3. Averages with different letters indicate statistical significance according to the LSD post hoc test $F_{(8, 18)} = 14.46$; $p < 0.001$.

3.7. Optimization of mycelial biomass

Four different media were used to optimize the growth of *K. pfeilii*. Fungal biomass was recorede after growth in various liquid media (Modified MMN, modified FNT, FNT, PGY no yeast and PGY. The results are illustrated in Figure 3.9.

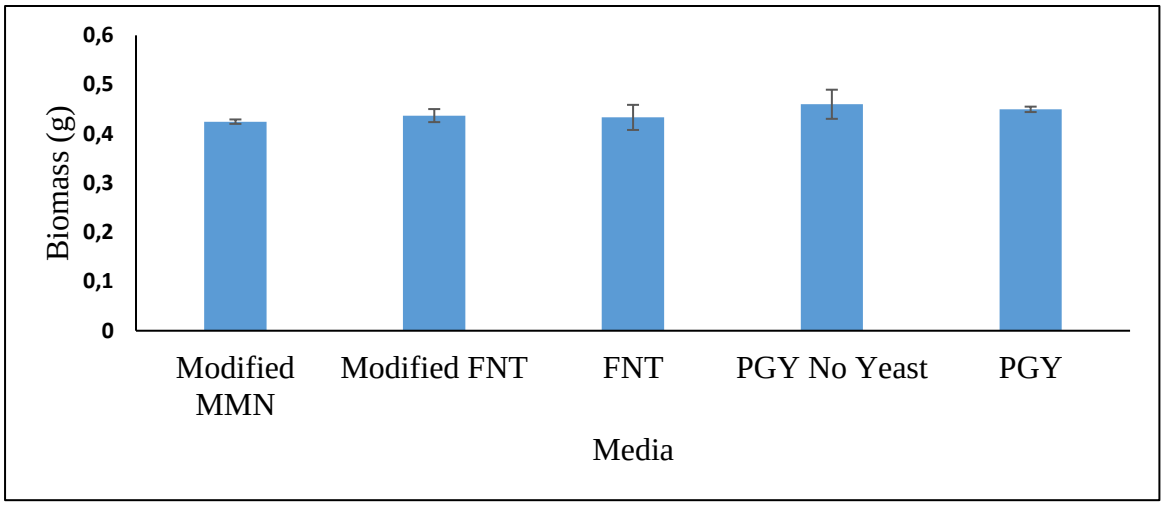

Figure 3.9: Effects of various media on mycelial biomass production of *Kalaharituber pfeilii* in liquid culture. Column represent means ± standard deviation, n=3, $F_{(1,4)}$=1.664; p=0.234. No significance was found.

3.8 Rhizosheath bacterial diversity

Stipagrostis ciliata var. *capensis* (host plant of *K. pfeilii*) with roots covered in rhizosheath were collected from the Northern Cape from plants in proximity to where truffles were collected (Fifure 3.10). The rhizosheath was used for root staining and the remaining was carefully removed leaving only the vascular cylinder behind and used for molecular determination of the bacterial communities.

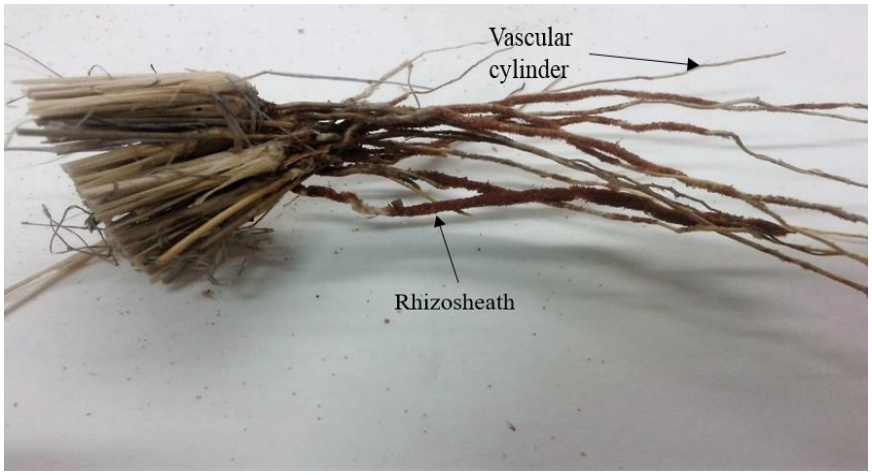

Figure 3.10: *Stipagrostis ciliata* var. *capensis* with rhizosheath collected from the Northern Cape.

3.8. 1 Mycorrhizal association found in roots.

Roots of *Stipagrostis ciliata* var. *capensis* showing evidence of mycorrhizal colonization (Figure 3.11).

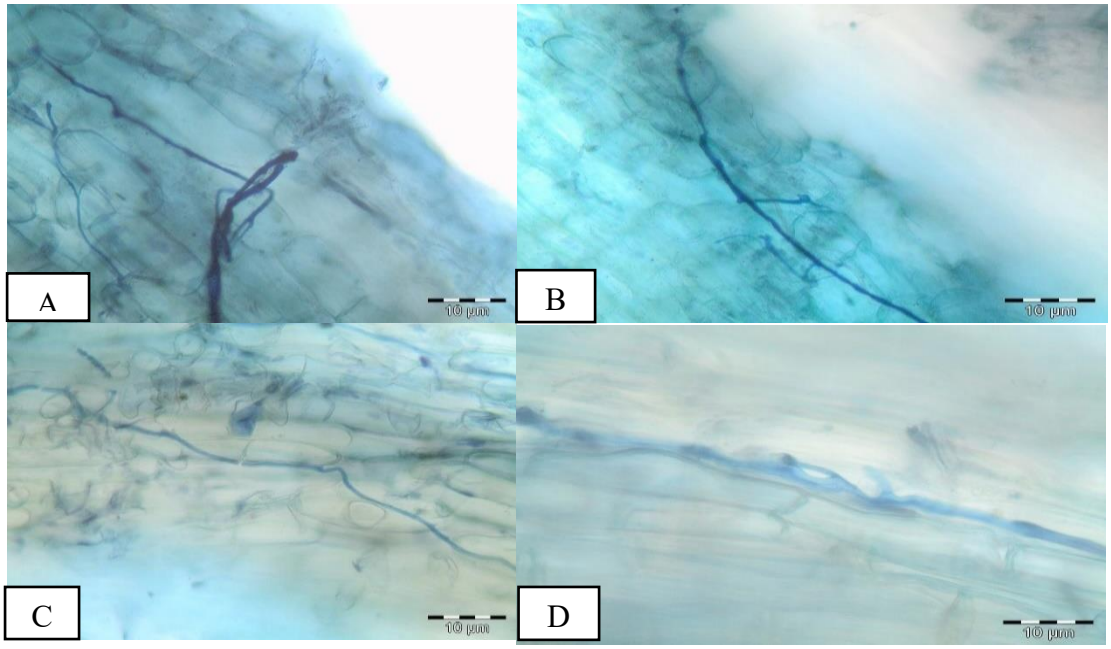

Figure 3.11: Qualitative analysis of stained roots of *Stipagrostis ciliata* var. *capensis* showing intraradical hyphae.

3.8.2 Assessment of bacterial diversity using Next Generation Illumina Sequencing and bioinformatics analysis

Fungal DNA was successfully extracted from the rhizosheath.This was followed by successful PCR amplification of the 16S region using Miseq primers for paired end bacterial 16S rDNA community sequencing on the Illumina Miseq platform. A band size of approximately 50bp was observed on the gel (Figure 3.12). Amplicons were sent for Illumina sequencing.

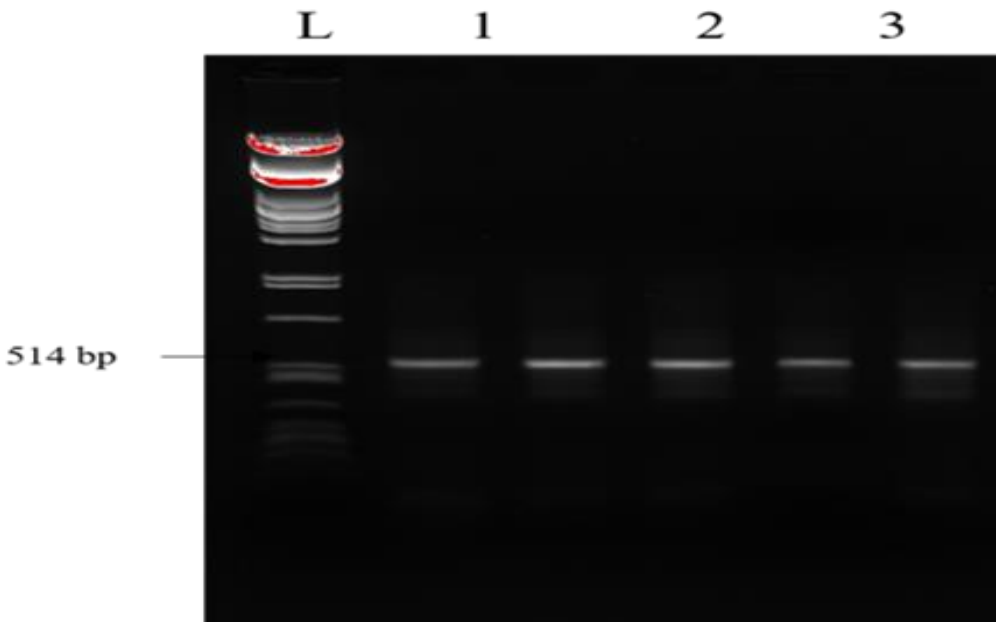

Figure 3.12: PCR products. DNA was eletrophoresed in 1% agrose gel stained with 2 µl ethidium bromide (5mg/ml) and run in TBE buffer pH 8 at 80 volts for 1 hr. Lane L- 100 bp lambda *Pst* ladder, Lane 1- 3 shows rhizosheath amplicons. PCR replicated sufficient amplicons after combination and clean up.

The product obtained in Figure 3.12 was sent for Illumina sequencing. The data was received back from sequencing unit in a zipped raw fastq file and was deposited into zip7 to unzip the file. The fastaq file was converted into a fasta file and analyzed using mothur software on the CHPC platform (https://www.chpc.ac.za/).

The top 25 phyla most abundant phyla were selected of which 13 % of those sequences were unclassified bacteria. Actinobacteria, Proteobacteria, Bacteroidetes, Acidobacteria, Plactomycetes, and Chloroflexi were the dominant bacterial phyla (Figure: 3.14).

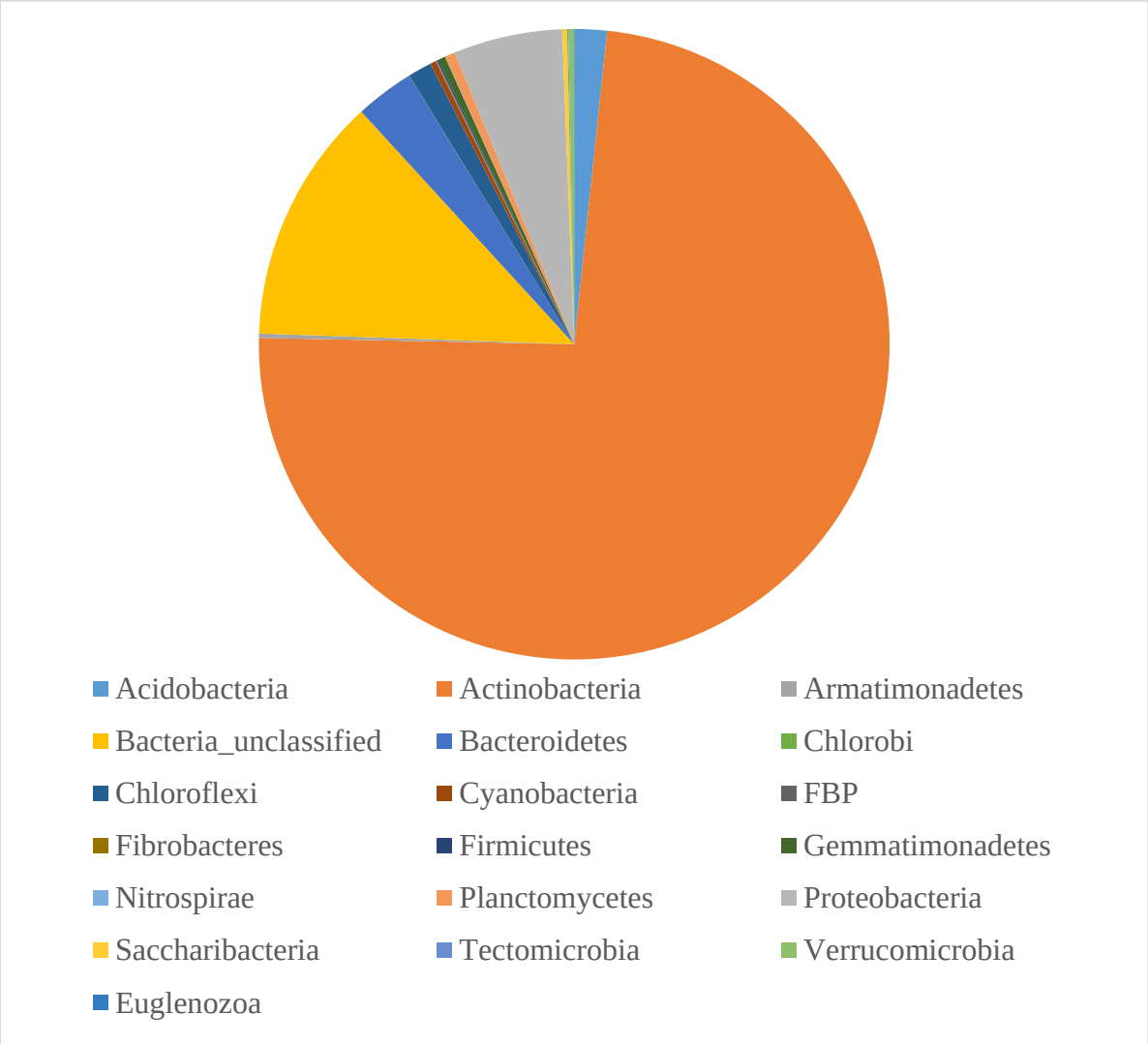

Figure 3.14: Relative abundance of the most dominant bacterial phyla associated with the rhizosheath of *Stipagrostis ciliata* var. *capensis*.

The top 25 OTUs were obtained in this study (Table 3.3) were submitted to a BLAST search on the NCBI GenBank database. Sequence similarity of ≥95 was found. Among these 12 OTU sequences had a 100% match to those found in the database for known bacterial sequences. Another 12 had a 98-99% similarity and only 24 had the lowest sequence similarity at 96% only for a genus level assignment (Table 3.4) (Landeweer et al., 2002).

Table 3.3: Identification of the top 25 bacterial OTUs using the NCBI-BLAST database.

OTU No.	Query cover	E-value	% Identity	GenBank Accession	Species name
1	100%	5e-80	100%	KY952635.1	*Amycolatopsis sp.*
2	100%	5e-80	100%	CP023445.1	*Actinosynnema pretiosum*
3	100%	5e-80	100%	NR_145619.1	*Kutzneria chonburiensis*
4	100%	5e-80	100%	KX358644.1	*Streptomyces chrestomyceticus*
5	100%	1e-76	99%	MF624488.1	*Amycolatopsis sp.*
6	100%	5e-80	100%	NR_152656.1	*Actinocrispum wychmicini*
7	100%	1e-75	98%	KY297007.1	*Pseudonocardia sp.*
8	100%	5e-80	100%	KY569410.1	*Streptomyces miharaensis*
9	100%	5e-80	100%	KY454621.1	*Streptomyces sp.*
10	100%	5e-80	100%	KY908432.1	*Streptomyces sp.*
11	100%	5e-75	98%	JQ977448.1	*Micromonospora sp. Ms20*
12	100%	2e-78	99%	KM349892.1	*Geodermatophilaceae AT04-02*
13	100%	5e-80	100%	LC342007.1	*Actinobacteria bacterium*
14	100%	1e-76	99%	AY234499.1	*Bacterium Ellin5082*
15	100%	5e-80	100%	LC333394.1	*Micromonospora sp. CHM1-11*
16	100%	2e-78	99%	NR_125543.1	*Actinoplanes subtropicus*
17	100%	2e-73	98%	KY445680.1	*Flavitalea sp.*
18	100%	5e-75	98%	KR184502.1	*Kineosporia sp. I13A-00002*
19	100%	5e-80	100%	NR_151866.1	*Phytohabitans kaempferiae*
20	100%	5e-75	98%	NR_125543.1	*Actinoplanes subtropicus*
21	100%	5e-80	100%	LC212912.1	*Streptomyces sp. 3SS3_06*
22	100%	5e-75	98%	KY952635.1	*Amycolatopsis sp.*
23	100%	1e-76	99%	NR_144572.1	*Actinoplanes cibodasensis*
24	97%	1e-66	96%	JN180129.1	*Actinomadura glauciflava*
25	100	1e-75	98	GQ494028.1	*Actinomycetospora sp. YIM 68245*

These dominant bacterial groups are further represented as % relative abundance in Figure 3.15.

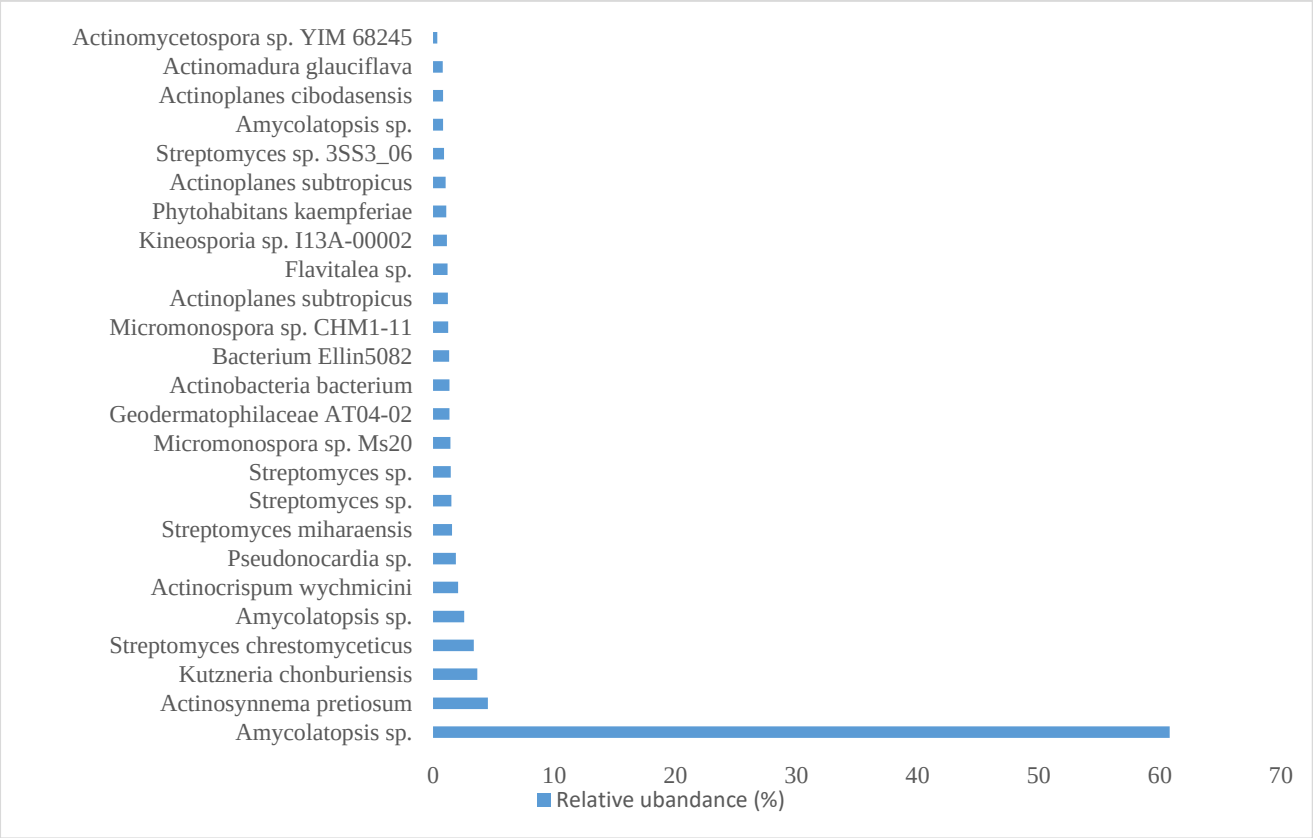

Figure 3.15: Relative abundance (%) of the most dominant OTUs identified in the rhizosheath

The Illumina bacterial analysis from the rhizosheath generated high quality sequences which were then distributed among 2863 OTUs. The bacterial OTUs were assigned into 1 phylum (Actinobacteria), 1 family (Geodermatophilaceae) and 23 genera. The Actinobacteria genera *Actinosynnema pretiosum, Kutzneria chonburiensis, Streptomyces chrestomyceticus, Amycolatopsis sp., Actinocrispum wychmicini, Pseudonocardia sp., Streptomyces miharaensis, Streptomyces sp., Micromonospora sp. Ms20, Bacterium Ellin5082, Micromonospora sp. CHM1-11, Actinoplanes subtropicus, Flavitalea sp., Kineosporia sp. I13A-00002, Phytohabitans kaempferiae, Streptomyces sp. 3SS3_06, Amycolatopsis sp., Actinoplanes cibodasensis, Actinomadura glauciflava, Actinomycetospora sp. YIM 68245, and Amycolatopsis sp.* were found in the rhizosheath of *Stipagrostis ciliata* var. *capensis* (Figure 3.15).

Chapter 4

4. Discussion

4.1 *Kalaharituber pfeilii* isolation

Kalahari truffles were collected from the Northern Cape Province, South Africa. Truffle isolation was successfully achieved on Fontana (FNT) medium as described by Adeleke (2007). During the first weeks of cultivation *in vitro* fungal growth was slow, this was also observed by Adeleke (2007) and Ntshakaza (2013). Bacterial contamination was noted and isolates often required subculturing. The presence of bacteria in the truffle ascocarp was also noted by both Adeleke (2007) and Ntshakaza, (2013). Adeleke and Dames (2014) molecularly identified several bacterial species which included *Leucobacter aluvunii*, *Stenotrophomas maltophilia*, *Phyllobacterium mysinacearum*, *Staphylococcus cohnii*, and *Pantoea dispersa.* Once subcultured plates were stored at 4°C and subcultured where necessary.

4.2 Molecular identification of culture

To ensure that the correct fungus had been isolated DNA was extracted from 7 truffle isolates and the universal fungal ITS region was PCR amplified using the universal fungal primers (ITS1F and ITS4) and sequenced. Comparative analysis of the sequences obtained with the GenBank database confirmed that 5 isolates had a 99% homology with *K. pfeilii*. The closest match in the GenBank database was AF301422.1 was assigned to all the sequences. This was deposited by Ferdman et al (2005) on NCBI. Ferdman et al., (2005) reallocated the Kalahari *Terfezia, T. pfeilii* to a different genus hence the Kalahari truffle became *Kalaharituber pfeilii* based on the observation that despite *T. pfeilii*'s incorperation in the ingroup it was considered far from the *Terfezia* clade meaning it that it belongs to a separate lineage.

4.3 Biological properties of *K. pfeilii*

4.3.1 Determination of antimicrobial activity

Screening for antimicrobial properties of Kalahari truffles could be a promising source of the novel therapeutic agents which are of clinical importance. The antimicrobial activity of *K. pfeilii* crude extracts was assayed using a Kirby-Bauer disc diffusion method (Figure 3.3). None of the truffle crude extracts (methanol, ethanol, water and DMSO) tested showed activity against the selected bacteria (*Bacillus subtilis*, *Pseudomonas putida*, *Staphylococcus aureus*, and *Escherichia coli*). Most studies first dry the truffle fruiting bodies prior to subject them into different tests, this delays the process of degradation of the active compounds (Hamza et al., 2016). However, in the present study, the truffles were not dried which may possibly explain the negative results.

As no inhibition from extracts was observed a well diffusion method was employed to determine whether actively growing mycelia produced any antimicrobial compounds. Again no activity was recorded against the test bacteria. These results differed from those obtained by Hussan and Al-Ruqaie (1999) who reported antimicrobial activity of truffle extracts from *Terfezia* sp. They reported methanol extracts of *Terfezia tirmania* to be effective against the Gram positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, however, ethanol extracts of the same truffle were noted to be effective against both Gram negative and Gram positive bacteria (Hussan and Al-Ruqaie 1999). In another study by Janakat et al., (2005), *Terfezia claveryi* extracts displayed antimicrobial activity against the Gram negative bacteria, *Pseudomonas aeruginosa*. In the present study, both Gram negative and Gram positive bacteria were tested. The results from this current study indicate that *K. pfeilii* does not produce biologically active antibacterial compounds.

The bacteria, however, were observed to have an antagonistic effect on mycelial growth of the fungi and may have been secreting some growth inhibitors i.e. lytic enzymes (chitinases), bacteriocins, antifungal compounds or siderophores. Adeleke and Dames, (2014) when they co-cultured the bacteria which were isolated from *K. pfeilii* ascocarps, reported some bacteria to have an inhibitory effect on the mycelium growth of the fungus. Mycelial growth is also influenced by bacterial volatile compounds including HCN and ammonia. Alhussaini et al., (2016) reported that aqueous extracts of *Terfezia claveryi* failed to inhibit *B. subtilis, P. putida*,

S. aureus, and *E coli*. Methanolic extracts of *T. claveryi* were found to be ineffective at 5% and 10% concetration, when applied as antimicrobial agents against the same bacterial isolates. Contrary to the results found in this study, Alhussaini et al., (2016) did observe that a zone of inhibition was detected at 20 and 40% concentration of methanol extracts.

4.4 Screening for enzyme activity

As a mycorrhizal fungus, the truffle mycelia interacts with a variety of host plants as well as other soil inhabitants and research has been conducted with a view of identifying potential sources of new bioactive compounds (Nadim et al., 2015). Fungal enzymes have a wide application in industry which includes the food, textile, and confectionary, cosmetic and leather industries and are preferred as they are considered to be more stable when compared to enzymes derived from other sources (Nadim et al., 2015).

Like any other living organisms, fungi, produce numerous enzymes required for various metabolic purposes such as hydrolysis and oxidation reduction reactions (Jonathan and Adeoyo, 2011). These enzymes are often secreted during truffle mycelium interaction with their host plants (Bedade et al 2017). During the developmental stages of mushrooms or truffles, enzymes play an important role in influencing their nutritional value, flavour, and shelf life (Jonathan and Adeoyo, 2011). Enzymes such as amylase, cellulase, laccase, protease and many others have been reported to be produced by mushroom fruiting bodies (Jonathan and Adeoyo, 2011). However, there are certain factors that may influence the production of enzymes by microorganisms and these include physical and chemical factors. Time, temperature and pH as well as access to micro- and macro-nutrients are examples of physical and chemical factors, respectively (Karim et al., 2015).

4.4.1 Protein utilization

This study showed that K. *pfeilii* had strong proteolytic abilities as indicated by the zone of clearance observed. Protease production has potential use at industrial level for a wide variety of industrial processes, such as food processing at a low cost (Novelli et al 2015). This was supported by the mycelial biomass produced in the liquid medium which had a reduced carbon

source. Protease production by other mycorrhizal fungi such as Ericoids has been documented. Leake and Read (1990) demonstrated the protease activity of two ericoid endophytes from soils of varying pH with BSA as a sole N source. These were *Hymenoscyphus ericae* and *Rhododthamnus chamaecistus,* with *Hymenoscyphus ericae* producing high levels of protease at acid pH values and *Rhododthamnus chamaecistus* at neutral pH levels. Proteases can be derived from various sources including microorganisms, plants and animals (Novelli et al., 2016). These enzymes play an important role in chemical and biochemical reactions which are involved in food, beverage, cosmetic, pharmaceutical and other related industries, as they hydrolyse peptide bonds of proteins and polypeptides (Beena and Geevarghese, 2010; Novelli et al., 2016). Proteases produced by microorganisms are generally intracellular in nature and are secreted into the medium (Savitha et al., 2011; de Souza et al., 2014).

4.4.2 Laccase

In this study, guaiacol was used as a laccase inducer to investigate the laccase production by *K. pfeilii*. Guaiacol is a phenol compound with an attached methoxy group, and is readily oxidized by the heme iron of peroxidases and therefore serves as a reducing co-substrate for cyclooxygenase reactions. It is a phenolic compound, yellowish in colour and found in nature and can be isolated from Guaiac and oxidation of lignin, guaiacum or wood creosote. This substrate did not stimulate the growth of truffle mycelium, and a negative reaction was recorded. This result is contrary to a study conducted on *K. pfeilii* from Namibia which showed laccase activity (Haileka, 2015). Similar results were expected in this study as this is the same species which grows in the same arid savannah biogeographic zone. However, the contradictory results reported here may also be due to desert species variability. In the above-mentioned study, laccase was isolated from the surface of the peridium of the Kalahari truffle as no activity could be detected from the glebal tissue. *K. pfeilii* was not grown in a culture media as they could not find an appropriate growth media for it, instead they blended the samples from the surface of peridium of *K. pfeilii* and collected the supernatant which was subsequently used to screen laccase activity with 2,2'-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as the laccase substrate (Haileka, 2015). This probably explains the negative results obtained in the present study as the samples from the current study were isolated from the glebal tissue. Nadim et al., (2015), obtained similar results to the current study

when investigating enzyme production of two truffle species, *Tuber aestivum* and *Tuber maculatum* with *Tuber aestivum* being negative for laccase production. Different fungi generally produce laccases throughout their secondary metabolism and are influenced by a number of factors which include carbon, nitrogen source, culture media as well as the presence of microelements (Brijwani et al., 2010; Yang et al., 2017). Certain fungi require excessive nitrogen concentration for laccase production, while in others laccase production is induced by nitrogen starvation (El-Batal et al., 2015). Nitrogen concentrations in both the FNT media (solid and liquid) were not adjusted and could explain the negative results. The enzymatic activity of ectomycorrhizal species are highly distinct among ECM lineages and climatic conditions as well as substrate availability are the main determinants (Courty et al., 2010; Tedersoo and Smith, 2013). The results obtained in the present study could be a result of different climatic conditions between South Africa and Namibia.

4.4.3 Cellulase

In this study, screening for cellulase production by *K. pfeilii* was carried out in CMC amended FNT medium. *K. pfeilii* was proved to be unable to assimilate CMC at 1% concentration this was supported by the inability to convert CMC into biomass. This was quite surprising as many mycorrhizal fungi have been reported to produce cellulase enzymes (Adeoyo et al., 2017). For instance cellulase activity of an ericoid mycorrhizal endophyte *Hymenoscyphus ericae* has been documented (Cairney and Burke, 1998). Another study by Bedade et al., (2017) showed that higher concentration of CMC reduced the production of cellulase in *Tuber maculatum* with an optimum CMC concentration of 0.5%. Cellulase activity would differ between different fungi (Jonathan and Adeoyo, 2011) and additionally the 1% concentration used in this study may have been too high. The results obtained from this study are similar to those obtained by Nadim et al., (2015) on *Tuber aestivum* which did not display cellulase activity.

Cellulolytic enzymes are ubiquitous in nature and are heavily involved in biodegradation processes (Jonathan and Adeoyo, 2011). These enzymes aid in the conversion of lignocellulosic materials into soluble materials which can be easily assimilated by fungi to stimulate vegetative growth and ultimately the development of the fruiting body (Jonathan and Adeoyo, 2011). In this study growth inhibition could have been due to the irregular circulation of oxygen and nutrients owing to the high viscosity of the medium which was observed after

adding 1% of CMC (Karim et al., 2015). This suggests that the concentration of CMC used in this study may have repressed cellulase production by *K. pfeilii*.

4.4.4 Starch utilization

Starch is ubiquitous in plant storage organs and is found profusely in nature as the second most abundant carbohydrate (Peters, 2006; Zhu et al., 2017). There is predominantly two well-known starch degrading pathways for the purpose of glucose production by fungi. A single enzyme glucoamylase facilitates the conversion of starch to glucose in one pathway. While ά-amylase and ά-glucosidase are the two enzymes responsible for starch hydrolyses in the alternate pathway. ά-amylase hydrolyses starch into maltose-oligosaccharide while ά-glucosidase hydrolyses maltose-oligosaccharide into glucose (Kusudu et al., 2008; Zhu et al., 2017).

In the present study screening for amylase production by *K. pfeilii* was carried out in FNT medium amended with 1% starch. *K. pfeilii* was positive for amylase activity (Figure 3.7). Ten fungal isolated of Nigerian mushrooms including *Agaricus blazei* sp., *Corilopsis occidentalis, Coriolus versicolor, Termitomyces clypeatus, Termitomyces globulus, Pleurotus tuber-regium, Podoscypha bolleana, Pogobomyces hynoides,* and *Nothopanus hygrophanus* have also been documented to be good amylase producers (Jonathan and Adeoyo, 2011). These enzymes have a broad range of application in the biotechnology sector, they are utilized in starch processing, production of bakery products, beer production and other cereal based beverages, flavouring production, yeast processing, sugar beet and distilled alcoholic beverages (Suganthi et al., 2011). This enzyme also has some important clinical application in the treatment of operation infection, removal of tumors and wound management (Azzopardi et al., 2016).

Starch and protein contents are of paramount importance in the determination of plant's health and fruit yield (Babu et al., 2015). Fungi require high levels of carbohydrates as a carbon source and as a growth factor, and starch is one form of carbohydrates. During fructification, huge amounts of mycelia are essential which aid in the transfer of nutrients to the fruiting body and act as a storage for these nutrients (Ohta, 1997). Even though ECM can be cultured apart from their host in the laboratory, they still cannot survive without their hosts in the field as they depend on the host in acquiring carbohydrates.

4.5 Optimization of mycelial biomass in various media

This aim of the present study was to maximize the production of *K. pfeilii* in liquid culture. Several studies have been carried out on optimization of ectomycorrhizal fungal growth using various nutrient sources in culture media (France and Reid, 1984; Holligan and Jennings, 1972; Mischiati and Fontana, 1993). The following media were used: FNT, modified FNT, modified MMN, PGY with yeast, and PGY supplemented with yeast to evaluate the production of biomass by *K. pfeilii*. Modified MMN was reported to be good culture media for fungus maintenance, while PGY was noted to be the most efficient media for biomass production (Rossi et al., 2011). Comparing the biomass production on the four culture media in figure 3.9 it is evident that all the media tested did not show a remarkable change in biomass yield. Hence, no statistically significant difference was observed in all four culture media studied. These results are different from those obtained by Rossi et al., (2011) where PGY was the most favourable media in comparison to MMN for the ectomycorrhizal fungi studied which was *Pisolithus microcarpus* thus, indicating species preference. The findings of the current study are also contrary to those found by Adeleke (2007), who reported that MMN did not support good growth of *K. pfeilii* and FNT was the most preferred media for the growth of this fungi.

4.6 Determination of substrate preference using Biolog FF Microplate

The present study aimed at optimizing the biomass production by *K. pfeilii* in liquid culture. Substrate utilization of *K. pfeilii* was assayed using the Biolog FF Microplate system. This system demonstrates the ability fungi to utilize 95 discrete carbon sources at the same time (Singh, 2009). The principle of this assay is based on the quantification of respiration through the reduction of the tetrazolium dye by succinate dehydrogenase (Pinzari et al., 2016). Purple colour formation is indicative of the dye reduction with maximum absorbance at 490 nm. However, the colour formation does not necessarily mean growth. Tenzer et al., (2003) showed that colour formation in some wells may occur even in the absence of cells because of the presence of required enzymes in supernatant or culture media. In the current study, blanked readings above 0.1 absorbance were regarded as positive for utilization (Table 3.2). Poor

colour development may be partly due to the slowness with which this fungus grows *in vitro*. Similar results have been reported by Rice and Currah (2005). The fungus was shown to utilize on D-Arabitol, Arbutin, Dextrin, Glycogen, Maltose, Maltotriose, D-Raffinose, L-Rhamnose, L-Sorbose, Stachyose, Sucrose, D-Tagatose, Xylitol, D-Xylose, Bromosuccinic Acid, p-Hydroxyphenylacetic Acid, L-Malic Acid, Sebacic Acid, Succinamic Acid, Succinic Acid Mono-Methyl Ester, N-Acetyl-LGlutamic Acid, L-Asparagine, L-Aspartic Acid and Uridine as sole carbon sources. In ECM fungi glycogen which is usually found in large quantities in the fungal hyphae, is a long-term carbohydrate storage much like in plants and is only utilized when the short term storage compounds such as trehalose is depleted (Nehls, 2008). Sucrose as a plant exudate is translocated to the plant-fungus interface from the plant root cells whereby it is hydrolysed by plant derived enzymes (Nehls et al., 2004). Out of the utilized substrates, 4 carbon sources were subsequently evaluated for biomass production of *K. pfeilii* in liquid media (Figure 3.10). These were xylose, malic acid, maltose and sucrose.

4.7 Determination of substrate preference using selected carbon sources

Effects of carbon sources on mycelial biomass production by *K. pfeilii* were investigated on FNT media amended with four different carbon sources including xylose, sucrose, malic acid or maltose (Figure 3.10). The results from the present study revealed that sucrose media and FNT/glucose plus sucrose were the most preferred carbon sources. This is in agreement with the results obtained by Adeleke (2007), these two carbon sources ensured the best mycelium biomass of *K. pfeilii*. However, Adeleke (2007) found glucose to be more efficient than sucrose, contrasting results were obtained in the current study as sucrose promoted more biomass in comparison to glucose. These contrasting results may be due to differences in incubation temperature 32°C/ 28°C. *K. pfeilii* growth was supported by all the carbon sources tested and this was confirmed by the mycelial biomass production, notwithstanding the fact that xylose, malic acid supported minimal mycelial growth. In the presence of FNT/glucose plus maltose an increased growth was recorded in comparison to media containing maltose alone. All the complex media used in this study did not significantly influence the mycelial biomass, except for the one with sucrose plus FNT/glucose which also showed a significant difference comparred to the reference media (FNT). Sucrose is the most affordable substrate and can be regarded as the most suitable carbon source for production of biomass by *K. pfeilii*.

The findings of the present study have provided new information on culture media optimization for improved biomass production by *K. pfeilii.*

4.8 Rhizosheath bacterial diversity

4.8.1 Assessment of bacterial diversity using Next Generation Illumina Sequencing and bioinformatics analysis

Identification of mycorrhizal association found in roots of the grass *S. ciliata var. capensis* demonstrated an endomycorrhizal association. Our findings are in line with Kagan-Zur et al., (1999), who reported *K. pfeilii* to form an endomycorrhizal association without Hartig net and sheath but with the presence of undifferentiated intracellular hyphae (Kurgan-Zur and Roth-Bejerano, 2008). Ntshakaza (2013) also looked at the type of mycorrhizal association present in the grass (*S. ciliata var. capensis*) and indicated that ectendomycorrhiza was the mycorrhizal type present in the roots of this grass. They also encountered difficulties in trying to focus the microscope due to the presence of the sand in the roots. The interference of the sand occurring in the rhizosheath with trying to find the mycorrhizal structures was also noted in the present study.

Identification of bacteria associated with the rhizosheath of *K. pfeilii* host plant *Stipagrostis ciliata var. capensis* was established using a next generation Illumina Miseq approach. This platform employs next-generation sequencing (NGS) for the identification of both culture dependent and culture-independent bacteria and analysis of 16S rDNA. The Illumina platform offers a quicker and simpler bacterial identification alternative to the traditional sequencing techniques and is more reliable. Illumina sequencing has received a great deal of attention in recent years, this is attributed to its lower cost per sequence when compared to other platforms (Caporaso et al., 2012; Degnan and Ochman, 2012).

The bacterial community of the rhizosheath was composed of 18 taxa mainly from Actinobacteria, Bacteroidetes, Proteobacteria, and Chloroflexi. The Actinobacteria were the most predominant taxon accounting for 73.65%. Unclassified taxa were the second most abundant accounting for 12.67%, followed by Proteobacteria at 5.58%, Bacteroidetes 3.02%, Acidobacteria 1.67%, Chloroflexi 1.22%, and Planctomycetes accounting for 0.52% and all

others were below 0.5%. The findings of this study are similar to the recent work done by van Aswegen (2017) on soil bacterial communities and diversity found associated with mycorrhizal fungi *Vachellia erioloba* (Camel thorn) which was collected from the Kalahari region. With the use of Illumina sequencing, van Aswegen (2017) recorded Actinobacteria and Proteobacteria as the most abundant bacterial phyla.

Actinobacteria, Proteobacteria and Firmicutes were also found in association with the *K. pfeilii* ascocarp (Adeleke and Dames, 2014). Desert soils have been reported to harbour a wide range of ubiquitous bacterial phyla including Actinobacteria, Bacteroidetes, and Proteobacteria (Fierer et al., 2009; Makhalanyane et al., 2015). These results also correspond with those shown in the Namibian Desert soil, with Actinobacteria being the most dominant bacteria (Makhalanyane et al., 2015). The predominance of these groups in arid environments is attributed to their capacity for sporulation, and a broad metabolismic capacity (Makhalanyane et al., 2015).

Proteobacteria which are Gram positive bacteria were also represented in our data, these are ubiquitous bacterial groups and are one of the prominent members of desert soil. Although present in low percentages, other bacterial lineages were also found in this study such as Gemmatimonadetes, Firmicutes, and Cyanobacteria which have been demonstrated to be more abundant in desert environments when compared to other biomes (Fierer et al., 2012).

The highest number of OTUs belonged to Actinobacteria. Actinobacteria have been observed to be the most abundant microbial taxon in subterranean environments (Cuezva et al., 2012). Actinobacteria have potential application in the fertilizer industry as natural fertilizer due to its influence on the soil fertility (Jog et al., 2016; Sathya et al., 2017). These bacteria secrete a cocktail of enzymes in addition to their capacity to solubilize phosphate and produce siderophore (Sathya et al., 2017). They have been reported to have plant growth promoting traits. Actinobacteria are Gram positive bacteria and one of the largest bacterial phyla known. This phylum consists of six classes, twenty-five orders, fifty two families and two-hundred and thirty two genera (Schumann 2000; Sathy et al., 2017). These are a group of soil bacteria found inside plant tissues or in the rhizosphere which aid in the plant growth promotion by forming a relationship with the plant roots (Tariq et al., 2014). Generally, plant growth promoting bacteria (PGPB) improves the health of the plant by regulating the detrimental effects of the pathogenic agents originating from bacteria, fungi and nematodes (Gupta et al., 2016; Tariq et al., 2014, Tariq et al., 2017). They do this through the production of growth inhibitors such as

antibiotics, siderophores, bacteriocins and lytic enzymes which disintegrate the pathogens cells (Tariq et al., 2017). Lytic enzymes such as protease, chitinases, lipase and glucanases are responsible for the disintegration of the fungal cell wall (Neeraja et al., 2010). Plant growth promoting actinobacteria have a capacity to affect plant growth by reducing the deleterious impact that may be caused by external stresses (Sathya et al., 2017). This is established through nutrient competition, secretion of low molecular inhibitory substances including secondary metabolites (Sathya et al., 2017). They also improve plant health indirectly by influencing the surroundings of the plant through selectively excluding and reducing the plant pathogens inhabiting the rhizosphere (Singh et al., 2017; Ahemad and Kibret, 2014).

Actinobacteria have been documented to have a helper effect on mycorrhizal symbioses by influencing the elongation of the symbiotic fungal hyphae. Adeleke and Dames, (2014) reported the presence of Actinobacteria in the ascorcap of *K. pfeilii*. The helper effect of Actinobacteria has been studied extensively with different host plants (Solan, 2007; 2009; 2015). This effect has been noticed with symbiotic associations such as *Ochetophila trinervis* and *Frankia* when they were inoculated with three actinobacterial species (*Streptomyces MM40, Actinoplanes ME3 and Micromonospora MM18*) (Solan, 2007). When these three isolates were co-inoculated with rhizobium and studied in another symbiotic system, the plants displayed an increased growth and nodulation in comparison to those with single inoculation (Solan et al., 2009; Solan et al., 2015). Soil microoganisms are an integral part of desert ecosysytems especially where plants are sparsely distributed to maintain the stability and productivity of the desert environments (Pointing et al., 2007; Makhalanyane et al., 2015).

The genus *Streptomyces* is the most dominant genus found in the present study. *Streptomyces* is the biggest antibacterial genus, it accounts for 80% of natural products produced (Manivasagan et al., 2013). Siderophores are crucial for plant nutrition as well as plant protection as they play a role in regulating plant pathogens (Sathya et al., 2017). *Streptomyces* have also been noted to be major producers of secondary metabolites, producing a broad range of compounds encompassing polyene, macrolides, actinomycins, aminoglucosides, streptothricins, anthracylines, cyclopolylactones and quinoxaline peptides (Berdy, 2012; Nicolaou et al., 2009). Over 3000 compounds with pesticidal and herbicidal activity have been reported in the literature. These secondary metabolites are used preferentially to those of fungal origin as they pose less of a phytotoxic threat (Sathya et al., 2017).

Pseudonocardia sp. have been isolated from soil as well as from the aquatic environments and have been observed to have some antimicrobial properties (Sujada et al., 2014), particularly against the microfungal parasite *Escovopsis* (Cafaro et al., 2011). *Pseudonocardia* and *Micromonospora* are crucial in xylan hydrolysis through the production of xylanases. *Micromonospora* have some antimicrobial properties as a defence mechanism against pathogens (Hirsch and Valdés, 2010).

Micromonospora are endophytic actinobacteria and have been isolated from the aquatic environments, animals, plant tissues and from arid habitats (Mohammadipanah and Wink, 2016). These actinobacterial species contain bioactive compounds which qualify them to be considered as potential sources of biocontrol agents, biofuels and plant growth products (Talukdar et al., 2016).

The *Amycolatopsis* genus belongs to the family *Psuedonocardiaceae* and currently consists of 69 species (Tan and Goodfellow, 2012). It has been isolated from different environments including natural caves, catacombs, salt mines, and ocean sediments as well as in plant roots as they have been noted to be more frequent in arid soil (Lee, 2006; Groth et al., 2007; Tatar et al., 2013; Bian et al 2009; Duangmal et al., 2011; Kim et a., 2002; Zucchi et al., 2012; Işık et al., 2018). This genus is well known for its secondary metabolites as well as its potential as a source of antibiotics which generally contributes to plant health. (Sphohn et al., 2014; Chen et al., 2016). They produce rifamycin (*Amycolatopsis mediterranei*) and vancomycin (*Amycolatopsis orientalis* NRRL 2452) which are the drugs used for the treatment of tuberculosis and inactive meningitis (Spohn et al., 2014). *Actinosynnema pretiosum* is one of the major producers of a secondary metabolite called ansamitocin an antitumor agent which has a great application in clinical research (Gao et al., 2014)

Actinosynnema pretiosum is a novel actinobacteria species. This is a synnema-forming bacterium. Generally known, for production of ansamycin which is a class of antibiotics, including ansamitocin P-3, geldanamycin, and rifamycin. These compounds have been reported to have strong antitumor and antiviral activity (Higashide et al., 1977; Du et al., 2017).

4.9 Conclusion and recommendations

Aim 1: Assess biological properties of *Kalaharituber pfeilii*

Kalaharituber pfeilii is endemic to the Kalahari and is often found in slightly calcareous sands in association with shrubs and grasses (Trappe et al., 2008). The present study demonstrated that the *K. pfeilii* extracts as well as the mycelia does not have antimicrobial activity, in fact, bacteria tended to show an anantagonistic effect against the fungus. Enzymes play a significant role in biological, clinical, biochemical, and industrial processes. Production of amylases and proteases suggest the ability of *K. pfeilii* to degrade and utilise starch and proteineous substrates. Enzymes of microbial origin are in high demand in biotechnological industries due to their low price, high production, availability, stability and diversity (Banerjee and Ray, 2017). This data supports ongoing need to investigate fungal enzymes for use in biotechnological processes such as breaking down starch, hydrolysis of protein based stains in fabrics and modifying the structure of cellulose fibre to increase the colour brightness and softness of cotton in the detergent industries (Li et al., 2012). The findings of the present study necessitates further investigation and characterization of these enzymes for further exploration in terms of industrial application. Future studies should look at optimization of nutrient sources for enzyme production and using a more biochemical approach to assess breakdown products. Adeoyo et al., (2017) looked at maximizing enzyme production of ericoid fungi and successfully optimized conditions and fully improve the production. They used different pH values ranging from 3 to 8, checked different carbon, and nitrogen sources as well as metal ions at various concentrations. A similar approach could be employed to optimize enzyme production by *K. pfeilii. K. pfeilii* produces extracellular enzymes such as proteases into the environment in order to breakdown the complex molecules which then the fungi reabsorb and allocate them to their host plant roots. The interactions between soil particles and enzymes are essential in improving the nutrient absorption by the plants as well as promoting the nutrient cycling of soil organic matter (Wang et al., 2014).

Aim 2: Optimisation of fungal growth in liquid medium

Fungal growth in liquid media displayed a preference for sucrose as a carbon source. These findings enable the production of this the truffle mycelium at a larger scale. These results were slightly different from the findings of Adeleke (2007) where glucose was the most preferred

carbon source. Adeleke (2007) did report that sucrose and glucose promoted the growth of the fungus which was found to be the case in the present study.

Aim 3: Assessment of rhizosheath bacterial diversity

The scientific evidence collected from the present study has demonstrated for the first time the diversity of the bacteria found in the rhizosheath of *Stipagrostis ciliata* var. *capensis*, of which Actinobacteria was encountered to be the most dominant bacterial phyla, this strengthen the literature about Actinobacteria being the most dominant phyla in Desert habitats (Mohammadipanah and Wink, 2016). The bacterial species found in the rhizosheath may influence the development of *K. pfeilii* fruiting body. For instance, Sbrana et al., (2002) demonstrated that the spore forming *Bacillus* sp. isolated from *Tuber borchii* had enhanced its growth by 78%. Further studies can explore the effects/functions of these bacteria in the development of the Kalahari truffle. Isolation of these rhizosheath inhabiting actinobacteria not only could provide more information of truffle development but also provide sources of compounds that can be further developed for agricultural or industrial purposes.

The purpose of this study was to determine bacterial communities associated with the host plant of *K. pfeilii* which is endemic to the Kalahari region of South Africa. A vast number of taxonomic groups of bacteria was obtained after Illumina sequencing. The present investigation had to rely on the forward primer sequence as the reverse primer sequence was of poor quality. A significant number of bacterial OTUs were found in the present study. This Next Generational Sequencing technology is an important tool in understanding the interaction of *K. pfeilii* and the bacteria found in the present study.

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Appendices

Appendix 1

Fontana medium preparation for 1L (adeleke, 2007)

6.5g D-glucose

4.65g Peptone

0.33g Potassium dihydrogen orthophosphate, KH2PO4

150 µl of 1% Magnesium sulphate heptahydrate, MgSO4.7H2O

150 µl of 1% Iron chloride, FeCl2

150 µl of 1% Zinc sulphate, ZnSO4

150 µl of 1% Magnessium sulphate, MgSO4

150 µl of 1% Calcium Chloride, CaCl2

15g Bacteriological gar

pH adjusted to

Modified FNT (Adeleke, 2007)

3.25 g D-glucose

4.65g Peptone

0.33g Potassium dihydrogen orthophosphate, KH2PO4

150 µl of 1% Magnesium sulphate heptahydrate, MgSO4.7H2O

150 µl of 1% Iron chloride, FeCl2

150 µl of 1% Zinc sulphate, ZnSO4

150 µl of 1% Magnessium sulphate, MgSO4

150 µl of 1% Calcium Chloride, CaCl2

15g Bacteriological gar

Modified Melin- Norkrans (MMN) medium preparation for 1L (Marx, 1969)

10 g Glucose

3 g malt extract

0.05 g $CaCl_2$

0.025 g NaCl

0.25 g $(NH_4)_2PO_4$

0.5 g KH2PO4

0.15 g $MgSO_4.7H_2O$

100 µg/l thiamine

1.2 ml $FeCl_3$ ((1%, w/V)

Modified Pridham-Gottlieb (PGY) medium preparation for 1L (Kuerk, 1996)

10 g glucose

3.33 g peptone

0.67 g yeast extract

1.0 g NH_4NO_3

0.264 g KH_2PO_4

0.628 g K_2HPO_4

0.33 g $MgSO_4.7H_2O$

0.0021 g $CuSO_4$

0.0006 g $MnCl_2$.4H2O

0.0005 g $ZnSO_4.7H_2O$

0.004 g $FeSO_4.7H_2O$

Appendix 2

Molecular identification of mycelium cultures

Sequence obtained from Inqaba Biotechnologies for fungi

>AF301422.1 *Kalaharituber pfeilii*

ATCATTATTGAGTAAGCTTTATTGTAGCTTTCTCTCTTATCCCTTTGTTTACTTTACCCTGTTGCTTCCA

CTGGACAGTGTGAGCTTTGCTGGCAGTTGAAGAAGTTCAATTGTAGGCAAGTGAGCCCTCTGGTTTTGGT

GCACTCGGTACCATTGCTGGGGAGTTTGCCGGTGGGTAGCCCCTTTTATAATCAAAACCTGTGTAATAGA

GAAACCTTTTTGTCTGATATTAAATGAAATAAAATGAAAAAGAATAAAACTTTCAACAACGGATCTCTAG

GCTCTTGCATCGATGAAGAACGCAGTGAATTGCGATAAGTAATGTGAATTGCAGAATCTCGTGAATCATC

GAATCTTTGAACGCACATTGCGCCCTATGGTATTCCGTAGGGCATGCCTGTCTGAGCGTCAGCATCACCT

CTCATAAGCAGCCATTTATTTCTTTGAGTGGTTCTGTATTTGAGGACTCATTGGATAAGAAGGTTTTACT

CCTATGGGTGAATTCTTCTATCCAGAAAGTTATAGGCAGTACTGGTTAGTTCTTCTGTACTGGGCGTAAT

AATTTACTTTTATTCTCGTCTAGAAAAGGTGAATAGGTGCTTGCCTTGAACCCACAAGTTATGTTAACTG

GGTGACCTCAGATCAGGTAGGGATACCCGCTGAACTTAA

MOTHUR LOGFILE

Linux version

Using ReadLine

Running 64Bit Version

mothur v.1.38.1

Last updated: 8/9/2016

by

Patrick D. Schloss

Department of Microbiology & Immunology

University of Michigan

pschloss@umich.edu

http://www.mothur.org

When using, please cite:

Schloss, P.D., et al., Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol, 2009. 75(23):7537-41.

Type 'help()' for information on the commands that are available

Type 'quit()' to exit program

Batch Mode

mothur > fastq.info(fastq=Viwe_S76_L001_R1_001.fastq)

10000

20000

30000

34763

Output File Names:

Viwe_S76_L001_R1_001.fasta

Viwe_S76_L001_R1_001.qual

[WARNING]: your sequence names contained ':'. I changed them to '_' to avoid problems in your downstream analysis.

mothur > summary.seqs(fasta=Viwe_S76_L001_R1_001.fasta, processors=24)

Using 24 processors.

	Start	End	NBases		Ambigs		Polymer	NumSeqs
Minimum:	1	51	51	0	3	1		

2.5%-tile:	1	300	300	0	4	870
25%-tile:	1	301	301	0	4	8691
Median:	1	301	301	0	4	17382
75%-tile:	1	301	301	0	5	26073
97.5%-tile:	1	301	301	0	8	33894
Maximum:	1	301	301	0	119	34763
Mean: 1	300.897		300.897	0	4.65518	
# of Seqs:	34763					

Output File Names:

Viwe_S76_L001_R1_001.summary

It took 0 secs to summarize 34763 sequences.

mothur > trim.seqs(fasta=Viwe_S76_L001_R1_001.fasta, qfile=Viwe_S76_L001_R1_001.qual, qwindowaverage=20, minlength=150)

mothur > classify.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.fasta, template=silva.nr_v128.align, taxonomy=silva.nr_v128.tax, cutoff=80, count=Viwe_S76_L001_R1_001.trim.good.count_table)

Using 24 processors.

Generating search database... DONE.

It took 321 seconds generate search database.

Reading in the silva.nr_v128.tax taxonomy... DONE.

Calculating template taxonomy tree... DONE.

Calculating template probabilities... DONE.

It took 524 seconds get probabilities.

Classifying sequences from Viwe_S76_L001_R1_001.trim.unique.good.fasta ...

[WARNING]: mothur reversed some your sequences for a better classification. If you would like to take a closer look, please check Viwe_S76_L001_R1_001.trim.unique.good.nr_v128.wang.flip.accnos for the list of the sequences.

It took 34 secs to classify 15171 sequences.

It took 1 secs to create the summary file for 15171 sequences.

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.nr_v128.wang.taxonomy

Viwe_S76_L001_R1_001.trim.unique.good.nr_v128.wang.tax.summary

Viwe_S76_L001_R1_001.trim.unique.good.nr_v128.wang.flip.accnos

mothur > remove.lineage(fasta=Viwe_S76_L001_R1_001.trim.unique.good.fasta, count=Viwe_S76_L001_R1_001.trim.good.count_table,

taxonomy=Viwe_S76_L001_R1_001.trim.unique.good.nr_v128.wang.taxonomy, taxon=unknown)

[NOTE]: The count file should contain only unique names, so mothur assumes your fasta, list and taxonomy files also contain only uniques.

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.nr_v128.wang.pick.taxonomy

Viwe_S76_L001_R1_001.trim.unique.good.pick.fasta

Viwe_S76_L001_R1_001.trim.good.pick.count_table

mothur > summary.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.fasta, count=Viwe_S76_L001_R1_001.trim.good.pick.count_table)

Using 24 processors.

	Start	End	NBases		Ambigs	Polymer	NumSeqs
Minimum:	1	150	150	0	3	1	
2.5%-tile:	1	156	156	0	3	371	
25%-tile:	1	198	198	0	4	3703	
Median:	1	220	220	0	4	7405	
75%-tile:	1	240	240	0	5	11107	
97.5%-tile:	1	250	250	0	6	14439	

Maximum:	1	250	250	0	8	14809
Mean:	1	216.054	216.054	0	4.26133	

# of unique seqs: 14807

total # of seqs: 14809

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.summary

It took 0 secs to summarize 14809 sequences.

mothur > summary.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.fasta, count=Viwe_S76_L001_R1_001.trim.good.count_table, processors=24)

Using 24 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	150	150	0	3	1
2.5%-tile:	1	156	156	0	3	380
25%-tile:	1	196	196	0	4	3794
Median:	1	219	219	0	4	7587
75%-tile:	1	240	240	0	5	11380
97.5%-tile:	1	250	250	0	6	14794
Maximum:	1	250	250	0	8	15173
Mean:	1	215.274	215.274	0	4.26349	

# of unique seqs: 15171

total # of seqs: 15173

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.summary

It took 1 secs to summarize 15173 sequences.

mothur > summary.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.fasta, count=Viwe_S76_L001_R1_001.trim.good.pick.count_table, processors=24)

Using 24 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	150	150	0	3	1
2.5%-tile:	1	156	156	0	3	371
25%-tile:	1	198	198	0	4	3703
Median:	1	220	220	0	4	7405
75%-tile:	1	240	240	0	5	11107
97.5%-tile:	1	250	250	0	6	14439
Maximum:	1	250	250	0	8	14809
Mean:	1	216.054	216.054	0	4.26133	

# of unique seqs: 14807

total # of seqs: 14809

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.summary

It took 0 secs to summarize 14809 sequences.

mothur > align.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.fasta, reference=silva.nr_v128.align, flip=T)

Using 24 processors.

Reading in the silva.nr_v128.align template sequences... DONE.

It took 206 to read 190661 sequences.

Aligning sequences from Viwe_S76_L001_R1_001.trim.unique.good.pick.fasta ...

[WARNING]: Some of your sequences generated alignments that eliminated too many bases, a list is provided in Viwe_S76_L001_R1_001.trim.unique.good.pick.flip.accnos. If the reverse compliment proved to be better it was reported.

It took 30 secs to align 14807 sequences.

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.align

Viwe_S76_L001_R1_001.trim.unique.good.pick.align.report

Viwe_S76_L001_R1_001.trim.unique.good.pick.flip.accnos

mothur > summary.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.align, count=Viwe_S76_L001_R1_001.trim.good.pick.count_table)

Using 24 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1046	1067	10	0	2	1
2.5%-tile:	13129	21590	156	0	3	371
25%-tile:	13129	21991	198	0	4	3703
Median:	13129	22091	220	0	4	7405
75%-tile:	13129	22529	240	0	5	11107
97.5%-tile:	13129	22549	250	0	6	14439
Maximum:	43096	43116	250	0	8	14809
Mean:	13143.9	22198	216.017	0	4.26065	

# of unique seqs: 14807

total # of seqs: 14809

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.summary

It took 4 secs to summarize 14809 sequences.

mothur > summary.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.align, count=Viwe_S76_L001_R1_001.trim.good.pick.count_table, processors=24)

Using 24 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1046	1067	10	0	2	1
2.5%-tile:	13129	21590	156	0	3	371
25%-tile:	13129	21991	198	0	4	3703
Median:	13129	22091	220	0	4	7405
75%-tile:	13129	22529	240	0	5	11107
97.5%-tile:	13129	22549	250	0	6	14439
Maximum:	43096	43116	250	0	8	14809
Mean:	13143.9	22198	216.017	0	4.26065	

# of unique seqs: 14807

total # of seqs: 14809

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.summary

It took 2 secs to summarize 14809 sequences.

mothur > screen.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.align, count=Viwe_S76_L001_R1_001.trim.good.pick.count_table, optimize=start-end, criteria=95)

Using 24 processors.

Optimizing start to 13129.

Optimizing end to 21777.

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.align

Viwe_S76_L001_R1_001.trim.unique.good.pick.bad.accnos

Viwe_S76_L001_R1_001.trim.good.pick.good.count_table

It took 5 secs to screen 14807 sequences.

mothur > filter.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.align, vertical=T, trump=.)

Using 24 processors.

Creating Filter...

Running Filter...

Length of filtered alignment: 269

Number of columns removed: 49731

Length of the original alignment: 50000

Number of sequences used to construct filter: 14021

Output File Names:

Viwe_S76_L001_R1_001.filter

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.fasta

mothur > summary.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.fasta, count=Viwe_S76_L001_R1_001.trim.good.pick.count_table)

Using 24 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	269	153	0	3	1
2.5%-tile:	1	269	166	0	3	371
25%-tile:	1	269	166	0	4	3703
Median:	1	269	166	0	4	7405
75%-tile:	1	269	166	0	5	11107
97.5%-tile:	1	269	174	0	8	14439
Maximum:	1	269	174	0	8	14809
Mean:	0.946924	254.723	157.222	0	3.94416	

# of unique seqs: 14021

total # of seqs: 14809

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.summary

It took 1 secs to summarize 14809 sequences.

mothur > unique.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.fasta, count=Viwe_S76_L001_R1_001.trim.good.pick.good.count_table)

14021 12569

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.count_table

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.fasta

mothur > summary.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.fasta, count=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.count_table)

Using 24 processors.

	Start	End	NBases		Ambigs	Polymer	NumSeqs
Minimum:	1	269	153	0	3	1	
2.5%-tile:	1	269	166	0	3	351	
25%-tile:	1	269	166	0	4	3506	
Median:	1	269	166	0	4	7012	
75%-tile:	1	269	166	0	4	10518	

97.5%-tile:	1	269	167	0	6	13673
Maximum:	1	269	174	0	8	14023
Mean:	1	269	166.035	0	4.16523	

# of unique seqs: 12569

total # of seqs: 14023

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.summary

It took 0 secs to summarize 14023 sequences.

mothur > pre.cluster(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.fasta, count=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.count_table, diffs=2)

Using 24 processors.

Processing group Viwe_S76_L001_R1_001:

12569 10112 2457

Total number of sequences before pre.cluster was 12569.

pre.cluster removed 2457 sequences.

It took 20 secs to cluster 12569 sequences.

It took 21 secs to run pre.cluster.

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.fasta

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.count_table

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.Viwe_S76_L001_R1_001.map

mothur > summary.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.fasta, count=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.count_table)

Using 24 processors.

	Start	End	NBases		Ambigs	Polymer	NumSeqs
Minimum:	1	269	153	0	3	1	
2.5%-tile:	1	269	166	0	3	351	
25%-tile:	1	269	166	0	4	3506	
Median:	1	269	166	0	4	7012	
75%-tile:	1	269	166	0	4	10518	
97.5%-tile:	1	269	167	0	6	13673	
Maximum:	1	269	174	0	8	14023	
Mean:	1	269	166.036	0	4.16658		

# of unique seqs: 10112

total # of seqs: 14023

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.summary

It took 0 secs to summarize 14023 sequences.

mothur > quit()

mothur > summary.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.fasta, count=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.count_table, processors=24)

Using 24 processors.

	Start	End	NBases		Ambigs	Polymer	NumSeqs
Minimum:	1	269	153	0	3	1	
2.5%-tile:	1	269	166	0	3	351	
25%-tile:	1	269	166	0	4	3506	
Median:	1	269	166	0	4	7012	
75%-tile:	1	269	166	0	4	10518	
97.5%-tile:	1	269	167	0	6	13673	
Maximum:	1	269	174	0	8	14023	
Mean:	1	269	166.036	0	4.16658		

# of unique seqs: 10112

total # of seqs: 14023

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.summary

It took 0 secs to summarize 14023 sequences.

mothur > chimera.vsearch(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.fasta, count=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.count_table, dereplicate=t)

Using 24 processors.

/apps/chpc/bio/mothur/1.38.1.1/blast/bin/vsearch file does not exist. Checking path...

Found vsearch in your path, using /apps/chpc/bio/vsearch/2.4.2/bin/vsearch

Checking sequences from Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.fasta ...

90873 here

It took 6 secs to check 0 sequences from group Viwe_S76_L001_R1_001.

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.denovo.vsearch.pick.count_table

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.denovo.vsearch.chimeras

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.denovo.vsearch.accnos

mothur > remove.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.fasta, count=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.count_table, accnos=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.denovo.vsearch.accnos)

[NOTE]: The count file should contain only unique names, so mothur assumes your fasta, list and taxonomy files also contain only uniques.

Removed 1161 sequences from your fasta file.

Removed 1232 sequences from your count file.

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.fasta

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.count_table

mothur > count.groups(count=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.count_table)

Viwe_S76_L001_R1_001 contains 12791.

Total seqs: 12791.

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.count.summary

mothur > classify.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.fasta, count=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.count_table, template=silva.nr_v128.align, taxonomy=silva.nr_v128.tax, cutoff=80)

Using 24 processors.

Reading template taxonomy... DONE.

Reading template probabilities... DONE.

It took 13 seconds get probabilities.

Classifying sequences from Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.fasta ...

It took 16 secs to classify 8951 sequences.

It took 0 secs to create the summary file for 8951 sequences.

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.nr_v128.wang.taxonomy

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.nr_v128.wang.tax.summary

mothur > dist.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.fasta, cutoff=0.15)

Using 24 processors.

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.dist

It took 44 seconds to calculate the distances for 8951 sequences.

mothur > cluster(column=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.dist,

count=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.count_table, cutoff=0.15, method=furthest)

**********#****#****#****#****#****#****#****#****#****#****#

Reading matrix: ||||||||||||||||||||||||||||||||||||||||||||||||||

****

It took 82 seconds to cluster

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.fn.unique_list.list

mothur > quit()

mothur > make.shared(list=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.fn.unique_list.list, count=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.count_table)

unique

0.02

0.03

0.04

0.05

0.06

0.07

0.08

0.09

0.10

0.11

0.12

0.13

0.14

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.fn.unique_list.shared

mothur > quit()

mothur > get.oturep(column=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.dist, count=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.count_table, list=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.fn.unique_list.list, fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.fasta, sorted=group, label=0.03-0.05-0.10)

**********#****#****#****#****#****#****#****#****#****#****#

Reading matrix: ||||||||||||||||||||||||||||||||||||||||||||||||||

****

0.03 7631

0.05 5217

0.10 2329

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.fn.unique_list.0.03.rep.count_table

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.fn.unique_list.0.05.rep.count_table

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.fn.unique_list.0.10.rep.count_table

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.fn.unique_list.0.03.rep.fasta

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.fn.unique_list.0.05.rep.fasta

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.fn.unique_list.0.10.rep.fasta

mothur > quit()

mothur > get.seqs(fasta=Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.fn.unique_list.0.03.rep.fasta, accnos=VIWE.accnos)

Selected 25 sequences from your fasta file.

Output File Names:

Viwe_S76_L001_R1_001.trim.unique.good.pick.good.filter.unique.precluster.pick.fn.unique_list.0.03.rep.pick.fasta

mothur > quit()

Appendix 3

Staining solutions (Smith and Dickson, 1997)

5% KOH

100g KOH

2L Distilled water

Alkaline H2O2

3ml NH4OH

3ml 30% H2O2

594ml Distilled water

Lactoglycerol Trypan Blue Stain

Lactic acid: Glycerol: Water (13:12:16)

440ml Lactic acid

406.2ml Glycerol

541.6ml Distilled water

0.694ml Trypan blue

Lactoglycerol destain

440ml lactic acid

406.2ml glycerol

541.6ml distilled water