BIOPROSPECTING FOR AMYLASES, CELLULASES AND XYLANASES FROM ERICOID ASSOCIATED FUNGI, THEIR PRODUCTION AND CHARACTERISATION FOR THE BIO-ECONOMY

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By

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Abstract

South Africa is one of the most productive areas for ericaceous plants with about 850 identified species in the Cape Floral Region. The Albany Centre of Endemism where all fungi used in this study were isolated from, falls within this region. Ericaceous plants interact with some fungi via an association called the ericoid mycorrhizal (ERM) association. All fungi used in this study were isolated from roots of six ericaceous plants; *Erica cerinthoides*, *Erica demissa*, *Erica chamissonis*, *Erica glumiflora*, *Erica caffra* and *Erica nemorosa*. Fungal enzymes are known to play a significant role in the food, brewing, detergent, pharmaceutical and biofuel industries. The enzyme industry is among the major sectors of the world, and additional novel sources are being explored from time to time. This study focussed on amylases (amyloglucosidase, AMG), cellulases (endoglucanase) and xylanases (endo-1,4-β-xylanase) production from ERM fungal isolates. Out of the fifty-one (51), fungal isolates screened, ChemRU330 (*Leohumicola* sp.), EdRU083 and EdRU002 were among the fungi that had the highest activities of all the enzymes. They were tested for the ability to produce amylases and cellulases under different pH and nutritional conditions that included: carbon sources, nitrogen sources and metal ions, at an optimum temperature of 28°C in a modified Melin-Norkrans (MMN) liquid medium.

Cellulase specific activity of 3.99, 2.18 and 4.31 (U/mg protein) for isolates EdRU083, EdRU002 and ChemRU330, respectively, was produced at an optimal pH of 5.0. For amylase, ChemRU330 had the highest specific activity of 1.11 U/mg protein while EdRU083 and EdRU02 had a specific activity of 0.80 and 0.92 U/mg protein, respectively, at the same pH with corresponding biomass yield of 113, 125 and 97 mg/50 ml, respectively. Increased enzyme activities and improved mycelial biomass production were obtained in the presence of supplements such as potassium, sodium, glucose, maltose, cellobiose, tryptone and peptone, while NaFe-EDTA and cobalt inhibited enzyme activity. ChemRU330 was selected to determine the consistency and amount of amylase, cellulase and xylanase formed after several *in vitro* subculturing events. AMG and endo-1,4-β-xylanase were found to have the most consistent production throughout the study period. The AMG was stable at 45°C (pH 5.0), retaining approximately 65% activity over a period of 24 h. The molecular mass of AMG and endo-1,4-β-xylanase were estimated to be 101 kDa and 72 kDa, respectively. The K_m and k_{cat} were 0.38 mg/ml and 70 s^{-1}, respectively, using soluble starch (AMG). For endo-

1,4-β-xylanase, the K_m and V_{max} were 0.93 mg/ml and 8.54 U/ml, respectively, using beechwood xylan (endo-1,4-β-xylanase) as substrate.

Additionally, crude extracts of five root endophytes with unique morphological characteristics were screened for antibacterial properties and was followed by determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). *L. incrustata* (ChemRU330) and *Chaetomium* sp. extracts exhibited varying degrees of inhibition against two Gram-positive and Gram-negative bacteria. The crude extract of *L. incrustata* was the most effective which was found to inhibit *Staphylococcus aureus* (MIC: 1 mg/ml), *Bacillus subtilis* (MIC: 2 mg/ml) and *Proteus vulgaris* (MIC: 16 mg/ml). The *L. incrustata* displayed potential for antibacterial production and could be considered as an additional source of new antimicrobial agents in drug and food preservation. Also, the three isolates used for enzyme production were identified to genus and species levels, i.e., *Leohumicola incrustata* (ChemRU330), *Leohumicola* sp. (EdRU083) and *Oidiodendron* sp. (EdRU002) using both ITS and *Cox1* DNA regions. The molecular analysis results indicated that these ERM mycorrhizal fungi were similar to those successfully described by some researchers in South Africa and Australia.

Therefore, this study opens new opportunities for exploring ERM fungal biomolecules for the bio-economy. The promising physicochemical properties, starch and xylan hydrolysis end-products, and being non-pathogenic make AMG and endo-1,4-β-xylanase potential candidates for future applications as additives in the food industry for the production of glucose, glucose syrups, high-fructose corn syrups, and as well as the production of bio-ethanol. Finally, the findings of this study revealed that it is possible to produce hydrolytic enzymes from ERM fungi *in vitro* using chemically defined media.

Declaration

I, Olusegun Richard Adeoyo (15A6437), declare that this thesis is my original work. It is being submitted for the degree of Doctor of Philosophy in Microbiology in the Department of Biochemistry and Microbiology, Faculty of Science, Rhodes University, South Africa. It has not been previously submitted for any degree for examination in any other university.

17-11-2017

Olusegun Richard Adeoyo

Dedication

I dedicate this study to Almighty God and my late parents (Mr and Mrs Polinus Adeoyo) for who have been my sources of inspiration. How I wish you were alive to witness this moment of joy. May your gentle souls rest in perfect peace.

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Firstly, I thank God, the giver of life for granting me the wisdom to complete this study and thesis. I would like to express my sincere gratitude to my supervisor Professor Joanna Dames and co-supervisor Professor Brett Pletschke for their guidance, patience and support throughout the course of the project. I also want to thank Dr Christine Bizabani for granting access to all isolates used for this study.

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°C	degrees celcius
µl	microliter
µM	micromolar
µmol	micromole
+	positive sign
-	negative sign
#	number
AMG	amyloglucosidase
ANOVA	analysis of variance
BPNPG7	benzylidene blocked 4-nitrophenyl maltoheptaoside
BLAST	basic local alignment search tool
bp	basepair
Ca	calcium
CMC	carboxylmethyl cellulose
Co	cobalt
Cox1	cytochrome oxidase subunit 1
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
DNS	dinitrosalicylic
DSE	dark septate endophyte
dNTP	deoxyribose nucleotide phosphate
ECM	ectomycorrhizal
ERM	ericoid mycorrhizal
EDTA	ethylinediamine tetraacetic acid
Fe	iron
NaFe-EDTA	ethylenediaminetetraacetic acid ferric sodium
Fig	figure
g	gram
g	gravitational force
h	hour
Hg	mercury
IAA	indoacetic acid
ITS	internal transcribed spacer

kDa	kilodalton
LSD	least significant difference
K	potassium
M	molar
MBC	minimum bacteriacidal concentration
MIC	minimum inhibitory concentration
MEGA7	Molecular Evolutionary Genetics Analysis Version 7.0
Mg	magnesium
min	minute
ml	millilitre
mM	millimolar
Mn	manganese
MMN	Modified Melin-Norkran
MW	molecular weight
N	nitrogen
Na	sodium
NCBI	National Centre for Biotechnology Information
OTU	operational taxonomic unit
P	phosphorus
PCR	polymerase chain reaction
rDNA	recombinant DNA
rpm	revolution per minute
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
sp.	species
TLC	thin-layer chromatography
U	unit
Zn	zinc

CHAPTER 1

1.0 Introduction and literature review

Ericoid and related microbial root endophytes can produce some bioactive substances that include amylases, cellulases, xylanases, proteases and phosphatases which break down specific substrates in soils and subsequently facilitate the release of unavailable nutrients for plant uptake (Burke and Cairney, 1997a; Gibson and Derek, 2005; Kusuda et al., 2007; Maijala, 2000). Most studies conducted on the extracellular enzymes from mycorrhizal fungi emphasised their role in supplying nutrients to host plants and the plating method is often used to determine activity. But beyond these, there is evidence that some ericoid, ectomycorrhizal and dark septate endophytic fungi have the potential of producing a good number of hydrolytic enzymes *in vitro*.

Enzymes are biological substances produced by living cells which act as biocatalysts that bring about specific biochemical reactions. When enzymes are produced, they help to accelerate the biochemical reactions inside (intracellular) or outside (extracellular) the cell. Enzymes can be obtained from some common soil-borne fungi (e.g., *Aspergillus* spp., *Penicillium* spp. and *Rhizopus* spp.), root endophytic and mycorrhizal fungi (Lee et al., 2014; Rodriguez et al., 2009). The extracellular or intracellular enzymes can perform both protective and degradative functions within the rhizosphere. They can either oxidise extracellular toxic soluble phenolic metabolites to insoluble polymerised products (oxidoreductases) or hydrolyse polymeric substrates to monomeric products (hydrolases). For example, fungi can utilise enzymes to break down substrates such as starch and cellulose into glucose which can readily be assimilated by mycorrhizal and root endophytic fungi to supply the required nutrients for growth and survival of the host plant (Caldwell et al., 2000).

Researchers (Bergero et al., 2000; Hou and Guo, 2009; Mitchell and Gibson, 2006; Smith and Read, 2008; Vohnik et al., 2012) have shown that most plants in natural ecosystems form a symbiosis with mycorrhizal fungi and fungal endophytes (Table 1.1). Fungal symbionts can have a dramatic effect on plant fitness, evolution, and ecology (Cairney, 2000). They regulate nutrient and carbon cycles, and influence soil structure (van der Heijden et al., 2015) thereby determining

the plant communities in a given area (Clay and Holah, 1999). Mycorrhizal fungi grow and colonise plant roots and the rhizosphere, while endophytic fungi reside within plant tissues, sometimes growing within roots, stems, and leaves and sporulate on host-tissue at senescence (Stone et al., 2004). Fungi found growing around and within roots are termed root endophytic fungi (Figure 1.1). Molecular and biotechnological techniques have been extensively applied to the study of mycorrhizal and root endophytic fungi, and the knowledge gained has substantially modified the view of researchers on the biology, evolution, and biodiversity of mycorrhizal fungi (van der Heijden et al., 2015). Therefore, this study focuses on enzymes from ericoid mycorrhizal and associated root endophytic fungi, their characterisation and production for the bio-economy and related studies.

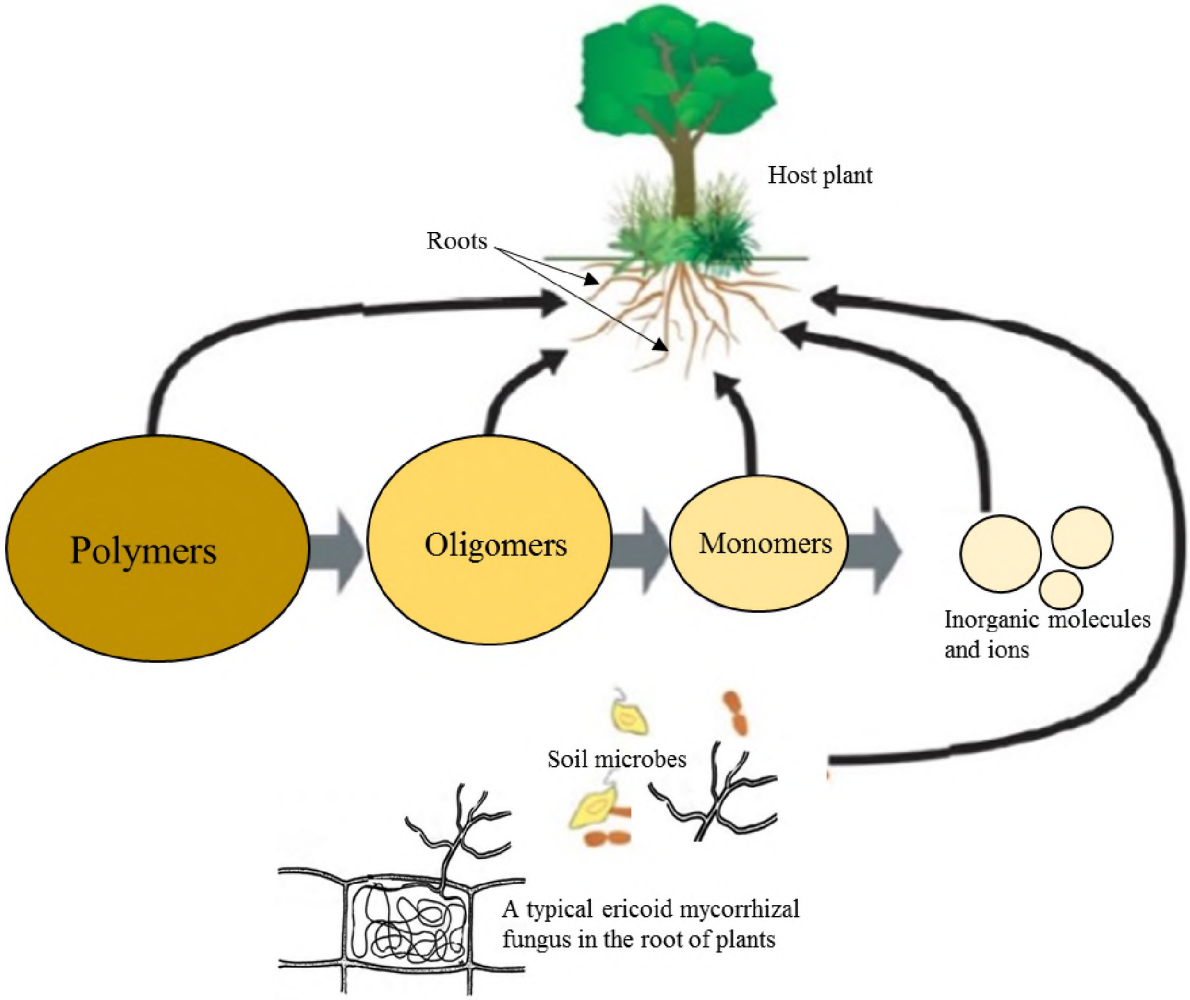


Figure 1.1. Proposed scheme of plant nutrient sources in which soil microbes and root-derived enzymes contribute to depolymerisation and mineralisation of organic matter. Plants acquire inorganic nutrients directly from natural nutrients via mycorrhizal symbionts and also take up organic compounds and microbes (Modified from Schmidt et al., 2013).

1.1. Ericaceous plants

Ericaceae is a family of plants found in harsh edaphic conditions (Schumann and Kirsten, 1992). Ericaceae has a subfamily called Ericoidae, which consists of twenty-five genera that include *Rhododendron, Erica, Calluna, Empetrum* and *Rhodothamnus* (Schumann and Kirsten, 1992). The genus *Erica* is the second largest genus in the Ericoideae, with about 865 identified species worldwide (McGuire and Kron, 2005). Erica species are widely distributed in Africa, Middle East, Madagascar and Europe (McGuire and Kron, 2005). Plants belonging to this group usually play a significant role in ecosystem development and soil formation (Chapin et al., 2002, 1994). They add to available nutrients, and their rhizospheres harbour a diversity of microorganisms (Hawkes et al., 2007) that include ericoid fungi (Figures 1.1 and 1.3). The distribution pattern of mycorrhizal fungi in newly developing ecosystems cannot be predicted with exactness but is believed to be driven by two main factors: Firstly, the edaphic conditions and plant community composition; secondly, the ecosystem disturbances (Dickie et al., 2013). Researchers (Mandyam and Jumpponen, 2005; Sieber, 2007; Smith and Read, 2008) have shown that most plants in natural ecosystems form a symbiosis with mycorrhizal fungi and fungal endophytes.

1.2. Ericoid mycorrhizal (ERM) fungi

The relationship that exists between ericoid mycorrhizal fungi and ericaceous plants is described as obligate with the formation of several distinctive mycorrhizal categories and endophyte relations (Smith and Read, 2008). Plants belonging to Ericaceae have a distinct mycorrhizal association, where fungi colonise fine roots (hair roots) and produce hyphal complexes in epidermal cells (Figure 1.2 and Table 1.1) (Smith and Read, 2008). Culturable ERM fungi that have been isolated are mainly from the phylum of Ascomycota (Smith and Read, 2008) with *Scytalidium vaccinii* as the first isolated and identified ericoid mycorrhizal fungus (Dalpe et al., 1989; Vrålstad et al., 2002).

Ericaceous plants are naturally found on well-drained and acidic soils in temperate climatic zones; as well as at high elevations in the mountainous areas of the tropic, where they can become a dominant plant community (Martino et al., 2000). The survival of ericaceous plants

under nutrient-stressed conditions is thought to depend on the formation of the mycorrhizal symbiosis (Table 1), and the evolution of the association is noted to be mediated by the selective and competitive advantages conferred by fungal colonisation (Straker, 1996).

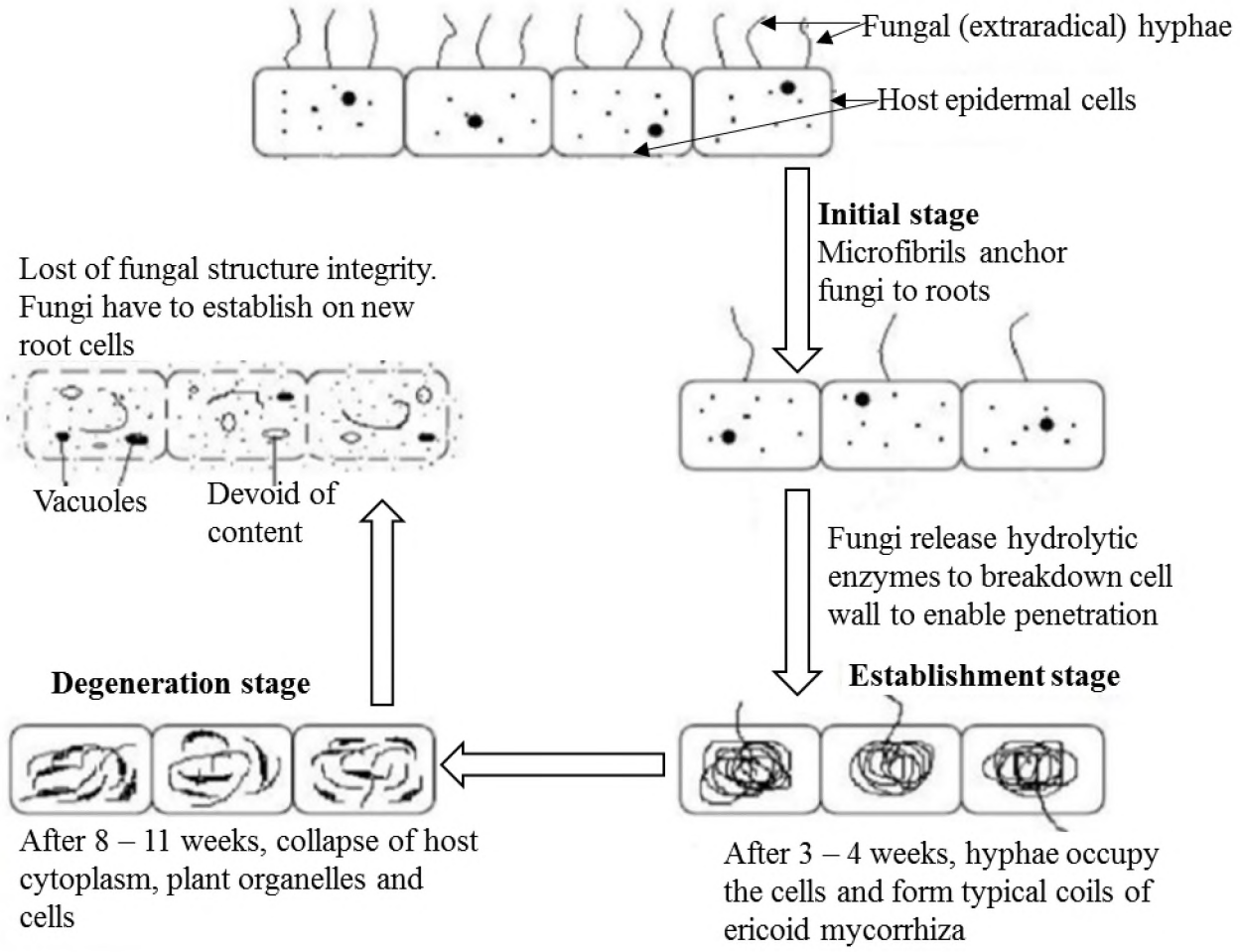


Figure 1.2. Series of events that occur during the formation of the ericoid mycorrhizal association in the Ericaceae hair roots. The stages include initiation, establishment, and degeneration, lasting up to 11 weeks (Modified from Bizabani, 2015).

The ability of ERM to degrade complex substrates (e.g., starch and cellulose) have been studied and performed over the years by some authors (Bending and Read, 1997; Cairney and Burke, 1998). Their results indicated that the enzymatic degradation of organic polymers in the soil and the transfer of some of the resulting products to the root is a significant benefit to the growth and development of ericaceous plants (Table 1.1) (Smith and Read, 2008). Subsequently, host plants can access nutrients from the unavailable organic sources. The production and activity of

enzymes from mycorrhizal fungi influence the development of the host plant (Figure 1.2) and confers on their host the ability to compete successfully with other plant species. For example, *Calluna vulgaris* is the most common ericaceous species in the oceanic North West of Europe, where it was found to form nearly a pure plant community (Rodwell, 2001). The competitive ability allows *C. vulgaris* to compete over *Nardus stricta* as demonstrated using pot cultures under different growth conditions (Genney, 2000). There is now a concerted effort geared towards the study of ERM fungi because of their ability to grow on polluted sites contaminated by heavy metals (Martino et al., 2000) which could suggest the possibility of using them for bioremediation purposes.

1.3. Ectomycorrhizal (ECM) fungi hyphae surrounding the plant cells within the root cortex

ECM are dominant members of the soil microbial community in temperate and boreal forests. Tropical rainforests harbour the highest number of trees, and different ecological studies have attributed this to the presence of ECM fungi (Hawley, 2006; Leigh et al., 2004). They form a fungal mantle (sheath) surrounding the plant's roots and an intercellular network of hyphae (Figure 1.3 and Table 1.1) forming a 'Hartig' net around cortical root cells (Smith and Read, 2008). They mainly belong to members of Basidiomycota, Ascomycota and some Zygomycota (Rinaldi et al., 2008). Genera such as *Pisolithus, Suillus, Paxillus, Cantharellus, Cortinarius, Laccaria, Rhizopogon, Hebeloma*, *Russula, Amanita, Cenococcum* and *Thelephora* are common ECM fungi (Cairney and Chambers, 1999). Some ECM fungal species form symbiotic associations with a broad range of hosts, e.g. *Pisolithus* sp. (Cairney and Chambers, 1999), while *Suillus* is associated mainly with Pinaceae (Dahlberg and Finlay, 1999). *Suillus* species possess different traits that make them suited for co-invasion with *Pinus* invasions these include a large number of fruit bodies, spores resistant to environmental factors, dispersal mode, and rapid rate of colonising roots of pines (Hayward et al., 2015). Another trait suggested in host invasion by mycorrhizal fungi is the ability of these fungi to break down substrate with the help of specific enzymes involved in the degradation of woody substrates (Cullings and Courty, 2009). ECM can as well facilitate the acquisition of some vital mineral nutrients in the establishment of plants on sites contaminated by toxic metals (Meharg and Cairney, 2000).

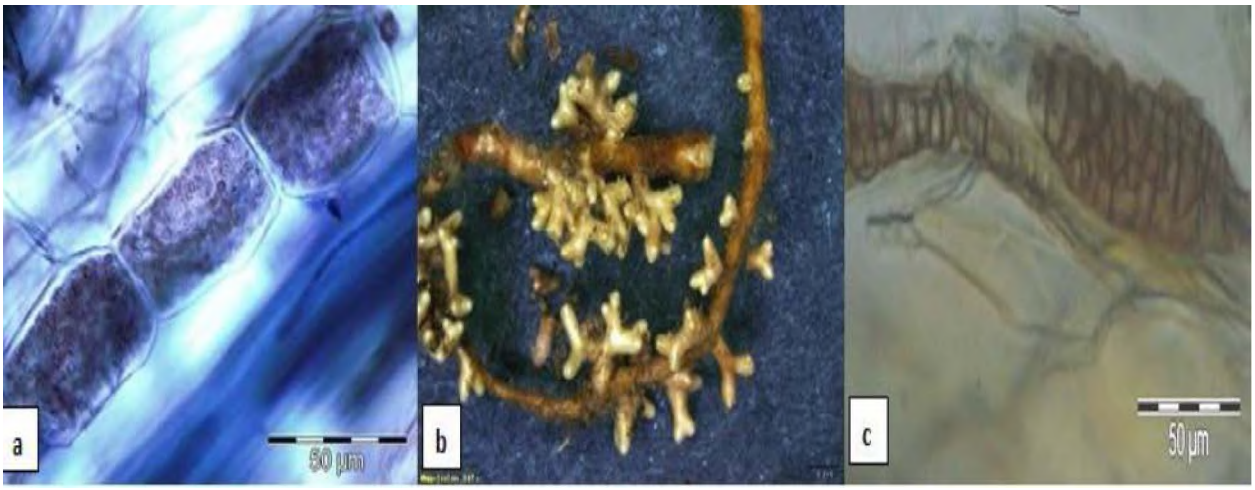


Figure 1.3. Typical ericoid, ectomycorrhizal and root endophytic fungal structures observed under the light microscope. (a) Ericoid coils within the individual cells, (b) ectomycorrhizal mantle surrounding roots, (c) dark septate root endophyte within individual cells (Photograph credits: Dr Christine Bizabani and Veronique Chartier-Fitzgerald).

1.4. Endophytic fungi

The word endophytes refer to fungi belonging mainly to the Ascomycota, which usually colonise healthy plant tissue causing no immediate or overt adverse effects (Pawłowska et al., 2014; Sunitha et al., 2013). It should be noted that many endophytes may cause diseases under stressed conditions. An endophytic fungus can inhabit all available tissues including leaves, roots, stems, twigs, barks, fruits, flowers and seeds within the host system. Root endophytic fungi are those mainly found in the roots of plants (Figure 1.1). Fungal endophytes exhibit complex web interactions with host plant and have been extensively studied as rich sources of new bioactive natural products (Sunitha et al., 2013). They can produce some vital enzymes that include cellulases, amylases, laccases, and pectinases (Choi et al., 2005). Starch-degrading endophytic fungi such as *Gibberella pulicaris* and *Acremonium* sp. have been reported (Marlida et al., 2000).

Dark septate endophytic (DSE) fungi can form symbiotic associations with Ericaceae (e.g. *Phialocephala fortinii*) and produce extracellular enzymes (Vohnik et al., 2003). DSE are conidial or sterile ascomycetous fungi that colonise living plant roots without causing apparent tissue disorganisation (Jumpponen and Trappe, 1998). DSE colonisation has been recorded in about 600 plant species which represent about 320 genera and 114 families (Jumpponen and

Trappe, 1998). They are widely distributed in soil and most common in environments with intense abiotic stress (Mandyam and Jumpponen, 2005). Some DSE (*Cadophora*, *Leptodontidium*, *Phialophora,* and *Phialocephala*) isolated from roots of poplar trees on metal-polluted sites (Berthelot et al., 2016), produced auxin while some accelerated plant growth by the release of volatile organic compounds (VOCs) (Berthelot et al., 2016). The ability of metal-resistant DSE strains producing both soluble and volatile compounds can discern the possibility of using them for phytoremediation and enzyme production.

Table 1.1. Plant-mycorrhizal/root endophytic fungal relationships

	Ericoid mycorrhizal fungus	**Ectomycorrhizal fungus**	**Root endophytic fungus**	**Reference(s)**
Hyphae	Hyphae cover root tips and colonise the rhizodermis forming hyphal coils.	Hyphae include root tips creating 'Hartig' net and do not penetrate cells of cortex in root tips. Hyphal 'Hartig' net surrounds cortical cells.	Hyphae in root tips penetrate cells of the cortex	Smith and Read, 2008
Host plant	Ericaceous plants (common genera are *Erica, Calluna, Pieris, Empetrum*, *Vaccinium,* and *Rhododendron*).	Commonly found on trees and many smaller perennial plants (Pinaceae, e.g. cedars, firs, hemlocks, larches, pines, and spruces. Fagaceae, e.g. oak and beech Betulaceae, e.g. birch).	All plant roots with mycorrhizal fungi.	Bano and Ashfaq, 2013; Jumpponen and Trappe, 1998
Soil condition	Mostly found in acidic soil.	Mostly found in the soil with moderate pH.	All soil types.	Mandyam and Jumpponen, 2005
Benefit	Enhanced nutrient uptake particularly from organic sources.	Enhance nutrient absorption and protects plants against nematodes and soil pathogens.	Secrets bioactive metabolites such as alkaloids, flavonoids, and phenolics that inhibit soil pathogens.	Lee et al., 2014; Solaiman and Mickan, 2014

1.5. Other mycorrhizal fungi

Arbuscular, ectendomycorrhizal (arbutoid and monotropoid) and orchid mycorrhizal fungi are among other mycorrhizal fungi that exist in the rhizosphere. Arbuscular mycorrhizal (Vesicular-Arbuscular Mycorrhizas, VAM or AM) associations are ubiquitous endomycorrhizal fungi, that associate with more than 74% of all terrestrial plants (Brundrett, 2004, 2009). They belong to the phylum Glomeromycota and can produce arbuscules, hyphae, and vesicles within root cortex cells (Redecker et al., 2013; Schubler et al., 2001).

Ectendomycorrhizas consist of arbutoid and monotropoid fungi that are characterised by a hyphal sheath, Hartig net and intracellular hyphal colonisation (i.e. they show the characters of both ectotrophic as well as endotrophic mycorrhizal fungi) (Selosse et al., 2007; Setaro et al., 2006). Arbutoid mycorrhizal fungi can be found in the Ericaceae subfamily Arbutoideae, while monotropoid fungi occur with the Ericaceae subfamily Monotropoideae and Orchidaceae. In orchid mycorrhizal associations, coils of hyphae (pelotons) penetrate the root cortex, root tubers in the plant family Orchidaceae (Dearnaley, 2007).

Recently, cavendishioid and sheathed ERM associations have been discovered with roots of plants (Smith and Read, 2008). Cavendishioid mycorrhizal (CVM) is characterised by the unclamped hyphal sheath, mantles with intercellular fungal tissue that resembles a Hartig net and hyphal colonisation in cortical cells (Setaro et al., 2006). Sheathed ERM, on the other hand, has clamped hypha and does not possess any structure that resembles a Hartig net that is present in CVM associations (Vohník et al., 2012).

1.6. Microbial enzymes and fungi

Microbial enzymes are known to play crucial roles in biocatalysis. This encourages their use for various industrial applications. Their use as biotechnological sources of industrially relevant enzymes has stimulated renewed interest in the exploration of extracellular enzymatic activity in several microorganisms (Buzzini and Martini, 2002) such as mycorrhizal fungi. Over 500

essential industrial products have been produced using enzymes (Chandel et al., 2013). The production of extracellular enzymes in microorganisms is significantly mediated by some factors which include temperature, pH, aeration and medium constituents. The relationship between these variables has a remarkable effect on the final production of the enzymes (Table 1.3).

DSE such as *Periconia* and *Microdochium* fungi have been tested for their enzymatic capabilities. These fungi tested positive for amylases, cellulases (Table 1.2), polyphenol oxidases and gelatinases (Mandyam and Jumpponen, 2005).

1.6.1. Amylases

Amylases are enzymes which hydrolyse starch molecules to produce various products that are composed of glucose units (Rani, 2012). These enzymes are of high significance to modern biotechnology with applications ranging from food to bioethanol production (Aiyer, 2005; Das et al., 2011). Amylases are one of the most common biomolecules which account for about 25% of the world's enzyme production (Gurung et al., 2013).

Amylases are capable of hydrolysing α-1,4-glucosidic bonds of amylose, amylopectin, glycogen and their degradation products (Gurung et al., 2013). They hydrolyse bonds between adjacent glucose units to produce products typical for the particular enzyme involved. *Tricholoma matsutake* has been reported to produce high α-amylase activity when the production medium was supplemented with rice powder (Table 1.3) (Kusuda et al., 2004). Hur et al. (2001) indicated that the amylase activity of *T. matsutake* was higher with starch originated from barley grain than from other sources. Microbial amylases have almost replaced chemical hydrolysis in the starch processing industry. Today, amylases have impacted the world enzyme market positively (Gurung et al., 2013). Several amylase preparations are now available for various industrial applications.

Amylases are broadly classified into α, β, and γ subtypes (Figure 1.4) viz. α-amylase (also called 1,4-α-D-glucan glucanohydrolase, glycogenase, EC 3.2.1.1) cleaves at random locations on the starch chain, to yield maltotriose and maltose, and glucose from amylose and amylopectin

(Gopinath et al., 2017). β-amylase (also called 1,4-α-D-glucan maltohydrolase, glycogenase, saccharogen amylase, EC 3.2.1.2) catalyses the hydrolysis of α-1,4 glycosidic bond from the non-reducing end to yield two glucose units (maltose) (Das et al., 2011). Amyloglucosidase (AMG), (also called γ-amylase, glucoamylase, glucan 1,4-α-glucosidase, exo-1,4-α-glucosidase, EC 3.2.1.3) cleaves α-1,6 glycosidic linkages, as well as the last α-1,4 glycosidic linkages at the nonreducing ends of amylose and amylopectin to produce glucose (Saranraj and Stella, 2013).

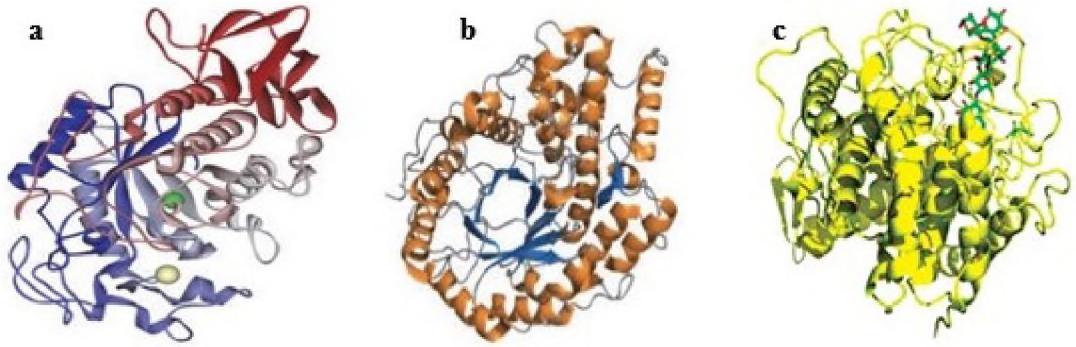


Figure 1.4. The computer simulated three-dimensional amylase structure: (a) alpha-amylase, (b) beta-amylase (Gopinath et al., 2017) and (c) amyloglucosidase (Bott et al., 2008).

1.6.2. Cellulases

There are some studies aimed at obtaining novel microorganisms that are capable of producing cellulases with higher specific activities and greater stability. ERM and related fungal species have been demonstrated to produce or secrete extracellular hydrolytic enzymes in agar media with appropriate nutrient composition (Varma and Bonfante, 1994).

Cellulase has a broad range of industrial applications such as in the textile, laundry, pulp and paper industries, in fruit juice extraction, and animal feed additives, as well as in biofuel production (Bhat, 2000). Cellulases can be used for extraction and clarification of fruit (Table 1.3) and to increase the yield of juices (de Carvalho and da Silva, 2010).

Cellulase may cleave either glucose dimers from the end of the cellulose polymer (exoglucanase) or randomly fragment the polymer into smaller molecules by internal digestion (endoglucanase). These two types of actions usually take place simultaneously, but the amount of each enzyme expressed and rate of activity differs between microbial species (Singh et al., 2007). Hydrolysis

of cellulose is carried out by three components of cellulose: endo-(1,4)-β-glucanase (EC 3.2.1.4), exo-(1,4)-β-glucanase (EC 3.2.1.91) and β-glucosidases or cellobiase (EC 3.2.1.21) (Sharada et al., 2013). The endoglucanase cleaves internal β-1,4-glucan linkages in cellulose randomly (Schulein, 2000), which opens up the cellulose molecules structure for cellobiohydrolase to hydrolyse the bonds at the nonreducing end of the crystalline cellulosic chain to produce cellobiose. The cellobiases then split the disaccharide units, converting them into glucose (Gautam et al., 2011). A 61 glycoside hydrolase (GH61) family, structurally similar to 33 carbohydrate-binding module (CBM33) proteins, has been discovered (Horn et al., 2012). It promotes the efficiency of cellulases by acting on the surfaces of the insoluble substrate and introduces chain breaks in the polysaccharide chains without the need of first "extracting" these chains from their crystalline matrix (Horn et al., 2012).

1.6.3. Xylanase

Xylanases are enzymes that degrade β-1,4-xylan, a linear polysaccharide found as hemicellulose in plant cell walls (Wipusaree et al., 2011). Microbial enzymes have been involved in the hydrolysis of xylan, which is a significant step towards the degradation of most lignocellulosic material (Beg et al., 2001). A β-1,4-endoxylanase has been purified and characterised from *Hymenoscyphus ericae* (Table 1.2), and the enzyme was reported to have an isoelectric point of 4.85 to 5.20 and a molecular weight of 58.4 kDa (Burke and Cairney, 1997a). The hydrolysis of lignocellulosic materials usually requires the synergistic action of different enzymes because of the structural heterogeneity of its components (Polizeli et al., 2005). It has been reported that supplementation of xylanase with acetyl xylan esterase enhances the solubilisation of hemicellulose to a certain level and increases the subsequent hydrolysis of cellulose (Zhang et al., 2011). The xylanase produced in this study belongs to GH11 family which can cleave the internal β-1,4-xylosidic linkages only. The structure of this endoxylanase (Figure 1.5) has been described using a thermophilic fungus, *Thermomyces lanuginosus* (Juturu and Wu, 2011). The endo-1,4-β-xylanase (EC 3.2.1.8) initiates the degradation of xylan into xylose and xylooligosaccharides of varying sizes (Collins et al., 2005; Hwang et al., 2012). Xylanases can be used for the bioconversion of lignocellulosic materials to produce higher value products, e.g.

biofuel (Kumar et al., 2008). Xylanases have also been noted to be generated concurrently with production of cellulases (Jampala et al., 2017).

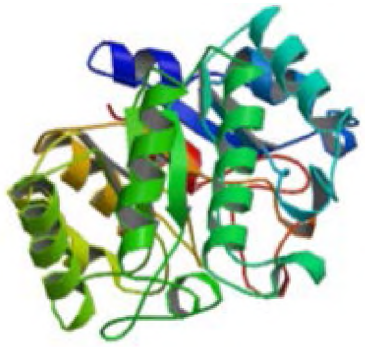


Figure 1.5. A structural front view of endo-xylanase from *Thermoascus aurantiacus* (Juturu and Wu, 2011).

Table 1.2. Some selected mycorrhizal and endophytic fungal enzymes, their sources, relationship with the host plants and methods of detection [RE = root-endophyte]

Fungus	Enzyme	Source	Relation -ship	Method for detection	Reference(s)
Hymenoscyphus ericae	Cellulase	Ericaceous plant	ERM	*p*-nitrophenol based substrate	Burke and Cairney, 1997a
Acremonium sp.	Amylase	Ericaceous plant	RE	Plating	Mandyam et al., 2010
Phialophora finlandia	Cellulase	*Pinus strobus*	DSE	Plating	Caldwell et al., 2000
Periconia macrospinosa	Amylase, Cellulase	Tallgrass	DSE	Plating	Mandyam et al., 2010
Suillus variegatus	Cellulase	Ericaceous plant	ECM	Plating	Burke and Cairney, 1998
Mortierella hyaline	Cellulase, Xylanase	*Osbeckia stellata*	RE	Plating, DNS	Bhagobaty and Joshi, 2012
Paecilomyces variabilis	Amylase, Xylanase	*Osbeckia chinensis*	RE	Plating, DNS	Bhagobaty and Joshi, 2012
Hymenoscyphus ericae	Endoxylanase	Ericaceous plant	ERM	DNS	Burke and Cairney, 1997a
Lyophyllum shimeji	Glucoamylase	Barley	ECM	Somogyi–Nelson	Kusuda et al., 2004
Cylindrocephalum sp.	Amylase	*Alpinia calcarata*	RE	Plating, DNS	Sunitha et al., 2012
Tricholoma matsutake	Amylase	*Pinus densiflora*	ECM	Somogyi-Nelson	Hur et al., 2001
Leohumicola sp.	Endoglucanase	Ericaceous plant	ERM	DNS	Adeoyo et al., 2017

Table 1.3. Some already known hydrolytic enzymes from mycorrhizal and endophytic fungi, their unique enzymatic properties, and potential applications

Enzyme (fungal source)	Properties	Potential application	Reference(s)
Cellobiohydrolase (*Cortinarius* sp.)	2.413 nkatal/mg protein when MMN medium was supplemented with cellobiose	To clarify fruit juice	Burke and Cairney, 1998
Cellulase (*Coriolus versicolor*)	Highest enzyme activity (0.74 U/ml) when supplemented with peptone at pH of 6.8	To clarify fruit juice	Jonathan and Adeoyo, 2011
α-amylase (*Tricholoma matsutake*)	Most at pH 5.0-6.0 towards soluble starch, and stable at 50^{o}C	Hydrolyse starch into fermentable sugars	Kusuda et al., 2003
Cellulase (*Heterobasidion annosum*)	Addition of CMC into culture media produced clearing on CMC-agar plates	Plant growth stimulator	Maijala, 2000
α-Amylase (*Tricholoma matsutake*)	Higher activity levels when production medium was supplemented with corn starch (11.5 U/ml), rice powder (13.8 U/ml), and flour containing gluten (12.5 U/ml)	Hydrolyse starch into fermentable sugars	Kusuda et al., 2007
Endoxylanase (*Hymenoscyphus ericae*)	pH optimum for activity was 4.5	Plant growth stimulator	Burke and Cairney, 1997a

1.7. Carbohydrate substrates (starch, carboxymethylcellulose and xylan)

Starch is a major carbohydrate reserve of all higher plants (Encarnación et al., 2011). In some cases, starch accounts for as much as 70% of the undried plant material. Starch is a polysaccharide substance commonly found in cereal grains (corn, wheat, rice, oats, barley) as well as potatoes (tuber crops). The native starch granule is made up of two polysaccharides amylose, 20-30% and amylopectin, 70-80% (Chung and Liu, 2009). Starches with varying amylose and amylopectin contents are of interest in food industries because of their ability to improve the quality, texture, and stability of starch-based food products (Schirmer et al., 2013). Amylose (Figure 1.6a) is long linear chains composed of α-1,4-linked D-glucose units with a few branches (Bijttebier et al., 2008; Hizukuri et al., 1981). Amylopectin (Figure 1.6b) has a larger molecular weight, shorter chains of α-1,4-linked D-glucan, and is highly branched through additional α-1,6-linked D-glucose linkages (Bijttebier et al., 2008). Starch is insoluble in cold water but swells reversibly to a limited extent through hydrogen bonding. Gelatinisation is a process of disrupting the molecular orders within the starch granule which result in granular swelling and solubilisation of starch by heating to a particular temperature.

Cellulose is the most abundant renewable biopolymer (Bhat, 2000). The renewable value of cellulose as an energy source has made cellulose hydrolysis a relevant area for research (Bhat, 2000). Cellulose is the carbohydrate that forms the wooden parts and cell walls of plants, and the use of cellulose as biodegradable raw material in various applications is a proposed solution to various environmental and recycling challenges (Kovacs et al., 2010). Carboxymethylcellulose (CMC) is commonly used as sodium salt Na-CMC (Figures 1.7 a and b) and is one of the most widely used cellulose derivatives (Lopez et al., 2014).

Xylan is an abundant biopolymer in the biosphere and a major hemicellulosic polysaccharide found in the plant cell wall (Timell and Syracuse, 1967). Xylan (Figure 1.7c) is one of the residues released during wood processing (Prade, 1996), it possesses a complex structure consisting of β-1,4-D-xylopyranose backbone with side groups on the 2- or 3-position (Hansen and Plackett, 2011).

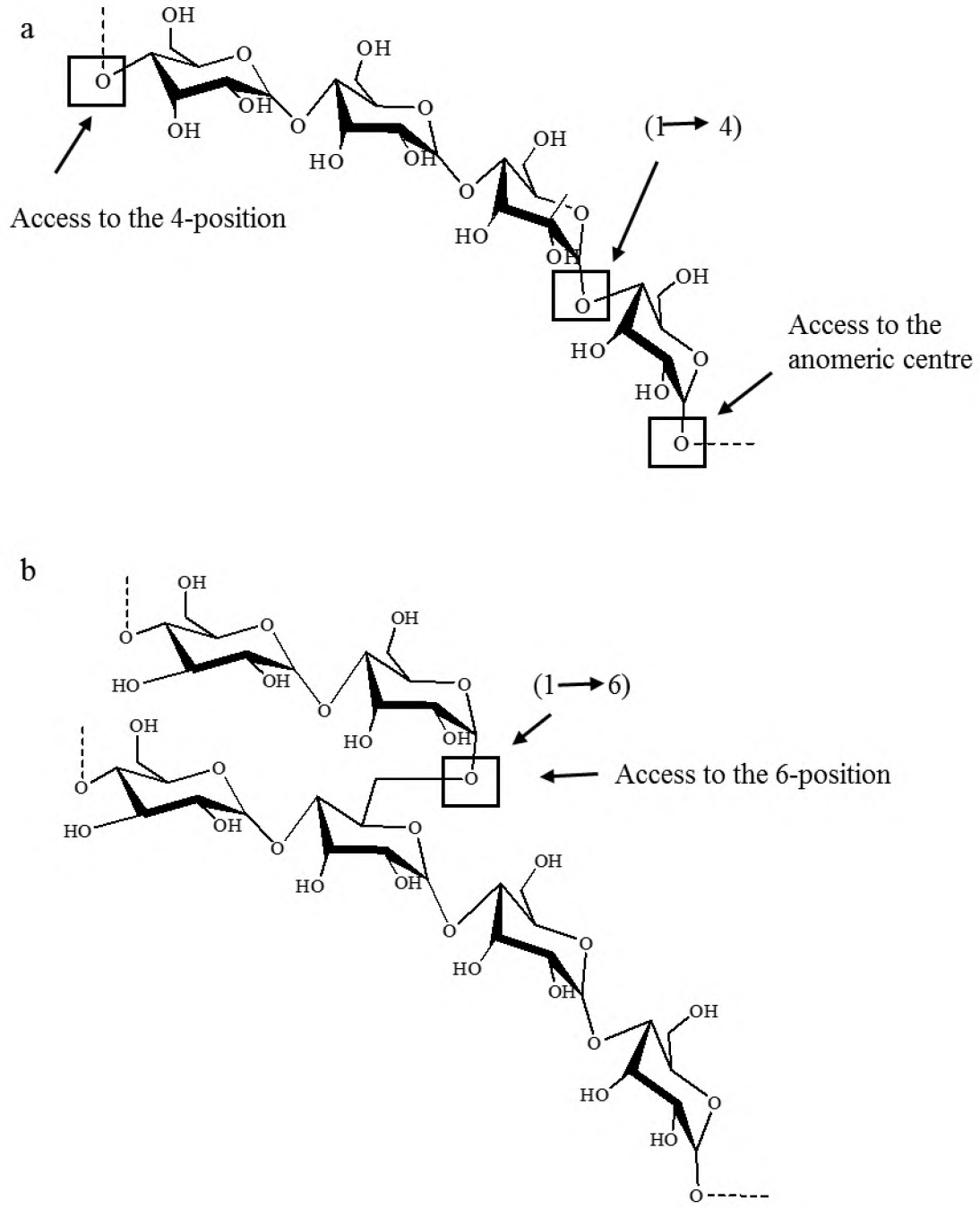


Figure 1.6. Structures of two components of starch (a) amylose and (b) amylopectin (Modified from Damager et al., 2010).

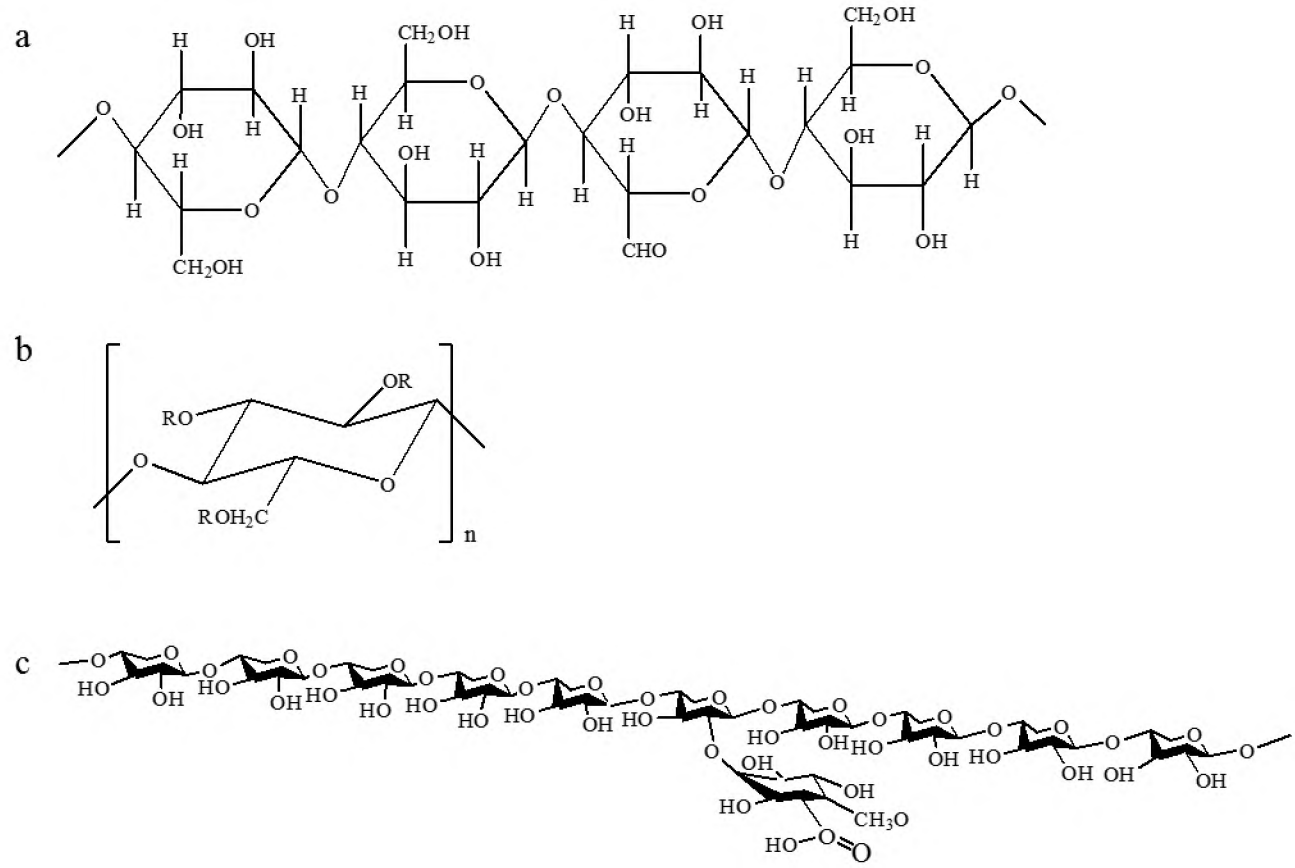


Figure 1.7. Carbohydrate structures: (a) cellulose, (b) monomer structure of sodium carboxymethylcellulose structure, R can be H or CH2COONa. The degree of substitution (DS) is defined as the average number of CH_2COONa groups per monomer of 3 (Modified from Lopez et al., 2014) and (c) xylan (4-*O*-methyl glucuronoxylan, Megazyme).

1.8. Mycorrhizal fungi: potential sources of enzymes for the bio-economy

Microbial enzymes can be secreted into the fermentation broth by the producer (e.g., ERM, DSE and ECM fungi), enhancing fast downstream processing of the biocatalyst as compared to those obtained from other sources (e.g., plants and animals) and encouraging an increase in production. The shift from chemical processes to biological processing, attained by using fungal enzymes rather than chemical processes in industries has significantly eliminated most negative impacts associated with the use of chemicals on the environment (Lange, 2014). Enzyme activity has been investigated in some ERM, DSE and ECM fungi (Table 1.2) using plating methods (Baldrian, 2009; Baldrian and Valášková, 2008; Peretto et al., 1993; Sunitha et al., 2013). The highest cellulase activity of 0.74 units/ml was recorded for *Coriolus versicolor* when production

medium was supplemented with 1% (w/v) peptone at a temperature of 28°C (Table 1.3) (Jonathan and Adeoyo, 2011). Burke and Cairney (1997a) produced, purified and characterised a β-l,4-endoxylanase from the ericoid mycorrhizal fungus (*H. ericae*). This enzyme had an isoelectric point of 4.85-5.20 and a molecular weight of 58.4 kDa (Burke and Cairney, 1997a). The pH optimum for activity was 4.5 and was stable between pH 3.5-4.0 (Burke and Cairney, 1997a).

Africa is blessed with several unique habitats, harbouring a rich microbial diversity that includes ERM, DSE and ECM fungi. Despite the enormous potential of enzymes for biotechnology innovation, the continent is not exploiting these microbial resources to the fullest. Hence, research groups should be established to identify and produce biometabolites from these domestic microbial resources and test their suitability for commercial production. The estimate of future demand for microbial enzymes is encouraging, with future markets of $7.1 billion expected in 2018 (BCCReseach, 2014; OECD, 2009). Bio-economy involves the production of renewable biological resources and their conversion into food, feed, bio-based products and bioenergy using biotechnological techniques.

1.9. Mycorrhizal fungi in ecosystems

Enzymes that are present in the soil are involved in all activities relevant to agriculture and biochemical transformations in the soil environment (Rao et al., 2014). Enzyme activities in soil often provide a distinct biological assessment of soil function, through catalysis of the soil biological processes (Das and Varma, 2011). Mycorrhizas are among the fungi that can produce enzymes (Table 1.2), which support the utilisation of nitrogen and phosphorus in the soil permitting access to valuable compounds embedded within the dead plant tissues (Cairney and Burke, 1998). Thus they support host plants in acquiring nutrients and alleviating various stresses associated with their growth and development (Smith and Read, 2008). They connect host plants to the nutrient source, enabling the flow of energy-rich compounds required for nutrient mobilisation (Finlay, 2008). Some mycorrhizal fungi mobilise nutrients from multiple organic sources as a strategy for the development of nutrient cycling models, particularly in a poor soil (Burke et al., 2011). Therefore, ectomycorrhizas and ericoid associated fungi are

among those mycorrhizal fungi that can improve the acquisition of mineral nutrients in agricultural soils by producing the needed enzymes capable of breaking down complex substrates into smaller forms.

1.10. Antimicrobial properties of root endophytic fungi

Several recent reports on drug resistance to disease-causing pathogens have necessitated the need to exploit the environment for newer bio-control agents. Scientists are now involved in the screening for new antimicrobial agents from new natural sources, and mycorrhizal fungi, as well as other root fungal endophytes, could be an additional source of new antimicrobials. An understanding of the interaction that exists between some root endophytes, saprobes and the pathogenic organism is valuable to the development of a healthy environment. *Agaricus arvensis, Gymnopilus purpuratus* and *Phaeogyroporus beniensis* are some of the fungi that have been isolated from the root of *Pinus radiata* (Garrido et al., 1982). Extracts from these fungi were found to inhibit the growth of *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, and *Escherichia coli* UCCL51 (Garrido et al., 1982). Moreover, extracts from *Lepista nuda* isolated from a *Pinus* sp. was also found to inhibit *Proteus mirabilis* and *Pasteurella multocida* (Alves et al., 2012). Some mycorrhizal fungi are reported to protect against root invading pathogens. These include the host - pathogen - mycorrhizal fungus: (i) *Picea mariana* - *Cylindrocladium floridanum* - *Paxillus involutus* (Morin et al., 1999); (ii) *Pinus banksiana* – *Fusarium oxysporum* – *Laccaria laccata* (Chakravarty and Hwang, 1991); (iii) *Pinus banksiana* – *Fusarium moniliforme* – *Paxillus involutus* (Hwang et al., 1995); (iv) *Pinus resinosa* – *Fusarium oxysporum* – *Laccaria laccata* (Chakravarty et al., 1991); (v) *Pseudotsuga menziesii* - *Fusarium oxysporum* - *Laccaria laccata* (Sylvia and Sinclair, 1983); (vi) *Pinus wallichiana* – *Fusarium oxysporum, Rhizoctonia solani* – *Pisolithus tinctorius, Laccaria laccata* (Dar et al., 2011).

1.11. Research question

Are ericoid associated mycorrhizas and root endophytes capable of producing amylases, cellulases and xylanases for commercial purposes?

1.12. Hypothesis

If the right conditions of growth are met, some ericoid mycorrhizas and root endophytic fungi can be used in the production of the desired amylases, cellulases and xylanases using chemically defined media.

1.13. Motivation

Africa has a vast number of untapped natural resources; these include mycorrhizal fungi and their metabolites which could further enhance the bio-economy development of the region. Ericoid mycorrhizas and related fungi are known to support plant growth in impoverish soil, facilitate the survival of plants in heavy metal contaminated soils and hence, the need to further investigate some other properties associated with them to improve human welfare. This study focuses on the production of enzymes that could be added to the already available enzymes, encouraging the production of these biocatalysts on a commercial scale from novel sources.

1.14. Aims and thesis outline

This thesis seeks to: (i) determine the enzyme-producing potentials of some ericoid mycorrhizal fungi for the bio-economy, (ii) produce, purify and characterise the best-performing enzyme from a novel isolate, (iii) identify the best isolate to species level.

These aims were achieved and discussed under the following thesis chapters:

- ✓ Chapter 1. A general introduction and literature review of the current study.
- ✓ Chapter 2. The objective was to determine the growth of ERM fungi on modified Melin-Norkrans media, screen for the best amylase and cellulase-producing ericoid mycorrhizal fungi.
- ✓ Chapter 3. The objective was to optimise the production of amylase, cellulase, and mycelial biomass of some selected ericoid mycorrhizal fungi. The effect of sequential subculturing on

the production of amylase, cellulase and co-produced endoxylanase are monitored after several subculturing and separation from the symbiotic partner (host plant).

- ✓ Chapter 4. The objective was to subject endoxylanase co-produced with cellulase to partial purification and characterisation.
- ✓ Chapter 5. The objective was to purify and characterise amyloglucosidase (AMG) produced from *Leohumicola* sp. (ChemRU330).
- ✓ Chapter 6. The objective was to evaluate the antibacterial properties of some ericoid mycorrhizal and related fungi by performing minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) assays.
- ✓ Chapter 7. The objective was to carry out molecular identification of some enzyme-producing ericoid associated fungi to species level using the mitochondrial cytochrome c oxidase subunit 1 (*Cox1*)-encoding gene as a molecular marker and nuclear rDNA internal transcribed spacer (ITS) region (encompasses the noncoding transcribed spacers ITS1 and ITS2 and the 5.8S rDNA gene).
- ✓ Chapter 8. The discussion and conclusion based on overall results presented in all chapters

CHAPTER 2

2.0 Screening for amylase and cellulase producing ericoid fungi

2.1. Introduction

The mycorrhizal fungal type found in the ecosystem and soil environment has distinctive natural characteristics and innate selectivity conferred on them to aid the development of a plant community (Read, 1991). ERM and ECM fungi are commonly found at high to mid altitudes and latitudes (Read, 1991). Their survival in these environments has been linked to their ability to secrete bioactive compounds such as enzymes, which facilitate the breakdown of complex substrates into smaller and available forms (Burke and Cairney, 1997b). The use of microbes to hydrolyse starch and cellulose is an essential step towards the efficient utilisation of lignocellulosic and starchy waste materials found in nature (Das and Varma, 2011; Ja'afaru, 2013). The most predominant polysaccharide in plant cell walls is made up of 40.6–51.2% cellulose, 28.5–37.2% hemicelluloses, and 13.6–28.1% lignin (Pauly and Keegstra, 2008). Starch is an insoluble, non-structural carbohydrate composed of α-glucose polymers that occur in plants and algae in the form of granules (Pfister and Zeeman, 2016). Fungal enzymes break down these composite materials into smaller products with the help of amylases, cellulases, and xylanases (Schwarze, 2007; Webster and Weber, 2007).

Fungal enzymes have been extensively exploited for industrial applications for many years; they have also shown promising potential for biotechnological use in the bio-economy. Hence, considerable efforts are geared towards characterising better enzymes from novel microbial sources. This work aimed to screen enzymes produced by ERM isolates collected from the root of ericaceous plants occurring in the Albany Centre of Endemism, Grahamstown, South Africa and monitoring the production of these enzymes from *Leohumicola* sp. over a period of 18 months. Starch and cellulose agar screening methods were chosen to assess different enzyme activities. These methods usually facilitate rapid detection of either a positive or negative enzyme

producer and are useful in screening a large number of fungal isolates for enzyme production (Pointing, 1999). The application of amylases and cellulases has been considered for the bioconversion of starchy and lignocellulosic materials such as agricultural wastes to produce bio-ethanol, and assist in baking, fruit juice extraction, beverage preparation and animal feed (Ja'afaru, 2013). Therefore, screening for novel amylolytic and cellulolytic enzymes, and procedures to evaluate their potential as biocatalysts for the bio-economy deserves considerable attention.

2.2. Materials and methods

2.2.1. Fungal isolates and their cultivation

Fungal isolates used for this study were cultured from roots of ericaceous plants (*Erica cerinthoides*, *Erica nemorosa*, *Erica demissa* and *Erica chamissonis*) (Bizabani, 2015). *Leohumicola* sp. (Isolate code ChemRU330 / Genbank accession number KC979127 / South African National Collection of Fungi accession number PPRI 17268), and two unidentified fungi belonging to Hyaloscyphaceae (EdRU083 / PPRI 17261) and Leotiomycetes (EdRU002 / PPRI 17284) were among the fifty-one (51) ericoid associated fungi obtained from the Mycorrhizal Research Laboratory, Rhodes University, Grahamstown. These isolates were selected at random irrespective of their taxonomic status. They were maintained on potato dextrose agar (PDA) and malt extract agar (MEA) throughout the study period.

2.2.2. Media and mycelial preparation

PDA and MEA were prepared according to the manufacturer's specification while Modified Melin Norkrans (MMN) media had the following composition (g l^{-1}): malt extract 3.0; glucose 1.0; $(NH_4)_2HPO_4$ 0.25; $MgSO_4 \cdot 7H_2O$ 0.15; $CaCl_2$ 0.05; NaCl 0.025; $ZnSO_4 \cdot 7H_2O$ 0.003; thiamine-HCl 100 µg; 1.2 ml of $FeCl_3$ (1% w/v) and 1.5% agar (Marx, 1969). Autoclaving was conducted at 121°C for 15 min, and chloramphenicol (0.05 g/l) was added to the cooled medium (approximately 45°C) before pouring into plates to prevent bacterial growth. Mycelial plugs (5 mm) were regularly subcultured and incubated at 28°C for three weeks and stored at 4°C.

2.2.3. Screening for amylase producing ericoid mycorrhizal fungi

The basal medium (MMN) was amended with 1% w/v soluble starch (Merck, Cat # 101252) as the sole carbon source in the medium (Caldwell et al., 2000). About 20 ml of the medium was placed into each Petri dish and allowed to set and solidify before inoculating with a 5 mm mycelial plug of each mycorrhizal fungus. The plates were incubated at 28°C for three weeks. After incubation, plates were flooded with iodine solution (10% v/v) to immediately observe any clear zones (halo) around the colonies. Agar plates showing maximum hydrolysis clearance (halo) and distinguishing morphological characteristics such as the colour and size of the colonies were selected for further studies. The strength of activity was described and classified based on the diameter of the hydrolytic zone.

2.2.4. Screening for cellulase producing ericoid mycorrhizal fungi

The basal medium (MMN) was amended with 1% w/v carboxymethylcellulose sodium salt (Na-CMC, Sigma-Aldrich, Cat # C4888) (Caldwell et al., 2000). About 20 ml of the medium was placed into each Petri dish and allowed to set and solidify before inoculating with a 5 mm mycelial plug of each mycorrhizal fungus. The plates were incubated at 28°C for three weeks, and zones of clearance were viewed by flooding the plates with an aqueous solution of Congo red (0.1% w/v) for 15 min. After draining the dye, plates were flooded with 1.0 M NaCl for another 15 min and then stabilised with 1 M HCl (Teather and Wood, 1982). The appearance of a yellow area around the fungal colony indicated cellulase activity. The strength of activity of each of the isolate was classified based on the diameter of the hydrolytic zone [- = negative result, + = minimum activity (1-9 mm) ++ = moderate activity (10-14 mm), +++ = maximum activity (15 mm and above), ND = activity not determined].

2.2.5. Relative activity (RA)

The RA was carried out in triplicate using fresh samples, and each replicate was examined for the presence of a clear zone around the colony, and the diameters of the colony and the clear zone (activity zone) were measured. The measurement was repeated in two mutually orthogonal dimensions, and the mean value calculated (Krishnan and Convey, 2016). The RA was calculated using the following formula:

$$RA = \frac{\text{Clear zone diameter - Colony diameter}}{\text{Colony diameter}}$$

2.2.6. Statistical analysis

All experiments were conducted in triplicate and analysed using one-way analysis of variance (ANOVA). Error bars were represented as the standard errors of the means (SEM).

2.3. Results

2.3.1. Substrate utilisation and enzyme activity (clearance zone)

All the 51 isolates used in this study grew on MMN medium, though at different growth rates with different colony formation. Scanty to dense hyphae were noticed with some isolates, and better growth rates were observed in medium supplemented with soluble starch and Na-CMC for most of the species. The starch and cellulose-containing substrates were broken-down to varying degree by the hydrolytic enzymes, which was indicated by the level of clearance zone observed across the board. Positive activity for both amylase and cellulase production was discovered in 33% of the isolates, while 43 and 59% exhibited a positive reaction for amylase and cellulase activities, respectively (Table 2.1).

Table 2.1. Screening of ericoid associated fungal isolates for enzymes production

S/N	Isolate	Host plant	Amylase		Cellulase	
			Activity strength	Cleared zone diameter (mm)	Activity strength	Cleared zone diameter (mm)
1.	Caf721,	*Erica caffra*	-	0	++	12±1.15
2.	Caf928	*Erica caffra*	+++	18±1.45	-	0
3.	CafRU024	*Erica caffra*	+++	15±1.20	++	11±0.58
4.	CafRU078	*Erica caffra*	-	0	+	6±1.33
5.	CafRU082	*Erica caffra*	-	0	++	12±0.88
6.	CafRU48	*Erica caffra*	++	11±1.00	+++	18±0.88
7.	CafRU500	*Erica caffra*	-	0	+	4±0.88
8.	CafRU0826	*Erica caffra*	ND	0	-	0
9.	Chem008	*Erica chamissonis*	-	0	-	0
10.	Chem009	*Erica chamissonis*	++	14±0.58	ND	0
11.	Chem038	*Erica chamissonis*	-	0	-	0
12.	Chem077	*Erica chamissonis*	-	0	+	7±0.88
13.	Chem081	*Erica chamissonis*	-	0	-	0
14.	ChemRU009	*Erica chamissonis*	++	12±0.88	++	13±1.45
15.	ChemRU018	*Erica chamissonis*	-	0	-	0
16.	ChemRU330	*Erica chamissonis*	++	12±1.53	+++	17±1.53
17.	EdRU002	*Erica demissa*	++	11±1.67	+++	15±1.15
18.	EdRU003	*Erica demissa*	-	0	-	0
19.	EdRU016(09)	*Erica demissa*	-	0	+	4±0.88
20.	EdRU030	*Erica demissa*	++	11±3.00	++	12±0.88
21.	EdRU0151(000)	*Erica demissa*	++	10±1.00	++	11±0.67
22.	EdRU051(D7)	*Erica demissa*	-	0	+	6±1.15
23.	EdRU060	*Erica demissa*	-	0	-	0
24.	EdRU081	*Erica demissa*	-	0	-	0

25.	EdRU083	*Erica demissa*	+++	15±0.33	++	11±2.33
26.	EdRU096	*Erica demissa*	+	6±0.33	ND	0
27.	EdRU104	*Erica demissa*	-	0	+	5±0.88
28.	EdRU200(060)	*Erica demissa*	-	0	+++	17±0.67
29.	EdRU90	*Erica demissa*	++	10±1.45	+++	16±0.88
30.	Glum001	*Erica glumiflora*	+	7±0.58	+++	16±0.58
31.	Glum002	*Erica glumiflora*	+++	14±0.41	-	0
32.	Glum038	*Erica glumiflora*	-	0	ND	0
33.	Glum82	*Erica glumiflora*	+++	21±1.53	++	14±0.67
34.	Glum828	*Erica glumiflora*	++	10±0.58	++	11±0.88
35.	GlumRU033	*Erica glumiflora*	-	0	-	0
36.	GlumRU059	*Erica glumiflora*	-	0	-	0
37.	Ncf91	*Erica caffra*	-	0	-	0
38.	Nem13	*Erica nemorosa*	+++	16±1.20	++	13±0.33
39.	Nem15	*Erica nemorosa*	-	0	++	12±1.00
40.	Nem24	*Erica nemorosa*	++	12±0.88	+++	20±1.20
41.	Nem36	*Erica nemorosa*	-	0	++	13±0.58
42.	Nem42	*Erica nemorosa*	++	11±1.20	+++	20±1.45
43.	Nem44	*Erica nemorosa*	-	0	-	0
44.	Nem50	*Erica nemorosa*	++	12±0.88	+++	19±2.33
45.	Nem54	*Erica nemorosa*	-	0	-	0
46.	Nem60	*Erica nemorosa*	-	0	+++	19±1.76
47.	Nem62	*Erica nemorosa*	-	0	ND	0
48.	Nem69	*Erica nemorosa*	-	0	-	0
49.	Nem81	*Erica nemorosa*	+++	16±0.88	++	10±0.58
50.	Nem83	*Erica nemorosa*	+	7±0.33	+	4±0.33
51.	Nem88(17)	*Erica nemorosa*	ND	0	+++	16±1.76

The values are represented as means ± SEM, n = 3 per treatment, S/N = serial number.

- = Negative result
+ = Minimum activity (1-9 mm)
++ = Moderate activity (10-14 mm)
+++ = Maximum activity (15 mm and above)

ND = Activity not determined

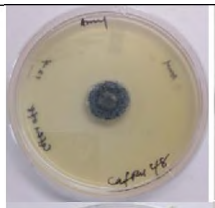
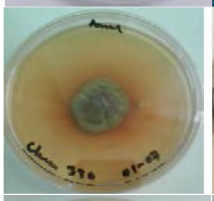
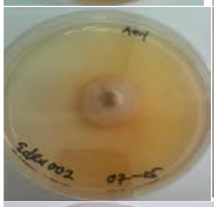
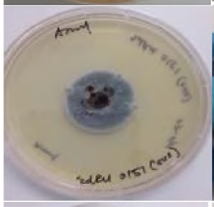
2.3.2. Amylase activity on soluble starch agar

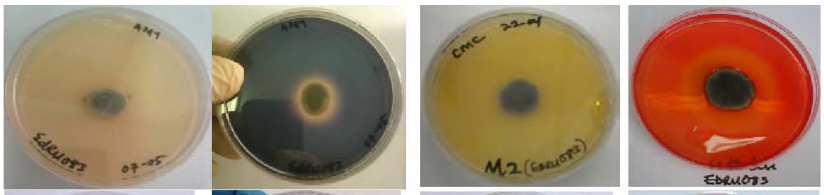
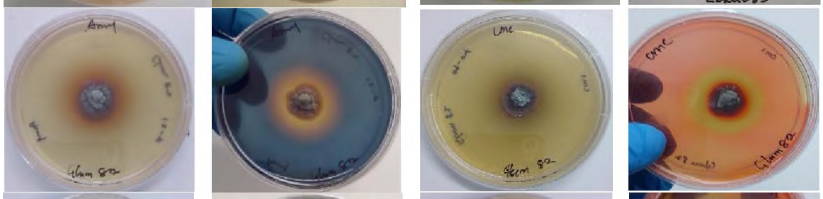
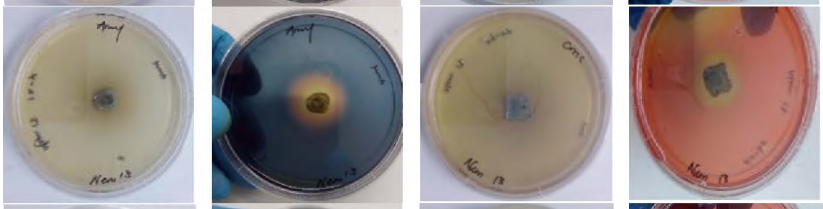
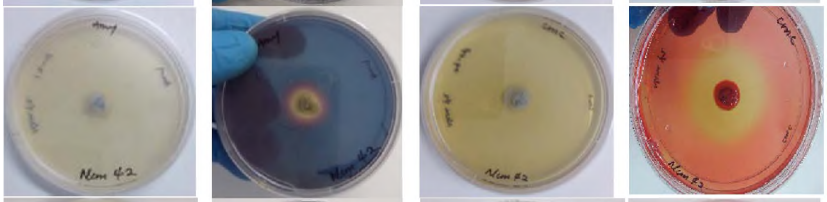
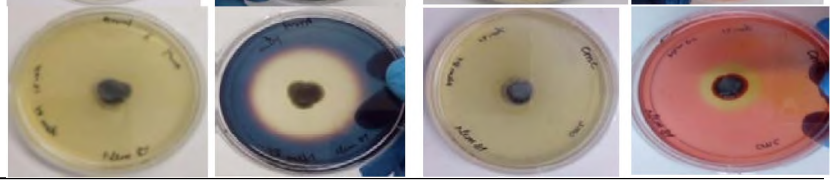
In this study, hydrolysis zones in the starch medium were spotted with clearance zone indicating the degradation of starch under the growing hyphae (Table 2.2). A total of twenty-two (22) out of fifty-one isolates tested (43%) were positive for amylase production. Isolates Caf928, CafRU024, EdRU083, Glum002, Glum82, Nem13 and Nem81 showed highest amylase activities, followed by CafRU48, Chem009, ChemRU009, ChemRU330 EdRU002, EdRU030, EdRU0151(000), EdRU90, Glum828, Nem24, Nem44, and Nem50. The remaining isolates showed little or no activity. The enzyme activities of CafRU0826 and Nem88(17) were not determined, this was due to lack of sufficient colony growth on the agar medium.

2.3.3. Cellulase activity on carboxymethylcellulose (CMC) agar

Table 2.1 shows that 59% tested fungi exhibited a positive reaction on CMC agar. The highest activity was observed with CafRU48, ChemRU330, EdRU200(060), EdRU90, Glum001, Nem24, Nem42, Nem50, Nem60 and Nem88(17). Moderate enzyme activities were recorded for isolates Caf721, CafRU024, CafRU082, CafRU0826, ChemRU009, EdRU002, EdRU030, EdRU0151(000), EdRU083, Glum82, Glum828, Nem13, Nem15, Nem36 and Nem81. The remaining isolates showed little or no activity. Activities (for Chem009, EdRU096, Glum038, and Nem62) were not determined, due to insufficient colony formation on the medium.

Table 2.2. Detection of amylase and cellulase production when the solid mineral medium was supplemented with either soluble starch or Na-CMC as the only carbon source

S/N	Isolate	AMYLASE		CELLULASE	
		Before flooding with iodine	After flooding with iodine	Before flooding with Congo red	After flooding with Congo red
1.	CafRU48				
2.	ChemRU009				
3.	ChemRU330				
4.	EdRU002				
5.	EdRU0151(000)				
6.	EdRU030				

7.	EdRU083	
8.	Glum82	
9.	Nem13	
10.	Nem42	
11.	Nem50	
12.	Nem81	

2.4. Discussion and conclusion

The results from screening indicated the presence of hydrolytic enzymes (amylases and cellulases) produced by ERM fungi obtained from roots of ericaceous plants found in the Albany Centre of Endemism, Grahamstown. The findings revealed that isolates, CafRU024, CafRU048, ChemRU330, EdRU002, EdRU083, EdRU90, Glum82, Nem13, Nem24, Nem50 and Nem81 had the highest amylase and cellulase activities. This corroborates the report that some fungi excreted significant amounts of amylolytic and cellulolytic enzymes in culture media, while others excreted little or no enzyme activity on the medium (Begum and Absar, 2009).

High amylase and cellulase activities can be found in culture filtrates only in the stationary phase of growth in submerged cultures where enzymes were released by autolysis (Begum and Absar, 2009). In this study, the emphasis was on agar screening method indicating that enzymes were actively secreted into the environment. Microbial enzymes can be classified according to their localisation, extracellular and cell-bounded (intracellular and surface-bound) (Begum and Absar, 2009). It has been suggested, that a limited zone of clearance beneath the colony can be considered as an indicator of intracellular enzyme production (Choi et al., 2005). Some of these fungi were able to degrade starch and Na-CMC substrates because they possess the synergistic endo or exo-catalytic system that facilitated the breakdown processes (Saxena et al., 2015; Schmidt, 2006).

In conclusion, the study revealed that agar screening method could be a good technique for providing fast information on amylolytic or cellulolytic ability of fungal isolates when a large number of isolates are available for study (Florencio et al., 2012; Tan and Leong, 1986). Moreover, these findings confirmed that the incorporation of an inducing substrate such as soluble starch or Na-CMC into a fermentative basal medium could be a necessity for the production of amylolytic or cellulolytic enzymes. Therefore, isolates ChemRU330, EdRU002 and EdRU083 were selected for enzyme production in liquid MMN medium in the next chapter.

CHAPTER 3

3.0 Optimisation of culture conditions for mycelial biomass and enzyme production, and monitoring the effect of sequential sub-culturing

3.1. Introduction

Enzymes occur in all living organisms and hence in mycorrhizal fungi. Every microbial cell will produce an enzyme capable of hydrolysing or oxidising a specific substrate (Nigam, 2013). The type of enzyme produced by an individual species will vary markedly. Therefore, there is the need to further explore the environment for additional microbial enzymes that can complement or improve the enzyme production for the bio-economy. Hydrolases, which constitute a class of enzymes are widely distributed in nature from bacteria to higher eukaryotes that include mycorrhizal fungi (Moreno et al., 2013). Scientists are now developing programs to isolate, produce, and characterise novel hydrolytic enzymes with different properties to those of conventional enzymes.

Extracellular proteins secreted by mycorrhizal fungi can degrade starch and cellulose components because they harbour enzymes to cleave the types of glycosidic linkages present in the substrate. Biomass resources, such as starchy and cellulosic materials of plant origin, are the most abundant renewable resources on earth (Ali et al., 2013). Amylases from ericoid fungi will help in degradation of starch (Hur et al., 2001), cellulases are involved in degradation of cellulose (Baldrian and Valášková, 2008), while endoxylanases coproduced with cellulases will help in the hydrolysis of hemicellulose (Elisashvili et al., 2008).

Extracellular enzymes are essential to fungi in breaking down large complex polysaccharides into smaller sugars. The soils of temperate, boreal forests and heathlands are characteristically

enriched with soil microbes that include ERM and DSE fungi. The roots of ericaceous plants harbour these fungi, conferring eco-physiological benefits to the host (Bizabani and Dames, 2015). The primary function of any mycorrhizal fungus is to facilitate the breakdown of complexed substrates into utilisable forms as nutrients, while the fungus acquires a photosynthetic carbon supply as either glucose or sucrose from the host for the completion of their life cycle (Burke et al., 2011; Pritsch and Garbaye, 2011). *Hymenoscyphus ericae* (ERM) has been widely investigated for its ability to grow on a variety of complex organic substrates such as starch or carboxymethylcellulose (CMC) (Burke and Cairney, 1997a; Cairney and Burke, 1998; Leake and Miles, 1996).

In vitro, extracellular enzymes production in mineral salt media can be assayed to determine the activity of the enzyme produced per millilitre of the filtrate, per minute. Assessing the production of amyloglucosidase, endoglucanase and endoxylanase *in vitro* involve the use of mineral salt medium amended with either 1% soluble starch or CMC. The idea is to mimic the natural pattern of output where hydrolysis of starch or cellulose takes place because of the action of amylolytic or cellulolytic microorganisms in the soil or plants (Doolotkeldieva and Bobusheva, 2011; Kusuda et al., 2007). The saprophytic ability of ERM fungi has been confirmed by culture-based method (Cairney and Meharg, 2003; Meharg and Cairney, 2000) as well as in soils, where their biomolecules unlock organic complexes to release mineral nutrients for plant uptake.

Amyloglucosidase hydrolyses starch to produce glucose (Adeniran and Abiose, 2010), while endoglucanase is one of the enzymes that make up the cellulase complex (Doolotkeldieva and Bobusheva, 2011), and xylanase can be co-produced with cellulase by both ERM and DSE fungi *in vitro* (Jampala et al., 2017). Other vital enzymes such as laccases, pectinases, and proteases may also be produced from mycorrhizal fungi (Burke et al., 2014; Choi et al., 2005; Elisashvili et al., 2008) but for this study, only amylase and cellulase were selected for testing and production. In this chapter, the effect of pH and nutritional factors on amylase, cellulase and mycelial biomass production of selected ERM and DSE fungi in a mineral salt liquid medium was investigated. The data reported on endoglucanase in this study has been published in AMB Express (Adeoyo et al., 2017).

3.2. Materials and methods

3.2.1. Fungal isolates and their cultivation

Fungal isolates used for this study were cultured from roots of ericaceous plants (*Erica demissa* and *Erica chamissonis*) (Bizabani, 2015). *Leohumicola* sp. (Isolate code ChemRU330 / Genbank Accession Number KC979127 / The South African National Collection of Fungi Accession Number PPRI 17268), and two unidentified fungi belonging to Hyaloscyphaceae (EdRU083 / KF225587 / PPRI17261) and Leotiomycetes (EdRU002 / KF225581 / PPRI17284) were obtained from Mycorrhizal Research Laboratory, Rhodes University, Grahamstown. The selection of these three isolates was based on their strength of enzyme activity (both amylase and cellulase) and morphological characteristics (e.g., colony size, growth rate and exudate colour). These isolates were preserved on PDA for further studies.

3.2.2. Effect of sequential subculturing on the survival and enzyme activity of Leohumicola sp. (ChemRU330)

A 5 mm mycelial plug of *Leohumicola* sp. was cultivated on a soluble starch (amylase) or carboxymethylcellulose (cellulase) MMN agar medium and sequentially subcultured at three-week intervals for 24 times. This was to show if there will be any variation in the activity of the enzyme of interest after production over various subculturing events. The mycelial plug of each subculture was then used to inoculate a 50 ml mineral salt medium of soluble starch or carboxymethylcellulose-containing MMN as a primary carbon source. Enzyme production was allowed to proceed at 28^{o}C for three weeks after which activity (U/ml) was determined according to the DNS method of Miller (1959) and the relative activity calculated.

Relative activity = Initial enzyme activity X 100%
Final enzyme activity

3.2.3. Production of amylase and cellulase/xylanase enzymes

Soluble starch (1%, w/v) was added to 150 ml Erlenmeyer flask for amylase production while carboxymethylcellulose (1%, w/v) was added to a 150 ml Erlenmeyer flask for cellulase or xylanase production in a salt solution having the composition: (g l^{-1}): malt extract 3.0; $(NH_4)_2HPO_4$ 0.25; $MgSO_4.7H_2O$ 0.15; $CaCl_2$ 0.05; NaCl 0.025; $ZnSO_4.7H_2O$ 0.003; thiamine-HCl 100 µg l^{-1} and 1.2 ml of $FeCl_3$ (1%, w/v). Production medium of 50 ml was distributed into each 150 ml flask; the contents were thoroughly mixed and was sterilised at 121°C for 15 min. The sterilised medium was inoculated with two discs of 5 mm mycelial plugs of ERM. The non-inoculated medium was used as a control. Growth was allowed to proceed at 28°C in the dark for three weeks in a rotary incubator shaker at 150 rpm. After 21 d of growth, the cultures were homogenised using IKA's ULTRA-TURRAX homogeniser (20,000 rpm), and centrifugation was performed at 10,000 x *g* for 15 min to obtain crude enzyme filtrates (supernatant) using Beckman Coulter Avanti-J high-speed centrifuge. The pellet containing the mycelia was resuspended in 10 ml water and filtered using a vacuum suction filtering system to retain the mycelia on a filter paper (Whatman no. 1).

3.2.4. Protein estimation

Analysis of the concentration of protein was determined according to the method of Bradford (Bradford, 1976) using a standard comprising of bovine serum albumin (BSA, Sigma-Aldrich, Lot # 127H1078). This method measures the interaction between the proteins' amino acids and the dye (Coomassie Blue G-250) in an acid solution. The reading was carried out at 595 nm on a UV/Visible spectrophotometer (BioTek's Synergy™ Mx Microplate Reader). The values of absorbance were compared with a standard curve for bovine serum albumin. Each assay was performed in triplicate, and Bradford's 96 well plate assay protocol modified by Sigma-Aldrich

technical bulletin, 2013 was used (A 150 µl of a 1–10 µg/ml protein BSA standard was used). A 150 µl of the protein standards were added to separate wells in the 96 well plate, the blank and the sample. A 150 µl Bradford reagent (Sigma, Lot # SLBB8933V) was added to each well being used and mixed on a shaker for 30 seconds. Samples were incubated at room temperature for 30 min. After that, the absorbance was read at 595 nm. A graph of the net absorbance versus the protein concentration was plotted, and the unknown sample concentration was extrapolated from the standard curve (Bradford, 1976).

3.2.5. Enzyme assay

The enzyme assay was conducted using dinitrosalicylic acid (DNS, Sigma-Aldrich, Lot # MKBS4991V) assay method described by Miller (1959). A 1% w/v of either soluble starch (AMG) or CMC (endoglucanase) or beechwood (endoxylanase) was each added to a 10 ml buffer (pH 5.0) of either sodium acetate (AMG) or citrate-phosphate (endoglucanase) in a Schott bottle. The mixture was stirred with a magnetic stirrer until completely dissolved. The crude endoglucanase extract was concentrated using Amicon Ultra-15 filter unit (Sigma) with a molecular weight cut-off of 10 kDa, while AMG crude extract was not concentrated before use. A 100 µl of each enzyme with controls (substrate and enzyme) was added to separate 300 µl of the corresponding substrate in triplicate while blank sample contained 400 µl buffer. All samples were incubated at 37°C for 30 min except if otherwise stated, followed by centrifugation at 6000 × *g* for 2 min. After centrifugation, a 150 µl supernatant was then withdrawn and transferred to a new Eppendorf tube, and 300 µl DNS reagent was added. Then, was followed by boiling on a heating block at 100°C for 5 min and cooled on ice for 5 min. A 250 µl of each blank, controls and samples were distributed into a 96-well plate and read with the aid of a spectrophotometer (BioTek's Synergy™ Mx Microplate Reader) at a wavelength of 540 nm. A unit of enzyme activity was expressed as 1 μmol of glucose (AMG and endoglucanase) or xylose (endoxylanase) equivalent released per minute per milliliter of enzyme solution.

3.2.6. Determination of fungal biomass

The mycelial biomass was expressed as dry weight by drying in a hot air oven at 80^{o}C for 16 h

(Sunitha et al., 2012).

3.2.7. Effect of temperature manipulation on enzyme and biomass production

A 50 ml mineral salt medium was adjusted to pH of 5.0 using HCl and NaOH. The medium was sterilised at 121^{o}C for 15 min and was inoculated with two discs of 5 mm mycelial plugs. The medium was incubated at either 20, 24, 28, or 32^{o}C with an agitation speed of 150 rpm in rotary shaker incubator, and the non-inoculated medium was used as a control for 3 weeks. Enzyme activity was determined after obtaining a cell-free filtrate (Jonathan and Adeoyo, 2011).

3.2.8. Effect of pH manipulation on enzyme and biomass production

The pH of a 50 ml mineral salt medium was adjusted to either 3.0, 4.0, 5.0, 6.0, 7.0 or 8.0 using HCl and NaOH. The medium was sterilised at 121^{o}C for 15 min and was inoculated with two discs of 5 mm mycelial plugs. The medium was incubated at 28^{o}C with an agitation speed of 150 rpm in rotary shaker incubator, and the non-inoculated medium was used as a control for 3 weeks. Enzyme activity was determined after obtaining a cell-free filtrate (Jonathan and Adeoyo, 2011).

3.2.9. Effect of metal ion manipulation on enzyme and biomass production

The mycelia of collected fungi were grown in a liquid medium similar to that used in the previous experiment. The liquid medium was supplemented with 0.1% (w/v) K^{+}, Na^{+}, Mn^{+2}, Zn^{+2}, or Co^{+2} supplied in sulphate form while FeNa-EDTA (ferric EDTA) was provided as ethylenediaminetetraacetic acid iron (III) sodium salt to investigate the effect of metal ions. The incubation was carried out at 28°C for three weeks, and enzyme activity was determined as described previously (Gautam et al., 2011).

3.2.10. Effect of carbon source manipulation on enzyme and biomass production

The effect of carbon sources (1.0% w/v) on the enzyme production was carried out using glucose, maltose, sucrose, cellobiose and starch as supplements. The incubation was performed at 28°C for 3 weeks, and enzyme activity was determined as described previously (Gautam et al., 2011).

3.2.11. Effect of nitrogen source manipulation on enzyme and biomass production

The mycelia of collected fungi were cultured in a liquid medium similar to that used in the previous experiment. Nitrogen sources (1.0% w/v) supplemented into the liquid medium included either peptone, yeast extract, malt extract, tryptone or sodium nitrate ($NaNO_3$). The incubation was performed at 28°C for 3 weeks, and enzyme activity was determined as described previously (Gautam et al., 2011).

3.2.12. Statistical analysis

All experiments were conducted in triplicate and analysed using one-way ANOVA. A significant difference between the means of each treatment was determined using least significant difference (LSD) test at the 0.05 level of significance. LSD analysis was conducted using software by Assaad et al. (2014), and error bars were represented as the standard errors of the means (SEM).

3.3. Results

3.3.1. Effect of sequential sub-culturing on the survival and enzyme activity of Leohumicola sp.

The monitoring of enzyme production in liquid MMN medium after sequentially sub-culturing and inoculating with *Leohumicola* sp. (ChemRU330) for 24 times on a solid medium (PDA) was investigated. The fungus was able to utilise either starch or CMC as carbon for growth and survival *in vitro* throughout the study period. It should be noted that the study focused on enzyme production in a liquid MMN medium for all investigations. The monitoring of enzymatic activity

showed that the production of AMG and endoxylanase production was consistent with the relative activity of approximately 100% (Figure 3.1), while cellulase (endoglucanase) production starts to decrease after 11th to 24th sequential subcultures at 68 to 18%.

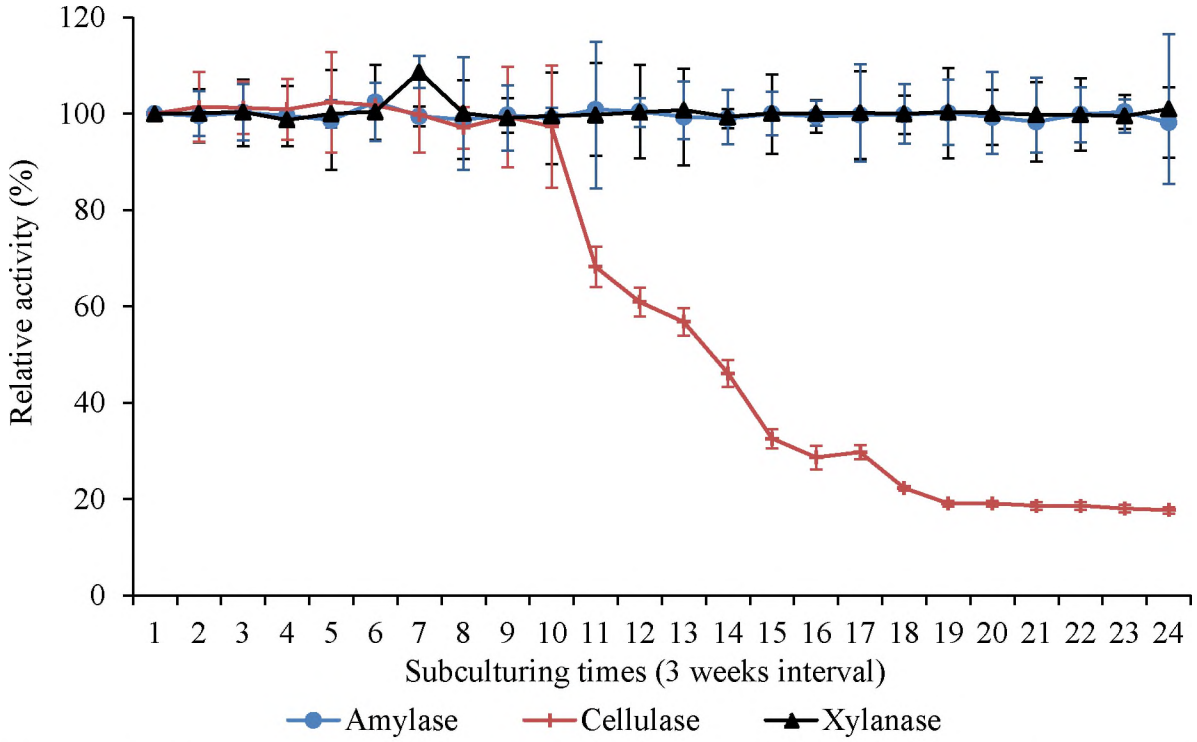
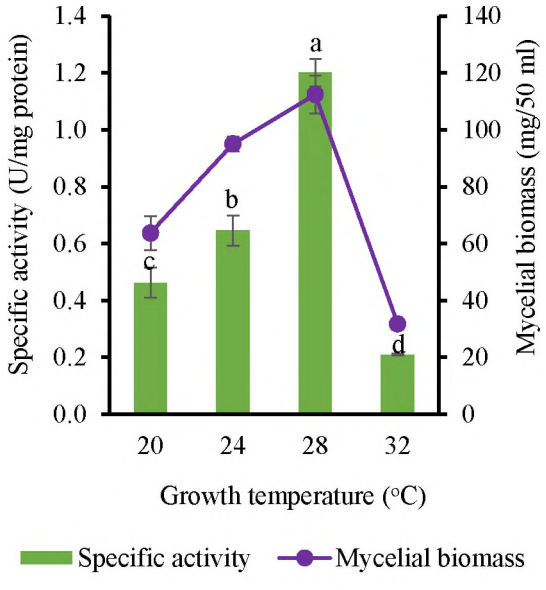


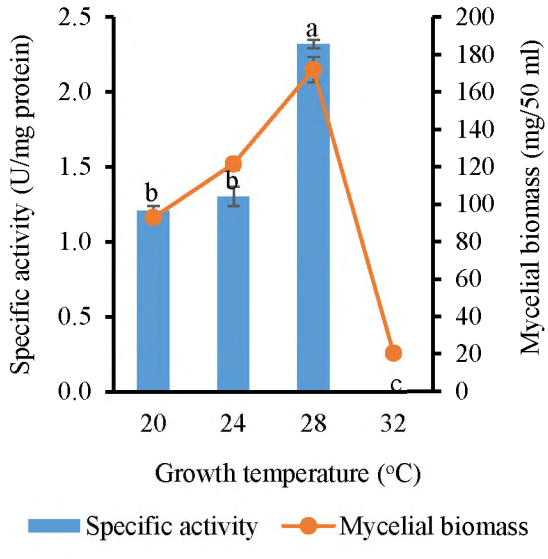
Figure 3.1. Monitoring enzyme production in liquid MMN medium after sequentially subculturing and inoculating with *Leohumicola* sp. (ChemRU330) for 24 times on a solid medium (PDA). All error bars indicate the standard errors of the means (SEM).

3.3.2. Effect of different temperature on AMG and endoglucanase production

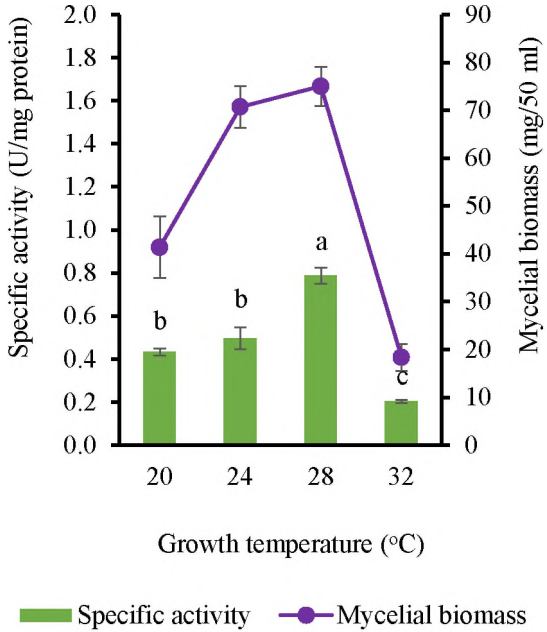
In this study, the effect of temperature on enzyme production was investigated, the enzyme reaction was carried out at various temperatures for 3 weeks on a rotary shaker. A temperature range from 20 to 32^{o}C was used to study the effect of temperature on the hydrolytic enzyme and mycelial biomass production (Figure 3.2). The optimum temperature was found at 28^{o}C, followed by 24^{o}C and 20^{o}C, while at 32^{o}C least AMG and endoglucanase production was recorded with all the three isolates (ChemRU330, EdRU083 and EdRU002) used for the experiment. No amylase activity was detected with isolate EdRU002 at 32^{o}C, while no cellulase activity was detected for both sample ChemRU330 and EdRU002 at the same temperature. The results showed that the hydrolytic enzyme production is affected by temperature.



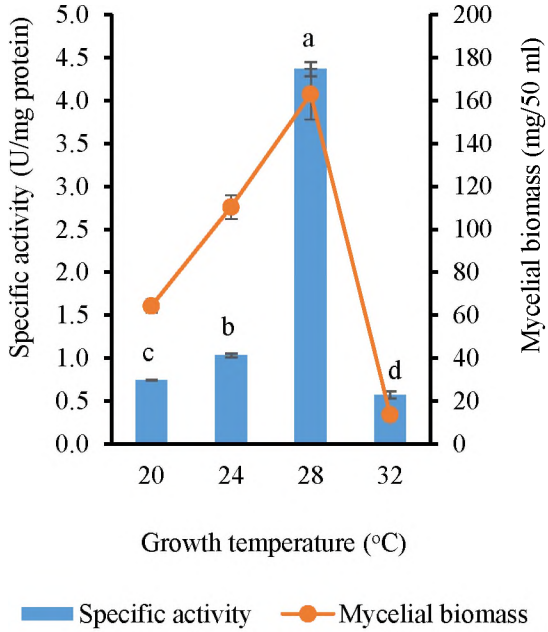
(ii) ChemRU330



(v) ChemRU330



(i) EdRU083



(iv) EdRU083

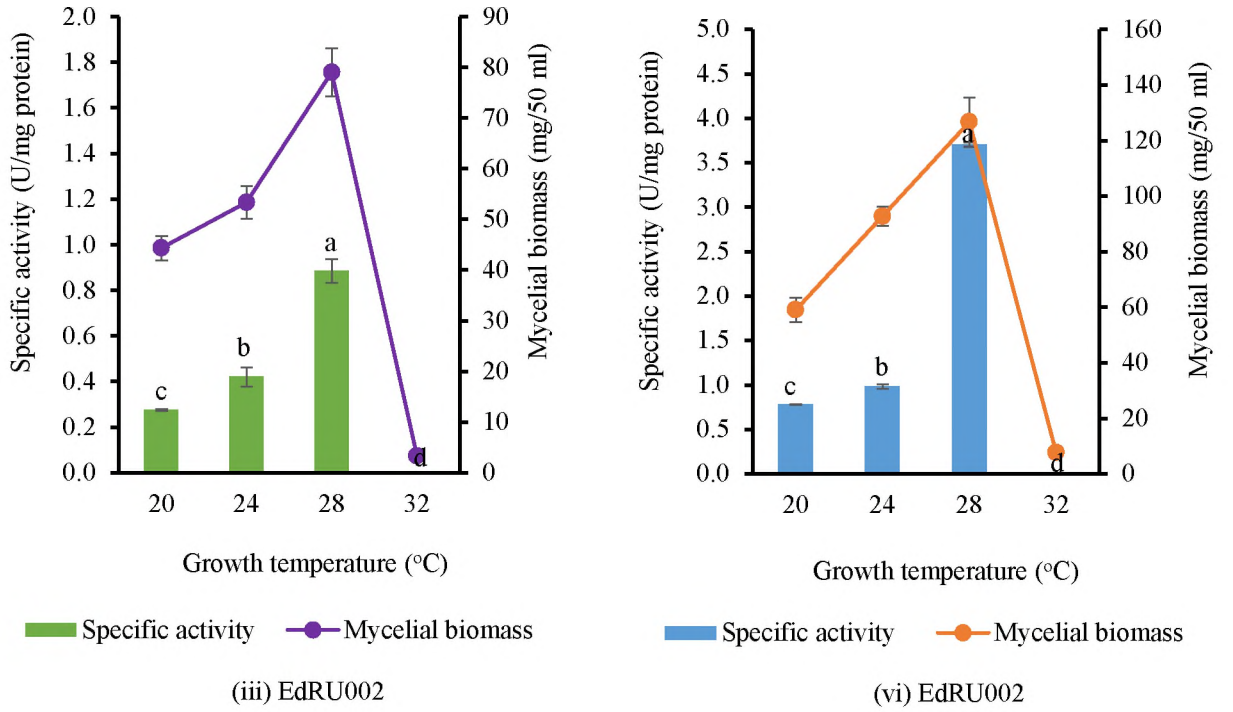
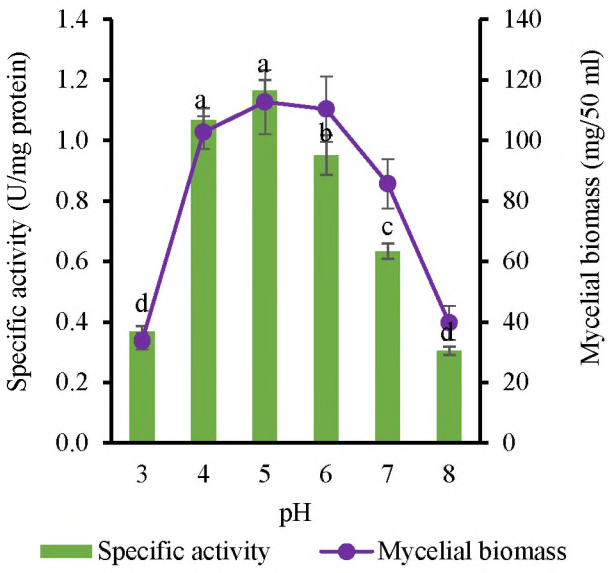


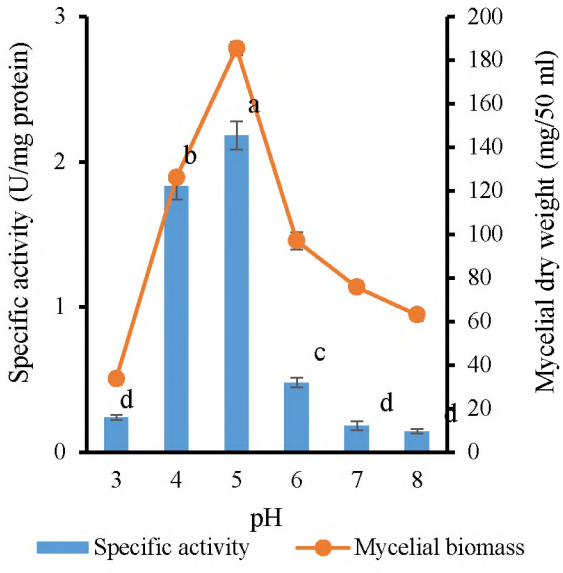
Figure 3.2. Effect of different temperature on AMG (i, ii, and iii) and endoglucanase (iv, v, and vi) production. All treatments were performed in triplicate (p-value < 0.05). Means without a common superscript letter differ as analysed by one-way ANOVA and the LSD test while the error bars indicate the standard errors of the means (SEM).

3.3.3. Effect of different pH on AMG and endoglucanase production

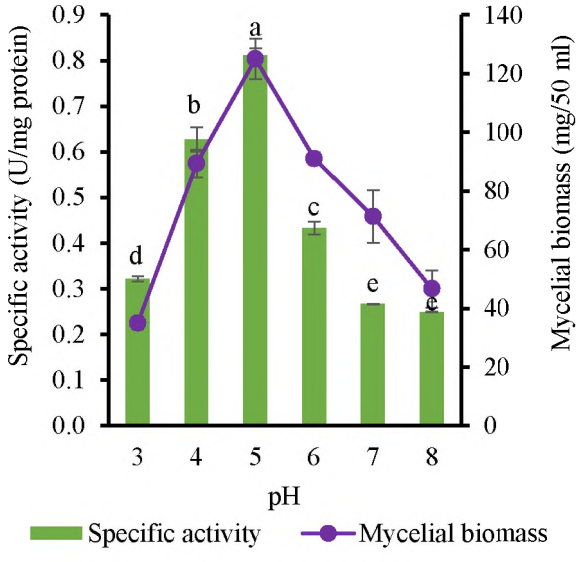
The effect of pH on enzyme and biomass production was conducted using HCl or NaOH at various pH (3.0, 4.0, 5.0, 6.0, 7.0 and 8.0) at 28°C for three weeks. The AMG activity was conducted using sodium acetate buffer (pH 5.0), while endoglucanase activity was determined using a citrate-phosphate buffer (pH 5.0). Maximum enzyme activity and biomass yield were obtained between pH 4.0–6.0 (Figure 3.3) except ChemRU330 that showed a significant decrease in endoglucanase production at pH 6.0. Further increase or decrease in pH beyond this resulted in a significant decline in both AMG and endoglucanase production and biomass yield for all isolates.



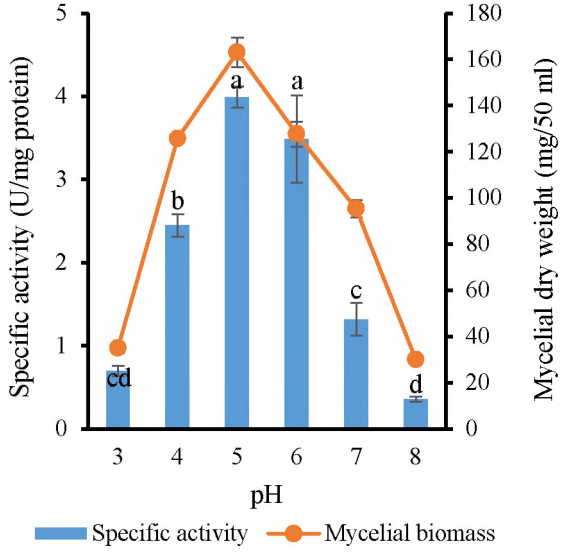
(ii) ChemRU330



(v) ChemRU330



(i) EdRU083



(iv) EDRU083

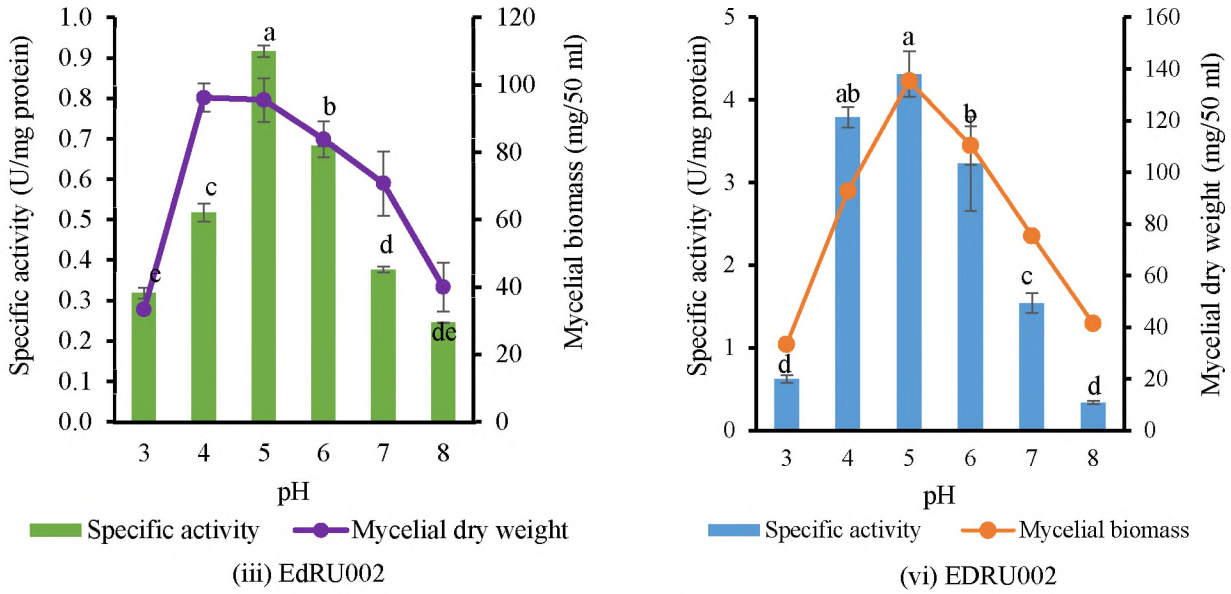
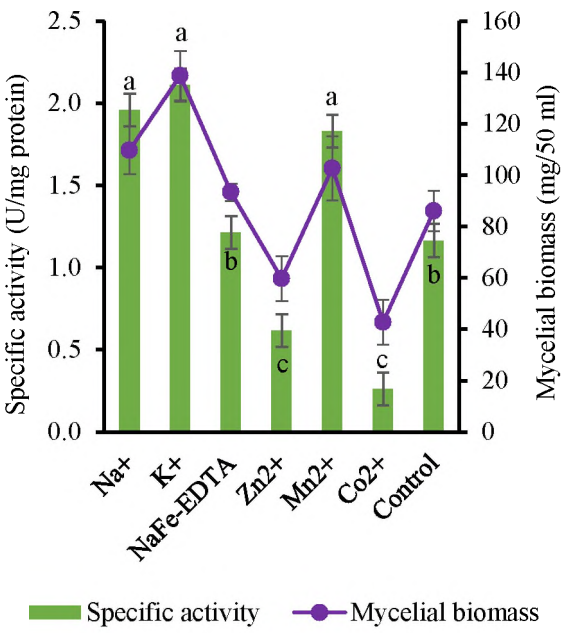


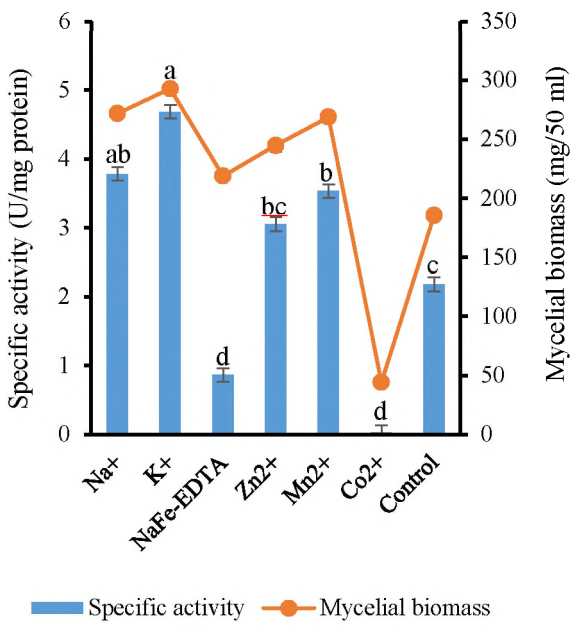
Figure 3.3. Effect of different pH on AMG (i, ii, and iii) and endoglucanase (iv, v, and vi) production. All treatments were performed in triplicate (p-value < 0.05). Means without a common superscript letter differ as analysed by one-way ANOVA and the LSD test while the error bars indicate the standard errors of the means (SEM).

3.3.4. Effect of different metal ions on AMG and endoglucanase production

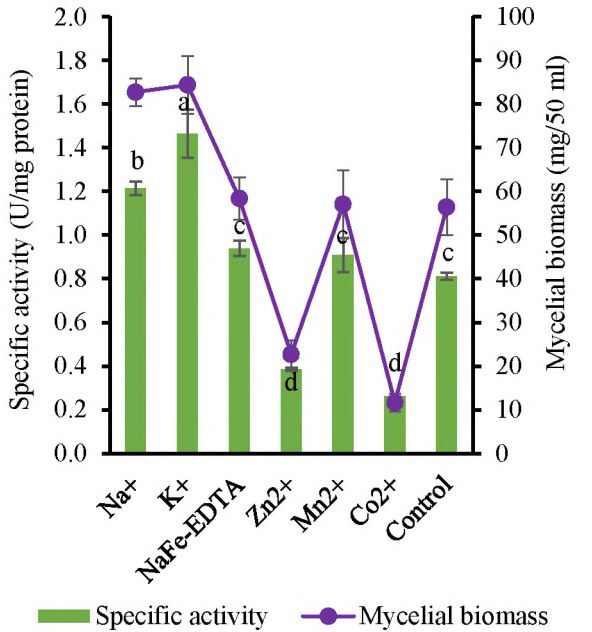
The effect of metal ions on the secretion of AMG, endoglucanase, and mycelial biomass was significant when either 0.1% Na^{+}, K^{+}, Mn^{2+}, Zn^{2+}, NaFe-EDTA or Co^{2+} was added to the liquid medium (Figure 3.4). The metal ions used in this experiment were so selected to complement those already present in the basal mineral salt medium. The activity was significant when the medium was amended with potassium and sodium for all tested isolates, while a significantly low activity was recorded when cobalt was added to the liquid medium (Figure 3.4).



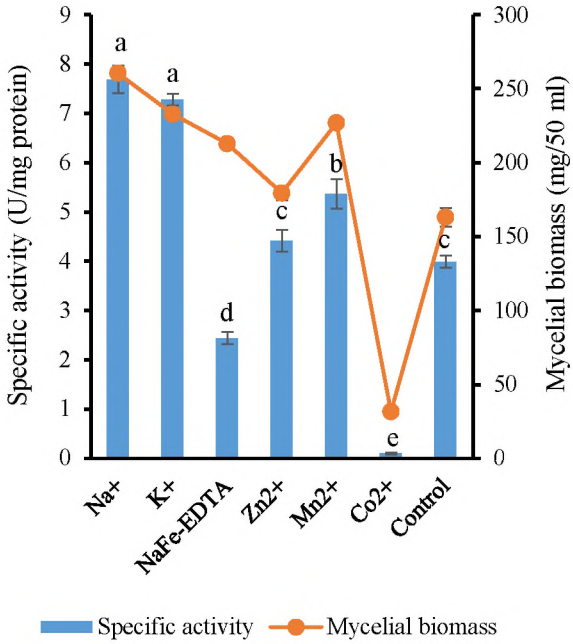
(ii) ChemRU330



(v) ChemRU330



(i) EdRU083



(iv) EDRU083

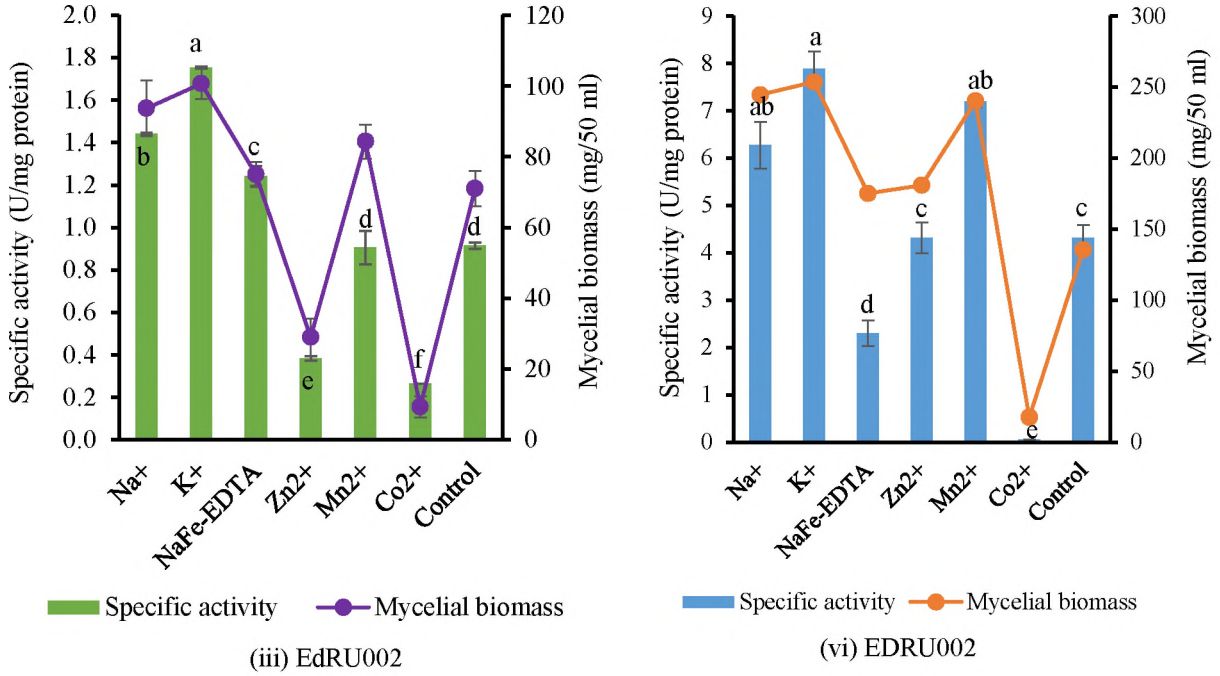
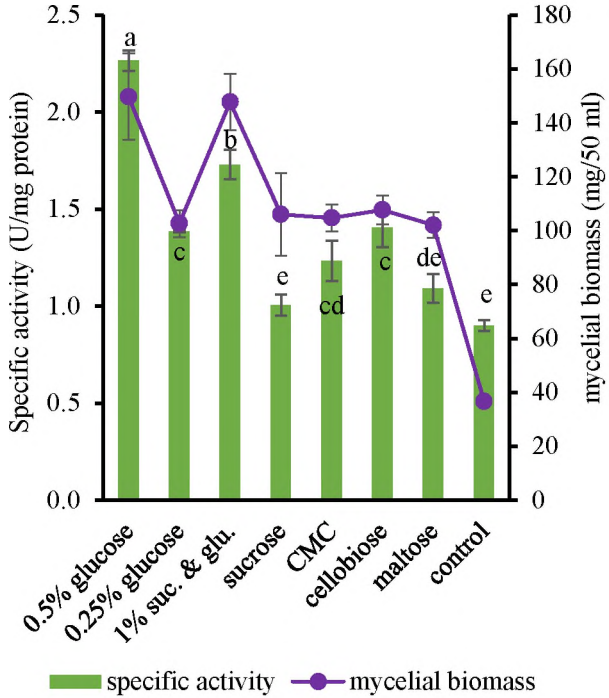


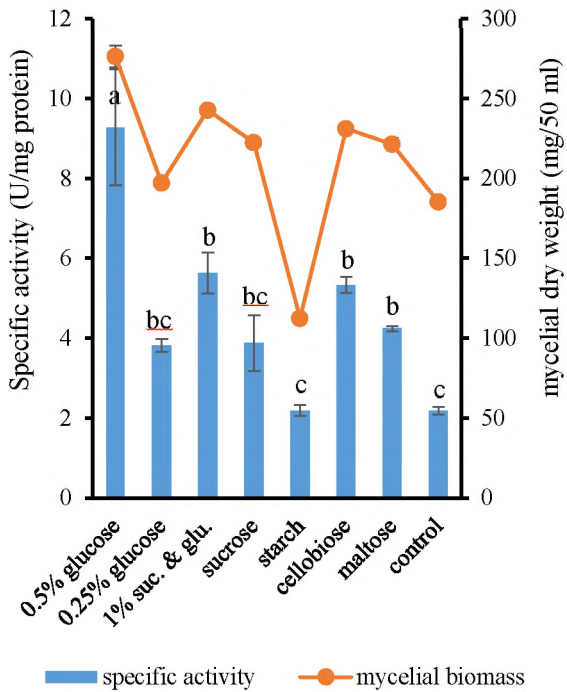
Figure 3.4. Effect of different metal ions on AMG (i, ii, and iii) and endoglucanase (iv, v, and vi) production. All treatments were performed in triplicate (p-value < 0.05). Means without a common superscript letter differ as analysed by one-way ANOVA and the LSD test while the error bars indicate the standard errors of the means (SEM).

3.3.5. Effect of different carbon sources on AMG and endoglucanase production

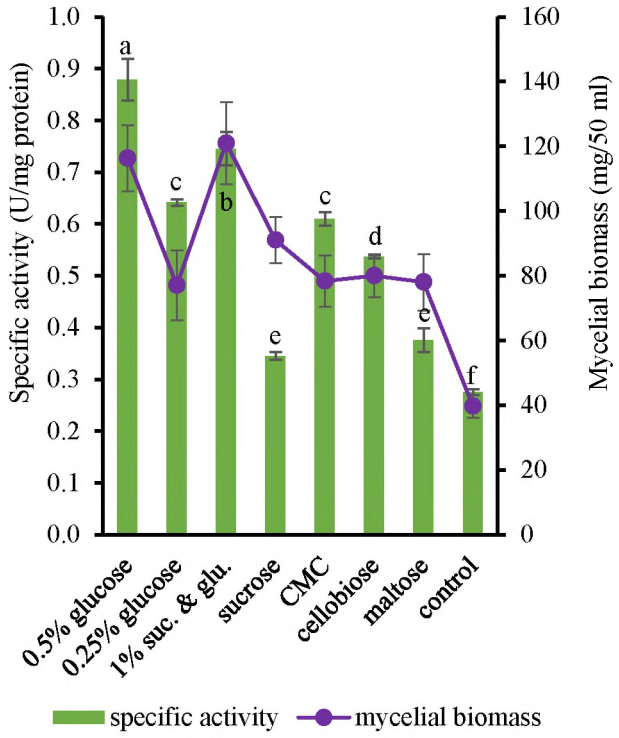
Ericoid fungi were able to grow moderately in all the tested carbon sources, and increased AMG, endoglucanase production and yield of biomass were recorded. Glucose (0.5%) gave the highest enzyme activity followed by 1.0% cellobiose, 1.0% maltose, 1.0% sucrose/glucose (ratio 1:1), 0.25% glucose, 1.0% sucrose and 1.0% starch (Figure 3.5). There was a significant difference in both enzyme activity and mycelial biomass yield when the production medium was amended with 0.5% glucose and least when 1.0% starch was used as a supplement for endoglucanase but higher for AMG production (Figure 3.5). The importance of a suitable carbohydrate source in the culture medium is important for the enzyme production and biomass yield of all isolates. Among all parameters used, glucose plays an important role in increasing the mycelial biomass yield for better AMG and endoglucanase production (Figure 3.5).



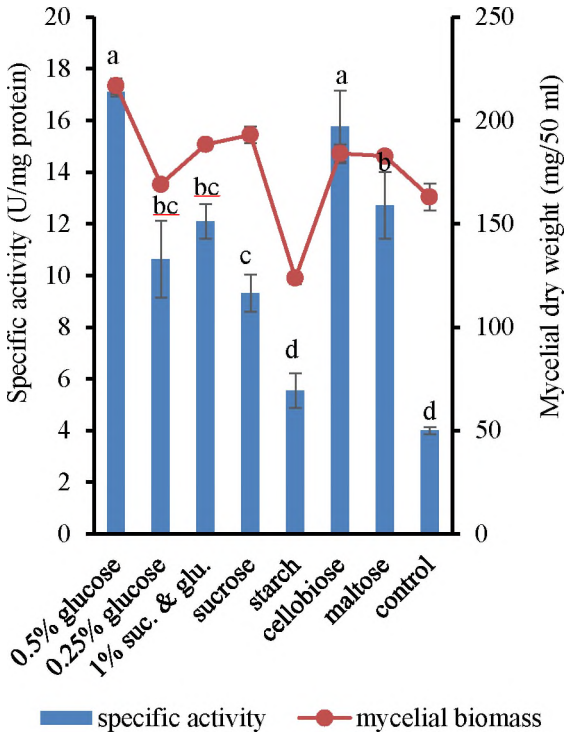
(ii) ChemRU330



(v) ChemRU330



(i) EdRU083



(iv) EDRU083

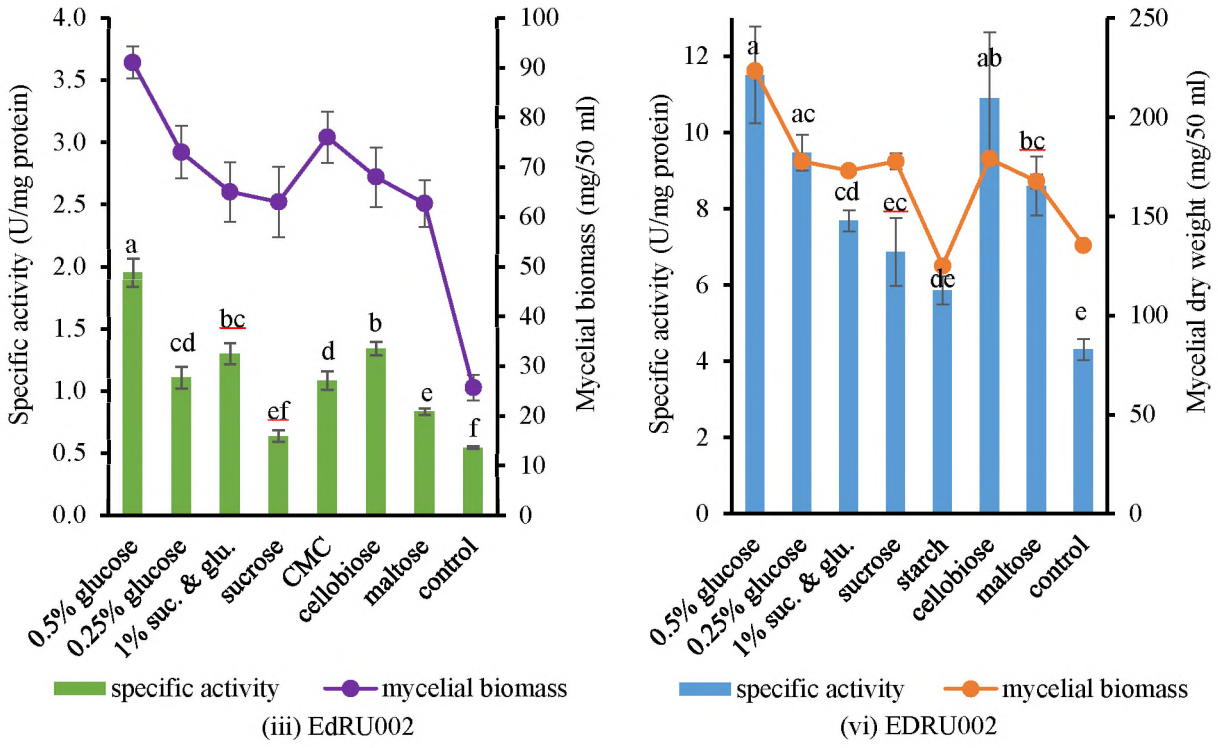
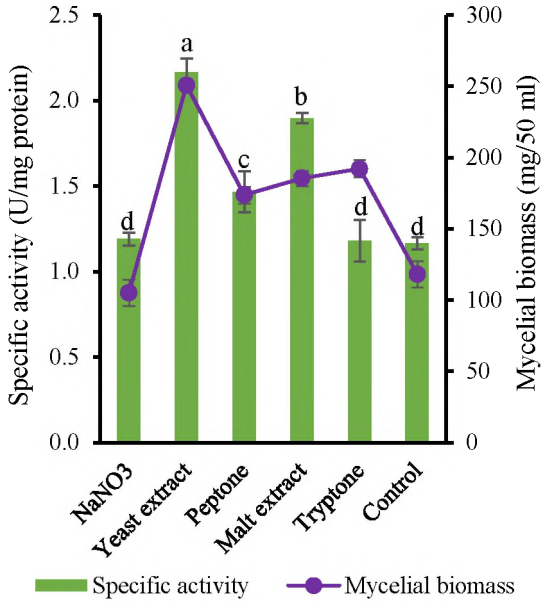


Figure 3.5. Effect of different carbon sources on AMG (i, ii, and iii) and endoglucanase (iv, v, and vi) production. All treatments were performed in triplicate (p-value < 0.05). Means without a common superscript letter differ as analysed by one-way ANOVA and the LSD test while the error bars indicate the standard errors of the means (SEM).

3.3.6. Effect of different nitrogen sources on AMG and endoglucanase production

Nitrogen source had an inducing effect on the secretion of AMG, endoglucanase and biomass yield of all tested isolates. A significant increase in endoglucanase activity was noted when 0.1% tryptone was added to the liquid basal medium for ChemRU330 and EdRU083 isolates while 0.1% peptone was significant for isolate EdRU002 (Figure 3.6). Minimum endoglucanase activity was obtained when yeast extract or malt extract was used as a supplement. For AMG production, highest activity was observed with yeast extract followed by malt extract for ChemRU330 and EdRU002, while yeast extract was followed by peptone, malt extract and tryptone for isolate EdRU083. The sodium nitrate had no noticeable effect on AMG production across all the isolates investigated.



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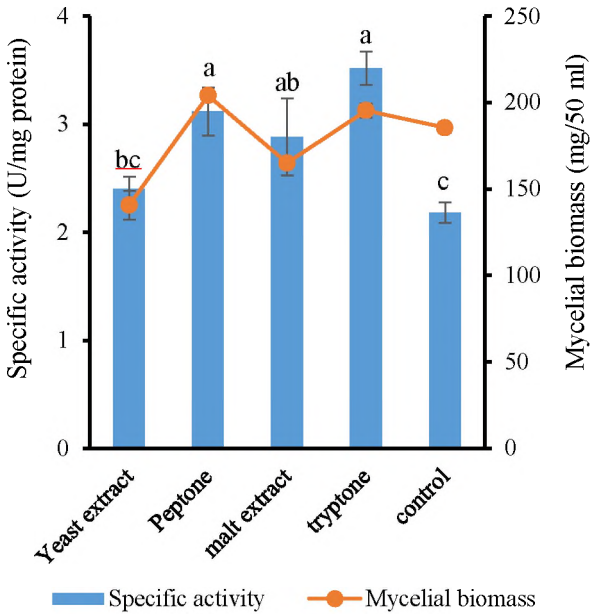
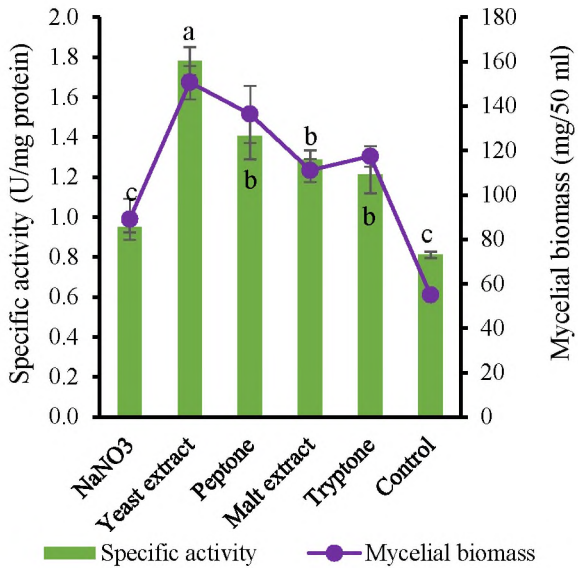
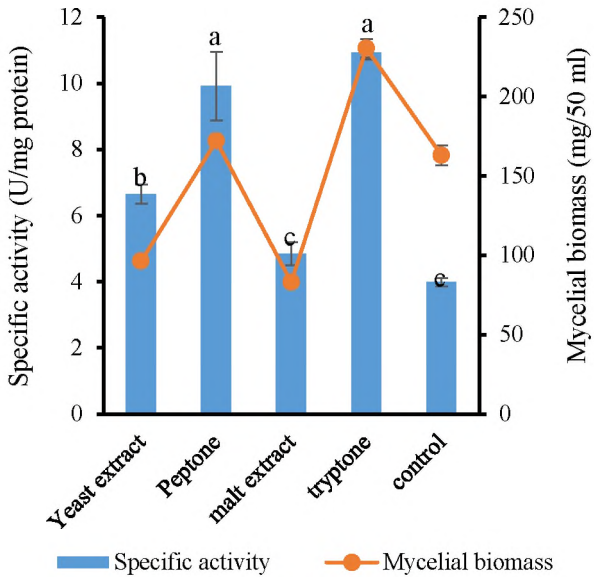


(v) ChemRU330



(i) EdRU083



(iv) EDRU083

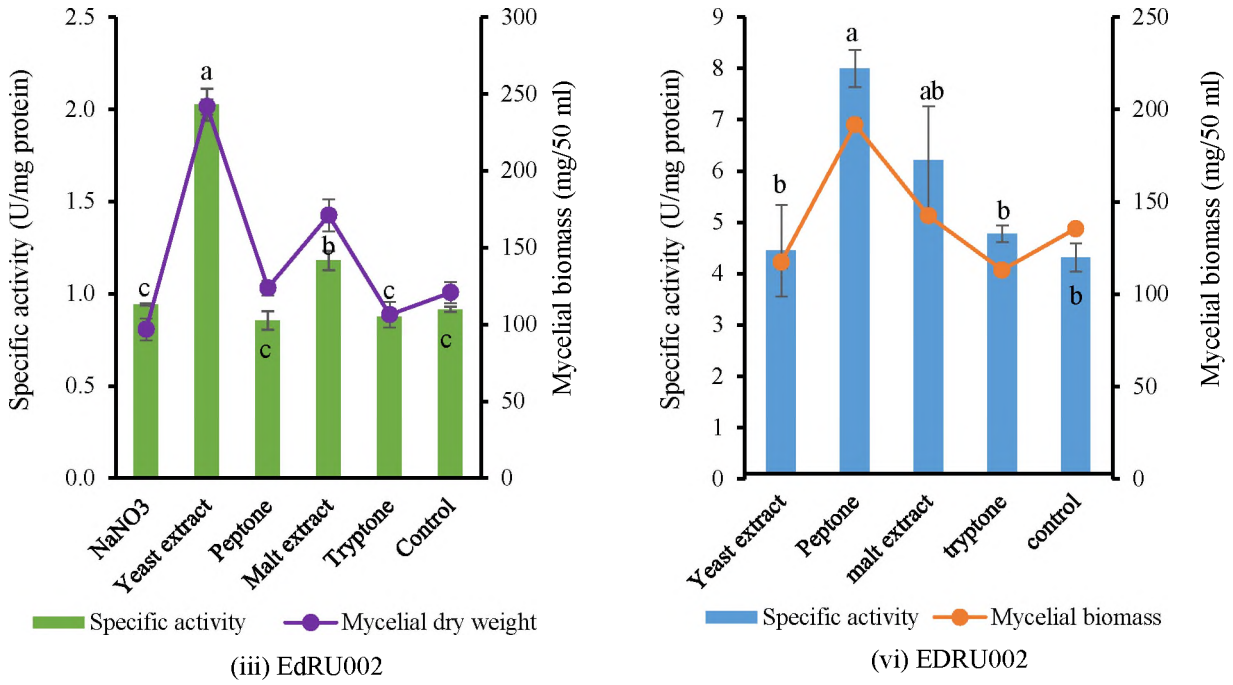


Figure 3.6. Effect of various nitrogen sources on AMG (i, ii, and iii) and endoglucanase (iv, v, and vi) production. All treatments were performed in triplicate (p-value < 0.05). Means without a common superscript letter differ as analysed by one-way ANOVA and the LSD test while the error bars indicate the standard errors of the means (SEM).

3.4. Discussion and conclusion

The consistency in enzyme production was demonstrated by the activities of the AMG and the endoxylanase at 100% even after several sequential subculturing events (Figure 3.1). Hence, ERM could be used for the production of some specific enzymes for the bio-economy. The monitoring results showed a decrease in the cellulase activity after the twelveth sequential *Leohumicola* sp. subculturing, while AMG and endoxylanase were produced maximally throughout the study period. A similar effect of subculturing on the survival and enzyme activity of *Neocallimastix hurleyensis* (a rumen anaerobic fungus) has been reported (Ekinci et al., 2006). The report showed that there was a significant decrease in cellulase enzyme activities after 31 consecutive subculturing *N. hurleyensis* isolate grown in a medium containing glucose as the sole energy source. These findings indicated that the consistency in enzyme production could be affected by the change in a natural environment where the organisms were found, and the effect

was enzyme-specific. Therefore, there is the need first to examine the consistency of production to enable proper choice of the enzyme when exploring for new biomolecules from the environment.

The AMG and endoglucanase production differed for different isolates at a different temperature. This investigation revealed that the best temperature for enzyme production was recorded at 28^{o}C [Figures 3.2 i, ii, iii (AMG), iv, v, and vi (endoglucanase)]. The variation observed with other temperatures could be as a result of the followings: (i) low temperature could not allow sufficient biomass production and (ii) inactivation at a high temperature that destroys amino acid or hydrolyses the peptide chain. The temperature of incubation played a significant role in the metabolic activities of these microorganisms. Hence, I acknowledged that slight changes in temperature could affect enzyme production and the optimal temperature for ERM growth is at 28°C. An experiment elsewhere corroborates these findings that growth and production of microbial secretomes ultimately depend on the temperature of cultivating medium which could lead to poor growth and reduced enzyme yield (Norouzian, 2008).

The pH of a growing medium is one of the most crucial factors influencing microbial growth, biomolecule production and transport of various constituents across the cell membrane (Lynd et al., 2002). Thus, accounts for the rate and quantity of growth, reproduction, and morphology of microorganisms in a liquid medium. High enzyme activity values were obtained for isolates EdRU083, *Leohumicola* sp. (ChemRU330) and EdRU002 at a pH of 5.0 (Figure 3.3). A similar result was reported by Yazdi and co-workers (1990) who recorded an optimal pH of 4.5 for endoglucanase activity (crude extract) from *Neurospora crassa*. Conversely, El-Hadi and coworkers (2014) in their investigation observed that a maximum CMCase production of 0.23 U/ml was obtained at pH 7.0 in a liquid medium and assay temperature of 37^{o}C for *Aspergillus hortai*. In specific term, the effect of pH on endoglucanase production by these fungi (Figures 3.3d, e, and f) also aligns with the observations made by Lynd et al. (2002). Their review showed that *A. hortai* exhibited both endoglucanase and filter paper (FPase) activities at the pH between 4.0 and 7.0 for most microbes (Lynd et al., 2002). AMG production was also affected by pH difference. A pH of 5.0 was the best pH for AMG production across all isolates tested;

Leohumicola sp. produced the highest activity at the same pH with corresponding biomass yield (Figure 3.3).

The AMG and endoglucanase activity for most isolates used in this study were increased when either Na^+, K^+ or Mn^{2+} was added to the production medium, followed by Zn^{2+}, NaFe-EDTA and least when Co^{2+} was used (Figures 3.4 i, ii, iii, iv, v, and vi). The least activity and mycelial biomass were recorded for both NaFe-EDTA and Co^{2+}; this may be as a result of their toxic effect on these fungi during assimilation processes. The findings of Baldrian and Gabriel (2002) confirmed that a white-rot fungus (*Pleurotus ostreatus*) needed trace amounts of either Cd, Mn or Zn for enzyme secretion. Similarly, some other findings revealed that metal ions in higher concentrations were potent inhibitors of enzymatic reactions (e.g. cellulase activity) in white-rot fungi (Baldrian, 2003; Naraian et al., 2010). Based on current results (Figure 3.4), cobalt ion and ferric EDTA concentration of 0.1% or more was inhibitory to the production of endoglucanase and biomass yield of ERM fungi. For AMG production (*Leohumicola* sp.), only cobalt and zinc were inhibitory to the production with least activity values (Figure 3.4).

The three ERM fungi selected for this study had highest enzyme activities (AMG, endoglucanase) and mycelial biomass when the production medium was amended with 0.5% glucose (Figures 3.5 i, ii, iii, iv, v, and vi). Conversely, least AMG, endoglucanase activity and mycelial yield were obtained when there was no other carbon supplementation (control). This aligns with the findings of Cao and Crawford (1993), where four strains of an ectomycorrhizal fungus, *Pisolithus tinctorius* were investigated for carbon nutrition, and for the production of hydrolytic and cellulolytic enzymes. Glucose, maltose and cellobiose supported the rapid mycelial growth of all the four strains studied. As observed in this study, ERM enzyme expression was heavily influenced by the substrate they colonised. Also, the presence of an inducible cellulolytic enzyme complex in some ectomycorrhizal fungi, grown *in vitro* with similar medium composition already reported (Cao and Crawford, 1993). Recently, Bizabani and Dames (2016) reported high biomass production when some ericaceous root-associated fungi (*Meliniomyces* sp., *Acremonium implicatum*, *Leohumicola* sp., *Cryptosporiopsis erica*, *Oidiodendron maius* and an unidentified Helotiales fungus) were grown on glucose and cellobiose. Thus, we affirmed that either glucose or cellobiose (Figure 3.5) is sufficient at

improving enzyme activity when compared with other fermentable sugars, and their incorporation into AMG and endoglucanase production media should be encouraged to improve the ERM and DSE mycelial production.

Organic nitrogen sources have been reported to have an inducing effect on the secretion of amylases and cellulases (Jonathan and Adeoyo, 2011). Figure 3.6 shows that the effect was more pronounced when the production medium was supplemented with either yeast extract (AMG), or peptone/tryptone (endoglucanase). These observations were similar to the findings of Jonathan and Adeoyo (2011) who reported a high cellulase activity and mycelial biomass when the production medium was supplemented with peptone, recording values of 0.74 U/ml and 116 mg/30 ml, respectively, for *Coriolus versicolor*. The report of El-Hadi et al. (2014) also confirmed the role of organic nitrogen (e.g. peptone) at increasing enzyme activity during optimisation of cultural and nutritional conditions for carboxymethylcellulose production by *Aspergillus hortai*, stating that organic nitrogen sources were preferable for improving endoglucanase activity. Similarly, Gautam et al. (2011) reported that either peptone or yeast extract (1.0% w/v) was found to be the best nitrogen source for the production of cellulase by *Aspergillus niger* and *Trichoderma* sp.

In conclusion, the potential of using ericoid fungi in producing improved mycelial biomass, AMG, and endoglucanase activity *in vitro* under various pH and nutritional conditions were investigated to maximise production. This study provides useful information that can assist in improving the mycelial biomass yield of some selected starter cultures for better mycorrhizal fungus inoculum production, while the AMG and endoglucanase production can contribute to the improvement of starch and cellulose bioconversion. The bio-enzymes obtained from these fungi represent a potential additional source of AMG and endoglucanase for industrial applications. Based on the information currently available, this is the first report which shows the potential of ericoid fungi for commercial AMG and endoglucanase production. Hence, a mineral salt liquid medium (e.g., MMN) of pH 5.0 with 0.1 - 0.5% glucose, 1.0% peptone or tryptone and either (0.1%) sodium, potassium or manganese sulphate is adequate for the production of either endoglucanase or endoxylanase from ERM and DSE fungi at 28°C. For AMG production, addition of 0.1 - 0.5% glucose, 0.3 - 1.0% yeast extract and 0.5 g/l KH_2PO_4 (pH 5.0) to the basal medium (MMN) would be sufficient for its production at 28°C. *Leohumicola* sp. (ChemRU330)

was found to be suitable for further study based on its good responses to enzymes production and optimisation. Also, the fungus showed the ability to consistently produce biocatalysts of interest when grown in a nutrient-rich medium without the help of their symbiotic partner (host) for carbon supply, an attribute similar to those of currently used saprophytic fungi.

CHAPTER 4

4.0 Partial purification and characterisation of endoxylanase from *Leohumicola* sp. (ChemRU330)

4.1. Introduction

Plant cell walls consist of three major renewable resources viz cellulose, hemicellulose, and lignin. (i) Cellulose is an insoluble polymer composed of β-D-glucopyranosyl residues linked by β-1,4-glycosidic bonds. (ii) Hemicellulose is the second largest component of lignocellulose, which is made up of different carbon sugars (5 or 6) such as xylose, arabinose, galactose, glucose, and mannose. (iii) Lignin is a complex polyphenol (*p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol) intimately interconnected with the hemicelluloses forming a network of orderly microfibrils (Corral and Villaseñor-Ortega, 2006). On average, the cellulose, hemicellulose and lignin content of plant cell wall biomass comprises 40%, 33% and 23%, respectively (Corral and Villaseñor-Ortega, 2006). Xylan is the most abundant of the hemicelluloses which have a linear backbone of β-1,4-linked D-xylopyranose residues which is further substituted, depending on plant sources to a varying degree with glucuronopyranosyl, 4-*O*-methyl-D-glucopyranosyl, α-l-arabinofuranosyl (Sakthiselvan et al., 2014).

Xylan is an essential component of hemicellulosic polysaccharide in cell walls of most plants, making up to 7-12% and 15-30% of the total dry weight in softwood from gymnosperms and hardwood from angiosperms, respectively (Saha, 2003). Hardwood hemicellulose mainly consists of *O*-acetyl-L-4-*O*-methyl-glucuronic acid xylan, e.g., the content of this hemicellulose in birchwood is approximately 35% (Chen, 2014). Xylan hemicellulose in softwoods is 4-*O*-methyl-glucuronic acid arabinose-xylan with almost no acetyl (Chen, 2014). Beechwood (BW) xylan from agricultural residues is an inexpensive and abundant raw material that could be used

for oligosaccharide production. BW xylan consists of a backbone of *p*-1,4-linked D-xylopyranose residues, with side chains of 4-*O*-methyl-glucuronic acid attached to the C-2 position of xylose and *O*-acetyl groups at C-2 or C-3 positions (Freixo and De Pinho, 2002).

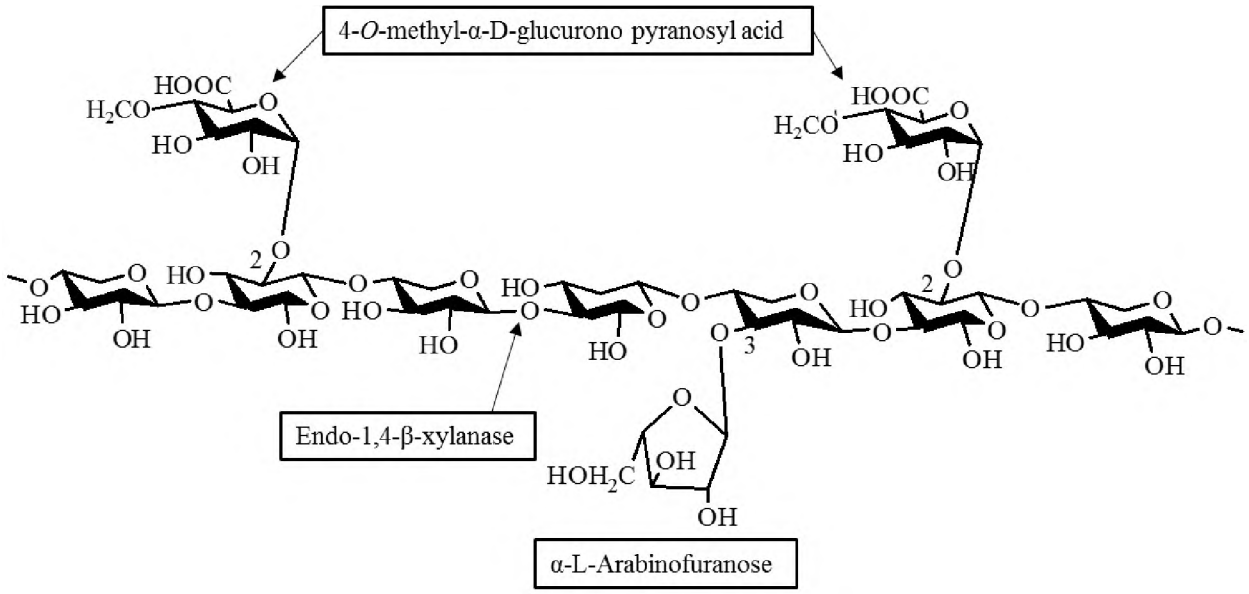


Figure 4.1. Action site of xylan-degrading endo-1,4-β-xylanase with numbers showing carbon atoms to which chemical groups are linked including acetyl chains (Modified from Corral and Villaseñor-Ortega, 2006).

Table 4.1. Enzymes involved in the hydrolysis of hemicellulose

Enzyme	Mode of action
Endoxylanase	Hydrolyses interior β-1,4-xylose linkages of the xylan backbone yielding various 1,4-β-D-xylooligosaccharides
Exoxylanase (endo-1,4-β-xylanase)	Hydrolyses β-1,4-xylose linkages releasing xylobiose
β-Xylosidase	Releases xylose from xylobiose and short chain xylooligosaccharides
α-Arabinofuranosidase	Hydrolyses terminal nonreducing α-arabinofuranose from arabinoxylans
α-Glucuronidase	Releases glucuronic acid from glucuronoxylans
Acetylxylan esterase	Hydrolyses acetylester bonds in acetyl xylans
Ferulic acid esterase	Hydrolyses feruloylester bonds in xylans

p-Coumaric acid esterase	Hydrolyses *q*-coumaryl ester bonds in xylans

(Modified from Saha, 2003).

A typical xylanase mode of action on an arabinose-4-*O*-methylglucuronoxylan is represented in Figure 4.1; it shows the xylanolytic action of an endoxylanase (Corral and Villaseñor-Ortega, 2006). Table 4.1 shows the enzymes required for a complete process of xylan degradation and their modes of action. Some authors have evaluated the prospects of using fungal xylanases for the hydrolysis of various lignocellulosic substrates (Corral and Villaseñor-Ortega, 2006; Goncalves et al., 2015). Xylanase is a complex of endoxylanase (E.C 3.2.1.8), β-xylosidase (E.C 3.2.1.37), α-glucuronidase (E.C 3.2.1.139), α-arabinofuranosidase (E.C 3.2.1.55) and acetyl xylan esterase (E.C 3.1.1.72) (Juturu and Wu, 2011). Endoxylanases catalyse the random hydrolysis of xylan to produce various short xylooligosaccharides (XOS), while β-xylosidase releases xylose residues from the non-reducing ends of XOS (Yun et al., 2015). Endo-1,4-β-xylanase is the most important xylan degrading enzyme, it removes or cleaves the internal glycosidic linkages of the heteroxylan backbone, leading to a decreased degree of polymerisation of the substrate (Corral and Villaseñor-Ortega, 2006). Endo-1,4-β-xylanase cleaves the main chain only near a substituted region and acts only at uninterrupted sequences (Corral and Villaseñor-Ortega, 2006). Some novel xylanases degrade xylan at high temperatures and have contributed to the development of a highly efficient system for lignocellulose conversion (Goncalves et al., 2015).

Purification of proteins usually involves the use of three or more separation steps in sequence, and the first one should be easy to use with large quantities of materials (Nielsen and Borchert, 2000). Precipitation techniques are used most commonly during early stages of a purification sequence. For many years, ammonium sulphate [$(NH_4)_2SO_4$] fractionation has often been used to purify proteins from the crude filtrates of their sources partially and the concentrations of $(NH_4)_2SO_4$ used can be modified to achieve optimal separation (Duong-Ly and Gabelli, 2014). Ammonium sulphate is the most common salt used in enzyme purification because it combines many useful features, such as salting out effectiveness, high solubility and low price. In principle, the fractionation method depends on the ability of high concentrations of ammonium sulphate to bind available water molecules and thus prevent the solvation of proteins (Duong-Ly

and Gabelli, 2014). Moreover, ammonium sulphate fractionation provides a crude purification of proteins, and it is typically involved in protein purification protocols (Wang et al., 2007). However, protein precipitates obtained from salt fractionation have a high salt content, which requires desalting before the subsequent analysis. One way to remove excess salt is by dialysing protein samples against a buffer low in salt content (Wang et al., 2007).

Xylanases are glycoside hydrolases (GH), and sequences based on GH classification has placed xylanase in two major families, 10 and 11. Other related GH families include 5, 8, 30 and 43 (Chakdar et al., 2016; Hong et al., 2014). Xylanases belonging to the GH10 family are composed mostly of endo-1,4-β-xylanases with a few endo-1,3-β-xylanases (biose, triose, and tetraose are the primary products, whereas the xylose, pentraose, and other oligosaccharides with more than five xylose units are produced in small quantities). They can hydrolyse cellulose and aryl β-D-cellobiosides, and the products of their action. GH10 xylanases cleave the β-1,4-linkages that precede a β-1,3-linkage on both sides, but not the ones that immediately follow β-1,3-linkages. GH11 members are monospecific, as they consist exclusively of an appropriate enzyme that cleaves internal β-1,4-xylosidic linkages only. Their catalytic ability is lower than those enzymes in the GH10 class. The action of the GH11 family enzymes is solely on D-xylose containing substrates, and they cannot cleave cellulose or aryl β-D-cellobiosides (Chakdar et al., 2016). The GH11 family cleaves un-substituted regions of the backbone because they cannot attack the xylosidic linkage towards the nonreducing end (next to a branched xylose). Most bacterial xylanases belong to the GH10 family while fungal xylanases majorly belong to the GH11 family (Liu et al., 2012).

Interest in carbohydrate-active enzymes has increased over the years because of their potential application. Xylanases are used in food, feed, bleaching (pulp and paper) industries, and could also be utilised in improving the effectiveness of detergent cleaning, biochemical and biofuel production (Juturu and Wu, 2011; Yun et al., 2015). The objective of this chapter was to determine the characteristics and apparent activity of a partially purified endoxylanase. The choice of xylanase for characterisation was motivated by its consistency in production during a production monitoring study (Chapter 3).

4.2. Materials and methods

4.2.1. Fungal isolates, production and activity assay

The *Leohumicola* sp. (ChemRU330) isolate was used, the production of xylanase was carried out as described earlier (Chapter 3) and enzyme activity was measured using 1% beechwood (BW) xylan (Lot # 141202) in acetate buffer (pH 5.0) for 1 h at 50°C. The released, reducing sugars were assayed using the DNS method of Miller (1959). One unit of activity was defined as the amount of endoxylanase that librated 1 µmol of xylose per minute under standard assay condition. All values are expressed as the standard errors of the mean (±SEM), obtained from triplicate experiments.

4.2.2. Determination of protein content

The protein content was estimated according to the method of Bradford (1976). Protein concentration was determined using bovine serum albumin (BSA, Lot # 127H1078) as a standard. The protein optical density was measured at 595 nm (Bradford, 1976).

4.2.3. Ammonium sulphate precipitation and dialysis of crude enzyme extract

The crude enzyme filtrate was concentrated and optimised using the ammonium sulphate precipitation method (Kamble and Jadhav, 2012). The precipitation was carried out by diluting 120 ml crude enzyme extract partially into $(NH_4)_2SO_4$ with a concentration of 80% (w/v). The pellet and filtrate were separated by centrifugation at 6000 x *g* for 15 min at 4°C. The precipitated enzyme in the pellet was diluted with 10 ml of acetate buffer (pH 5.0). Dialysis of the partially purified enzyme was performed by using a pre-treated dialysis bag (10 kDa cut-off). The partially purified enzyme (10 ml) was dialysed against 0.1 M acetate buffer (pH 5.0) at 4°C with three changes of the buffer according to the method described by Kusuda et al. (2004).

4.2.4. TLC analysis of hydrolytic products

The hydrolysed products of xylan were analysed by the thin-layer chromatography (TLC) using silica gel plates 60G F254 HPTLC (Merck, Darmstadt Germany). BW xylan was used as the substrate; the reaction mixture was incubated at 50°C for 24 h. An aliquot (80 µl) of the sample was collected after boiling and centrifugation at 6000 x *g* for 5 min. A 5 µl of each aliquot with xylo-oligomer standard, substrate, and enzyme blanks were spotted onto TLC plates. The plates were subsequently developed with n-butanol: acetic acid: water (2:1:1, v/v/v) (Yun et al., 2015). Plates were developed twice for 3 h 15 min, and then 2 h 13 min. The resultant plate was briefly submerged in a staining solution of methanol containing 5% phosphoric acid, and 0.3% α-naphthol, and heated for 10 minutes at 120°C in an oven to visualise the xylo-oligomers before taking photographs. The xylooligosaccharide mixture consisted of xylose (Sigma-Aldrich, Lot # SLBK7809V), xylobiose (Megazyme, Lot # 130502), xylotriose (Megazyme, Lot # 140803), xylotetraose (Megazyme, Lot # 150604), xylopentaose (Megazyme, Lot # 150605), and xylohexaose (Megazyme, Lot # 151206).

4.2.5. Enzyme zymography

The zymogram analysis was performed using a modified zymographic method (Ratanakhanokchai et al., 1999). The culture supernatant in the sample application buffer was boiled for 2 min at 95°C and was followed by electrophoresis on a 10% sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) gel containing 1% BW xylan. After electrophoresis, the gel was soaked in 2.5% (v/v) Triton X-100 with gentle shaking which removed the SDS and renatured the proteins in the gel for 45 min at 4°C. The gel was then washed with 0.01 M acetate buffer (pH 5.0) and incubated for 1 h at 50°C. The gel was soaked in 0.1% Congo red solution for 30 min at room temperature and washed with 1 M NaCl until the excess dye was removed from the active band. A Bio-Rad ChemiDoc X-Ray Spectrometer (XRS) system was used to capture photographic images.

4.2.6. The effect pH of endoxylanase activity and stability

The optimum pH for xylanase activity was obtained by assaying the partially purified enzyme in buffer at different pH (1.0-9.0, interval 1.0 unit) prepared in 0.1 M buffer having pH values of 1.0, 2.0 (hydrochloric acid-potassium chloride); 3.0, 4.0, and 5.0 (citrate-phosphate); 6.0, 7.0 (phosphate); 8.0 and 9.0 (Tris-HCl). For enzyme stability, the enzyme was preincubated in acetate buffer (pH 5.0) at 37°C. The optimum pH was determined by assaying enzyme activity at 37°C for 1 h using the DNS method (Miller, 1959).

4.2.7. The effect of temperature on endoxylanase activity and stability

The optimum temperature was obtained by incubating the enzyme under different temperatures (4, 20, 30, 40, 50, 60, 70, and 80°C, while stability was tested by pre-incubating the enzyme at 37, 50, 60, and 70°C. The enzyme activity was determined every 6 h for 30 h. Enzyme activity was assayed using the DNS method (Miller 1959) at pH 5.0 (0.1 M).

4.2.8. Effect of metal ions and chemicals on the activity of the endoxylanase

Metal ions such as K^{+}, Na^{+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+} Al^{3+}, Hg^{2+}, Cd^{2+}, and Mn^{2+} (all supplied in chloride form) were each applied to determine the effect on the enzyme activity. Each of the metal ions was used at a concentration of either 1, 5, or 10 mM, while incubation was performed at 50°C for 1 h. The following compounds; sodium azide (NaN_3), ammonium chloride (NH_4Cl), ethylenediaminetetraacetic acid (EDTA), SDS, dimethyl sulfoxide (DMSO), and indole-3-acetic acid (IAA) were also tested for their inhibitory effect on endoxylanase activity.

4.2.9. Substrate specificity assay

A 10 mg/ml each of either BW xylan, CMC, starch, glycogen, microcrystalline cellulose (Avicel) and chitin was used to determine substrate specific for xylanase action. Each substrate was incubated with the xylanase extract at 50°C for 1 h (pH 5.0). Activities were determined as previously described.

4.2.10. Kinetic parameters

Initial reaction rates using BW xylan as substrate were determined at substrate concentrations of 1.0-10.0 mg/ml in 0.1 M acetate buffer (pH 5.0) at 50°C. The kinetic constants, K_m and V_{max}, were estimated using a Michaelis-Menten plot using KaleidaGraph curve fitting software (Golicnik, 2011).

4.2.11. Statistical analysis

All experiments were conducted in triplicate and analysed using one-way ANOVA. Error bars were represented as the standard errors of the means (SEM).

4.3. Results

4.3.1. Partial purification of endoxylanase

The effect of sequential subculturing on coproduced xylanase from *Leohumicola* sp. indicated that xylanase activity was optimal throughout the study period (Chapter 3). The enzyme purification is summarised in Table 4.2. The protein was precipitated with 80% ammonium sulphate, dialysed against acetate buffer and concentrated using an Amicon ultrafiltration unit. The enzyme was partially purified to 49.6 fold with a specific activity of 1.57 U/mg protein and a recovery yield of 77%. Figure 4.2 shows the SDS-PAGE and zymogram of the crude extract from *Leohumicola* sp. The partially purified sample gave a single band of yellow against the red colour of the Congo red used for gel staining. The molecular weight of the endoxylanase was estimated by plotting a relative migration distance (R_f) graph based on the electrophoretic mobilities of endoxylanase and of the reference standard (R_f) on SDS-PAGE with the

corresponding zymographic position. The partially purified endoxylanase was observed to have a molecular weight of 72 kDa.

Table 4.2. Purification table of endo-1,4-β-xylanase from *Leohumicola* sp. (ChemRU330)

Step	Protein (mg/ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude	245.93	29511.11	936.90	0.03	1.0	100
Dialysis	41.50	460.68	725.35	1.57	49.6	77

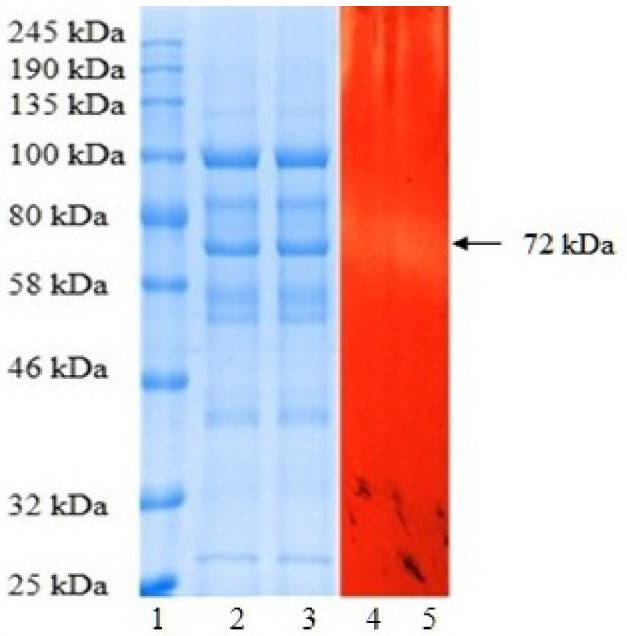


Figure 4.2. The SDS-PAGE and endo-1,4-β-xylanase zymogram of a *Leohumicola* sp. partially purified enzyme prepared according to the method described by Ratanakhanokchai et al. (1999). Lane 1 contains a colour pre-stained standard, broad range (BioLabs), lanes 2-3 contain partially purified enzyme and lanes 4-5 contain β-1,4-endoxylanase zymogram (Congo red staining). Note that the 1,4 glycosidic bond is formed between the carbon-1 of one monosaccharide and carbon-4 of the other monosaccharide.

4.3.2. Thin layer chromatographic analysis of the xylanase

Beechwood xylan (1%) was incubated with the enzyme for 24 h to assure maximum hydrolysis. The mixtures were analysed by thin layer chromatography (TLC), and the hydrolytic products were compared to those of the standards. The result showed that the tested enzyme liberated varying smaller-sized linear xylooligosaccharides with R_f values corresponding to those of xylobiose, xylotriose, xylotetraose, xylopentaose, xylohexaose and other higher oligomers (Figure 4.3).

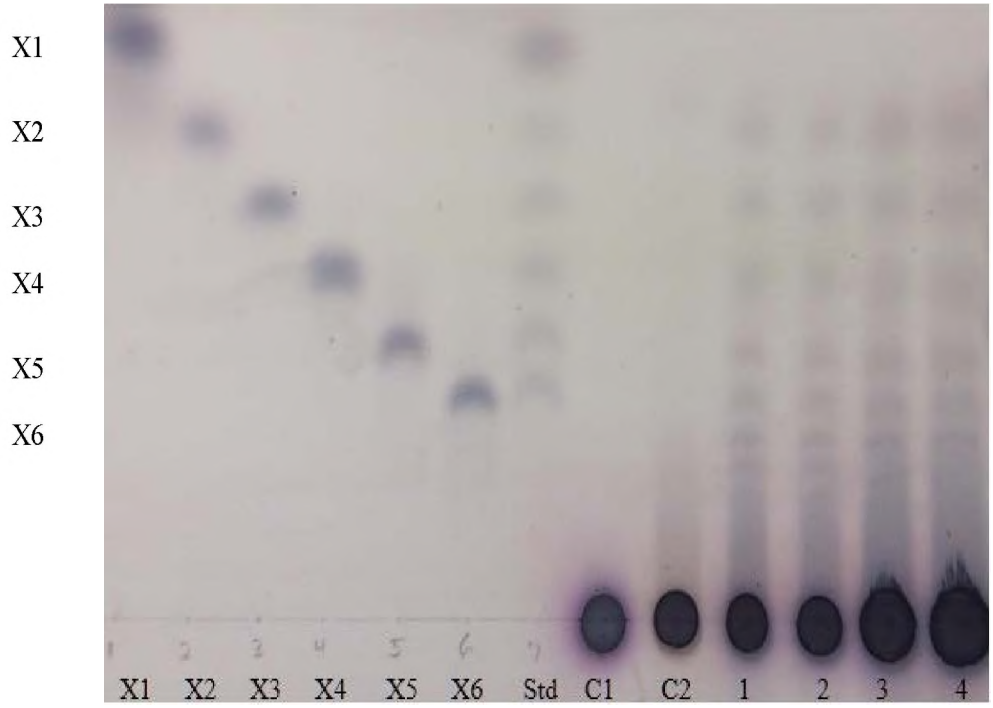


Figure 4.3. Thin-layer chromatograms of hydrolyzates using endo-1,4-β-xylanase from *Leohumicola* sp. Plates were developed twice with n-butanol: acetic acid: water (2:1:1, v/v/v). The plates were briefly submerged in methanol containing 5% (v/v) sulfuric acid and 0.3% α-naphthol. Standards (Std); xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6), substrate control (C1), enzyme control (C2).

4.3.3. The effect pH of endo-1,4-β-xylanase activity and stability

The pH of any medium plays a crucial role in influencing enzyme production and activity. The effect of pH on endoxylanase activity was evaluated by adjusting the pH values to 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0. The assay was performed at 37°C for 1 h. Figure 4.4 shows that

pH 5.0 had the highest activity of 1.11 U/mg protein. Low enzyme activity was found at a pH of 2.0 and 8.0 while the activity at the pH of 1.0 was significantly low. The optimum pH for the endoxylanase activity from *Leohumicola* sp. was 5.0, and the activity gradually decreased outside this pH. Also, it was observed that the enzyme activity was stable at pH of 5.0 for 6 h (Figure 4.5), after which the activity started to decrease gradually in a similar faction to those of other pHs. After 24 h of pre-incubation, the enzyme retained over 70% of its activity at a pH of 5.0 at 37°C.

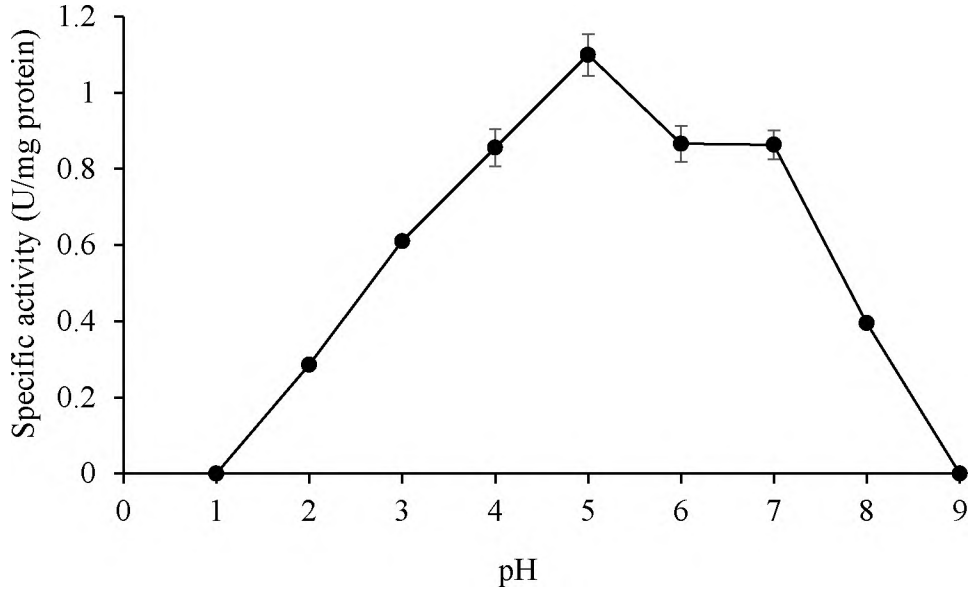


Figure 4.4. Effect of pH on endo-1,4-β-xylanase activity. The enzyme was incubated at 37°C for 1 h. All error bars are represented as the standard errors of the means (SEM).

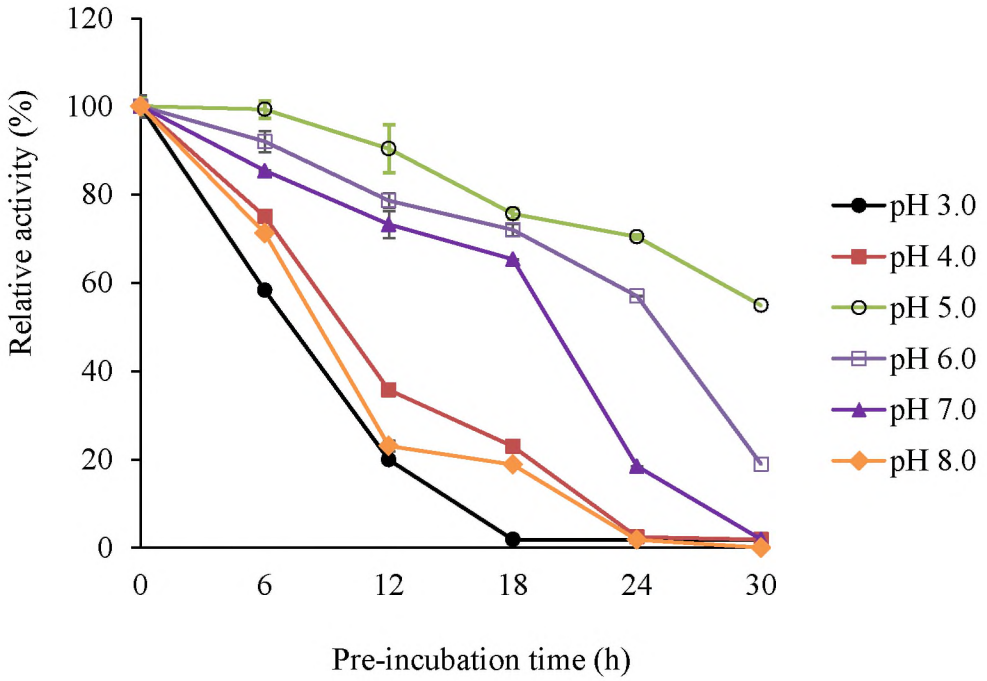


Figure 4.5. Effect of pH on the stability of endo-1,4-β-xylanase. The enzyme was incubated at 37°C for 1 h. All error bars are represented as the standard errors of the means (SEM).

4.3.4. Effect of temperature on endo-1,4-β-xylanase activity

The effect of temperature on endoxylanase activity was investigated over a range of temperatures (4, 20, 30, 40, 50, 60, 70, and 80°C). Figure 4.6 shows that the optimal temperature was obtained between 60°C (1.69 U/mg protein) and 50°C (1.62 U/mg protein). The xylanase was very stable at 37°C. However, the activity of endoxylanase gradually decreased after 1 h at 50°C and retained over 69% activity after 120 min, while at 60 and 70°C, the enzyme activity sharply decreased with an increased pre-incubation period (Figure 4.7).

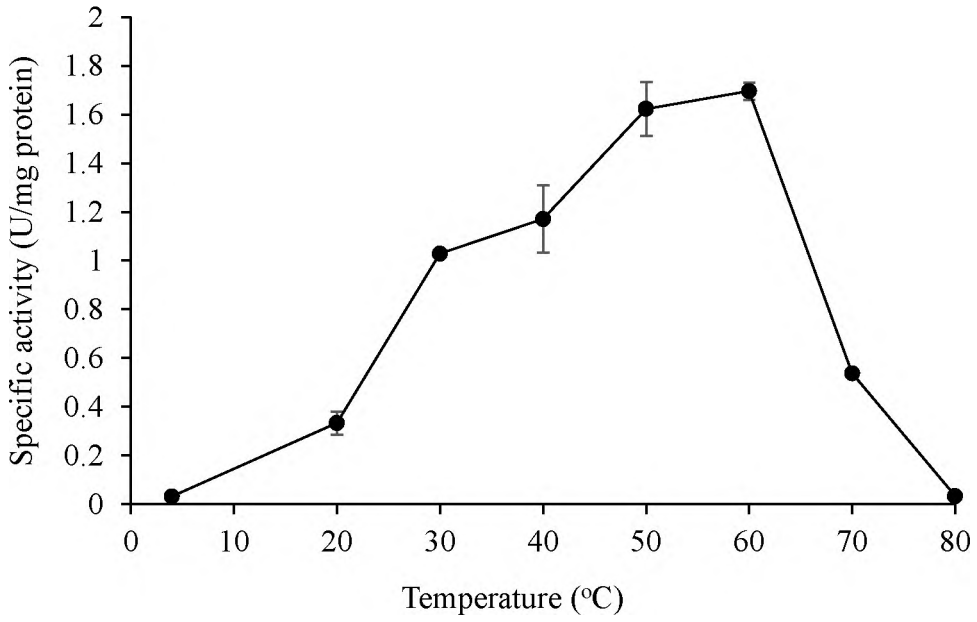


Figure 4.6. Effect of temperature on the endo-1,4-β-xylanase activity. The enzyme was incubated at 4 to 80°C for 1 h (pH 5.0). All error bars are represented as the standard errors of the means (SEM).

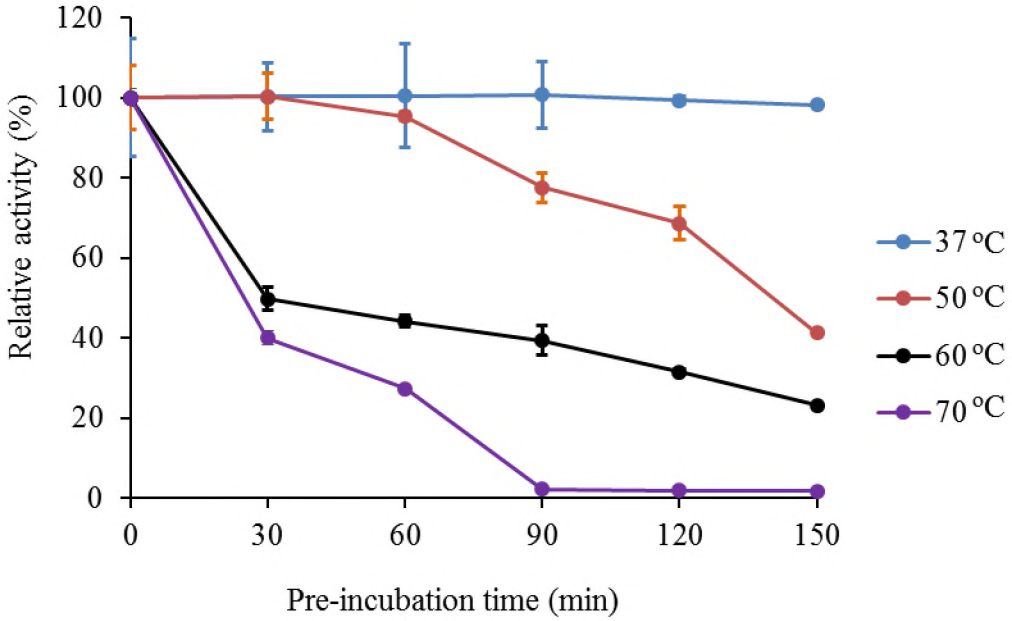


Figure 4.7. Thermal stability of the endo-1,4-β-xylanase activity. The enzyme was pre-incubated for 30, 60, 90, 120, and 150 min before the assay was carried out at 50°C for 1 h (pH 5.0). All error bars are represented as the standard errors of the means (SEM).

4.3.5. Effect of metal ions and chemicals on the activity of the endo-1,4-β-xylanase

Metal ions used in this investigation were supplied in chloride forms (K^+, Na^+, Ca^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+} Al^{3+}, Hg^{2+}, Cd^{2+} and Mn^{2+}). Metal ions such as Cu^{2+}, Al^{3+}, Hg^{2+} and Cd^{2+} inhibited enzyme activity significantly at a concentration of 1, 5 and 10 mM, while cobalt had an effect only at a concentration of 10 mM (Figure 4.8). Also, for most chemicals used in this experiment (Figure 4.9), only SDS showed an inhibitory effect on endoglucanase activity at 1, 5 and 10 mM concentrations.

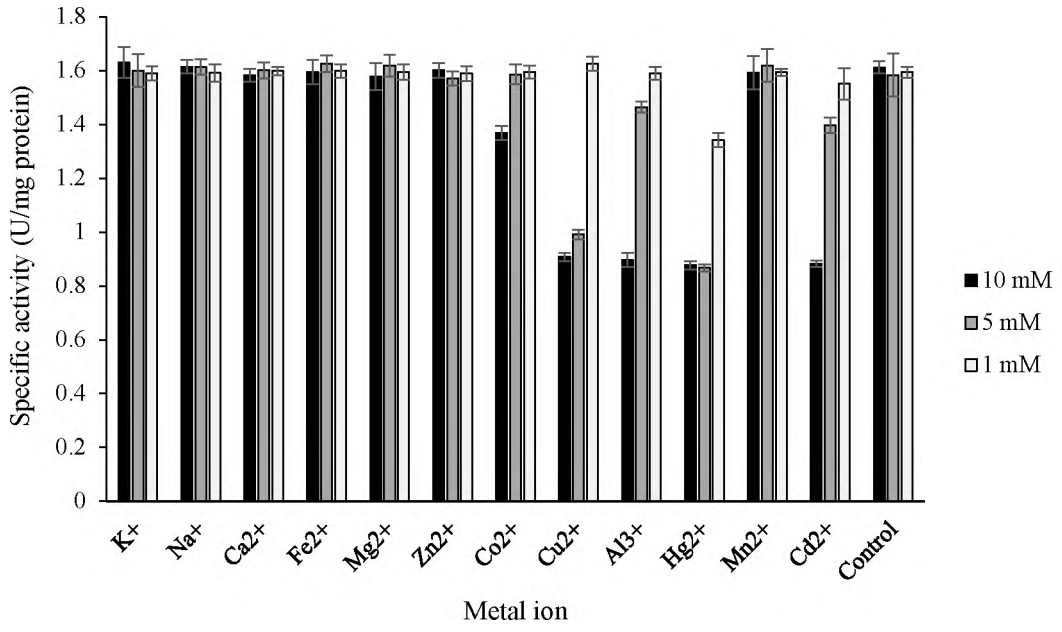


Figure 4.8. Effect of metals on the endo-1,4-β-xylanase activity. The enzyme was incubated in either 1, 5, or 10 mM of K^+, Na^+, Ca^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+} Al^{3+}, Hg^{2+}, Cd^{2+}, and Mn^{2+} at 50°C for 1 h (pH 5.0). All error bars are represented as the standard errors of the means (SEM).

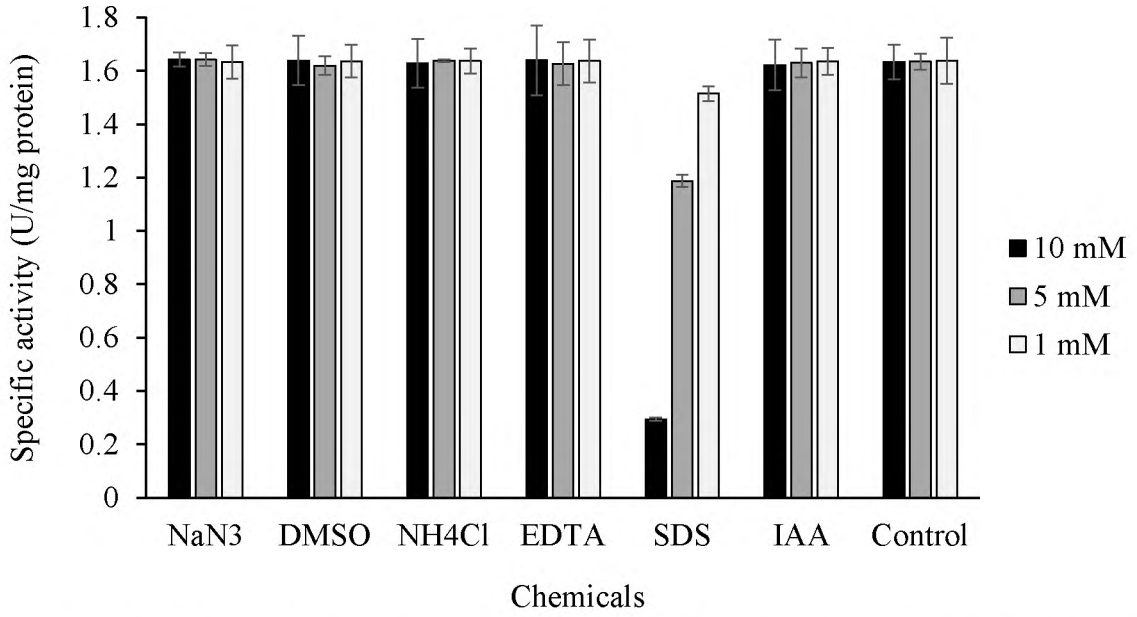


Figure 4.9. Effect of chemicals on the endo-1,4-β-xylanase activity. The enzyme was incubated in either 1, 5, or 10 mM of NaN_3, DMSO, NH_4Cl, EDTA, SDS and IAA at 50°C for 1 h (pH 5.0). All error bars are represented as the standard errors of the means (SEM).

4.3.6. Substrate specificity

The efficient utilisation of xylanases relies on a proper understanding of their substrate specificity and the complex structures of heteroxylans. A good number of studies on the three-dimensional structures of xylanases from different GH families in complex with the substrate provide insight into the various mechanisms through which xylanases bind and hydrolyse structurally different heteroxylans and xylooligosaccharides (Pollet et al., 2010). The substrate (1%) of either BW xylan, CMC, starch, glycogen, Avicel and chitin was used was to determine specificity. Figure 4.10 shows that endo-1,4-β-xylanase was specific for two substrates - BW xylan and CMC with values of 1.61 and 0.42 U/mg protein, respectively. The enzyme could not degrade starch, glycogen, Avicel and chitin.

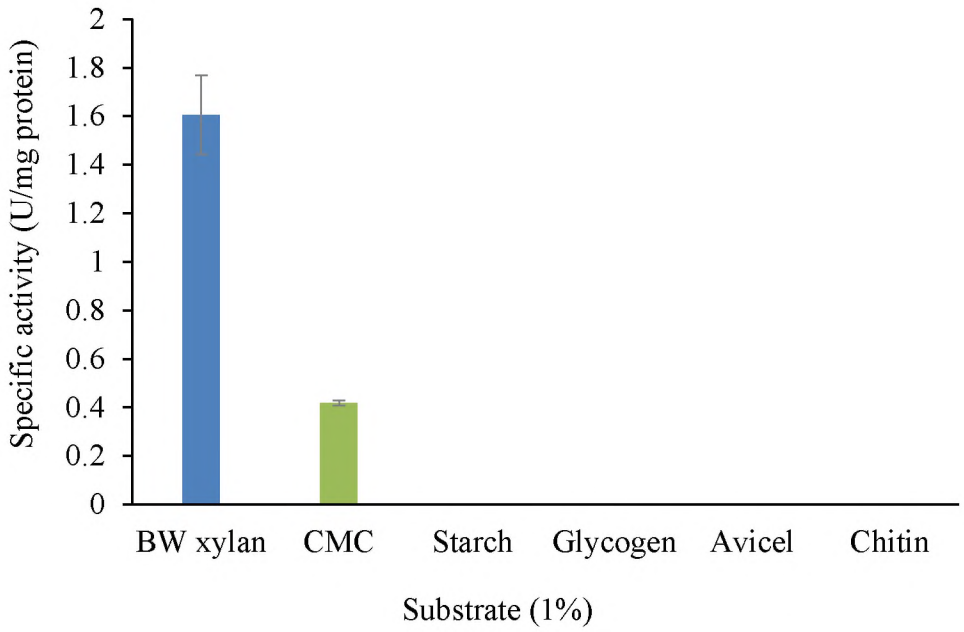


Figure 4.10. Substrate specificity of endo-1,4-β-xylanase activity. All error bars are represented as the standard errors of the means (SEM).

4.3.9. Kinetics study

Lineweaver-Burk plots help to eliminate the difficulty experienced in drawing a hyperbola, locating the asymptotes accurately, identifying the relationship among hyperbola and deviation from expected curve by plotting initial velocity V_o against the log of the substrate [S]. The Hanes-Woolf plots, on the other hand, help to accurately analyse data graphically, eliminating the incorrect impression of experimental errors which are sometimes associated with Lineweaver-Burk plots (double reciprocal plots). Michaelis-Menten plots are better expressed using non-linear regression software such as KaleidaGraph (Golicnik, 2011). Figure 4.11 shows the kinetic parameters for the endo-1,4-β-xylanase of *Leohumicola* sp. K_m and V_{max} values of 1.04 mg/ml and 8.7 U/ml, respectively were obtained when 1% BW xylan was used as a substrate and incubated at 50°C (pH 5.0). The k_{cat} (turnover number) was 2.79 s^{-1}, and the catalytic efficiency (k_{cat}/K_m) was 2.70 s^{-1} mg^{-1} ml^{-1}.

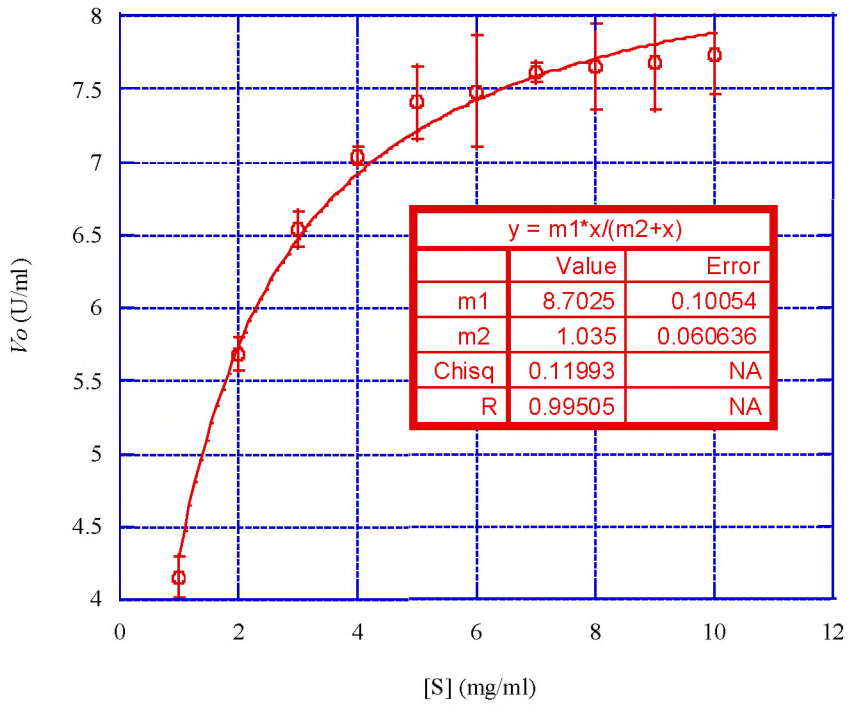


Figure 4.11. Michaelis-Menten plot of partially purified endo-1,4-β-xylanase enzyme versus beechwood xylan concentration. All error bars are represented as the standard errors of the means (SEM) (m1 = V_{max} and m2 = K_m).

4.4. Discussion and conclusion

The endo-1,4-β-xylanase from *Leohumicola* sp. revealed a single protein band with a molecular weight (MW) of 72 kDa (Figure 4.2). This is corroborated by a report on an endo-1,4-β-xylanase which were from several fungi displayed exclusive single subunit protein structure with an MW ranging from 8.5 to 85 kDa (Polizeli et al., 2005). The capacity of endo-1,4-β-xylanase to degrade the substrates (BW xylan) was demonstrated by analysing the hydrolysates through a TLC method. The qualitative identification of the XOS was conducted, the TLC analysis resolved the reaction into different XOS, but no XOS was produced where the enzyme was not included. Figure 4.3 shows that xylobiose, xylotriose, xylotetraose, xylopentaose, xylohexaose and other higher oligomers were produced. The hydrolytic profile observed in the *Leohumicola* sp. was similar to that of *Aspergillus niger* (Takahashi et al., 2013) and *Penicillium oxalicum* (Liao et al., 2015). The substrate specificity is a key indicator to determine the efficiency and products of hydrolysis because not all of the xylosidic linkages in the heteroxylans are readily accessible to particular xylanase (Gírio et al., 2010). The degradation profile of BW xylan by

xylanase monitored by the TLC analysis revealed that the hydrolytic products were longer xylooligomers with the degree of polymerisation (DP) starting from xylobiose to higher DP. This result indicated that the xylanase produced from *Leohumicola* sp. was endo-type xylanase with similar catalytic properties to those in the GH11 family, a characteristic feature of fungal xylanases (Liu et al., 2012).

Xylanases obtained from fungal sources are known to be active and stable in the acidic range of pH (Burke and Cairney, 1997a). The pH study revealed that pH 5.0 was the optimum and stable at a pH between 5.0 and 7.0. Also at this pH, the enzyme retained 70% of its activity after incubation for 24 h at 37°C. This is better than a report that an endo-1,4-β-xylanase from the ericoid mycorrhizal fungus, *Hymenoscyphus ericae* had an optimum pH of 4.5 and stable between pH 3.5-4.0 (Burke and Cairney, 1997a) because most industrial enzymes are produced within this pH range. According to Burke and Cairney (1997a), one of the environmental factors regulating enzyme activity at the mycorrhizal-host root interface was pH, which tends to reduce enzyme activity and growth when outside the optimum or permissible range. Also, endoxylanase of *Leohumicola* sp. had optimal activity at a temperature of 60°C (pH 5.0), was more stable at 50°C, retaining about 69% activity after 2 h. These results confirmed that the endoxylanase from *Leohumicola* sp. compared well with those of commonly used *Trichoderma* and *Aspergillus* spp. (Subramaniyan and Prema, 2002).

The effects of metal, detergent, and other chemicals were evaluated, and the partially purified enzyme showed strong stability in the presence of most metals used (K^+, Na^+, Ca^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+} and Mn^{2+}) except for Cu^{2+} Al^{3+}, Hg^{2+} and Cd^{2+} (Figure 4.8). The study was at variance with a report that endoxylanase was inhibited in the presence of Co^{2+} and Mn^{2+} (Chen et al., 2006; Hmida-Sayari et al., 2012). The findings revealed that the effect of metal ions was concentration dependent and at a concentration of 5 mM and more the effect becomes significant. SDS (a detergent) affected the enzyme activity across all the three different concentrations used whereas other reagents showed no noticeable effects.

The endo-1,4-β-xylanase from *Trichoderma reesei* had K_m value of 2.1 mg/ml and 1.4 mg/ml of birchwood xylan and BW xylan, respectively (He et al., 2009). The K_m value reported for

Leohumicola in this investigation was 0.93 mg/ml (Figure 4.11). The kinetic parameters for the endo-1,4-β-xylanase of *Leohumicola* sp. compared well with those reported for those of other fungal endoxylanases (Abbas et al., 2012). Hence, further purification of this enzyme to homogeneity would show far more accurate results.

In conclusion, having considered the unique properties of this enzyme (high thermal stability, stable in the pH range of 5.0-7.0, and a good xylanolytic efficiency against the substrate) as well as the possibility of purifying it to homogeneity for a better catalytic efficiency, the xylanase from the *Leohumicola* sp. (ChemRU330) can be considered an attractive candidate for future industrial applications. For example, it can be used to convert agricultural residues into other useful bio-based products.

CHAPTER 5

5.0 Purification and characterisation of an amyloglucosidase from *Leohumicola* sp. (ChemRU330)

5.1. Introduction

Ericaceae is a family of plants that can alter their physiological or morphological features in environments characterised by nutrient deficiencies (Cairney, 2002). The survival of ericaceous plants in these habitats depends strongly on the symbiotic associations that exist between them and mycorrhizal fungi, where nutrients are made available from soil organic matter through the assistance of hydrolytic enzymes. Ericoid mycorrhizal (ERM) fungi have been found to produce hydrolytic enzymes (Cairney and Burke, 1998, 1994), which include the production of amyloglucosidase. Other reports have also indicated that the enzymatic degradation of organic polymers in the soil and the transfer of some of the resulting products to the root is a significant benefit to the growth and development of ericaceous plants (Smith and Read, 2008).

Amyloglucosidase (AMG, glucoamylase, EC 3.2.1.3) is an enzyme that is capable of hydrolysing the α-1,4 glycosidic bonds from the non-reducing ends of starch to produce glucose. It is also an exo-acting enzyme that catalyses the production of β-D-glucose from the non-reducing ends of substrates that include starch, and maltooligosaccharides by consecutively hydrolysing α-1,4 and α-1,6 linkages (Riaz et al., 2012; Sauer et al., 2000). Starch-degrading AMG has gained more importance among industrial enzymes because of its role in starch degradation and manufacturing of sugar in modern biotechnological applications (Omemu et al., 2008).

Starch is one of the most abundantly distributed polysaccharides produced by plants, and it accounts for about 70% of the undried plant material (Aiyer, 2005). Starch is a significant reserve carbohydrate of all higher plants. The shape and size of starch granules are often characteristic of the plant species from which they are extracted (corn, wheat, sorghum, rice, cassava, potato, arrowroot and the pith of sago palm) (Aiyer, 2005). Corn has mostly been used as a source of starch which can be extracted by a wet milling process (Singh and Eckhoff, 1996). Steeping helps to soften the corn kernels for grinding facilitates disintegration of the protein matrix that encapsulates the starch granules in the endosperm, and sodium metabisulfite or related compounds, help to disrupt the protein matrix and limit undesirable fermentation during steeping (Sheriff et al., 2012; Singh and Eckhoff, 1996). Heating starch in water helps in weakening the hydrogen bonds holding its granules together, thus permits them to swell and gelatinise. The swelling capacity of starch is dependent on the amylopectin content, and amylose

acts as a diluent and inhibitor of swelling (Singh et al., 2003). Gelatinisation occurs when water diffuses into the starch granule, causing swelling due to hydration of the amorphous phase leading to loss of crystallinity and order of its molecule (Jimenez et al., 2012).

Starch is made up of two molecular weight polymers, amylose and amylopectin. Separation of starch components involves the addition of a polar solvent (n-butanol), which causes the insoluble (amorphous) amylose complex to separate from the soluble (crystalline) amylopectin fraction (Aiyer, 2005; Alcázar-Alay and Meireles, 2015). Amylose is a linear chain of glucose residues linked by α-1,4 bonds that can be readily degraded by α-amylase to maltose (Aiyer, 2005). Amylose precipitates spontaneously because of its molecular shape and structure; it aggregates in aqueous solution and the linear chains align themselves by hydrogen bonding. Amylopectin, on the other hand, is a branched polymer with α-1,4-linked and α-1,6-linked glucose residues; and in aqueous solutions, amylopectin is relatively stable due to branched molecules, and no aggregates are formed (Singh et al., 2003).

Other amylase-degrading substrates that can be used for amylase activity studies include glycogen and a nonreducing end-blocked p-nitrophenyl maltoheptaoside (BPNPG7) (Figure 5.1). Glycogen is a polymer of glucose (approximately 120,000 glucose residues) and is a primary carbohydrate storage form in animals. The polymer is composed of units of glucose linked α-(1,4) with branches occurring α-(1,6) at about every 8-12 residues. BPNPG7 is an oligosaccharide containing a blocking group at the non-reducing end, which prevents exoglucosidase from attacking the substrate molecule. Benzylidene blocked 4-nitrophenyl maltoheptaoside (BPNPG7) comprises a *p*-nitrophenyl group at the reducing end of maltoheptaoside and a 4,6-linked-*O*-benzylidine group at the non-reducing end of the oligomer. BPNPG7 is a substrate for endo-acting α-amylase, but it is not hydrolysed by exo-acting enzymes such as amyloglucosidase, α-glucosidase or β-amylase (McCleary et al., 2002).

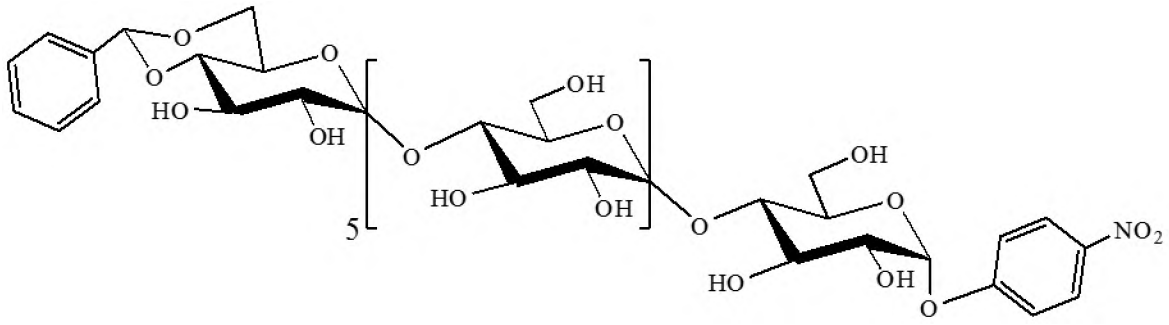


Figure 5.1. Ceralpha substrate: blocked *p*-nitrophenyl maltoheptaoside (BPNPG7) (Adopted from Cornaggia et al., 2016).

AMG is an essential enzyme popularly used in the production of ethanol, glucose syrups, and also can be used to upgrade barley mash for beer production (Aiyer, 2005; Pavezzi et al., 2008; Zambare, 2010). Hence, AMG is an economically viable enzyme, and its effectiveness in hydrolysing starch and some oligosaccharides into β-D-glucose has been widely reported in *Aspergillus Rhizopus* and some *Trichoderma* species. Earlier in 1977, glucoamylase from *Mucor rouxianus* was reported (Yamasaki et al., 1977), and in 1978, glucoamylase was also said to be produced from an ectomycorrhizal fungus *Lentinus edodes* (Yamasaki and Suzuki, 1978). More recently, this enzyme has been reported to be produced by a few microorganisms - *Aspergillus niger* (Slivinski et al., 2011), *Aspergillus awamori* (Negi and Banerjee, 2009), *Rhizopus oryzae* (Morita and Fujio, 2000), as well as two other ectomycorrhizal fungi, *Tricholoma matsutake* and *Lyophyllum shimeji* (Hur et al., 2001; Kusuda et al., 2004). Kusuda and co-workers in 2004 characterised an extracellular glucoamylase from *Lyophyllum shimeji*, stating that the enzyme was most active at around 40°C. This study aimed to purify and characterise AMG from *Leohumicola* sp. (ChemRU330), an ericoid mycorrhizal fungus.

5.2. Materials and methods

5.2.1. Enzyme production and activity assay

The ChemRU330 isolate was used for this investigation, the production of amylase was carried out as described in the previous chapter. Enzyme activities were measured using 1% soluble starch in acetate buffer (pH 5.0) for 1 h at 45°C except if otherwise stated. The released, reducing sugars were assayed using the DNS method (Miller, 1959). One unit of activity was defined as the amount of AMG that liberated 1 µmol of glucose per minute under standard assay condition. All values are expressed as the standard errors of the mean (±SEM), obtained from triplicate experiments.

5.2.2. Determination of protein content

The protein content was estimated according to the method of Bradford (1976). Protein concentration was determined using bovine serum albumin (BSA) as a standard. The protein optical density was measured at 595 nm (Bradford, 1976).

5.2.3. Ammonium sulphate precipitation

A 120 ml volume of the crude enzyme was brought to 80% saturation with solid ammonium sulphate according to the method of Kusuda et al. (2004). The mixture was left overnight at 4°C on a magnetic stirrer. The pellet obtained from the centrifuged mixture was re-dissolved in 20 mM sodium acetate buffer (pH 5.0) to make up a volume of 10 ml.

5.2.4. Dialysis of the partially purified enzyme

A pre-treated dialysis bag was used for the dialysis (10 kDa cut-off) of the enzyme collected after ammonium sulphate precipitation. The partially purified enzyme (10 ml) was dialysed against 20 mM sodium acetate buffer (pH 5.0) at 4°C with three changes of a buffer according to the method described by Kusuda et al. (2004).

5.2.5. Gel filtration

A vertical glass chromatographic column (1.5 x 50 cm) was packed using Sephadex G-100. The dialysed enzyme solution (1.5 ml) was used after it was concentrated by ultrafiltration (Amicon Ultra-100 centrifugal filter device; cut-off 10 kDa; Lot # R5HA14338). Gel filtration chromatography was carried out using sodium acetate buffer (20 mM, pH 5.0), at a flow rate of 1.2 ml/min. All fractions collected were subjected to analysis by measuring the absorbance at 280 nm, followed by the activity assay, the active fractions (fractions no. 36–40; total volume, 5ml) were then pooled together.

5.2.6. Identification of hydrolytic products using thin layer chromatography (TLC)

Soluble starch was used as the substrate. The mixture was incubated in a dry heating block at 45°C for 1 min up to 24 h and samples were removed at various time intervals for analysis. Samples were then boiled for 5 min at 100°C to denature the proteins and then centrifuged at 6000 x *g* for 5 min. The supernatant (80 µl) was used for TLC analysis. Identical volumes (5 µl) of the supernatant were applied to Silica Gel 60G F254 HPTLC plates (Merck, Darmstadt, Germany). Plates were developed twice with n-butanol: acetic acid: water (2:1:1, v/v/v). Then, to detect carbohydrates, plates were briefly submerged in methanol containing 5% (v/v) sulfuric acid and 0.3% α-naphthol. Plates were then air dried, heated at 120°C for 10 min and observed for the bands formed by various hydrolytic products.

5.2.7. AMG activity staining (zymography)

The enzyme from crude extract was analysed by its native protein pattern using the co-polymerisation method (Martinez et al., 2000). Sample was diluted to a ratio of 1:1 in sample buffer (0.125 M Tris; 20% v/v glycerol; 0.04% v/v bromophenol blue) and boiled at 95°C for 5 min. The resolving gel (12%) consisted of:- polyacrylamide 4.0 ml, 1.5 M Tris-HCl buffer (pH 8.8) 2.5 ml, distilled water 2.25 ml, 2% w/v soluble starch 1.25 ml, 10% w/v sodium dodecyl sulfate (SDS) 100 µl, 10% w/v ammonium persulfate 50 µl, and N,N,N′,N′-Tetramethylethylenediamine (TEMED) 5 µl. The stacking gel (4%) consisted of:- polyacrylamide 0.65 ml, 0.5 M Tris-HCl buffer (pH 6.8) 1.25 ml, distilled water 3.05 ml, 10%

w/v SDS 50 µl, 10% w/v ammonium persulfate 25 µl, and TEMED 5 µl. Starch-SDS-PAGE (PAGE - polyacrylamide gel electrophoresis) was carried out in two phases viz. gels were first subjected to a constant voltage of 30 V for 30 min to ensure the tracking dye (bromophenol blue) enters the separating gel. After this, gels were finally subjected to a constant voltage of 100 V for 75 min. Electrophoresis was carried out at a temperature of 0 – 2°C to allow the enzyme to migrate in-actively without hydrolysing the starch. After electrophoresis, the gel was washed in distilled water and then incubated in 0.1 M phosphate-citrate and 0.05 M NaCl buffer (pH 6.0) for 3 h at 39°C. Again, gels were washed and fixed in 12% trichloroacetic acid (TCA) for 10 min, followed by another washing and gently shaking (50 rpm) in 2.5% w/v Triton X-100 for 1 h at 4°C to remove SDS and restore activity. Finally, the gels were stained with Lugol solution (6.7 mg/ml KI and 3.3 ml/ml I_2), and photographs were taken.

5.2.8. Molecular weight determination by SDS-PAGE electrophoresis

SDS-PAGE was performed using 8 x 10 x 0.75 cm gels in a Mini-Protean II (Bio-Rad) gel apparatus. Samples were treated with reducing (containing 2-mercaptoethanol) sample buffer and boiled for 5 min before loading the gel. After electrophoresis, proteins in the gel were visualised by staining with Coomassie Blue R-250 (Laemmli, 1970).

5.2.9. Effect of incubation period

The enzyme production medium was set up as described earlier and was incubated for different periods of 1-10 weeks at 28°C. Activity was determined at the end of every week.

5.2.10. Effect of pH on activity and stability of AMG

The optimum pH for the enzyme was determined by incubating the enzyme with the substrate (1%, w/v) prepared in 0.1 M buffer having pH values of 1.0, 2.0 (hydrochloric acid-potassium chloride); 3.0 (citrate); 4.0, 5.0 (acetate); 6.0, 7.0 (phosphate); 8.0 and 9.0 (Tris-HCl) at a temperature of 45°C for 1 h. After this, the enzyme activity was measured. The pH stability was assessed by pre-incubating the enzyme in buffers of different pH (3.0, 4.0, 5.0, 6.0, 7.0 and 8.0)

at 37°C for 6, 12, 18, 24 and 30 h before determining the residual activity by the standard procedure.

5.2.11. Effect of temperature on activity and stability of AMG

The optimum pH for the enzyme was determined by incubating enzyme with the substrate (1%, w/v) prepared in 0.1 M acetate buffer (pH 5.0) (Bai et al., 2013). The enzyme was incubated at different temperatures of 4, 20, 30, 40, 50, 60, 70 and 80°C for 1 h, after which, the enzyme activity was measured. The temperature stability was conducted by pre-incubating the enzyme at different temperatures (37, 45, 50, 60, and 70°C) for 6, 12, 18, 24 and 30 min before determination of the residual activity by the standard procedure.

5.2.12. Effect of metal ions and other chemicals on the activity of AMG

The effect of metal ions on enzyme activity was carried out using a concentration of either 1, 5, or 10 mM at a temperature of 45°C for 1 h in 20 mM sodium acetate buffer (pH 5.0). Metal ions used include Mg^{2+}, Na^{+}, K^{+}, Al^{3+}, Mn^{2+}, Ca^{2+}, Fe^{2+}, Co^{2+}, Hg^{2+}, and Cd^{2+} which were all supplied in chloride form. Also, NaN_3, NH_4Cl, EDTA, SDS, DMSO, and IAA were tested for their inhibitory effect on AMG activity.

5.2.13. Laboratory scale extraction of starch from corn

Extraction of starch from corn (*Zea mays*) was performed as described by Sheriff et al., (2012) with slight modification. 10 g each of the cleaned yellow corn grains were steeped in 60 ml of 1% sodium metabisulfite solution at 45°C for 72 h. This was followed by manual removal of the pericarp and germ. The separated endosperms were placed in a 250 ml centrifuge tube with 100 ml distilled water and homogenised using a vortex type tissue homogeniser (Ultra Turrax, 170W, 20000 rpm) for 2 min. The homogenised slurry was filtered under vacuum using a muslin cloth with several washes until the wash water became clear with a total volume of 1000 ml. The starch slurry was subjected to two sedimentation methods: (a) centrifuged (Beckman Coulter Avanti-J high-speed) at 6000 x *g* for 30 min at 4°C, (b) allowed to settle at 4°C for 2 h, and the

supernatant drained. The raw starch was rinsed with 500 ml of distilled water, drained three times and the sediment air dried (Ji et al., 2004; Sheriff et al., 2012). The starch yields were determined as described by Ji et al., (2004).

$$\text{Starch yield (\%)} = \frac{\text{Dry weight of starch recovered from extraction}}{\text{Dry weight of whole corn kernels}} \times 100$$

5.2.14. Substrate specificity assay

The substrates (1%) of each of starch soluble (Merck, Lot # F1922052618), CMC (Sigma-Aldrich, Lot # SLBN8656V), Avicel (Sigma-Aldrich, Lot # BCBP6787V), BW xylan (Megazyme, Lot # 141202) and colloidal chitin (Sigma, Cat # C6767-100G) were used to assay the catalytic ability of AMG. The AMG also was tested on some common amylase-degrading substrates - amylose (Sigma-Aldrich, Lot # SLBN5941V), amylopectin (Sigma-Aldrich, Lot # BCBQ7009V), glycogen (Sigma-Aldrich, Lot # SLBF8199V) and blocked *p*-nitrophenyl-α-D-maltoheptaoside (BPNPG7) which is a substrate specific for measuring and analysing α-amylase activity (Megazyme, Lot # 160902-3) and cornstarch (raw). Also, a α-amylase from porcine pancreas (Sigma-Aldrich, Lot # SLBJ9505V) was used as a positive control test for α-amylase activity.

5.2.15. Enzyme kinetics (substrate affinities)

A Michaelis-Menten type substrate saturation curve was used to determine the K_m and V_{max} values of the AMG by measuring the rate of soluble hydrolysis under standard assay conditions. The reaction mixture contains 20 mM acetate buffer (pH 5.0), with the soluble starch substrate at concentrations ranging from 0 to 10 mg/ml. The Michaelis constant (K_m) was determined using starch, amylose, amylopectin, glycogen and cornstarch (crude) as substrates. Results were analysed using KaleidaGraph software.

5.2.16. Statistical analysis

All experiments were conducted in triplicate and analysed using one-way ANOVA. Error bars were represented as the standard errors of the means (SEM).

5.3. Results

5.3.1. Enzyme activity and purification of enzyme

The *Leohumicola* sp. (ChemRU330) produced AMG when grown on a mineral medium containing starch at a temperature of 28°C for 42 d (pH 5.0). Table 5.1 summarises the purification steps of the AMG solution prepared from the cell-free culture filtrate (120 ml). Partial purification of the AMG by ammonium sulphate [$(NH_4)_2SO_4$] precipitation was the first step of the purification procedure; the filtrate was precipitated at 80% saturation. The elution profile on Sephadex G-100 showed that peak (b) (Figure 5.2) exhibited high AMG activity, while other peaks did not show any amylase activity.

Table 5.1. Purification table of AMG from *Leohumicola* sp. (ChemRU330)

Purification Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification fold	Yield (%)
Cell-free filtrate	234.709	271.080	1.155	1.0	100
$(NH_4)_2SO_4$	12.152	192.139	15.812	13.70	71
Dialysis	6.848	147.207	21.496	18.61	54
Gel filtration	2.698	73.326	27.178	23.50	27

5.3.2. Enzyme characterisation

Fractions 36-40, with the highest AMG activity, were pooled for further studies (Figure 5.2). Figure 5.3 shows the pattern of TLC of AMG hydrolysis from *Leohumicola* sp. The reaction time ranged from 1 to 10 min (Figure 5.3a), 10 min to 90 min and 24 h (Figure 5.3b) at 45°C. TLC showed a single band after analysis, indicating that the *Leohumicola* sp. releases a single isoform of the AMG and produces only a band indicating the presence of glucose as the product of hydrolysis when the enzyme hydrolysed the substrate (starch) at 45°C.

SDS-PAGE was performed to confirm the homogeneity of the enzyme using a 12% gel. Figure 5.4 shows the results of SDS-PAGE and zymography, a single band with a molecular weight estimated to be 101 kDa using a standard curve of log (MW) versus R_f was obtained. The band of a protein associated with AMG activity was observed as a clear zone on a nondenaturing gel analysis of the AMG and was found at one position, thereby indicating the absence of isozymes (Figure 5.4). Figure 5.5 shows the result of gel filtration on SDS-PAGE to obtain a single homogeneous protein band (101 kDa).

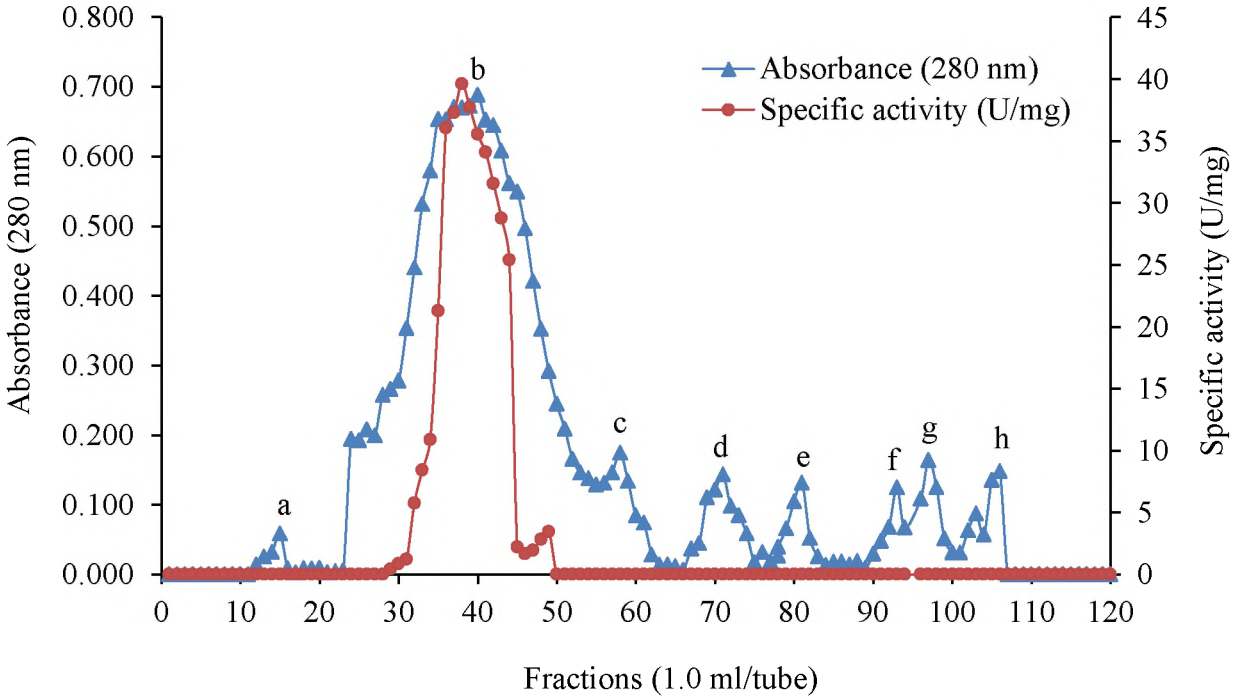


Figure 5.2. Chromatogram of the crude enzyme fractions of *Leohumicola* sp. on a Sephadex G-100 chromatographic column (Flow rate: 1.2 ml/min).

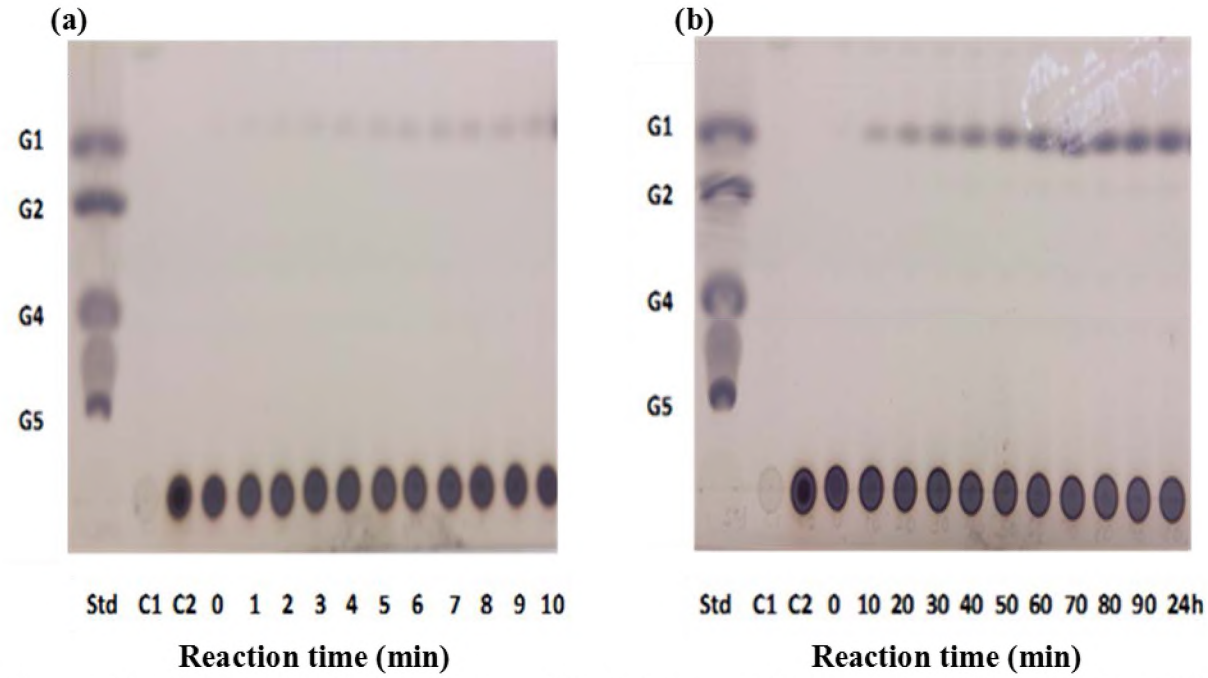


Figure 5.3. Thin-layer chromatograms of hydrolysates. Plates were developed twice with n-butanol: acetic acid: water (2:1:1, v/v/v). The plates were briefly submerged in methanol containing 5% (v/v) sulfuric acid and 0.3% α-naphthol. Standard (Std): glucose (G1), maltose (G2), maltotetraose (G4), and maltopentaose (G5), substrate control (C1), enzyme control (C2), reaction time: 0 – 90 min and 24 h.

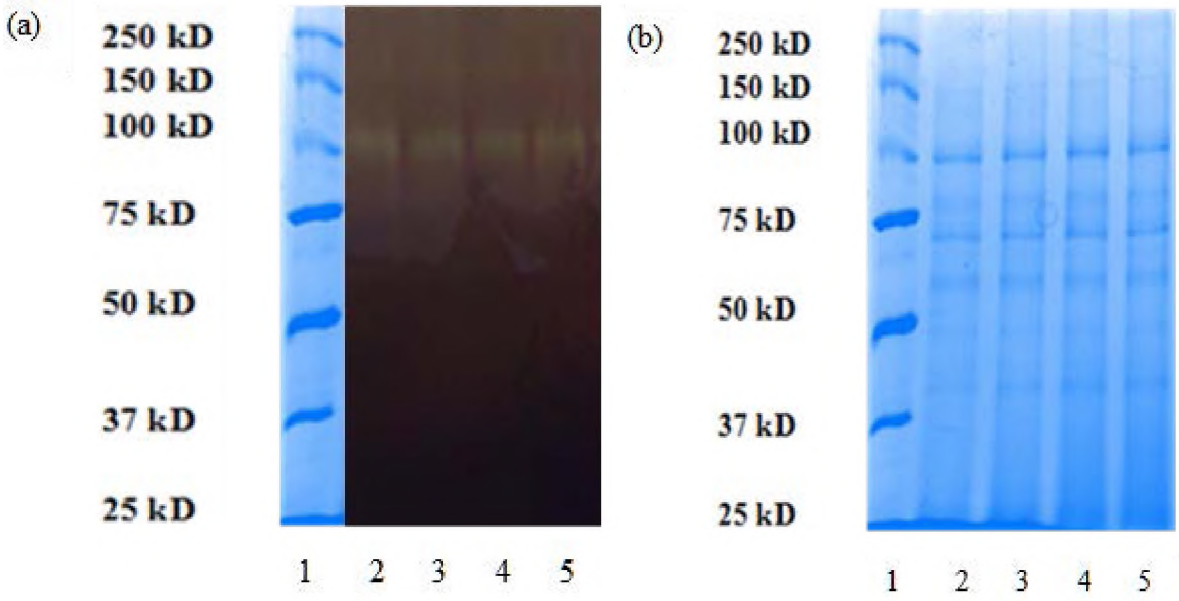


Figure 5.4. Zymogram and SDS-PAGE analysis of the AMG prepared according to the method described by Martinez et al. (2000). (a) Lane 1 is precision plus unstained protein standard (BIO-RAD), lanes 2-5 is a crude enzyme of *Leohumicola* sp (iodine staining); (b) Lane 1 is precision plus unstained protein standard (BIO-RAD), lanes 2-5 is SDS-PAGE of crude AMG.

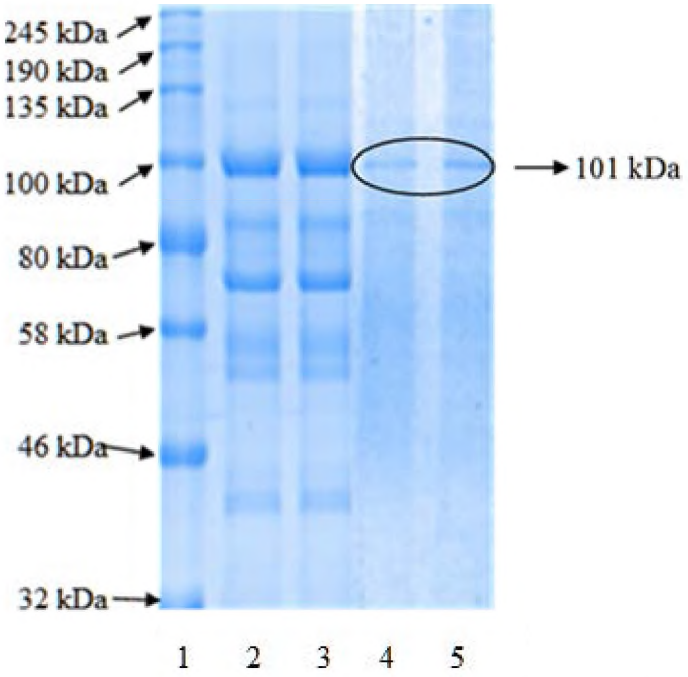


Figure 5.5. 12% SDS-PAGE analysis of the AMG (Laemmli, 1970): lane 1 is colour pre-stained standard, broad range (BioLabs); AMG crude enzyme (lanes 2-3) and the post-gel filtration chromatography protein bands (lanes 4-5).

5.3.3. Effect of production period

Figure 5.6 indicated that as the incubation period increased, the enzyme activity increased up to the optimum point where it remained constant for a while and started to reduce gradually. The optimum production and test periods on AMG activity were obtained after 6 weeks with 38.9 U/mg protein. The enzyme was produced optimally between 6 to 8 weeks; activity started to decrease gradually beyond 9 and 10 weeks (37.4 and 35.1 U/mg protein).

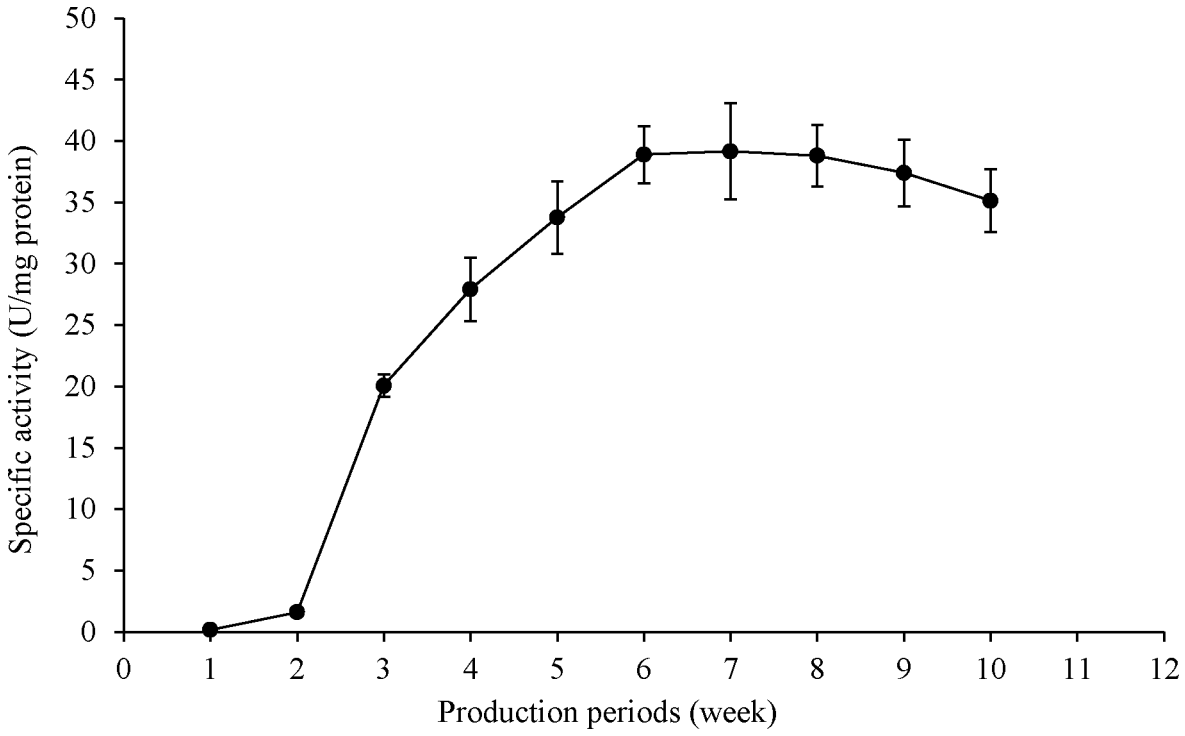


Figure 5.6. Effect of production period on AMG activity. The enzyme was incubated at 45°C for 1 h. All error bars are represented as the standard errors of the means (SEM).

5.3.4. Effect of pH on enzyme activity and stability

After gel filtration, fractions 36-40, corresponding to the peak of AMG activity, were pooled and tested using reaction mixtures buffered at different pH values ranging from 1.0 to 9.0, for optimal activity (Figure 5.7). The results showed that the highest activity of the purified enzyme was obtained at pH 4.0, and more than 50% activity was retained between pH 4.0 to 6.0. At pH 5.0 (37°C) the enzyme was 100% stable over a period of 24 h, (Figure 5.8). Hence, the enzyme displayed normal activity under acidic pH. AMG activity was also observed to be stable (above 50%) up to 6 h at a pH of 7.0 (37°C) but dropped rapidly after 12 h (Figure 5.8). The stability of a range of pH between 4.0-7.0 could be one of the factors for its consideration for industrial applications.

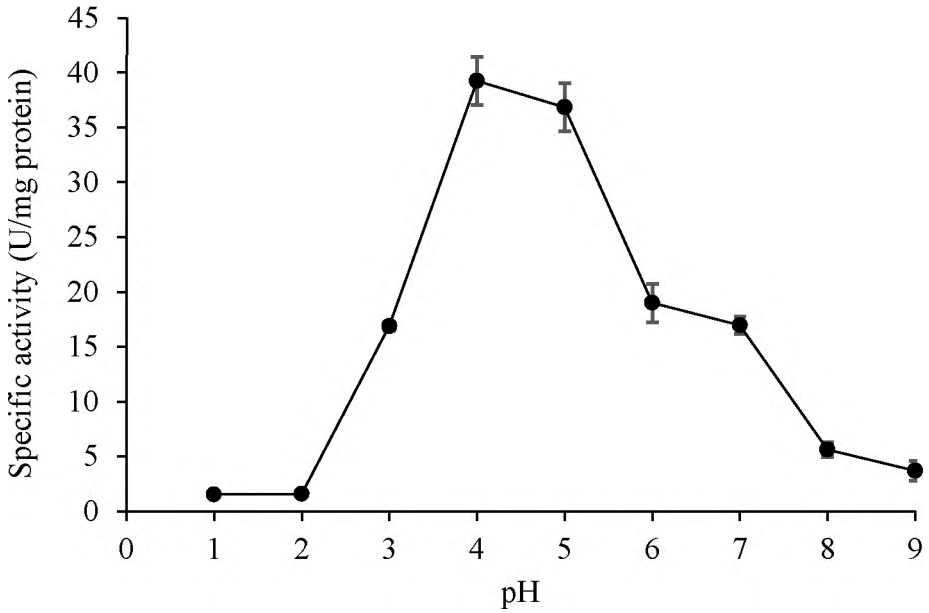


Figure 5.7. Effect of pH on AMG activity. The enzyme was incubated at 45°C for 1 h. All error bars are represented as the standard errors of the means (SEM).

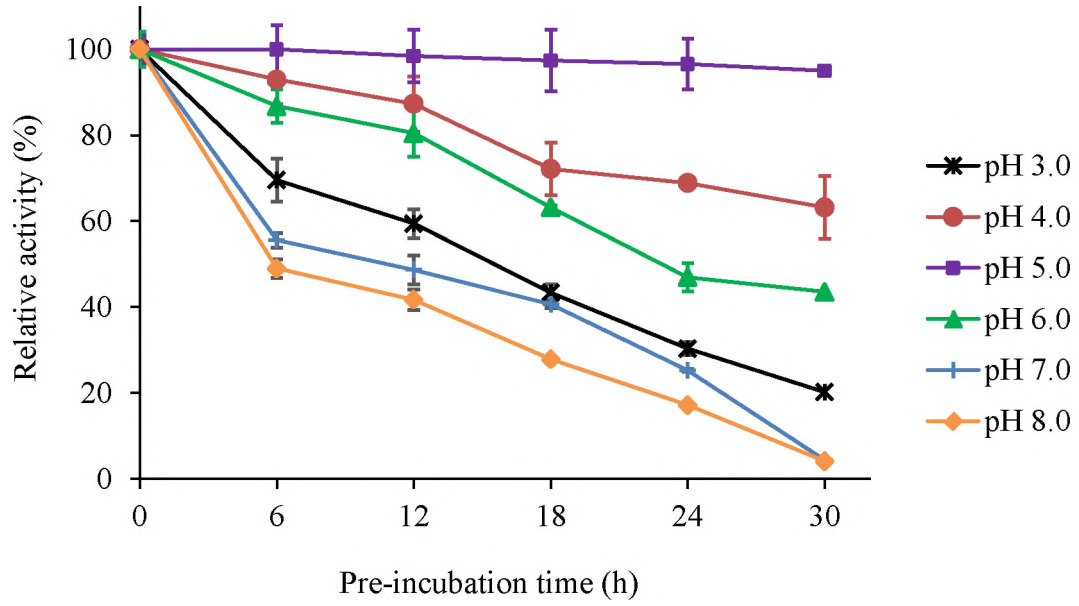


Figure 5.8. Effect of pH on AMG stability. The enzyme was pre-incubated (37°C) for 6, 12, 18, 24, and 30 h before the assay was carried out at 45°C for 1 h. All error bars are represented as the standard errors of the means (SEM).

5.3.5. *Effect of temperature on AMG activity and stability*

The effect of temperature on AMG showed that the enzyme reached its optimum activity at a temperature of 50°C (Figure 5.9). Lowest activities were observed at 4°C and 70°C, while no apparent activity was recorded at 80°C (Figure 5.9). The enzyme also displayed its highest stability at 37 and 45°C and was the least stable at 70°C (Figure 5.10). The enzyme retained at least 65% of its original activity at a temperature between 37°C and 45°C up to 24 h, while less than 60% activity was retained up to 12 h at a temperature of 50°C (Figure 5.10).

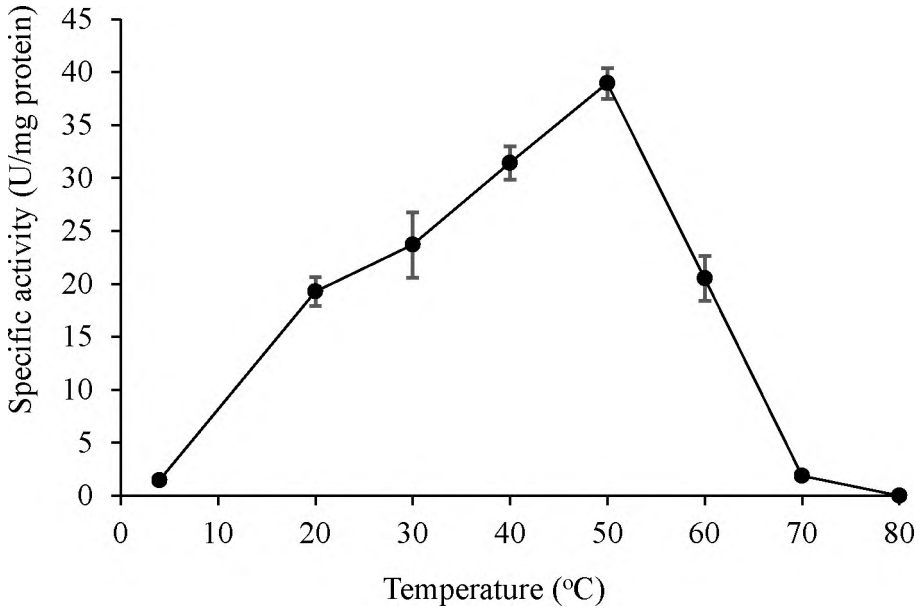


Figure 5.9. Effect of temperature on AMG activity. The enzyme was incubated at temperatures between 4 to 80°C for 1 h (pH 5.0). All error bars are represented as the standard errors of the means (SEM).

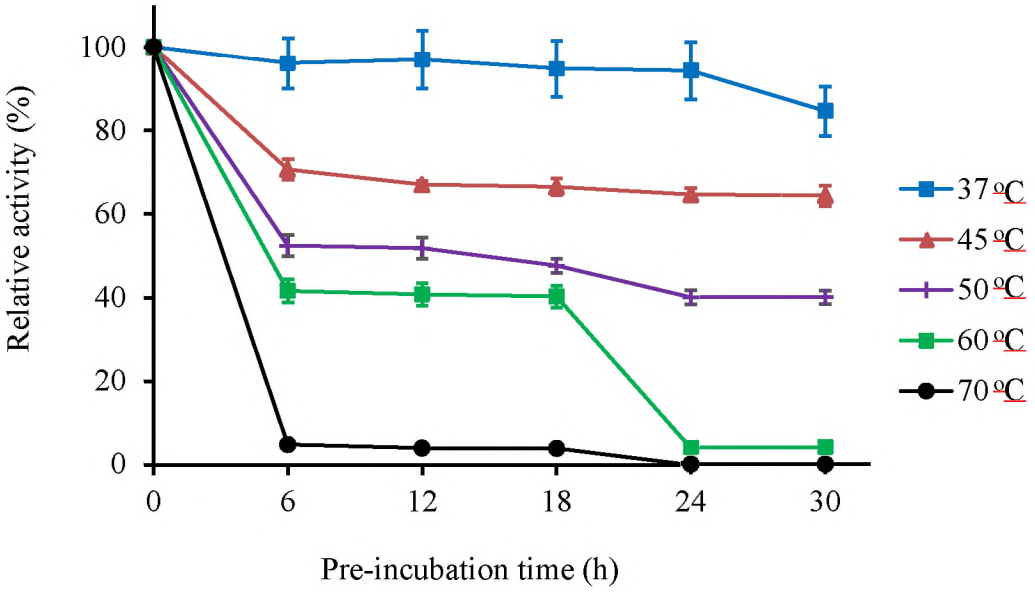


Figure 5.10. Effect of temperature on AMG stability. The enzyme was incubated at temperatures of 37, 45, 50, 60, and 70°C (pH 5.0). All error bars are represented as the standard errors of the means (SEM).

5.3.6. Effect of metal ions and chemicals on the activity of AMG

Table 5.2 shows the effect of metal ions on AMG activity measured at pH 5.0 and a temperature of 45°C. Assays were performed with the addition of each metal ion with the concentration ranging from 1 – 10 mM. The chloride salts of these metals were used. Manganese (Mn^{2+}) and calcium (Ca^{2+}) had the highest relative activity values of 112 and 106% at 10 mM concentration. Cobalt (Co^{2+}), mercury (Hg^{2+}), and cadmium (Cd^{2+}) all inhibited the enzyme, with values of 77, 0.45, 4%, respectively, at the same concentration. Other metal ions tested did not have any major effect on AMG activity. Table 5.3, on the other hand, shows the influence of other chemicals on AMG activity. SDS had the highest inhibitory effect on the various concentrations used, followed by EDTA at a concentration of 10 mM only, while other tested chemicals were not inhibitory to AMG activity.

Table 5.2. Effect of the presence of metal ions on AMG activity

Metal Ion	% Relative activity (10 mM)	% Relative activity (5 mM)	% Relative activity (1 mM)
Mg^{2+}	98 ± 4.7	99 ± 6.33	100 ± 3.87
Na^{+}	102 ± 9.78	100 ± 7.14	101 ± 6.14
K^{+}	96 ± 5.66	99 ± 6.74	101 ± 6.97
Al^{3+}	86 ± 3.05	95 ± 5.68	100 ± 4.27
Mn^{2+}	112 ± 7.41	102 ± 2.8	100 ± 4.98
Ca^{2+}	106 ± 5.95	100 ± 2.17	100 ± 2.31
Fe^{2+}	98 ± 4.7	100 ± 1.18	100 ± 1.28
Co^{2+}	77 ± 3.51	95 ± 2.93	100 ± 3.95
Hg^{2+}	0.45 ± 0.05	59 ± 0.19	92 ± 2.23
Cd^{2+}	4 ± 0.23	66 ± 2.17	100 ± 3.16

The values are represented as means ± SEM, n = 3 per treatment.

Table 5.3. Effect of the presence of other chemicals on AMG activity

Other reagent	% Relative activity (10 mM)	% Relative activity (5 mM)	% Relative activity (1 mM)
NaN_3	100 ± 7.28	102 ± 4.99	101 ± 5.28
NH_4Cl	101 ± 1.69	101 ± 4.25	101 ± 8.05
EDTA	92 ± 5.59	102 ± 9.5	102 ± 6.73
SDS	5.37 ± 0.32	39.8 ± 1.64	87.9 ± 4.48
DMSO	99.7 ± 8.12	101 ± 1.45	102 ± 3.12
IAA	99.6 ± 7.69	102 ± 8.88	102 ± 4.83

The values are represented as means ± SEM, n = 3 per treatment.

5.3.7. Substrate specificity study

The substrate specificity study was conducted using starch, CMC, Avicel, beechwood xylan and colloidal chitin. The result indicated only starch was a substrate of choice for AMG activity and no activity was recorded for the other four tested substrates (Figure 5.11). However, in Figure 5.12 where some amylase-degrading substrates were employed, it was noticed that the enzyme

hydrolysed all the substrates except BPNPG7 which could only be acted upon by α-amylase (Figure 5.13). The results also showed that starch, amylose, amylopectin, glycogen and raw corn starch were sufficiently hydrolysed.

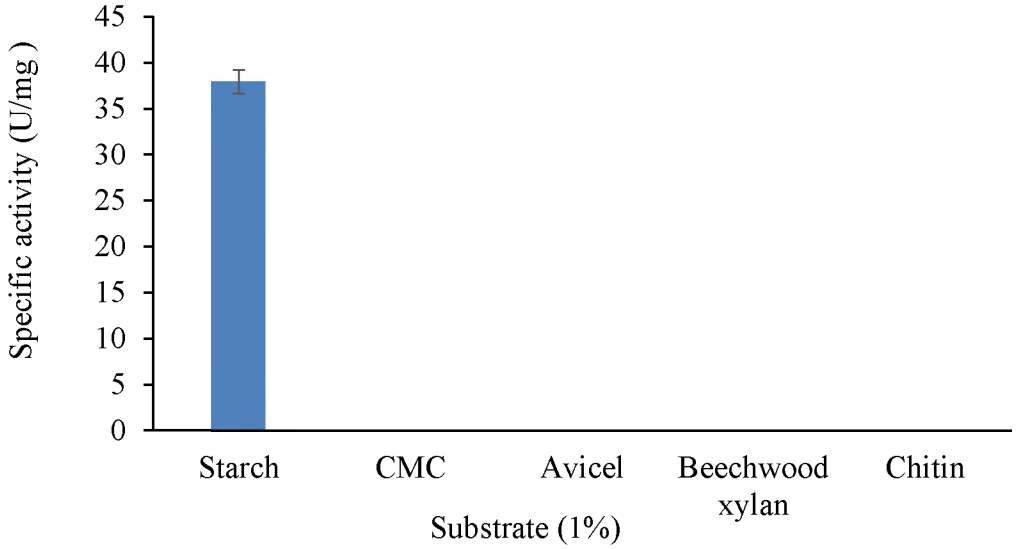


Figure 5.11. Substrate specificity study and determining AMG activity on different substrates to confirm for other co-produced enzymes. All error bars are represented as the standard errors of the means (SEM).

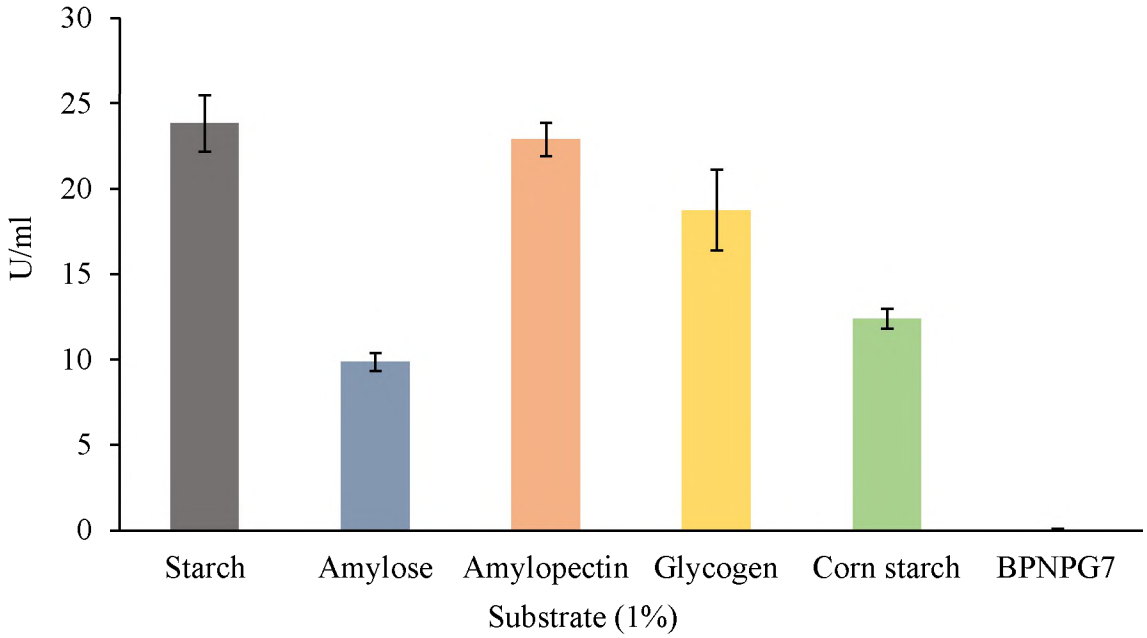


Figure 5.12. Substrate specificity study and determining AMG activity on the various amylase-degrading substrate to investigate AMG affinity for each substrate. All error bars are represented as the standard errors of the means (SEM).

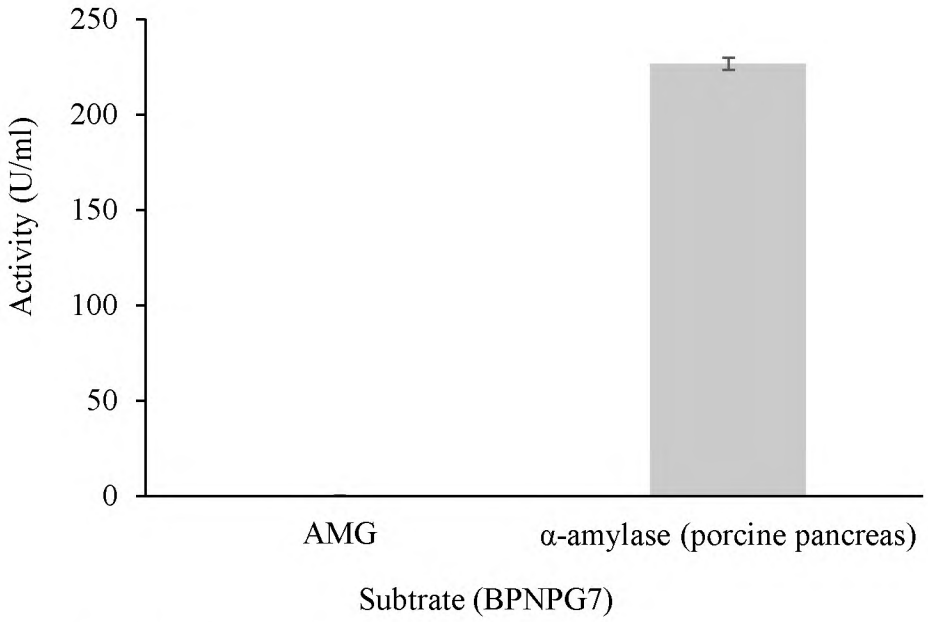


Figure 5.13. Catalytic ability of AMG (*Leohumicola* sp.) and α-amylase (porcine pancreas) on BPNPG7 (blocked *p*-nitrophenyl-α-D-maltoheptaoside). All error bars are represented as the standard errors of the means (SEM).

5.3.8. Starch yield (corn)

The result of starch yield in this investigation indicated that an average starch yield of 14.3% was obtained with the slurry centrifugation method, while sedimentation at 4°C for 2 h gave a 22% starch yield after 72 h of steeping and homogenisation (Table 5.4).

Table 5.4. The sedimentation methods and raw starch yield from corn after steeping and homogenisation

Sedimentation method	**Starch yield (%)**
Sedimentation at 4°C	22.0±2.06
Centrifugation at 6000 x *g*	14.3±1.63

All values are presented as means ± SEM, n = 3 per treatment.

5.3.9. Enzyme kinetics (substrate affinities)

Figures 5.14 to 5.18 show the results of Michaelis-Menten plots using KaleidaGraph. K_m represents the Michaelis constant of the AMG enzyme and V_{max}, the maximum velocity achieved by the AMG enzyme-starch reaction. The result indicated that AMG displayed the highest affinity with a K_m value of 0.38 mg/ml on soluble starch, followed by glycogen, amylopectin, amylose and raw corn starch with the K_m values of 0.70, 0.76, 1.05 and 1.77 mg/ml, respectively. The k_{cat} values of 73, 70, 66, 57, and 32 s^{-1} were obtained for raw corn starch, soluble starch, amylopectin, glycogen and amylose, respectively (Table 5.5). The K_m and V_{max} were used to demonstrate the affinities between some starch-degrading substrates and the AMG. In this study, a comparison was made between starch, amylose, amylopectin, glycogen and crude corn starch to determine the affinity of AMG with these substrates (Table 5.5).

Table 5.5. Kinetics study of AMG against various substrates analysed using Michaelis-Menten plots

Substrate	**K_m (mg/ml)**	**V_{max} (µmol/min/ml)**	**Catalytic constant, k_{cat} (s^{-1}) = $\frac{V_{max}}{[E_t]}$**	**Specificity constant (s^{-1} mg^{-1} ml^{-1}) = $\frac{k_{cat}}{K_m}$**
Starch (soluble)	0.38	22.56	70	184
Amylose	1.05	10.11	32	30
Amylopectin	0.76	21.29	66	87
Glycogen	0.70	18.40	57	81
Raw corn starch	1.77	23.52	73	41

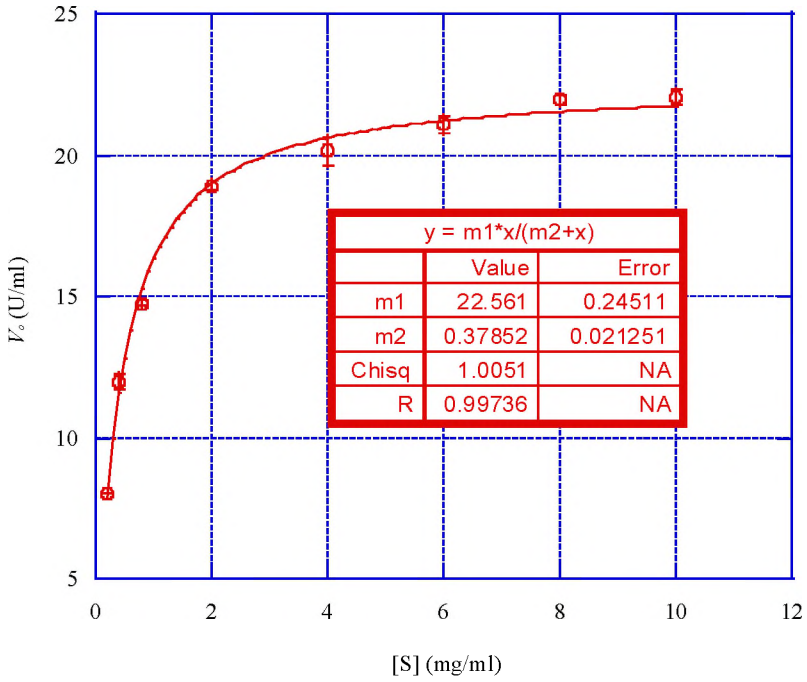


Figure 5.14. Michaelis-Menten type kinetics of purified AMG enzyme versus soluble starch concentration. All error bars are represented as the standard errors of the means (SEM) (m1 = V_{max} and m2 = K_m).

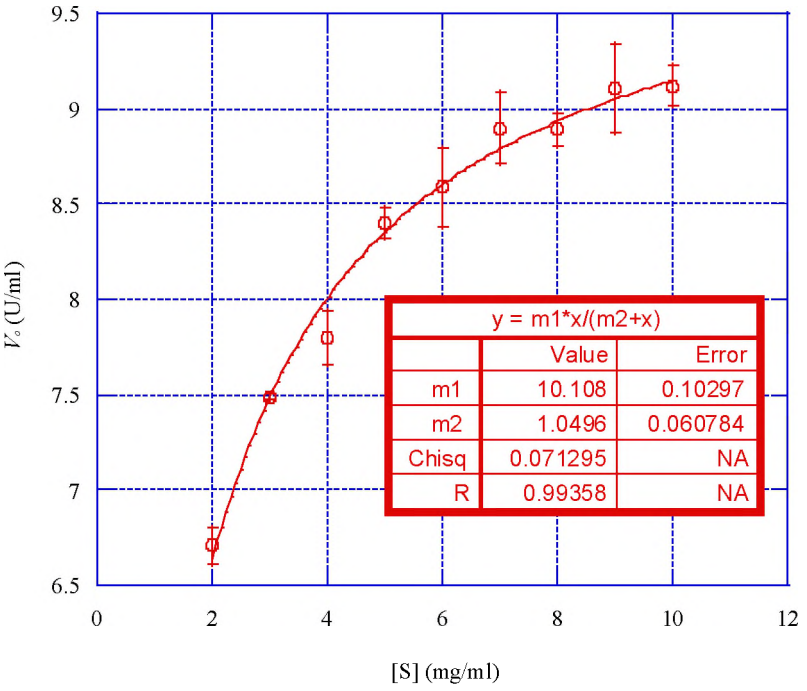


Figure 5.15. Michaelis-Menten type kinetics of purified AMG enzyme versus amylose concentration. All error bars are represented as the standard errors of the means (SEM) (m1 = V_{max} and m2 = K_m).

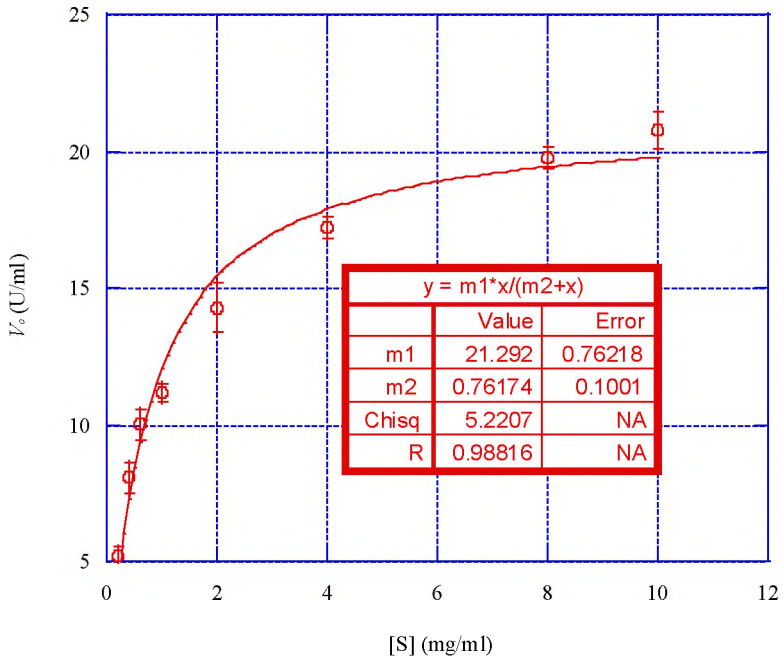


Figure 5.16. Michaelis-Menten type kinetics of purified AMG enzyme versus amylopectin concentration. All error bars are represented as the standard errors of the means (SEM) (m1 = V_{max} and m2 = K_m).

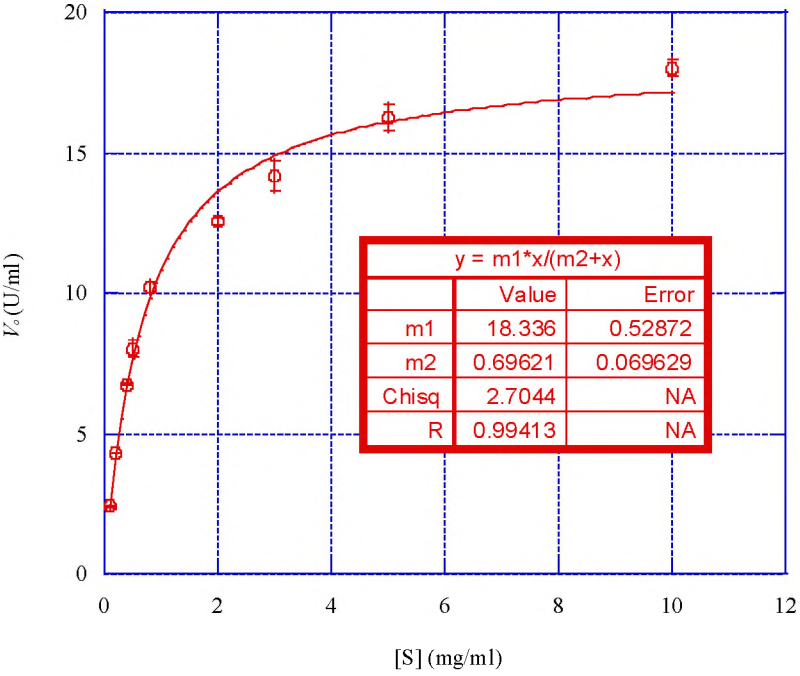


Figure 5.17. Michaelis-Menten type kinetics of purified AMG enzyme versus glycogen concentration. All error bars are represented as the standard errors of the means (SEM) (m1 = V_{max} and m2 = K_m).

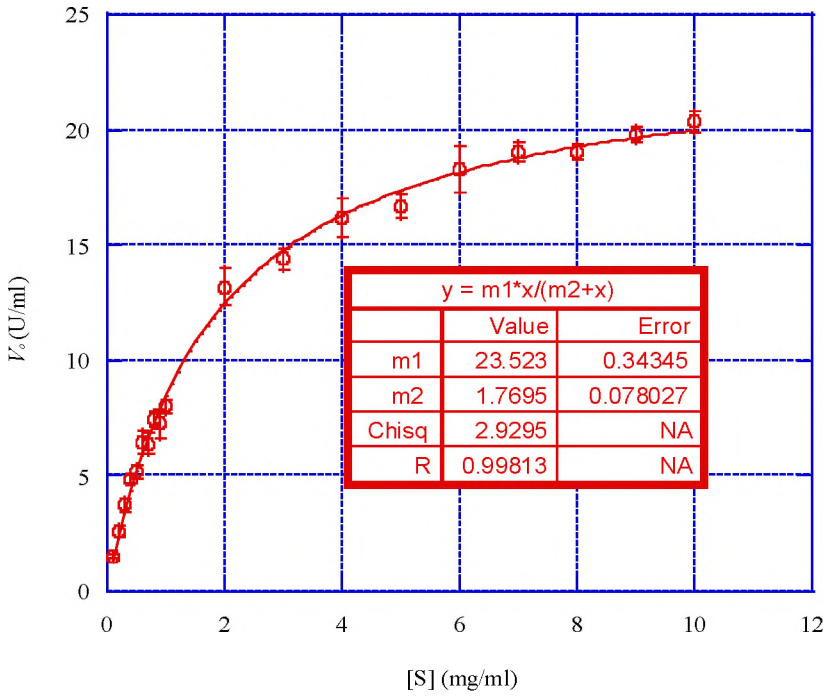


Figure 5.18. Michaelis-Menten type kinetics of purified AMG enzyme versus corn starch (raw) concentration. All error bars are represented as the standard errors of the means (SEM) (m1 = V_{max} and m2 = K_m).

5.4. Discussion and conclusion

AMG enzyme was produced under liquid-state fermentation conditions using a modified Melin-Norkrans medium. The elution profile on Sephadex G-100 was sufficient to obtain a single protein band which confirmed the purity of the enzyme (Figure 5.5). Size-exclusion chromatography (gel filtration) is an established chromatographic technique for separating proteins according to their size. This method is suitable for the purification and molecular weight estimation of proteins (Nielsen and Borchert, 2000). In gel filtration, the separation depends on the different abilities of the sample component to enter the pores within the gel beads. Molecules larger than the pores in the beads will move through the column quickly in the shortest time; however, small molecules that can enter the pores freely are retarded and therefore move more slowly down the column by their molecular weight. Thus, molecules are eluted from the column in decreasing size order (Nielsen and Borchert, 2000). Other resins that can be used are Superdex, Sepharose and Superose. The TLC patterns of the hydrolysis products demonstrated

that only glucose was produced as an end product of enzyme activity (Figure 5.3). Starch hydrolysates were analysed by thin TLC using silica gel plates, where oligosaccharides spots were identified by comparing their R_f values with those similarly obtained for spots of glucose, maltose, maltotetraose, and maltopentaose (reference standards) (Campos and Felix, 1995).

The study corroborates the reports of some researchers that fungi can produce one or more isoforms of AMG, depending on the species being tested (Kusuda et al., 2004; Negi and Banerjee, 2009; Slivinski et al., 2011). The result of the crude extract of *Leohumicola* sp., using the starch-SDS-PAGE zymography method of Martinez et al. (2000) revealed the formation of a single amylolytic band on the starch-SDS gel. After gel filtration, the enzyme was found to be homogeneous on SDS-PAGE (Figure 5.5), and also the SDS-PAGE result revealed many protein bands with no amylase activity except a single band of AMG which had a molecular weight of 101 kDa (Figure 5.5). This agrees with some observations where the molecular weights of AMG in most fungi were reported to be in a range of 11.5 - 118 kDa (Hur et al., 2001; Kusuda et al., 2004; Nguyen et al., 2002; Slivinski et al., 2011; Yamasaki et al., 1977; Yamasaki and Suzuki, 1978).

In this study, all enzyme activities were expressed as specific activity (U/mg protein) except if otherwise stated. All experiments were performed in triplicate and the standard errors of the means calculated. The optimum and pH stability assays (Figures 5.6 and 5.7) showed that the optimum pH for AMG was pH 4.0 and that the enzyme was most stable at a pH of 5.0. A change in the pH of 1.0 unit resulted in decreased activity. These results were similar to the report of Slivinski and co-workers (2011), who stated that the pH stability range for *Aspergillus niger* was within 4.0-6.0 at temperatures between 40-60°C (Riaz et al., 2012; Slivinski et al., 2011). Kusuda et al. (2004) also reported that the glucoamylase obtained from an ectomycorrhizal fungus (*Lyophyllum shimeji*) was most active at around 40°C (pH 5.0) and stable at pH 4.5-6.5 for 30 min at 37°C. The AMG from *Leohumicola* sp. was found to exhibit stable activity at a temperature of 37°C and 45°C, retaining over 65% of AMG activity up to 24 h at 45°C (Figure 5.9), while activity declined with an increase in temperature. The stability of the AMG at 45°C was an improvement, considering the report of Hur et al. (2001) who reported that the α-amylase and glucoamylase from *Tricholoma matsutake* were only stable at 4°C to 30°C for 30 min.

The inhibition, activation, and stabilisation of microbial enzymes by metal ions are concentration dependent (Coolbear et al., 1992). The maximum AMG activity occurred at a concentration of 10 mM for manganese and calcium ions, at this concentration, both metal ions were stimulatory to the AMG activity of *Leohumicola* sp. indicating that bivalent cations were preferred for the active and stabilisation sites (Table 5.2). This agrees with the findings of Kusuda et al. (2004) that AMG was activated in the presence of calcium ions. Moreover, most of the metal ions (salt) used were not inhibitory except for some metal ions at high concentrations. This stability could be a function of the enzyme source (ERM fungus), which has been reported to be tolerant to metal ions and being able to grow in soil contaminated with some of these metals (Martino et al., 2000). Metal ions such as Co^{2+}, Hg^{2+}, Cd^{2+} and Al^{3+} have been reported to have an inhibitory effect on biomolecules (Tamás et al., 2014). The inhibitory effect of these metal ions (Co^{2+}, Hg^{2+} and Cd^{2+}) on the AMG enzyme activity was significant to varying degrees across all the concentrations tested (Table 5.2) and more pronounced at a concentration of 10 mM.

It has been suggested that inhibition by Hg^{2+} and other heavy metal ions are not only related to binding to the thiol groups but may be the result of interactions with tryptophan residues or the carboxyl groups of amino acids in the enzyme (Selvakumar et al., 1996). For other chemicals, the inhibitory effect of SDS on the AMG activity was the most significant (Table 5.3), this merely indicated that SDS is a denaturant (Amako and Yasunaka, 1974), while other tested chemicals were not inhibitory to the AMG activity. Also, SDS is an anionic detergent known for its undesirable effect on enzymes activities, and only limited cases of enzyme activation have been reported (Diamantidis et al., 2000). EDTA slightly inhibited activity at a concentration of 10 mM; this could be explained by the activity activation noticed with Mn^{2+} and Ca^{2+} ions. Both cations may act as co-factor required to increase the AMG activity (Annamalai et al., 2011).

There was no noticeable hydrolytic activity against CMC, Avicel, beechwood xylan and colloidal chitin (Figure 5.11). Therefore, the specificity noticed with AMG appeared to be restricted to the α-(1,4 or 1,6) glycosidic linkages and not those of β-(1,3 or 1,4) linkages. Figure 5.13 showed the catalytic ability of AMG from *Leohumicola* sp. and α-amylase from porcine pancreas on BPNPG7 which is a substrate specific for the determination of α-amylase activity.

The result revealed that the AMG was an exo-acting enzyme that could not hydrolyse BPNPG7, a substrate specific for only alpha-amylase (Cornaggia et al., 2016; McCleary et al., 2002; Megazyme, 2017). Enzyme kinetics can be expressed using the K_m and V_{max}, where K_m is the substrate concentration at $½V_{max}$, while V_{max} is the maximum rate achieved by an enzyme at enzyme-substrate saturation point. In another word, it reveals the number of substrate molecules converted into a product by an enzyme per unit time when the enzyme is fully saturated with substrate (Kaur et al., 2014). An enzyme with a small value of K_m means it will achieve its maximum catalytic efficiency at low substrate concentration. Thus, the lower the value of K_m, the more tightly the enzyme binds the substrate. Determining K_m and V_{max} values require the rate of catalysis (reaction velocity) for different substrate concentrations, which is V at various values of [S] (Kaur et al. 2014).

Raw starch was generated from corn through the sedimentation technique before a substrate affinity study (Table 5.5). The highest starch yield of 22% obtained by sedimentation at 4°C was used as a substrate (raw corn starch). This corroborates the report that sedimentation (allowing the slurry to stand at 4°C for at least 2 h) resulted in a greater starch yield than starch extracted by using the centrifugation method (Ji et al., 2004; Sheriff et al., 2012). Table 5.5 shows that AMG (*Leohumicola* sp.) had a high affinity with all substrates tested with the highest affinity value on starch, followed by glycogen, amylopectin, amylose and raw corn starch. These results agree with the report of Slivinski et al. (2011) who observed similar substrate affinities where less substrate was required to reach enzyme-substrate saturation. Furthermore, some reports on glucoamylase by *L. shimeji* (ECM fungus) and *Rhizopus oryzae* revealed that the amylase readily hydrolysed the α-1,4 glucosidic linkage (amylose) and α-1,6 glucosidic linkage (amylopectin) into glucose (Abdelwahab, 2015; Hur et al., 2001; Kusuda et al., 2004). Conversely, the AMG could not hydrolyse BPNPG7 because it is an exo-acting enzyme. It is worthy to note that AMG hydrolysed the raw starch extracted from corn with a K_m value of 1.77 mg/ml and k_{cat} of 73 s^{-1} which showed the potential of the AMG for industrial application for hydrolysing starch-based feedstock. K_m values of 0.38, 0.70, 0.76, 1.05 and 1.77 mg/ml obtained for soluble starch, glycogen, amylopectin, amylose and raw corn starch, respectively, clearly indicated that soluble starch was the substrate of choice for AMG hydrolysis. All results obtained from the substrate affinity study agree with the report of Michelin et al. (2008). Furthermore, the AMG turnover

number (k_{cat}) was 70 s^{-1} on soluble starch compared well with those of *Humicola* and *Aspergillus* species. Riaz et al. (2007) reported a k_{cat} value of 69 s^{-1} for *Humicola* sp., and also a much higher k_{cat} value (343 s^{-1}) was reported for *Aspergillus niger* (Riaz et al., 2012). The K_m and k_{cat} values reported in this study were better than those reported by some authors. For example, 4.5 mg/ml and 0.75 s^{-1} were reported for *Tetracladium* sp. (Carrasco et al., 2017); 3.8 mg/ml and 41.7 s^{-1} for *Paecilomyces variotii* (Michelin et al., 2008).

In conclusion, the approach of sourcing enzymes from the environment proved to be useful in producing novel enzymes that are suitable for the bio-economy. The *Leohumicola* sp. is just one of many ericoid associated fungi that could be employed for the production of enzymes for the bio-economy. This present study describes, for the first time, the purification and characterisation of an AMG from *Leohumicola* sp. AMG is highly sought-after in industries for the production of glucose, fructose syrup and bioethanol. Therefore, the results presented in this study showed that this enzyme exhibits some promising properties such as temperature stability over an extended period, stable to a wide range of chemicals and its high substrate affinity also makes it an attractive candidate for future industrial applications.

CHAPTER 6

6.0 Evaluation of the antibacterial properties of some ericoid mycorrhizas

6.1. Introduction

Mycorrhizal fungi are members of root endophytes that are capable of complex web interactions with host plants (especially the ECM). They have been studied as new sources of novel bioactive natural products. Fungi are known for their different critical processes that include viz decomposition of organic materials, recycling and transportation of nutrients in the soil environment. Thus they are major components of the ecosystem.

Endophytes are abundant and useful sources of genetic diversity, and new species capable of protecting plants against pathogens, herbivore, and insect attack. Although, some can cause diseases to plants when the soil environment is stressed. These are an outstanding and diverse group of fungi which colonise healthy plant tissues without causing any symptoms (Pawłowska et al., 2014). They produce some secondary metabolites, which confer ecological benefits to their host plants (Kusari et al., 2013). Some of these bioactive compounds are alkaloids, flavonoids, terpenoids, phenols, tannins, cardiac glycosides, steroids, and saponins (Devi et al., 2012). The metabolites produced by endophytic fungi are primarily used as a source of drugs. The use of fungi for the production of bioactive compounds dates back to the first time when penicillin was discovered from *Penicillium notatum* (Fleming, 1929). The discovery of novel bioactive compounds is now being encouraged to solve the problem of pathogen-drug resistance. It's been shown that *Alternaria alternata, Geotrichium albida, Penicillium frequentans,* and *Thielaviopsis basicola* have antibacterial activity when screened for antimicrobial and phytochemical properties (Bhardwaj et al., 2015).

Bioactive compounds from mushrooms have been found to have antiviral, antidiabetic, antitumor, antioxidant, radical scavenging and antibacterial effects (Zavastin et al., 2016). For

example, basidiomycetes such as *Boletus edulis, Fomes fomentarius, Inonotus obliquus, Fomitopsis officinalis,* and *Piptoporus betulinus* have been used to treat gastrointestinal disorders and cancers (Nowacka et al., 2015; Zavastin et al., 2016). Searching for microorganisms with unique and effective properties is highly desirable nowadays because of the burden of contending with diseases (including cancers) that affect human welfare. Therefore, there is a growing interest in investigations of natural antibacterial agents, which can provide a viable alternative capable of inhibiting pathogenic organisms.

Issues such as antimicrobial resistance are worldwide, the development of new drugs and the search for novel natural products will go a long way to support and improve human health. Antibiotic-resistant has been reported with some strains of bacteria such as *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*, the report indicated that antibacterial resistance jeopardises the efficient use of drugs in prevention and treatment of different infections caused by microorganisms (WHO, 2014). The Gram-positive bacterium, *S. aureus* is a pathogen that has received considerable attention due to a combination of nasal carriage and the bacterial immuno-evasive strategies it possesses. It can cause a range of illnesses, from minor skin infections (pimples, impetigo, boils, cellulitis, carbuncles, and abscesses), to life-threatening diseases (pneumonia, meningitis, osteomyelitis, and endocarditis) (Das et al., 2014).

The resistance of pathogenic bacteria to antimicrobial agents has been attributed to two major properties. Firstly, intrinsic properties, such as the innate ability to degrade/inactivate a particular antibiotic, possession of an efflux system for expelling antibiotics out of the cell, and alteration of the antibiotic target site (Adimpong et al., 2012; European Food Safety Authority, 2012). Secondly, acquiring resistance genes through plasmids, transposons, and mutation of a native gene (Adimpong et al., 2012; European Food Safety Authority, 2012).

Symbionts, such as mycorrhizal fungi and other free-living endophytes form a critical component of the rhizosphere, where different kinds of antagonistic, parasitic interaction occur. Mycorrhizal fungi establishment and survival of hyphal networks, ensure a sustainable supply of nutrients to their host plant, and they protect against invading pathogens (Jha et al., 2008). The culture filtrate of mycorrhizal fungi, e.g. *Suillus collinitus, Hebeloma mesophaeum* and *Paxillus*

sp. are reported to exhibit an antagonistic effect on mycelial growth and spore germination of *Fusarium oxysporum* and *Pythium vexan*; this antifungal activity has been attributed to water-soluble phenolic production (Yamaji et al., 2005).

Phenolic compounds (polyphenols) are secondary metabolites that play a crucial role in maintaining human health and are known for their antioxidant properties (Aberoumand and Deokule, 2008). Phenolic compounds consist of an aromatic ring bearing one or more hydroxyl substituent; they range from a simple molecule to complex polymerised compounds (Aberoumand and Deokule, 2008). Many phenolic compounds are found in nature, such as in plants, foods and microorganism (particularly in endophytic fungi) and can be categorised into different classes (Figure 6.1). They include phenolic acids, flavonoids, tannins, hydroxybenzoic (vallinilic, gallic and syringic acids) and hydroxycinnamic acids (caffeic, ferulic, coumaric and sinapic acids) (Balasundram et al., 2006). Phenolic compounds have health benefits derived from consuming high levels of fruits and vegetables (Parr and Bolwell, 2000). Polyphenols provide a lot of micronutrients in the human diet, which is evident in the prevention of cancer and cardiovascular diseases (Manach et al., 2004).

Minimum inhibitory concentration (MIC) is a term used in resistance surveillance, comparative testing of new antibacterial agents and the establishment of susceptibility of organisms where disc test is insufficient and unreliable for clinical management (EUCAST, 2000). MIC involves a dilution test of microorganisms where investigations are carried out to determine their ability to produce visible growth on a series of broth dilutions or agar dilutions (increase geometrically) of the antimicrobial agent with varying concentrations. The lowest concentration of the bioactive agent that prevents the appearance of any visible growth is the MIC. Minimum bactericidal concentration (MBC) on the other hand is the lowest concentration of an antimicrobial agent that is needed to achieve a bactericidal effect and leading to a 100% reduction in the initial microbial density. Among many sources of natural bioactive substances, mycorrhizas constitute a huge and almost unexplored group. Because many micro and macro-fungi are already valuable sources of bioactive compounds, we were encouraged to assess the antibacterial potential of ChemRU330 isolate belonging to the ERM fungi. The aim of the present study (Chapter 6) is to determine the antibacterial properties of ChemRU330 isolate by performing MIC and MBC assays.

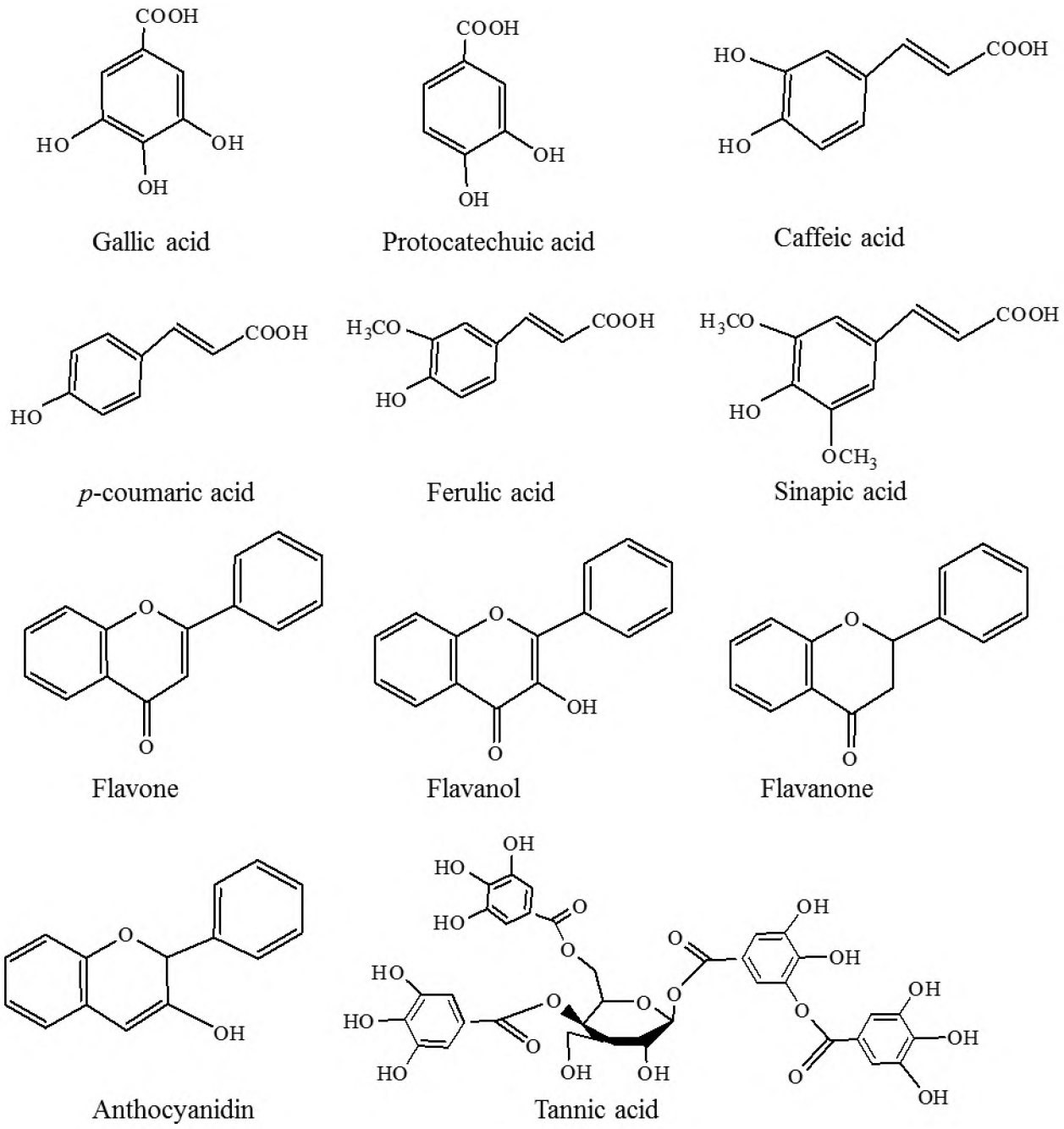


Figure 6.1. Structures of some bioactive compounds (Modified from Balasundram et al., 2006). Hydroxybenzoic acids (gallic acid and protocatechuic acid); hydroxycinnamic acids (caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid); flavonoids (flavone, flavanol and flavanone, anthocyanidin); tannins (tannic acid).

6.2. Materials and methods

6.2.1. Fungal cultivation and production of antibacterial agent

Five fungal isolates were used for the investigation. These were *Acremonium implicatum* (Nem 83), *Chaetomium* sp. (Ncf91), *Leohumicola* sp. (ChemRU330), two unidentified fungi belonging to Hyaloscyphaceae (EdRU083), Leotiomycetes (EdRU002) were obtained from Rhodes University, Grahamstown. These isolates were preserved on PDA as stated in other chapters. For each actively growing fungal endophyte, two 5 mm mycelial mat was cultivated in a liquid MMN medium. The organisms were incubated at 28°C for three weeks, with continuous shaking at 150 rpm on a rotary shaker. After the incubation, the fermentation broth of each fungus was homogenised and filtered through Whatman no. 1 filter paper to obtain cell-free crude filtrates.

6.2.2. Test bacteria

The crude extract of each of the fungal isolate was screened for antibacterial activity using some bacterial strains as indicator organisms. The indicator bacteria included both Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli*, *Serratia marcescens*, *Proteus vulgaris*, *Shigella sonnei,* and *Klebsiella pneumoniae*) bacteria were obtained from the undergraduate laboratory of the Department of Biochemistry and Microbiology, Rhodes University, Grahamstown. The bacterial isolates were already identified cultures used for undergraduate demonstration. All cultures were adjusted to 0.5 McFarland standards, which is visually comparable to a microbial suspension of approximately 1.5 x 10^8 CFU/ml.

6.2.3. Preparation of a 0.5 McFarland turbidity standard

McFarland turbidity standard was prepared by mixing 9.95 ml of 1% sulfuric acid (H_2SO_4) and barium chloride dihydrate ($BaCl_2.2H_2O$), 0.05 ml of 1.175%, to obtain a solution with specific optical densities (Figure 6.2). McFarland turbidity standard facilitates an optical density comparable to the amount of a bacterial suspension $1.5x10^8$ colony forming units (CFU/ml),

Absorbance value of between 0.08 to 0.12 at 625 nm. The density of the suspension of bacterial cells was compared to the McFarland turbidity standard by holding the McFarland turbidity standard and suspension in front of light against a white background with contrasting black lines. The bacterial density was adjusted to the density of the McFarland turbidity standard using either saline or bacterial suspension.

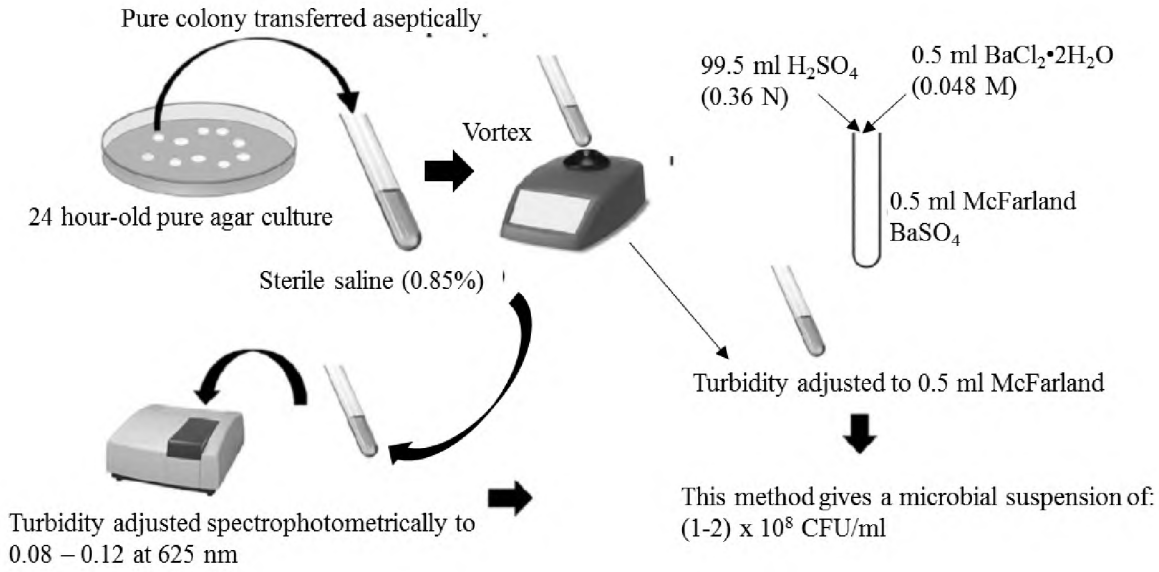


Figure 6.2. The 0.5 McFarland microbial inoculum preparation by the direct colony suspension according to Clinical and Laboratory Standard Institute (CLSI) guidelines (Modified from Balouiri et al., 2016)

6.2.4. Preliminary screening (using crude fungal filtrate)

The initial screening of antibacterial activity was conducted using a well-dilution method. The nutrient agar (NA) and Luria-Bertani (LB) media were poured into separate Petri plates and inoculated with 50 µl of the bacterial suspension ($1.5x10^8$ CFU/ml) and spread uniformly by using a sterile glass spreader. Wells (5 mm) were made on the agar media with a sterile core borer, 50 µl crude filtrate of each of the fungal isolate was placed into each separate well, and the controls [positive (chloramphenicol) and negative (uninoculated broth)]. Parafilm was used in sealing the plates before incubation at 37°C for 24 h. Plates showing antibacterial activity were

confirmed by visualisation, followed by measurement of inhibition zones. The average of three repeated trials was taken to evaluate the antibacterial activity (Hema et al., 2015).

6.2.5. Extraction and concentration of bioactive metabolites

The bioactive compounds of the fungal filtrate(s) showing inhibition against some test organisms (bacterial species) after preliminary screening were then extracted by solvent extraction procedure using ethyl acetate as the organic solvent. To the filtrate, an equal volume of extracting solution (ethyl acetate) was added (ratio 1:1), this was mixed thoroughly for 10 min and kept for 5 min to obtained two clear immiscible layers. The upper tier (bioactive compound) was separated using a separating funnel. The extracting solvent was then evaporated, and the resultant compound was dried in a rotary vacuum evaporator (Buchi Rotavapor® R-200 Rotavapor System) to yield the crude metabolite (Sharma et al., 2016). The crude extract was then dissolved in distilled water and kept at 4^{o}C. A known weighed crude extract was dissolved in distilled water before use to obtain a particular concentration.

6.2.6. Determination of MIC and MBC (cell viability assay using MTT)

The dried crude extract of ChemRU330 was adjusted to a final concentration of 16 mg/ml using a sterile distilled water as the diluent. The test was carried out in duplicate. The MIC was determined using the micro broth dilution method in a 96-well microplate. The test organisms were grown for 24 h at 37^{o}C. 100 µl of bacterial liquid culture [(optical density adjusted to match 0.5 McFarland standard (1.5×10^{8} CFU/ml)], then distributed into a 96-well microtiter plate. The crude extract preparations were diluted to contain different concentrations ranging from 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 mg/ml dilutions, and were added to the wells containing the bacterial cells. A 5 µg/ml chloramphenicol was used as positive control, and sterile distilled water was used as negative control for the test microorganisms, and the microtiter plates were incubated at 37°C for 24 h (Sarker et al., 2007). A 40 µl (0.2 mg/ml) thiazolyl blue tetrazolium bromide [methylthiazolyldiphenyl-tetrazolium bromide (MTT)] was added and at 37°C for 30 min to detect the MIC. The presence of viable bacterial cells reduced the yellow dye to a pink colour. MIC is the lowest concentration that prevented change and inhibited bacterial growth. MBC was determined by removing a portion of liquid (50 µl) from each well without

colour change and placing it on NA, and LB agar then incubated at 37°C for 24 h. The lowest concentration that yielded no growth after this culturing was regarded as the MBC. All experiments were performed in duplicate (Alves et al., 2012).

6.2.7. Phytochemical screening

Phytochemical screening was conducted on the ethyl acetate extract to check for the presence of the following secondary metabolites – alkaloids, flavonoids, phenols, saponins, steroids, cardiac glycosides, tannins, and terpenoids. Alkaloids: an 80 mg of solid fungal extract was dissolved in 4 ml 2N HCl. The mixture was divided into two portions, one portion was treated with equal amount of Wagner's reagent, and the second portion was treated with equal amount of Mayers reagent. Reactions showing the appearance of a brown precipitate indicated the presences of alkaloids. Flavonoids (zinc hydrochloride reduction test): to a test-tube containing 1 ml of fungal crude extract, 5-10 drops of dilute HCl, 0.5 g of zinc turnings were added, and the solution was boiled for 2 min. A reddish pink or dirty brown colouration of the solution indicated the presences of flavonoids in the extract. Phenols: a 40 mg crude extract was dissolved in 2 ml of distilled water. Then, a few drops of neutral 5% $FeCl_3$ solution was added. A dark green colour indicated the presence of phenolic compounds. Saponins: The crude extract (1 ml) was combined with 5 ml water and shaken for 2 min. The saponins are known to possess frothing activity, the volume of froth was recorded every 10 min. Froth more than 1.5 cm indicated a positive result. Steroids: steroid content was detected by using the Liebermann-Burchard reaction method. A 1 ml of the crude extract was placed in a tube containing an acetic anhydride, and a few drops of sulphuric acid (H_2SO_4) was added. A bluish-green ring indicated the presence of steroids. Cardiac glycosides: a 1 ml $FeCl_3$ reagent (a mixture of 1 volume of 5% $FeCl_3$ solution and 99 volumes of glacial acetic acid) was added to 1 ml of the crude extract and was later treated with a few drops of H_2SO_4, carefully placed in a dropwise manner along the sides of the test tube. The appearance of greenish blue colour within a few min indicated a positive result. Tannins: the already prepared alcoholic $FeCl_3$ reagent was mixed with the crude extract. The mixture produced a bluish-black colour, which disappears on the addition of a few drops of H_2SO_4 to produce a yellowish brown precipitate indicates a positive result. Terpenoids: a 1 ml crude extract was added to 1 ml of chloroform. Followed by addition of 1or 2 drops of concentrated

H_2SO_4 to form a layer. A reddish-brown precipitate at the interface indicated that terpenoids were present (Devi et al., 2012; Singh et al., 2015).

6.3. Results

6.3.1. Preliminary screening

Table 6.1 shows the results of initial screening for bioactive compound producing isolates. Out of all the five fungi employed for this study, only ChemRU330 and *Chaetomium* sp. (Ncf91) showed the potential for antibacterial production. In this experiment, crude extract from each isolate was used. No noticeable inhibition was observed with *Acremonium implicatum* (Nem83) and two other unidentified fungi belonging to Hyaloscyphaceae (EdRU083), Leotiomycetes (EdRU002), while only *Chaetomium* sp. had a slight activity against *Serratia marcescens* (Table 6.1). On the other hand, ChemRU330 inhibited two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and had mild activity against a Gram-negative bacterium, *Proteus vulgaris.* Figures 6.3 – 6.5 also show the zones of inhibition of ChemRU330 against *S. aureus, B. subtilis,* and *P. vulgaris.*

Table 6.1. Preliminary screening for antimicrobial activity of fungal isolates from root endophytes against some bacterial species

	Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*	*Sarratia marcescens*	*Proteus vulgaris*	*Shigella sonnei*	*Klebsiella pneumoniae*
Acremonium implicatum	-	-	-	-	-	-	-
Chaetomium sp.	-	-	-	+	-	-	-
ChemRU330	++	+++	-	-	+	-	-
EdRU83	-	-	-	-	-	-	-
EdRU002	-	-	-	-	-	-	-

- = no activity (5 mm); + = slight activity (6 - 10 mm); ++ = good activity (11-15 mm);
- +++ = very good activity (< 15 mm).

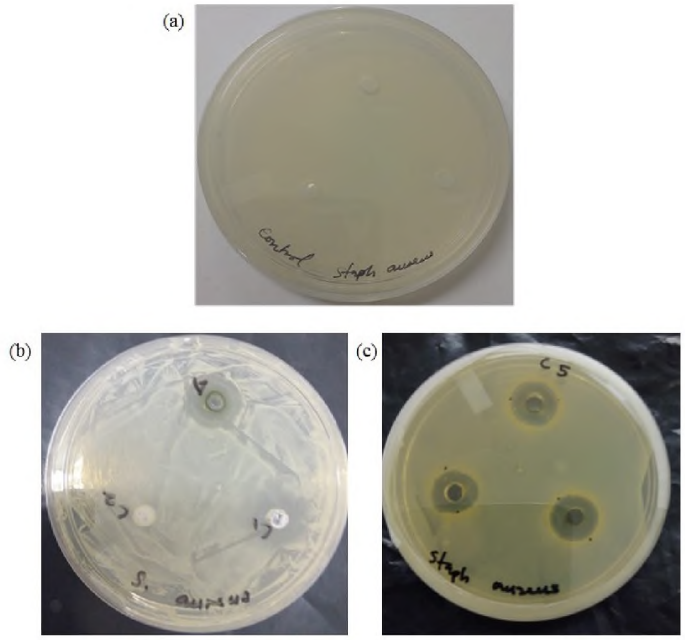


Figure 6.3. Agar well-diffusion method using *Staphylococcus aureus* against the crude extract ChemRU330: (a) plate with uninoculated extract, (b) plates with positive control (well-C1), negative control (well-C2), crude extract (well-A), and (c) plate with concentrated extract (16 mg/ml) in all the three wells.

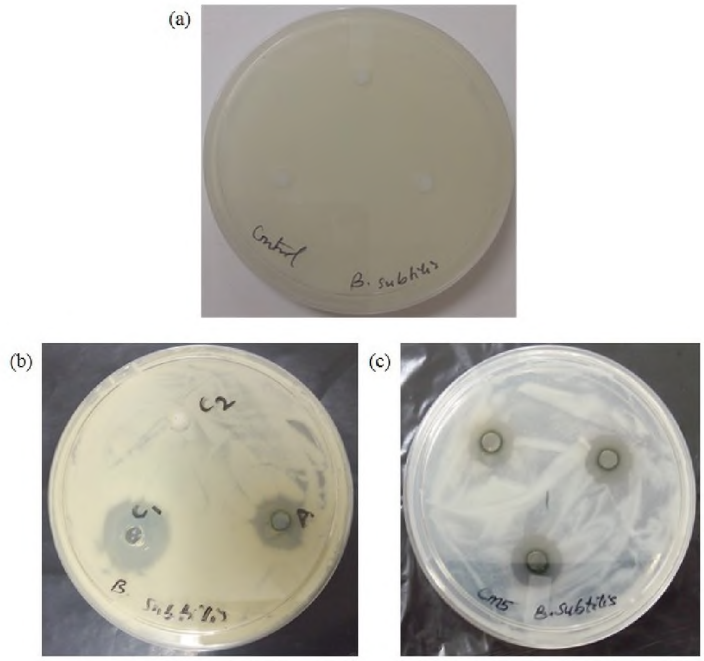


Figure 6.4. Agar well-diffusion method using *Bacillus subtilis* against the crude extract of ChemRU330: (a) plate with uninoculated extract, (b) plates with positive control (well-C1), negative control (well-C2), crude extract (well-A), and (c) plate with concentrated extract (16 mg/ml) in all the three wells.

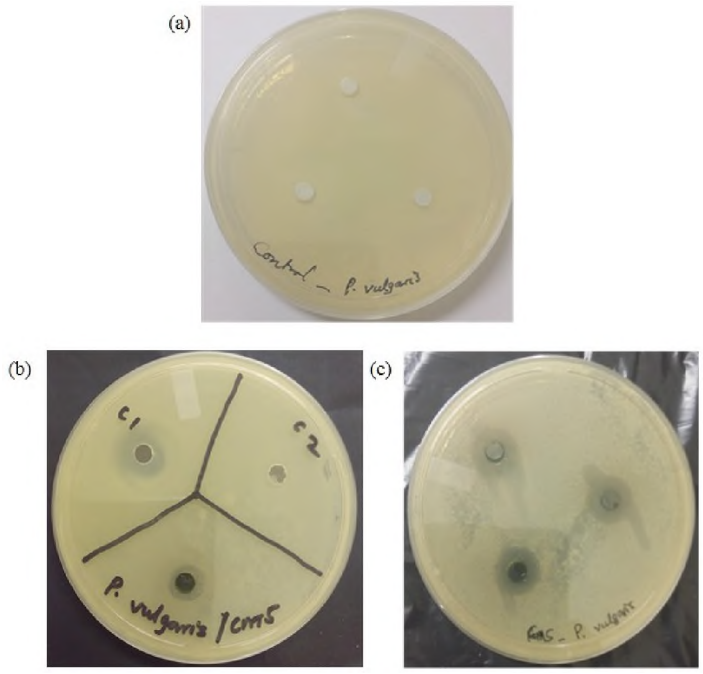


Figure 6.5. Agar well-diffusion method using *Proteus vulgaris* against the crude extract of ChemRU330: (a) plate with uninoculated extract, (b) plates with positive control (well-C1), negative control (well-C2), crude extract (well-A), and (c) plate with concentrated extract (16 mg/ml) in all the three wells.

6.3.2. Determination of MIC and MBC (cell viability assay using MTT)

Table 6.2 shows that the MIC of the extracts ranged between 2 and 16 mg/ml. *S. aureus* had the lowest MIC (1 mg/ml), followed by *B. subtilis* (2 mg/ml), and *P. vulgaris* (16 mg/ml) (Figure 6.6). The results of the MBC showed that the *L. incrustata* extract completely inhibited *B. subtilis* and *S. aureus* at the concentrations of 2 and 4 mg/ml, respectively, while in the case of *P. vulgaris*, there was no growth (MBC = 0). The MBC was confirmed by sampling from wells showing no visible growth as indicated by colour change and when subcultured on NA/LB media.

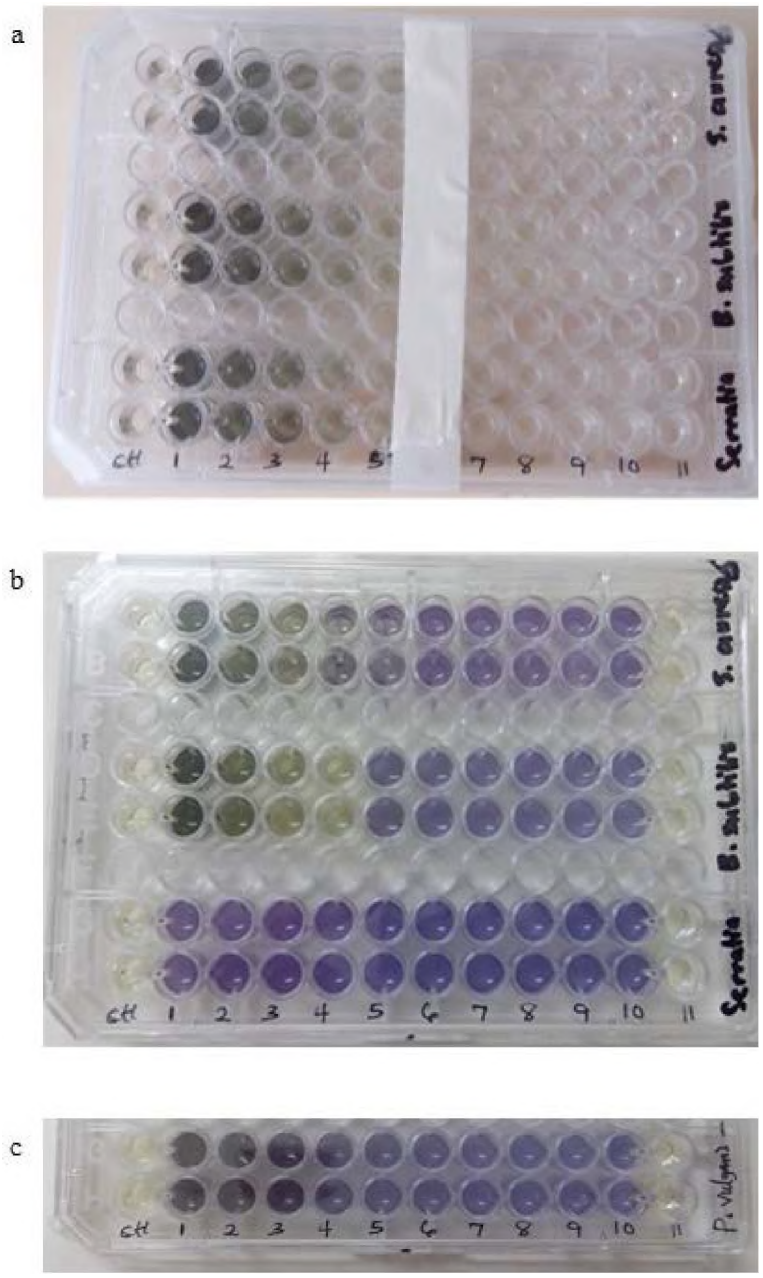


Figure 6.6. Determination of MIC for ChemRU330 against *Staphylococcus aureus, Bacillus subtilis, Serratia marcescens* and *Proteus vulgaris*. Figure 6.6a is before the addition of MTT dye while Figures 6.6 b and c show results after MTT dye was added. Column ctl – confirms no contamination occurred while preparing the plates. Column 11 is a negative control – shows no change of MTT natural yellow colour. The highest extract concentration incorporated into the plate is 16 mg/ml (column 1), and the lowest performed through twofold serial dilution is 0.03125 mg/ml (column 10).

Table 6.2. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of ChemRU330 extract against four bacterial isolates

Test organism	MIC (mg/ml)	MBC (mg/ml)
Staphylococcus aureus	1.0	4.0
Bacillus subtilis	2.0	2.0
Sarratia marcescens	0	0
Proteus vulgaris	16.0	0

6.3.3. Qualitative phytochemical screening

Eight bioactive secondary metabolites that included alkaloids, flavonoids, phenols, saponins, steroids, cardiac glycosides, tannins, and terpenoids were tested for in this experiment (Table 6.3). The result indicated that only alkaloids, flavonoids, phenols, saponins, cardiac glycosides, and terpenoids were present while steroids and tannins were absent.

Table 6.3. Qualitative determination of a ChemRU330 extract for secondary metabolites

S/N	Phytochemical compound	Result
1.	Alkaloids	Positive
2.	Flavonoids	Positive
3.	Phenols	Positive
4.	Saponins	Positive
5.	Steroids	Negative
6.	Cardiac Glycosides	Positive
7.	Tannins	Negative
8.	Terpenoids	Positive

6.4. Discussion and conclusion

Some bacteria used were susceptible to the crude extract of ChemRU330. The results obtained are promising and even bacteriostatic for a Gram-negative bacterium (*P. vulgaris*). The presence of some phenolic compounds (phenolic acids and tannins) in the extracts was responsible for the bioactivity. Some authors had already related the antimicrobial activity of different natural matrixes with the presence and content of phenolic compounds (Alves et al., 2012; Alves et al., 2013; Chowdhury et al., 2015; Sarker et al., 2007). It was observed that the majority of the extracts did not show antimicrobial activity against the tested bacterial cells except for extract from ChemRU330 and *Chaetomium* species that slightly inhibited *S. marcescens*. This agrees with the results reported earlier on *E. coli*, *K. pneumonia* and *P. aeruginosa* (Alves et al., 2012; Barros et al., 2008) who reported negative activity against some of these organisms.

The method microculture tetrazolium assay (MTA) was originally used to probe the relationships between the cell survival, growth and differentiation; this relies on the cellular reduction of a tetrazolium salt to their intensely coloured formazans in mammalian cells (Balouiri et al., 2016). Tetrazolium salt reagent has now been used to distinguish live and dead bacteria because only live bacteria can convert dye into an insoluble purple formazan. This method was adopted for quantifying dead cells by MTT assay in the MIC method of bacterial differentiation (Patel et al., 2013). There are suggestions that the lower the MIC, the more sensitive and promising the extract (Alves et al., 2012; Gbolagade et al., 2007).

The MBC was investigated by removing 50 µl of the suspensions from the MIC wells, which did not show any growth (i.e. no colour change) after incubation, and plates without bacterial growth were taken as the MBC. This aligns with a report on MBC which described it as the lowest concentration of extract that inhibited 100% bacterial growth (Cohen et al., 1998). The increasing resistance rate of pathogenic microbes against various marketed drugs is highly problematic and increases the need for the discovery of new antimicrobials. In this chapter, the antimicrobial potential of a mycorrhizal fungus (ChemRU330) was investigated to determine the MIC and MBC. The results showed that the fungus contains antibacterial compounds which should be explored to complement the already available drugs for active disease treatment. Also,

this isolate (ChemRU330) revealed the presence various bioactive compounds that include phenolics, alkaloids, flavonoids, cardiac glycosides and terpenoids. The production of phenolic compounds and other bioactive compounds in endophytic fungi (Bhardwaj et al., 2015; Devi et al., 2012) and mushrooms (Alves et al., 2012; Nowacka et al., 2015) have been vastly reported.

In conclusion, bioactive compounds produced by endophytes are highly rated because they possess superior biosynthetic capabilities, presumably due to their gene recombination with the host while residing within the healthy plant tissues (Li et al., 2005). A high proportion of endophytic fungi (80%) produce bioactive compounds in tests for antibacterial, fungicidal and herbicidal activities (Schulz et al., 2002). The progressive exploration of new and novel antimicrobial compounds is principally aimed at overcoming the difficulties associated with resistant pathogens (Petersen et al., 2004). Thus, root endophytic fungi such as the ERM fungi should be extensively researched, encouraged, and considered as additional sources of new antimicrobial agents in drug and food preservation.

CHAPTER 7

7.0 Molecular identification of some ericoid associated isolates

7.1. Introduction

Erica species are plants commonly found in harsh edaphic conditions, belonging to the family Ericaceae and subfamily Ericoideae (Bizabani, 2015). The subfamily consists of twenty-five major genera such as *Rhododendron, Erica, Calluna, Empetrum* and *Rhodothamnus* (Bizabani, 2015). The genus *Erica* is the second largest genus in the Ericoideae, with approximately 865 identified species globally (McGuire and Kron, 2005). Ericaceous plants can be found in Africa, Middle East, Madagascar and Europe (McGuire and Kron, 2005). *Erica* species reproduce both sexually and asexually (Ojeda, 1998; Ojeda et al., 2005). The sexual reproduction of *Erica* species involves seed germination while in the asexual sprouting is involved. The association that exists between ericaceous plants and mycorrhizal fungi is obligated. Thus, forming a unique, distinctive relation (Smith and Read, 2008). The fungal association plays a significant role in determining taxonomy, ensuring the growth of the host plants under harsh conditions (Setaro et al., 2006).

These relationships are characterised by the morphological structures formed by the fungi within the hair roots. The ericoid mycorrhizal (ERM) association is the most common root-fungus relationship in the ericaceous family (Smith and Read, 2008; Martino et al., 2007). Root-fungal interactions that result in no apparent morphological structures are also common in the Ericaceae. They occasionally co-occur with other mycorrhizal associations (Sadowsky et al., 2012) in non-ericaceous host plants (Chambers et al., 2008). Ericaceae roots have fine roots known as 'hair roots'. The ERM fungi penetrate individual cortical cells of the hair roots, forming thin hyphal coils (Bergero et al., 2003; Smith and Read, 2008). It has been suggested that coexisting plants in natural habitats act as a repository for ERM inocula (Chambers et al., 2008). Fungal host recognition is facilitated by the secretion of mucilage via the root tip (Peterson and Massicotte, 2004). Degrading hydrolytic enzymes such as cellulase and pectinase break down the epidermal cell walls and allows the fungi to penetrate the hair root cells. After that, fungal hyphae colonise the epidermal cells to form ericoid coils within individual epidermal

cells (Smith and Read, 2008). *Leohumicola* species are distinguished members of ericoid mycorrhizal fungi (Nguyen and Seifert, 2008). They produce two-celled aleurioconidia (single cell conidia formed by projection from the conidiophores) with a spherical to ellipsoidal, dark-brown terminal cells coupled with slightly thickened walls, and cylindrical or cupulate basal cells. They grow slowly on PDA, MEA and MMN media and are naturally found in soil, particularly, at the root of the ericaceous plants (Nguyen and Seifert, 2008).

Hambleton et al. (2005) described four *Leohumicola* species (*L. verrucosa*, *L. minima*, *L. terminalis*, and *L. lenta*) belonging to a new genus of heat-resistant hyphomycetes. Nuclear ribosomal small subunit (SSU) ribosomal DNA sequences have been used to reveal that *Leohumicola* is a monophyletic group in the Leotiomycetes, distinct from *Humicola* and *Trichocladium* (Sordariales), and *Thermomyces* (Eurotiales) (Hambleton et al., 2005). Internal transcribed spacer sequence (ITS) aids in recognition of some *Leohumicola* species with unique cultural and morphological characteristics (Hambleton et al., 2005). The ITS region and cytochrome oxidase subunit I (*Cox1*) mitochondrial gene sequences have been used to identify *Leohumicola* species and the results obtained gave satisfactory resolution (Chen et al., 2009; Nguyen and Seifert, 2008). Molecular techniques have helped a great deal in unravelling the ERM fungal diversity when compared to cultural methods of identification. The molecular analysis allows for precise individual taxonomic identification of sporulating and non-sporulating fungi that are difficult to identify based on cultural and morphological characters. The objective of this study was to molecularly identify to species level the three key isolates used in this study. The molecular identification is coming at the later stage of the thesis because we wanted to identify to species and genus levels these unique fungal isolates which have been previously studied by Bizabani (2015).

7.2. Materials and methods

7.2.1. Fungal isolates

Pure fungal mycelia of *Leohumicola* sp. (ChemRU330), and two unidentified fungi belonging to Hyaloscyphaceae (EdRU083) and Leotiomycetes (EdRU002) were obtained and grew on PDA as described earlier. These are some of the isolates already reported by Bizabani (2015).

7.2.2. DNA extraction

Genomic DNA was extracted from pure fungal mycelia using the ZR Fungal/Bacterial DNA Mini-Prep kit (Catalogue # D6005) according to manufacturer's instructions. DNA concentration was determined by NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) and preparations were diluted to make 1–5 ng/µl of DNA template. The integrity of the isolated DNA was evaluated by electrophoresis in a 1% (w/v) agarose gel at 100 V for 75 min in 1X Tris-borate-EDTA (TBE) buffer, stained with 2 µl (concentration 0.5 µg/ml) ethidium bromide and visualised under a Bio-Rad ChemiDoc X-Ray Spectrometer (XRS) system.

7.2.3. Amplification of the ITS and Cox1 gene regions

The method described by Nguyen and Seifert (2008) was used, where the ITS region was amplified using the following primers ITS1, ITS4 and ITS5 (White et al., 1990) and KAPA *Taq* ReadyMix (2X). The KAPA *Taq* ReadyMix (2X) is a ready-to-use cocktail containing all components for PCR, except primers and template. The 2X ReadyMix contains KAPA *Taq* DNA Polymerase (1 U per 50 µl reaction), KAPA *Taq* Buffer, dNTPs (0.2 mM of each dNTP at 1X), $MgCl_2$ (1.5 mM at 1X) and stabilisers (Kapabiosystems, 2013). PCR master mix was made up in a total reaction volume of 50 µl comprising of 25 µl KAPA *Taq* ReadyMix PCR kit (KAPA Biosystems, Catalogue # KK1006), 5 µl template DNA, 2 µl each of both primers (forward and reverse), and 16 µl of water. Amplification was conducted in an automated Applied Biosystems 2720 Thermal Cycler. The cycling parameters used were as follows: initial denaturation at 95°C

for 3 min, 40 cycles at 95°C for 45 s (denaturing), annealing temperature at 60°C for 45 s, followed by extension at 72°C for 1.5 min, and finally 72°C for 8 min (final elongation). After that, electrophoresis was used as previously described to determine the size of the amplified bands. The *Cox1* gene region was amplified using primers designed for the *Cox1* gene of the Pezizomycotina, PezizF (5′-TCAGGRTTAYTAGGWACAGCATTT-3′) and PezizR (5′-ACCTCAGGRTGYCCGAA GAAT-3′) (Nguyen and Seifert, 2008). The PCR amplification was conducted in a total reaction volume of 25 µl comprising of 12.5 µl KAPA HiFi HotStart ReadyMix PCR kit (KAPA Biosystems, Catalogue # KK2605), 5 µl template DNA, 0.75 µl each of both primers (forward and reverse), and 6 µl of water. Amplification was conducted in an automated Applied Biosystems 2720 Thermal Cycler. The following parameters were used: 95°C for 3 min (initial denaturation), 40 cycles at 95°C for 1 min (denaturation), 51°C for 1 min (annealing), 72°C for 1.5 min (extension), then 72°C for 8 min (final elongation) (Nguyen and Seifert, 2008). After that, electrophoresis was used as previously described to determine the size of the amplified bands.

7.2.4. Sequencing and analysis of the ITS and Cox1 gene regions

After amplification of ITS and *Cox1* barcode regions, the PCR products clean-up were performed using a Wizard SV gel, and PCR clean-up kit (Promega, Catalogue # A9281) and the protocol outlined by the manufacturer of the kit was followed. The purified PCR products were sent to Inqaba Biotechnology, Pretoria, South Africa for Sanger sequencing. The sequencing reaction was carried out using ITS1, ITS4 and ITS5 (ITS region); pezizF and pezizR (*Cox1* gene) (Beeck et al., 2014; Porter and Golding, 2011). Nucleotide sequence chromatograms were analysed and edited using Chromas Lite software and compared to sequences in National Centre for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov (Altschul et al. 1997) and UNITE http://www.unite.ut.ee (Kõljalg et al., 2005) databases using Basic Local Alignment Search Tool (BLAST) program. Sequences derived from the study and their respective closest matches with homology greater than 95% were pre-aligned in Chromas version 2.6.4 (www.technelysium.com.au) before alignment using BioEdit sequence alignment editor version 6 (Hall, 1999).

7.2.5. Phylogenetic analysis

To test for phylogenetic relationships, the ChemRU330 sequence and those of species in the genera *Leohumicola* (available in GenBank) were aligned using the ClustalX Version 1.81 (Thompson, 1997). Phylogenetic analysis of *Cox1* barcode region was performed using molecular evolutionary genetics analysis version 7 (MEGA 7) (Kumar et al., 2016). The evolutionary history was inferred using Neighbor-Joining (NJ) statistical method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches (Felsenstein, 1985). Bootstrap support values above 50% from 1000 replicates search. The evolutionary distances were computed using Maximum Composite Likelihood method (Tamura et al., 2004) and were expressed as the units of the number of base substitutions per site. The analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 628 positions in the final dataset. *Myxotrichum deflexum* was chosen as an outgroup to analysis because of its position as near neighbour to *Leohumicola* clade in the 18S analyses (Hambleton et al., 2005).

7.3. Results

7.3.1. Molecular identification

In the current study, ChemRU330, EdRU083 and EdRU002 were some of the isolates previously studied using ITS barcode region (ITS1F and ITS4) (Bizabani, 2015). ChemRU330 was identified as *Leohumicola* sp. while EdRU083 and EdRU002 are two unidentified fungi belonging to Hyaloscyphaceae and Leotiomycetes, respectively (Bizabani, 2015). To give an explanation to the source of the isolates used in this study, Table 7.1 which shows the identification of isolates from six *Erica* species classified in operational taxonomic units (OTUs) by Bizabani (2015) was included.

Here, *Cox1* gene and ITS (ITS1 and ITS4) barcode regions were used to identify them to species and genus levels. The BLAST analysis was sufficient to resolve the organism to a particular name of the ERM fungus. ChemRU330, EdRU083, and EdRU002 (Figure 7.2) were identified to have percentage similarities of 99 (GenBank), 97.13 and 99.55% (UNITE), respectively. *Cox1* gene sequences matched reference *Leohumicola* sequences in GenBank using BLAST searches. The closest match to isolate ChemRU330 is with accession number EU678437 and a barcode region of cytochrome oxidase subunit 1 (*Cox1*) of 628 bp (Figures 7.3 and 7.4). EdRU083 (*Leohumicola* sp.) and EdRU002 (*Oidiodendron* sp.) closest GenBank matches are EU678450 and DQ398091, respectively. For EdRU083 isolate, we could not infer that the isolate belongs to *Leohumicola atra* which its closest match in GenBank with just 92% coverage. The quality of DNA sequences obtained from ITS5 and ITS4 was insufficient and was disregarded. The internal transcribed spacer sequence was estimated as 590 bp for ITS1-ITS4 (Figures 7.2 and 7.4). The ITS sequence reads obtained in the current study had between 495-523 bp which were sufficient to allow taxonomic identification of these fungi to genus level.

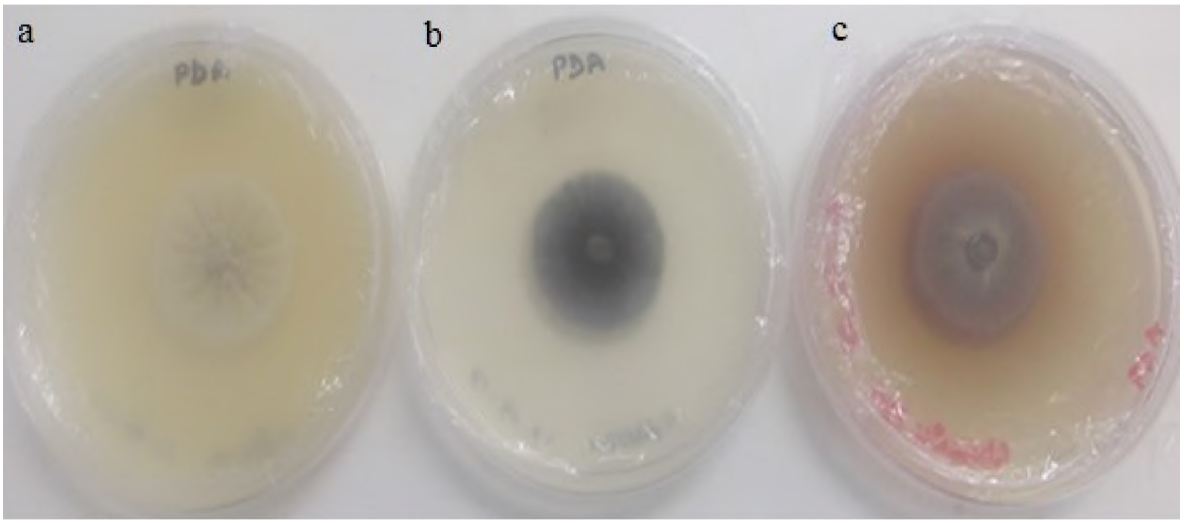


Figure 7.1. The photographs showing pure cultures of some ericoid associated fungi on potato dextrose agar (PDA) after 4 weeks of incubation at 28^{o}C (a = EdRU002, b = EdRU083 and c = ChemRU330).

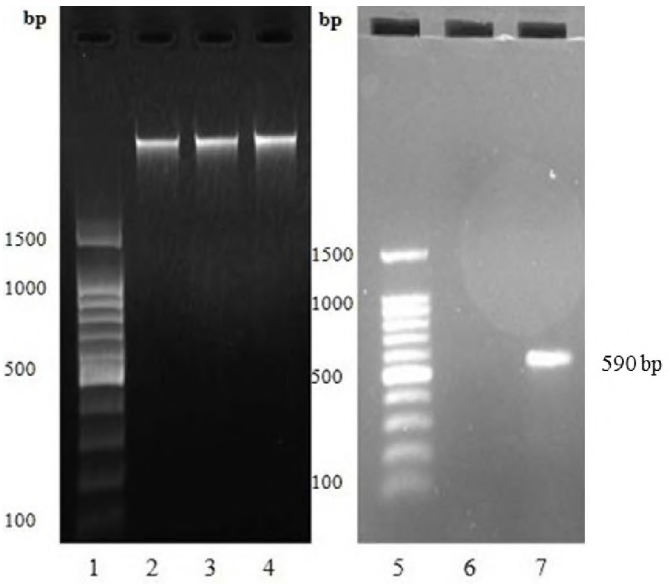


Figure 7.2. PCR products from DNA extracted from ChemRU330, amplified using primers ITS1 and ITS4, stained with ethidium bromide and separated by agarose gel electrophoresis. Lanes 1 and 5 are 100 kb DNA ladder; lanes 2 to 4 are genomic DNA; lane 6 is primer control; lane 7 is amplified ITS sequence.

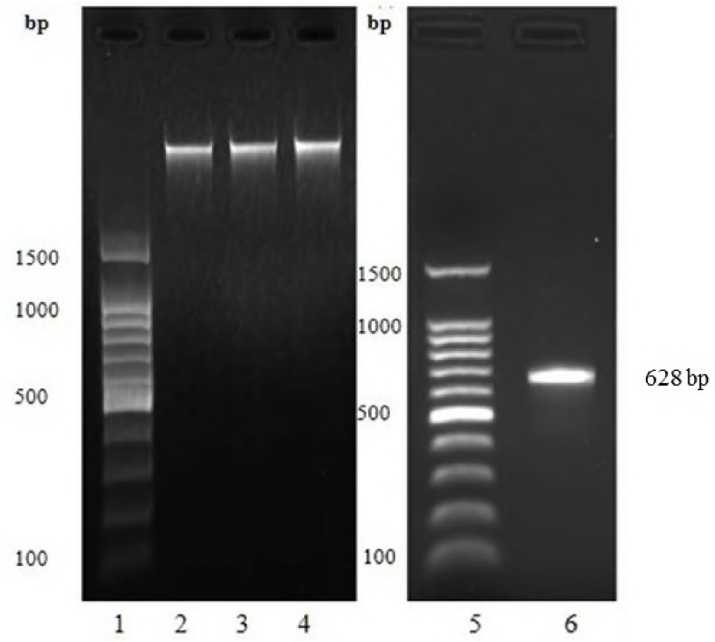


Figure 7.3. PCR products from DNA extracted from ChemRU330, amplified using primers PezizF-PezizR, stained by ethidium bromide and separated by agarose gel electrophoresis. Lanes 1 and 5 are 100 kb DNA ladder; lanes 2 to 4 are genomic DNA; lane 6 is amplified *Cox1* gene sequence.

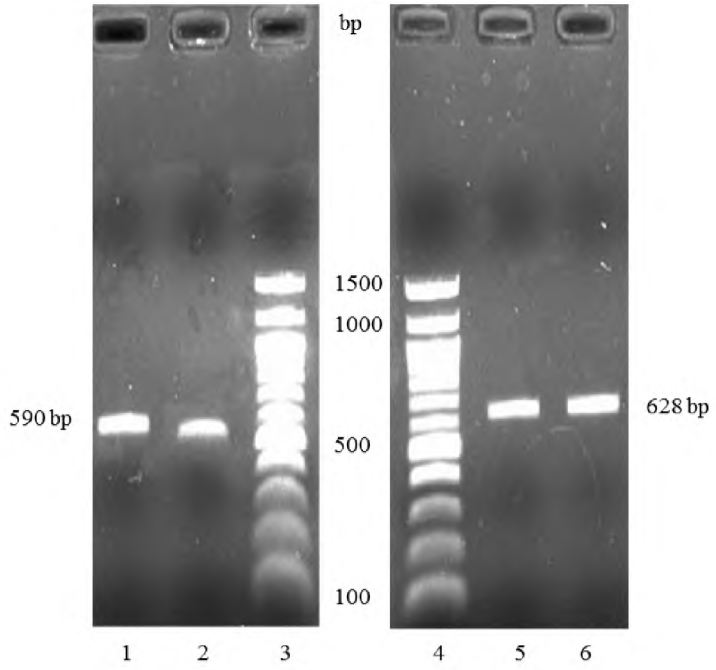


Figure 7.4. PCR products from DNA extracted from two ericoid mycorrhizas, amplified using primers ITS1-ITS4 (lanes 1-2) and PezizF-PezizR (lanes 5-6), stained with ethidium bromide and separated by agarose gel electrophoresis. Lanes 3 and 4 are 100 kb DNA ladder, lanes 1 and 5 (EdRU083), lanes 2 and 6 (EdRU002).

Table 7.1. Identification of isolates from six *Erica* species classified in operational taxonomic units (OTUs) (Adopted from Bizabani, 2015)

OTUs	No. of isolates	Genbank Accession OTU Representative	Closest BLAST match	GenBank Accession closest match	Coverage / Similarity (%)	Source of closest match	Host plant species
2	7	KC979127	*Leohumicola* sp.	JX912155	100/98	*Erica carnea* (Austria)	*E. nemorosa, E. caffra, E. chamissonis*
8	8	KM678348	*Oidiodendron cf. maius*	KC180732	100/95	*Gaultheria poeppigii* (Arg entina)	*E. nemorosa, E. caffra, E. chamissonis, E. demissa, E. cerinthoides, E. glumiflora.*

Table 7.1. Identification of some ericaceous plant roots associated fungi

Isolate	Primer target region	Genbank accession no.	Closest BLAST match	GenBank accession closest match	Percentage coverage / similarity (GenBank)	Percentage coverage / similarity (UNITE)	Source of closest match
ChemRU330	*Cox1*	MF374380	*Leohumicola incrustata*	EU678437	100/99	ND	South Africa
	ITS	MG209608	*Leohumicola* sp.	KM678361	97/99	99.38	South Africa
EdRU083	*Cox1*	MG132079	*Leohumicola atra*	EU678450	100/92	ND	South Africa
	ITS	MG209609	Helotiales	FN298703	99/97	97.13	Australia
EdRU002	*Cox1*	NA	NA	NA	NA	NA	NA
	ITS	MG209610	*Oidiodendron* sp.	DQ398091	ND	99.55	Australia

ND = not detected, NA = not applicable.

7.3.2. Phylogenetic analysis

The maximum composite likelihood analysis of *Cox1* sequences alignment revealed that the *Leohumicola* species form a monophyletic group of consensus trees (Figure 7.5). The *Cox1* sequence analysis was supported by the bootstrap value of 78%, thus inferred that the isolates (ChemRU330) belong to the *Leohumicola* clade (Figure 7.5). The phylogenetic tree further showed that all *Leohumicola* species formed a single clade including EdRU083 isolate, while phylogenetic relationships inferred from the ITS region sequences (not shown) did not give sufficient information.

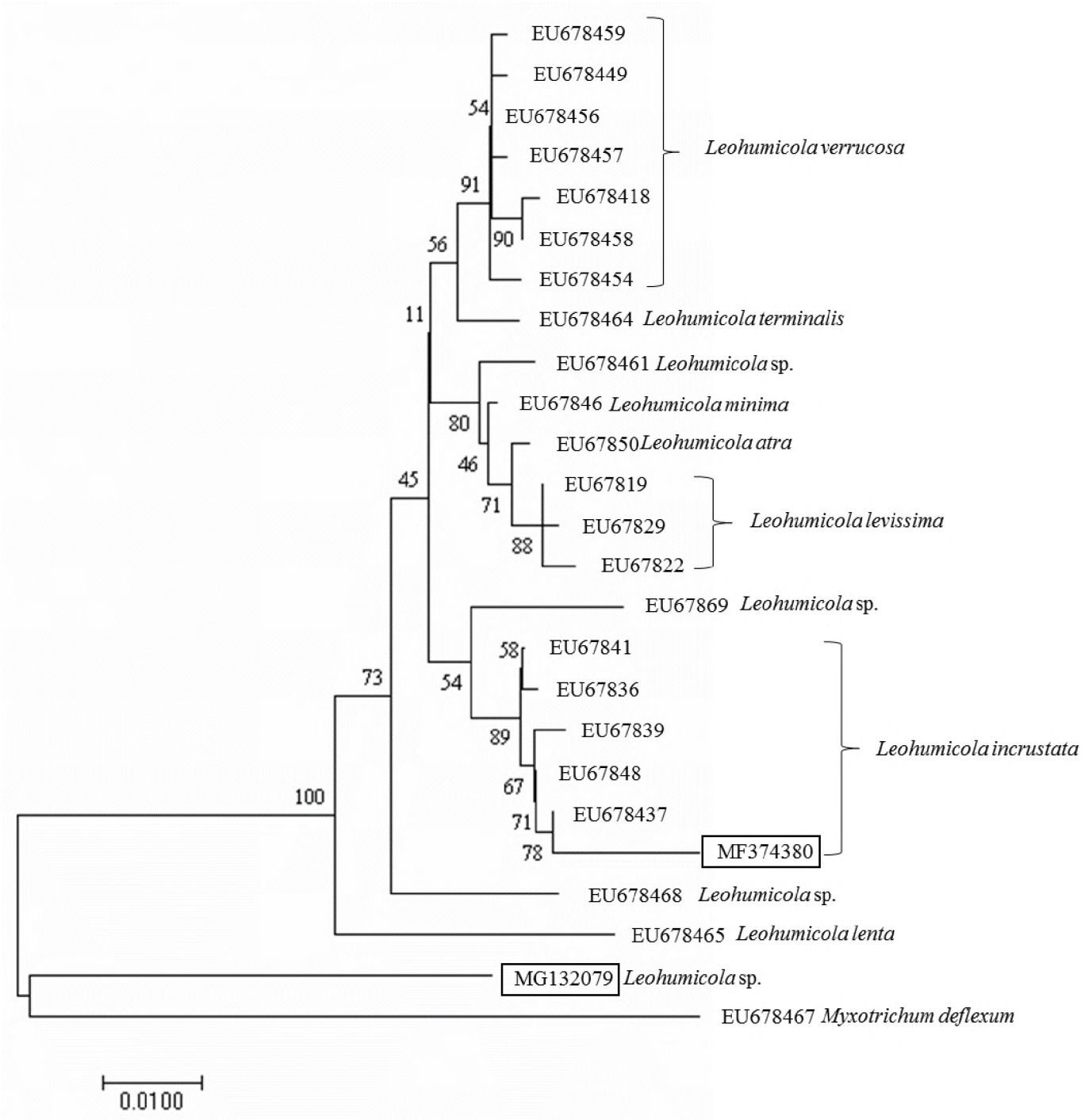


Figure 7.5. Phylogenetic tree generated from cytochrome oxidase 1 (*Cox1*) gene sequences using the Neighbour-Joining statistical method.

7.4. Discussion and conclusion

A sizable number of Ericaceae root-associated fungi have been identified through culture-based techniques and molecular analysis (Bizabani, 2015). But, there are still limited reports available on the identity of *Leohumicola* species using sequences generated through the ITS barcode region analysis. Subsequently, the *Cox1* barcode region was adopted to facilitate identification to species level allowing the specific name of the organism to be inferred. ChemRU330 isolate was inferred to be *L. incrustata*. Similar, Nguyen and Seifert (2008) reported three new species (*L. levissima, L. atra and L. incrustata*) from the United States and South Africa using both the ribosomal internal transcribed spacer (ITS) and cytochrome oxidase 1 (*Cox1*) as DNA barcodes for the identification of *Leohumicola* species, and also this observation was made by Hambleton et al. (2005). Other two isolates belonging to Hyloscyphaceae (EdRU083) and Leotiomycetes (EdRU002) used earlier for the production of amylase and cellulase enzymes (Chapter 3) were inferred to be *Leohumicola* and *Oidiodendron* sp., respectively. All results obtained in this investigation were either obtained from GenBank or UNITE databases. A large proportion of the ERM fungi and related fungi are now being identified using both GenBank and UNITE databases (Hambleton et al., 2005; Toju et al., 2012). It should be noted that UNITE database contains ITS sequences generated from identified fungal sporocarp voucher specimens (Kõljalg et al., 2005) and are suitable for identifying mycorrhizal fungi.

For phylogenetic relationships, the taxonomic status of two isolates (ChemRU330 and EdRU083) were inferred only from the *Cox1* gene sequences while ITS sequences generated in this study were inadequate for this purpose and were disregarded. Also, the results here showed that NJ statistical method is capable of producing a reasonably accurate parsimonious tree (Tamura et al., 2004). Finally, there is the need to explore the soil and rhizosphere to discover more species of ERM associations due to their role in the environment and especially the nutritional benefit that ericaceous plants derive from them, and now the possibility of using them to produce enzymes for the bio-economy.

CHAPTER 8

8.0 General discussion and conclusion

8.1. Discussion

The bio-economy is the knowledge-based production and application of renewable resources to make products, processes and services available for various economic sectors (European Commission, 2015). It makes a significant contribution by linking economic growth with environmental sustainability (European Commission, 2015). Our approach to this involved the use of mycorrhizal fungi (root endophytes) for the production of amylases, cellulases and xylanases to hydrolyse agro-based materials. This study was an opportunity for profiling some ERM fungi and examining their potential for the bio-economy.

Enzymes are vital for processing industrial, pharmaceutical and biotechnological products (Sanchez and Demain, 2011). Enzyme production has been tested in some ERM fungi other than *H. ericae*, root endophytic fungi and ECM fungi using agar screening methods (Peretto et al., 1993; Read and Perez-Moreno, 2003; Smith, 2009). *Leohumicola incrustata* (ChemRU330) was carefully selected and exploited in this study to determine its suitability for the bio-economy.

This study revealed that a comprehensive method of bioprospecting for new enzymes should involve the use of agar screening (Chapter 2) and optimisation of growth conditions to ensure optimum yield. The sequential subculturing assessment provided an insight into the consistency of producing biocatalysts of interest after several transfers (*in vitro* subculturing events) and separation from the symbiotic partner. Here, the emphasis was placed on an ericoid associated fungus with the ability to produce enzymes that would be secreted consistently without recourse to the symbiotic partner. Amylases and xylanases (co-produced with cellulases) were constantly produced throughout the monitoring period, hence their selection for purification and characterisation. For cellulases, there was a decreased production after several subculturing events. This could be attributed to the change in environment and form/type of carbon source supplied to the ERM fungus in the compounded medium (e.g., may prefer the form of carbon

provided by its symbiotic partner for cellulose production). This study suggested that production of amylases, cellulases and xylanases from *L. incrustata* might be under different regulatory controls. Enzyme production and activity by microorganisms can either be affected positively or negatively by the forms/types of energy sources in the growth medium (Srinivasan et al., 2001). Therefore, the regulatory mechanisms suggest that the cellulase system was repressed by the presence of cellulose while soluble starch and cellulose induced amylases and xylanases production, respectively, over a period (Beguin and Aubert, 1994). However, little or no information is available about the enzyme regulatory mechanisms of ERM fungi, and further investigations are required.

In Chapter 3, optimisation of nutrient parameters for maximisation of amylase and cellulase production was performed using a multi-objective approach for obtaining best yield regarding the mycelial biomass and the corresponding enzymes. Process parameters such as temperature, pH, different carbon and nitrogen supplements, and metal ions were investigated to improve amylase and cellulase activities using a one-factor at a time approach. This showed the effectiveness of selected ERM fungal isolates in the production of amylases and cellulases. The tested strains responded differently to various environmental and nutritional conditions, thus displaying enzyme activities that correlated with mycelial biomass yields. The *L. incrustata* (ChemRU330), *Leohumicola* sp. (EdRU083) and *Oidiodendron* sp. (EdRU002) were able to produce significantly higher biomass and enzyme activity in glucose when compared to their respective controls. This could be because these organisms require glucose as a source of energy for growth which is typically supplied by their symbiotic partners (ericaceous plants) in the natural environment (Rodriguez et al., 2009; Smith and Read, 2008).

Most microorganisms are capable of producing cellulases and xylanases. However, fungi (e.g. *Trichoderma*, and *Aspergillus* species) are mostly used in industries. They can be cultivated in mineral salt media, and production is induced by incorporation of required substrates (Bhat, 2000; Jampala et al., 2017; Kang et al., 2004; Sohail et al., 2009; Takahashi et al., 2013). Moreso, an MMN medium composition of pH 5.0 with 0.5% glucose, 1% yeast extract or peptone and either calcium or manganese ion was adequate for the production of either amylases or cellulases from ERM and related root endophytic fungi. Concurrent production of cellulase

and xylanase in liquid culture of *L. incrustata* was also observed. In the present study, all the three enzymes mentioned can be produced by the indigenous *L. incrustata* strain which was capable of growing on mineral salt media. *L. incrustata* utilised either glucose, cellobiose, maltose, CMC or starch, but not Avicel as a carbon source, for growth and biocatalyst production. Additionally, this investigation provides valuable information that could be valuable in the production of mycelial biomass of selected starter cultures for better mycorrhizal fungal inoculum production.

Xylanases play a significant role in processing biomass through advancement in biotechnology. The design and the application of xylanases require an understanding of the activities of these enzymes. A large number of microbes are capable of producing xylanases; however, fungi are considered most active against the hemicellulose which is a natural polymer. *L. incrustata* is a mycorrhizal fungus capable of producing endo-1,4-β-xylanase, and its usefulness for commercial production was tested for the first time. Chapter 4 revealed that the partially purified endo-1,4-β-xylanase exhibited tolerance to a varied range of pH values (pH 4.0-7.0) and was stable at 50^{o}C. Therefore, could be considered for inclusion in future industrial pentose production, fruit-juice clarification, bio-bleaching in textile, paper and pulp industries, improving rumen digestion and the bioconversion of lignocellulosic agricultural residues to ethanol (Nigam, 2013). However, further purification to homogeneity will improve its efficiency.

Starch processing in industrial settings requires an enzyme with excellent thermostability, raw-starch degradation ability and high glucose yield (Hua et al., 2014). *Aspergillus niger* is one of the most widely used microbes for the production of AMG with the optimal enzyme temperatures ranging between 45–65°C (Michelin et al., 2008). The AMG from *L. incrustata,* as observed in this study (Chapter 5), had an optimum temperature and stability comparable to those of currently used saprophytic fungi. The AMG can function at temperatures that would decrease the possibility of microbial contamination in large-scale industrial reactions of prolonged duration. The enzyme retained stability over a prolonged period of processing at a temperature ranging from 45-50^{o}C. Although the catalytic rate of *A. niger* AMG is higher than the one reported in this study, *L. incrustata* AMG compared well with most saprophytic fungi and better than those of other fungi such as *P. variotii* and *Tetracladium* sp. (Carrasco et al.,

2017; Michelin et al., 2008). Also, the AMG directly hydrolysed raw starch to glucose as the sole product, this is crucial for starch processing and will reduce energy consumption during the production of starch-based industrial products.

Additionally, analysis of the antibacterial properties of selected root-associated fungal isolates was conducted in chapter 6. Some ECM fungi and mushrooms are known producers of antibacterial compounds (Gbolagade et al., 2007; Sharma et al., 2014), while yeasts, phycomycetes and slime moulds rarely produce bioactive compounds (Bérdy, 2005). In this study, *L. incrustata* has shown the ability for its inclusion as a bioactive compound producer (Chapter 6). The results disclosed that the tested bioactive compounds had varying antimicrobial effects against pathogenic bacteria. Both Gram-negative and Gram-positives bacteria were observed to have varying degrees of antimicrobial susceptibility following 24 h of incubation. These variations showed the differences in cell surface structures between Gram-negative and Gram-positive bacteria (Puupponen-Pimia et al., 2001). The outer membrane of Gram-negative bacteria functions as a preventive barrier against hydrophobic compounds (Puupponen-Pimia et al., 2001). The results of this study demonstrated that the sensitivity of the pathogenic bacteria to phenolic compounds depends on bacterial species and the bioactive compounds. Thus, the usage of *L. incrustata* antimicrobial compounds could be alternatives for chemicals used in the preservation of food. However, further experimentation is required to determine the structures and individual components of the bioactive compounds using nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS) methods (Liu et al., 2012).

The three isolates (ChemRU330, EdRU83 and EdRU002) used for the production of enzymes and antibacterial agents (Chapter 3-6) were identified to genus and species levels using both ITS and *Cox1* gene barcode regions. The molecular analysis results indicated that these ERM mycorrhizal fungi were similar to those successfully described by some researchers in South Africa and Australia. The PezizF/PezizR primer pairs resulted in robust amplification reactions for all DNA extracted.

For clarity sake, we only worked with some of the fungi already isolated by Bizabani (2015) from Albany Centre of Endemism, South Africa, and a pot trial as demonstrated by Bizabani (2015) showed that the *Leohumicola* sp. (ChemRU330) belongs to ericoid mycorrhizal fungi. Also, the current study is exploratory, and the suitability of these fungi for the bio-economy and bioremediation is an ongoing project.

8.2. Future perspectives

The advancement of modern biotechnology (e.g., protein engineering) has provided a platform for purifying and stabilising enzymes. Their application to fungal amylases and xylanases studies are advantageous and could lead to better stability and effectiveness towards starch and xylans hydrolysis which would make these biomolecules amenable to the specific industrial application. sAlso, increased awareness about environmental implications of the use of chemicals in many industries has opened up a way to explore new microbial enzymes for more energy efficient, cost-effective, and environmentally friendly means. This would help to speed up the research and understanding of mycorrhizal enzyme production. The upsurge of bioinformatics and access to complete genome sequences make it possible to design amylases and xylanases with industrially desirable features. Consequently, for future study, the following can be performed to improve efficiency: (i) molecular characterisation of the DNA sequences of AMG and endoxylanase; (ii) molecular cloning and expression of the ERM mycorrhizal genes. The recombinant DNA techniques will help in cloning the AMG gene into a competent cell of either *E. coli* or *Saccharomyces cerevisiae*, which can facilitate production under reduced growth periods. Though this method has not been applied to study any ERM biocatalyst genes, using the knowledge of earlier successful technologies (e.g., *A. niger* and *Lentinula edodes*), could direct this line of research (Hmida-Sayari et al., 2012). Some other AMG-encoding fungal gene already cloned include; *A. awamori* (Goto et al., 1994), *A. oryzae* (Hata et al., 1991) and *Neurospora crassa* (Stone et al., 1993).

8.3. Conclusion

The bioprospecting for amylases, cellulases and xylanases in mycorrhizal fungi provides essential characteristics that would further enhance their use in the agricultural sector, could play a significant role in bioremediation purposes, as well as encourage their use in food and biodiesel industries. This study is a pilot investigation to producing mycorrhizal enzymes for commercial purposes. Most studies on mycorrhizal biocatalysts have focused mainly on their use as a strategy for nutrient acquisition and the role they play in ecosystems. There is a need for future research into these biomolecules which will be beneficial to humanity. The physicochemical properties, hydrolysis end-products, and being non-pathogenic make AMG and endo-1,4-β-xylanase potential candidates for future applications as additives in the food industry for the production of glucose, glucose syrups, high-fructose corn syrups, as well as the production of ethanol. Finally, the results underpin the hypothesis that if the right conditions of growth are met, some ericoid mycorrhizas and root endophytic fungi can be used in the production of the desired amylases, cellulases and xylanases using chemically defined media.

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Appendices

Appendix A

Modified Melin Norkrans (MMN) Marx, 1969

Glucose	10.0 g
Malt extract	3.0 g
Ammonium tartrate [$(NH_4)_2C_4H_4O_6$]	0.25 g
KH_2PO_4	0.5 g
$MgSO_4.7H_2O$	0.15 g
$CaCl_2$	0.05 g
$FeCl_3$ (1% solution)	1.2 ml
NaCl	0.025 g
Thiamine-HCl	100.0 µg
Agar	1.5%
Distilled water	1.0 L

Autoclave at 121°C for 15 min

Appendix B

Dinitrosalicylic acid (DNSA) reagent

Sodium hydroxide (NaOH)	1.0 g
Potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$)	20.0 g
3,5-Dinitrosalicylic acid ($C_7H_4N_2O_7$)	0.2 g
Phenol (C_6H_6O)	0.05 g
Sodium metabisulfite ($Na_2S_2O_5$)	100 ml

Bradford assay protocol

Preparation of 10 ml of a 1–10 µg/ml protein sample

1 mg/ml = 0.001 g BSA

10 mg / 10 ml = 0.01 g BSA

Therefore, dissolve 0.01 g BSA in 10 ml buffer

Add, 100 µl BSA to 9900 µL buffer

Final concentration (BSA stock) = 10 µg/ml

BSA concentrations were prepared as shown in table bellow

Protein concentration (µg/ml)	Volume of BSA stock (µl)	Volume of buffer (µl)
0	0	1000
1	100	900
2	200	800
4	400	600
6	600	400
8	800	200
10	1000	0

A 96-well microassay protocol

1. The Bradford reagent was gently mixed in the bottle and brought to room temperature.

2. Protein standards were prepared in buffer ranging from 1–10 µg/ml using a BSA standard.

3. A 150 µl of each protein standard was added to separate tubes, and to the blank, 150 µl of buffer was added.

4. The unknown sample(s) with an approximate concentration of 1–10 µg/ml was prepared, and 150 µl of each sample was added to separate tubes.

5. A 150 µl of the Bradford reagent was added to each of the tube and mixed.

6. The samples were incubated at room temperature for 30 minutes.

7. Samples were transferred into a 96-well microtitre plate, and the absorbance read at 595 nm.

8. The net absorbance was plotted against the protein concentration of each standard to construct a standard cure.

9. The protein concentration determination of the unknown samples were extrapolated from the standard curve.

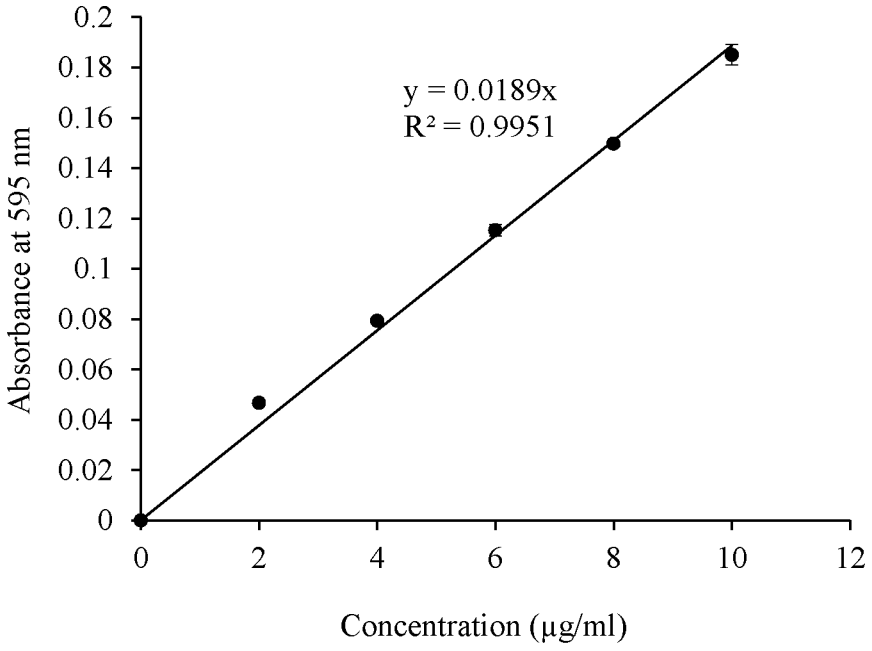
Glucose/xylose standard

A 10 mg/ml stock solution was prepared using 0.1 g glucose/xylose in 10 ml citrate phosphate buffer (pH 5.0), and various concentrations of glucose were prepared as shown in the table below. A magnetic rod was inserted into the Schott bottle containing the substrate and was placed on a revolver until the substrate were each dissolved.

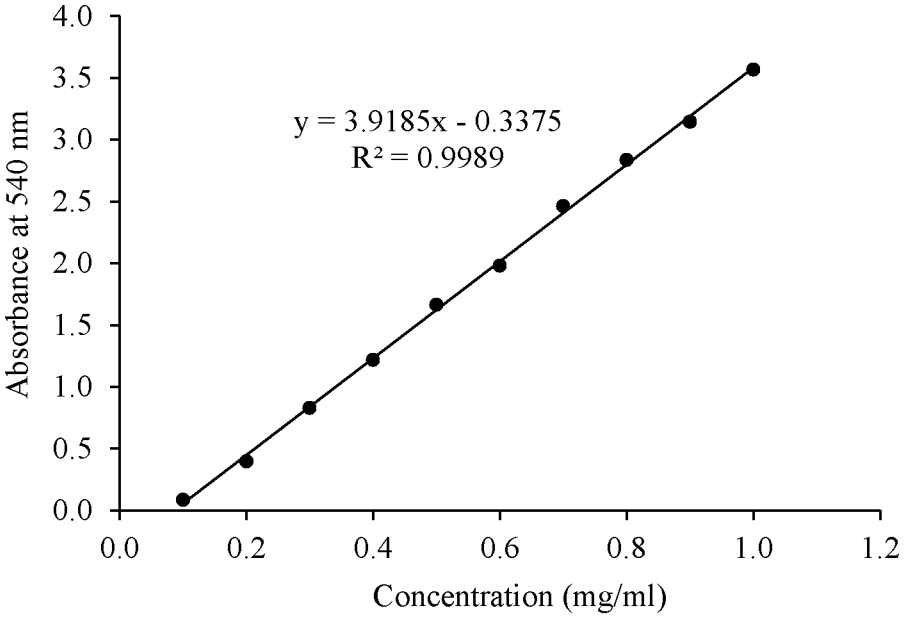
Glucose/xylose concentrations table

Concentration (mg/ml)	Volume of stock used (µl)	Volume of buffer (µl)
0	0	1000
0.1	10	990
0.2	20	980
0.3	30	970
0.4	40	960
0.5	50	950
0.6	60	940
0.7	70	930
0.8	80	920

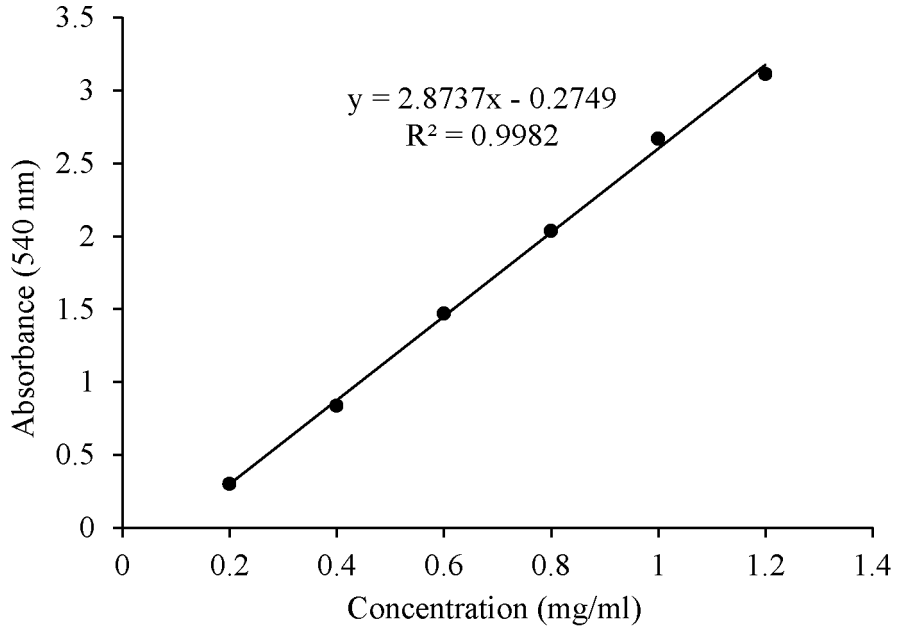
0.9	90	910
1.0	100	900



Protein standard curve (BSA)



Glucose standard curve



Xylose standard curve

Appendix C

SDS-PAGE of proteins and reagent recipes

10X Running buffer

Tris base	30.0 g
Glycine	144.0 g
SDS	10.0 g
Distilled water	1000 ml
pH	8.3

4X Stacking gel buffer

Tris base	60.5 g
Distilled water	850 ml

The pH was adjusted to 6.8 with 6M HCl. Water was added to make 1000 ml and was stored at 4°C.

4X Resolving gel buffer

Tris base	181.5 g

Distilled water	850 ml

The pH was adjusted to 8.8 with 6M HCl. Water was added to make 1000 ml and was stored at 4°C.

2X SDS-PAGE sample buffer

4X stacking gel buffer	2.0 ml
Glycerol	1.6 ml
10% SDS	3.2 ml
2-mercaptoethanol	0.8 ml
1.0% bromophenol blue	0.4 ml

n-Butanol (H_2O-saturated)

n-butanol	50 ml
Distilled water	50 ml

Ammonium persulfate (10%)

Ammonium persulfate	1.0 g
Distilled water	10 ml

The stock solution was prepare every 2-3 weeks.

30% Acrylamide stock solution

Acrylamide	29.22 g
Bisacrylamide	0.78 g
Distilled water	100 ml

The stock solution was filtered and store at 4°C.

Coomassie gel stain

Coomassie blue R250	1.0 g
Methanol	450 ml
Distilled water	450 ml
Glacial acetic acid	100 ml

Coomassie gel destain

Methanol	100 ml
Glacial acetic acid	100 ml
Distilled water	800 ml

Appendix D

DNA Extraction protocol (Zymo Research)

1. About 40 mg wet fungal mycelia were weighed and placed in a ZR BashingBead™ lysis tube (0.1 and 0.5 mm). A 750 µl lysis solution was added to the tube.
2. The tube was secure in a bead beater fitted with a 2 ml tube holder assembly and was processed at maximum speed for 5 min.
3. The ZR BashingBead™ lysis tube (0.1 and 0.5 mm) was centrifuged in a microcentrifuge at 10,000 x *g* for 1 min.
4. A 400 µl supernatant was transferred to a Zymo-Spin™ IV spin filter (orange top) in a collection tube. The base of the filter was snapped off and centrifuged at 7,000 x *g* for 1 min.
5. A 1,200 µl of genomic lysis buffer was added to the filtrate in the collection tube from Step 4.
6. An 800 µl was transferred off the mixture from Step 5 to a Zymo-Spin™ IC column in a collection tube and centrifuge at 10,000 x *g* for 1 min.
7. The flow-through was discarded from the collection tube and step 6 repeated.
8. A 200 µl DNA pre-wash buffer was added to the Zymo-Spin™ IC column in a new collection tube and centrifuged at 10,000 x *g* for 5 min.
9. A 500 µl g-DNA wash buffer was added to the Zymo-Spin™ IC column and centrifuged at 10,000 x *g* for 1 min.
10. The Zymo-Spin™ IC column was transferred to a clean 1.5 ml microcentrifuge tube, and 50 µl DNA elution buffer was added directly to the column matrix. The sample was incubated at room temperature for 1 min and then centrifuged at 10,000 x *g* for 30 seconds to elute the DNA.

DNA purification protocol (Promega)

A. Dissolving the gel slice

1. DNA band was excised from the gel and placed in a 1.5 ml microcentrifuge tube.
2. A 10 µl membrane binding solution per 10 mg of gel slice was added. The mixture was vortexed and incubated at 50–65°C until gel slice was completely dissolved.

B. Processing PCR Amplifications

1. An equal volume of membrane binding solution was added to the PCR amplification.

Binding of DNA

1. SV minicolumn was into the collection tube.
2. The dissolved gel mixture or prepared PCR product was transferred to the Minicolumn assembly and incubated at room temperature for 1 min.
3. The sample was centrifuge at 16,000 × *g* for 1 min. The flowthrough was discarded, and SV minicolumn reinserted into the collection tube.

Washing

4. A 700 µl membrane wash solution (ethanol added) was added to the SV minicolumn. Centrifuged at 16,000 × *g* for 1 min. The flowthrough was discarded, and SV minicolumn reinserted into the collection tube.
5. Step 4 was repeated with 500 µl membrane wash solution. Centrifuged at 16,000 × *g* for 5 min.
6. The collection tube was emptied and the column assembly recentrifuged for 1 min with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

Elution

7. The SV minicolumn was carefully transferred to a clean 1.5 ml microcentrifuge tube.
8. A 50 µl of nuclease-free water was added to the minicolumn and incubated at room temperature for 1 min. Centrifugation was performed at 16,000 × *g* for 1 min.
9. The minicolumn was discarded and DNA stored at –20°C.

Agarose gel (1%)

Agarose powder	1 g
Distilled water	100 ml

EDTA (0.5 M, pH 8.0)

Disodium EDTA.2H2O	186.1 g
Distilled water	800 ml

Stir vigorously on a magnetic stirrer and adjust the pH with NaOH pellets.

TE (Tris/ EDTA) Buffer (pH 8.0)

Tris/ HCI (10 mM), EDTA (10 mM)

Preparation of 5X TBE (Tris-EDTA) Buffer

Tris base	5.3 g
Boric acid	27.5 g
EDTA (0.5M, pH 8.0)	20 ml
Distilled water	1000 ml

1X TBE Buffer (working solution)

5X TBE	200 ml
Distilled water	800 ml

Primers information

S/N	Primer	Sequence	Tm^{o}C
1	PezizF	5'-TCAGGRTTAYTAGGWACAGCATTT-3'	53.9
2	PezizR	5'-ACCTCAGGRTGYCCGAAGAAT-3'	58.2
3	ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	59.6
4	ITS4	5'-TCCTCCGCTTATTGATATGC-3'	52.1

>*Cox1* [ChemRU330]

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