SCREENING THE GUT OF DUNG BEETLES AND DUNG BEETLE LARVAE FOR HEMI-CELLULOLYTIC FUNGI AND ENZYMES FOR APPLICATION IN THE BIOFUEL INDUSTRY

BY

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DECLARATION

I declare that the thesis hereby submitted to the University of Limpopo for the degree of Doctor of Philosophy in Microbiology has not been previously submitted by me for a degree at this or any other university; that it is my work in design and execution and that any material herein contained has been duly acknowledged.

Signature

Date

23 May 2021

Dedication

I dedicate this work to the ALMIGHTY GOD, the journey was long and hard, from losing my parents to watching my older brother Christopher suffers the after effect of several strokes, to sitting on the side of the bed with my husband on life support, to supervisor stepping out of the study and the passing away of a co-supervisor, watching the hit of COVID19. I think about it and I wonder how I got here. Father God you alone are faithful and trustworthy.

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SUMMARY

Biofuel production from lignocellulose material is an attractive alternative to fossil fuel. The use of lignocellulose material for biofuel production is imperative because of the numerous advantages that it offers. Biofuel is environmentally friendly and in developing countries such as South Africa, it has the potential to reduce the use of imported fuel and create jobs. Currently, several constraints are affecting the implementation of biofuel. One of the constraints is the cost-effectiveness and the efficiency of the enzymes involved in the enzymatic degradation of lignocellulose polymers to monomers, which can further be fermented to bioethanol. The potential way to reduce enzymatic degradation cost could be by supplementing the fungal enzymes with accessory enzymes such as endo-xylanase. The enzyme production cost is also dependent on the carbon source used. Lignocellulose materials that are regarded as waste must be assed for their use as enzyme inducer carbon sources and as biomass for biofuel production. This is a potential route that will reduce enzyme and biofuel production costs. Biofuel production cost can further be reduced by finding a yeast that can ferment xylose and ferment in the presence of inhibitors released during lignocellulose pretreatment. This study sought to tackle the enzymatic hydrolysis constraints and also search for xylose-fermenting yeast by exploring the gut microbiota of dung beetle. The gut of the dung beetle has recently received great attention since it is proposed to be a bioreactor for lignocellulolytic microorganisms that can be used in biofuel applications. This is because dung beetles feed on the dung of herbivorous animals and the dung is composed of 80% undigested plant material. In this study the guts of four Scarabaeidae dung beetles *Kheper nigroanaeus Boheman, Heteronitis castelnaui*, *Pachylomerus femoralis*, *Anachalcos convexus* and dung beetle larvae, *Euoniticellus intermedius* were screened for hemicellulolytic fungi and xylose-fermenting yeast. Hundred and thirty-two yeast isolates and two-hundred and twenty-two filamentous fungi were isolated and identified using ITS and D1/D2 regions. The yeast isolates were assigned to 8 genera and 18 species, *Trichosporon* was the most dominant genus while *Candida tropicalis* was the most dominant specie. Some of the yeast isolates were identified as uncultured fungi. This yeast must be characterised to be certain if they are novel species. The fungal isolates were assigned to 12 genera and 25 species, *Aspergillus* was the most dominant genus while *Hypocrea lixii* was the most dominant specie. The yeast isolated could assimilate

xylose and could grow at a maximum temperature of 40 °C. Furthermore, these yeast isolates could also grow in the presence of 3 g/L acetic acid. Most of the fungal isolates had xylanolytic activity. The phylogenetic analysis revealed close genetic relatedness between isolates from the different dung beetle species and dung beetle larvae. The profile of the fungal genera was similar in the different dung beetles. Both guts and the larvae had *Aspergillus*, *Hypocrea, Trichoderma, Talaromyces and Penicillium*. The filamentous fungi that showed good xylanolytic activity were further screened for their ability to produce xylanase enzyme using thatch grass as an inductive carbon source. Thatch grass was selected in this study since it is in-house plant-based biomass. Thatch grass is abundantly available in South Africa; it is used for animal grazing but the more it grows it loses its nutritional content. Once it reaches this stage, it is no longer used and most of it is burnt. The fire from burning grass contains higher levels of nitrogen-containing chemicals that pollute the environment. Its compositional analysis (cellulose 46%, hemicellulose 27% and lignin 10%) also attributed to its selection as potential inductive carbon and attractive lignocellulose biomass for biofuel production. The higher xylanase activity of 283.43, 270 and 287.03 nkat/ml were observed from *Aspergillus fumigatus* L1XYL9 (*Euoniticellus intermedius* larvae), *Hypocrea lixii* AB2A3 and *Neosartotya sp* AB2XYL20 (*Anachalcos convexus*), respectively. This was achieved when acid pretreated thatch grass was used as an inductive carbon source. *Aspergillus fumigatus* L1XYL9 (*Euoniticellus intermedius* larvae), *Hypocrea lixii* AB2A3 and *Neosartotya sp* AB2XYL20 (*Anachalcos convexus*) showed xylanase activity of 393,22, 313,06 and 200 nkat/ml when grown on synthetic xylan. *Neosartotya sp* AB2XYL20 showed higher xylanase activity on thatch grass. The suitable production process for xylanase enzyme on acid pretreated thatch grass was assessed by conducting a comparative study on solid-state and submerged fermentation using L1XYL9 (*Euoniticellus intermedius* larvae), *Hypocrea lixii* AB2A3 and *Neosartotya sp* AB2XYL20 (*Anachalcos convexus*) as the best xylanase producer on acid pretreated thatch grass. The strain showed better xylanase activity when submerged fermentation was used. In this study, *Hypocrea lixii* AB2A3 was selected for further studies since it was the most dominant species and also showed good xylanase activity. Thatch grass was pretreated differently to evaluate the suitable chemical for pretreating thatch grass. Thatch grass was pretreated with dilute sulphuric acid 1.2% and maintained the pH of 5.5 by using sodium hydroxide while another batch was pretreated the same way and was washed with distilled water till

pH of 5.5. The other batch was then pretreated with ammonium solution and was also washed with distilled water to maintain a pH of 5.5. The above-mentioned pretreated thatch grass was tested as an inductive carbon source as well as untreated thatch grass. The xylanase activity was determined to assess a good inductive carbon. All the thatch grass pretreated and washed with distilled water showed very low xylanase activity. The untreated thatch grass resulted in lower xylanase activity as compared to xylanase activity achieved when pretreated thatch grass was used. Parameters such as agitation speed and initial inoculum size were also assessed during xylanase production by *Hypocrea lixii* AB2A3 on acid pretreated thatch grass. Xylanase activity increased from 525 nkat/ml (Inoculum size 2×10^6 spore/ml and agitation speed 150 rpm) to 584.8 nkat/ml (Inoculum size 2×10^6 spore/ml and agitation speed 200 rpm). The crude xylanase from *Hypocrea lixii* AB2A3 was used to hydrolyse acid pretreated thatch grass. This resultant in xylose yield of 138 mg/g of substrate and glucose yield of 49 mg/g of substrate. Crude xylanase was mixed with commercial celluclast™. This enzyme mixture resulted in a xylose yield of 128 mg/g substrate and a glucose yield of 549 mg/g of substrate. The results obtained in this study show that indeed gut of the dung beetles and dung beetle larvae are a rich source of microorganisms that can play an important role in biofuel application and remediating the environment by degrading plant-based biomass regarded as waste into valuable products. It is imperative to evaluate the gut microbiota of dung beetles from different regions in South Africa for their application in the biofuel industry to reinforce its implementation. Thatch grass is a potential inductive carbon and lignocellulose biomass for biofuel production.

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CHAPTER 1

1.1. INTRODUCTION

Fossil fuel is a restricted energy source that will eventually run out. Currently, the world is dependent on fossil fuels such as coal, gas, and petroleum (Ragauskas *et al.*, 2006 & Prasad *et al.*, 2019). Burning fossil fuels contributes to the emission of greenhouse gases and this leads to global warming, which has an adverse effect on the environment (IPCC, 2014 & Carbon Footprint, 2018). As fossil fuel becomes limited, its price will increase, and this will consequently increase food prices worldwide. Escalating food prices will have a bigger impact on the socio-economic factors in developing countries, especially those in the Sub-Saharan region as compared to developed countries (Charles *et al.*, 2018).

Biofuel is an alternative fuel that could be used in the transportation industry (Lynd *et al.*, 2017 & Ciolacu, 2018). It is produced from biomass derived from plant-based material. The advantages of biofuels are that it is environmentally friendly, renewable, sustainable, and has the potential to meet transportation fuel requirements in both developing and developed countries. Furthermore, the use of biofuels will add value by stimulating economic growth, especially in a country such as South Africa with vast farming opportunities (Mutombo and Numbi, 2019). Bioethanol is one type of biofuel, where ethanol is produced by fermenting simple sugars and starch components from plant material using microorganisms.

Plant material, especially lignocellulose material, is an ideal source for bioethanol production and will not have an impact on the environment and the economy (Kumar and Sharma, 2017). Lignocellulosic biomass includes agricultural and municipal waste, waste from the pulp and paper industry as well as dedicated energy crops, wood, and grasses that do not compete directly with food and fiber production (Prasad *et al.*, 2019). Lignocellulose is composed of cellulose, hemicellulose, and lignin. The cellulose and hemicellulose fractions are especially rich in carbohydrates that can be fermented into ethanol (Ciolacu, 2018).

Three processes are crucial in the conversion of lignocellulosic biomass to ethanol. These processes include pretreatment of the biomass, enzymatic hydrolysis of the exposed polysaccharides, and fermentation of the released sugars. Pretreatment alters the structure of the lignocellulose complex and exposes the cellulose, hemicellulose, and lignin to be accessible to enzymatic hydrolysis. The enzymatic hydrolysis releases sugars from polysaccharide, which are then fermented into ethanol (Anwar *et al*., 2014).

Enzymatic hydrolysis is an effective, viable, convenient and environmentally friendly process that can result in the release of up to 90% of the sugars under optimised conditions (Badhan *et al.*, 2007). Cellulases and hemicellulases work in synergy during enzymatic hydrolysis to degrade cellulose and hemicellulose to simple sugar. The cellulase enzyme complex is composed of endoglucanases, cellobiohydrolases and β-glucosidases. On the other hand, the β-1,4 endoxylanase and β-xylosidase enzymes hydrolyse xylan, which is the major hemicellulose (Badhan *et al.*, 2007 & Van den Brink and De Vries, 2011). For successful and efficient enzymatic hydrolysis, both cellulase and hemicellulase enzymes are required to hydrolyse the lignocellulosic plant biomass. Conversely, the production cost of these enzymes is very high and this hinders the industrialisation of ethanol production from lignocellulosic biomass (Bussamra *et al*., 2015 & Costa *et al.*, 2016). The mining of microorganisms that produce novel lignocellulolytic enzymes on cheap substrates can mitigate the current high costs associated with the production of these enzymes (Gonçalves *et al.*, 2016; Ajijolakewu *et al.*, 2017 & Garcia *et al.*, 2018). Inexpensive substrates such as agro-industrial residues are used in the production of cellulases and hemicellulases instead of commercially purified, such as cellulose and xylan. The hydrolytic activity of enzymes isolated from agro-industrial residues is potentially better than those isolated on synthetic media when hydrolysing lignocellulosic material (Uday *et al.*, 2017; Souza *et al.*, 2018 & Siqueira *et al.*, 2020).

All the fermentable sugars released during enzymatic hydrolysis need to be fermented to ethanol to ensure the process is commercially viable. Microorganisms, such as *Saccharomyces cerevisiae* and *Zymomonas mobilis,* are capable of fermenting glucose but cannot ferment xylose (Canilha *et al.*, 2012). Pentose fermenters include yeast strains such as *Scheffersomyces stipitis, Scheffersomyces shehatae and Pachysolen tannophilus,* which naturally ferments xylose to ethanol (Saha, 2003;

Mussatto *et al*., 2012; Urbina and Blackwell, 2012 & Coda *et al*., 2013), although not as effectively as *S. cerevisiae f*erments glucose. These xylose-fermenting yeast are sensitive to inhibitors formed during the pre-treatment process such as furfural, hydroxymethylfurfural and acetic acid (Verduyn *et al*., 1985; Lee *et al*., 2003 & Kwak *et al*., 2019). Notably, *S. stipitis* is currently the best xylose-fermenting yeast (Jeffries *et al*., 2007 & Agbogbo and Coward-Kelly, 2008), however, it has a low tolerance for ethanol and inhibitors, and this hinders its use in large-scale bioethanol production from xylose in industry. There is still a need to find a yeast that can ferment the lignocellulosic hydrolysate (both glucose and xylose) efficiently with high yields and high tolerance to ethanol, sugar concentration, and inhibitors released during the pretreatment step (Moyses *et al.,* 2016).

Dung beetles feed on plant roots, decaying organic matter, and animal waste of very low nutritional value (Rojas-Jiménez and Hernández, 2015). This suggests that the gut of these insects could be a rich source of cellulolytic and hemicellulolytic microorganisms that can degrade the polysaccharides commonly found in plant matter (Zhang and Jackson, 2008). This makes the gut of the dung beetles a possible bioreactor that could be useful in the lignocellulose bioethanol industry (Huang *et al.*, 2010).

1.2. AIMS AND OBJECTIVES

1.2.1. Aim

The aim of this study was to investigate the gut of adult dung beetles and dung beetle larvae for hemicellulolytic fungi and xylose-fermenting yeast.

1.3. Objectives

The objectives of this study were to:

i. Isolate and identify fungal strains from the gut of dung beetles that can grow on different substrates.
ii. Isolate and identify all yeast isolates that can ferment xylose.
iii. Screen for fungal isolates for xylanase activity on xylan.
iv. Screen isolates to identify the best hemicellulolytic fungi and xylose-fermenting yeast.

v. Screen the best hemicellulolytic fungi for xylanase production on pre-treated thatch grass.

vi. Hydrolyse the pre-treated thatch grass using the isolated xylanase.

1.4. Research questions

- Literature suggests that the gut of a dung beetle should be a rich bioreactor of microbiota such as hemicellulolytic fungi and xylose-fermenting yeast. Are the gut of dung beetles and larvae in South Africa a rich source of lignocellulose-degrading microorganisms with potential application in the biofuel industry?
- Can the use of thatch grass as a carbon inducer of the gut microbiota with xylanolytic activity increase their xylanolytic activity?
- Can the resultant crude xylanase preparation result in the complete degradation of hemicellulose in thatch grass?

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CHAPTER 2

2.1. LITERATURE REVIEW

2.1.1. Renewable energy

Renewable energy is produced from natural and sustainable sources and it is receiving more attention worldwide due to insecurity in the energy supply, air pollution due to the use of fossil fuel, resource diversification, and the depletion of fossil fuels. Fossil fuel is not renewable; unsustainable and not readily available. Fossil fuel is predicted to be depleted in the next 40 – 50 years, due to a high consumption rate (Zabed *et al.*, 2017). However, renewable energy as an alternative energy source is sustainable, renewable, and environmentally friendly. The availability of plant biomass for biofuel production (a renewable energy source) in South Africa is abundant since the country is rich in forestry and available land for agriculture (Mutombo and Numbi, 2019). The use of biofuel in developing countries will lead to job creation and less dependence on imported fuel (Naicker and Thopil, 2019).

Biofuel is produced from organic material (biomass), such as plants and animal wastes (Demirbas *et al*., 2009). Bioethanol is a type of biofuel, which is ethanol produced by fermenting simple sugars and starch components from plant material using microorganisms. Ethanol in its pure form can be used as a vehicle fuel or can be blended with petroleum fuel (Stamenković *et al.*, 2020).

Bioethanol is divided into three generations, which depend on the type of substrate used for ethanol production (Table 2.1**)**. **First-generation bioethanol** is produced from food-based plant sources, such as starch from maize, mostly produced in the United States of America, and sucrose from sugar cane, commonly found in Brazil (Mączyńska *et al.*, 2019). Other substrates used in first-generation bioethanol include soybeans, potato, beet, coconut, camelina and cassava (Binod *et al.*, 2019). The production and use of first-generation bioethanol in developing countries, such as South Africa, will harm economic growth due to increased food prices. The South African government has therefore restricted the use of certain food crops, such as maize and sugarcane, for the production of biofuels (Stafford *et al.*, 2019).

Second-generation bioethanol is produced from inedible plants or the inedible parts of food crops and waste material. This includes lignocellulosic residues from agricultural and forestry sectors, as well as dedicated energy crops (Priefer *et al.*, 2017). Food production is not affected if energy crops are cultivated on marginal land (Amigun *et al*., 2011). Lignocellulose is omnipresent, generally available at a low cost, high in carbohydrates and does not influence food production (Taha *et al.*, 2016 & Bhatia *et al.*, 2017). The limitation in the use of second-generation bioethanol production is the cost of converting lignocellulose to ethanol. Thus far the cost and the effectiveness of the enzymes involved in enzymatic degradation of lignocellulose to simple sugars are of concern (Passos *et al*., 2018 & Chandel *et al.*, 2019).

Third-generation bioethanol is produced from algae. Various microalgae, such as *Chaetocero scalcitrans, Isochrysisgal bana, Nanochloropsis sp., Schizochytrium limacinum, Chlorella species, Scenedesmus,* and *Botryococcus braunni* have been identified for third-generation bioethanol production (Robak and Balcerek, 2018 & Prasad *et al.*, 2019). Bioethanol from microalgae is sustainable and does not impact the environment negatively or influence food production (Chew *et al.*, 2017). Currently, biofuel produced from algae is not economically feasible, due to the production, harvesting and processing costs that still need to be addressed (Chowdhury and Loganathan, 2019).

Table 2.1. The different generations of biofuels and biomass sources (Ciolacu, 2018 & Ghaemi *et al*., 2019).

Biofuel	**First-generation biofuel**	**Second-generation biofuel**	**Third-generation biofuel**
Type of substrate	Seeds, grains, or sugar	Lignocellulose biomass	Algae, seaweeds
Source of biomass	Wheat Barley Corn Potato Sugar beet Sugar cane Rapeseed Soybeans Sunflower Palm Coconut Jatropha	**Woody substrates** hardwood [birch, aspen] **Softwood** (spruce, pine) **Agricultural residues** (wheat straw, sugarcane bagasse, corn stover), **Dedicated energy crops** (switchgrass, willow, hemp, Miscanthus) **Weedy materials** (Eichhornia crassipes, Lantana camara), Municipal solid waste (food and kitchen waste).	microalgae

2.1.2. Lignocellulose

Lignocellulose is the most abundantly available renewable energy resource on earth. Lignocellulose consists mainly of cellulose, hemicellulose and lignin (Figure 2.1). These polymers interact with each other in a hetero-matrix to different degrees and varying relative composition depending on the type, species and exact composition depend on the origin of the lignocellulosic material (Table 2.2) (Menon and Rao, 2012; Anwar *et al*., 2014 & Zaiton and Norfaryanti, 2019). Sources of lignocellulosic biomass include agricultural and forest residues (e.g., palm empty fruit bunch, sugarcane bagasse, wheat straw and leaves, coconut husks, corn stover, empty fruit bunches, and wood), energy crops (empty fruit bunches and different types of grasses) and industrial wastes (waste paper) (Bhatia *et al.*, 2017). The structure of lignocellulose makes it very recalcitrant and this hinders the hydrolysis by enzymes (Zhang *et al.*, 2016 & Mohapatra *et al.*, 2017). This structural robustness of plant cell walls is due to the lignin and hemicellulose linkage (Ghaemi *et al*., 2019). Several factors affect the structure of lignocellulose, namely, (1) the amount of lignin (2) the hemicellulose coating of the cellulose fibers (3) the degree of cellulose crystallinity and (4) cellulose surface area and strength of cellulose fibers. Addressing these factors will reduce the strength of the lignocellulose structure and make it vulnerable to hydrolysis.

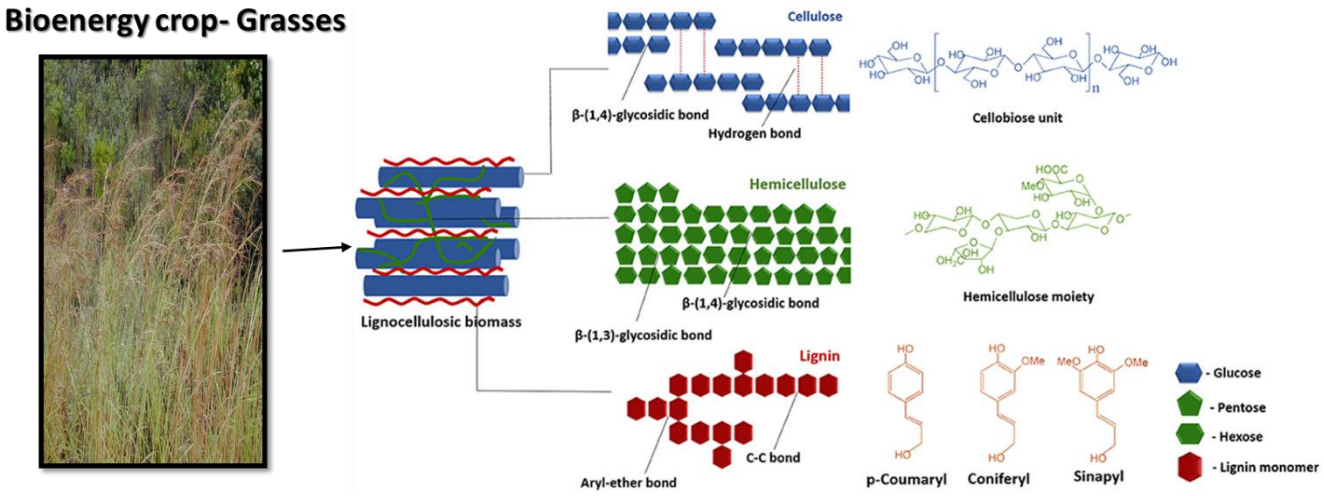

Figure 2.1. Schematic representation of the structure of lignocellulose biomass (Cellulose, hemicellulose, and lignin), modified from Kumar *et al*. (2009)

Table 2.2. The composition of cellulose, hemicellulose, and lignin in different lignocellulosic biomass substrates (Ghaemi *et al*., 2019).

Types of plant biomass	Lignocellulosic biomass	Cellulose (%)	Hemicellulose(%)	Lignin (%)
Agricultural waste	● Wheat straw	30	50	15
	● Corn stover	39 – 42	22 – 28	18 – 22
Forest waste	● Hardwoods	40 – 55	24 – 40	18 – 25
	● Softwoods	45 – 50	25 – 35	25 – 35
Energy crops	● Empty fruit bunch	41	24	21.2
	● Switchgrass	45	31.4	12
	● Grasses	25 – 40	35 – 50	10 – 30
	● Coastal Bermuda grass	25	35.7	6.4
Industrial waste	● Waste paper from chemical pulps	60 – 70	10 – 20	5 – 10

2.1.2.1. Cellulose

Cellulose is a linear, crystalline polymer of β-D-glucose units. It is rigid in structure and resists degradation. Cellulose is the main constituent of plant cell walls (up to 50% of the plant cell wall). This polymer is a polycrystalline aggregate consisting of crystalline and non-crystalline or amorphous components. As represented in Figure 2.2 cellulose is formed by crystalline regions separated by amorphous or less crystalline regions. Hemicelluloses and lignin are amorphous substances, whereas cellulose has ordered (crystalline) regions. Cellulose fibers have repeating glucose units linked by β-1,4 glycosidic bonds that are tightly bound together to form the crystalline structure with inter-and intramolecular bonds cellulose with a hydroxyl group bonded together, display their hydrophobic property and are insoluble in water. The crystalline cellulose consists of a higher percentage of cellulose as compared to the amorphous cellulose. The amorphous cellulose is readily available for degradation as compared to cellulose in its crystalline form (Menon and Rao, 2012; Tian *et al.*, 2018 & Ghaemi *et al*., 2019).

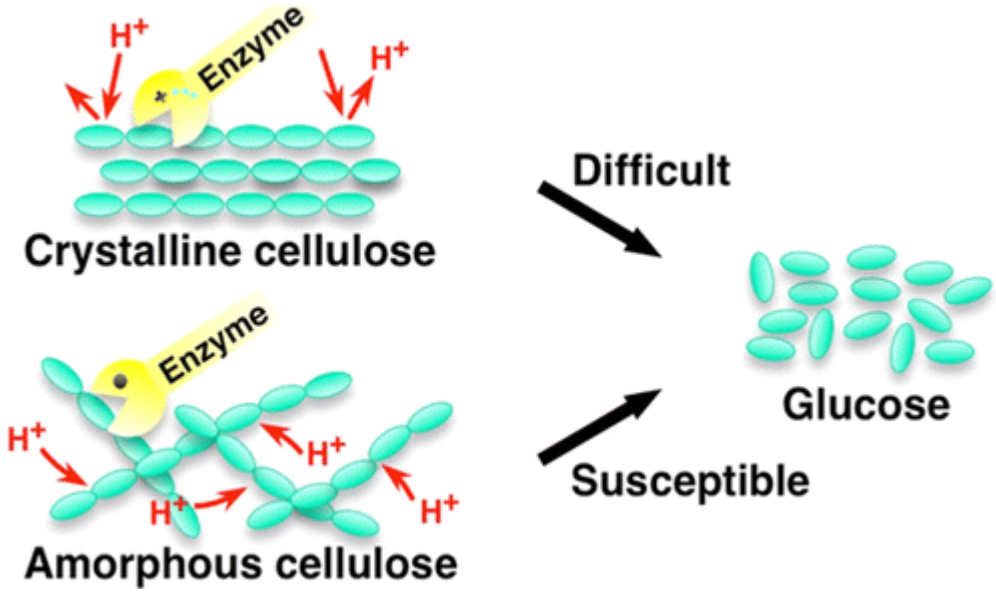

Figure 2.2. The difference between crystalline and amorphous regions of cellulose (Hattori and Arai, 2016).

2.1.2.2. Hemicellulose

Hemicellulose in lignocellulosic biomass consists of linear and branched heteropolymers including xylan, glucomannan, galactan, and arabinan. Hemicellulose is the second most abundant polymer in lignocellulose biomass after cellulose (Table 2.2). Unlike cellulose, hemicellulose has a low degree of polymerization, without crystalline regions. Therefore, hemicellulose is easily degraded into monosaccharides such as arabinose, xylose, galactose, fructose, mannose, dextrose or glucuronide, glucuronic, galacturonic, and 4-O-Me-glucuronic acids (Beg *et al.*, 2001; Girio *et al.*, 2010 & Chen *et al.*, 2017). Hemicellulose interacts with cellulose through hydrogen bonds and covalent bonds (mainly α-benzyl ether linkages) with lignin, and ester linkages with acetyl units and hydroxycinnamic acids (Figure 2.1) (Glasser *et al*., 2000).

Hemicellulose has a more heterogeneous structure than cellulose (Figure 2.3 A, B). This polysaccharide forms a complex network of bonds that gives structural strength by connecting cellulose fibers into microfibrils. The composition of hemicellulose varies in different plants such as softwood, hardwood and annual plants such as grasses, wheat straw, and corn stover (Table 2.2). Hemicelluloses found in grasses consist mainly of glucuronoxylans (O-acetyl-4-O-Methyl-D-glucuronoxylan) or generally known as xylan (Álvarez *et al*., 2016). The main monosaccharides in the glucuararonoxylans are D-xylose and L-arabinose. It is therefore important to convert xylan to monosaccharides to improve the amount of ethanol produced from lignocellulose to reduce costs (Ren and Sun, 2010 & Álvarez *et al.,* 2016).

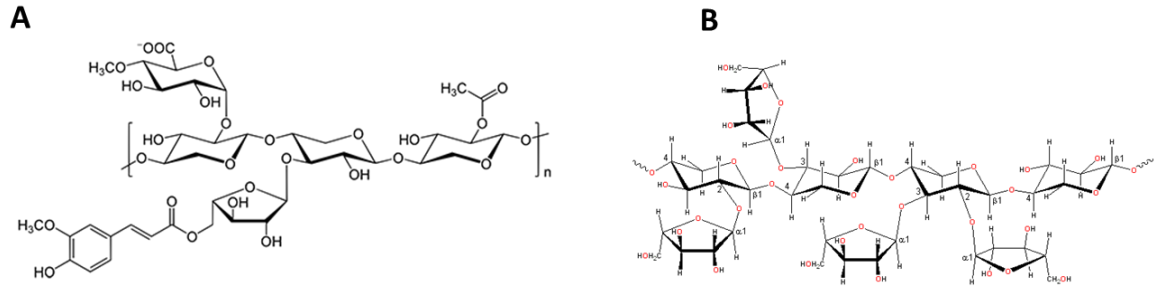

Figure 2.3. The structure of hemicellulose. A – Xylan; B- Arabinoxylan (https://www.e-education.psu.edu/egee439/node/664).

2.1.2.3. Lignin

Lignin is a rigid aromatic, amorphous, and hydrophobic polymer (Chen *et al.*, 2018) (Figure 2.1). Lignin interlinks and connects with the cellulose in the same manner as hemicellulose. The lignin in the lignocellulose biomass accounts for about 15 – 30% and the composition is influenced by the type of the plant species. It accounts for about 24 – 33% in softwoods, 19 – 28% in hardwoods, and 15 – 25% in grasses (Lu *et al.*, 2017). The main purpose of the lignin in the plant material is for structural support, transport of water and nutrients (Laurichesse and Averous, 2014). The protection that the lignin offers to the plant material serves as a defense barrier against enzymatic degradation of the wood biomass. This slows down enzymatic hydrolysis due to the unproductive binding of the enzymes to the lignin (Alvarez *et al.,* 2016). The removal of lignin during the pre-treatment process is crucial and will enhance enzymatic hydrolysis, which enhances bioethanol production (Chen *et al.*, 2018 & Zhang *et al.*, 2020).

2.1.3. Bioethanol production from lignocellulosic biomass

Lignocellulose material cannot be converted directly to bioethanol due to the crystalline structure of cellulose and the physical lignin barrier throughout the structure. This property hinders the enzymatic attack and results in slowing the hydrolysis of the lignocellulosic biomass (Loelovich, 2016). The hydrolysis step is required to release monosaccharides for fermentation to bioethanol by yeast. Several steps are needed in the bioethanol production process, which is pretreatment of the lignocellulose biomass, enzymatic hydrolysis of the polymers, fermentation of the released sugars and purification (Figure 2.4).

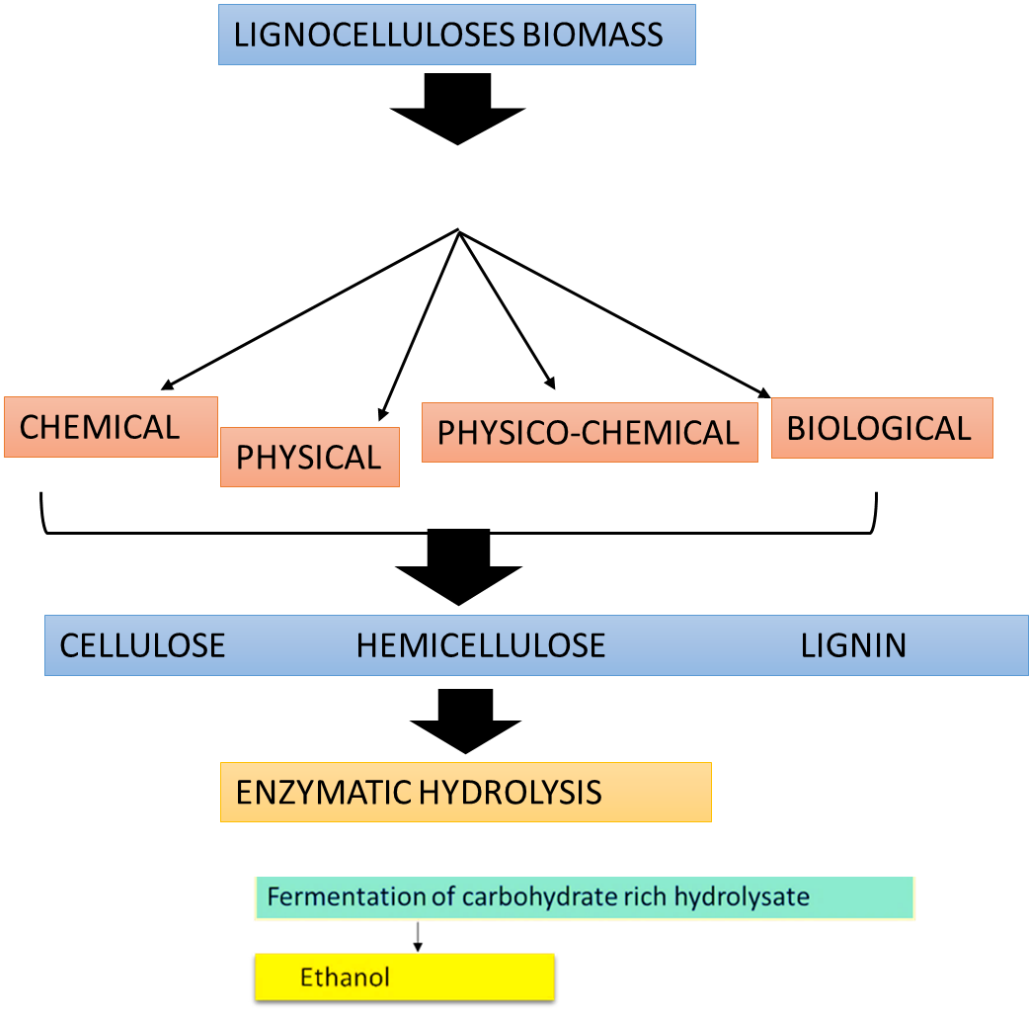

Figure 2.4. A schematic representation of the processes involved in the production of bioethanol from lignocellulose biomass modified from Prasad *et al*. (2019).

2.1.3.1. Pretreatment

Pretreatment alters the structure of the lignocellulose complex and its importance is to remove or degrade the lignin and expose the cellulose for enzymatic hydrolysis. The pretreatment processes are categorized into **Physical, Chemical, Physiochemical** and **Biological** pretreatment methods. Depending on the type of the lignocellulose biomass utilised, one or a combination of pretreatments can be used. It is significant to choose a proper pretreatment method that will aid in reducing the cost of bioethanol production. The selected method should be rapid, cost-effective, limit sugar loss, and the production of inhibitors (Woiciechowski *et al.*, 2020). Several studies have revealed the effectiveness of a proper pretreatment process on different

lignocelluloses and resulted in increased product titre (Binod and Pandey, 2015; Fei *et al.*, 2020 & Gaweł *et al.,* 2020).

2.1.3.1.1. Physical pretreatment

The physical pretreatment method is the most common and widely used in the pretreatment of lignocellulosic biomass. Physical pretreatment methods are normally used in combination with other non-physical pretreatment methods. The physical pretreatment method is considered environmentally friendly since there are no chemicals used (Inoue *et al.*, 2008). There are a variety of physical pretreatments and are classified into two categories: mechanical comminution which includes chipping, cutting, grinding, or milling, and irradiation which includes gamma rays, electron beam (EB), and microwave pretreatments. In most studies, the mechanical comminution that includes grinding, shearing, or milling of the lignocellulose biomass is the most preferred (Taherzadeh and Karimi, 2008 & Sun *et al.*, 2016).

The mechanical comminution processes are necessary for reducing polymerization, particle size, increase the surface area and the porosity of the lignocellulosic biomass (Sun and Cheng, 2002; Hendriks and Zeeman, 2009; Hu *et al.*, 2014; Saini *et al*., 2015 & Amin *et al.*, 2017). Grinding and milling of the lignocellulose are effective in altering the natural ultrastructure of the lignocellulose biomass (Taherzadeh and Karimi, 2008). Some studies have shown that reducing the particle size of different lignocellulose biomass (rice straws, sugarcane bagasse including different grasses) using grinding and milling increases the extraction yield of the sugars (Kelsall *et al*., 2003; Menegol *et al.*, 2016 & Dalena *et al.*, 2018). This method has some limitations, which include high energy input, and the inability to remove lignin that negatively affects enzymatic degradation of the cellulose and hemicellulose. Another advantage of using comminution is that there are no inhibitors formed during this process (Da Silva *et al.*, 2010). It has been reported that the comminution of hardwood requires more energy consumption than agricultural residues (Cadoche and Lopez, 1989). Grinding and milling of the lignocellulose biomass such as grass are usually combined with chemical pretreatment (Tsai *et al.*, 2018). Depending on the type of chemical pretreatment, the combination assists in degrading the lignin polymer (Zheng *et al.*, 2017).

2.1.3.1.2. Chemical pretreatment

The **diluted acid** and **alkaline pretreatment** methods are chemical pretreatment methods that are often employed and will also be utilised in this study (Taherzadeh and Karimi, 2008; Aslanzadeh *et al.*, 2014 & Martínez *et al.*, 2015).

Dilute acid chemical pretreatment method

The main purpose of the acid-catalyzed pretreatment method is to solubilise the hemicellulose and lignin fraction to expose the cellulose to enzymatic hydrolysis. This results in a high ethanol concentration after fermentation. The acid-catalyzed pretreatment method uses different acids such as sulfuric acid, hydrochloric acid, phosphoric acid and nitric acid. Sulfuric acid and phosphoric acid are the most studied and the most preferred because they are effective and inexpensive (Dahadha *et al.*, 2017 & Deshavath *et al.*, 2017). Dilute acid pretreatment can be performed in two ways, at a high temperature (around 180 °C) for a short time or at a lower temperature (<120 °C) for 30 – 90 minutes. The effective concentration of the acids used to pretreat lignocelluloses biomass is below 4%. This pretreatment method has been used in the pretreatment of a variety of lignocellulose biomass such as herbaceous crops, agricultural residues, hardwoods, softwoods and grasses (Cao *et al.*, 2018).

In addition, after pretreating the biomass, the biomass is usually neutralised. Neutralisation of the biomass after pretreating with acids is very important. One of the important facts for neutralizing has been shown by neutralising biomass pretreated with phosphoric acid using a basic chemical such as NaOH or ammonia has shown to be beneficial. This is because the hydrolysate forms sodium or ammonia phosphate that is used as a nutrient for microorganisms in the fermentation step. The disadvantage associated with the use of dilute acid pretreatment process includes high cost of the reactors because acids used are very corrosive, the formation of gypsum during neutralisation, the formation of inhibitory by-products (furfurals, 5-hydroxymethylfurfural, phenolic acids, and aldehydes), the formation of aldehydes and it solubilised hemicellulose into monosaccharide (Mosier *et al.*, 2005; Gámez *et al.*, 2006; Alvira *et al*., 2010 & Sahoo *et al.*, 2018).

Alkaline pretreatment

The alkaline pretreatment process solubilises the lignin polymer in the lignocellulose biomass. The different bases used in the alkaline pretreatment include NaOH, KOH, N_2H_2, $Ca(OH)_2$, and ammonium hydroxide (Mirmohamadsadeghi *et al.*, 2016). Compared with acid pretreatment, alkaline pretreatment works well at room temperature and natural atmospheric pressure and usually takes hours to days (Gaweł *et al.*, 2020). These methods usually generate less sugar degradation than acid pretreatment and most of the corrosive salts can be recovered. Furthermore, this method is effective on agricultural residues, herbaceous crops with low lignin content, and softwood with high lignin content (Bjerre *et al.*, 1996; Cui *et al.*, 2015 & Heggset *et al.*, 2016). The study by Mosier *et al.* (2005) noted the increase in digestibility of cellulose by using $Ca(OH)_2$ that removes the acetyl group from hemicellulose. Bali *et al.* (2015) studied the effect of different alkaline pretreatments and found NaOH to be the most effective. Furthermore, this method is preferred and highly recommended in the pretreatment of grasses (Han *et al.*, 2011 & Tsai *et al.*, 2018). Grasses have high xylan content and are extracted by alkaline dissolution followed by alcohol precipitation. Several studies have revealed the effectiveness of extracting cellulose and hemicellulose from different types of grass when using NaOH as an alkaline pretreatment agent (Luo *et al.*, 2012; Martin, 2012; Stoklosa and Hodge, 2012 & Sahoo *et al.*, 2018b). In addition, the concentration of inhibitors produced during alkaline pretreatments is generally lower when compared to those released in acid pretreatments (Jönsson and Martín, 2016).

2.1.3.1. Enzymatic hydrolysis

Enzymatic hydrolysis is a biological process that uses enzymes to break down polysaccharides (hemicellulose and cellulose) released after the pretreatment process. The monomers released during the enzymatic hydrolysis can then be fermented into ethanol and other useful products by microorganisms (Casey *et al.*, 2013). Enzymatic hydrolysis is dependent on various factors that include species diversity, chemical composition complexity, the efficiency of the utilised pretreatment method and mode of enzyme action. Enzymatic hydrolysis in grasses is mostly affected by cellulose crystallinity and the presence of hemicellulose (Yoshida *et al.*, 2014). Small quantities of commercial enzymes were used successfully to increase

the efficiency of enzymatic hydrolysis of different grasses but are not economically feasible due to the high cost of the enzymes (Kumar and Murthy, 2011; Tutt and Olt, 2011; Meineke *et al*., 2014 & Thomsen *et al.*, 2016).

The major limitation in the industrialisation of bioethanol production from lignocellulose biomass is the production cost of the enzyme complexes. Lignocellulolytic enzymes, such as cellulases, hemicellulase and ligninases work together or individually to degrade the lignocellulose biomass to fermentable monomers. One major approach that increases enzymatic efficiency and the enzymatic rate is the use of cellulase and xylanase that work synergistically to co-hydrolyse cellulose and hemicellulose. This will increase the amount of reducing sugar that will subsequently increase ethanol production because both the hexose and pentose sugars will be available for fermentation. The degradation of hemicellulose by xylanases also makes the cellulose polymer accessible for enzymatic attack (Losordo *et al.*, 2016 & Lynd *et al.*, 2017). The study by Ajijolakewu *et al.* (2017) demonstrated that a 91.7% saccharification yield from hydrolysis of alkaline-treated oil-palm-empty-fruit bunches biomass was achieved with the synergistic activity of hemicellulase from the novel mycoparasitic *Trichoderma asperellum* and commercial cellulase. This was higher than a 77.9% or 12.2% yield when no hemicellulase was used or used without the addition of cellulase.

Lignocellulolytic enzymes are also used in other applications such as the conversion of fruits and vegetables into different food commodities, from texturizing to flavoring, etc. Due to this demand, there is a need to increase the production of cellulases and xylanases (Liu *et al*., 2016). Different approaches have recently been introduced which involve the isolation of microorganisms with lignocellulolytic activity and screening of the enzyme activity on inexpensive agro-industrial substrates (Shrestha *et al.*, 2015).

Cellulases

Cellulases are part of the glycoside hydrolases family that consists of three different classes of enzymes. **Endocellulase** (1,4-β endoglucanase (EC. 3.2.1.4) randomly cleaves the glycosidic linkages on cellulose and is more active on the amorphous part of cellulose. **Exocellulase** (exo-1,4-β-glucanase (EC. 3.2.1.91)) acts on the ends of the cellulose chain and releases β-cellobiose as the end-product while the **Cellobiase** (β-glucosidase) (EC. 3.2.1.21) cleaves cellobiose to release glucose (Brune, 2014; Wu *et al.*, 2016; Bohra *et al.*, 2019 & Prasad *et al.*, 2019) (Figure 2.5).

Cellulases are inducible enzymes produced by several microorganisms. The enzymes are used in several industries, such as cotton processing, paper recycling, juice extraction, as part of detergents and animal feed additives (Singhania *et al.*, 2010). Fungi are well-known cellulose decomposers that are responsible for 80% of cellulose depolymerisation on earth. Most cellulose-degrading fungi belong to *Aspergillus*, *Penicillium*, *Chaetomium*, *Trichoderma*, *Fusarium, Stachybotrys, Cladosporium, Alternaria, Acremonium, Ceratocystis, Myrothecium*, and *Humicola* species (Mehrotra and Aneja, 1990; Wood, 1985). Anaerobic bacteria produce cellulosomes (protein complex) that are linked to the cell surface. Fungi release the cellulases into the extracellular medium as free molecules (Doi, 2004 & Juturu and Wu, 2014). The anaerobic bacteria are usually not considered because the individually active enzyme from species is difficult to isolate. The well-known species that are considered as hypercellulase producers are *Aspergillus, Trichoderma, Penicillium* and *Sclerotium*. These fungi have been used for commercial production of cellulase enzymes (Sreedharan, 2016).

The best-studied cellulase producer is *Trichoderma reesei* (Reczey *et al.*, 1996 & Singhania *et al.*, 2006). This fungus showed a remarkable ability to degrade native crystalline cellulose. *Trichoderma reesei* has two genes that encode for exoglucanase, eight for endoglucanases, and seven for glucosidases (Eggeman and Elander, 2005 & Aro *et al*., 2005). Cellulases have also been isolated from other species such as *Trichoderma longibrachiatum* (Shaibani *et al.*, 2012)*, Trichoderma viride* (Nathan *et al.*, 2014)*, Penicillium* echinulatum (Scholl *et al.*, 2015), and *Thermoascus aurantiacus* (Jain *et al.*, 2015 & Bischof *et al*., 2016). Studies have shown that the cellulase enzyme from one microorganism is usually not efficient. This is exhibited by *T. reesei* which produces low levels of β-glucosidase and this results in inefficient cellulose hydrolysis (Cherry and Fidantsef, 2003). *Aspergillus terreus* is used for the commercial production of β-glucosidase (Mohapatra *et al.*, 2017). Due to this inefficiency, several commercial cocktails that have a mixture of all the enzymes required for hydrolysis are available. These cocktails have been used for the hydrolysis of different types of plant biomass including different types of grasses, to increase the rate and enzymatic efficiency in releasing monomers (glucose) that can be fermented to ethanol. The use of commercial enzymes is a major limitation economically and affects cellulase enzyme application in the industry due to the cost and low yield of cellulase production.

Research in discovering novel microbial species secreting higher levels of cellulases is still an emerging area of research (Sarkar *et al*., 2012). This will assist in the development of economically viable strategies that are applicable on a large scale (Vodovnik *et al*., 2018; Chen *et al*., 2020 & Claes *et al*., 2020).

Recent studies have concentrated on the isolation of cellulase enzymes using cheap agro-industrial substrates to reduce production costs. This was shown successfully by the study by Kumar and Parikh, (2015) who reported the isolation and enzymatic hydrolysis of cellulose-producing strains from rice straw and sugarcane bagasse that are available in abundance in Asian countries. In addition, there is a wide range of studies that have shown successful isolation of cellulase enzymes and enzymatic hydrolysis of several lignocellulose biomass using agro-industrial residues as substrate (Wongwatanapaiboon *et al.*, 2012 & Shrestha *et al.*, 2015). This includes sugarcane bagasse, straw of wheat, rice, corn, wheat bran, corn stover and different grasses (Zhou *et al.*, 2008 & Binod *et al*., 2010). The standardised cellulose loading for lignocellulose hydrolysis varies between 10 and 30 FPU/g cellulose and is based on the pretreatment method. Conversely, equal activities under the standard conditions may not lead to equal hydrolysis rates or yields. This is due to different pretreatment methods that have a different effect on different biomass, with different cellulose structures and composition of hemicellulose and lignin. As a result, standardisation is difficult when pretreated agro-industrial residues are used (Pryor and Nahar, 2010) since these materials differ vastly. Studies by Wongwatanapaiboon *et al*. (2012) used *T. reesei* with cellulase and xylanase activity to hydrolyse different types of grasses resulting in total reducing sugars of 500 – 600 mg/g of substrate, while Belalal, (2013b) achieved total reducing sugars of 350 mg/g from the degradation of rice straw.

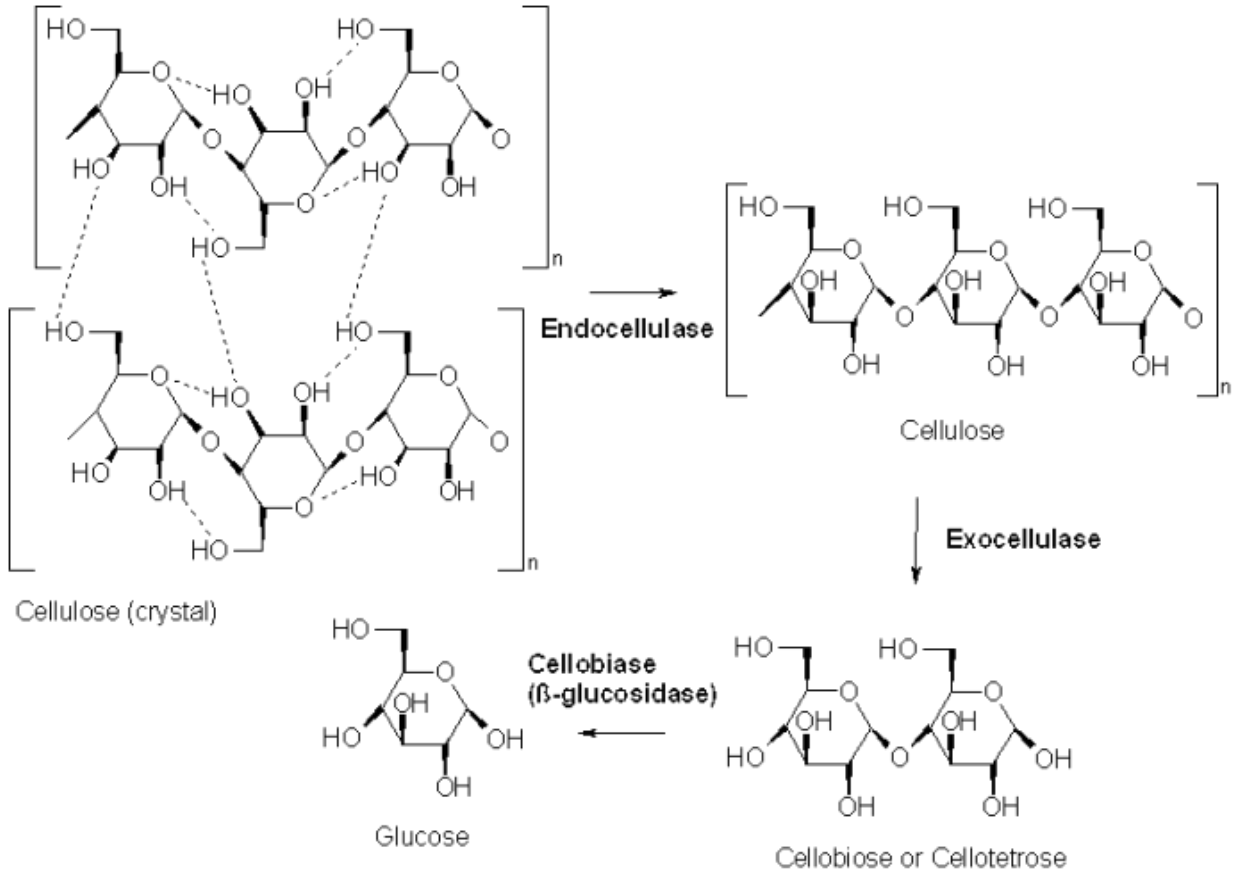

Figure 2.5. Schematic representation of the degradation of complex cellulase enzymes (https://www.e-education.psu.edu/egee439/node/669).

Hemicellulases

The hemicellulose fraction consists predominantly of xylan. Xylan is degraded into fermentable sugars by endo-β-1,4-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37). The endo-β-1,4-xylanase acts randomly on the internal bonds of xylan to release xylooligosaccharides, followed by β-xylosidase to release xylose. Several accessory enzymes including α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.139), α-galactosidase (EC 3.2.1.22), acetyl xylan esterase (EC 3.1.1.72) and ferulic acid esterase (EC 3.1.1.73) assist in the degradation of xylan (Kumar and Wyman, 2000 & Van dyk and Pletschke, 2012). These enzymes work in synergy to degrade the hemicellulose polymer (Figure 2.6).

Xylanase enzymes are widely used in various industries such as wastewater treatment, pulp and paper industry, clarification of fruit juices and wines; improvement of the texture of bread and the formulation of animal feed additives (Enshasy *et al.*,

2016 & Walia *et al*., 2017). In addition to the demand for xylanase enzymes in other industries, the world energy crisis has led to high demand for xylanase production. Xylanases assist in the bioconversion of lignocellulosic materials to produce ethanol and other valuable products, including xylitol (Beg *et al.*, 2001 & Juodeikiene *et al.*, 2011).

Xylanases are produced by a wide variety of microorganisms (fungi and bacteria) (Subramaniyan and Prema, 2002; Ajijolakewu *et al.*, 2017 & Lee *et al*., 2018). Filamentous fungi are mostly used in the production of commercially available xylanase enzymes due to the efficient excretion of these enzymes into the medium. Most reports have shown that xylanases are mostly produced by mesophilic and thermophilic microorganisms (Polizeli *et al.*, 2005). The most reviewed and studied genera for xylanase enzyme production are *Aspergillus, Trichoderma,* and *Penicillium* on an industrial scale (Cano and Palet, 2007 & Zhang and Sang, 2015). The low yields and high production costs are still a major bottleneck in the application of xylanase enzymes. This has led researchers into the isolation of microorganisms with novel hemicellulolytic activity (Orencio-Trejo *et al.*, 2016). Recent studies have focused on the isolation of new filamentous fungi that produce xylanases with novel characteristics. Microorganisms with novel hemicellulolytic activity are important to increase the rate and efficiency of enzymatic hydrolysis of lignocellulose biomass. The xylanase enzyme activity usually varies in different lignocellulose substrates. The enzyme stability also differs from one microorganism to the next, but generally, the stability is at a pH range of 4.5 – 5.5 and a temperature range of 40 – 55 °C (Binod and Pandey, 2015; Menegol *et al.*, 2017; Kumar *et al*., 2017 & Gautam *et al.*, 2018) (Table 2.3).

Other studies have demonstrated the importance of cultivating xylanolytic microorganisms in the same substrate to be hydrolysed. This assists in the isolation of specific enzymes that are optimal for the hydrolysis of that substrate (Sørensen *et al.*, 2011). This method is cost-efficient since enzyme selection and substrate hydrolysis uses the same substrate. Efforts in the isolation of microorganisms producing xylanase on cheap, abundantly available agro-industrial residues are important to improve enzymatic hydrolysis of the lignocellulose biomass that will make biofuel production effective. The xylanase enzymes isolated in this manner have higher hydrolytic activity (Delabona *et al.*, 2012; Lynd *et al.*, 2017 & Souza, *et al.,*

2018). Shrestha *et al.* (2015) isolated thirty fungal strains from decaying leaves of *Miscanthus* and sugarcane, and the fungi were found to produce xylanases, exocellulases, endocellulases, and β-glucosidases. The conversion ability of the isolated fungi was found to be better than that of native *T. reesei*. The xylanase from *P. chrysogenum* F-15 showed lower enzymatic activity on commercial xylan than on hemicellulose from agro-industrial biomass. The xylanase enzyme from this strain resulted in successful hydrolysis of aqueous ammonia solution pretreated corn cob powder (Zhang and Sang, 2015). The study by Rojas-Jiménez and Hernández, (2015) isolated fungi and bacteria with lignocellulolytic activity from the gut of wood-feeding insects and found *Trichoderma* species to be in abundance. One main limitation that affects the type of isolated species is the cultivation-dependent approach, based on artificial media, which covers only a small proportion of the total microbial diversity present in a particular niche area.

Recently several studies (Table 2.3) have shown a successful production of xylanase using grasses as a carbon source. Menegol *et al.* (2017) showed the highest xylanase enzyme activity when using elephant grass (*Pennisetum purpureum*) as a substrate. Conversely, a study by Scholl *et al*. (2016) showed negative results for xylanase enzyme production when elephant grass (*Pennisetum purpureum Schum*.) pretreated with a steam explosion was used as a carbon source. The negative results were due to the washing of the grass after the pretreatment process.

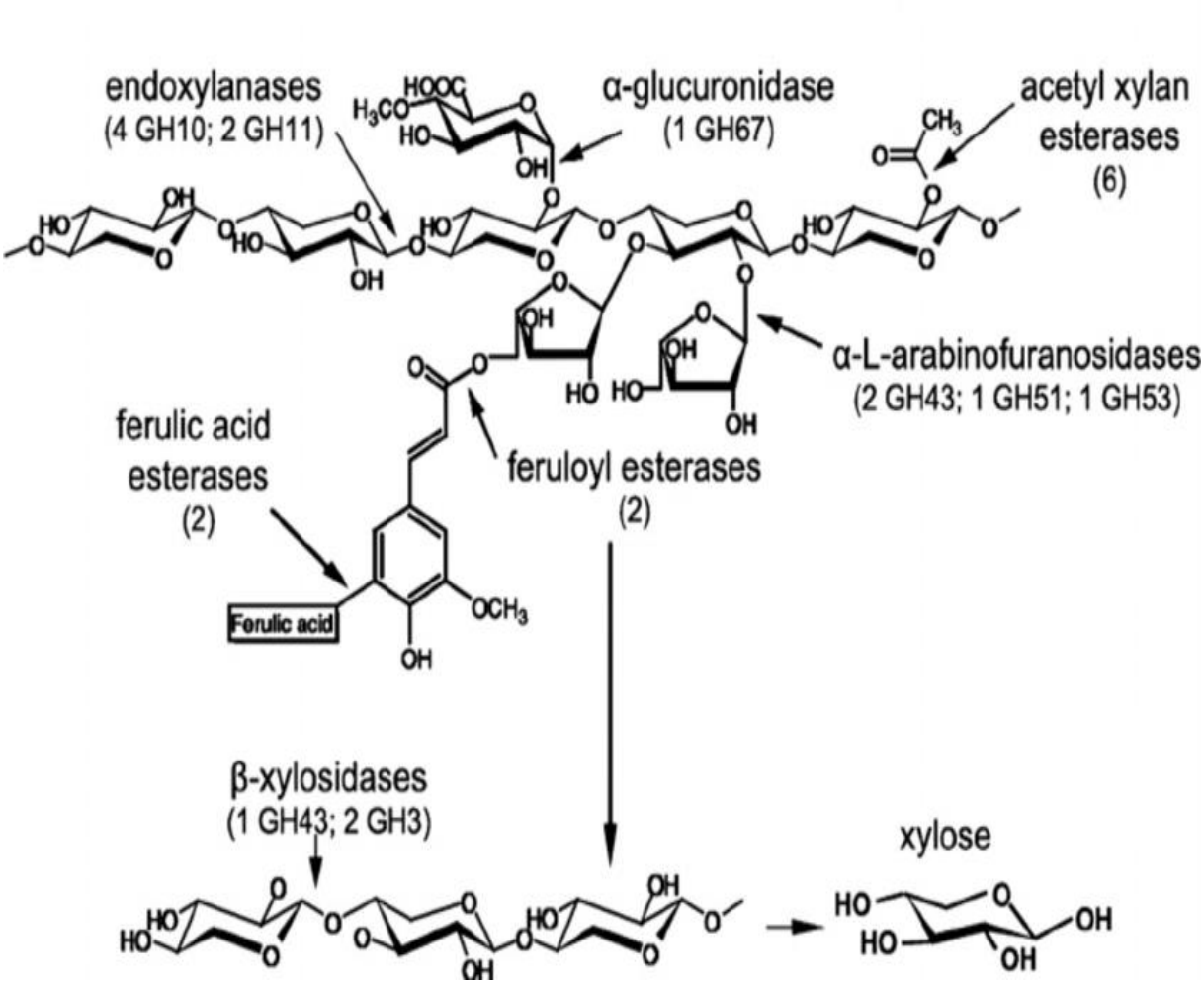

Figure 2.6. Schematic representative of hemicellulase enzyme degradation of the hemicellulose (Sun *et al*., 2014).

Table 2.3. Production of xylanase enzyme from a variety of grasses.

Type of grass used as a carbon source	Microorganisms used	Production Processes (SSF or SmF)	Xylanase activity	Reference
Elephant grass (*Pennisetum purpureum*)	*Penicillium echinulatum*	SSF	(372.62 U.g-1 dry matter)	(Menegol *et al.*, 2017)
Rice straw	*Schizophyllum commune* ARC-11	SSF	4288.3 IU/gds	(Gautam *et al.*, 2018)
Paddy straw	*Thermomyces lanuginosus*	SmF	51.36 U mL^{-1}	(Kumar *et al*., 2017)
Thatch grass	*Hypocrea lixii* AB2A3	SmF	35.08 U mL^{-1}	This study

Ligninases

Ligninases break down lignin and are also called the lignin-modifying enzymes. There are different types of lignin-modifying enzymes including phenol oxidases (laccases) and heme peroxidases as lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP) (Figure 2.7.) (Ciolacu, 2018). The study of these enzymes was intensified (Liu *et al.*, 2020) due to their vast applications. These enzymes are applied in the biotransformation of lignocellulose biomass to use in biofuels, bio pulping; bioleaching of paper pulps; decolorizing; detoxifying Kraft bleach plant effluents; and degradation of highly toxic environmental chemicals such as dioxins, polychlorinated biphenyls, various dye pollutants, and polyaromatic hydrocarbons (Varnai *et al.*, 2010). The major drawback of delignification is the long residence time and space required (Chandra *et al.*, 2007 & Varnai *et al*., 2010).

Table 2.4. Comparison of xylanase activity from different microorganisms, and their effect on enzymatic hydrolysis of different lignocellulose biomass pretreated with different methods.

Enzyme source	Pretreatment method	Xylanase activity	Optimum Activity		Substrate	Enzymatic efficiency	Reducing sugar	Xylose (mg/g dry substrate)	Reference
			pH	°C					
T. asperellum USM SD4	Alkaline Pretreatment	186.3 U/ml	5.3	50	Oil-palm-empty-fruit bunches (OPEFB)	91.7 %	ND	ND	(Ajijolakewu *et al.*, 2017)
T. reesei	NaOH pretreatment	6.23 U/ml	5	ND	*Saccharum spontaneum*	79.61%	350 mg/g	ND	(Kataria and Ghosh, 2014)
A foetidus MTCC 4898	Ammonia solution	8450 U/g	5.3	50	Wheat straw	ND	4.9 g/L	ND	(Chapla *et al.*, 2010)

A terreus D34	Mild alkaline	3,921 U/g	5	50	Rice straw (RS)	82.8% ± 1.0%	0.733 g g− 1	ND	(Kumar and Parikh, 2015)
P. chrysogenum QML-2	Aqueous ammonia solution	1734.78 U/g)	4.5	50	Corn cob	ND	553.94	236.63	(Zhang and Sang, 2015)
Roryzae UC2	ND	213.99 U/g	5	50	Raw oil palm frond leaves (OPFL)	75.95 %	ND	1.44	(Ezeilo *et al.,* 2019)
H.lixii AB2A3	Acid pretreatment	35.08 U mL^{-1}	ND	ND	Thatch grass	59%	ND	138.7	This study

T = *Trichoderma*

A= *Aspergillus*

R= *Rhizopus*

P = *Penicillium*

ND= Not Determined

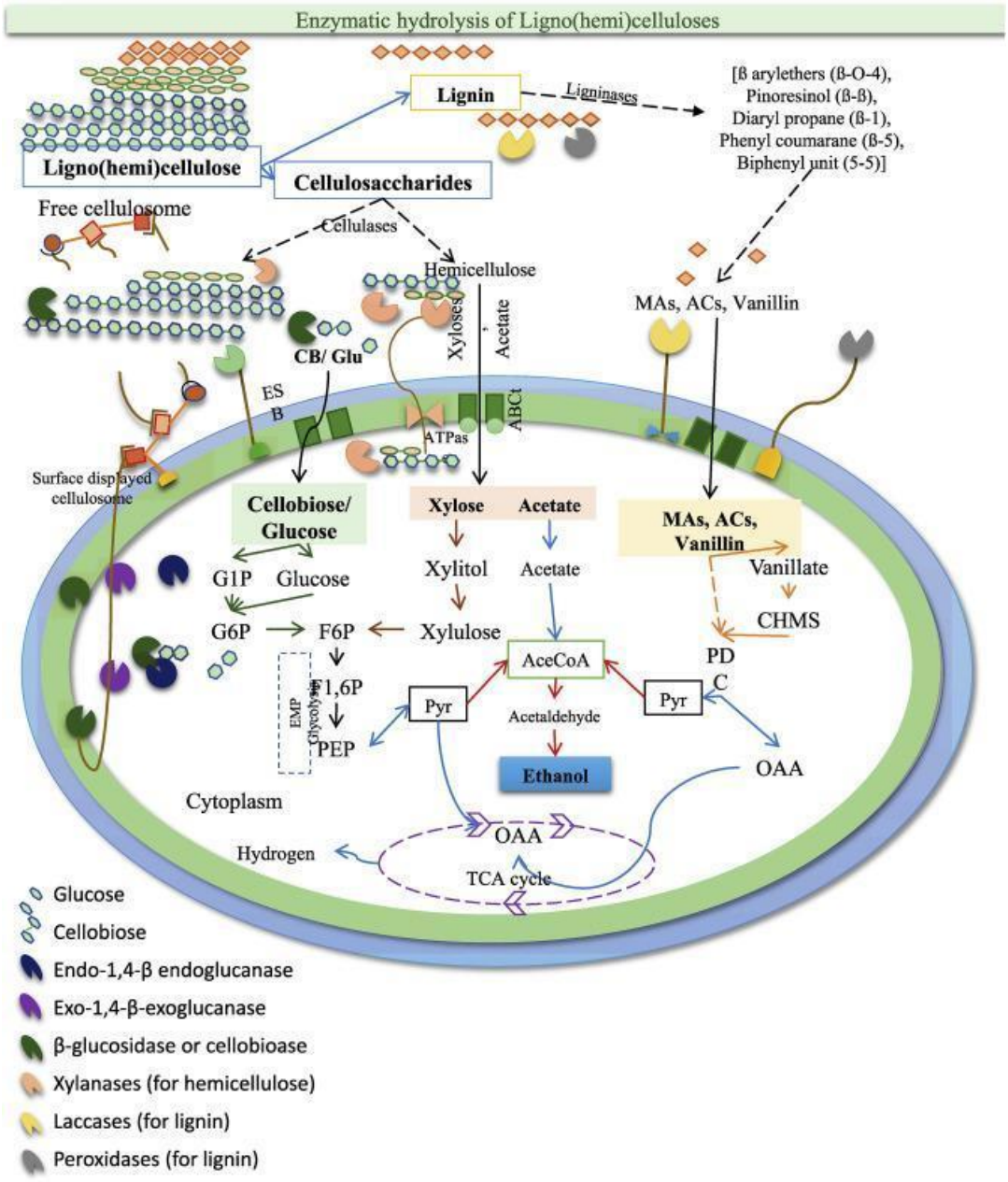

Figure 2.7. Overall schematic representation of lignocellulolytic enzymes involved in the hydrolysis of lignocellulose biomass. [abbreviations: ABCt: ABC transporters; ESB: Extracellular binding protein, CB: Cellobiose, Glu: Glucose, G1P: Glucose-1-phosphate, GP6: Glucose-6-phosphate, F6P: Fructose-6-phosphate, F1,6P: Fructose-1,6-phosphate, PEP: Phosphoenol-pyruvate, Pyr: Pyruvate, OAA: Oxaloacetic acid, AceCoA: Acetyl-CoA, TCA: Tricarboxylic acid, MA: Monoaryls, AC: Aromatic compounds, PDC: 2-pyrone-4,6-dicarboxylate, CHMS: 4-carboxy-2-hydroxymuconate-6-semialdehyde] (Prasad *et al.*, 2019).

2.1.4. Lignocellulolytic enzyme production

The carbon source is important when isolating microorganisms producing cellulases and hemicellulases. Glucose acts as a restrainer while other polysaccharides and oligosaccharides act as inducers. The use of cheap agro-industrial residues is favorable for the isolation of the (Hemi) cellulase-producing microorganisms. The cheap substrate for the production of lignocellulolytic microorganisms can reduce the cost associated with enzyme production (Singhania *et al.*, 2010). Some reports have shown that the fermentation process used for the production of cellulases and xylanases affects the efficiency of the produced enzyme during hydrolysis. The process that results in the production of an effective enzyme must be used, as this is a priority in increasing the effectiveness and rate of enzymatic hydrolysis (Liu *et al.*, 2020). Submerged Fermentation (SmF) and Solid-State Fermentation (SSF) are used in the production of extracellular enzymes for the bioethanol industry.

2.1.4.1. Submerged Fermentation

Submerged Fermentation (SmF) is the fermentation operated in the presence of excess liquid (Figure 2.8) (Singhaniaa *et al.*, 2010). This process is attractive and has widely been used in enzyme production. Submerged fermentation has a well-developed bioreactor, instrumentation, and process control (Amorim *et al*., 2019). During the operation of this fermentation process, several parameters can be controlled such as temperature, agitation, aeration, foam and pH (Vaidyanathan *et al.*, 1999). Novozymes, which is the world-leading enzyme producer, uses SmF for cellulase enzyme production (Hansen *et al.*, 2015 & Krull *et al.*, 2013). About 80 – 90% of commercially available xylanase enzymes are produced using submerged fermentation (Polizeli *et al.*, 2005). Several reports have exhibited successful xylanase and cellulase production using submerged fermentation and these enzymes have largely been produced from fungi such as *Trichoderma* and *Aspergillus* species (Chandel *et al.*, 2012). *Aspergillus niger* produced a higher xylanase activity on wheat bran used as a carbon source (30.4 ±3.1 IU/g dry Wheat Bran) at 32 ºC shaking at 250 rpm (Amorim *et al*., 2019). Xylanase activity of 17.48 ± 0.86 U/mL was also reported when sunflower stalks were used as a carbon source. In this study, *Aspergillus sp* was cultivated at 40 ºC, pH 6.0, and an agitation rate of 100 rpm (de Souza *et al.*, 2020a). Scholl *et al.* (2015) found that *P. echinulatum* showed potential

for the simultaneous production of xylanase and cellulase when elephant grass was used as a substrate in SmF. The review by Hansen *et al.* (2015) has compared lignocellulolytic enzyme production from different species using submerged and solid-state fermentation. The favorable conditions that result in a higher yield of produced enzymes during submerged fermentation depend on the type of microorganism and the substrate used.

Filamentous fungi are morphologically complex, and in nature, they show different structural forms throughout their life cycles. Their structural forms and morphology are also affected by different factors such as the nature of the inoculum, the chemical factors in the medium, and physical factors such as temperature, pH and mechanical forces (Kossen, 2000). Filamentous fungi in submerged culture show a different morphology such as dispersed mycelial and dense linked mycelial masses called pellets (Papagianni, 2004). Submerged fermentation has several limitations especially during high substrate loading when agro-industrial residues are used. There is usually a mass transfer and agitation problem during cultivation and this is due to non-uniform mixing. The uniform homogenisation is also affected by the increase in viscosity of the culture broth during cultivation for enzyme production by filamentous fungi (Antecka *et al.*, 2016).

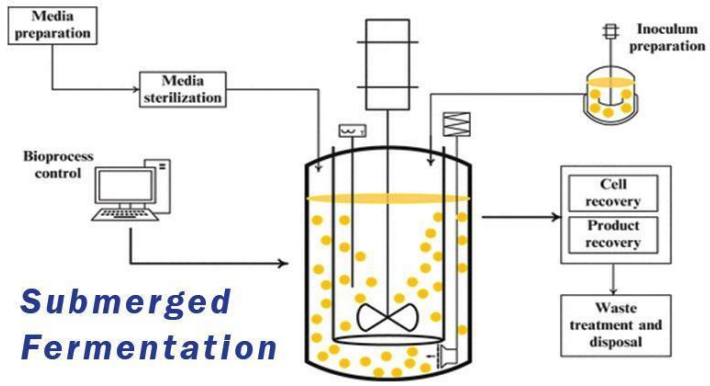

Figure 2.8. Schematic representation of submerged fermentation using a bioreactor (Manan and Webb, 2017).

2.1.4.2. Solid-State Fermentation

Solid-State Fermentation (SSF) involves solid substrates in the absence of free water, but the substrate should have sufficient moisture that will support the growth of the microorganisms (Figure 2.9). SSF has recently gained more attention in the enzyme

production industry. This is due to the conditions employed in this fermentation that mimic the natural environment which favors the growth of filamentous fungi. Several advantages make SSF more attractive than conventional SmF. SSF is cost-effective, no need for nutrient solubilisation, no rigorous control of many parameters during fermentation, high volumetric productivity, low energy requirements, release less wastewater, no foam generation, and relative ease in the recovery of end products (Manan and Webb, 2017 & Liu *et al.*, 2020). Some studies indicated that enzyme production in SSF was higher compared to submerged fermentation. The study by Liu *et al*. (2020) compared characteristics of extracellular enzymes secreted by *P. chrysosporium* during SSF and SmF concluded that more cellulase was secreted when SSF was used compared to SmF. This is due to lower catabolite repression in SSF (Kriaa and Kammoun, 2016 & Zhao *et al.*, 2019).

Factors to be considered in SSF include the type of substrate and the moisture content. Several agro-industrial residues that have been used in SSF as substrate and resulted in higher enzyme production include wheat bran, sorghum stover, corn cob, soybean meal including different types of grasses. The use of these agro-industrial residues in enzyme production could help in reducing the production cost (Khanahmadi *et al.*, 2018 & Ezeilo *et al.,* 2019). The study by Subsamran *et al.* (2019) found higher lignocellulolytic enzyme production when SSF was used as the fermentation process when vertiver grass was used as the substrate. Several studies have shown variation when the same microorganisms and substrate are used. This is because several important parameters cannot be controlled in SSF compared to SmF and is a limitation when using this process (Kang *et al.*, 2004 & Bansal *et al.*, 2012).

Moisture content in SSF is very crucial and can affect process efficiency. Moisture content that is too high will result in oxygen limitation. Conversely, if the water content is too low, it will hinder microbial growth (Raghavarao *et al.*, 2003). Delabona *et al.* (2013) investigated the effect of initial moisture content on two Amazon rainforest *Aspergillus* strains cultivated on agro-industrial residues (grasses, wheat bran and wheat stover). The author concluded that moisture content plays a crucial role in the effectiveness of the SSF.

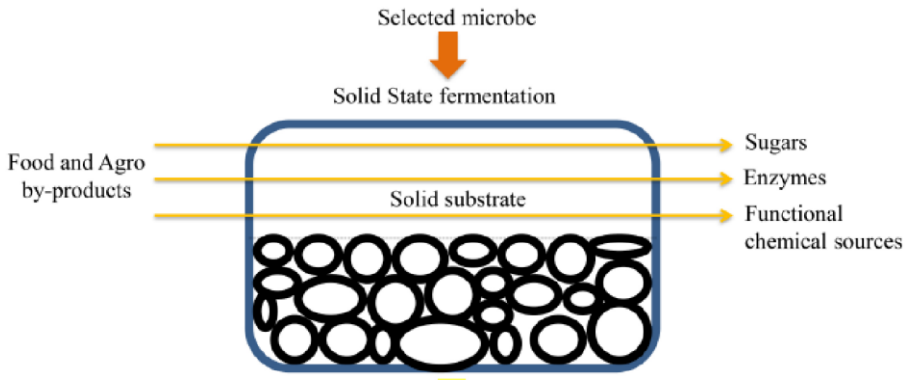

Figure 2.9. Representation of Solid-state fermentation (Manan and Webb, 2017).

2.1.5. Dung beetles and the gut of the microflora

Cellulases and hemicellulases are found in a wide range of natural environments and are produced by a wide range of organisms such as microbes, plants, insects and animals (Zahura *et al.*, 2011 & Zhang *et al.*, 2011). The dung from herbivores consists of undigested plant fragments, intestinal secretions (including mucus and dead epithelial cells), bacteria, fungi and other undigested material (Holter and Scholtz, 2007). Several studies have shown that the microorganisms found in the guts of dung beetles have a symbiotic relationship with the host. Therefore, the gut of the dung beetle should contain a rich source of microorganisms that can degrade polysaccharides commonly found in plant material (Zhang and Jackson, 2008 & Rojas-Jiménez and Hernández, 2015).

The order Coleoptera is the largest order of insects. The family Scarabaeidae is comprised of over 37,000 species of beetles where most are herbivorous or saprophagous (Thiyonila *et al.*, 2018). The beetles from this order are referred to as “true” beetles and this beetle utilizes dung either as dwellers, tunnellers, or rollers. Tunnelers are beetles, such as *Phanaeus vindex*, that consume the dung and burrow into the soil. The manure dwelling beetles, such as *Aphodius* species, ingest the dung pat and deposit eggs in the dung. The dung rolling beetles break the pat into brood balls that are rolled to a suitable site and bury it. Several studies have revealed that dung beetles harbor microorganisms with cellulolytic activity. This includes dung beetles such as *Reticulitermes flavipes*, *Anoplophora glabripennis*, *Tenebrio molitor*, and *Pachnoda marginata* (Amore *et al*., 2013). There are some novel enzymes and genes that have been reported in *Reticulitermes flavipes* such as β-glucosyl

ceramidase, α-mannosidase, glycosyl hydrolase gene, trehalase and RfBGluc-1 beta-glucosidase.

Suh *et al*. (2008) reported the isolation of yeast strains capable of fermenting xylose and cellobiose from *Odontotaenius disjunctus* (pallisade dung beetle). The yeast strains isolated from *Odontotaenius disjunctus* beetle included the xylose-fermenting yeast strains such as *Scheffersomyces stipitis*, *Scheffersomyces shehatae,* and *Candida maltosa* while the cellobiose-fermenting yeast strain was *Scheffersomyces ergatensis* (Urbina *et al.*, 2013). Lignocellulolytic fungi such as *Trichoderma, Bionectria*, and *Trametes* were isolated from the gut of the wood-feeding dung beetles belonging to Coleoptera (Rojas-Jiménez and Hernández, 2015). These studies show that lignocellulose-degrading insects are a potential source of pentose fermenting yeast and hemicellulolytic microorganisms and enzymes, which may be useful in biofuel production (Huang *et al.*, 2010).

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CHAPTER 3

Screening and characterisation of xylanolytic and xylose-fermenting fungi isolated from the gut of dung beetles and dung beetle larvae

ABSTRACT

Research on renewable biotechnologies for bio-fuels application has reached new heights. This is highlighted by the extensive domination of current biotechnological research in the mining of novel enzymes from animal and insect gut microbiomes. This study explored the diversity and composition of the gut-microbiota for hemicellulolytic and xylose-fermenting fungi from Scarabaeidae dung beetles and dung beetle larvae in South Africa. The exploited dung beetles were *Kheper nigroanaeus Boheman, Heteronitis castelnaui*, *Pachylomerus femoralis* and *Anachalcos convexus* and *Euoniticellus intermedius* larvae. Three hundred and fifty-four (222 are molds and 132 yeast isolates) fungi were isolated and identified using ITS1/2 and D1/2 sequences. Phylogeny of the filamentous fungi with xylanolytic activity was analysed using ITS rDNA sequences. The predominant yeast species from the *Kheper nigroanaeus Boheman* and *Heteronitis castelnaui* dung beetle were *Candida tropicalis* and *Malassezia globosa*, respectively. Yeast isolated could assimilate xylose but could not ferment it effectively. *Candida tropicalis* Y9 was the highest xylose-fermenting yeast producing 1.11 g/L of ethanol. The dominant hemicellulolytic fungi from *Pachylomerus femoralis* and *Anachalcos convexus* belong to the genus *Aspergillus* while the dominant genus from *Euoniticellus intermedius* larvae belong to the genus *Hypocrea.* High levels of xylanase activity of 393.22 and 392 nkat/ml were produced by *Aspergillus fumigatus* L1XYL9 (*Euoniticellus intermedius* larvae) and *Hypocrea lixii* AB2A3 (*Anachalcos convexus*), respectively. The results from this study shows that the gut of dung beetles in South Africa are a rich bioreactor of hemicellulolytic fungi " for dung beetles/larvae. While the yeast isolates from these dung beetles need to be manipulated to increase ethanol yield.

3.1. INTRODUCTION

Most fungi that are industrialised and play an important role in biotechnology were isolated from insects. The fungi and insects have a symbiotic relationship. The fungal biodiversity of insects such as wood-feeding termites, honeybees and mosquitoes has been extensively studied (Huang *et al.*, 2010 & Urbina *et al*., 2013). There is a dearth of information regarding the diversity and composition of the gut microbiota of the dung beetle with hemicellulolytic and xylose-fermenting fungi (Franzini *et al.*, 2016 & Nwaefuna *et al.*, 2021). The depletion of fossil fuel and the effect of global warming caused by pollution has ignited the interest in biomass fuel produced from lignocellulose matter. Lignocellulose biomass is plant-derived material such as dedicated energy crops (grass, herbaceous plants, trees), agricultural residues (straw and corn stover; bagasse, husks, shells and cobs) and forestry residues (branches and leaves, and sawdust and cutter shavings) (Stafford *et al.*, 2019). The composition of lignocellulose varies between different sources in terms of lignin (15 – 20%), hemicellulose (25 – 30%) and cellulose (40 – 50%) (Ciolacu, 2018).

Lignocellulose biofuel production requires three processes namely, pre-treatment, saccharification of the polysaccharides (cellulose and hemicellulose) and fermentation of the resultant sugars to value-added products such as bioethanol (Wahlström and Suurnäkki, 2015). The pretreatment process is pivotally performed prior to saccharification since it degrades lignin exposing the polysaccharides to saccharification. Several available pretreatment processes have been used successfully (Ciolacu, 2018). Enzymatic saccharification is preferred over acid saccharification since it is environmentally friendly with the potential for almost complete conversion of the polymers. Enzymatic saccharification is the breakdown of glycosidic bonds in polymers found in lignocellulose material into fermentable sugars. This process requires lignocellulolytic enzymes, such as cellulase and hemicellulase enzyme complexes to hydrolyse the polymer (Cellulose and hemicellulose). Cellulose is a homopolysaccharide of β-1,4-linked D-glucose residues. Endoglucanases, randomly cleave internal β-1,4-glycosidic bonds, while the cellobiohydrolases release cellobiose from the reducing and non-reducing ends of the cellulose chain and finally β-glucosidases which cleave cellobiose into glucose units (Chen *et al.*, 2020).

Hemicellulose consists of heterogeneous groups of polysaccharides, with xylan being the major component consisting of β-1,4-D-xylose linked units substituted with O-acetyl, arabinose, ferulic acid, p-coumaric acid, and uronic acid. Degradation of hemicellulose require the synergistic action of endo-xylanase (endo- 1,4-β-xylanase, E.C.3.2.1.8), β-xylosidase (xylan-1,4-β-xylosidase, E.C.3.2.1.37), β-glucuronidase (β-glucosiduronase, E.C.3.2.1.139), β-arabinofuranosidase (β-l-arabinofuranosidase, E.C.3.2.1.55) and acetyl xylan esterase (E.C.3.1.1.72). The endo-xylanases and β-xylosidases are the major enzymes responsible for the complete hydrolysis of xylan. (Uday *et al.*, 2016 & Claes *et al.*, 2020).

Fungi are well-known for their remarkable ability to produce extracellular enzymes that are important in biotechnological application. Mesophilic fungi such as *Aspergillus* and *Trichoderma* are well known for their ability to produce xylanase enzymes that are used commercially. Other species include *Talaromyces* (Orencio-Trejo *et al.*, 2016), *Rhizomucor pusillus* (Hüttner *et al*., 2018), and *Rasamsonia emersonii* (Martínez *et al.*, 2016). There is an increasing emphasis on more research on xylanase production because the addition of xylanase in enzymatic saccharification will degrade xylan exposing the cellulose to cellulase attack, in turn, this will increase the rate and the efficiency of the process (Kumar and Sharma, 2017 & Tian *et al*., 2018).

The gut microbiota of dung beetle/larvae represent a novel source for bioprospecting of xylanase enzymes to not only degrade plant biomass into biofuels, but also for the production of industrial value-added products, and bioremediation of pollutants. Dung beetles are known to feed on wet and dry dung of herbivore animals. Notably, the dung beetles of the Scarabaeidae family feed on mammalian dung that is rich in lignocellulose material. This dung consists of 80% indigestible material such as cellulose, hemicellulose, lignin, tannin, chitin and other waste materials and therefore a well-regulated symbiotic relationship exists with microorganisms (Chapman, 2006; Shi *et al.,* 2010; Thiyonila *et al.*, 2018 & Nwaefuna *et al.*, 2021). The gut microbiota of the dung beetles plays a role in the degradation of lignocellulosic biomass for nutrient production, detoxification and protection from pathogens (Engel and Moran, 2013 & Suárez-Moo *et al.*, 2020). The aforementioned reflect the importance of exploiting Scarabaeidae gut microbiota for hemicellulolytic microorganisms and xylose-fermenting yeast (Nwaefuna *et al.*, 2021). Dung beetles are a major group of insects (Coleoptera: Scarabaeidae) with approximately 370 000 species identified worldwide

and 8000 species reported from the Kruger National Park in South Africa (Thiyonila *et al.*, 2018 & http://www.krugerpark.co.za/krugerpark-times-20-dung-beetle-18101.html). This study will be the first in determining the fungal diversity in the gut of dung beetles associated with the degradation of hemicelluloses and yeast capable of fermenting xylose.

The filamentous fungi with lignocellulolytic activity which have been isolated from the gut of different wood-feeding beetle larvae from the Scarabaeidae family were from the genus *Trichoderma, Bionectria*, and *Trametes* (Rojas-Jiménez and Hernández, 2015). Novel yeast strains isolated from different dung beetles include *Wickerhamomyces mori, Scheffersomyces parashehatae* and *Wickerhamiella allomyrinae* (Hui *et al*., 2013; Suh *et al*., 2013 & Ren *et al.,* 2014). Urbina *et al*. (2013) reported a wide variety of xylose and cellobiose fermenting yeast strains such as *Phaffomyces, Scheffersomyces, Spathaspora, Spencermartinsiella* and *Sugiyamaella* that were isolated from the gut of various species of passalid beetles. Different insects have unique fungal species due to their geographic distribution, diet, habitat, climatic conditions and type of the insect (Gonzalez, 2014 & Urbina *et al*., 2013). Microorganisms from the gut of various dung beetles could potentially be beneficial in biofuel production (Lemke *et al.*, 2003; Starmer and Lachance, 2011 & Rojas-Jiménez and Hernández, 2015).

Fermentation of pentose sugars is another factor that is key in reducing the cost of second-generation biofuel production. This includes the capability of yeast that can ferment the pentose sugars released from hemicellulose hydrolysis. There is a need for efficient xylose-fermenting yeast with high ethanol production, tolerance to high ethanol concentrations and tolerance of the inhibitors released during pretreatment (Kwak *et al.*, 2019 & Moremi *et al.*, 2020). *Saccharomyces cerevisiae* is a well-known yeast that ferments glucose effectively and can tolerate a high concentration of ethanol but cannot ferment xylose. There are a wide variety of species that can ferment xylose but do not ferment it as effectively as glucose. These yeast strains include *Scheffersomyces stipitis, Scheffersomyces shehatae* and *Pachysolen tannophilus.* Currently*, Scheffersomyces stipitis* is the best xylose-fermenting yeast but has a low tolerance for ethanol and for other inhibitors released during pretreatment of lignocellulose. This has hindered its use as an industrial strain for large-scale bioethanol production (Agbogbo and Coward-Kelly, 2008).

The suggestion that the gut of the dung beetle could be a rich source for hemicellulolytic fungi and xylose-fermenting yeast, this study thus sought to explore the diversity and composition of the gut-microbiota for hemicellulolytic fungi and xylose-fermenting yeast from Scarabaeidae dung beetles and dung beetle larvae in South Africa.

3.2. MATERIALS AND METHODS

3.2.1. Collection and dissection of dung beetles

Three dung beetle larvae (*Euoniticellus intermedius*) (Figure 3.1) and six dung beetles (*Pachylomerus femoralis* and *Anachalcos convexus*) (Figure 3.2) were received from Prof K Rumbold, School of Molecular and Cell Biology at Witwatersrand University. Dung beetles were also collected from the Kruger National Park from the dung of two different herbivores (impala and rhino) (Figure 3.3). The dung beetles collected from the Kruger National Park were identified by Prof M Byrne, School of Animal, Plant and Environmental Sciences at Witwatersrand University, South Africa. The beetles were kept alive on the dung of the herbivores until dissection. The beetles were surface sterilised by washing in 70% ethanol for 10 minutes and subsequently washed with sterile distilled water before dissection. The dung beetles were dissected by placing individual beetles on a flamed-sterilised glass slide. Sterile forceps were used to remove the elytra which allowed easy access to the gut content of each beetle (Figure 3.4) (Lemke *et al*., 2003). The entire gut of the dung beetles and dung beetle larvae were homogenised by suspending the content in 10 ml of a 0.7% saline solution.

Figure 3.1. *Euoniticellus intermedius* dung beetle's larvae (Mabhegedhe, 2017) were received from Witwatersrand University, South Africa. The gut consists of the Mid-gut (M) and Hindgut (H) while (A) represent the anal opening.

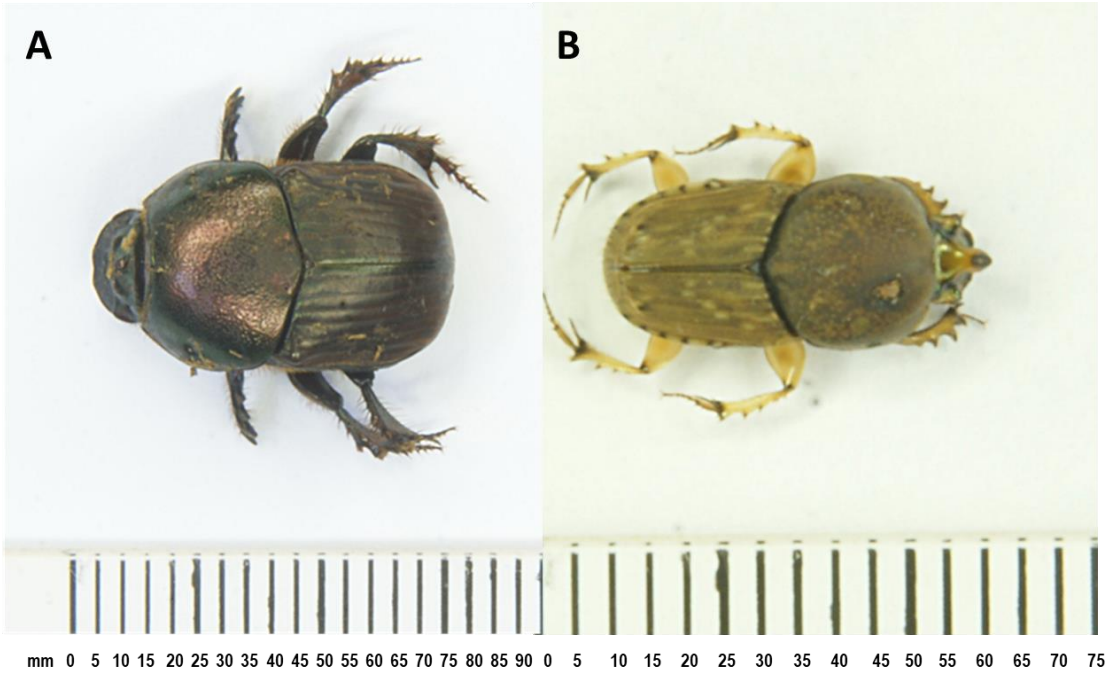

Figure 3.2. Dung beetles received from Witwatersrand University, South Africa. A: *Pachylomerus femor*alis dung beetle B: *Anachalcos convexus* dung beetle.

Figure 3.3. Dung beetles were collected from the Kruger National Park, South Africa. A: *Heteronitis castelnaui* dung beetle from rhino dung; B: *Kheper nigroanaeus Boheman* dung beetle from impala dung.

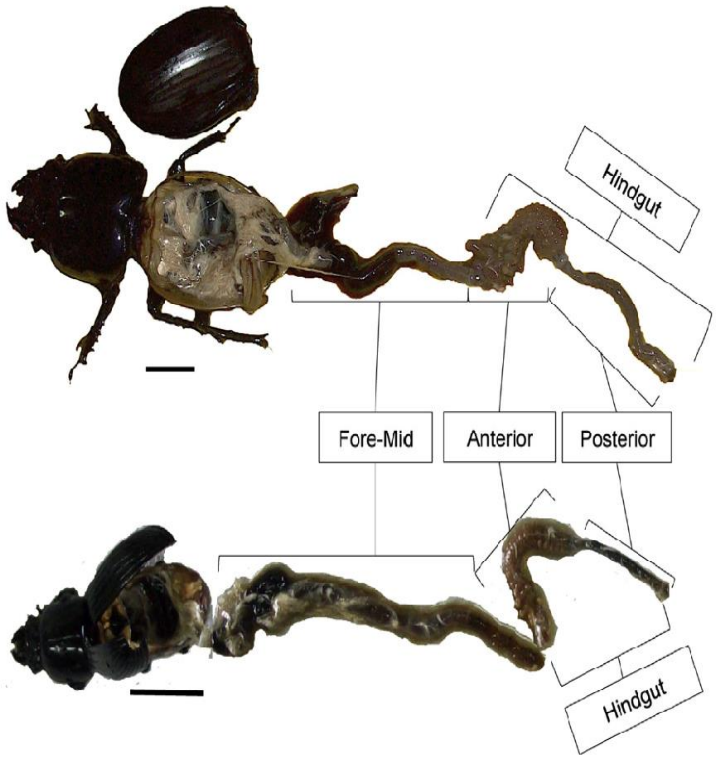

Figure 3.4. Representation of the dissected beetles and the sections of the gut that were used for isolation of the fungi (Urbina, *et al.*, 2013).

3.2.2. Isolation of yeast from the gut of the dung beetles

One milliliter of the homogenised solution from *Kheper nigroanaeus Boheman* (impala dung) and *Heteronitis castelnaui* (rhino dung) was poured on a plate with 0.67% Yeast Nitrogen Base (YNB) with amino acids containing 2% xylose as carbon source media and 0.2% chloramphenicol to inhibit bacterial growth. The plates were incubated at 30 °C for up to 5 days. Different morphotypes were picked and purified by re-streaking on the same media until pure colonies were obtained. The purified isolates were suspended in YM broth (20 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 0.2 g/L chloramphenicol) supplemented with 15% glycerol and then preserved at -80 ºC. Physiological characteristics were carried out following the methods of Yarrow (1998), Barnett *et al*. (2000) & Kurtzman *et al*. (2011).

3.2.3. Isolation of filamentous fungi from the gut of the dung beetles

One milliliter of the homogenised solutions obtained from the beetles (*Pachylomerus femoralis* and *Anachalcos convexus*) and dung beetle larvae (*Euoniticellus intermedius*) was plated on 0.67% YNB with amino acids containing 2% xylan as carbon source and 0.2% chloramphenicol to inhibit bacterial growth. The plates were incubated at 30 °C for up to 5 days. Different morphotypes were purified several times by placing a 10 x 5 mm agar block repeatedly on YM plates. The process was repeated until pure colonies were obtained.

The purified fungal isolates were inoculated on YM plates by placing a 10 x 5 mm agar block with the fungus on the plates. The plates were incubated at 30 °C for 5 days. Agar blocks were then transferred to YM slants and re-inoculated every 30 days. Preservation of fungal isolates for a longer period (years) was performed by preparing and inoculating YM media with the fungal isolates. The YM plates were incubated at 30 °C for 5 days, after which the spores were dislodged with a hockey stick by adding 1 ml of sterile distilled water. Approximately 1 ml of the spore solution was mixed with 1 ml of a 30% glycerol solution in 2 ml cryo-tubes and stored at −80 °C.

3.2.4. ITS and D1/D2 sequencing

All fungal isolates were sent to Inqaba Biotechnical Industries (Pty) Ltd, South Africa for ITS and D1/D2 sequencing. DNA was extracted using the ZR Fungal DNA MiniPrepTM Kit (Zymo Research) according to the manufacturer's instructions. The ITS1-5.8S-ITS2 region was amplified using PCR primers ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). Amplification was carried out in 25 µl reactions using the EconoTaq Plus Green Master Mix (Lucingen). The following PCR conditions were used: 35 cycles including an initial denaturation at 95 °C for 2 minutes. Subsequent denaturation at 95 °C for 30 seconds, annealing at 50 °C for 30 seconds and extension at 72 °C for 1 minute. A final extension at 72 °C for 10 minutes was followed by holding at 4 °C. Additionally, the D1/D2 domain of the 26 S rDNA region was amplified using primers NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') as described above. DNA sequencing was done using ABI V3.1 Big dye according to the manufacturer's instructions on the ABI 3500 XL Instrument (Makhuvele *et al*., 2017).

3.2.5. Identification of fungal isolates and phylogenetic analysis

The DNA sequence data obtained were cleaned, trimmed and aligned using Bio edit software (http://www.mbio.ncsu.edu/bioedit/page2.html). Alignments were performed using the Muscle software as implemented in the MEGA 5.2 program. The ITS and D1/D2 sequences were used to identify the fungal isolates. The ITS and D1/D2 sequences were compared to sequences in the Genbank database using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/ BLAST/). A phylogenetic tree based on the ITS domains was constructed using MEGA software version 7.0 (Kumar *et al.*, 2018). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Bootstrap analysis (Felsenstein, 1985) was performed from 1000 replications to determine confidence levels of the clades, and only values > 50% were recorded on the resulting tree (Kumar *et al.*, 2018).

3.2.6 Screening for D-xylose-fermentation in partially aerobic condition

A single colony of the yeast isolates that was maintained on YM media were inoculated in media containing 0.67% YNB with amino acids supplemented with 2% xylose. This was done in test tubes closed with lids containing Durham tubes. The initial cell density was adjusted to an OD_{600} of 0.05. The inoculated tubes were incubated at 30 ºC on an orbital shaker at 100 rpm for up to 14 days and observed every 24 hours for the qualitative production of gas, which was indicative of fermentative ability (Kurtzman and Fell, 1998). *Scheffersomyces stipitis* NRRL Y-7124 was used as a positive control.

Pre-cultures for yeast isolates that were positive in the fermentation test were inoculated into 250 ml Erlenmeyer flasks with 25 ml fermentation medium (20 g/L xylose, 4.5 g/L yeast extract, 7.5 g/L peptone, 1 g/L $MgSO_4$, 3 g/L KH_2PO_4, 0.2 g/L chloramphenicol) at pH 5 (Modified Kurtzman *et al.,* 2011). The flasks were incubated at 30 °C for 24 hours on a rotary shaker at 200 rpm. The flasks were used to inoculate 100 ml of fresh xylose fermentation media with the cell density adjusted to an OD_{600nm} of 0.1. The flasks were incubated at 30 °C and 200 rpm on a rotary shaking incubator for 48 hours. Samples (2 ml) were centrifuged for 5 minutes at 2000 x g and 4 °C after which the supernatants were filtered through a 0.22 µm syringe filter and stored at −20 °C until analysis was performed on a Gas Chromatograph. All experiments were performed in duplicates. *S. stipitis* (NRRL Y-7124) was used as a positive control.

3.2.7. Screening yeast isolates for acetic acid- and thermo-tolerance

The xylose-fermenting yeast isolates were tested for their ability to grow in the presence of 1, 2, 3, 5, 7 and 10 g/L acetic acids on YM agar plates. All plates were incubated at 30 ºC for 48 hours. The maximum growth temperatures for the yeast isolates were determined using YM slants. Slants were incubated at 35, 37, 40 and 45 ºC. The maximum temperature for growth is considered the highest temperature where growth still occurred (Makhuvele *et al*., 2017).

3.2.8. Screening filamentous fungal isolates for xylanase activity

A 15mm X 2mm block of agar was cut from 5-day old fungal culture plates and inoculated into test tubes containing 5 ml 0.67% YNB with amino acids with 1% beechwood xylan (Sigma) as the sole carbon source. The test tubes were incubated on a rotary shaker at 100 rpm at 30 °C. Samples were taken after 72 hours for enzyme analysis. Quantitative analysis for xylanase activity was performed using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). The method determined the amount of reducing sugar (xylose) released during the hydrolysis of xylan. The xylanase enzyme activities were expressed in katals per milliliter (nkat/ml), where 1 katal is the amount of enzyme needed to produce 1 mol of reducing sugar from the substrate per second. All experiments were performed in triplicates.

3.2.9. Determination of ethanol content

Ethanol was measured by capillary gas chromatography using a Shimadzu (Kyoto, Japan) GC 2010 Plus apparatus equipped with Autoinjector AOC 20i (Shimadzu) and Autosampler AOC 20S (Shimadzu), a flame ionization detector (FID) and a Zebron ZB wax Plus 30 M (Phenomenex, USA) column (30 m, 0.25 mm ID and film thickness of 0.25 µm). The column flow rate was maintained at 1.29 ml/min. The oven temperature was maintained at 70 °C for 1 minute then increased to 140 °C and finally maintained at 250 °C for the duration of the analysis. The injection temperature was 200 °C and the injection volume was 1 µl. The detector was maintained at 250 °C. Nitrogen gas at a flow rate of 30 ml/min was used as the carrier gas. Peak detection and integration were done using GC Solutions software from Shimadzu (Kyoto, Japan). Known ethanol standards were included.

3.3. RESULTS

A total of one hundred and thirty-two yeast strains, able to utilise xylose, were isolated from the gut of the dung beetles *Kheper nigroanaeus* Boheman (45 yeast isolates) and *Heteronitis castelnau* (87 yeast isolates). The yeast isolates were screened for xylose fermentation, growth at elevated temperature and growth in the presence of acetic acid. A total of two hundred and seventeen xylanase-producing filamentous fungi were isolated from the guts of *Pachylomerus femoralis* (35 fungal isolates), *Anachalcos convexus* (118 fungal isolates) and the larvae of the dung beetle *Euoniticellus intermedius* (69 fungal isolates).

3.3.1. Isolation, identification and characterisation of xylose-fermenting yeast

One hundred and thirty-two yeast strains (Appendix, Table 3.1) were isolated and identified from the gut of *Kheper nigroanaeus* Boheman (*K. nigroanaeus Boheman)* and *Heteronitis castelnau (H. castelnau).* The yeast isolates belonged to eight genera representing both Ascomycota and Basidiomycota. The Basidiomycota was represented by *Cryptococcus, Mallassezia, Trichosporon and Rhodotorula,* while the Ascomycota included *Candida, Debaryomyces* and *Meyerozyma*. Species representing *Candida* were the most prevalent representing the Ascomycota while the most prevalent representative from the Basidiomycota was the species from *Trichosporon*. *Candida* and *Trichosporon* genus were common in both guts of the dung beetles *K. nigroanaeus Boheman* and *H. castelnau.*

Eighty-seven yeast isolates belonging to 14 species were isolated from the gut of the dung beetle *H. castelnau* (Figure 3.5.A) compared to 44 yeast strains (5 species) from *K. nigroanaeus Boheman* (Figure 3.5.B). In the gut of *H. castelnau* dung beetle, 38 strains belonged to the genus *Trichosporon* representing four species that are *T. inkin (*20*%), T. debeurmannianum* (1%)*, T. ovoides* (17%), *and T. cutaneum (7%)* (Figure 3.5). *T. inkin* Y1 and Y2 were positive xylose fermenters that were deduced by the gas formation in the Durham tubes.

Twenty yeast isolates were associated with *Mallassezia globosa* representing 22% of the yeast isolates from *H. castelnau* (Figure 3.5. A). Only *M. globosa* Y128 could produce gas during xylose fermentation. *Candida* was represented by 20 yeast strains belonging to *C. tropicalis* (11%), *C. parapsilosis* (1%) and *C. ranongensis* (7%). Nine

strains belonging to *C. tropicalis* (Y103, Y147, Y155, Y156, Y158, Y159, Y162, Y3, and Y9) and one strain for *C. ranongensis* (Y8) formed gas in the Durham tube during xylose fermentation.

Rhodotorula mucilaginosa Y233, *Cryptococcus saitoi* Y121 and *C. laurentii* Y19 were unable to ferment xylose. *C. laurentii* Y19 was able to ferment xylose. Two yeast isolates identified as uncultured fungi (Y100 and Y86) and yeast isolates belonging to *Sordariomyceta* sp. Y132, *Coniochaeta polymorpha* Y127 and *Debaryomyces hansenii* Y38 were not able to ferment xylose.

Forty-four yeast strains representing 5 yeast species were isolated and identified from the gut of the dung beetle *K. nigroanaeus Boheman* (Figure 3.5.B). The most prevalent yeast from the gut of this dung beetle belong to genus *Trichosporon* (21 isolates) represented by five different species *T. inkin* (2%), *T. debeurmannianum* (9%), *T. asahii* (9%), *T. insectoum* (5%) and *T. faecale* (2%). *Trichosporon ovoides* Y62 was the only species that produced gas during xylose fermentation.

Fifteen yeast strains belonged to *Candida tropicalis* with 9 strains fermenting xylose (Y150, Y152, Y154, Y161, Y163, Y164, Y165, Y7 and Y61). Seven yeast strains were associated with *Malassezia globosa* and none of the strains could ferment xylose. *Meyerozyma guilliermondii* Y63 and *C. saitoi* Y94 could also not ferment xylose (Figure 3.5.B).

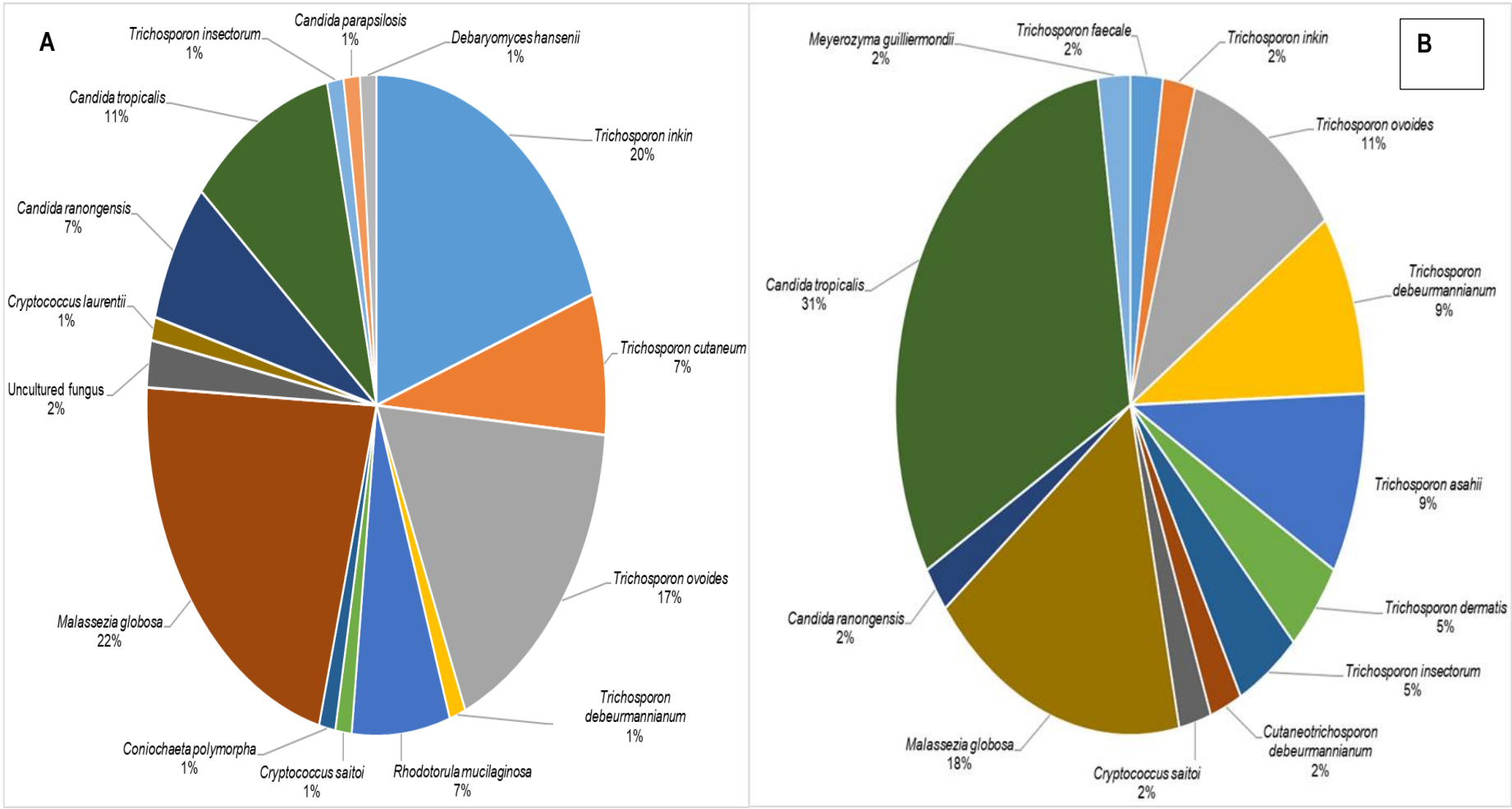

Figure 3.5. Distribution of yeast species isolated from the gut of the dung beetle *H. castelnau* (A) and *K. nigroanaeus Bohem* (B)

3.3.2. Isolation, identification and characterisation of filamentous fungi

Two hundred and eighteen xylanase-producing filamentous fungi were isolated from the gut of *Pachylomerus femoralis*, *Anachalcos convexus* and dung beetle larvae of *Euoniticellus intermedius*. The filamentous fungal isolates were identified using the ITS and D1/D2 region and were represented by ten different genera namely, *Aspergillus, Hypocrea, Trichoderma, Penicillium*, *Mucor*, *Neosartorya*, *Rhizopus, Talaromyces*, *Taifanglania and Byssochlamys.* The most prevalent genus was *Aspergillus* (82 strains) followed by *Hypocrea* (53 strains), *Penicillium* (28 strains), *Trichoderma* (27 strains*)* and *Neosartorya* (11 strains), while the less dominant isolates belonged to *Talaromyces* (7 strains) Rhizopus (3 strains), Mucor (4 strains) with *Byssochlamys, Taifanglania ,* and *Emericella* represented by 1 strain each (Figure 3.6).

Most filamentous fungi were isolated from the gut of the dung beetle *A. convexus* (118 strains). The most dominant genus in this dung beetle was *Aspergillus*, followed by *Hypocrea* and *Penicillium* (Figure 3.6 A). The dung beetle larvae (*E. intermedius*) were represented by 10 different genera (65 strains). The most dominant genus in the larvae was the genus *Hypocrea* followed by *Aspergillus* and *Neosartorya* (Figure 3.6.B). Thirty-five filamentous fungal strains were isolated from the gut of the adult dung beetle *P. femoralis. Aspergillus* was the most dominant genus followed by *Trichoderma* and *Penicillium* (Figure 3.6.C).

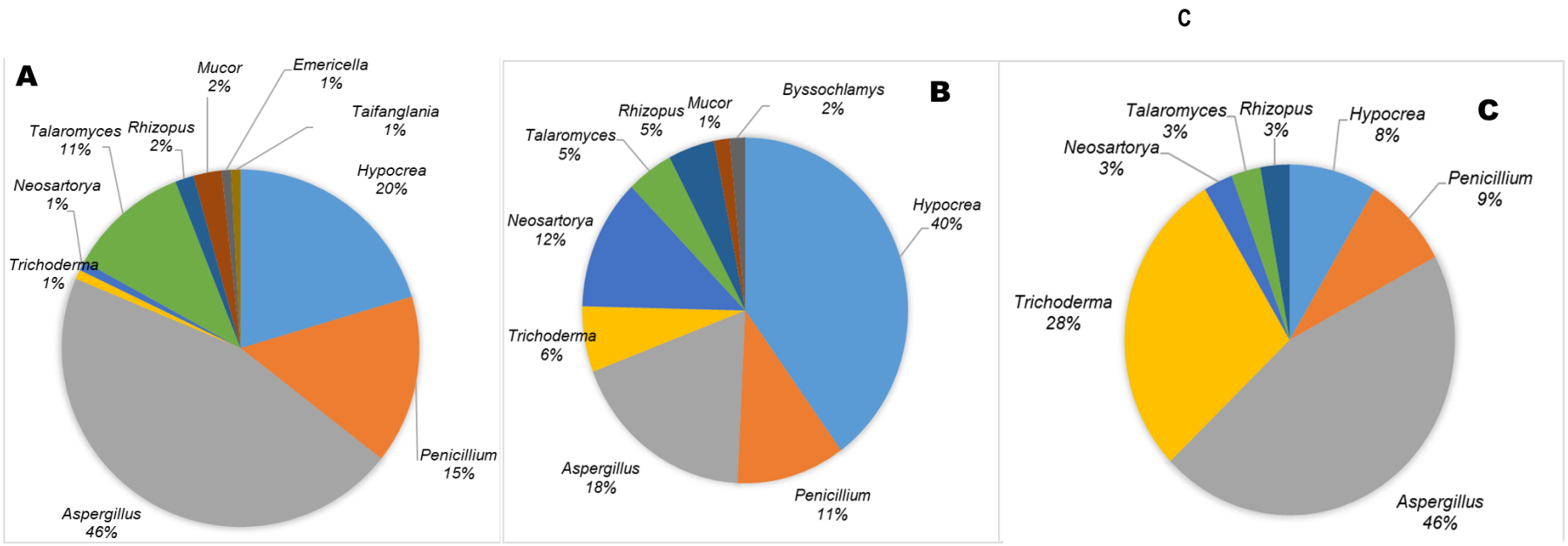

Figure 3.6. The filamentous fungi isolated from the gut of dung beetle and dung beetle larvae. (**A**) gut microbiome from *A. convexus* (**B**) microbiome from *E. intermedius* Larvae and (**C**) gut microbiome from *P. femoralis.*

3.3.2. Phylogenetic Analysis

The phylogenetic trees were constructed using the ITS sequence of the filamentous fungi isolated from the dung beetles (*Pachylomerus femoralis* and *Anachalcos convexus)* and dung beetle larvae (*Euoniticellus intermedius*) (Fig 3.7 – 3.10). Distinct clusters were apparent for different fungal species. This was done to evaluate any evolutionary relationships between different species with xylanolytic activity isolated from these beetles and the larvae.

The species isolated from the gut of dung beetle *A. convexus* formed several clades namely *Trichocomaceae, Penicillium*, *Aspergillus* and *Trichoderma* (Figure 3.7). The family *Trichocomaceae* split into three separate families which were *Aspergillaceae*, *Trichocomaceae*, and *Thermoascaceae*. These were reflected in the phylogenetic analysis by *Trichocomaceae* clade I composed of *Talaromyces, Aspergillus* and *Penicillium* species (Figure 3.7). *Aspergillus* and *Penicillium* species are known to have close genetic relations. The Mucoraceae clade was presented by *Hyphomucor, Mucor* and *Rhizopus* genera. The relationship among the species was well supported by branches > 50%.

The results for phylogenetic analysis (Figure 3.8) showing the relationship of species isolated from the gut of dung beetle *P. femoralis.* There are only two clades deduced from the phylogenetic analysis namely *Trichocomaceae* and *Trichoderma*. The species *Rhizopus microsporus* is a sister taxon with an outgroup.

Phylogenetic analysis (Figure 3.9) showing the relationship of species isolated from the dung beetle larvae (*E. intermedius).* Different clades were shown in this phylogenetic tree where three monophyly groups for *Penicillium* were deduced. Other clades were *Neosartorya, Aspergillaceae, Talaromyces*, *Trichoderma* and *Byssochlamyces*. These clades, beside *Trichoderma,* are part of the *Trichocomaceace* family. The other clade deduced was *Mucoraceae clade* made of *Mucor circinelloides* and *Rhizopus* sp.

All the fungal isolates from the two different species of dung beetles and dung beetle larvae were used to construct a best-scoring maximum likelihood tree to evaluate the relatedness of the species isolated. Different monophyletic groups revealed in the three phylogenetic trees were present. The species such as *Hypocrea lixii,*

Trichoderma sp., *Aspergillus niger, Neosartorya fischeri* and *Talaromyces helicus* from the gut and the larvae showed relatedness as they formed clades with strains from the gut of both the dung beetle and the larva. The *Trichocomacea* family was reflected on the phylogenetic tree that combined different species from the gut and larvae of the dung beetles (Figure 3:10). The deduced branches were well-supported with the bootstrap > 50%. The best scoring maximum likelihood tree showed that the fungal species isolated from the guts of *P. femoralis* and *A. convexus* and dung beetle larvae (*E*. *intermedius*) are closely related (Figure 3.10). The figure for the phylogenetic trees are also displayed in the **Appendix 1**

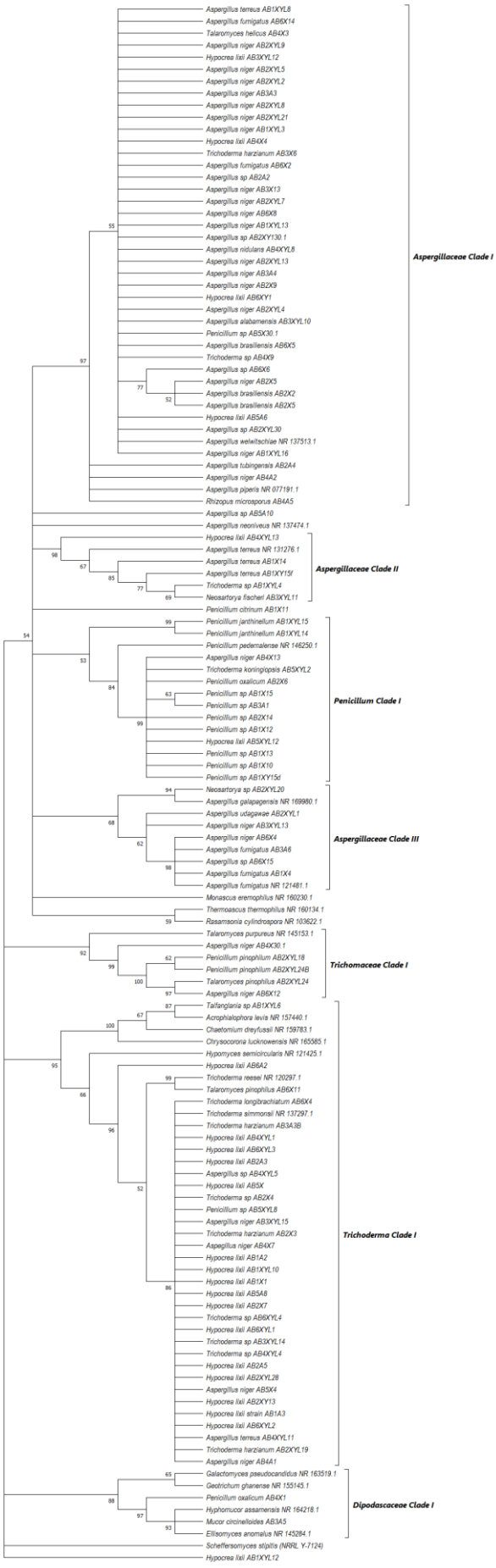

Figure 3.7. Best-scoring Maximum Likelihood tree using ITS sequence of different strains isolated from the gut of dung beetle *A. convexus*. Only branches with more than 50% bootstrap support are shown. *S. stipitis* NRRL-Y-7124 was used as an outgroup.

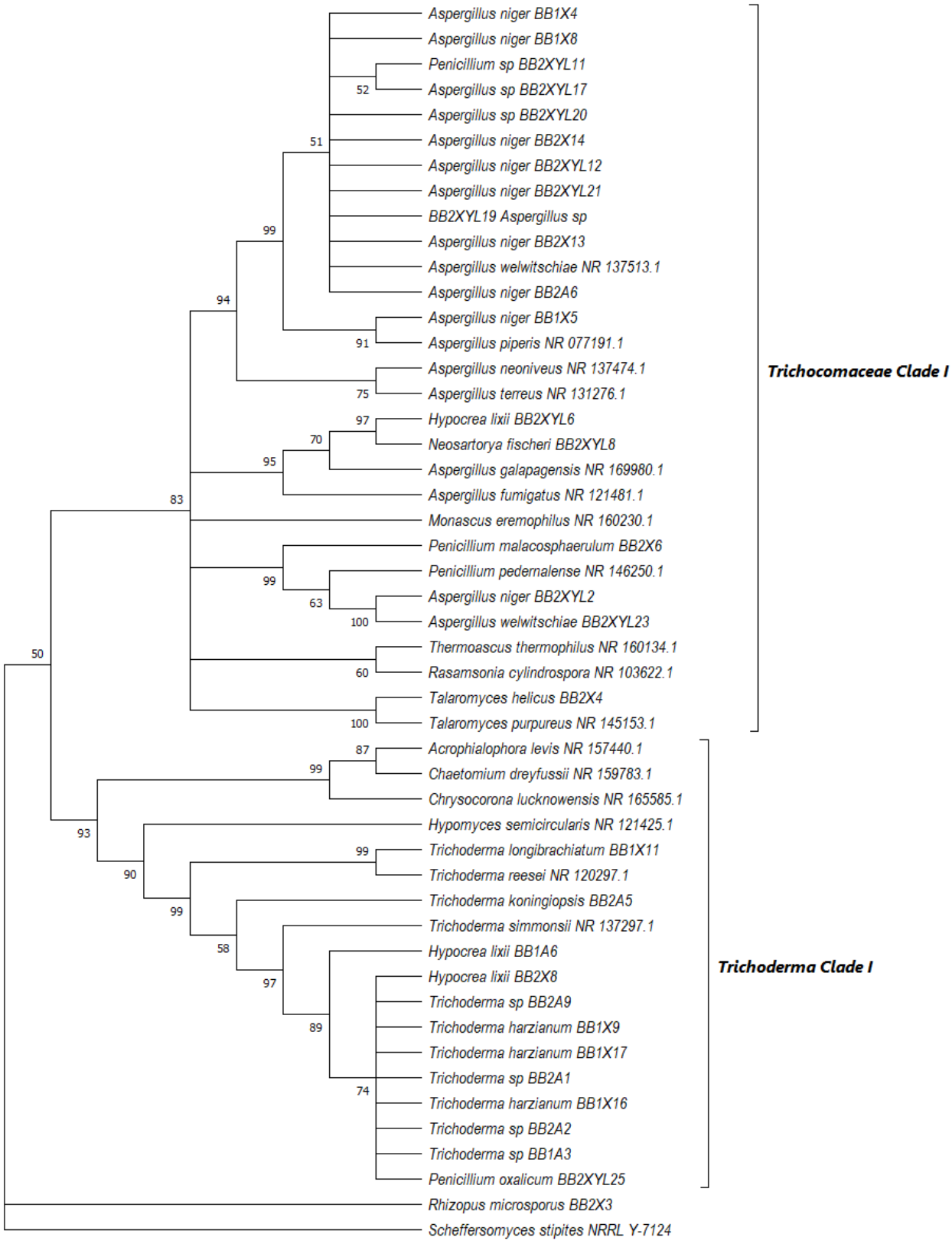

Figure 3.8. Best-scoring Maximum Likelihood tree using ITS sequence of different strains isolated from the gut of dung beetle *P. femoralis*. Only branches with more than 50% bootstrap support are shown. *S. stipitis* NRRL-Y-7124 was used as an outgroup.

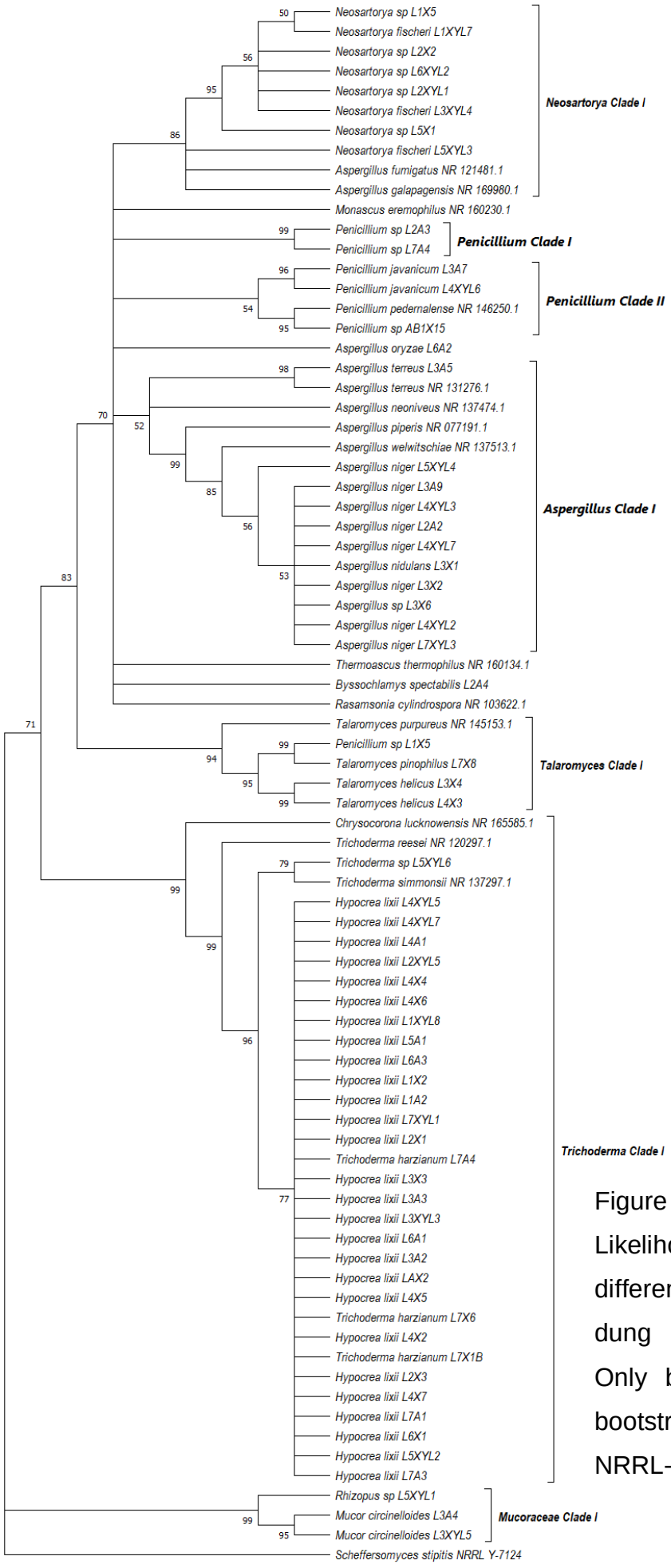

Figure 3.9. Best-scoring Maximum Likelihood tree using ITS sequence of different strains isolated from the gut of dung beetle larvae (*E. intermedius)*. Only branches with more than 50% bootstrap support are shown. *S. stipitis* NRRL-Y-7124 was used as an outgroup.

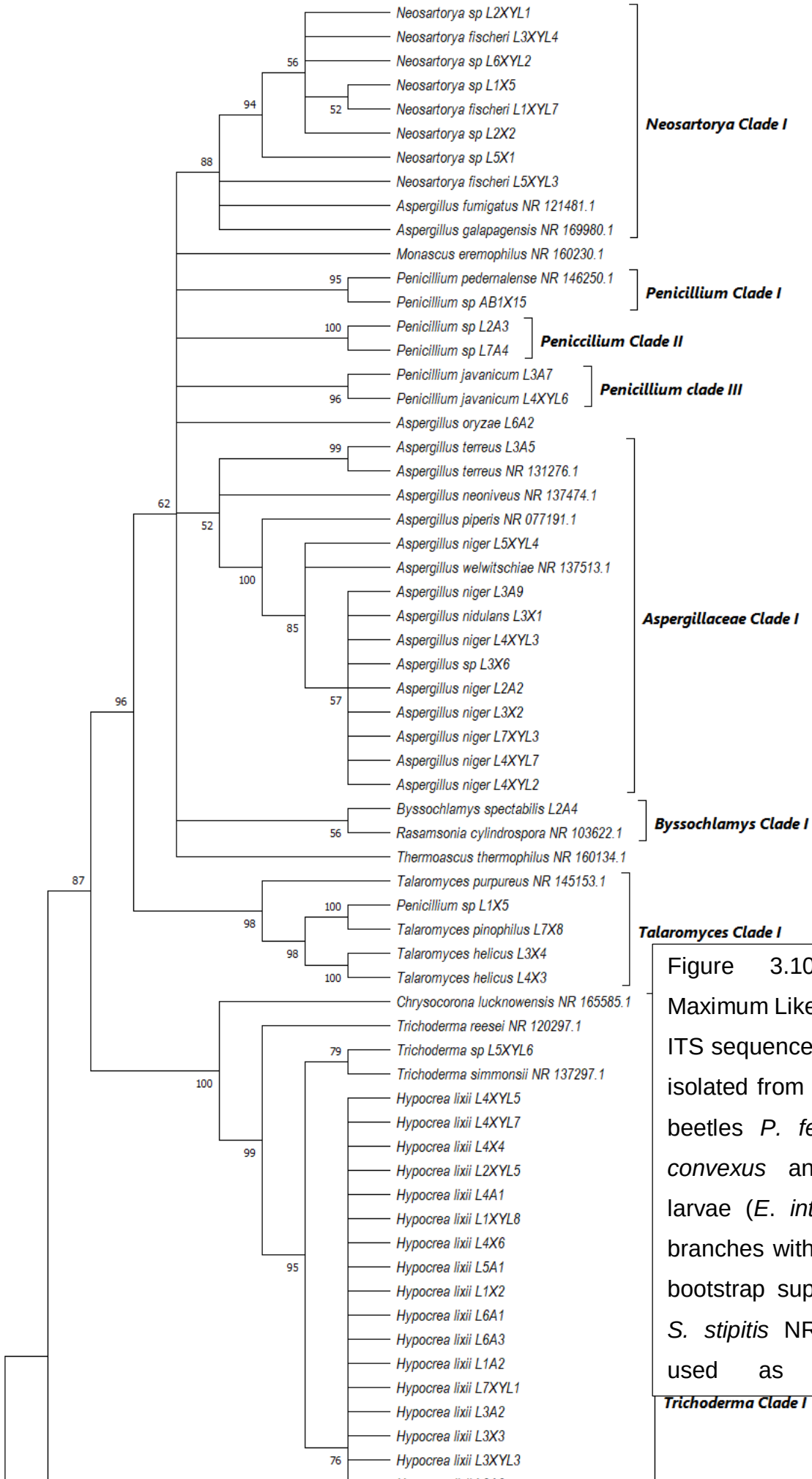

Figure 3.10. Best-scoring Maximum Likelihood tree using ITS sequence of fungal strains isolated from the guts of dung beetles *P. femoralis* and *A. convexus* and dung beetle larvae (*E. intermedius*). Only branches with more than 50% bootstrap support are shown. *S. stipitis* NRRL-Y-7124 was used as an outgroup.

3.3.3. D-xylose Fermentation

Twenty-six yeast strains fermented xylose and produced between 0.15 and 1.11 g/L ethanol (Figure 3.11) with 13 strains isolated from *K. nigroanaeus Boheman* and 13 strains from *H. castelnau*. None of the yeast strains were able to produce similar or higher ethanol concentrations when compared to *S. stipitis* NRRL Y-7124.

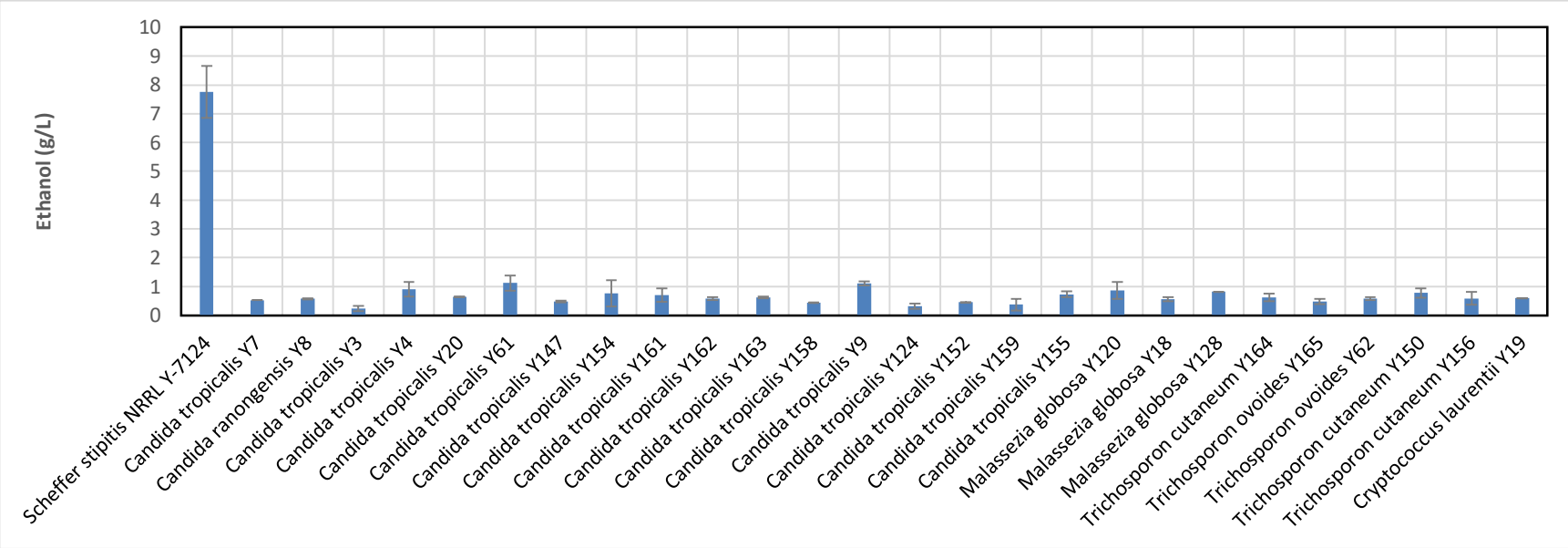

Figure 3.11. Ethanol produced by yeast strains isolated from the gut of the dung beetles *H. castelnau* and *K. nigroanaeus Boheman* during growth on xylose as the sole carbon source.

3.3.4. Growth at elevated temperature and in the presence of inhibitors

The 26 xylose-fermenting yeast strains were screened for the ability to grow at elevated temperatures (Table 3.2). All the yeast strains except *M. globosa* Y120 (37 ºC) were able to grow at 40 °C and in the presence of 3 g/L acetic acids.

Table 3.2. Xylose-fermenting yeast from dung beetles (*H. castelnau* and *K. nigroanaeus Boheman*) screened for the ability to grow in the presence of acetic acid and at 40 °C.

Yeast strains	**Growth in the presence of 3 g/L Acetic acid**	**Growth at 40 ºC**
Candida tropicalis Y7	**+**	**+**
Candida tropicalis Y8	**+**	**+**
Trichosporon faecale Y18	**+**	**+**
Papiliotrema laurentii Y19	**+**	**+**
Candida tropicalis Y20	**+**	**+**
Cutaneotrichosporon debeurmannianum Y62	**+**	**+**
Trichosporon ovoides Y128	**+**	**+**
Candida tropicalis Y161	**+**	**+**
Candida tropicalis Y162	**+**	**+**
Candida tropicalis Y163	**+**	**+**

Candida tropicalis Y164	**+**	**+**
Candida tropicalis Y165	**+**	**+**
Candida tropicalis Y3	**+**	**+**
Candida tropicalis Y4	**+**	**+**
Malassezia globosa Y120	**-**	**-**
Candida tropicalis Y150	**+**	**+**
Candida tropicalis Y147	**+**	**+**
Candida tropicalis Y154	**+**	**+**
Candida tropicalis Y155	**+**	**+**
Candida tropicalis Y156	**+**	**+**
Candida tropicalis Y158	**+**	**+**
Candida tropicalis Y9	**+**	**+**
Candida tropicalis Y124	**+**	**+**
Candida tropicalis Y152	**+**	**+**
Candida tropicalis Y159	**+**	**+**

Key: **+ Growth**

No growth

3.3.5. Xylanase production by filamentous fungi isolated from dung beetles and dung beetle larvae

All the filamentous fungal strains (218) isolated from the gut of *Anachalcos convexus* and *Pachylomerus femoralis* and the larvae of *Euoniticellus intermedius* were screened for xylanase activity using xylan as substrate. Two hundred and four filamentous fungi showed xylanase activity higher than 50 nkat/ml (Figure 3.12 – 3.14). All the filamentous fungi with xylanase activity of less than 50 nkat/ml were excluded from the figure. The xylanase producing filamentous fungi belonged to the following 12 genera namely *Aspergillus* (78), *Hypocrea* (51), *Penicillium* (27), *Trichoderma* (26), *Talaromyces* (4), *Neosartorya* (6), *Mucor* (4), *Geotrichum* (3), *Rhizopus* (2), *Byssochlamys,* and *Taifanglania* (1).

The dung beetle *Pachylomerus femoralis,* resulted in 22 fungal strains with xylanolytic activity higher than 50 nkat/ml belonging to genus *Neosartorya, Hypocrea, Penicillium*, *Aspergillus* and *Talaromyces* (Figure 3.12). *Aspergillus* was the dominant genus in the gut of the dung beetle *Pachylomerus Femoralis. Trichoderma* sp. (BB2A1 and BB2A3) produced the highest xylanase activity (both producing 195.83 nkat/ml). This was followed by *Penicillium oxalicum* BB2XYL23 (90.96 nkat/ml), *Aspergillus niger* BB2XYL21 (170.73 nkat/ml) and *Talaromyces helicus* BB2X4 (145.60 nkat/ml).

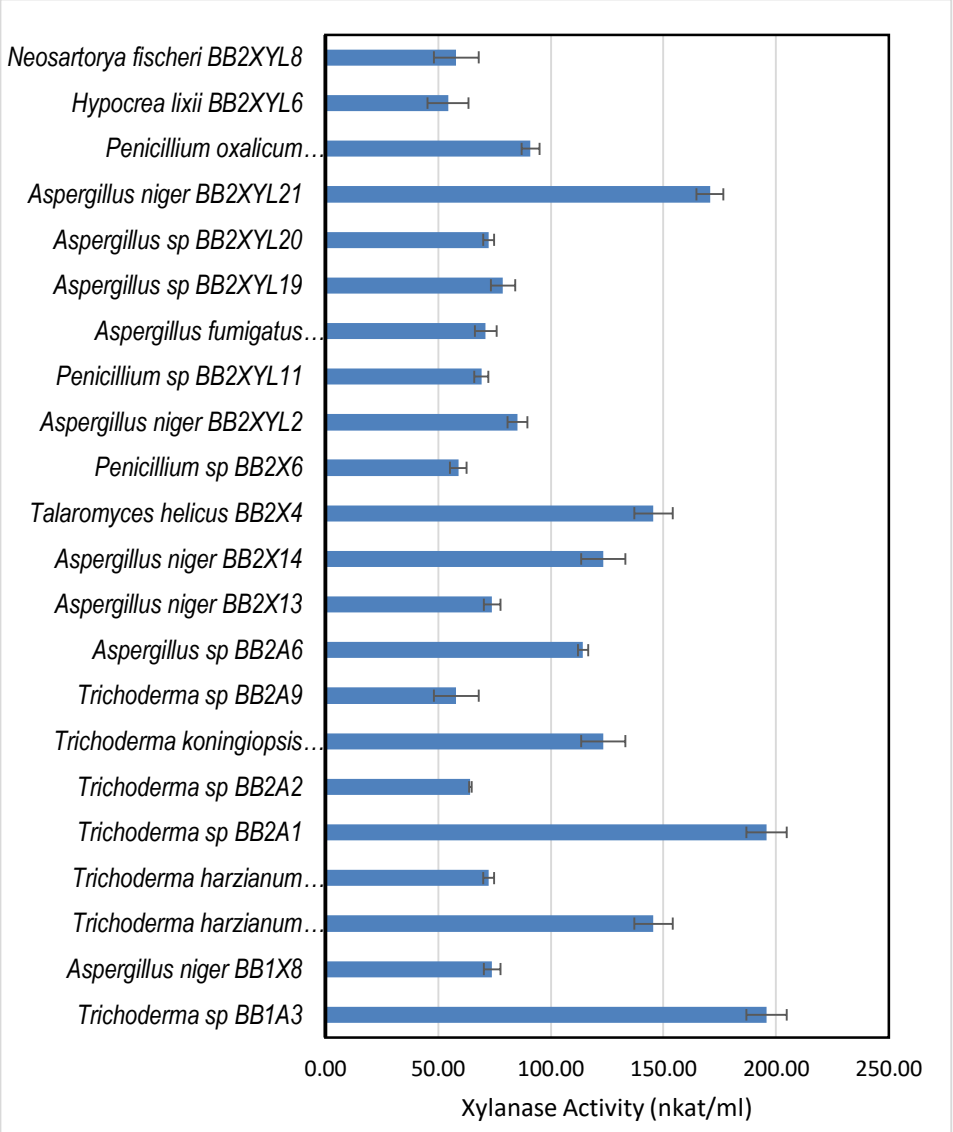

Figure 3.12. Xylanase activity by filamentous fungi isolated from the gut of the dung beetle *P. femoralis.*

Thirty-five filamentous fungi isolated from *E. intermedius* larvae showed xylanase activity higher than 50 nkat/ml (Figure 3.13). *Hypocrea lixii* was the most dominant species with xylanase activities ranging from 68.66 – 211.89 nkat/ml. Two filamentous fungi with the highest xylanase activity were *Byssochlamys spectabilis* L2A4 and *Aspergillus fumigatus* L1XYL9 (311.42 and 393.22 nkat/ml, respectively) (Figure 3.13).

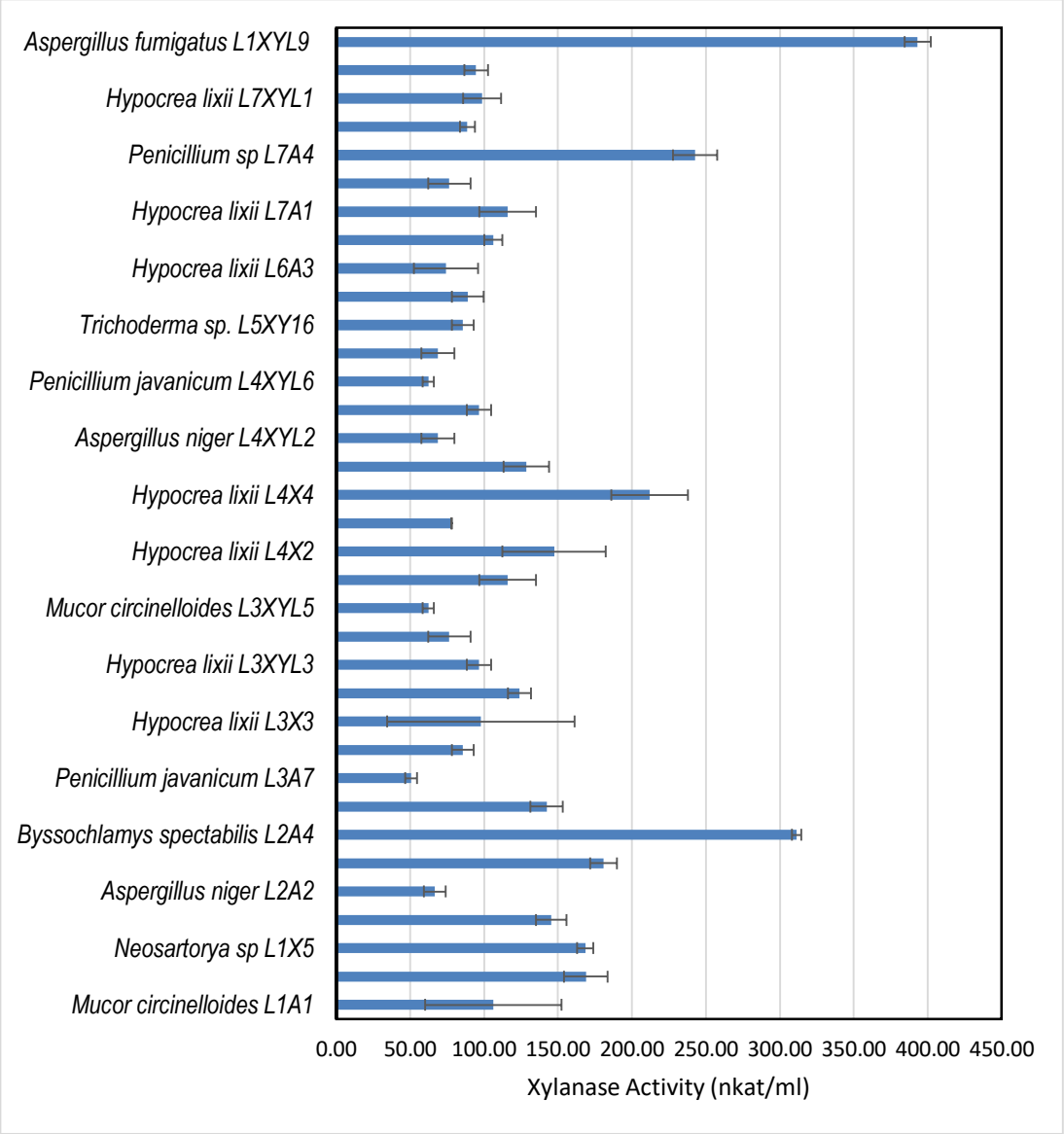

Figure 3.13. Xylanase activity by filamentous fungi isolated from the dung beetle *Euoniticellus intermedius* larva.

The dung beetle *Anachalcos convexus* had the most fungi with xylanase activities higher than 50 nkat/ml. The highest xylanase activity was produced by *Hypocrea lixii* AB6XYL2 and AB2A3 with xylanase activity of 392.53 and 313.06 nkat/ml, respectively (Figure 3.14). While other species that showed good xylanase activity in the gut of *A. convexus,* were *Aspergillus* sp. AB2XYL30 (245.99 nkat/ml), *Penicillum janthinellum* AB1XYL14 (245.99 nkat/ml) and *Trichoderma* sp. AB1XYL4 (207.39 nkat/ml).

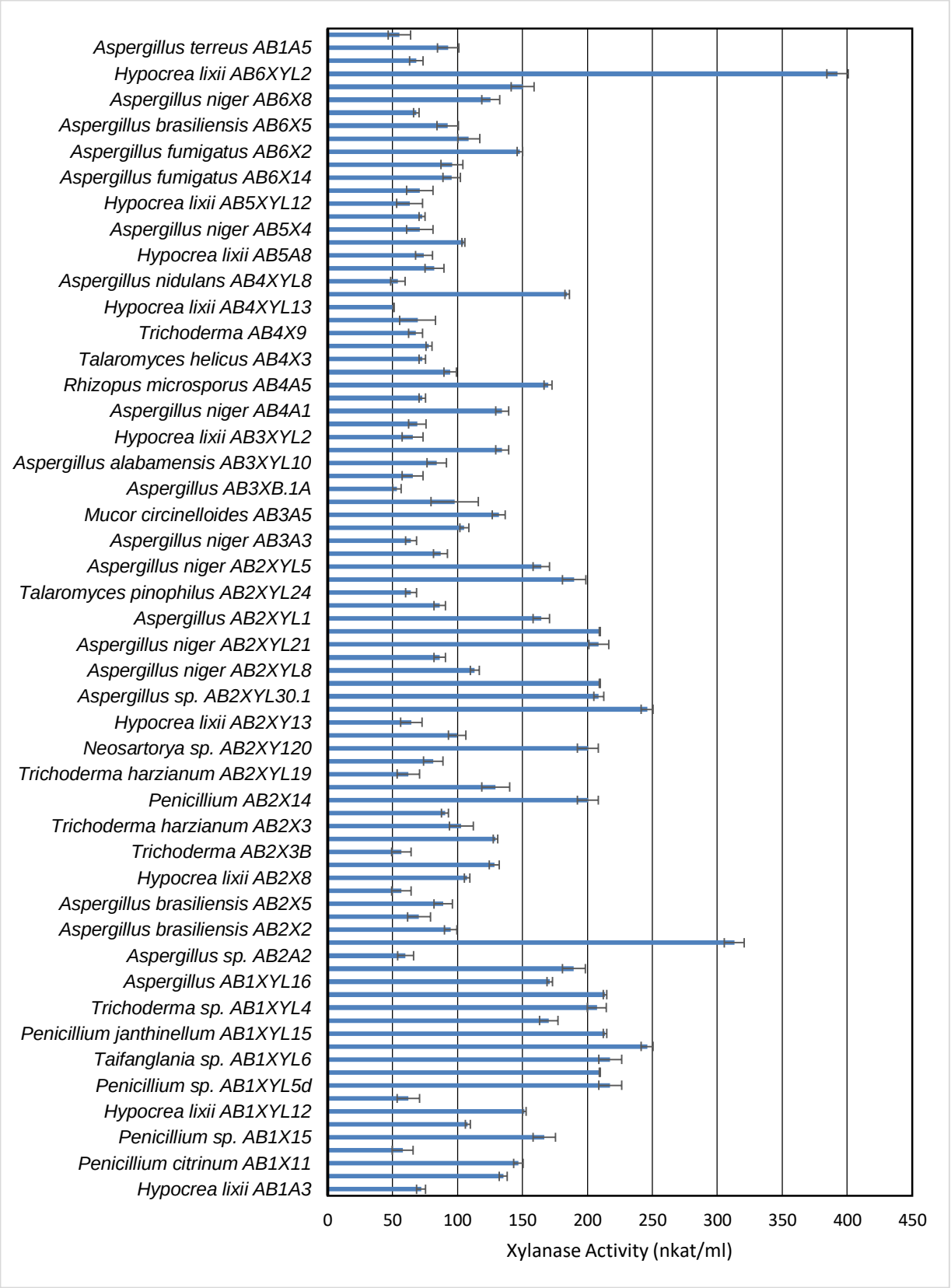

Figure 3.14. Xylanase activity by filamentous fungi isolated from the gut of the dung beetle *A. convexus.*

The fungi producing more than 50 nkat/ml xylanase activity from the gut of the two dung beetles *A. Convexus* and *P. femoralis* and one dung beetle larvae *E. intermedius* were selected for future investigations (Figure 3.15). From the gut of *Anachalcos convexus* with higher xylanase activity of 392.53 nkat/ml. While from the gut of *P. femoralis Trichoderma* sp. (BB2A1 and BB2A3) were two species with higher xylanase activity of 195.83 nkat/ml. Furthermore, from *E. intermedius* larvae *Aspergillus fumigatus* L1XYL9 showed higher xylanase activity 393.53 nkat/ml (Figure 3.15). Other unique species such as *Talaromyces helicus* BB2X4 from *P. femoralis*, *Neosartorya* sp. AB2XYL20 and *Rhizopus microsporus* AB4A5 from *A. convexus* were also selected.

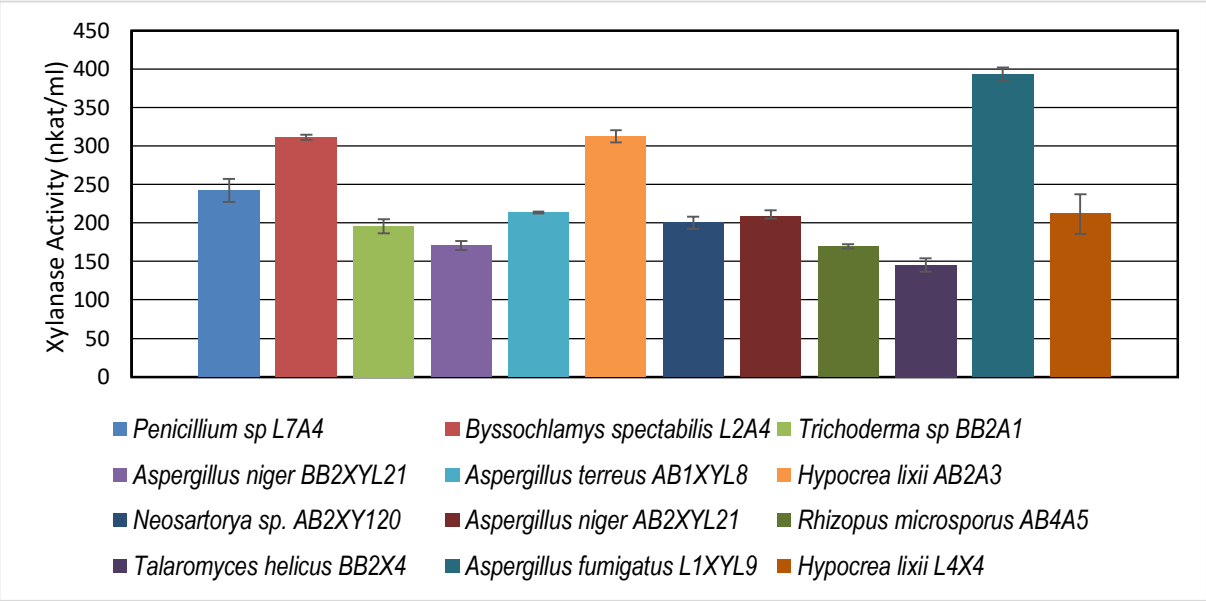

Figure 3.15. Filamentous fungi isolated from the gut of *P. femoralis* and *A. convexus* and dung beetle larvae *E. intermedius* with xylanase activities higher than 145 nkat/ml.

3.4. DISCUSSION

Second-generation bioethanol is produced from lignocellulose material. Bioethanol is an important alternative to fossil fuel since it is environmentally friendly and renewable. Lignocellulose is the predominant component of woody plant material, soil organic material and decaying wood. Lignocellulose is the most abundant form of biomass in terrestrial ecosystems. Currently, the process of second-generation bioethanol production is not economically feasible. Strategies to reduce the production cost of second-generation bioethanol include lowering the cost of enzymes used in enzymatic saccharification and fermentation of all the sugars that are released during the saccharification process (Chen *et al.*, 2017 & Mohapatra *et al.*, 2017). As aforementioned, searching for fungi with hemicellulolytic activity and yeast capable of fermenting xylose is important. Fungi are well-known for their ability to produce extracellular enzymes while yeast is known for its ability to ferment monomers and most of them are regarded as safe. The gut of the dung beetle has been reported to be a rich bioreactor for hemicellulolytic microorganisms and xylose-fermenting yeast isolates (Rojas-Jiménez and Hernández, 2015 & Nwaefuna *et al.*, 2021). This study aimed at exploring the diversity and composition of the gut-microbiota for hemicellulolytic microorganisms and xylose-fermenting yeast isolates from Scarabaeidae dung beetles and dung beetle larvae in South Africa.

3.4.1. Diversity and xylose fermentation of yeast isolates from the gut of *Kheper nigroanaeus Boheman* (Impala dung) and *Heteronitis castelnaui* (Rhino dung).

There has not been any yet reported on the diversity of yeast strains inhabiting the gut of *Kheper nigroanaeus Boheman* (impala dung) and *Heteronitis castelnaui* (rhino dung). The symbiotic relationship between yeast and insects is through ingestion or interaction with the environment or yeast colonising the gut. Some reports have demonstrated that endogenous gut proteinases enzymes and microorganisms with hemicellulolytic activity are involved in the degradation of recalcitrant organic material in the gut of scarab dung beetle (Gonzalez, 2014). Food particles that are made of cellulose, hemicellulose, pectin and polysaccharides are degraded by enzymatic and fermentation processes in the gut of scarab dung beetles. The gut of the scarab beetle is therefore regarded as a small bioreactor (Huang *et al.*, 2010 & Nwaefuna *et al.*,

2021). Yeast is generally preferred over other varieties of microbes because of their ability to grow at low pH resulting in reduced risk of contamination and easier downstream processing due to larger cell size (Qin *et al.*, 2017). Most yeast strains are known for the ability to assimilate xylose but very few can ferment it. The yeast isolates from the gut of both dung beetles in this study were capable of assimilating xylose but produced low ethanol yields.

The dominant genus was *Trichosporon,* while *Candida tropicalis* was the dominant species from the gut-inhabiting *Kheper nigroanaeus Boheman* (impala dung) and *Heteronitis castelnaui* (rhino dung) microbiome when xylose was used as the sole source of carbon. Kunthiphun *et al.* (2016) observed similar dominance by *Trichosporon* sp. from the gut of *Heliocopris bucephalus Fabricius* (Coleoptera, Scarabaeidae) dung beetle. This infers mutualistic relationship between *Trichosporon* species and scarabaeidae dung beetles. In this study, only *T. inkin* was able to ferment xylose while yeast strains belonging to *Trichosporon* were negative for xylose fermentation. None of the *Trichosporon* isolates showed any xylanolytic activity. There are recent studies that have focused on the association between *Trichosporon* and insects. Most of these studies have reported several *Trichosporon* species with the ability to degrade and utilise wood components (Urbina *et al.*, 2013; Kunthiphun *et al.*, 2016 & Moubasher *et al*., 2017). In this study, *Trichosporon species such as Trichosporon cutaneum* and *Trichosporon asahii* were some of the interesting species found in the gut of *Kheper nigroanaeus Boheman* (impala dung) and *Heteronitis castelnaui* (rhino dung). This species has been reported for its use in lipid production that is used in biodiesel production (Hu *et al.*, 2011). *Trichosporon* species may have an association with dung beetles by assisting with the degradation of wood components found in the dung of herbivore animals.

The frequent occurrence of *Candida tropicalis* in the gut of dung beetles was reported by Urbina *et al*. (2013) and Ali *et al.* (2017). *Candida tropicalis* was also found inhabiting the dung of wild herbivores isolated from the Kruger National Park (Makhuvele *et al.*, 2017). *Candida tropicalis* may be transferred from the substrate to the gut of the dung beetle where it has a symbiotic relationship with the insects. The digestive tract of the scarabaeidae dung beetle consists of three regions: foregut, midgut and hindgut (also referred to as the fermentation chamber). All the sugars released during hydrolysis of woody particles are fermented in the hindgut (Huang *et*

al., 2010). The presence of *Candida tropicalis* in this study was expected since the whole alimentary tract was used for the isolation of xylose-fermenting yeast strains. Surprisingly, some of the isolated *Candida tropicalis* strains could not ferment xylose and those that fermented xylose produced little ethanol yield. Lower or no production of ethanol could be attributed to the yeast producing other metabolites such as xylitol. *Candida tropicalis* is well-known for its ability to ferment xylose to xylitol and ethanol (Urbina *et al.*, 2013). *Candida tropicalis* Y9 produced the most ethanol (1.11 g/L) (Figure 3.12), which is higher than 0.6 g/L ethanol that was reported by Lorliam *et al.* (2013). In this study, xylitol production was not tested which makes it difficult to know if most of the xylose were converted to xylitol.

Some of the yeast strains that are found in the gut of dung beetles are acquired by ingestion or interaction with conspecifics and environment. Makhuvele *et al*. (2017) reported that *Malassezia* sp. was not part of the yeast isolates from rhino dung. While in this study several *Malassezia globosa* was found in the gut of *Heteronitis castelnaui* dung beetle that was harvested from rhino dung and in the gut of *Kheper nigroanaeus Boheman* that was harvested from impala dung. The relationship between *Malassezia globosa* and dung beetles is currently unknown. According to my knowledge this is the first report on the presence of *Malassezia globosa* in the gut of dung beetles.

Insect gut microbiomes differ from one insect species to the other, this is because there is a difference in gut structures in the life cycles of the insects and the diet as well (Franzini *et al.*, 2016). *Meyerozyma guilliermondii* Y63 was present in the gut of *Kheper nigroanaeus Boheman* and absent in the gut of *Heteronitis castelnaui.* This reflects the importance of exploring the gut microbiome of different dung beetles in the mining of microorganisms with hemicellulolytic activity and capable of fermenting xylose. *Meyerozyma guilliermondii* Y63 is well known for its ability to ferment different carbon sources including xylose (Thiyonila *et al.*, 2018 & Suárez-Moo *et al.*, 2020). In this study *M. guilliermondii* Y63 was able to assimilate xylose and was not positive for xylose fermentation. *Meyerozyma guilliermondii* UFV-1 was reported to be present in the gut of beetles associated with the family Passalidae. This current study recommended the yeast for its potential for xylitol production in biorefineries (Silveira, da *et al.*, 2020). *Debaryomyces hansenii* Y38 is another interesting and desirable yeast isolated from the gut of *Heteronitis castelnau* (rhino dung), but absent from *Kheper nigroanaeus Boheman* (Impala dung). This yeast is widely distributed in nature

and is known for its ability to assimilate cellobiose (Lopes *et al.*, 2018). It is unclear if this yeast could have been transferred from the dung or if it has some symbiotic relationship with the beetle (Urbina *et al.,* 2013).

3.4.3. Growth at elevated temperature and in the presence of inhibitors from the gut of *Kheper nigroanaeus Boheman* (Impala dung) and *Heteronitis castelnaui* (Rhino dung)

The lignocellulose biomass used in second-generation bioethanol requires pretreatment before enzymatic hydrolysis. This step results in the formation of inhibitors that affect the growth of microorganisms, especially during fermentation, with acetic acid often found to be present (Mattam *et al.*, 2016; Kim, 2018 & Yang *et al.*, 2018). All the yeast strains in this study except *M. globosa* (Y120) could grow in the presence of 3 g/L of acetic acid. All the *Candida tropicalis* strains screened could tolerate acetic acid. Similar observations were reported by Mattam *et al.* (2016) and Sagia *et al.* (2020) that *Candida tropicalis* could tolerate inhibitors found in lignocellulosic hydrolysates. *Candida tropicalis* was also reported to exhibit better inhibitor tolerance than *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* and was linked to the capacity for detoxification/degradation of these inhibitors (Sagia *et al.*, 2020). This is an important characteristic for second-generation bioethanol production.

Enzymatic hydrolysis during bioethanol production normally occurs at around 50 ºC (Thomsen *et al.*, 2016). Therefore, yeast able to ferment at higher temperatures will reduce cooling costs during this process (Nweze *et al*., 2019). Furthermore, high-potential thermotolerant yeast is a key factor for successful ethanol production at high temperatures. All the yeast strains screened for thermotolerance could not grow at a temperature ranging from 45 – 50 ºC. *Candida tropicalis* Y9 was able to ferment xylose, could tolerate 3 g/L acetic acid and be capable of growing at 40 ºC. It has been reported that the ability of *Candida tropicalis* (Y9) to ferment xylose could be improved through evolutionary adaptation (Saini *et al.*, 2017 & Moremi *et al.*, 2020).

3.4.4. Diversity and xylanolytic activity of filamentous fungi isolated from the gut of the dung beetle *Anachalcos Convexus and Pachylomerus Femoralis* and dung beetle larvae from *Euoniticellus intermedius*

The symbiotic relationship between microorganisms and insects has long been studied. This was to assist to gain an understanding of their evolutionary diversification. Fungal mutualism with insects plays an important role in the developmental stages of the insects and their fitness. This is achieved by providing nitrogen compounds, degrading high molecular weight molecules and producing pheromones for mating and communication (de León *et al*., 2016). Recently, these microorganisms have been studied for their lignocellulolytic activity that is beneficial in biofuel application (Thiyonila *et al.*, 2018). Although microorganisms from the insects have been widely studied their isolation from the gut of dung beetles is very limited.

In this study, the fungal species found in the guts of different dung beetles *Anachalcos Convexus* and *Pachylomerus Femoralis* and the dung beetle *Euoniticellus intermedius* larvae were similar. The phylogenetic analysis revealed that the species from the guts of the different dung beetles and dung beetle larvae were closely related. The relatedness of all fungal species isolated from the gut dung beetle and dung beetle larvae showed that the strains might share some characteristics unless lost during evolutionary changes. According to the study by Suárez-Moo *et al.* (2020), the difference in species composition could be due to the type of diet that the dung beetle feeds on (Franzini *et al.*, 2016), the environment (Ng *et al.*, 2018), and also the social interactions (Martinson *et al.*, 2012). The species isolated from the gut of the dung beetle *Pachylomerus Femoralis,* the *Euoniticellus intermedius* larvae deduced a monophyletic group. The *Taifanglania* sp. AB1XYL6 isolated in this study did not show any xylanase activity. But this fungal strain formed a paraphyletic group with genus *Acrophialophora* which is a thermotolerant soil fungus that is widely distributed in temperate and tropical regions (Barros *et al.*, 2010). *Acrophialophora nainiana* has been reported for its ability to produce cellulase and xylanase enzymes (Barros *et al.*, 2010). Since it shares a common recent ancestor with *Taifanglania* sp. and they may be paraphyletic because *Taifanglania* sp, lost some of its character (lignocellulolytic activity) that it shared with *Acrophialophora*. Evolutionary adaptation can assist and improve this species. Genus *Taifanglania* further forms a monophyletic group with the genus *Chaetomium*. Genus *Chaetomium* is known for its ability to colonise different

substrates. Most species from this genus are capable of producing cellulase that degrades cellulose resulting in the production of different bioactive metabolites (Wang *et al.*, 2016).

The dominant species in the gut of *A. convexus* and *P. femoralis* dung beetle is *Aspergillus niger*. *Hypocrea lixii* was found to be dominant in the gut of E*. intermedius* larvae. *Hypocrea lixii* is the teleomorph of *Trichoderma harzianum*. *Trichoderma and Aspergillus* species are well-known for their ability to degrade polymers (Bischof *et al*., 2016). Their dominance and presence in the gut of these dung beetles were expected since these dung beetles feed on the dung of herbivorous animals and dung is made up of 80% of plant material. The presence of *Aspergillus niger* in the gut of insects was in agreement with the study by Moubasher *et al*. (2017). Most of the *Aspergillus niger* isolated in this study were positive for xylanase enzyme production. *Hypocrea lixii* (*Trichoderma harzinum*) was also the frequently occurring species from the gut microbiome of both dung beetle and dung beetle larvae. *T. harzinum* is well-known for its ability to produce xylanase enzymes (Uday *et al.*, 2016 & Ahmed *et al*., 2007). This was in agreement with the findings in this study since *T. harzinum* was positive when screened for xylanase production. The co-operative metabolism between *Aspergillus niger* and *Hypocrea lixii* in the degrading of the polymers such as cellulose and hemicellulose reflects a symbiotic association that the gut microbiomes have with a dung beetle.

Other interesting species isolated from the gut of *A. convexus* and *P. femoralis* dung beetle and E*uoniticellus intermedius* larvae included species such as *Aspergillus terreus* (Moubasher *et al*., 2017) *Rhizopus oryzae* (Pandey *et al*., 2016), *Penicillium echinulatum* (Scholl *et al.*, 2015), and *Talaromyces helicus* (Varriale *et al.*, 2018) that were reported to be effective in the biofuel industry. In this study, they also showed good xylanase activity.

The species that showed higher xylanolytic activity from the gut microbiome in this study was *Aspergillus fumigatus* L1XYL9 with xylanase activity of 393.22 nkat/ml followed by *Hypocrea lixii* AB2A3 with xylanase activity of 392 nkat/ml. Filamentous fungi are well-known for the ability to produce xylanase enzymes and are widely used in the production of commercial enzymes in industry. This includes species such as *Trichordema reesei* and *Aspergillus niger* (Bischof *et al*., 2016 & Li *et al.*, 2020). The

xylanase activity produced by *Aspergillus fumigatus* L1XYL9 in this study was higher than that produced by *Aspergillus fumigatus* GGV – BT 03 (1.67 nkat/ml) reported by Ire *et al*. (2021), *Aspergillus fumigatus* Z5 (250.05 nkat/ml) by Liu *et al*. (2013) and *Aspergillus fumigatus* MA28 (140,86 nkat/ml) by Bajaj and Abbass (2011). While *Hypocrea lixii* (*Trichoderma harzianum)* produced xylanase activity that was high as compared to *Trichoderma harzianum* E-58 that showed xylanase activity of 80 nkat/ml (Ahmed *et al.*, 2007).

3.5. CONCLUSION AND RECOMMENDATION

The results in this study deduced that both the filamentous fungi and yeast populations associated with the guts of the dung beetle/larvae are highly diverse in terms of the number of species while phylogenetic analysis showed their close relatedness. The study is in agreement with the report that the gut of the dung beetle is a rich bioreactor with hemicellulolytic microorganisms and xylose-fermenting yeast. The gut habitats have consortia that are acting synergistically to provide many of the nutritional needs of the beetle host. And also to degradation and fermentation of lignocellulosic materials is reflected by the high percentage of filamentous fungi and yeast genera with the xylanolytic ability and xylose-fermenting yeast. The yeast isolates in this study reflected characteristics that are required for yeast that can effectively ferment monomers from second-generation biofuel production (Nwaefuna *et al.*, 2021). This yeast could assimilate xylose but could not ferment it effectively and required to go through evolutionary adaptation to manipulate its ability to ferment xylose. This is the first study to report the distribution of xylanase-producing fungal species inhabiting the gut of the *Anachalcos Convexus* and *Pachylomerus Femoralis*, *Kheper nigroanaeus Boheman* (Impala dung) and *Heteronitis castelnaui* (Rhino dung) and *Euoniticellus intermedius* larvae.

Further gut microbiota with xylanolytic activity will be screened for xylanase production using thatch grass. Thatch grass was chosen since it is abundantly available in South Africa. Its use as inducer carbon for xylanase production will lower the cost for xylanase enzyme production. This will add to efforts that are being made in making biofuel production economically feasible.

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CHAPTER 4

XYLANASE PRODUCTION ON THATCH GRASS BY FUNGI ISOLATED FROM DUNG BEETLES AND THE USE OF CRUDE XYLANASE IN ENZYMATIC SACCHARIFICATION OF THATCH GRASS

ABSTRACT

Disposal of plant-based biomass that is not being used often affects the environment negatively. The use of this plant-based biomass that have been regarded as waste have now received attention from the biofuel industry. Enzymatic conversion is a preferred method for the complete degradation of the plant-based residues into valuable products. Enzymatic saccharification is hindered by the overall process due to the high costs of enzyme production. Supplementation of xylanase to cellulase in enzymatic saccharification has received attention as it increases production yield. In this study, thatch grass was used as a carbon source for inducing xylanase production by filamentous fungi with xylanolytic activity isolated from the gut of dung beetle and dung beetle larvae. Supplementation of the crude xylanase enzyme with commercial celluclast™ during enzymatic saccharification of thatch grass was evaluated. Several factors such as pretreatment method (acid & washed, acid & unwashed and ammonium solution), different fermentation processes (submerged and solid-state fermentation), agitation and inoculum size tested for submerged fermentation and initial moisture content evaluated in solid-state fermentation. Xylanase production was further evaluated on untreated thatch grass and in the bioreactor. Twelve filamentous fungi that showed good xylanase activity isolated from the gut of dung beetles (*Pachylomerus femoralis* and *Anachalcos convexus*) and dung beetle larvae (*Euoniticellus intermedius*) belonged to genus *Aspergillus, Trichoderma, Hypocrea, Neosartorya, Talaromyce, Penicillium* and *Rhizopus*. *Hypocrea lixii* AB2A3, *Aspergillus fumigatus* L1XYL9 and *Neosartorya* sp AB2XYL20 with xylanase activity of 525.77, 304 and 549 nkat/ml, respectively were selected as best producers of xylanase. Higher xylanase production was observed in submerged fermentation (agitation speed of 200 rpm and inoculum size of 2 x 10^6 spores/ml) than in solid-state fermentation at all evaluated moisture levels. Acid & unwashed pretreated thatch grass was a good inducer for *Hypocrea lixii* AB2A3. Xylanase production by *Hypocrea lixii* AB2A3 in the bioreactor was very low as compared to xylanase production in the flask.

Enzymatic saccharification of acid pretreated thatch grass using crude xylanase from *Hypocrea lixii* AB2A3 resulted in maximum xylose production of 128 mg/g after 72 hours with an enzymatic saccharification efficiency of 59%. Supplementation of crude xylanase from *Hypocrea lixii* AB2A3 with commercial cellulase celluclast™ resulted in maximum glucose production of 549 mg/g with enzymatic saccharification efficiency of 60%. While when commercial celluclast™ was used unison it resulted in glucose production of 528 mg/g with enzymatic saccharification efficiency of 54%. In conclusion, instead of disposing unused thatch grass by burning it causing pollution, it can be used as an inducer for the production of enzymes, such as xylanase, to especially reduce costs in biofuel production.

4.1. INTRODUCTION

Enzymes play a vital role in biotechnological applications. The use of enzymes is preferred since synthetic chemical catalysts have a negative environmental impact. Enzymes recently received attention in the conversion of plant-based biomass that is regarded as waste in the production of high-value products such as biofuels, animal feeds, chemicals and enzymes. Similarly, xylanases have received attention for their application in various industries such as the animal feed industry, biofuel industry, baking industry, pulp and paper industry, liquefaction of fruits and vegetables and also in the clarification of beer and juices (Kumar *et al*., 2017). In the biofuel industry, xylanases are used for the efficient saccharification of hemicelluloses.

Two processes are vital in the production of biofuel products from plant-based biomass. These processes are the pretreatment process and enzymatic saccharification. Pretreatment of the plant-based biomass is vital in exposing the cellulose, thus making the substrate accessible to enzymes. Different pretreatment processes have been employed in the treatment of grasses. Physicochemical pretreatments have been reported to be highly effective (Mohapatra *et al.*, 2017). Scholl *et al.* (2015) reported negative results for xylanase production when the steam explosion was used in unison as a pretreatment process for elephant grass. Different pretreatment methods have different effects on the substrate and influence enzyme production. Acids such as sulfuric, nitric or hydrochloric solubilise hemicelluloses and allow exposure of cellulose to enzymatic attack (Mohapatra *et al.*, 2017 & Ciolacu, 2018). Conversely, the alkaline pretreatment methods tend to remove the lignin and thus reduce the degree of cellulose crystallinity. In this study, the steam explosion method was used with acids and then with alkaline solution to pretreat grinded thatch grass. Makhuvele *et al*. (2017) successfully isolated microorganisms with hemicellulolytic activity after pretreating the thatch grass with sulfuric acid that reduced hemicellulose in lignocellulosic materials. Sodium hydroxide is usually used to adjust the pH of the pretreated material. Sodium hydroxide removes lignin (Mohapatra *et al.*, 2017). A suitable pretreatment method is vital in enzyme production.

Plant-based biomass is mainly composed of cellulose, hemicellulose and lignin. Xylan consisting mainly of D-xylose is the most common hemicellulose and is the second most abundant biopolymer found in nature after cellulose (Ciolacu, 2018). Plant-based

biomass such as thatch grass is rich in cellulose and hemicellulose polymers. Hemicellulose polymer is known to influence the effectiveness of enzymatic hydrolysis of the cellulosic polymer. Commercial cellulase complex has a low content of hemicellulase enzymes and is not effective in enzymatic hydrolysis. Supplementation of accessory enzymes such as xylanase has commonly been reported to result in the complete degradation of lignocellulose biomass into fermentable sugars (Hu *et al.,* 2011). The limitation of using accessory enzyme is the high dosage that still results in increased production cost (Kumar *et al*., 2017). This makes bioethanol production from second-generation biofuel not economically feasible (Bussamra *et al*., 2015). One approach to reducing costs is to search for microbes producing these enzymes more efficiently (Uday *et al.*, 2016; de Almeida *et al.*, 2019 & de Souza *et al.*, 2020). In addition, the production cost can be lowered by the production of xylanase and cellulase from microbes produced on plant-based residues rather than expensive synthetic media. Furthermore, the enzymes produced on the plant-based material are potentially more effective than when produced from synthetic media (Uday *et al.*, 2017; Souza *et al.*, 2018 & Siqueira *et al.*, 2020). Hemicellulose is found on the outer surface of cellulose fibers and also diffuses into the interfibrillar space using the fiber pores. This polymer together with lignin act as a physical barrier that limits the accessibility of the cellulase enzymes to the cellulose (Kuila *et al.*, 2017 & Ciolacu, 2018). Xylanase is capable of degrading β (1,4) glycosidic bonds between xylopyranosyl units of xylan releasing xylooligosaccharides and xylose (Walia *et al.*, 2017). Filamentous fungi such as *Aspergillus, Trichoderma* and *Penicillium* are well-known xylanase producers (Rojas-Jiménez and Hernández, 2015, Kumar and Parikh, 2015 & Ajijolakewu *et al.*, 2017).

Thatch grass (*Hyparrhenia hirta*) belongs to the family Graminae (Poaceae) (Mohapatra *et al.*, 2017). *Hyparrhenia hirta* consists of 46% cellulose, 24% hemicellulose and 10% lignin (Ncube *et al*., 2013). *Hyparrhenia hirta* is known for its hard basal tussock, rough narrow leaves and has a scanty panicle of pairs of white villous racemes. The grass can yield up to 40 Mg/ha/year, which is high when compared to corn that can yield 7 Mg/ha/year. *Hyparrhenia hirta* is in abundance in Southern Africa and was also introduced in other parts of the world. It is widely distributed in open grassland and on rocky slopes, can also grow along rivers and on most soil types, but it grows well in well-drained, lighter textured granites to heavy

black stony soils. *Hyparrhenia hirta* forms dense stands in disturbed areas (uncultivated lands and roadsides). Currently, thatch grass is used for animal grazing however, its nutritional value decreases at later stages of growth and as it dries out. The decrease in nutritional content makes it unsuitable as an animal feed, at this point it is used for roof thatching. Nowadays, there is very little use of this grass for roof thatching then most of it remains unused and then is disposed of, through burning, which in turn causes air pollution (Mashau, 2009). Thatch grass is potentially the ideal feedstock for bioethanol production in the South African context since it is renewable, abundant and requires minimal maintenance.

Submerged (SmF) and solid-state fermentation (SSF) is commonly used in the production of xylanases. Solid-state cultivation is a process where insoluble substrates are fermented with sufficient moisture but without free water (Papagianni *et al*., 2001). Solid-state cultivation was the preferred cultivation process when using agricultural residues. Properties that make SSF attractive include high product concentration, less aeration and no agitation during enzyme production (Krishna, 2005). Another important factor is that SSF imitates the natural environment for enzyme production. Elephant grass was successfully used in cellulase and xylanase production using SSF (Scholl *et al.*, 2015). The limitation of SSF is the large-scale feasibility of the process. This includes the control of operational parameters (pH, temperature and aeration) and adequate reactor design. Water content is another important operational parameter that affects process efficiency. Higher moisture content results in void spaces in the solids filled with water, resulting in oxygen limitation. Conversely, low moisture content hinders microbial growth (Delabona *et al.*, 2013). It is thus crucial to identify the optimal moisture content for each solid substrate to favor microbial growth and increase the production of metabolites. Submerged Fermentation (SmF) operates in the presence of excess liquid (Singhaniaa *et al*., 2010). Submerged fermentation is widely used in industries in the production of commercial enzymes. This cultivation process consists of a well-developed bioreactor, instrumentation and process control (Amorim *et al*., 2019). Parameters such as pH and agitation are better controlled in SmF compared to SSF (Liu *et al.*, 2020). Effective enzyme production is dependent on the microorganisms and type of substrate used (Leite *et al.*, 2021). Evaluation of some parameters in submerged fermentation is important for a feasible xylanase production process. Several significant parameters that can be evaluated to increase

enzyme production yield includes parameters such as type of substrate, regulation of nutrients concentration in media (carbon, nitrogen, trace elements, vitamins and amino acids and physical parameters (temperature, pH, agitation speed, aeration, inoculum sizes and incubation period). Furthermore, in enzyme production, it is important to select the most suitable fermentation process and optimise it to increase enzyme production yield (Kumar *et al.,* 2017).

Two studies on the use of thatch grass in enzyme production emerged from our laboratory. Makhuvele *et al*. (2017) reported successful isolation of microorganisms from the dung of herbivorous animals with xylanolytic activity when thatch grass was used as an inducer carbon but the author highlighted that the xylanase activity was very low and could have been affected by the highly lignified thatch grass. While Ncube *et al.* (2013) reported a lower production yield of thatch grass using crude xylanase by *Aspergillus niger*. From the conclusion drawn by both authors, the research question to be answered in this study was if the gut inhabiting microbiome have a xylanolytic activity which was probably responsible for breaking down dung that is made of 80% un-pretreated plant-based material, can thatch grass be a good inducer carbon that will mimic the natural habitat of this microorganism and results in the production of very effective xylanases that can completely degrade hemicellulose polymer in thatch grass into valuable products that can be used in the biofuel industry?

This current investigation sought to study xylanase production by gut microbiota isolated from two dung beetles (*Anachalcos Convexus* and *Pachylomerus Femoralis)* and dung beetle larvae (*Euoniticellus intermedius*) using thatch grass as inducer. Several parameters were evaluated during the xylanase production by *Hypocrea lixii* AB2A3 and its use in enzymatic saccharification. The production of xylanase was studied in the bioreactor. Whereas the enzymatic saccharification efficiency was studied by supplementation of crude xylanase with commercial cellulase^{TM}1.5L.

4.2. MATERIAL AND METHODS

4.2.1. Fungal strains

Filamentous fungi isolated from the gut of dung beetles (*Pachylomerus femoralis* and *Anachalcos convexus*) and dung beetle larvae (*Euoniticellus intermedius*) were used in this study (Table 4.1). The fungi were grown on YM plates (3 g/L yeast extract, 5 g/L peptone,3 g/L malt extract, 15 g/L bacteriological agar, 0.2 g/L chloramphenicol) for 5 days at 30 °C. After 5 days a 10 x 5 mm agar block was cut and placed on YM slants. The slants were incubated at 30 °C for 5 days and then kept at 4 °C until use.

Table 4.1. Xylanase-producing fungi isolated from the gut of the dung beetle *Anachalcos Convexus* and *Pachylomerus femoralis* and dung beetle larvae of *Euoniticellus intermedius*.

Anachalcos Convexus.	***Pachylomerus femoralis.***	***Euoniticellus intermedius larvae.***
Aspergillus terreus AB1XYL8	*Trichoderma* sp. BB2A1	*Penicillium* sp. L7A4
Hypocrea lixii AB2A3	*Aspergillus niger* BB2XYL21	*Byssochlamys spectabilis* L2A4
Neosartorya sp. AB2XY120	*Talaromyces helicus* BB2X4	*Aspergillus fumigatus* L1XYL9
Aspergillus niger AB2XYL21		*Hypocrea lixii* L4X4
Rhizopus microsporus AB4A5		

4.2.2. Thatch grass collection

Thatch grass was collected in the Limpopo province (South Africa), Tshivhuyuni Mashamba village (23.23 39ºS, 39.0810ºE). The grass was allowed to dry completely (until the color changes from green to brown). The air-dried grass was milled to a fine powder and stored in air-tight buckets. The composition of the thatch grass is indicated in Table 4.2 as previously determined by Ncube *et al*. (2013).

4.2.3. Pre-treatment of thatch grass for xylanase production

4.2.3.1 Dilute acid pretreatment

A total of 200 g milled thatch grass was mixed with a 2000 ml solution consisting of 1.2% H_2SO_4 and autoclaved for 60 minutes at 120 °C. The pH of the solution was adjusted to 5 with 5M NaOH (Makhuvele *et al*., 2017). The pre-treated grass solution was dried at 50 °C and stored in bottles at room temperature for later use.

In another approach (acid pretreated thatch grass followed by alkaline pretreatment), the same procedure was followed, but the acid pretreated thatch grass solution was filtered through a sieve to collect the liquor as acid hydrolysate. The residual thatch grass after treatment was washed with distilled water until neutrality and dried at 50 °C. The residual thatch grass biomass was pretreated using a 15% (w/v) slurry of the washed dried residual biomass in 2% NaOH and autoclaved at 121° C, 15 lbs for 15 minutes. After pre-treatment, the substrate was thoroughly washed with water until it reached a neutral pH and dried at 50 °C (Patel *et al.*, 2017). The dried pretreated thatch grass was then stored in sterile bottles at room temperature until used.

4.2.3.2. Ammonium solution pre-treatment

Thatch grass was pretreated using the method described by Patel *et al*. (2017). Milled thatch grass was mixed with a 15% ammonia solution in the ratio of 1:4.5 (w/v) and then autoclaved at 121° C, 15 lbs for 60 minutes. After pretreatment, the substrate was thoroughly washed with distilled water until neutral pH and was dried at 60 °C. The completely dried pretreated thatch grass was stored in sterile bottles at room temperature until used.

4.2.4. Fungal inoculum preparation

Filamentous fungi were grown on YM agar plates (3 g/L yeast extract, 5 g/L peptone,3 g/L malt extract, 15 g/L bacteriological agar, 0.2 g/L chloramphenicol) for 5 days at 30 °C. Spores were harvested from 5 days old YM plates by pipetting 10 ml of 0.1% filter sterilised Tween 80 solution onto the culture plates. A glass rod was used to gently dislodge the spores from the agar surface. A standard spore count was performed with a Neubauer bright-line counting chamber as described by Wolk *et al.* (2000). The spore suspension was adjusted to 1 x 10^6 spores/ml during inoculation.

4.2.5. Production of xylanase on acid pretreated thatch grass in Submerged fermentation

Erlenmeyer flasks (250 ml) containing 2 g of the acid pretreated thatch grass and 50 ml Mandels media (Mandels and Weber, 1969) (2 g/L K_2HPO_4, 0.5 g/L KCl, 0.0365 g/L FeSO4, 0.075 g/L $MgSO_4.7H_2O$, 7 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 1 g/L Yeast extract) were autoclaved at 121°C for 20 minutes and allowed to cool. Each flask was inoculated with the fungal spore (Table 4.1) inoculum to give a final concentration of 1 x 10^6 spore/ml of media. Flasks were incubated in a shaking incubator at 30 °C at 150 rpm for 120 hours. For the time-course analysis of enzyme production, 1 ml of the suspension was aseptically withdrawn every 24 hours. The suspension was centrifuged at 10 000 rpm for 5 minutes. The clear supernatant containing the crude enzyme was immediately used for xylanase activity determination. All results are expressed as the means of at least three independent experiments.

4.2.6. Submerged versus solid-state fermentation

Selected filamentous fungi *Hypocrea lixii* AB2A3, *Aspergillus fumigatus* L1XYL9 and *Neosartorya* sp. AB2XYL20 that showed high xylanase activity from section **4.2.5** were screened for xylanase activity in submerged and solid-state fermentation. These strains were further screened for endoglucanase activity.

4.2.6.1. Production of xylanase in submerged fermentation

The submerged fermentation process was done for the above-mentioned selected fungal strains (**4.2.6**) as described in section **4.2.5.** with modification. Different inoculation sizes used 2 x 10^6 spores/ml instead of 1 x 10^6 spore/ml of xylanase

production as mentioned in section **4.2.5**. For the time-course analysis of enzyme production, 1 ml of the suspension was aseptically withdrawn every 24 hours. The suspension was centrifuged at 10 000 rpm for 5 minutes. The clear supernatant containing the crude enzyme was immediately used for xylanase and endoglucanase activity. All results are expressed as the means of at least three independent experiments.

4.2.6.2. Production of xylanase in solid state fermentation

Selected fungal strains (**4.2.6**) were screened for xylanase production in solid-state fermentation (SSF). Erlenmeyer flasks (250 ml) containing 2 g of acid pretreated thatch grass and Mandel's media (Mendels & Sternberg, 1979) were prepared separately and autoclaved at 121 °C for 20 minutes and allowed to cool. The acid pretreated thatch grass was hydrated to achieve a 44 & 54% moisture content using Mendel's media. Each flask was inoculated with a fungal spore inoculum to give a final concentration of 2 x 10^6 spores/ml of media. The flasks were shaken to mix the contents and incubated at 30 °C for 10 days. A flask was removed every 24 hours and the enzymes were extracted by adding 50 ml of a 50 mM acetate buffer pH 5 onto the fermented substrate. After 30 minutes the mixture was centrifuged at 1500 x g for 20 minutes at 4 °C to obtain a clear supernatant. The clear supernatant containing the crude enzyme was immediately used for xylanase and endoglucanase activity determination. The cultures were prepared in triplicates.

4.2.7. Evaluation of the effectiveness of the treatment of thatch grass and methods used for xylanase production on different fermentation platforms

For further studies, one fungal strain was selected. Hy*pocrea lixii* AB2A3 was selected for further studies as it was found to be the most dominant species in the guts of dung beetles and showed good xylanase activity on thatch grass.

4.2.7.1. Effect of agitation speed during xylanase enzyme production

Erlenmeyer flasks (250 ml) containing 2 g of acid pretreated thatch grass with 50 ml of Mandel's media (Mandels and Weber, 1969) (2 g/L K_2HPO_4, 0.5 g/L KCl, 0.0365 g/L FeSO4, 0.075 g/L $MgSO_4.7H_2O$, 7 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 1 g/L Yeast extract) were prepared. The media was autoclaved at 121 ºC for 20 minutes and allowed to cool before inoculation. Each flask was inoculated with spore suspensions

of *Hypocrea lixii* AB2A3 to give a final concentration of 2 x 10^6 spore/ml of media. Duplicates of each flask were prepared and incubated into two separate incubators at 30 ºC and a different agitation speed of 150 while the other incubator was at 200 rpm for 120 hours. A 1 ml suspension of the culture was aseptically withdrawn every 24 hours into eppendorf tubes. The solution was centrifuged at 10 000 rpm for 5 minutes. The clear supernatant containing the crude enzyme was immediately used for xylanase activity determination as described in 5.2.4. All results are expressed as the means of at least three independent experiments.

4.2.7.2. Xylanase production on untreated, acid and alkaline pretreated thatch grass

Erlenmeyer flasks (250 ml) containing 2 g of either acid and washed, acid and unwashed and ammonium pretreated thatch grass and untreated thatch grass with 50 ml of Mandel's media (Mandels and Weber, 1969) (2 g/L K_2HPO_4, 0.5 g/L KCl, 0.0365 g/L FeSO4, 0.075 g/L $MgSO_4.7H_2O$, 7 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 1 g/L Yeast extract) were prepared. The media was autoclaved at 121 ºC for 20 minutes and allowed to cool before inoculation. Each flask was inoculated with the fungal spore inoculum of *Hypocrea lixii* AB2A3 to give a final concentration of 2 x 10^6 spores/ml of media. Flasks were incubated in a shaking incubator at 30 °C at 200 rpm for 120 hours. For the time-course analysis of enzyme production, 1 ml of the suspension was aseptically withdrawn every 24 hours. The suspension was centrifuged at 10 000 rpm for 5 minutes. The clear supernatant containing the crude enzyme was immediately used for xylanase activity determination. All results are expressed as the means of at least three independent experiments.

4.2.7.3. Xylanase production using a Bioflo 115 Bioreactor

Hypocrea lixii AB2A3 was grown in 3 L vessel bench bioreactors (BioFlo 115 New Brunswick Bioreactor). The bioreactor vessel was filled with 1 L of the Mandels media (Mandels and Weber, 1969) described in section 4.2.5. was prepared. The medium was sterilised (at 121 ºC for 20 minutes), and after cooling, 2 x 10^6 spore/ml was added to the medium. The pH was maintained at 5.0 using 2 M NaOH, the temperature was set at 30 ºC. The airflow rate was maintained at 1.0 vvm at an agitation speed of 200 rpm corresponding to the peripheral speed of 0.52 m/s. One milliliter of the culture was aseptically withdrawn every 24 hours into Eppendorf tubes. The cultures were

centrifuged at 10 000 rpm for 5 minutes. The clear supernatant containing the crude enzyme was immediately used for xylanase activity determination.

4.2.8. Preparation of enzymes for enzymatic saccharification of acid pretreated thatch grass

Erlenmeyer flasks (500 ml) containing 4 g of acid pretreated thatch grass with 100 ml of Mandel's media (Mandels and Weber, 1969) (2 g/L K_2HPO_4, 0.5 g/L KCl, 0.0365 g/L FeSO4, 0.075 g/L $MgSO_4.7H_2O$, 7 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 1 g/L Yeast extract) were prepared. The media was autoclaved at 121 ºC for 20 minutes and allowed to cool before inoculation. Six flasks were prepared and each flask was inoculated with spore suspensions of *Hypocrea lixii* AB2A3 to give a final concentration of 2 x 10^6 spore/ml of media and incubated at 30 ºC in the rotary incubator at 200 rpm. After 72 hours the flask contents were added into 50 ml centrifuged tubes and centrifuged at 4000 rpm for 20 minutes at 4 ºC. The clear supernatant containing the crude enzyme and also the commercial celluclast™ were screened for xylanase, *β*-Glucosidase, endoglucanase and total cellulase activity using the assay described at 4.2.10. Protein concentrations were determined by Lowry *et al.* (1951), using bovine serum albumin (BSA) as the standard. All results are expressed as the means of at least three independent experiments.

4.2.9. Enzymes assay

Xylanase enzyme activity was determined using 1% (w/v) xylan from beechwood solution according to Bailey *et al*. (1992). The amount of reducing sugar released was determined using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959), using xylose (Sigma) as the standards. A xylanase enzyme activity was expressed in katals per milliliter (nkat/ml).

***β*-Glucosidase enzyme activity** was determined according to the method described by Herr, (1979). The reaction mixture contained 25 µl of 10 mM *ρ*-nitrophenyl *β*-d-glucopyranoside (pNPG) in 0.05 M citrate buffer pH 4.8. The mixtures were incubated at 50 ºC for 15 minutes. The reaction was stopped by adding 100 µl of 0.25 M sodium carbonate. The activity of the enzyme, indicated by the release of *ρ*-nitrophenol, was determined at 420 nm using Beckman Coulter, DU_ 720 UV/Vis spectrophotometer

(Ghose, 1987). One unit of β-glucosidase activity was defined as the amount of enzyme liberating one μmole of ρ-nitrophenol under the assay conditions.

Endoglucanase enzyme activity in the culture supernatant was determined according to the method described by Ghose (1987). The reaction mixture contained 25 µl of 1% CMC in 0.05 M Na-acetate buffer, pH 5.0. The mixture was incubated at 50 ºC for 30 minutes and the released reducing sugar was estimated as indicated in the assay for total activity. The amount of reducing sugar released was determined using the 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959), using glucose as the standard. An endoglucanase enzyme activity was expressed in katals per milliliter (nkat/ml). The total cellulase activity was determined by **Filter Paper assay (FPase)** using Whatman No.1 filter paper strip with a dimension of 1 × 6.0 cm equivalent to 50mg of the substrate according to Ghose (1987). At least two dilutions were made, one dilution that releases slightly less than 2.0 mg and the other dilution releasing more than 2.0 mg. The reaction mixture contained 1.0 ml of 0.05 M Na-citrate, pH 5.0, filter paper strip, and 0.5 ml of crude enzyme diluted accordingly. The mixture was incubated at 50 C for 60 min. The released reducing sugar was estimated by the addition of 3,5-dinitrosalicylic acid (DNS) with glucose as standard. The absorbance was read at 540 nm by using Beckman Coulter, DU_ 720 UV/Vis spectrophotometer. The assay was performed in duplicate including controls. Filter paper activity (FPU) is defined as 0.37 divided by the amount of enzyme required to liberate 2.0mg of glucose from the filter paper strip (≈ 50 mg) in 1 h (Legodi *et al.*, 2019).

4.2.10. Enzymatic hydrolysis of acid pretreated thatch grass

Enzymatic saccharification of acid pretreated thatch grass was performed following a modified method by Patel *et al*. (2017). Enzyme loading used was 46 U/g for crude xylanase and 8 FPU/g for commercial celluclast™. A cocktail of commercial cellulase (8 FPU/g) and crude xylanase (46 U/g) was also tested. The assay consisted of acid pretreated thatch grass at a solid loading of 2.5% (dry matter, w/v) in 250 ml screw-cap tubes with enzymes diluted in 50 mM sodium citrate buffer (pH 4.8) containing 0.1%Tween-80 to the final volume of 40ml. Controls were kept for each reaction in which the active enzyme was replaced with the heat-inactivated enzyme. The reaction system was fortified with 10% sodium azide. The reaction was carried out at 50 ºC in

a water bath. The samples were withdrawn at 12 hour intervals and heated in a boiling water bath (100 ºC) to inactivate the enzyme and stop the reaction. The reaction mixture was then centrifuged at 10000xg to collect the clear supernatant. The supernatant was used to detect monomeric sugars (glucose and xylose) using HPLC.

4.2.11. High-Performance Liquid Chromatography Analysis

Thatch grass hydrolysate samples were analysed using a Shimadzu HPLC with a Aminex HPX-87C column (300 x 7.8 mm, Ca^{2+}, particle 9 µm) (Biorad, Hercules, CA, USA). The Aminex HPX-87C column was fitted with an Aminex Resin Microguard, Carbo C column (Biorad, Richmond CA, USA). The detection of eluents was done using a RID 10A refractive index detector (Shimadzu, Kyoto, Japan). The samples were filtered through a 0.2 µm syringe filter. Glucose and xylose were used as standards to determine the retention time of sugars in the samples separated on the column. The Aminex HPX-87C column temperature was maintained at 85 °C with degassed deionised distilled water at a flow rate of 0.6 ml/min as the mobile phase for the Aminex HPX-87C column. The initial glucose present was subtracted from the final glucose obtained at each sampling time. Peak detection and integration were done using LC solutions software from Shimadzu (Tokyo, Japan). Peak height was used for the analysis of the results using Sigmaplot. The conversion (%) was calculated using the following equations (van Dyk and Pletschke, 2012).

$$\text{Conversion (\%)} = \frac{\text{Glucose (g/L)} \times 0.90 \times 100}{\text{Initial glucan (g/L)}}$$

$$\text{Conversion (\%)} = \frac{\text{Xylose (g/L)} \times 0.88 \times 100}{\text{Initial glucan (g/L)}}$$

4.3. RESULTS

4.3.1. Thatch grass composition

Thatch grass composition is depicted in Table 4.2 as determined by Ncube *et al*. (2013). The chemical composition of the raw thatch grass material consists of 23.81% hemicellulose, 10.24% lignin and 46.27% cellulose. The lignocellulosic components were determined using the detergent approach. The total dry weight percentage of all tested components is higher than 100%. In the detergent approach, the Neutral Detergent Fibre (NDF) presents an estimate in the structural components of the plant (cell wall) that are insoluble in neutral detergent. While Acid Detergent Fibre (ADF) presents the plant material that is insoluble in acid detergent, this plant material is not easily digestible. Hemicellulose concentrations were obtained as the difference between NDF and ADF (23.81%). The cellulose component is the difference between the ADF and acid detergent lignin (ADL) (46.27%). The ADL is the residue obtained after the ADF fraction treatment with sulfuric acid. Crude Fiber (CF) is another analytical method that has been widely used for years for the characterisation of the fiber fraction. CF only “measures an incomplete and variable fraction of the fibrous carbohydrate components” (Hindrichsen *et al.*, 2006). The final dry weight of CF which is 76.02% represents cellulose content at 60% to 80% and lignin content at 4% to 6%.

Table 4.2: Composition of thatch grass (Ncube *et al.*, 2013).

Component	**% Dry weight**
Ash	5.42
Nitrogen	0.5
Crude protein	3.13
Crude fiber	76.02
NDF*	80.32
ADF**	56.51
ADL***	10.24

Hemicellulose	23.81
Cellulose	46.27

Key: * Neutral Detergent Fibre (NDF),

**Acid Detergent Fibre (ADF),

***Acid Detergent Lignin (ADL)

4.3.2. Xylanase enzyme production on pretreated thatch grass in submerged fermentation

The fungal strains indicated in Table 4.1 were screened for xylanase activity using acid pretreated thatch grass as an inductive carbon source. All the filamentous fungi showed good xylanase activity ranging from 77.3 to 287.03 nkat/ml (Figure 4.1**)**. *Talaromyces helicus* BB2X4 and *Neosartorya* sp. AB2XYL20 produced the highest xylanase activity of 234.57 & 287.03 nkat/ml, respectively, after 48 hours. *Aspergillus fumigatus* L1XYL9 and *Hypocrea lixii* AB2A3 produced the highest xylanase activity of 285.43 and 270 nkat/ml, respectively, after 72 hours. *Aspergillus niger* BB2XYL21 produced the lowest xylanase activity of 77.3 nkat/ml after 120 hours. *Neosartorya* sp. AB2XYL20, *Aspergillus fumigatus* L1XYL9 and *Hypocrea lixii* AB2A3 were selected for further studies.

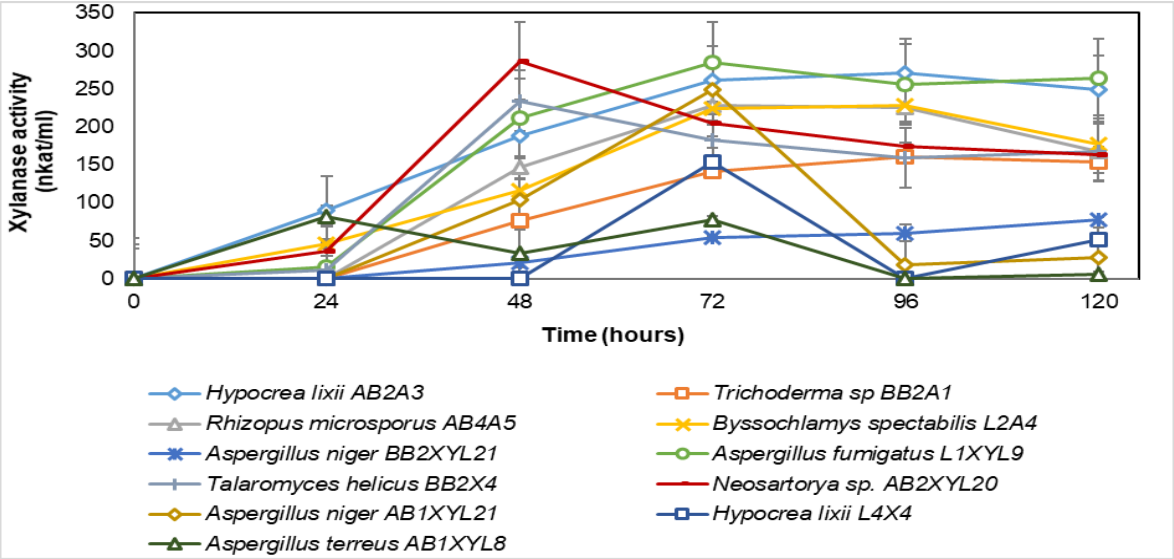

Figure 4.1. Xylanase production by fungi previously isolated from dung beetles and dung beetle larvae in submerged fermentation using acid pre-treated thatch grass as carbon source.

4.3.3. Comparative studies on xylanase on acid pretreated thatch grass

Neosartorya sp. AB2XYL20, *Aspergillus fumigatus* L1XYL9 and *Hypocrea lixii* AB2A3 showed good xylanase activity when acid pretreated thatch grass was used as an inductive carbon source. The fungal strains were selected for comparative studies using solid-state and submerged fermentation. The strains were further screened for endoglucanase activity. Xylanase activity was measured as an indicator for higher/lower xylanase production by each strain during xylanase production on acid pretreated thatch grass.

4.3.3.1. Xylanase production on acid pretreated thatch grass in submerged fermentation

The xylanase activity obtained from *Hypocrea lixii* AB2A3, *Aspergillus fumigatus* L1XYL9 and *Neosartorya* sp. AB2XYL20 was different compared to the xylanase activity depicted in Figure 4.1. The xylanase activity ranged from 304 to 543 nkat/ml. *Hypocrea lixii* AB2A3 and *Neosaryorta* sp. BB2XYL20 showed higher xylanase activity of 529.77 and 543.80 nkat/ml respectively after 48 hours. *Aspergillus fumigatus* L1XYL9 showed lower xylanase activity of 304 nkat/ml as compared to *Hypocrea lixii* AB2A3 and *Neosartorya* sp. AB2XYL20 (Figure 4.2).

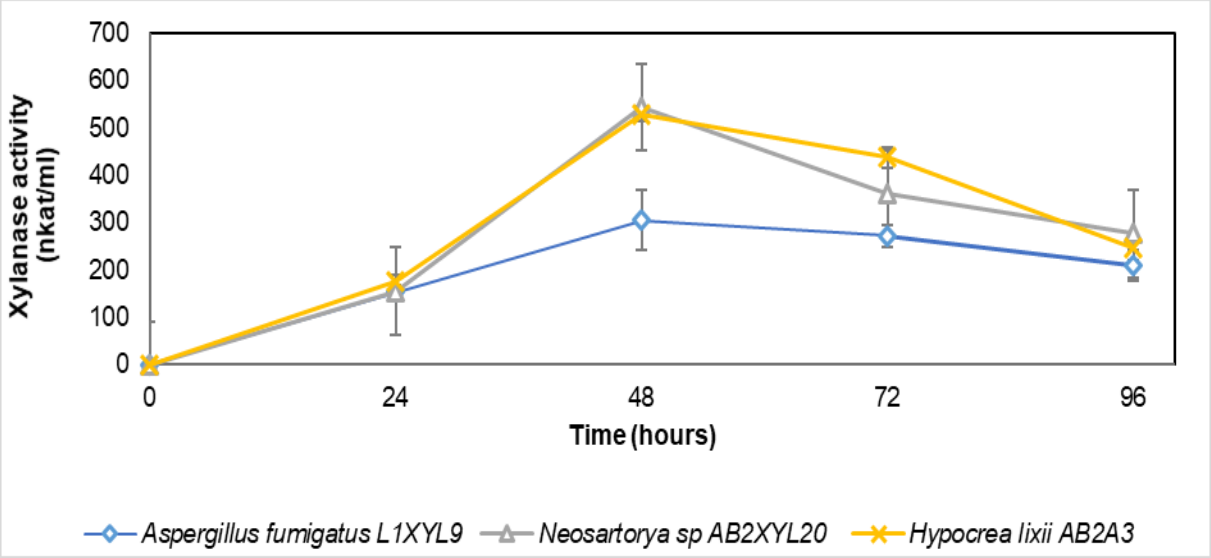

Figure 4.2: Xylanase production in submerged fermentation using acid pretreated thatch grass as the substrate.

Hypocrea lixii AB2A3, *Aspergillus fumigatus* L1XYL9 and *Neosartorya* sp. AB2XYL20 were further screened for endoglucanase activity. The endoglucanase activity was low when compared to that of the xylanase activity (Figure 4.2). The endoglucanase activity ranged from 0.37 to 3.8 nkat/ml. *Hypocrea lixii* AB2A3 showed higher endoglucanase activity of 3.59 nkat/ml after 48 hours while *Neosartorya* sp. AB2XYL20 showed higher endoglucanase activity (3.8 nkat/ml) after 96 hours (Figure 4.3).

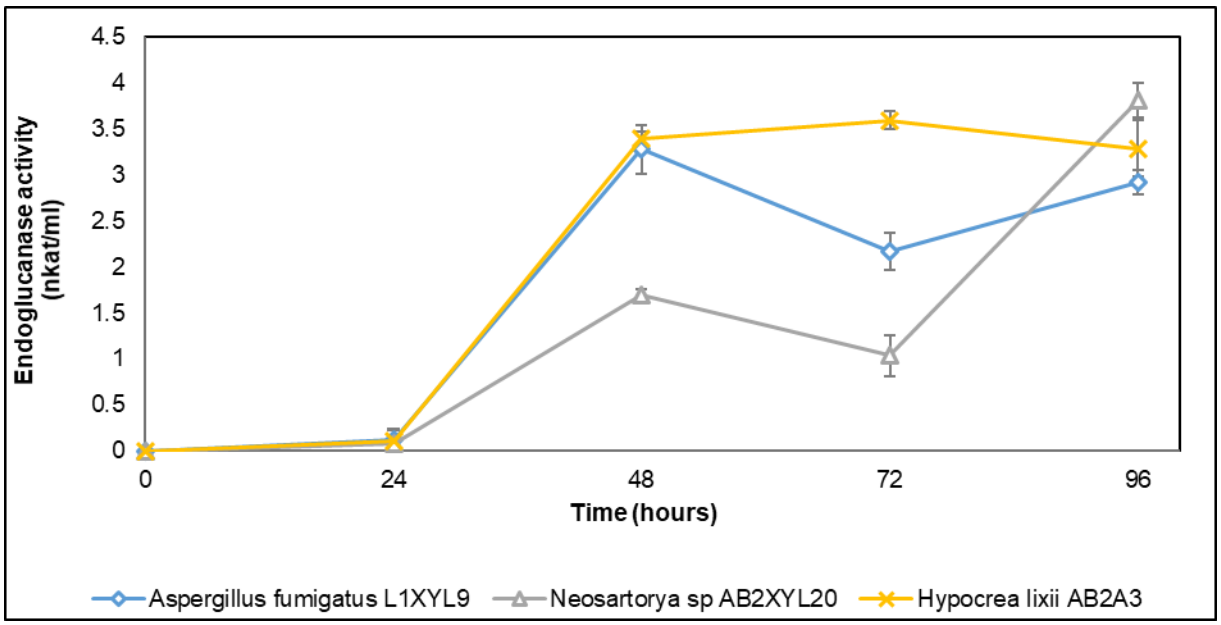

Figure 4.3. Endoglucanase activity in submerged fermentation using acid pretreated thatch grass as substrate.

4.3.3.2. Xylanase production by selected fungi on thatch grass in solid state fermentation

Production of xylanase produced by fungi in solid state Fermentation depends largely on the moisture level of the substrate, which may vary from one fungal strain to another. Two different moisture levels (F1 = 44% and F2 = 54%) were used in solid state fermentation for the production of xylanase in this study (Figure 4.4 & Figure 4.5).

The xylanase activity from the three selected strains ranged from 0 – 168 nkat/ml after 96 hours. When the substrate level moisture was maintained at 44%, *Hypocrea lixii* AB2A3 produced the highest xylanase activity of 93.23 nkat/ml after 96 hours.

When the substrate level moisture was maintained at 54%, *Neosartorya* sp. AB2XYL20 and *Aspergillus fumigatus* L1XYL9 produced the highest xylanase activity of 168.82 and 110.23 nkat/ml, respectively after 96 and 120 hours. *Hypocrea lixii* AB2A3 produced a xylanase activity of 31 nkat/ml after 96 hours.

Xylanase activity in solid-state fermentation was lower compared to that observed from the same strains in submerged fermentation (Figure 4.3). For example, *Neosartorya* sp. AB2XYL20 F2 produced a xylanase activity of 168 nkat/ml in solid-state fermentation compared to 543.80 nkat/ml for submerged fermentation.

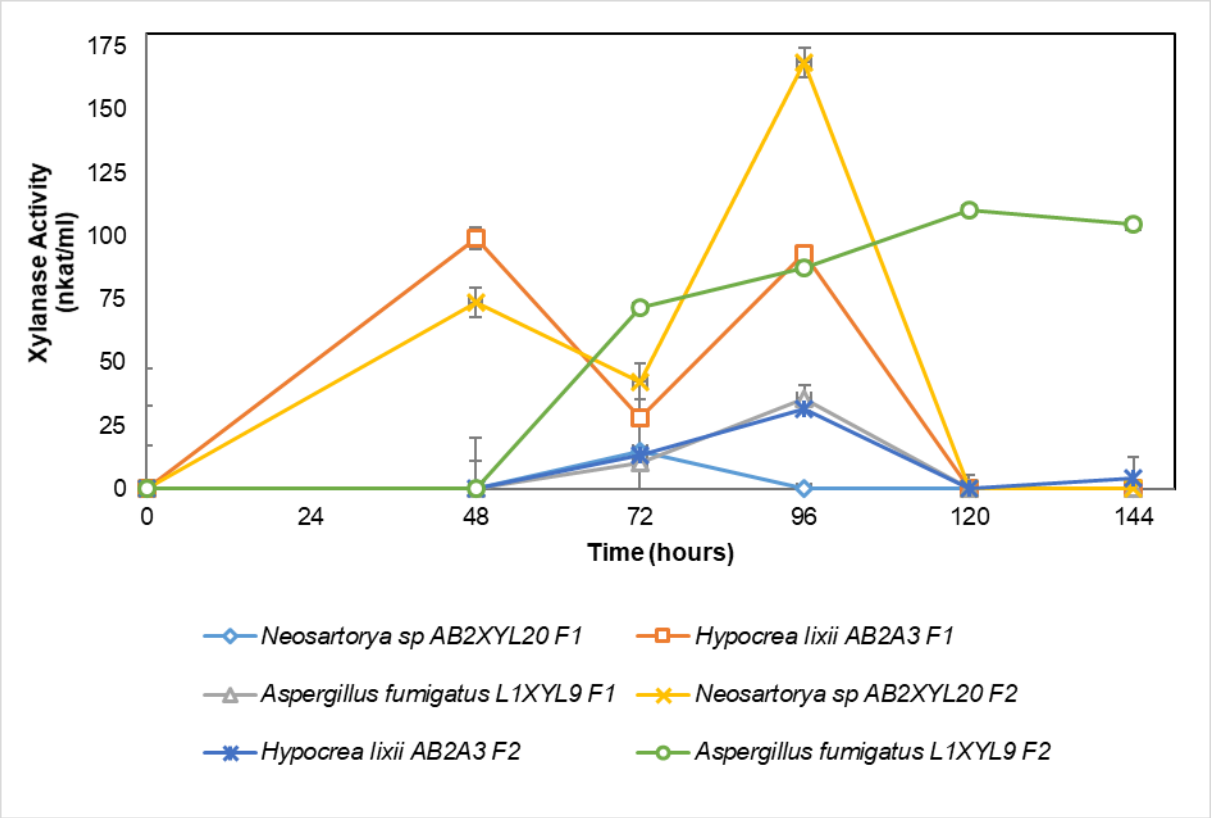

Figure 4.4. Xylanase activity on acid pretreated thatch grass at different moisture levels (F1 = 44% and F2 = 54%) using solid state Fermentation.

The three selected fungal strains *Hypocrea lixii* AB2A3, *Aspergillus fumigatus* L1XYL9 and *Neosartorya* sp. AB2XYL20 showed endoglucanase activity ranging from 1 – 29 nkat/ml. *Neosartorya* sp. AB2XYL20 produced the highest endoglucanase activity of 29.71 nkat/ml, after 96 hours, at 54% moisture content (Figure 4.5). *Neosartorya* sp. *AB2XYL20*, *Hypocrea lixii* AB2A3 and *Aspergillus fumigatus* L1XYL9 produced an endoglucanase activity of 6.5 nkat/ml at both moisture levels, after 72 hours.

Remarkably, the selected fungal strains did not produce good endoglucanase activity on submerged fermentation (Figure 4.3) compared to solid-state fermentation. *Neosartorya* sp. AB2XYL20 produced an endoglucanase activity of 29.71 nkat/ml at 54% moisture content on solid-state fermentation compared to 3.8 nkat/ml for submerged fermentation.

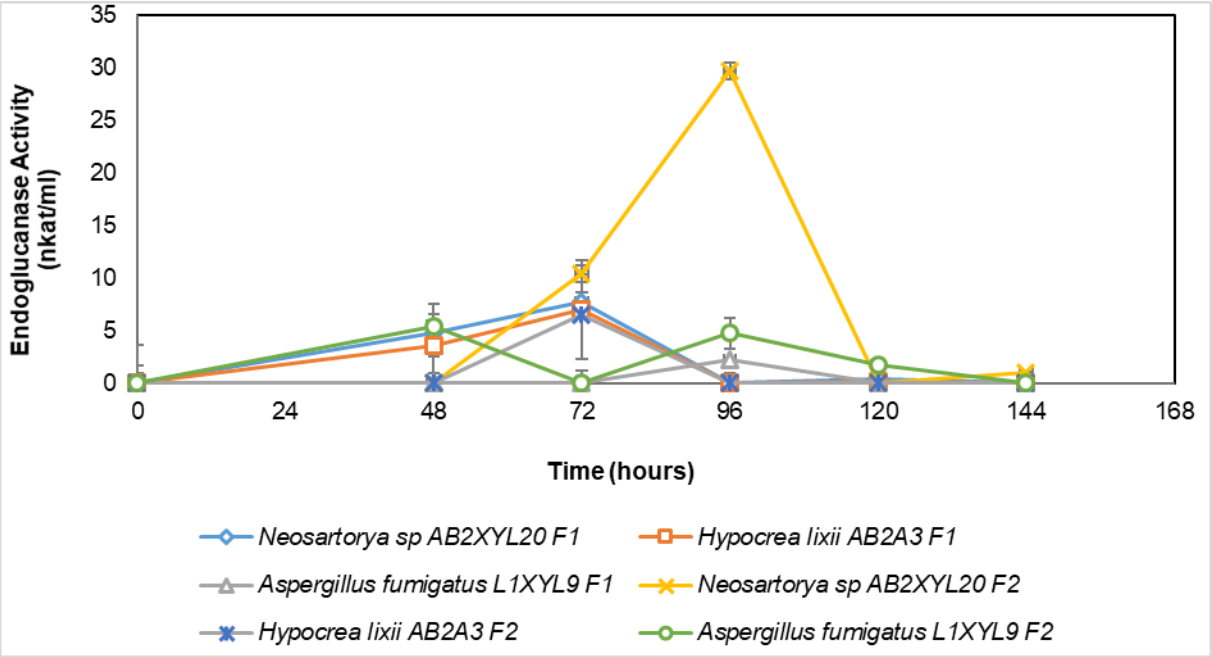

Figure 4.5. Endoglucanase activity on acid pretreated thatch grass at different moisture levels (F1 = 44% and F2 = 54%) using solid state Fermentation.

4.3.4. Evaluation of the effectiveness of the treatment of thatch grass and methods used for xylanase production on different fermentation platforms.

4.3.4.1. Effect of agitation on xylanase production on acid pretreated thatch grass in submerged fermentation

Xylanase production by *Hypocrea lixii* AB2A3 was studied using different agitation speeds (150 – 200 rpm) in the incubator (Figure 4.6). Higher xylanase activity of 584.8 nkat/ml was achieved at 200 rpm after 96 hours compared to 537.39 nkat/ml at 150 rpm after 72 hours.

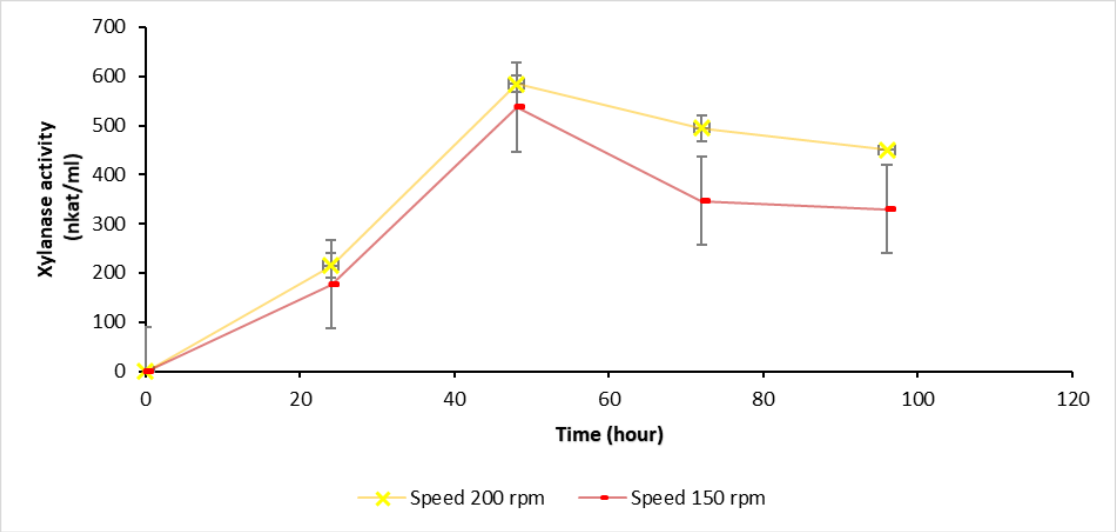

Figure 4.6. Effect of different agitation speed for xylanase production by *Hypocrea lixii* AB2A3 on acid pretreated thatch grass.

4.3.4.2. Xylanase production on acid and alkaline pretreated and untreated thatch grass

Three pretreatment methods were compared to each other and untreated thatch grass using *Hypocrea lixii* AB2A3. The three pretreatments included thatch grass pretreated with acid, thatch grass pretreated with acid and washed and thatch grass pretreated with an ammonium solution.

Pretreatment efficiency was evaluated by determining which pretreatment results in higher xylanase activity. Acid and washed pretreated thatch grass was not a good xylanase inducer. This was depicted by lower xylanase activity of 25 nkat/ml (Figure 4.7) as compared to 529 nkat/ml (Figure 4.2) produced by *Hypocrea lixii* AB2A3 on acid and unwashed pretreated thatch grass. Xylanase activity was also screened on ammonium pretreated thatch grass. *Hypocrea lixii AB2A3* showed lower xylanase activity of 64 nkat/ml after 72 hours.

The xylanase activity of *Hypocrea lixii* AB2A3 was also screened using untreated thatch grass as a carbon source and gave xylanase activity of 164.59 nkat/ml, which is lower compared to the xylanase activity of 529 nkat/ml (Figure 4.2) when acid pretreated thatch grass was used. However, the xylanase activity on the untreated thatch grass was higher compared to thatch grass pretreated with an ammonium

solution and thatch grass pretreated with acid and washed with distilled water (Figure 4.7).

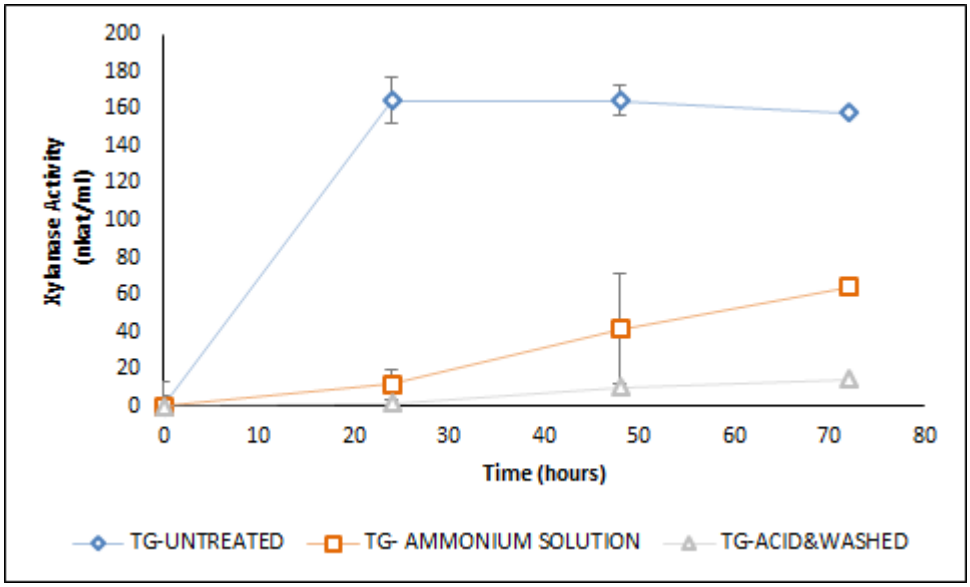

Figure 4.7: A comparison of xylanase production by *Hypocrea lixii* AB2A3 on acid, ammonium pretreated and untreated thatch grass (TG: Thatch grass).

4.3.4.3. Submerged fermentation of the selected fungal isolates for xylanase enzyme production of thatch grass in the bioreactor

Xylanase production by *Hypocrea lixii* AB2A3 was studied in the bioreactor to evaluate if the conditions that resulted in higher xylanase activity in the shake flask will result in higher xylanase activity in the bioreactor. *Hypocrea lixii* AB2A3 produced the highest xylanase activity of 53.29 nkat/ml after 24 hours (Figure 4.8) while higher xylanase activity in the shake flask was 529.77 nkat/ml (Figure 4.2).

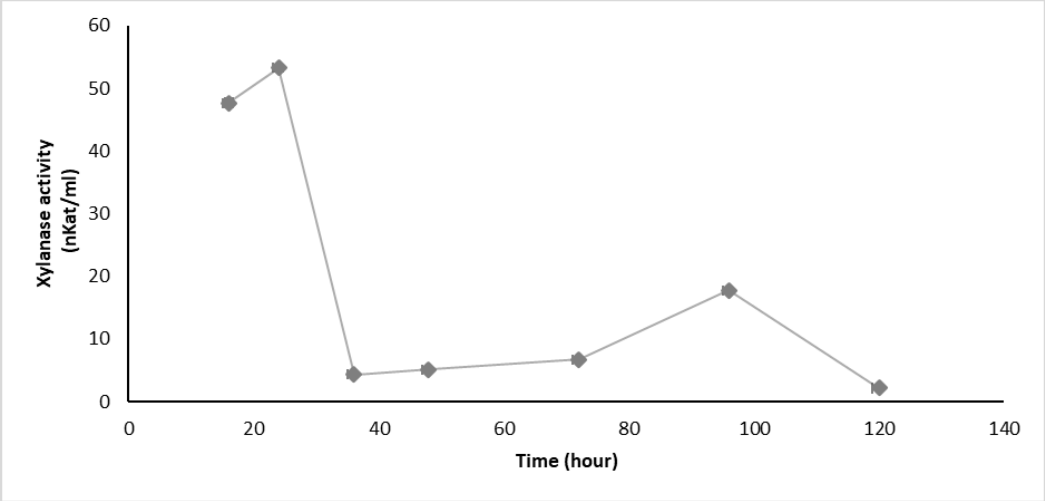

Figure 4.8. Xylanase production by *Hypocrea lixii* AB2A3 on acid pretreated thatch grass using submerged fermentation in the bioreactor.

4.3.5. Determination of specific activities of the enzyme preparations

The specific activities of the commercial and crude glycoside hydrolase preparations used in this study were determined and compared (Table 4.3). All enzymes demonstrated substantial differences in their specific activities towards tested substrates. Crude xylanase by *Hypocrea lixii* AB2A3 displayed the highest xylanase activity. Crude xylanase by *Hypocrea lixii* AB2A3 had below detectable levels of filter paper activity and contained very low endoglucanase activity. This reflected its low saccharification ability towards cellulose polymer. The cellulase enzymes cocktail celluclast™ showed the highest total cellulase activity but low levels of xylanase activity, which inferred low saccharification ability on xylan. Crude xylanase by *Hypocrea lixii* AB2A3 and celluclast™ showed similar β-glucosidase activity. The cellobiose is released by cellulase enzyme complex from the reducing or non-reducing ends of cellulose. The β-glucosidases hydrolyses cellobiose to glucose.

Table 4.3. Composition of the enzymes used.

Enzyme	**Endo-β-1, 4-glucanase (nkatml)**	**Total Cellulase (FPU /ml)**	**β-glucosidase (nkat/ml)**	**Xylanase (nkat/ml)**
Crude Xylanase enzyme[a] (2mg/ml)	4.23	0.16	8.26	494.76
Celluclast[b]™ (1mg/ml)	ND	65.14	8.3	57.01

Key:

[a] Crude xylanase activity from AB2A3 *Hypocrea lixii*

[b] Commercial cellulose Celluclast[b] ™

4.3.6. Enzymatic saccharification and efficiency of acid pretreated thatch grass for the production of fermentable sugars

Enzymatic saccharification of acid pretreated thatch grass was done by using crude xylanase by *Hypocrea lixii* AB2A3 (46 U/g) alone (Figure 4.9) and supplementing it with commercial celluclast™ (8 FPU/g) (Figure 4.10). Enzymatic saccharification efficiency using both enzymes on acid pretreated thatch grass was also assessed (Figure 4.10). Maximum glucose of 549 mg/g was produced after 48 hours by supplementation of crude xylanase with celluclast™ (8 FPU/g) with a saccharification efficiency of 60%. While enzymatic saccharification of acid pretreated thatch grass with celluclast™ (8 FPU/g) unison resulted in a maximum glucose yield of 528 mg/g after 48 hours with a saccharification efficiency of 54% (Figure 4.10).

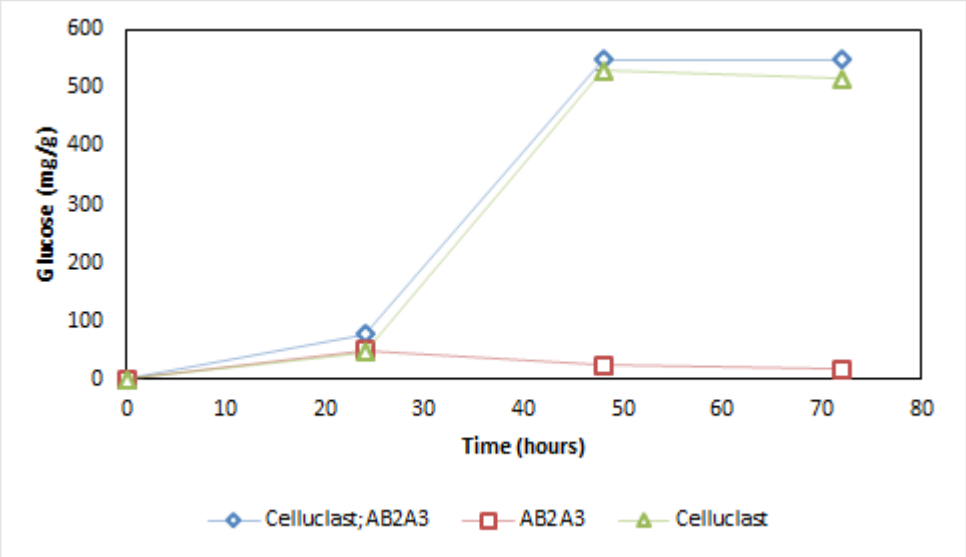

Figure 4.9. Glucose released during enzymatic saccharification of acid pretreated thatch grass using Celluclast™ and crude xylanase from *Hypocrea lixii* AB2A3.

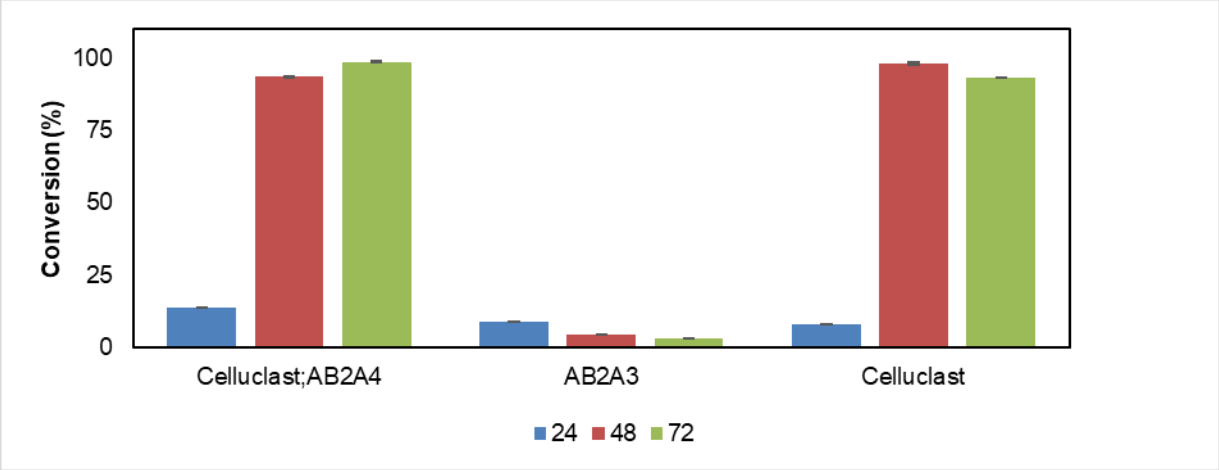

Figure 4.10. Conversion of glucan in acid pretreated thatch grass using Celluclast™ and crude xylanase by *Hypocrea lixii* AB2A3.

The maximum xylose yield was 138.7 mg/g after 72 hours produced from saccharification of acid pretreated thatch grass with crude xylanase from *Hypocrea lixii* AB2A3 unison and the crude xylanase resulted in saccharification efficiency of 59%. Enzymatic saccharification of the acid pretreated thatch grass using a cocktail of celluclast™ and crude xylanase from *Hypocrea lixii* AB2A3 produced xylose concentration of 128 mg/g after 72 hours (4.11). This cocktail resulted in enzymatic saccharification efficiency of 55% (4.12).

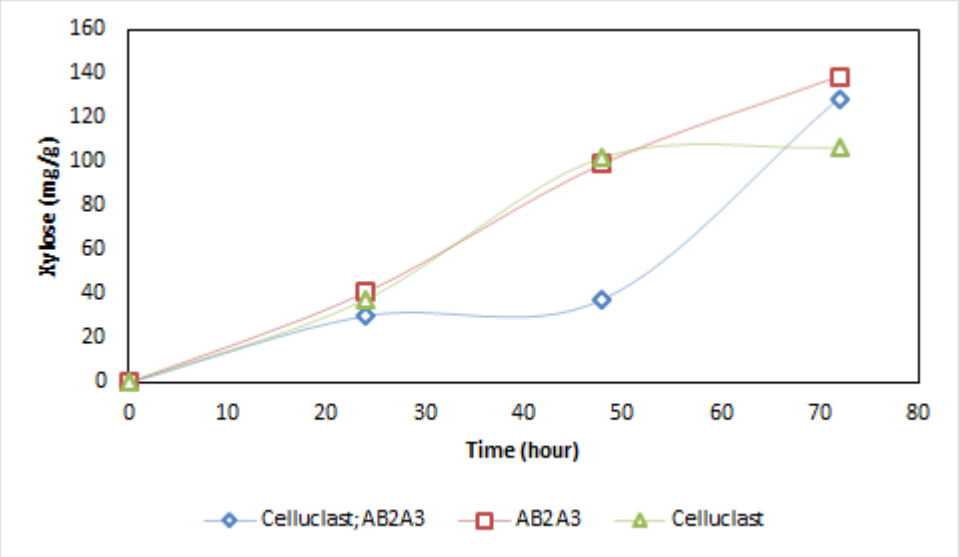

Figure 4.11. Xylose released during saccharification of acid pretreated thatch grass using Celluclast™and crude xylanase by *Hypocrea lixii* AB2A3.

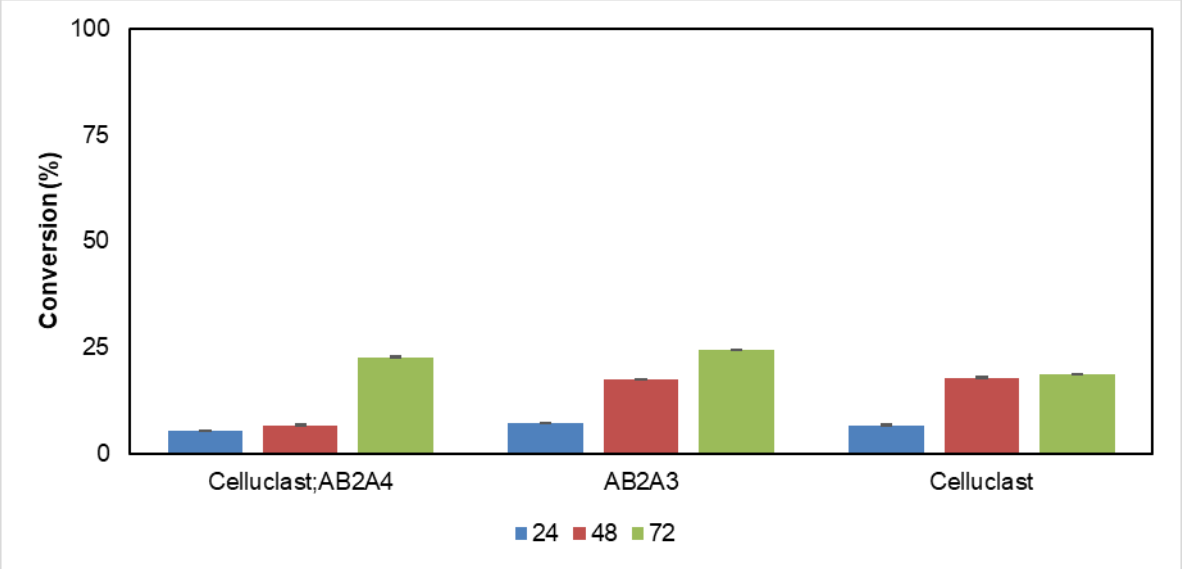

Figure 4.12. Conversion of xylan in acid pretreated thatch grass using celluclast™ and AB2A3 crude enzyme to xylose.

4.4. DISCUSSION

The search to reduce the cost of biofuel production has led researchers to explore microorganisms with hemicellulolytic activity. Hemicellulase, specifically xylanase, has received much attention. Finding enzymes and fungi capable of effectively breaking down hemicellulose could potentially improve the current conversion technology used in the lignocellulosic biofuel industry (Ajijolakewu *et al*., 2016 & Uday *et al*., 2017). The use of a cheap substrate, such as thatch grass to produce xylanase will reduce the production cost of bioethanol. While the application of thatch grass as feedstock in a variety of bioprocesses is an alternative low-cost substrate, that also promises to cut the pollution problems due to their inappropriate disposal. Their use in the industry, such as the biofuel industry, requires that they are degraded into fermentable sugars. Enzymatic hydrolysis is attractive since it is environmentally friendly and does not result in the production of inhibitors that hinder fermentation. The process requires effective enzymes that result in complete degradation of the lignocellulose polymers to increase product yield. The supplementation of xylanase with commercial cellulase in enzymatic hydrolysis has been reported to increase cellulose accessibility to enzymatic degradation (Ajijolakewu *et al.*, 2017).

The use of thatch grass as an inducer carbon of the gut microbiota with xylanolytic activity can result in increased xylanolytic activity? Can the resultant crude xylanase result in the complete degradation of hemicellulose thatch grass?

This study sought to study xylanase production by isolated fungi from the gut of two dung beetles (*Anachalcos Convexus* and *Pachylomerus Femoralis)* and dung beetle larvae (*Euoniticellus intermedius*) using thatch grass as inducer. Several parameters were evaluated during the xylanase production process by *Hypocrea lixii* AB2A3 and its use in enzymatic saccharification. Production of xylanase by filamentous fungi *Hypocrea lixii* AB2A3 in the bioreactor was studied. Enzymatic saccharification efficiency was studied by supplementation of crude xylanase by *Hypocrea lixii* AB2A3 to commercial cellulase^{TM} 1.5L during enzymatic saccharification of acid pretreated thatch grass.

4.4.1. Thatch grass composition and its use as inductive carbon

Different grasses are potential lignocellulosic materials to be used in second-generation biofuel. Most studies concentrated on cellulosic ethanol production using grass as a carbon source with little attention given to the use of grass as the carbon source for xylanase enzyme production (Mohapatra *et al.*, 2017 & de Almeida *et al.*, 2019). Thatch grass is an inexpensive substrate that is abundantly available and renewable in South Africa. The composition analysis of thatch grass indicated that it is a rich source for cellulose and hemicellulose at 46 and 23%, respectively (Ncube *et al.*, 2013). The most commonly used herbaceous biomass in biofuel production is *Miscanthus* sp. The lignocellulose content of *Miscanthus* sp. consists of 40 – 60% cellulose, 20 – 40% hemicellulose and 10 – 30% lignin. Therefore, thatch grass compares well with *Miscanthus* as a possible source in the production of enzymes and bioethanol (Wang *et al.*, 2018). There are also reports indicating that the composition of cellulose, hemicellulose and lignin in grasses are better suited for ethanol production as compared to other agro-industrial waste (Mohapatra *et al.*, 2017 & de Almeida *et al.*, 2019).

4.4.2. Xylanase production on acid pretreated thatch grass using submerged and solid-state fermentation

Xylanases are inducible enzymes and the use of inexpensive, abundantly available agricultural residues to induce xylanases production is important in industries (Kumar *et al*., 2017 & de Souza *et al.*, 2020). In this study acid pretreated thatch grass was used as the inducer carbon source for the production of xylanase since it was successfully used in our laboratory for isolation of different fungal species with lignocellulolytic activity from dung (Makhuvele *et al.*, 2017). Xylanase activity on acid pretreated thatch grass was low compared to when commercial xylan was used as the carbon source (Chapter 3). This could be attributed to the pretreatment that released by-products that affect microbial growth while commercial xylan is a pure substrate (de Souza *et al.*, 2020).

Neosartorya sp. AB2XYL20, *Aspergillus fumigatus* L1XYL9 and *Hypocrea lixii* AB2A3 showed good xylanase activity of 543.80, 304 and 529.77 nkat/ml, respectively on acid pretreated thatch grass. The xylanase activity by *Aspergillus fumigatus* L1XYL9 cultivated with acid pretreated thatch grass residue is comparable to previous reports.

Makhuvele *et al*. (2017) reported a xylanase activity of approximately 320 nkat/ml by *Aspergillus* sp. cultivated on acid pretreated thatch grass. The xylanase activities of *Aspergillus fumigatus* Z5 on rice straw (Liu *et al*., 2013) and *Aspergillus fumigatus* MA28 on wheat bran (Bajaj and Abbass, 2011) were reported to be 250,05 nkat/ml, and 140,86 nkat/ml respectively.

Neosartorya is the teleomorph of *Aspergillus.* In this study, a xylanase activity of 543.80 nkat/ml was observed for *Neosartorya* sp. AB2XYL20. Somboon *et al.* (2020) reported xylanase activity of *Neosartorya* sp. of 183 nkat/ml with birchwood xylan used as carbon source. This xylanase activity on purified xylan was lower compared to xylanase activity on acid pretreated thatch grass as observed in this study. Furthermore, *Neosartorya* sp. AB2XYL20 showed higher xylanase activity on acid pretreated thatch grass than when cultivated on xylan. This shows that acid pretreated thatch grass was a good inducer carbon source for this strain. *Aspergillus* species are known for their ability to produce higher xylanase enzyme (de Souza *et al.*, 2020).

Hypocrea lixii AB2A3 produced a xylanase activity of 529 nkat/ml on acid pretreated thatch grass. *Hypocrea lixii* is the teleomorph of *Trichoderma harzinum. Trichoderma reesei* is a well-known species for commercial cellulase and xylanase enzyme production (Bischof *et al*., 2016). Higher xylanase activity was reported on different agro-industrial residues, which is higher compared to the xylanase activity by *Hypocrea lixii* AB2A3 in this study. A much higher xylanase activity of 917 nkat/ml was achieved by *T. harzianum* LZ117 after 144 h with pretreated corn stover as substrate. (Li *et al.*, 2019). Xylanase production by *Hypocrea lixii* AB2A3 was observed in a shorter period of 48 hours which makes this strain more cost-effective to utilise. *Hypocrea lixii* AB2A3, *Aspergillus fumigatus* L1XYL9 and *Neosartorya* sp AB2XYL20 indicated very low endoglucanase activity. This inferred that thatch grass was not a good carbon inducer source for the production of endoglucanase, although thatch grass has a higher cellulose composition fraction than hemicellulose. Induction of enzymes have specificities to lignocellulosic biomass used as carbon source, thus the same species can produce different enzymes on the different inductive carbon source. The higher xylanase activity was also attributed to the use of inoculum size of 2 x 10^6 spore/ml while lower inoculum size of 1 x 10^6 spore/ml resulted in xylanase activity of 287.03, 270 and 283.43 nkat/ml by *Neosartorya* sp. AB2XYL20, *Hypocrea lixii* AB2A3 and *Aspergillus fumigatus* L1XYL9, respectively.

Tai *et al.* (2019) reported increased xylanase activity to 5.23 U/g from 2.93 U/g by *Aspergillus niger* DWA8 using 1×10^6 spores/mL of inoculum using agricultural waste (oil palm frond [OPF]) as substrate. Ezeilo *et al*. (2019) reported increased xylanase activity of 213.99 U/g by *Rhizopus oryzae* UC2 when 2×10^8 spore's/g inoculum size was used using agricultural waste (oil palm frond [OPF]) as substrate. Other studies reported higher xylanase activity by *Penicillium chrysogenum* and *Aspergillus foetidus* when inoculum sizes of 0.5 x 10^6 and 1.0 x 10^8 spores/g were used, respectively (Chapla *et al.*, 2010; Zhang and Sang, 2015b & Ezeilo *et al*., 2019). The effect of inoculum size during xylanase production on acid pretreated thatch grass has not been studied, thus in this study, this parameter was studied to determine if they have any effects on xylanase production. The correct inoculum size must be administered for xylanase production in submerged fermentation using filamentous fungi. Since surplus inoculum size can result in the starvation of fungal cells while insufficient inoculum load affects enzyme production by the decrease in mycelia biomass or due to rapid nutrient exhaustion (Ezeilo *et al.*, 2019).

4.4.3. The effect of initial moisture level in xylanase production on solid-state fermentation

Solid-state fermentation has recently received much attention with the increase in the use of agricultural residues as substrate. Solid-state fermentation mimics the natural environment of the enzyme-producing microbes (mostly fungi) and works well with agricultural residues (Leite *et al.*, 2021). In solid-state fermentation, enzyme production by fungal species is mainly influenced by moisture level and the biomass used (Deswal *et al*., 2011). Therefore, it is vital to optimise the initial moisture level of the substrate to achieve the maximum yield of the desired product. Due to difficulties in controlling different parameters in solid-state fermentation the xylanase activity fluctuated as observed in Figures 4.4 & 4.5.

A higher moisture level of 54% achieved higher xylanase activity for *Neosartorya* sp. AB2XYL20, *Hypocrea lixii* AB2A3 and *Aspergillus fumigatus* L1XYL9. Ang *et al.* (2013) reported higher xylanase activity at an initial moisture level of 70% from *Aspergillus fumigatus SK1* when untreated oil palm trunk (OPT) was used as substrate. Mostly higher xylanase activity is reported at the initial moisture level of agro-industrial and agricultural residues of 70 – 80% (Khanahmadi *et al.*, 2018). Menegol *et al.* (2017)

reported high xylanase activity of 621.16 nkat/ml by *Penicillium echinulatum* strain 9A02S1 at initial moisture of 67% when a mixture of 75% untreated elephant grass and 25% wheat bran were used as solid substrate. The higher xylanase activity at low initial moisture level in this study was in agreement with the study by Ezeilo *et al*. (2019), higher xylanase activity of 356,72 nkat/ml by *Rhizopus oryzae* UC2 was reported at initial moisture level of 40% when oil palm frond leaves were used as solid substrate.

Neosartorya sp. AB2XYL20, *Hypocrea lixii* AB2A3 and *Aspergillus fumigatus* L1XYL9 were further screened for endoglucanase activity. Endoglucanase enzyme is part of the cellulolytic enzymes complex. Mesophilic fungus, *Trichoderma reesei* is one of the most thoroughly studied because of its highly efficient cellulolytic systems (Soliman *et al*., 2015). *Neosartorya* sp. AB2XYL20 produced a higher endoglucanase activity at 44% moisture level and *Hypocrea lixii* AB2A3 produced a higher endoglucanase activity at 54% moisture level when compared to values obtained for submerged fermentation. This is comparable to results obtained by Delabona *et al.*, (2013), observed increased endoglucanase activity of 130 nkat/ml at 50% moisture level. Subsamran *et al.* (2019) also observed that the optimal moisture content for cellulase enzyme complex was 56.45% in SSF using vertiver grass as a solid substrate.

Surprisingly in this study submerged fermentation was the optimal process for the production of xylanase enzyme from *Neosartorya* sp. AB2XYL20 and *Hypocrea lixii* AB2A3 using acid pretreated thatch grass. This was unexpected since filamentous fungi are reported to produce higher enzyme activities in SSF. Furthermore, Ncube *et al.* (2013) reported that solid-state fermentation was an optimal process for xylanase production when thatch grass was used as solid substrate. The morphology of fungi is complex and plays an important role that determines the production yield. Fungi shows different morphological forms such as dispersed mycelial filaments to pellets that are densely interwoven mycelial masses. Different fungal species reflect different morphology which could be due to genetic material, the nature of the inoculum, the chemical (medium constituents) and physical (temperature, pH, mechanical forces) culturing conditions (Papagianni, 2004 & Veiter *et al.,* 2018). The study by Steel *et al*. (1954) & Kristiansen and Bullock, (1988) reported that filamentous growth of *Aspergillus niger* resulted in production of pectic enzyme while its pelleted form resulted in citric acid production. The higher xylanase activity in submerged as

compared to solid-state fermentation in this study could also be attributed to the type of morphology formed during SmF which differed in SSF. Furthermore, in SmF fungal strains are exposed to hydrodynamic forces and in SSF growth is rendered to the surface of the solid matrix (Papagianni *et al*., 2001).

4.4.4. **Evaluation of the effectiveness of the treatment of thatch grass and methods used for xylanase production on different fermentation platforms**

4.4.4.1. The influence of agitation speed on xylanase production by Hypocrea lixii AB2A3 using acid pretreated thatch grass as a carbon source

Agitation is an important physical parameter during enzyme production of plant-based residues using filamentous fungi. Agitation assists in the mixing of cells, assisting in homogenising the chemical and physical conditions in the medium. In this study xylanase activity of 584.8 nkat/ml was observed when agitation speed of 200 rpm was used during incubation of *Hypocrea lixii* AB2A3 in acid pretreated thatch grass. While at 150 rpm a xylanase activity of 537.39 nkat/ml was obtained. Conversely, Ravichandra *et al*. (2016) reported a high xylanase activity of 1216.91 nkat/ml at an agitation speed of 150 rpm by *Aspergillus fumigatus* RSP-8 (MTCC 12039).

The observed high xylanase activity at 200 rpm could be attributed to improved oxygen transfer between the different phases resulting in an increased enzyme production yield. The higher agitation speed also lessens the size of mycelial aggregates which results in oxygen being more easily accessible to the cells (Jafari *et al*., 2007). The study by Bakri *et al*. (2011) reported the importance of agitation speed in xylanase enzyme production by *Aspergillus niger* SS in the bioreactor. Enzyme production in a bioreactor is preferred since the different parameters can be controlled and assist in increasing enzyme production. In this study, xylanase production by *Hypocrea lixii* AB2A3 on acid pretreated thatch grass resulted in a lower xylanase activity of 53.29 nkat/ml as compared to xylanase activity of 584.8 nkat/ml in the shake flask. The lower xylanase activity in this study could be due to the shearing force in the fermenter since this can disrupt the fragile fungal biomass. The review by Namvar *et al.* (2014) also highlighted that when filamentous fungi are cultivated in the bioreactor they usually result in lower enzyme yield due to the shearing force.

4.4.4.2. The influence of acid, alkaline pretreated and untreated thatch grass on xylanase production

Hypocrea lixii AB2A3 was used to determine the best pretreatment method on thatch grass for xylanase production. Pretreatment with diluted sulphuric acid (at a lower concentration of 1.2%) was used in this study because it was previously used successfully in our laboratory for the screening of lignocellulolytic microorganisms from the dung of wild herbivores (Makhuvele *et al.*, 2017)**.** Furthermore, the study by Menegol *et al.* (2016) reported that a lower sulphuric acid concentration in the pretreatment of elephant grass was more effective than a high concentration of sulphuric acid. *Hypocrea lixii* AB2A3 xylanase activity on acid pretreated thatch grass with washing decreased significantly to 25 nkat/ml. Extensive washing is preferred after acid pretreatment for removal of potential inhibitors that are generated during acid pretreatments, such as acetic acid, furfural and 5-hydroxymethyl furfural (HMF) (Kumar and Sharma, 2017). The presence of these inhibitors/acids might denature the enzymes involved in enzymatic hydrolysis or affect microbial growth involved in fermentation. Scholl *et al.* (2015) also reported negative results for the production of xylanases when steam explosion pretreated elephant grass was washed and used as an inductive carbon source. Similarly, Yong *et al.* (2018) reported that the xylanase activity of *Trichoderma harzianum* decreased drastically from 183.37 to 41.34 nkat/ml on acid pretreated rice straw washed twice and four times, respectively. The study by Yong *et al*. (2018) reported that washing reduces inhibitors formed during pretreatment but the loss in xylanase activity showed that monosaccharides were also lost during this process.

The alkaline pretreatment method has been reported to be the preferred and highly recommended in the pretreatment of grasses. This is because grasses consist of a high xylan content and are extracted by alkaline dissolution which is followed by alcohol precipitation (Tsai *et al.*, 2018). In this study, thatch grass was pretreated with an ammonium solution. The use of ammonia is also preferred because it is safe to handle, does not pollute the environment, is not corrosive and can be recycled due to its high volatility (Patel *et al*., 2017). Xylanase activity from the thatch grass pretreated with the ammonium solution was lower (64 nkat/ml) compared to when the acid pretreated thatch grass (529 nkat/ml) was used. The lower xylanase activity of *Hypocrea lixii* AB2A3 was not expected since acid pretreatment degrades the lignin

and the hemicellulose by breaking the internal lignin and hemicellulose bonds (Behera *et al.*, 2014). This study was in agreement with the study by Aldo *et al.* (2016), who reported lower enzyme activity on alkaline pretreated elephant grass.

In this study xylanase activity by *Hypocrea lixii* AB2A3 on untreated thatch grass was higher when compared to xylanase activity on the washed acid pretreated thatch grass and ammonium pretreated thatch grass, but lower than the acid pretreated thatch grass. This was in agreement with the study by Scholl *et al*. (2015), who reported higher xylanase activity of 950 nkat/ml on untreated elephant grass by *Penicillium echinulatum* S1M29. The better results for acid pretreatment (unwashed) could be due to the breakage of lignin bonds and also results in a decrease in crystallinity index (Crl) and degree of polymerization (DP) of grasses (Mohapatra *et al.*, 2017). This was not expected since acid pretreatment has been reported to result in the production of inhibitors that can affect microbial growth which can further affect enzyme production (Sun *et al.*, 2016; Chen *et al.*, 2017 & Tsai *et al.*, 2018).

4.4.5. Enzymatic hydrolysis of acid pretreated thatch grass by crude xylanase from *Hypocrea lixii* AB2A3

Plant-based biomass such as thatch grass is an attractive alternative for biofuel production and exploitation of its use in the production of other valuable products is of global interest. Thatch grass requires involvement of several lignocellulolytic enzymes for its degradation into monomers. Hydrolytic enzyme efficiency at low enzyme loading is affected by synergetic enzyme interaction and this plays an important role in the complete degradation of plant-based biomass (Kumar and Parikh, 2015). Supplementation of xylanase in enzymatic hydrolysis of pretreated lignocellulose material with cellulase reduces the loading amount of cellulase enzymes. Enzymatic efficiency by supplementation of xylanase during enzymatic hydrolysis of steam pretreated corn stover using lower cellulase loading was increased from 45% to 80% (Hu *et al*., 2011). Crude xylanase produced by *Hypocrea lixii* AB2A3 and commercial celluclast™ were subjected to enzymatic saccharification of acid pretreated thatch grass unison and together. The blend of crude xylanase and commercial celluclast™ resulted in a glucose concentration of 549 mg/g and percentage conversion of 60% which is higher than 54% percentage conversion achieved when celluclast™ is used alone. The percentages of xylan conversion into xylose by crude xylanase unison was

59%. Degradation of xylan to its monomers is a more complex process compared to cellulose degradation. Conversion of xylan requires several enzymes such as β-Xylosidase, α-Arabinofuranosidase together with other accessory enzymes such as α-galactosidases, β-galactosidases and acetyl esterases. The higher xylan conversion efficiency shows that the crude enzyme blend from *Hypocrea lixii* AB2A3 has a better balance between β-Xylosidase, α-Arabinofuranosidase, α-galactosidases, β-galactosidases and acetyl esterases. Higher glucose production is probably attributed to xylanase degrading hemicellulose giving cellulase access to cellulose degradation (Kumar and Sharma, 2017). The synergistic action of hemicellulases plays a major role in the degradation of plant based biomass and their absence negatively affects the hydrolysis process. Xylanase is the most important hemicellulase enzyme in hemicellulose degradation but the presence of β-xylosidases and α-arabinofuranosidase increases the production yield from hemicellulose. The synergetic effect of enzymatic hydrolysis of grasses using cellulase and xylanase is a new concept. Congruent with the current observation, Liu *et al*. (2020) mentioned that extracellular enzymes that are produced using submerged fermentation are more effective in enzymatic hydrolysis than those in solid-state fermentation. This effectiveness of enzymes from submerged fermentation was reported to be attributed to the carbohydrate-binding module “because more xylanases bound with the substrate at the beginning of hydrolysis”.

4.5. CONCLUSION AND RECOMMENDATION

This study showed, for the first time, that thatch grass can be an efficient inductor for xylanase production in different filamentous fungi isolated from the gut of dung beetles and dung beetle larvae. The acid pretreatment method and submerged fermentation is preferred for xylanase production from thatch grass. *Hypocrea lixii* AB2A3 and *Neosartorya sp* AB2XYL20 are good xylanase producers on acid pretreated thatch grass. Xylanase produced from *Hypocrea lixii* AB2A3 is effective in the hydrolysis of hemicelluloses in thatch grass. Thatch grass is also a potential lignocellulose biomass for biofuel production. Enzymatic hydrolysis of thatch grass can further be improved by optimising enzymatic hydrolysis such as (enzyme loading, hydrolysis temperature, etc.). Optimisation of these parameters will result in increased yield that can be further fermented to valuable products such as ethanol.

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CHAPTER 5

5.1. CONCLUSION AND RECOMMENDATION

The results observed in this study have highlighted the gut of dung beetles and dung beetle larvae to be a bioreactor with diverse fungal species that displayed good xylanolytic activity and yeast species that could assimilate xylose. Thatch grass can be used in industries for production of enzymes to lower enzyme production cost. It can further be used for biofuel production that will also play role making biofuel production economically feasible. The use of thatch grass in biofuel production wil further lower enviromental pollution that is caused by burning it. In South Africa there is still a gap in the exploitation of gut microbiota and their application in biofuel applications. It is imperative to study the gut microbiota from dung beetles from different geographic regions, that are known to feed on different diets to further explore for microorganisms that can be used in biofuel production.

APPENDIX 1

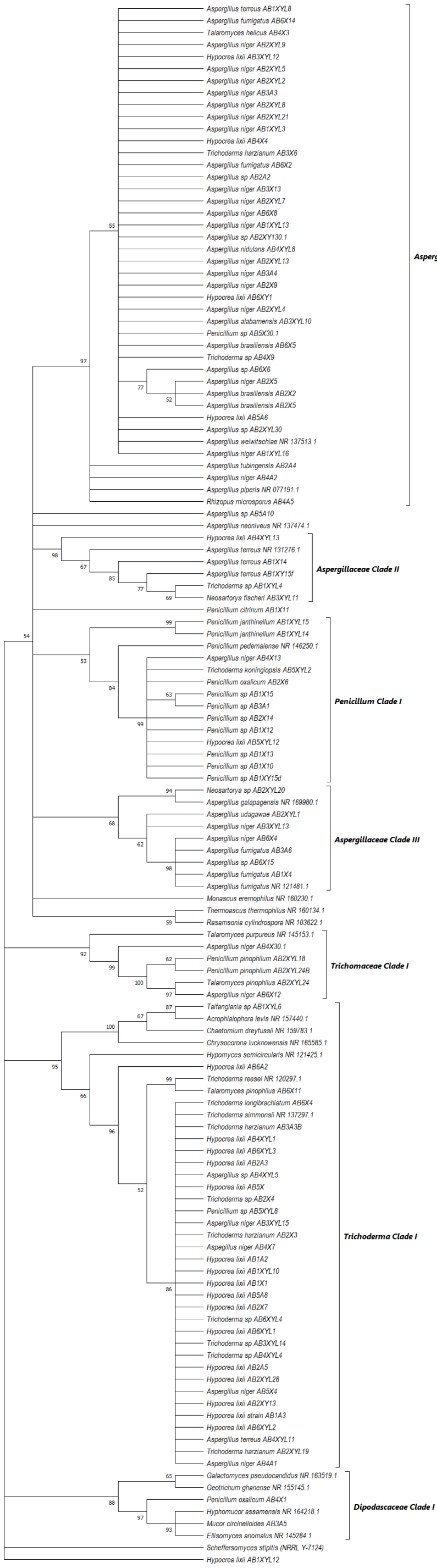

Figure 3.7. Best-scoring Maximum Likelihood tree using ITS sequence of different strains isolated from the gut of dung beetle *A. convexus*. Only branches with more than 50% bootstrap support are shown. *S. stipitis* NRRL-Y-7124 was used as an outgroup.

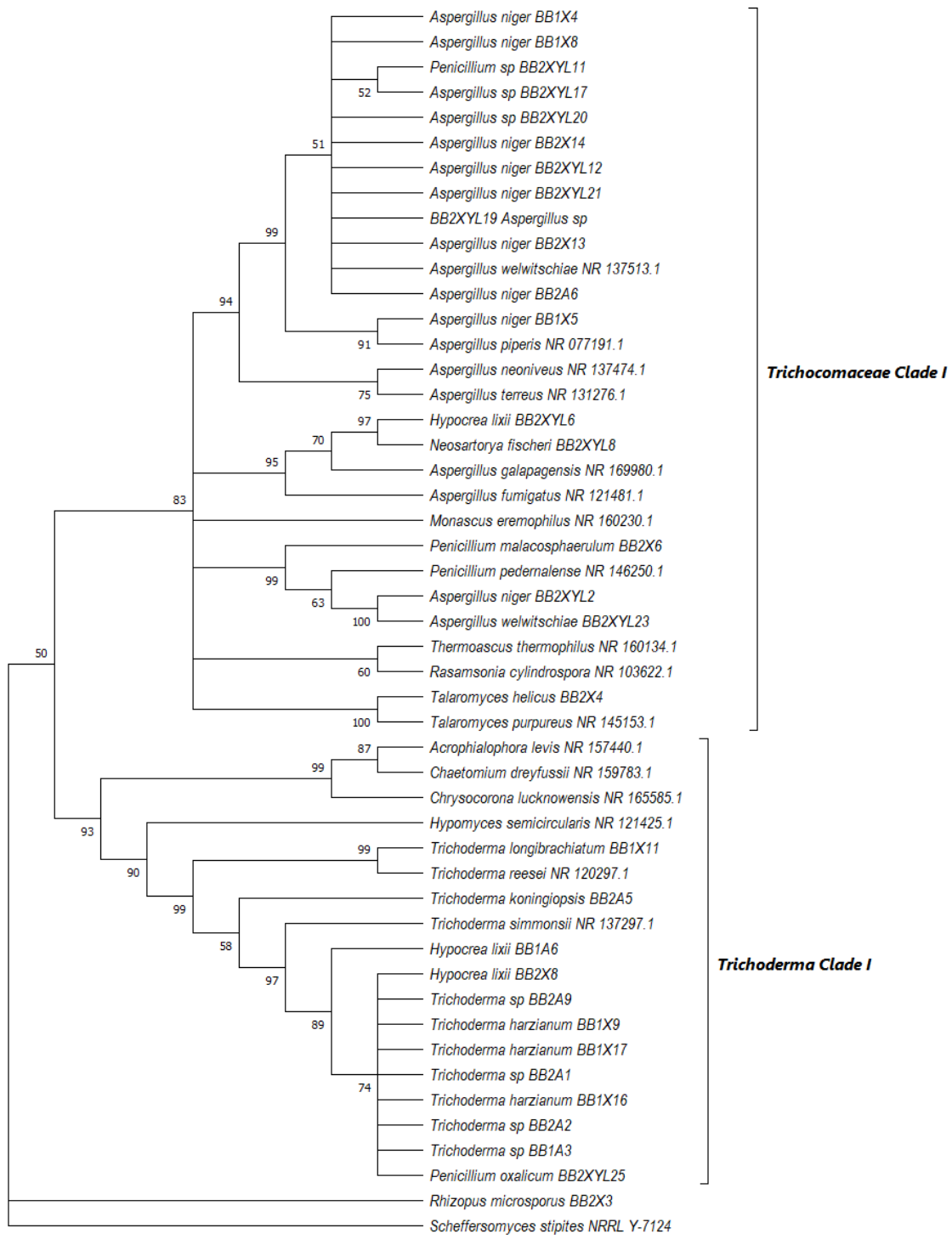

Figure 3.8. Best-scoring Maximum Likelihood tree using ITS sequence of different strains isolated from the gut of dung beetle *P. femoralis*. Only branches with more than 50 % bootstrap support are shown. *S. stipitis* NRRL-Y-7124 was used as an outgroup.

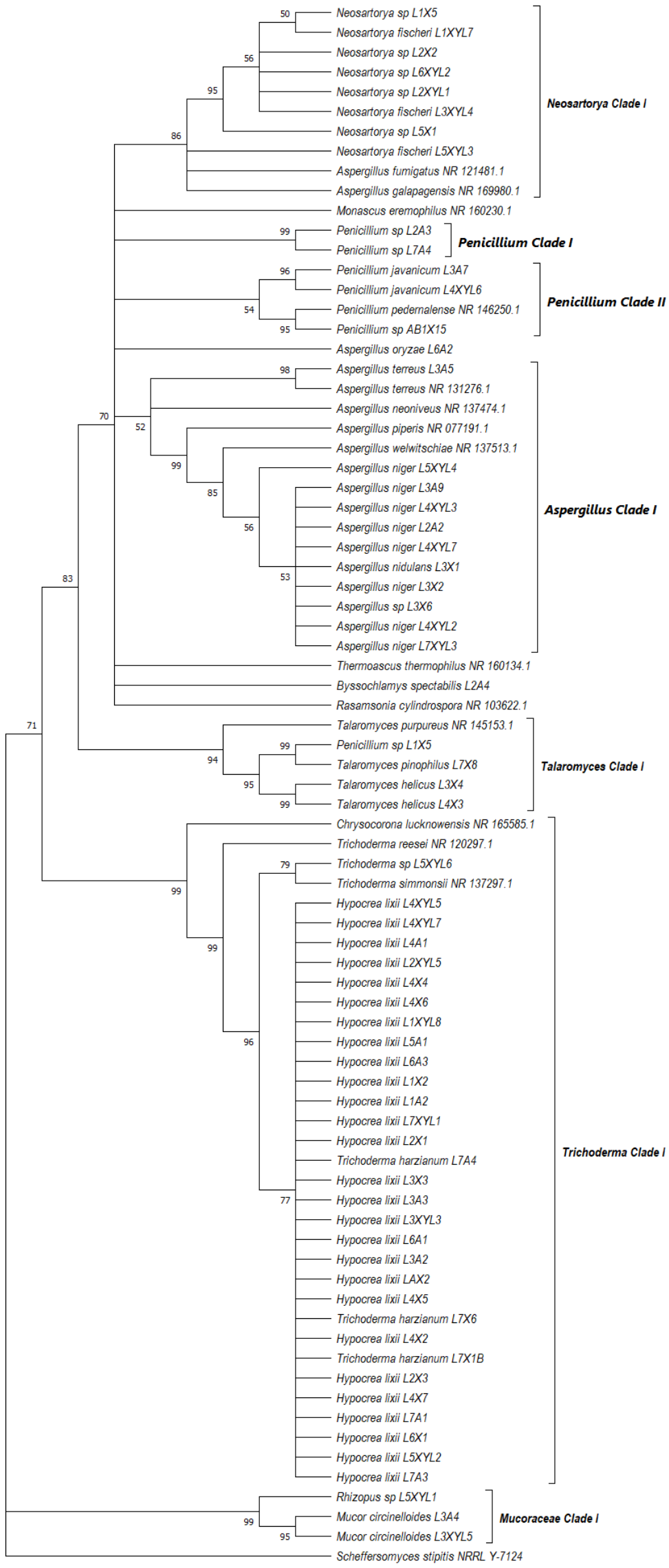

Figure 3.9. Best-scoring Maximum Likelihood tree using ITS sequence of different strains isolated from the gut of dung beetle larvae (*E. intermedius)*. Only branches with more than 50% bootstrap support are shown. *S. stipitis* NRRL-Y-7124 was used as an outgroup.

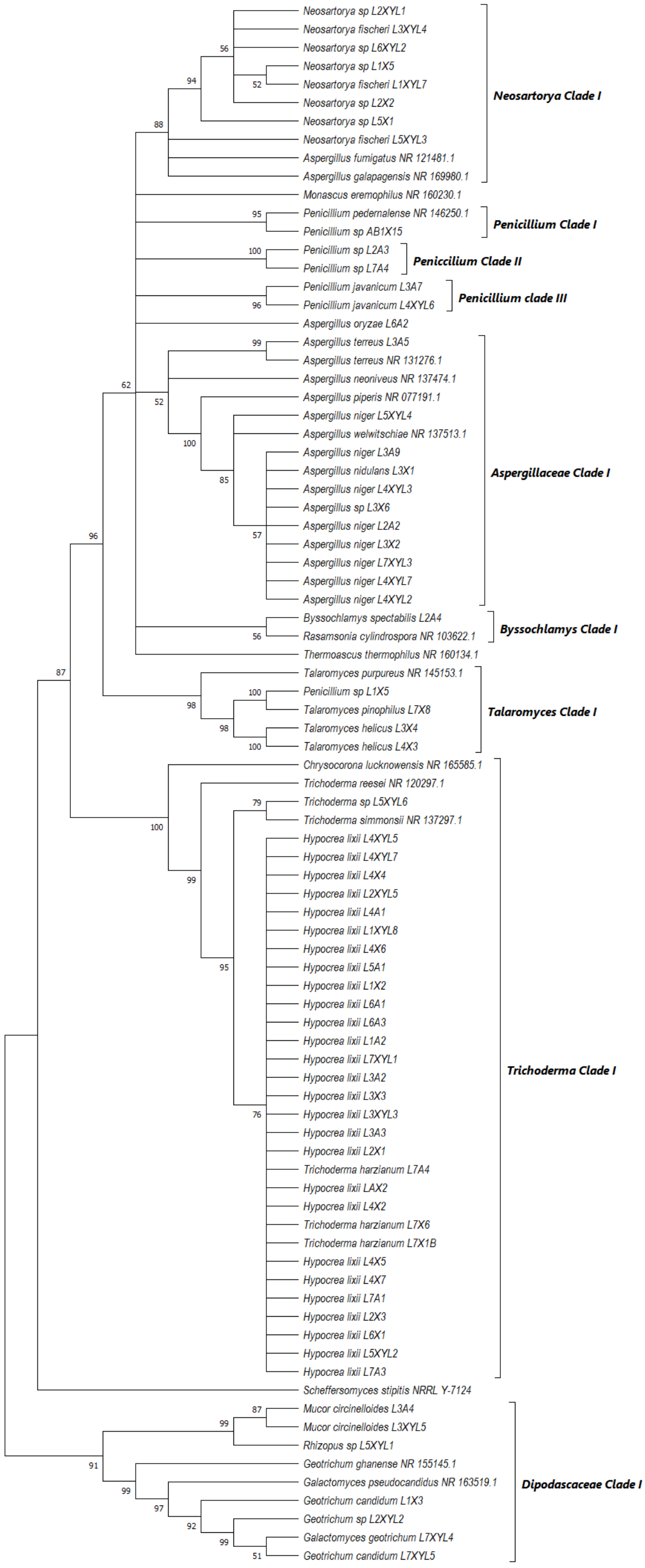

Figure 3.10. Best-scoring Maximum Likelihood tree using ITS sequence of fungal strains isolated from the guts of dung beetles *P. femoralis* and *A. convexus* and dung beetle larvae (*E. intermedius*). Only branches with more than 50% bootstrap support are shown. *S. stipitis* NRRL-Y-7124 was used as an outgroup. **Appendix 1.**

Table 3.1. Xylose fermentation, thermotolerate and tolerance to inhibitors of yeasts isolated from the gut of Heteronitis castelnaui (Rhino) and Kheper nigroanaeus Boheman (Impala) dung beetles.

Heteronitis castelnaui (Rhino)					**Kheper nigroanaeus Boheman (Impala)**				
Yeast isolates	**Strain name**	**Xylose**	**40 ºC**	**Acetic acid**	**Yeast isolates**	**Strain name**	**Xylose**	**40 ºC**	**Acetic acid**
Y1	*Trichosporon inkin*	-	-	-	Y114	*Malassezia globosa*	-	-	-
Y10	*Trichosporon cutaneum*	-	-	-	Y115	*Malassezia globosa*	-	-	-
Y11	*Trichosporon inkin*	-	-	-	Y116	*Cutaneotrichosporon debeurmannianum*	-	-	-
Y111	*Malassezia globosa*	-	-	-	Y117	*Trichosporon dermatis*	-	-	-
Y113	*Trichosporon debeurmannianum*	-	-	-	Y119	*Trichosporon asahii*	-	-	-
Y118	*Malassezia globosa*	-	-	-	Y12	*Trichosporon asahii*	-	-	-
Y121	*Cryptococcus saitoi*	-	-	-	Y120	*Malassezia globosa*	+	+	+
Y123	*Malassezia globosa*	-	-	-	Y122	*Malassezia globosa*	-	-	-
Y125	*Malassezia globosa*	-	-	-	Y124	*Candida tropicalis*	+	+	+
Y127	*Coniochaeta polymorpha*	-	-	-	Y126	*Malassezia globosa*	-	-	-
Y128	*Malassezia globosa*	+	-	-	Y131	*Trichosporon ovoides*	-	-	-
Y129	*Rhodotorula mucilaginosa*	-	-	-	Y14	*Trichosporon debeurmannianum*	-	-	-
Y13	*Trichosporon ovoides*	-	-	-	Y148	*Candida tropicalis*	-	-	-
Y130	*Trichosporon ovoides*	-	-	-	Y15	*Malassezia globosa*	-	-	-
Y132	*Trichosporon ovoides*	-	-	-	Y150	*Candida tropicalis*	+	+	+
Y133	*Trichosporon ovoides*	-	-	-	Y152	*Candida tropicalis*	+	+	+
Y135	*Trichosporon ovoides*	-	-	-	Y153	*Candida tropicalis*	-	-	-
Y136	*Trichosporon ovoides*	-	-	-	Y154	*Candida tropicalis*	+	+	+
Y137	*Malassezia globosa*	-	-	-	Y157	*Trichosporon ovoides*	-	-	-
Y138	*Trichosporon ovoides*	-	-	-	Y16	*Trichosporon asahii*	-	-	-
Y139	*Malassezia globosa*	-	-	-	Y161	*Candida tropicalis*	+	+	+
Y140	*Malassezia globosa*	-	-	-	Y163	*Candida tropicalis*	+	+	+
Y141	*Trichosporon ovoides*	-	-	-	Y164	*Candida tropicalis*	+	+	+
Y142	*Malassezia globosa*	-	-	-	Y165	*Candida tropicalis*	+	+	+
Y143	*Malassezia globosa*	-	-	-	Y20	*Candida tropicalis*	+	+	+
Y144	*Malassezia globosa*	-	-	-	Y231	*Trichosporon debeurmannianum*	-	-	-
Y145	*Malassezia globosa*	-	-	-	Y29	*Trichosporon debeurmannianum*	-	-	-
Y146	*Candida tropicalis*	-	-	-	Y30	*Trichosporon debeurmannianum*	-	-	-
Y147	*Candida tropicalis*	+	+	+	Y39	*Trichosporon asahii*	-	-	-
Y151	*Trichosporon ovoides*				Y4	*Candida tropicalis*	+	+	+
Y155	*Candida tropicalis*	+	+	+	Y40	*Candida ranongensis*	-	-	-
Y156	*Candida tropicalis*	+	+	+	Y48	*Trichosporon insectorum*	-	-	-
Y158	*Candida tropicalis*	+	+	+	Y49	*Malassezia globosa*	-	-	-
Y159	*Candida tropicalis*	+	+	+	Y5	*Trichosporon dermatis*	-	-	-
Y160	*Uncultured fungus*	-	-	-	Y51	*Trichosporon insectorum*	-	-	-
Y162	*Candida tropicalis*	+	+	+	Y54	*Trichosporon faecale*	-	-	-
Y17	*Trichosporon cutaneum*	-	-	-	Y6	*Trichosporon ovoides*	-	-	-
Y18	*Malassezia globosa*	+	+	+	Y61	*Candida tropicalis*	+	+	+
Y19	*Cryptococcus laurentii*	+	+	+	Y62	*Trichosporon ovoides*	+	+	+
Y2	*Trichosporon inkin*	-	-	-	Y63	*Meyerozyma guilliermondii*	-	-	-
Y22	*Trichosporon inkin*	-	-	-	Y64	*Malassezia globosa*	-	-	-
Y23	*Trichosporon inkin*	-	-	-	Y69	*Trichosporon inkin*	-	-	-
Y233	*Rhodotorula mucilaginosa*	-	-	-	Y7	*Candida tropicalis*	+	+	+
Y24	*Candida ranongensis*	-	-	-	Y94	*Cryptococcus saitoi*	-	-	-
Y26	*Trichosporon cutaneum*	-	-	-	Y97	*Trichosporon ovoides*	-	-	-

Y27	*Trichosporon cutaneum*	-	-	-
Y28	*Trichosporon inkin*	-	-	-
Y3	*Candida tropicalis*	+	+	+
Y31	*Candida ranongensis*	-	-	-
Y32	*Trichosporon inkin*	-	-	-
Y33	*Candida ranongensis*	-	-	-
Y34	*Trichosporon inkin*	-	-	-
Y35	*Trichosporon inkin*	-	-	-
Y36.1	*Trichosporon inkin*	-	-	-
Y36.2	*Trichosporon inkin*	-	-	-
Y38	*Debaryomyces hansenii*	-	-	-
Y41	*Candida ranongensis*	-	-	-
Y42	*Trichosporon inkin*	-	-	-
Y43	*Trichosporon cutaneum*	-	-	-
Y44	*Trichosporon cutaneum*	-	-	-
Y45	*Malassezia globosa*	-	-	-
Y46	*Trichosporon inkin*	-	-	-
Y47	*Malassezia globosa*	-	-	-
Y50	*Candida ranongensis*	-	-	-
Y52	*Trichosporon inkin*	-	-	-
Y55	*Trichosporon insectorum*	-	-	-
Y56	*Trichosporon inkin*	-	-	-
Y59	*Rhodotorula mucilaginosa*	-	-	-
Y60	*Trichosporon inkin*	-	-	-
Y65	*Malassezia globosa*	-	-	-
Y67	*Malassezia globosa*	-	-	-
Y70	*Trichosporon cutaneum*	-	-	-
Y74	*Candida parapsilosis*	-	-	-
Y75	*Malassezia globosa*	-	-	-
Y76	*Rhodotorula mucilaginosa*	-	-	-
Y78	*Malassezia globosa*	-	-	-
Y8	*Candida ranongensis*	+	+	+
Y81	*Rhodotorula mucilaginosa*	-	-	-
Y86	*Uncultured fungus*	-	-	-
Y9	*Candida tropicalis*	+	+	+
Y91	*Trichosporon ovoides*	-	-	-
Y92	*Trichosporon ovoides*	-	-	-
Y93	*Rhodotorula mucilaginosa*	-	-	-
Y95	*Trichosporon ovoides*	-	-	-
Y96	*Trichosporon ovoides*	-	-	-
Y98	*Trichosporon ovoides*	-	-	-
Y99	*Trichosporon ovoides*	-	-	-