ISOLATION AND CHARACTERIZATION OF LIGNOCELLULOSE BIODEGRADING ENZYMES FROM MARINE WOODBORERS: POTENTIAL IN BIOETHANOL PRODUCTION

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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LIST OF ABBREVIATIONS AND SYMBOLS

CBH	Cellobiohydrolase
CBP	Consolidated bioprocessing
CE	Capillary electrophoresis
CMC	Carboxymethyl cellulose
COI	Mitochondrial Cytochrome C oxidase subunit I gene
CTAB	Cetyltrimethylammonium bromide
DEAE	Diethylaminoethyl
DNS	Dinitrosalicylic acid
DP	Degree of polymerization of cellulose
F_a	Fraction of β-glucosidic bond accessible to cellulose
FGB	First-generation bioethanol
FID	Flame ionization detector
F_{RE}	Fraction of the reducing end to all anhydroglucose units of cellulose, 1/DP
GH	Glycoside hydrolase
HPLC	High performance liquid chromatography
IEF	Iso-electric focusing

Lac	Laccase
LiP	Lignin peroxidase
Mn	Manganese
MnP	Manganese dependent peroxidase
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethelene glycol
PMSF	Phenylmethylsulfonylfluoride
p-NPG	p-Nitrophenyl β-D-glucopyranose
SCP	Single-cell proteins
SDA	Sabouraud Dextrose Agar
SDS-PAGE	Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis
SGB	Second-generation bioethanol
SmF	Submerged fermentation
SSF	Solid-state fermentation
TKP	Tamarind kernel polysaccharide
TLC	Thin layer chromatography

ABSTRACT

Marine woodborers are members of phylum Mollusca, class bivalvia, families Teredinidae and Pholadidae; and phylum Arthropoda, class crustacea, family Sphaeromatidae. They have a close association with tropical mangrove habitats where they consume lignocellulose and play a role in nutrient cycling. They represent a rich source of lignocellulolytic enzymes that can be harnessed for conversion of biomass into simple sugars and other monomers for a variety of uses including bioethanol production.

Enzymatic degradation of lignocellulose has emerged as the most prominent technology for conversion of biomass into monomer sugars for subsequent fermentation into bioethanol. This is an ideal approach for degrading cellulose because of its mild reaction conditions (pH between 4.8–5.0 and temperature between 45–50 °C), it does not present corrosion problems in the reactors and results in high sugar yields.

This study isolated lignocellulolytic enzymes from marine woodborers in the Kenyan coast, investigated their potential in bioethanol production and characterized the enzyme with highest activity.

Three species of woodborers from marine mangrove plants were identified and relations between them and the host mangrove plants (*Avicennia, Sonneratia, Rhizophora*) described. Marine woodborers *Dicyathifer mannii* (Wright, 1866), *Sphaeroma terebrans* (Bate, 1866) and *Cirolana sp.* occur on submerged parts of roots (proproots, pneumatophores), stems and branches. *D. mannii* was found mostly on *Rhizophora* but also on *Sonneratia*, whereas *S. terebrans* and *Cirolana sp.* were found exclusively on *Avicennia*.

Crude gut extracts were obtained from *D. mannii* and *S. terebrans* (*Cirolana sp.* were not obtained in enough numbers for crude gut extraction). These were tested for lignocellulolytic activity. *D. mannii* crude extracts showed an appreciable endoglucanase (CMCase) activity of up to 50.7 ± 1.51 U/ml , xylanase activity of 35.52 ± 1.54 U/ml and Lip activity of up to 34.65 ± 0.12 U/L (1 U represents 1 micromol of glucose released min^{-1}). *D. mannii* is implicated as a source of these enzymes for industrial use.

To determine the bacterial and fungal diversity within the woodborers' digestive tracts, bacteria and fungi from the digestive tracts of *D. mannii*, *S. terebrans* and a *Cirolana sp.* were cultured and investigated. The bacteria and fungi were identified by sequencing the fragments of 16S rRNA and ITS gene respectively, with subsequent phylogenetic analysis. Four strains, *Lysinibacillus boronitolerans* (from *D. mannii* and *S. terebrans*), *L. fusiformis* (from *S. terebrans* and *Cirolana sp.*), *L. sphaericus* and *L. xylanilyticus* (both from *Cirolana sp.*) had similarity to known 16S rRNA sequences of 98–99 %.

Various strains of Ascomycetes fungi were identified from the digestive tracts of the woodborers. *Aspergillus niger* was isolated from the digestive tracts of both *D.mannii* and *S. terebrans*. In addition, *Neosartorya fischeri* and *A. fumigatus* were present in *D. mannii* whereas *Botryotinia fuckeliana* was found in *S. terebrans* digestive tract. *A. costaricaensis* and *A. fumigatus* were present in *Cirolana sp*. while *Penicillium sp*. was isolated from *D.manni* and *Cirolana sp.* digestive tract. The fungi had similarity to known ITS sequences of 95–100 %. Existence of bacterial and fungal groupings symbiotically associated with woodborers gut is proposed.

Pure bacterial and fungal isolates from each of the woodborers as well as mixed cultures for each woodborer were induced to produce lignocellulolytic enzymes. Substrates used for induction were carboxymethylcellulose sodium salt (CMC), Whatson No. 1 filter paper (FP), beechwood xylan, *Rhizophora* wood dust, D (+)-cellobiose and avicel cellulose. While there was generally low ligninolytic activity in both bacterial and fungal isolates, cellulolytic and hemicellulolytic activity was significantly high in both pure bacterial and fungal isolates as well as in mixed cultures. The highest bacterial enzyme activity was β-glucosidase (94.55 U/ml) shown by *L. boronitolerans* from *S. terebrans* cultured in a medium containing avicel cellulose as a carbon source. In contrast, xylanase activity was highly exhibited (up to 91.7 U/ml) by *L. xylanilyticus* from *Cirolana sp.* in medium containing cellobiose. The highest fungal activity was β-glucosidase (54.77 U/ml) shown by *A. niger* from *D. mannii* gut in a medium with mixed sudstrates. Wood, FP and CMC did not sufficiently induce production of β-glucosidase by the fungal isolates. CMCase production was significantly induced by xylan beechwood substrate.

Since *D. mannii* had shown to have the most lignocellulolytic efficacious extracts, the ability of the culture filtrate of its gut microbial community to biodegrade wheat straw into fermentable sugars for ethanol production was investigated. 24 hours fermentations by 0.3 % *Saccharomyces cerevisiae* of 3 % wheat straw degradation reaction mixture with *D. mannii* gut microbial filtrate (previously incubated for 1 hour at 50 ^{0}C in 0.1M sodium acetate buffer, pH 5.0) yielded 0.98 mg/100ml supernatant.

The highest *D. mannii* microbial community lignocellulolytic activity was xylanase. Consequently, xylanase from the culture filtrate of *D.mannii* gut microbial community was isolated and purified. The purified enzyme showed a single band on SDS polyacrylamide gel electrophoresis (SDS-PAGE) with an apparent molecular weight

of ≈ 20 kDa. The enzyme was moderately thermostable with optimum activity at 50 °C and pH 5.0. It had a high affinity for xylan beechwood with K_m and V_{max} values of 0.4 % (w/v) and 128.2 μm ml^{-1} min^{-1}, respectively. This is the first report on production, purification and characterization of xylanase from *D.mannii* gut microbial community.

Key words: Woodborer, lignocellulose, biodegradation, lignocellulolytic enzyme, and bioethanol.

CHAPTER ONE

1.0 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Marine woodborers are pests that voraciously consume lignocelluloses and pose a problem to timber constructions in the sea. These organisms represent a rich source of lignocellulolytic enzymes that can be harnessed for conversion of biomass into simple sugars and other monomers.

Lignocellulose is the major structural component of plants. It is composed of a mixture of carbohydrate polymers (cellulose and hemicellulose) tightly bound to lignin, a complex polymer of phenylpropanoid molecules. Biodegradation of lignin is achieved by enzymes lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and laccase (Lac) or monophenol oxidase. These liberate polysaccharides cellulose and hemicellulose. On the other hand, hydrolysis of cellulose is achieved by an enzyme complex containing at least three types of glucanases, namely, endoglucanase (endo-1-4-β-D-glucanase), exoglucanase (1,4-β-D-glucan-cellobiohydrolase), and β-D-glucosidase or cellobiase (β-D-glucoside glucanohydrolase). Hydrolysis of xylan, the chief type of hemicellulose, is achieved by endo-1-4- β- xylanase and β-xylosidase among other such enzyme complex. These enzymes are mainly produced by microbes (fungi, yeast and bacteria), marine algae and invertebrates (protozoans, crustaceans, insects and snails). Lignin modifying enzymes find applications in biobleaching of pulp and decolouration of textile dyes, whereas cellulases and hemicellulases find applications in animal feed, manufacture of bread, bioethanol and xylitol production among other uses.

Lately, there has been significant interest in the enzymes responsible for lignocellulose degradation in terms of understanding their ecological role as well as biotechnological potential (Reddy, 1995). Their lignocellulose biodegradation activity enables conversion of biomass into simple sugars, which can be fermented to release bioethanol. In addition, the enzymes can be utilised in animal nutrition by breaking down of the bonds within indigestible lignin in feed thereby increasing bioavailability. The enzymes have also been reported to depolymerise lignin as well as decolorize textile dyes, and therefore can be used in biobleaching of pulp and decolouration of dyes (Jimenez *et al*., 1997; Beauchemin *et al*., 2003, Boer *et al*., 2004).

There are three types of marine woodborers along the Indian coasts, namely, Shipworms, Piddocks and Pill Bugs (Santhakumaran, 1996). Shipworms (Family Teredinidae) are molluscs class bivalvia, whose classification has for a long time been entirely dependent on shells and pallets. Turner (1966a, 1966b) brought out the importance of the anatomy of shipworms in the systematics of this group, particularly in generic classification. Characters of systematics value for species identification are nature of the shell valves, tubes (internal lining of the burrows), pallets (a pair of calcareous organ situated at the posterior end of the animal which is used to plug the entry hole during adverse conditions or when the borer is disturbed) and siphons. Of these, the morphological variations exhibited by the pallets are remarkable and almost all the species can be identified from their pallets (Turner, 1966a, 1966b, 1971).

Piddocks are also molluscs Family Pholadidae (class bivalvia) that are classified based on the shape of shell valves, nature and arrangement of accessory plates (protoplax,

mesoplax, metaplax and hypoplax), presence or absence of callum in the adult stage, presence or absence of apophysis, and the morphology of the siphons. In some members (subfamily Martesiinae), the young and adult are different morphologically, the former having an anteriorly beaked and widely gaping shell and the latter having this gape closed by a calcareous deposit, the callum (Turner, 1971). The nature of the chitinous lamellae on the posterior slope of the shell, when present, also helps in species separation.

Pill Bugs are arthropods in the class crustacea; order isopoda and family Sphaeromatidae whose characters of taxonomic value in members of species are the number and disposition of large tubercles on the dorsal posterior part of body, posterior part of the telson and shape of the epistome. Of these, the arrangement of the large tubercles is strikingly different and shows variations characteristic of each species (Pillai, 1961).

Burrows produced by each of the three types of borers are also characteristic of its occupant (Figure 1.1). Shipworms bore deep into the wood making long tunnels almost parallel to the grain whereas burrows of pholads are pear-shaped, superficial and nearly at right angle to the grain. Pill bugs produce cylindrical burrows on the wood surface at right angle to the grain. Sometimes the juveniles start working from the main parent tunnel leaving side branches.

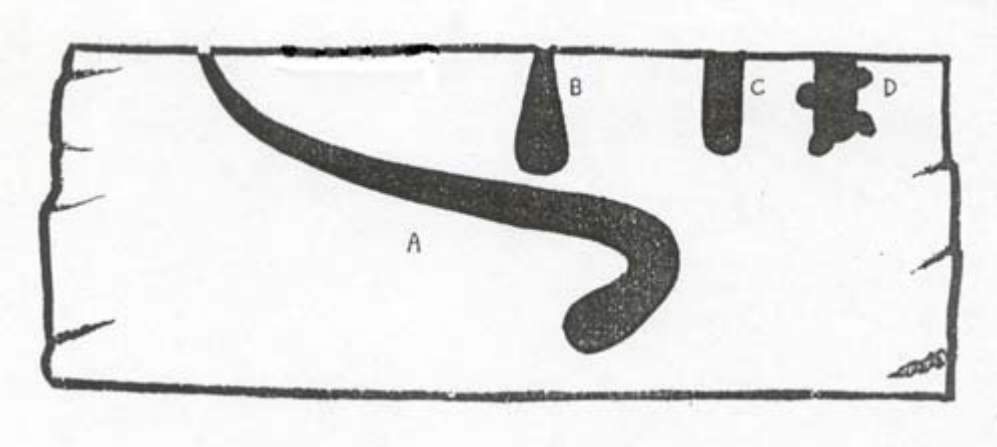


Figure 1.1 Burrows produced by marine woodborers: (A) Shipworms; (B) Piddocks; (C) Pill bugs; (D) Pill bugs with juveniles (Santhakumaran, 1996).

This study identified marine woodborer in the Kenyan Coast, isolated lignocellulolytic enzymes from the woodborers, investigated their potential in bioethanol production and characterized the enzyme with highest activity.

1.1.1 Rationale

It has been observed that marine woodborers voraciously consume lignocellulose. Thus, these organisms represent a rich source of lignocellulolytic enzymes that can be used in bioethanol production. Lignocellulolytic enzymes can convert biomass into simple sugars that are then fermented to release ethanol (Isaacs, 1984). Besides, they can also be harnessed for use in animal nutrition. This is by using the enzymes to break down bonds within indigestible lignin thereby increasing bioavailability (Beauchemin *et al*., 2003). In addition, the enzymes can be used in biobleaching of pulp and decolourization of textile dyes as they have been reported to depolymerise lignin as well as decolorize synthetic dyes (Jimenez *et al*., 1997; Boer *et al*., 2004).

Lignocellulolytic activity has been investigated in many organisms including bacteria, fungi and termites (Karnchanatat *et al*., 2008; Sahay *et al*., 2008; Tokuda *et al*., 2009; Desai *et al*., 2011; Bholay *et al*., 2012). However, little is known about the lignocellulolytic enzymes from marine woodborers.

The role played by woodborers along the Kenyan coast has not been studied, especially the biodeterioration of vegetation. Therefore, understanding the marine woodborers' biodiversity and their host preference in Kenya will be also be useful in restoration of mangrove ecosystem, environmental conservation and management.

First-generation bioethanol (FGB) is converted from edible sources such as corn and sugarcane. These require agricultural intensification to cater for food as well as fuel sources. Lignocellulosic biomass include raw material from hardwood, softwood, grasses, agricultural residues, newsprint, office paper, municipal solid wastes among others (Lee, 1997). It is by far the most abundant material in nature and it is renewable. Production of bioethanol from these materials constitutes the second-generation bioethanol (SGB). Therefore, SGB offers great promise to replace fossil fuels without causing the feud of food-fuel supply as they are derived from non-edible sources such as lignocellulose biomass (Sun and Cheng, 2002; Tan *et al*., 2008).

Bioethanol is a bio-fuel that can act as a substitute to use of firewood and charcoal at household levels. Its production from readily available biomass waste will reduce destruction of forests that act as carbon dioxide sinks. Carbon dioxide contributes greatly to greenhouse gases that lead to global warming and subsequent climate change.

Climate change and diminishing oil supplies are issues of acute concern for most nations of the world. This is why the European Commission has called for expansion

of the supply of renewable energies and alternative fuels in order to meet the carbon-dioxide reduction targets specified in the Kyoto Protocol (Kim *et al*., 2010).

The aforementioned justified the need to harness lignocellulolytic enzymes from marine woodborers, which can be used in the conversion of biomass into simple sugars that can be fermented to release bioethanol (Isaacs, 1984).

1.1.2 Hypothesis

The gut of marine woodborers contains lignocellulolytic enzymes capable of digesting lignocellulose.

1.1.3 Objectives

1. To isolate lignocellulolytic enzymes from marine woodborers.
2. To investigate potential of the lignocellulolytic enzyme(s) in bioethanol production.
3. To characterize the lignocellulolytic enzyme with the highest activity.

1.2 Literature Review

Marine woodborers are members of two invertebrate Phyla. These are phylum Mollusca, class bivalvia, families Teredinidae and Pholadidae; and phylum Arthropoda, class crustacean, order Isopoda, families Sphaeromatidae and Cirolanidae.

1.2.1 Class Bivalvia

Bivalves have evolved from shallow burrowers in soft substrata (protobranchia) to filter feeding lamellibranchiata. From some group of protobranchia evolved yet another subclass of bivalves, the septibranchia that are carnivorous and scavengers.

Important features of bivalvia are shells, mantle and foot. A typical bivalve has a shell consisting of two similar more or less oval, usually convex valves, which are attached and articulate dorsally with each other. It also has a mantle, which greatly overhang the body, forming a sheet of tissue lying beneath the valves. The edge of the mantle bears three folds, namely, inner (largest) that contains radial and circular muscles, middle (sensory in function) and outer (secretion of the shell) that lay the first calcareous layer. The entire mantle surface secretes the remaining calcareous portion.

Digestion in protobranchia is extracellular in the stomach, and absorption occurs in the digestive glands. Lamellibranchiata use finer particles as food in filter feeding on gills, extracellular digestion in the stomach, as well as within the digestive gland. Carnivorous septibranchia digestion occurs in the muscular stomach lined with chitin that acts as a crushing gizzard. Proteases from the digestive gland initiate extracellular digestion in the stomach. The digested material is conveyed into the ducts of the digestive diverticula for further digestion (intracellular).

All boring bivalves (peat, clay, sandstone, coral, coralline rock and wood) begin excavation after the larva settles and slowly enlarges and deepens the burrow with growth. The animal is forever locked within its burrow and only the siphons project to the small surface opening. Some boring bivalves hold to the side of the burrow by byssal threads (tough protein threads secreted by the foot). Some rotate within the burrow (i.e. change position) and as a result the burrow section is round. Others remain attached in one place, and the burrow tube takes the shape of the shell. Much of the sediment produced by the drilling is taken into the mantle cavity and ejected with pseudofaeces through inhalant siphon (Barnes, 1987).

Many boring bivalves inhabit wood and the most specialized woodborers are the shipworms, members of the family Teredinidae. They bore deeply into the wood using two small anteriorly placed roughened shell valves that are small in relation to the rest of the body. The valves are rotated with respect to the foot, carving a circular burrow, which is then lined by calcareous secretion of the mantle. It ingests a certain amount of wood, but it still possesses the filter feeding mechanism found in other bivalves (George and George, 1979). Most adult teredinids are obligate wood consumers with life history strategies that vary considerably between species. They attack mangrove wood and timber swept into the sea by rivers, and they play an important ecological role in the reduction of sea-borne wood. They typically spend their entire lives in a tunnel in a single piece of wood. They eventually cause the wood in which they live and on which they feed to disintegrate by their tunnelling (Cragg *et al*., 2009).

The body of shipworm is greatly elongated and cylindrical. The shell is reduced to two small, anterior valves. Cutting of the wood is accomplished by opening and rocking motions of the valves while the anterior end of the body is attached to the burrow by the small foot. The mantle enclosing the greater part of the body behind the valve produces a calcareous lining within the tunnel. Calcareous pallets plug the long delicate siphon open at the surface of the wood, and the burrow entrance when the siphon is retracted. The burrow increases with the growth of the shipworm that fills it, and may reach a length of 18 cm to 2 meters depending on the species. The life span is one to several years also depending on the species.

Shipworms use the excavated sawdust for food. The stomach is provided with a caecum for sawdust storage, and a section of digestive gland is specialized for handling wood particles. Wood-boring bivalves appear to lack highly developed microbial communities within their guts (Liu and Walden, 1970). Symbiotic bacteria

housed within a special organ that opens into the oesophagus provide cellulose digestion and also, by fixing nitrogen, compensate for the low-protein diet. (Waterbury *et al*., 1983; Distel, 2003; Lechene *et al*., 2007). The other boring bivalves include Pholadidae (Barnes, 1987; Sing and Sasekumar, 1994).

The majority of bivalves including shipworms are dioecious with two gonads that encompass the intestinal loop. They are simple gonadoducts as there is no copulation. In lamellibranchiata, the gonadoducts open into the mantle cavity. Gametes are shed into mantle cavity where fertilization and brooding of the eggs occur. The development of the free-swimming trochophore, succeeded by a veliger larva, is typical in marine bivalves. The shipworm larvae settle on wood. *Dicyathifer mannii* (Wright) belongs to family Teredinidae. It is a wood-burrowing bivalve with long worm-like body in calcareous tube secreted inside the burrow.

1.2.2 Order Isopoda

A striking characteristic of isopoda is a dorso-ventrally flattened body. The head is usually shield shaped and the terga of the thoracic and abdominal segments tend to project laterally. A carapace is absent, although the first one and two thoracic segments are fused with the head. The abdominal segments may be distinct or fused to varying degree. The last abdominal segment is almost always fused with the telson. The abdomen is usually the same width as thorax, so that the two regions may not be clearly demarcated dorsally. The first antennae are short and uniramous, or vestigial. They have sessile compound eyes. The first pair of thoracic appendages is modified to form maxillipeds; the remaining seven pairs are legs usually adapted for crawling. Isopod pleopods are used for gas exchange.

Most isopods are 5-10 mm in length. The colouration is usually drab, shades of grey being the most common. In many species, chromatophores adapt the body colouration to the background. Most isopods are adapted to crawling, others burrowing. Limnoria species tunnel through wood, *Sphaeroma terebrans* bore into prop roots of mangroves (Barnes, 1987).

Isopoda have evolved ability to roll up in a ball. Most of them are scavengers and omnivores, but some tend towards herbivorous diet. Wood-boring marine isopods feed on wood, and their hepatopancreatic secretions include cellulase. At settling, the wood-boring species of Limnoria are attracted to the fungi in the wood (Geyer and Becker, 1980). The fungi add nitrogen to their largely cellulose diet. In the woodlice, cellulose digestion results from bacteria, and the hindgut play a major role in the digestive process (Hassall and Jennings, 1975).

The blood in isopoda contains haemocyanin and the excretory organs are maxillary glands. Their gonads are paired and separate. After copulation, the eggs are fertilized in the oviduct. The eggs are usually brooded in a marsupium composed of sternal processes of the abdominal segments, the oostegites, in which the eggs develop to maturation and hatch into a post-larval stage that leaves the marsupium. Most isopods produce one to two broods each year and live for 2-3 years (Barrati *et al*., 2011; Baratti *et al*., 2005; Barnes, 1987).

Males leave the burrows after copulation, while females remain inside the burrow even after the offspring are released from the brood pouch. The mother spends most of the time in the hole, blocking the entrance with her telson and creating a flow of water with her pleopods to oxygenate the environment and provide a food supply (Barrati *et al*., 2011).

1.2.3 Lignocellulose

Lignocellulose is a major structural component of plants. It's a complex substrate composed of a mixture of carbohydrate polymers (cellulose and hemicellulose) and lignin. The carbohydrate polymers are tightly bound to lignin mainly by hydrogen bonds but also by some covalent bonds. Lignin is a complex polymer of phenylpropanoid molecules. The biological process degrading lignocellulose requires delignification to liberate cellulose and hemicellulose from their complex with lignin and depolymerisation of the carbohydrate polymers to produce free sugars. The removal of lignin and releasing fermentable sugars requires pretreatment of lignin followed by enzymatic or acidic hydrolysis of the carbohydrates. In the past decade, most of the research has been focused on the development of this method and significant progress has been made by thermal, mechanical, and chemical pretreatment and enzymatic hydrolysis (Tengerdy and Szakacs, 2003).

Lignin-degrading enzymes are oxidoreductive enzymes. They play an important role in degradation and transformation of polymeric substances, which have practical application in biobleaching of pulp, decolouration of textile dyes and bioremediation of polluted environment (Castillo *et al.,* 1997) among other uses. Lignin peroxidases are useful in the treatment of colored industrial effluents and other xenobiotics as it has bioremediation potential to decolourize the effluents (Buzzini *et al.,* 2006). During hydrolysis of lignocellulosic biomass, the major constituents in enzyme hydrolysates are glucose and xylose released from cellulose and xylan, respectively. Cellulases and xylanases find applications in animal feed, manufacture of bread, ethanol and xylitol production among other uses.

Cellulose, the major component of plant cell wall, is a linear polysaccharide composed of 30-15,000 β -D-glucopyranosyl units linked by β -1,4-glucosidic bonds (Figure

1.2). Cellulose is arranged in fibres that have a fully extended flat conformation and are tightly packed into microfibrils by hydrogen bonds and van der Waals interactions (Parthasarathi *et al*., 2011; Somerville *et al*., 2004). It is reported to consist of 24 to 36 chains based on scattering data (Fernandes *et al*., 2011) and information about the cellulose synthase (Endler and Persson, 2011), respectively. These fibres are also known as microcrystalline cellulose and they are interrupted by short amorphous regions (Kolpak and Blackwell, 1976). These crystalline microfibrils are non-soluble and enzymatic saccharification is challenging. Consecutive sugars along chains in crystalline cellulose are rotated by 180 degrees, meaning that the disaccharide (cellobiose) is the repeating unit. In nature crystalline cellulose is found as parallel chains in the form of α and β, where β is the predominant form in plants. While its recalcitrance to enzymatic degradation may pose problems, one big advantage of cellulose is its homogeneity. Complete depolymerization of cellulose yields just one product, glucose.

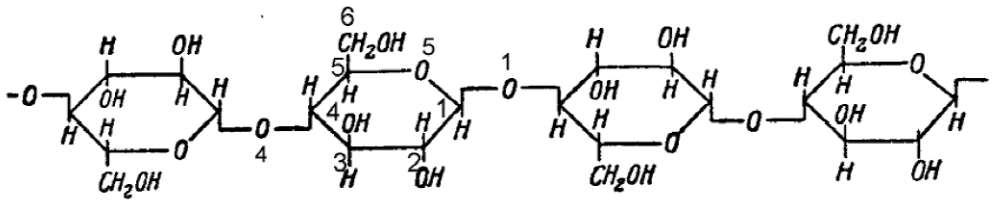


Figure 1.2. Chemical constitution of cellulose as a linear chain of 1-4-linked -D-glucopyranose units and numbering of carbon and oxygen atoms (Haworth, 1932)

The production of cellulolytic enzymes occurs almost exclusively in microorganisms, bacteria and fungi. Cellulolytic enzymes are also found in plants where they participate in leaf abscission, ripening of fruits, and cell wall growth (Loopstra et al.,

1998). Few reports can also be found on cellulolytic enzymes in higher eukaryotes e.g. in termites (Watanabe et al., 1997). To get reduced carbon compounds required for energy production, microorganisms produce cellulolytic enzymes to degrade the insoluble cellulose into soluble oligomers. These oligomers can be either directly taken up and metabolized or can be degraded to glucose and then taken up. Cellulolytic enzymes for industrial purposes most often come from filamentous fungi such as *Aspergillus nige*r, *Humicola insolens*, and *Trichoderma reesei*. Enzymes from *T. reesei* are primarily used in cellulose-to-ethanol pilot plants.

Xylan is the major constituent of hemicellulose found in the cell walls of plants and some green and red algae. It is next to cellulose the most abundant polysaccharide present in the plant cell wall. It is a heteropolymer consisting of a backbone of β-1,4- linked D-xylose residues which can be modified by various substituents: 1,2-linked α-D-glucuronic acid or 4-O- methyl-α-D-glucuronic acid residues can be present, as well as 1,2- and 1,3-linked α-L- arabinose residues. In some cases these L-arabinose residues are esterified with ferulic and *p-coumaric* acid, enabling cross-linking of the xylan to the lignin-matrix (Biely, 1985; Sunna and Antranikian, 1997). Depending on the source of the plant material, the D-xylose residues in the backbone can be modified by acetylation at the C-2 or C-3 position (Wilkie, 1979; Joseleau *et al*., 1992). Rice bran xylan is rich in arabinose whereas birchwood xylan possesses a significant amount of glucuronate and low arabinose content (Kormelink and Voragen, 1993). Acetate groups are not present in softwood xylan (Gregory *et al*., 1998).

Examples xylans include L-arabino-D-xylan (wheatflour), L-arabino-D-glucurono-D-xylan (grass) and D-glucurono-D-xylan (wood). Hemicelluloses are present in all layers of the plant cell wall but are concentrated mainly in the primary and secondary

layers where they occur closely associated with cellulose and lignin. Therefore unless the hemiceluloses can be properly utilized, biomass conversion based on cellulose utilization alone has little chance of becoming economically attractive due not only to the lost revenue from hemiceluloses but also to the added waste disposal costs.

The more common type of xylan is a (1→4)-β-D-xylan, a linear polymer of β-D-xylopyranose residues linked by (1→4) glycosidic bonds. This polysaccharide backbone is often decorated by additional sugars, forming complex polymers such as arabinoxylan and glucuronoxylan (Figure 1.3). Arabinoxylans have been identified in wheat, rye, barley, oat, rice and sorghum as well as in some other plants (pangola grass, bamboo shoots and rye grass). Glucuronoxylans and glucuronoarabinoxylans are located mainly in the secondary wall and function as an adhesive by forming covalent and non-covalent bonds with lignin, cellulose and other polymers essential to the integrity of the cell wall.

The known xylanolytic fungi are mostly ascomycetes. Particular attention has been granted to the enzymes produced by *Aspergilli* (de Vries and Visser, 2001) and *Trichoderma* (Wong and Saddler, 1992), while the xylanolytic system of *Penicillia,* also a genus with a large number of species producing xylanases, has not been reviewed.

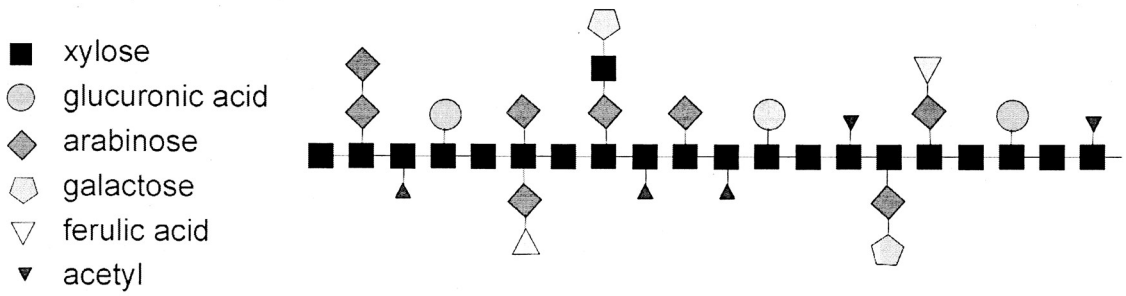


Figure 1.3. Schematic presentation of xylan (de Vries and Visser, 2001)

1.2.3.1 Biological delignification process

Lignin (Figure 1.4) is a complex, variable, hydrophobic, cross-linked, three-dimensional aromatic polymer of *p*-hydroxyphenylpropanoid units connected by C–C and C–O–C links. Biosynthetically, lignin arises from three precursor alcohols: *p*-hydroxycinnamyl (coumaryl) alcohol, which gives rise to *p*-hydroxyphenyl units in the polymer; 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol, the guaiacyl units; and 3, 5- dimethoxy-4-hydroxycinnamyl (sinapyl) alcohol, the syringyl units. Co-polymerization of these alcohols, as well as free radicals, produce the heterogeneous, optically inactive, cross-linked, and highly polydisperse polymer. There are over 10 interphenylpropane linkage types, including four that predominate (Kirk, 1987). The major components of lignocellulose and their biodegrading fungal enzymes are shown in Table 1.1.

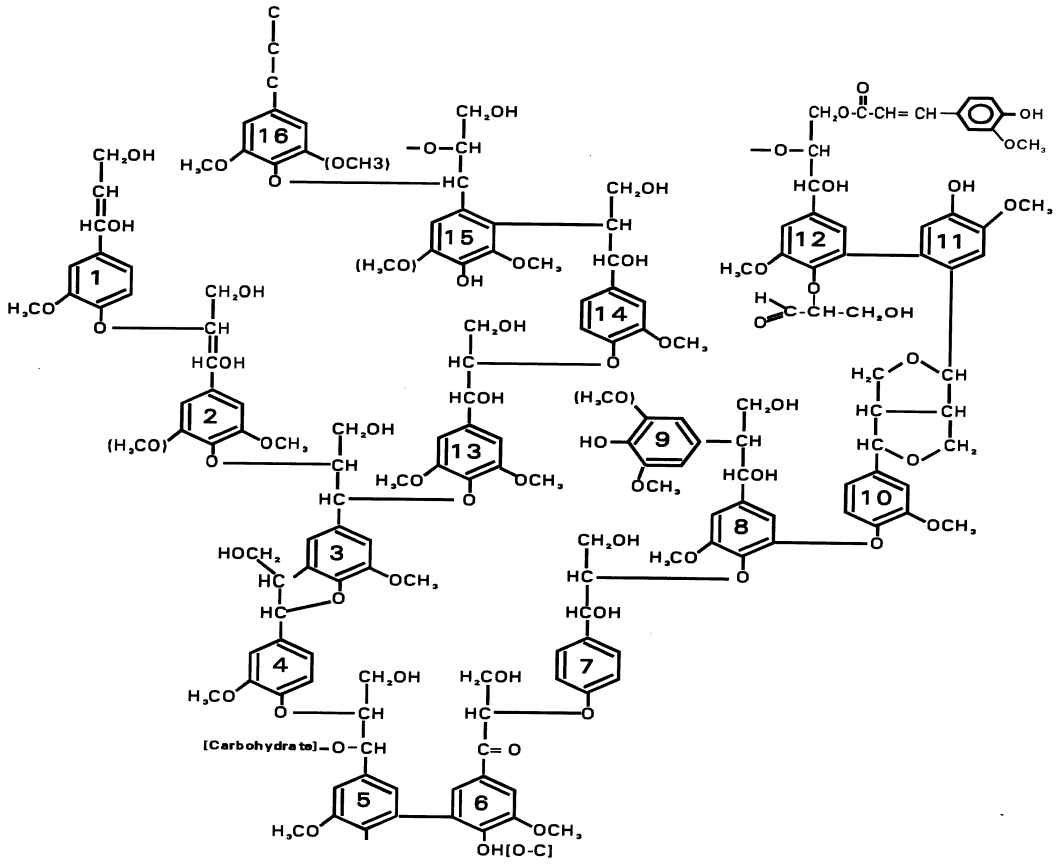


Figure 1.4. Schematic structural formula for lignin illustrating major interunit linkages and other features (Lee, 1997).

In the polymerization process, secondary reactions lead to cross-linking between lignin, cellulose and hemicelluloses. Lignin is extremely resistant to chemical and enzymatic degradation. Fungi achieve biological degradation, most efficiently by white-rot basidiomycetes, but also by certain actinomycetes. Lignin degradation by these organisms is a secondary metabolic process, occurring under low levels of nutrient nitrogen and requiring the presence of a carbon source such as glucose or cellulose. An extracellular lignin peroxidase (LiP), in the presence of H_2O_2, can degrade lignin by bringing about oxidative cleavage of the C–C backbone, oxidation and hydroxylation of benzylic methylene groups, oxidation of phenols and benzyl

alcohols, etc. Reaction of the LiP with H_2O_2 generates a high-redox-potential porphyrin cation radical (oxferryl complex) which can extract a single electron from an aromatic ring in the lignin substrate to generate aromatic cationic radicals; this is followed by a variety of spontaneous degradative reactions via radical and cation intermediates.

Other enzymes implicated in aerobic lignin degradation include Mn-dependent peroxidase (MnP) and laccase (monophenol oxidase). MnP oxidizes Mn^{2+} to Mn^{3+} that acts as a unique redox couple, and in turn oxidizes phenolic substrates to phenoxy radicals as laccase does, which undergo subsequent reactions to yield final products (Zouari-Mechichi *et al*., 2006; Lopez *et al*., 2007). Laccases catalyze the one-electron oxidation of a vast amount of phenolic substrates. Molecular oxygen serves as the terminal electron acceptor and is thus reduced to two molecules of water (Figure 1.5).

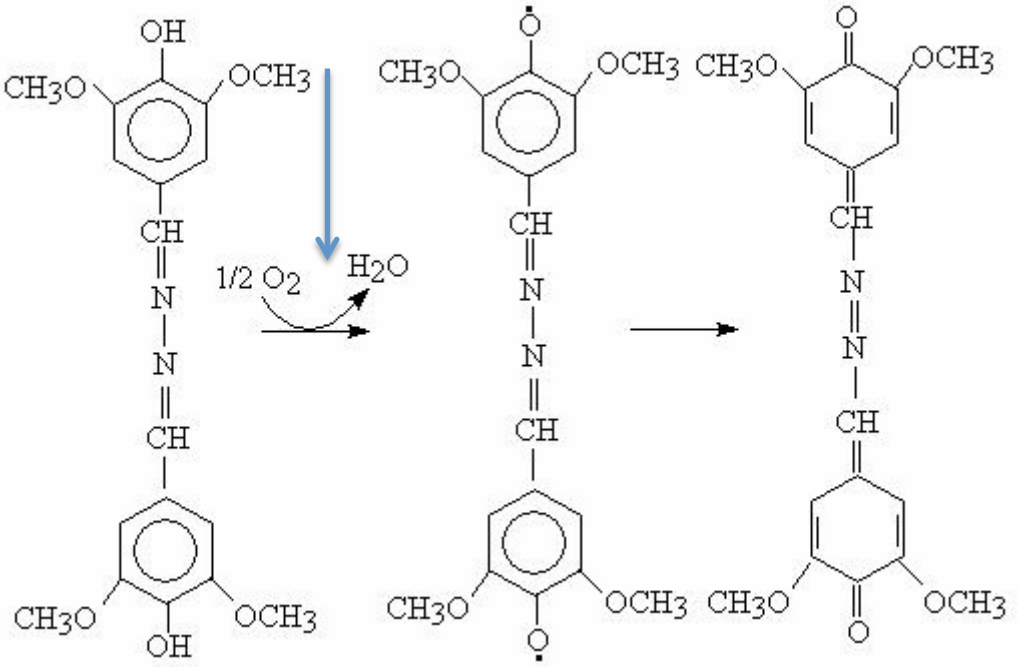


Figure 1.5. Catalyzed oxidation of syringaldazine by laccase (↓) to the corresponding quinone

Galliano *et al*. (1991) reported the enhancement in lignin solubilization with added glucose oxidase, resulting presumably from preventing the repolymerization of the radicals formed by the action of lignin-degrading enzymes. Effect of alcohol on LiP, MnP, and laccase was also investigated (Niku-Paavola *et al*., 1990). Activities of LiP and MnP were increased by veratryl alcohol while both benzyl alcohol and veratric acid improved laccase production. The heat stability of LiP was studied by Barclay *et al*. (1990) and the results showed that there was no significant loss in LiP activity at temperature of 37 °C, or below but activity was rapidly decreased at temperature between 50 and 60 °C.

Table 1.1. Components of lignocellulose and their biodegrading fungal enzymes

	Cellulose	Hemicelluloses	Lignin
% Wood	40-50	20-40	20-35
Monomer	D-anhydro-glucopyranose	Xylose Mannose Plus other pentoses and hexoses	Coniferyl alcohol p-coumaryl alcohol sinapyl alcohol
Polymeric structure	β-*O*-4 linked linear chains	β-*O*-4 linked linear chains, with substituted side chains	Dehydrogenative polymerization to an amorphous polymer
Major enzyme involved in degradation	Endoglucanase (E.C.3.2.1.4) Cellobiohydrolase (E.C.3.2.1.91) β-glucosidase (E.C.3.2.1.21)	Endoxylanase β-xylosidase (and other hydrolases)	Lignin peroxidase (E.C.1.11.1.7) Mn dependent peroxidase (E.C.1.11.7) Laccase (E.C.1.10.3.2)

Adapted from Pointing (1999).

1.2.4 Lignocellulolytic Enzymes

1.2.4.1. Ligninolytic enzymes

Lignin has been reported to be degradable by several ligninase fungal enzymes. These are lignin peroxidase, Mn-dependent peroxidase, and laccase or mono-phenol oxidase (Zouari-Mechichi *et al*., 2006; Lopez *et al*., 2007).

1.2.4.2. Cellulolytic enzymes

Cellulase enzymes are produced by a number of microbes, including fungi, yeast, and bacteria. The enzymatic hydrolysis of cellulose takes place under the action of a cellulolytic enzyme complex containing at least three types of glucanase (Figure 1.6). These are endoglucanase (endo-1-4-β-D-glucanase), exoglucanase (1,4-β-D-glucan-cellobiohydrolase), and β-D-glucosidase or cellobiase (β-D-glucoside glucanohydrolase) (Wood *et al*., 1989; Watanabe and Tokuda, 2010). There is possibility of mutants with both cellulase and cellobiase activity (Romero *et al*., 1999; Brijwan *et al*., 2010). Enzymatic properties and primary structures of many cellulases have been investigated and cellulase genes from widely differing origins have been cloned and sequenced (Hamada *et al*., 1999). However, little is known about the lignocellulolytic enzymes from marine woodborers.

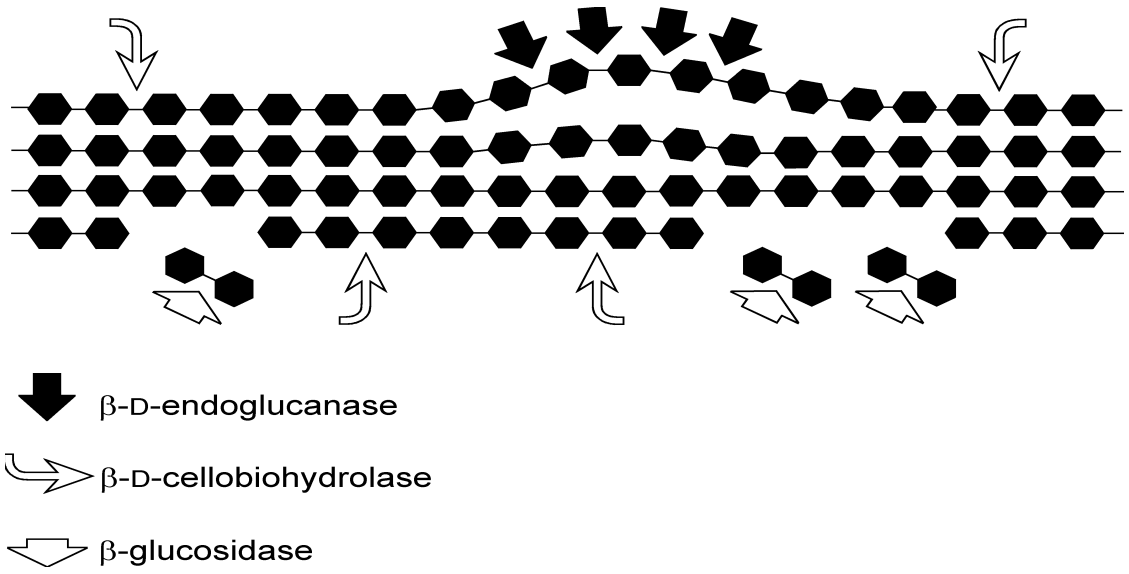


Figure 1.6. Cellulose structure and enzymes involved in cellulose degradation.

1.2.4.2.1 Endoglucanases

Endoglucanases cleave intramolecular β-1, 4-glucosidic linkages randomly, and their activities are often measured on a soluble high DP cellulose derivative, such as CMC. The modes of actions of endoglucanases and exoglucanases differ in that endoglucanases decrease the specific viscosity of CMC significantly with little hydrolysis due to intramolecular cleavages, whereas exoglucanases hydrolyze long chains from the ends in a progressive process (Irwin *et al*., 1993; Teeri, 1997; Zhang and Lynd, 2004). Endoglucanase activities can be measured based on a reduction in substrate viscosity and/or an increase in reducing ends determined by a reducing sugar assay. Because exoglucanases also increase the number of reducing ends, it is strongly recommended that endoglucanase activities be measured by both methods (viscosity and reducing ends). However, the carboxymethyl substitutions on CMC make some glucosidic bonds less susceptible to enzyme action.

Soluble oligosaccharides and their chromophore-substituted substrates, such as p-nitrophenyl glucosides and methylumbelliferyl-β-D-glucosides, are also used to measure endoglucanase activities based on the release of chromophores or the formation of shorter oligosaccharide fragments, which are measured by HPLC or TLC (van Tilbeurgh and Claeyssens, 1985; Bhat *et al*., 1990; Claeyssens and Aerts, 1992; Zverlov *et al*., 2002a, 2002b, 2003, 2005).

Endoglucanase activities can also be easily detected on agar plates by staining residual polysaccharides (CMC, cellulose) with various dyes as these dyes are adsorbed only by long chains of polysaccharides (Hagerman *et al*., 1985; Rescigno *et al*., 1994; Fulop and Ponyi, 1997; Piontek *et al*., 1998; Kim *et al*., 2000; Murashima *et al*., 2002; Jang *et al*., 2003; Ten *et al*., 2004). However, these methods are semi-quantitative, and are well suited to monitoring large numbers of samples (Sharrock, 1988).

1.2.4.2.2 Exoglucanases

Exoglucanases cleave the accessible ends of cellulose molecules to liberate glucose and cellobiose. *Trichoderma reesei* cellobiohydrolase (CBH) I and II act on the reducing and non-reducing cellulose chain ends, respectively (Teeri, 1997; Teeri *et al*., 1998; Zhang and Lynd, 2004). Avicel has been used for measuring exoglucanase activity because it has the highest ratio of F_{RE}/F_a among insoluble cellulosic substrates. During chromatographic fractionation of cellulose mixtures, enzymes with little activity on soluble CMC, but showing relatively high activity on avicel, are usually identified as exoglucanases. Since amorphous cellulose and soluble cellodextrins are substrates for both purified exoglucanases and endoglucanases, unlike endoglucanases and β- glucosidases, there are no substrates specific for exoglucanases within the cellulose mixtures (Sharrock, 1988; Wood and Bhat, 1988).

Van Tilbeurgh *et al*. (1982) found that 4-methylumbelliferyl-β-D-lactoside was an effective substrate for *T. reesei* CBH I, yielding lactose and phenol as reaction products, but it was not a substrate for *T. reesei* CBH II (van Tilbeurgh and Claeyssens, 1985) and some endoglucanases (van Tilbeurgh *et al*., 1982). *T. reesei* EG I, structurally homologous to CBH I, also cleaves 4-methylumbelliferyl- β-D-lactoside, but these enzymes can be differentiated by adding cellobiose, an inhibitor that strongly suppresses cellobiohydrolase activity (Claeyssens and Aerts, 1992). *T. reesei* CBH II does not hydrolyze 4- methylumbelliferyl-β-D-aglycones of either glucose or cellobiose units, but does cleave 4-methylumbelliferyl- β-D-glycosides with longer glucose chains (van Tilbeurgh and Claeyssens, 1985).

Deshpande *et al*. (1984) reported a selective assay for exoglucanases in the presence of endoglucanases and β- glucosidases. This assay is based on the following: (1) exoglucanase specifically hydrolyzes the aglyconic bond of p-nitrophenyl-β-D-cellobioside to yield cellobiose and p-nitrophenol, (2) β-glucosidase activity is inhibited by D-glucono-1, 5-δ-lactone (Holtzapple *et al*., 1990), and (3) the influence of exoglucanase hydrolysis activities must be quantified in the assay procedure in the presence of added purified endoglucanases. However, this technique has its own limitations: (1) CBH II activity cannot be measured using p-nitrophenyl-β-D-cellobioside, (2) the specific activity of the available purified endoglucanases may not be representative of all existing endoglucanases in the mixture, and (3) the product ratio from endoglucanase actions may be influenced by the presence of exoglucanases (Zhang *et al*., 2006).

1.2.4.2.3 β-D-glucosidases

β-D-glucosidases hydrolyze soluble cellobiose and other cellodextrins with a DP up to 6 to produce glucose in the aqueous phase. The hydrolysis rates decrease markedly

with increase in DPs (Zhang and Lynd, 2004). The term “cellobiase” is often misleading due to this key enzyme's broad substrate specificity beyond a DP of 2. β-D-glucosidases are very amenable to a wide range of simple sensitive assay methods, based on colored or fluorescent products released from p-nitrophenyl β-D-1,4-glucopyranoside (Deshpande *et al*., 1984; Strobel and Russell, 1987), β-naphthyl-β-D-glucopyranoside, 6-bromo-2-naphthyl-β-D-glucopyranoside (Polacheck *et al*., 1987), and 4-methylumbelliferyl- β-D-glucopyranoside (Setlow *et al*., 2004). In addition, β-D-glucosidase activities can be measured using cellobiose, which is not hydrolyzed by endoglucanases and exoglucanases, and using longer cellodextrins, which are hydrolyzed by endoglucanases and exoglucanases (Gong *et al*., 1977; Ghose, 1987; McCarthy *et al*., 2004; Zhang and Lynd, 2004).

1.2.4.3 Hemicellulolytic enzymes

The main enzymes involved in xylan degradation are the xylanases (Figure 1.7). These enzymes are produced mainly by microorganisms that break down plant cell walls, but are also present in marine algae, protozoans, crustaceans, insects, snails and seeds of land plants (Sunna and Antranikian, 1997). Hemicellulolytic enzymes include; endo-β-1, 4-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, acetylesterase and feruloyl- or p-coumaroyl esterase (Coughlan and Hazlewood 1993). A full set of enzymes required to release all the constituents of xylan has not yet been isolated from a single bacterial species, but some filamentous fungi secrete an effective xylan-hydrolysing enzyme mixture (Schafer *et al.,* 1996).

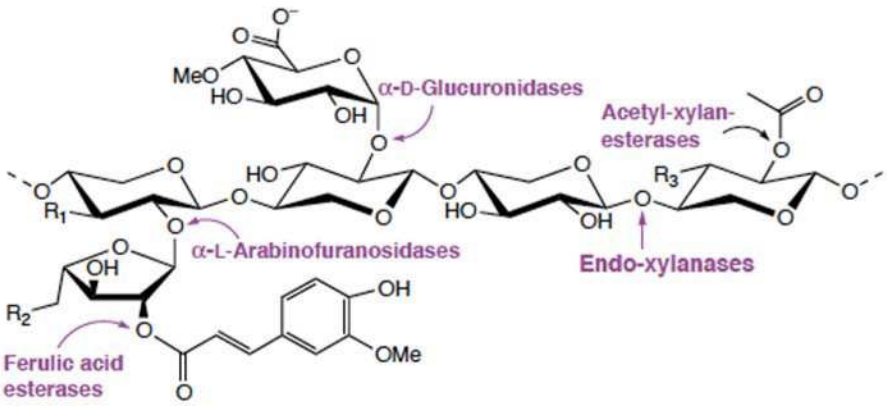


Figure 1.7. Enzymes involved in the degradation of xylan (Shallom & Shoham, 2003).

Cellulases and hemicellulases are glycoside hydrolases, which are extremely common enzymes with roles in nature including degradation of biomass such as cellulose and hemicellulose, and in normal cellular function (e.g., trimming mannosidases involved in N-linked glycoprotein biosynthesis) among others. Together with glycosyltransferases, glycosidases form the major catalytic machinery for the synthesis and breakage of glycosidic bonds. Glycoside hydrolases are found in essentially all domains of life. In prokaryotes, they are found both as intracellular and extracellular enzymes that are largely involved in nutrient acquisition.

Glycoside hydrolases are classified into EC 3.2.1 as enzymes catalyzing the hydrolysis of O- or S-glycosides. Glycoside hydrolases can also be classified according to the stereochemical outcome of the hydrolysis reaction: thus they can be classified as either retaining or inverting enzymes (Sinnott, 1990). Glycoside hydrolases can also be classified as exo or endo acting; dependent upon whether they act at the (usually non-reducing) end or in the middle, respectively, of an oligo or polysaccharide chain. Glycoside hydrolases may also be classified by sequence or structure based methods (Henrissat *et al*., 1995).

A classification system for glycosyl hydrolases, based on sequence similarity, has led to the definition of more than 100 different families (Davies and Henrissat, 1995; Bairoch, 1999; Coutinho and Henrissat, 1999). They are typically named after the substrate that they act upon. Thus glucosidases catalyze the hydrolysis of glucosides and xylanases catalyze the cleavage of the xylose based homopolymer xylan. Glycoside hydrolase (GH) family 11 consist of xylanases (Fig.1.8).

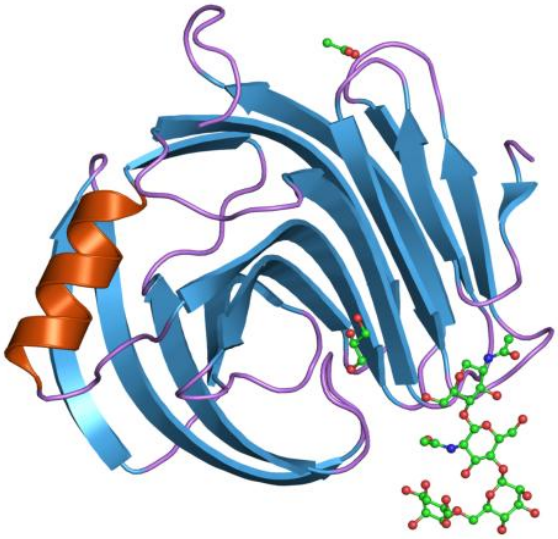


Figure 1.8. β-1, 4-xylanase, a glycoside hydrolase from *Nonomuraea flexuosa* (Hakulinen *et al*., 2003)

Cellulases and hemicellulases make up a large portion of the world‘s industrial enzymes due to their wide range of uses in various industries including chemicals, fuel, food, brewing and wine, animal feed, textile and laundry, pulp and paper, and agriculture. Xylanases are used in the baking industry to improve the texture, volume and shelf life of bread and for wheat separation. The paper and pulp industry is one of

the largest consumers of hemicellulases, ligninases and other lignocellulolytic enzymes. Cellulases and hemicellulases have also been used in the animal feed industry to reduce the fibre content to improve feed utilization, milk yield and body weight gain in cattle and sheep (Howard *et al*., 2003).

Lignocellulose can be used to produce chemicals such as biofuels. It is a cheap energy source for fermentation, improved animal feed and human nutrients (Figure 6.3) (Howard *et al*., 2003; Gravatis, 2004; Madson and Tereck, 2004). The enzymatic hydrolysis of lignocellulose, hemicellulose and cellulose results in production of hexose and pentose sugars as well as various lignin monomers. The sugars from this hydrolysis can be used in human nutrients, or in fermentation. Glucose is a common substrate used in the fermentation processes for industrial products such as organic acids, amino acids, vitamins and several bacterial and fungal polysaccharides. Xylose, produced from saccharification of hemicellulose, is used in the production of xylitol and furfural. Xylitol is used as an artificial sweetener in food, in teeth hardening, and as an anti-microbial agent in toothpaste and chewing gum. Furfural is used in manufacturing furfural-phenol plastics, varnishes and pesticides (Howard *et al*., 2003; Gravatis, 2004).

The fermentation of the sugars from cellulose and hemicellulose degradation generates products such as ethanol, acetone, butanol, glycerol, acetic acid, citric acid and fumaric acid. These chemicals, along with aromatic compounds produced from the hydrolysis of lignin, can be used to make other organic chemicals, which in turn can be used to produce various chemical products including polymers and resins (Howard *et al*., 2003). Ethanol and butanol produced from degraded and fermented lignocellulose can be used as biofuels.

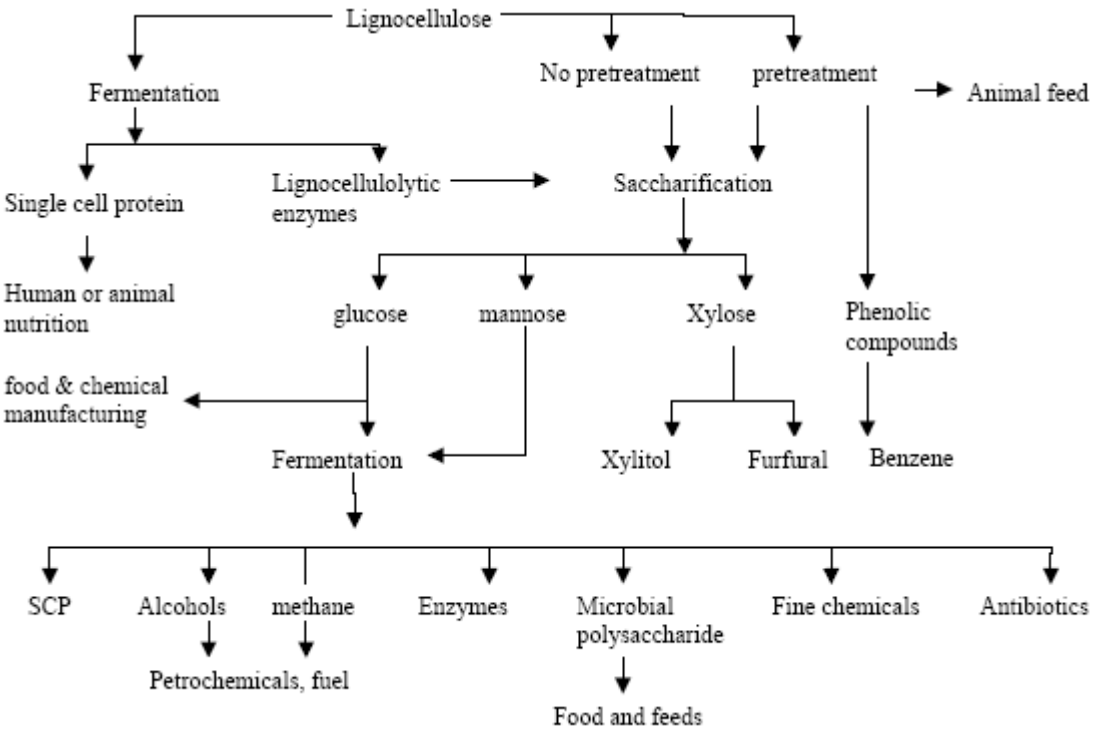


Figure 6.3. Lignocellulose bioconversion processes into valuable bioproducts

1.2.5 Bioethanol Production

Generally, bioethanol converted from edible source is called first-generation bioethanol (FGB). However, the drawback of FGB stems from the edible feedstock utilized, which includes corn and sugarcane. In this respect, second-generation bioethanol (SGB) offers great promise to replace fossil fuels without causing the feud of food-fuel supply as they are derived from non-edible sources such as lignocellulose biomass, which comprises mainly of cellulose, hemicellulose and lignin (Sun and Cheng, 2002).

Lignocellulosic biomass in nature is by far the most abundant raw material from hardwood, softwood, grasses, and agricultural residues. The additional raw materials of potential interest are newsprint, office paper, municipal solid wastes, etc. (Lee, 1997).

These long-chain polysaccharides can be hydrolyzed to produce a mixture of pentoses (C5) and hexoses (C6), which can be further converted to ethanol. Agricultural intensification is not needed as in FGB where edible agricultural crops such as sugarcane and corn are used as feedstock (Tan *et al*., 2008).

The use of enzymatic hydrolysis of cellulose is environmentally friendly compared the use of strong acids and alkali (Duff and Murray 1996, Fang *et al.* 2009). The high cost of cellulases is, however, the most significant barrier to the economical production of bioethanol from cellulosic biomass (Fang *et al.* 2008). Consolidated bioprocessing (CBP) is a promising strategy to reduce costs, through the production of cellulolytic enzymes, hydrolysis of biomass, and fermentation of the resulting sugars to bioethanol in a single process step via a cellulolytic microorganism or consortium (Lynd *et al.* 1996, Lynd *et al.* 2002). The development of CBP organisms may be achieved by engineering non-cellulolytic microorganisms, which exhibit high product yields and tolerance so that they are able to utilize cellulose.

Climate change and diminishing oil supplies are issues of acute concern for most nations in the world. With regard to climate change, the European Commission has called for expansion of the supply of renewable energies and alternative fuels in order to meet the CO_2-reduction targets specified in the Kyoto Protocol (Kim *et al*., 2010). This justifies the need to harness lignocellulolytic enzymes that can be used in the conversion of biomass into simple sugars that can be fermented to release ethanol (Isaacs, 1984).

1.2.5.1 Methods used in lignocellulose pretreatment and bioethanol production

Pretreatment of lignocellulosic biomass is aimed at increasing the accessible surface area and decrystallizing cellulose, partial depolymerization of cellulose, solubilizing hemicellulose and/or lignin, modification of the lignin structure, maximizing the

enzymatic digestibility of the pretreated material, minimizing the loss of sugars, and minimizing capital and operating costs of the process (Holtzapple and Humphrey, 1984; Margeot *et al*., 2009). Pretreatment technologies fall in four broad categories, namely physical (mechanical), physicochemical, chemical and biological.

Mechanical pretreatment include milling or grinding, which increase the surface area by reducing the size of biomass. It is environment friendly because it does not required chemicals addition and inhibitors are not generated, but high power is required by the milling machines and consequently high energy costs (Inoue *et al*., 2008; Mtui, 2009; Silva *et al*., 2010).

Physicochemical reactions occur at high temperature and pressure (Taherzsadeh and Karimi, 2007), require high control operation conditions and therefore costly. They include pyrolysis, microwave pretreatment, steam, ammonia and CO_2 explosion and hot water treatment. Pyrolysis requires high temperatures (more than 300°C) to degrade cellulose rapidly into H_2, CO, and residual char (Kumar *et al*., 2009). After the separation of char, the recovered solution is primarily composed by glucose, which can be eventually fermented for ethanol production (Sakar *et al*., 2012). Microwave pretreatment, has the advantage of the short reactions times and homogeneous heating of the reaction mixture (Balcu *et al*., 2011). Steam explosion or hydrothermal explosion is a thermochemical process, where lignocellulosic material is exposed to steam (Chornet and Overend, 1991). There is minimum, or in some cases, no chemical addition, therefore environmental friendly (Kaar *et al*., 1998). Biomass and steam are maintained at high temperature in a reactor, promoting the hemicellulose hydrolysis followed by a quickly decompression ending the reaction (Agbor *et al*., 2011). Steam explosion treatment yields high solubility of the hemicellulose (producing mainly oligosaccharides) with low lignin solubility (Mosier *et al*., 2005).

Ammonia fiber explosion (AFEX) is an alkaline thermal treatment that exposes the lignocellulosic material to high temperature and pressure followed by fast pressure release. Its main advantages are the efficient lignin removal and less generation of inhibitors, retaining appreciable amount of carbohydrates in the substrates. It is simple with short-time process but causes material structure change leading to decomposition of monosaccharides (Sakar *et al*., 2012). It however results in an increase of water holding capacity and digestibility of substrates (hemicellulose and cellulose) by enzymes, leading to high sugars recovery (Kumar et al., 2009; Sakar *et al*., 2012). CO_2 explosion forms carbonic acid, increasing the hydrolysis rate of the pretreated material, which lead to higher conversion yields (Sun and Cheng, 2002). It does not cause the formation of inhibitors because milder temperature is used during the process, preventing any appreciable decomposition of monosaccharides. The process is nontoxic, noninflammable, and environmental friendly. However, it is a method with hard operation and process complexities (Hamelinck *et al*., 2005; Kumar et al., 2009).

Hot Water method employs hot water under high pressure in the biomass hydrating the cellulose and removes a considerable part of hemicellulose fraction. It has the advantage of no use of chemicals and consequently not necessary to use corrosion-resistant materials in the hydrolysis reactor. In addition, it does not require reduction of the size of the raw material (Taherzsadeh and Karimi, 2008).

Chemical methods include acid and alkaline pretreatment, oxidative delignification, ozonolysis, organosolv process and wet oxidation. They degrade hemicellulose or remove lignin, loosening the structural of lignocellulose. Acid pretreatment solubilization of hemicellulose occurs at high temperatures, or at high concentrated acid, releasing pentose sugars (Mosier et al., 2005; Alvira *et al*., 2010), and facilitating

the enzymatic hydrolysis of remaining substrate (cellulignin) (Taherzsadeh and Karimi, 2008). The most commonly used acid is H_2SO_4, but HCl (Laopaiboon *et al*., 2010), phosphoric acid (Calvalho *et al*., 2004), nitric acid (Rodriguez-Chong et al., 2004), and oxalic acid (Chandel *et al*., 2011). They contact with biomass promoting hemicellulose breakdown. It has the advantage of operating at low and medium temperatures and consequently decreasing of energy costs (Girio *et al*., 2010). However, in high concentration of acid problems can occur with equipment corrosion and expensive costs of maintenance (Alvira *et al*., 2010), and also after this pretreatment, it is necessary to neutralize the hydrolysate before fermentations (Mosier *et al*., 2005). Another disadvantage of this process is the possibility of formation of other by-products that are considered inhibitory to microbial fermentation, like furans, furfural, carboxylic acids, formic levulinic and acetic acids, and phenolic compounds. Detoxification is therefore required to remove these inhibitory compounds to increase the fermentability of hydrolysates (Palmqvist and Hahn-Hagerdal, 2000).

Alkaline Pretreatment is a delignification process, in which a significant amount of hemicellulose is also solubilized. It uses various bases, including sodium hydroxide, calcium hydroxide (lime), potassium hydroxide, ammonia hydroxide, and sodium hydroxide in combination with hydrogen peroxide or other chemicals (Zheng *et al*., 2009). The action mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and other components (Sun and Cheng, 2002). This process utilizes lower temperatures and pressures than other pretreatment technologies, but pretreatment times are long (Zheng *et al*., 2009). Compared with acid-based pretreatment processes, alkaline processes causes less sugar degradation, and many of the caustic salts can be recovered and/or regenerated (Kumar et al., 2009). Alkaline pretreatment of lignocellulosic materials causes

swelling, leading to an increase in internal surface area, decrease in the degree of polymerization and crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure making cellulose and hemicellulose available for the enzymatic degradation (Zheng *et al*., 2009; Sakar *et al*., 2012). Reactor costs are lower than those for acid technologies but the use of expensive salts in high concentrations is a significant disadvantage that raises environmental concerns and may lead to prohibitive recycling, wastewater treatment, and residual handling costs (Hamelinck *et al*., 2005; Zang *et al*., 2009).

Oxidative delignification involve lignin degradation catalyzed b peroxidase enzyme with the presence of H_2O_2 (Sun and Cheng, 2002). Ozonolysis uses ozone to degrade the lignin and hemicellulose fractions from lignocellulosic materials such as wheat straw, bagasse, peanut, pine, cotton straw, and poplar sawdust (Kumar et al., 2009). Ozone is a powerful oxidant, soluble in water and is readily available. It is highly reactive towards the compounds incorporating conjugated double bonds and functional groups with high electron densities. Therefore, the most likely biomass constituent to be oxidized is lignin due to its high content of C=C bounds (Garcia-Cubero *et al*., 2009). It does not produce toxic residues for the downstream processes, and the reactions are carried out at ambient temperature and pressure (Vidal and Molinier, 1988). However, a large amount of ozone is required, making the process expensive (Sun and Cheng, 2002).

Organosolv process involves a strong inorganic acid which acts as a catalyst, promoting the breakdown of lignin-lignin and carbohydrates-lignin bonds from the biomass (Holtzapple and Humphrey, 1984). When the lignin is removed, the superficial area and volume of the material are also increased considerably, facilitating the enzyme accessibility and consequently improving the efficiency of the process to

achieve fermentable sugars (Koo *et al*., 2011). The organosolv process uses fewer amounts of chemicals to neutralize the hydrolyzate and generates few amounts of wastes compared with other similar process (Taherzsadeh and Karimi, 2008). Chemicals such as NaOH or Na_2SO_3 could be used as catalyst ((Ruzene *et al*., 2007). High efficiency for lignin removal coupled with the high pressure of carbon dioxide has been observed using this process (Pasquini et al., 2005).

Wet oxidation is an oxidation process in the presence of oxygen or catalyzed air, where the most used catalyst is the sodium carbonate (Carvalheiro *et al*., 2008). Wet oxidation allows obtaining high yields of biomass conversion into monosaccharides with low formation of furan and phenolic aldehydes.

Biological pretreatment methods use wood degrading microorganisms like bacteria and brown rot, white rot, and soft rot fungi in degradation of lignin and hemicellulose making the biomass more amenable to enzyme digestion (Chandel *et al*., 2007; Zheng *et al*., 2009; Sakar *et al*., 2012;). They have the disadvantage of loss of a considerable amount of carbohydrates during long pretreatment (Kumar et al., 2009).

The most effective microorganism for biological pretreatment of lignocellulosic materials is white rot fungi (Sakar *et al*., 2012). These microorganisms degrade lignin through the action of lignin-degrading enzymes such as peroxidases and laccase (Kumar *et al*., 2009). Brown rot fungi mainly attack cellulose, while white and soft rot fungi attack both cellulose and lignin (Sun and Cheng, 2002). The process is

environmental friendly because of its low energy use and mild environmental conditions (Hamelinck *et al*., 2005). Its main disadvantages are low efficiency, considerable loss of carbohydrates, long residence time, requirement of careful control of growth conditions, and restraints in its application space. In addition, most

ligninolytic microorganisms solubilize or consume lignin as well as a considerable fraction of hemicellulose and cellulose

The advantages of biological delignification over the previous methods may include mild reaction conditions, higher product yields and fewer side reactions, and less energy demand and less reactor resistance to pressure and corrosion.

Enzymatic hydrolysis is an ideal approach for degrading cellulose into reducing sugars because mild reaction conditions (pH between 4.8–5.0 and temperature between 45–50°C) can be used; it does not present corrosion problems in the reactors and result in high sugar yields. However, enzymatic hydrolysis depends on optimized conditions for maximal efficiency (hydrolysis temperature, time, pH, enzyme loading, and substrate concentration) and suffers from end-product inhibition and biomass structural restraints. To overcome the end-product inhibition and reducing the time, hydrolysis and fermentation can be combined, so-called simultaneous saccharification and fermentation (SSF) or simultaneous saccharification and cofermentation (SSCF) (Duff and Murray, 1996; Milagres *et al*., 2011).

1.2.6 Isolation, Purification and Characterization of Lignocellulolytic Enzymes

Rodionova *et al*. (1977) isolated and separated β-glucosidases from *Geotrichum candidum* cellulase preparation by means of DEAE-Sephadex A-50 chromatography, gel filtration through P-150 Biogel and chromatography on CM-cellulose, and then were fractionated by isoelectric focusing (IEF).

An automated micro preparative electrophoresis CE system Model 230 HPEC, which combines continuous sample elution, on-line detection and automated fraction collector, was used for isolation and recovery of *Aspergillus niger* glucoamylase (Zaidi and Rosenblum, 1992). SDS-PAGE was also used for preparative purposes

when multiple forms of cellulase from *Trichoderma reesei* were separated (Sprey, 1987; Messner *et al., 1988*).

Homogeneity of 1, 4-β-D-xylanase from *Thermoanaerobacterium sp*. was tested using PAGE (Shao *et al*., 1995). The same method was described for, β-D-xylosidase (syn. α-L-arabinosidase) from *Thermoanaerobacter ethanolicus* (Shao and Wiegel, 1992).

Yazdi *et al*. (1990), studied cellulase production by the cell-1 mutant of *Neurospora crassa*. Eight enzymes (three exoglucanases, four endoglucanases, and one β-glucosidase) were identified and characterized by gel filtration, ion exchange chromatography, and chromatofocusing. After purification, each of the proteins ran as a single band in polyacrylamide gel electrophoresis, using both native and denaturing gels. The molecular weights of the proteins were found to be between 70,000 and 22,000 daltons, and all were glycosylated, with carbohydrate contents ranging between 5.6 % and 36 %.

In a study on extracellular cellulolytic enzyme system of *Aspergillus japonicas* (Kundu *et al*., 1988), the crude culture filtrate of *A. japonicus* exhibited marked cellulose and xylan degrading activities. Affinity chromatography of the crude enzyme preparation on concanavalin A (Con A)-Sepharose 4B resolved it into three fractions. The eluted fraction A contained CMCase (CMCase I) and xylanase activities; fraction B showed only CMCase activity (CMCase II); and fraction C exhibited CMCase (CMCase III) and xylanase activities. On further purification by gel filtration, only fraction B imparted a homogeneous preparation that gave a single band on polyacrylamide gel electrophoresis at pH 8.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of this homogeneous form showed the molecular weight about 57, 000 daltons. Sephadex G-100 column chromatography also

supported the molecular weight. A low molecular weight endoglucanase, of about 12 700 daltons, was obtained from fraction A by Sephadex G-75 column chromatography whereas the molecular weight of the endoglucanase from fraction C was higher (77 000 daltons). The silver ion (Ag^{1+}) strongly inhibited the endoglucanase I activity but had no effect on II and III. Hg^{2+} inhibited all the three forms. The pH optimum for endoglucanase I, II and III was 4.5. The endoglucanase II showed the highest temperature optimum of 65°C whereas those of endoglucanase I and III were 50°C and 55°C, respectively. The first two forms had the least activity toward tamarind kernel polysaccharide (TKP) but the last form, endoglucanase III, was highly reactive to it.

Cellobiohydrolases I and II were purified to homogeneity from culture filtrates of a thermophilic fungus, *Chaetomium thermophile* var. *coprophile*, by using a combination of ion exchange and gel filtration chromatographic procedures. The molecular weights of cellobiohydrolase I and II were estimated to be 60,000 and 40 000. The enzymes were found to be glycoproteins containing 17 and 22.8 % carbohydrate, respectively. The two forms differed in their amino-acid composition mainly with respect to methionine, threonine, alanine, and arginine.

For a number of years now, lignocellulolytic activity has been investigated in several organisms. Bacteria and several fungi have been reported to exhibit ligninolytic enzyme (Lac, MnP and LiP) activity (Arora *et al*., 2002; Zouri-Mechichi *et al*., 2006; Quaratino *et al*., 2007; Lopez *et al*., 2007; Sahay *et al*., 2008; Fakoussa and Frost, 1999; Desai *et al*., 2011; Bholay *et al*., 2012). Cellulolytic and Xylanolytic enzymes have also been demonstrated from bacteria and several fungi (Gong *et al*., 1977; Romero *et al*., 1999; Suna and Antranikian, 1997; Karnchanatat *et al*., 2008). Schafer

et al. (1996) demonstrated hemicellulose-degrading bacteria and yeasts from termite gut.

Enzymatic properties and primary structures of many cellulases have been investigated and cellulase genes from widely differing origins have been cloned and sequenced (Hamada *et al*., 1999, Karnchanatat *et al*., 2008). Digestive β-glucosidases from wood-feeding termites have been cloned and sequenced (Tokuda *et al*., 2002; Tokuda *et al*., 2009). However, little is known about the lignocellulolytic enzymes from marine woodborers.

1.3. Research Design and Thesis Outline

Marine woodborers consume lignocellulose and represent a rich source of potential lignocellulolytic enzymes that can be harnessed for conversion of biomass into simple sugars and other monomers for a variety of uses including ethanol production. This PhD research aimed at isolating lignocellulolytic enzymes from the gut of marine woodborers, investigating potential of the lignocellulolytic enzyme(s) in bioethanol production and characterizing the lignocellulolytic enzyme with the highest activity.

In order to achieve the aforementioned research objectives, a series of field and laboratory studies were conducted. The materials and methods used are described in Chapter 2. The first study in Chapter 3 describes the morphological and molecular identification of the woodborers and subsequent analysis. Three species; *Dicyathifer mannii* (Wright, 1866), *Sphaeroma terebrans* (Bate, 1866) and *Cirolana sp. were* identified. Host preference pattern was observed during collection of the specimens, and this was therefore also analyzed.

In the second study in Chapter 4, lignocellulolytic activities of gut extracts from marine woodborers *D. mannii* and *S. terebrans* were investigated in an effort to seek

the species with the most lignocellulolytic efficacious extracts. *Cirolana sp.* was not obtained in numbers that could constitute enough gut extract for analysis. The lignocellulolytic activities investigated were lignin biodegradating enzymes; lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and laccase (Lac) or monophenol oxidase. Cellulolytic enzymes investigated were; glucanases endoglucanase (endo-1-4-β-D-glucanase), exoglucanase (1,4-β-D-glucan-cellobiohydrolase), and β-D-glucosidase or cellobiase (β-D-glucoside glucanohydrolase). Endo-1-4- β- xylanase was investigated in the hydrolysis of xylan, the chief type of hemicellulose.

The enzymes that digest lignin could either be extracellular enzymes produced by microorganisms in the gut or secreted by glands in the woodborer digestive tract and released into the gut for action. Therefore, there was need to culture microorganisms from the gut of these woodborers (Chapter 5), and investigate their lignocellulolytic activities (Chapter 6) in the third and fourth study respectively.

Chapter 5 determined microorganisms' diversity of woodborers' digestive tracts by culturing bacteria and fungi from the gut contents and carrying out morphological and molecular identification of the microorganisms, with subsequent phylogenetic analysis. In Chapter 6, pure bacterial and fungal isolates obtained in Chapter 5, as well as their mixed cultures were induced to produce lignocellulolytic enzymes in a variety of substrates. The chapter also investigated ethanol production from wheat straw hydrolysate obtained by degradation reaction of *D. mannii* gut microbial filtrate on ground and sieved wheat straw as substrate.

In the fifth study, the *D. mannii* microbial community from chapter 6 was screened for lignocellulolytic enzymes and was found to have moderate β–glucosidase and high

xylanase extracellular enzymes. For this reason, xylanase was recommended for isolation, purification and characterization in Chapter 7. The xylanase was purified by ammonium sulphate precipitation, gel filtration (sephadex G-200) and anion exchange chromatography (DEAE-Sephacel). Chapter summaries, general discussion, conclusions and recommendations are presented in Chapter 8.

CHAPTER TWO

2.0. MATERIALS AND METHODS

2.1. Study Area

Mangrove forests are very interesting intertidal ecosystems, appearing as islands of different shapes, separated from each other by sandy or rocky shores of various sizes. In the Kenyan coast, there is discontinuous distribution of mangroves, often limited to very narrow creeks and bays (Figure 2.1).

Adult woodborers were collected from wood in mangroves of Mida Creek (North coast), Jomvu Kuu within Tudor Creek (island) and Gazi Bay (south coast) in the intertidal region along the Kenyan coast (Figure 2.2, 2.3 and 2.4 respectively).

2.1.2. Description of Study Sites

Mida Creek or Watamu Marine National Reserve (3°20'S, 40°00'E) is situated 100 km north of Mombasa in Kilifi district. The reserve was established in 1968, which contains natural elements such as mangroves, coral reefs, and mud flats and is a sanctuary for shorebird populations. Seven of the 9 mangrove species described in Kenya are found in Mida creek and they occupy a total area of 1746 hactares. The dominant species are *Rhizophora mucronata* Lamk. (Rhizophoraceae), Ceriops tagal (Perr.) C.B. Robinson (Rhizophoraceae) and *Avicennia marina* (Forsk.) Vierh. (Avicenniaceae). There is no obvious zonation that is displayed by the dominant mangrove species in Mida Creek. *A. marina and Lumnitzera racemosa* Willd. occupy the landward zone, whereas mostly *C. tagal* and *R. mucronata* mosaic covers the middle zone. Wherever present, *Sonneratia alba* Sm. occupies the seaward margin, but is replaced by tall *A. marina* and *R. mucronata* along small creeks (Kairo *et al*., 2002).

Jomvu Kuu is a section of Tudor Creek (4°01'48S 39'40'12 E). Tudor creek bounds Mombasa island on the northwest and extends some 10 km inland. The creek has two main seasonal rivers, Kombeni and Tsalu, draining an area of 550 km^2 (450 and 100 km^2 respectively) with average freshwater discharge estimated at 0.9 m^3 s^{-1} during the inter-monsoon long rains. It has a single narrow sinuous inlet with a mean depth of 20 m, that broadens out further inland to a central relatively shallow basin (5 m) fringed by a well-developed mangrove forest mainly composed of *R. mucronata* (Rhizophoraceae), *A. marina* (Avicenniaceae) and *Sonneratia alba* Sm. (Sonneratiaceae). The basin has an area of 6.37 km^2 at low water spring and 22.35 km^2 at high water spring. Mangrove forests occupy 8 km^2 of the creek. Like Mida Creek, there is no obvious zonation displayed by the dominant mangrove species in Tudor Creek. *A. marina* and *L. racemosa* Wild. occupy the landward zone, whereas mostly *Ceriops tagal* (Perr) C. B. Robinson and *R. mucronata* mosaic covers the middle zone and occupies the seaward margin whenever present. Along small creeks, *S. alba* is replaced by *A. marina* and *R. mucronata* (Mohamed *et al*., 2009).

Gazi Bay (4"25'S, 39"30'E) is 50 km south of Mombasa. The bay is 18 km^2 sheltered from the Indian Ocean by a peninsula to the east and a fringing coral reef to the south. Mangrove vegetation, penetrated by two tidal creeks, covers 6.61 km^2 of the bay. The western creek has an inland continuation as the seasonal river Kidogweni; the eastern creek has no such freshwater input. Eight mangrove species are found in Gazi Bay: *R. mucronata* and *C. tagal* account for over 60 % of the vegetation cover, and the others are *S. alba* (Sm.), *Bruguiera gymnorrhiza* (L.), *Avicennia nzarirza* (Forssk.) Vierh., *Lumnitzera racemosa* Wild., *Xylocarpus granatum* Koen. and *Heriteria littoralis* Dryand ex *H.* Ait. The mangrove species occur in clear zonation patterns, with belts parallel to the low-water line. In general, *S. alba* forms the outermost zone towards the

open water, followed by pure stands of *R. mucronata* or mixed stands of *R. mucronata* and *B. gymnorrhiza,* and in turn these stands are followed by pure or mixed stands of *C. tagal* and *A. marina.* Along the river Kidogweni and other creeks, *A. marina* usually replaces *S. alba.* On the seaward side, the mangrove forest is bordered by intertidal and subtidal areas covered with seagrasses (Slim *et al*., 1996; Bosire *et al*., 2003).

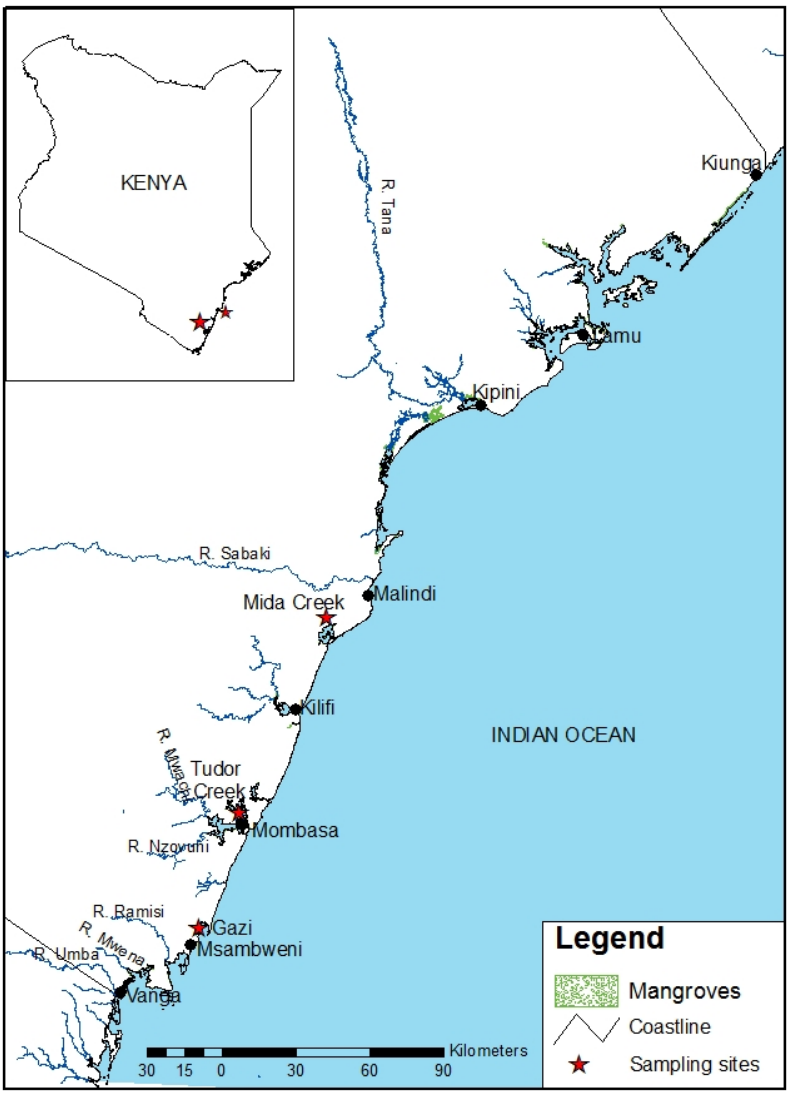


Figure 2.1. Map of the Kenyan Coast showing sampling sites

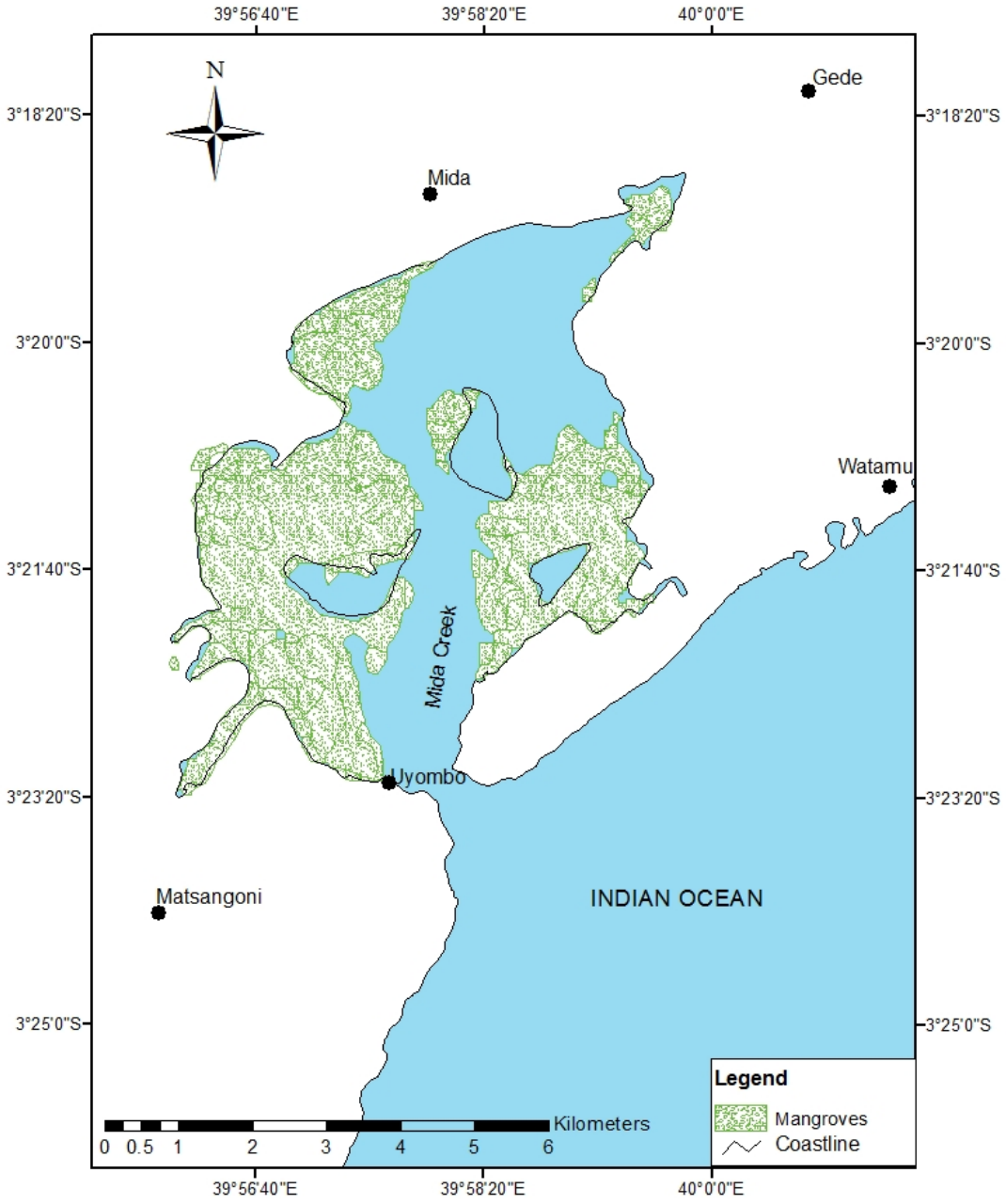


Figure 2.2. Mida Creek mangroves

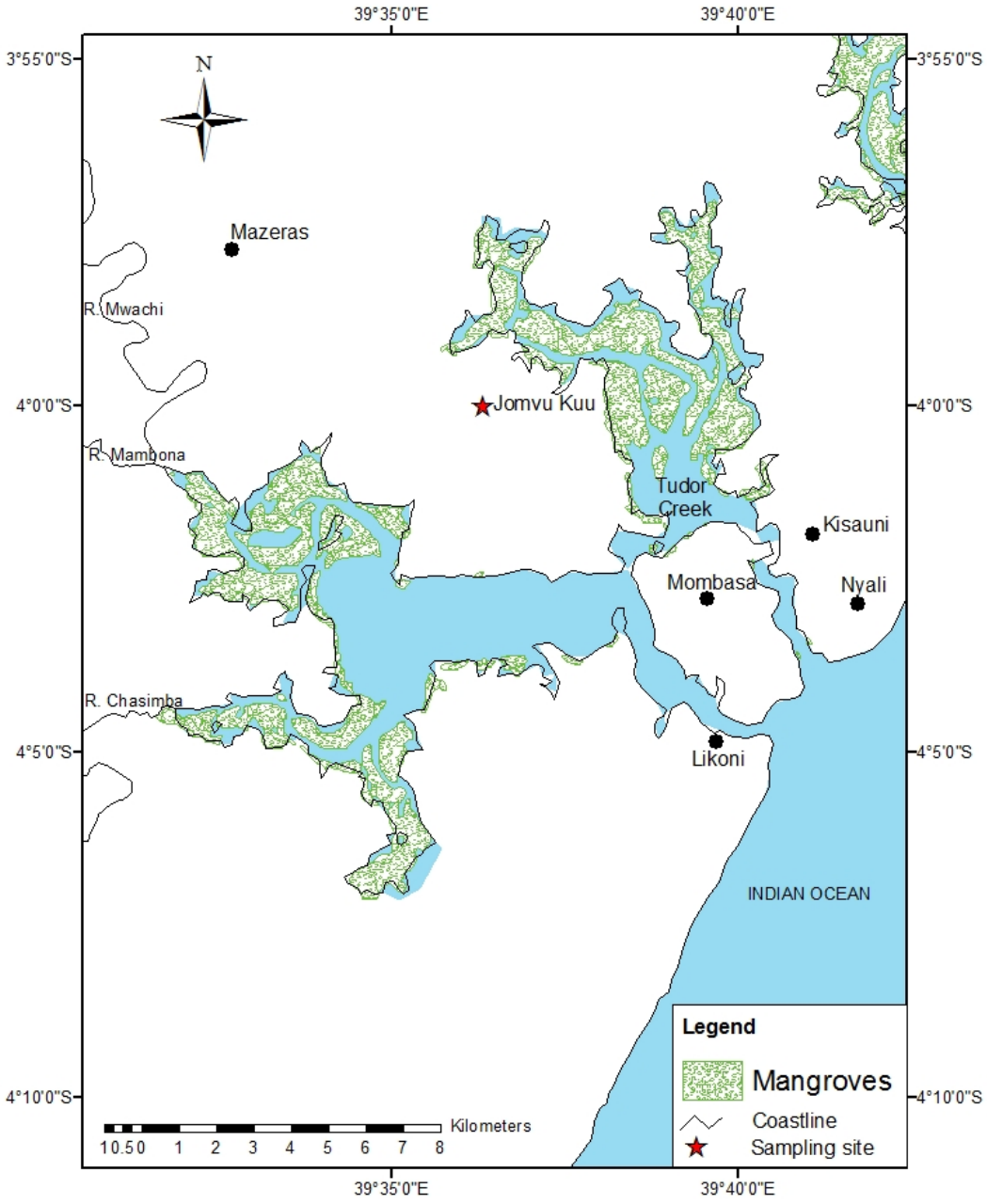


Figure 2.3. Tudor Creek mangroves

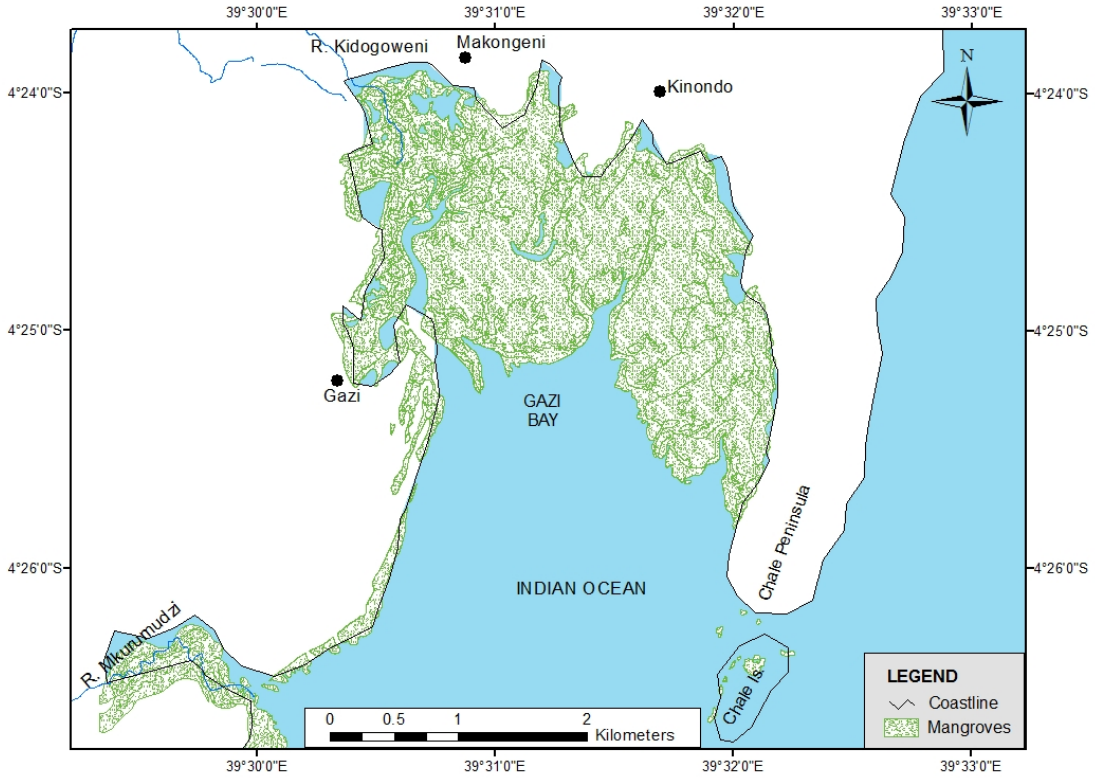


Figure 2.4. Gazi Bay mangroves

2.2. Collection and Preservation of Marine Woodborers

2.2.1. Collection Strategy

Collections of woodborers were made from Mida Creek, Tudor Creek (Jomvu Kuu) and Gazi Bay between January 2011 and March 2013. The collection efforts at each site lasted from 1–3.5 h, with two to three people observing and noting the number of attacked plants, and collecting sample specimens of woodborers encountered as they walked haphazardly through each site (Gilbert *et al*., 2008). Infested mangrove wood including those of decaying logs were identified to species level by experts. The woodborers were collected from *R. mucronata*, *S. alba* and either *A. marina* or *A. nzarirza* mangrove wood in the 3 sampling sites. The borers were collected from submerged parts of roots (proproots, pneumatophores), stems and branches in the mangrove ecosystem. They were immediately transported alive to the Kenya Marine

and Fisheries Research Institute laboratory where specimens for morphological and molecular identification were obtained.

The molluscan borers were extracted by carefully cutting open the damaged wood samples and best specimens were obtained when the borers were dissected out as soon as the wood was removed from the water. For later extraction, the destroyed wood pieces were submerged in 70 % alcohol and then transported to the laboratory wrapped in cotton and clothe soaked in alcohol. Specimens removed from the wood were preserved in a mixture of four parts 70 % alcohol and one part glycerine. This way the periostracal margins of the pallets were kept soft and pliable and in case the alcohol evaporated, the glycerine kept the pallets moist for some more time.

The crustacean borers on the other hand were collected by keeping the infested wood samples in a trough of diluted seawater or by adding a little formalin. The change in the milieu and the traces of formalin forced the borers out of their burrows and specimens were easily collected with a fine brush or forceps. The crustacean borers were also preserved in alcohol. No formalin was used in preservation as it slowly dissolves the tubercles on the dorsal surface of the animals, rendering species identification difficult. Specimen for molecular identification were preserved in absolute ethanol until DNA extraction, which was carried out from thoracic legs (pereopods) for isopods and the muscle below the head in bivalves.

The woodborers were identified based on macroscopic and microscopic morphological characteristics through comparison with appropriate literature (Kuhne, 1971; Harrison and Holdich 1984). This was confirmed by molecular identification. Voucher specimens are deposited in the National Museums of Kenya.

2.3. Morphological and Molecular Identification of Woodborers

2.3.1. Morphological Identification of Woodborers

Shipworms (Teredinidae) morphological identification was based entirely on shells and pallets. Characters of systematics value for species identification were nature of the shell valves, tubes (internal lining of the borrows which sometimes gets thickened as a tube particularly at the posterior end), pallets (a pair of calcareous organ situated at the posterior end of the animal which is used to plug the entry hole during adverse conditions or when the borer is disturbed) and siphons. Of these, the morphological variations exhibited by the pallets are remarkable and almost all the species can be identified from their pallets (Turner, 1966a, 1966b, 1971).

Pill-bugs (Sphaeromatidae and Cirolanidae), characters of taxonomic value were the number and disposition of large tubercles on the dorsal posterior part of body, posterior part of the telson and shape of the epistome. Of these, the arrangement of the large tubercles is strikingly different and showed variations characteristic of each species (Pillai, 1961).

In addition to morphological characteristics, burrows produced by each of the above two types of borers are also characteristic of its occupant. Shipworms bore deep into the wood making long tunnels almost parallel to the grain. On the other hand, pill-bugs produce burrows cylindrical on the wood surface at right angle to the grain with juvenile burrows working from the main parent tunnel leaving side branches.

2.3.2. Molecular Identification of Woodborers

2.3.2.1. DNA preparation and mitochondrial cytochrome C oxidase subunit 1 (CO1) gene fragment analysis

Extraction of DNA was performed using Quick-gDNA^{TM} Miniprep extraction kit

(Zymo Research) according the manufacturer's instruction. The obtained DNA was stored at $^{-}20^{0}$C.

The mitochondrial cytochrome c oxidase subunit I gene (COI) was amplified using Phusion High-Fidelity DNA polymerase (Thermo Scientific) following the manufacturer's instructions with slight modifications, using the following forward and reverse primers; 5'-ggtcaacaaatcataaagatattgg-3' and 5'-taaacttcagggtgaccaaaaaatca-3'.

The PCR was performed in a total volume of 50 µl using 28.95 µl distilled water, 1.0 µl dNTPs (10 mM), 10 µl 5x GC phusion buffer, 2 µl MgCl (25 mM), 2.4 µl forward primer (10 mM), 2.4 µl reverse primer (10 mM), 0.75 µl dimethylsulphoxide (DMSO), 0.5 µl Phusion DNA polymerase (0.02 U/µl) and 4.0 µl DNA template. The PCR profile included initial denaturation step at 95 ^{0}C for 3 minutes followed by 45 cycles of 95 ^{0}C (30 seconds) denaturation, 50 ^{0}C (30 seconds) annealing, 72 ^{0}C (1min) extension and a final 72 ^{0}C (7 min) extension.

The PCR product was analysed on 1% agarose gel electrophoresis containing 0.5 mg/ml ethidium bromide. The amplification patterns were viewed with DNA gel viewer (Bio-Rad) and cleaned using QIAquick PCR purification kit according to manufacturer's instruction. The purified DNA was quantified with a nanodrop spectrophotometer. The clean PCR product was sequenced with an ABI sequencing kit (Big Dye Terminator Cycle Sequencing, Applied Biosystems).

Preparation reaction for big dye PCR consisted of Big dye (0.5 µl), 5X seq buffer (1.75 µl), 10 mM primer (1.0 µl), PCR product (4.0 µl) and water (2.75 µl). The PCR profile was 96 ^{0}C for 1 min followed by 25 cycles of 96 ^{0}C for 30 seconds, 50 ^{0}C for 30 seconds and 60 ^{0}C for 4 minutes. Clean up (ethanol/ sodium acetate precipitation of

extended sequencing products) consisted of nuclease free water (24.5 µl), 3M NaOAc pH 5.2 (3.0 µl) and absolute ethanol (62.5 µl). To analyse the gene, 10 µl of HI-DI formamide was added and samples denatured at 96 ^{0}C for 3 minutes. The samples were electrophoresed on 3130xl Genetic Analyzer (Applied Biosystems®).

2.3.2.2. DNA sequence analysis

The electropherograms were viewed using Finch TV Ver. 1.5 and aligned using DNA Baser software Ver. 3.5 to correct apparent anomalies. To search for available matches with published sequences of other invertebrates, we aligned the sequences on BLAST at the NCBI web site.

2.4. Species Specificity Analysis

Species preference data was analysed by non-parametric statistics chi-square test using SPSS Ver. 16.0 to calculate the probability that each wood borer would be found on a particular host at the observed frequency or less, or the observed frequency or more, in a host tree species abundant habitat. The expected distribution of collections among the three host species, assuming no host preference, was estimated from combined counts of infested living and dead trees in the three sampling sites.

2.5. Chemicals (Enzymes, Substrates, Buffers and Reagents)

Laccase (from Trametes versicolor), Mn-dependent peroxidase (MnP), lignin peroxidase (LiP), β-glucosidase from *Aspergillus niger*, p-Nitrophenyl β-D-glucopyranose (pNPG), 4-hydroxy-3, 5-dimethoxybenzaldehyde azine (syringaldazine), D (+)- xylose, microcrystalline cellulose (Avicel PH101), carboxymethylcellulose (CMC) sodium salt, D (+)- cellobiose, 3,4-dimethoxybenzyl alcohol (veratryl alcohol), Sephadex® G-200, polyethylene glycol (PEG) 8000,

DEAE–Sephacel and beechwood xylan were all purchased from Sigma-Aldrich, USA. All other chemicals were of analytical grade.

2.6. Extraction and Preparation of Crude Gut Extracts

35 *D. mannii* and 200 *S. terebrans* were surface sterilized with 70 % ethanol, rinsed in distilled water and allowed to air dry for 1 minute. Under aseptic conditions, the entire guts were separately removed with a blade and a pair of tweezers and immediately washed in sodium phosphate buffer (0.1 M, pH 6.2). The tissues were pooled separately and homogenized (5 min) in 100 ml of chilled sodium phosphate buffer (0.1 M, pH 6.2) using an electric homogenizer. The homogenates were centrifuged at 12,000 rpm for 30 minutes at 4 °C. The resultant supernatant was stored in aliquots of 5 ml at ⁻20 °C (Foster *et al*., 1999) and used for screening for lignocellulolytic enzymes.

2.7 Determination of Lignocellulolytic Activities of the Extract

Lignocellulolytic activities were determined spectrophotometrically using a Beckman Coulter^{tm} UV/VIS spectrophotometer (DU® 530 Life Science). The least count of absorbance measurement was 0.001 (Risna and Suhurma, 2002; Sahay *et al*., 2008; Yadav *et al*., 2009; Singh *et al*., 2011;).

2.7.1. Ligninolytic Activity

Lignin peroxidase (LiP) was determined by the peroxide-dependent oxidation of 10mM veratryl alcohol to veratraldehyde in 125mM tartrate buffer, pH 3.0 with 2mM H_2O_2 (controls without H_2O_2 were included), according to Orth *et al*. (1993). The molar extinction coefficient value of 9300 M^{-1} Cm^{-1} for veratraldehyde at 310nm was used for calculating the enzyme units. All enzyme assays were carried out in triplicate. The results were also interpreted as percent discolouration compared to the control calculated as (A_{310} for control – A_{310} for test / A_{310} for control) x 100 (Denise *et al.,*

1996).

Manganese-dependent peroxidase (MnP) was assayed by oxidation of phenol red, which was measured by monitoring the A_{610} (ε = 22.0 mM^{-1} cm^{-1}) at room temperature (Camarero *et al*., 1999; Salame *et al*., 2012). The reaction mixture contained 250 mM lactate, 2mM $MnSO_4$, 0.5 % bovine serum albumin, 1mg/ml of phenol red, and 0.5 ml of supernatant in 20 mM sodium succinate buffer (pH 4.5) in a total volume of 1 ml. The reaction was initiated by the addition of H_2O_2 to final concentration of 2mM and was stopped after 1 min with 50 µl of 10 % NaOH. Omitting $MnSO_4$ from the reaction mixture carried out control assays of phenol red oxidation in the absence of Mn^{2+}. MnP activity was calculated by subtracting the value for phenol red-oxidizing activity in the absence of Mn^{2+} from the value for the activity obtained in the presence of manganese. All enzyme assays were carried out in triplicate.

Laccase (Lac) was analyzed by monitoring the oxidation of 0.25 mM syringaldazine in 100mM citrate–phosphate buffer at pH 5.2 (Fakoussa and Frost, 1999; Lopez *et al*., 2007). Heat inactivated (100 ^{0}C for 15 minutes) supernatant and enzyme substrate controls were included. Lac activity was determined by the increase in the absorbance due to the formation of tetramethoxy-azo-bis-methylenequinone, resulting from the oxidation of syringaldazine as described by Leonowicz and Grzywnowickz (1981). The activity was assayed in mixed reactions containing supernatant, citrate-phosphate buffer (pH 5.2, 0.1 M) and syringaldazine 0.25 mM in methanol. An increase in absorbance at 530 nm (ε = 65000 M^{-1} cm^{-1}) was followed at 25 ^{0}C to determine laccase activity in international units (IU) (Salmones and Mata, 2002). All enzyme assays were carried out in triplicate.

All ligninolytic enzyme activity was expressed as IU/ml. An international unit IU (or U) is defined as the amount of enzyme activity, which catalysed the transformation of 1 micromole of substrate per minute under standard conditions. This was calculated using the formula:

Enzyme Activity (U/ml) = (A* V) / (t * ε * v), where A = Absorbance at corresponding wavelength, V = Total volume of reaction mixture (ml), v = enzyme volume (ml), t = Incubation time (min) and ε = Corresponding Extinction Coefficient (M^{-1} cm^{-1}) (Desai *et al*., 2011).

2.7.2. Cellulolytic Activity

The crude extract was assayed for cellulose activity by incubating crude enzyme solution with substrate and measuring amount of reducing sugar released. For general cellulase (FPase) activity, the substrate was Whatman No. 1 filter paper in 0.05M sodium citrate buffer (pH 4.8). For CMCase (endoglucanase) and avicelase (exoglucanase) activity determination, the substrates were 1 % (w/v) CMC sodium salt and 1 % avicel cellulose respectively, in 50mM sodium acetate buffer (pH 5.0). Boiling at 100 ^{0}C for 15 min stopped the reactions. The amount of reducing sugar released was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). Heat inactivated (100 ^{0}C for 15 minutes) supernatant and enzyme substrate controls were included. Glucosidase activity was assayed with p-NPG as the substrate according to Chen *et al*. (2010). All the enzyme assays were carried out in triplicate.

One unit of cellulase activity is defined as the amount of enzyme that released 1 µmol/ml of glucose equivalents per minute (Libmond and Savoie, 1993). Glucose was used as standard.

2.7.3. Hemicellulolytic Activity

The crude enzyme extract was assayed for xylanase activity by incubating 0.1 ml of crude enzyme solution with 0.1 ml of 0.5 % (wt v^{-1}) Beechwood xylan freshly suspended in 0.1 M pH 5.5 acetate buffer. The reaction mixture was incubated at 40 °C for 30 minutes and then completed to 1.0 ml by adding 0.8 ml distilled water (Sherief *et al*., 2010). The amount of reducing sugar released was determined by DNS method (Miller, 1959) against boiled enzyme using D-xylose as standard. All enzyme assays were carried out in triplicate. One unit of xylanase is defined as the amount of enzyme that catalysed the release of one µ mole of D-xylose per ml per min under the assay conditions.

2.8. Culturing of Microorganisms from the Gut of the Woodborers

One *D. mannii*, three *S. terebrans,* and three *Cirolana sp*. were surface sterilized with 70 % ethanol, rinsed in distilled water and allowed to air dry for 1 minute. Under aseptic conditions, the entire gut of *D. mannii* and three guts each from the two isopoda species were separately removed with a blade and a pair of tweezers and mixed with 1 ml 0.85 % sodium chloride to obtain inoculums.

Bacteria were cultivated in nutrient agar and subsequent culturing and plating carried out to obtain pure colonies. The cultures were incubated at 37 ^{0}C for 3 days. Fungi were cultivated on sabouraud dextrose agar (SDA) medium and incubated at 30 ^{0}C for 2-5 days. A single agar disc was cut from the actively growing colony margin of a culture to inoculate each assay medium in subsequent culturing and plating to isolate pure colonies.

2.9. Morphological and Molecular Identification of Bacteria

A light microscope with Leica ICC 50 camera (Leica Microsystems) connected to a windows computer was used to view the cells of each culture, and Gram stain was used in identifying the cultures. A colony was picked from a nutrient agar plate, placed in a drop of water on a slide and fixed by passing the slide through a flame. The Gram stain involved staining with crystal violet and then iodine for 30 seconds each, and counterstaining with safranin for 1 minute. This resulted in Gram-positive cells appearing purple and Gram-negative cells appearing pink.

2.9.1. DNA Preparation and 16S rRNA Fragment Analysis

Bacteria were lysed and proteins removed by digestion with proteinase K. Cell wall debris, polysaccharides and residual proteins were removed by selective precipitation with cetyltrimethylammonium bromide (CTAB), and purification completed by phenol: chloroform extraction. Finally the DNA was recovered from the resulting supernatant by isopropanol precipitation, resuspended in nuclease free water and stored at $^{-}20\ ^{0}C$.

The 16S rRNA gene was amplified using DNA polymerase (Invitrogen) using the following two bacterial specific primers; 27F gagtttgatcctggctcag and 1492R ggttaccttgttacgact. The polymerase chain reaction (PCR) was performed in a total volume of 25 µl using 13.7 µl distilled water, 1.0 µl dNTPs (10 mM), 2.5 µl PCR buffer, 2 µl MgCl (25 mM), 1.5 µl forward primer (10 mM), 1.5 µl reverse primer (10 mM), 0.8 µl Taq polymerase (5U/ µl) and 2.0 µl DNA template. The PCR profile included initial denaturation step at 95 ^{0}C for 5 minutes followed by 45 cycles of 95 ^{0}C (30 seconds) denaturation, 50 ^{0}C (30 seconds) annealing, 72 ^{0}C (2 minutes) extension and a final 72 ^{0}C (10 minutes) extension.

The PCR product was analysed on 1 % agarose gel electrophoresis containing 0.5 mg/ml ethidium bromide. The amplification patterns were viewed with DNA gel viewer (Herolab) and cleaned using QIAquick PCR purification kit according to manufacturer's instruction. The clean PCR product was sequenced with an ABI sequencing kit (Big Dye Terminator Cycle Sequencing, Applied Biosystems).

Preparation reaction for big dye PCR consisted of big dye (0.5 µl), 5X seq buffer (1.75 µl), 10 mM primer (1.0 µl), PCR product (4.0 µl) and water (2.75 µl). The PCR profile was 96^0C for 1 min followed by 25 cycles of 96 ^{0}C for 30 seconds, 50 ^{0}C for 30 seconds and 60 ^{0}C for 4 minutes. Clean up (ethanol/ sodium acetate precipitation of extended sequencing products) consisted of nuclease free water (24.5 µl), 3M NaOAc pH 5.2 (3.0 µl) and absolute ethanol (62.5 µl). To analyse the gene, 10 µl of HI-DI formamide (Applied Biosystems) was added and samples denatured at 96 ^{0}C for 3 min. The samples were electrophoresed on 3130xl Genetic Analyzer (Applied Biosystems®).

2.9.2. Bacterial DNA Sequence Analysis

The electropherograms were viewed, aligned and edited using BioEdit software. The 16S rRNA sequences were compared with the closest sequences deposited in the GenBank (NCBI) public database (http://www.ncbi.nlm.nih.gov/BLAST) using the BLASTn software.

2.10. Morphological and Molecular Identification of Fungi

Morphological identification of fungal isolates on SDA plates was done using macromorphological features (observed with naked eye or stereo microscope) and micromorphological features (seen through compound microscope). Macromorphological features included conidial colour, mycelia colour, reverse colour (colour under colonies), type of hyphae, and fruit bodies. Fungal isolates from SDA

plates were stained with lactophenol blue stain for studying micromorphological characteristics. The slides were observed under a microscope (Olympus BX51). The photographs of the slides were used in identification of the fungi. The shape and size of vesicles, shape, size and surface texture of conidia, stipe and ascospores differentiated various isolates from each other.

2.10.1. DNA Preparation and Internal Transcribed Spacer (ITS) Fragment Analysis

Extraction of DNA was performed using Quick-gDNA^{TM}Miniprep extraction kit (Zymo Research) according the method described by the manufacturer. The eluted DNA was stored at $^{-}20\ ^{0}C$.

The rDNA internal transcribed spacer (ITS) regions were amplified using Dream Taq DNA polymerase (Thermo Scientific) following the manufacturer's instructions with slight modifications. ITS primers used were; ITS1 5'-tccgtaggtgaacctgcgg-3' and ITS4 5'-tcctccgcttattgatatgc-3'. The PCR was performed in a total volume of 25 µl using 13.7 µl distilled water, 1.0 µl dNTPs (10 mM), 2.5 µl PCR buffer, 2 µl MgCl (25 mM), 1.5 µl forward primer (10 mM), 1.5 µl reverse primer (10 mM), 0.8 µl Taq polymerase (5U/ µl) and 2.0 µl DNA template. The PCR profile included initial denaturation step at 95 ^{0}C for 5 minutes followed by 45 cycles of 95 ^{0}C (30 seconds) denaturation, 50 ^{0}C (30 seconds) annealing, 72 ^{0}C (1minute) extension and a final 72 ^{0}C (10 minutes) extension.

The PCR product was analysed on 1 % agarose gel electrophoresis containing 0.5 mg/ml ethidium bromide. The amplification patterns were viewed with DNA gel viewer (Herolab) and cleaned using QIAquick PCR purification kit according to manufacturer's instruction. The clean PCR product was sequenced with an ABI sequencing kit (Big Dye Terminator Cycle Sequencing, Applied Biosystems).

Preparation reaction for big dye PCR consisted of big dye (0.5 µl), 5X seq buffer (1.75 µl), 10mM primer (1.0 µl), PCR product (4.0 µl) and water (2.75 µl). The PCR profile was 96 ^{0}C for 1 minute followed by 25 cycles of 96 ^{0}C for 30 seconds, 50 ^{0}C for 30 seconds and 60 ^{0}C for 4 minutes. Clean up (ethanol/ sodium acetate precipitation of extended sequencing products) consisted of nuclease free water (24.5 µl), 3M sodium acetate pH 5.2 (3.0 µl) and absolute ethanol (62.5 µl). To analyse the gene, 10 µl of HI-DI formamide was added and samples denatured at 96 ^{0}C for 3 min. The samples were electrophoresed on 3130xl Genetic Analyzer (Applied Biosystems®).

2.10.2. Fungal DNA Sequence Analysis

The electropherograms were viewed, aligned and edited using BioEdit software. To search for available matches with published sequences of other fungi, the sequences were aligned on BLASTn at the NCBI web site.

2.11. Phylogenetic Analysis

Fungal and bacterial nucleotide sequences were aligned with ClustalW and phylogenetic trees were constructed using the neighbour-joining method provided in the MEGA 5.2.2 software using maximum likelihood algorithm (Tamura *et al*., 2011). The significance of the junctions was established using the bootstrap method (1000 replicates) and junctions with greater than 50 % significance were labelled.

2.12. Induction of Woodborers' Gut Microbiota to Produce Enzymes

One *D. mannii*, three *S. terebrans,* and three *Cirolana sp*. were surface sterilized with 70% ethanol, rinsed in distilled water and allowed to air dry for 1 minute. Under aseptic conditions, the entire gut of *D. mannii* and the guts of the 2 isopod species were separately removed with a blade and a pair of tweezers and mixed with 1 ml 0.85 % sodium chloride to obtain inoculums.

Bacteria were cultivated in nutrient agar and subsequent culturing and plating carried out to obtain pure colonies. The bacterial isolates were cultured in liquid culture medium containing 0.1M citrate-phosphate buffer, 5 g/L carbon source (carboxymethylcellulose sodium salt (CMC)/ filter paper / beechwood xylan/ *Rhizophora* wood dust/ cellobiose/ avicel cellulose) and 0.2 g/L yeast extract. The cultures were incubated at 37 ^{0}C for 3 days. Solubilisation of insoluble carbon sources indicated degrading activity. Proteinase inhibitor PMSF (phenylmethylsulfonylflouride) was added to the culture media. The media were homogenized and centrifuged at 12,000 rpm for 30 minutes at 4 ^{0}C and the supernatant (crude extract) stored at $^{-}$20 ^{0}C.

Fungi were cultivated on sabouraud dextrose agar (SDA) medium and incubated at 30 ^{0}C for 2-5 days. A single agar disc was cut from the actively growing colony margin of a culture to inoculate each assay medium in subsequent culturing and plating to isolate pure colonies.

The fungal isolate were cultured in liquid medium containing 0.1M citrate-phosphate buffer, pH 4, 10 g/L yeast nitrogen base and 5 g/L carbon source (carboxymethylcellulose sodium salt (CMC)/ filter paper / beechwood xylan/ *Rhizophora* wood dust/ cellobiose/ avicel cellulose). Solubilisation of insoluble carbon sources indicated degrading activity. Proteinase inhibitor PMSF (phenylmethylsulfonylflouride) was added to the culture media. The culture media were homogenized and centrifuged at 12,000 rpm for 30 minutes at 4 ^{0}C and the supernatant (crude extract) stored at $^{-}$20 ^{0}C. The extracts were used in determination of lignocellulolytic activities as in 2.7 above.

2.13. *Dicyathifer mannii* Microbial Community

Wheat straw used as a natural substrate was dried, milled and sieved. 3 g of the substrate was moistened with 100 ml of 0.1M citrate- phosphate buffer pH 4.8 in 500 ml Erlenmeyer flasks and the medium was sterilized by autoclaving at 121 ^{0}C for 20 minutes. One *D. mannii* was surface sterilized with 70 % ethanol, rinsed in distilled water and allowed to air dry for 1 minute. Under aseptic conditions, the entire gut was removed with a blade and a pair of tweezers and mixed with 1 ml 0.85 % sodium chloride to obtain an inoculum. This was inoculated in the wheat straw wet culture in 500 ml Erlenmeyer flasks. The cultures were incubated at 27 ± 2 ^{0}C on a shaker at 180 rpm. The culture of microbial community growing in the liquid culture of wheat straw was harvested after 120 hours, and centrifuged at 12,000 rpm for 30 min at 4 ^{0}C. Supernatants were analyzed for cellulolytic and hemicellulolytic activity determination as well as bioethanol production.

2.14. Bioethanol Production

Ability of the culture filtrate of gut microbial community from *D.mannii* to biodegrade wheat straw into sugars for ethanol production was investigated according to Lever *et al*. (2010), with modifications. Wet cultures were prepared by mixing 50 ml filtrate and 50 ml of 3 % ground wheat straw in 0.1M sodium acetate buffer pH 5.0 and incubating at 50 ^{0}C for 1 hour. A control consisting of uninoculated culture filtrate was run with the experiment. 0.3 % yeast (*Saccharomyces cerevisiae*) was then added to the hydrolysed reaction mixture for fermentation and incubation was carried out in stoppered 500 ml Erlenmeyer flasks at room temperature (25 ± 2 ^{0}C) for 24 hours without agitation. After 24 hours, the mixture was homogenized, centrifuged and the supernatant was used for alcohol content determination using gas chromatography.

1µl standard sample with known concentration (5.7mg/100 ml deionized water) was injected into the gas chromatography (Shimadzu 2010). A FID detector measured the quantity of the components that exited the column. The standard sample peak retention time (appearance time) and area were recorded. 1µl of culture supernatant with unknown concentration of ethanol was then injected and peak retention time and area were compared to the standard sample to calculate the concentration. Ethanol concentrations were expressed as % (w/v).

2.15. Enzyme Purification and Characterization

2.15.1. Purification of Enzyme

Isolation and purification of enzyme was guided by the highest cellulolytic and hemicellulolytic activity (xylanase activity). The purification involved several steps with measurement of xylanase activity as well as total protein at each step. All the purification procedures were carried out at 4 ^{0}C unless otherwise stated. Finely powdered $(NH_4)_2SO_4$ was added to the culture supernatant to 80 % saturation. After 2 hours on ice, the precipitate formed was collected by centrifugation at 10,000xg for 30 min, resolubilized in 50 mM sodium acetate buffer (pH 5.5) and dialysed overnight in the same buffer to remove $(NH_4)_2SO_4$. After dialysis unsolubilized protein was removed by centrifugation at 10,000 × *g* for 10 min and the clear supernatant was concentrated using polyethylene glycol (PEG) 8000. The concentrated culture filtrate was loaded onto the gel filtration chromatography (Sephadex G-200) at room temperature (25 ^{0}C). The column was equilibrated and washed with 50 mM sodium acetate buffer; pH 5.5 at a flow rate of 0.3 ml/ minute. Active fractions were pooled and concentrated by PEG 8000. The concentrated enzyme (5 ml) was applied to an anion exchange DEAE–Sephacel equilibrated with 50 mM sodium acetate buffer (pH 5.5). The enzyme was eluted in the same buffer with a linear gradient of 0–1.0 M NaCl

in 400ml of the same buffer at a flow rate of 1ml/min. The active fractions were pooled, dialyzed and concentrated by lyophilization.

2.15.2. Determination of Protein Concentration

Protein was quantified by the Lowry method (Lowry *et al.,* 1951) using bovine serum albumin as standard.

2.15.3. Effect of pH and Temperature on Xylanase Activity

2.15.3.1. Optimum pH and pH stability

Measuring the xylanase activity of enzyme on xylan beechwood substrate at 25 ± 2 ^{0}C, and varying pH from 4.0 to 9.0 in universal buffer for 30 minutes evaluated optimum pH. Percentage of enzyme activity was estimated considering 100 % the highest activity detected in the assay. The effect of pH on stability of the enzymes was estimated by incubating enzyme and substrate in varying pH from 4.0 to 9.0 (universal buffer) with buffer solution for 24 hours at 25 ^{0}C and measuring residual activity. Residual activity was expressed as a percentage of the original activity.

2.15.3.2. Optimum temperature and thermostability

Optimum temperature was evaluated by measuring xylanase activity of enzyme on xylan beechwood substrate over temperature range from 30^0 to 70 ^{0}C at optimum pH. Percentage of enzyme activity was estimated considering 100 % the highest activity detected in the assay. Thermostability was measured by evaluating residual activity at optimum pH after incubation of enzyme and substrate for 120 minutes at various temperatures from 30-70 ^{0}C.

2.15.4. Molecular Weight Determination

Molecular weight was determined on a 15 % Sodium DodecylSulphate-polyacrylamide gel electrophoresis (SDS-PAGE) calibrated with molecular weight markers from 14.3 to 66 kDa (Dalton Mark VI®, Sigma). This was performed using the Bio-Rad Mini Protean II apparatus, employing the discontinuous buffer system of

Laemmli (1970). Proteins were detected by staining with Coomassie Brilliant Blue R-250 (Merck, Darmstadt, Germany).

2.15.5. Zymogram Analysis

This analysis was performed by the method of Tseng *et al.* (2002) with slight modification. 0.1 % of beechwood xylan was incorporated into running polyacrylamide gel (15 %). After electrophoresis, the gel was washed three times for 30 minutes at 4 ^{0}C in 100mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.0) containing 25 % isopropanol for the first two washes to remove SDS, and then incubated in the same buffer for 10 min at 37 ^{0}C. The zymogram was prepared by soaking the gel in 0.1 % Congo red solution for 15 minutes at room temperature (25 ± 2 ^{0}C), then washed with 1 M NaCl and 0.5 % acetic acid introduced into it to expose the xylanase active bands that contrasted the dark background.

2.15.6. Substrate Concentration and Suitability

The substrate specificity of the purified enzyme was determined using 1 % (w/v) low viscosity CMC, avicel and beechwood xylan as substrates (Mamo *et al.,* 2006; Gao *et al*., 2012). The reaction was carried out in 50 mM sodium acetate buffer (pH 5.0) at 50 °C for 30 minutes. The total amount of reducing sugars in the reaction was determined by the DNS method.

2.15.7. Kinetic Studies

Enzyme kinetic parameters of purified protein were obtained by measuring the rate of hydrolysis of xylan at various concentrations (0.1–2.5 %) (w/v) at optimum temperature for 30 min in 50 mM sodium acetate buffer (pH 5.0). The K_m (substrate concentration that yields a half-maximal reaction rate) and V_{max} (maximum velocity of the reaction at saturated substrate conditions) values were obtained under optimal conditions for enzyme activity. The K_m and V_{max} parameters were determined from the

linear regression from double-reciprocal plots according to Lineweaver and Burk (1934) and Michaelis-Menten plots using Microsoft® Excel 2011, using the following equation;

Michaelis–Menten equation:

$V = V_{max}[S]/K_m + [S]$

Taking the reciprocal gives

$1/V = K_m + [S]/V_{max}[S] = K_m/V_{max}\ 1/[S] + 1/V_{max}$

Where V is the reaction velocity (the reaction rate), K_m is the Michaelis–Menten constant, V_{max} is the maximum reaction velocity, and [S] is the substrate concentration.

2.16. Data Analysis

Rate of enzyme activity can be measured as disappearance of reactant or accumulation of product (Nelson and Cox, 2004). This was monitored spectrophotometrically as change in absorbance. To quantify enzyme activity;

Rate of reaction

= Concentration of substrate disappearing per unit time (mol L^{-1} min^{-1})

= Concentration of product produced per unit time (mol L^{-1} min^{-1})

Enzyme activity

= Moles converted per unit time

= Rate × reaction volume

1 international unit (IU or U) of enzyme activity is defined as enzyme quantity required for consuming 1 µmol of substrate or producing 1 µmol of product per minute (Manole *et al*., 2008). Data was represented as means ± S.D.

CHAPTER THREE

3.0 MANGROVE WOODBORERS: A CASE STUDY OF THE KENYAN COAST. IS THERE SPECIES PREFERENCE?

3.1. Introduction

The ability to consume wood as food (xylotrophy) is unusual among animals. In terrestrial environments, termites and other xylotrophic insects are the principle wood consumers while in marine environments wood-boring bivalves and isopods fulfil this role.

By their tunnelling, woodborers eventually cause the wood in which they live and on which they feed to disintegrate (Cragg *et al*., 2009). Bores and tunnels produced by the woodborers greatly increase the surface area available for fungal and bacterial decay process, which, in turn, accelerate the disintegration of wood into smaller pieces. The service rendered by woodborer community in the process of wood disintegration thereby cleansing the mangrove ecosystem from unwanted trash-wood is very significant, but when this phenomenon occurs on living vegetation, their role tends to be negative. They are then viewed as pests that warrant control measures.

The role played by woodborers along the Kenyan coast has not been studied, especially the biodeterioration of vegetation by various marine woodborers. They are also capable of extending their destructive activities to drift wood and wooden boats. Besides, there is need to monitor woodborer distribution and abundance in the mangroves because of their vulnerability in the event the sea level rises. Incase the forests recede or migrate inland, bench-marks can be established against which such changes can be measured keeping these organisms as indicator species. Therefore, the

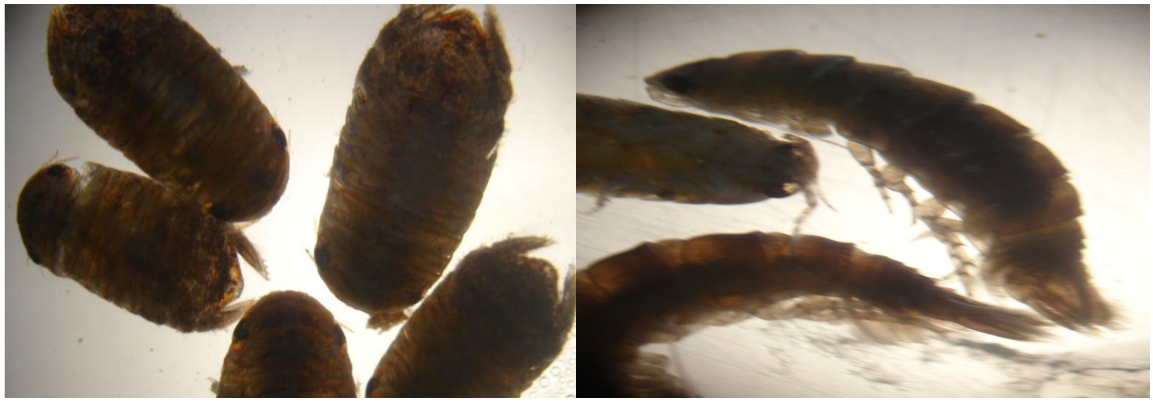
present investigation might be useful in mangrove ecosystem restoration, environmental conservation and management.

3.2. Mangrove Woodborers

3.2.1. Morphological and Molecular Identification of Marine Woodborers

The mangrove woodborers were found at temperatures ranging from 24 ^{0}C to 28.6 ^{0}C, salinity of between 35 ‰ and 36 ‰. They could withstand abrupt changes in water conditions, particularly temperature and salinity. Combining DNA barcoding and anatomotyping, 3 taxa of woodborers were identified from the three sampling sites (Figure 3.1). They were identified as *Dicyathifer (Teredo) mannii* (Wright, 1866), *Sphaeroma terebrans* (Bate, 1866) and *Cirolana sp.*

D. mannii morphological features included long cylindrical bodies with two small anterior valves and a paired of posterior pallet. They were enclosed in tunnels with calcareous lining. Cytochrome oxidase gene 1 (CO1) homology of *D. mannii* confirmed the morphological identification. The highest nucleotide similarity score was with *Dicyathifer mannii*, partial sequence, 99 % identity, NCBI accession No. JF899180.1. *S. terebrans* morphological features included an almost spherical body and round epistome, with prominent tubercles on the dorsal posterior part of the body and posterior part of the telson. *Cirolana sp.* were swift moving, with a body different from that of *S. terebrans* as it was slender and had no prominent tubercles, with a tapering epistome. It was not possible to obtain good sequences for *S.terebrans* and *Cirolana sp*. despite repeated attempts with various reaction conditions.



A B

C

Figure 3.1. Woodborer morphology: A, *Sphaeroma terebrans* (X10) Phylum Arthropoda, Class Crustacea, Order Isopoda. B, *Cirolana sp.* (X10) Phylum Arthropoda, Class Crustacea, Order Isopoda. C, *Dicyathifer (Teredo) mannii* Phylum Mollusca, Class Bivalvia, Family Teredinidae.

The burrows produced by the above three species differed (Figure 3.3 & 3.4). Those produced by *D. mannii* were long tunnels parallel to the grain. *S. terebrans* and *Cirolana sp.* (whenever present) co-existed in the same burrows. These were cylindrical on the wood surface with branches in the wood giving a honeycomb appearance.

Figure 3.3. Sphaeroma terebrans and Cirolana sp. burrows

Figure 3.4. *Dicyathifer mannii* burrows

Sampling for diversity was used where all the attacked plant species encountered were identified and counted, noting which woodborer attacked them. *S. terebrans* were found in hundreds, *D. mannii* in tens and few *Cirolana sp*. in a single attacked plant. 50 infested tree host species were observed in the three sampling sites of mangrove forest. *D. mannii* infested 25, *S.terebrans* 16 and *Cirolana sp.* 9 tree host species respectively (Table 3.1). The woodborer species were found multiple times showing strong host preferences, each being found exclusively (or nearly so) on only one of the three mangrove host species (Table 3.2). Severe damage of mangrove wood by the woodborers was also observed (Figure 3.5).

Table 3.1. Woodborers collected from mangrove wood

Wood borer	Host plant	No. of attacked plants			Total No. Observed
		Gazi bay	Tudor Creek	Mida Creek	
D. mannii	*R.mucronata*	8	7	5	20
	Sonneratia alba	2	1	1	4
	Avicennia sp.	1	0	0	1
	Total	11	8	6	25
S. terebrans	*R.mucronata*	0	0	0	0
	Sonneratia alba	0	0	0	0
	Avicennia sp.	7	5	4	16
	Total	7	5	4	16
Cirolana sp.	*R.mucronata*	0	0	0	0
	Sonneratia alba	0	0	0	0
	Avicennia sp.	5	4	0	9
	Total	5	4	0	9
	Grand total	23	17	10	50

Woodborers collected from mangrove wood at Gazi Bay, Tudor Creek and Mida Creek along the Kenyan coast.

Most of the specimens identified were extracted from dead wood; however, *D. mannii* was also found in living roots of *Rhizophora.* Generally they had entered dead wood then invaded living tissue. Comparison of the woodborers found in mangrove habitats between the three study sites showed few strong differences. In all the three sampling sites, only three species of mangroves were attacked and by the same woodborers. However, *Cirolana sp.* was not encountered at Mida Creek. *S. terebrans* and *Cirolana sp.* were found exclusively on *Avicennia sp.*, whereas *D. mannii* was found mainly on *R. mucronata* but also on *S. alba*.

3.2.2. Species Host Specificity

Host specificity can be defined in many different ways (Thomas *et al*., 2005). Here the term host specificity is used to refer to the ability of woodborers to develop and live within the wood of particular host mangrove species, but to have reduced or no development in the wood of others. Analysis of data obtained for the three taxa of woodborers from the 50 attacked trees in the three study sites showed strong evidence for tree host preference. Within mangrove habitats of the study sites, we found significant host preferences for all three woodborer species that were sufficiently common for statistical testing (Table 3.2).

D. mannii was much more common on *R. mucronata* (and much less common on *S. alba*) than expected by chance. *S. terebrans* and *Cirolana sp.* (whenever present) were more common on *A. marina* than expected. Chi-square test analysis of the data from each of the three study sites for the three most common mangrove species (*R. mucronata, A. marina,* and *S. alba*) was significant with P-values ranging from 0.0007 to 0.0067 (Table 3.2). *D. mannii* and *S. terebrans* were present across the three sites whereas *Cirolana sp.* was observed at Gazi Bay and Tudor Creek only.

Table 3.2. Host preference of various species of mangrove woodborers

Woodborers	Gazi bay					Tudor Creek					Mida Creek				
	R. mucronata	*S. alba*	*Avicennia sp.*	X2(df)	p-value	*R. mucronata*	*S. alba*	*Avicennia sp.*	X2(df)	p-value	*R. macronata*	*S. alba*	*Avicennia sp.*	X2(df)	p-value
D. mannii	**8**	2	1			**7**	1	0			**5**	1	0		
S. terebrans	0	0	**7**			0	0	**5**			0	0	**4**		
Cirolana sp	0	0	**5**			0	0	**4**			-	-	-		
Total	8	2	13	19.30(4)	0.0007	7	1	9	17.00(4)	0.0019	5	1	4	10.00(2)	0.0067

Host (*Rhizophora mucronata, sonneratia alba* and *Avicennia sp*) preference of various species of mangrove woodborers. P-values are for within study sites tests and indicate the probability of observing a sample statistic as extreme as the test statistic (significant at $\alpha=0.05$). Emboldened values indicate that a species is significantly more common on that host than expected.

A

B

C

D

Figure 3.5. Mangrove wood deterioration by borers: A, heavily fragmented piece of wood with vacant tunnels created by extensive teredinid boring activity; B, C and D, tree trunks severely damaged by isopod borers.

3.3. Discussion and Conclusions

This study identified three mangrove woodborer taxa in the mangrove forests along the Kenyan coast. It also demonstrates that there is strongly host species-preferring tendency in the woodborers along the Kenyan coast. Chi-square tests indicated that the 50 observed woodborers showed a significant degree of host selectivity, infecting their most commonly used host species more frequently than expected by chance. The host specific *S. terebrans* and *Cirolana* occur only on *Avicennia sp*. *D. mannii* occurs mostly on *Rhizophora* plants, but also on *Sonneratia*. *Cirolana sp.* appears to be uncommon occurring only in 9 out of 50 observations. The two most abundant woodborer species were *D.mannii*, and *S. terebrans*, each found in 50 % and 32 % of the observed infested mangrove host plants respectively.

The observed specificity is not a function of local host density, as the studied mangrove forests were host abundant. Host specificity of borers can probably be attributed to the interrelationship that may exist between microorganisms and marine borers. The microflora that colonise wood substratum are known to influence and facilitate larval settlement of sedentary marine organisms (Mitchell and Kirchman, 1984). The woodborers might require certain specific types of microflora, especially fungi, to induce settlement, attachment and metamorphosis (Santhakumaran and Sawant, 1998). Besides, mangrove plant species that showed no attack could be having natural immune mechanisms or stress-induced responses that prevented attack. This need to be investigated as it may provide opportunities for evolving methods of controlling wood-boring organisms and curbing the problem of biodeterioration.

The choice of study sites is a representative of the Kenyan coast, Mida Creek (North coast), Tudor Creek (island) and Gazi Bay (south coast). The three study sites are separated by 50-100 km but the difference in woodborers in mangrove habitat was

small. Except for *Cirolana sp.*, there was overlap with woodborers collected from mangroves of the three study sites.

Sphaeromatidae and Teredinidae species that we collected have been described elsewhere in the world on multiple hosts. *Sphaeroma terebrans* (Bate, 1866) has been reported to live mainly in aerial roots of the mangrove *Rhizophora mangle* in tropical and subtropical regions. *Sphaeroma* taxa have also been reported to be present in single holes bored in the pneumatophores of another mangrove tree, *Sonneratia alba* (Harrison and Holdich, 1984; Villalobos *et al*., 1985; Barrati *et al.*, 2011). In this study however, *S. terebrans* was exclusively observed in *Avicennia sp*. According to Cragg (1993, 2007) teredinids inhabit a *Rhizophora-dominated* forest in Papua New Guinea. In addition, Brearly *et al*. (2003) reported that *D. mannii* was found in *Rhizophora*, *Avicennia* and *Bruguiera* mangrove plants in Australia but was most abundant in *Rhizophora*. This is in agreement with this study as *D. mannii* was observed mainly on *Rhizophora sp.*

In all the observations and collections, *Cirolana sp.* was not found in burrows on their own. They were always found in association with *S. terebrans*. Cirolanidae have not been reported to be woodborers. Since they are found in *S. terebrans* burrows, they could be mistaken to be woodborers while in essence, they could be scavengers. This could mean that they are *S. terebrans* natural enemies. Keable (2001) describes scavenging species of *Cirolana* from the Australian coast. Bowman *et al.* (1981) also reported that an exotic *Cirolana sp*., *C. arcuata*, was found in the company of *Sphaeroma sp*., *S.quoyana* in the San Francisco bay, North America.

This study has two limitations: first, without experimental manipulations we are unable to distinguish between preferences for a particular host or site and aversion to

others. Second, we were unable to determine relative densities of all roots (proproots, pneumatophores), stems and branches of potential host plants, whether or not they had woodborers. Using overall relative abundances is the preferred approach for testing host specificity (Gilbert and Sousa, 2002; Gilbert *et al*., 2007). But by observing all the infested tree host species encountered, and noting which woodborer was found within them, relative frequencies of host species that supported the particular woodborers were tested, and the results should be robust. Besides, the collection was spread over a long period of time and therefore was not affected by seasonal patterns.

This study recommends monitoring of woodborer community to give an overall idea of the intensity of the problem and enable removal of the source of infestation at the very onset. It also recommends study of natural bio-resistance of the mangrove plants that show no attack as well as investigations on effect of fungal metabolite on larval settlement in the fungi- borer interrelationships and specificity theory. Lastly, it would be rewarding if further studies were done to establish the possibility of the *Cirolana sp.* being natural enemies of *S. terebrans* as they could be used in the natural control of wood-boring pests.

CHAPTER FOUR

4.0 LIGNOCELLULOLYTIC ACTIVITIES OF CRUDE GUT EXTRACTS OF MARINE WOODBORERS *DICYATHIFER MANNII* AND *SPHAEROMA TEREBRANS*

4.1. Introduction

Lignocellulolytic activity has been investigated in several organisms including bacteria and several fungi (Fakoussa and Frost, 1999; Arora *et al*., 2002; Zouri-Mechichi *et al*., 2006; Lopez *et al*., 2007; Quaratino *et al*., 2007; Sahay *et al*., 2008; Desai *et al*., 2011; Bholay *et al*., 2012). Cellulolytic and Xylanolytic enzymes have also been demonstrated from bacteria, several fungi and termite gut (Gong *et al*., 1977; Schafer *et al*., 1996; Suna and Antranikian, 1997; Romero *et al*., 1999; Karnchanatat *et al*., 2008). Enzymatic properties and primary structures of many cellulases have been investigated and cellulase genes from widely differing origins have been cloned and sequenced (Hamada *et al*., 1999; Tokuda *et al*., 2002; Karnchanatat *et al*., 2008; Tokuda *et al*., 2009). However, little is known about the lignocellulolytic enzymes from marine woodborers.

The present investigation reports on lignocellulolytic activities of gut extracts of two marine woodborers, *D.mannii* and *S. terebrans*, for potential in industrial applications.

4.2. Determination of Lignocellulolytic Activities of Crude Gut Extracts

All the gut extracts from the two woodborers showed ligninolytic, cellulolytic and xylolytic activities. Generally, among the lignin modifying enzymes, there was a high lignin peroxidase activity in the crude gut extracts obtained from all the woodborers screened whereas laccase and manganese peroxidase activities were relatively low. MnP activity ranged from 1.64 ± 0.02 U/L in *S. terebrans* from Gazi Bay to 4.73 U/L

in *S. terebrans* from Tudor Creek. This was the lowest ligninolytic activity. It was followed by Lac activity ranging from 0.36 ± 0.03 U/L in *S. terebrans* from Gazi Bay to 21.16 ± 0.59 U/L in *D. mannii* from the same study site. LiP activity was the highest ranging from 17.65 ± 0.08 U/L in *D. mannii* from Tudor Creek to 34.65 ± 0.12 U/L in *D. mannii* from Gazi Bay (Table 4.1). The highest ligninolytic activity (LiP activity of 34.65 U/L) exhibited 68.9 % discolouration.

Table 4.1. Lignin modifying enzyme activity of crude gut extracts

Lignin modifying enzyme activity of crude gut extracts (U/L)			
Isolate	Lac (A_{530})	LiP (A_{310})	MnP (A_{610})
DT	8.75 ± 0.23	17.65 ± 0.08	3.76 ± 0.02
DG	21.16 ± 0.59	34.65 ± 0.12	3.15 ± 0.02
DM	10.32 ± 0.28	18.26 ± 0.26	2.41 ± 0.00
ST	4.44 ± 0.32	22.67 ± 0.61	4.73 ± 0.00
SG	0.36 ± 0.03	25.14 ± 0.12	1.64 ± 0.02
SM	3.18 ± 0.22	21.99 ± 0.65	3.71 ± 0.00

Data is mean of triplicates ± SD; Woodborers D, *Dicyathifer mannii* and S, *Sphaeroma terebrans*; sampling sites T, Tudor Creek, G, Gazi bay and M, Mida Creek; enzymes Lac, Laccase, LiP, Lignin Peroxidase and MnP, Manganese Peroxidase.

There was generally high cellulolytic and hemicellulolytic activities by *D. mannii sp.* from all the three sampling sites (Table 4.2). Fpase activity ranged from 2.22 ± 0.11 U/ml in *S. terebrans* from Gazi Bay to 34.78 ± 0.71 U/ml in *D.mannii* from Tudor Creek. Avicelase activity ranged from 1.78 ± 0.24 U/ml in *S. terebrans* from Tudor Creek to 17.5 ± 1.4 U/ml in *D.mannii* also from Tudor Creek. β-glucosidase activity ranged from 10.77 ± 0.74 U/ml in *S. terebrans* from Mida Creek to 22.31 ± 0.46 U/ml in *D. mannii* from Gazi Bay. Xylanase activity ranged from 6.66 U/ml in *S. terebrans* from both Mida Creek and Gazi Bay to 35.52 ± 1.54 U/ml in *D. mannii* from Gazi Bay. CMCase activity ranged from 2.59 ± 0.13 U/ml in *S. terebrans* from Gazi Bay to 50.7 ± 1.51 U/ml in *D. mannii* from the same study site. The highest cellulolytic/hemicellulolytic activity was therefore endoglucanase (CMCase) activity of 50.7 U/ml in *D. mannii* sampled from Gazi Bay. This was followed by xylanase activity of 35.5 U/ml in *D. mannii* also sampled from Gazi Bay.

Table 4.2. Cellulolytic and hemicellulolytic activity spectrum of gut extracts

Cellulolytic /hemicellulolytic activity spectrum of woodborers' crude gut extracts (U/ml)					
Woodborer	Fpase	CMCase	Avicelase	β-glucosidase	Xylanase
DT	34.78 ± 0.71	37.37 ± 1.83	17.5 ± 1.4	17.39 ± 0.21	26.64 ± 1.15
DG	34.41 ± 1.02	50.7 ± 1.51	15.02 ± 0.59	22.31 ± 0.46	35.52 ± 1.54
DM	31.82 ± 0.13	27.75 ± 1.64	15.8 ± 0.42	13.91 ± 0.61	28.86 ± 1.54
ST	6.29 ± 0.50	8.51 ± 0.65	1.78 ± 0.24	11.77 ± 0.92	8.88 ± 0.39
SG	2.22 ± 0.11	2.59 ± 0.13	2.00 ± 0.33	11.47 ± 0.68	6.66 ± 0.00
SM	2.96 ± 0.17	12.95 ± 1.03	3.63 ± 0.37	10.77 ± 0.74	6.66 ± 0.00

Data is mean of triplicates ± SD; woodborers D, *Dicyathifer mannii* and S, *Sphaeroma terebrans*; sampling sites T, Tudor Creek, G, Gazi bay and M, Mida Creek.

4.3. Discussion and Conclusions

This study demonstrated that the gut extracts of marine woodborers *D. mannii* and *S. terebrans* exhibit lignocellulolytic activities. The highest activities (LiP and endoglucanase) were shown by *D. mannii*, a wood boring teredinid. *D. mannii* gut extract had better overall lignocellulolytic activities than that of *S. terebrans*. The highest lignocellulolytic activity exhibited was endoglucanase (CMCase) of 50.7 ± 1.51 from *D. mannii* sampled from Gazi Bay.

In a recent study, Kalmis *et al*. (2008) investigated ligninolytic enzyme activities of different fungal species (six commercial and 13 wild) in solid and liquid culture media. Highest Lac activity of 941.66 ± 1.67 U/L was obtained from wild *Pleurotus ostreatus*-4 (PO-4) after 12 days cultivation, the highest MnP activity of 267.63 ± 0.55 U/L was from wild *P. eryngii* (PE-1) after 10 days and the highest LiP activity of 17.84 ± 0.11 U/L was from a commercial strain of *P. sajor-caju* (PS) after 14 days cultivation.

While Lac and MnP activity in the current study is not comparable with the activity maxima obtained in their study, the highest LiP activity of 34.65 ± 1.16 U/L obtained in this study is much higher (two times) than that from their LiP activity maxima. Also, in another study to investigate bacterial LiP activity on industrial effluents (Bholay *et al.,* 2012), the activity ranged between 30 % to 76 % discolouration while in this study the highest LiP activity exhibited 68.9 %. In addition, the highest endoglucanase activity of 50.7 U/ml observed in this study is appreciable as it is comparable to a purified endoglucanase activity (50.2 U/mg) from *Penicillium notatum NCIM NO-923* produced under mixed solid-state fermentation of waste

cabbage and Bagasse (Das *et al.,* 2012). Therefore *D. mannii* is implicated as a good source of these enzymes for industrial use.

The lignocellulolytic activities observed in this study are as a result of lignocellulolytic enzymes, but it is not clear whether the enzymes are produced by gut microbiota symbionts or they are endogeneous, produced by glands in the digestive tract. Ability of Teredinidae to feed on wood is thought to depend on intracellular bacterial endosymbionts contained within specialized cells (bacteriocytes) of their gills. These bacterial endosymbionts are thought to produce cellulolytic enzymes that aid the host in digestion of wood (Distel, 2003), and they are known to fix nitrogen (Lechene *et al.,* 2007; Waterbury *et al.,* 1983) that may supplement the host's nitrogen deficient diet. Lignin degrading enzymes are essentially extracellular in nature due to the large and complex structure of lignin that cannot enter the cell for intracellular action. This implies that enzymes that digest lignin are either produced by microorganisms in the gut or are secreted by glands in the digestive tract and released into the gut for action. Therefore, there is need to culture microorganisms from the gut of these woodborers and investigate their lignocellulolytic activities. For lignin modifying enzymes, these microorganisms could be potential sources of commercial enzymes for industrial use in biobleaching of pulp, decolouration of textile dyes and bioremediation of polluted environment (Castillo *et al.,* 1997). The microorganisms could also be the source of cellulolytic and xylanolytic enzymes for use in animal feed, manufacture of bread, ethanol and xylitol production among other uses.

CHAPTER FIVE

5.0 CULTURABLE GUT MICROBIOTA OF MARINE WOOD BORING INVERTEBRATES *DICYATHIFER MANNII*, *SPHAEROMA TEREBRANS* AND *CIROLANA SP*.

5.1. Introduction

Chapter 4 demonstrated lignocellulolytic activities of *D. mannii* and *S. terebrans* crude gut extracts. The enzymes that digested lignocellulose could either be produced by microorganisms in the gut or were secreted by glands in the digestive tract and released into the gut for action. Therefore, there was need to culture microorganisms from the guts of these woodborers and investigate their lignocellulolytic activities. The present investigation cultured bacteria and fungi from *D. mannii*, *S. terebrans* and *Cirolana sp*. guts and determined their biodiversity.

5.2. Marine Woodborers Culturable Gut Microbiota

5.2.1 Morphological and Molecular Identification of Bacteria

Four different species of bacteria were identified; *Lysinibacillus fusiformis* from *S. terebrans* and *Cirolana sp*., *L. sphaericus* and *L. xylanilyticus* from *Cirolana sp* whereas *L. boronitolerans* was obtained from *D. mannii* and *S. terebrans* guts. They were identified by the fact that they were aerobic, rod-shaped and gram-positive cells. *Lysinibacillus* are Gram-positive, rod-shaped, and round-spore-forming bacterial genus of the family Bacillaceae. 16S rRNA fragment analysis confirmed the morphological identification of the bacteria (Table 5.1), and their gene sequence phylogenetic analysis is shown in Fig. 5.1.

Table 5.1. Identification of bacteria from woodborers' gut

Best blastn 16S rRNA Match			
Isolate	**NCBI ACCESSION**	**SPECIMEN**	**% IDENTITY**
St 1	NR 041276.1	*Lysinibacillus boronitolerans*	98
St 2	NR 042072.1	*Lysinibacillus fusiformis*	98
St 3	NR 042072.1	*Lysinibacillus fusiformis*	98
Csp 1	NR 042072.1	*Lysinibacillus fusiformis*	98
Csp 2	KF 208475.1	*Lysinibacillus xylanilyticus*	99
Csp 3	JX 144693.1	*Lysinibacillus sphaericus*	99
Dm 1	NR 041276.1	*Lysinibacillus boronitolerans*	98

Best blastn matches of the 16S region to database sequences are shown; bacteria isolated from St, *Sphaeroma terebrans*, Csp, *Cirolana sp.*, and Dm, *Dicyathifer mannii*.

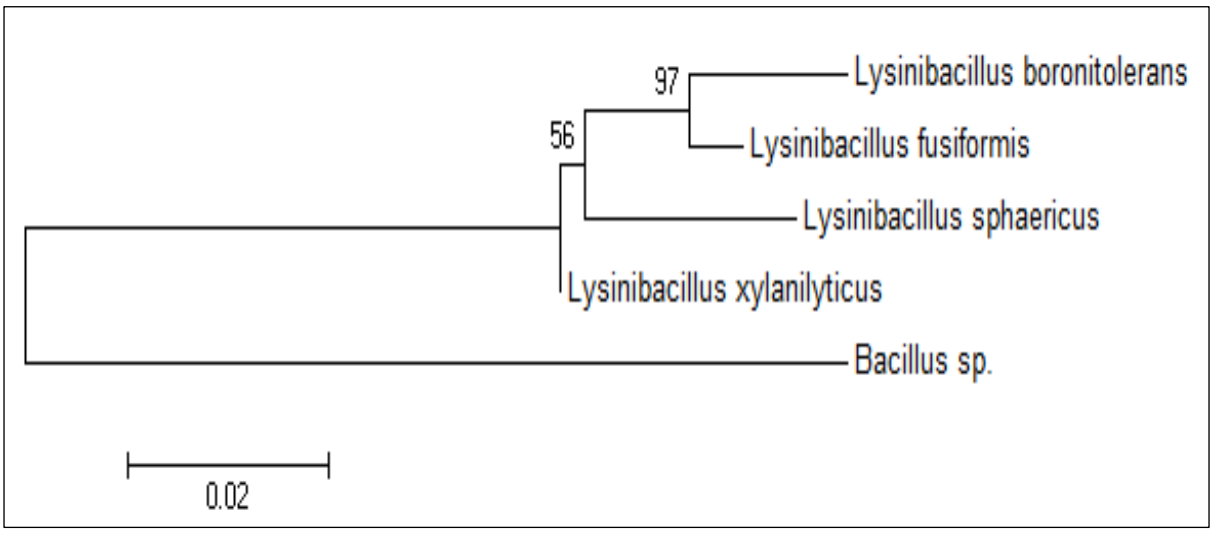


Figure 5.1. Phylogenetic tree based on 16S rRNA gene sequence

Neighbour-Joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of the bacterial isolates and some other related taxa. Bootstrap values (>50 %) based on 1000 replications are shown at branch nodes. *Bacillus sp*. (Accession No. AJ000648.1) was used as an out-group. Bar, 0.02 substitutions per nucleotide position.

5.2.2 Morphological and Molecular Identification of Fungi

Aspergillus niger strains and *Botryotinia fuckeliana* were isolated from *S. terebrans* gut while *A. costaricaensis* and *A. fumigatus* were isolated from *Cirolana sp. A. niger, Neosartorya fischeri, A. fumigatus* and *Penicillium sp.* were isolated from *D. mannii*. The rDNA internal transcribed spacer (ITS) region analysis confirmed the morphological identification of the fungi (Table 5.2). Their subsequent phylogenetic analysis is shown in Fig. 5.2.

Table 5.2. Identification of fungi from woodborers' gut

Best blastn ITS Match				
Isolate	**Taxon**	**NCBI ACCESSION**	**SPECIMEN**	**% IDENTITY**
St 1	*Aspergillus sp.*	NT 166520.1	*Aspergillus niger*	96
St 2	*Aspergillus sp.*	NT 166520.1	*Aspergillus niger*	99
St 3	*Aspergillus sp.*	NT 166520.1	*Aspergillus niger*	95
St 4	*Botryotinia sp.*	NW 001814456.1	*Botryotinia fuckeliana*	100
St 5	*Aspergillus sp.*	NT 166520.1	*Aspergillus niger*	95
Csp 1	*Aspergillus sp.*	NR103604.1	*Aspergillus costaricaensis*	99
Csp 2	*Aspergillus sp.*	NC 007197.1	*Aspergillus fumigatus*	99
Dm 1	*Aspergillus sp.*	NT 166520.1	*Aspergillus niger*	98
Dm 2	*Aspergillus sp.*	NT 166520.1	*Aspergillus niger*	98
Dm 3	*Aspergillus sp.*	NW 001509767.1	*Neosartorya fischeri*	99
Dm 4	*Aspergillus sp.*	NT 166520.1	*Aspergillus niger*	98
Dm 5	*Aspergillus sp.*	NC 007197.1	*Aspergillus fumigatus*	99
Dm 6	*Penicillium sp*	nd	nd	nd

Best blastn matches of the internal transcribed spacer (ITS) region to database sequences are shown; fungi isolated from St, *Sphaeroma terebrans*, Csp, *Cirolana sp*, and Dm, *Dicyathifer mannii*; nd, not determined due to consistent failure to obtain good sequences despite repeated attempts with various reaction conditions.

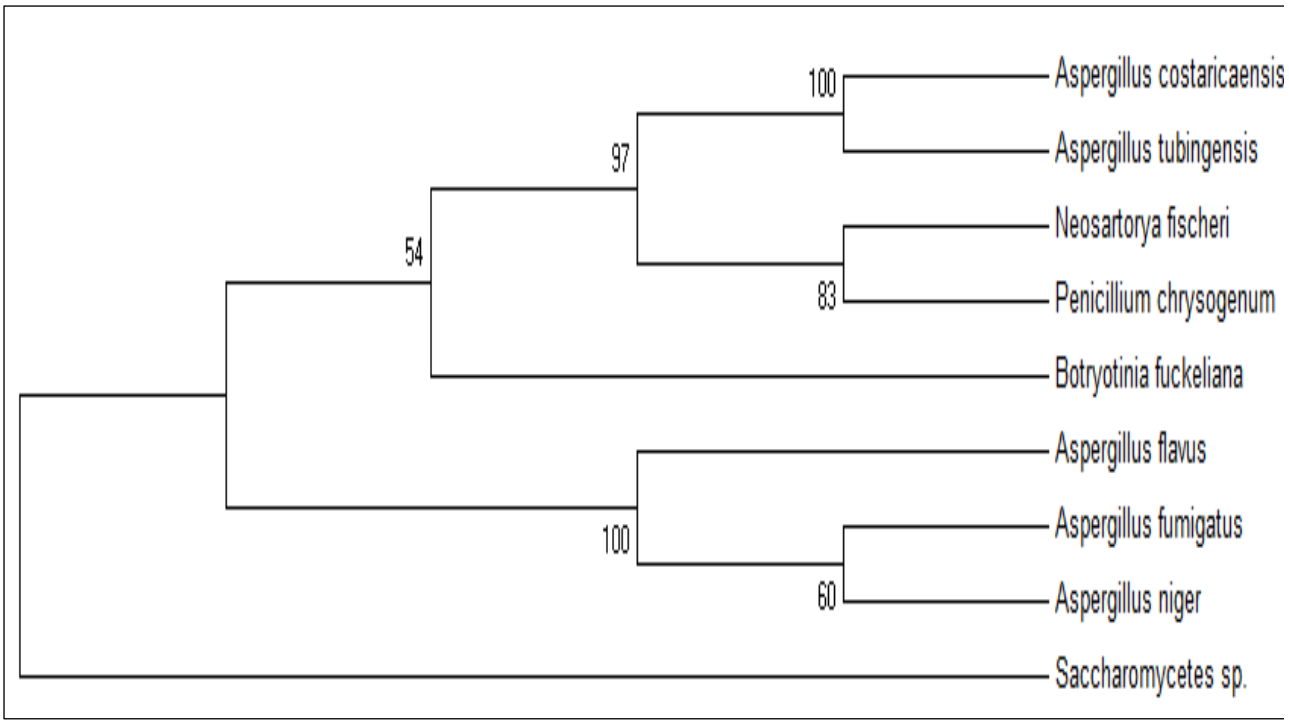


Figure 5.2. Phylogenetic tree based on ITS gene sequence

Neighbour-Joining phylogenetic tree based on ITS gene sequences showing the positions of the fungal isolates and some other related taxa. Bootstrap values (>50 %) based on 1000 replications are shown at branch nodes. *Saccharomycetes sp*. (Accession No. JQ993372.1) was used as an out-group.

5.3. Discussion and Conclusions

Bacteria isolated from the digestive tracts of the woodborers belong to the genus *Lysinibacillus*. Four strains, namely *L. boronitolerans* (from *D. mannii* and *S. terebrans*), *L. fusiformis* (from *S. terebrans* and *Cirolana sp.*), *L. sphaericus* and *L. xylanilyticus* (both from *Cirolana sp.*) had similarity to known 16S rRNA sequences of 98–99 % (Table 5.1).

It is possible that bacteria establish a mutual symbiosis within the digestive tract of the woodborers. Bacteria inhabit the soil or wood and develop considerably when there are easily degradable organic nutrients. The bacterial community inside the digestive tract of woodborers may pertain to at least four physiological groups: plant growth promoters, free-living nitrogen fixers, biocides and phosphate solubilizers. Lysinibacillus is commonly found in soil (Ahmed *et al*., 2007) and has been isolated from plant tissues (Melnick *et al*., 2011), from fermented plant seed products (Parkouda *et al*., 2010, Nam *et al*., 2012), from insect gut (Maji *et al*., 2012), and even from puffer fish liver specimens (Wang *et al*., 2010). *L. xylanilyticus* is associated with forest humus (Lee *at al*., 2010). The diversity of bacterial communities within the digestive tracts of woodborers may depend on host plant, climate, soil type and organic matter. Therefore, existence of bacterial groupings symbiotically associated with woodborers gut is proposed.

Fungi isolated from the digestive tracts of the woodborers were different strains of Ascomycetes. *A. niger* was isolated from the digestive tracts of *S. terebrans* and *D.mannii*. In addition, *N. fischeri*, *Penicillium sp*. and *A. fumigatus* were isolated from *D. mannii* digestive tract whereas *A. costaricaensis* and *A. fumigatus* were isolated from *Cirolanna sp*. digestive tract. They had similarity to known ITS sequences of 95–100 % (Table 5.2).

The fungi in the woodborers gut may have originated from the wood they ingest or the soil in which the host plants grow. Many *Penicillia* are soil fungi, and grow in a variety of organic substances, particularly dead plant materials (Hamlyn *et al*., 1987). Lee *et al*. (2011) has reported wood-inhabiting *Penicillium* strains. Lindblad, 2000; Gilbert *et al*., 2002; Gilbert & Sousa, 2002 and Ferrer & Gilbert, 2003 have reported a large number of fungi that inhabit wood where they play the role of wood decay. El

Shanshoury *et al*. (1994) reported a number of cellulolytic microorganisms associated with wood boring marine isopods; amongst these were *A. niger* and *A. candidus*. Ectomycorrhizal fungi improve host performance by enhancing nutrient and water uptake from the soil and protect host roots from pathogens and toxic compounds (Smith and Read, 1997).

Not many species of symbiotic microorganisms were found in the gut of woodborers in this study. The mechanism of wood digestion in marine bivalves differs from that found in terrestrial wood consumers. Terrestrial organisms that consume wood as food contain within their digestive tracts communities of symbiotic microorganisms that are thought to aid in the digestion and metabolism of wood (Haigler and Weimer, 1991). Wood-boring bivalves appear to lack such highly developed microbial communities within their guts (Liu and Walden, 1970). In the case of teredinids, the ability to feed on wood is thought to depend on intracellular bacterial endosymbionts contained within specialized cells (bacteriocytes) of their gills. These bacterial endosymbionts are thought to produce cellulolytic enzymes that aid the host in digestion of wood (Distel, 2003). The bacteria are also known to fix nitrogen (Waterbury *et al*., 1983; Lechene *et al*., 2007) that may supplement the host's nitrogen deficient diet. These intracellular bacteria constitute a consortium of closely related species (Distel *et al*., 2002; Luyten *et al*., 2006), only one (*Teredinibacter turnerae*) has been grown in pure culture. This species was however not isolated in our study. Members of Xylophagainae have also been shown to harbor bacterial endosymbionts within their gills (Distel and Roberts, 1997) although none has yet been cultivated (Distel *et al*., 2011).

Cragg *et al*. (1999) reported wood degrading tunnelling bacteria, and soft-rotting ascomycete and deuteromycete fungi in the wood ingested by boring crustaceans. The

gut contents of *Limnoria* (a wood-boring isopod) consisted of wood particles and associated microorganisms (bacteria and fungi) that were associated with tunnelled wood. However, the authors did not specify whether these microorganisms were wood-degraders.

The growth of bacteria and fungi inside the digestive tracts of woodborers is of industrial importance in biodegradation of lignocellulose. Investigation of the functional characteristics and role in the host organism is required to confirm the symbiotic status of the microorganisms associated with the woodborers digestive tract.

CHAPTER SIX

6.0 LIGNOCELLULOLYTIC ACTIVITIES OF CULTURABLE MARINE WOODBORERS' GUT MICROBIOTA: POTENTIAL IN BIOETHANOL PRODUCTION

6.1. Introduction

Pure bacterial and fungal isolates from *D. mannii*, *S. terebrans* and *Cirolana sp*. guts, as well as mixed cultures for each the woodborers, were induced to produce lignocellulolytic enzymes. Substrates used for induction were carboxymethylcellulose sodium salt (CMC), Whatson No. 1 filter paper (FP), beechwood xylan, *Rhizophora* wood dust, D (+)-cellobiose and avicel cellulose. These were in media containing single substrates as well as mixed substrate media. The focus of the present work was to explore the lignocellulolytic activities of culturable bacteria and fungi from the gut of woodborers. Also, since *D. mannii* had shown to have the most efficacious lignocellulolytic extracts, the ability of its gut microbial community to biodegrade wheat straw for subsequent ethanol production was investigated.

6.2. Lignocellulolytic Activities of Woodborers' Gut Microbiota

6.2.1. Culturable Marine Woodborers' Gut Microbiota

Host species identification (Chapter 3), bacterial and fungal identification (Chapter 5) are summarized in Table 6.1.

Table 6.1. Bacteria and fungi isolated from woodborers' guts

	Host Species	Isolate	Scientific Name
Bacterial Isolates	*Sphaeroma terebrans*	1a	*Lysinibacillus boronitolerans*
		1b	*Lysinibacillus fusiformis*
		1c	*Lysinibacillus fusiformis*
	Cirolana sp.	2a	*Lysinibacillus fusiformis*
		2b	*Lysinibacillus xylanilyticus*
		2c	*Lysinibacillus sphaericus*
	Dicyathifer mannii	ma	*Lysinibacillus boronitolerans*
Fungal Isolates	*Sphaeroma terebrans*	1a	*Aspergillus niger*
		1b	*Aspergillus niger*
		1c	*Aspergillus niger*
		1d	*Botryotinia fuckeliana*
		1e	*Aspergillus niger*
	Cirolana sp.	2a	*Aspergillus costaricaensis*
		2b	*Aspergillus fumigatus*
	Dicyathifer mannii	ma	*Aspergillus niger*
		mb	*Aspergillus niger*
		mc	*Neosartorya fischeri*
		md	*Aspergillus niger*
		me	*Penicillium sp.*

6.2.2. Induction of Woodborers' Gut Microbiota to Produce Enzymes

The cellulosic substrates successfully induced the microorganisms to produce cellulolytic and xylanolytic enzymes, but not ligninolytic enzymes. The crude enzyme extracts from these microorganisms were screened for lignocellulolytic activities and the activities compared between different carbon sources.

6.2.3. Lignocellulolytic Activities of Gut Microbiota Extracts

6.2.3.1. Ligninolytic activity assays

There was generally low lignin modifying enzyme activity in both bacterial and fungal isolates (data not shown).

6.2.3.2. Cellulolytic and Hemicellulolytic activities of the crude bacterial extract

Bacterial isolates showed significantly high cellulolytic (β-glucosidase) and hemicellulolytic (xylanase) activities. β-glucosidase activity was the highest (94.55 U/ml) shown by *Sphaeroma terebrans* isolate 1 (*L. boronitolerans*) cultured in a medium containing avicel cellulose substrate as a carbon source. Xylanase activity was also relatively high (up to 91.7 U/ml) by isolates in media containing cellobiose and xylan beechwood substrates. Highest Xylanase activity was shown by all the 3 bacteria species (*L. fusiformis, L. xylanilyticus and L. sphaericus*) isolated from *Cirolana sp*. in media containing cellobiose substrate (Table 6.2).

Table 6.2. Cellulolytic and hemicellulolytic activity of bacterial isolates

Culture	Isolate	β-Glucosidase	Xylanase
C_1W	*L. fusiformis*	11.86 ± 0.22	11.03 ± 0.34
C_2W	*L. xylanilyticus*	28.92 ± 0.06	13.43 ± 0.3
C_3W	*L. sphaericus*	4.64 ± 0.08	11.32 ± 0.22
D_1W	*L. boronitolerans*	17.9 ± 4.9	13.32 ± 0.45
S_2F	*L. fusiformis*	9.53 ± 0.35	13.21 ± 0.4
S_3F	*L. fusiformis*	11.25 ± 0.18	2.21 ± 0.25
C_2F	*L. xylanilyticus*	10.51 ± 0.46	-
C_3F	*L. sphaericus*	13.9 ± 0.75	0.55 ± 0.29
S_1CM	*L. boronitolerans*	19 ± 0.38	0.59 ± 0.34
S_2CM	*L. fusiformis*	31.57 ± 0.43	0.51 ± 0.25
C_2CM	*L. xylanilyticus*	11.92 ± 0.25	0.52 ± 0.26
S_1A	*L. boronitolerans*	94.55 ± 8.35	0.5 ± 0.25
S_3A	*L. fusiformis*	13.64 ± 0.76	-
C_1A	*L. fusiformis*	14.62 ± 0.37	0.49 ± 0.24
D_1A	*L. boronitolerans*	15.84 ± 0.23	11.12 ± 0.25
S_2X	*L. fusiformis*	10.45 ± 0.08	-

C_2X	*L. xylanilyticus*	10.92 ± 0.17	-
C_3X	*L. sphaericus*	3.79 ± 0.08	79.71 ± 0.8
D_1X	*L. boronitolerans*	22.39 ± 0.7	89.4 ± 1.43
S_1CE	*L. boronitolerans*	27.31 ± 0.59	79.78 ± 0.13
S_2CE	*L. fusiformis*	29.77 ± 3.07	88.66 ± 0.46
S_3CE	*L. fusiformis*	6.31 ± 0.18	88.6 ± 0.39
C_1CE	*L. fusiformis*	1.55 ± 0.11	90.14 ± 1.18
C_2CE	*L. xylanilyticus*	1.5 ± 0.06	91.7 ± 1.35
C_3CE	*L. sphaericus*	28.57 ± 0.71	90.44 ± 1.43
D_1CE	*L. boronitolerans*	8.4 ± 0.55	84.37 ± 2.22

Data is mean of triplicates (U/ml); bacterial isolates from the gut of woodborers D, *Dicyathifer mannii*; S, *Sphaeroma terebrans* and C, *cirolana sp.*; cultured in media containing substrates W, wood; CM, carboxymethylcellulose; A, avicel cellulose; X, xylan beechwood and CE, cellobiose. (-) No activity.

β-Glucosidase production was induced in all the substrates used while xylanase was induced moderately in wood and FP and but highly induced in xylan (2 isolates) and cellobiose substrates. All the bacteria isolates from the 3 woodborers showed an appreciably high xylanase induction in media containing cellobiose substrate (Figure 6.1).

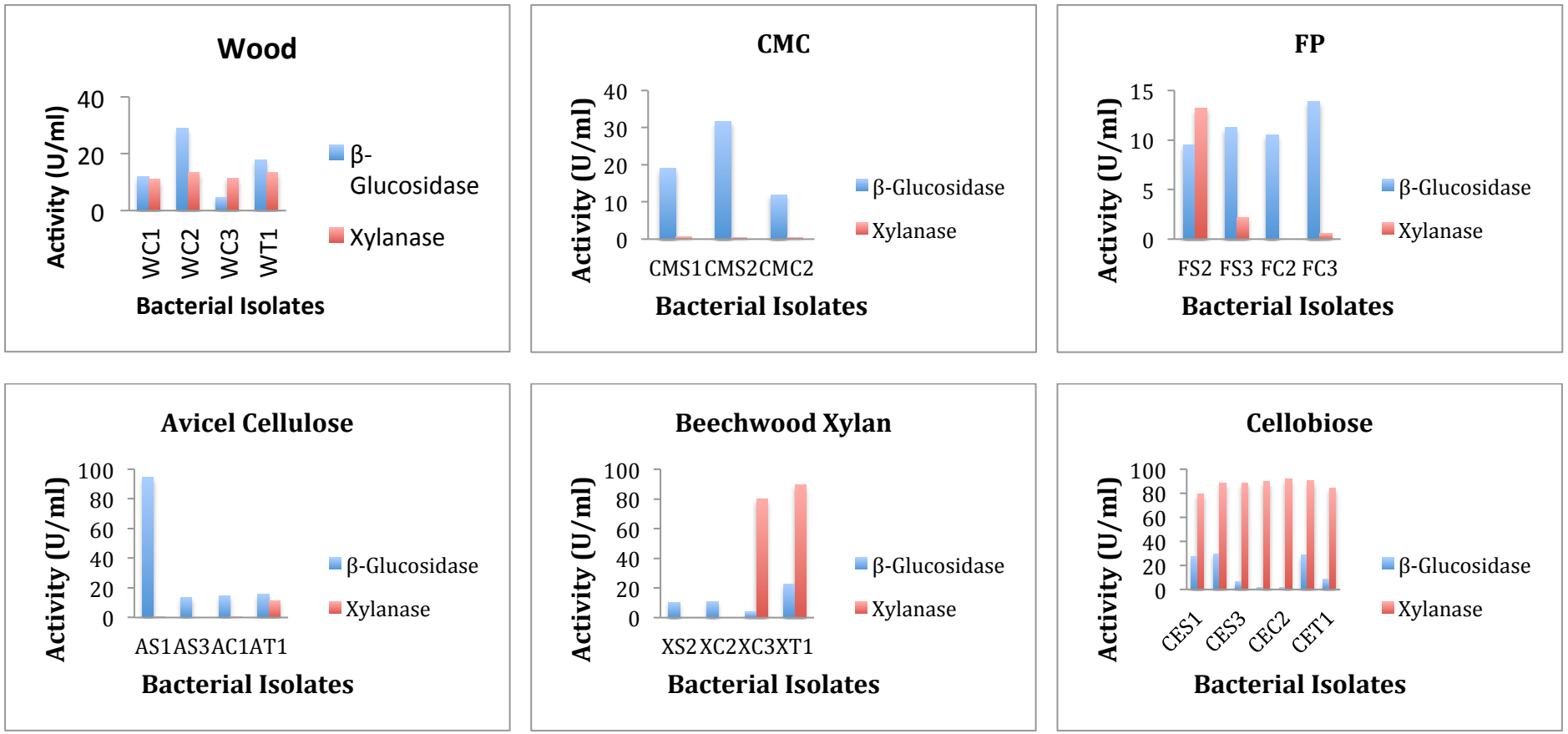


Figure 6.1. Comparison of β-glucosidase and xylanase activities (U/ml) of the crude supernatants of cultures grown in enrichment media containing 0.5 % (w/v) wood, CMC, FP, Avicel®, xylan beechwood and cellobiose as sole carbon sources. U = µmol reducing sugar produced/min.

6.2.3.3. Cellulolytic and hemicellulolytic activities of the crude fungal extract

Inducement of cellulolytic and hemicellulolytic enzyme production by fungal isolates on a number of substrates was successful. Their activities were however relatively lower compared to bacterial isolates. They showed significantly high cellulolytic (CMCase or endoglucanase and β-glucosidase) activities and hemicellulolytic (xylanase) activity. The fungal isolate with the highest cellulolytic activity was β-glucosidase activity of 38.34 U/ml shown by *Aspergillus niger* obtained from the gut of *S. terebrans* and cultured in a medium containing avicel as a sole carbon source. The next highest β-glucosidase activity was by another *A. niger* strain (33.62 U/ml) also from *S. terebrans* cultured in medium containing xylan beechwood substrate. The highest xylanase activity (17.02 U/ml) was exhibited by yet another *A. niger* strain from *D. mannii* cultured in a medium containing cellobiose, whereas the highest CMCase activity (14.14 U/ml) was exhibited by *A. costaricaensis* from *Cirolana* cultured in a medium containing xylan beechwood (Table 6.3). FPase and Avicelase activities were very low in all the fungal isolates (data not shown).

Table 6.3 Cellulolytic and hemicellulolytic activity of fungal isolates

Culture	Isolate	CMCase	β-glucosidase	Xylanase
S_1CM	*A. niger*	3.57 ± 0.67	0.44 ± 0.01	10.58 ± 0.56
S_1A	*A. niger*	2.35 ± 0.14	16.84 ± 2.22	2.37 ± 0.25
S_2A	*A. niger*	2.33 ± 0.15	18.87 ± 0.61	3.09 ± 0.03
S_3A	*A. niger*	0.7 ± 0.36	38.34 ± 0.56	2.44 ± 0.22
S_4A	*B. fuckeliana*	1.46 ± 0.2	11.32 ± 0.3	3.33 ± 0.22
S_5A	*A. niger*	9.33 ± 0.88	14.18 ± 0.28	1.56 ± 0.11
C_1A	*A. costaricaensis*	1.42 ± 0.03	19.6 ± 0.39	1.61 ± 0.53
C_2A	*A. fumigatus*	0.12 ± 0.05	17.76 ± 0.5	0.89 ± 0.44
D_2A	*A. niger*	2.87 ± 0.08	17.6 ± 1.81	2.66 ± 0.22
D_5A	*Penicillium sp.*	2.48 ± 0.28	16.69 ± 0.61	2.37 ± 0.25
S_1X	*A. niger*	7.36 ± 0.61	17.78 ± 0.08	13.84 ± 1.43
S_2X	*A. niger*	10.6 ± 0.53	33.62 ± 1.8	11.77 ± 0.59
S_3X	*A. niger*	10.66 ± 0.51	6.11 ± 0.61	11.92 ± 0.72
S_4X	*B. fuckeliana*	9.7 ± 0.28	16.21 ± 0.24	12.8 ± 0.56
S_5X	*A. niger*	11.55 ± 0.4	8.37 ± 0.56	14.65 ± 0.45
C_1X	*A. costaricaensis*	14.14 ± 0.28	20.72 ± 0.37	13.32 ± 1.11
C_2X	*A. fumigatus*	11.01 ± 0.7	21.22 ± 0.65	10.21 ± 1.18
D_1X	*A. niger*	7.25 ± 0.56	21.09 ± 0.56	14.5 ± 1.12

D_2X	*A. niger*	9.51 ± 0.57	17.19 ± 0.65	11.54 ± 0.59
D_3X	*N. fischeri*	11.32 ± 1.18	14.15 ± 0.25	0.93 ± 0.17
D_4X	*A. niger*	10.4 ± 1.68	14.4 ± 1.51	11.69 ± 0.56
D_5X	*Penicillium sp.*	9.51 ± 0.57	18.67 ± 0.2	4.22 ± 0.22
S_1CE	*A. niger*	7.88 ± 0.59	15.76 ± 0.3	15.77 ± 0.23
S_2CE	*A. niger*	1.41 ± 0.28	18.65 ± 0.19	11.1 ± 1.11
S_3CE	*A. niger*	0.11 ± 0.03	13.45 ± 0.16	16.36 ± 0.9
S_4CE	*B. fuckeliana*	0.43 ± 0.59	12.81 ± 0.09	12.73 ± 0.34
S_5CE	*A. niger*	1.67 ± 0.56	14.66 ± 0.33	10.51 ± 0.56
C_1CE	*A. costaricaensis*	0.22 ± 0.06	15.46 ± 0.12	5.03 ± 0.56
C_2CE	*A. fumigatus*	0.13 ± 0.03	11.72 ± 0.1	7.4 ± 1.7
D_1CE	*A. niger*	0.13 ± 0.04	16.05 ± 0.21	13.99 ± 0.59
D_2CE	*A. niger*	0.14 ± 0.03	18.17 ± 0.13	17.02 ± 0.92
D_3CE	*N. fischeri*	0.88 ± 0.2	18.72 ± 0.29	15.32 ± 0.8
D_4CE	*A. niger*	0.13 ± 0.04	21.93 ± 0.36	14.65 ± 1.18
D_5CE	*Penicillium sp.*	0.13 ± 0.03	15.3 ± 0.26	16.06 ± 0.56

Data is mean of triplicates (U/ml); fungal isolates from the gut of woodborers D, *Dicyathifer mannii*; S, *Sphaeroma terebrans* and C, *cirolana sp.*; cultured in media containing substrates CM, carboxymethylcellulose; A, avicel cellulose; X, xylan (beechwood) and CE, cellobiose.

While avicel cellulose, xylan bechwood and cellobiose substrates showed sufficient inducement of CMCase, β-glucosidase and xylanase production by many fungal isolates, CMC showed sufficient inducement of only *A. niger* from *S.terebrans* gut. Interestingly, CMCase (endoglucanase) production was significantly induced by xylan beechwood substrate (Figure 6.2).

It was observed that fungal isolates grew slowly in media containing wood and FP as a sole carbon source. The resulting enzyme activities of these cultures were very low (data not shown), indicating that wood and FP were not good carbon sources for the production of cellulolytic and hemicellulolytic enzymes from the isolates.

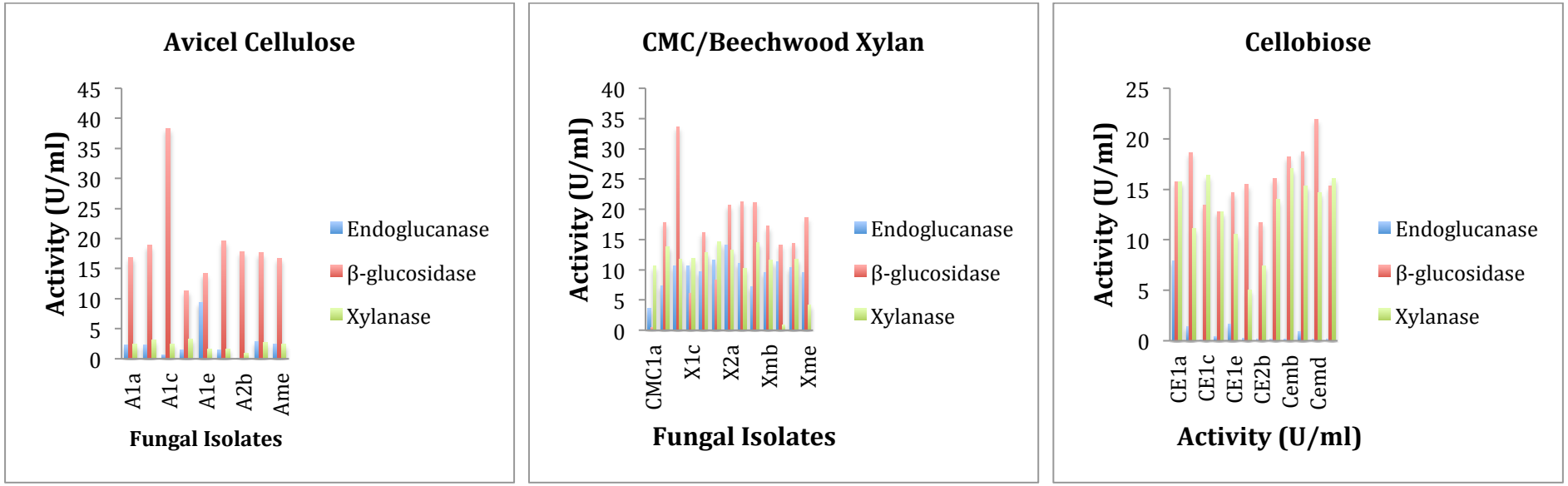


Figure 6.2. Comparison of CMCase (endoglucanase), β-glucosidase and xylanase activities (U/ml) of the crude supernatants of cultures grown in enrichment media containing 0.5 % (w/v) Avicel®, CMC or xylan beechwood and cellobiose as sole carbon sources. U = µmol reducing sugar produced/min.

6.2.3.4. Cellulolytic and hemicellulolytic activities of bacterial mixed substrate cultures

Bacterial isolates grown in mixed substrate cultures showed some appreciable β-glucosidase (highest 11.19 U/ml by *L. fusiformis* from *S. Terebrans gut*) and Xylanase (highest 10.76 U/ml by *L. xylanilyticus* from *Cirolana* gut) activities (Table 6.4), but low FPase, CMCase and Avicelase activity (Figure 6.3). These activities were however lower compared to those of bacteria grown in media with single substrates as sole carbon sources (Table 6.2). Some synergism was expected in mixed cultures, but the results (Iso1, Iso2 and 1,2&3) did not indicate any synergism (Figure 6.4).

Table 6.4. Bacterial mixed substrate cultures cellulase & hemicellulase activity

Culture	Isolate	β-glucosidase	Xylanase
1a	*L. boronitolerans*	6.82 ± 0.01	9.89 ± 0.01
1b	*L. fusiformis*	7.24 ± 0.04	7.55 ± 0.44
1c	*L. fusiformis*	9.39 ± 0.06	9.34 ± 1.43
2a	*L. fusiformis*	11.19 ± 0.02	8.46 ± 0.95
2b	*L. xylanilyticus*	11.13 ± 0.04	10.76 ± 1.56
2c	*L. sphaericus*	11.10 ± 0.00	8.70 ± 0.39
ma	*L. boronitolerans*	1.59 ± 0.11	10.37 ± 0.72
Iso1	Mixed	11.05 ± 0.15	10.29 ± 1.69
Iso2	Mixed	0.39 ± 0.01	8.63 ± 1.45
1,2&ma	Mixed	3.29 ± 0.06	8.81 ± 1.47

Data is mean of duplicates (U/ml); bacterial isolates from the gut of woodborers 1, *Sphaeroma terebrans;* 2, *Cirolana sp.* and m, *Dicyathifer mannii*; cultured in media containing 0.1 % (w/v) each of wood, FP, CMC, Avicel®, xylan and cellobiose.

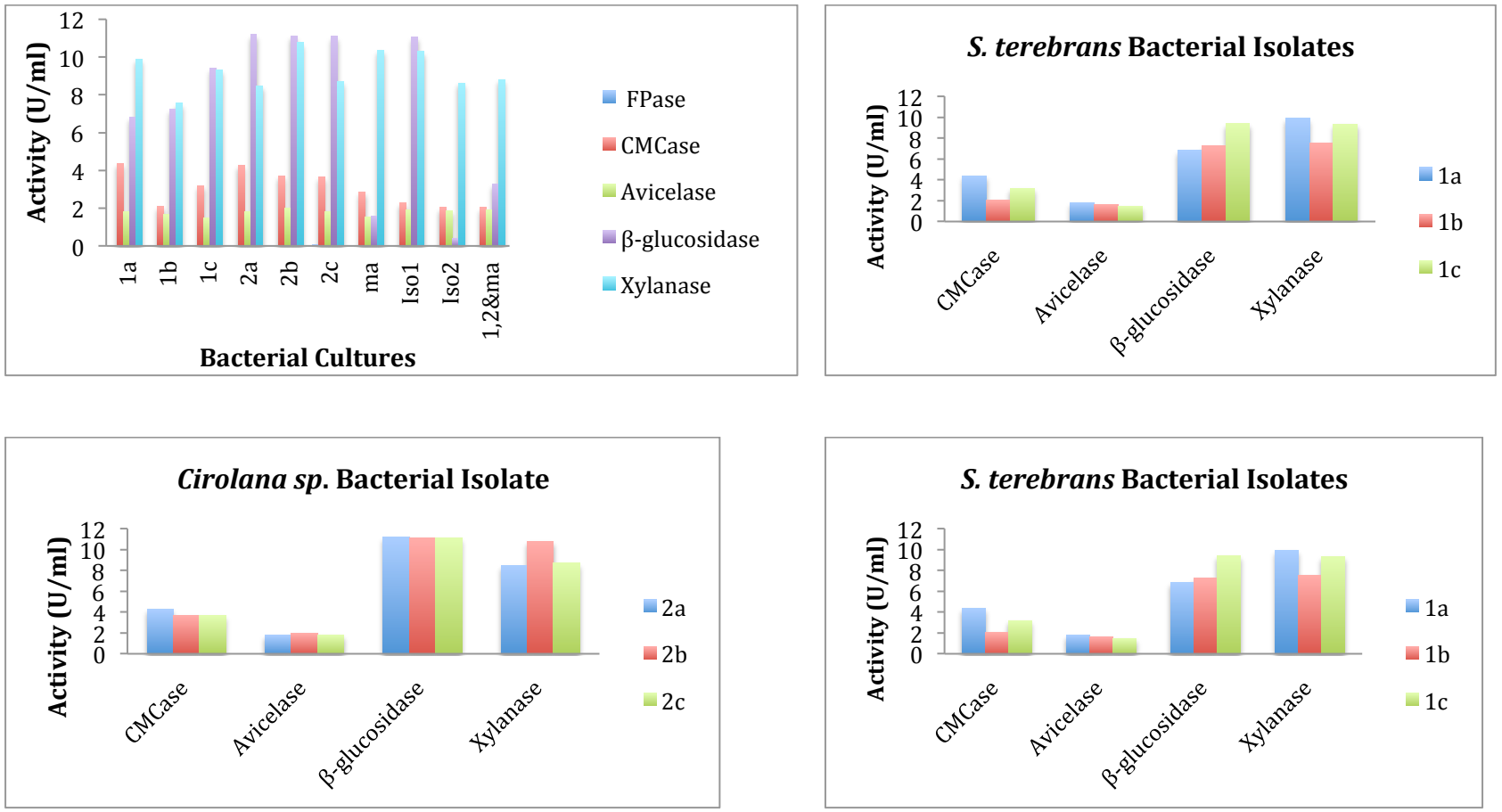


Figure 6.3. **Comparison of FPase, CMCase, Avicelase, β-glucosidase and xylanase activities (U/ml) of crude supernatants of bacterial cultures grown in enrichment media containing 0.1 % (w/v) each of wood, FP, CMC, Avicel®, xylan and cellobiose. U = µmol reducing sugar produced/min.**

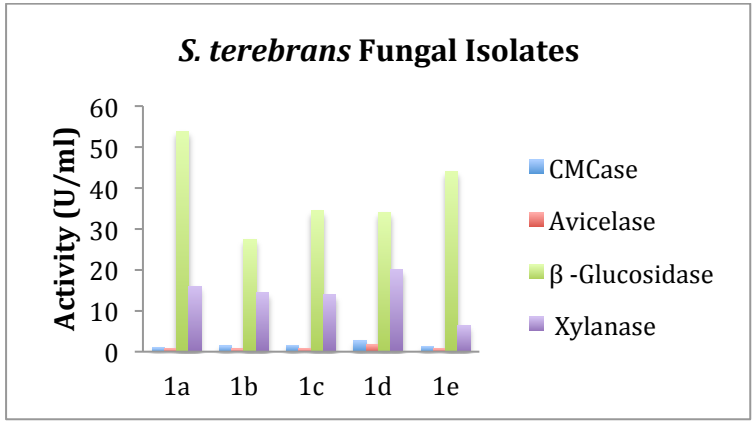
6.2.3.5. Cellulolytic and hemicellulolytic activities of fungal mixed substrate cultures

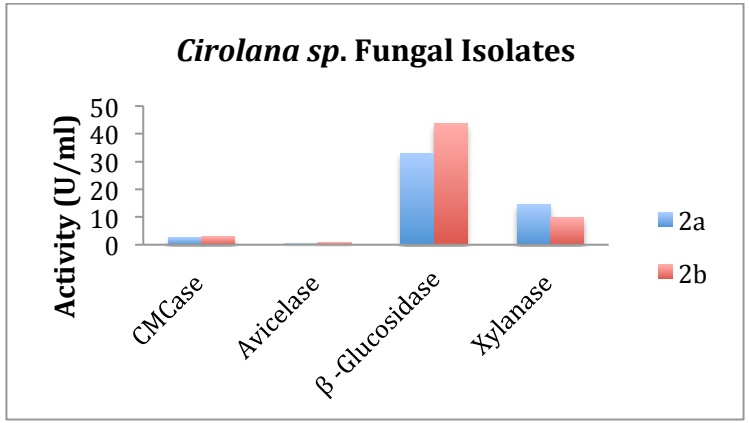
Cellulolytic and hemicellulolytic activities of the crude fungal extracts from mixed substrate cultures (Table 6.5, Figure 6.4) were much higher than those of bacterial isolates in the same media (Table 6.4). In addition, they were much higher than those of the same fungal isolates in media with single substrates (Table 6.3). The highest activities were those of β-glucosidase with the highest of 54.77 U/ml (*Aspergillus niger* from *D. mannii* gut), and xylanase with highest of 20.42 U/ml (*Penicillium sp.* from *D. mannii* gut). β-glucosidase and xylanase activity maxima are summarized in Table 6.6.

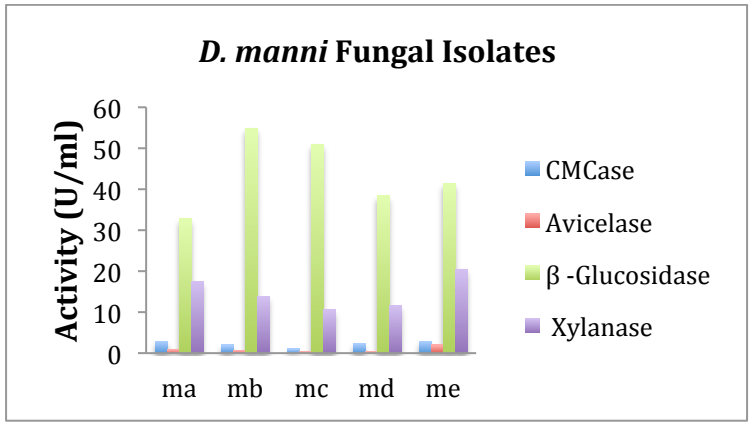
Table 6.5. Fungal mixed substrate cultures activity

Culture	Isolate	β-glucosidase	Xylanase
1a	*A. niger*	53.73 ± 0.18	15.95 ± 0.05
1b	*A. niger*	27.48 ± 0.39	14.4 ± 0.36
1c	*A. niger*	34.38 ± 0.06	13.85 ± 0.39
1d	*B. fuckeliana*	33.98 ± 0.01	19.97 ± 0.02
1e	*A. niger*	44.12 ± 0.42	6.36 ± 0.43
2a	*A. costaricaensis*	33.02 ± 0.42	14.51 ± 0.2
2b	*A. fumigatus*	43.75 ± 0.94	9.97 ± 0.02
ma	*A. niger*	32.8 ± 0.73	17.51 ± 0.01
mb	*A. niger*	54.77 ± 0.3	13.93 ± 0.08
mc	*N. fischeri*	50.84 ± 0.26	10.58 ± 0.01
md	*A. niger*	38.4 ± 0.66	11.59 ± 0.0
me	*Penicillium sp.*	41.52 ± 0.18	20.42 ± 0.32
Iso1	Mixed	28.74 ± 0.19	18.6 ± 0.07
Iso2	Mixed	51.06 ± 0.04	16.92 ± 0.0
MM	Mixed	20.15 ± 0.01	15.22 ± 0.14

Data is the mean of duplicates (U/ml); fungal isolates from the gut of woodborers 1, *Sphaeroma terebrans;* 2, *Cirolana sp.* and m, *Dicyathifer mannii*; cultured in media containing all the substrates.







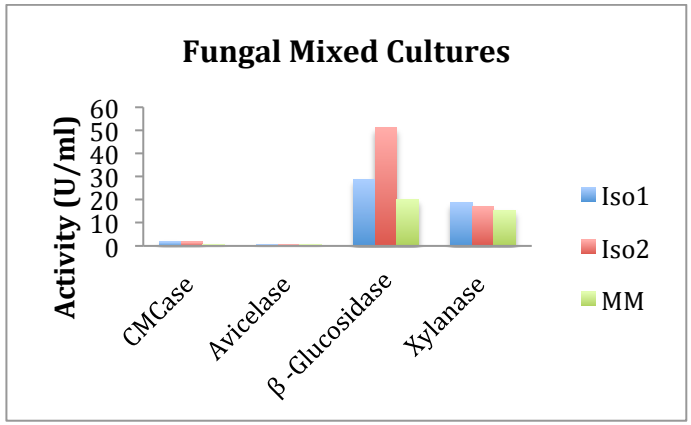


Figure 6.4. Comparison of CMCase, Avicelase, β-glucosidase and xylanase activities (U/ml) of crude supernatants of fungal cultures grown in enrichment media containing 0.1 % (w/v) each of wood, FP, CMC, Avicel®, xylan and cellobiose. U = µmol reducing sugar produced/min.

Table 6.6. Activity maxima of woodborers' gut microbiota

Culture	Substrate	Isolate	Activity	Units (U/ml)	±	SD
S_1A	Avicel	*L. boronitolerans*	β -glucosidase	94.55	±	8.35
C_2CE	Cellobiose	*L. xylanilyticus*	Xylanase	91.70	±	1.35
C_3CE	Cellobiose	*L. sphaericus*	Xylanase	90.44	±	1.43
C_1CE	Cellobiose	*L. fusiformis*	Xylanase	90.14	±	1.18
D_1X	Xylan	*L. boronitolerans*	Xylanase	89.40	±	1.43
S_2CE	Cellobiose	*L. fusiformis*	Xylanase	88.66	±	0.46
S_3CE	Cellobiose	*L. fusiformis*	Xylanase	88.60	±	0.39
D_1CE	Cellobiose	*L. boronitolerans*	Xylanase	84.37	±	2.22
S_1CE	Cellobiose	*L. boronitolerans*	Xylanase	79.78	±	0.13
C_3X	Xylan	*L. sphaericus*	Xylanase	79.71	±	0.80
D_2	Mixed	*A. niger*	β -glucosidase	54.77	±	0.30
S_1	Mixed	*A. niger*	β -glucosidase	53.73	±	0.18
C Fungi	Mixed	Mixed	β -glucosidase	51.06	±	0.04
D_3	Mixed	*N. fischeri*	β -glucosidase	50.84	±	0.26
S_5	Mixed	*A. niger*	β -glucosidase	44.12	±	0.42
C_2	Mixed	*A. fumigatus*	β -glucosidase	43.75	±	0.94
D_5	Mixed	*Penicillium sp.*	β -glucosidase	41.52	±	0.18
D_4	Mixed	*A. niger*	β -glucosidase	38.40	±	0.66
S_3A	Avicel	*A. niger*	β -glucosidase	38.34	±	0.56
S_3	Mixed	*A. niger*	β -glucosidase	34.38	±	0.06

S_4	Mixed	*B. fuckeliana*	β -glucosidase	33.98	± 0.01
S_2X	Xylan	*A. niger*	β -glucosidase	33.62	± 1.80
C_1	Mixed	*A. costaricaensis*	β -glucosidase	33.02	± 0.42
D_1	Mixed	*A. niger*	β -glucosidase	32.80	± 0.73
S_2CM	CMC	*L. fusiformis*	β -glucosidase	31.57	± 0.43
S_2CE	Cellobiose	*L. fusiformis*	β -glucosidase	29.77	± 3.07
C_2W	Wood	*L. xylanilyticus*	β -glucosidase	28.92	± 0.06
S Fungi	Mixed	Mixed	β -glucosidase	28.74	± 0.19
C_3CE	Cellobiose	*L. sphaericus*	β -glucosidase	28.57	± 0.71
D_5	Mixed	*Penicillium sp.*	Xylanase	20.42	± 0.32
S_4	Mixed	*B. fuckeliana*	Xylanase	19.97	± 0.02
S Fungi	Mixed	Mixed	Xylanase	18.60	± 0.07
D_1	Mixed	*A. niger*	Xylanase	17.51	± 0.01
D_2CE	Cellobiose	*A. niger*	Xylanase	17.02	± 0.92
C Fungi	Mixed	Mixed	Xylanase	16.92	± 0
S_3CE	Cellobiose	*A. niger*	Xylanase	16.36	± 0.9
D_5CE	Cellobiose	*Penicillium sp.*	Xylanase	16.06	± 0.56
S_1	Mixed	*A. niger*	Xylanase	15.95	± 0.05
S_1CE	Cellobiose	*A. niger*	Xylanase	15.77	± 0.23
D_3CE	Cellobiose	*N. fischeri*	Xylanase	15.32	± 0.80

Data is mean of triplicates (U/ml); bacterial and fungal isolates from the gut of woodborers D, *Dicyathifer mannii*; S, *Sphaeroma terebrans* and C, *cirolana sp.*; cultured in media containing substrates CM, carboxymethylcellulose; A, avicel cellulose; X, xylan (beechwood); CE, cellobiose; W, *Rhizophora mucronata* wood; and Mixed, a mixture of substrates.

6.3. Ethanol Production

Ethanol production was determined by its elution time compared against a standard sample of pure ethanol (5.7 mg/100 ml water) and the concentrations were calculated based on the peak areas of the known concentration of ethanol. 0.98 mg/100 ml supernatant yield was observed in 24 hours fermentations of 0.3 % *S.cerevisiae* on 3 % wheat straw degradation reaction mixture with *D. mannii* gut microbial filtrate incubated for 1 hour at 50 ^{0}C (Fig. 6.5).

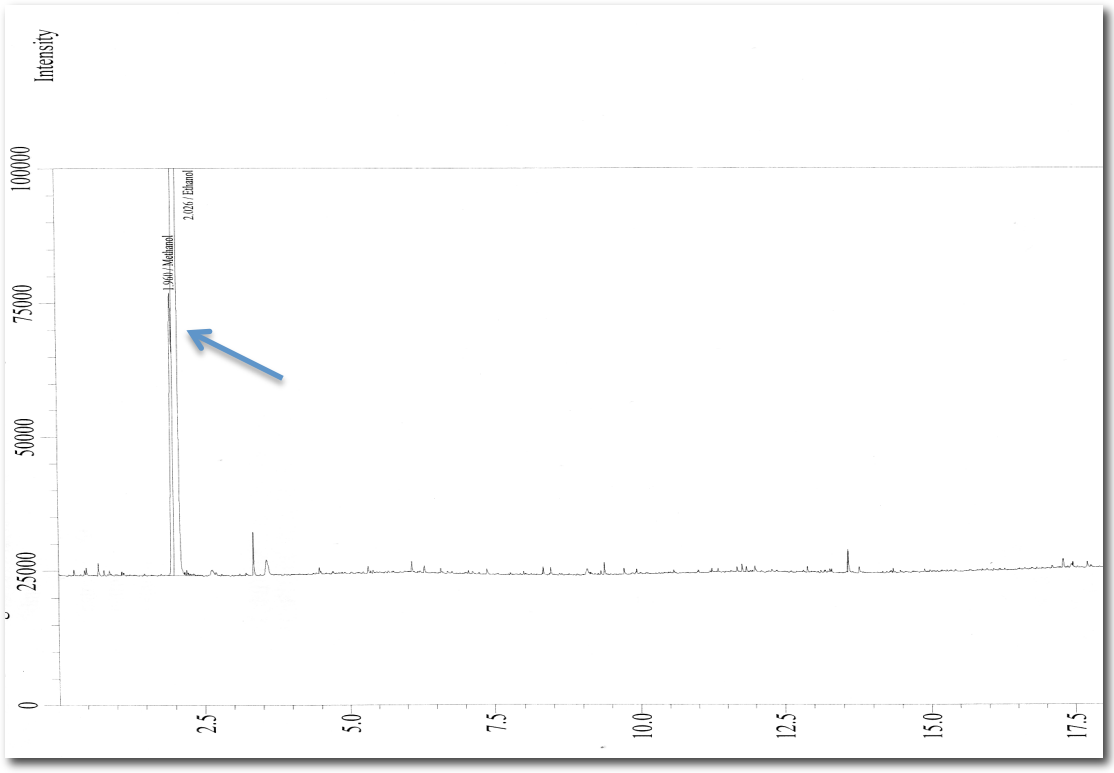


Figure 6.5A Gas chromatography profile of ethanol standard control (5.7 mg/100ml water)

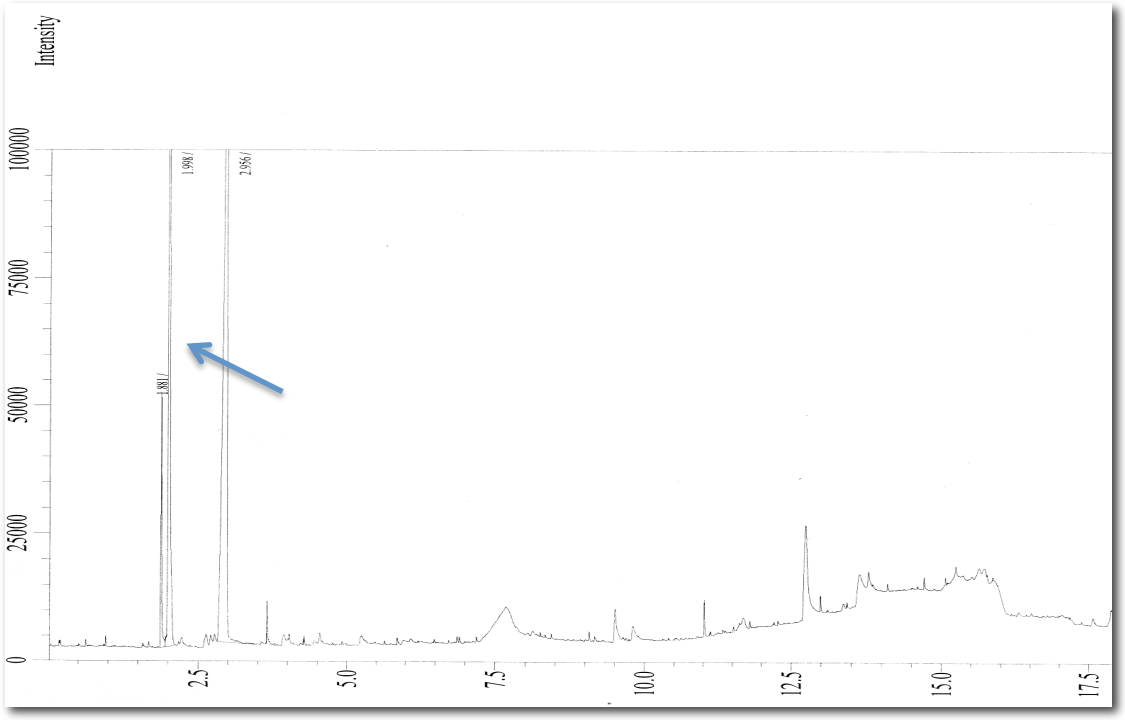


Figure 6.5B Gas chromatography profile of ethanol from the fermentation reaction.

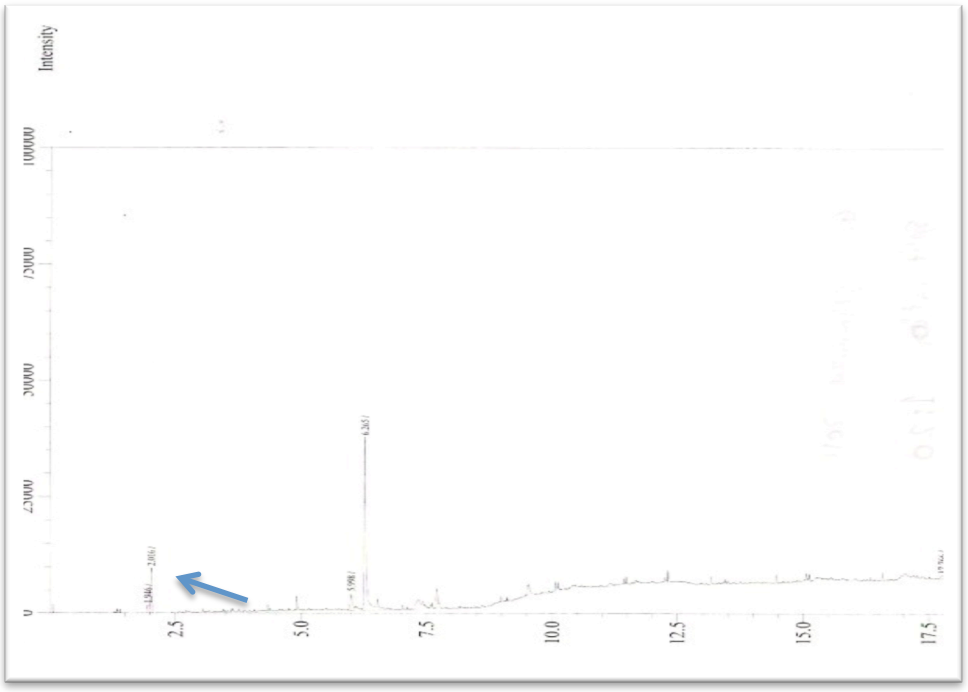


Figure 6.5C Gas chromatography profile of uninoculated control

6.4. Discussion and Conclusions

β-glucosidase and xylanase activities are the highest lignocellulolytic activities exhibited by both bacterial and fungal isolates. Bacterial isolates showed higher activities than fungal isolates and with all the substrates, but highest induction occurred in avicel, cellobiose and xylan (Table 6.6). High CMCase activity was shown in fungal isolates only. The results are discussed here in relation to the role of the aerobic bacteria and fungi from woodborers gut, and the possible application of the enzyme in lignocellulose bioconversion processes.

Bacteria and fungi are known to produce cellulase isoforms (Glick and Pasternak, 1989; Kubicek *et al*., 2008). Among them, mycelial fungi constitute a unique group of microorganisms showing prominent lignocellulolytic activity. The enzymes produced

by a number of bacteria and fungi are mostly extracellular (Sunna and Antranikian, 1997). Ray (1959) reported a cellulase complex isolated from the isopod wood boring *Limnoria* digestive tract capable of hydrolysing wood cellulose.

Bacterial genus Lysinibacillus is Gram-positive, rod-shaped, and round-spore-forming bacteria in the family Bacillaceae. Organisms in this genus were previously regarded as members of the genus Bacillus, but the taxonomic status of these microorganisms was changed to the genus Lysinibacillus in 2007 (Ahmed *et al*., 2007). Howard et al. (2003) reported cellulolytic bacteria with hemicellulase and cellulase activities isolated from a variety of sources; *Bacillus subtilis* from soil (mannan endo-1, 4-β-mannosidase; endo-β-1, 4-mannanase; endo-α-1, 5- arabinanase; endo-galactanase), *Bacillus macerans* from soil (1,3-1,4-β-D-glucan glucanohydrolase), another *Bacillus sp.* from soil (1,3-β-D-glucan glucanohydrolase), *Bacillus pumilus* from soil and dead plant (endo-1-4-β-xylanase), *Bacillus polymyxa* (β-glucosidase). Therefore, from these studies and our current study, we conclude that Bacillaceae are promising sources of cellulolytic and hemicellulolytic enzymes.

The known xylolytic fungi are mostly ascomycetes, particularly *Aspergilli* (de Vries and Visser, 2001), *Trichoderma* (Wong and Saddler, 1992) and *Penicillia* (Hamlyn *et al.*, 1987; Chavez *et al*., 2006; Liu *et al*., 2013). Many *Penicillia* are soil fungi, and grow in a variety of organic substances, particularly dead plant materials. They produce extracellular hydrolases such as pectic enzymes, lipases, proteases, cellulases and xylanases (Hamlyn *et al.*, 1987). *Penicillium* species (P. *chrysogenum, P. corylophilum, P. duclauxii, P. funiculosum,* and *P. oxalicum)* have also been reported to produce endoxylanases (Abdel-Sater and El Said, 2001) and exoglucanases (Medeiros *et al*., 2003). Bradner et al. (1999) also reported endoxylanase from *P.*

hirsutum and *P. commune.* In addition, Shimokawa *et al*. (2012) reported lignocellulolytic enzymes from *A. tubingensis.* From all these studies and our study, it can be concluded that *Aspergilli* and *Penicillia* constitute a rich source of enzymes for the biodegradation of cellulose and xylan.

Other lignocellulolytic activities that have been reported include wood-inhabiting penicillium strains (Lee *et al*., 2011) with xylanase and β-xylosidase activity of 158 U/ml and 6.25 U/ml respectively; *Trichoderma harzianum* with 146 U/ml cellulase activity on basal salt medium (BSM) supplemented with 0.5 % CMC, 147 U/ml and 168 U/ml from BSM supplemented with flours of potato and banana respectively (Rubeena *et al*., 2013); *Trichoderma viride* in 0.5 % CMC-BSM with 137 U/ml cellulase activity, which was enhanced to 173 U/mL at 1.25% CMC concentration (Neethu *et al*., 2012); Penicillium species with cellulolytic and xylanolytic activities of 87 U/ml (Jorgensen *et al*., 2004; Krogh *et al*., 2004; and Jorgensen *et al*., 2005); and endoxylanase activity of 31.5 IU/mg, 24.7 IU/mg and 8 IU/mg from xylan, wheat bran and wheat straw respectively (Rakotoarivonina *et al*., 2012). In this study, *L. boronitolerans* and *L. xylanilyticus* exhibited β –glucosidase and xylanase activities of 94.55 U/ml and 91.70 U/ml from 0.5 % Avicel and Cellobiose respectively, which falls within the range of these reported activities.

In this study, we induced lignocellulolytic enzymes in liquid cultures. Majority of the reports on commercial production of these enzymes utilize submerged fermentation (SmF), because of its ease of controlling the conditions. However, in nature, the growth and lignocellulose utilization of aerobic microorganisms harbouring these enzymes probably resemble solid-state fermentation (SSF) than a liquid culture (Neethu *et al*., 2012). Besides, Tengerdy (1996) reported ten fold reduction in

production cost by SSF than SmF. There is need therefore to investigate production of these enzymes in SSF.

The results of the current study expand our knowledge on lignocellulolytic enzyme systems from gut microbiota. Their genomic features and systematic studies on their enzyme systems need to be investigated to facilitate directed strain engineering for improved performance.

The present study proved that bacterial and fungal strains of *Lysinibacilli* and *Aspergilli* used have a high potential for β-glucosidase and xylanase production. We therefore conclude that *Lysinibacilli* and *Aspergilli* present interesting advantages that make them good models for studying physiological approaches to enzyme production and lignocellulose degradation, with the aim of developing the key enzymes. For this reason, either β-glucosidase or xylanase were recommended for isolation, purification and characterization (Chapter 7).

Fungal isolates cultured on wood, FP and CMC (except for 1 isolate) exhibited very low cellulolytic and hemicellulolytic activities, but when grown on xylan, avicel and cellobiose they were able to degrade these substrates with appreciable activity Therefore there is need of an optimisation study to determine the growth conditions under which the best cellulolytic and hemicellulolytic enzyme activities are obtained.

In this study, 0.98 mg/100 ml supernatant yield was observed in 24 hours fermentations of 0.3 % *S.cerevisiae* on 3 % wheat straw degradation reaction mixture with *D. mannii* gut microbial filtrate previously incubated for 1 hour at 50 ^{0}C. In another study, Chen *et al*. (2009), inserted and expressed *Zymomonas mobilis* genes encoding essential enzymes involved in the fermentation pathway, alcohol

dehydrogenase II (adh II) and pyruvate decarboxylase (pdc), into E. coli, resulting in increased cell growth and ethanol production. After 72 hours under microaerobic conditions, ethanol concentrations of 30 g/L were obtained on 10% glucose. Considering the fact that the wheat straw in this study was not pretreated for delignification, and a short reaction time as well as fermentation period was used, the obtained yield is commendable.

Therefore, biodegradation of lignocellulosic agro-industrial residues by means of microbial community is a promising approach providing efficient biomass decomposition for subsequent conversion to value- added products.

CHAPTER SEVEN

7.0 CELLULOLYTIC AND HEMICELLULOLYTIC SYSTEM OF *DICYATHIFER MANNII* GUT MICROBIAL COMMUNITY: ISOLATION, PURIFICATION AND CHARACTERIZATION OF XYLANASE

7.1. Introduction

In Chapter 4, *D. mannii* had shown to have the most efficacious lignocellulolytic extracts. In addition to that, the highest *D. mannii* microbial community lignocellulolytic activity was xylanase. Consequently, xylanase from the culture filtrate of *D.mannii* gut microbial community was isolated, purified and characterized.

The present study reports for the first time the purification and characterization of xylanase from *D. mannii* microbial community. This extracellular enzyme was produced using liquid culture fermentation under shake conditions with wheat straw as inducement.

Haruta *et al.* (2002) constructed the first effective microbial community with high cellulose-degradation ability. Some research on the structure and application of microbial community has been made (Kato *et al*., 2004; Kato *et al*., 2005; Liu *et al*., 2006; Wang *et al*., 2006). These studies reported prospective and potential applications of microbial communities in industry. Thus the need to produce, purify and characterize xylanase from *D. mannii* gut microbial community for industrial use.

7.2. *D. mannii* Gut Microbial Community

7.2.1. Activities of Crude *D. mannii* Gut Microbial Culture Supernatant

D. mannii microbial community was successfully grown on wet wheat straw culture at 30 ^{0}C for 5 days on a shaker at 180 rpm. Clear supernatant solutions obtained after centrifugation of the wet culture were assayed for FPase, CMCase, avicelase, β–glucosidase and xylanase activity. Evidently, moderate β–glucosidase and high xylanase extracellular enzymes were secreted (Fig. 7.1). For this reason, proceeding purification steps were xylanase activity guided.

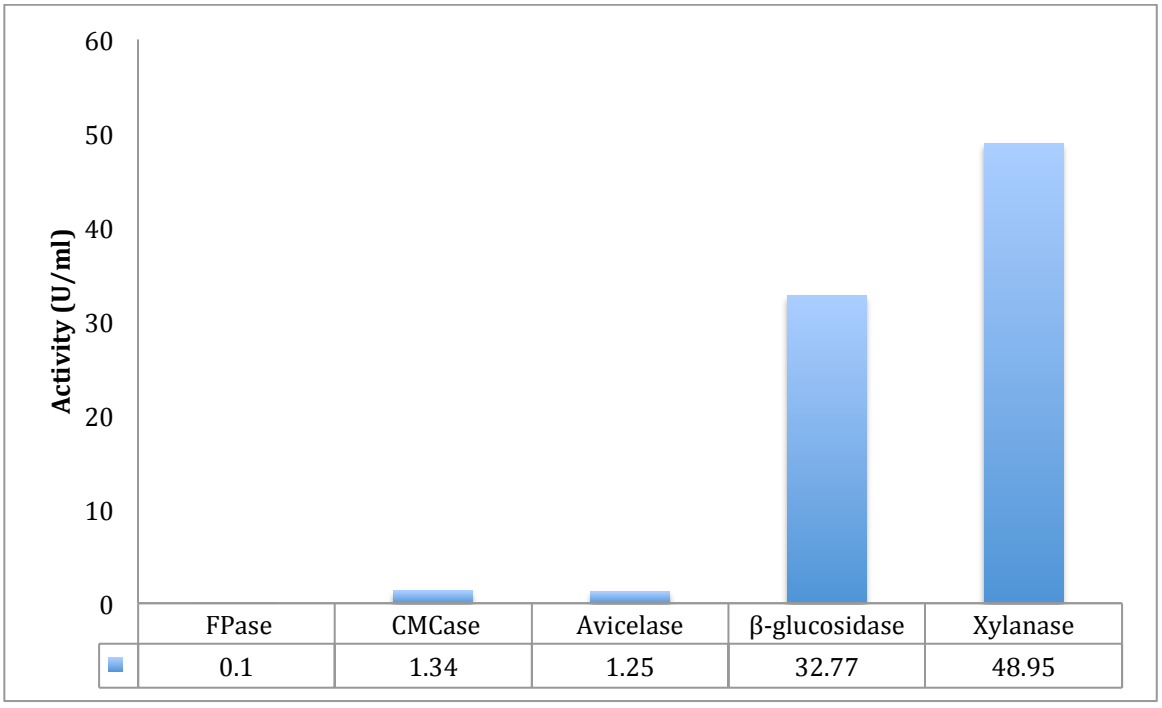


Figure 7.1. Activity of the crude culture supernatant

1 U is the amount of enzyme producing 1 µmol of xylose or glucose equivalents per min under the given conditions.

7.2.2. Isolation and Purification of Xylanase

The procedure for the purification of extracellular xylanase from microbial community is shown in Table 7.1. After 80 % ammonium sulphate precipitation, sephadex G-200 and DEAE–Sephacel ion-exchange chromatography, the purified xylanase protein appeared as a single band on SDS-PAGE and had the molecular weight of ≈ 20 kDa (Fig.7.2) with a specific activity of 16.67 U/mg. This protocol afforded 5.8-fold purification of xylanase from the culture filtrate with a yield of 72 %. The pure xylanase showed a relatively clear band on the zymogram gel, detected by Congo red staining, indicating it was active xylanase (Fig. 7.2C). The amount of protein in the crude fractions and purification steps fractions were estimated by the Lowry protein assay using bovine serum albumin (BSA) as a standard.

Table 7.1. Purification of xylanase from *D. mannii* gut microbial community

Purification step	Total protein (mg/ml)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture supernatant	17.08	48.95	2.86	100	1
$(NH_4)_2SO_4$ ppt	6.88	42.01	6.11	85	2.14
Sephadex GF	4.23	40.06	9.47	81	3.3
DEAE-Sephacel	2.12	35.33	16.67	72	5.8

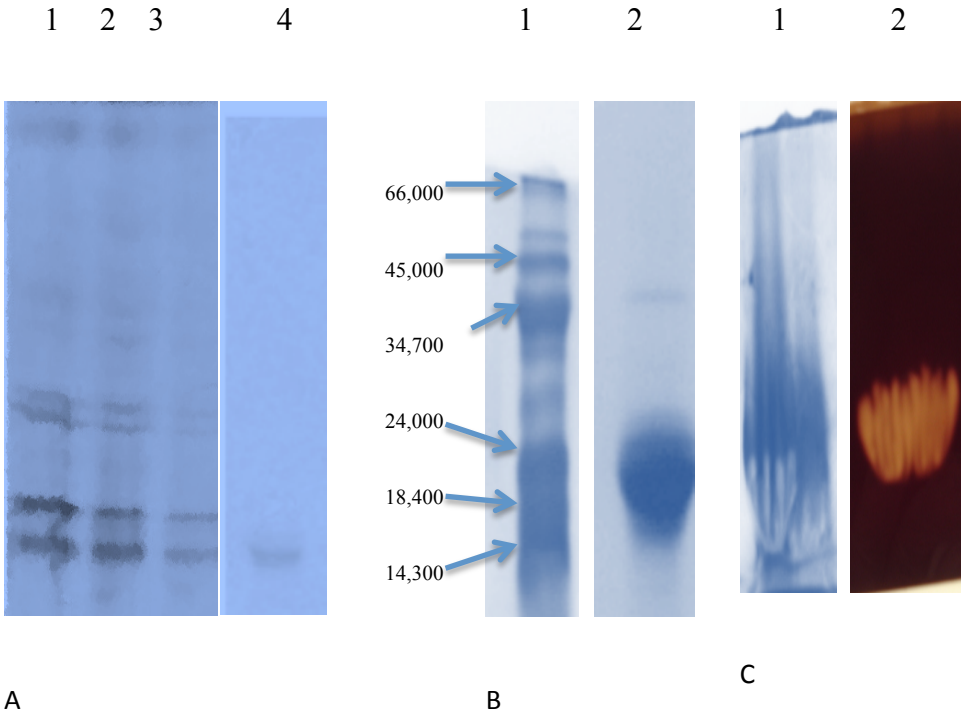


Figure 7.2. SDS-PAGE profile of the purified enzyme: A, SDS-PAGE gel of purification steps; 1, Crude culture supernatant; 2, $(NH_4)_2SO_4$ precipitate; 3, Sephadex Gel Filtration; 4, DEAE-Sephacel Anion Exchange Chromatography. B, SDS-PAGE gel of purified xylanase from *D. mannii* gut microbial community. Lane 1, Molecular marker protein (14.3 to 66 kDa); Lane 2, Purified enzyme. C, Zymogram profile of purified xylanase from *D. mannii* microbial community. Lane 1, stained with Coomassie blue R-250, Lane 2, stained with 0.1 % Congo red.

7.2.3. Effect of pH and Temperature on Xylanase Activity

7.2.3.1. Optimum pH and pH stability

The activities of xylanase from *D. mannii* gut microbial community at various pH values were measured using beechwood xylan as the substrate. The reaction pH was adjusted to 4.0–9.0 with various universal buffers. The xylanase showed enzyme activity over a pH range of 5.0–6.0 at 50 °C (Fig.7.3), with the maximum activity at pH 5.0. The enzyme showed a good pH stability of 74.6-77.2 % relative activity at pH 5.0 to 8.0.

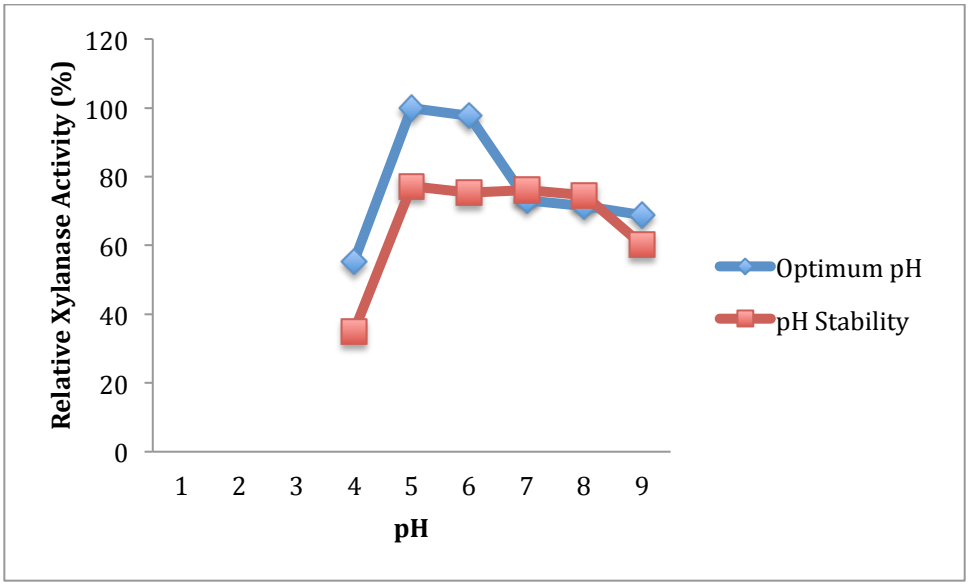


Figure 7.3. Effect of pH on xylanase activity

The pH optimum (◆), expressed in percent of maximum, was determined by measuring the activity at 50 °C for 30 minutes using universal buffers of different pH, and assayed by the DNS method. The pH stability (■) was determined by incubating the purified enzyme at different pH values for 24 hours at 50 °C. The residual activity is shown as a percent of the original activity.

7.2.3.2. Optimum temperature and thermostability

Initial reaction rates for the purified xylanase were determined at temperatures between 30 and 70 ^{0}C for 30 minutes at pH 5.0 and the optimum temperature was 50 °C (Fig.7.4). The xylanase activity dropped at temperature values above 50 °C, and only 13.7 % of activity was detected at 70 °C. Thermostability studies of xylanase showed that after 120 minutes incubation at various temperatures, more than 70 % of residual xylanase activities were retained at optimum temperature of 50 °C and pH 5.0.

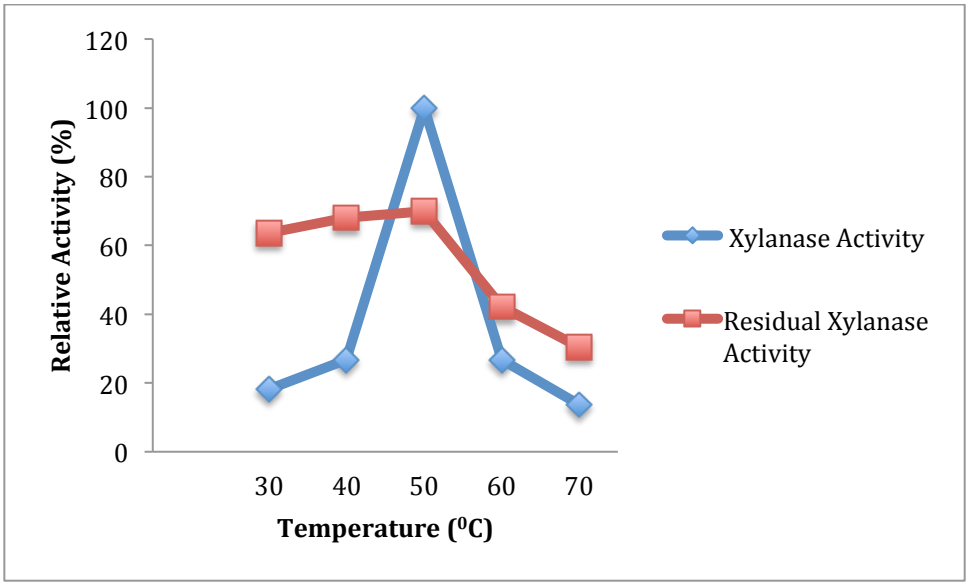


Figure 7.4. Xylanase optimal temperature

The optimal temperature of the xylanase was determined by incubating the protein for 30 min at different temperatures (30–70 ^{0}C) in 1 % beechwood xylan solution prepared in 50 mM sodium acetate buffer, pH 5.0. Residual activity was measured after 120 minutes.

7.2.4. Substrate Specificity and Suitability

The substrate specificity of the enzyme was determined by performing the assay with different substrates (Table 7.2). Compared to the enzyme activity on beechwood xylan (100 % relative activity), this xylanase showed much lower relative activities of 8.1 % on avicel cellulose and 4.4 % on CMC.

Table 7.2. Michaelis-Menten analysis of substrate affinity of xylanase

Michaelis-Menten	Beechwood Xylan	95 % CI		AVICEL PH101	95 % CI		CMC	95 % CI	
		LCL	UCL		LCL	UCL		LCL	UCL
Vmax	128.2 ± 3.74	118.8	114.4	10.38 ± 0.74	8.885	11.88	5.653 ± 0.48	4.675	6.631
Km	0.36 ± 0.02	0.27	0.45	0.2770 ± 0.06	0.16	0.39	0.3835 ± 0.09	0.21	0.56

V_{max}, U/ml ± SE; K_m, % (w/v); Data is Triplicates, n=12; CI, Confidence Interval; LCL, Lower Confidence Limit; UCL, Upper Confidence Limit.

7.2.5. Kinetic Parameters

The rate dependence of enzymic reaction on beechwood xylan 0.1-2.5 % (w/v) concentrations in 50 mM sodium acetate buffer (pH 5.0) at 50 ^{0}C followed by double reciprocal (Lineweaver-Burk) kinetics plot of data showed apparent K_m and V_{max} values of 0.4 % (w/v) and 128.2 µmol ml^{-1} min^{-1}, respectively (Fig.7.5). These values were used in the Michaelis-Menten equation to produce Michaelis-Menten plot for beechwood xylan (Fig.7.6).

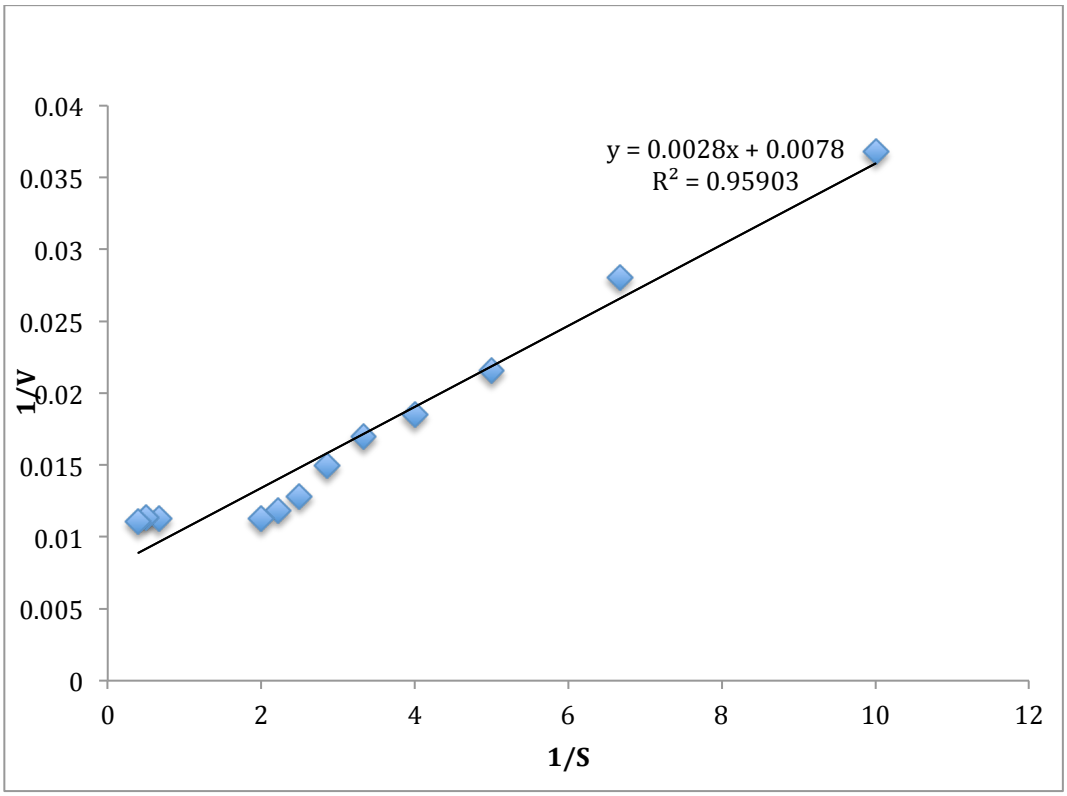


Figure 7.5. Lineweaver-Burk plot for xylanase activity

Effect of various concentrations of beechwood xylan on xylanase from *D. mannii* gut microbial community. Reciprocal values V, velocity (U/ml); S, substrate conc. (%).

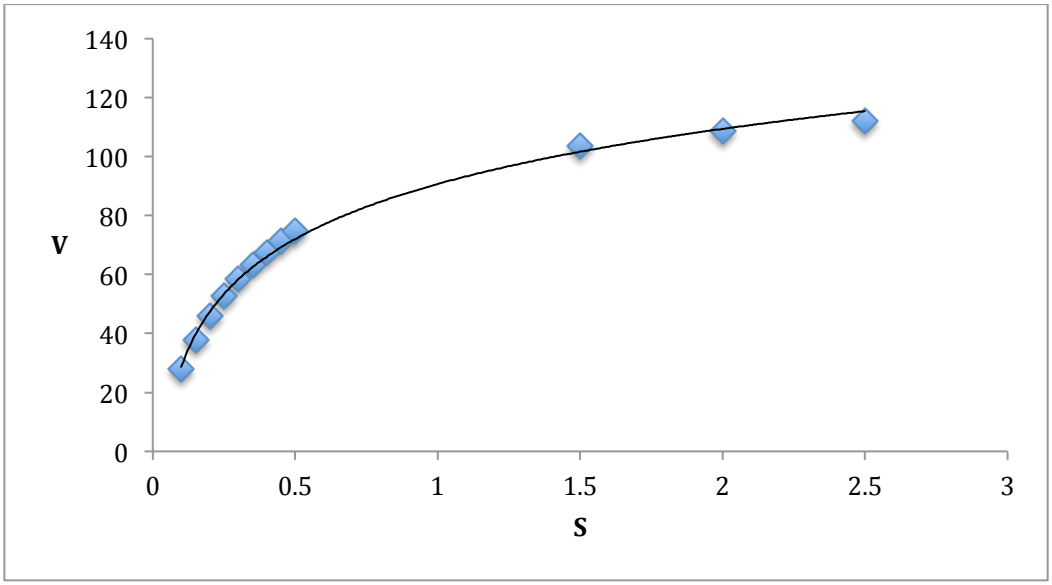


Figure 7.6. Michaelis-Menten plot for xylanase activity

Xylanase activity with different Beechwood Xylan concentrations at pH 5.0. V, velocity (U/ml); S, substrate conc. (%).

7.3. Discussion and Conclusions

In Chapter 5, the bacteria *Lysinibacillus boronitolerans* and fungi colonies of Ascomycetes; *Aspergillus niger, Neosartorya fischeri*, *Penicillium sp*. and *A. fumigatus* were detected in *D. mannii* gut microbial community by 16S rRNA gene and internal transcribed spacer (ITS) region analysis, respectively. Xylanases secreted by bacteria from genera *Bacillus* have been well studied (Beg *et al*., 2001). The use of microbial community for enzyme production has proved effective in this work, and this microbial community and the purified enzyme have prospective and potential application in industry.

Despite an increased knowledge of microbial xylanolytic systems in the past few years, further studies are required to achieve a complete understanding of the

mechanism of xylan degradation by microorganisms and their enzymes. The enzyme system used by microbes for the metabolism of xylan is the most important tool for investigating the use of the second most abundant polysaccharide (xylan) in nature. Recent studies on microbial xylanolytic systems have generally focussed on induction of enzyme production under different conditions, purification, characterization, molecular cloning and expression, and use of enzyme predominantly for pulp bleaching (biobleaching) (Beg *et al*., 2001). This is the first report on production, purification and characterization of xylanase from *D. mannii* gut microbial community.

Xylanases are known to be inducible enzymes (Cai *et al*., 2003) and generally, efficient production of xylanolytic enzymes is known to be dependent upon the choice of an appropriate inducing substrate and the medium composition (Kulkarni *et al*., 1999). Generally, xylanases are induced in most microorganisms during growth on substrates containing xylan (Purkarthofer et al., 1993). In a shake flask culture, wheat straw was capable of playing a key role in the regulation of xylanase biosynthesis.

When xylanase production was examined at different temperatures, maximum enzyme activity was noticed at 50 ^{0}C but at higher temperatures the enzyme production decreased considerably. The results suggest that the enzyme is mesophilic. A number of reports have indicated that the optimal temperatures for activity of most xylanases ranged between 30 and 50 ^{0}C (Haltrich *et al*., 1996; Subramaniyan and Prema, 2002). In this study, xylanase production by the microbial community was observed in the pH range of 3.0 - 9.0. Maximum xylanase activity occurred at pH 5.0. After 24 hours activity remained fairly stable at pH 5-.0-8.0 with 74.6-77.2 % relative activity. The results in this study indicate that the optimal pH for xylanase activity was 5.0 and it is acidophilic in nature. Other studies have indicated that xylanases are produced at

initial pH lower than 7.0 (Subramaniyan and Prema, 2002; Ghosh *et al*., 1993).

The xylanase isolated in the current study is a single subunit protein of ≈20 kDa. It is therefore, similar in size to xylanases from fungi such as *Paecilomyces themophila* (Li *et al*., 2006) and *Penicillium citrinum* (Tanaka *et al*., 2005). This group of xylanases that is characterized by a small molecular weight and lack of or low cellulase activity has been classified under glycoside hydrolase (GH) family 11 of xylanases, a group viewed favourably in the pulp and paper industry (Henrissat, 1991; Henrissat and Bairoch, 1993). Absence of cellulase activity is attractive in the paper and pulp industry, as the cellulose required for paper manufacture is not degraded during the enzymatic bleaching. The small size of the enzyme is also advantageous as it facilitates easy penetration of the woody structures in the application of the xylanase (Haki and Rakshit, 2003).

Thermal stability is a desirable property of xylanases when considering their industrial application. The optimal temperature for the purified enzyme was found to be 50 ^{0}C and the activity dropped considerably after that. Residual activity also peaked at 50 ^{0}C with 70 % relative activity after 120 min incubation. The xylanase in the current study also had a high affinity for Xylan beechwood and very low affinity for cellulosic substrates, and also a low K_m of 0.4 % (w/v). Low K_m for substrate is important for industrial saccharification. It has been widely reported that many cellulase-free xylanases produced by mesophilic microorganisms lack high thermostability (Subramaniyan and Prema, 2002). These results indicate that for industrial application of the xylanase from *D. mannii* microbial culture in the current study, the suitable temperature range is 45.5-50.5 ^{0}C. These observations suggest that the xylanase produced by the culture may not be thermostable at temperatures commonly used in

the bleaching of pulp, but may be used in processes operated at moderate temperatures and pH which may include preparation of baked cereal food products, saccharification of agro-residues, aiding extraction and clarification of fruit juices (Chidi *et al*., 2008). Therefore, molecular cloning and expression of xylanase from *D. mannii* for industrial use, is recommended.

CHAPTER EIGHT

8.0 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

8.1. General Discussion and Chapter Summary

Lignocellulosic materials are abundant and renewable. Therefore, they can be valuable resources to use as raw materials in many biotechnological processes. One identified bottleneck in the enzymatic release of glucose from the cellulose fraction in lignocellulose is the enzyme cost. Dependence on depleting fossil fuel reserves and growing public concern over detrimental environmental effects associated with their use, have sparked great interest in alternative fuel sources (Li *et al.* 2009). The development of technologies for the conversion of renewable lignocellulosic biomass to bioethanol is emerging as one of the most popular alternatives to fossil fuels. Over the last three decades, extensive research has been conducted on the different approaches for the conversion of the cellulose fraction of lignocellulose to bioethanol (Sun and Cheng 2002). These approaches differ mainly in the method of hydrolysis of cellulose to fermentable sugars and the subsequent fermentation of the liberated sugars to produce bioethanol (Lynd *et al.* 1991).

Chapter 1 is introduction and a review of literature and highlights the need for the development of technologies for the production of alternatives to fossil fuels. In this chapter, known woodborers' ecology and digestive physiology is discussed. Lignocellulose structure and its delignification to liberate polysaccharides, and their depolymerization into monomers by lignocellulolytic enzymes are discussed. Lignocellulose, more specifically its cellulose fraction, is evaluated as feedstock for the production of bioethanol. This chapter also highlights methods of isolation, purification and characterization of lignocellulolytic enzymes.

Chapter 2 describes the study area, Mida Creek, Tudor Creek and Gazi Bay along the Kenyan coast. It also describes the collection of the woodborers and materials and methods used in the field and laboratory studies to achieve the stipulated objectives.

Chapter 3 describes morphological and molecular identification of woodborers. Three species were identified as *Dicyathifer mannii* (Wright, 1866)*, Sphaeroma terebrans* (Bate, 1866) and *Cirolana sp.* Host plant species (*Avicennia, Sonneratia, Rhizophora*) specificity was also evaluated, and the woodborers appear to have host preference.

Chapter 4 demonstrated that the gut extracts of marine woodborers *D. mannii* and *S. terebrans* exhibit lignocellulolytic activities. The lignocellulolytic activities investigated were lignin biodegradation enzymes; lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and laccase (Lac) or monophenol oxidase. Those that hydrolyse cellulose were glucanases endoglucanase (endo-1-4-β-D-glucanase), exoglucanase (1,4-β-D-glucan-cellobiohydrolase), and β-D-glucosidase or cellobiase (β-D-glucoside glucanohydrolase). Endo-1-4- β- xylanase was investigated in the hydrolysis of xylan, the chief type of hemicellulose. *D. mannii* crude extracts showed an appreciable endoglucanase (CMCase) activity of up to 50.7 ± 1.51 U/ml, xylanase activity of 35.52 ± 1.54 U/ml and Lip activity of up to 34.65 ± 0.12 U/L (1U represents 1 micromol of substrate transformed min^{-1}).

Chapter 5 determined microorganisms' diversity of woodborers' digestive tracts. Bacteria isolated on nutrient agar and fungi isolated on sabouraud dextrose agar (SDA) were identified by 16S rRNA and ITS gene barcoding respectively, with subsequent phylogenetic analysis. Four strains of bacteria were isolated from the digestive tracts of the woodborers; namely *L. boronitolerans* (from *D. mannii* and *S. terebrans*), *L. fusiformis* (from *S. terebrans* and *Cirolana sp.*), *L. sphaericus* and *L. xylanilyticus*

(both from *Cirolana sp.*). They had similarity to known 16S rRNA sequences of 98–99 %. A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showed that the bacteria are closely related members of the genus *Lysinibacillus.* Different strains of Ascomycetes fungi were also isolated. *Aspergillus niger* was isolated from the digestive tracts of *D.mannii* and *S. terebrans*. In addition, *Neosartorya fischeri, A. fumigatus* and *Penicillium sp.* were isolated from *D. mannii* whereas *Botryotinia fuckeliana* was isolated form *S. terebrans* digestive tract. *A. costaricaensis* and *A. fumigatus* were isolated from *Cirolana sp*. digestive tract. The fungi had similarity to known ITS sequences of 95–100 %. Existence of bacterial and fungal groupings symbiotically associated with woodborers is proposed.

In Chapter 6, pure bacterial and fungal isolates obtained in Chapter 5, as well as their mixed cultures were induced to produce lignocellulolytic enzymes in a variety of substrates. The chapter also investigated ethanol production from wheat straw hydrolysate obtained by degradation reaction of *D. mannii* gut microbial culture supernatant on ground and sieved wheat straw as substrate. The microorganisms showed general low ligninolytic activities but commendable cellulolytic and hemicellulolytic activities. β-glucosidase and Xylanase activities were the highest activities exhibited by both bacterial and fungal isolates. The highest was bacterial β-glucosidase activity (94.55 U/ml) shown by *Lysinibacillus boronitolerans* from *S. terebrans* gut cultured in a medium containing avicel cellulose. Xylanase activity was also relatively high (up to 91.7 U/ml) cultured in media containing cellobiose and xylan. The highest fungal activity was β-glucosidase (54.77 U/ml) shown by *Aspergillus niger* from *D. mannii* gut cultured in media containing mixed substrates (wood, FP, CMC, Avicel, xylan and cellobiose). 0.98 mg/100 ml supernatant yield was observed in 24 hours fermentations of 3 % wheat straw degradation reaction

mixture with *D. mannii* gut microbial filtrate (previously incubated for 1 hour at 50 ^{0}C) by 0.3 % *Saccharomyces cerevisiae*

In this study, 0.98 mg/100 ml supernatant yield was observed in 24 hours fermentations of 3 % wheat straw degradation reaction mixture with *D. mannii* gut microbial culture supernatant (previously incubated for 1 hour at 50 ^{0}C) by 0.3 % *S.cerevisiae*. In another study, Chen *et al*. (2009), inserted and expressed *Zymomonas mobilis* genes encoding essential enzymes involved in the fermentation pathway, alcohol dehydrogenase II (adh II) and pyruvate decarboxylase (pdc), into E. coli, resulting in increased cell growth and ethanol production. After 72 hours under microaerobic conditions, ethanol concentrations of 30 g/L were obtained on 10 % glucose. Considering the fact that the wheat straw in this study was not pretreated for delignification, and a short reaction time as well as fermentation period was used, the obtained yield is commendable.

In Chapter 7, an active lignocellulose degrading microbial consortium from *D. mannii* gut was bred on a liquid culture of wheat straw. β–glucosidase and Xylanase were constitutive and inducible in the culture at 30 ^{0}C for 5 days on a shaker at 180 rpm. Enzyme production was tested using different carbon sources and the culture supernatant showed moderate β–glucosidase and a high xylanase activity. Xylanase was purified to homogeneity by ammonium sulphate precipitation, Sephadex G-200 gel filtration and DEAE-Sephacel anion exchange chromatography, with 72 % yield from the liquid culture. A single band of activity was detected by SDS-PAGE zymogram analysis with an apparent molecular weight of ≈20-kDa. Activities of up to 49 ± 1.43 U ml^{-1} (1 U represents 1 micromol of glucose equivalents released min^{-1}) were obtained. The optimum temperature of the enzymatic activity was around 45.5-50.5 °C. The enzyme was stable for 120 min at 50 ^{0}C, maintaining 63-70 % of the

initial activity. The pH optimum of the xylanase activity was 5.0-6.0, and it had K_m and V_{max} values of 0.4 % (w/v) and 128.2 μmol ml^{-1} min^{-1}, respectively. This Chapter reports the isolation, purification and characterization of the xylanase from *D. mannii* gut microbial community and its potential implication for use in bleaching of pulp, animal feed, manufacture of bread, and xylitol production industries.

The size of the purified enzyme (≈20 kDa) as revealed by SDS-PAGE, and optimum activity at 50° C in pH 5.0, suggest that the enzyme is small, mesophilic and acidophilic in nature. A group of xylanases that is characterized by a small molecular weight and lack or low cellulase activity has been classified under glycoside hydrolase (GH) family 11 of xylanases, a group viewed favourably in the pulp and paper industry (Henrissat, 1991; Henrissat and Bairoch, 1993). Absence of cellulase activity is attractive in the paper and pulp industry, as the cellulose required for paper manufacture is not degraded during the enzymatic bleaching. The small size of the enzyme is also advantageous as it facilitates easy penetration of the woody structures in the application of the xylanase (Haki and Rakshit, 2003). The xylanase in the current study also had a high affinity for xylan beechwood and very low affinity for cellulosic substrates, and in addition, a low K_m of 0.4 % (w/v). Low K_m for substrate is important for industrial saccharification. However, thermal stability is a desirable property of xylanases when considering their industrial application. These results indicate that for industrial application of the xylanase from the culture in the current study, the suitable temperature range is 45.5-50.5 ^{0}C, this may not be thermostable at temperatures commonly used in the bleaching of pulp, but may be used in processes operated at moderate temperatures and pH which may include preparation of baked cereal food products, saccharification of agro-residues, aiding extraction and clarification of fruit juices (Chidi *et al*., 2008).

8.2. Conclusions

1. This research identified three woodborer species from the Kenyan Coast as *Dicyathifer mannii* (Wright, 1886), *Sphaeroma terebrans* (Bate 1886) and *Cirolana sp.* The host specific *S. terebrans* and *Cirolana sp.* occur only on *Avicennia sp*. *D. mannii* occurs mostly on *Rhizophora* plants, but also on *Sonneratia*. The most frequent marine species were *D. mannii* and *S. terebrans*.
2. This research successfully isolated lignocellulolytic enzymes from woodborers' guts. *D. mannii* had the most efficacious lignocellulolytic gut extracts and therefore, the most suitable source for lignocellulolytic enzymes.
3. The study also demonstrated that the lignocellulolytic enzymes are inducible in a variety of carbon sources including natural ones like ground *Rhizophora* wood dust and wheat straw. The bacterial and fungal strains of *Lysinibacilli* and *Aspergilli* used have a high potential for β-glucosidase and xylanase production.
4. The lignocellulolytic enzymes were able to biodegrade lignocellulose in wheat straw for subsequent bioethanol production.
5. The study revealed the highest lignocellulolytic enzyme as xylanase, which was successfully purified and characterized. The enzyme has a small molecular weight, is mesophilic and acidophilic in nature.

   Therefore, biodegradation of lignocellulosic agro-industrial residues using *D. mannii* gut microbial community is a promising approach in providing efficient biomass decomposition for subsequent conversion to value-added products, especially bioethanol production.

8.3. Recommendations

1. The study recommends monitoring of woodborer community to give an overall idea of the intensity of the problem and enable removal of the source of infestation at the very onset. It also recommends study of natural bio-resistance of the mangrove plants that show no attack as well as investigations on effect of fungal metabolite on larval settlement in the fungi-borer interrelationships and specificity theory, as well as possibility of woodborers' natural enemies for their control.
2. The presence of bacteria and fungi inside the digestive tracts of woodborers is of industrial importance in biodegradation of lignocellulose. Investigation of the functional characteristics and role in the host organism is required to confirm the symbiotic status of the microorganisms associated with the woodborers digestive tract. These microorganisms could be potential sources of commercial enzymes for industrial use in biobleaching of pulp, decolouration of textile dyes and bioremediation of polluted environment (Castillo *et al.,* 1997) in the case of lignin modifying enzymes; and in animal feed, manufacture of bread, bioethanol and xylitol production for cellulolytic and hemicellulolytic enzymes (Bhat, 2000; Gray *et al.,* 2006).
3. *Lysinibacilli* and *Aspergilli* in the current study present interesting advantages that make them good models for studying physiological approaches to enzyme production and lignocellulose degradation, including inhibition studies, with the aim of developing key lignocellulolytic enzymes. Their genomic features and systematic studies on their enzyme systems need to be investigated to facilitate directed strain engineering for improved performance.
4. Molecular cloning and expression of xylanase isolated and purified from *D. mannii* gut

microbial community for industrial use is therefore recommended.

8.4 Future Perspectives: Microbial Metagenomics

Cultured microorganisms have been the most common resource for biotechnological exploration of novel natural products and processes (Bull *et al*., 2000). These microbial compounds include a wide range of important industrial lignocellulolytic enzymes. Microorganisms from environmental samples have traditionally been accessed by isolation and cultivation. However, in the last 2 decades, scientists have realised that only a small percentage (1-10 %) of the microbial diversity is retrievable from an environmental sample by current culturing techniques (Pace, 1996; Hugenholtz *et al.,* 1998).

Culture-independent molecular biology-based methods involving the direct isolation of the total microbial community DNA (metagenome) provide an opportunity to explore the metabolic potential of organisms that cannot be isolated by cultivation. The strategy involves cloning large DNA fragments (20kb up to >500kb) from an environmental sample followed by analysis of the resulting metagenomic libraries to search for novel biological activities (Handelsman *et al.,* 1998; Lorenz *et al.,* 2002). This technique had been used to detect a wide range of biocatalysts from unculturable biodiversity (Streit *et al*., 2004; Steele and Streit, 2005). To date there are only a few reports of metagenome-derived cellulases (Healy *et al.,* 1995; Rees *et al.,* 2003; Voget *et al.,* 2003).

One of these reports is on Cel5A, a soil metagenome-derived cellulase. This has been shown to be an endoglucanase (endo 1,4-β-glucan hydrolase), which is highly active towards soluble forms of cellulose (Voget *et al.,* 2006). This novel cellulase is remarkably stable over a wide pH and temperature range. It also withstands high salt

concentrations, and is therefore an ideal candidate for industrial applications.

The application of lignocellulolytic enzymes in biorefineries and ethanol production requires improved enzymes, the use of low cost resources and the manufacture of enzymes on-site to overcome the need for purification and stabilisation of enzyme preparations to reduce production costs. To address these problems, several studies have focused on the discovery of new lignocellulolytic enzymes, including screening for new enzyme-producing microorganisms, random mutagenesis of bacterial and fungal strains as well as genetic engineering of microorganisms (Hayward, *et al.,* 1999; Picart, *et al.,* 2007).

The use of metagenomics to explore the vast biocatalytic potential unearthed in the uncultured portion of the biodiversity is a promising tool for the discovery of new and versatile lignocellulolytic enzymes, especially from extreme environments, including guts of xylotrophic invertebrates, which usually harbour many enzymes with industrially relevant characteristics. Currently, a wide range of different approaches are being exploited for the development of commercially significant and low cost technologies to facilitate the production of enzymes with industrial utility.

Since only approximately 1-10 % of all microbes in the biosphere are culturable under normal laboratory conditions (Pace, 1997; Rappe *et al.,* 2003), culture independent approaches should be embraced to exploit the collective genomes of microbial communities (Schloss and Handelsman, 2003).

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APPENDICES

1.0 Woodborers DNA PCR Gel Photo

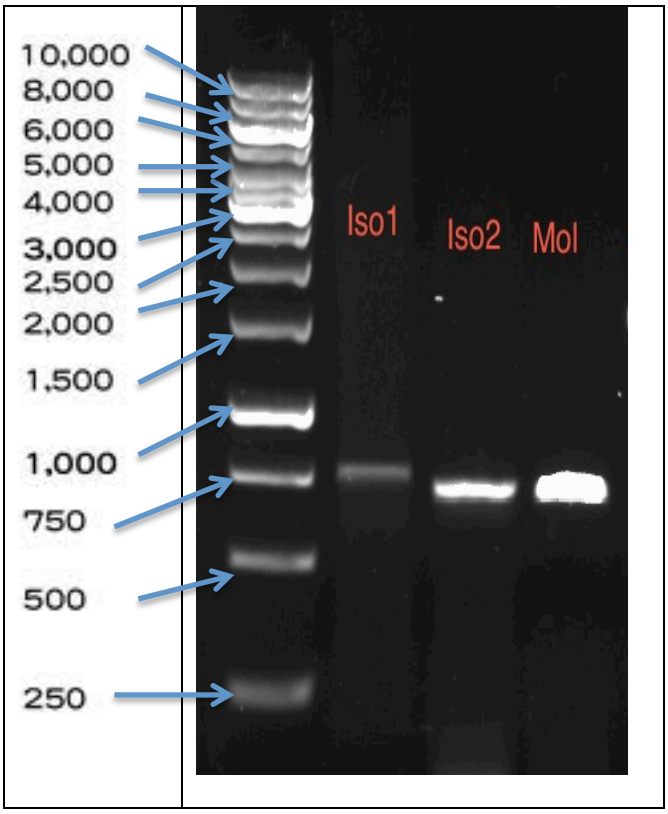

Figure A1. Agarose gel of woodborers PCR product

Lane 1, ladder 1kb; Iso1, *S. terebrans*; Iso2, *Cirolana* sp; Mol, *D. mannii.*

2.0 Sugar Standard Curves

Enzyme activity was measured by the reducing sugars formed in a modified dinitrosalicylic acid (DNS) method (Miller, 1959) using glucose and xylose standard curves. The DNS reagent was composed of the following:

1 g sodium hydroxide, 1 g 3,5-dinitrosalicylic acid (DNS), 0.2 g phenol, 0.05 g sodium metabisulphite in 100 ml distilled water.

40 % potassium-sodium tartrate (Rochelle salt).

1ml of DNS Reagent was added to 1ml of sample and heated at 90-100 ^{0}C for 5-10 minutes. 0.3ml of 40 % potassium-sodium tartrate was then added and solution cooled to room temperature in a cold water bath. Absorbance readings were taken at 575 nm for glucose and 540 nm for xylose. The reducing sugar standard curves were generated with concentrations of glucose between 0 and 0.8 mg/ml and xylose between 0 and 0.1 mg/ml.

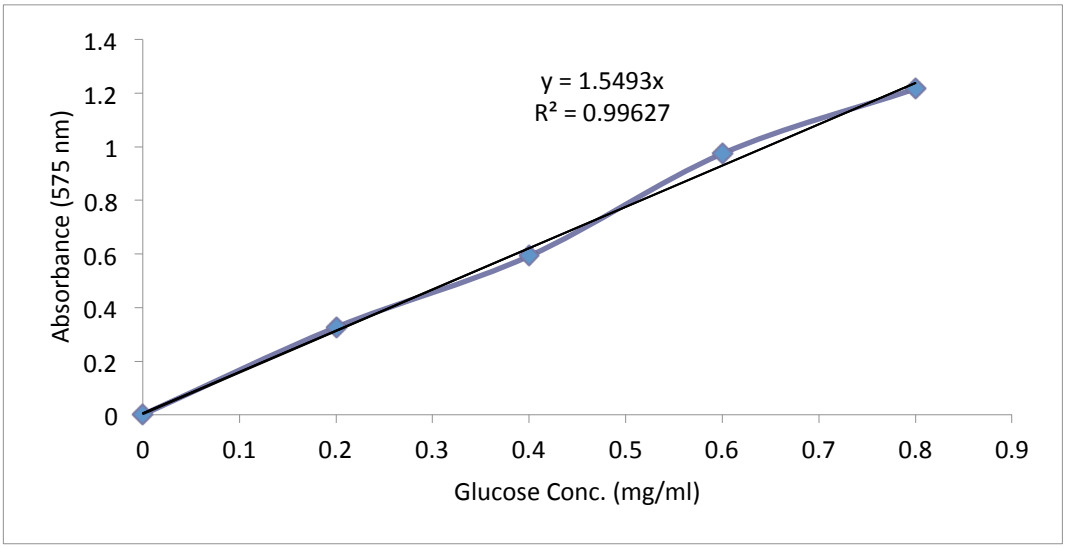

Figure A2. Standard curve of absorbance as a function of glucose concentration

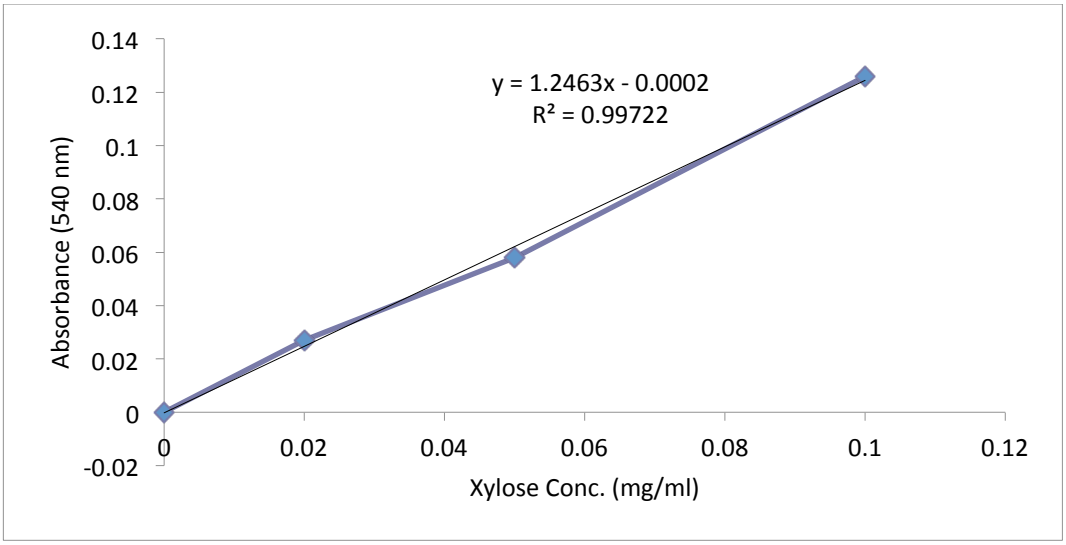

Figure A3. Standard curve of absorbance as a function of xylose concentration

3.0 Media Preparation for Bacterial and Fungal Growth

Table A1. Bacterial growth media

Nutrient agar (1L)	
Meat extract	1g
Yeast extract	2g
Peptone	5g
Sodium chloride	5g
Agar	15g

28 g of nutrient agar was added to 1L of distilled water, mixed with magnetic stirrer and autoclaved at 121 ^{0}C for 20 minutes. This was cooled and aseptically poured onto plates. The plates were inoculated and covered with parafilm and kept at 39^{0}C.

Table A2. Fungal growth media

Sabouraud Dextrose Agar (SDA) (1L) pH 5.6	
Mycological peptone	10g
Glucose	40g
Agar	15g

65 g of SDA was added to 1L of distilled water, mixed with magnetic stirrer and autoclaved at 121 ^{0}C for 20 minutes. This was cooled and aseptically 1g antibiotic added to inhibit bacterial growth, then poured onto plates. The plates were inoculated and covered with parafilm and kept at 30^{0}C.

4.0 Bacteria and Fungi DNA PCR Gel Photos

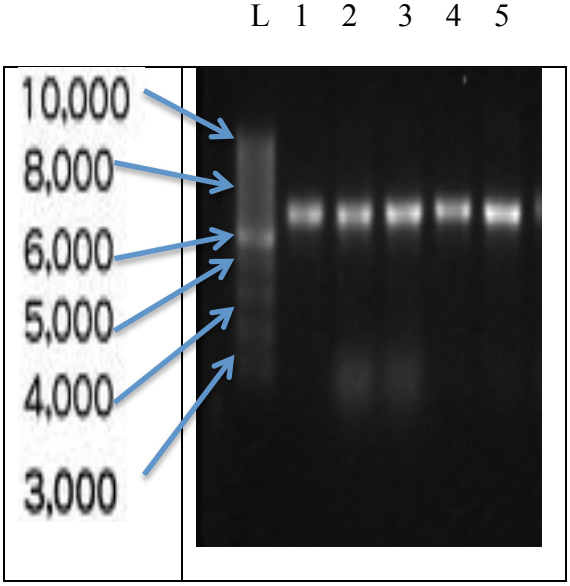

Figure A4. Agarose gel of PCR products of fungal isolates from *S.terebrans* gut

L, ladder 1kb; Lane 1, sp a; Lane 2, sp b; Lane 3, sp c; Lane 4, sp d; and Lane 5, sp e.

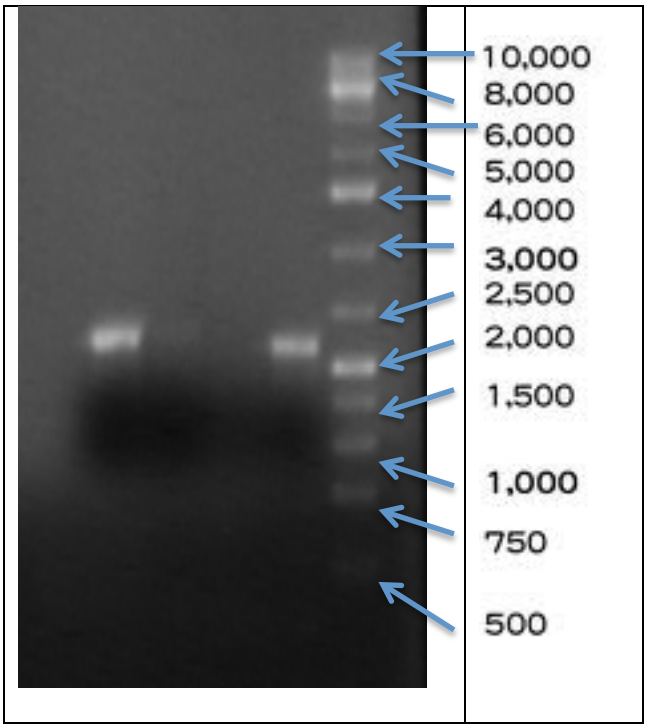

Figure A5. Agarose gel of PCR product of fungal isolates from C*irolana sp.* gut

L, ladder 1kb; Lane 1, sp a; and Lane 2, sp b.

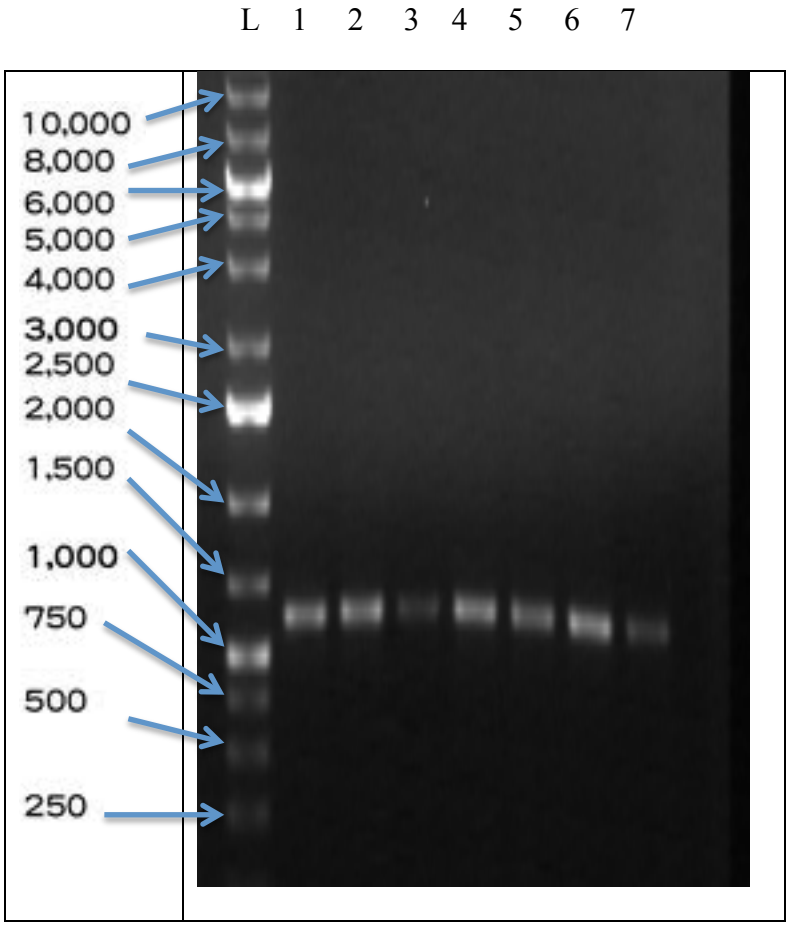

Figure A6. Agarose gel of PCR products of fungal isolates from *Teredo mannii* gut

L, ladder 1kb; Lane 1, sp a; Lane 2, sp b; Lane 4, sp c; Lane 5, sp d; and Lane 6, sp e.

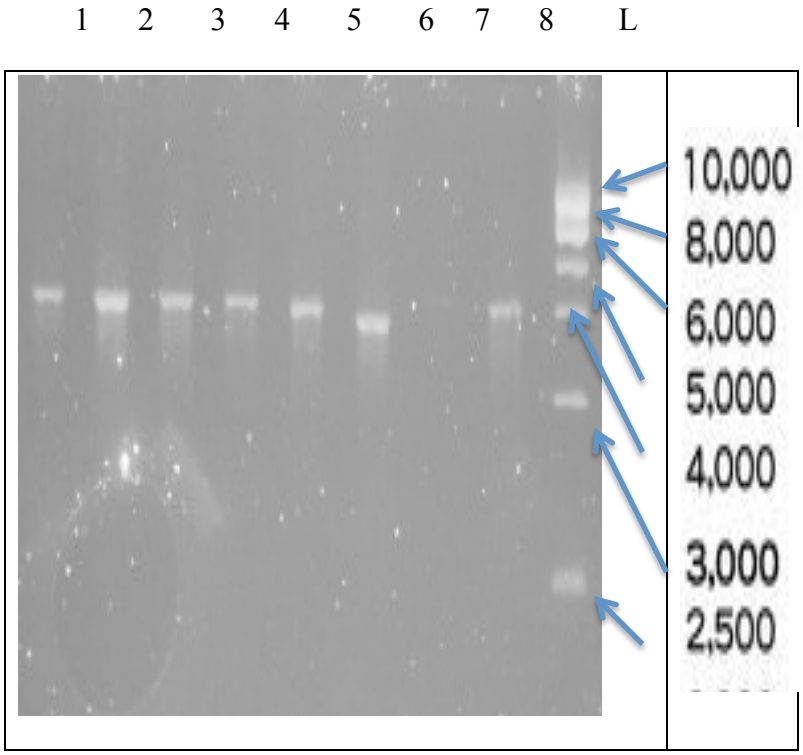

Figure A7. Agarose gel of bacterial isolates PCR product from the woodborers

L, ladder 1kb; Lane 1, *S.terebrans* 1; Lane 2, *S.terebrans* 2; Lane 3, *S.terebrans* 3; Lane 4, *Cirolana sp* 1; Lane 5, *Cirolana sp* 2; Lane 6, *Cirolana sp* 3; and Lane 8, *Teredo mannii*.

5.0 *D. mannii* Gut Microbial Crude Culture Supernatant Activity

Table A3. Activity of *D. mannii* gut microbial Community

FPase	CMCase	Avicelase	β–glucosidase	Xylanase
0.1 ±0.01	1.34 ±0.56	1.25 ±0.9	32.77 ±0.3	48.95 ±1.43

Cellulolytic and hemicellulolytic activity (U/ml) of the crude culture supernatant of *D. mannii* gut microbial community. Data is mean of triplicates.

6.0 SDS-PAGE

Table A4. Laemmli gel for SDS-PAGE

10 ml separating gel, 5 ml stacking gel solution		
	15 % separating gel	4 % stacking gel
Acrylamide stock solution (30 % Acrylamide, 0.8 % Bis-Acrylamide)	5 ml	0.67 ml
1.5M Tri-HCl, pH 8.8	2.5 ml	-
0.5M Tri-HCl, pH 6.8	-	1.25 ml
10% SDS solution	0.1 ml	55 µl
Deionized water	2.35 ml	3.00 ml
10% Ammonium persulphate (APS) solution	50 µl	25 µl
NNN'N'-Tetramethylethylene-diamine (TEMED)	3.3 µl	2.5 µl
FINAL VOLUME	10.00 ml	5.00 ml

Table A5. Dalton Mark IV® (Sigma) for SDS-PAGE

Lysozyme	14,300
β-lactoglobulin	18,400
Trypsinogen	24,000
Pepsin	34,700
Egg albumin	45,000
Bovine albumin	66,000

A lyophilized mixture of bovine and egg albumins, β-lactoglobulin, lysozyme, trypsinogen (PMSF treated), and pepsin.

7.0 Xylanase Purification & Characterization

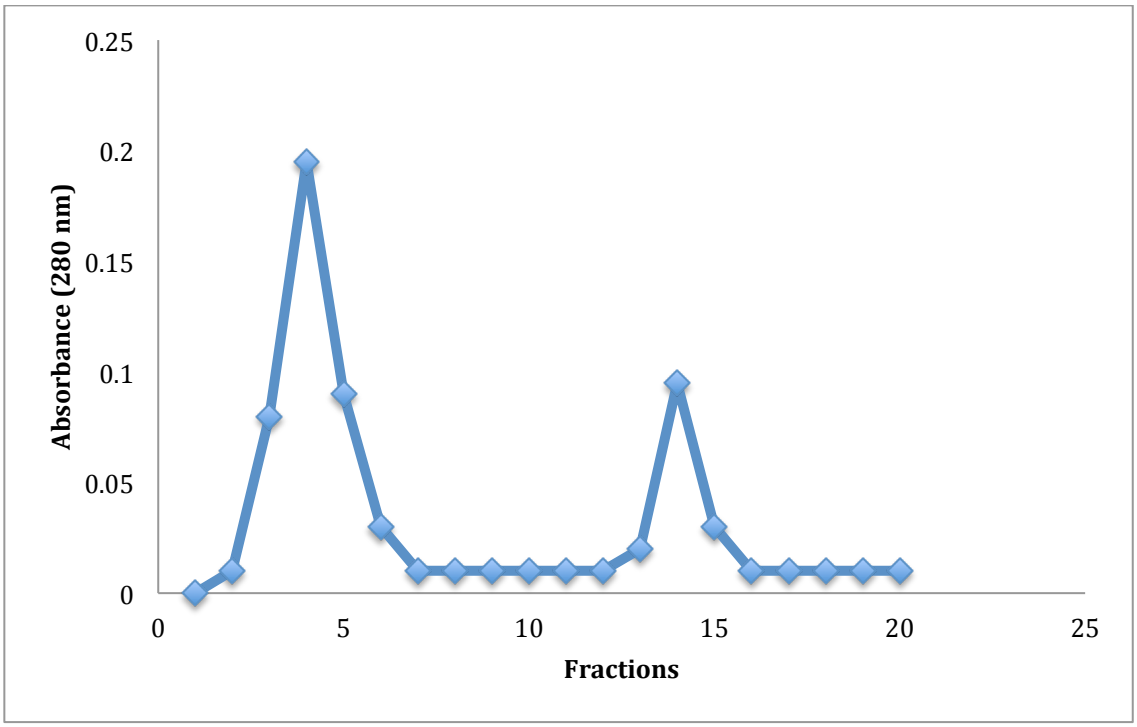

Figure A8. Sephadex G-200 gel filtration chromatography A_{280} profile

A_{280} of fractions of Sephadex G-200 Gel filtration chromatography of the ammonium sulphate precipitated culture supernatant from *D. mannii* gut microbial community.

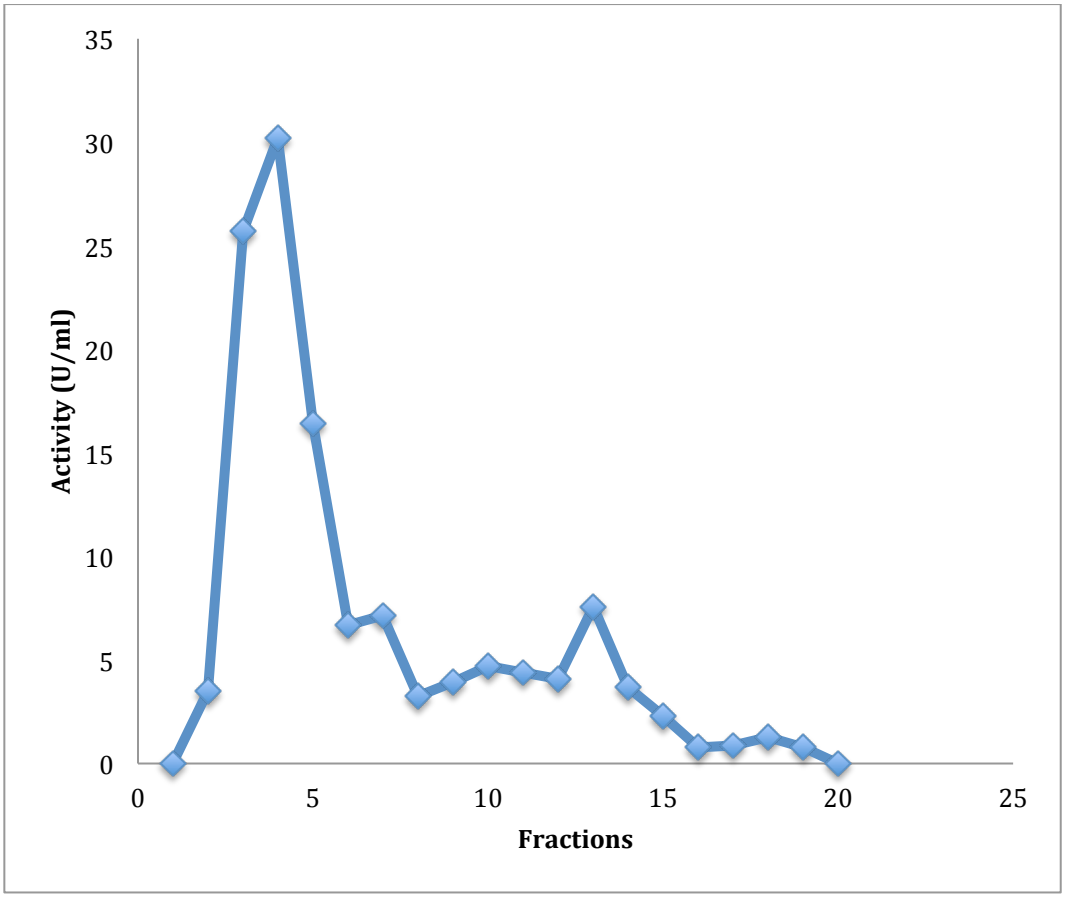

Figure A9. Activity of sephadex G-200 gel filtration chromatography fractions

Xylanase activity of fractions from sephadex G-200 gel filtration chromatography of the ammonium sulphate precipitated culture supernatant from *D. mannii* gut microbial community.

Table A6. Results obtained during optimum pH determination.

pH	Initial Activity (U/ml)	Optimum Activity (%)	Residual Activity (U/ml)	% Residual Activity
pH 4	32.76	55.27	11.47	35.01
pH 5	59.27	100.00	45.75	77.20
pH 6	57.84	97.60	43.61	75.40
pH 7	43.29	73.05	32.93	76.07
pH 8	42.36	71.48	31.58	74.55
pH 9	40.84	68.92	24.62	60.28

Initial activity obtained after incubation of xylanase and beechwood xylan substrate in universal buffer with different pH values at 50 ^{0}C for 30 minutes, residual activity is percent of the original activity after 24 hours incubation.

Table A7. Results obtained during optimum temperature determination

TEMP ^{0}C	Xylanase Activity	% Activity	Residual Activity	% Residual Activity
30	8.25	18.25	5.25	63.64
40	12.11	26.79	8.25	68.13
50	45.21	100.00	31.65	70.01
60	12.11	26.79	5.11	42.20
70	6.21	13.74	1.89	30.43

Xylanase activity obtained after incubation of xylanase at different temperatures (30–70 ^{0}C) in 1 % beechwood xylan solution prepared in 50 mM sodium acetate buffer, pH 5.0. Residual activity was measured after 120 minutes.

Folin-Lowry standard curve

Folin-Lowry assay method was used in protein detection. The standard curve for this assay was generated using various concentrations of BSA from 0 to 0.8 mg/ml.

Reagent A: 2% Na_2CO_3, 0.4 % NaOH

Reagent B: 0.5 %Copper Sulphate, 1 % Potassium-Sodium Tartrate

Solution 1: 100 ml of Reagent A, 2ml of Reagent B

Solution 2: Commercial Folin- Ciocalteau phenol diluted 1:1 with distilled water.

50 µl volume of sample was added to a solution containing 1 ml of solution 1 and incubated at room temperature for 15 minutes in the dark. 125 µl of solution 2 was added and the mixture incubated for a further 30 minutes at room temperature. The absorbance was read at 750 nm to obtain the standard curve.

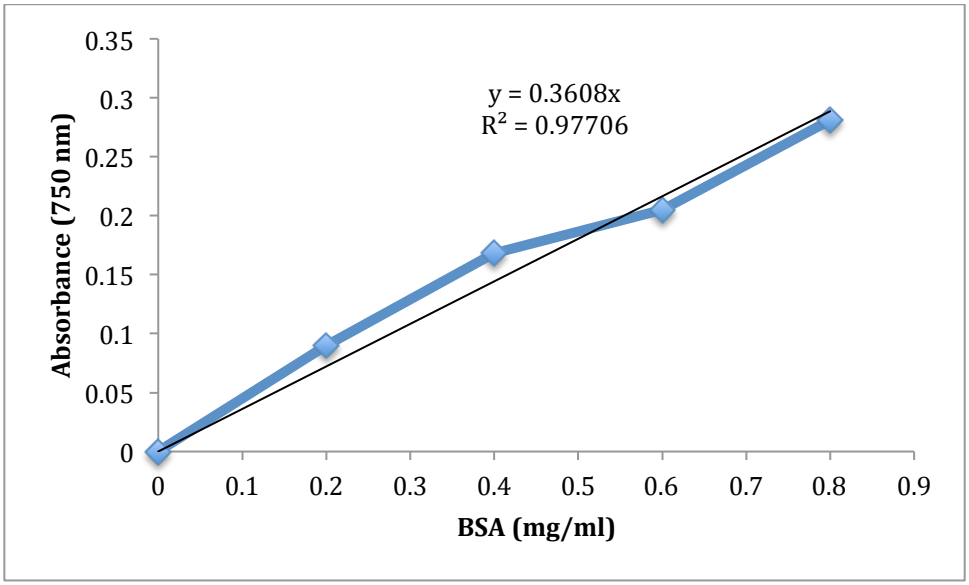

Figure A10. Folin-Lowry standard curve