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

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

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This thesis is dedicated to my dear husband Mr.

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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

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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

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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.