BIOETHANOL PRODUCTION BY *SACCHAROMYCES CEREVISIAE* USING LIGNOCELLULOSE SUBSTRATES SACCHARIFIED BY FUNGAL ISOLATES FROM KARURA FOREST RESERVE, NAIROBI COUNTY, KENYA

KAMANDE STEPHEN MWANIKI (BPHL. BIOTECHNOLOGY)

I56/CTY/PT/38083/2017

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE (BIOTECHNOLOGY) IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA UNIVERSITY

JANUARY, 2025

DECLARATION

I, Stephen Mwaniki Kamande declare that this thesis is my original work and has not been presented for degree or other awards in any other institution or university.

Signature ………………………………………. Date ……………………………………

Stephen Mwaniki Kamande - I56/CTY/PT/38083/2017
Department of Biochemistry, Microbiology and Biotechnology

SUPERVISORS

We confirm that the work reported in this thesis was carried out by the candidate under our supervision

Signature ………………………………………. Date …………………………………….

Dr. George Isanda Omwenga

Department of Biochemistry, Microbiology and Biotechnology

Kenyatta University

Signature ………………………………………. Date …………………………………….

Prof. Mathew Piero Ngugi

Department of Biochemistry, Microbiology and Biotechnology

Kenyatta University

DEDICATION

This thesis is highly dedicated to Almighty God who has blessed me with wisdom and knowledge, as well as my family who have continuously supported and encouraged me. I also dedicate this work to my loving parents Simon and Florence as well as my siblings. Thank you all for always supporting and believing in my dreams.

ACKNOWLEDGMENTS

I am delighted to express my profound appreciation to DAAD and HELP secretariats for awarding me the scholarship that helped me to achieve this dream.

I am extremely grateful to my thesis supervisor Dr. George Omwenga of the school of Pure and Applied Sciences, Department of Biochemistry, Microbiology and Biotechnology at Kenyatta University. His immense knowledge, experience and availability has encouraged me. When doing my research, I could always consult him whenever I had a problem. I would like to acknowledge my second supervisor Prof. Mathew Ngugi of the school of Pure and Applied Sciences, Department of Biochemistry, Microbiology and Biotechnology at Kenyatta University. I appreciate his insightful criticism of this thesis.

Finally, a lot of appreciation to my family for their steadfast financial assistance and consistent prayers during my study. This accomplishment would not be possible without them.

TABLE OF CONTENTS

DECLARATION ..... **ii**
DEDICATION ..... **iii**
ACKNOWLEDGMENTS ..... **iv**
TABLE OF CONTENTS ..... **v**
LIST OF FIGURES ..... **x**
LIST OF TABLES ..... **x**
ABBREVIATIONS AND ACRONYMS ..... **xiii**
ABSTRACT ..... **xiv**
CHAPTER ONE **1**
INTRODUCTION **1**
1.1 Background Information 1
1.2. Statement of the Problem 3
1.3. Justification of the Study 4
1.4 Null Hypotheses 5
1.5 Objectives 6
1.5.1 General Objective 6
1.5.2 Specific Objectives 6
1.6 Significance of the Study 6
CHAPTER TWO **8**
LITERATURE REVIEW **8**
2.1. Lignocellulose 8
2.1.1. Cellulose 8
2.1.2. Hemicellulose 9
2.1.3 Lignin 10
2.2 Fermentation methods of lignocellulose conversion to cellulase enzymes and bioethanol 11
2.2.1 Submerged Fermentation (SmF) 11
2.2.2 Solid State Fermentation (SSF) 12

2.3 Pretreatment 13
2.3.1 Chemical pretreatment 13
2.3.2 Physical pretreatment 14
2.3.3 Biological method 14
2.4 Optimization of cellulase enzymes production 15
2.5 Applications of cellulase enzymes 16
2.5.1 Biofuel 16
2.5.2 Food processing industry 19
2.5.3 Animal feed industry 19
2.5.4 Agriculture industry 19
2.6 Substrates 20
2.6.1 Maize cobs 21
2.6.2 Sawdust (cypress and eucalyptus) 22
2.6.2.1 Differences between softwood (cypress) and hardwood (eucalyptus) 22
2.7 Cellulase producing organisms 23
2.8 Cellulolytic enzymes 24
2.8.1 Endo β-glucanase (carboxymethyl cellulases) 24
2.8.2 Exo, 4-β-glucanase (Cellobiohydrolase – CBH) 24
2.8.3 β-glucosidase (Cellobiase) 25
2.9 Enzymatic saccharification of maize cobs 25
2.10 Bioethanol production from maize cobs 26
CHAPTER THREE 27
MATERIALS AND METHODS 27
3.1 Collection of samples 27
3.1.1 Sampling area 27
3.1.2 Sampling technique 27
3.2 Experimental design 28
3.3 Screening and isolation of cellulase producing fungi 28
3.3.1 Preparation of 1% CarboxyMethyl Cellulose (CMC) agar 28
3.3.2 Screening and isolation of fungal isolates 28

3.3.3 Preparation of Potato Dextrose Agar (PDA) 29
3.3.4 Culture maintenance 29
3.4 Molecular identification of fungi 30
3.4.1 Fungal genomic DNA extraction 30
3.4.2 PCR amplification 31
3.4.3 Sequencing of PCR amplicons 31
3.4.4 Phylogenetic analysis 32
3.5 Solid State Fermentation (SSF) for cellulase enzymes production. 32
3.5.1 Preparation of substrates 32
3.5.2 Preparation of inoculum and inoculation 33
3.5.3 Effect of time of incubation on cellulase enzyme production 33
3.5.4 Effect of substrate pretreatment on cellulase enzyme production 33
3.6 Lignocellulose composition determination 34
3.6.1 Extractives 34
3.6.2 Hemicellulose 34
3.6.3 Lignin 34
3.6.4 Cellulose 35
3.7 Enzyme extraction 35
3.8 Cellulase enzyme assays 35
3.8.1 Preparation of DNS reagent 35
3.8.2 Preparation of Potassium Sodium Tartrate (PST) solution 36
3.8.3 Preparation of standard glucose stock solution 36
3.8.4 Preparation of standard 1mM p-nitrophenol (pNP) stock solution 36
3.8.5 Total cellulase activity (FPase) 36
3.8.6 Endo-glucanase assay 37
3.8.7 Exo-glucanase assay 37
3.8.8 β glucosidase assay 38
3.9 Bioethanol production 39
3.9.1 Production of cellulase enzymes for saccharification of maize cobs 39

3.9.2 Concentration of crude enzymes 39
3.9.3 Saccharification of maize cobs 40
3.9.4 Estimation of reducing sugar by DNS Method (Miller, 1959) 40
3.9.5 Preparation of *Saccharomyces Cerevisiae* inoculum 41
3.9.6 Saccharification and fermentation of maize cobs 41
3.10 Determination of bioethanol 41
3.10.1 Preparation of acidified potassium dichromate 41
3.10.2. Preparation of ethanol standards 42
3.10.3 Determination of bioethanol by acidified dichromate (Mushimiyimana and Tallapragada, 2016)……… 42
3.11 Data management and statistical analysis 42
CHAPTER FOUR 44
RESULTS 44
4.1 Cellulase activity of isolated fungi 44
4.2.1 Gel electrophoresis 45
4.2.2 Phylogenetic analysis 45
4.3 Effect of time of incubation on cellulase production 47
4.3.1 Effect of time of incubation on total cellulase activity (FPase) 47
4.3.3 Effect of time of incubation on endoglucanase activity 53
4.3.4 Effect of time of incubation on β-glucosidase activity 56
4.4 Effect of substrates pretreatment on cellulase production 59
4.4.1 Effect of substrates pretreatment on Total cellulase activity(FPase) 59
4.4.2 Effect of substrates pretreatment on exoglucanase activity 63
4.4.3 Effect of substrates pretreatment on endoglucanase activity 66
4.4.4 Effect of substrate pretreatment on β-glucosidase activity 70
4.4.5 Effect of pretreatment on chemical composition of maize cobs, cypress and eucalyptus substrates 74
4.5 Bioethanol production from maize cobs substrate 76
4.5.1 Saccharification of maize cobs with cellulases of isolated fungi 76

4.5.2 Production of bioethanol from maize cobs by simultaneous hydrolysis and fermentation processes. 78

CHAPTER FIVE 80

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS 80

5.1 Discussion..... 80

5.1.1 Screening and isolation (CMC- Congo red), and maintenance of cellulase producing fungi on PDA 80

5.1.2 Molecular identification and phylogenetic analysis of cellulase producing fungi 80

5.1.3. Cellulases production and optimization 81

5.1.4 Limitation of the study 87

5.2 Conclusions 87

5.3 Recommendations 88

5.3.1 Recommendations from the study 88

5.3.2 Recommendations for future research..... 88

REFERENCES..... 90

APPENDIX 105

Appendix I: Sequences of the isolates 105

Appendix II: Research approval 106

Appendix III: NACOSTI 107

LIST OF FIGURES

Figure 2.1 Schematic structure of cellulose. 9

Figure 2.2 Schematic structure of hemicellulose. 10

Figure 2.3. Primary precursors molecule of lignin. 11

Figure 2.4. Schematic representation of lignocellulose biomass pretreatment. 14

Figure 2.5. Mechanism of cellulase action 25

Figure 3.1: Map of Karura Forest Reserve 27

Figure 4.1 Cellulase activity of isolated fungi 44

Figure 4.2 Photographic image of an agarose gel indicating the amplification of the ITS target 45

Figure 4.3 Phylogenetic tree for *Xylaria sp.* km01, *Xylaria sp.* km03, *Nemania sp.*…km02 *Cyathus sp*. Km04, *Podoscypha bolleana* km05 and *Podoscypha petalodes* km06.......... 46

Figure 4.4 FPase activities of (A) *Xylaria* sp. km01, (B) *Nemania* sp. km02, (C) *Xylaria* sp. km03, (D) *Cyathus sp*. km04, (E)*Podoscypha bolleana* km05 and (F) *Podoscypha petalodes* km06 cultured on untreated cypress, eucalyptus and maize cob substrates. 49

Figure 4.5 Exoglucanase activities of (A) *Xylaria* sp. km01, (B) *Nemania* sp. km02, (C) *Xylaria* sp. km03, (D) *Cyathus sp*. ..... km04, (E) *Podoscypha bolleana* km05 and (F) *Podoscypha petalodes* km06 cultured on untreated cypress, eucalyptus and maize cob 52

Figure 4.6 Endoglucanase activities of (A) *Xylaria* sp. km01, (B) *Nemania* sp. km02, (C) *Xylaria* sp. km03, (D) *Cyathus* ......*sp*. km04, (E) *Podoscypha bolleana* km05 and (F) *Podoscypha petalodes* km06 cultured on untreated cypress, eucalyptus and maize cob substrates.. 55

Figure 4.7 β-glucosidase activities of (A) *Xylaria* sp. km01, (B) *Nemania* sp. km02, (C) *Xylaria* sp. km03, (D) *Cyathus sp*. ..... km04, (E) *Podoscypha bolleana* km05 and (F) *Podoscypha petalodes* km06 cultured on untreated cypress, eucalyptus and maize cob substrates.. 58

Figure 4.8 FPase activities of (A) *Xylaria sp*. km01, (B) *Nemania sp.* km02, and (C) *Xylaria sp*. km03, (D) *Cyathus sp*. ..... km04, (E) *Podoscypha bolleana* km05 and (F) *Podoscypha petalodes* km06 cultured on pretreated cypress, eucalyptus and maize cob substrates. 62

Figure 4.9 Exoglucanase activities of (A) *Xylaria* sp. km01, (B) *Nemania* sp. km02, and (C) *Xylaria* sp. km03, (D) *Cyathus.sp*. km04, (E) *Podoscypha bolleana* km05 and (F) *Podoscypha petalodes* km06 cultured on pretreated cypress, eucalyptus and maize cob substrates.. 65

Figure. 4.10 Endoglucanase activities of (A) *Xylaria* sp. km01, (B) *Nemania* sp. km02, and (C) *Xylaria* sp. km03, (D) *Cyathus sp.* km04, (E) *Podoscypha bolleana* km05 and (F) *Podoscypha petalodes* km06 cultured on pretreated cypress, eucalyptus and maize cob substrates.. 69

Figure. 4.11 β-glucosidase activities of (A) *Xylaria* sp. km01, (B) *Nemania* sp. km02, and (C) *Xylaria* sp. km03, (D) *Cyathus sp*. km04 (E) Podoscypha *bolleana* km05 and (F) *Podoscypha petalodes* km06 cultured on pretreated cypress, eucalyptus and maize cob substrates.. 73

Figure 4.12 Reducing sugars yield (mg/ml) produced by saccharomyces cerevisiae using untreated maize cob hydrolysate at........ different concentration by cellulases of (A) *Xylaria* sp. km01, (B) *Nemania* sp. KM02, (C) *Xylaria* sp. KM03 (D) *Cyathus* sp. km04, (E) *Podoscypha boleana* km06, and (D) *Podoscypha petalodes*.................... 77

Figure 4.13 Ethanol yield (mg/ml) produced by saccharomyces cerevisiae using untreated maize cob hydrolysate at differentconcentration by cellulases of (A) Xylaria sp. km01, (B) *Nemania* sp. KM02, (C) *Xylaria sp.* KM03 (D) *Cyathus* sp. km04, (E) *Podoscypha boleana* km06, and (D) *Podoscypha petalodes*. Values are..79

LIST OF TABLES

Table 2.1. Dry Matter (DM) Distribution in Corn Residue 21
Table 4.1 Clear zone dimension of cellulolytic activity of fungal isolates 45
Table 4.2 Effect of pretreatment on chemical composition of maize cobs, cypress and eucalyptus substrates 75

ABBREVIATIONS AND ACRONYMS

CBH	Cellobiohydrolase
CMC	Carboxymethyl cellulose
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic acid
FPase	Total cellulase activity
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PST	Potassium sodium solution
SmF	Submerged fermentation
SSF	Solid state fermentation

ABSTRACT

Lignocellulose from plant biomass is the most available organic compound in nature that can be used by the microbes for the cellulases production, reducing sugars and bioethanol. However, its recalcitrant to hydrolysis and use of lignocellulolytic enzymes to convert this plant biomass into fermentable sugars and for biofuel production is paramount. Push for clean environment and renewable energy is health concerns emanating from fossil fuels The main goal of the study was to to investigate bioethanol production by saccharomyces cerevisiae using lignocellulose substrates saccharified by fungal isolates from karura forest reserve, nairobi county, kenya. A total of 20 of fungal samples were obtained from Karura forest reserve growing on decaying biomass based on their morphological variation. Six fungi from collected sample were selected after screening using 1% CMC- congo red agar for cellulolytic activities based on zonal inhibition and the isolates selected for the study. Characterization by molecular technique (ITS1 and ITS4 primers) was used and biological sequences analyzed using BLAST algorim and MEGA II software to identify the six isolates, as *Xylaria sp.* km01, *Nemania sp.* km02, *Xylaria sp.* km03, *Cyathus sp.* KM04, *Podoscypha bolleana* km05 and *Podoscypha petalodes* km06. The phylogenetical analysis revealed divergence of isolated fungi and classified them as either ascomycota or basidiomycota. The six isolates were cultured for cellulases production and enzymes used for saccharification under SSF process. Data obtained were recorded in triplicates and statistically analyzed using one-way ANOVA at $P \leq 0.05$ significant level on R software. Any significant differences in the factors affecting enzyme and ethanol production was determined by Tukey's HSD Post Hoc test. Effect of time of incubation was investigated and maize cobs substrate produced the highest cellulase activity. *Xylaria sp*.km01 recorded the maximum FPase activity at 16.7±0.34 IU/ml on the 9th day of incubation. *Xylaria sp*. km03 produced the highest exoglucanase activity at 8.32±0.23 IU/ml while *P. petalodes* km06 produced the highest endoglucanase activity at 28.7±1.2 IU/ml on the 6th day of incubation. *Podosycpha boleana* km05 produced the highest β-glucosidase activity at 6468±210 IU/g on the 12th day of incubation. Effect of pretreatment on substrates was also investigated and maize cobs produced the highest cellulase activity. *Xylaria sp*.km01 produced the highest FPase at 20.1±1.31 IU/ml, exoglucanase activity at 9.35±0.77 IU/ml and endoglucanase activity 35.8±1.19 IU/ml while *P. boleana* km05 produced the highest β-glucosidase activity at 5111±101 IU/g when pretreated with 0.1M HCl at 121°c for 15 min. Saccharification and Fermentation of maize cob substrate of low and high loading concentration (4%,8%,18% and 20%). Hydrolysis by cellulase of *P. petalodes* km06 recorded the maximum of 10.63±0.70 g/l reducing sugars with 8%, whereas cellulase of *P. bolleana* km05 and *Saccharomyces cerevisiae* produced higher bioethanol of 37.3±0.72 g/l with 20% during fermentation period at 72 hours and 96 hours respectively. These findings show that both cellulolytic enzymes and bioethanol can be produced from local microbes using agro waste and the technology harnessed for creating income. Maize cobs, therefore performed as the best substrate for cellulases and bioethanol production

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Kenya population grows with time and require increasing energy demand with limited fossil fuel (oil, coal and gas) reserves to sustainably meet this demand (World Bank, 2010). The projected depletion of fossil fuels and the increase in global warming have led countries to explore the use of non-conventional and natural energy sources (Tuo, 2013; Zhao *et al.,* 2016). Non convectional and natural energy sources can be produced from wind, solar, and biomass (Park *et al*., 2012). WHO (2016) estimates over four (4) million people lose their live from diseases caused by air pollution from using wood or agricultural residues for cooking. Kenya is also facing the threat of environmental pollution due to inadequate disposal mechanism of municipal waste (NEMA, 2015). The goal of Kenya Vision 2030 is to transform the country into a modern industrial hub that provide good quality life for citizens by 2030 in a safe and clean environment (Vision 2030). Agricultural residues as a result of human activities pose an environmental concern, a problem usually aggravated by biomass burning (Iqbal *et al.,* 2013). Biomass provides for more than 90% of rural household energy needs in Kenya and accounts for 68% to the country's total energy consumption (Government of Kenya. Bioethanol Strategy (2010-2013). However, the recalcitrant nature of biomass is hindrance to its full utilization. Use of microbial enzymes and pretreatment are key aspects of improving saccharification of biomass for biofuel production in solid-state fermentation (Azhar *et al,* 2017). Lignocellulose primarily consists of cellulose, hemicellulose and lignin and form

major structural component of plant biomass (Acharya *et al.,* 2010; Deswal *et al.,* 2011; Montoya *et al.,* 2012).

Cellulose have glucose molecules linked together by β-(1–4) glycosidic bonds with repeating cellobiose units (Sawatdeenarunat et al, 2015). Cellulose hydrolysis catalyticaly by the action of three enzymes endoglucanase, exoglucanase and β-glucosidase. Endoglucanase breaks the noncovalent bonds in the amorphous structure of cellulose, while exoglucanase cleaves two glucose units (cellobiose) from the end of the chain and finally cellobiose is hydrolyzed by β - glucosidase (Guimarães *et al,* 2012). The recalcitrance of lignocellulose to saccharification has limited their use for biofuel production (Blaschek and Ezeji, 2010; Parawira and Tekere, 2011). It is therefore necessary to focus on isolating and characterizing new microorganisms from local sources as sources of lignocellulolytic enzymes that hydrolyze lignocellulose biomass to reduce the cost and process of producing biofuels, among other products in today's market (Isroi *et al*., 2011; Kuhad *et al.,* 2011; Sainz, 2011). Cellulosic biomass widely used in SSF includes, sugarcane bagasse, wheat (bran and straw), rice (bran, straw, and husk) maize bran, soy hull, sawdust, and corncobs among others (Jacob *et al* 2006). The choice of the substrate in a solid-state fermentation is dependent on many factors such as the availability and the cost of the substrates.

Different micro-organisms secret cellulolytic enzymes in nature, which can be purified and used to breakdown lignocellulose to simple fermentable sugars for biofuels production. *Trichoderma sp.* and *Aspergillus sp*. are commonly utilized species to source lignocellulases (Pandey *et al,*1999). Despite numerous reports of isolation of lignocellulolytic enzymes from microorganisms in other parts of the world, the biotechnological benefit of novel lignocellulolytic enzymes from Kenyan microbial communities has not been fully exploited.

Plant biomass like agricultural and forestry residues are renewable natural source of organic matter that can serve as low cost source of feedstock that could be utilized to produce enzymes and alternative fuel among other commodity products. (Acharya *et al.*, 2010).

Bioethanol is produced from the sugar component of agro-waste through fermentation under controlled environmental conditions releasing carbon dioxide (Sun *et al* 2002). The fermentation process is anaerobically and follows the Embden-Meyerhoff pathway (EMP) which is catalyzed by bacterial and fungal enzymes (Sun *et al* 2002). In SSF process, the substrates are first hydrolyzed by chemical means or with cellulase enzymes to reducing sugars, followed by fermentation by ethanologenic microorganisms to produce bioethanol (Martin *et al,* 2002). Bioethanol production from corn starch, though faces challenges due to the shortage of edible crops, but this can be substituted by using non-edible food crops and agro-waste. This would therefore facilitate proper farming for food security.

1.2. Statement of the Problem

Increasing energy demand with limited fossil fuel (oil, coal and gas) have led to an increase in global warming resulting countries to explore the use of non-conventional and natural energy sources (Tuo, 2013; Zhao *et al.,* 2016). Push towards clean environment and renewable energy is driven by health concerns emanating from fossil fuels and climatic change from global warming. Lignocellulosic biomass can be converted into fermentable sugars for biofuel production, however its recalcitrant to hydrolysis and use of cellulolytic enzymes are currently imported and are expensive. Several fungi utilized lignocellulose as source of energy, but only few strains have potential of producing a complex of

lignocellulolytic enzymes and biofuel (Carvalho *et al.,* 2008). Despite lignocellulosic waste being potential source of raw materials for cellulolytic enzymes production due to its availability, degradability and environmentally friendly, the organic waste remains a global challenge as well as untapped resource (Zhang, 2011). In Kenya only 10% of the plant biomass is utilized for energy, Government of Kenya. Bioethanol Strategy (2010-2013). Fungi that degrade lignocellulose are important in degrading the plant biomass into fermentable sugars and biofuels. Due to continuously use of fossil fuels, the enzymatic degradation of lignocellulose feedstock into residual sugars for clean and cost friendly biofuel production is considered as the option due to demand of clean energy (Martinez *et al.*,2008). Despite some progress in fermentation process for enzyme production from lignocellulose feedstock, production cost is still a challenge for industrial scaling up (Merino *et al.*,2007). Sourcing of lignocellulolytic enzymes from local microorganisms will significantly solve the challenges encountered with high cost of imported enzymes.

1.3. Justification of the Study

Bioethanol as a renewable energy source, can contribute to energy security, a resilient transportation system, and reduce emissions in the country as envisaged in the Energy Policy and Energy Act, and to meeting the country's other national goals covered under Vision 2030 such as agriculture, health and commerce (Government of Kenya. (2020). Bioethanol Strategy (2020-2023). Plant biomass from forest reserves may be source of microorganisms that are ideal for producing cellulolytic enzymes for actively degrading decaying materials and recycling nutrient in soil (Sailendra *et al*, 2014) thus, the choice of Karura forest. Fermentation conditions optimization for production of cellulase enzymes for higher enzyme yield and activity had necessitated the adaptation of solid state fermentation for the

cellulolytic enzymes production. Naturally, production of biofuels through non-conventional method that is expected to significantly reduce the net CO_2 emission (IEA, 2013) is probably the next important step. Use of agricultural waste for production of biofuel would establish a commercial use for such waste and mitigate the problems caused by environmental pollution due to their poor disposal in Kenya. Cultivation of maize crops, eucalyptus and cypress trees will be considered as viable farming.

1.4 Null Hypotheses

i. Fungi isolated from Karura forest have no relationship with cellulases production
ii. Time of incubation has no effect on cellulolytic enzymes production from selected fungi using eucalyptus, cypress and maize cob substrates under solid state fermentation.
iii. Pre-treatment (alkali and acid) has no effect on cellulolytic enzymes production by selected fungi using eucalyptus, cypress and maize cob substrates under solid state fermentation.
iv. Substrate concentration has no relationship with saccharification of maize cob into fermentable sugars using cellulolytic enzymes produced by selected fungi.
v. Saccharified maize cob hydrolysate has no relationship with bioethanol production using *saccharomyces cerevisiae*.

1.5 Objectives

1.5.1 General Objective

This study was carried out in order 'to investigate bioethanol production by *saccharomyces cerevisiae* using lignocellulose substrates saccharified by fungal isolates from Karura forest reserve, Nairobi county, Kenya'

1.5.2 Specific Objectives

i. To determine the cellulase activities of isolates on 1% CMC - Congo red agar
ii. To determine the effect of time of incubation on production of cellulolytic enzymes using eucalyptus, cypress and maize cob substrates under solid state fermentation.
iii. To determine the effect of pre-treatment of eucalyptus, cypress and maize cob substrates for cellulolytic enzyme production by selected fungi under solid state fermentation.
iv. To determine saccharification potential of crude cellulolytic enzymes produced by selected fungi to saccharifize maize cobs substrate into fermentable sugars.
v. To determine bioethanol production potential of saccharifized maize cobs hydrolysate using *Saccharomyces cerevisiae.*

1.6 Significance of the Study

Biofuel is an important form of energy for Kenya, contributing 68% of the country's final energy demand for diverse needs. It is estimated that biofuel output will significantly increase by 2030 targeting the use of renewable energy in the transport sector and in clean cooking interventions (Government of Kenya. (2020). Bioethanol Strategy (2020-

2023). The vision 2030 in Kenya has environmental conservation in its agenda with the goal of having a secure, clean and sustainable environment for its citizens by the year 2030. This research will contribute greatly because it will address possibility of producing biofuel locally and thus reducing overdependence on fossil fuel. This study will isolate cellulases producing fungi and their potential implication for sustainable bioconversion of plant biomass to biofuel using cypress, eucalyptus and maize cobs.

CHAPTER TWO

LITERATURE REVIEW

2.1. Lignocellulose

Lignocellulose residues represent the abundant natural sources of potential carbohydrates that could be utilized for fermentation to produce several products like enzymes, reducing sugars and ethanol among other commodity products (Poletto *et al*., 2013; Sawatdeenarunat *et al.,* 2015). Lignocellulose mainly composed of cellulose, hemicelluloses and lignin which interact to form micro fibrils which are main constituent of the plant cell wall (Mussatto and Teixeira, 2010). The lignocellulose composition varies with the maturity and different sources of plant (Lopez *et al.,* 2010; Feria *et al.,* 2011).

2.1.1. Cellulose

Cellulose consist of glucose molecules linked together by β-(1-4) glycosidic bond with repeating cellobiose units as (Li *et al.,* 2014). Cellulose molecules (about 30 units) are packaged into elementary fibrils, which are reassembled into micro fibrils, finally assembled to cellulose fibers (Moon *et al*., 2011). The structure of cellulose conformations (secondary and tertiary) and its closely association with hemicellulose, lignin and other adjacent elements make it recalcitrant to hydrolysis (Yang *et al*., 2011). Cellulose is the largest organic polymer residue which occur in nature (Poletto *et al*., 2013). Generally, cellulose forms crucial layer in structure of plant cell wall due to its toughness nature making it water-insoluble material that (Habibi *et al*., 2010). Insoluble cellulose material is degraded into simple sugar units by chemicals and extreme temperature (Tabet and Aziz, 2013). Cellulose

polymer is a homopolysaccharide (unbranched) of D-glucose molecules (an hydro) of a high molecular weight. The D-glucose molecules have covalent bond between the C4 of one glucose ring (with both hemiacetal unit hydroxyl group at each end) due to its directional chemical asymmetry (Habibi *et al*., 2010) and the oxygen bond to the C1 of the adjacent glucose ring (Figure 2.1). Each molecule of D-glucose in the chain is rotated at 180° to form a long straight chain due to the angle of the bond (Moon *et al.,* 2011; Tabet and Aziz, 2013).

a) Non reducing end b) Repeating cellulose units c) Reducing end

Figure 2.1 Schematic structure of cellulose (Cave and Walker, 1994).

2.1.2. Hemicellulose

The second lignocellulose component is hemicellulose representing about 20-35% of lignocellulose (Feria *et al.,* 2011). Hemicellulose is a highly branched heteropolymer that contains Hexoses (D-Galactose, L-Galactose and D-Mannose), Pentose (D-Xylose and L-Arabinose) and Uronic acids (D-Glucuronic acid) (Burton *et al.,* 2010). Basically, hemicellulose being a complex material work closely with the cellulose material and link both cellulose polymer and lignin polymer, to provide rigid cell wall. (Nishiyama, 2009; Scheller and Ulvskov, 2010).

Majority of hemicelluloses are alike to cellulose polymer in structure due to the presence of (1,4) β-D-pyranosyl residues at their backbone (O'Neill and York, 2003).

Hemicelluloses are synthesized in the Golgi apparatus and through surface vesicles transported to the cell wall (Cosgrove, 2005). The hemicellulose residue is relatively small structural units of about 150 – 200 monomer units as compared to cellulose (Tabet and Aziz, 2013). The hemicellulose polymer is a hetero polymeric molecule non crystalline in nature with different molecules and several side chains that are branched, that, unlike in cellulose (Gurunathan *et al*., 2015). Due to this nature, it is therefore easily broken down by either chemical or enzymatic mean, (O'Neill and York, 2003; Guimarães, 2012).

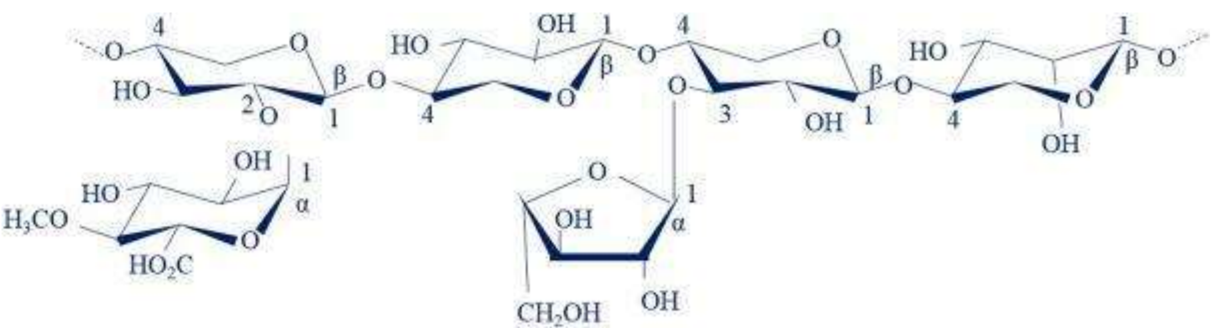

Figure 2.2 Schematic structure of hemicellulose (Gurunathan *et al*., 2015).

2.1.3 Lignin

Lignin is a heterogeneous 3-dimensional aromatic molecule and amorphous in nature and third lignocellulose component. The lignin polymer developed during the secondary plant cell wall development, a process referred to as matrix lignification. Structure of lignin makes the cell wall strengthened and thus provide support. The lignin structure is hydrophobic making it water impermeable acting as a sealant and thus controlling water to the cell. It also resists degradation of microorganisms via their cellular enzymes (Collinson and Thielemans, 2010; Vanholme *et al*., 2010). Lignin polymer composed of trans-p-coumaryl alcohol, trans-p-sinapyl alcohol and trans-p-coniferyl alcohol which are

derived from p-cinnamic acid synthesized through the phenylpropanoid pathway. The precursor molecules have three-carbon side chain with two R group aromatic ring of a hydroxyl group (figure 2.3). The composition of these monomers differs from species to species (Vanholme *et al.,* 2010).

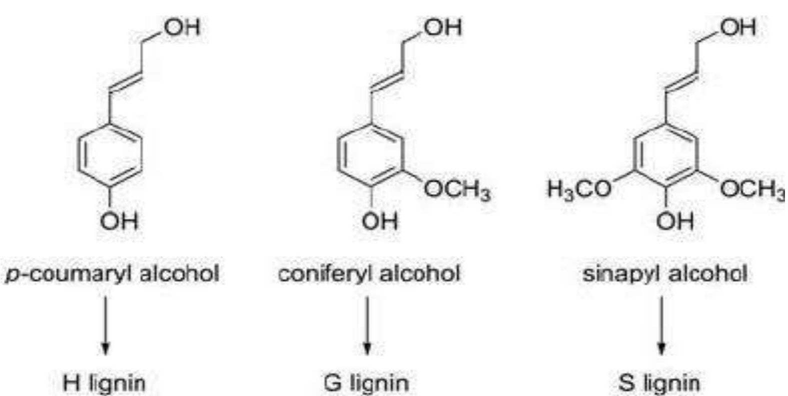

Figure 2.3. Primary precursors molecule of lignin. (Collinson and Thielemans, 2010)

2.2 Fermentation methods of lignocellulose conversion to cellulase enzymes and bioethanol

The two main methods used in most studies are SMF and SSF technologies (Jacob and Prema, 2006; Vintilla *et al.,* 2009).

2.2.1 Submerged Fermentation (SmF)

Submerged fermentation is a method of fermentation where the enzymes, substrate and other materials required for the production of a biomolecule are immersed in a liquid nutrient medium. In this method, the microorganisms grow floating on the surface of the nutrient medium and form small aggregates in the fermenters to produce useful products which may be either intracellularly or extracellularly (Carlile *et al.,* 2001). SmF provides

several advantages such as higher productivity and yields, lower labor costs, and lower contamination risk. On the other hand, its expensive due to high cost of required media, low volumetric productivity, more efflux is generated and oxygen circulation is not so effective. In SmF, microorganisms are grown on soluble e.g. xylan, pectin, mannan or insoluble substrates like wheat bran, rice bran, wheat straw that are dissolved or submerged in liquid media.

In SmF the process parameters like pH, temperature, agitation, aeration and foam are very important factors which affect the growth of microorganism and product yield can be easily controlled. SmF is primarily used in the extraction of secondary metabolites that need to be used in liquid form. SmF technology can be either continuous, fed-batch or batch culture systems. (Vaidyanathan *et al.,* 1999).

2.2.2 Solid State Fermentation (SSF)

SSF involves the growth of microorganisms on moist particles of solid materials in beds in which the spaces between the particles are filled with a continuous gas phase. (Archana and Satyanarayana, 1997; Pandey *et al.,* 2004). Advantages in SSF includes, less microbial contamination, and easy downstream processing. on the hand, it's not suitable for bacteria that require high moisture content, presence of higher impurity, and controlling & measuring of parameters is difficult in solid-state fermentation. The main factors affecting SSF include the carbon and nitrogen sources, the particle size of the substrate, the moisture and water activity, temperature, aeration and pH of the media as well as pretreatment strategy (Pandey *et al* 2004).

2.3 Pretreatment

Pretreatment primarily aim at disruption of recalcitrant lignocellulose to access to more cellulosic materials that could be converted to fermentable sugars. These structures are major obstacle in effective utilization of cellulose from lignocellulose biomass due to the resistance to hydrolysis. Cellulose has hydrophobic surfaces of thick layer containing water and this prevent penetration of enzyme molecules and products degradation. (Matthews *et al.,* 2006). Studies reveal a large spectrum of pre-treatment protocols for hydrolysis, however only a few of these have been developed and utilized. These pre-treatment processes have been investigated for their suitability and efficiency towards lignocellulose biodegradation and depends mainly on the biomass choice and selection.

2.3.1 Chemical pretreatment

In chemical pretreatment method, the commonly used chemicals include NaOH, Perchloric acid ($HClO_4$), Peroxyacetic acid ($C_2H_4O_3$), H_2SO_4, HCOOH, freezing with ammonia and using solvent like ethylene di-amine and n-butylamine (Weil *et al.,* 1994; Martinez *et al.,*2005). However, different chemicals utilized in the pretreatment procedures pose an initial drawback affecting the bioconversion of lignocellulose. Alkali pretreatment with 0.1M NaOH was done to dissolves hemicelluloses by destroying the links of lignin and other polymers that is recalcitrant (Yamashita *et al.* 2010). Acid pretreatment with 0.1M HCL was done to hydrolyze the cellulose and hemicellulose (Khalid, 2010).

Figure 2.4. Schematic representation of lignocellulose biomass pretreatment.

(Hsu *et al*., 1980)

2.3.2 Physical pretreatment

Physical pretreatment methods included milling, grinding and explosion of steam (Weil *et al.,* 1994; Smith *et al.,* 1999). Physical pretreatment reduces the size of particles of substrate for easy enzyme degradation. Use of heat break the bond attaching the three lignocellulose polymers and free them, separating pentose sugar to increase cellulose. Pretreatment with heat could produce some inhibitors that interfere with microbial degradation of substrate biomass (Hartree *et al.,* 1987).

2.3.3 Biological method

Biological delignification is another interesting unconventional pretreatment method. One of the application of this method employs fungi that selectively degrades lignin content. Research to remove lignin content from sugarcane bagasse, paddy straw, and sawdust was attempted with several organisms (*Cyathus sp, Streptomyces viridosporus, Phelebia tremellosus, Pleurotus Xoridaand Peurotus* and *cornucopiae strain*) (Kuhad and Singh, 1993; Chaudhary *et al.,* 1994). Biological delignification method has several

merits such as less-energy input, less cost and increased product formation. On the other hand, it demerits in that it requires longer time for pretreatment and the risk of degrading residual sugars.

Through several research, there is a consensus for the successful pre-treatment should optimize the enzymatic convertibility, reduce sugars loss, optimize products formation, no addition of toxic chemicals to the fermentation system, reduce energy use, use harmful chemicals, low cost equipment and be ease to scale up. Due to recalcitrant nature of selected substrates and the need to dissolves hemicelluloses by destroying the links of lignin and other polymers and to hydrolyze the cellulose and hemicellulose, this study utilized both physical and chemical pretreatment methods.

2.4 Optimization of cellulase enzymes production

To optimize cellulase enzymes production, its paramount to screen and evaluate nutritional and environmental requirements for production of enzymes. Although, the cultural condition enzymes production may differ due differences in organisms used. Other factors affecting the activity of enzyme are varying conditions such as pH, aeration, nitrogen, substrate specificity and temperature. To characterize enzyme production, it therefore requires more information about their optimum level (Bhat, 2000; Parry *et al.,* 2002). In this study time of incubation and pretreatment were the only two factors optimized for cellulases production. Most fungal isolates exhibit optimal enzyme activity on different day of incubation. However, the rate of activity is not a simple linear function of the time of incubation. Chemical pretreatment improves the conversion rate and production of enzymatic hydrolysis to some extent; however, the high input and low

conversion efficiency are still the main challenges encountered by most researchers in various countries.

2.5 Applications of cellulase enzymes

2.5.1 Biofuel

Over usage of natural fossil energy on earth could be main obstacle to the realization of generational goal. looking for another source of energy that is environmental friendly, easy to generate and able to sustain is therefore an ideal strategy to deal with this obstacle. Regeneration of bioethanol as biofuel is significantly an integral part in the market mainly due to its use in chemical industry as feedstock, additives and primary fuel. Bioethanol comprises approximately 99% of biofuels in the USA (Farrell *et al.,* 2007).

First generation biofuel is from edible feed stocks such as maize cobs and bagasse for bioethanol and oil generated from seed such as soybean oil, palm oil, rapeseed oil and sunflower oil for biodiesel through chemical processes. However, these biofuels emit greenhouse gas making them less beneficial as compared to fossil fuels and need significantly more energy from fossil fuels to cultivate and produce (Khan *et al.,*2021). These biofuels pose potential challenges on food commodities due to their production. The danger of organic chemicals and materials made it difficult for the choice of bio refinery model which should consider environmental conditions. Presently these operate with largely limited product materials like cellulose, ethanol, and biofuels (Naik *et al.,*2010). Feedstock that were converted to biofuels are known as first generation feedstock and include rapeseed oil, soybean oil, palm oil, sunflower oil, corn, sugarcane, wheat, and

sugar beet. Biodiesel are produced from extracted oils via transesterification process while bioethanol is produced by direct fermentation (Awogbemi *et al.,*2021). High oil prices in the last decade has contributed cost-competitive liquid biofuels with petroleum-based fuels. This development has attracted more research and product formation (Naik *et al.,*2010). Biodiesel, bioethanol and biogas are the first commercial used biofuels (Naik *et al.,*2010).

Second generation biofuel are commonly from agricultural residual waste and are carbon neutral or negative impact on CO2 production (Naik *et al*.,2010). These types of biofuels are produced from agricultural residues or cellulosic residues such as wood, leaves and grass which can be grown on marginal land. These biofuels are produced from cellulose conversion into simple sugar units, and then be ultimately fermented to alcohol. Cellulose materials that thrives alongside consumable crops could be utilized for biomass. However, this agricultural method ultimately competes for nutrients from the soil and this would lead to another cost of applying fertilizer. This method is chemically and economically costly, time consuming, need for sophisticated equipment and larger-scale facilities (Khan *et al.,*2021). Lignocellulose waste are commonly used as feedstock for second generation biofuels. Pretreatment of lignocellulose before conversion is always recommended. Through pretreatment process, physical method, biological method and chemical method are commonly adopted. After treatment, microbial hydrolysis, fermentation process and distillation process are applied for the transforming lignocellulose to biofuels (Awogbemi *et al.,*2021).

Third generation biofuel originate from non-edible food feedstock. The biofuels are produced from aquatic cultivated feedstock such as algae. Algae are good examples of feedstock for third generation biofuels and are subdivided into macroalgae and microalgae. Microalgae minute unicellular organisms that obtain their food through photosynthesis and generate a lot of substrate than terrestrial plant for industrial application (Awogbemi *et al.* ,2021). Algae have exhibited as potential feed stocks for high yield biofuel production and have ability to fix CO2, which is a good way for reducing CO2 emissions (Khan *et al.,* 2021). Algae use CO2 emitted through photosynthesis, use for growth and releasing oxygen to the environment. Algae has also advantage of quick growth in most environmental condition and using it as raw material is a way mitigating the reduction of CO2 in the environment. Open pond cultivation, photo bioreactors and heterotrophic aerobic fermenters are used to artificially produced microalgae and macroalgae biomass. Algal biomass has fast rate to acclimatize to environments with potential challenges and therefore widely used as a sustainable biofuel feedstock. (Naik *et al*.,2010; Awogbemi *et al.,*2021).

Fourth generation biofuel originated from genetically engineered algae, though at its advanced modification stage (Awogbemi *et al.,* 2021). These bioengineered microorganisms considered for fourth generation biofuel includes algae, yeast, fungi and cyanobacteria (Khan *et al*., 2021). Genetically modified algae easy to cultivate by metabolically increasing carbon uptake and producing increased oil contents. There is ease in the subsequent processes such as fermentation and harvesting procedures. Application of metabolically bioengineered algae in biofuel production have enhanced lipid content to some species of algae. Species of algae utilized in this application

includes, *Botryococcus braunii, Chaetoceros calcitrans, Chlorella species, Isochrysis galbana, Nannochloropsis*, and *Schizochytrium limacinumm* (Awogbemi *et al.,* 2021). The first generation biofuels have demerit over the second, the third and the fourth generation biofuels because of food security, however, still in the experimental phase and not yet commercialized (Khan *et al.,*2021).

2.5.2 Food processing industry

Cellulase enzymes have a broad range of potential applications in food processing industry. Use of enzymes in fruit and vegetable juices for better extraction, stabilization and clarification which is paramount. A complex of enzymes (cellulases, xylanases, and pectinases) have significantly been applied to extract fruit and vegetable juices for hgher yield (Minussi *et al.,* 2002; Carvalho *et al.,* 2008). β-glucanase is another example use of enzyme in brewing industry. This enzyme is used to hydrolyze the glucans in the grain to improve the productivity and efficiency during filtration, hence improving the brewed product appearance such as beer before starting brewing (Celestino *et al.,* 2006).

2.5.3 Animal feed industry

The use of hemicellulases and cellulases in the animal feed is paramount due to their ability to enhance value of the animal feed and animal performance (Dhiman *et al.,* 2002). Enzymatic treatment of fodder and grain by cellulases and xylanases can significantly enhance their nutritional value (Godfrey and West, 1996).

2.5.4 Agriculture industry

Various enzyme preparations of cellulases with different enzymes combinations have latent applications in agriculture industry. Example is the use of hemicellulases and

pectinases for healthy crops growth and plant diseases control (Bhat, 2000). Fungal ß-glucanases have potential of degrading plant pathogen in the cell walls. Many cellulases producing fungi such as *Trichoderma sp., Geocladium sp., Chaetomium, sp* and *Penicillium sp.* can be useful in farming by facilitating rapid growth, improve seed viability, enhance flowering, improve rooting and enhancing productivity (Bailey and Lumsden, 1998).

The demand of cellulases is therefore increasing which has high activity and specific activity, due to the wide industrial application. Research on cellulase system in microbes should therefore be investigated. From these findings, this study was therefore designed to screen the native fungi isolates and hyper cellulases producer and better substrate for cellulase production

2.6 Substrates

Lignocellulose plant biomass (agricultural residues) are the substrates used for enzymes and bioethanol production. The ability of Lignocellulose plant biomass to have significant soluble sugars and substances to enhance fungal growth make it an attractive choice (Sun *et al.,* 2004; Rosales *et al.,* 2005; Kachlishvili *et al.,* 2006; Winquist *et al.,* 2008; Elisashvili *et al.,* 2009). Agro-wastes use as source of raw material in fermentation process could significantly save cost and reduce environmental pollution. Researches revealed that fungi species that degrade lignocellulose are the potential candidates for producing enzymes. They are reported to produce significantly high enzyme activity as compared to those cultured on commercially defined substrate (Bollag and Leonowicz, 1984; Elisashvili *et al.,* 2006; Songulashvili *et al.,* 2007). Maize cobs, cypress and

eucalyptus sawdust are three of these agricultural wastes considered for the study due to their predominance.

2.6.1 Maize cobs

Maize cobs are waste products potential source of feedstock for biofuels production. Corn plant consist of approximately 50% of corn stover residues and is commonly used biomass as feedstock in fermentation process. From this corn stover, more than 66% of reducing sugars could be obtained from portions of cellulose and hemicellulose which account for about 38% and 28% respectively (Graham *et al.,* 2007). Scaling up ethanol production

was achieved and became promising when using corn stover hydrolysates (Blaschek and Ezeji, 2010).

Table 2.1. shows dry matter (DM) composition in corn stover which was done after harvesting. Proper consideration should be done for the removal of corn stover after harvesting due to depletion of limited available nutrients (Follet, 2001; Wilhelm *et al.,* 2004). The harvesting of corn stover should therefore be considered due to the merits and demerits of the crop on agricultural (Wilhelm *et al.,* 2004).

Table 2.1. Dry Matter (DM) Distribution in Corn Residue (Myers & Underwood, 1992)

Corn residue (%)	Moisture (%)	Residue DM basis (%)
Stalk	70-75	50
Leaf	20-25	20
Cob	50-55	20
Husk	45-50	10

2.6.2 Sawdust (cypress and eucalyptus)

Sawdust is a complex waste material due to its heterogeneity in structural and compositional conformity. The three polymers of sawdust contain approximately 90-98% of cellulose materials, hemicellulose polymer and lignin polymer. The remaining portions are ash content and extractives (Fengel, and Wegener, 1989). Sawdust material differ both in plant species and within same plant due to genetic and climatic conditions. Plant cell wall have lumen in innermost, a primary wall connected to neighboring cells via middle lamella and three secondary walls to form multi-layer system. These layers have different relative amount of cellulose content and micro fibrils orientation as well as hemicellulose and lignin contents.

2.6.2.1 Differences between softwood (cypress) and hardwood (eucalyptus)

There exist physiological and chemical differences between both hardwood and softwood. The resistance to microbial attack is due to these variations. Softwood consist of long and slender cells referred to as tracheid which account for approximately 90-95% (Fengel and Wegener, 1989). Tracheid remain intact and give softwood tree its structural appearance. Hemicellulose in softwood have more mannan sugar that contains fewer acetyl groups and therefore require strong treatment. Lignin content is more in softwood compared to hardwood, however it could differ in distribution. The interaction between lignin and other components (cellulose and hemicellulose) could also differ. Due to these variations, its more complicated to degrade softwood than hardwood (Grethlein, 1984; Ramos *et al* 1992).

On the other hand, hemicellulose in hardwood contains more xylans (Grethlein, 1984; Ramos *et al* 1992). Hardwood contains open pores referred to as vessels which aids in heat transfer, transport of enzymes and other molecules through the matrix. Due to presence of these vessels, its easily rendered to degradation than softwood (McMillan, 1994).

2.7 Cellulase producing organisms

The occurrence of active cellulase systems is well distributed within the fungi and bacteria (aerobic and anaerobic). Cellulase enzymes are either extra-cellularly produced in aerobic fungi or as a complex structure (cellulosome) that is bound to the cell membrane in anaerobic bacteria (Clostridia). Fungal strains are mostly used in cellulase production studies. It is because of their capacity to produce a larger amount of extracellular cellulases while growing in selected substrate (Maki *et al*., 2009).

Fungi isolates, *Xylaria s p. isolates, Nemania sp. isolate, Cyathus sp., P. bolleana strain and P. petalodes* are well characterized to produce cellulases. *Xylaria sp* and *Nemaniasp* are genus of ascomycetes fungi commonly found growing on decaying plant biomass. *Cyathus* sp. and *Podoscypha sp* are genus of fungi in the family bascomycetes with widespread distribution in tropical regions (Kirk *et al* 2008). These organisms grow naturally in decaying plant tissues. Product characteristics and development of fermentation process are considered when selecting the preferred organisms.

Generally, fungi are well-known agents for decomposing organic matter (Carlile *et al*., 2001). Cellulose degrading microbes (both aerobic bacteria and fungi) degrade cellulose

to produce significant cellulase enzymes extracellularly and are easily recovered from substrate (Rapp and Beerman, 1991; Schwarz, 2001). In some cases, cellulases presented together in the microbial cell, although they act individually and work synergistically need during hydrolysis (Bond and Stutzenberger, 1989; Wachinger *et al*., 1989).

2.8 Cellulolytic enzymes

Cellulase enzymes are a class of enzymes that work synergistically to breakdown 1, 4-glycosidic bonds to produce glucose units from cellulosic residues (Ezekiel *et al.,* 2010). Cellulose degradation synergistically is done through the catalytic action of the three enzymes, endoglucanase, exoglucanase and beta glucosidase (Sawatdeenarunat *et al.,* 2015).

2.8.1 Endo β-glucanase (carboxymethyl cellulases)

Endo β-glucanase cleave randomly in the internal D-glycosidic linkages preferentially in the amorphous units of the cellulose, to easily rendering cellulose more prone for exo-glucanase cleavage to form free ends chain (Guimarães, 2012).

2.8.2 Exo, 4-β-glucanase (Cellobiohydrolase – CBH)

CBH hydrolyze cellulose to produce cellobiose units. CBH I cleave cellulose from the reducing end while CBH II cleaves from the non-reducing end. The presence of cellobiose, byproduct of substrate breakdown will however prevent the activity of cellobiohydrolase (Nachiyar, 2011).

2.8.3 β-glucosidase (Cellobiase)

β-glucosidase catalyze the breakdown of cellobiose and oligosaccharides into glucose monosaccharides (Xie *et al.,* 2007; Kumar *et al.,* 2008). However, the production or presence of glucose competitively inhibit β-glucosidase activity (Guimarães, 2012).

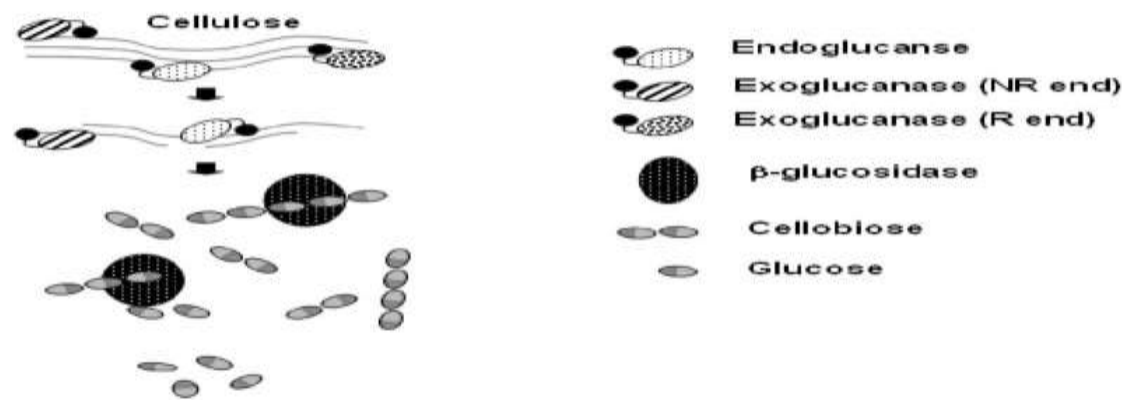

Figure 2.5. Mechanism of cellulase action (Sukumaran,2008)

2.9 Enzymatic saccharification of maize cobs

Saccharification is a process of enzymatic hydrolysis of cellulosic materials into simple sugars. This project main objective is producing bioethanol from maize cobs in a saccharification process by cellulase enzymes of fungal isolates and subsequently fermentation by microorganisms such as *Saccharomyces cerevisiae.* However, several technical challenges hinder saccharification process due to presence of recalcitrant materials in plant cell-wall and to degrade this cell wall by both microbial enzymes and mechanical means is a major concerned. This is a big challenge to produce residual sugars from cellulose substrate (Carpita *et al.,* 2001). Increasing the rates of cellulose hydrolysis, various bottlenecks effect need to be elucidated.

Other than product inhibition, other factors that contribute to substrate conversion decreasing rates are highlighted in the existing models. These includes, cellulose adsorption, hydrolysis susceptible bond location on substrate surface, enzyme substrate complex formation, cellobiose units formed after hydrolyzing bond, cellobiose units repetitive cleaving until desorption and cellobiose hydrolysis into separate glucose units. There are experimental studies these models based on above factors and their sensitivity in depicting the hydrolysis kinetics accurately (Bansal, 2009). Maize crop is an important crop, and more than 40% of the agricultural land is planted with maize crop which is an important staple food for Kenyans. (BASF SE –Kenya agricultural solutions, 2020). Furthermore, maize cobs are used as fuel alternative in most households in rural areas in Kenya. Bioethanol production from agro waste has been done with different sources of enzymes from different organisms for lignocellulose saccharification and fermentation (EkenSaracoglu and Arslan, 2000; Ohgren *et al.,* 2006).

2.10 Bioethanol production from maize cobs

In this process the maize cobs are first hydrolyzed by cellulase enzymes to residual sugars, followed by fermentation by *Saccharomyces cerevisiae* under controlled temperature to degrade residual sugars into reducing sugars to produce bioethanol. (Martín *et al.,* 2002). Bioethanol production from corn cob, though faces challenges due to the shortage of edible crops, but this can be substituted by using non-edible food crops and agro waste. This would therefore facilitate proper farming for food security. Serious environmental concern posed by fossil fuels can be overcome by substituting bioethanol production from agricultural wastes.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection of samples

3.1.1 Sampling area

The sampling area for the present study was Karura forest reserve, located north of central Nairobi (-1.250916S 36.845914 E) along Kiambu road. The reserve is an ideal geographical site of indigenous tree with many tree species.

Figure 3.1: Map of Karura Forest Reserve (source:https://www.google.com/maps/search/karura+forest+coordinates+map/; accessed 15 June 2016)

3.1.2 Sampling technique

A total of 20 fungal samples from unidentified decaying plant biomass were collected from different sites in the forest. The distribution of the sample units was carried out selectively by locating the sites with fungal species found growing on decaying plant biomass with good morphological variation. The fruit bodies that includes all parts of the fungus, representing a range of developmental stages, were carefully selected, collected and placed into sample

envelopes and labelled before transporting them to the Microbial Biotechnology Laboratory, Department of Biochemistry, Microbiology and Biotechnology in Kenyatta University.

3.2 Experimental design

Locally available substrates (cypress, eucalyptus and maize cobs) for production of cellulase enzymes by the six isolates were used. To examine their performance, a multifactorial experimental design was used in the study. The tests were done in triplicates for reproducibility. The three independent variables for this study were the six isolated fungi, three substrates and four pretreatment methods while the dependent variables were cellulase enzymes and ethanol.

3.3 Screening and isolation of cellulase producing fungi

3.3.1 Preparation of 1% CarboxyMethyl Cellulose (CMC) agar

Carboxymethylcellulose agar preparation was done according to the protocol developed by legodi *et al*., 2019. One (1 g/l) of Carboxymethyl cellulose powder, 1(g/l) of glucose, 15(g/l) of agar powder and 0.1 (%) Congo red solution were put in volumetric flask and filled up to one litre with distilled water. The agar was sterilized at 121$^{\mathrm{o}}$c for 20 minutes. After cooling 2.5 ml of gentamycin antibiotic solution (100mg/l) was added before dispensing into sterile petri dishes.

3.3.2 Screening and isolation of fungal isolates

The fruiting body and hyphae of the fungi were cut into 0.5 mm by 0.5 mm in size and sterilized with 1.5% sodium hypochlorite for 30 second, rinsed 5-7 times with sterile distilled

water before culturing on screening media (Tilay 2012). Selected fungi based on their morphological variations were cultured on 1% CMC-Congo red medium at $25^{O}C$ for 7 days and monitored daily to visualize the hydrolysis zones. (Sukesh, 2010; Tilay 2012). Zone of inhibition also known as a zone of clearing or a halo assay, refers to the clear zone surrounding the fungal growth. If the observed zone of inhibition was greater than or equal to the size of the standard zone, the isolate was considered to be positive. Conversely, if the observed zone of inhibition was smaller than the standard size, the isolate was not selected. Six cultures with large clearing zones were selected for fermentation

3.3.3 Preparation of Potato Dextrose Agar (PDA)

PDA was used for sub culturing and culture maintenance. PDA was prepared according to Amadi, 2021. 200mg of irish potato were chopped into small sizes and put into 1-liter conical flask, 500 ml of distilled water was added and the flask covered with aluminum foil, then boiled on hot plate. After boiling the broth was decanted into a clean conical flask and 20g of dextrose and 15g of agar powder added. The agar was sterilized at $121^{O}c$ for 20 minutes. After cooling 2.5 ml of gentamycin antibiotic solution (100mg/l) was added before dispensing into sterile petri dishes.

3.3.4 Culture maintenance

Six fungal isolates were preserved on PDA medium at room temperature and their viability checked after every 14 days during. After study the fungal isolates were stored at low temperature, at 4°C for future studies.

3.4 Molecular identification of fungi

Selected fungi with cellulolytic activity were sub-cultured in PDA medium and identified using molecular techniques. Molecular characterization was based on the PCR amplification of the conserved nucleotide sequence of ribosomal internal transcribed spacer (ITS) using gene coding for the 18S, 5.8S, and 28S rRNA.

3.4.1 Fungal genomic DNA extraction

Mycelia of the six fungal strains was used for genomic DNA isolation by Graham method (Graham *et al*. 2010). Fungal mycelia were scraped out with the help of a fine sterile scalpel followed by freezing in liquid nitrogen and put in about 100 µl of extraction buffer solution [100mM Tris-HCl, pH 8.0, 60mM EDTA, pH8.0] in a sterile mortar and pestle and ground into fine powder. The powder was placed in 2000ul microcentrifuge tubes and about 400 µl of lysis buffer [400mM Tris-HCl, pH 8.0, 60mM EDTA, pH 8.0, 150mM NaCl, 1% SDS] added and the contents left at 25°c for 10 minutes. This was followed by adding 10 µl of Proteinase K [20mg/ml], mixed gently and incubated at 65°c for 15 minutes in a water bath. An equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added and centrifuged at 13,200 rpm for 5 minutes at 4°c.Then the supernatant was transferred to a fresh tube and an equal volume of 3M Sodium acetate and isopropyl alcohol was added. The tubes were briefly mixed by invasion. The tubes were centrifuged at 13,200 rpm for 10 minutes, and the supernatant was discarded. The resultant DNA pellet was washed with 300 µl of 70% ethanol by spinning at 10,000 rpm for 1 minutes,

the supernatant was discarded. The DNA pellet was air dried and dissolved in 50 µl of 1 x Tris-EDTA, pH 8.0. The quality of DNA was checked on 1% agarose gel.

3.4.2 PCR amplification

The internal transcribed spacer of ITS region of 5.8S rRNA was amplified using ITS4 (R) 5'TCC TCC GCT TAT TGA TAT GC 3'for reverse strand and ITS 86 (F) 5' GTG AAT CAT CGA ATC TTT GAA 3' for forward strand (Tony *et al*., 2009). Amplification was done in 22 µl reaction mixture (3µl of fungal DNA template, 0.4 µl Taq polymerase, and 4.0 µl buffer with dNTPs and $MgCl_2$, 4 µl of both primers and 10.6ul of PCR H_2O). The themocycler was initiated with denaturation process at $95^{O}C$ for 3 minutes, followed by 32 cycles of second denaturation at $95^{O}C$ for 30 seconds, annealing at $55^{O}C$ for 2 minutes and elongation at $72^{O}C$ for 1 minute. Final elongation was carried out at $72^{O}C$ for 10 minutes before maintaining at $4^{O}C$ (Delgado-Serrano *et al*., 2016). The quality of amplicons was viewed on 1.2 % agarose gel.

3.4.3 Sequencing of PCR amplicons

The amplified DNA samples were packed in 1.0 ml micro centrifuge tubes and sent to Microgen Inc. in Netherlands for sequencing using the Sanger deoxy chain termination method. The method involves 3 basic steps, chain termination PCR, size separation of DNA fragments by gel electrophoresis and interpreting sequencing data (Delgado-Serrano *et al.,* 2016).

3.4.4 Phylogenetic analysis

Phylogenetic analysis of 5.8S rRNA region of fungal isolates were inferred using the Neighbor-Joining method and the distance computed using p-distance method, Nei M. and Kumar S. (2000). The bootstrap values are represented by figures at the nodes with sequences of *related* strains obtained from NCBI BLAST were included in MEGA11 software package, Tamura *et al*. (2013). The phylogenetic tree was created to a matrix of pairwise distances estimated by the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985).

3.5 Solid State Fermentation (SSF) for cellulase enzymes production.

SSF was used to produce cellulase enzymes from four cellulolytic fungi using three locally available substrates as carbon sources.

3.5.1 Preparation of substrates

Maize cobs, eucalyptus and cypress sawdust were used as substrates (carbon source) for production of cellulase enzymes. These substrates were dried, then grounded into fine powder by an electric mill and sieved with using SA3-2 sieve. The fine powder obtained was used in SSF. Five (5) g of each substrate powder was weighed and dispensed into 350 ml bottles and 10 ml basal salt solution (1.25 g/l $NaNO_3$, 1.0 g/l KH_2PO_4, 0.05 g/l $CaCl_2$. 6H2O, 0.1 g/l $MgSO_4$.7H2O, 0.1g/l NaCl) was added to each bottle. The bottles were covered with cotton wool and sterilized at 121° C for 15 minutes.

3.5.2 Preparation of inoculum and inoculation

Inoculum was prepared from fungal cultures grown on PDA medium for seven (7) days. Spores were suspended in 20 ml of 5% D-glucose solution at pH 4.8. The number of spores were determined using Neubauer chamber with 0.4% trypan blue stain (Alfenore *et al.,* 2002). The yeast cells loading concentration used in the 20 ml of medium was adjusted to 1.0 x 106 cells / ml. For fungal that did not sporulate, mycelium was scrapped from PDA plate and blended in a sterilized blender for 30 seconds. The resulting mycelium extract was used as inoculum. Two (2) mls of the spore suspension or mycelia extract was transferred into each bottle in a biosafety cabinet, then thoroughly mixed with substrates and incubated at room temperature.

3.5.3 Effect of time of incubation on cellulase enzyme production

SSF process was done for 15 days of fermentation period to determine the effect of time of incubation on cellulase enzyme production. Samples were taken at 3 days' intervals and of cellulase enzyme activity determined as described in section 3.8. All assays were carried out in triplicates.

3.5.4 Effect of substrate pretreatment on cellulase enzyme production

Maize cobs, cypress and eucalyptus sawdust were pretreated using 0.1M NaOH (w/v) and 0.1M HCl (v/v) at 60°C for 2 hours and at 121^{0}C for 15 minutes. After pretreatment, the substrates were washed severally with distilled water until the neutral pH. The substrates were then dried at 70°C until constant weight. The pretreated substrates were inoculated with the six cellulolytic fungi as described in section 3.5.2 and after 5 days of fermentation, samples were analyzed as described in section 3.8.

3.6 Lignocellulose composition determination

The amount of lignocellulose composition in cypress, eucalyptus and maize cobs substrates were estimated as proposed by Yang *et al*. (2006) modified method.

3.6.1 Extractives

Determination of the amount of extractives (materials that are not part of the cellular structure) in substrate was done using acetone. 60 mL of acetone was used in 1 g/w of substrate and boiled at 55 °C for 2 h. The sample was filtered with muslin cloth and dried in an oven at 110 °C to obtain constant weight. The amount of dried sample was measured to determine the amount of extractives.

3.6.2 Hemicellulose

The amount of hemicellulose in substrate was extracted using 18% (w/v) Sodium Hydroxide. 100ml of Sodium Hydroxide solution was put to 1 g/w of free extractives substrate and boiled at 80 °C for 2 h. The sample was thoroughly rinsed with deionized water to free sodium ion up to pH 7. Filtered sample with muslin cloth was placed in an oven at 110 °C to obtain constant weight. The amount of dried sample was measured to determine the amount of hemicellulose.

3.6.3 Lignin

The amount of lignin in biomass was extracted using 98 % (v/v) sulphuric acid. 30ml of H_2SO_4 (sulphuric acid) solution was put in 1 g/w of free extractives and left at ambient temperature for 24 h. The biomass sample was then boiled at 80 °C in a fume hood for 1 h. Filtered sample with muslin cloth was thoroughly washed with deionized water to remove sulfate ion in the solid residue. 10 % of Barium chloride solution was used to test

the presence of sulfate ion through the process of titration. The solid residue was placed in an oven at 110 °C to obtain constant weight. The amount of dried sample was measured to determine the amount of lignin content.

3.6.4 Cellulose

Total lignocellulose component of the biomass sample used in this study was 1 g. The amount from the difference between the initial weight of the sample with the weight of others three component, was measured to determine the amount of cellulose content.

3.7 Enzyme extraction

The extracted of crude enzyme was done by adding 50ml of 50mM citrate buffer pH 4.8 to all bottles. Culture bottles were vigorously shaken and left to stand at RT for 2 hours. The mixture was sieved through muslin cloth and the filtrate collected, centrifuged for 10 minutes at 10,000 rpm. A total of 50ml of crude enzyme was collected a plastic bottles from each 350 ml fermentation bottle and stored in a freezer under -20°c.

3.8 Cellulase enzyme assays

3.8.1 Preparation of DNS reagent

10g/w of 3,5-Dinitrosalicylic acid, 0.5g/w of sodium sulfite, 10g/w of sodium hydroxide and 2g/w of phenol were dissolved in 1000 ml distilled water was added. The solution was completely dissolved before was stored in a room temperature.

3.8.2 Preparation of Potassium Sodium Tartrate (PST) solution

Four hundred (400g) of potassium sodium tartrate powder was measured and transferred in a clean 1000 ml conical flask and dissolved in 1000ml distilled water. The solution was completely dissolved before was stored in a room temperature.

3.8.3 Preparation of standard glucose stock solution

To a 100ml conical flask, 20 g glucose was dissolved in 100ml of distilled water. The mixture was stirred using magnetic stirrer to dissolve completely. A linear serial dilution of glucose solutions (0.2, 0.4, 0.6, 0.8 and 1.0) was prepared from stock solution.

3.8.4 Preparation of standard 1mM p-nitrophenol (pNP) stock solution

To make 1mM p-nitrophenol (pNP) standard curve stock solution, to a 10ml conical flask 0.0139 g of pNP was dissolved in 10 ml of Tris buffer, pH 7. A linear serial dilution of p-nitrophenol solutions (10, 20, 30, 40 and 50) was prepared from stock solution.

3.8.5 Total cellulase activity (FPase)

A piece of Whatman's filter paper (1x6cm^2) approximately 50mg was placed in a 2000 ul microcentrifuge tube. Five hundred (500) ul of 5mM citrate buffer pH 4.8 was added followed by adding 500 ul of crude enzyme extract in 50mM citrate buffer, pH4.8 and then incubated at 50ºC for 1 hour. The reaction was terminated by adding 700 ul 3,5-Dinitrosalicylic acid (DNS) reagent after incubation, tubes were boiled for 5 minutes followed by adding 300 ul sodium potassium tartrate after cooling. Reducing sugars produced during the reaction were assayed in triplicates using spectrophotometer at 540 nm and calculated using a standard glucose curve y= a + bx (Guimarães, 2012; Mafe *et al.,* 2014). One unit of FPase was defined as the amount of enzyme that required to release

1μmol of glucose (reducing sugar equivalents) per minute per unit. Formulae for calculating FPase activity in International units (IU) (Rahnama *et al* 2014)

$$= \frac{final\ abs - c}{m} \times \frac{df}{sample\ volume} \times \frac{1}{time} \times \frac{1000\ \mu g}{1\ mg} \times \frac{1\mu mole}{180.16\mu g}$$

Where c = intercept, m = slope obtained from glucose standards

3.8.6 Endo-glucanase assay

Five hundred (500) ul of crude extract was added to a solution of 500 ul 2% CMC in a 2000 ul microcentrifuge tube, then the reaction mixture was incubated at 50ºC for 30 minutes. After incubation, about 700ul DNS reagent was added to terminate the reaction, boiled for 5 minutes followed by adding 300 ul sodium potassium tartrate after cooling. Reducing sugars produced during the reaction were assayed by the DNS method at 540 nm in triplicates and estimated by a standard glucose curve (Guimarães, 2012; Mafe *et al.,* 2014). One unit of endo-glucanase activity was defined as the amount of enzyme releasing 1 μmol of glucose (reducing sugar equivalents) per minute per unit. Formulae for calculating endoglucanase activity in International units (IU) (Rahnama *et al* 2014)

$$= \frac{final\ abs - c}{m} \times \frac{df}{sample\ volume} \times \frac{1}{time} \times \frac{1000\ \mu g}{1\ mg} \times \frac{1\mu mole}{180.16\mu g}$$

Where c = intercept, m = slope obtained from glucose standards

3.8.7 Exo-glucanase assay

Five hundred (500) ul of the crude extract was added to solution of 500 ul of 1.25% microcrystalline (Avicel) solution in 100 mM sodium acetate buffer pH 4.8 in a 2000 ul

microcentrifuge tube, the reaction mixture was incubated at 50°C for 2 hours. About 700ul DNS reagent was added to terminate the reaction, boiled for 5 minutes followed by adding 300 ul sodium potassium tartrate after cooling. Reducing sugars produced during the reaction was assayed by the DNS method at 540 nm in triplicates and estimated by a standard glucose curve (Guimarães, 2012; Mafe *et al.,* 2014). One Unit of exoglucanase activity was defined as the amount of enzyme required to release one µmol of glucose (reducing-sugar equivalents) per minute per unit. Formulae for calculating exoglucanase activity in International units (IU) (Rahnama *et al* 2014)

$$= \frac{final\ abs - c}{m} \times \frac{df}{sample\ volume} \times \frac{1}{time} \times \frac{1000\ \mu g}{1\ mg} \times \frac{1 \mu mole}{180.16 \mu g}$$

Where c = intercept, m = slope obtained from glucose standards

3.8.8 β glucosidase assay

One hundred (100) ul crude enzyme was added to a solution containing 100 ul of 5 mM para-nitro phenol-β-Gl-D-glucopyranoside (pNPG) and 400ul of 100 mM sodium acetate buffer, pH 4.8 solution in a 2000 ul microcentrifuge tube, the reaction mixture was incubated at 50°C for 15 minutes. 800ul ml glycine buffer, pH 10.8 was added to terminate the reaction (Guimarães, 2012; Mafe *et al.,* 2014). The amount of para-nitrophenol produced was measured at 420 nm in triplicates and quantified using a standard Para-nitrophenol curve (Mafe *et al*., 2014). One unit of β-glucosidase activity was defined as the amount of enzyme required to release 1ul mol of para-nitrophenol per minute per unit under assay condition. Formulae for calculating β-glucosidase activity in International units (IU) (Rahnama *et al* 2014)

$$= \frac{final\ abs - c}{m} \times \frac{df}{sample\ volume} \times \frac{1}{time} \times \frac{1000\ \mu g}{1\ mg} \times \frac{1\mu mole}{139.1\mu g}$$

Where c = intercept, m = slope obtained from para-nitro phenol standards

3.9 Bioethanol production

Bioethanol was produced from lignocellulosic maize cobs through saccharification using FPase crude enzymes from fungal isolates followed by the fermentation with *Saccharomyces cerevisiae*. Effect of time of incubation and substrate loading concentration were determined to optimize both reducing sugars and bioethanol yields.

3.9.1 Production of cellulase enzymes for saccharification of maize cobs

Total cellulase enzyme (5 IU/ml FPase) was utilized for saccharification process from already harvested crude enzymes stored in freezer under -20°c on both pretreated and untreated maize cobs substrate.

3.9.2 Concentration of crude enzymes

Freeze-drying is a process in which water is sublimated by the direct transition of water from solid (ice) to vapor, thus omitting the liquid state, and then desorbing water from the “dry” layer Typically, the process involves 3 steps: (1) freezing: to convert water into ice; (2) primary drying: to remove ice via sublimation; and (3) secondary drying: where unfrozen water is removed via desorption (Nowak *et al* 2020). Crude enzyme from maize cob substrate with high enzyme activity was concentrated five-fold by freeze drying and used for saccharification process on maize cobs substrate.

3.9.3 Saccharification of maize cobs

Enzymatic hydrolysis of maize cobs with low (4% and 8%) and high (18% and 20%) loading substrate concentration was carried out in 50 ml Falcon tube with total volume of 20 ml containing crude enzyme extract, 50 mM sodium citrate buffer, pH 4.8, 1 ml of both gentamycin (2mg/50ml) and Griseofulvin (500 mg/50 ml) to inhibit microbial contamination for all six fungi. The crude enzyme concentration of FPase 5 IU/g was used for saccharification. The saccharification was done for 72 hours at room temperature using a shaker at 150 rpm. Commercial cellulase enzymes (5 IU/g of Fpase) were used as control during the saccharification process. During this process, 500 ul from the reaction mixture were withdrawn from the SSF medium at every 12 hours' intervals (12,24,36,48,60 and 72) and the supernatant analyzed by DNS method to evaluate the effect of incubation time and substrate loading concentration on reducing sugar yield.

3.9.4 Estimation of reducing sugar by DNS Method (Miller, 1959)

The reducing sugars produced during saccharification were determined by DNS method. The solution mixture with 500 ul of crude enzyme and 500 ul of DNS reagent in 2 ml micro centrifuge tubes was boiled at $100^{O}C$ for 5 minutes. 300 ul of sodium potassium tartrate was added to the mixture after cooling and the absorbance read at 540 nm. Calibration was done with standard glucose solutions as discussed in subsection 3.7.3 (($y = a + bx$), ($R^2 = 0.998$)). Where a = intercept b = slope obtained from glucose standard and R^2 = degree of freedom

3.9.5 Preparation of *Saccharomyces Cerevisiae* inoculum

Saccharomyces cerevisiae, is the most widely utilized to metabolizes glucose to ethanol in industrial application as commercial baker's yeast in the fermentation. The activation of the yeast was done using 1g of baker's yeast inoculated on 5% malt extract broth plate at $25^{O}C$ for 48 h to activate the yeast. Pure yeast colony was inoculated in the PDA agar plate. Colonies were multiplied by culturing in a sterile 50 ml of 5% malt extract broth and incubated at $25^{O}c$ in a shaker at 150 rpm for 24 h Suh *et al.* (2007). The total viable yeast cells were then determined using Neubauer chamber with 0.4% trypan blue stain (Alfenore *et al.,* 2002). The yeast cells loading concentration used in the 20 ml of maize cobs hydrolysate was adjusted to 1.0×10^{6} cells / ml.

3.9.6 Saccharification and fermentation of maize cobs

The crude enzyme extracts from the six fungal isolates was used to saccharify maize cobs to produce residual sugars to produce ethanol in 50 ml falcon tubes as described in section 3.9.4. 200 ul of baker's yeast was transferred to each 50 ml tubes containing 20 ml of maize cobs substrate devoid antifungal agent. The tubes were then incubated at $37^{O}C$ under stationary environmental conditions for 96 hours. During fermentation period, 500ul samples was withdrawn at 12 h intervals for four days

3.10 Determination of bioethanol

3.10.1 Preparation of acidified potassium dichromate

For 1000 ml of potassium dichromate(VI) solution, 20 g of potassium dichromate(VI) was dissolved in 800 ml distilled water, in a conical flask. 100 ml of absolute sulfuric

acid was slowly added to the solution, with cooling. Distilled water was filled to the 1000 mark.

3.10.2. Preparation of ethanol standards

Ethanol standards were prepared from 2% v/v ethanol stock solution. 2 ml of absolute ethanol was dissolved in 100 ml distilled water in a conical flask. Different concentrations were then prepared (0.2, 0.4, 0.8, 1.2, 1.6, and 2.0) from stock solution.

3.10.3 Determination of bioethanol by acidified dichromate (Mushimiyimana and Tallapragada, 2016)

After fermentation, the maize cobs hydrolysate was sieved with muslin cloth, then the supernatant centrifuged at 10,000 rpm for 10 minutes. 500 ul acidified potassium dichromate reagent was added into 500 ul of hydrolysate in 2 ml micro centrifuge tube and heated at $60^{O}C$ for 5 min. after cooling 200 ul of sterile distilled water was added to the mixture and the absorbance read at 600 nm. Calibration was performed using ethanol standard solutions as discussed in subsection 3.10.2 (($y = a + bx$), ($R^2 = 0.998$)). Where a = intercept b = slope obtained from ethanol standard and R^2 = degree of freedom.

3.11 Data management and statistical analysis

Molecular data analysis by comparing isolates sequences with the sequences in the nucleotide database (NCBI) using the BLAST technique. The MEGA software version X program was used for phylogenetic analysis and multiple alignment using CLUSTAL W (Kumar et al 2018). UV-Spectrophotometry data of enzyme activity and ethanol yield obtained was entered into an excel spreadsheet. Enzymes activity was converted into

international enzyme units. The data was imported in R software version 3.5.1 (R. Core Team, 2018) Data were statistically analyzed using one-way ANOVA at $P \leq 0.05$ significant level. Any significant differences in the factors affecting enzyme and ethanol production was determined by Tukey's HSD Post Hoc test.

CHAPTER FOUR

RESULTS

4.1 Cellulase activity of isolated fungi

This study successfully isolated 20 fungal isolates from decaying plant biomass. The cellulolytic activities of these isolates were tested based on the clear zone formation on the minimal media containing 1% CMC- Congo red agar (Figure 4.1). The result of the screening showed that six isolates had cellulolytic activities indicated by large clear zone formation around the colony (Table 4.1). The dimension of the clear zone ranged from 0.580 cm^2 to 0.700 cm^2. Six isolates which were most potential as cellulase producers were *Xylaria sp.* km01, *Nemania sp.* km02, *Xylaria sp*. km03, *Cyathus sp*. km04, *P. bolleana* km05 and *P. petalodes* km06 respectively.

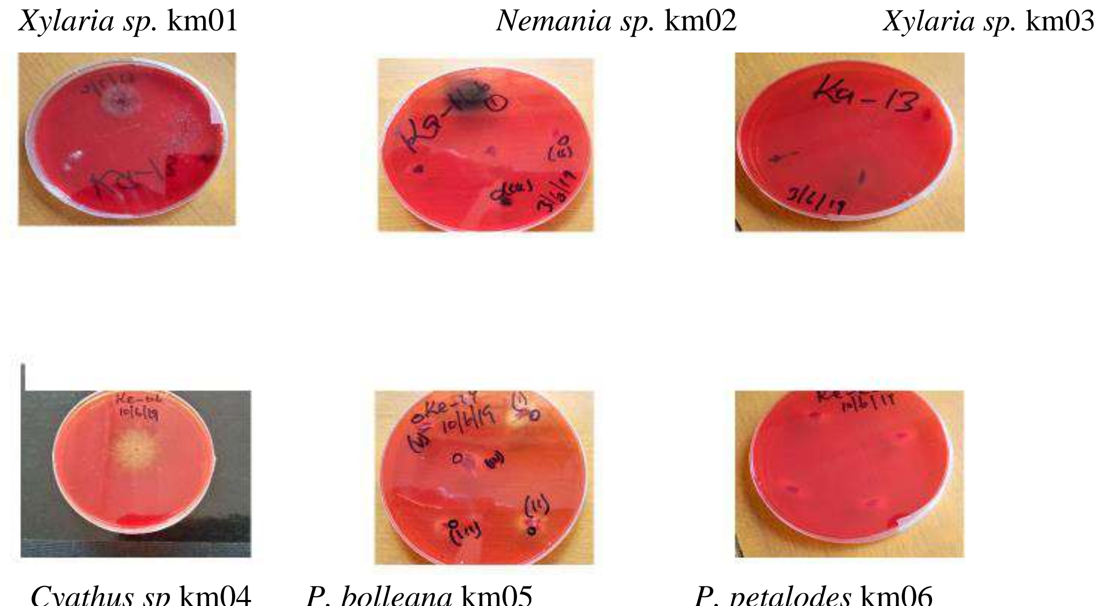

Figure 4.1 Cellulase activity of isolated fungi

Table 4.1 Clear zone dimension of cellulolytic activity of fungal isolates

Isolate code	Isolate name	Clear zone dimension (cm^2)
Km01	*Xylaria sp*	0.630
Km02	*Nemania sp*	0.580
Km03	*Xylaria sp*	0.640
Km04	*Cyathus sp*	0.700
Km05	*Podoscypha bolleana*	0.595
Km06	*Podoscypha petalodes*	0.590

4.2 Molecular identification

4.2.1 Gel electrophoresis

Figure 4.2 show gel electrophoresis separation results of *Xylaria sp.* km01, *Nemania sp.*km02, *Xylaria sp*. km03, *Cyathus sp*. km04, *P. bolleana* km05 and *P. petalodes* km06 amplified ITS region of 5.8S rRNA fragments according to their sizes of ~650 bp. DNA samples were loaded into wells (indentations) at one end of a gel, and an electric current applied to pull them through the gel.

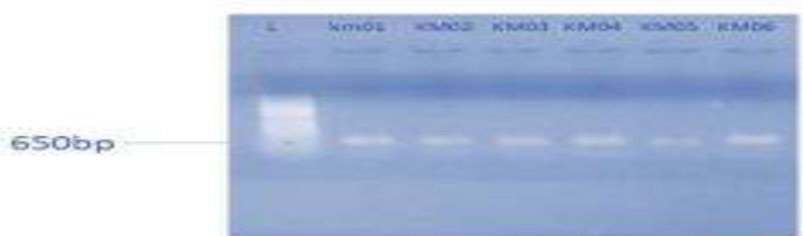

Figure 4.2 Photographic image of an agarose gel indicating the amplification of the ITS target

4.2.2 Phylogenetic analysis

Figures 4.3, show general phylogenetic trees generated with sequences of isolates obtained from the amplification of the ITS region of 5.8S rRNA and some sequences from the NCBI GenBank database for comparison using BLASTN. The outcome of the

submitted sequences affirmed that km01, km02 and km03 speciess belonged to ascomycota species and km04, km05 and km06 to basidiomycota. Based on the analysis of ITS gene sequence, the isolates km01, km02, km03, km04, km05, and km06 were identified as *Xylaria sp., Nemania sp., Xylaria sp., Cyathus sp., P. bolleana and P. petalodes.* The result of phylogenetic analysis (figure 4.3) showed that two strains of *Xylaria sp*. (km01 and *Xylaria sp*. km03) and of *Podoscypha sp. (P. bolleana* km05 and *P. petalodes* km06) were in one group (sister taxa), however *Xylaria sp*. are closely related to *Nemania sp.* km02, while *Cyathus sp*. km04 was not closely related with the isolates.

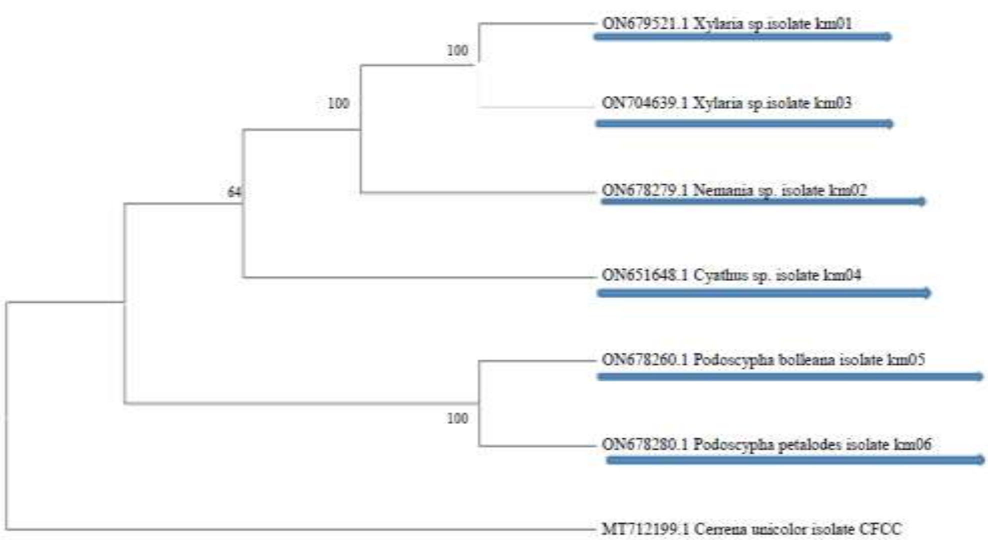

Figure 4.3 Phylogenetic tree for *Xylaria sp.* km01, *Xylaria sp.* km03, *Nemania sp.* km02 *Cyathus sp*. Km04, *Podoscypha bolleana* km05 and *Podoscypha petalodes* km06

Phylogenetic tree showing the relationships among the isolates of *Xylaria sp*. Km01, *Nemania sp*. Km02, *Xylaria sp*. Km03 *Cyathus sp.* Km04, *Podoscypha bolleana* km05

and *Podoscypha petalodes* km06 based on the ITS rRNA gene. Cerrena unicolor isolate CFCC was used as an outgroup.

4.3 Effect of time of incubation on cellulase production

The effect of fermentation period on total cellulase activity (FPase), exoglucanase, endoglucanase and β-glucosidase activity was investigated on the six cellulase producing fungi. Fermentation process was done at 25°c using cypress, eucalyptus and maize cobs substrates under SSF process. The crude enzyme was extracted on different days (3,6,9,12 and15) during fermentation and bioassays carried out for cellulase activity.

4.3.1 Effect of time of incubation on total cellulase activity (FPase)

Figure 4. 4 shows the total cellulase activity (FPase) of *Xylaria sp.* KM01*, Nemania sp.* KM02 *Xylaria sp.* KM03*, Cyathus sp.* KM04*, P. bolleana* KM05 *and P. petalodes* KM06 cultured on cypress, eucalyptus and maize cobs substrates for 15 days and samples taken at 3 days' interval for analysis. There was a significant effect of time of incubation on Fpase activity of the six cellulase producing fungi cultured on three substrates. The highest Fpase activities for *Xylaria sp.* KM01*, Nemania sp.* KM02*, Xylaria sp.* KM03*, Cyathus sp.* KM04 and *P. bolleana* KM05 were recorded on the 3rd day of incubation on cypress and eucalyptus substrates which were significantly higher than Fpase recorded on the remaining days of incubation. *Cyathus sp.* KM04 and *P. bolleana* KM05 recorded the highest Fpase activities on the 3rd day and the 15th day of incubation on maize cobs. The highest Fpase activities for *Xylaria sp.* KM01*, Nemania sp.* KM02 *Xylaria sp.* KM03 were recorded on the 9th day of incubation on maize cobs.

The highest Fpase activity for *P. petalodes* KM06 was recorded on the 3rd day of incubation on cypress and maize cobs substrates and on the 9th day of incubation on eucalyptus. Fpase activity for *Xylaria* sp. KM01 cultured on the three substrates were not significantly different on the 3rd day of incubation, but were significantly higher on maize cobs substrate on the 6th, 9th, 12th, and 15th days of incubation compared to those recorded for cypress and eucalyptus substrates. *Nemania sp.* KM02, *Xylaria sp.* KM03*,* *Cyathus sp.* KM04*, P. bolleana* KM05 *and P. petalodes* KM06 recorded significantly higher Fpase activities on maize cobs substrate compared to cypress and eucalyptus substrates over the 15-day incubation period.

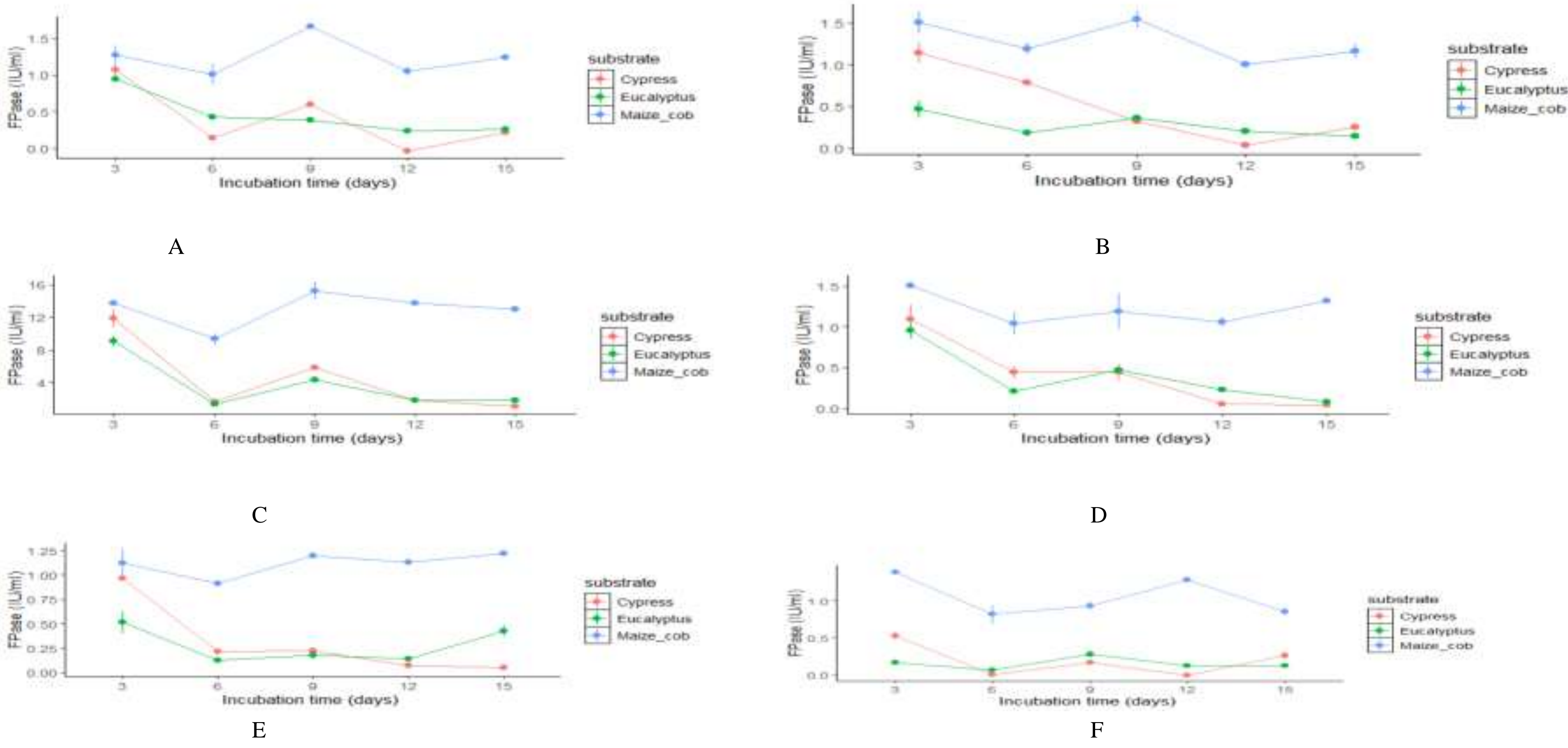

Figure 4.4 FPase activities of (A) *Xylaria* sp. km01, (B) *Nemania* sp. km02, (C) *Xylaria* sp. km03, (D) *Cyathus sp*. km04, (E) *Podoscypha bolleana* km05 and (F) *Podoscypha petalodes* km06 cultured on untreated cypress, eucalyptus and maize cob substrates. Values are means of three replicates ± SEM.

4.3.2 Effect of time incubation on exoglucanase activity

Figure 4.5 shows the exoglucanase activity of *Xylaria sp.* KM01*, Nemania sp.* KM02 *Xylaria sp.* KM03*, Cyathus sp.* KM04*, P. bolleana* KM05 *and P. petalodes KM06* cultured on cypress, eucalyptus and maize cobs substrates for 15 days and samples taken at 3 days' interval for analysis.

There was a significant effect of time of incubation on exoglucanase activity of the six cellulase producing fungi cultured on three substrates. The highest exoglucanase activities for *Xylaria sp.* KM01 was recorded on the 3rd day of incubation on cypress and eucalyptus substrates. Exoglucanase activity of *Xylaria sp.* KM01 cultured on maize cobs peaked at 6.47±0.28 IU/ml, recorded on the 9th day of incubation which however was only significantly higher than that recorded on 15th day of incubation.

The exoglucanase activity recorded on cypress and eucalyptus substrates was significantly higher than that recorded on the remaining days of incubation, while on eucalyptus substrate was only significantly higher than that recorded on the 6th day of incubation. *Xylaria sp.* KM03*, Cyathus sp.* KM04*, P. bolleana KM05* and *P. petalodes* KM06 were recorded on the 3th day of incubation on cypress and eucalyptus substrates and for *Nemania sp*. KM02 recorded on 3rd day of incubation on maize cobs substrate.

On cypress and eucalyptus substrates, the highest exoglucanase activities for *Xylaria sp.* KM01*, Xylaria sp.* KM03*, Cyathus sp.*KM04 and *P. bolleana* KM05 and *P. petalodes*

KM06 were recorded on the 3rd day of incubation. Significantly higher exoglucanase activity for *Nemania sp*. KM02 was recorded on the 6th day of incubation on cypress and on the 9th day of incubation on eucalyptus respectively. Higher exoglucanase activity for *P. bolleana* KM05 was recorded on the 3rd day of incubation in cypress and on the 15th day of incubation in eucalyptus respectively. Exoglucanase activities for *Xylaria* sp. KM01, *Xylaria* sp. KM03, *Cyathus sp.* KM04 and *P. bolleana* KM05 cultured on the cypress and maize cobs substrates were not significantly different on the 3rd day of incubation, but significantly higher on maize cobs substrate on the 6th, 9th, 12th, and 15th days of incubation compared to those recorded on cypress and eucalyptus substrates. *Nemania sp.* KM02 *and P. petalodes* KM06 recorded significantly higher exoglucanase activities in maize cobs substrate compared to cypress and eucalyptus substrates over the 15-day incubation period.

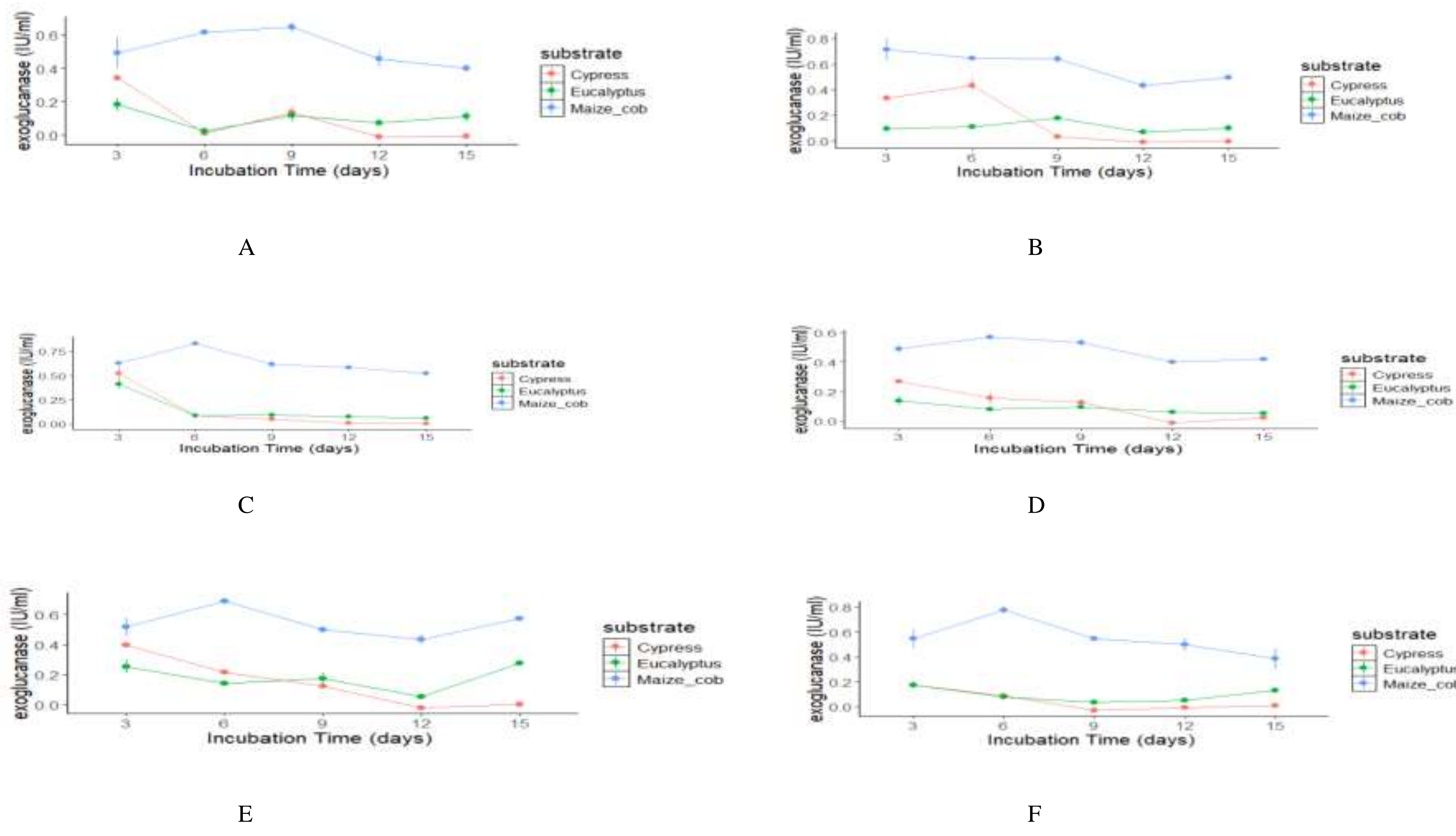

Figure 4.5 Exoglucanase activities of (A) *Xylaria* sp. km01, (B) *Nemania* sp. km02, (C) *Xylaria* sp. km03, (D) *Cyathus sp*. km04, (E) *Podoscypha bolleana* km05 and (F) *Podoscypha petalodes* km06 cultured on untreated cypress, eucalyptus and maize cob substrates. Values are means of three replicates ± SEM.

4.3.3 Effect of time of incubation on endoglucanase activity

Figure 4.6 shows the endoglucanase activity of *Xylaria sp* .KM01*, Nemania sp. KM02, Xylaria sp.* KM03*, Cyathus sp.* KM04*, Podoscypha bolleana* KM05 *and Podoscypha petalodes* KM06 cultured on cypress, eucalyptus and maize cobs substrates for 15 days and samples taken at 3 days' interval for analysis.

There was a significant effect of time of incubation on endoglucanase activity of the six cellulase producing fungi cultured on three substrates. The highest endoglucanase activities for *Xylaria sp.* KM01 and *Xylaria sp.* KM03 were recorded on the 3rd day of incubation on cypress and eucalyptus substrates, but on maize cob substrate they recorded highest on the 9th day and on the 6th day of incubation respectively. *Cyathus sp.* KM04 recorded the highest endoglucanase activity on the 3rd day of incubation for all three substrates. *Nemania sp*. KM02 recorded the highest endoglucanase activity on the 3rd day of incubation on maize cobs and eucalyptus substrates, but on the 9th day of incubation on cypress sawdust.

The highest endoglucanase activities for *P. bolleana* KM05 and *P. petalodes* KM06 were recorded on 6th day and 15th day of incubation on maize cobs and eucalyptus substrates, but on the 3rd day and 15th day on cypress sawdust respectively. Endoglucanase activities for *Xylaria* sp. KM01, *Nemania sp*. KM02 and *Xylaria* sp. KM03 cultured on the three substrates were not significantly different on the 3rd day of incubation, but significantly

higher on maize cobs substrate on the 6th, 9th, 12th, and 15th days of incubation compared to those recorded on cypress and eucalyptus substrates. *Cynthus sp.* KM04*, P. bolleana KM05 and P. petalodes* KM06 recorded significantly higher endoglucanase activities on maize cobs substrate compared to cypress and eucalyptus substrates over the 15-day incubation period.

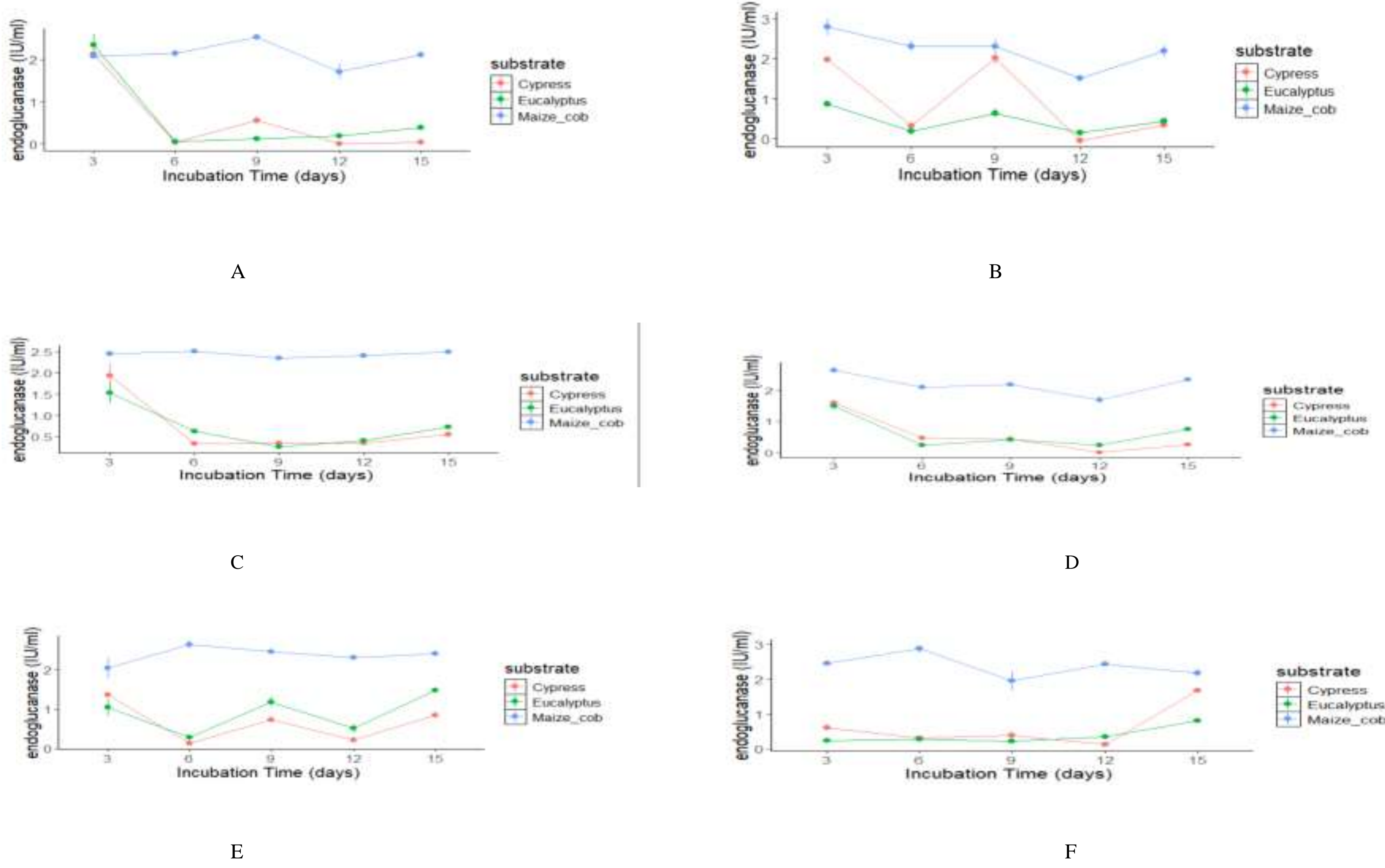

Figure 4.6 Endoglucanase activities of (A) *Xylaria* sp. km01, (B) *Nemania* sp. km02, (C) *Xylaria* sp. km03, (D) *Cyathus sp*. km04, (E) *Podoscypha bolleana* km05 and (F) *Podoscypha petalodes* km06 cultured on untreated cypress, eucalyptus and maize cob substrates. Values are means of three replicates ± SEM.

4.3.4 Effect of time of incubation on β-glucosidase activity

Figure 4.7 shows the β-glucosidase activity of *Xylaria sp.* KM01*, Nemania sp. KM02, Xylaria sp.* KM03*, Cyathus sp.* KM04*, Podoscypha bolleana* KM05 *and Podoscypha petalodes* KM06 cultured on cypress, eucalyptus and maize cobs substrates for 15 days and samples taken at 3 days' interval for analysis. There was a significant effect of time of incubation on β-glucosidase activity of the six cellulase producing fungi cultured on three substrates. The highest β-glucosidase activities for *Xylaria sp.* KM01 and *Cyathus sp.* KM04 were recorded on the 9th day of incubation and those for *Xylaria sp.* KM03 and *P. petalodes* KM06 were recorded on the 12th day of incubation on the three substrates. *Nemania sp.*KM02 recorded the highest β-glucosidase activity on the 6th day on eucalyptus substrate, the 9th day on cypress substrate and the 12th day on maize cob substrate.

P. bolleana KM05 recorded highest β-glucosidase activity on the 9th day on cypress substrate and on the 12th day on eucalyptus and maize cob substrates. β-glucosidase activity for *Cyathus* sp. KM04 cultured on the three substrates were not significantly different on the 9rd day of incubation, but significantly higher on maize cobs substrate on the 6th, 9th, 12th, and 15th days of incubation compared to those recorded on cypress and eucalyptus substrates. All the six fungi recorded significantly higher endoglucanase

activities on maize cobs substrate compared to cypress and eucalyptus substrates over the 15-day incubation period.

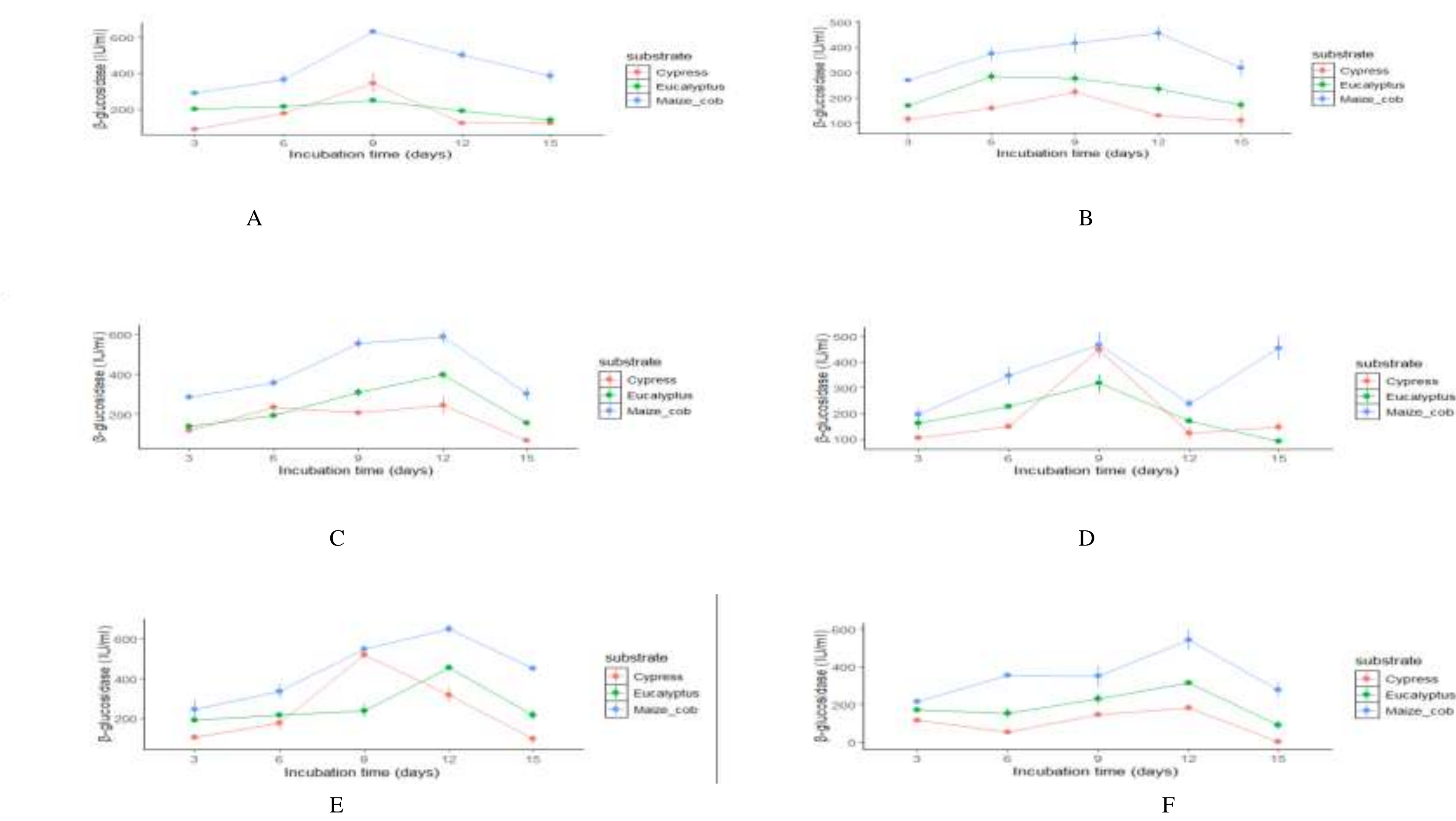

Figure 4.7 β-glucosidase activities of (A) *Xylaria* sp. km01, (B) *Nemania* sp. km02, (C) *Xylaria* sp. km03, (D) *Cyathus sp*. km04, (E) *Podoscypha bolleana* km05 and (F) *Podoscypha petalodes* km06 cultured on untreated cypress, eucalyptus and maize cob substrates. Values are means of three replicates ± SEM.

4.4 Effect of substrates pretreatment on cellulase production

The six cellulase producing fungi were studied to determine the effect of pretreatment on total cellulose activity (Fpase), exoglucanase, endoglucanase and beta glucosidase activity. The pretreatment was carried out at 60°C and 121°C using 0.1M HCl and 0.1M NaOH on cypress, eucalyptus and maize cob substrates under SSF process. The crude enzyme was extracted on 5th day during fermentation and bioassays carried out for cellulase activity.

4.4.1 Effect of substrates pretreatment on Total cellulase activity(FPase)

Figure 4.8 shows total cellulase activity (Fpase) of *Xylaria sp* .KM01*, Nemania sp.* KM02*, Xylaria sp.* KM03*, Cyathus sp* .KM04*, P. bolleana* KM05 *and P. petalodes* KM06 cultured on cypress, eucalyptus and maize cobs substrates pretreated with 0.1M HCl at 60°C and 121°C and 0.1M NaOH at 60°C and 121°C. The six fungi were cultured for 5 days and crude enzyme extract analysis. There were significant effects of substrate pretreatment on total cellulase activities (Fpase) recorded for the six cellulase producing fungi. Significantly higher fpase activities were recorded on untreated and pretreated maize cobs substrate as compared to untreated and pretreated cypress and eucalyptus sawdust ($P \leq 0.05$) except for *Nemania sp*. KM04. *Xylaria sp.* KM01 recorded the highest Fpase activity on untreated cypress sawdust which however was not significant from that recorded on cypress sawdust pretreated with 0.1M HCl at 121°C for 15 minutes. On eucalyptus the highest Fpase activity was recorded when pretreated with 0.1M NaOH at

121°C for 15 minutes which was only not significant when pretreated with 0.1M HCl at 60°C for 2 hours. While on maize cobs recorded the highest Fpase activity when pretreated with 0.1M HCl at 121°C for 15 minutes which was only not significant when pretreated with 0.1M HCl at 60°C for 2 hours. *Nemania sp.* KM02 recorded the highest Fpase activity on pretreated maize cobs with 0.1M HCl at 121°C for 15 minutes which was only not significant with untreated substrate. While on eucalyptus the highest Fpase activity was recorded when pretreated with 0.1M NaOH at 60°C for 2 hours which was only not significant to that pretreated with 0.1M NaOH at 121°C for 15 minutes.

On cypress, *Nemania sp.* KM02 and *Xylaria sp.* KM03 recorded highest Fpase activity was recorded when pretreated with 0.1M NaOH at 60°C for 2 hours which was not significant to other treatments. *Xylaria sp.* KM03 recorded the highest Fpase activity on pretreated maize cobs with 0.1M NaOH at 121°C for 15 minutes which was only not significant to that pretreated with 0.1M HCl at 121°C for 15 minutes. While on eucalyptus the highest Fpase activity was recorded when pretreated with 0.1M NaOH at 121°C for 15 minutes which was only not significant to that pretreated with 0.1M NaOH at 60°C for 2 hours.

Cyathus sp. KM04 recorded the highest Fpase activity on pretreated substrates with 0.1M HCl at 121°C for 15 minutes which was significant as compared to the other

pretreatments. *P. bolleana sp.* KM05 recorded the highest Fpase activity on pretreated cypress and maize cobs with 0.1M HCl at 121°C for 15 minutes which was only not significant to that pretreated with 0.1M HCl at 60°C for 2 hours. While on eucalyptus the highest Fpase activity was recorded when pretreated with 0.1M 0.1M HCl at 60°C for 2 hours which was significant as compared to other pretreatments. *P. petalodes sp.* KM06 recorded the highest Fpase activity on pretreated cypress and maize cobs with 0.1M HCl at 121°C for 15 minutes which was only not significant when pretreated with 0.1M NaOH at at 60°C and 0.1M HCl at 60°C for 2 hours respectively.

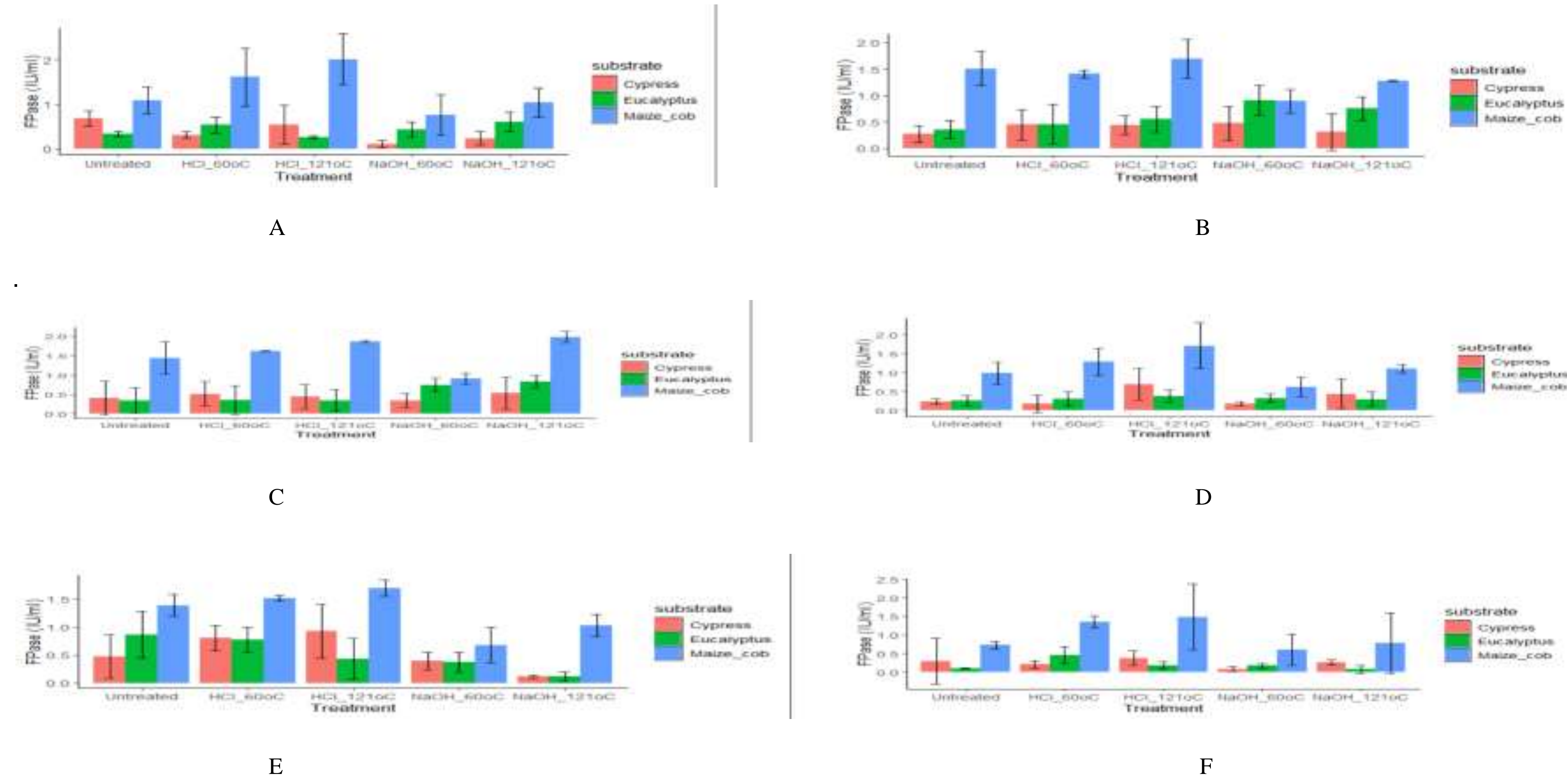

Figure 4.8 FPase activities of (A) ***Xylaria sp*****. km01, (B)** ***Nemania sp.*** **km02, and (C)** ***Xylaria sp*****. km03, (D)** ***Cyathus sp*****. km04, (E)** ***Podoscypha bolleana*** **km05 and (F)** ***Podoscypha petalodes*** **km06 cultured on pretreated cypress, eucalyptus and maize cob substrates. Values are means of three replicates ± SEM.**

4.4.2 Effect of substrates pretreatment on exoglucanase activity

Figure 4.9 shows exoglucanase activity of *Xylaria sp.* KM01*, Nemania sp.* KM02*, Xylaria sp.* KM03*, Cyathus sp.* KM04*, P. bolleana* KM05 *and P. petalodes* KM06 cultured on cypress, eucalyptus and maize cobs substrates pretreated with 0.1M HCl at 60°C and 121°C and 0.1M NaOH at 60°C and 121°C. The six fungi were cultured for 5 days and crude enzyme extract used to determine the exoglucanase activity.

There were significant effects of substrate pretreatment on exoglucanase activity recorded for the six cellulase producing fungi. Significantly higher exoglucanase activities were recorded in untreated and pretreated maize cobs substrate as compared to untreated and pretreated cypress and eucalyptus sawdust ($P \leq 0.05$) except *P. bolleana* KM05. *Xylaria sp.* KM01 recorded the highest exoglucanase activity on pretreated eucalyptus and maize cob with 0.1M HCl at 60°C and 121°C, however not significant when pretreated with 0.1M HCl at 121°C and 60°C respectively. While on cypress the highest exoglucanase activity was recorded when pretreated with 0.1M NaOH at 121°C for 15 minutes. *Nemania sp.* KM02 recorded the highest exoglucanase activity on pretreated cypress with 0.1M NaOH at 121°C for 15 minutes which was not significant when treated with 0.1M NaOH at 60°C while on eucalyptus the highest exoglucanase activity was recorded when pretreated with 0.1M NaOH at 60°C which was significant to other pretreatments. *Xylaria sp.* KM03 recorded the highest exoglucanase activity on pretreated and maize cob with 0.1M HCl at 121°C for 15 minutes which was not significant as compared when pretreated with0.1M HCl at

$60^{O}C$ for 2 hours. On cypress and eucalyptus, the highest exoglucanase activities were recorded when pretreated with 0.1M NaOH at $121^{O}C$ for 15 minutes which were not significant as compared when pretreated with 0.1M HCl at $121^{O}C$ for 15 minutes.

Cyathus sp. KM04 recorded the highest exoglucanase activity on pretreated cypress with 0.1M NaOH at $60^{O}C$ for 2 hours which was not significant to other pretreatments. While on eucalyptus the highest exoglucanase activity was recorded when pretead with 0.1M HCl at $121^{O}C$ for 15 minutes which was not significant as compared to that pretreated with 0.1M HCl at $60^{O}C$ and 0.1M NaOH at $121^{O}C$.

P. bolleana sp. KM05 recorded the highest exoglucanase activity on pretreated cypress and eucalyptus with 0.1M HCl at $60^{O}C$ for 2 hours, however it was only significant when pretreated with 0.1M NaOH at $121^{O}C$ for cypress while on eucalyptus it was significant as compared to other pretreatments. While *P. petalodes sp.* KM06 recorded the highest exoglucanase activity on pretreated cypress and eucalyptus with 0.1M HCl at $60^{O}C$ for 2 hours, however it was only not significant when eucalyptus was pretreated with 0.1M HCl at $121^{O}C$ and on cypress it was significant as compared to other pretreatments.

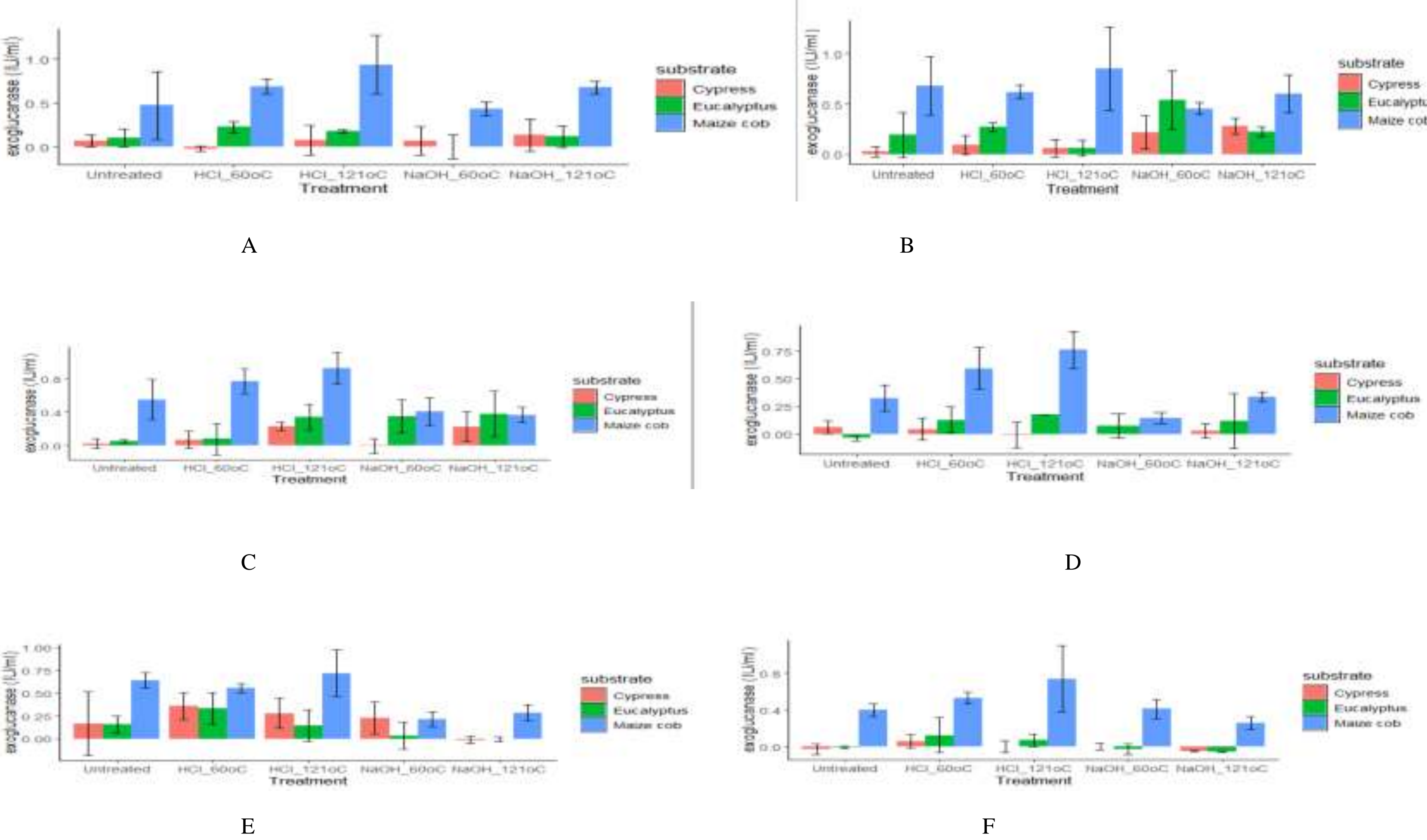

Figure 4.9 Exoglucanase activities of (A) *Xylaria* sp. km01, (B) *Nemania* sp. km02, and (C) *Xylaria* sp. km03, (D) *Cyathus sp*. km04, (E) *Podoscypha bolleana* km05 and (F) *Podoscypha petalodes* km06 cultured on pretreated cypress, eucalyptus and maize cob substrates. Values are means of three replicates ± SEM.

4.4.3 Effect of substrates pretreatment on endoglucanase activity

Figure 4.10 shows endoglucanase activity of *Xylaria sp.* KM01*, Nemania sp.* KM02*, Xylaria sp.* KM03*, Cyathus sp.* KM04*, P. bolleana* KM05 *and P. petalodes* KM06 cultured on cypress, eucalyptus and maize cobs substrates pretreated with 0.1M HCl at 60°C and 121°C and 0.1M NaOH at 60°C and 121°C. The six fungi were cultured for 5 days and crude enzyme extract used to determine the endoglucanase activity. There were significant effects of substrate pretreatment on endoglucanase activity recorded for the six cellulase producing fungi.

Higher endoglucanase activity was recorded by *Xylaria sp.* KM01 when pretreated maize cobs 0.1M HCl at 121°C for 15 minutes which was significant compared to other pretreatments. On pretreated cypress the highest endoglucanase activity was recorded with 0.1M HCl at 121°C for 15 minutes which was only significant to that pretreated with 0.1M NaOH at 60°C for 2 hours. While on eucalyptus the highest endoglucanase activity was recorded with 0.1M NaOH at 121°C for 15 minutes which was not significant to other pretreatments.

Nemania sp. KM02 recorded the highest endoglucanase activity on pretreated maize cobs with 0.1M HCl at 121°C for 15 minutes which was only significant that pretreated with 0.1M NaOH at 60°C for 2 hours. On cypress and eucalyptus, the highest endoglucanase activities were recorded with untreated substrates, however on cypress it was significant

compared to other pretreatment while on eucalyptus it was only significant when pretreated with 0.1M HCl at $121^{O}C$ for 15 minutes. *Xylaria sp.* KM03 recorded the highest endoglucanase activities on pretreated maize cobs and cypress with 0.1M NaOH and 0.1M HCl at $121^{O}C$ for 15 minutes respectively and were significant compared to other pretreatments.

Cyathus sp. KM04 recorded the highest endoglucanase activity on pretreated cypress and eucalyptus with 0.1M HCl at $121^{O}C$ for 15 minutes, however on cypress was significant compared to other pretreatments while on eucalyptus it was only significant to that pretreated with 0.1M HCl at $60^{O}C$ for 2 hours.

The two strains of *Podoscypha sp.* recorded the highest endoglucanase activity on pretreated maize cobs with 0.1M HCl at $121^{O}C$ for 15 minutes which was not significant as compared to that pretreated with 0.1M HCl at $60^{O}C$ for 2 hours and untreated substrate for *P. bolleana sp.* KM05. On cypress and eucalyptus, *P. bolleana sp.* KM05 recorded the highest endoglucanase activities when pretreated with 0.1M NaOH at $121^{O}C$ for 15 minutes and 0.1M HCl at $60^{O}C$ for 2 hours respectively which were significant compared to other pretreatments. *P. petalodes sp.* KM06 recorded the highest endoglucanase activity on pretreated cypress with 0.1M HCl at $121^{O}C$ for 15 minutes which was significant as compared to that pretreated with 0.1M NaOH at $121^{O}C$ for 15 minutes while eucalyptus

recorded the highest endoglucanase activity when pretreated with 0.1M HCl at $60^{o}C$ for 2 hours which was significant compared to other pretreatments.

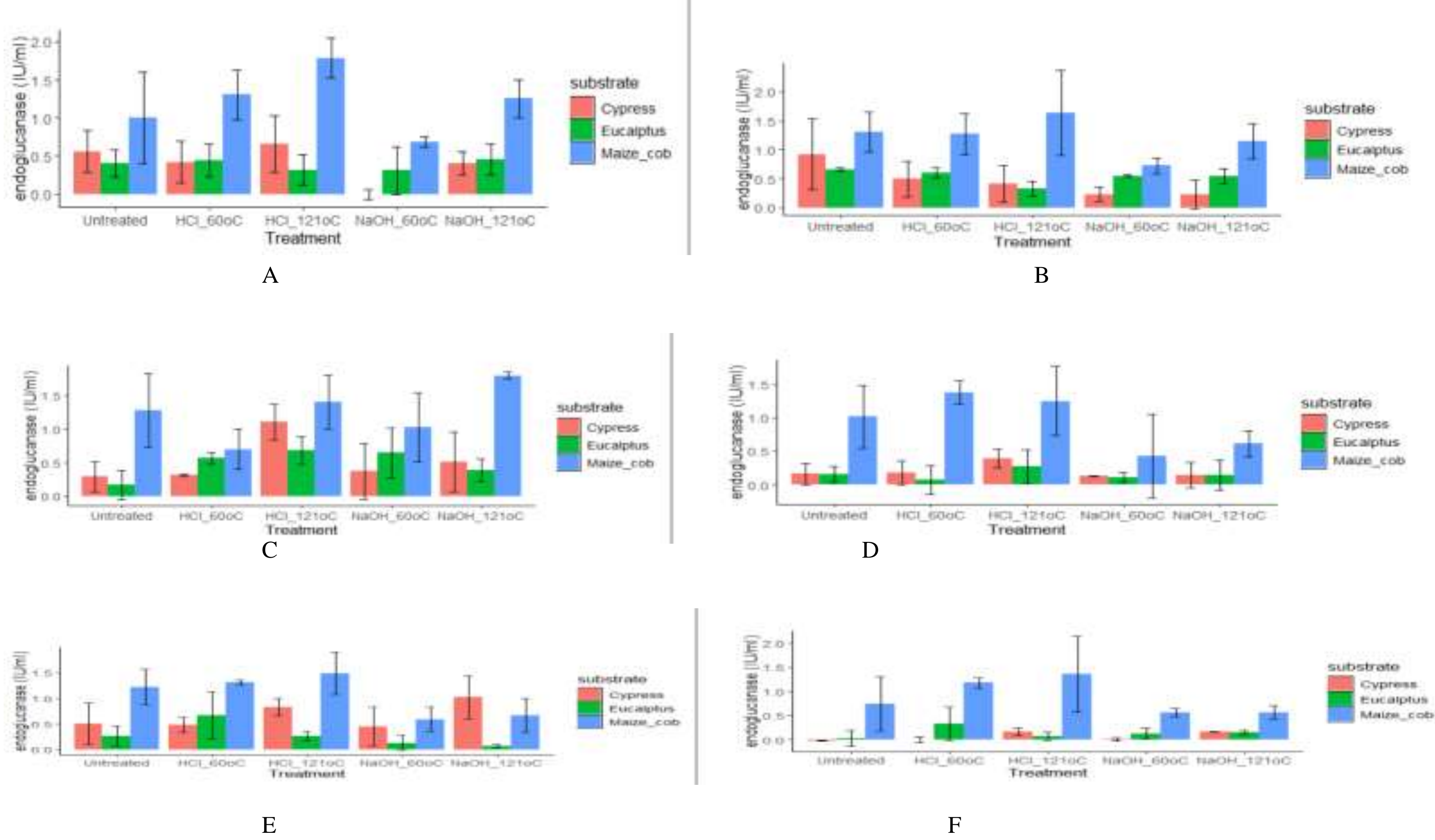

Fig. 4.10 Endoglucanase activities of (A) *Xylaria* sp. km01, (B) *Nemania* sp. km02, and (C) *Xylaria* sp. km03, (D) *Cyathus sp*. km04, (E) *Podoscypha bolleana* km05 and (F) *Podoscypha petalodes* km06 cultured on pretreated cypress, eucalyptus and maize cob substrates. Values are means of three replicates ± SEM.

4.4.4 Effect of substrate pretreatment on β-glucosidase activity

Figure 4.11 shows β-Glucosidase activity of *Xylaria sp.* KM01*, Nemania sp.* KM02*, Xylaria sp.* KM03*, Cyathus sp.* KM04*, P. bolleana* KM05 *and P. petalodes* KM06 cultured on cypress, eucalyptus and maize cobs substrates pretreated with 0.1M HCl at 60°C and 121°C and 0.1M NaOH at 60°C and 121°C. The six fungi were cultured for 5 days and crude enzyme extract used to determine the β-glucosidase activity.

There were significant effects of substrate pretreatment on β-glucosidase activities recorded for the six cellulase producing fungi. Significantly higher β-glucosidase activity was recorded in pretreated maize cobs as compared to cypress and eucalyptus sawdust. *Xylaria sp.* KM01 recorded significantly higher β-glucosidase activity on pretreated maize cobs with 0.1M HCl at 121°C for 15 minutes. On cypress the highest β-glucosidase activity was recorded when pretreated with 0.1M NaOH at 121°C for 15 minutes, which was only not significant to that pretreated with 0.1M HCl at 121°C for 15 minutes. While on eucalyptus the highest β-glucosidase activity was recorded when pretreated with 0.1M NaOH at 60°C for 2 hours, however not significant to that pretreated with 0.1M HCl at 60°C for for 2 hours and untreated substrate.

Nemania sp. KM02 recorded the highest β-glucosidase activity on pretreated maize cobs with 0.1M HCl at 60°C for 2 hours which only significant to that pretreated with 0.1M NaOH at 121°C for 15 minutes. On cypress the highest β-glucosidase activity was

recorded when pretreated with 0.1M HCl at 121°C for 15 minutes which was only not significant to that pretreated with 0.1M NaOH at 121°C for 15 minutes. While on eucalyptus the highest β-glucosidase activity was recorded with untreated substrate which was not significant as compared to the pretreated substrate.

Xylaria sp. KM03 recorded significantly higher β-glucosidase activity on pretreated maize cobs with 0.1M NaOH at 121°C for 15 minutes. On cypress, it also recorded significantly higher β-glucosidase activity was recorded when pretreated with 0.1M HCl at 60°C for for 2 hours. While on eucalyptus the highest β-glucosidase activity was recorded when pretreated with 0.1M NaOH at 121°C for 15 minutes which was not significant as compared to the other pretreatments.

Cyathus sp. KM04 recorded the highest β-glucosidase activity on pretreated maize cobs with 0.1M HCl at 121°C for 15 minutes which was only not significant to that pretreated with 0.1M HCl at 60°C for for 2 hours. On cypress the highest β-glucosidase activity was recorded when pretreated with 0.1M NaOH at 121°C for 15 minutes, however not significant to that pretreated with 0.1M HCl at 121°C for 15 minutes and 0.1M NaOH at 60°C for for 2 hours. While on eucalyptus the highest β-glucosidase activity was recorded when pretreated with 0.1M NaOH at 121°C for 15 minutes which was not significant as compared to the other pretreatments.

P. bolleana KM05 recorded significantly higher β-glucosidase activity on pretreated maize cobs and cypress with 0.1M HCl at 121°C for 15 minutes. While on eucalyptus the highest β-glucosidase activity was recorded when pretreated with 0.1M HCl at 60°C for 2 hours which was not significant as compared to the that pretreated with both 0.1M HCl and 0.1M NaOH at 121°C for 15 minutes.

P. ptalodes KM06 recorded significantly higher β-glucosidase activity on pretreated maize cobs with 0.1M HCl at 121°C for 15 minutes. On cypress the highest β-glucosidase activity was recorded with untreated substrate which was only significant to that pretreated with 0.1M HCl at 60°C for 2 hours. While on eucalyptus the highest β-glucosidase activity was recorded when pretreated with 0.1M NaOH at 121°C for 15 minutes which was only not significant as compared to the untreated substrate.

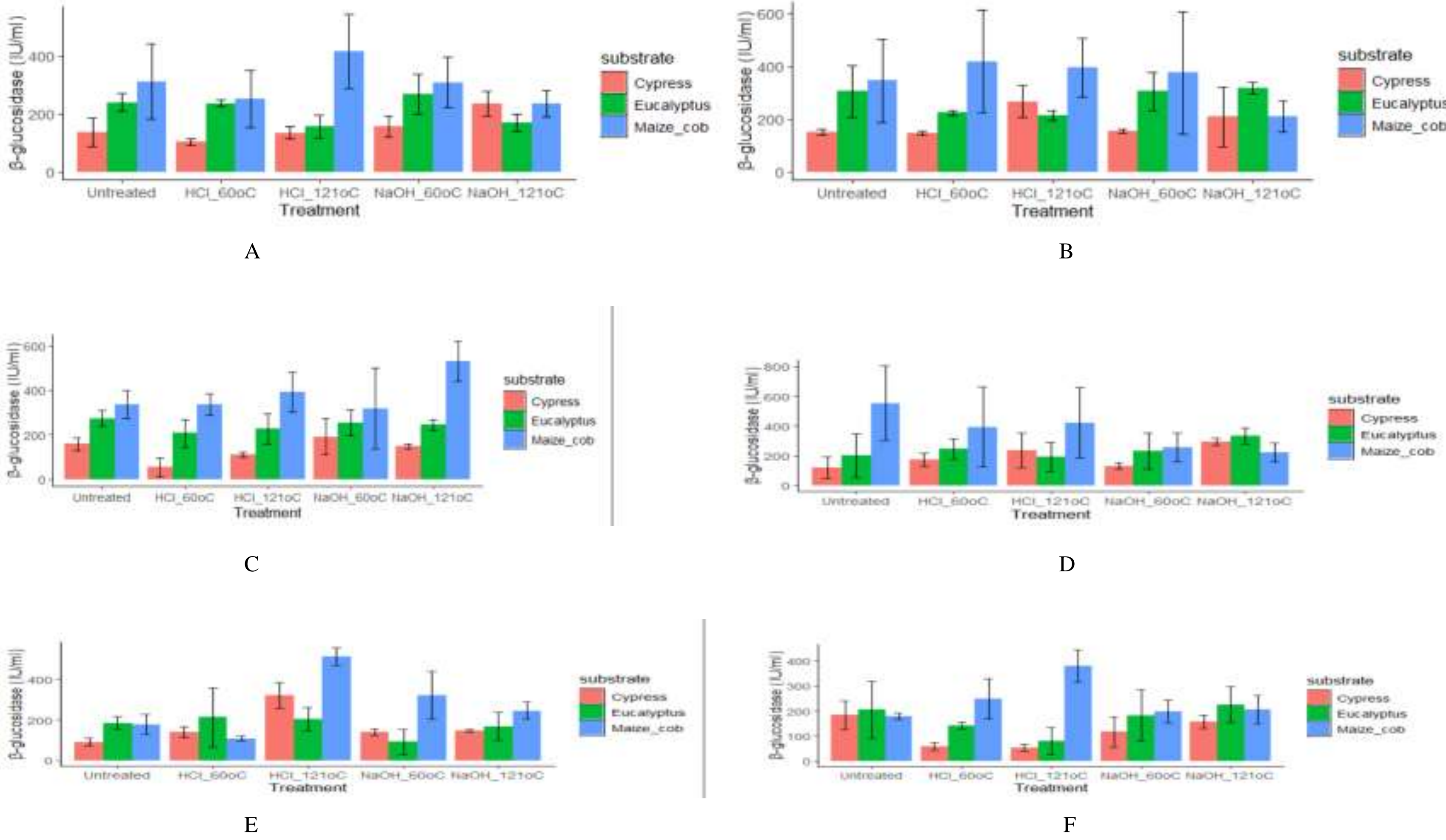

Fig. 4.11 β-glucosidase activities of (A) *Xylaria* sp. km01, (B) *Nemania* sp. km02, and (C) *Xylaria* sp. km03, (D) *Cyathus sp*. km04 (E) Podoscypha *bolleana* km05 and (F) *Podoscypha petalodes* km06 cultured on pretreated cypress, eucalyptus and maize cob substrates. Values are means of three replicates ± SEM.

4.4.5 Effect of pretreatment on chemical composition of maize cobs, cypress and eucalyptus substrates

The results in table 4.2 show the pretreatment effect on substrates with dilute acid and alkali on the composition of maize cobs, cypress and eucalyptus substrates. The chemical treatment of the substrates with 0.1M HCl at $121^{O}C$ lead to a significant reduction of lignin and hemicellulose of the three substrates as compared to that of untreated substrate, while the amount of cellulose in the three substrates significantly increased as compared to that of untreated (control). Pretreatment of the three substrates with 0.1M NaOH at $60^{O}C$ resulted in no significant change in cellulose, hemicellulose and lignin as compared to untreated biomass. Pretreatment of maize cobs substrate with 0.1M HCl at $121^{O}C$ lead to a significant decrease of lignin content and hemicellulose content as compared to that of untreated maize cobs substrate and lead to a significant enhanced cellulose content as compared to that of control. Pretreatment of the three substrates with 0.1M NaOH at $121^{O}C$ resulted in a significant change in amount of cellulose in eucalyptus and maize cobs substrates whereas the use of 0.1M NaOH at $60^{O}C$ resulted in a significant reduction in hemicellulose in maize cobs compared to untreated biomass

Table 4.2 Effect of pretreatment on chemical composition of maize cobs, cypress and eucalyptus substrates

Substrate	Biomass contents (%w/w)	HCl_121^{o}c	HCl_60^{o}c	NaoH_121^{o}c	NaoH_60^{o}c	Control
Cypress	Extractives	8.33 ± 0.58^{a}	6.67 ± 0.58^{b}	7.33 ± 0.58^{ab}	6.67 ± 0.58^{b}	6.33 ± 0.58^{b}
	Cellulose	47.89 ± 2.76^{a}	45.89 ± 2.03^{ab}	44.78 ± 1.99^{bc}	43.0 ± 1.99^{c}	42.33 ± 2.0^{c}
	Hemicellulose	22.0 ± 1.0^{b}	23.33 ± 0.58^{ab}	24.33 ± 1.53^{ab}	24.67 ± 0.58^{a}	25.67 ± 0.58^{a}
	Lignin	22.0 ± 1.0^{b}	23.33 ± 0.58^{ab}	24.33 ± 1.53^{ab}	24.67 ± 0.58^{a}	25.67 ± 0.58^{a}
Eucalyptus	Extractives	8.0 ± 1.0^{a}	6.67 ± 0.58^{ab}	6.67 ± 0.58^{ab}	5.67 ± 0.58^{b}	5.0 ± 1.0^{b}
	Cellulose	47.0 ± 1.0^{a}	46.0 ± 1.0^{a}	44.67 ± 0.5^{ab}	42.67 ± 1.53^{bc}	40.67 ± 0.58^{c}
	Hemicellulose	23.0 ± 1.0^{d}	24.33 ± 0.58^{cd}	25.25 ± 0.5^{bc}	27.0 ± 1.0^{ab}	29.0 ± 1.0^{a}
	Lignin	22.0 ± 22.0^{b}	22.67±0.58b	22.67 ± 1.53^{ab}	23.33 ± 0.58^{ab}	25.33 ± 0.58^{a}
Maize-cobs	Extractives	12.0 ± 1.0^{a}	11.0 ± 1.0^{a}	11.33 ± 0.58^{a}	10.0 ± 1.0^{a}	10.33 ± 0.58^{a}
	Cellulose	51.33 ± 0.58^{a}	48.2 ± 1.0^{b}	47.0 ± 1.0^{b}	46.67 ± 1.0^{bc}	44.67 ± 0.58^{c}
	Hemicellulose	30.0 ± 1.0^{b}	33.67 ± 0.58^{b}	32.67 ± 0.58^{b}	33.67 ± 0.58^{b}	35.33 ± 0.58^{a}
	Lignin	20.67 ± 0.58^{b}	22.0 ± 2.0^{b}	22.67 ± 1.53^{ab}	23.33 ± 0.58^{ab}	25.33 ± 0.58^{a}

Values are means of three replicates ± SEM and are expressed as (% w/w). Means expressed with different superscript small letter in the same row are significantly different at $P < 0.05$

4.5 Bioethanol production from maize cobs substrate

4.5.1 Saccharification of maize cobs with cellulases of isolated fungi

The results show the amount of sugars produced during 72 hours of the fermentation period (Figure 4.12). Maize cob hydrolysis by crude cellulases of isolated fungi produced higher reducing sugars. Significantly higher reducing sugars were produced at 8% substrate loading. cellulase of *P. petalodes* KM06 yielded the highest amounts of sugar at 10.63±0.70 mg/ml, *P. bolleana* KM05 produced yields of 10.37±0.84 mg/ml, *Xylaria sp*. KM03 yielded amounts of sugar at 10.17 ± 0.37 mg/ml, *Cyathus sp*. produced amounts of KM04 9.63±0.40 mg/ml, *Xylaria sp*. KM01 produced yields of 9.48 ± 0.13 mg/ml, whereas *Nemania sp*. KM02 produced 9.41 ± 0.27 mg/ml respectively after 72 h of fermentation period. It was observed that the sugar yields increased steadily reaching the peak after 72 h of fermentation at all the concentrations. Significantly higher reducing sugars were produced by commercial cellulase (positive control), however, low level of sugar recorded in negative control.

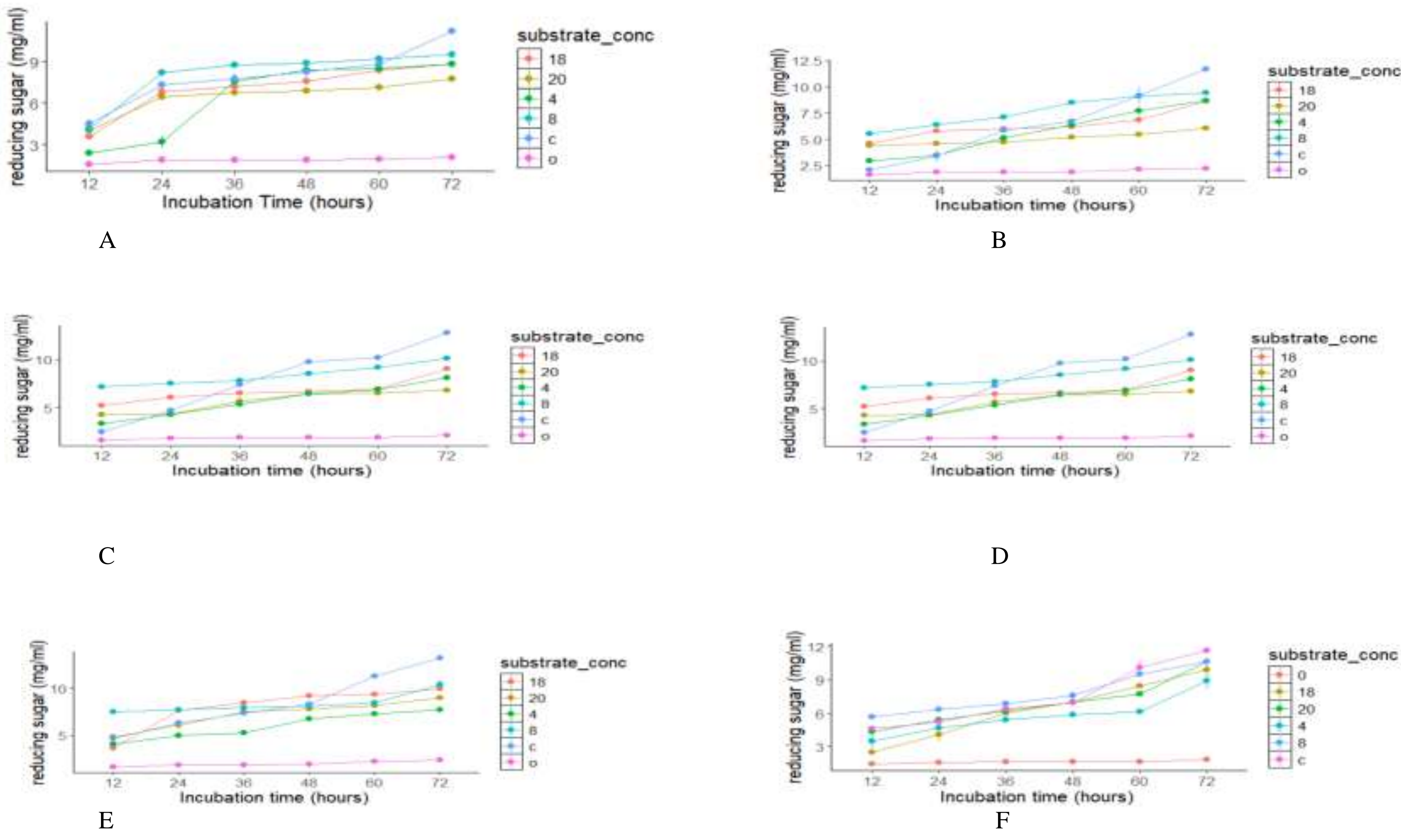

Figure 4.12 Reducing sugars yield (mg/ml) produced by saccharomyces cerevisiae using untreated maize cob hydrolysate at different concentration by cellulases of (A) *Xylaria* sp. km01, (B) *Nemania* sp. KM02, (C) *Xylaria* sp. KM03 (D) *Cyathus* sp. km04, (E) *Podoscypha boleana* km06, and (D) *Podoscypha petalodes*. Values are means of three replicates ± SEM.

4.5.2 Production of bioethanol from maize cobs by simultaneous hydrolysis and fermentation processes.

Figure 4.13 shows the amount of ethanol yield (mg/ml) produced from untreated maize cobs hydrolysate of different concentrations with simultanoues fermentation by *Saccharomyces cerevisiae* during the fermentation period (Table 2). Hydrolysis of maize cobs by cellulases of *Xylaria sp.* KM01*, Nemania sp* .KM02*, Xylaria sp.* KM03*, Cyathus sp.* KM04, *P. bolleana* KM05 *and P. petalodes* KM06 recorded higher amounts of ethanol after 96 h of fermentation. At 8 % concentration, cellulases of *Xylaria sp.* KM03 produced the higher amounts of ethanol at 28.59 ± 1.58 mg/ml, *Nemania sp.* KM01 produced yields of 28.72 ± 3.82 mg/ml, *Xylaria sp.* KM02 produced amounts of 25.75 ± 0.21 mg/ml, whereas *Cyathus sp.* KM04 recorded lower yields at 23.22±0.98 mg/ml respectively after 96 h of fermentation period. At 20% substrate loading, cellulases of, *P. bolleana* KM05 produced highest amounts of ethanol at 37.30±0.72 mg/ml, while *P. petalodes* KM06 recorded yields at 24.42±0.35mg/ml. It was observed that there was a steady increase in ethanol yield with the maximum produced after 96 h of fermentation at all the concentrations.

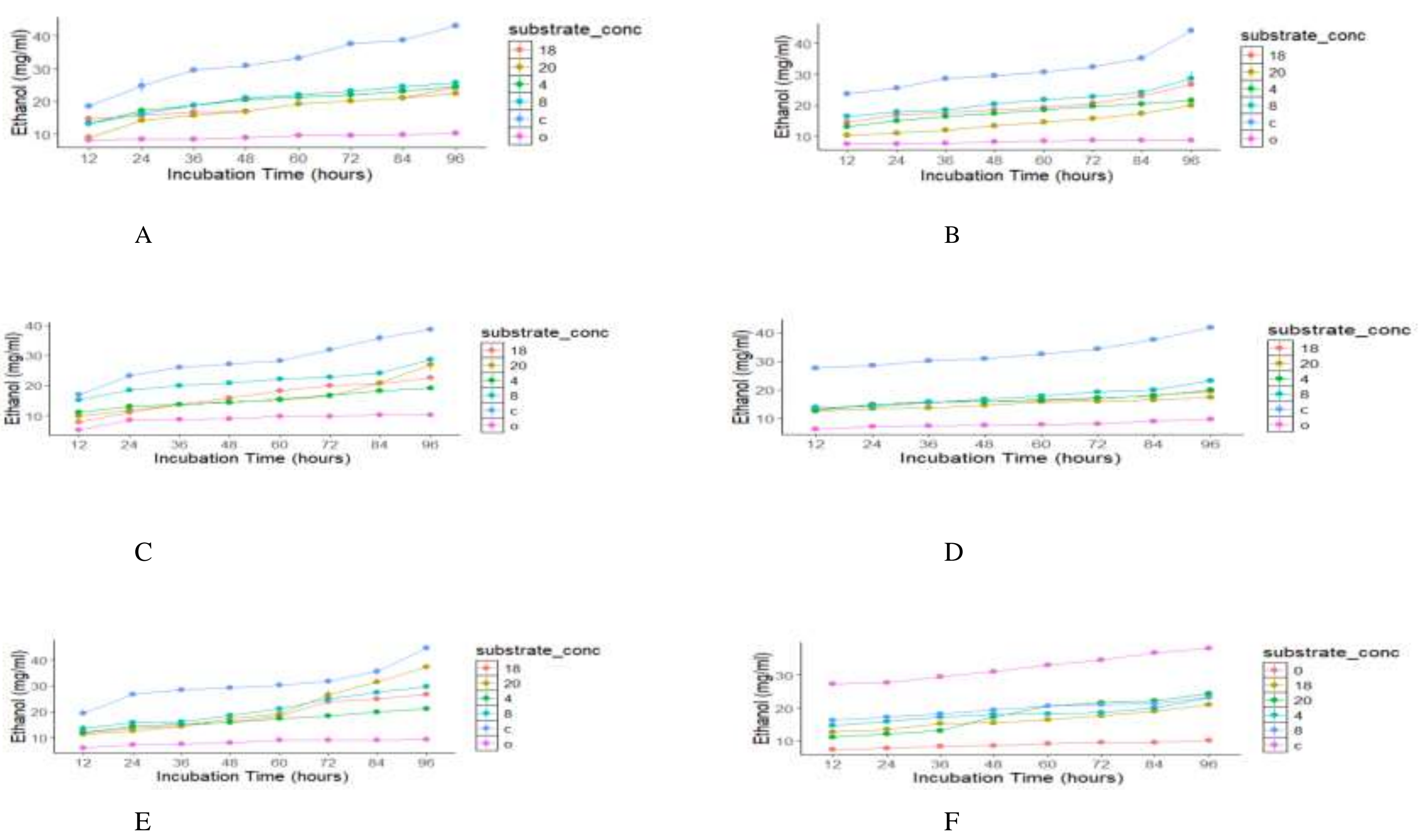

Figure 4.13 Ethanol yield (mg/ml) produced by saccharomyces cerevisiae using untreated maize cob hydrolysate at different concentration by cellulases of (A) Xylaria sp. km01, (B) *Nemania* sp. KM02, (C) *Xylaria sp.* KM03 (D) *Cyathus* sp. km04, (E) *Podoscypha boleana* km06, and (D) *Podoscypha petalodes*. Values are means of three replicates ± SEM.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Screening and isolation (CMC- Congo red), and maintenance of cellulase producing fungi on PDA

In this study, three methods were used to screen for cellulolytic activity of the fungal isolates, fungal growth on carboxy methyl cellulose, clearing of carboxy methyl cellulose zone in agar (hydrolysis zone) and production of reducing sugars. Six fungal isolates exhibited cellulase activity. The result of cellulolytic activity screening based on the clear zone dimension on the CMC media showed that six isolates, namely: *Xylaria sp.* KM01*, Nemania sp.*KM02*, Xylaria sp.* KM03*, Cyathus sp.* KM04*, P. bolleana* KM05 *and P. petalodes* KM06 had the highest cellulolytic activities. These findings were similar to that recorded by Naik *et al*. (2012); Zakpaa *et al.,* (2009).

5.1.2 Molecular identification and phylogenetic analysis of cellulase producing fungi

Molecular identification was based on the detection of conserved sequences in the 5.8S rRNA region and the amplification of the ITS2 region (Tony *et al*., 2009). ITS4 R and ITS86 F primers were used to identify these fungal isolates by comparing sequences to those in NCBI databases using BLAST with accuracy ranging from 98 % to 100 %. Xylaria *sp.* KM01; Genebank accession number: ON679521*, Nemania sp.*KM02; Genebank accession number: ON678279*, Xylaria sp.* KM03; Genebank accession number: ON704639 belong to ascomycetes and *Cyathus sp.* KM04; Genebank accession

number: ON651648, *P. bolleana* KM05; Genebank accession number: ON678260, *and P. petalodes* KM06: Genebank accession number: ON678280 belong to basidiomycetes (ncbi.nlm.nih.gov/blast)

5.1.3. Cellulases production and optimization

The optimal time of incubation for cellulase production depends on the type of substrate used and the fungal species involved (Ilyas *et al.,* 2012). In this study, time of incubation significantly affected cellulase activities from the 3rd day of incubation. The highest FPase activity by all six fungal isolates were recorded on maize cob. *Xylaria sp*. km01 produced the highest activity (16.7 ± 0.034 IU/ml), *Nemania sp*. km02 yielded (15.4 ± 0.01 IU/ml), followed by *Xylaria sp.* KM03 (15.2 ± 0.11 IU/ml) on the 9th day of incubation respectively. *Cyathus sp.* km04 and *P. bolleana* km05 recorded (15.1±0.39 IU/ml and 13.8±0.25 IU/ml) on the 3rd day respectively. However, *P. petalodes* km06 recorded (12.2±0.11 IU/ml) on 15th day of incubation. These results were similar to Gautam *et al.,* 2011 findings on *Aspergillus niger* which showed the highest cellulase activities were recorded after 5 days of incubation period cultured on a corn cob. However, contrary when they recorded a trend in which cellulase activities by *Trichoderma viride* cultured on corn cob peaked on the 3rd day of incubation and decreased afterward.

Exoglucanase activity produced by the six isolates also recorded higher yields on maize cob. *Xylaria sp*. km03 produced the highest activity (8.3 ± 0.23 IU/ml) and *P. petalodes* km06 yielded (7.79±0.26 IU/ml) on the 6th day of incubation. *Nemania sp*. km02 produced (7.16 ±

0.89 IU/ml) on the 3rd day of incubation. *P. bolleana* km05 recorded (6.89±0.2 IU/ml) on 6th whereas, *Xylaria* sp. km01 recorded (6.47 ± 0.28 IU/ml) on the 9th day of incubation. *Cyathus sp*. km04 produced lower level (5.69±0.17 IU/ml) on 6th of incubation. These results were similar to Gautam *et al.,* 2011 findings on Aspergillus niger which showed the highest exoglucanase activity, was recorded after 5 days of incubation and a similar trend in which cellulase activities by *Trichoderma viride* peaked on the 3rd day of incubation when cultured on a corn cob. Exoglucanase activity by *Xylaria* sp. km03 recorded a steady decrease after 3rd day of incubation on cypress and eucalyptus substrates. This could be attributed to fungal species and substrates used for growth (Yadav *et al.,* 2016).

Endoglucanase produced by the six fungal strains recorded higher activity on maize cob. *P. petalodes* km06 recorded highest (28.7±1.2 IU/ml) on 6th day of incubation. *Nemania sp.* km02recorded (28.0 ± 2.12 IU/ml) on the 3rd day of incubation. *Cyathus sp*. km04 recorded (26.4±1.04 IU/ml) on 3rd day of incubation while *P. bolleana* km05 26.3±0.89 IU/ml) on 6th *Xylaria sp*. km01 (25.3 ± 0.52 IU/ml) on the 9th day of incubation. *Xylaria sp*. km03 yielded (25.1± 0.1 IU/ml) on the 6th day of incubation. These results were similar to Gautam et al., 2011 findings on Aspergillus niger which showed the highest cellulase activities were recorded after 5 days of incubation period cultured on a corn cob. However, endoglucanase activities by *Xylaria sp*. km01 and km03 recorded a steady decrease after 3rd day of incubation on cypress and eucalyptus substrates. This could be attributed to fungal species and substrates used for growth (Yadav *et al.,* 2016). High viscosity of the substrate,

decreases the oxygen supply to the organisms. It could also be inhibitory effects of accumulated cellobiose generated by the action of cellulases (Yue *et al.,* 2004).

The highest level of β-glucosidase activity was produced on maize cobs by the six studied fungi. *P. bolleana* km05 recorded highest (646.8±21.0 IU/ml) on 12th day of incubation. *Xylaria sp*. km01 produced (629.7 ± 20.2 IU/ml) on the 9th day of incubation. *Xylaria sp*. km03 produced (597.2 ± 29.7 IU/ml) on the 12th day of incubation. *P. petalodes* km06 yielded (544.1±55.6 IU/ml) on 12th day of incubation. *Cyathus sp.* km04 produced (466.3±47.3 IU/ml) on 9th day of incubation and *Nemania sp*. km02 recorded (455.9 ± 28.7 IU/ml) on the 12th day of incubation. Gautam *et al.,* 2011 recorded similar findings in which cellulase activities by *Trichoderma viride* cultured on corn cob peaked after the 5th day of incubation and decreased afterward. These differences could be due to the difference in the type and composition of substrates (Bhat *et al.,* 2015). It could be also attributed to the maximum metabolic activity of the fungus attributed to the genetic make-up of the fungal strains as a result of adaptations to different habitats, and the concentration of soluble sugar in the substrate (Yoon *et al.,*2014). The low cellulase activities could be a result of an inhibitory effect of accumulated cellobiose (Yue *et al*., 2004).

The effect of pretreatment on FPase production from different investigations on various substrates has been done. In this study, highest FPase activity was recorded on pretreated maize cobs; *Xylaria sp* km01 (20.1 ± 1.31 IU/ml), *Cyathus sp.* km04 (17.1 ± 1.40 IU/ml), *Nemania sp*. km02 (16.9 ± 0.85 IU/ml), *P. bolleana* km05 (16.9 ± 0.34 IU/ml) and *P. petalodes* km06 (14.9 ± 2.05 IU/ml) when pretreated with 0.1M HCl at 121°C for 15

minutes. Lee *et al*.2011 reported similar findings when corn stover was pretreated with dilute HCl at 121°C for 20min with the highest glucose conversion. This was contrary with *Xylaria sp* km03 (19.8 ± 0.33 IU/ml) recorded the maximum activity when pretreated with 0.1M NaOH at 121°C for 15 minutes. Similar finding was reported by Ojumu *et al.,* 2003 where cellulase activity by *Penecillum decumbens* recorded significantly higher on corn cob pre-treated with the dilute NaOH at 121°C for 20min.

The highest exoglucanase activity was produced on pretreated maize cobs; *Xylaria sp.* km01 (9.35 ±0.77 IU/ml), *Xylaria sp.* KM03 (9.28 ± 0.44 IU/ml), *Nemania sp*. km02 (8.52 ± 0.96 IU/ml), *Cyathus sp.* km04 (7.62 ± 0.39 IU/ml), *P. petalodes* km06 (7.41 ± 0.84 IU/ml) and *P. bolleana* km05 (7.21± 0.59 IU/ml) when pretreated with 0.1M HCl at 121°C for 15 minutes. Similar finding was reported by Ojumu *et al.,* 2003 where cellulase enzyme produced by *Penicillum decumbens* recorded significantly higher when corn cob pre-treated with the dilute HCl. Pretreatment increased exoglucanase activity to about 40 % higher by *Penicillum decumbens*.

The highest endoglucanase activity was produced on pretreated maize cobs; *Xylaria sp* km01 (35.8 ± 1.19 IU/ml), *Nemania sp.* km02 (32.6 ± 3.4 IU/ml), *P. bolleana* km05 (29.7 ± 1.91 IU/ml), *Xylaria sp* km03 (28.0 ± 1.85 IU/ml), *P. petalodes* km06 (27.1 ± 3.61 IU/ml) and *Cyathus sp.* km04 (27.6 ± 0.80 IU/ml) recorded the maximum activity when pretreated with 0.1M HCl at 121°C for 15 minutes. Similar finding was reported by Ojumu *et al.,* 2003 where cellulase produced by *Penicillum decumbens* recorded significantly higher on corn cob pre-treated corn cob with the dilute HCl.

Higher β-glucosidase enzyme activity was recorded on pretreated maize cobs; *Xylaria sp.* km03 (588.6 ± 64.2 IU/ml) recorded the maximum activity when pretreated with 0.1M NaOH at 121°C for 15 minutes. *P. bolleana* KM05 (511.1 ± 10.1 IU/ml), *Cyathus sp.* km04 (421.7 ± 55.7 IU/ml), *Xylaria sp* km01 (4157 ± 294 IU/g) and *P. petalodes* km06 (379.2± 14.7 IU/ml) recorded the maximum activity when pretreated with 0.1M HCl at 121°C for 15 minutes. *Nemania sp*. KM02 (418.7 ± 44.9 IU/ml) recorded the maximum activity when pretreated with 0.1M HCl at 60°C for 2 hours. Similar finding was reported by Ojumu *et al.,* 2003 where β-glucosidase produced by *Penicillum decumbens* recorded significantly higher on corn cob pre-treated corn cob with the dilute HCl.

This differences could be due to difference in pretreatment conditions. (Forrest *et al.,* 2010; Gao *et al.,* 2013; Ma *et al.,* 2010; Timung *et al.,* 2015; Yan *et al.,* 2015). Pretreatment, disrupt the biomass increasing the surface area between the biomass and enzymes, thus dissolution of hemicellulose to initiate the loosening of the lignin complex structure of lignocellulose (Ma *et al.,* 2010; Xia *et al.,* 2013).

The concentration of substrate (4%, 8%, 18% and 20%) influenced the yields of reducing sugar and ethanol. High yield of reducing sugars was optimized on substrate loading with 8% at 72 hours of saccharification for all the six fungi in this study. The amounts of reducing sugars recorded by *Podoscypha petalodes* km06(10.63±0.84 g/l), *Podoscypha bolleana* km05 (10.37±0.84 g/l), *Xylaria sp.* km03 (10.17±0.37 g/ml), *Cyathus sp.* km04 (9.63±0.4 g/l), *Xylaria sp.* km01 (9.48±0.13 g/l) and *Nemania sp.* km02 (9.41±0.27 g/l) were the

maximum in 72 hours of saccharification period. Liming and Xuelang, 2004 and Ouyang *et al.,* 2009 reported similar studies with records of 90.4 % glucose and 49.99 % glucose respectively by *Trichoderma reesei* when grown on corn cobs at different substrate loading. Vyas *et al.* (2005) also reported a significant higher yields with corn cob substrate concentration ranging from 1 to 5% from isolated *A. niger.* Filtrates. Similar finding had recorded an increased yield of 19.4 g/l reducing sugars on untreated corn cob at 8%, Ogunbayo *et al*., (2016). Saliu and Sani, 2012 recorded a yield of 7.63 mg/ml of reducing sugar produced on hydrolysis of corn cob with cellulases of *A. niger* within 48 h. The maximum yield of reducing sugars was as a result of successful saccharification process from cellulose biomass and fungal species (Saliu and Sani, 2012)

High yield of ethanol was optimized on substrate loading with 8% and 20% at 96 hours of fermentation for all the six fungi in the study, with the highest produced by *P. bolleana* km05 (37.3±0.72 g/ml) at 20%, *Nemania sp.* km02 (28.72±3.82 g/ml), *Xylaria sp.* km03 (28.59±1.58 g/ml), *Xylaria sp.* km01 (25.75±0.21 g/ml) all at 8%, *P. petalodes* km06 (24.51±1.23 g/ml) with at% and *Cyathus sp.* km04 (23.22±0.98 g/ml) at 8% respectively.

High yield of ethanol was due to time of incubation and substrate loading optimization during fermentation period. Lawford and Rousseau, (2003) also recorded similar trend. The presence of fermentable sugar in negative control also recorded low ethanol yield. However, yeast believed to use residual sugars from the media and produced other products. Furthermore, residual sugars became used up for ethanol production (Nester *et al* 2001). The bioethanol yield generally increases during the fermentation period with maximum bioethanol production at 96 hours of incubation. As ethanol level increases, it

reduces the pH that favours *Saccharomyces cerevisiae* to converts the reducing sugars to ethanol in the medium and also provides acidic environment to wade off bacterial contamination during fermentation process.

Nester *et al* 2001 recorded similar results, however, the fermentation did not proceed after 96 hrs., the projection was a decreases afterwards as the yeast assume to consumes the nutrients from the medium and reduces the amount of reducing sugar as the fermentation proceeds. Yeast could also be affected by slow hydrolysis of glucose. Similar finding was recorded with a fibrous residues of 5% dry matter loading and with liquid material of 7.5% substrate loading which fermented well. This demonstrated that the fibrous material has high negative effect than liquid media (Lemaresquier 1987; Narendranath 1997).

5.1.4 Limitation of the study

i. Feedback inhibition of cellulase biosynthesis due to formation of cellobiose
ii. Cost of production

5.2 Conclusions

From this study it can be concluded that,

i. *Xylaria sp.* km01, *Nemania sp.*km02, *Xylaria sp.* km03, *Cyathus sp.* km04, *P. bolleana* km05 *and P. petalodes* km06 can be used to produce cellulase enzymes.
ii. Cypress, eucalyptus and maize cobs substrates can be used to produce cellulase enzymes at different optimum period of time

iii. The use of 0.1M HCl for pretreatment on cypress, eucalyptus and maize cobs substrates can be used to produce cellulase enzymes by the six fungi isolates

iv. Cellulase enzymes by six fungal isolates can be used to hydrolyzed maize cobs to fermentable sugars (10.63±0.84 g/ml by *Podoscypha petalodes* km06)

v. Saccharified maize cobs can be used to produce ethanol by *saccharomyces cerevisiae*. (37.3±0.72 g/ml by *P. bolleana* km05)

5.3 Recommendations

5.3.1 Recommendations from the study

i. Maximum level of cellulase activity can be achieved from the 3rd day of incubation by the six isolates

ii. Maize cobs as substrates can be more preferred than cypress and eucalyptus to produce cellulase

iii. Use of 0.1M HCl pretreatment on cypress, eucalyptus and maize cobs substrates can be used to achieve higher cellulase production by the six fungi isolates.

iv. Saccharification of maize cobs by cellulase of *Podoscypha petalodes* km06 can produce higher reducing sugars yields than other isolates

v. Relatively higher ethanol yields by *saccharomyces cerevisiae* can be achieved by hydrolysis of maize cobs by cellulase of Podoscypha bolleana km05

5.3.2 Recommendations for future research

From this study, further research suggestions include:

i. Reduction in the cost of cellulase production and the improvement of the performance of cellulases to make them more effective, so that less enzyme is needed.
ii. Research to produce other lignocellulolytic enzymes like xylanase and lignin modifying enzymes
iii. Optimizing growth conditions or processes for cellulase and bioethanol production
iv. Purification of cellulose enzymes to assess the effect of protease inhibitors on cellulase activity and stability.
v. Protein engineering and microbial genetics to improve the properties of the cellulases is probably an area to focus to advance in more research in cellulase technology.

REFERENCES

Acharya, B.K., Mohana, S., Jog, R., Divecha, J., Madamwar, D. (2010). Utilization of anaerobically treated distillery spent wash for production of cellulases under solid state fermentation. *Journal of Environmental Management*, 91:2019 – 2027.

Acharya, P.B., Acharya, D.K. and Modi, H. A. (2008). Optimization for cellulase production by Aspergillus niger using saw dust as substrate. *African Journal of Biotechnology*, 7(22): 4147-4152.

Alfenore, S., Molina-Jouve, C., Guillouet, S., Uribelarrea, J. L., Goma, G. and Benbadis, L. (2002). Improving ethanol production and viability of Saccharomyces cerevisiae by avitamin feeding strategy during fed-batch process. *Applied Microbiology and Biotechnology,* 60(1):67-72.

Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J. (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. *Bioresource Technology*, 101(13):4851 – 4861.

Amadi O. C. (2012). Use of starch containing tubers for the formulation of culture media for fungal cultivation. *Africa Journal of Microbiology Research,* 6(21):213-222

Antoni, D., Zverlov, V.V. and Schwarz, W.H. (2007). Biofuels from microbes. *Applied Microbiology Biotechnology,* 77(1): 23–35.

Archana, A. and Satyanarayana, T. (1997). Xylanase production by *thermophilic Bacillus licheniformis* A 99 in solid state fermentation. *Enzyme Microbiology Technology*, 21: 12- 17.

Arora, D. K. and Bhatnagar, D. (2003). Handbook of fungal biotechnology. *CRC press*. 471-493.

Awogbemi, O., Kallon, D.V.V., Onuh, E.I. and Aigbodion, V.S. (2021). An overview of the classification, production and utilization of biofuels for internal combustion engine applications. *Energies,* 14(18): 5687.

Ayyappa Kumar Sista Kameshwar and Wensheng Qin (2018). Purification and Characterization of the Total Cellulase Activities (TCA) of Cellulolytic Microorganisms. *Cellulases-Methods in Molecular Biology*, (255-269)

Bansal, P., Hall, M., Realff, M.J., Lee, J.H. and Bommarius, A. S. (2009). Modeling cellulose kinetics on lignocellulosic substrates. *Biotechnology Advances*, 27: 833-48.

Bhat, M. and Bhat, S. (1997). Cellulose degrading enzymes and their potential industrial applications. *Biotechnology Advances*, 15: 583-620.

Bhoosreddy, C.L. (2014). Comparative study of cellulase production by *Apsergillus niger* and *Trichoderma viride* using solid state fermentation on cellulosic substrates

corncob, cane bagasse and sawdust. *International Journal for Science Resource.*, 3: 324–326

Bilal Ahmad Bhat, G. R. Khan and K. Asokan (2015). Role of substrate effects on the morphological, structural, electrical and thermoelectrical properties of V2O5 thin films. *Resources Advances*., 5: 552-602

Blaschek HP and Ezeji TC. (2010). Science of alternative feedstock.: A handbook of Biomass to Biofuels: Strategies for global industries. Wiley: United States, 112-128.

Bollag JM & Leonowicz A. (1984). Comparative studies of extracellular fungal laccases. *Applied and Environmental Microbiology*, 48(4): 849-854.

Bommarius, A.S., Katona, A. and Cheben, S.E. (2008). Cellulase kinetics as a function of cellulose pretreatment. *Metabolic Engineering*, 10: 370–381.

Cannel, E. and Moo-Young, M. (1980). Solid state fermentation systems. *Process Biochemistry* 15:27.

Carlile, M. J., Watkinson, S. C. and Gooday, G. W. (2001). The fungi. *Academic Press, London, UK*. pp. 164-168, 269–275, 476-479, 507-514.

Carpita, N., Tierney, M. and Campbell, M. (2001). Molecular biology of the plant cell wall: searching for the genes that define structure, architecture and dynamics. *Plant Molecular Biology,* 47: 1–5.

Carvalheiro F, Duarte LC & Gírio FM (2008). Hemicellulose biorefineries: a review on biomass pretreatments. Journal of Scientific and Industrial Research, 67(11): 849–864.

CaveI. D.WalkerJ. F. C. (1994). Stiffness of Wood in Farown Plantation Softwood: The Influence of Microfibril angle. *Forest Product Journal*, 4, 454-348

Celestino, K.R.S., Cunha, R.B. and Felix, C.R. (2006). Characterization of a β-glucanase produced by Rhizopus microsporus var. microsporus, and its potential for application in the brewing industry. *BMC Biochemistry*, 7: 1-9.

Chaudhary, L.C., Singh, R., and Kamra, D.N. (1994). Bio delignification of sugar cane bagasse by *Pleurotus Xorida* and *Pleurotus cornucopiae*. *Indian Journal of Microbiology*, 34:55-54.

Chen, H., He, Q., Liu, L. (2011). Cellulase production from the corn stover fraction based on the organ and tissue. *Biotechnology and Bioprocess Engineering*, 16: 867 – 874.

Collinson, S.R. and Thielemans, W. (2010). The catalytic oxidation of biomass to new materials focusing on starch, cellulose and lignin. *Coordination Chemistry Reviews*, 254:1854-1870.

Cosgrove, Daniel J. (2005). Plant cell wall extensibility: connecting plant cell growth with cell wall structure, mechanics, and the action of wall-modifying enzymes. *Journal of Experimental Botany* VO 67 IS 2 SP 463 OP 476

Couri, S., Terzi, S.C., Pinto, S.C., Freitas, S.P.A. and Costa, A.C.A. (2000). Hydrolytic enzyme production in solid-state fermentation by *Aspergillus niger* 3T5B8. *Process Biochemistry*, 36: 255–261.

De Oliva-Neto, P. and Yokoya, F. (1997). Effects of nutritional factors on; growth of Lactobacillus Fermentum mixed with *Saccharomyces cerevisiae* in alcoholic fermentation. *Revista de Microbiologia*, 28: 25-31

Delgado-Serrano L, Restrepo S, Bustos JR, Zambrano MM & Anzola JM (2016). Mycofier: a new machine learning-based classifier for fungal ITS sequences. *BioMed Central Research Notes*, 9: 402.

Deswal, D., Khasa, Y.P., Kuhad, R.C., (2011). Optimization of cellulose production by a brown rot fungus Fomitopsis sp. RCK under solid state fermentation. *Bioresource Technology*, 102: 6065– 6072.

Doelle, H.W., Mitchell, D.A. and Rolz, C.E. (1992). Solid Substrate Cultivation. Elsiever Science Publication ltd; London & New York; *Bioresource Technology*,466.

Dogaris, I., Vakontios, G., Kalogeris, E., Mamma, D. and Kekos, D. (2009). Induction of cellulases and hemicellulases from Neurospora crassa under solid-state cultivation for bioconversion of sorghum bagasse into ethanol. *Industrial crops and products*, 29: 404 411.

Elisashvili V & Kachlishvili E (2009). Physiological regulation of laccase and manganese peroxidase production by white-rot Basidiomycetes. *Journal of Biotechnology*, 144: 37,42. *Energy Information Administration (EIA), 2013. International Energy Outlook 2013.*

Elisashvili V, Kachlishvili E, Tsiklauri N, Metreveli E, Khardziani T & Agathos SN (2009). Lignocellulose-degrading enzyme production by white-rot Basidiomycetes isolated from the forests of Georgia. *World Journal of Microbiology and Biotechnology,* 25: 331-339.

Elisashvili V, Penninckx M, Kachlishvili E, Asatiani M & Kvesitadze G (2006). Use of Pleurotus dryinus for lignocellulolytic enzymes production in submerged fermentation of mandarin peels and tree leaves. *Enzyme Microbial Technology*, 38:998-1004.

Enoch Y. Park, Kazuya Naruse, Tatsuya Kato (2012). Improvement of cellulase production in cultures of Acremonium cellulolyticus using pretreated waste milk pack with cellulose targeting for biorefinery, Bioresource Technology, Volume 102, Issue 10, Pages 6120 - 6127

Farrell A.E, Plevin, R.J., Turner, B.T., Jones, A.D., O'Hare, M. and Kammen, D.M. (2006). Ethanol can contribute to energy and environmental goals. *Science* 113: 506–508.

Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.

Feria, M.J., Lopez, F., Garcia, J.C., Perez, A., Zamudio, M.A.M. & Alfaro, A., (2011). Valorization of Leucaena leucophala for energy and chemicals from autohydrolysis. *Biomass and Bioenergy*, 35: 2

Follett RF (2001): Soil management concepts and carbon sequestration in cropland soils. *Soil and Tillage Research,* 61(1): 77-92.

G Matthews, AK Emo, G Funke, M Zeidner, RD Roberts, PT Costa Jr, 2006 Emotional Intelligence, Personality, and Task-Induced Stress. *Journal of Experimental Psychology: Applied* 12 (2): 96

Ganesh D. Saratale & Siddheshwar D. Kshirsagar & Vilas T. Sampange & Rijuta G. Saratale & Sang-Eun Oh & Sanjay P. Govindwar & Min-Kyu Oh, (2014). Cellulolytic Enzymes Production by Utilizing Agricultural Wastes Under Solid State Fermentation and its Application for Biohydrogen Production: *Applied Biochemistry and Biotechnology* 174:2801-2817.

Gautam SP, Bundela PS, Pandey AK, Khan J, Awasthi MK, Sarsaiya S, (2011). Optimization for the production of cellulase enzyme from municipal solid waste residue by two novel cellulolytic fungi. *Biotechnology Resources International*,81:04-25.

Ghose TK, (1987). Measurement of cellulase activities. Pure Appl Chem 1987; 59:257-68.

Golla, N, Pradeep, R, and A. Sridevi, (2016). Chemical Pretreatment of Agricultural Feedstock for Enhanced Production of Cellulase by Mutant Fungus, Aspergilus Niger.*Journal of Applied Biotechnology and Bioengineering;* 1: 2572-8466

Government of Kenya (2010). Bioethanol Strategy (2010-2013)

Graham RL, Nelson R, Sheehan J, Perlack RD & Wright LL (2007). Current and potential US corn stover supplies. *Agronomy Journal,* 99(1):1-1.

Graham, G. C.; Meyers, P. and Henry, R. J. A. (1994). Simplified method for the preparation of fungal genomic DNA for PCR and RAPD analysis. *Bio Techniques,* 16:48-50.

Grethlein, H.E., Allen, D.C. and Converse, A.O. (1984). A comparative study of the enzymatic hydrolysis of acid-pretreated white pine and mixed hardwood. *Biotechnology Bioengineering*, 26:1498-1505

Guimarães, L.H. (2012). Carbohydrates from Biomass: Sources and Transformation by Microbial Enzymes. Comprehensive Studies on Glycobiology and Glycotechnology. Carbohydrates Technologies. Chapter 20 .441-458.

Guimarães, L.H. (2012). Carbohydrates from Biomass: Sources and Transformation by Microbial Enzymes. In C. Changed. Carbohydrates - Comprehensive Studies on Glycobiology and Glycotechnology. Carbohydrates Technologies. Chapter 20 .441-458.

Habibi, Y., Lucia, L.A. & Rojas, O.J., (2010). Cellulose nanocrystals: Chemistry, biomass pyrolysis based on three major components: hemicellulose, cellulose and lignin, *Energy &Fuels*, 20(1), 388-393.

Harmsen, P.F.H., Huijgen, W.J.J., Bermudez Lopez, L.M., Bakker, R.R.C. (2010). Literature review of physical and chemical pretreatment processes for lignocellulosic biomass. *Biosynergy journal*, 1184-1230

Hartree, M.M., Yu, E.K.C., Reid, I.D. and Saddler, J.N. (1987). Suitability of aspen wood biologically delignified with Pheblia tremellosus for fermentation of ethanol or butanol. *Applied Microbiology and Biotechnology*, 26:120–125

Hideno, A., Inoue, H., Tsukahara, K., Yano, S., Fang, X., Endo, T., Sawayama, S. (2011). Production and characterization of cellulases and hemicellulases by Acremonium cellulolyticus using rice straw subjected to various pretreatments as the carbon source. *Enzyme Microbiology Technologogy,*48:162 – 168.

Himmel, M.E, Ruth, M.F. and Wyman, C.E. (1999). Cellulase for commodity products from cellulosic biomass. *Current Opinion in Biotechnology*, 10: 358–64.

Hsu, T.A., Ladisch, M.R., Tsao, G.T. (1980). Alcohol from cellulose. *Chemical Technology,* 10(5): 315–319

IEA. (2013). World Energy Outlook 2013 [Assessment report]. International Energy Agency.https:// www.iea.org/reports/world-energy-outlook-2013

Ihrmark, K., Bodeker, I. T. M., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J. (2012). New primers to amplify the fungal its2 region - evaluation by 454 sequencing of artificial and natural communities. *FEMS Microbial Ecol*ogy, 82:666-677.

Ilyas, U., Ahmed, S. Majeed, A. and Nadeem, M. (2012). Biohydrolysis of Saccharum spontaneum for cellulase production by Aspergillus terreus. *African Journal of Biotechnology,* (21): 4914-4920.

Iqbal, H.M.N., Kyazze, G., Keshavarz, T. (2013). Advances in the valorization of lignocellulosic materials by biotechnology: An overview. *Bio Resources*, (2):3157 3176.

Isroi, Millati R, Syamsiah S, Niklasson C, Cahyanto MN, Lundquist K& Taherzadeh MJ (2022). Biological pretreatment of lignocellulose with white-rot fungi and its applications: A review. *BioResources,* 6(4): 5224–5259

Jabasingh, S.A., Nachiyar, C.V. (2011). Response surface approach for the biodegradation of pretreated coir pith using *Aspergillus nidulans* SU04 for cellulase production. *Second International Conference on Sustainable Energy and Intelligent System.*

Jacob, N. and Prema, P. (2006). Influence of Mode of Fermentation on Production of Polygalacturonase by a Novel Strain of *Streptomyces lydicus*. *Food Biotechnol*ogy 44 (2):263– 267.

Kachlishvili E, Penninckx MJ, Tsiklauri N & Elisashvili V. (2006). Effect of nitrogen source on lignocellulolytic enzyme production by white-rot basidiomycetes under solid-state cultivation. *World Journal of Microbiology and Biotechnology*, 22:391-397

Khalid, I. (2010). Chemical Changes in 15 Year-old Cultivated Acacia Hybrid Oil Heat Treated "at 180, 220 and 220°C", *International Journal of Chemistry*, 2: 97-107.

Khan, S.U., Al-Khaled, K., Aldabesh (2021). Bioconvection flow in accelerated couple stress nanoparticles with activation energy: *Bio-Fuel Applications,*11:3331.

Kim, S.W., Kang and Lee, J.S. (1997): Cellulase and xylanase production by *Aspergillus niger* KKS in various bioreactors. *Bioresource Technology*, 59: 63-67.

Kimura M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16:111-120.

Kiranmayi, M.U., Poda, S., Charyulu, P.B.B.N., Vijayalakshmi, M., Krishna, P.V. (2011). Studies on influence of natural biowastes on cellulase production by *Aspergillus niger*. *Journal of Environmental Biology,*32:695 – 699.

Kirk PM, Cannon PF, Minter DW, Stalpers JA (2008). Dictionary of the Fungi. Wallingford, UK: *CAB International*. (10th ed.) 551.

KJ Shinners, BN Binversie, RE Muck, PJ Weimer (2007). Comparison of wet and dry corn stover harvest and storage. *Biomass and Bioenergy* 31 (4): 211-221.

Kuhad, R.C. and Singh, A. (1993): Lignocellulose biotechnology: Current and future prospects. *Critical Reviews in Biotechnology*, 13: 98–118.

Kuhad, R.C., Singh, A. and Eriksson, K.E. (1997). Microorganisms and enzymes involved in the degradation of plant fiber cell walls. *Advances Biochemical Engineering Biotechnology*, 57: 45–125.

Kumar, A.K., Parikh, B.S. (2015). Cellulose-degrading enzymes from Aspergillus terreus D34 and enzymatic saccharification of mild-alkali and dilute-acid pretreated lignocellulosic biomass residues. *Bioresource of Bioprocess*, 2: 7

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular biology and evolution,* 35(6): 1547.

Legodi L. M., D. La Grange, E. L. Jansen van Rensburg, I. Ncube (2019). "Isolation of Cellulose Degrading Fungi from Decaying Banana Pseudostem and Strelitzia alba",*Enzyme Research,* vol. 2.

Lee, H.V., Hamid, S.B.A., Zain, S.K., (**2014).** Conversion of lignocellulosic biomass to nano cellulose: Structure and chemical process. *Scientific World* Journal., 1 – 20.

Lemaresquier, H. (1987). Inter-relationships between strains of Saccharomyces cerevisiae from the Champagne area and lactic acid bacteria. *Application of Microbiol*ogy, 4: 91-94

Li ZJ, Ji XJ, Kan SL, Qiao HQ, Jiang M, Lu DQ, Wang J, Huang H, Jia HH, Ouyang PK, Ying HJ (2010). Past, present, and future industrial biotechnology in China. *Advances of Biochemial Engineering and Biotechnology,* 122:1–42

Li. H, Pu Y, Kumar R, Ragauskas AJ & Wyman CE 2014. Investigation of lignin deposition on cellulose during hydrothermal pretreatment, its effect of cellulose hydrolysis, and underlying mechanisms. *Biotechnology and Bioengineering,* 111(3):485-492

Mafe, O., Pensupa, N., Roberts, E. & Du, C., (**2014**). Advanced Generation of Bioenergy. In C.Lin & R. Luque, eds. Renewable Resources for Biorefineries. *Royal Society of Chemistry* (RSC).117-45.

Maki M. LeungK. T. QinW. (**2009**). The Prospects of Cellulase-Producing Bacteria for the Bioconversion of Lignocellulosic Biomass. *International Journal of Biological Sciences*, 295.

Maki, M., Leung, K.T. and Qin, W. (2009). The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass (Review). *International Journal Biological Science,* 5: 500-516.

Markets and Markets, (2015). Industrial enzymes market by type (carbohydrases, proteases, non-starch polysaccharides & others), application (food & beverage, cleaning agents, animal feed & others), brands & by region - *Global Trends & Forecasts* of Polyunsaturated Fatty Acids Producing Marine Bacteria. *Biotechnology Research International*, 542721: 1–8.

Martín, C., Galbe, M., Wahlbom, C.F., Hahn-Hägerdal, B., Jönsson, (2002). L.J. Ethanol production from enzymatic hydrolysates of sugarcane bagasse using recombinant xylose utilizing *Saccharomyces cerevisiae*. *Enzyme and Microbial Technology*, 31:274–282.

Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D (2008). Genome sequencing and analysis of the biomass degrading fungus *Trichoderma reesei* (syn. Hypocrea jecorina). *National Biotechnol*ogy, 26:553-560.

Matthews, J.F, Skopec, C.E., Mason, P.E., Zuccato, P., Torget, R.W., Sugiyama, J., Himmel, M.E. and Brady, J.W. (2006). Computer simulation studies of microcrystalline cellulose Iβ. *Carbohydrate Research*, 341: 138-152.

McCarthy, J.K., Uzelac, A., Davis, D.F. and Eveleigh, D.E. (2004). Improved catalytic efficiency and active site modification of 1, 4-Beta-D-glucan glucohydrolase a from *Thermotoga neapolitana* by directed evolution. *Jornal of Biological Chemistry*, 279: 11495 502.

McMillan, J.D. (1994). Pretreatment of lignocellulosic biomass. In: Enzymatic conversion of biomass for fuels production. M.E. Himmel, J.O. '3 Baker and R.P. Overend (eds.) ACS symp. Ser. 566. *American Chemical Society*, Washington, DC, Chapter 15:292-324)

Merino ST, Cherry J (2007). Progress and challenges in enzyme development for biomass utilization. Edited by: Lisbeth O. Springer, Springer Berlin Heidelberg. *Biofuels,*95-120.

Michael Ladisch, Nathan Mosier, Charles Wyman, Bruce Dale, Richard Elander, Y Y Lee, Mark Holtzapple (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology*,96 (6): 673-686.

Miles, E. A. and Trinci, A. P. J. (1983). Effect of pH and temperature on morphology of batch and chemostat cultures of *Penicillium chrysogenum*. *Soc*iety *of Mycology*, 81 (2): 193-200.

Miller, G.L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Journal of Analytical Chemistry*, 31: 426-428.

Mohammed, I.Y., Abakr, Y.A., Kazi, F.K., Yusup, S., Alshareef, I. & Chin, S.A., (**2015).** Comprehensive characterization of Napier grass as a feedstock for thermochemical conversion. *World Journal of Energies,* 8: 3403-17.

Montoya, S., Orrego, C.E., Levin, L., (**2010**). Growth, fruiting and lignocellulolytic enzyme production by the edible mushroom Grifola frondosa (maitake). *World Journal of Microbiology and Biotechnology,* 28: 1533 – 1541.

Moon, R.J., Martini, A., Nairn, J., Simonsen, J. & Youngblood, J., (**2011**). Cellulose nanomaterials review: structure, properties and nanocomposites. *Chemical Society Reviews,* 40:3941-94.

Moo-Young, M., Moreira, A. and Tengerdy, R. (1983). Principles of solid state fermentation. Fungal Biotechnology, Smith, J., D. Berry and B. Kristiansen (Eds.). *Edward Arnold Publishers, London*, 117-144.

Mushimiyimana, I., and P. Tallapragada (2016). Bioethanol production from agro wastes by acid hydrolysis and fermentation process. *Journal of Scientific and Industrial Research*,75:383-388.

Mussatto SI & Teixeira JA. (2010). Lignocellulose as raw material in fermentation processes. In Méndez-Vilaz A (Ed.). Current Research, Technology and Education Topics in *Applied Microbiology and Microbial Biotechnology,* Formatex, pp. 897-907.

Myers DK & Underwood, JF. (1992). Report number: AGF-003-92. The Ohio State University Extension.

Nachiyar, C.V., Jabasingh, S.A. (2011). Response surface approach for the biodegradation of pretreated coir pith using *Aspergillus nidulans* SU04 for cellulase production. *Second International Conference on Sustainable Energy and Intelligent System.*

Naik, G.P., Poonia, A.K. and Chaudhari, P.K. (2021). Pretreatment of lignocellulosic agricultural waste for delignification, rapid hydrolysis, and enhanced biogas production: A review. *Journal of the Indian Chemical Society*, 98(10):100-147.

Narendranath, N. V., Hynes, S. H„ Thomas, K. C. and Ingledew, W. M. (1997). Effects of lactobacilli on yeast-catalyzed ethanol fermentations. *Application of Environment and Microbiology,* 63:4158-4163.

Nathan Mosier, Charles Wyman, Bruce Dale, Richard Elander, YY Lee, Mark Holtzapple, Nester, E.W., Anderson, D.G., Roberts, C.E., Pearsall, N.N and Nester, M.T. (2001). Dynamics of prokaryotic growth in Microbiology. *A human perspective*. 3rd edition. McGraw-Hill,New York, 87-108.

Nema (2014). National Solid Waste Management Strategy

Nishiyama Y. (2009). Structure and properties of the cellulose micro fibril. *Journal of Wood Science* 55: 241-249

Nowak D, Jakubczyk E. (2020). The Freeze-Drying of Foods-The Characteristic of the Process Course and the Effect of Its Parameters on the Physical Properties of Food Materials. Foods. Oct 18;9(10):1488.

Ojumu TV, Solomon BO, Betiku E, Layokun SK, Amigun B. (2003). Cellulase Production by Aspergillus flavus Linn isolate NSPR 101 fermented in sawdust, bagasse and corn cob. *African Journal of Biotechnology,* 2:150–152.

Ojumu TV, Solomon BO, Betiku E, Layokun SK, Amigun B. (2003). Cellulase Production by Aspergillus flavus Linn isolate NSPR 101 fermented in sawdust, bagasse and corn cob. *African Journal of Biotechnology,* 2:150–152.

O'Neill, M.A., and York, W.S. (2003). The composition and structure of plant primary cell walls. The Plant Cell Wall, J.K.C. Rose, ed (*Boca Raton, FL: CRC Press*), 1–54.

Ouyang J, Li X, Ying H, Yong Q. (2009). Enhanced enzymatic conversion and glucose saccharification of water hyacinth through microwave heating with dilute acid pretreatment for biomass energy utilization. *Energy* 61, 158–166.

Pandey, A., Francis, F., and Soccol, C. R. (2004). General aspects in solid state fermentation, in: *Concise Encyclopedia of Bioresource Technology*, edited by Ashok Pandey, Haworth Press, New York,702-718.

Pandey, A., Nigam, P., Selvakumar, P. and Soccol, C.R. (1999). Solid state fermentation for the production of industrial enzymes. *Current Science,* 77:149- 162.

Pandey, S.; Shrivastava, M.; Shahid, M.; Kumar, V.; Singh, A.; Trivedi, S.; Srivastava, Y.K. (2015). *Trichoderma species* cellulases produced by solid state fermentation. *Journal of Data Mining, Genome and Proteome,* 6:170.

Pandya B & Albert S. (2014). Evaluation of *Trichoderma reesei* as a compatible partner with some white-rot fungi for potential bio-bleaching in paper industry. *Annals of Biological Research*, 5:43-51.

Papagianni, M. (1995). Morphology and citric acid production of *Aspergillus niger* PM1 in submerged fermentation, Ph.D. Thesis, University of Strathclyde, Glasgow, Scotland.

Parawira W & Tekere M (2011). Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: Review. *Critical Reviews in Biotechnology,* 31(1): 20-31

Park JW, Takahata Y, Kajiuchi T & Akehata T. (2012). Effects of non-ionic surfactant on enzymatic hydrolysis of used newspaper. *Biotechnology Bioengineering*, 39: 117-120.

Park S, Ransom C, Mei C et al (2011). The quest for alternatives to microbial cellulase mix production: corn stover-produced heterologous multi-cellulases readily deconstruct lignocellulosic biomass into fermentable sugars. *Journal of Chemical Technology and Biotechnol*ogy, 86:633– 641.

Parry, N.J., Beever, D.E., Owen, E., Vandnberghe, J., Beeumen, V. and Bhat, M. (2002). Biochemical characterization and mechanism of action of a thermostable ß glucosidase purified from *Thermoascus aurantiacu*, *Biochemistry Journal*, 353: 117- 127

Pérez-Guerra, N., Torrado-Agrasar, A., López-Macias, C. and Pastrana, L. (2003). Main characteristics and applications of solid substrate fermentation. *Electronic Journal of Environmental Agriculture and Food Chemistry,* 2 (3): 343-350.

Poletto Patricia, Gabriela N. Pereira, Carla R.M. Monteiro, Maria Angélica F. Pereira (2009). Production via two step enzymatic hydrolysis of corn cob residue from xylooligosaccharides producer's waste. *BioResources*; 4:1586–99.

Rachel A. Burton, Helen M. Collins, Natalie A. J. Kibble, Jessica A.Smith, Neil Shirley, Stephen A. Jobling (2011). Over-expression of specific *HvCslF* cellulose synthase-like genes in transgenic barley increases the levels of cell wall (1,3;1,4)-β-d glucans and alters their fine structure. *Plant biotechnology journal* Volume 9, Issue2 Pages 117-135

Rahnama, N., Foo, H.L., Abdul Rahman, N.A. (2014). Saccharification of rice straw by cellulose from a local *Trichoderma harzianum* SNRS3 for biobutanol production. *BMC Biotechnology,*14:103.

Raimbault, M. (1998). General and microbiological aspects of solid substrate fermentation. *Electronic Journal of Biotechnology*, 1(3): 174 189.

Ramos, L.P., Breuil, C. and Saddler, J.N. (1992). Comparison of steam pretreatment of eucalyptus, aspen, and spruce wood chips and their enzymatic hydrolysis. *Application of Biochemistry and Biotechnol*ogy, 34/35: 37-48 23.

Roberto, I.C., Mussatto, S.I. and Rodrigues, R.C.L.B. (2003). Dilute-acid hydrolysis for optimization of xylose recovery from rice straw in a semi-pilot reactor. *Industrial Crops Production,* 17: 171–176.

Rosales E, Couto RS & Sanromán A (2005). Reutilization of food processing wastes for production of relevant metabolites: application to laccase production by *Trametes hirsuta*. *Journal of Food Engineering*, 66: 419-423.

Sadhu, S., Saha, P., Sen, S.K., Mayilraj, S. and Maiti, T.K. (2013). Production, purification and characterization of a novel thermotolerant endoglucanase (CMCase) from *Bacillus* strain isolated from cow dung. *Springer plus*, 25: 2:10.

Sailendra G, Devi SS, Kalita MC, Talukdar NC. (2014). Population, diversity and characteristics of cellulolytic microorganisms from the Indo-Burma Biodiversity hotspot. *Springerplus,* 28;3:700.

Sainz MB. (2011). Commercial cellulosic ethanol: the role of plant-expressed enzymes. Biofuels, 237–264.

Saitou N. and Nei M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution,* 4:406-425.

Saleem, Ram Naraian, Roshan L. Gautam, (**2018**). Hand book in New and Future Developments in Microbial. *Biotechnology and Bioengineering.*

Santoni, I., Callone, E., Sandak, A., Sandak, J., Dirè, S., (**2015**). Solid state NMR and IR chacaterization of wood polymer structure in relation to tree provenance. *Carbohydrate Polymer*. 117, 710 – 721.

Sawatdeenarunat, C., Surendra, K.C., Takara, D., Oechsner, H., Khanal S.K. (**2015).** Anaerobic digestion of lignocellulosic biomass: Challenges and opportunities. *Bioresource Technol*ogy, 178-186

Scheller HV, Ulvskov P. (2010). Hemicelluloses. *Annals Review of Plant Biology*, 61: 263–289 self-assembly and applications. *Chemical Reviews,* 110:3479-500.

Shen, X. and L. Xia. (2004). Production and immobilization of cellobiase from *Aspergillus niger* ZU-07. *Process Biochemistry*, 39: 1363–1367.

Sidnei E. Bordignon, Débora de Oliveira (2013). Xylooligosaccharides: Transforming the lignocellulosic biomasses into valuable 5-carbon sugar prebiotics, Process Biochemistry, Volume 91,2013, Pages 352-363.

Singh, A. & Hayashi, K. (1995). Microbial cellulase, protein architecture, Molecular properties & biosynthesis. *Advanced Microbial Application,* 40: 1-44.

Smith, B.G. and Harris, P.J. (1999). The polysaccharide composition of Poales cell walls: Poaceae cell walls are not unique. *Biochemical Systematics and Ecology*, 27: 33–53

Solomon BO, Amigun B, Betiku E, Ojumu TV & Layokun SK (1999). Optimization of cellulase production by Aspergillus flavus Linn isolate NSPR 101 crown on bagasse. *Journal of National Society of Chemistry*, 16: 61-68.

Songulashvili G, Elisashvili V, Wasser SP, Nevo E & Hadar Y (2007). Basidiomycetes laccase and manganese peroxidase activity in submerged fermentation of food industry wastes. *Enzyme and Microbial Technology,* 41: 57-61.

Sorensen, A., Lübeck, M., Lübeck, P.S. and Ahring, B.K. (2013). Fungal β-Glucosidases: A bottleneck in industrial use of lignocellulosic materials. *Biomolecules,* 3: 612-631.

Subramaniyam, R. and Vimala, R. **(2012).** Solid State and Submerged Fermentation for the Production of Bioactive Substances: A Comparative Study. *International Journal of Science and Nature*, 3:480-486.

Suh S, Zhang N, Nguyen N, Gross S, Blackwell M. (2007). Lab manual for yeast study. Mycology lab. Louisiana state university. *Statistical assistance*, 200-208.

Sukesh K. (2010). An Introduction to Industrial Microbiology. *S. Chand Publishing:* New Delhi, PP.22

Sun X & Guo L. (2012). Endophytic fungal diversity: review of traditional and molecular techniques. *Journal of Mycology,* 3(1): 65–76.

Sun, Y.and Cheng, J. (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology*, 83[2]: 1–11

T Gurunathan, S Mohanty, SK Nayak (2015). Composites Part A: *Applied Science and Manufacturing,*77: 1-25.

Tabet, T.A. & Aziz, F.A. (**2013**). Cellulose Microfibril Angle in Wood and Its Dynamic Mechanical Significance. In T.G.M. van de Ven & L. Godbout, ds. Cellulose - Fundamental Aspects. *International Technology*, 113-142.

Talebnia, F., Karakashev, D. & Angelidaki, I. (**2010).** Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. *Bioresource Technology,* 101:4744-4753.

Tamura K., Stecher G., and Kumar S. (2021). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution.*

Thekra Al-Ka'aby (2012). Optimization for cellulase production by Aspergillus niger using sawdust as substrate *Journal of Babylon University/Pure and Applied Sciences*/ No. (5) Vol. (20)

Thomson, A.M., Calvin, K.V., Smith, J.S., Kyle, P.G., Volke, A., Patel, P., Delgado-Arias, S., Bond-Lamberty, B., Wise, M.A., Clarke, L.E. and Edmonds, J.A. (2011). RCP4.5: a pathway for stabilization of radiative forcing. *Climatic Change*, 109: 77-94.

Tilay A & Annapure U (2012). Novel Simplified and Rapid Method for Screening and Isolation of Polyunsaturated Fatty Acids Producing Marine Bacteria. *Biotechnology Research International*, 542721: 1–8.

Tony Vancov, Brad Keen (2009). Amplification of soil fungal community DNA using the ITS86F and ITS4 primers, FEMS Microbiology Letters, Volume 296, Issue 1, Pages 91–96.

Tuo H. (2013). Energy and exergy-based working fluid selection for organic Rankine cycle recovering waste heat from high temperature solid oxide fuel cell and gas turbine hybrid systems. *International Journal of Energy Resources,* 37(14):1831–1841.

Vanholme, R., Demedts, B., Morreel, K., Ralph, J. & Boerjan, W. (2010). Lignin biosynthesis and structure. *Plant Physiology Journal*, 153, 895-905.

Verduyn, C., Postma, E., Scheffers, W.A. and van Dijken, J.P. (1990). Energistics of Saccharomyces cerevisiae in anaerobic glucose-limited chemostat cultures. *Journal of General Microbiology,* 136: 405-412.

Victor Ojumu Tunde, Solomon Bamidele Ogbe, Betiku Eriola, Layokun Stephen Kolawole and Amigun Bamikole (2003). Cellulase Production by *Aspergillus flavus* Linn Isolate NSPR 101 fermented in sawdust, bagasse and corncob bagasse and corncob *African Journal of Biotechnology* 2(6):150-152

Viniegra-Gonzalez, G., Favela-Torres, E., Aguilar, C. N., Romero-Gomez, S. J., Diaz Godinez, G. and Augur, C. (2002). Advantages of fungal enzyme production in solid state over liquid fermentation systems *Biochemical Engineering Journal*,13:157-167.

Vintila, T., Dragomirescu, M., Jurcoane, S., Vintila, D., Caprita, R. and Maniu, M. (2009). Production of cellulase by submerged and solid-state cultures and yeasts selection for conversion of lignocellulose to ethanol. *Romanian Biotechnological Letters*. 14 (2): 4275- 4281.

Weil, J., Westgate, P., Kohlman, K. and Ladish, M.R. (1994). Cellulose pretreatment of lignocellulosic substrate. *Enzyme Microbiology Technology*, 16: 1002–1004.

WHO. (2016). Stakeholder meeting on Building the Health and Energy Platform of Action WHO; World Health Organization. http://www.who.int/airpollution/news/health-and energy platform-of-action/en/

Wilhelm WW, Johnson JM, Hatfield JL, Voorhees WB & Linden DR (2004). Crop and soil productivity response to corn residue removal. *Agronomy Journal*, 1:1-7.

Winquist E, Moilanen U, Mettälä A, Leisola M & Hatakka A (2008). Production of lignin modifying enzymes on industrial waste material by solid-state cultivation of fungi. *Biochemical Engineering Journal*, 42: 128-132.

Woese, C. R. (2000). Interpreting the universal phylogenetic tree. *National Academy & Science USA* 97: 8392–8396.

World Development Report (2010). Development and Climate Change (2010) © Washington, DC http://hdl.handle.net/10986/4387License:CCBY3.0IGO Google Scholar

Xia, A., Cheng, J., Song, W., Yu, C., Zhou, J., and Cen, K. (2007). Enhancing enzymatic saccharification of water hyacinth through microwave heating with dilute acid pretreatment for biomass energy utilization. *Energy* 61, 158–166.

Xie Wei Xiwen Liu, Lichuang Cao, Jianhua Zeng, Yuhuan Liu (2019). Improving the cellobiose-hydrolysis activity and glucose-tolerance of a thermostable β-glucosidase through rational design, *International Journal of Biological Macromolecules*, Volume 136,2019, Pages 1052-1059.

Yamashita, Y., Shono, M., Sasaki, C. and Nakamura, Y. (2010). Alkaline peroxide pretreatment for efficient enzymatic saccharification of bamboo. *Carbohydrate Polymer,* 79: 914–920.

Yang B, Dai Z, Ding S-Y, & Wynman CE. (**2011**). Enzymatic hydrolysis of cellulosic biomass. *Biofuels Journal*, 2(4): 421–450

Yang H., Yan R., Chen H., Zheng C., Lee D.H., Liang D.T. (2006). In-depth investigation of biomass pyrolysis based on three major components: hemicellulose, cellulose and lignin, *Energy & Fuels*, 20(1), 388-393.

Zakpaa HD, Mak-Mensah EE, Johnson F S (2009). Production of bio-ethanol from corn cobs using *Aspergillus niger and saccharomyces cerevisiae* in simultaneous saccharification and fermentation *African Journal of Biotechnology,* 8: 3018-22.

Zhang X., Jing, X. & Bao, J. (2009). Inhibition Performance of Lignocellulose Degradation Products on Industrial Cellulase Enzymes During Cellulose Hydrolysis. *Application of Biochemistry and Biotechnology Journal*, 159: 696–707.

Zhang, Y.H.P., Himmel, M.E. and Mielenz J.R. (2006). Outlook for cellulase improvement: Screening and selection strategies. *Biotechnology Advances*, 24: 452–481.

Zhang, Y-H.P. (2011). What is vital to advance economically competitive biofuels Production. *Process Biochemistry*, 46: 2091–2110.

Zhao C, Ma Z, Shao Q, Li B, Ye J, Peng H. (**2016**). Enzymatic hydrolysis and physiochemical characterization of corn leaf after H-AFEX pretreatment. *Energy Fuels*, 28(7) :4288-4293

Zhao C, Ma Z, Shao Q, Li B, Ye J, Peng H. (**2016**). Enzymatic hydrolysis and physiochemical characterization of corn leaf after H-AFEX pretreatment. *Energy Fuels*, 28(7) :4288–4293

Zhao X, Zhang L, Liu D (2012). Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. *Biofuels, Bioproduct & Biorefining* 10: 1002-1331

Zych D. (2008). The viability of corn cobs as a bioenergy feedstock. *A report of the West Central Research and Outreach Center,* University of Minnesota.

APPENDIX

Appendix I: Sequences of the isolates

> ON679521.1 Xylaria sp. isolate km01
ttgtgaatca tcgaatcttt gaacgcacat tgcgcccatt agtattctag tgggcatgcc tgttcgagcg tcatttcaac cctcaagccc ccgttgcttg gtgttgggag cctacggggt cacccgtagc tcctcaaagt tagtggcgga gtcggttcgc actctagacg tagtagattt tcacctcgcc tgtagtgccg gaccggtccc ctgccgtaaa acacccctta ttccaaaggt gacctcggat catgtagct

> ON678279.1 Nemania sp. isolate km02
gtgaatcatt cgaatctttg aacgcacatt gcgcccatta gtattctagt gggcatgcct gttcgagcgt catttcaacc cttaagccct tgttgcttag cgttgggagc ctacggcacc gtagctcccc aaagtcagtg gcggagtcgg ctcacactct agacgtagta atttctcacc tcgcctatag ttggaccggt cccttgccgt aaaacgccct agttttaaaa ggtgacct

> ON704639.1 Xylaria sp. isolate km03
tttttgtgaa tcatcgaatc tttgaacgca cattgcgccc attagtattc tagtgggcat gcctgttcga gcgtcatttc aaccctcaag cccccgttgc ttggtgttgg gagcctacgg ggtcacccgt agctcctcaa agttagtggc ggagtcggtt cgcactctag acgtagtaga ttttcacctc gcctgtagtg ccggaccggt cccctgccgt aaaacaccc

> ON651648.1 Cyathus sp. isolate km04
tttgtgaatc atcgaatctt tgaacgcacc ttgcgctctt tggtattccg aagagcatgc ctgtttgagt gtcattaaat tctcaaccca ctacatttgt ttatcaagtg aagtgcgtgt tggatgtggg ggttgcgggc ttcatttagt tgaggtcggc tcctttgaaa tgcattagcg ggaatctttg ttgtgccgct tctattgggt gtgataatta tctacgccta ggaaggtggc tgacactagg gtttggcaac aaaccggggt tctgcttcta atcgtccatt cacttggacg taagacatct atacggatga ccgcacg

> ON678260.1 Podoscypha bolleana isolate km05
tttctgtgaa tcatcgaatc tttgaacgca ccttgcactc cttggtattc cgaggagtat gcctgtttga gtgtcatggt attctcaatt ctaataactt ttgttatcgg aattggactt ggaggttttt tgccggtgct tcggcggctg gctcctctta aatgcattag tgtgaatcaa gttacacatt actcagtgtg ataattatct gcgctgtgtt tcaatgttga ctttataagt gttcatgctt ataacccgtc tgttgactca gacactttac tatgaaatct gacctcaaatcatgtagaca cgcagtccct

> ON678280.1 Podoscypha petalodes isolate km06
attcttttgt gaatcatcga atctttgaac gcaccttgca ctccttggta ttccgaggag tatgcctgtt tgagtgtcat ggtattctca attctaataa cttttgttat cggaattgga cttggaggtt ttttgccggt gcttcggcgg ctggctcctc ttaaatgcat tagtgtgaat caagttacac attactcagt gtgataatta tctgcgctgt gtttcaatgt tgactttata agtgttcact gcttataacc cgtctgttga ctcagacacg tatgaac

> MT712199.1 Cerrena unicolor isolate CFCC
gggctatgat ttatggcaga gttgtagctg gccccaatcg ggtatgtgca cactttgttc attccattct catacacctc tgtgcacttt tcataggttt agttatggat gaggcacttt tatagtgtct tggaagtgac tatcctatgt attttacaaa cgcttcagtt ttagaatgtc attcgcgtat aacgcaataa atacaacttt cagcaacgga tctcttggct ctcgcatcga tgaagaacgc agcgaaatgc gataagtaat gtgaattgca gaattcagtg aatcatcgaa tctttgaacg caccttgcgc cccttggtat tccgaggggc atgcctgttt gagtgtcatg gtattctcaa taccctaaat ctttgcggat gagggtgtat tggatttgga ggtttttgca ggcaatattc attgtcagct cctcttaaat acattagcag agatattact gctactctcc agtgtgataa ttgtctacac tgttagtagt gcggtataat caaagtcttt gcttctaatc gtcttcggac aattctttga catctgacct caaatcaggt aggactaccc gctgaactta agcatatcaa aagggggag gaaa

Appendix II: Research approval

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 020-8704150

Internal Memo

FROM: Dean, Graduate School **DATE:** 19th August, 2020

TO: **Mr. Kamande Stephen** **REF:** I56/CTY/PT/38083/2017
C/o Department of Biochemistry, Microbiology & Biotechnology

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

This is to inform you that Graduate School Board, at its meeting on **1st July, 2020,** approved your Research Proposal for the M.Sc. Degree entitled, **"Production of Lignocellulotytic Enzyme by Wild Mushrooms Collected from Karura Forest in Kenya Using Lignocellulose Substrates Under Solid State Fermentation."**

You may now proceed with your Data collection, subject to clearance with the Director General, National Commission for Science, Technology & Innovation.

As you embark on your data collection, please note that you will be required to submit to Graduate School completed Supervision Tracking and Progress Report Forms per semester. The forms are available at the University's Website under Graduate School webpage downloads.

Thank you.

JULIA GITU
FOR: DEAN, GRADUATE SCHOOL

CC: Chairman, Department of Biochemistry, Microbiology & Biotechnology

Supervisors:

1. Dr. George Omwenga
   C/o Biochemistry, Microbiology & Biotechnology
   Kenyatta University

2. Dr. Mathew P. Ngugi
   C/o Biochemistry, Microbiology & Biotechnology
   Kenyatta University

Appendix III: NACOSTI

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY & INNOVATION

Ref No: **985936**

Date of Issue: **13/January/2025**

RESEARCH LICENSE

This is to Certify that Mr.. Stephen Mwaniki Kamande of Kenyatta University, has been licensed to conduct research as per the provision of the Science, Technology and Innovation Act, 2013 (Rev.2014) in Nairobi on the topic: PRODUCTION OF LIGNOCELLULOTYTIC ENZYME BY WILD MUSHROOMS COLLECTED FROM KARURA FOREST IN KENYA USING LIGNOCELLULOSE SUBSTRATES UNDER SOLID STATE FERMENTATION for the period ending : 13/January/2026.

License No: **NACOSTI/P/25/415063**

985936

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

See overleaf for conditions