UTILIZATION OF BANANA PSEUDOSTEM FOR PRODUCTION OF CELLULOLYTIC ENZYMES AND BIOETHANOL

by

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DECLARATION

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

Signature _____

Date _____

DEDICATION

This work is dedicated to my beloved parents; Lekau and Manhlapile and my family; Nyamo, Kamogelo (Marotola), Manhlapile, Lekau and Mafisha for the unconditional love and support throughout the period of this study.

ABSTRACT

In an effort to align the current research with the country's biofuel strategy, the aim of the study was to utilize banana pseudostem in the production of fungal cellulolytic enzymes and bioethanol through fermentation of the banana pseudostem hydrolysate. The selection of microorganisms was based on the ability of the fungi to grow on agar containing Avicel (microcrystalline cellulose) followed by assaying for cellulases in the form of endoglucanase and total cellulase activity. Ten fungal isolates obtained from screening process showed positive endoglucanase activity on carboxymethyl cellulose – Congo Red agar plate. The six fungal isolates selected based on high cellulase activity belonged to *Trichoderma* and *Aspergillus* genera.

In submerged fermentation (SmF), the maximum cellulase and endoglucanase production under optimal conditions by all fungal isolates was achieved in media with an initial of pH 6.5 at 30 °C. Under these conditions, the total cellulase activity was 9.79 filter paper units (FPU)/mL and endoglucanase activity 45.2 U/mL for *Trichoderma longibrachiatum* LMLUL 14-1 and total cellulase activity of 7.7 FPU/mL and endoglucanase activity of 32.7 U/mL for *Trichoderma harzianum* LMLUL 13-5. These cellulase activities were higher than in the crude enzymes system for all *Aspergillus fumigatus*. The production conditions for maximum β-glucosidase varied amongst the *Aspergillus* spp. For example, *Aspergillus fumigatus* LMLUL 13-4 had produced higher β-glucosidase activity in a medium with an initial pH of 6.5 and at an incubation temperature of 30 °C whereas *A*. *fumigatus* LMLUL 13-1 had produced higher β-glucosidase activity at an initial pH of 7.0 and at 35 °C.

Solid state fermentation (SSF) to produce cellulase enzymes system was influenced by temperature, nature of the substrate (i.e. moisture, modification) and culturing technique/strategy (i.e. monoculture versus co-culture). Higher cellulase enzymes system was produced under the conditions of 30 °C, 75% moisture content of untreated (native) BPS and pH 6.5. All the fungi investigated, produced thermo-tolerant and acidophilic cellulase and endoglucanase, whilst β-glucosidase is both acidophilic and alkaliphilic. The cellulase enzymes complex of *T. harzianum* LMLBP07 13-5 is most stable, followed by *A*. *fumigatus* LMLPS 13-4 and the least stable cellulase enzymes complex was for *T*. *longibrachiatum* LMLULSA 14-1.

For the pretreatment of BPS, the material was first subjected to three different pretreatment conditions; namely alkaline (3% NaOH), acid (5% H_2SO_4) and hot water (autoclave method) pretreatment to remove lignin and loosen the cellulose structure. After the pretreatments, alkaline method exposed more cellulose than other pretreatments methods. The alkaline pretreated BPS contained 52.3% cellulose, 10.8% hemicellulose and 8.7% lignin, which is 2.3-fold more cellulose and 0.48-fold less hemicellulose as well as 0.6-fold less lignin to the native BPS.

The enzymatic saccharification of the alkaline pretreated BPS at different substrate loadings at 50 °C for 76 hours by an individual crude cellulase enzymes system from *T*. *longibrachiatum* LMLSAUL 14-1 and *T*. *harzianum* LMLBP07 13-5 cultures were used at a final concentration of 10 FPU/g. Saccharification released maximum glucose of 43.5 g/L and 20.1 g/L form alkaline pretreated BPS by crude cellulase enzymes from *T*. *longibrachiatum* LMLSAUL 14-1 and *T*. *harzianum* LMLBP07 13-5 measured at the highest solid loading.

The production of bioethanol was carried out in separate hydrolysis and fermentation (SHF). Fermentation of nutrient supplemented BPS hydrolysate with an initial pH of 5.0 by *S. cerevisiae* UL01 occurred at 30 °C for 48 hours. The maximum ethanol concentration obtained after fermentation was 17.6 g/L corresponding to ethanol yield of 60% of the maximum theoretical yield. In conclusion, banana pseudostem is a suitable alternative substrate for the production of second-generation bioethanol.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BPS	Banana pseudostem
CF	Co-fermentation
SSF	Solid state fermentation
SmF	Submerged fermentation
CBP	Consolidated bioprocessing
SSCF	Simultaneous sachharification and co-fermentation
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
NaOH	Sodium hydroxide
H_2SO_4	Sulphuric acid
CO_2	Carbon dioxide
H_2O_2	Hydrogen peroxide
NH_3	Ammonia
KOH	Potassium hydroxide
CaOH	Calcium hydroxide
NH_4OH	Ammonium hydroxide
Na_2S	Sodium sulphide
Na_2SO_3	Sodium sulphite
H^+	Hydrogen ion
OH^-	Hydroxyl ion
$MgSO_4$	Magnesium sulphate
KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	Di-potassium hydrogen phosphate
NH_4SO_4	Ammonium sulphate
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
$Fe.SO_4.7H_2O$	Ferrous sulphate heptahydrate
KCl	Potassium chloride
$Na_3C_6H_5O_7$	Sodium citrate
H_2O	Water
$C_2H_3NaO_2$	Sodium acetate
YNB	Yeast nitrogen base

GC	Gas chromatography
HPLC	High performance liquid chromatography
FTIR	Fourie transform infrared spectroscopy
FID	Flame ionization detector
RID	Refractive index detector
Endo	Inner
Exo	Outer
Rpm	Revolution per minute
EGs	Endoglucanases
CBHs	Cellobiohydrolases
BGs	β-glucosidases
GHs	Glycoside hydrolases
CAzy	Carbohydrate-active enzyme
CBD	Catalytic binding domain
DNS	3,5-Dinitrosalicyclc acid
pNPG	ρ-nitrophenyl β-D-glucopyranoside
LiPS	Lignin peroxidase
GLOX	Glyoxal oxidase
AAO	Aryl alcohol oxidase
MnPs	Manganese peroxidase
FPA	Filter paper activity
FPU	Filter paper unit
U	Unit
CMCase	Carboxy methyl cellulase
FPase	Filter-paperase
HMF	5-hydroxymethyl-2-furaldehyde
DNA	Deoxyribonucleic acid
ITS	Internal transcribed spacer
PCR	Polymerase chain reaction
<	Less
>	More
B	Beta
µL	Microlitre

G	Gram
Mg	Microgram
Mm	Micrometre
Mg	Miligram
mL	Mililitre
L	Litre
°	Degrees centigrade
%	Percentage
Fig.	Figure
w/v	Weight per volume
v/v	Volume per volume
EtOH	Ethanol
DP	Degree of polymerization
GHG	Greenhouse gas
PM	Particulate matter

CHAPTER 1

GENERAL INTRODUCTION

Tapping into valuable agricultural wastes from the existing farmland might preserve the rich diversity of plants considered to be important and desirable from being destroyed by Man's attempts to produce biofuel.

1.1. Overview

The South African government, through the Department of Energy (DoE), has launched a quest for cleaner (or green) renewable and sustainable energy. Such renewable energy would include energy from biomass, wind, solar and small-scale hydro energy plants (DoE, 2015). The principal goal in developing renewable energy is to mitigate the climate change effect, for energy security and rural development (GBEP, 2008). In an effort to support this process, the government granted a fuel levy tax reduction of 30% for biofuels (BFAP, 2005).

Biofuels include bioethanol, biodiesel and biogas which are all derived from the biochemical interaction of microorganisms and organic matter (Gasparatos *et al*., 2013). These biofuels are classified as first, second and third generation technology (Lee & Lavoie, 2013; Nigam & Singh, 2011): The first generation biofuels are produced from food biomass such as starch, sugar and oil crops while those of the second generation are produced from a variety of non–food lignocellulosic biomass and municipal solid wastes. The third generation on the other hand are produced from micro-algae and seaweeds.

The production of first generation biofuels is in an advanced state with the processing and production technologies well understood. Some of the emerging countries like Brazil, China, Thailand, and Colombia etc. have successfully implemented strategies on first generation biofuel industry (Eisentraut, 2010). The leading countries in the first generation technology for biofuel production are the United State of America (USA) and Brazil, with production volumes, increased at a steady pace since 2007 http://www.ethanolrfa.org/resources/industry/statistics/world/

The state of bioethanol production in S.A., particularly bioethanol dates back to the 1920s when ethanol derived from sugarcane was mixed with petrol until the 1960s due to low international crude oil prices (DoME, 2007). Between the 1970s and 1990 S.A. developed the Fischer-Tropsch process to convert both coal and natural gas to petroleum as a fuel source in response to sanctions put on the apartheid government (Winkler, 2006). Since 2008, the government of S.A. has adopted the biofuel industry strategy which envisages the production of bioethanol at a quantity of about 400 million litres, i.e. 2% of the total national fuel consumption (Fundira & Henley, 2017). The selected feedstock for the production includes sugarcane and sugar beet for the bioethanol, and sunflower, canola and soya, for biodiesel. However, based on DoE (2013), grain sorghum and sugarcane have been identified as the most appropriate commercial crops for bioethanol due to the fact that large volumes of these crops are already grown nationally and sufficient experience exists to allow for expansion of those crops production.

Currently, bioethanol is more costly as compared to the cost of fossil fuel simply because large scale production of bioethanol is based on food crops such as sugarcane and maize (corn), (Karmee, 2016). Therefore, there is a demand for alternative, untapped and abundant renewable biomass in the development of bioethanol. Lignocellulosic biomass presents an opportunity especially for Africa to intensify investment and also attract investments for research and development of renewable energy from non-food biomass for bioethanol production. Lignocellulosic biomass consists of three polymeric compounds: cellulose entrapped in hemicellulose and lignin. According to Nigam and Singh (2011), biofuels produced from lignocellulosic biomass also have the advantage of generating low GHG in the environment.

Second-generation bioethanol production process requires the production of cellulases or the availability of cellulases, pretreatment of lignocellulosic biomass, saccharification (hydrolysis) of biomass, fermentation and distillation. Cellulases are increasingly becoming important in the biofuel industry (Yan & Wu, 2013). Cellulases, namely exoglucanases, endoglucanases and β-glucosidase hydrolyse β-1,4 glycosidic linkages in the cellulose chains. These enzymes are synthesised and secreted by numerous microorganisms including fungi, bacteria, actinomycetes etc.

The efficiency of enzymes secretion and composition is different and diverse from one microorganism to another, even at the strain level (Jayasekara & Ratnayake, 2019). Cellulases are secreted either as free extracellular or cell surface bound enzymes.

The synthesis of cellulases in *Trichoderma* is inducible and it can be affected by various carbon sources such as cellulose, lactose, cellobiose 7A, 8A, 9A and in the presence of 5% glycerol, a catabolite repressor. In the presence of glycerol as carbon source, the levels of the enzymes are reduced (Montenecourt *et al*., 1981). The control of cellulase production in filamentous fungi is beyond the transcription and translation processes of the structural genes. The hypersynthetic capacity and the greater ability of the microorganism to transport the proteins to the external environment will cause overproduction of cellulases (Montenecourt *et al*., 1981).

The information about the mechanism of secretion of proteins in filamentous fungi is steadily becoming available. The proposed secretory pathway of proteins in animal cells involves a series of processing steps; glycosylation, packaging into secretory vesicles, movement to the site of exportation and release to the external environment. Fungi lack Golgi apparatus – subcellular structures essential for processing extracellular proteins in mammalian cells. The absence of Golgi in fungi has necessitated the need to identify the processing structures and sequences of events involved in the protein synthesis and transportation to the external environment (Montenecourt *et al*., 1981). Gosh *et al*. (1982) revealed a Golgi-like apparatus in fungi which do not have a stacked appearance similar to the eukaryotes and some fungi showed many individual endoplasmic reticulum associated saccules. A thorough knowledge of secretory pathway of cellulases will assis in the selection of organisms which efficiently secrete cellulases, and selection of organisms whose secretory pathways have less frequent mutations. Further information on secretory pathways of cellulases in different organisms is reviewed by Yan & Wu (2013).

The S.A. government has endorsed the development and use of second generation biofuel technologies (DoME, 2006). Due to constraints related to additional availability of land, the second generation biofuel industries should focus on agricultural and forestry residues as an available source of lignocellulosic biomass without a need for additional land cultivation (Eisentraut, 2010). South Africa's

agriculture and forestry industry generate a substantial amount of lignocellulosic residues such as maize stover, sugarcane bagasse, wheat straw, saw mill and paper mill sludge (Lynd *et al*., 2003). The utilisation of these residues by the biofuel industry in the production of bioethanol would lessen the current constrains on the use of food-biomass. The choice of raw material depends on the location and availability of the feedstock, among other factors. Globally, many countries including S.A. have embarked on research to study the utilisation of lignocellulosic materials for the production of secondary generation biofuels (Parawira & Tekere, 2011).

In order to meet or exceed the required target for bioethanol, South Africa has taken advantage of the established agricultural sectors such as sugar, sweet sorghum, etc. These agricultural sectors generate massive waste during the processing of crops for various uses. For instance, the sugar sector produces approximately 27 million tons of sugarcane. For every 100 tons of sugarcane 13 tons and 15 tons of sugarcane bagasse are produced. Bagasse is a waste product containing starch and can be used to produce bioethanol (BFAP, 2005). However, the conversion of bagasse to ethanol ratio is not high. Thus, about 15 tons of bagasse could produce around 3.4 tons of ethanol. Therefore, bagasse yields a lower percentage of ethanol than sugar, i.e. 5.7 tons of ethanol (BFAP, 2005).

Over a decade ago, South Africa's sorghum industry with a marginal increase in the land used for plantation of sorghum has experienced a great increase in the total quantity that is produced/or harvested. About 340 000 tons of sorghum have been harvested from 120 000 hectares of land during planting season of 2003/2004. There are three types of products resulting from sorghum processing, which can be used for bioethanol production. Such products include grains, sugar juice extract and the bagasse (BFAP, 2005).

Bananas are tropical plants that are grown under sub-optimal, sub-tropical conditions in South Africa and are available throughout the year (DAFF, 2011). Bananas and plantains are staple diets in other parts of Africa. The banana plant is herbaceous of the genus *Musa* and cultivated for mainly fruit production. There are about 25 – 80 species of the genus Musa (Tock, *et al*., 2010). However, during harvesting and post-harvesting of the banana fruit, a massive waste (banana biomass) is produced in the form of leaves, rachis, pseudostem, rejected banana fruits and peels. This

banana biomass can be classified as amylaceous (i.e. banana pulp and banana fruit) and lignocellulosic biomass such as leaves, rachis, and pseudostem (Velásquez-Arrendondo *et al*., 2010; Tock *et al*., 2010). Approximately 80% of the banana biomass generated is made up of banana pseudostem (Tock, *et al*., 2010). According to Santa-Maria *et al*. (2013) little research efforts have been undertaken in the bioconversion of banana biomass into ethanol (Santa-Maria *et al*., 2013).

In the quest to explore the alternative available natural resources in S.A., and to diversify the feedstock for the production of bioethanol, the current study had identified banana pseudostem to be an attractive and a potential biomass due to its reported high holocellulose and low lignin composition (Li *et al*., 2010; Abdul Khalil *et al*., 2006). Holocellulose refers to the sum of cellulose and hemicellulose present in the biomass. Low lignin of the biomass is believed to require less severe processing, also called pretreatment. The availability and abundance of banana pseudostem in S.A will be discussed under the literature review.

The view of this study is that the use of banana pseudostem would benefit both the biofuel and agriculture industry if the technology becomes successful and does not negatively impact on the food (fruit) chain for the following reasons: (a) Eventually the production of bioethanol will also boost the profit of the farmers, including the local small household production. (b) It will create additional jobs in the farming sector to improve the social well-being of the neighbouring communities in Limpopo and other provinces. (c) The focus on banana pseudostem will ensure the preservation of the biodiversity (plant diversity) while aiming at reducing greenhouse gas (GHG) emissions. (d) The banana pseudostem is a renewable, under-utilised agricultural waste that is available throughout the year and its cost is likely to be relatively low. (e) The use of pseudostem would contribute additional volumes of bioethanol to the country's production volume.

It is a known fact that the geographic location of the Cavendish cultivar of *M acuminate* or any other *Musa* species could possibly influence the chemical compositions of lignocellulose content in the banana pseudostem. For that reason, only evaluate the potential of the banana pseudostem from the Cavendish cultivar of *Musa acuminate* grown at Allesbeste farm in the area of Tzaneen in Limpopo suitability as a feedstock for bioethanol production will be evaluated.

Unlike the first generation of technology, which is relatively uncomplicated, the second generation of technology has challenges. The main challenge of the second generation technology for bioethanol production is the complexity and recalcitrant nature of the lignocellulosic biomass during processing steps such as pretreatment and hydrolysis (Balan, 2014; Nigam & Singh, 2011).

1.2. Research hypothesis

The hypothesis of this study is that through the selection of fungal isolates that inhabit and are adapted to banana plantation site or to related herbaceous plant, a potential fungal isolate(s) that produce cellulases that effectively hydrolyse banana pseudostem to fermentable sugars can be obtained, leading to the successful production of ethanol

1.3. Aim and objectives of the research

The aim of the study is to utilize banana pseudostem in the production of fungal cellulolytic enzymes and ethanol through fermentation of the banana pseudostem hydrolysate.

Specific objectives were:

i. To screen and select for fungi that produce cellulase and to optimise the production conditions in submerged fermentation.
ii. To produce cellulase using banana pseudostem as a substrate in solid state fermentation and optimise the conditions.
iii. To evaluate the efficiency of different pretreatment methods such as hot-water, alkaline and acid pretreatments in increasing the susceptibility of banana pseudostem to hydrolysis.
iv. To hydrolyse the pretreated banana pseudostem with the “in-house” produced cellulases.
v. To produce ethanol from banana pseudostem hydrolysate using separate hydrolysis and fermentation.

CHAPTER 2

LITERATURE REVIEW

2.1. Banana production

The banana plant is a large annual herbaceous flowering plant of the genus *Musa* (Fig. 2.1). It is a tall arborescent monocotyledon with a false stem (pseudostem) consisting of leaf sheaths and underground true stem (corm) that is able to produce suckers through vegetative reproduction (Mukhopadhyay *et al*., 2008). The pseudostem is a clustered cylindrical aggregation of leaf stalk bases, Fig. 2.1 (Tock *et al*., 2010; Mukhopadhyay *et al*., 2008).

Figure 2.1. The Banana plant (Source: image is taken by the author at Allesbeste farm)

The plant is native to India and eastern Asia (Malaysia and Japan) and some varieties are genetically linked with other varieties from Africa (Nelson *et al*., 2006). Banana is cultivated over 130 countries along the tropical and subtropical region of

Capricorn (Mohapatra *et al*., 2010). According to the Agri-food business development centre, S.A was ranked 30th country in the world in banana production (DARD, 2016). Over the last years, banana the second largest fruit produced globally has contributed approximately 16% of the world's total fruit production (FAO, 2009). In the developing world, banana is the fourth most important food after rice, wheat and maize (INIBAP, 2000).

South Africa is a relatively small banana grower in terms of global hectares. Banana and other subtropical fruits are cultivated mainly in three provinces; Mpumalanga, Limpopo and Kwa-Zulu Natal. Based on the annual average quantity produced during the period 2015/2016, the country produced 401400 tons of bananas as shown in Table 2.1 (DAFF, 2016; DAFF, 2015).

Table 2.1. The production of subtropical fruit from 2011/12 to 2015/16 (DAFF, 2016).

Fruit types	**Annual seasons in tons**				
	2011/12	2012/13	2013/14	2014/15	2015/16
Avocados	88100	87000	97700	98200	82800
Bananas	371300	392300	463400	424400	401400
Pineapples	108700	96800	96700	95800	98900
Mangoes	65100	52600	57600	75700	41000
Papayas	12700	14900	13700	15900	11100
Granadillas	0.500	0.800	0.700	0.700	0.700
Litchis	7800	5600	8300	8300	7500
Guavas	23700	33600	31600	31900	30200

The main banana cultivar grown in Limpopo province is "Pisang Awak", ABB genome (70%), followed by "Cavendish", AAA genome, (30%) of *Musa acuminate* (De Beer & Sigawa, 2010). The banana plant requires 10 – 14 months depending on the geographic climate condition and soil type for it to bear fruit (Chaurasia *et al*., 2017). It bears banana fruit once in its life cycle and for every cycle of production; four times of wastes are generated. Banana wastes range from rotten fruit, peels, fruit-bunch stem, leaves, pseudostem and rhizome (Abdullah *et al*., 2014). After the harvesting of the banana fruit, banana pseudostem becomes available in abundance

as waste which is either left to rot at the local dumpsite and pollute the surrounding environment or left to decompose at the plantation to serve as organic soil fertiliser Fig. 2.2.

Figure 2.1. A - Fresh banana pseudostem after the harvest of banana fruit in banana plantation at the farm, Allesbeste in Tzaneen area, Limpopo, South Africa. B - Decomposing banana pseudostem.

The common practices regarding management of banana waste generated by local communities in the villages are that banana wastes are discarded into rivers, lake and near by the road thereby causing environmental problems, Fig. 2.3.

Figure 2. 2. Disposal of banana pseudostem after the harvest of banana fruit by the local community in Mankweng area, Limpopo, South Africa. A - Fresh cut pseudostem. B - Old sun-dried pseudostem.

The generated banana waste is approximately 88% of the whole banana plant (Low *et al*., 2015; Elanthikkal *et al*., 2010) and about 60 – 80 t/ha is made of banana pseudostem alone (Meena *et al*., 2015). The banana waste has the potential to be utilised as biomass for biofuel production and other metabolites such as enzymes. Bananas are available throughout the seasons of the year and this, in turn, implies that the banana pseudo-stem will be available all year round. An alternative environmental remediation of agricultural wastes, particularly banana pseudostem is to produce bioethanol (Kannahi & Megala, 2017).

2.2. Chemical composition of banana pseudostem

The moisture content of fresh banana pseudostem is about 94 – 96 % (Farjana Begum *et al*., 2014 – 2015; Li *et al*., 2010). The pseudostem is a lignocellulosic material made up of cellulose, hemicellulose and lignin as the major components. The cellulose content is 31 – 44%, hemicellulose 12 – 33% and lignin 6 – 14%, Table 2.2. The chemical composition of BPS shows higher holocellulose (cellulose + hemicellulose) content more than other agricultural residues such as rice straw, wheat straw, corn stover etc. that are used for bioethanol production and it also contains low lignin compared to woody biomass as shown in Table 2.2. The amount

of each polymer in each plant is variable in different portions of the same plant and may be influenced by the cultivar age of a plant and the geographic location of cultivation (Jahn *et al*., 2011).

Table 2.1. Chemical composition of lignocellulosic biomass (adapted from Sun and Cheng 2002).

Plant biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40 – 55	25 – 40	18 – 25
Softwood stems	45 – 50	25 – 35	23 – 35
Nut shells	25 – 30	25 – 30	30 -40
Corn cobs	45	35	15
Grasses	25 – 40	35 – 50	10 – 30
Paper	85 – 99	0	0 – 15
Wheat straws	30	50	15
Sorted refuse	60	20	20
Leaves	15 – 20	80 – 85	0
Cotton seed hairs	80 – 95	5 – 20	0
News papers	40 – 50	25 – 40	18 – 30
Waste paper from chemical pulps	60 – 70	10 – 20	5 – 10
Solid cattle manure	1.6 – 4.7	1.4 – 3.3	2.7 – 5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12
Swine waste	6.0	28	N/A
Primary wastewater solids	8 – 15	N/A	24 – 29
***Banana pseudostem**	31 – 44	12 – 33	6 – 13

*Souza *et al*., 2017; Li *et al*., 2016; Farjana Begum *et al*., 2014 – 2015; Gabhane *et al*., 2014; Thakur *et al*., 2013; Li *et al*., 2010; Bilba *et al*., 2007; Oliveira *et al*., 2007; Cordeiro *et al*., 2004

2.3. Applications of banana Pseudostem

Banana by-products such as pseudostem, leaves, inflorescence, fruit stalk/rachis *etc*. are undervalued and often regarded as agricultural waste (Padam *et al*., 2012). The overall waste generated by a single plant can make up approximately 80% of the total plant mass (Shah *et al*., 2005). A huge portion of the waste is made up of the pseudostem. Some of the pseudostems generated during harvest are left to rot in the plantation site to replenish some of the nutrients in the soil (Kennedy 2009), whereas most of the pseudostem are dumped at the nearby site (dump site) and left unutilised.

Given the need to continuously create and invent new products with value added applications in various industries, some researchers have paved a new and alternative approach to develop bio-products by recycling banana wastes (Padam *et al*., 2012). The pseudostem has application in the making of ropes, crafts, textile, paper and boards (Abdullah *et al*., 2014; Uma *et al*., 2005). The pseudostem fibers are also used to reinforce epoxy composite and the fibers increased the tensile strength of the epoxy by 40% (Maleque *et al*., 2005). Paul, (2015, Ph.D. thesis) used the sap from banana pseudostem as a precursor to develop bio-resin using pseudostem fibers as the reinforcement and thus forming a biodegradable bio-composite for the automotive industry, e.g. Bumper. The pseudostem fibers are comparable in their physical strength and cellulose content to the other fibrous by-products (Uma *et al*., 2005).

In the textile industry, banana pseudostem fibers were used for making traditional handcrafts and clothes (Kennedy, 2009). In the paper and pulp industry, pseudostem fibers from *Musa acuminate* colla cv. *cavendish* was found to have a high burst index and breaking length, alone or in combination with other common pulps (Cordeiro *et al*., 2005). In making greaseproof paper (Marella *et al*., 2014; Goswami *et al*., 2005), the pseudostem pulp from *Musa parasidica* L. showed increased burst index, tensile index, tear index and oil resistibility when compared with bamboo pulp (Goswami *et al*., 2005).

Heavy metals (lead, chromium, cadmium, mercury and zinc) pose a health threat to humans because the availability of these metals in wastewater might contaminate

drinking water systems, boreholes, dams *etc*. (Metcheva *et al*., 2010). Cleaning an environment contaminated with heavy metals is a costly exercise and therefore, cheaper alternative absorbers are required (Padam *et al*., 2012). *Musa species* have been used to generate the fibres that are used for bioremediation and as natural water purifiers (Uma *et al*., 2005). Noeline *et al*. (2005) have found that formaldehyde polymerised banana pseudostem is an effective absorber in cleaning lead (II) in a batch reactor, while a carboxylated banana pseudostem have been reported to be a good mercury (II) absorber (Anirudhan *et al*., 2007). Becker *et al*. (2013) used banana pseudostem as an adsorbent to remove chromium (Cr IV) from aqueous solution. The fibres extracted from banana pseudostem, stalk and leaves were used to remove acid green dye from aqueous solution and the fibers possessed adsorption capacity of 8 to 18 mg/g (Karim *et al*., 2016).

Recently, there has been a growing interest in using banana agro-waste in enzyme and biofuel production in order to generate energy. Dabhi *et al*. (2014) have used banana pseudostem as a substrate to produce cellulolytic enzymes under solid state fermentation by a bacterial consortium and other enzyme production by *Aspergillus niger* (Bhavsar & Bhalerao, 2012) and *Pleurotus* species, namely *P*. *ostreatus* and *P*. *sajor-caju* (Reddy *et al*., 2003). Banana pseudostem was also used for biogas production (Li *et al*., 2016; Pei *et al*., 2014). Several studies have also demonstrated the potential use of banana pseudostem as a substrate in the production of bioethanol (Guerrero *et al*. 2018; Souza *et al*., 2014; Souza *et al*., 2013; Filho *et al*., 2013; Reddy *et al*., 2011; Reddy *et al*., 2009). Souza *et al*. (2014) found that the acid (2% H_2SO_4) hydrolysis of pseudostem can efficiently release glucose. However, the inhibitory compound such as 5-hydroxymethyl-2-furaldehyde (HMF) formed during the process can result in low ethanol level. It is thus imperative to optimise saccharification and fermentation processes to obtain high ethanol level (Souza *et al*., 2014). The focus should be on the development of efficient enzymatic cocktail formulations to achieve maximum saccharification and the use of more robust yeast strains the tolerate inhibitors to increase the ethanol yield (Guerrero *et al*., 2018)

2.4. Lignocellulosic biomass

Lignocellulose cell walls are comprised of cellulose, hemicellulose, and lignin in varying proportions. However, this ratio differs amongst the type of plants, such as

hardwood, softwood and herbaceous plants. In addition to the three major components, lignocellulose contains a small amount of pectin, nitrogenous compounds and ash (Chen, 2014). In plant cell walls, the cellulose micro-fibrils are encrusted in lignin and hemicellulose in a complex architecture as illustrated in Fig. 2.4 (Mussatto & Teiseira, 2010). This, together with crystallinity of cellulose makes untreated cellulose biomass recalcitrant to enzymatic hydrolysis in order to release fermentable sugars (Wu *et al*., 2011a; Sánchez, 2009).

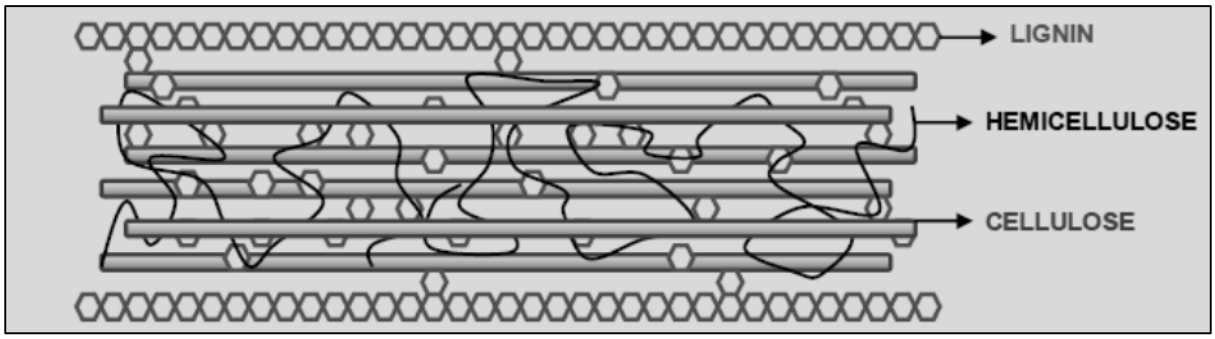

Figure 2.4. A schematic of lignocellulosic plant cell wall structure whereby the cylinders correspond to cellulose fiber bundles that are surrounded by hemicellulose- the darker bold lines and the rings represent lignin (Mussatto & Teiseira, 2010).

2.4.1. Cellulose structure

Cellulose is a fibrous, tough water insoluble substance which is found in the protective cell wall of plants, particularly in stalks, stems, trunks and all woody portions of plant tissues (O'sullivan, 1997). Cellulose is a linear polymer of glucose units that are linked by β-1,4-glycosidic bonds to form cellobiose. This is depicted by its empirical formula $(C_6H_{10}O_5)_n$ and it's chemical structural illustrated in Fig. 2.5 (Sandgren *et al*., 2005).

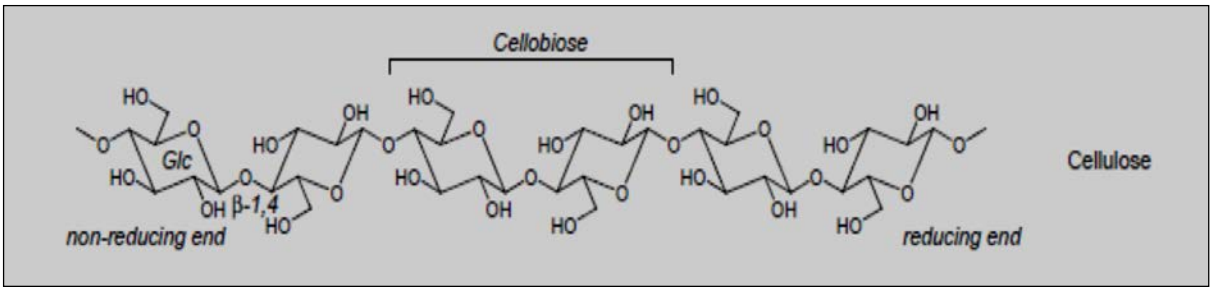

Figure 2.3. Chemical structure of cellulose (Sandgren *et al*., 2005).

The end of the glucan chain with anomeric carbon that is not linked to ~~next~~ glucose is referred to as reducing end of the polymer. The other end of the polymer is called non-reducing end (Sandgren *et al*., 2005). The length of the cellulose chains, i.e. the degree of polymerisation (DP) varies from 2000 glucose units or even less units in the primary wall and up to 15000 or more units in the secondary walls (Quiroz-Castaneda & Folch-Mallol, 2013; Sandgren *et al*., 2005; Cowling, 1958). The molecules of cellulose are bound by hydrogen bonds or van der Waals forces as depicted in Fig. 2.6 (Sánchez, 2009; Cowling, 1958), which hold cellulose into microfibrils form. This hydrogen bonding within the cellulose chains may act to determine the "straightness" of the chain. Inter-chain hydrogen bonds might introduce order or disorder into the structure depending on the bonds regularity (Sánchez, 2009; O'sullivan, 1997).

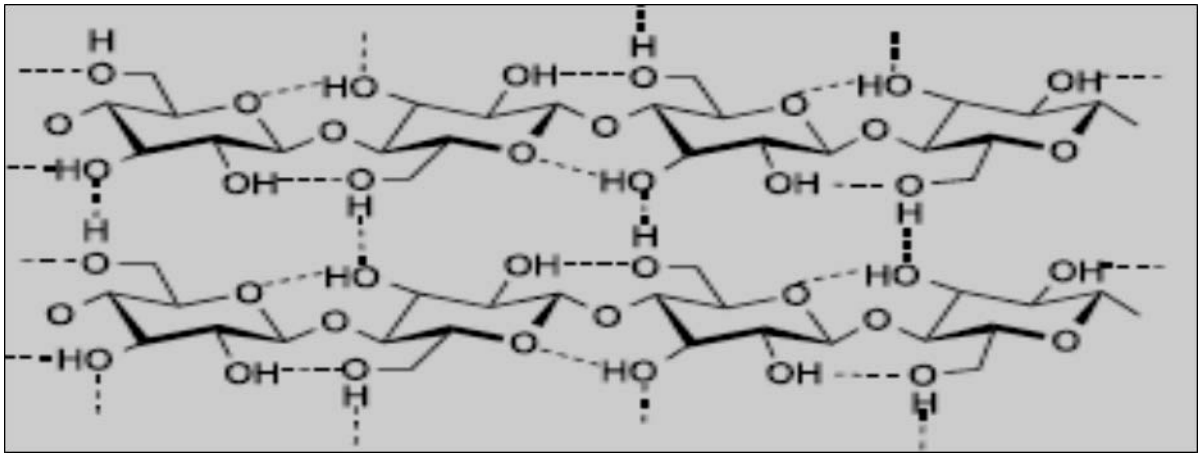

Figure 2.6. Cellulose chains held together by hydrogen bonds (Sánchez, 2009).

Chain regions that are containing highly orientated (ordered) are called crystalline and those regions that are less ordered are called amorphous as shown in Fig. 2.7. These regions are interspersed along the cellulose chains (Lynd *et al*., 2002; Cowling, 1958).

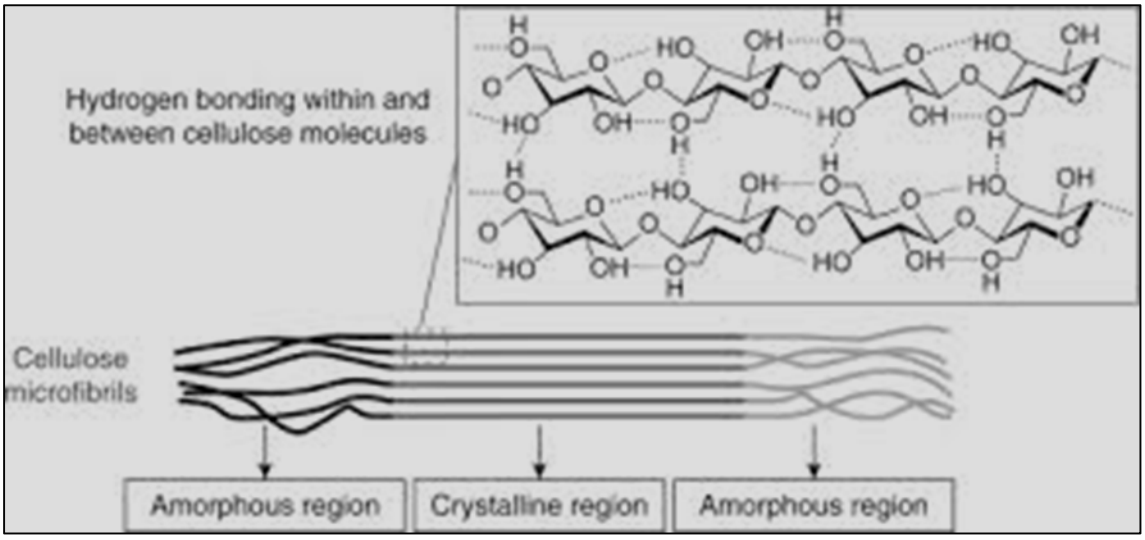

Figure 2.7. Homopolymer structure of crystalline and amorphous cellulose (https://www.sciencedirect.com/topics/chemistry/homopolysaccharide/31/Jan/2019).

The crystalline nature of cellulose implies a structural order in which all of the atoms are fixed in discrete positions with respect to one another. When individual micro-fibrils are tightly packed, it prevents the penetration by enzymes including the small molecules of water (Lynd *et al*., 2002). Twists and bends in the cellulose fibrils prevent an orderly arrangement in the amorphous regions (Quiroz-Castaneda & Folch-Mallol, 2013).

2.4.2. Hemicellulose structure

Hemicellulose is the second most abundant renewable source that can be converted to useful end products (Satyanarayana *et al*., 2012). Hemicelluloses are heterogeneous polymers which are easily hydrolysed by acids to their monomeric components made up of pentoses (D-xylose and D-arabinose), hexoses (D-glucose, D-mannose and D-galactose) and sugar acids. (Pérez *et al*., 2002; Sjöholm *et al*., 2000; Sjostrom, 1993). Hemicellulose found in hardwood differs from hemicellulose in softwood. Hardwood contains mainly glucuronoxylan while softwood contains mostly glucomannans (Chen, 2014; Kumar *et al*., 2008; Pérez *et al*., 2002; Jeffries, 1994). Hemicelluloses are classified according to the main sugar residue in the backbone, for example, xylans, mannans, and glucans with xylans and mannans being the most prevalent (Saha, 2003; Pérez *et al*., 2002). Xylan is the principal component of hemicellulose in most plant cell walls comprising about 1/3 of the total

plant biomass; it has a β-1-4-D-xylopyranose backbone with a variety of side chains (Prade, 1996). The composition and linkages of the side chains determine the specific type of xylan (Gielkens *et al*., 1997). Xylan types include linear homoxylan, arabinoxylan, glucuronoxylan and glucunoarabinoxylan (Saha, 2003). The exact chemical composition and structural features of hemicellulose differ across plant species, subcellular location and developmental stage of a plant (Saha, 2003).

Grasses (graminaceous plants) containing a high level of α-L-arabinofuranosides and acetyl groups linked to β-1-4-D-xylan backbone by α-(1-2,3) linkages, are termed arabinoxylans. The esterified acetyl groups are attached to the hydroxyl group of carbon 2 or carbon 3. The hardwood (Angiosperm) xylans are highly substituted with 4-*O*-methyl-glucuronic acid and acetyl groups, hence are called glucuronoxylans, Fig. 2.8a. The 4-*O*-methyl-glucuronic acid is linked to the xylan backbone by α-(1-2) glycosidic bonds while the ester linked acetyl groups are attached to hydroxyl groups of carbon 2 or 3 (Pérez *et al*., 2002; Sjöholm *et al*., 2000; Jeffries, 1994). In contrast, the softwood (Gymnosperm) xylans are not acetylated, but the xylan backbone is linked at carbon 2 with 4-*O*-methyl-α-D-glucuronic acid and carbon 3 with α-L-arabinofuranosyl moiety. In softwood, hemicellulose is dominated by galactoglucomannan making up 15 – 20% of dry weight and with xylan constituting about 7 – 10% of the biomass dry weight (Sjöholm *et al*., 2000). The softwood glucomannan has galactose side branch linked by α-(1-6) linkages to the main mannose chain, Fig. 2.8b (Pérez *et al*., 2002; Jeffries, 1994).

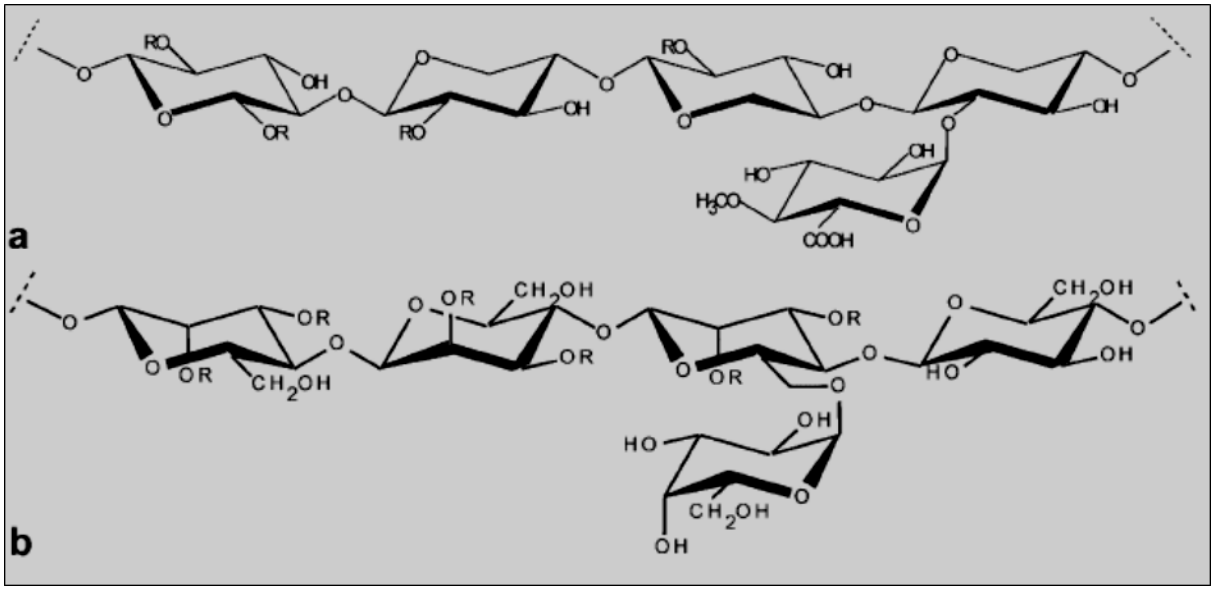

Figure 2.4. (a) A structure of O-acetyl-4O-methylglucuronoxylan from angiosperms. (b) A structure of O-acetyl-galactoglucomanna from gymnosperms (Perez *et al*., 2002).

2.4.3. Lignin and its structure

Lignin is the third plant polymer and provides the flexibility and strength required ~~for~~ by plants. Lignin is aromatic and hydrophobic polymer synthesised from one, two, or three different phenyl-propanoids namely ρ-coumaryl alcohol (ρ-hydroxyphenyl propanol), sinapyl alcohol (syringyl propanol) and coniferyl alcohol (quaiacyl propanol), as shown in Fig. 2.9 (Boerjan *et al*., 2003), which are singly derived from the amino acid phenyalamine through the enzymatic process (Leisola *et al*., 2012; Jeffries, 1994). These phenyl-propane units are linked to each other by irregular coupling of C-C and C-O (Chen, 2014). The proportions of the three structural monomers vary in different families of plants (Chen, 2014; Leisola *et al*., 2012).

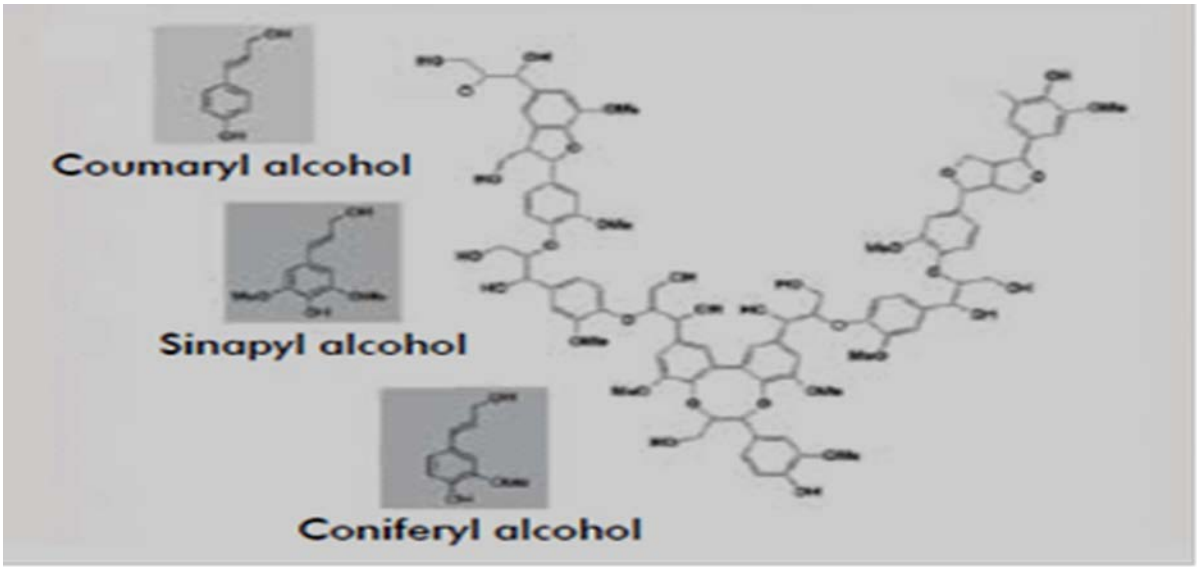

Figure 2.5. Structure of lignin formed by the polymerization of the three phenolic alcohols, (Boerjan *et al*., 2003).

In softwood, the principal constituent of lignin is coniferyl alcohol, whereas the lignin of hardwood is composed of coniferyl and sinapyl alcohols (Chen, 2014; Perez *et al*., 2002; Jeffries, 1994). Grass lignin contains all the three phenolic alcohols. The lignin inter-monomer linkages are similar in all softwoods, hardwoods and grasses (Jeffries, 1994). The polymerization of phenolic alcohols produces a heterogeneous structure as shown in Fig. 2.9, whose basic units are linked by C-C and aryl-ether linkages with aryl-glycerol β-aryl ether being the predominant structure (Perez *et al*., 2002).

2.5. Lignocellulosic biomass processing

Current advances in industrial biotechnology offer great opportunities for economic use of agricultural and industrial residues, which are an abundant source of lignocellulose material (Yousuf, 2012). The residual plant biomass considered as waste can be converted into various value added products including biofuels, chemicals, improved animal feeds and human nutrients (Howard *et al*., 2003). However, the inherent structure of lignocellulosic biomass; i.e. cellulose, hemicellulose and lignin intertwined into a tight network present a major challenge in the processing (hydrolysis) of such biomass to fermentable sugars (Dekker, 2016; Yousuf, 2012).

The presence of lignin, an aromatic biopolymer, acts as a barrier by shielding the polysaccharides from microbial attack and enzymatic (cellulase and hemicellulase) accessibility to the carbohydrates (Dekker, 2016). Therefore, pretreatment is necessary for the polysaccharide components to become accessible to hydrolytic enzymes, which will yield monosaccharide sugars, mainly glucose and xylose (Dekker, 2016). The term pretreatment refers to a process of inducing a structural change in lignocellulosic biomass from its native (original) form, which is recalcitrant to enzymatic hydrolysis, into a form that is prone to hydrolysis (Lynd *et al*., 2002).

Lignocellulosic biomass is processed for ethanol production through three key stages (Balat *et al*., 2008), namely: (1) Removal of lignin to expose hemicellulose and cellulose prior to hydrolysis. (2) Hydrolysis of cellulose and hemicellulose to produce sugars which includes glucose, xylose, arabinose, pentose, galactose and mannose. (3) Fermentation of sugars to ethanol.

2.5.1. Pretreatment of lignocellulosic biomass

Pretreatment methods are reported to have improved the digestibility of lignocellulosic biomass such as perenial grass and agricultural wastes. For example, Wu *et al*. (2011a) had attributed the improvement in the enzymatic digestibility of the alkali pretreated sugarcane stem (bagasse) to the disruption of carbohydrate-lignin complex. The significance of pretreatment in cellulosic bioethanol production has been realised and many of the pretreatment strategies have been developed to enhance the reactivity of cellulose and to increase the yield of sugar monomers, i.e. glucose and xylose (Brodeur *et al*., 2011). The ultimate goal of pretreatment methods is to improve the rate of enzymatic hydrolysis and increase yields of fermentable sugars from cellulose by altering or removing structural barriers (Dekker, 2016; Ding *et al*., 2012). The pretreated biomass is more easily hydrolysed than the raw biomass even though the amount of the lignin content in both materials was approximately the same (Agbor *et al*., 2011). Hemicellulose is amorphous and more easily hydrolysed than cellulose. The structural features of cellulose such as the degree of crystallinity, DP and the surface area are known to limit accessibility to enzymes and affect the rate of enzymatic hydrolysis of cellulose (Dekker *et al*., 2016).

It has been noted that not all the pretreatment efforts achieve substantial delignification (lignin removal); the structure of lignin may be altered without removal due to changes in chemical properties of lignin (Agbor *et al*., 2011). Delignification causes swelling in biomass, disrupts the lignin structure, increases cellulose internal surface area and accessibility of cellulose fibers to cellulolytic enzymes (Agbor *et al*., 2011). The extent of delignification is considered as a key indicator for the selection of the pretreatment conditions required for efficient hydrolysis of sugarcane bagasse (Wu *et al*., 2011a). Figure 2.10 illustrates the structure of lignin (outer thick black lines) surrounding the cellulose microfibrils (inner semi-straight lines) and hemicellulose (irregular lines coiled around the cellulose). Upon pretreatment, the lignin seal is broken and the oligosaccharides from either hemicellulose or cellulose are dissolved and cellulose is partially disrupted (Hsu *et al*., 1980).

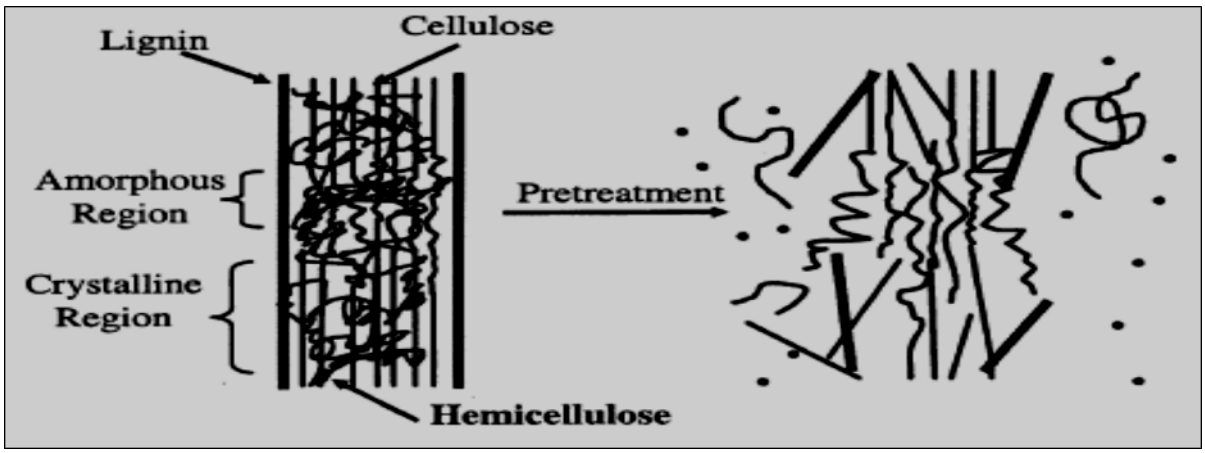

Figure 2.10. Schematic representation of the effect of pretreatment on cellulose structure (adapted from Hsu et al., 1980).

According to some authors (Brodeur *et al*., 2011; Kumar *et al*., 2009; Mosier *et al.,* 2005), pretreatment method applied on lignocellulose biomass must ensure that; (1) lignin structure is broken. (2) the biomass surface area available for enzymatic attack is increased by increasing pore size. (3) Cellulose crystallinity is reduced in order to enhance enzymatic digestibility of biomass. (4) high levels of fermentable sugars are attained. (5) degradation or loss of carbohydrates is avoided. (6) the formation of inhibitory byproducts to subsequent hydrolysis and fermentation process is minimised. Pretreatment methods that completely disrupt the highly-ordered cellulose structure and the lignin carbohydrate complex or remove lignin or increase surface area accessible to enzymes have been shown to promote hydrolysis and

speed up the rate and the extent of hydrolysis of cellulose in various pretreated lignocellulose biomass (Dekker, 2016).

Pretreatment methods are also classified as physical, chemical, biological, hydrothermal and a combination of a hybrid method combining either chemical or physical method with hydrothermal. Chemical pretreatment uses dilute acid (H_2SO_4, H_3PO_4, HCl etc), alkaline (NaOH, H_2O_2, NH_3 etc) and gases (CO_2, SO_2, Ozone etc), whereas physical method involves milling and grinding, and microwave irradiation. The hydrothermal method includes steam pretreatment and hot water pretreatment (Dekker, 2016; Menon & Rao, 2012; Kumar *et al*., 2009; Sun & Cheng, 2002). These pretreatment methods impact on lignocellulosic biomass differently, especially regarding the structure and chemical composition of the same biomass. For instance, hydrothermal and acidic pretreatments solubilise hemicellulose fraction whereas, alkaline pretreatment removes lignin.

Pretreatment such as milling retains the biomass initial chemical composition of the lignocellulose. Steam or hydrothermal and acidic pretreatments do not reduce cellulose crystallinity significantly, while ionic-liquid based technique can change crystalline cellulose form into amorphous cellulose form and significantly increase the enzymatic hydrolysis rates and yields (Silva *et al*., 2013; Kumar *et al*., 2009). These pretreatment methods have been extensively reviewed (Rabemanolontsoa & Saka, 2015; Chaturvedi & Verma, 2013; Menon & Rao 2012; Agbor *et al*., 2011; Brodeur *et al*., 2011; Hendriks & Zeeman, 2009).

2.5.1.1. Chemical pretreatment of lignocellulosic biomass

Chemical pretreatment method has been developed extensively and used primarily for delignification of lignocellulosic material. The most commonly used chemical pretreatments include acid and alkali based approaches (Bensah & Mensah, 2013). Hemicelluloses are unstable to chemical pretreatments and can be easily removed under mild conditions. However, the overall effect of mild pretreatment of the biomass on enzymatic hydrolysis can depend on the chemical composition of lignocellulosic materials, especially on the structure and content of lignin which becomes difficult to extract (Ioelovich & Morag, 2012). Many of the pretreatment methods have a common goal which is either to remove lignin or alter its structure

and sometimes to solubilize the hemicellulose. The choice of pretreatment should consider the overall compatibility of feedstocks, enzymes and subsequent fermenting organisms (Menon & Rao, 2012).

(a) Acid pretreatment of lignocellulose biomass

Dilute acid method is developed for biomass pretreatment and it significantly improves the efficiency of subsequent enzymatic hydrolysis step (Binod *et al*., 2011). Dilute acid pretreatment allows for the deconstruction of lignocellulosic biomass structure and the release of sugar monomers, mostly derived from hemicellulose. The amorphous and branched nature of hemicellulose makes it more easily accessible to hydrolysis agents. This structure allows for the diffusion of acids which accelerate the hydrolytic process. Therefore, dilute acid pretreatment selectively removes and hydrolyses hemicellulose (Silva *et al*., 2013). This fact is also dependent on the processing conditions, which can be mild or severe. Dilute acid pretreatment of lignocellulosic biomass is an effective method which predominantly affects hemicellulose with little impact on lignin degradation (Silverstein *et al*., 2007).

Dilute sulphuric acid (0.5% v/v) pretreatment improved cellulose and lignin content of wheat straw with a great reduction of hemicellulose at high temperature (160 °C) and treatment time of 10 minutes (Agrawal *et al*., 2015). Zhang *et al*. (2011) also reported an increase of cellulose and lignin content in H_2SO_4 (1% w/v and 180 °C in an autoclave for 10 min) pretreated sweet sorghum bagasse and about 90% solubilised hemicellulose. At a temperature of 130 °C, 0.5% H_2SO_4 and on a longer time period, hemicellulose was completely solubilised in banana pseudostem with biomass loss of approximately 60%. About 25 – 35% of lignin was removed during H_2SO_4 pretreatment of banana pseudostem (Idrees *et al*., 2013). The differences in H_2SO_4 pretreated biomass (Wheat straw and banana pseudostem) could be attributed to the incubation period since the lignin content in their untreated biomass is similar.

According to Anwar *et al*. (2014), apart from chemical agents used in the pretreatment process, temperature and time of treatment are important factors that impact on the alteration of the biomass structure. Dilute acid pretreatment of rice straw and sugarcane bagasse using 1.5 % v/v H_2SO_4 at 121 °C for 10 – 60 min had increased cellulose content in rice straw from 40 to 60% and 35 to 49.9% in

sugarcane bagasse and almost completely solubilised hemicellulose (Kumar & Parikh, 2015). The loosening of structure and increase of cellulose content could potentially lead to achieving higher biomass digestibility. However, the increased lignin content could hinder enzymatic hydrolysis (Ioelovich & Morag, 2012). Dilute acid pretreatment (140 °C, 40 min and 0.98% w/v H_2SO_4) of Poplar biomass resulted in the production of inhibitory compounds namely, furfural, acetic acid, formic acid and 5-hydroxymethyl-2-furaldehyde (HMF) that hindered both enzymatic saccharification and fermentation processes. The inhibitory effect of these compounds was minimised by washing the pretreated Poplar biomass with water and recovery of 5.3 times more glucose and increased ethanol yield was attained (Frederick *et al*., 2013).

(b) Alkaline pretreatment of lignocellulosic biomass

During alkaline pretreatment, the lignocellulosic biomass undergoes two reactions, namely solvation and saponification. These reactions cause the structure of lignocellulose to swell, decrease the degree of polymerisation thereby making the lignocellulose components more accessible to enzymatic degradation and microbial attack. The pretreatment also causes the solubilisation, redistribution and condensation of lignin leading to a modification of crystalline cellulose. The effectiveness of alkaline pretreatment depends on the physical structure and composition of biomass and the pretreatment condition (Sindhu *et al*., 2015). Alkaline pretreatment increases biomass utilisation rate and yield of sugar after enzymatic hydrolysis more than both untreated and acid pretreated biomass (Ioelovich & Morag, 2012). The most common used alkali solutions for pretreatment are sodium hydroxide (NaOH), calcium hydroxide (CaOH), ammonium hydroxide (NH_4OH) and potassium hydroxide (KOH), (Sindhu *et al*., 2015). Cao *et al*. (2012) compared the effects of four different pretreatments methods with the aim of enhancing enzyme digestibility of sweet sorghum bagasse, using sodium hydroxide (both dilute 2% NaOH and concentrated 20% NaOH), hydrogen peroxide (H_2O_2) in combination with alkaline solutions (2% NaOH – 5% H_2O_2), and autoclaving. It was observed that concentrated NaOH (20%) resulted in a high (83.7%) loss in biomass dry matter and high solubilisation of hemicellulose in sweet sorghum bagasse (Cao *et al*., 2012).

Wu *et al*., (2011b) reported an increased loss of hemicellulose in sweet sorghum bagasse at higher concentration of NaOH and low temperature, 25 °C. Sodium hydroxide (2% v/v) autoclaving followed by immersion in hydrogen peroxide pretreatment had greatly increased the cellulose content of the sorghum bagasse, solubilised more of hemicellulose and removed lignin better, compared to all other methods investigated. Combination of alkaline solutions (NaOH – H_2O_2) in the pretreatment improved the solubilisation of hemicellulose and removal of lignin while it retained its high cellulose content (Cao *et al*., 2012). Soaking of rice straw and barley straw in aqueous ammonia (15 wt%, weight percent) at a moderate temperature revealed that enzymatic digestibility of both biomass at concentration of 5 wt% were enhanced by 85% and 95%, respectively (Park & Kim, 2012). Sodium sulphide (Na_2S), sodium sulphite (Na_2SO_3) and NaOH both at a concentration of 0.5% v/v and high temperature (130 °C) have been shown to remove lignin in banana pseudostem completely resulting in more than 90% enzymatic hydrolysis (Idrees *et al*., 2013). Alkaline pretreatment (80 °C, 39 min, 018 g NaOH and 0.06 g lime per gram of raw biomass) of wheat straw resulted in 93.1% conversion of cellulose to glucose after enzymatic hydrolysis (35 FPU/g) and which achieved 80.3% of monosaccharides namely, glucose, xylose and arabinose (Jaisamut *et al*., 2013)

In another study, mild alkaline pretreatment (NaOH 0.5% v/v, room temperature, 24 hours) of rice straw had increased cellulose content from 40% to 60% while that for sugarcane bagasse increased from 35 to 49.9% (Kumar & Parikh, 2015). Lignin removal in rice straw and sugarcane bagasse was 61% and 45%, respectively. There was also a marginal loss in hemicellulose for both biomass (Kumar & Parikh, 2015). Wang *et al*. (2010) showed that a concentration of NaOH (2 – 3%) and time (60 – 90 min) removed 80 – 85% of lignin and solubilised approximately 60% of hemicellulose with little effect on cellulose (glucan) content of coastal Bermuda grass. Low *et al*. (2015) also investigated various concentrations of NaOH (1, 2, 4 & 7% w/v) at room temperature on delignification of banana pseudostem. Their results revealed that NaOH (4% w/v) increased cellulose content to 73%, with greater delignification than all other concentrations with an increase in accessibility of the carbohydrate. The concentration of NaOH (7% w/v) has been shown to reduce the amount of holocellulose (cellulose plus hemicellulose) content (Low *et al*., 2015).

Other authors have reported that at higher concentration of NaOH, a greater delignification of lignin occurs concomitantly with solubilisation of hemicellulose (Low *et al*., 2015; Cao *et al*., 2012; Wu *et al*., 2011b). The loss of hemicellulose is attributed to the amorphous structure of hemicellulose which makes it easily degradable. These findings by Cao *et al*. (2012) on sweet sorghum bagasse (20% NaOH) and Low *et al*., 2015 on banana pseudostem (7% NaOH) pretreatment suggest that for each biomass, it is imperative that critical concentration is determined due to differences in lignocellulose matrix and composition. The effect of temperature, time, and chemical concentration on the alteration of the biomass structure differs from one biomass to another due to the level of crystallinity in the material which can also be influenced by age of the biomass.

Generally, chemical pretreatment methods have been extensively studied on different types of biomass. However, these methods are costly due to requirements of chemical and high energy input in the process and in addition, the need for specialised reactors that are resistant to corrosion. As a result, a moderate or low cost efficient pretreatment process is essential for economic viability of lignocellulosic bioethanol production.

(c) Hydrothermal pretreatment of lignocellulosic biomass

The hydrothermal method is a relatively mild pretreatment that requires no catalysts and it is a non-corrosive process. According to Iroba *et al*. (2013), water and heat are not sufficient to disrupt the lignin or create pores on the biomass matrix for subsequent hydrolysis. On the contrary, Jőnsson & Martin, (2016) and Taherzadeh & Karimi (2008) stated that under high pressure water penetrates into biomass, hydrates cellulose and removes most of the hemicellulose and minor part of lignin. The solubilisation of hemicellulose is catalysed by hydronium ions resulting from water auto-ionisation (dissociation) (Jőnsson & Martin, 2016). Thus, the auto-dissociation equilibrium constant (Kw) of water is equal to 0.01×10^{-12} at 25 °C and 6×10^{-12} at 230 °C. The values above represent the dissociation of water into H^+ and OH^-. At high temperatures, more concentration of catalytic protons are generated through the dissociation process (Ladisch *et al*., 2013).

Van Walsum *et al*. (1996) reported the hydrothermal pretreatment (220 °C, 2 min) of sugarcane bagasse, aspen chips (3 mm) and mixed hardwood-flour (-60+70 mesh) which yielded 90% conversion to ethanol at an enzyme load of 30 FPU/g in 75 h. Hydrothermal pretreatment of herbaceous material (Prairie cord grass) resulted in 97.96% of pretreatment conversion rate and 94.53% of enzymatic hydrolysis (15 FPU/g and 60 U/g β-glucosidase) conversion rate in studies by Lei *et al*. (2013). Liquid hot water pretreatment (180 °C for 20 min, solid: liquid ratio 1:10) of reed resulted in cellulosic conversion rate of 82.59% after enzymatic hydrolysis, 30 FPU/g (Lu *et al*., 2012). In another study by Ko *et al*. (2014), the LHW pretreatment severity (180 – 210 °C for 5 – 15 min) on the properties of hardwood lignin and enzymatic hydrolysis of cellulose revealed that structural changes in lignin occurred and lignin content was increased, which led to reduced enzymatic hydrolysis (40 FPU/g) of cellulose.

Another advantage of hydrothermal (e.g. autoclave) pretreatment is that it minimises the loss in biomass dry matter (Cao *et al*., 2012). However, at high temperatures (185 – 250 °C) hydrothermal pretreatment produces acetic acid from the thermally labile O-acetyl groups attached to the xylose residues that make up hemicellulose (heteroxylan) polymer (Dekker, 2016) and the catalytic action of water results in hydrolysis of cellulose to monosaccharides which are later degraded to aldehydes and humic substances (Ladisch *et al*., 2013; Gromov 2016). Depending upon the conditions such as temperature and time, the hemicellulose fraction can be hydrolysed into mono- and oligosaccharide (Dekker, 2016). The formation of fermentation inhibitors could be minimised by controlling the pH close to neutral values (Jőnsson & Martin, 2016). Most of the inhibitors and hemicellulose were found in the pre-hydrolysate which is a liquid fraction obtained after pretreatment (Lei *et al*., 2013).

(d) Biological pretreatment of lignocellulosic biomass

Chemical pretreatment requires high energy input and generates effluent that may be hazardous to the environment. As such, biological pretreatment becomes an alternative method that alters the structure of lignocellulosic biomass. Biological pretreatment is performed by culturing microorganisms (e.g. fungi and actinobacteria) which then synthesize and secrete hydrolytic enzymes and oxidative

enzymes (Chaturvedi & Verma, 2013; Pérez *et al*., 2002; Ilmén *et al*., 1997) which enable them to utilise the complex plant polysaccharides for growth. In nature, fungi colonize the plant debris and in a symbiotic relationship with other microorganisms, they secrete assortment of proteins and complex of enzyme systems to hydrolyse plant polysaccharides. However, not all microorganisms are able to secrete a significant amount of such complex enzyme systems for biotechnological applications (Benoliel *et al*., 2013).

Three different wood decaying fungi include brown-rot, white-rot and soft-rot fungi. The soft-rot fungi's hyphae occupy the decaying, cylindrical cavities within the secondary walls of wood cells and rarely present in the lumens, unlike the brown-rot and white-rot (Cowling, 1958). The white-rot fungi have the capability to utilise both the carbohydrates polysaccharides (i.e. cellulose and hemicellulose) and lignin, whereas the brown-rot fungi which evolved from white-rot fungi can selectively degrade carbohydrates polysaccharides of wood, leaving lignin-rich residue in the soil (Canam *et al*., 2013; Cowling, 1958). White-rot fungi and *Streptomyces* species can achieve delignification of a plant biomass (Berrocal *et al*., 1997). Efficient degradation of lignin depends on the lignolytic enzymes such as lignin peroxidase (LiPs), manganese peroxidase (MnPs) and laccase (Sindhu *et al*., 2015). During the pretreatment, microorganisms require extracellular hydrogen peroxide (H_2O_2) to support the oxidative turnover of LiPs and MnPs responsible for lignolysis. The H_2O_2 can be provided by extracellular oxidases that reduce molecular oxygen to H_2O_2 with synergistic oxidation of co-substrate. The most studied and characterised extracellular H_2O_2-generating enzymes are glyoxal oxidase (GLOX) and aryl alcohol oxidase (AAO) (Souza, 2013). The biological pretreatment is considered inexpensive and easy to operate. However, a large scale operation leads to higher operational costs for the following reasons: (1) the process requires a large sterile area and maintenance of sterility during the process. (2) carbohydrates - sugar monomers from the hydrolysis of cellulose and hemicellulose are consumed by the cultured microorganism(s) leading to lower product output.

Du *et al*., (2011) reported an 82% hydrolysis yield after 28 days of biological pretreatment of corn stalks by *Irpex lacteus*, a white-rot fungus capable of producing both hydrolytic and oxidative enzymes. The drawback of biological pretreatment is

the long residence time (10 – 44 days) and it becomes inconvenient for industrial application (Chaturvedi & Verma, 2013; Agbor *et al*., 2011). However, biological pretreatment can be a suitable method of biomass containing low lignin content (Agbor *et al*., 2011) and the by-products produced during the pretreatment have a less inhibitory effect to the subsequent hydrolysis step (Sindhu *et al*., 2015).

A white-rot fungus that poses high selective degradation for lignin over cellulose is essential to fungal pretreatment of lignocellulosic biomass for bioethanol production (Wan & Li, 2012). Previously, Reddy *et al*. (2003) reported two white-rot fungi, *Pleurotus ostreatus* and *P*. *sajor-caju* with low levels of endoglucanase (CMCase), cellulase (FPase) and high laccase activities. Improvement of such strains through genetic engineering or mutagenesis and even screening for natural cellulase deficient white-rot fungus would be a breakthrough for biological pretreatment without loss of fermentable sugars.

2.5.2. Hydrolysis of lignocellulosic biomass

This is the production step of lignocellulosic hydrolysate (fermentable sugars – glucose). In the hydrolysis process, the sugars are released from carbohydrate chains. The hydrolysis of hemi-(cellulose) chains can be achieved by acid hydrolysis and enzymatic hydrolysis.

2.5.2.1. Acid hydrolysis of lignocellulosic biomass

Acid hydrolysis is an important method for the recovery of sugar monomers from cellulose and hemicellulose polymers from lignocellulosic biomass (Anwar *et al*., 2014). Concentrated mineral acids such as H_2SO_4 and HCl are commonly used in hydrolysis. The concentrated acid disrupts the hydrogen bonding between cellulose chains thereby converting it to a completely amorphous state. De-crystalized cellulose forms homogenous gelatin with the acid and in this form cellulose is extremely susceptible to hydrolysis. Homogeneous gelatin – acid mixture reacts in the presence of water at modest temperature to provide rapid hydrolysis to glucose (Binod *et al*., 2011; Kumar *et al*., 2009). Hydrolysis of lignocellulose using concentrated acid achieves near-theoretical sugar yields and results in less

degradation of products than the commonly used dilute acid hydrolysis process (Moe *et al*., 2012; Binod *et al*., 2011).

The molecular mechanism of acid-catalysed hydrolysis of cellulose (cleavage of β-glycosidic bond) follows the pattern illustrated in Fig. 2.11 (Xiang *et al*., 2003). Acid hydrolysis proceeds in three steps. The reaction starts with a proton from acid interacting rapidly with the glycosidic oxygen linking two sugar units thereby forming a conjugate acid. The cleavage of the C-O bond and breakdown of conjugate acid to the cyclic carbonium ion take place, which adopts a half-chair conformation. After a rapid addition of water, free sugar and a proton are liberated (Xiang *et al*., 2003). While concentrated acid hydrolysis results in more released fermentable sugars, the process is toxic, corrosive and hazardous. It requires reactors that are resistant to corrosion (Chaturvedi & Verma, 2013; Binod *et al*., 2011).

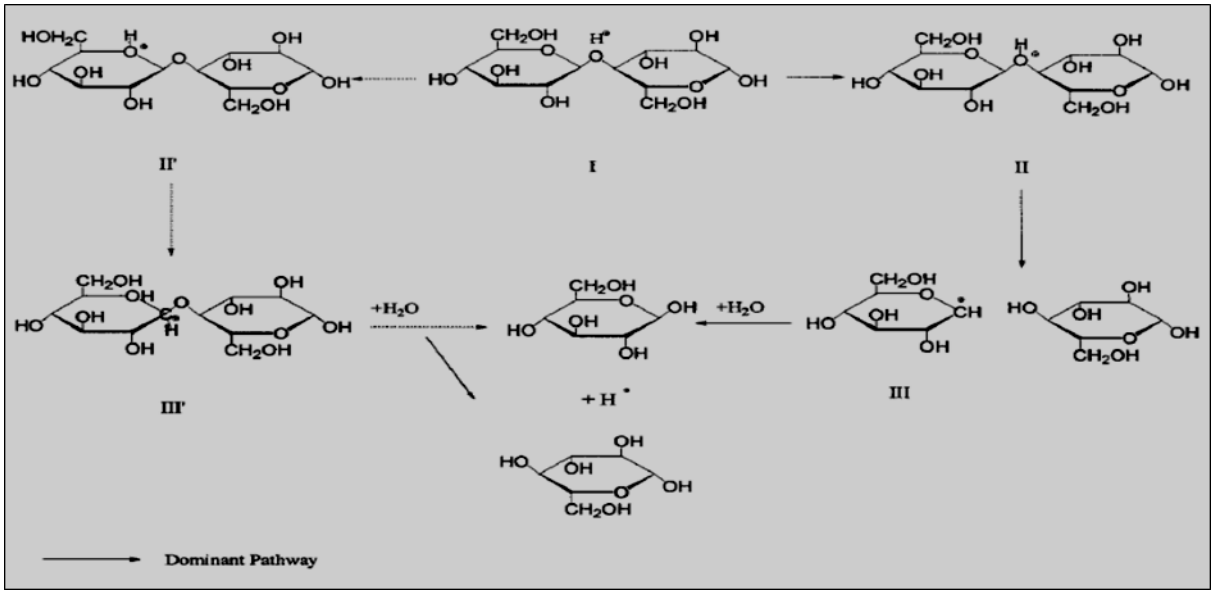

Figure 2.6. Mechanism of acid catalysed hydrolysis of β-1-4 glucan (Xiang *et al*., 2003).

These drawbacks make the downstream process expensive (Binod *et al*., 2011).
Monosaccharide products can be further reduced to undesirable products. The process conditions are crucial in preventing undesirable reactions, which could promote a decrease in monosaccharide yields by the formation of sugar-derived inhibitory compounds outlined below;
With I: anhydro glucose plus H* radical

II: anhydro glucose intermediate including O* radical (with high energy)

II': anhydro glucose intermediate including O* radical (without high energy)

III: fragment from anhydro unit includes C* radical

III': anhydro glucose intermediate includes C* radical

Concentrated acid pretreatment of lignocellulose biomass solubilises hemicellulose completely and cellulose, leaving lignin unaffected (Anwar *et al*., 2014; Chaturvedi & Verma, 2013). The mechanism of acid hydrolysis reaction of hemicellulose is described in Fig. 2.12 below by (Herrera *et al*., 2002).

(1) The diffusion of protons through the wet lignocellulosic matrix
(2) The protonation of the ether-oxygen link between sugar monomers
(3) The breakage of the ether bond and generation of a carbocation as an intermediate
(4) The solvation of the carbocation with water
(5) The regeneration of protons and the cogeneration of sugar monomers, oligomers and polymers, depending on the ether connection that is broken.
(6) Distribution of the products in the liquid phase
(7) Restart of the process from step 2

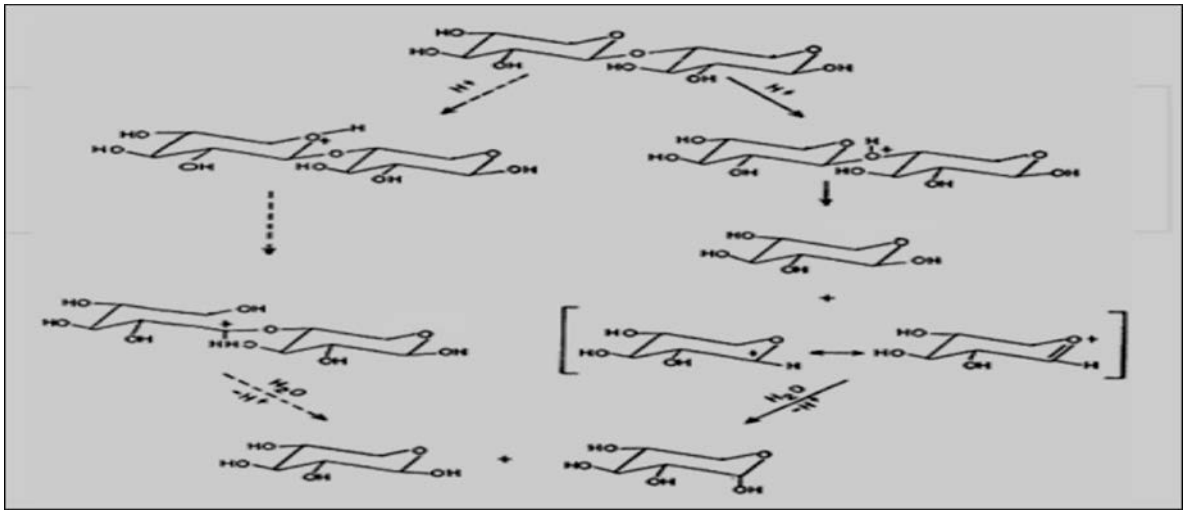

Figure 2. 12. A simplified mechanism of acid catalysed hydrolysis of hemicellulose (Herrera *et al*., 2002).

In acid hydrolysis, the final product, mainly sugar (glucose or xylose) does not pose an inhibitory effect on the hydrolysis reaction.

2.5.2.2. Enzymatic hydrolysis of lignocellulosic biomass

Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes system. The term “Cellulase enzymes system” refers to multiple enzymes involved in complete hydrolysis of cellulose namely; exoglucanase (exo-1,4-β-glucanases, EC 3.2.1.91) endoglucanase (endo-1,4-β-glucanases, EC 3.2.1.4) and β-glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) Enzymatic hydrolysis is more desirable than the use of inorganic catalysts, because enzymes are highly specific and can function in mild process conditions. However, there are limitations to the use of enzymes in industrial processes. Firstly, most enzymes are relatively unstable at high temperatures. Secondly, the cost of enzyme production and purification are high and it is difficult to recover them from the reaction mixtures. These limiting factors had prompted extensive research for cellulases with improved thermo-stability (Verardi *et al*., 2012; Taherzadeh & Karimi, 2007). Enzymatic hydrolysis of cellulose to glucose by cellulase occurs under mild conditions of pH 4.5 – 5.0 and temperature 40 – 50 °C. Effective enzymatic hydrolysis of cellulosic biomass is more influenced by the type (i.e. structural features of cellulose) and concentration of biomass or substrate (Yang *et al*., 2011; Sun & Cheng, 2002) and often the cellulase loading is in the range of 7 to 33 FPU/g substrate (Sun & Cheng, 2002). Major drawbacks of enzymatic hydrolysis are the inhibitory effect of the product (glucose) on hydrolysis reaction and long hydrolysis time (Taherzadeh & Karimi, 2007). The next section provides a description of the enzymes involved in depolymerisation of cellulose and hemicellulose polymers. The depolymerisation of cellulose is effected by cellulases whereas hemicellulose involves a group of enzymes called hemicellulases.

2.5.2.2.1. Cellulase and their mode of action

Cellulase is a class of enzyme with different specificities to catalyse the hydrolysis of glycosidic bonds within cellulose (Khan *et al*., 2016; Jecu, 2000). The enzymatic hydrolysis of cellulose involves exoglucanases, endoglucanases, and β-glucosidases which exhibit high specificity for the β-1.4 glycosidic linkages (Jecu, 2000). Cellulolytic enzyme system from filamentous fungi, particularly *Trichoderma reesei* contains two exoglucanases or cellobiohydrolases (CBH1 and CBH2), four endoglucanases (EG1, EG2, EG3 and EG4) and one β-glucosidase (Kumar *et al*., 2008). For ~~a~~ complete hydrolysis of cellulose, the three enzymes act synergistically

to catalyse the hydrolysis process, Fig. 2.13 (Khan *et al*., 2016; Saini *et al*., 2015a; Yoon *et al*., 2014; Kumar *et al*., 2008). The efficiency of cellulose hydrolysis depends on the type of biomass and a balance of the cellulase enzymes (Khare *et al*., 2015).

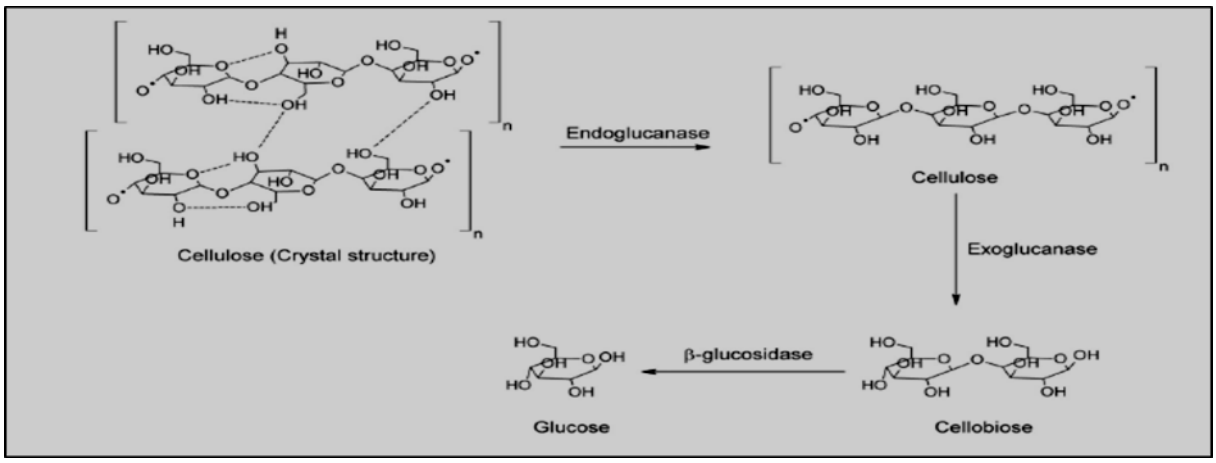

Figure 2.13. Hydrolysis action of endoglucanase, cellobiohydrolase and β-glucosidase on cellulose (Yoon *et al*., 2014).

(a) Endoglucanase

The endogucanase catalyses the random cleavage of internal bonds of the cellulose chain thereby generating new two ends, which is the reducing and non-reducing ends (Saini *et al*., 2015a; Kumar *et al*., 2008). The random cleavage of endoglucanase on the soluble cellulose derivatives (amorphous) causes a rapid decrease in chain length. When acting on cellodextrins-glucose polymers of varying length (i.e. two or more glucose monomers), the rate of hydrolysis increases with the degree of polymerisation within the limits of substrate solubility with cellobiose and cellotriose being the major final products.

(b) Exoglucanase

Exoglucanases attack the chain ends of cellulose molecule thereby releasing cellobiose (Saini *et al*., 2015a; Kumar *et al*., 2008). It also cleaves glucose units successively from the non-reducing end of glucan (cellulose). Exoglucanases are distinguished from β-glucosidase by their preference for substrate of longer chain length and by their inversion of their product (Saini *et al*., 2015a). Accumulation of cellobiose inhibits cellulase activity (Isaacs, 1984).

(c) β-glucosidase

β-glucosidases are active on cello-oligosaccharides up to cellohexaose and cellobiose to release glucose monomer units (Maitan-Alfenas *et al*., 2015; Saini *et al*., 2015a; Kumar *et al*., 2008; Isaacs, 1984). Most β-glucosidases are active on a range of β-dimers of glucose and their rate of hydrolysis of cellobiose decreases markedly as the degree of polymerisation of the substrate increases (Saini *et al*., 2015a). The end product of hydrolysis of glucose is a fermentable sugar and can be converted into ethanol by fermenting microorganisms. The excessive accumulation of glucose during hydrolysis leads to inhibition of β-glucosidase in a non-competitive mechanism (Isaacs, 1984).

2.5.2.2.2. The structure of fungal cellulase

Glycoside hydrolases (GHs) including cellulase have been classified into 115 families based on amino acids sequence similarities and crystal structures. A large number of cellulase genes are found in 13 GHs families, namely 1, 3, 5, 6, 7, 8, 9, 12, 26, 44, 45, 51 and 48. Cellulase like activities has also been proposed for families 61 and 74 (Schulein, 2000). Based on the Carbohydrate-Active Enzyme database (CAZy; http://www.cazy.org), the three-dimensional structures of more than 50 cellulases are available, with the exception of family GHs3. It is important to note that all cellulases cleave β-1,4-glucosidase bonds, but display a variety of topologies ranging from β-sheet to β/α-barrels, to α-helical proteins (Zhang & Zhang, 2013). Some families, e.g. GHs6 and GHs7 contain enzymes with great different and mechanistically synergistic activities. Other families such as GHs1 and GHs3 contain many enzymes with the same activity (i.e. cleavage of glycosidic bond in cellobiose), whilst some of these enzymes have subtle differences in substrate specificity (Payne *et al*., 2015). Family GH7 contains both CBH (Cle7A) and EG (Cel7B) based on the same protein fold, i.e. folded β-sheet sandwich. The endoglucanases that are found in GH families 5, 7, and 12 catalyze the hydrolysis of glycosidic bonds with retention of configuration (Payne *et al*., 2015).

Typically, fungal cellulase has two separate domains: a catalytic binding domain (CBDs) and non-catalytic carbohydrate (or cellulose) binding domain, which are

linked by a short poly-linker region to the catalytic domain at the N- or C-terminal, Fig. 2.14 (Sajith *et al*., 2016; Zhang & Zhang 2013; Kuhad *et al*., 2011).

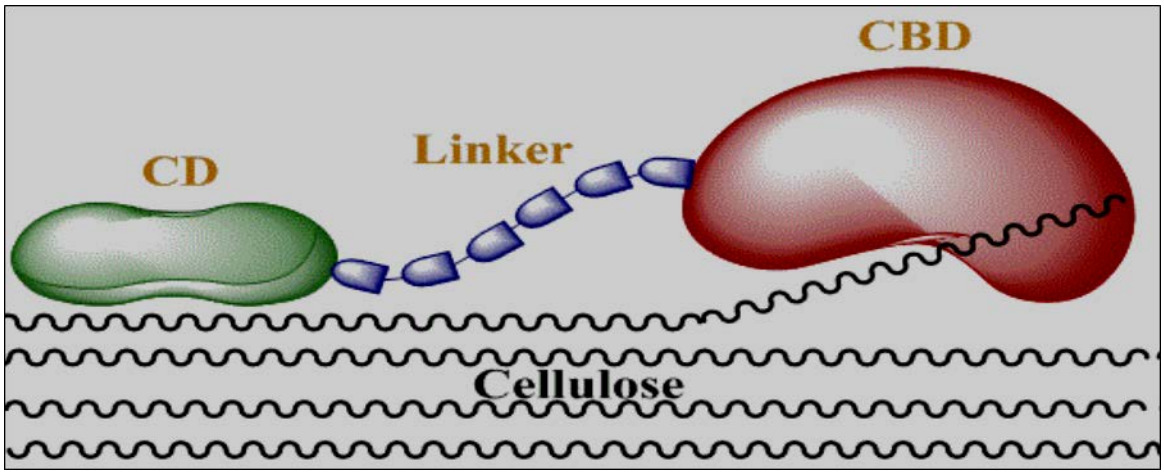

Figure 2.7. A schematic representation of the two-domain structure found in cellulases. The catalytic domain (CD) is linked to the cellulose binding domain (CBD) by a distinct linker region, which is often glycosylated (Sajith *et al*., 2016).

The active site of the catalytic domain may be topological, tunnel, cleft or pocket in shape in order to allow efficient hydrolysis of the substrate (Hildén & Johansson, 2004). A comparison of the GH family of either bacterial or fungal species reveals quantitative patterns in linker characteristics. For instance, multimodular fungal GH6 and GH7 cellulase exhibit significant differences in linker length, with GH6 cellulase linker average length of 42 and GH7 30 amino acid residues (Payne *et al*., 2015). The amino acid content of bacterial and fungal linkers differed significantly with higher Proline found in bacterial cellulase linkers (Payne *et al*., 2015) and higher Serine-Threonine content in fungal cellulase linker (Sajith *et al*., 2016; Payne *et al*., 2015; Kuhad *et al*., 2011).

Cellobiohydrolase (CBHs) is the most studied exoglucanase. Different CBH with catalytic domain/or modules belongs to GHs families 5, 6, 7, 9, 48 and 74 glycoside hydrolases. Aerobic fungal CBH belongs to GH6 and GH7 only, whilst anaerobic fungal CBH is in GH family 48. In contrast, aerobic bacterial CBHs falls in GH families 6 and 48, while anaerobic bacterial CBHs are in GH family 9 as well as family 48. The most significant topological feature of CBH's catalytic module is the tunnel structure which is formed by two surface loops, Fig. 2.15. In the case of family

7 CBH, the tunnel may cover entirely the active site or only part of the active site may be covered by family 48 CBH (Zhang & Zhang, 2013).

In general, fungal endoglucanases possess a catalytic domain with or without a CBD, whilst bacterial endoglucanase may possess multiple CBD and other domains with unknown functions. The catalytic domains of most endoglucanases have a cleft/grove-shaped active site which allows the endoglucanase to bind and cleave the cellulose chain to generate soluble cellodextrins or insoluble cellulose fragments (Zhang & Zhang, 2013). Figure 2.15 shows the crystal structure of GH6 *Thermobifida fusca* endoglucanase Cel6A (*Tf*Cel6A) and *Humicola insolens* exoglucanase Cel6A (*Hi*Cel6A).

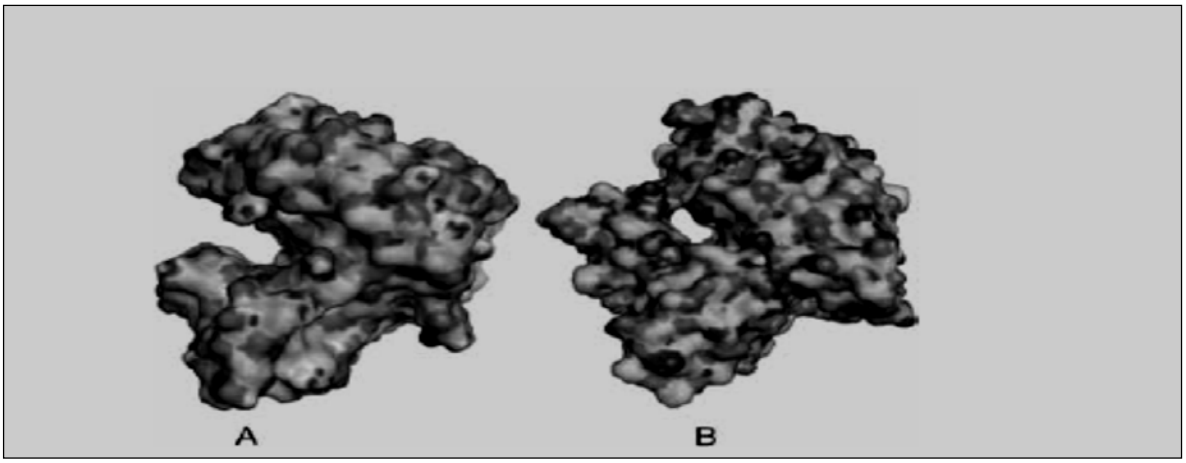

Figure 2.8. Crystal structures of GH6 endoglucanase and exoglucanase. (A) The structure of endoglucanase TfCel6A, which exhibit a deep cleft at the active site. (B) The structure of exoglucanase HiCel6A, in which the active site bears an extended loop that forms a tunnel (Zhang & Zhang, 2013).

2.5.2.2.3. Hemicellulase and their mode of action

Hemicellulases are classified according to: (1) the substrate they act upon, (2) the bonds they cleave and (3) their patterns of product formation. However, a greater variety exists among the endo-xylanases and β-xylosidases (Jeffries 1994). Hemicellulases are various enzymes responsible for the degradation of hemicellulose polymer. For instance, in hardwood, xylan is the main carbon backbone, in which its degradation requires endo-1,4-β-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase and acetyl xylan esterase acting together on

different heteropolymer sites, as shown in Fig. 2.16 (Kumar *et al*., 2008). In softwood, the glucomannans backbone is cleaved by β-mannanase and β-mannosidase (Kumar *et al*., 2008). A notable distinction is that endo-1,4-β-xylanase (EC 3.2.1.8) produce oligosaccharides from random cleavage of xylan whilst xylan 1,4-β-xylosidase (EC 3.2.1.37) acts on xylan oligosaccharides to produce xylose. Some endo-xylanases show a greater specificity towards straight chain substrates and others have specificity for frequent side chains or branching chains (Jeffries, 1994).

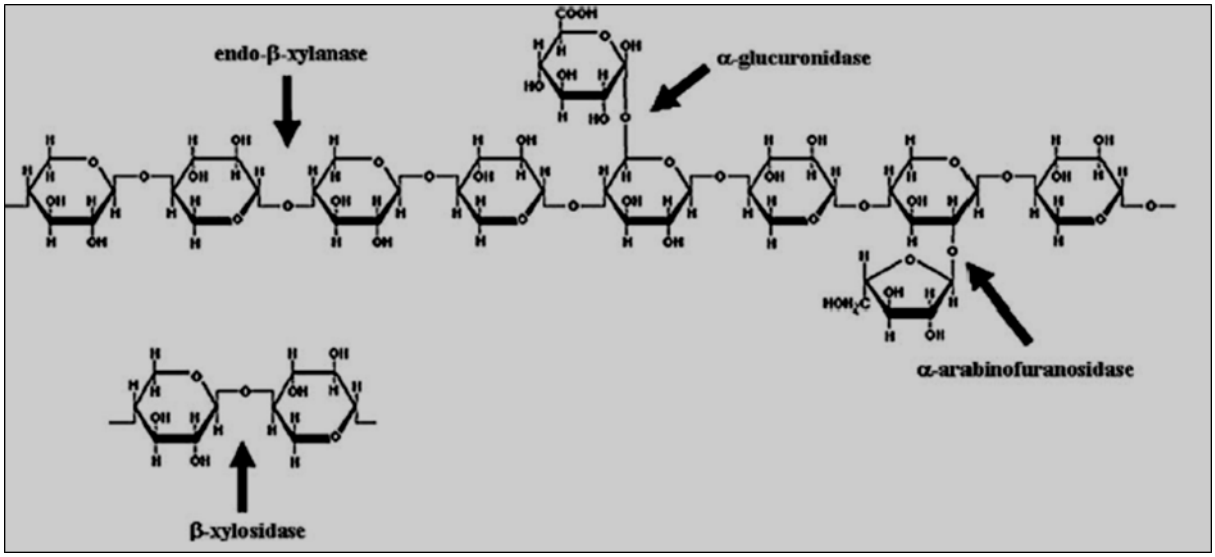

Figure 2.16. Chemical structure of hemicellulose and target site of enzymatic hydrolysis of the polymer (Kumar *et al*., 2008).

The end products in the hydrolysis are mixtures of sugars mainly xylose, glucose, arabinose, galactose and mannose and these sugar mixtures vary from one plant to another.

2.6. Fermentation

The fermentation process involves the use of microorganisms to produce various products such as enzymes (proteins), bioethanol, acids, and other by-products. In this section, only the literature on enzyme and ethanol production will be reviewed.

2.6.1. Microorganisms in enzymes (cellulase and hemicellulose) production

Microorganisms are an attractive source of enzymes because they can be cultured in large numbers in a relatively short period by established fermentation techniques. However, the production of enzymes is influenced by the genetic make-up of the microorganisms, nutrition, and environmental factors (Olaniyi & Oyesiji, 2015; Norouzian, 2008). Many microorganisms in nature, mostly bacteria and fungi are capable of producing biomass degrading enzymes, Table 2.3. Screening and selection of cellulase producing microorganisms from nature is one way of getting novel cellulases. Newly isolated microorganisms are potential sources of new genes encoding enzymes with unique properties (Khan *et al*., 2016).

Cellulolytic enzymes that are secreted by such microbial population belong to classes of glycoside hydrolases (GHs), (Yang *et al*., 2011; König *et al*., 2002). More emphasis will be on the fungi owing to the advanced progress made in the “know-how” of enzymes they produce. The importance of fungi had been recognised in several biotechnological industries for their improvement of various aspects of the final product through the secreted enzymes (Soares *et al*., 2012). Fungal cell factories produce approximately 40 – 50% of the value of industrial enzymes (Paloheimo *et al*., 2016).

Trichoderma species are soil borne with greenish-yellow to red spores with potential to colonise plant materials and efficiently utilise the substrate in contact and possess the ability to survive under different environments (Schuster & Schmoll, 2010; Samuels, 1996).

Table 2.3. Microbial community with cellulolytic abilities (Adapted from Khan *et al*., 2016)

Microorganisms	Examples of cellulase producers
Fungi	Soft-rot fungi
	Aspergillus niger; A. nidulus; A. oryzae; A. terreus; Fusarium solani; F. oxysporum; Humicola insolens; H. grisea; Trichoderma longibrachiatum; T. harzianum; T. reesei; T. atroviride; Chaetomium cellulyticum; C. thermophilum; Neurospora crassa; Penicillium fumigosum; P. occitanis; P. brasilianum; P. decumbens; P. echinulatum; Melanocarpus albomyces; Thermoascus aurantiacus; Mucor cirnelloides
	Brown-rot fungi
	Coniophora puteana; Lanzites trabeum; Poria placenta; Tyromyces palustris; Fomitopsis sp.
	White-rot fungi
	Phanerochaete chrysosporium; Sporotrichum thermophile; Tramets versicolor; Agaricus arvensis; Pleurotus ostreatus; Phlebia gigantean
Bacteria	Aerobic bacteria
	Acinetobacter junii; A. anitratus; Acidothermus cellulolyticus; Anoxybacillus sp; Bacillus subtilis; B. pumilus; B. amyloliquefaciens; B. licheniformis; B. circulan; B. flexus; Bacteriodes sp; Cellulomonas biazotea; Cellvibrio gilvus; Eubacterium cellulosolvens; Geobacillus sp; Microbispora bispora; Paenibacillus curdlanolyticus; Pseudomonas cellulose; Salinvibrio sp; Rhodothermus marinus
	Anaerobic bacteria
	Acetivibrio cellulolyticus; Butyrivibrio fibrisolvens; Clostridium thermocellum; C. cellulolyticum; C. acetobutylium; C. papyrosolvens; Fibrobacter succinogens; Ruminococcus albus
Actinomycetes	*Cellulomonas fimi, C. biazotea, C. uda; Streptomyces drozdowiczii; S. lividans; Thermomonospora fusca; T. curvata*

The kingdom fungi have approximately 200 species of *Aspergillus* which produce a great number of extracellular enzymes with biotechnological applications. Such species can be isolated from soil, decomposing plants and air. Some of the most studied *Aspergillus* species include *A*. *flavus*, *A*. *niger*, *A*. *oryzae*, *A*. *nidulus*, *A*. *fumigatus*, *A*. *clavatus*, *A*. *glaucus*, *A*. *ustus* and *A*. *versicolar* (Soares *et al*., 2012).

Most commercial cellulase is produced by *Trichoderma spp*. with a few of the enzymes from *A. niger* (Taherzadeh & Karimi, 2007). Cellulase produced by *Trichoderma sp*. lacks sufficient β-glucosidase activity and requires the addition of β-glucosidase to fully convert cellobiose into glucose (Agrawal *et al*., 2015; Ha *et al*., 2011) and *Aspergillus ssp*. produce large quantities of β-glucosidase into the medium (Sørensen et al., 2011; Sternberg *et al*., 1977).

The past decades have seen growing interests both from academic and industrial researchers in using cellulase for the production of second generation bioethanol with substantial investment by various government institutions in the development of enzymes, and discovery of other microbial strains in nature. With the emergence of biotechnology tools such as bioinformatics, mutagenesis, gene-deletion (gene-knockout) *etc*., it has become possible to screen and improve or develop new microorganisms (enzyme source) that offers more desirable traits, including higher specific activities, improved thermal stability, resistance to inhibitors and improved synergism amongst various enzymes such as cellulase, hemicellulose, pectinase and proteinase activities that maximise sugar yields at low cost (Paloheimo *et al*., 2016; Yang *et al*., 2011).

A continuing effort to exploit natural resources will lead to the discovery of new “multi-activity” microorganisms with all required enzymes for second generation bioethanol and low protease level or new genes encoding for novel cellulase with unique properties. Low protease producing strains are needed for successful and cost-effective industrial enzyme production business.

2.6.2. Enzymes (cellulase and hemicellulase) production

Enzymes have played important roles in the biotechnological processes involved in the production of food and beverages, detergent, clothing, paper industry, pharmaceuticals, dairy and biofuel industry (Soares *et al*., 2012). The production of

enzymes is achieved primarily through fermentation techniques. Such techniques are classified into submerged fermentation and solid state fermentation. The key essential steps involved in the production are: (1) Screening and selection of potential strains that produce cellulase. (2) Selection of inducing substrate and/or in the case of solid state fermentation a lignocellulosic substrate's cellulose content may be enriched by pretreatment. (3) Cultivation of selected strain for production of enzymes. (4) Downstream processing – harvesting, purification and product recovery.

2.6.2.1. Submerged fermentation

Submerged fermentation (SmF) technique is best suited for microorganisms that require high moisture content. Traditionally, vast majority of microbial industrial enzymes (> 75%) that are used in commercial applications are produced by SmF method in which the selected microorganisms (bacteria and fungi) are cultivated in liquid nutrient rich medium containing inducing substrate for a particular enzyme of interest (Khan *et al*., 2016; Paloheimo *et al.,* 2016; Subramaniyam & Vimala, 2012; Renge *et al*., 2012; Zhuang *et al*., 2007).

The substrate is utilised quickly and the bioactive compounds (e.g. enzymes, antibiotics *etc*.) are secreted into the broth. One other important advantage of SmF is the easy and perfect mixing that ensures that microorganisms, nutrients and metabolites are evenly distributed within the reactor (Gervais & Molin, 2003). Another advantage in SmF is easy purification of product (Khan *et al*., 2016). *T*. *harzianum* was able to produce endoglucanase activity of 168 U/mL when grown on 0.5% banana flour, an activity that was much higher than when CMC was used (146 U/mL) as a substrate (Rubeena *et al*., 2013).

Different *Trichoderma* species were also assessed for their abilities to produce cellulase enzymes. *T*. *reesei* strain FCBP-364, *T*. *viride* strain FCBP-142 and *T*. *harzianum* FCBP-325 were found to produce endoglucanase activity of 53.42/U/mL, 52.97 U/mL and 49.42 U/mL, respectively when CMC was used as carbon source (Shafigue *et al*., 2009). In other studies, *T*. *harzianum* produced cellulase enzymes system; exoglucanase (7.8 U/mL), endoglucanase (0.79 U/mL) and 0.92 U.mL of β-glucosidase (Ahmed *et al*., 2009). *T*. *harzianum* strain (L04) produced

endoglucanase (62.2 U/L.h), exoglucanase (10.2 U/L.h) and β-glucanase (52.0 U/L.h) using sugarcane bagasse (Benoliel *et al*., 2013). Another strain of *T*. *harzianum* IOC-3844 when grown on pretreated sugarcane bagasse produced cellulase activity of 445 U/L FPase, 6358 U/L endoglucanase and 742 U/L β-glucanase (Castro *et al*., 2010). Under optimised conditions using the statistical method, and central composite rotational design (CCRD) *T*. *harzianum* IOC-3844 produced cellulase with activity of 1225 U/L FPase, 27017 U/L endoglucanase and 609 U/L β-glucanase (Rocha *et al*., 2013).

Leghlimi *et al*. (2013) investigated the effect of temperature on the production of cellulase. They reported that *T*. *longibrachiatum* (GHL) produced 10.61 U/mL of endoglucanase activity, 2.04 (U/mL) FPase and 1.32 U/L β-glucosidase at 35 °C and *T*. *reesei* Rut C-30 had 13.67 U/mL of endoglucanse, 2.78 U/mL FPase and 0.62 U/ml of β-glucosidase at 30 °C. The results indicate that the stimulatory effect of temperature on the secretion of cellulase varies from one species to another within the same genus. Overall, the results indicate that improvement of these *Trichoderma* species could result in higher cellulase activity been produced.

The major drawback of SmF is the considerable costs involved in concentrating the enzymes from the aqueous medium (Zhuang *et al*., 2007). Submerged fermentation may be achieved through different fermentation systems namely, batch fermentation, semi-batch fermentation and continuous fermentation system. The most appropriate strategy of choice depends on the kinetic properties of the microorganism in addition to the aspects of process economics (Olsson & Hahn-Hägerdal 1996).

2.6.2.1.1. Batch fermentation

Batch fermentation is a fermentation process in which the medium containing the substrate and fermenting microorganism are introduced into the bioreactor under controlled environmental conditions and the system remains closed for the entire period of fermentation. The batch fermentation is a low cost and easy to operate with reduced risks of contamination (Paola *et al*., 2011; Olsson & Hahn-Hägerdal 1996).

2.6.2.1.2. Fed-batch fermentation

This is a process conceived for fermenting microorganisms prone to substrate inhibition (Andrietta *et al*., 2011). Fed-batch fermentation is a semi-open system in which periodic controlled addition of substrate (or at times substrate with other essential nutrients) occurs during the process at a concentration lower than the inhibition concentration of a substrate in order to obtain higher final product concentration. The fed-batch process provides better yields and productivity than the batch process (Paola *et al*., 2011; Olsson & Hahn-Hägerdal 1996). Application of Fed-batch system in the fermentation of lignocellulose hydrolysate is shown to have "in-situ" detoxification through the action of fermenting microorganism (Paola *et al*., 2011).

2.6.2.1.3. Continuous fermentation

In continuous fermentation, a substrate is constantly added to the reaction vessel and corresponding flow of fermented medium (product) is removed to keep the reaction volume constant. Moreover, the balance between feed and discharge is maintained for long and sufficient time to achieve steady-state operation with no changes in the onditions within the reactor. When compared to a batch system, continuous system's mode of operation offers a reduced vessel down time for cleaning and filling by providing volumetric productivity that can translate into smaller reactor volumes and lower capital investment plus ease of control at steady state (Brethauer & Wyman 2010; Olsson & Hahn-Hägerdal 1996).

2.6.2.2. Solid state fermentation

Solid state fermentation (SSF) is considered a three phase heterogeneous process comprising of solid, liquid and gaseous phase. It offers potential benefits for the microbial cultivation for bioprocesses and product development (Gervais & Molin, 2003; Thomas *et al*., 2013). In comparison with submerged fermentation, SSF has low capital costs for equipment, high volumetric productivity, produces less waste water (effluent), also produces relatively high concentrated product and shows reduced operational costs, Fig. 2.17 (Zhuang *et al*., 2007; Pandey, 2003; Pandey *et al*., 1999; Dueňas *et al*., 1995). In SSF the substrate is a solid material and the

substrate differs in composition, chemical nature, mechanical properties, particle size, water retention capacity etc. The substrate must also possess sufficient moisture to facilitate assimilation of nutrients to support microbial growth (Thomas *et al*., 2013). The solid material could either provide necessary carbon and other nutrients or it may be an inert material impregnated with rich nutritional medium to support growth of microorganisms growing on it (Pandey, 2000; Thomas *et al*., 2013). An example of the most utilised substrate in SSF is agro-industrial residues, which are of low cost and attractive for bioprocessing (Thomas *et al*., 2013). In the case of lignocellulosic substrate, it is generally a practice to pretreat the material either chemically or mechanically to make the substrate accessible for microbial growth (Pandey, 2000). The use of agricultural waste in bioprocesses may help to mitigate environmental problems caused by waste disposal (Reddy *et al*., 2003).

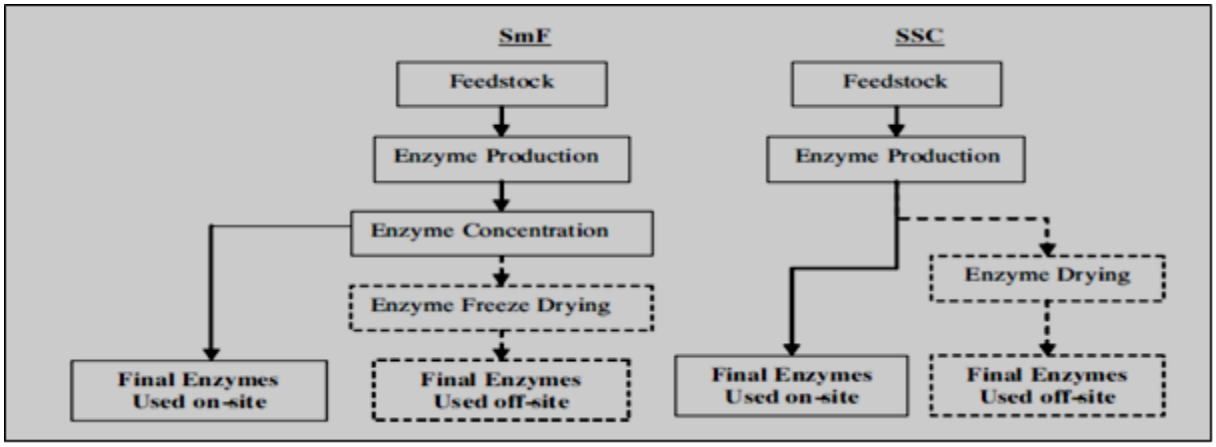

Figure 2.9. Flow charts of enzyme production using submerged fermentation (SmF) method compared to solid state fermentation, SSF (Zhuang *et al*., 2007).

Bacteria (*Bacillus sp*, *Pseudomonas sp* etc.), yeasts (*Endomicopsis burtonii*, *Schwanniomyces castelli* etc) and fungi (*Aspergillus sp*, *Fusarium sp*, *Trichoderma sp* etc) can grow on solid substrate; however, the filamentous fungi are the best adapted microorganisms (Raimbault, 1998). The hyphal mode of growth enables the fungi to penetrate into the pores or intra-particles of the solid substrate to access the available nutrients and secrete hydrolytic enzymes (Raimbault, 1998). Fungi grow better in the undisturbed substrate (non-stirred environment), hence this makes unstirred SSF the best choice (Lee, 1997) and the higher product titres in SSF are due to the fact that the process mimics the natural habitat of the microorganisms,

and the activity is increased (Pandey, 2003). The main enzymes produced by lignocellulolytic fungi are cellulase, hemicellulose, pectinase and lignases (Tengerdy & Szakacs, 2003). *Trichoderma* and *Aspergillus* species have been used the most for the production of cellulase, xylanase, pectinases, etc. while amylolytic enzymes have been produced by *Aspergillus* and *Rhizopus* (Pandey *et al*., 1999).

A major challenge in SSF is the mixing ability~~,~~ and high viscosity that results in great shear force that damage microbial cells during growth. The water mass transfer is strongly related to other parameters such as aeration and temperature. Aeration plays a crucial role in the attempt to improve microbial growth by controlling water content (therefore, water activity), removal of volatile compounds, and CO_2 together with the heat generated during the metabolism while providing O_2 for aerobic growth (Gervais & Molin, 2003). It is difficult to monitor and control parameters such as pH, temperature, dissolved gas etc. than in submerged fermentation (Lee, 1997).

Temperature directly influences microbial growth and secondary metabolite formation. During SSF, high heat accumulates in the process, which results in early cessation of growth and affects product formation. Solid state fermentation can be classified based on inoculum type; single (pure culture) or mixed culture. In pure culture, a single strain is used in fermentation whereas in a mixed culture using different microorganisms for biological processes, e.g. enzyme production or bioconversion of agro-industrial materials, different cultures are used (Bhargav *et al*., 2008). In monoculture, *A*. *fumigatus* SK1 cultivated on untreated oil palm trunk produced cellulolytic and xylanase enzymes (Ang *et al*., 2013). *T*. *longibrachiatum* produced xylanase activity of 592.7 U/g substrate (Azin *et al*., 2007).

In an effort to improve the production of hydrolytic enzymes, several studies investigated the effect of using mixed substrate and mixed culture amongst others to enhance the production levels and activities of the enzymes. In some instances, combinations of mixed substrate and mixed culture are used in SSF. Kilikian *et al*. (2014) reported cellulase activity of 10.6 FPU/g produced by *Myceliophthora thermophile* M77 grown on mixed substrates of soybean bran and sugarcane bagasse (10:90), initial moisture of 80% at 45 °C. The activity was 4.4 times higher than the production on a single substrate, wheat bran (Kilikian *et al*., 2014).

In mixed culture SSF, microbial strain compatibility is a critical factor and it has to be established case by case for each application. Once the microbial compatibility is established a cheaper substrate may be used in mixed culture SSF for production of enzyme without compromising on enzyme yields obtained in the case of single culture fermentation (Gutierrez-Correa *et al*., 1999). Dueňas *et al*. (1995) had found that co-culturing of *Trichoderma reesei* LM-UC4 and *Aspergillus phoeniers* QM239 in SSF yielded cellulase activity of 28 U/L.h whilst 10 U/L.h was attained in single SSF. Again, SSF by *T*. *reesei* LM-UC4 also lacked β-glucosidase activity (Dueňas *et al*., 1995). Co-culturing of *T. reesei* and *A*. *oryzae* resulted in maximum cellulase activity of 10.7 FPU/g.ds and β-glucosidase of 10.7 U/g.ds under the optimal conditions at 30 °C, pH 5 and 70% of moisture level (Brijwani *et al*., 2010).

2.7. Ethanol production

Ethanol (CH_3CH_2OH) is a liquid substance that is colourless, volatile and it has a slight odour. Ethanol production can be achieved by three processes (John, 1969 in Demirbaş, 2005) such as (1) chemical production – which involves hydration of alkanes (synthetic process), (2) biochemical process – involving fermentation of carbohydrates, and (3) hydrolysis and fermentation of lignocellulosic hydrolysate. A distinction is drawn between ethanol and bioethanol. Ethanol is synthesised by hydration of ethylene and bioethanol is produced by fermentation of sugars from biomass (e.g. maize and sugarcane), and lignocellulosic biomass (forest residues and agricultural wastes) (Roozbehani *et al*., 2013).

2.7.1. Chemical production of ethanol (Synthetic production of ethanol)

The ethanol is produced through indirect and direct hydration processes using sulphuric acid (H_2SO_4). The ethanol produced using the above processes is non-renewable. The indirect process begins with the hydrocarbon feedstock containing 35 – 95% ethylene being exposed to 95 – 98% H_2SO_4 to form mono-sulphate and subsequently hydrolysed with sufficient water to produce 50 – 60% aqueous sulphuric acid solution. The ethanol is then separated from dilute sulphuric acid. The resulting acid is concentrated and recycled back to the process. This process is summarised by chemical reactions below (Demirbaş, 2005):

$$CH_2=CH_2 + H_2SO_4 \rightarrow CH_3CH_2OSO_3H \quad (1)$$

$$CH_3CH_2OSO_3H + H_2O \rightarrow CH_3CH_2OH + H_2SO_4 \quad (2)$$

Alternatively ethanol can be produced from acetylene process through direct hydration of ethylene in the present of a catalyst such as H_2SO_4 and $HgSO_4$ (mercuric sulphate) to form acetaldehyde. Acetaldehyde can be readily reduced by catalytic hydrogenation to ethyl alcohol. The process is summarised below:

$$C_2H_2 + H_2O \rightarrow CH_3CHO \quad (3)$$

$$CH_3CHO + H_2 \rightarrow CH_3CH_2OH \quad (4)$$

The synthetic process has some advantages; more ethanol volumes can be produced in a short time and requires little or no purification process steps. Global interest to reduce greenhouse gas emission led to increasing demand in the usage of renewable resource for ethanol production through biochemical processes (Demirbaş, 2005).

2.7.2. Biochemical production of ethanol (bioethanol)

Saccharomyces cerevisiae has been studied extensively for its ability to produce ethanol through glycolysis (Embden-Meyerhof-Parnas, EMP pathway) – a process by which one molecule of glucose is metabolised and two molecules of pyruvate are produced (Madigan *et al*., 2000). Fermentation of glucose by yeast to produce ethanol involves a series of coordinated enzymatic reactions, Fig. 2.18. The enzymes involved in the fermentation of glucose include hexokinase, (HK), phosphoglucoisomerase, (PGI), phosphofructokinase, (PFK), fructose bisphosphate aldolase, (FBPA), triose phosphate isomerase, (TPI), glyceraldehydes-3-phosphate dehydrogenase, (GAPDH), phosphoglycerate kinase, (PGK), phosphoglyceromutase, (PGM), enolase, (ENO), pyruvate kinase, (PYK), pyruvate decarboxylase, (PDC) and alcohol dehydrogenase, (ADH) (Madigan *et al*., 2000).

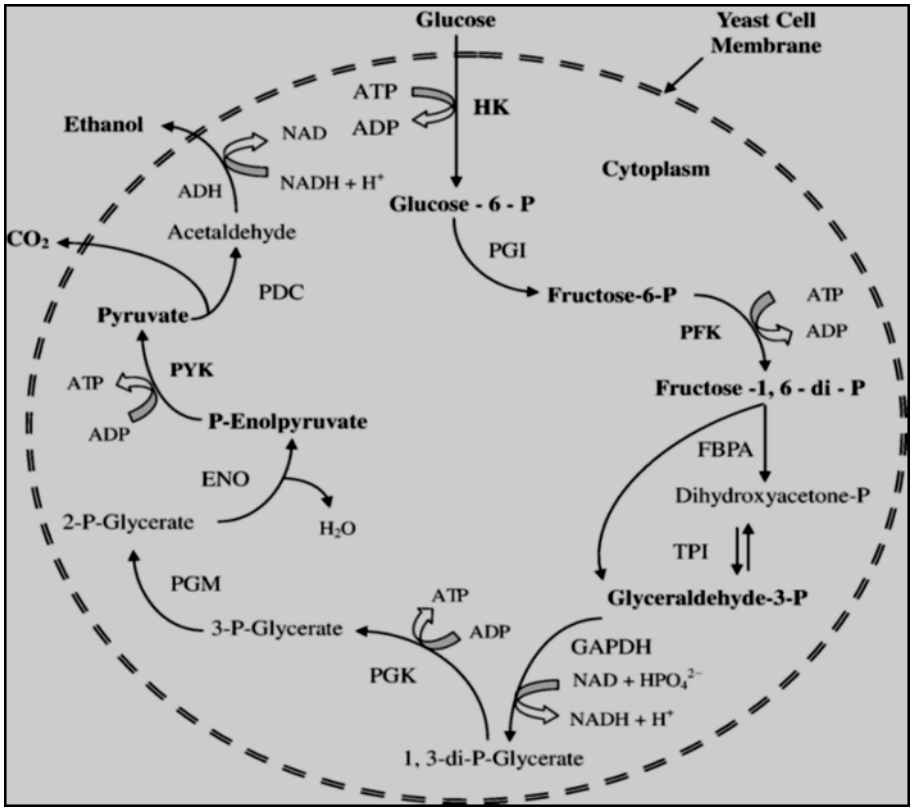

Figure 2.10. Metabolic pathway of ethanol fermentation in *S. cerevisiae* (Madigan *et al*., 2000).

The simplest form of reaction through which yeast produce ethanol is shown below (Balat, 2006):

(Glucose) $C_6H_{12}O_6 \rightarrow$ Ethyl alcohol $2(CH_3CH_2OH)$ + Carbon dioxide $2(CO_2)$

Under anaerobic conditions, the pyruvate is further reduced to ethanol with the release of CO_2. Other by-products that are produced during the process include glycerol (making up approximately 1% w/v), organic acids and higher alcohols at much lower levels (Bai *et al*., 2008; Ingledew, 1999). Theoretical yield is 0.511 g for ethanol and 0.489 for CO_2 on the basis of glucose metabolised. In the process, two ATPs produced are used to drive the biosynthesis of yeast cells which involves a variety of energy requiring reactions. Therefore, ethanol production is tightly coupled with yeast cell growth (Bai *et al*., 2008).

The sugar and starch crops are feedstock for first generation bioethanol while lignocellulosic is the feedstock for second generation bioethanol production. Bioethanol production from sugar based crops such as sugarcane, molasses, sugar

beet and sweet sorghum, involves direct assimilation of the simple sugars (glucose, fructose and sucrose) by the fermenting microorganisms and converting them into bioethanol. The fermentable sugars from sugar based crops are extracted by milling or crushing followed by fermentation (Devarapalli & Atiyeh, 2015). In contrast, starch and cellulose feedstock are made up of glucose units linked together by glycosidic bonds to form polymers and these feedstocks cannot be directly utilised to produce bioethanol by natural yeasts. A key step in the production of bioethanol from biomass is the conversion of complex carbohydrates (e.g. starch, cellulose and hemicellulose) through a process called saccharification to simple sugars that are fermented by microorganisms (Zhuang *et al*., 2007). Figure 2.19 summarises key processes involved in the bioethanol production through exploitation of renewable biomass such as sugar crops, starch crops and lignocellulosic material (Sriroth *et al*., 2012).

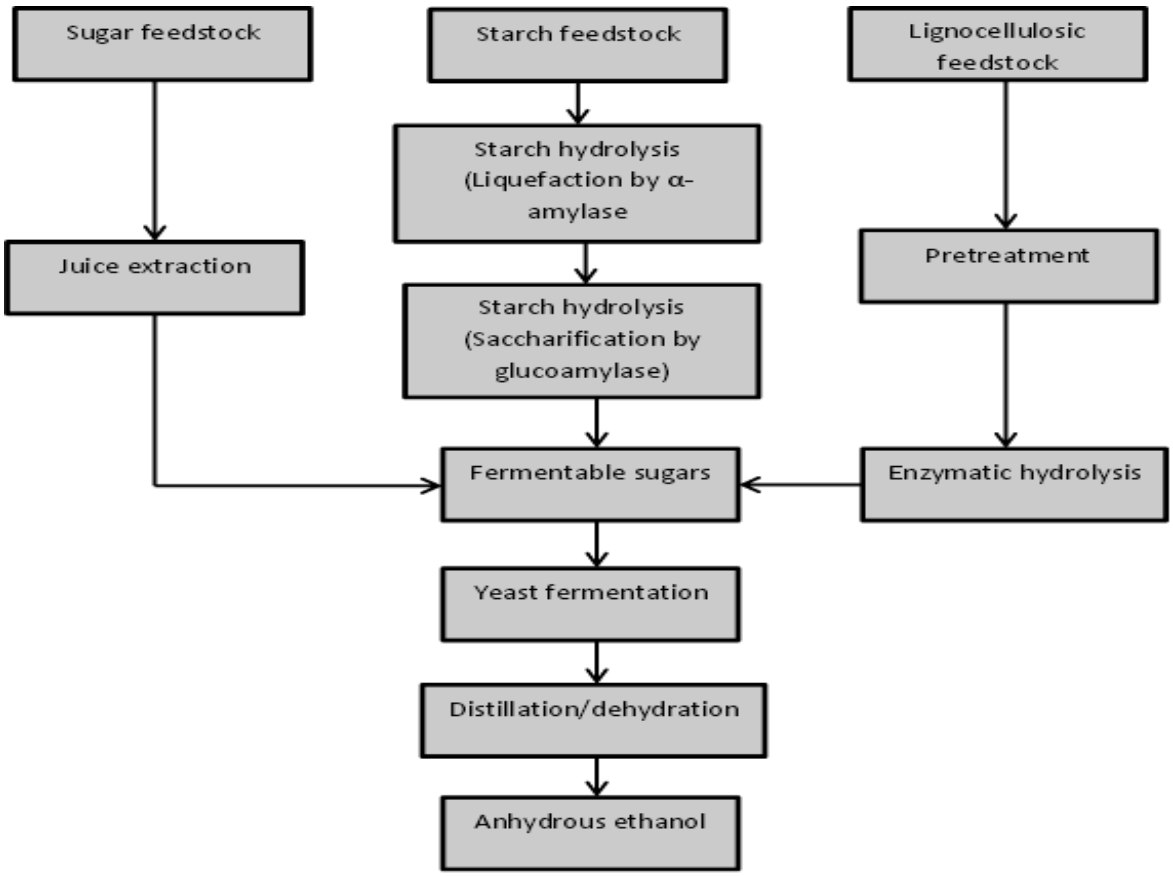

Figure 2.11. Schematic view of bioethanol production by fermentation process using sugar, starch and lignocellulosic feedstock (Sriroth *et al*., 2012).

2.7.2.1. Bioethanol production from sucrose containing materials

Sugar cane, either its cane juice or cane molasses is the most important feedstock used in tropical and sub-tropical countries for bioethanol production. European countries use beet molasses as a feedstock (Winner Network, 2002). The conversion of sucrose into ethanol is a simple process because it does not require hydrolysis step. The disaccharide (Sucrose, a dimer of fructose and glucose) can be broken down directly by the fermenting yeast cells and the other advantage is that conditioning of the cane juice or molasses favours the hydrolysis of sucrose (Cardona & Sánchez, 2007).

2.7.2.2. Bioethanol production from starch materials

Starch feedstock (corn and wheat) is most utilised for bioethanol production in North America and Europe. In tropical countries, other starchy crops as tubers (e.g. cassava) can be used for commercial production of bioethanol (Cardona & Sánchez, 2007). Starch is made up of 30% of long chains of D-glucose molecules linked together by α-1,4 glycosidic bonds(amylose) and about 70% of highly branched polymer with α-1,6 glycosidic bonds, amylopectin (Bothast & Schlicher, 2005; Badger, 2002). Bioethanol production from corn is a well-established technology (Devarapalli & Atiyeh, 2015). Starch is not directly assimilated and fermented by yeast.

The bioethanol production starts by corn undergoing either dry milling or wet milling process. Both starch obtained from the dry milling (producing about 67% of bioethanol) and wet milling making about 33% are treated the same way during the bioethanol production (Bothast & Schlicher, 2005). The conversion of starch to glucose begins firstly by thermostable α-amylase hydrolysing α-1,4 glycosidic linkage of starch polymer to soluble dextrins which is heated to over 100 °C to liquefy the mash. Secondly, glucoamylase with an optimum temperature of 65 °C is added to convert the liquefied starch to glucose. The resulting glucose can be readily fermented to bioethanol (Sriroth *et al*., 2012; Hahn-Hägerdal *et al*., 2006; Bothast & Schlicher, 2005). The last enzymatic stage (glucoamylase) can be performed either by separate hydrolysis and fermentation, SHF or simultaneous saccharification and fermentation, SSF (Bothast & Schlicher, 2005).

2.7.2.3. Bioethanol production from lignocellulosic material

Lignocellulose biomass is the most abundant raw material and a more complex substrate than starch (Lee, 1997). Lignocellulose comprises of cellulose, hemicellulose and lignin. The production of bioethanol using diverse conversion technologies and various renewable non-food biomass marks the commencement of sustainable energy (Devarapalli & Atiyeh, 2015). The use of non-food biomass for bioethanol production will require efficient utilization of the sugars present in lignocellulose, mainly glucose and xylose. Cellulose polymer, just like starch, consists of long chains of glucose molecules (6-carbon sugar) but with different structural configuration. On the other hand, hemicellulose is also comprised of long chains of sugar molecules (5-carbon sugars mainly xylose) and some glucose molecules (Demirbaş, 2005). Lignocellulosic hydrolysate contains a mixture of sugars with a high concentration of glucose (~ 70 – 100 g/L) and xylose (40 – 60 g/L). Different processes or strategies to obtain hydrolysate (fermentable sugars) and ultimately bioethanol are (1) Separate enzymatic hydrolysis and fermentation, SHF. (2) Simultaneous saccharification and fermentation, SSF. (3) Separate hydrolysis and co-fermentation, SHCF. (4) Simultaneous saccharification and co-fermentation, SSCF. (5) and consolidated bioprocessing, CBP.

i. Separate Hydrolysis and Fermentation

In SHF, the key advantage is that both hydrolysis and fermentation occur under the optimum conditions of each stage, while the drawback is the inhibition of cellulase activity by cellobiose and to a lesser extent by glucose. Various attempts to reduce inhibition of cellulase include the use of high concentration of enzyme, the supplementation of β-glucosidase during hydrolysis and removal of accumulated sugars by simultaneous saccharification and fermentation (Sun & Cheng, 2002).

ii. Simultaneous Saccharification and Fermentation

Simultaneous saccharification and fermentation is a batch system; it requires compatible fermentation and saccharification with a similar pH, temperature and optimum substrate concentration. This necessitates the use of thermo-tolerant yeasts capable of fermenting glucose to ethanol at a temperature above 40 °C,

which is closer to optima for the activity of the cellulase (Ballesteros *et al*., 2004). The optimum temperature for cellulase is often between 45 and 50 °C, whereas *S. cerevisiae* is becoming inactive at more than 40 °C (Taherzadeh & Karimi, 2007).

A commercial *Saccharomyces cerevisiae* had been reported to have produced an ethanol yield of 72.4% in SSF, which corresponded to 20.5 g/L ethanol (Öhgren *et al*., 2007). A similar ethanol yield of 71.2% by thermotolerant yeast, *Kluyveromyces marxianas* CECT10875 from 10% substrate concentration and an enzyme loading of 15 FPU/g substrate (Ballesteros *et al*., 2004). Zhang *et al*. (2013) reported ethanol yield of 69.49% under optimised condition, 37.5 °C, enzyme loading 30 FPU/g substrate, yeast concentration of 10 g/L and pH 4.6. In SSF the risk of contamination is minimised by the presence of accumulating ethanol during the process. SSF process has lower capital costs compared to SHF, since it requires a single vessel (Taharzadeh & Karimi, 2007). SSF showed distinct advantage over SHF with respect to reduction of total time required for the yeast to produce ethanol from wheat straw by 57% and 53% in non-detoxified and biodetoxified wheat straw hydrolysate, respectively (Saha *et al*., 2013)

Through evolutionary adaptation of an *S*. *cerevisiae* strain a significant improvement in fermentation performance was attained with ethanol titer of 71.4 g/L and ethanol yield of 80.34% at 37 °C and 30% solids content (Qureshi *et al*., 2015). Thermotolerant yeast *Klyveromyces marxianus* DBTIOC-35 is a promising strain for SSF because of its capability to produce high titer of ethanol at temperatures above 40 °C with maximum fermentation occurring at 45 °C (Saini *et al*., 2015b). *S*. *cerevisiae* lacks the ability to utilize xylose and arabinose during fermentation (Margeot *et al*., 2009). This high xylose and high cost of hydrolytic enzymes have hampered cellulosic ethanol production at a commercial scale (Yang *et al*., 2011; Ha *et al*., 2011). The high residual xylose is due to “glucose repression effect” that occurs during sequential assimilation of glucose then xylose and also as a result of the intolerance of fermenting microorganism to the already accumulated bioethanol. These effects are significant barriers to complete utilisation of mixed sugars in cellulosic hydrolysates (Ha *et al*., 2011).

Yeasts such as *Candida shehatae*, *Pachysolen tamophilus* and *Pichia stipitis* can ferment xylose. However, the ethanol yields and productivity by the natural pentose

fermenting yeasts are often significantly lower than the glucose fermenting *S. cerevisiae*, implying the need for improvement in xylose fermentation. Xylose transport and xylose reductase (XR) are key targets for improvement (Chu & Lee, 2007). Improvement through adaptation strategy by *S. cerevisiae* in xylose to enhance metabolism have been reported by Sonderegger & Sauer, (2003). The ethanol yields obtained through adaptation strategy are still low, 0.14 g/g (Pitkanen *et al*., 2005).

Since the pentose sugar (xylose) constitutes the second highest percentage of sugars available in the hydrolysate, its fermentation to bioethanol becomes important for the efficiency and the economics of cellulosic bioethanol. To realise the economic benefits of xylose fermentation, several microorganisms have been genetically engineered to improve the fermentability of the sugar (Demirbaş, 2005). While many microorganisms can convert glucose efficiently into ethanol, the conversion of pentose remains relatively inefficient. Therefore, it is imperative that the potential microorganisms involved in the bioconversion efficiently convert both hexoses and pentoses present in lignocellulosic hydrolysate into desired end products, e.g. bioethanol (Chu & Lee, 2007). Co-fermentation of both hexose and pentose through SHCF, SSCF and CBP could address the inefficient utilisation of pentose sugar.

iii. Separate hydrolysis and co-fermentation

Separate hydrolysis and co-fermentation are similar to SHF in that the conditions for hydrolysis are and co-fermentation are performed under independently different optimised state. The drawback of this process is initial high sugar concentration which when fermented might lead to high ethanol being produced. The high ethanol inhibits yeast performance thereby indirectly suppressing the utilisation of xylose by the fermenting yeast. Nielsen *et al*. (2016) had a strategy to improve the utilization of xylose by two stage fermentation. The first stage was to preferment the xylose rich hydrolysate of steam pretreated wheat straw separately followed by fed-batch fermentation with feed hydrolysate containing glucose. This had resulted in an ethanol yield of 0.423 g/g and xylitol yield of 0.036 g/g.

iv. Simultaneous Saccharification and Co-fermentation

An improvement to the SSF process is simultaneous saccharification and co-fermentation (SSCF), in which co-fermentation refers to the fermentation of both 6-carbon and 5-carbon sugars to ethanol (Teixeira *et al*., 2000). During lignocellulosic hydrolysate fermentation, the yeast *S*. *cerevisiae* preferentially uses glucose in preference to xylose and consequently more xylose remains unutilised. Katahira *et al*. (2006) constructed a recombinant yeast strain that can ferment xylose and cellooligosaccharides by integrating genes for intracellular expression of xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Pichia stipitis* and xylulokinase (XK) from *S*. *cerevisiae* and a gene for β-glucosidase (BGL I) obtained from *Aspergillus acleatus* expressing β-glucosidase on the cell surface. The recombinant strain was able to ferment both xylose and cellooligosaccharides making up total sugar of 73 g/L after 36 hours and produced 30 g/L ethanol.

Another challenge is that during simultaneous saccharification and fermentation the effective enzymatic hydrolysis is inhibited/or hampered by the accumulation of cellobiose and the compromised chosen temperature for the process (Hu *et al*., 2016). Traditionally, accumulation of cellobiose during saccharification is minimised by the addition of β-glucosidase to convert cellobiose to glucose, then ethanol is produced by the fermenting microorganism. Several strategies to overcome the bulk of residual xylose and accumulated cellobiose have been developed by several researchers. For instance, Ha *et al*. (2011) had developed a strategy to promote co-fermentation of hexose and pentose sugars by *S*. *cerevisiae*. This was achieved by combining an efficient xylose utilisation pathway with cellobiose transport system in order to by-pass problems associated with glucose repression, Fig. 2.20. The engineered yeast was able to co-ferment the two non-fermentable sugars present in lignocellulose hydrolysate synergistically into ethanol. The improved ethanol productivity had advanced the fermentation economics of lignocellulosic hydrolysate and may reduce the enzyme usage (including addition of β-glucosidase) and overall costs associated with cellulosic saccharification process (Ha *et al*., 2011). A thermo-tolerant industrial *S*. *cerevisiae* SyBE001603 capable of assimilating cellobiose at 42 °C was constructed. The strategy helped in expressing the genes encoding cellobiose transporter (CDT), which was discovered in *Neurospora crassa* and BGL

into the yeast. Overexpression of such genes accelerated cellobiose utilisation and the overall improvement also depended on the strain background (Hu *et al*., 2016).

Recently, Ko *et al*. (2016) reported that *S*. *cerevisiae* SXA-RZP-E strain expressing xylose isomerase had shown efficient co-fermentation of lignocellulosic hydrolysate with ethanol yield improvement ranging from 0.43 to 0.46 g/g sugar. Successful conversion of xylose concomitantly with glucose could possibly reduce the cost of bioethanol production.

v. Consolidated bioprocessing

The cost of cellulases associated with capital investment in the process can be reduced by another strategy called consolidated bioprocessing (CBP) process, which increases volumetric productivity by integrating cellulase production, cellulose hydrolysis and ethanol production in a single step process (Zhang & Zhang, 2013; Lynd *et al*., 2008). Consolidated bioprocessing requires a microorganism that combines properties that relate to both substrate utilisation and product formation. The desired substrate utilisation properties include the production of a hydrolytic enzyme system to facilitate high rates of hydrolysis and utilisation of the resulting hydrolysis products under anaerobic conditions. The desired product formation properties would include high product selectivity and concentrations (Lynd *et al*., 2002).

An engineered wine yeast strain K1-V1116 with genes encoding cellulase, β-glucosidase I (BGL I) from *A*. *aculeatus*, endoglucanase II (EG II) and cellobiohydrolase II (CBH II) both from *T*. *reesei* were able to ferment 63% of cellulose in 96 hours and produced 2.6% (v/v) of bioethanol titer (Khramtsov *et al*., 2011). Engineering cellulolytic enzymes with improved catalytic efficiency and enhanced thermo-stability will be economical key to commercialisation of lignocellulosic bioethanol (Zhang & Zhang, 2013). du Plessis, (2008) constructed *S. cerevisiae* strain co-expressing cellulase genes, endoglucanase I and II (egI or Cel5A and edII or Cel7B) of *Trichoderma ressei* QM6a for efficient hydrolysis of amorphous cellulose. The strain expressed a satisfactory level of cellulase activity. However, when additional synthetic codon optimised cellobiohydrolase gene (sCBHI) from *T*. *reesei* and β-glucosidase (*bgl*I) from *Saccharomycopsis fibuligera* were

introduced in *S*. *cerevisiae*, the overall cellulase activity was affected and the recombinant strain failed to produce sufficient glucose when grown on cellulose, therefore no ethanol produced (du Plessis, 2008). *T*. *reesei* has been considered to be an agent of CBP due to its ability to produce sufficient cellulases and it is already commercially established. However, the challenge with *T*. *reesei* is its low ethanol yield, production rate and ethanol tolerance (van Zyl *et al*., 2011; Xu *et al*., 2009).

The inefficient production of ethanol by *T*. *reesei* is not as a result of the absence of the relevant genes and pathways for efficient conversion process but notably due to the low expression of the genes or the activity of the enzymes encoded by the genes in the bioethanol pathway (Xu *et al*., 2009). Due to the need for microorganisms possessing high tolerance towards high ethanol and sugar and perhaps inhibitors that may be produced in the process, a filamentous fungi such as *Fusarium oxysporum* was proposed as a commercial competitive CBP agent or components of its enzymatic arsenal contributing to the development of robust CBP agent (Ali *et al*., 2016). Success in CBP development has the potential to lower the cost of biomass processing by eliminating operational and capital costs associated with specific enzyme production and more effective biomass solubilisation (Olson *et al*., 2011).

Currently, the CBP systems have improved hydrolysis and fermentation efficiencies, eliminated addition of hydrolytic enzymes and inhibition effects while requiring low energy for biological conversion of lignocellulosic biomass to biofuels (Mbaneme-Smith & Chinn, 2015). Recently, Yang *et al*. (2016) constructed a recombinant *sestc S*. *cerevisiae*, a strain which harboured a single-enzyme system three cellulase gene (*sestc*) by protoplast method to improve cellulase expression. The total activity (i.e. cellulase activity, endoglucanase, exoglucanase and xylanase) of the recombinant strain was 1.1 (27.5-fold), 378 (63-fold), 1.44 (24-fold) and 164 U/mL (19-fold), which was higher than the activity of the wild type. The engineered *S*. *cerevisiae* strain produced 8.1 g/L of ethanol, a 57.86-fold higher than 0.14 g/L of ethanol produced by wild type strain. Liu *et al*. (2016) also constructed *S*. *cerevisiae* that displayed BGL I, EG, CBH I and CBH II on the cell surface through a series of rational design. The cellulose-adherent strain displayed cellulose degradation mechanisms which differed from the free form cellulase mechanism. The strain directly produced ethanol of about 1.3 g/L while the wild type produced no ethanol in high-density cellulose

(100 g/L of MC6 substrate). A 7-fold increase in ethanol production was achieved with addition of 1.0 FPU/g biomass C-Tec2 leading to 18 g/L ethanol yield (Liu *et al*., 2016).

Overall, the bioethanol production from lignocellulosic biomass includes the following: biomass pretreatment, cellulose hydrolysis, fermentation of sugar (hexoses and/or including pentoses), separation and effluent treatment. All the fermentation strategies/techniques applicable to lignocellulosic hydrolysate are illustrated in Fig. 2.20 (Cardona & Sánchez, 2007). This includes SHF whereby co-fermentation of hexose and pentose occurs, SSF, SSCF and CBP. It is also important to note that commercialisation of bioethanol produced from lignocellulosic biomass is held back by the cost of the enzyme as well as their hydrolytic efficiency (Sindhu *et al*., 2015).

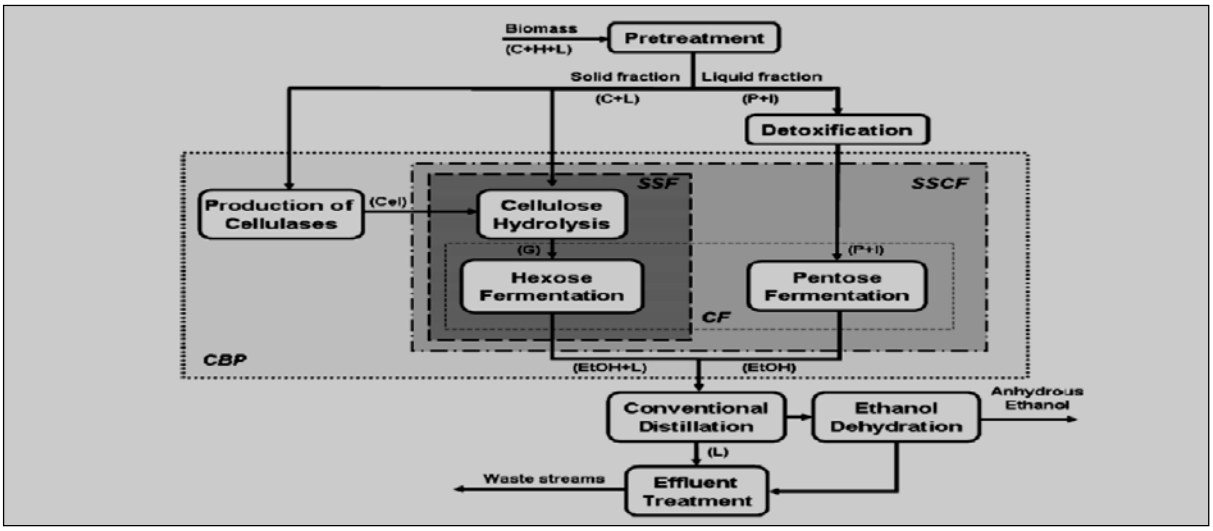

Figure 2.12. Generic block diagram of bioethanol production from lignocellulosic biomass. Possibilities for reaction-reaction integration are shown inside the shaded boxes: CF, co-fermentation; SSF, simultaneous saccharification and fermentation; SSCF, simultaneous saccharification and co-fermentation; CBP, consolidated bioprocessing. Main stream components: C, cellulose; H, hemicellulose; L, lignin; Cel, cellulase; G, glucose; P, pentose; I, inhibitors; EtOH, ethanol (Cardona & Sánchez, 2007).

2.7.3. Factors affecting bioethanol production from lignocellulosic biomass

During ethanol fermentation various stress factors such as nutrients deficiency, high temperature, pH<3.5, osmotic pressure (>25% sugar w/v), contamination and other yeast metabolic products such as ethanol accumulation, and organic acids affect the yeast fermentative strength. During the late phases of ethanol fermentation, these factors act synergistically and severely affect yeast cells leading to reduced yeast viability and vigor, as well as ethanol yield (Zabed *et al*., 2014; Ingledew, 1999).

The process of lignocellulosic bioethanol production generates inhibitory products during biomass pretreatment. The inhibitory compounds include phenolics, furans (furfurals and 5-Hydroxymethylfurfural, HMF), aliphatic acids and inorganic compounds. 5-Hydroxymethylfurfural is formed from the degradation of hexoses and furfural is formed from the degradation of pentoses (Kupiainen *et al*., 2014; Lenihan *et al*., 2010). Other compounds such as acetic acid and uronic acids are released from acetyl groups of hemicellulose and other acids such as formic (FA) and levulinic acids (LA) resulting from sugar degradation acidify hydrolysate as shown in Fig. 2.21 and eventually inhibit downstream biochemical processes (Jönsson & Martín, 2016; Kupiainen *et al*., 2014).

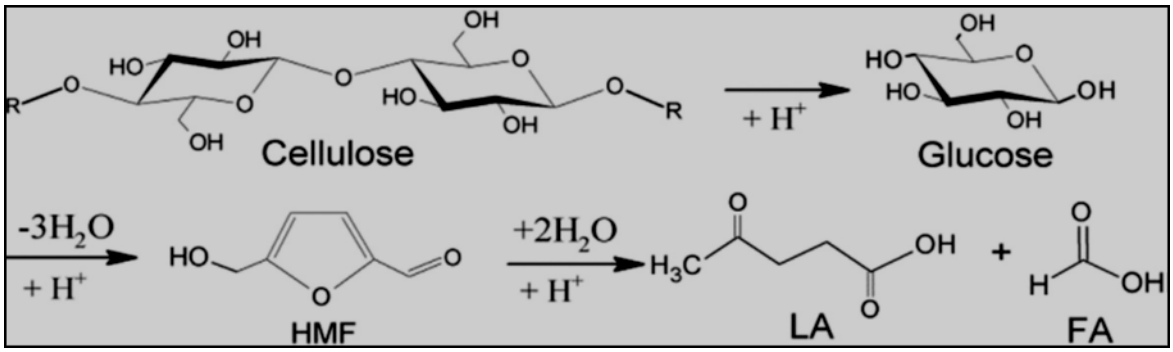

Figure 2.13. Illustrate the formations of inhibitory compounds during acid hydrolysis (Kupiainen *et al*., 2014).

Aromatic carboxylic acids are found within the group of the phenyl compounds which include both phenolic aromatic carboxylic acids such as ferulic acid and 4-hydroxybenzoic acid, and non-phenolic aromatic carboxylic acids like cinnamic acid. However, the aromatic carboxylic acids are present in the lignocellulosic hydrolysate in relatively low concentrations, but these compounds do have stronger inhibitory

effects on microbial growth than the aliphatic carboxylic acids (Jönsson & Martín, 2016). These inhibitory compounds slow down the yeast and other fermenting microorganism's metabolism and reduce the organism's glycolytic enzymatic activity leading to a premature stop of the fermentation process. It has been postulated that the compounds enter the cell's nucleus and bind to the replicating deoxyribonucleic acid (DNA) and arrest cell growth and reproduction (Lenihan *et al*., 2010).

Other compounds such as lignin derived aromatic aldehydes have relatively high toxicity at low concentration and the effects of these compounds as well as other aromatic compounds on fermenting microorganism vary and can also be predicted based on the functional groups (Jönsson & Martín, 2016). The levels of all these inhibitory compounds depend on the feedstock type and severity of the pretreatment.

In order to overcome these limiting factors for lignocellulosic bioethanol, such inhibitory compounds can be removed by microbial detoxification process (Parawira & Tekere, 2011) and through membrane filtration system or develop a robust fermenting microorganism(s) through adaptation strategies in the presence of inhibitory compounds. Ethanol accumulation during fermentation has an inhibitory effect on the fermenting microorganism.

2.7.4. Effects of ethanol on *Saccharomyces cerevisiae*

The advent of a robust CBP strain with a super hydrolysis machinery and high fermentation performance in high-density substrate will mean high ethanol level can be attained. Ethanol tolerance by fermenting microorganisms is an important trait in the economy of the bioethanol industry. High ethanol tolerance by yeast strain is prerequisite for high fermentation efficiency and consequently high ethanol yield (Hu *et al*., 2007). During ethanol fermentation various stress factors such as nutrient deficiency, high temperature, pH<3.5, osmotic pressure (>25% sugar w/v), contamination and other yeast metabolic products such as ethanol accumulation, and organic acids affect the yeast fermentative strength. During the late phases of bioethanol fermentation, these factors act synergistically and severely affect yeast cells leading to reduced yeast viability and vigor, as well as ethanol yield (Ingledew, 1999).

Understanding the mechanisms through which ethanol inhibits yeast cells is fundamental to exploiting the full potential of the yeast strains and the optimisation of fermentation process (Bai *et al*., 2008). The effects of ethanol on the fermenting microorganisms are multifaceted as illustrated in Fig. 2.22 and the response by *S. cerevisiae* are equally complex with alterations in gene expression enhancing the frequency of peptide mutations, altering metabolism, and denaturing intracellular proteins. The glycolytic enzymes and plasma membrane composition also change and lowering the rate of protein accumulation (Tran *et al*., 2011; Hu *et al*., 2007; D'Amore & Stewart, 1987).

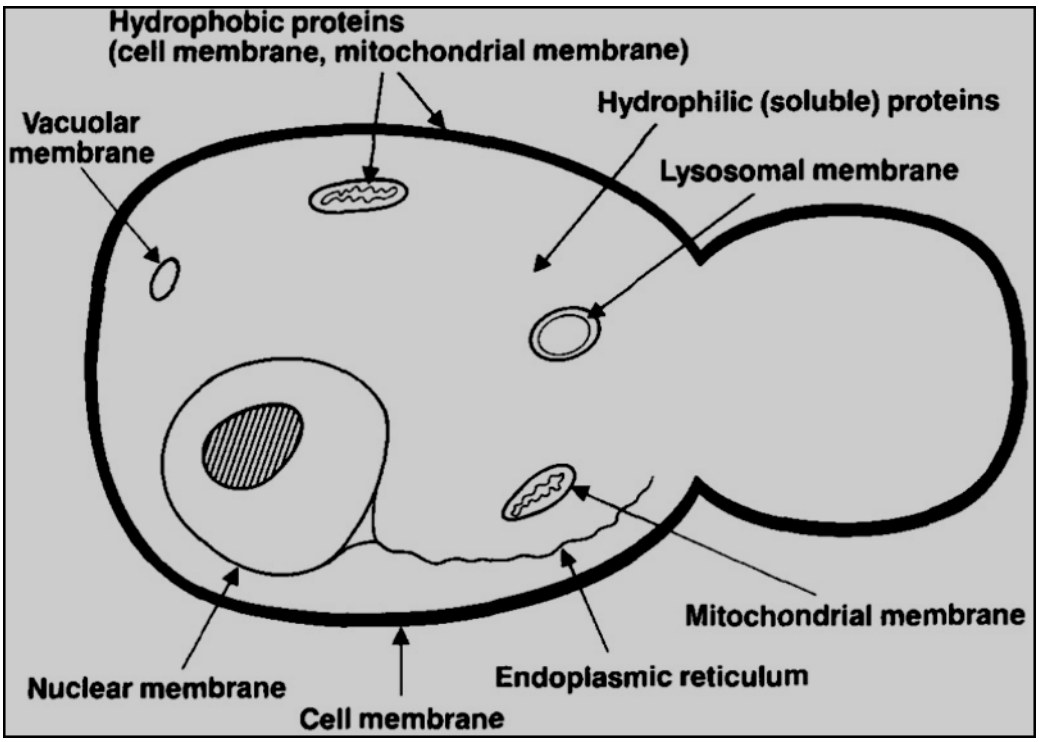

Figure 2.14. The complex phenomenon about ethanol inhibition with some possible sites in yeast cells at which ethanol could exert a significant influence on the yeast's vigor (D'Amore & Stewart, 1987).

A generally accepted view amongst many studies is that the mechanism of ethanol inhibition targets the membranes of some organelles and cells (D'Amore & Stewart, 1987). The membrane fluidity, which is related to its lipid composition, is altered in the presence of ethanol and consequently, membrane permeability to some ions (especially H^+) is significantly affected. As ions enter the cell, there is dissipation of the electro-chemical gradient across the membrane which in turn affects the

formation and maintenance of the proton driving force with a subsequent decrease in intracellular pH (Basso *et al*., 2011). Other effects of ethanol on yeast physiology during fermentation include growth inhibition and enzymatic inactivation which leads to a decreased cell viability (Basso *et al*., 2011

Traditionally, the improvements of ethanol production relied on mutagenized pool of a single strain (Thammasittirong *et al*., 2013), which potentially limit the effectiveness of the method (Snoek *et al*., 2015). Thammasittirong *et al*. (2013) improved ethanol tolerance of *S*. *cerevisiae* NRI by random UV-C mutagenesis. The mutant *S*. *cerevisiae* URNR56 produces a maximum ethanol concentration of 10.3% (v/v), ethanol productivity 1.7 g/L.h and theoretical yield of 98.7% from molasses medium at 37 °C, while for the *S*. *cerevisiae* NRI's (wild type) produced less ethanol with the corresponding values of 8.6% (v/v), 1.4 g/L.h and 83.3%, respectively.

Due to continued growing interests in research towards understanding the metabolic processes and the pathways associated with ethanol tolerance in *S*. *cerevisiae*, a system based network approach to ethanol tolerance was developed (Kasavi *et al*., 2014). With system based network approach 17 candidate genes with unknown biological functions were identified. Four genes (YDR307W, YHL042W, YPL264L and YMR215W) were randomly selected for functional test and deletion of YDR307W and YHL042W showed improved ethanol tolerance in the strains, whereas deletion of the other two genes YPL264L and YMR215W resulted in decreased cell viability when exposed to ethanol treatment for an extended period (Kasavi *et al*., 2014).

Recently, Snoek *et al*. (2015) explored a novel robot-assisted strategy that allows genome shuffling of multiple heterothallic parental yeast strains on a large scale. The strategy allowed selection of eight hybrids exhibiting superior fermentation performance over the commercial biofuel strain Ethanol Red. The best hybrid strain H1 fermented 32% (w/v) of glucose completely, yielding 18.7% (v/v) ethanol with ethanol productivity of 0.9 g/L.h and ethanol yield of 0.45 g/g glucose (Snoek *et al*., 2015). Therefore, the availability of yeast strains with increased fermentation performance could help to increase the productivity and economic viability of bioethanol production (Snoek *et al*., 2015).

CHAPTER 3

Screening and selection of fungi that produce cellulase and to optimise the production conditions in submerged fermentation

3.1. INTRODUCTION

Cellulase enzymes system are produced by a wide spectrum of microorganisms found in nature. Soil is inhabited by a large variety of microorganisms including bacteria, actinomycetes, algae and fungi. Microorganisms form a major component of soil (Sharada *et al*., 2013) and are associated with compost materials. Plant compost, for example, it is inhabited by microorganisms capable of degrading lignocellulosic polymers. The complete depolymerisation of cellulose requires synergistic activity between cellulolytic and non-cellulolytic microorganisms (Beguin & Aubert, 1994). The term “Cellulase enzymes system” refers to multiple enzymes involved in the hydrolysis of cellulose namely; exoglucanase (exo-1,4-β-glucanases, EC 3.2.1.91) endoglucanase (endo-1,4-β-glucanases, EC 3.2.1.4) and β-glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21). Screening and isolation of microorganisms that are producing cellulases are one of the pivotal ways of discovering novel cellulase enzymes with diverse properties (Juturu & Wu, 2014). Current commercial cellulases are mainly produced by *Trichoderma* and *Aspergillus* species. *Trichoderma* produces large quantities of endoglucanase, exoglucanase and relatively low levels of β-glucosidase (Schuster & Schmoll 2010), whereas *Aspergillus* produces large quantities of endoglucanase, β-glucosidase and low levels of exoglucanase (Ward *et al*., 2006).

Cellulose is a simple linear organic polymer of β-1-4 linked glucopyranose units. Cellulose chains have varying degrees of polymerization (DPs). In primary cell walls, the DP range is 5000 – 7500 glucopyranose units, whereas the DP in secondary cell walls is approximately 10000 and 15000 (O’Sullivan, 1997). Cellulose is abundantly available in materials, such as agro-wastes, municipal wastes, forest residues, *etc*. Enzymatic hydrolysis of cellulosic biomass to soluble fermentable sugars could potentially be a sustainable approach to develop an alternative fuel source and reduce global dependence on non-renewable fossil energy (Kuhad *et al*., 2011).

Enzymatic hydrolysis of cellulosic biomass is preferred over acid hydrolysis due to the absence of sugar degradation in the enzymatic process. The complete enzymatic hydrolysis of cellulosic biomass requires synergy between endoglucanases, exoglucanases and β-glucosidases. This action is often referred to as total cellulase, of which the activity is measured using insoluble substrates such as the Whatman No. 1 filter paper, cotton linter, microcrystalline cellulose (Avicel) or bacterial cellulose (Zhang *et al*., 2006). Endo-β-1-4 glucanase cleaves cellulose internally at the β-1-4 glycosidic linkage releasing oligosaccharide chains of different lengths, whereas exo- β-1-4 glucanase cleaves the oligosaccharide chains from either the reducing or non-reducing ends to release di-saccharides, trio-and tetra-saccharides. β-glucosidase cleaves the β-1-4 linkage in the di-saccharide (cellobiose) to release glucose molecules. It can also hydrolyse very short chains of β-1,4 oligoglucosides up to cellohexaose, but the reaction rate decrease with chain length (Saini *et al*., 2015a; Bhat & Bhat, 1997; Beguin & Aubert, 1994).

Technology for the utilization of cellulosic biomass for the production of bioethanol is progressing slowly due to high production cost of cellulases, the recalcitrant nature of cellulosic biomass and inefficient cellulases for the release of high levels of fermentable sugars (Zhuang *et al*., 2007). Research efforts have been undertaken to improve the efficiency of the known enzymes, identify new enzymes, and optimise enzyme mix preparations for cellulosic biomass hydrolysis (Merino & Cherry, 2007). Improving fungal hydrolytic activity and finding stable enzymes that are tolerant to extreme conditions have become a priority (Dashtban *et al*., 2009).

Agricultural composts are also the habitat of a variety of cellulose degrading microorganisms. Banana by-products, such as leaves, rachis and pseudostem contain high levels of cellulose (Li *et al*., 2010; Abdul Khalil *et al*., 2006). These cellulose rich materials are discarded after the harvest of the banana fruit and left to decay either in the plantation site or at the dumping site (Meena *et al*., 2015; Li *et al*., 2010). The decaying of plant material is facilitated by microorganisms, hence this study aimed to isolate fungi that secrete significant levels of cellulase system (endoglucanase, exoglucanase and β-glucosidase) with ability to hydrolyse cellulose obtainable from banana waste to fermentable sugars for the bioethanol industry. In

this study, a stringent isolation method has been applied to selectively isolate fungal species that produce cellulase enzyme system for the bioethanol industry.

3.2. MATERIALS AND METHODS

3.2.1. Sample collection and screening of fungi that produce cellulase

Upper soil samples were collected in clean plastic bags from decomposed banana agro-waste (dumpsite and plantations) at the Tzaneen Allesbeste farm, Limpopo, South Africa and from a decomposed *Strelitzia alba* plant at the University of Limpopo campus, South Africa. Fungal isolation was done by the serial dilution method. Three grams of soil from each site were suspended in 50 mL sterile distilled water. A 100 µL aliquot of soil suspension, after been diluted ten times, was spread plated onto a selective Avicel agar medium (2% Avicel, 1.5% agar, 5 mL chloramphenicol (50 mg/mL) and 0.67% Yeast Nitrogen Base (YNB) without amino acids). The Avicel agar plates were incubated at 30 ˚C until fungal growth was evident. The fungi were purified by the hyphal tip method. The tip (or edge) of mycelia growth was cut using a sterile scalpel and sub-cultured onto agar plates containing chloramphenicol to inhibit bacterial growth for three passages (Núñez - Trujillo *et al*., 2013; Ibatsam *et al*., 2012). The fungal isolates were maintained at 4 °C on malt extract agar (30 g/L malt extract, 20 g/L dextrose, 3 g/L peptone and 15 g/L agar).

The enzymatic screening for cellulase was based on Congo Red (CR) dye supplemented agar plate assay (Yoon *et al*., 2007). The composition of the medium was 1% carboxymethyl cellulose (CMC), 1.5% agar, 0.67% YNB without amino acids and 0.01% Congo Red. The ten fungal isolates obtained were cultured on CMC – CR agar and incubated at 30 °C for 72 – 96 hours. This method does not require any washing of the plates with sodium chloride solution. As growth develops the degradation (hydrolysis) of CMC results in the release of the bound CR dye. This is revealed by the appearance of a pale yellow halo zone surrounding the fungal colony. Intact CMC- bound CR dye has a red or dark red background depending on the percentage of dye used. The degree of degradation varies amongst fungal species or even at the strain level.

The second screening was based on cellulolytic hydrolysis of filter paper (total cellulase activity). Six fungal isolates were induced to produce cellulase using a synthetic medium described by Peixoto (2006) and Avicel as the sole source of carbon. The synthetic media consisted of 2 g/L K_2HPO_4, 0.5 g/L KCl, 0.01 g/L $FeSO_4.7H_2O$, 20 g/L Avicel, 0.15 g/L $MgSO_4.7H_2O$, 7 g/L KH_2PO_4, 1 g/L $(NH_4)SO_4$, and 1 g/L yeast extract. The pH of the medium was adjusted to 5.5 prior to sterilization. Inoculation was done by cutting approximately 0.5 x 0.5 cm of fungal growth on agar medium and adding it into 100 mL medium in 250 mL Erlenmeyer flasks. All the flasks were incubated at 30 ˚C for 7 days, while shaking at 150 rpm. A 5 mL sample was removed after every 24 hours of incubation and used for filter paper assays i.e. total cellulase activity.

3.2.2. Molecular identification of fungal isolates

The identification of the unknown fungi relied on the conserved nucleotide sequence of the gene (DNA) coding for the 18S, 5.8S and 28S rRNA by the amplification of the ribosomal internal transcribed spacer (ITS) region of the genomic DNA by Polymerase Chain Reaction (PCR) (White *et al*., 1990).

3.2.2.1. Genomic DNA isolation and purification

Fungal genomic DNA isolation and purification was done by following the procedure outlined in the ZR Fungal/Bacterial DNA Kit™ (Zymo Research, Catalogue No. D6005), according to the manufacturer's instruction.

3.2.2.2. Amplification of the gene for identification of the fungus

The ITS target region (i.e. ITS-5.8S-ITS fragment) was amplified using PCR primers, ITS-1 (5'-TCCGTAGGTGAACCTGAGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3)' (White *et al*., 1990; Wang *et al*., 2012; Benoliel *et al*., 2013). Amplification was carried out in 25 µL reactions using EconoTaq® PLUS GREEN 2X Master Mix (Lucigen).The following PCR conditions were used: 35 cycles including an initial denaturation step at 95 °C for 2 minutes. Subsequent denaturation was at 95 °C, 30 seconds, annealing at 50 °C for 30 seconds and extension at 72 °C for 1 minute. A final extension at 72 °C for 10 minutes was followed by holding at 4 °C. The PCR products were analysed on a 1% agarose gel.

3.2.2.3. Sequencing analysis

The amplicons (fragments) were extracted from the 1% agarose gel using Zymoclean™ Gel DNA Recovery Kit™ (Zymo Research, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse directions by using Applied Biosystems, ThermoFisher Scientific, Big Dye terminator kit v3.1 and purified using ZR-96 DNA Sequencing Clean-up Kit™ (Zymo Research, Catalogue No. D4050). The purified fragments were sequenced on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific). Species were identified by searching databases using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

3.2.3. Effect of initial pH medium and incubation temperature on cellulase production

The effect of initial pH on the production of cellulase was studied by adjusting the pH of the media in the range of 4.5 to 7.0. The pH adjustment was done using either 1M NaOH or 1M HCl solution. The effect of temperature on the production of cellulase was investigated at 30, 35 and 40 ˚C with the pH adjusted to 6.5 or 7.0 (based on cellulase activity determined during pH studies). Inoculation and incubation conditions were maintained as described above, in secondary screening. Five millilitre samples were harvested every 24 hours of incubation and used for enzyme assays. The crude enzyme was prepared by centrifugation of harvested sample using a micro-centrifuge at 12470 x g for 10 minutes at room temperature.

3.2.4. Cellulolytic activity assay

3.2.4.1. Cellulase (FPase)

The total cellulase activity was determined by filter paper assay using Whatman No.1 filter paper strip with a dimension of 1 x 6.0 cm equivalent to 50 mg of substrate according to Ghose (1987). At least two dilutions were made, one dilution that releases slightly less than 2.0 mg and the other dilution releasing more than 2.0 mg. The reaction mixture contained 1.0 mL of 0.05 M Na-citrate, pH 5.0, filter paper strip and 0.5 mL of crude enzyme diluted accordingly. The mixture was incubated at 50 °C for 1 hour. The released reducing sugar was estimated by addition of 3,5-dinitrosalicylic acid (DNS) with glucose as standard. The absorbance was read at

540 nm by using a Beckman Coulter, DU® 720 UV/Vis spectrophotometer. The assay was performed in triplicate including controls. Filter paper activity (FPU) is defined as 0.37 divided by the amount of enzyme required to liberate 2.0 mg of glucose from filter paper strip (≈ 50 mg) in 1 hour.

3.2.4.2. Endoglucanase assay

Endoglucanase activity in the culture supernatant was determined according to the method described by Ghose (1987). The reaction mixture contained 0.5 ml of 1% CMC in 0.05 M Na-acetate buffer, pH 5.0 and 0.5 mL of appropriately diluted crude enzyme. The mixture was incubated at 50 °C for 30 minutes and the released reducing sugar was estimated as indicated in the assay for total activity above. One unit of endoglucanase activity was defined as the amount of enzyme liberating one µmole of reducing sugar from CMC under the assay conditions.

3.2.4.3. β-glucosidase assay

β-glucosidase activity was determined according to the method described by Herr (1979). The reaction mixture contained 0.2 mL of 0.01 M *ρ*-nitrophenyl β-D-glucopyranoside (pNPG) in 0.05 M citrate buffer pH 4.8 and 0.2 mL of appropriately diluted enzyme solution. The substrate control contained 0.4 mL of 0.01 M pNPG in 0.05 M citrate buffer at pH 4.8. The mixtures were incubated at 50 ˚C for 30 minutes. The activity of the enzyme indicated by the released *ρ*-nitrophenol was estimated by addition of 0.05 M NaOH-Glycine buffer with *ρ*-nitrophenol used as standard and absorbance of the developed colour was read at 420 nm by using spectrophotometer (Beckman Coulter, DU® 720 UV/Vis). One unit of β-glucosidase activity was defined as the amount of enzyme liberating one µmole of *ρ*-nitrophenol under the assay conditions.

3.2.5. Calculations of enzyme activities

3.2.5.1. Cellulase (Filter paper activity, FPase)

Cellulase activity was determined by filter paper assay using Whatman No.1 according to Ghose (1987)

$$\mathrm{FPA}\left(\frac{\mathrm{FPU}}{\mathrm{mL}}\right) = \frac{0.37}{[\mathrm{Enz}]} \qquad (1)$$

,where [Enz] is the concentration of enzyme that release 2.0 mg of glucose from filter paper in 60 minutes.

3.2.5.2. Endoglucanase (CMCase) and β-glucosidase.

To estimate the activities of either endoglucanase and/or β-glucosidase based on the released reducing sugars or *ρ*-nitrophenol, the equation (2) was used (Rubeena *et al*., 2013).

$$\beta - \mathrm{glucosidase\ or\ CMCase}\ \left(\frac{\mathrm{U}}{\mathrm{mL}}\right) = \frac{\Delta \mathrm{E}\ \times \mathrm{Vf}\ \times \mathrm{Df}}{\varepsilon\ \times \Delta \mathrm{t}\ \times \mathrm{Venz}} \qquad (2)$$

,whereby ΔE is absorbance value at 540 nm, Vf is final volume, ε is extinction coefficient of glucose (slope), Δt is incubation time, Venz is the volume of crude enzyme and DF is dilution factor (if applicable).

3.2.6. Statistical analysis

All the experiments were done in triplicates. The data generated was statistically analysed by Two-way analysis of variance (ANOVA) test using MS Excel 2010. Differences were considered significant when probability value (p) was <0.05. The error bars in the graphs represent standard error, SE.

3.3. RESULTS

3.3.1. Screening and identification of fungi that produce cellulase

According to Yoon *et al*. (2007), the plate screening methods with dye coupled substrates provided a relatively straight forward and easy tool for specific detection of endoglucanase producing fungi. The results revealed that Ten of the isolated fungi were able to grow and secrete endoglucanase which hydrolysed CMC bound to CR dye. This was revealed by the appearance of pale-yellow “halo zone” around the fungal growth or colony and it was an indication of endoglucanase activity or CMC hydrolysis. However, only six fungal isolates indicated larger halo zones around growth. These six fungal isolates were subsequently identified using ITS sequencing. The identification of the selected six isolates revealed two different genera, namely *Trichoderma* and *Aspergillus*, Table 3.1. The *Trichoderma* species were *T. longibrachiatum* and *T. harzianum* and the *Aspergillus* species were *A. fumigatus*. The six fungal species were further evaluated for cellulase production in submerging fermentation.

A quantitative evaluation of cellulase production by the selected fungi, Table 3.1 was carried out in submerged fermentation using Avicel as a substrate at an initial pH of 5.5 at 30 °C. Maximum cellulase activity was observed after 96 hours for all the fungal strains, Fig 3.1. *T. longibrachiatum* LMLSAUL 14-1 produced 4.14 FPU/mL followed by *T. harzianum* LMLBP07 13-5 with an activity of 3.05 FPU/mL, A. *fumigatus* LMLPS 13-4 with an activity of 2.14 FPU/mL, A. fumigatus LMLPS 13-1 with an activity of 1.85 FPU/mL, A. *fumigatus* LMLBS02 13-2 an activity of 1.69 FPU/mL and *A. fumigatus* LMLBP06 13-3 an activity of 3 1.55 FPU/mL.

Table 3. 1. Fungal isolation and screening on Avicel and CMC-Congo Red agar.

Sampling site	Isolate code no.	Identification
Banana plantation site, Allesbeste farm, Tzaneen	[a]LMLBP07 13-5	*Trichoderma harzianum*
	[a]LMLBP06 13-3	*Aspergillus fumigatus*
Decomposing banana pseudostem, Allesbeste farm, Tzaneen	[b]LMLPS 13-1	*Aspergillus fumigatus*
	[b]LMLPS 13-4	*Aspergillus fumigatus*
Banana dumpsite, Allesbeste farm, Tzaneen	[c]LMLBS02 13-2	*Aspergillus fumigatus*
Decomposing *Strelizia alba*, University of Limpopo	[d]LMLSAUL 14-1	*Trichoderma longibrachiatum*

[a]BP refers to banana plantation site; [b]PS refers to Pseudostem; [c]BS refers to banana dumpsite outside plantation; [d]SAUL refers to *Strelitzia alba* at University of Limpopo

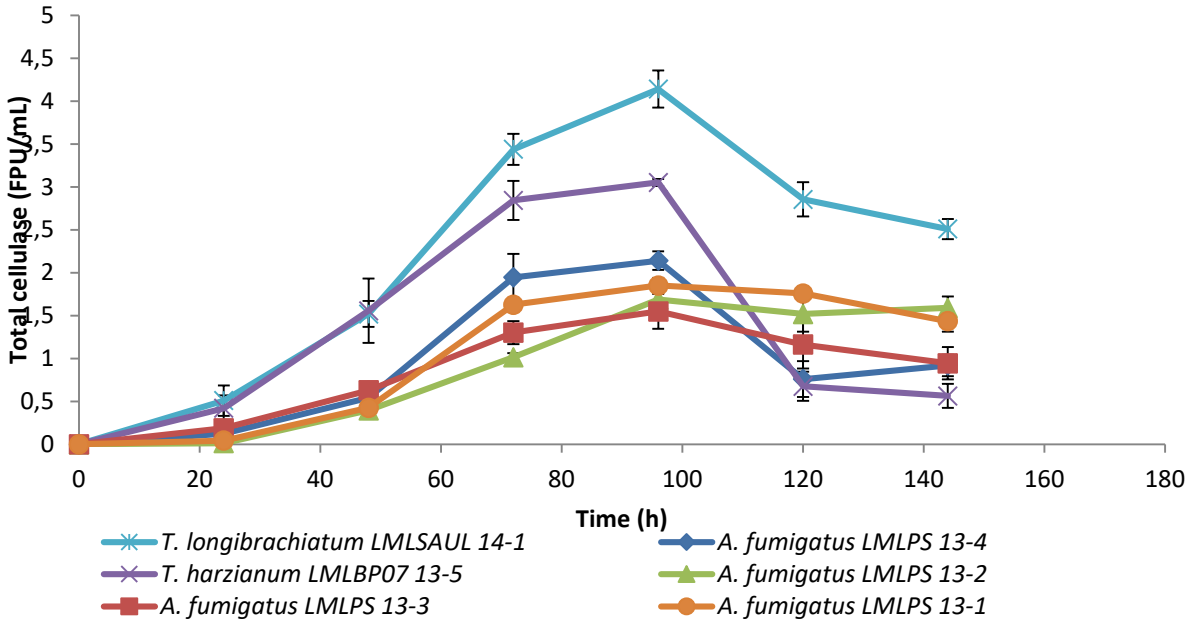

Figure 3.1. Cellulases production by isolated fungal strains at 30 °C and initial pH 5.5.

3.3.2. Effect of initial pH medium on cellulase production

The influence of pH on the production of cellulase was assessed for the six fungal strains at 30 °C, Fig. 3.2. Maximum cellulase activity was detected for all fungal isolates when the initial pH of the medium was 6.5, Fig. 3.2. The *Trichoderma longibrachiatum* LMLSAUL 14-1 strain produced the highest cellulase activity of 8.08 FPU/mL. This was followed by *T. harzianum* LMLBP07 13-5 with activity of 5.77 FPU/mL. *A. fumigatus* LMLBP06 13-3 and *A. fumigatus* LMLPS 13-1 produced maximum cellulase activity of 3.05 FPU/mL with the lowest cellulase activity of 2.04 FPU/mL by *A. fumigatus* LMLPS 13-4.

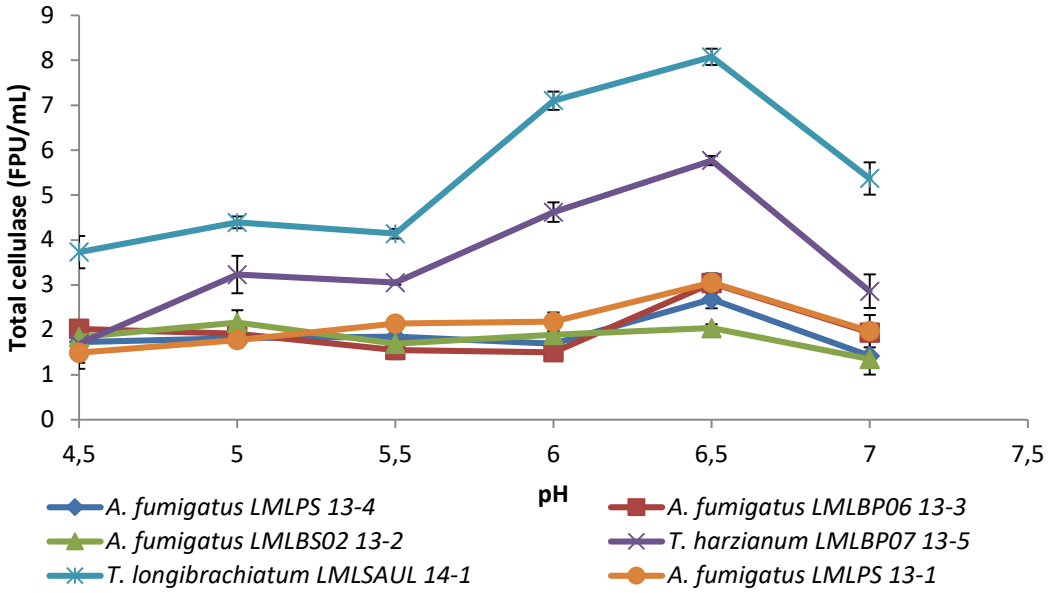

Figure 3.2. The effect of initial medium pH on the production of cellulases by isolated fungi at 30°C.

The production of endoglucanse activity was the highest at pH 6.5 for all the fungal strains (**Fig. 3.3**).

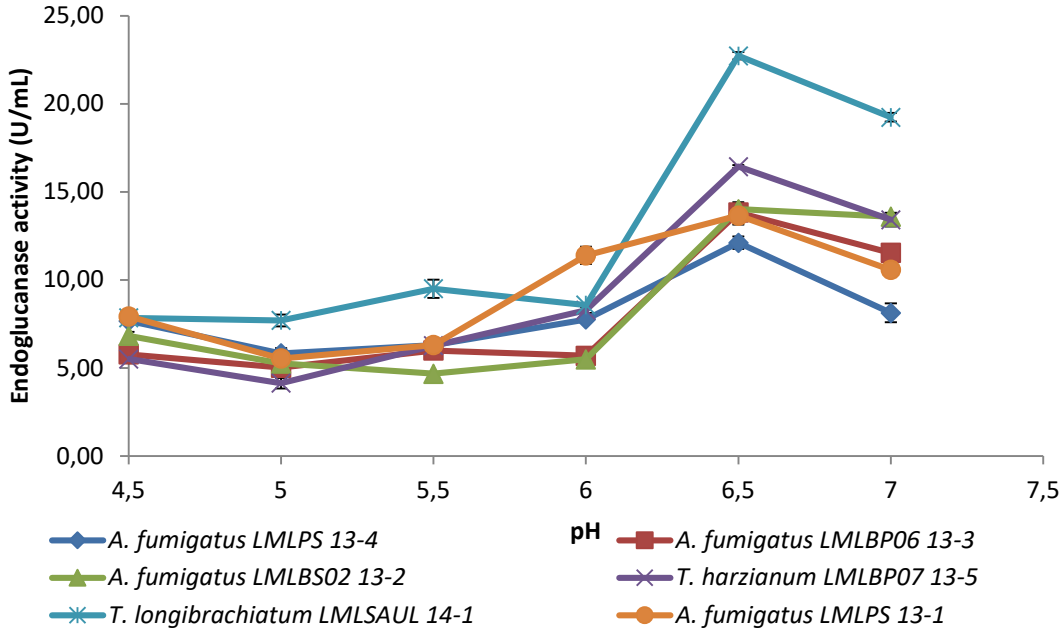

Figure 3.3. The effect of initial medium pH on the production of endoglucanase by the isolated fungal strains at 30 °C.

Trichoderma longibrachiatum LMLSAUL 14-1 and *T. harzianum* LMLBP07 13-5 had produced activities of 22.74 U/mL and 16.44 U/mL respectively, whereas *A. fumigatus* LMLPS 13-1; LMBS02 13-2 and LMBP06 13-3 produced equal amounts of 13.66 U/mL. The lowest activity 12.11 U/mL was noted for *A. fumigatus* LMLPS 13-4.

The levels of β-glucosidase produced with respect to initial medium pH differed between the fungal strains. *A. fumigatus* LMLPS 13-4 and *A. fumigatus* LMLBS02 13-2 produced higher activities of 37.73 U/mL and 34.07 U/mL at pH 7.0, respectively compared to the other isolates tested, Fig. 3.4. Some fungi were able to secrete maximum β-glucosidase at various pH values. *T. harzianum* LMLBP07 13-5 produced β-glucosidase activity of 25.69 U/mL over a pH range of 6.0 - 7.0 and *A. fumigatus* LMLPS 13-1 produced β-glucosidase activity of 23.52 U/mL over a pH range of 6.5 - 7.0, Fig. 3.4). The lowest β-glucosidase activity produced was 20.58 U/mL by *T. longibrachiatum* LMLSAUL 14-1.

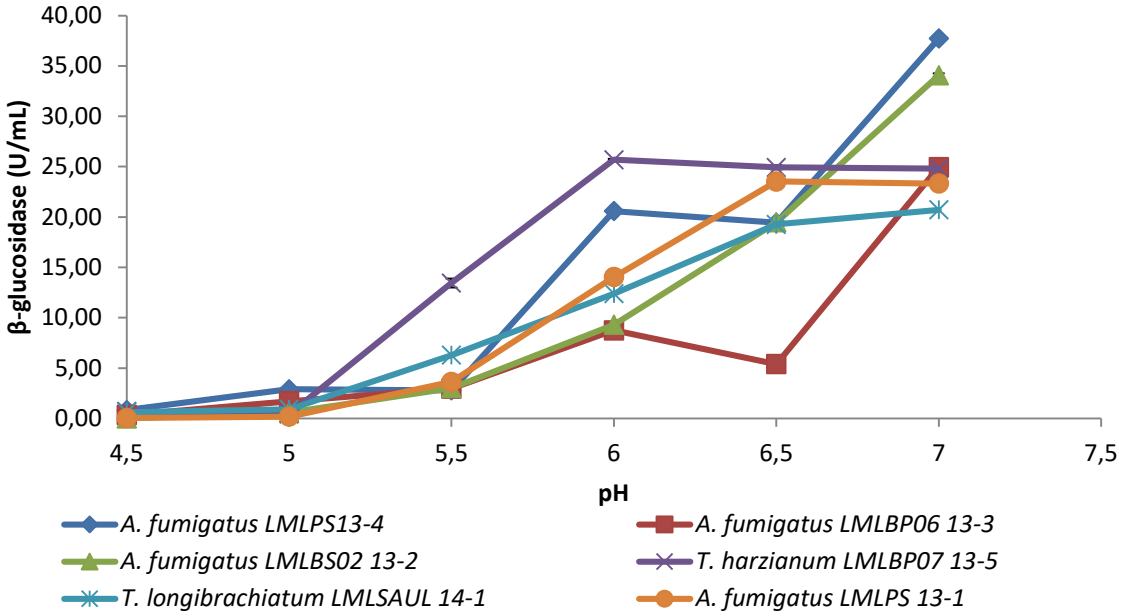

Figure 3.4. The effect of initial medium pH on the production of β-glucosidases by the isolated fungi at 30 °C.

3.3.3. Effect of incubation temperature on cellulase production

The production of cellulase with respect to changes in incubation temperature was also investigated at an initial medium pH of 6.5 and 7.0, Fig. 3.5 and 3.6. These pH values have shown to favour production of β-glucosidases by species of *Aspergillus*

and *Trichoderma*. At the initial medium pH of 6.5, an increase in incubation temperature led to a decrease in the level of total cellulase activity for *T. longibrachiatum* LMLUL 14-1 at 35 °C, Fig. 3.5. The *A. fumigatus* strains were able to produce cellulases at both 30 and 35 °C, with the exception of *T. harzianum* LMLUL 13-5. Cellulase activity in *A. fumigatus* LMLUL 13-1 increased by 1.8 fold at 35 °C, Fig. 3.5. Increasing the incubation temperature to 40 °C drastically reduced the cellulase production for *T. longibrachiatum* LMLUL 14-1, *A. fumigatus* LMLUL 13-1 and *A. fumigatus* LMLUL 13-3, Fig. 3.5. Generally, most fungal species cellulase activity levels decreased as the temperature increased to 40 °C.

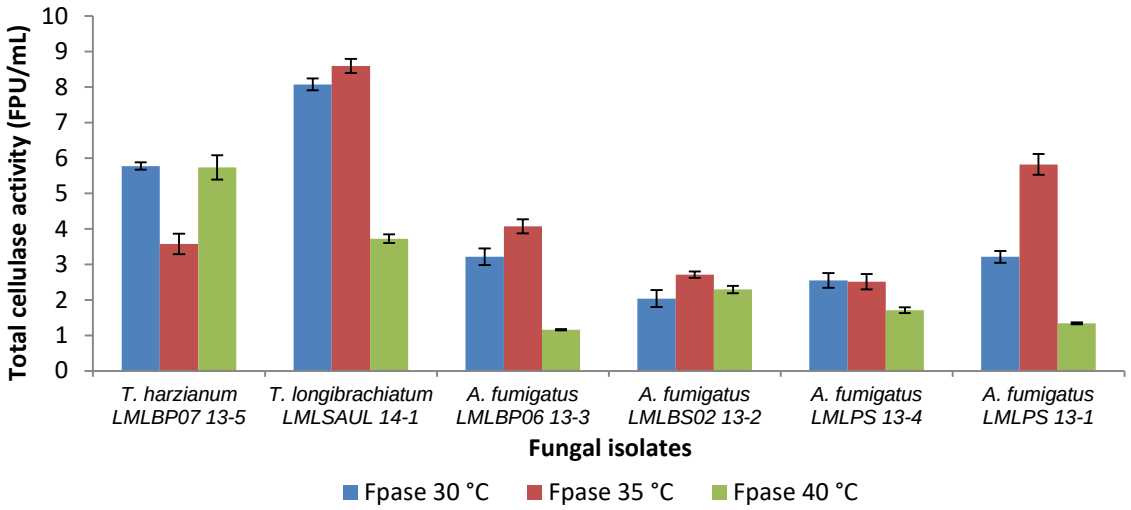

Figure 3.5. Effect of incubation temperature on the production of cellulase (total cellulase activity) by fungal strains at initial pH 6.5.

At initial medium pH of 7.0, a minimum temperature of 30 °C favoured high production of cellulase by *T. harzianum* LMLBP07 14-5, *T. longibrachiatum* LMLUL 14-1 and *A. fumigatus* LMLBS02 13-2, Fig. 3.6. The cellulase production by *A. fumigatus* LMLPS 13-1, *A. fumigatus* LMLBP06 13-3 and A. *fumigatus* LMLPS 13-4 improved at 35 °C, irrespective of initial pH tested, Fig. 3.5 and 3.6. The production of cellulase was reduced for all strains evaluated at 40 °C.

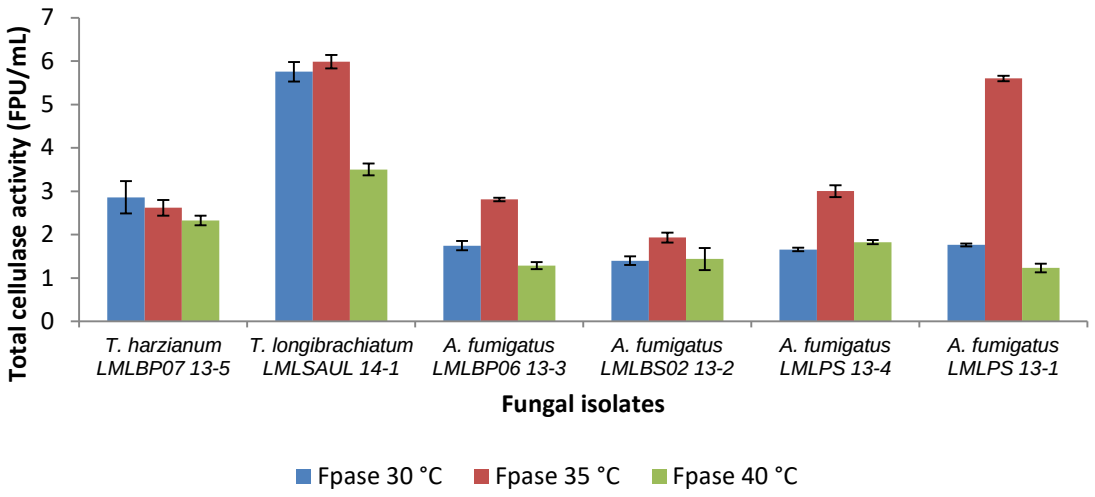

Figure 3.6. Effect of incubation temperature on the production of cellulases by fungal strains at initial pH 7.0.

All the *Trichoderma* and *Aspergillus* strains produced more endoglucanase at 30 °C with both at an initial medium pH of 6.5 and 7.0, Fig. 3.7 and 3.8), but initial medium pH of 6.5 favoured more enzyme production. An increase in temperature to 35 °C and 40 °C resulted in a 2-fold decrease of the endoglucanase activity in all fungal species, Fig. 3.7.

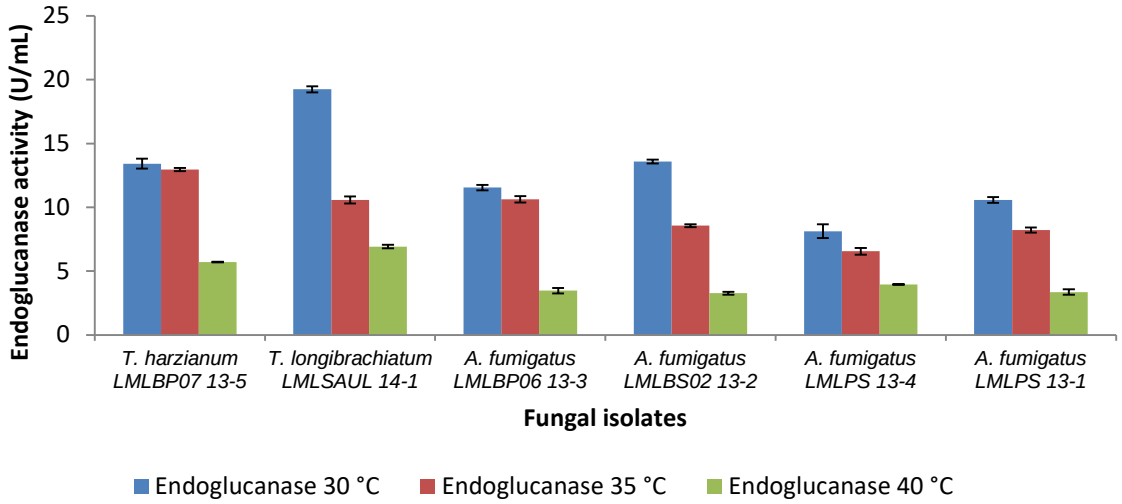

Figure 3.7. Effect of incubation temperature on the production of endoglucanase by fungal strains at initial pH 6.5.

As the initial medium changes to pH 7.0 a greater improvement in the production of endoglucanase was seen at 35 °C, Fig. 3.8.

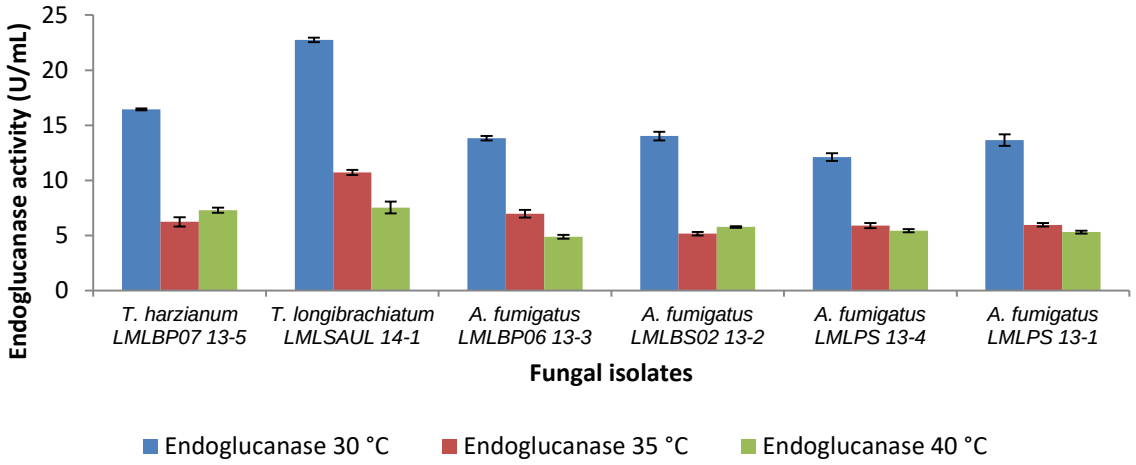

Figure 3. 8. Effect of incubation temperature on the production of endoglucanase by fungal strains at initial pH 7.0.

Trichoderma harzianum LMLBP07 13-5 had produced 2-fold higher endoglucanase activity at initial medium pH of 7.0 while other fungal species attained a slight increase, Fig. 3.8. At both pH of 6.5 and 7.0, higher temperatures caused a reduction in endoglucanase activity and severe reduction occurred at 40 °C, Fig. 3.7 and 3.8).

The production of β-glucosidase at an initial pH of 6.5 and 7.0 was dependent on the fungal species and production temperature. In a medium with an initial pH of 6.5, only *A*. *fumigatus* LMLPS 13-1 and *T*. *harzianum* LMLBP07 13-5 produced high levels of β-glucosidase activity of 23.52 U/mL and 24.90 U/mL, respectively at 30 °C. An increase in temperature from 30 – 40 °C led to improved production of β-glucosidase by some fungal species. For instance, *A. fumigatus* LMLBP06 13 - 3 showed 5-fold increase, i.e. from 5.38 to 25.35 U/mL of β-glucosidase activity at 40 °C and 5.2-fold increase of β-glucosidase at 35 °C, Fig. 3.9.

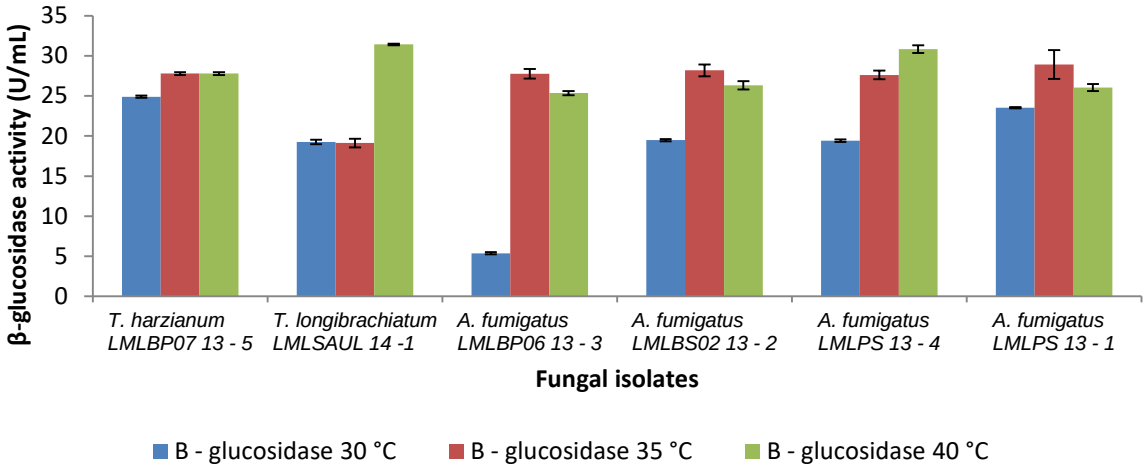

Figure 3.9. Effect of incubation temperature on the production of β-glucosidase by fungal strains at initial pH 6.5.

The production of β-glucosidase by *T. harzianum* LMLBP07 13-5 was optimal between 35 – 40 °C, whilst *T. longibrachiatum* LMLSAUL 14-1 produced β-glucosidase optimally at 40 °C. *A. fumigatus* LMLPS 13-4 attained maximum β-glucosidase activity at 30 °C, Fig. 3.9. Generally, at 40 °C the production of β-glucosidase by all fungal species was much higher than at 30 °C with the exception of *A. fumigatus* LMLPS 13-4. In a medium with an initial pH 7.0, an increase in temperature led to an improvement in the production of β-glucosidase, Fig.3.10.

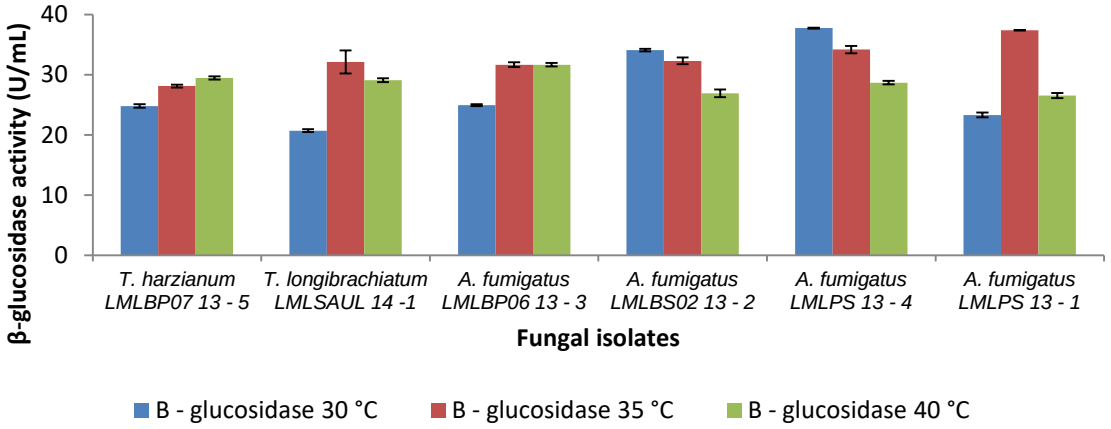

Figure 3.10. Effect of incubation temperature on the production of β-glucosidase by fungal strains at initial pH 7.0.

A maximum level of β-glucosidase was attained at 35 °C with *T. longibrachiatum* LMLSAUL 14-1 and *T. harzianum* LMLBP07 13-5 showing 1.55-fold increase and 1.13-fold increase, respectively. *A. fumigatus* LMLPS 13-4 and *A. fumigatus* LMLPS 13-1 also showed an increase of β-glucosidase activity at 35 °C (1.76-fold and 1.6-fold, respectively). *A. fumigatus* LMLBP06 13-3 showed a proportional increase of 1.27-fold with maximum β-glucosidase produced at 40 °C. Conversely, 40 °C led to a 1.26-fold decrease of β-glucosidase produced by *A. fumigatus* LMLBS02 13-2, Fig. 3.10. Generally, β-glucosidase activity was the highest at 35 °C and pH 7.0 for all strains tested.

3.4. DISCUSSION

3.4.1. Screening and identification of fungi that produce cellulase

Fungi and bacteria are associated with soil and decaying plant materials. In nature, fungi colonise the plant debris and in a symbiotic relationship with other microorganisms, they secrete an assortment of proteins and a complex of hydrolytic enzymes to hydrolyse plant polysaccharides for their survival. Filamentous fungi are sources of various commercial enzymes such as cellulases, pectinases and possess efficient enzymatic machinery for the degradation of lignocellulosic biomass (Álvarez *et al*., 2016). Cellulases have significant importance in the production of cellulosic ethanol (a second generation ethanol based on non-edible feedstocks). This non-food based fuel strategy could be realised by continuous search for microorganisms which are either hyper-producers or producers of novel enzymes which possesses unique traits such as the ability to withstand high pH, high temperature and the inhibitory compounds (Howard *et al*., 2003).

However, not all microorganisms are able to secrete a significant amount of hydrolytic enzymes for biotechnological applications. Hence, there is a need to screen and select hyper-secreting hydrolytic enzymes. In this study, three different methods for screening for cellulolytic activity were used including (1) microbial growth on cellulose agar, (2) clearing of cellulose in agar, and (3) reducing sugar production (or glucose), were performed. Growth on cellulose containing agar was useful for isolation of cellulolytic fungal strains. Ten fungal strains were selected based on fast and abundant growth on Avicel agar plates. Several authors have also

reported the use of microcrystalline cellulose to screen for cellulase producing microorganisms (Delabona *et al*., 2012; Andrade *et al*., 2011; Grigorevski-Lima *et al*., 2009). CMC–CR (0.01%) agar plate has been used to detect endoglucanase activity as indicated by the presence of a pale yellow zone around the colony. Larger halos are the result of higher enzyme activity (Zhang *et al*., 2006). However, at high CR dye concentrations, fungal growth is suppressed.

The application of rDNA genes for identification of fungal species is based on the detection of conserved sequences in 5.8S rDNA and 28S rDNA that enables the amplification of the ITS2 region between them (Turenne *et al*., 1999). The identification of the fungal species based on the amplification of the ribosomal internal transcribed spacer (ITS) region of the ribosomal DNA revealed that the organisms are *A. fumigatus* strains, *T. harzanium* and *T. longibrachiatum*. Other authors also used ITS region to identify *T*. *harzianum* strain (Benoliel *et al*., 2013) and *A*. *fumigatus* ECU0811 (Wang *et al*., 2012). This is because ITS regions are regarded as primary DNA barcode for the fungal kingdom and exhibit high reliability (Schoch *et al*., 2012). The *Aspergillus* and *Trichoderma* species are associated with agricultural compost (Sreenivase, 2012; Singh *et al*., 2012), and fruit spoilage (Al-Hindi *et al*., 2011; Llyas *et al*., 2007).

3.4.2. Effect of initial pH medium on cellulases production

Screening is often followed by process optimisation with various factors such as nutrient requirements, temperature, pH, agitation speed, *etc*. (Gautam *et al*., 2010; Shahriarinour *et al*., 2011) being investigated. In this study, the effect of initial pH and temperatures on the production levels of cellulase were investigated. Fungal strains belonging to *Trichoderma* and *Aspergillus* has shown the potential to produce cellulase over a pH range of 4.5 – 7 and temperature range of 30 – 40 °C, although the cellulase production levels varied from one fungal strain to another under the conditions used in this study. It has been reported that the expression of fungal genes are regulated by extracellular pH and many fungi exhibit growth and enzyme secretion over a wide pH range (Archer & Peberdy, 1997).

The results showed that initial pH of 4.5 – 5.5 drastically reduced the levels of cellulase and endoglucanase produced by all strains. This lower initial pH effect on

the production level of cellulases by *Trichoderma* and *Aspergillus* was also reported by Gautam *et al*. (2011) and Delabona *et al*. (2012). The pH conducive to support the production of cellulase enzymes system is also depended on the media composition. For instance, other authors reported maximum cellulase production at pH 3.0 (Andrade *et al*., 2011), pH 4.0 (Shafigue *et al*., 2009; Sohail *et al*., 2009; Sarkar & Aikat 2014), pH 5.0 (Das *et al*., 2013) and pH 5.5 (Ahmed *et al*., 2009) when different media were used. Our results showed that a pH above 5.5 favoured cellulase production by all *Trichoderma* and *Aspergillus* strains with an optimum at a pH of 6.5. Beyond pH 6.5 a drastic decrease in cellulase levels were observed. Gilna and Khaleel (2011) and Gautam *et al*. (2011; 2010) also reported maximum cellulase activity at pH 6.5. The maximum production of cellulase by *Aspergillus niger* was achieved at an initial pH of 6 when cultured on 10% rice straw (Aboul-Fotouh *et al*., 2016). Other findings (Ncube *et al*., 2012) showed no significant differences in the production of endoglucanase by *A*. *niger* FGSCA 733 over a pH range of 3 – 7. These findings illustrate that an optimum pH for maximum production of cellulase is dependent on fungal species and to some extent on the particular species investigated.

3.4.3. Effect of incubation temperature on cellulase production

Temperature has been reported to also influences microbial growth and enzyme production (Soni *et al*., 2010; Rubeena *et al*., 2013) and an optimal environment conducive to the production of cellulase will also be influenced by media nutritional composition (Shahriarinour *et al*., 2011). By increasing the temperature to 40 °C a significant reduction in endoglucanase activity was observed in all strains tested, Fig. 3.8 and 3.9. The optimum temperature for the production of cellulase enzymes system is also influenced by culturing conditions and fungal strains. Ncube *et al*. (2012) reported maximum production of endoglucanase at 40 °C by *A. niger* FGSCA 733. Leghlimi *et al*., (2013) reported optimum temperature for production of cellulase (FPA), endoglucanase and β-glucosidase by *T. longibrachiatum* (GHL) at 35 °C. *Aspergillus niger* MS82 was reported to produce sufficient endoglucanase at 30 °C and 35 °C with initial pH kept at pH 4.0 (Sohail *et al*., 2009). Other studies reported optimal cellulase and endoglucanase production at 45 °C (Gautam *et al*., 2011) and 60 °C (Andrade *et al*., 2011; Stewart & Parry, 1981).

A temperature of 40 °C did not severely affect the production of β-glucosidase, Fig. 3.9 and 3.10. Other authors reported maximum levels of β-glucosidase produced by *T*. *longibrachiatum* at 35 °C (Leghlimi *et al*., 2013) and *A*. *niger* MS82 at 25 °C.(Sohail *et al*., 2009). These discrepancies with regards to the optimum initial pH and production temperature on the levels of cellulases as measured by filter paper activity (FPU/mL) can be attributed to the genetic make-up of the fungal strains as a result of adaptations to different habitats. Furthermore, our reported high levels of total cellulase, endoglucanase activities and low level of β-glucosidase activity by *Trichoderma* and *vice versa* for β-glucosidase by *A. fumigatus* are in agreement with the reported levels of these fungal species by Stewart & Parry (1981).

3.5. CONCLUSION

All the *Trichoderma* and *Aspergillus* species produced substantial levels of the cellulase enzymes system (i.e. exoglucanase, endoglucanase and β-glucosidase) required in subsequent stages to complete hydrolysis of banana pseudostem. *Trichoderma* strains produced higher cellulase and endoglucanase levels while *A. fumigatus* strains produced higher β-glucosidase levels. The production of cellulase enzymes system seemed to be strongly influenced by the interactive effect of initial pH and incubation temperature on the microorganisms. Hence, the observed maximum production of cellulase by the fungi depended on the chosen initial pH of the medium and incubation temperature. These fungal species will be assessed for their ability to produce cellulase enzymes system when cultivated on banana pseudostem in solid state fermentation, SSF (**Chapter 4**).

CHAPTER 4

Production of cellulase enzymes system by *Trichoderma* and *Aspergillus* species cultivated on banana pseudostem in solid state fermentation after pretreatment

4.1. INTRODUCTION

Generally, fungi produce cellulase enzymes system which includes endoglucanase (EC 3.2.1.4 endo-1,4-β-glucanase), exoglucanase (EC 3.2.1.91 1,4-β-cellobiohydrolase) and β-glucosidase (EC 3.2.1.21), (Zhang et al., 2006; Bisaria & Ghose, 1981). The demand for cellulase enzymes is mostly fulfilled through submerged fermentation (SmF) processes with genetically modified strains of *Trichoderma*. However, the production costs of enzyme in SmF are high (Menon & Rao, 2012; Pandey *et al*., 1999). The cost of cellulase enzymes is the limiting factor for the feasible production of ethanol from fibrous biomass. (Zhuang *et al*., 2007). In efforts to reduce the cost of producing cellulase, solid state fermentation (SSF) can serve as an alternative to SmF. Numerical simulation for cost-effective production of cellulases indicated that unit costs for SSF were lower than SmF (Zhuang *et al*., 2007). Enzyme producers such as Dyadic, Novozymes and DuPont have reduced the production cost of cellulases from $2 in 2010 to $0.30 (Brooks & Tchelet, 2014). This reduction in enzyme cost is a breakthrough toward the commercialization of large scale-biomass-to-ethanol production (Liu *et al*., 2016; NREL, 2010). Remarkable progress has been made to reduce the cost of cellulases, however cellulase still presents a significant operational cost in cellulosic bioethanol production chain (Ellilä *et al*., 2017).

Solid state fermentation mimics the natural microbiological processes such as composting and ensiling (Singhania *et al*., 2009). This fermentation offers a high volumetric productivity and highly concentrated product (Pandey *et al*., 1999). Various microorganisms such as bacteria, fungus and actinomycetes, which possess different enzyme induction systems, have been used in the production of various hydrolytic enzymes (Moretti *et al*., 2012). The most commonly isolated species of cellulolytic fungi in composting materials are *Aspergillus*, *Penicillium*, *Rhizopus*,

Trichoderma, *Fusarium*, *Chaetomonium* and *Cladosporium* (Singh *et al*., 2012). In SSF, growth of microorganism(s) in the absence of free liquid can occur on the surface of a solid substrate (or nutrient impregnated solid material) or within the whole substrate depending on the porosity of the substrate (Gervais & Molin, 2003; Cannel & Moo-Young, 1980;). The production of enzymes system is influenced by the nature of the solid substrate, microorganism used and environmental conditions such as pH, temperature and moisture. (Moretti *et al*., 2012; Singhania *et al*., 2009; Pandey *et al*., 1999).

Industrial SSF processes have been developed for traditional food industries such as cheese, fermented vegetables, meat and other biotechnology products such as antibiotics and enzymes (Gervais & Molin, 2003). Solid state fermentation has advantages over SmF by utilizing less water and energy, produces minimum or less waste and can also produce a more concentrated product. However, when compared to SmF, SSF presents difficulty in heat and mass transfer due to limited diffusion through solid material (Mitchell *et al*., 2003). An uncontrolled SSF will lead to accumulation of heat and a decline in oxygen availability. This effect can negatively impact mesophilic aerobic microbe's activity and completely stop enzyme production (Zhuang *et al*., 2007).

The pretreatment (e.g. chemical or mechanical) of lignocellulosic biomass enable the biomass to be porous and easily accessible for both microbial growth in SSF and susceptible to enzymatic hydrolysis. The hydrolysis reaction increases the production of fermentable sugars than can be converted through biochemical activities of fermenting microorganisms to ethanol (Pandey *et al*., 2000). The high costs of cellulase hamper the commercialization of biomass bio-refineries. As it stands, large amount of cellulase is still required for biomass saccharification (Liu *et al*., 2016). For instance, approximately 100 g enzymes are needed per gallon of cellulosic ethanol production (Zhang *et al*., 2006). The amount of cellulase required for lignocellulosic biomass saccharification is one-fold higher than the process of starch saccharification (Balan, 2014). To overcome high cost cellulase, the production strategies of cellulase should increase enzyme volumetric activity, produce enzymes using cheaper substrate, produce enzymes preparations with greater stability for specific processes and producing cellulase with higher specific activity on a solid substrate (Zhang *et al*., 2006). Further more, On-site enzyme

production can also reduce the cost by approximately 30-70% of the total enzyme production costs (Brooks & Tchelet, 2014; Takimura *et al*., 2013).

The use of agricultural biomass such as banana waste as feedstock for ethanol production can potentially reduce the number of food crops such as corn and wheat used for the production of ethanol (Padam *et al*., 2014). Banana waste including leaves, rachis and pseudostem and contain considerable amounts of cellulose (Li *et al*., 2010; Abdul Khalil *et al*., 2006). These cellulose rich materials are discarded after the harvest of banana fruit and left to decay either at the plantation site or at a dumping site (Li *et al*., 2010). The utilisation of low cost agricultural residues such as banana waste in SSF to produce enzymes could mitigate waste disposal which contributes to environmental pollution (Singhania *et al*., 2009). Hence this study was aimed at using banana pseudostem as an inducing substrate for cellulase production by *Trichoderma* and *Aspergillus* species in the SSF process.

4.2. MATERIALS AND METHODS

4.2.1. Microorganisms and inoculum preparation

Three fungal species, namely *Trichoderma harzianum* LMLBP07 13-5, *Trichoderma longibrachiatum* LMLSAUL 14-1 and *Aspergillus fumigatus* LMLPS 13-4 previously isolated (details in section 3.2.1) and stored at 4 – 8 °C with periodic sub-culturing onto malt extract agar (MEA) were used in the study.

For inoculum preparation, the fungal species were grown on malt extract agar until spore formation at 30 °C. Spores from each fungus were harvested by adding 10 mL of 0.05% sterile Tween 80 solution (Merck) onto the culture plates and sterile swabs were then used to dislodge the spores from the agar surface. A standard spore count procedure was done using the Neubauer bright line (1/10 mm) counting chamber. Spore suspensions were adjusted to contain 1 x 10^8 spore/mL.

4.2.2. Collection and preparation of banana pseudostem

Fresh banana pseudostems (BPS) that remained after banana fruit harvest were collected from Allesbeste farm, Tzaneen, Limpopo province, South Africa. The BPS was washed of soil and other debris using tap water. The washed BPS was cut into small pieces (approximately 20 – 25 cm diameter and 10 cm height) and the outer,

and core bark (pith) separated into several blocks, Fig. 4.1. The blocks and piths were dried at 65 – 70 °C until constant mass and subjected to grinding using agricultural machine (Zhuans milling and crushing; Electric model). The ground material was sieved through a Universal Test Sieve with aperture 500 µm (Abdullah *et al*., 2014). The sieved ground particles (< 500 µm) were stored at room temperature (20 – 25 °C) in a sealed container until needed.

Figure 4.1. Shows banana plant (a), banana pseudostem (b), cut banana pseudostem (pith and outer portion of pseudostem).

4.2.3. Pretreatment of banana pseudostem

Three different pretreatment procedures were evaluated. One hundred and fifty grams of ground BPS was suspended in each pretreatment solutions, i.e. 3% NaOH (Filho *et al*., 2013); 5% H_2SO_4 (El-Zawawy *et al*., 2011; Gabhane *et al*., 2014; Lin *et al*., 2015) and H_2O, at a ratio of 1:10 (solid: liquid). The slurries were autoclaved at 121 °C, 15 PSI for 1 hour and cooled prior to washing with tap water until pH 7.0. The solid material was dried at 65 – 70 °C until constant mass and ground using Waring commercial blender (Model 32BL8) and stored at room temperature in sealed container until needed.

4.2.4. Solid state fermentation (untreated BPS)

Three milligrams of untreated BPS was moistened to 75% with synthetic medium as described by Peixoto (2006) in 250 ml Erlenmeyer flasks. The medium composition was as follows: 2 g/L K_2HPO_4, 0.5 g/L KCl, 0.01 g/L $FeSO_4.7H_2O$, 0.15 g/L $MgSO_4.7H_2O$, 7 g/L g KH_2PO_4, 1 g/L $(NH_4)SO_4$ and 1.2 g/L Yeast Extract. The pH of the medium was adjusted to 6.5 using1 M NaOH or 1 M HCl prior to sterilization at

121 °C, and 15 PSI for 1 hour. A 1 mL spore suspension (1 x 10^8 spores/ml) of *T. longibrachiatum* LMLSAUL 14-1, *A. fumigatus* LMLPS 13-4 and *T. harzianum* LMLBP07 13-5 were inoculated into separate 250 ml Erlenmeyer flasks that contained 3 g of untreated BPS. The flasks were incubated at 30 °C for 9 days without shaking. The whole content of each flask was sampled from day 3 up to day 9

4.2.5. Enzyme extraction

Enzyme extraction was carried out by modifying the method described by El-Shishtawy *et al*., (2015). The crude enzyme was extracted by adding 50 mL of 0.05 M sodium citrate buffer pH 4.8 to the fermented contents in flasks with intermittent shaking for 1 hour at room temperature (20 – 25 °C). The mixture was filtered and centrifuged at 3834 x g for 10 min and the supernatant was used for enzyme assays.

4.2.6. Determination of the effect of moisture level on the production of cellulase enzymes system

The effect of initial substrate moisture content was investigated in the range of 65 – 80% (v/w) at 30 °C under static conditions. Three grams (3 g) of untreated BPS was inoculated as indicated in section 4.2.4 for all fungal species. The fermentation progressed for a period of 9 days under static conditions. The whole flask content was used for enzyme extraction (see section 4.2.5).

4.2.7. Determination of the effect of temperature on the production of cellulase enzymes system

The effect of temperature on cellulose production was investigated in the range of 30 – 40 °C under static conditions and 75% initial moisture content of the substrate. A 3 g of untreated BPS was moistened with synthetic medium described above and inoculated as stated in section 4.2.4. The incubation temperature was as indicated herein. The fermentation continued for 9 days under static conditions. Enzyme extraction was done according to section 4.2.5.

4.2.8. Determination of the effect of different pretreatment methods on the production of cellulase enzymes system

Three grams of each pretreated BPS was moistened to 75% initial moisture content with synthetic medium and the culturing conditions were maintained as described under section 4.2.4.

4.2.9. Cellulolytic activity assays

4.2.9.1. Cellulase (FPase)

The cellulase activity was determined using the filter paper assay (FPase) with Whatman No.1 filter paper strip of 1 x 6.0 cm equivalent to 50 mg of substrate according to Ghose (1987). Details of the method are described in section 3.2.4.1.

4.2.9.2. Endoglucanase assay

Endoglucanase activity in the culture supernatant was determined according to the method described by Ghose (1987). Details of the method are described in section 3.2.4.2.

4.2.9.3. β-glucosidase assay

β-glucosidase was assayed according to the method described by Herr (1979). Details of the method are described in section 3.2.4.3.

4.2.10. Calculations of enzyme activities

4.2.10.1. Cellulase (Filter paper activity)

Filter paper activity was used to determine total cellulase activity of the crude enzyme using the equation (1);

$$\mathrm{FPA}\left(\frac{\mathrm{FPU}}{\mathrm{mL}}\right) = \frac{0.37}{[\mathrm{Enz}]} \qquad (1)$$

,where [Enz] is the concentration of enzyme that releases 2.0 mg of glucose from the 50 mg Filter paper in 60 minutes under the conditions of the assay (Ghose 1987). For cellulase activity in SSF, FPU/mL was converted to FPU/g d.s (d.s refer to dry substrate) using the equation 2 (Adney & Baker, 1996).

$$\text{FPA}\left(\frac{\text{FPU}}{\text{g d. s}}\right) = \frac{\frac{\text{FPU}}{\text{mL}} \times \text{total volume of fungal extract (mL)}}{\text{Dry weight of the substrate used in SSF (g)}} \quad (2)$$

4.2.10.2. Endoglucanase (CMCase) and β-glucosidase activities

To estimate the activities of either endoglucanase or β-glucosidase the equation (3) was used (Rubeena *et al*., 2013)

$$\beta - \text{glucosidase or CMCase}\left(\frac{\text{U}}{\text{mL}}\right) = \frac{\Delta\text{E} \times \text{Vf} \times \text{Df}}{\varepsilon \times \Delta\text{t} \times \text{Venz}} \quad (3)$$

To convert the U/mL to u/g d.s, the above equation becomes;

$$\text{CMCase or }\beta\text{glucosidase}\left(\frac{\text{U}}{\text{mL}}\right) = \frac{\frac{\text{U}}{\text{mL}} \times \text{total volume of fungal extract (mL)}}{\text{Dry weight of the substrate used in SSF (g)}} \quad (3)$$

4.2.11. Determination of the effect of temperature and pH on the activities of the cellulase enzymes system

The effect of temperature on the activities of cellulase enzymes system was investigated by assaying reaction mixture (i.e. enzyme plus relevant substrate) at various temperatures ranging from 40 – 80 °C and pH 5 in respective buffers for each enzyme. Relative activity (%) was determined by considering maximum activity as the standard reference. The effect of pH on the activities of cellulase enzymes system was determined at different pH values using respective substrates prepared in the following buffer solutions (0.1 M): sodium citrate buffer (pH 4.5 – 6); sodium acetate buffer (pH 6.5 – 8) and Tris-glycine buffer (pH 8.5 – 9.0). The reaction

temperature used was 60 °C. Relative activity (%) was determined by considering maximum activity as the standard reference (Moretti *et al*., 2012). The enzymes were assayed as outlined in section 3.2.4.1 to 3.2.4.3.

4.2.12. Determination of thermal and pH stability of cellulase enzymes system

Both thermal and pH stability followed a modified method by Santos *et al*. (2016). Thermal stability was investigated by pre-incubating the crude enzyme without substrate at various temperatures (40 – 80 °C) for 24 hours. Residual activity was determined at 60 °C and pH 6.5. Residual activity was expressed as a percentage of the crude enzyme activity without pre-incubation, which was considered 100%.

The pH stability of crude enzymes was evaluated by mixing the crude enzyme with buffer solutions as described in section 4.2.10 in equal proportions (i.e. 1:1 v/v). The solutions were incubated at room temperature (20 – 25 °C) for 24 hours without substrates. An aliquot was taken to determine the residual activities of the cellulase at 60°C. The enzymes assays were carried out as outlined in section 3.2.4.1 to 3.2.4.3. Residual activity was expressed as a percentage of the crude enzyme activity at the optimum temperature, which was considered 100% (Santos *et al*., 2016).

4.2.13. Statistical analysis

All the experiments were done in triplicates. The data generated was statistically analysed by Two-way analysis of variance (ANOVA) test using MS Excel 2010. Differences were considered significant when probability value (p) was <0.05. The error bars in the graphs represent standard error, SE.

4.3. RESULTS

4.3.1. The production of cellulase enzymes system

Solid state fermentation was used to produce cellulase enzymes system. Three filamentous fungi, *T. harzianum* LMLBP07 13-5, *T. longibrachiatum* LMLSAUL 14-1 and *A. fumigatus* LMLPS 13-4 were investigated for their ability to produce cellulase enzymes system in solid state fermentation of untreated BPS as inducing substrate. The amount of the cellulase enzymes system, namely cellulase (FPase), endoglucanse (CMCase) and β-glucosidase are shown in Table. 4.1.

The levels of the cellulase enzymes system produced varied amongst the fungal species. *T. longibrachiatum* LMLSAUL 14-1 produced the highest activities of cellulase enzymes system followed by *A*. *fumigatus* LMLPS 13-4. *Trichoderma harzianum* LMLBP07 13-5 produced the least amount of cellulase enzymes system, specifically cellulase and β-glucosidase exhibited the lowest activities when compared to the other two fungi.

Table 4.1. Production of cellulolytic enzymes by fungal species cultivated on solid state fermentation of untreated banana pseudostem as a substrate.

Fungi	**Total cellulase activity (FPU/g d.s)**	**Endoglucanase activity (U/g d.s)**	**β-glucosidase activity (U/g d.s)**
T. longibrachiatum LMLSAUL 14-1	75.04 ± 3.2648 (168 h)	111.35 ± 2.2290 (192 h)	235.83 ± 12.3296 (192 h)
T. harzianum LMLBP07 13-5	21.75 ± 3.9724 (120 h)	9.46 ± 0.5261 (144 h)	30.87 ± 8.1248 (144 h)
A. Fumigatus LMLPS 13-4	41.33 ± 5.0714 (216 h)	4.39 ± 4.1339 (120 h)	116.68 ± 17.8776 (72 h)

NB: Standard deviation ± values of independent triplicates

4.3.2. Effect of temperature on the production of cellulase enzymes system

The effect of temperature on cellulase enzymes system production was investigated over the range 30 – 40 °C, Fig. 4.2. In general, the temperature has a direct influence on the production of cellulase enzymes system by all fungal strains. Cellulase production by *T. longibrachiatum* LMLSAUL 14-1 showed a 0.47-fold decrease in activity at 40 °C (i.e. from 75 - 35.3 U/g d.s) while the optimum temperature for its production was attained at 30 °C. Conversely, endoglucanase level increased by 3.25-fold at 40 °C to a maximum of 363 U/g d.s, whereas a 0.93-fold decrease in the production of β-glucosidase was observed from 236 U/g d.s at 30 °C to 220 U/g d.s. at 40 °C, Fig. 4.2.

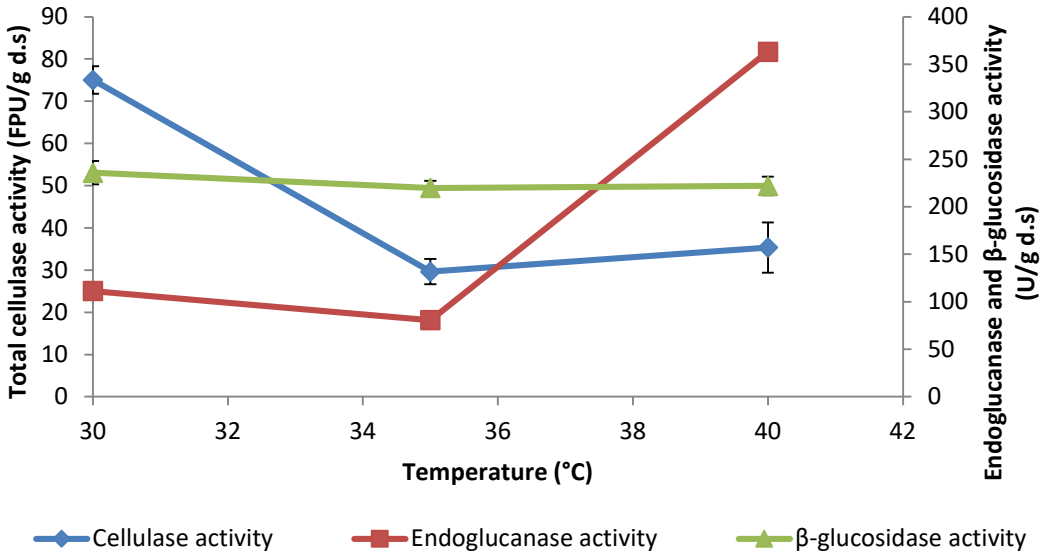

Figure 4.2. Effect of temperature on the production of cellulase enzymes system by *T. longibrachiatum* LMLSAUL 14-1 in solid state fermentation of untreated banana pseudostem.

The cellulase enzymes system produced by *T. harzianum* LMLBP07 13-5 is shown to increase proportionally by 1.8-fold with temperature from 22 FPU/g d.s at 30°C to 39.9 FPU/g ds. at 40 °C. The production of endoglucanase and β-glucosidase is also shown to increase by 4.2-fold to 40 U/g d.s and 4.8-fold to 164 U/g d.s, respectively as the temperature increase to 40°C, Fig. 4.3.

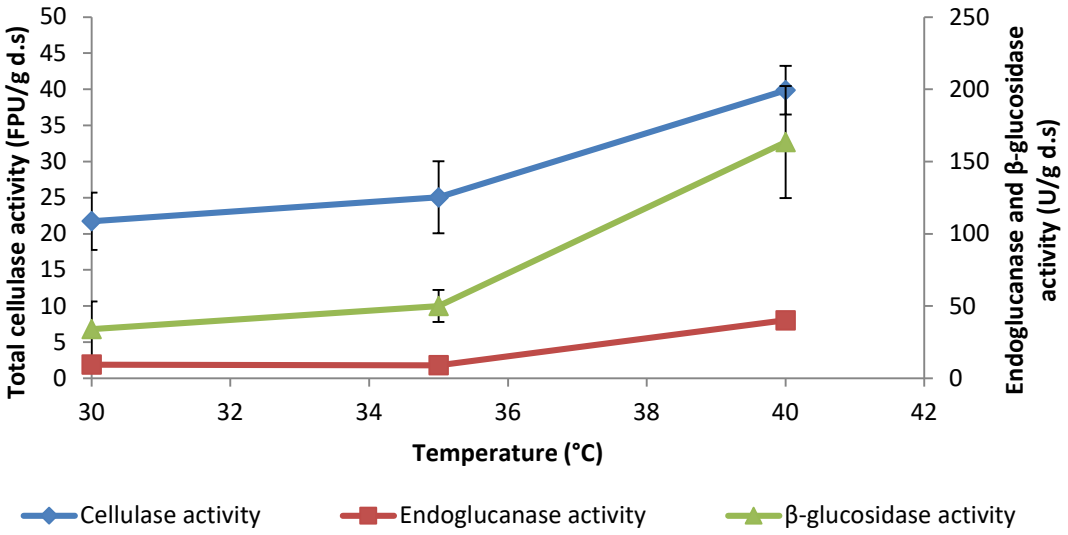

Figure 4.3. Effect of temperature on the production of cellulase enzyme system by *T. harzianum* LMLBP07 13-5 in solid state fermentation of untreated banana pseudostem

The cellulase enzymes system of *A. fumigatus* LMLPS 13-4 exhibited a different trend. The production of cellulase decreased by 0.8-fold from 41 FPU/g d.s at 30 °C to 33 FPU/g d.s at 40°C. On the contrary to the decreasing cellulase, the endoglucanase and β-glucosidase increased by 15.6-fold to 69 U/g d.s and by 2.4-fold to 276 U/g d.s, respectively Fig. 4.4.

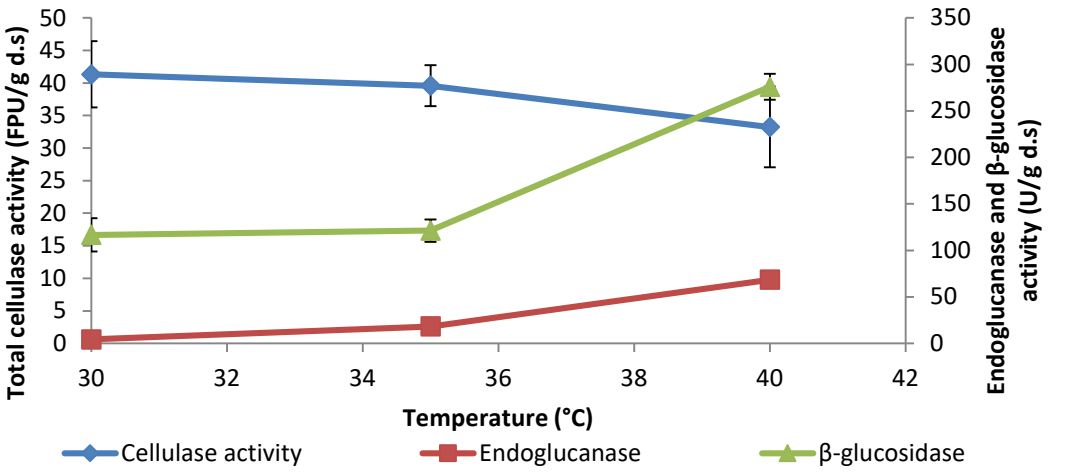

Figure 4.4. Effect of temperature on the production of cellulase enzymes system by *A. fumigatus* LMLPS 13-4 in solid state fermentation of untreated banana pseudostem.

4.3.3. Effect of initial moisture content of the banana pseudostem on the production of cellulase enzymes system

The moisture content of the substrate has been noted as a critical factor for growth support and enzyme production under SSF. The effect of initial substrate moisture content on the production of cellulase enzymes system was assessed in the range of 65 to 80%. The production of cellulase enzymes system by *T. longibrachiatum* LMLSAUL 14-1 in SSF is strongly dependent on the initial moisture level of the substrate Fig 4.5. The production of cellulase enzymes system was optimal at 75% moisture content.

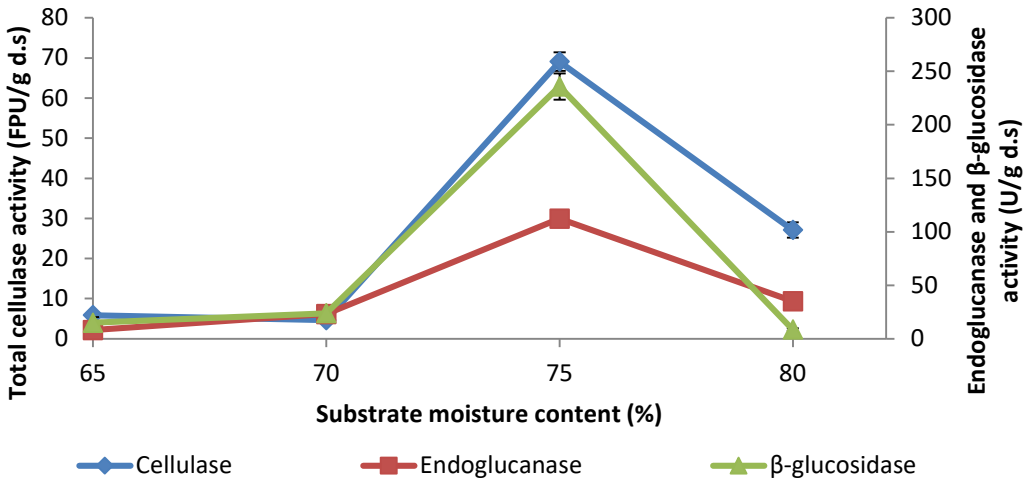

Figure 4.5. Effect of initial moisture content of banana pseudostem on the production of cellulase enzymes system by *T. longibrachiatum* LMLSAUL 14-1 in solid state fermentation of untreated banana pseudostem.

T. harzianum LMLBP07 13-5 has exhibited different moisture optima conducive for the production of cellulase enzymes system, Fig 4.6. Each enzyme showed specific moisture optimal for maximum production, unlike the trend exhibited by *T. longibrachiatum* LMLSAUL 14-1. The maximum production of cellulase (30 FPU/g d.s) occurred over 65 – 75% of initial moisture of the substrate. At 80% of initial moisture of the substrate, the cellulase was lower by 0.6-fold. The endoglucanase production remained low; irrespective of the initial moisture contents when compared with the endoglucanase levels attained by *T. longibrachiatum* LMLSAUL 14-1 and *A. fumigatus* LMLPS 13-4. Maximum β-glucosidase was produced at an initial substrate

moisture content of 70% (294.3 U/g d.s) and reduced to 0.7-fold at 80% moisture content.

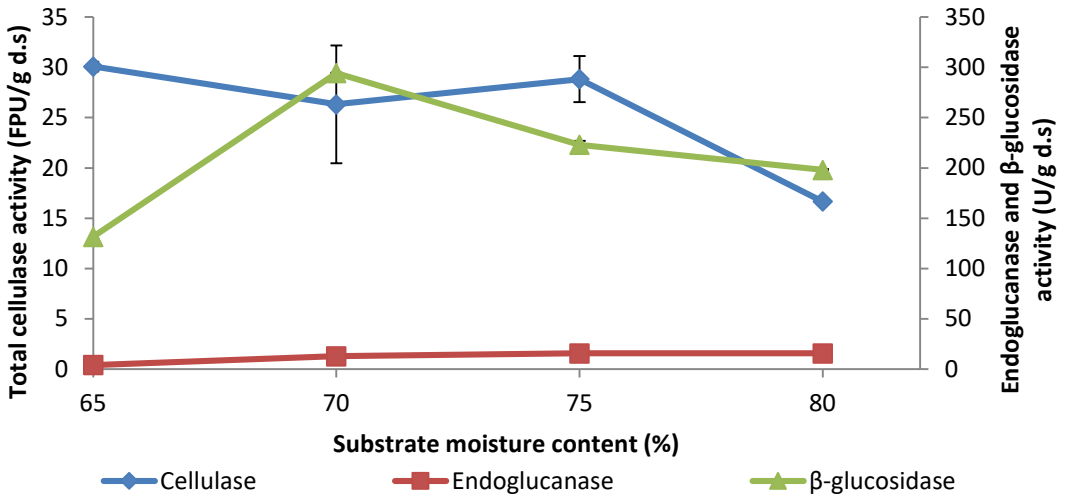

Figure 4.6. Effect of initial moisture content of banana pseudostem on the production of cellulase enzymes system by *T. harzianum* LMLBP07 13-5 in solid state fermentation of untreated banana pseudostem.

The production of cellulase enzymes system by *A*. *fumigatus* LMLPS 13-4 was also attained at different moisture contents as illustrated in Fig. 4.7. The maximum cellulase of 41.3 FPU/d.s was produced at 75% of the initial moisture content of the substrate. At 65% of initial moisture content of the substrate the production of endoglucanases reached 193 U/g d.s but decreased to 8.5 U/g d.s at 80% initial moisture content of the substrate. *Aspergillus fumigatus* LMLPS 13-4 showed a two-phase production trend with high levels of β-glucosidase (160 U/g d.s) occurring at 70% initial moisture content of substrate and 186.3 U/g d.s at 80% of initial moisture content of the substrate.

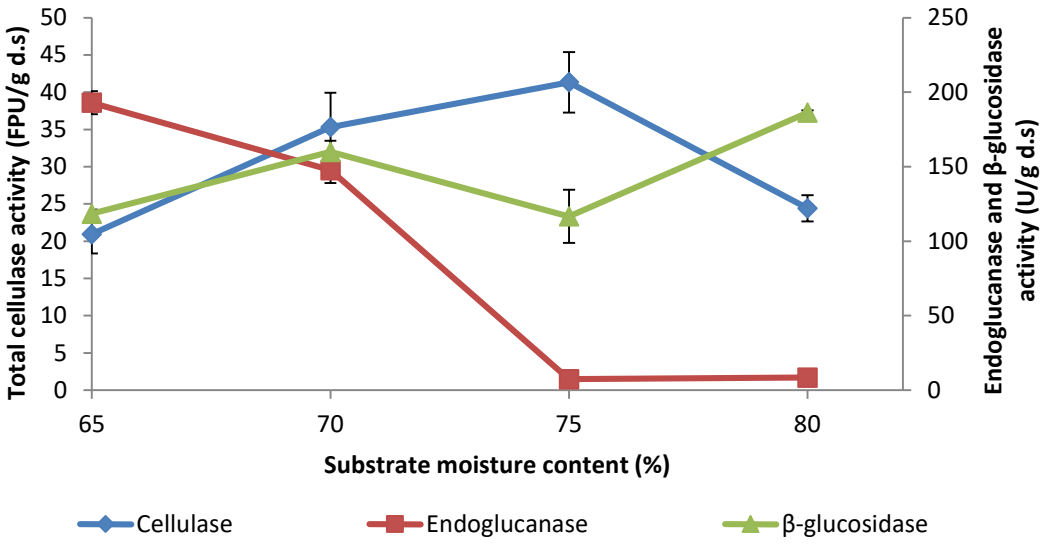

Figure 4.7. Effect of initial moisture content of banana pseudostem on the production of cellulase enzymes system by *A. fumigatus* LMLPS 13-4 in solid state fermentation of untreated banana pseudostem.

4.3.4. Effect of pretreated banana pseudostem on the production of cellulase enzymes system

Three pretreatment methods, namely thermo-dilute acid, thermo-alkaline, and hydrothermal (or hot water), were applied to BPS. The productions of cellulase enzymes system were influenced by the nature of the BPS substrate, Fig. 4.8. The pretreated BPS poorly induced the synthesis and secretion cellulase and endoglucanase of *T. longibrachiatum* LMLSAUL 14-1 when compared to untreated BPS substrate. The cellulase decreased by 0.3-fold to 21 FPU/g d.s in 3% NaOH pretreated BPS and 0.24-fold reduction to 16.6 FPU/g ds.s in both 5% H_2SO_4 and hot water pretreated BPS. The endoglucanase production decreased by 0.29-fold in SSF of 3% NaOH pretreated BPS. There is no significant change in β-glucosidase production between hot water and untreated BPS, Fig. 4.8.

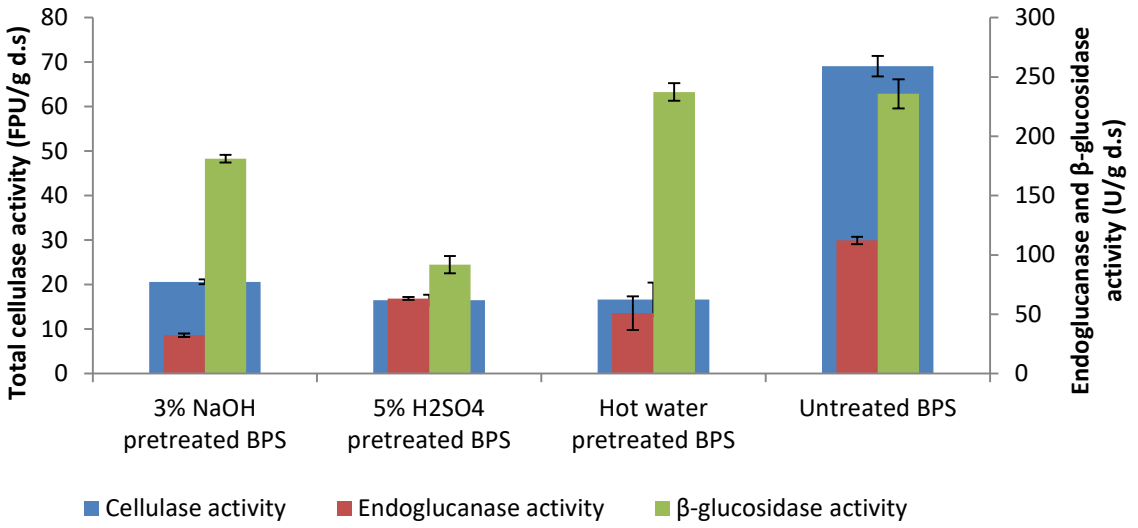

Figure 4.8. Effect of pretreatment methods on the production of cellulase enzymes system by *T. longibrachiatum* LMLSAUL 14-1 in solid state fermentation of the pretreated banana pseudostem.

The production of cellulase enzymes system by *T. harzianum* LMLBP07 showed that untreated BPS was more suitable for cellulase production as indicated by its high activity of 28.8 FPU/g d.s, Fig. 4.9. Hot water pretreated BPS led to a significant decrease in cellulase production with activity of 6.2 FPU/g d.s. The productions of endoglucanase levels were similar to untreated BSP. β-glucosidase level was produced in high quantities in SSF of pretreated BSP where 3% NaOH yielded the highest activity.

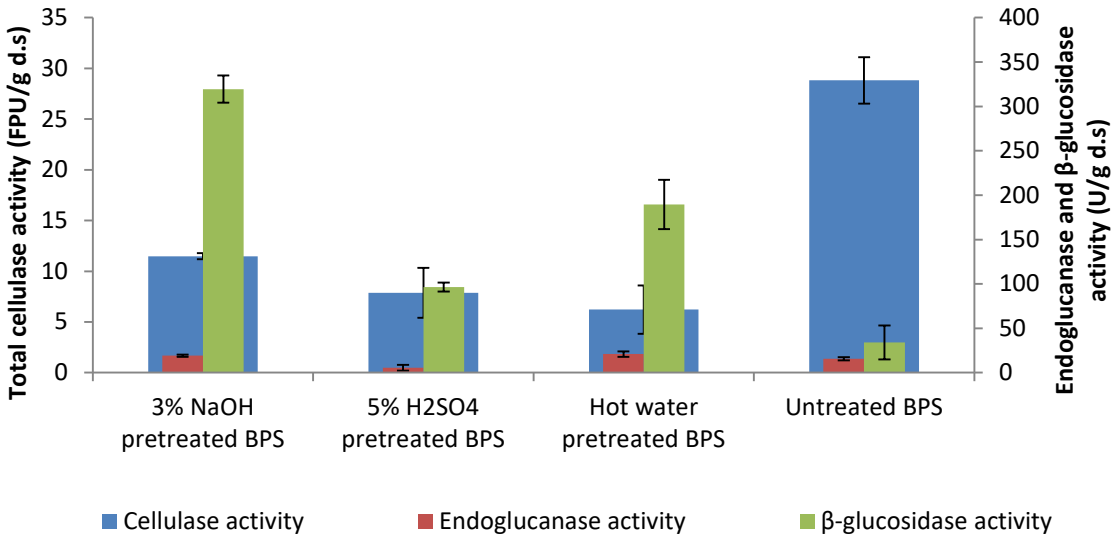

Figure 4.9. Effect of pretreatment methods on the production of cellulase enzymes system by *T. harzianum* LMLBP07 13-5 in solid state fermentation of pretreated banana pseudostem.

The highest cellulase produced for *A. fumigatus* LMLPS 13-4 was detected in untreated BPS (41 FPU/g d.s) followed by hot water pretreated BPS (24 FPU/g d.s). On the other hand, SSF of 5% H_2SO_4 pretreated BPS favoured the production of endoglucanase (45 U/g d.s) and β-glucosidase (260 U/g d.s), Fig. 4.10.

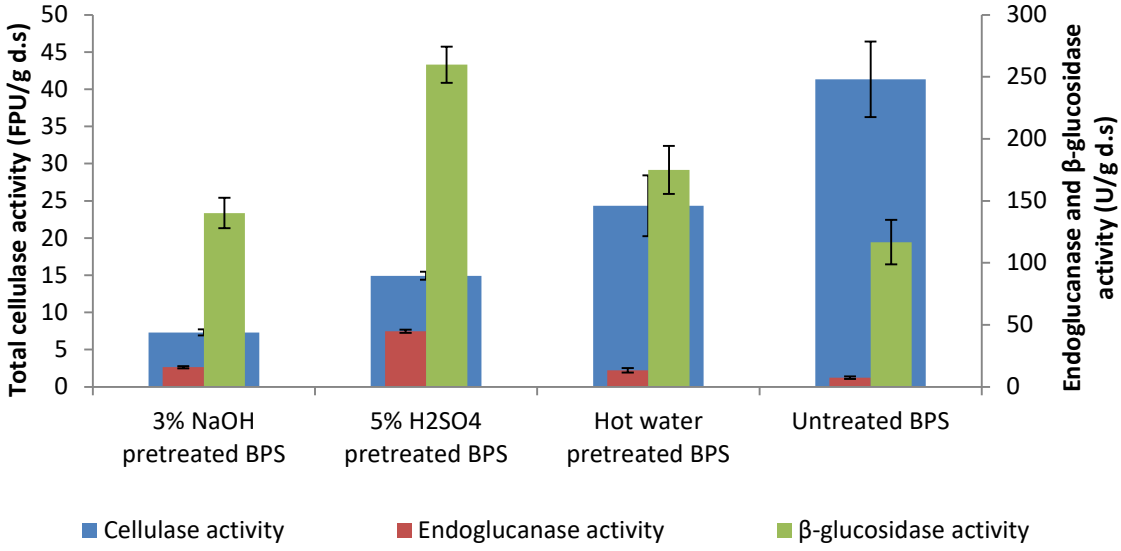

Figure 4.10. Effect of pretreatment methods on the production of cellulase enzymes system by *A. fumigatus* LMLPS 13-4 in solid state fermentation of pretreated banana pseudostem.

4.3.5. Effect of co-cultivation of *Trichoderma* and *Aspergillus* species on the production of cellulase enzymes system

Co-cultivation of *Trichoderma* and *Aspergillus* was assessed in SSF using untreated BPS as substrate in an attempt to increase hydrolytic activity of the cellulase enzymes activity. Co-culturing of *T. longibrachiatum* LMLSAUL 14-1 and *A. fumigatus* LMLPS 13-4 produced 61 FPU/g d.s, which is a 0.8-fold decrease in cellulase activity when compared to 75 FPU/g d.s obtained in monoculture of *T. longibrachiatum* LMLSAUL 14-1, Fig. 4.11. There was also a reduction of 0.47-fold in the β-glucosidase activity. In general, the co-cultivation of the above fungi did not yield improvement in production of cellulase enzymes system production.

On the contrary, co-cultivation of *T. harzianum* LMLBP07 13-5 and *A. fumigatus* LMLPS 13-4 yielded an improvement with 2.23-fold increase in cellulase and a 10-fold increase in endoglucanase production compared to a monoculture of *T. harzianum* LMLBP07 13-5. However, the production of β-glucosidase was affected with 0.48-fold decrease compared to monocultures, Fig. 4.11.

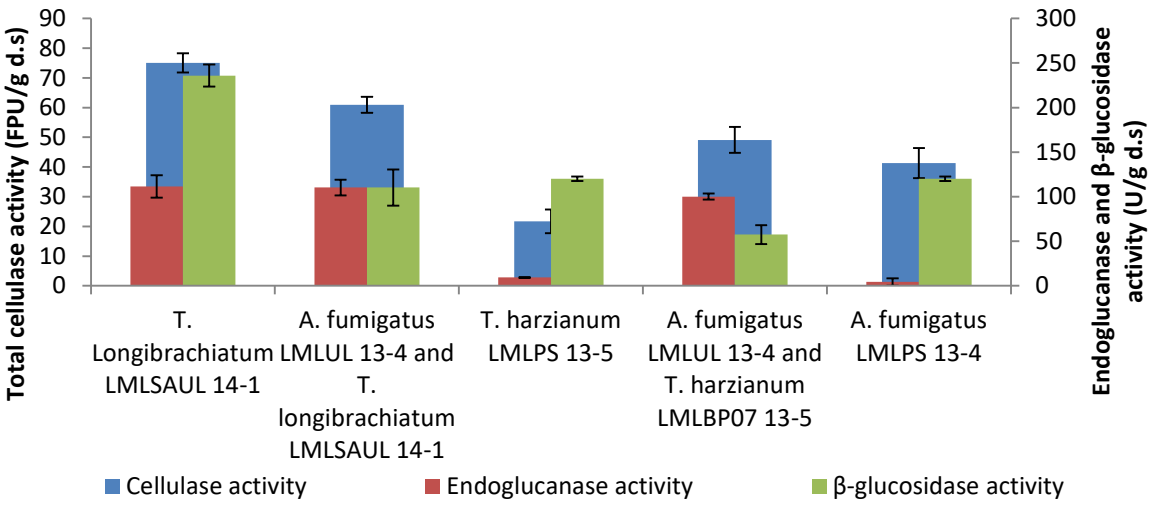

Figure 4.11. Effect of co-culturing the *Trichoderma* and *Aspergillus* strains on the production of cellulase enzymes system in solid state fermentation of untreated banana pseudostem.

4.3.6. Effect of temperature on the activities of cellulase enzymes complex

The fungi investigated produced the cellulase enzymes system, which is highly sensitive towards temperature. *T. longibrachiatum* LMLSAUL 14-1 exhibited maximum cellulase and β-glucosidase activity at 60 °C. The endoglucanase activity was optimal at 50 °C and gradually decreased as the temperature increased, Fig. 4.12.

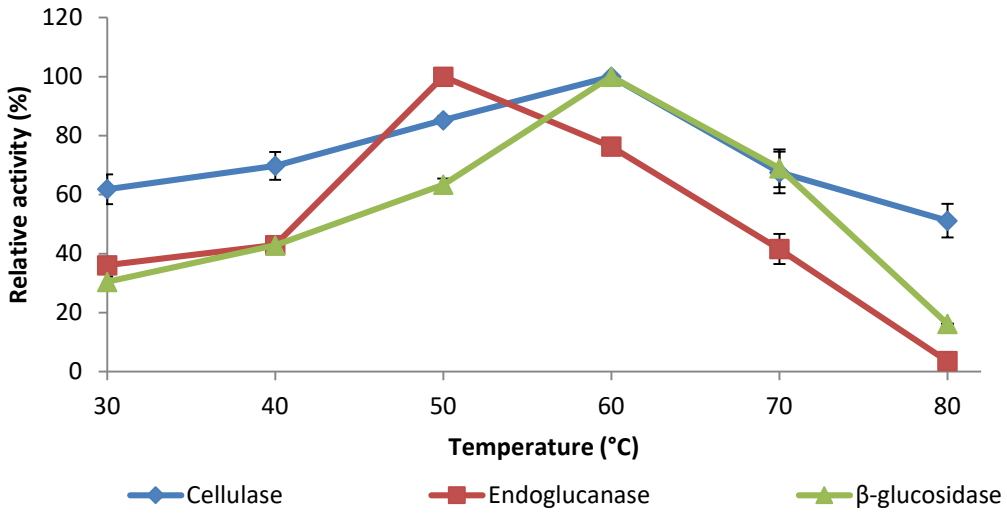

Figure 4.12. Effect of temperature on the catalytic activities of cellulase enzymes complex produced by *T. longibrachiatum* LMLSAUL 14-1 in solid state fermentation of untreated banana pseudostem.

With regard to cellulase enzymes system produced by *T. harzianum* LMLBP07 13-5, the cellulase exhibited dual peaks of activities, with maximum cellulase activity occurred at 40 °C followed by 88% activity at 70 – 80 °C. The cellulase activity was above 65% level across the temperature investigated. Endoglucanase activity was optimal at 70 °C. β-glucosidase activity was optimal at 60 °C and also achieved over 90% of β-glucosidase activity at 70 °C, Fig. 4.13. The β-glucosidase activity was drastically affected (15% activity) at 80 °C.

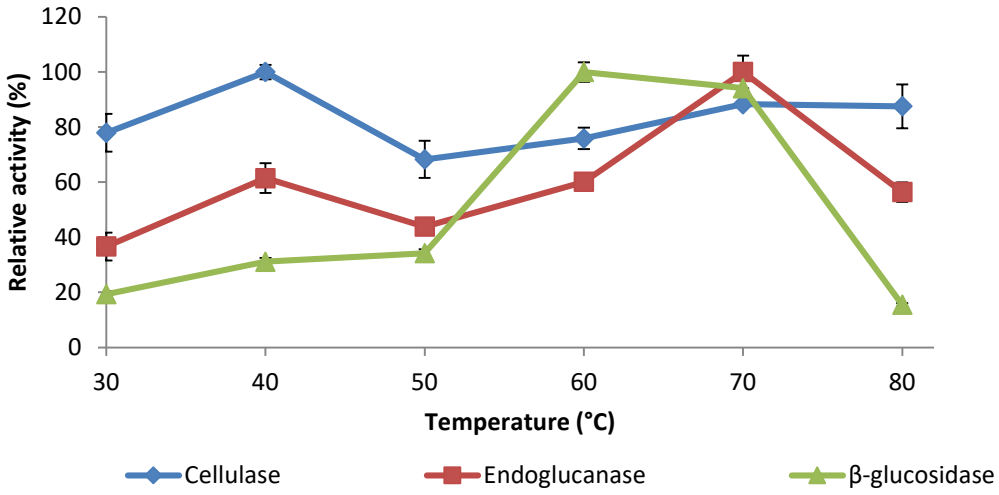

Figure 4.13. Effect of temperature on the catalytic activities of cellulase enzymes system produced by *T. harzianum* LMLBP07 13-5 in solid state fermentation of untreated banana pseudostem.

With *A. fumigatus* LMLPS 13-4, maximum the cellulase activity was measured at 60 °C, whereas endoglucanase activity was maximum at 50 - 60 °C. Higher temperatures negatively affected both cellulase and endoglucanase activities. The β-glucosidase activity was the maximum at 60 °C and 70 °C respectively, Fig. 4.14.

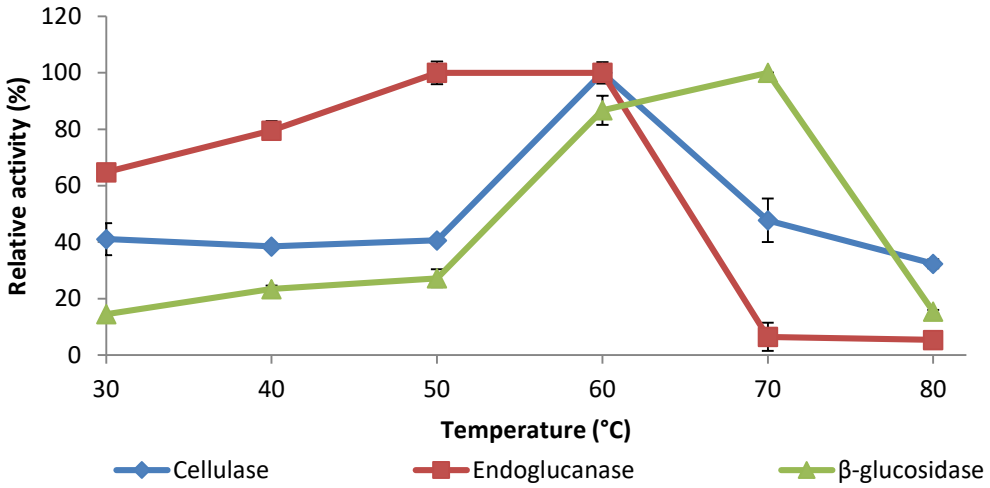

Figure 4.14. Effect of temperature on the catalytic activities of cellulase enzymes system produced by *A. fumigatus* LMLPS 13-4 in solid state fermentation of untreated banana pseudostem.

4.3.7. Effect of pH on the activities of the cellulase enzymes system

The cellulase produced by *T. longibrachiatum* LMLSAUL 14-1 exhibited over 55% of its activity between pH 5.5 – 7.5, with high activity (84%) at pH 6.5. Acidic pH (4.5 – 6.5) positively influenced endoglucanase activity with maximum activity at pH 5.5. Endoglucanase activity was sensitive to pH higher than 6.5. The β-glucosidase activity was not sensitive to pH and was active over a wide range, but peaked at pH 5.5, Fig. 4.15.

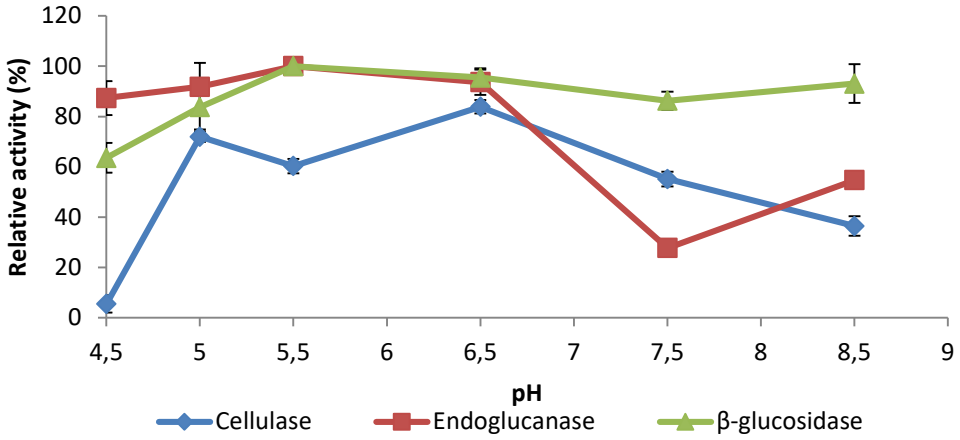

Figure 4.15. Effect of pH on the catalytic activities of cellulase enzymes system produced by *T. longibrachiatum* LMLSAUL 14-1 in solid state fermentation of untreated banana pseudostem.

The cellulase produced by *T. harzianum* LMLBP07 13-5 exhibited 100% activity at pH 4.5 and the activity slightly decreased to 90% at pH 5.5 – 6.5. At a pH level higher than 6.5, the cellulase activity gradually decreased to 54%. High endoglucanase activity was observed at pH 4.5 – 6.5, with maximum activity at pH 4.5. A pH higher than 6.5 also reduced the endoglucanase activity. The β-glucosidase activity remained above 85% from pH 5.5 – 8.5, Fig. 4.16.

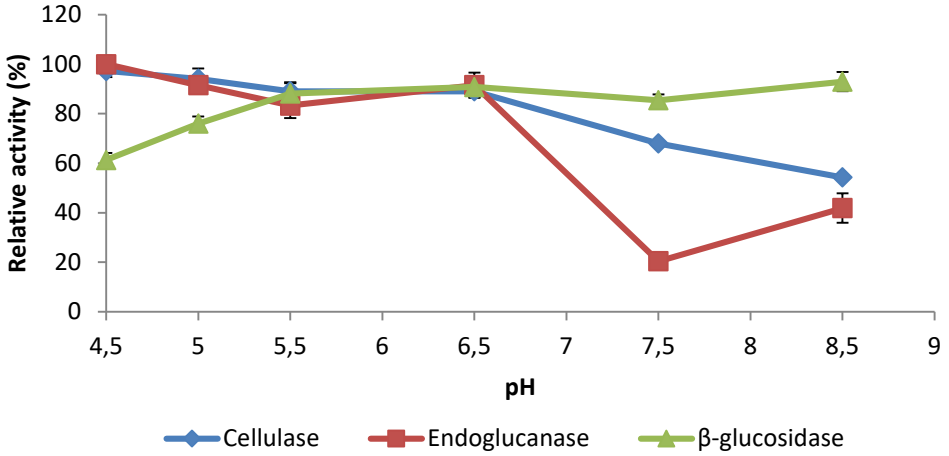

Figure 4.16. Effect of pH on the catalytic activities of cellulase enzymes system produced by *T. harzianum* LMLBP07 13-5 in solid state fermentation of untreated banana pseudostem.

Cellulase produced by *A. fumigatus* LMLPS 13-4 was high at pH 5.0 – 8.0, with 100% activity observed at pH 5.0. The activity was negatively affected at pH lower than 5.0 and higher than 8.0. Maximum endoglucanase activity was observed at pH 4.5 and over 70% activity was attained at pH 5.0 – 6.5. β-glucosidase activity (90 – 100%) was high at pH 5.5 – 8.5, Fig. 4.17.

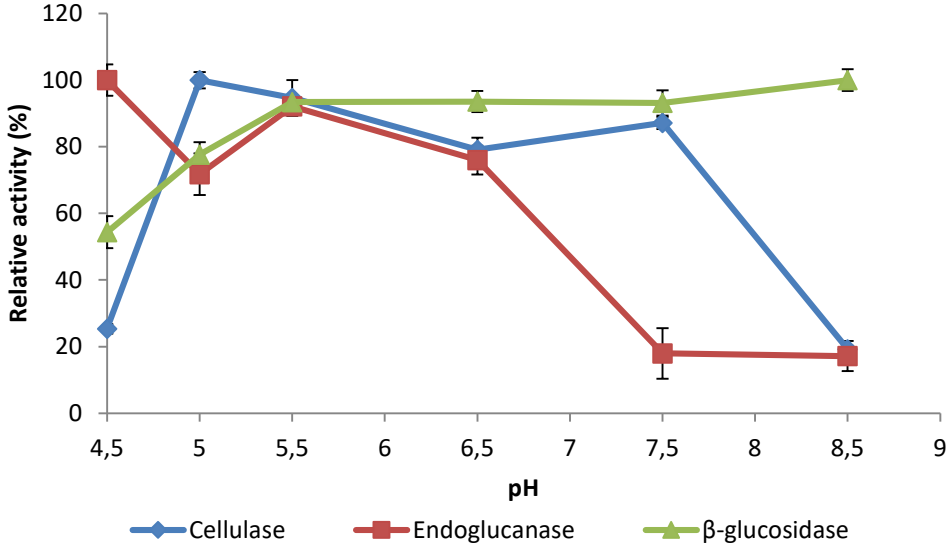

Figure 4.17. Effect of pH on the catalytic activities of cellulase enzymes system produced by *A.fumigatus* LMLPS 13-4 in solid state fermentation of untreated banana pseudostem.

4.3.8. Effect of temperature on the stability of the cellulase enzymes system

The thermal stability of cellulase enzymes system was investigated at optimum pH for each enzyme as indicated in section 4.2.11 and optimum temperature of 60 °C for the enzymatic assays. After 24 hours of incubation at respective temperatures (40 – 80 °C with 10 °C intervals), the residual activity of cellulase produced by *T. longibrachiatum* LMLSAUL 14-1 retained 54% of its activity at 40 °C and gradually decreased to less than 25% as temperature increased to 80 °C. As for endoglucanase, the enzyme retained 70% of its activity at 40 - 50 °C. A sharp decline in activity was observed at a temperature above 50 °C resulting in a 98% loss of activity, complete irreversible enzyme inactivation, Fig. 4.18. β-glucosidase was also inactivated at high temperature, retaining 80% of its activity at 40 °C, and drastically decreased to approximately 6% at 80 °C.

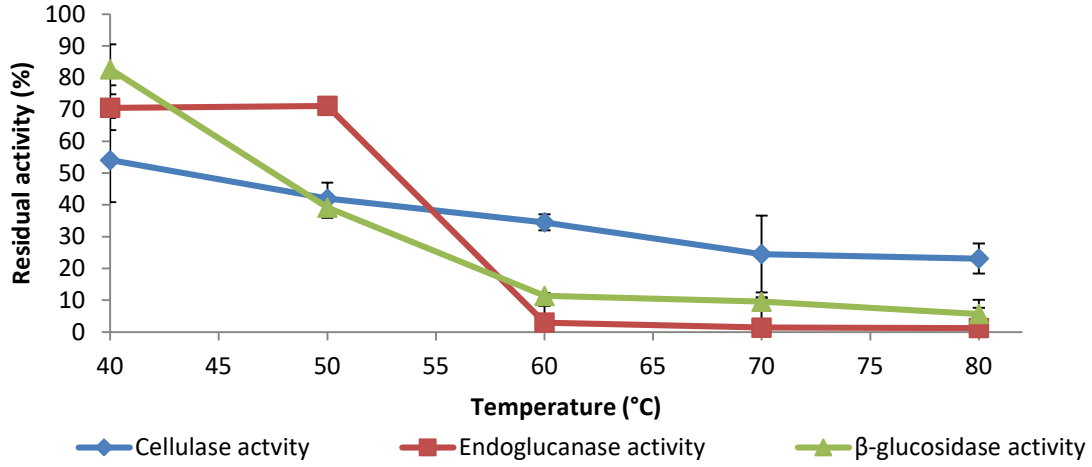

Figure 4.18. Thermal stability of cellulase enzymes system produced by *T. longibrachiatum* LMLSAUL 14-1 in solid state fermentation of untreated banana pseudostem.

The cellulase enzymes system of *T. harzianum* LMLBP07 13-5, particularly cellulase and endoglucanase enzymes showed enhancement of activities at 40 °C and 50 °C, Fig. 4.19. The cellulase lost 65% of its original activity at 60 °C with a decrease to below 20% at 80 °C. However, endoglucanase remained stable and retained over 80% of its activity up to 70 °C. β-glucosidase was stable up to 50 °C (>60%), but was rapidly inactivated to below 2% of original activity between 60 and 80 °C.

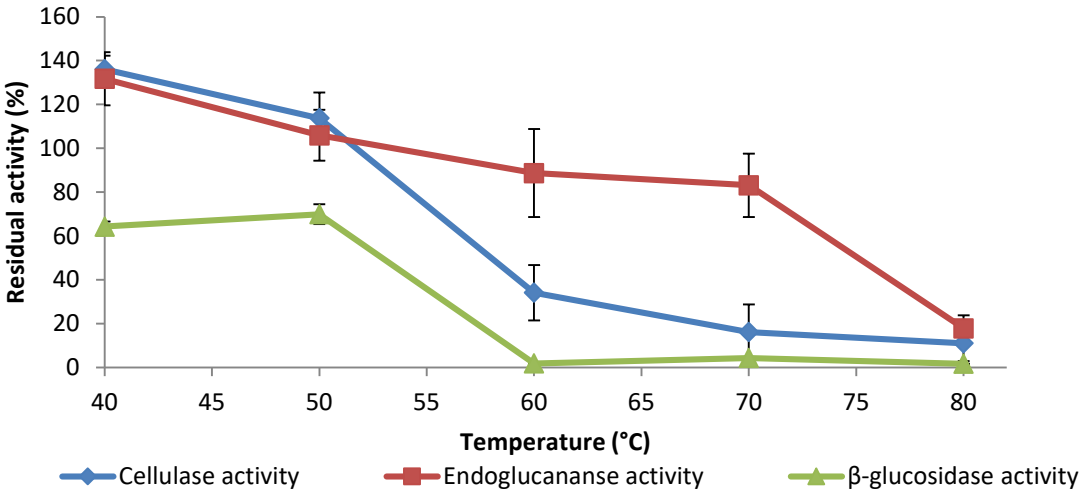

Figure 4.19. Thermal stability of cellulase enzymes system produced by *T. harzianum* LMLBP07 13-5 in solid state fermentation of untreated banana pseudostem.

Regarding *A. fumigatus* LMLPS 13-4, cellulase retained approximately 70% of its activity at 40°C and activity remained above 50% at 50 – 60 °C. Endoglucanase retained more than 50% activity throughout the temperature range tested, Fig. 4.20. The thermal stability of β-glucosidase activity was poor with only 42% of its activity retained at 40 °C. At higher temperatures, the β-glucosidase rapidly lost activity to below 1%.

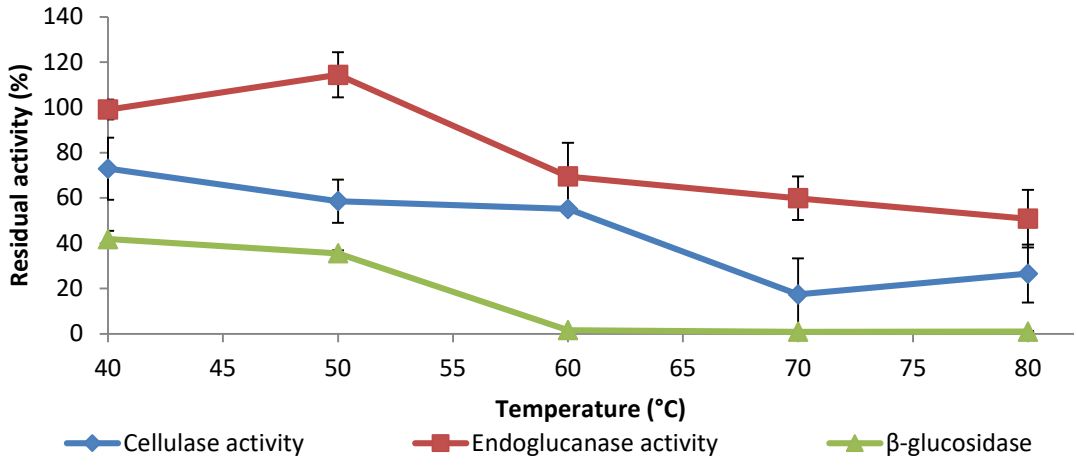

Figure 4.20. Thermal stability of cellulase enzymes system produced by *A. fumigatus* LMLPS 13-4 in solid state fermentation of untreated banana pseudostem.

4.3.9. Effect of pH on the stability of the cellulase enzymes system

For pH stability, crude preparations of the cellulase enzymes system were pre-incubated in the appropriate buffers at room temperature for 24 hours. After 24 hours, T. *longibrachiatum* LMLSAUL 14-1 cellulase retained over 80% of its activity at pH 5.0 – 5.5 but the activity decreased at a higher pH values. The stability of endoglucanase was poor, with 40% activity retained between pH 4.5 – 5.0 followed by further loss up to 21% of activity at pH 8.5. β-glucosidase remained stable, with over 80% of its activity retained at pH 4.5 – 5.5, Fig. 4.21.

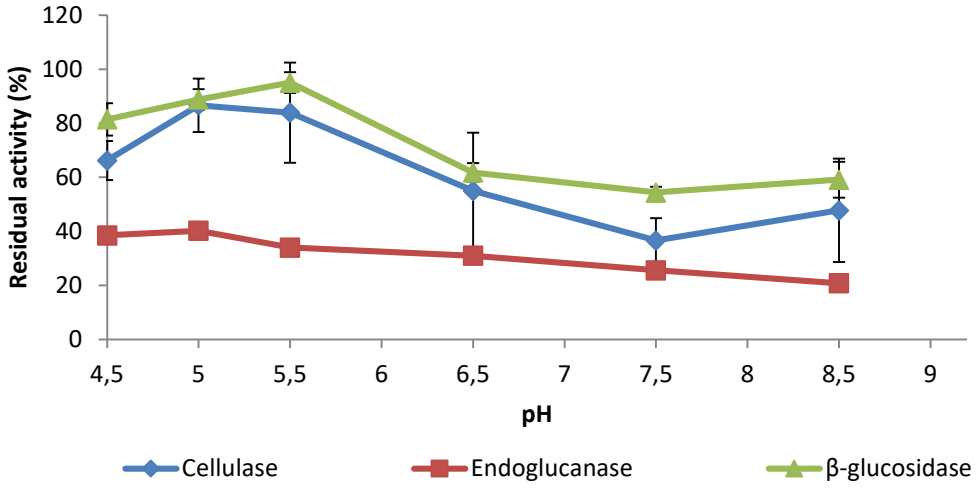

Figure 4.21. Effect of pH on the stability of the cellulase enzymes system produced by *T. longibrachiatum* LMLSAUL 14-1 in solid state fermentation of untreated banana pseudostem.

The cellulase produced by *T. harzianum* LMLBP07 13-5 was most stable in alkaline conditions, pH 7.5 – 8.5, retaining 90 – 110% of its activity. The endoglucanase retained 53% of its original activity at pH 4.5, with a gradual loss in activity as the pH increased to 8.5. The β-glucosidase of *T. harzianum* LMLBP07 13-5 was stable and retained over 70% of its activity at pH 4.5 – 6.5, with the highest activity (81%) retained at pH 5.0, Fig. 4.22.

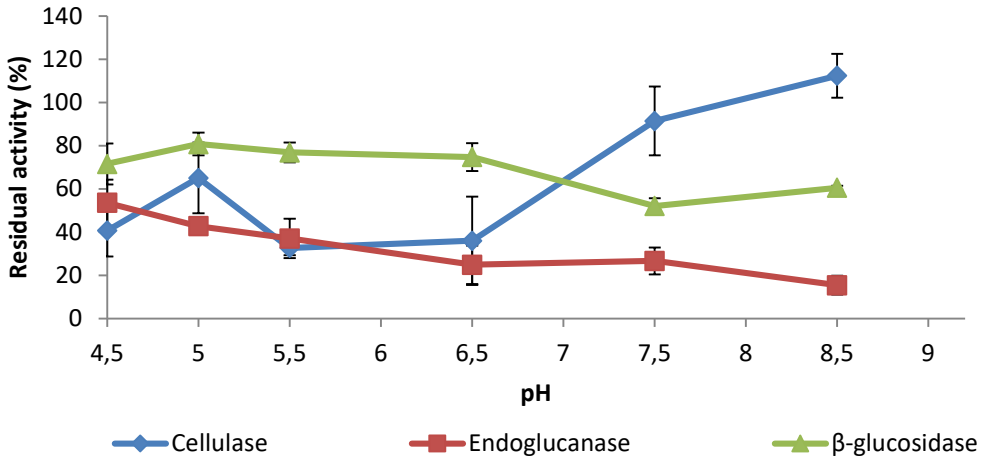

Figure 4.22. Effect of pH on the stability of the cellulase enzymes system produced by *T. harzianum* LMLBP07 13-5 in solid state fermentation of untreated banana pseudostem.

Aspergillus fumigatus LMLPS 13-4 produced cellulase with poor pH stability. The cellulase retained approximately 40% of its original activity across pH 4.5 – 6.5. The endoglucanase showed stability across the pH 4.5 – 8.5, with most activity (100 – 80%) retained in acidic conditions (pH 4.5 – 5.5). On the other hand, β-glucosidase showed stability at pH range 4.5 – 8.5 maintaining over 70% of its activity, Fig. 4.23.

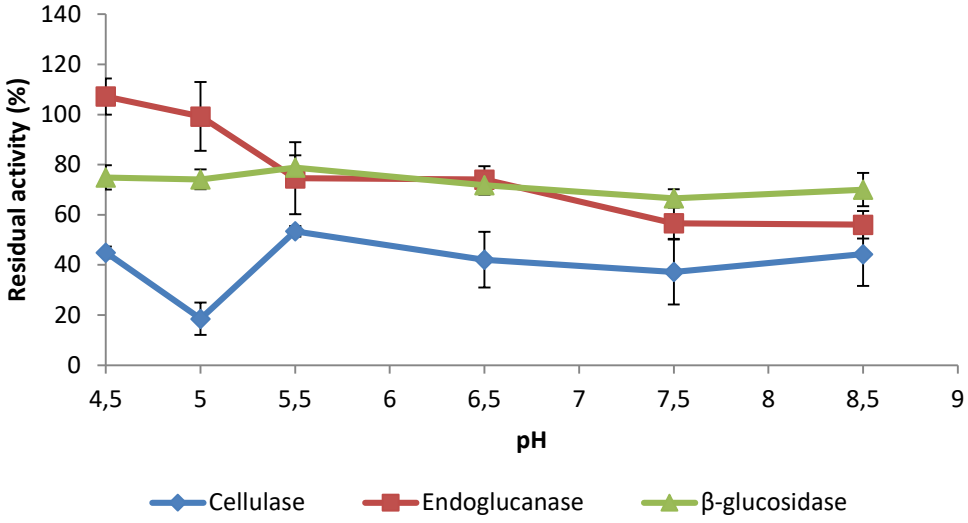

Figure 4.23. Effect of pH on the stability of the cellulase enzymes system produced by *A. fumigatus* LMLPS 13-4 in solid state fermentation of untreated banana pseudostem.

4.4. DISCUSSION

The production of enzymes involved in the degradation of lignocellulosic biomass is regulated at the transcriptional level (Stricker *et al*., 2008) and is also subject to the substrate used (Olsson *et al*., 2003). Several carbon sources including lactose, sophorose, L-sorbose *etc*. have shown to induce the formation and secretion of cellulase enzymes system, namely cellobiohydrolases, endoglucanases and β-glucosidases (Olsson *et al*., 2003; Sternberg & Mandels, 1979). However, industrial fermentation using the above carbon sources to produce cellulose degrading enzymes remains expensive. This study utilised banana pseudostem (BPS), an inexpensive agricultural waste product to produce cellulase enzymes system in solid state fermentation (SSF). The findings show that BPS has the ability to induce the production of these enzymes in all *Trichoderma* and *Aspergillus* species studied, Table 4.1.

4.4.1. Effect of temperature on the production of cellulase enzymes system by selected fungal species.

Temperature affects the growth and metabolic activities of microorganisms (Mrudula and Murugamnal, 2011). The production of cellulase enzymes system appears to be dependent on a particular temperature for specific enzymes that were evaluated. For instance, the maximum cellulase (FPase), endoglucanase and β-glucosidase of *T. longibrachiatum* LMLSAUL 14-1 were produced at different temperatures, Fig. 4.2. This trend suggests varying degrees for which temperature activate the different regulatory enzyme systems. The optimum temperature for the production of cellulases and β-glucosidases by *T. longibrachiatum* LMLSAUL 14-1 was 30 °C. At higher temperature, there was a decrease in synthesis and secretion of these enzymes. According to Jecu (2000), the decrease in enzyme production at such high temperatures could be due to the deactivation of a particular regulatory enzyme system. Conversely, the optimum temperature for the production of endoglucanases by *T. longibrachiatum* LMLSAUL 14-1 was found to be 40 °C. On the contrary Leghlimi *et al*. (2013) reported maximum cellulases (FPase) and endoglucanases produced by both *T. longibrachiatum* (GHL) and *T. reesei* Rut C-30 to be at 35 °C. Such differences are exacerbated by the chemical composition of the medium and other factors such as pH.

Trichoderma harzianum LMLBP07 13-5 was able to produce maximum cellulase enzymes system at one specific temperature. The optimum temperature for the production of cellulases, endoglucanases and β-glucosidases by the fungus was 40 °C, Fig. 4.3. Another fungus, *A. fumigatus* LMLPS 13-4 also produced higher endoglucanase and β-glucosidase activity at 40 °C, while its cellulase was higher at 30 °C, Fig. 4.4. Similarly, another study reported an optimum temperature for the production of cellulase enzyme system by *A*. *fumigatus* to be 40 °C (Sherief *et al*., 2010).

The differences in optimal temperatures for the production of cellulases, endoglucanases and β-glucosidases as observed in Fig. 4.2 and 4.4 were also reported in other studies. Pirota *et al*. (2016) also noted significant influence of temperature on the production of both endoglucanases and β-glucosidases. These authors reported a 2.3-fold increase in endoglucanase activity at 28 °C and higher β-glucosidase activity between 35 and 37 °C under static aeration and optimum moisture content of 70%. Liu *et al*. (2011) reported maximum production of cellulase and endoglucanase at 50 °C by *A*. *fumigatus* Z5 in SSF using corn stover. Another fungus, *A*. *fumigatus* fresenius (AMA), when cultivated on rice straw produced maximum cellulase at 45 °C (Soni *et al*., 2010).

Generally, *Trichoderma* and *Aspergillus* species are mesophilic and these organisms exhibited different optimum temperatures for the production of cellulase enzymes system. The differences in optimum temperature observed in this study when compared to other findings (Liu *et al*., 2011; Soni *et al*., 2010) might be influenced by natural habitat from which these fungal species were isolated and the type of substrate used for cultivation. It is also known that when an organism is subjected to certain environmental conditions it respond differently by activating regulatory mechanisms that trigger growth, conidiation and biosynthesis of secondary metabolites (Schmoll *et al*., 2010). This study revealed that temperature influences growth as well as spore formation (i.e. green spores at 30 °C to white spores at 40 °C) and synthesis of enzymes.

4.4.2. Effect of initial moisture content of the banana pseudostem on the production of cellulase enzymes system by the selected fungal species

Another important factor that affects the metabolic activities of fungi for efficient enzyme synthesis and secretion in SSF is the moisture content of the solid substrate. The results obtained showed that the production of cellulase enzymes system in SSF of untreated BPS was strongly influenced by the moisture content of the substrate. The optimum initial moisture content of BPS that supported maximum production of cellulase enzymes system by *T*. *longibrachiatum* LMLSAUL 14-1 was 75%, Fig. 4.5. Similarly, findings by Sherief *et al*. (2010) reported that *A*. *fumigatus* growing on a mixture of rice straw and wheat bran (1:1) produced the highest cellulase activity at an initial moisture content of 75%.

According to Lee (1997), too little moisture prevents fungal growth and too much ~~of~~ moisture clogs inter-particle spaces thereby inhibiting oxygen circulation resulting in compaction of the substrate or possibly contamination by bacteria. Depending on the type of substrate, some microorganisms respond positively to high/or low initial moisture of the substrate during synthesis of enzymes. For instance, a thermophilic fungi, *M. thermophile* M77 produced cellulase activity of 10.6 FPU/g d.s when cultivated on mixed substrate of soybean and sugarcane (10:90) with moisture adjusted to 80% and a temperature of 45 °C (Kilikian *et al*., 2014). A different production trend was observed for *T*. *harzianum* LMLBP07 13-5 whereby each enzyme of the cellulase enzymes system was produced at different initial moisture contents, Fig. 4.6. As a result of such differences, the optimum initial moisture content of BPS for cellulase production was between 65 and 75% and as well as 70% for β-glucosidase.

The endoglucanase production increased proportionally with an initial moisture content of BPS and remained high between 75% and 80%. The initial moisture content of BPS higher than 75% has reduced cellulases and β-glucosidases production by 0.53 and 0.67, respectively. *A. fumigatus* LMLPS 13-4 also responded differently under varying moisture contents of BPS for the production of cellulase enzymes system. The optimum initial moisture content conducive for maximum cellulases and endoglucanases production was 75% and 65%, respectively. The optimum initial moisture content of BPS for the production of β-glucosidase by *A*.

fumigatus LMLPS 13-4 was 80%, Fig. 4.7. Similarly, higher initial moisture content of substrates has been found to produce maximum cellulases. For instance, Delabona *et al*. (2013) also observed that an initial moisture content of 80% in untreated sugarcane bagasse enhanced the production of endoglucanase by both *A*. *niger* P47C3 and *A*. *fumigatus* P40M2. Ang *et al*. (2013) also reported cellulase activity of 3.36 FPU/g d.s, endoglucanase of 54.27 U/g d.s, and β-glucosidase of 4.54 U/g d.s produced by *A*. *fumigatus* SK1 grown on untreated palm oil trunk with an optimum initial moisture content of 80% at room temperature. Furthermore, *A*. *fumigatus* Z5 has been reported to produce cellulases activity of 139.9 FPU/g d.s and 325 U/g d.s endoglucanase when cultivated on corn stover as substrate with initial moisture content of 75 and 80% (Liu *et al*., 2011).

Pirota *et al*. (2016) reported optimum initial moisture of 80% in wheat bran that supported higher cellulases production by *A*. *oryzae* P27C3. In this case, the maximum cellulases activities produced were 0.48 FPU/g d.s and 0.14 FPU/g d.s under forced aeration and static aeration, respectively. Despite this improvement of the production of cellulases at 80% initial moisture content, Yoon *et al*. (2014) found that moisture content lower than 60% and higher than 80% was unfavourable for both fungal growth and cellulase production in SSF.

The results show that each fungus has a specific requirement for optimum moisture of the substrate to fully activate regulatory enzymes system involved in the synthesis and secretion of cellulase enzymes system. The initial moisture content of BPS is critical as it facilitates nutrients and oxygen uptake by the cultivated microorganism in SSF. These differences in the initial moisture content of the BPS on cellulase enzymes system secretion suggest that optimum moisture conducive for high cellulase induction depends on the adaptive mechanism of each fungus and substrate properties.

4.4.3. Effect of the pretreatment of the banana pseudostem on the production of the cellulase enzyme system

The pretreatment of BPS prior to SSF did not improve the production of the cellulase enzymes system instead it enhanced specific type of enzyme(s) by particular fungal strain(s). Different pretreated BPS showed an improvement of β-glucosidase by all

fungal species. There was an equal amount of β-glucosidase in SSF of hot water pretreated BPS and untreated BPS by *T. longibrachiatum* LMLSAUL 14-1. On the other hand, *T*. *harzianum* LMLBP07 produced a 9.4-fold increase of β-glucosidase in SSF of BPS pretreated with 3% NaOH, while SSF of BPS pretreated with 5% H_2SO_4 by *A*. *fumigatus* LMLPS 13-4 resulted in a 1.85-fold increase of β-glucosidase. These results confirm that each fungus responds differently to carbon source and regulatory systems that trigger biosynthesis of enzymes are activated differently as well in different organisms. Generally, SSF of untreated BPS increased the production of cellulase by all the fungal strains. This was due to high nutrients found in untreated BPS such as proteins, potassium, phosphorus and other micronutrients (Pereira *et al*., 2014).

Brijwani & Vadlani (2011) specifically reported that only alkali pretreated soybean hulls had a significant reduction in enzyme production by both monoculture and mixed cultures of *T*. *reesei* and *A*. *oryzae* compared to untreated, acid and steam pretreated soybean hulls. The possible explanation for the alkaline effect was that the inhibitory compounds that are generated during substrate pretreatment have a deleterious effect on microbial growth and enzyme production (Brijwani & Vadlani 2011). However, Sarkar & Aikat, (2012) reported that pretreatment of rice straw with 2% NaOH resulted in enhanced production of cellulase and endoglucanase. However, concentrations above 2% NaOH did not show an increase in the enzyme production in that study.

Pretreatment of other different agricultural waste substrates that includes sugarcane bagasse, cassava bagasse, and wheat bran and rice straw improved enzyme production more than the untreated substrates. Singhania *et al*. (2006) found that dilute alkaline pretreatment of substrates mentioned above induced higher cellulase production and pretreated sugarcane bagasse induced the most cellulase of 154.58 FPU/g d.s from *T*. *reesei* NRRL 11640. Thus, an in-depth understanding of the role of physicochemical characteristics of substrate on cellulase production in SSF would provide a comprehensive framework to facilitate cellulase production with enhanced hydrolytic activities.

4.4.4. Effect of co-cultivation of *Trichoderma* and *Aspergillus* species on the production of cellulase enzymes system

Another strategy to enhance the production of cellulase enzymes system with efficient hydrolytic properties was to use a combination of *Trichoderma* and *Aspergillus* species. The *Trichoderma* species are known as good cellulase producers but lack sufficient β-glucosidase activity. On the contrary, *Aspergillus* species produce high levels of β-glucosidase. The co-culturing of *T*. *longibrachiatum* LMLSAUL 14-1 and *A*. *fumigatus* LMLPS 13-4 negatively affected the levels of cellulase enzyme system produced when compared monocultures. However, co-culturing of *A*. *fumigatus* LMLPS 13-4 and *T*. *harzianum* LMLBP07 13-5 enhanced the production of cellulases and endoglucanases, with the exception of β-glucosidase, which decreased compared to a monoculture of the two fungi. The improved levels of cellulase and endoglucanse in co-culture could be attributed to dominance by *Trichoderma*. An increased level of β-glucosidases in co-culture of *A*. *oryzae* and *A*. *niger* was observed (Hu *et al*., 2011). Results from the current study suggest that co-culturing does not stimulate an increase in overall cellulase enzymes system secretion, but it may activate (or induce) specific enzymes. Fungal strain compatibility is also believed to promote better colonization and substrate penetration and possibly the synthesis of some metabolites by one organism may have a positive effect on the other organisms in terms of increased production of cellulolytic enzymes (Biswas *et al*., 2014; Tengerdy & Szakacs, 2003).

Other co-culturing studies have also shown an improvement in enzyme production. It was shown that co-culturing of the *T*. *reesei* QM 9414 mutant (*T*. *reesei* M) and *A*. *niger*, improved levels of β-glucosidase by 4-fold and 2-fold compared to monoculture on water hyacinth (Deshpande *et al.,* 2008). Ingale *et al*. (2014) also reported enhanced cellulase enzymes system production with cellulase activity of 4.05 FPU/g d.s and endoglucanase of 13.15 U/g d.s by *A*. *ellipticus* and *A*. *fumigatus* cultivated on pretreated BPS.

Dueñas *et al*. (1995) reported cellulase activity of 18.7 FPU/g d.s and β-glucosidase activity of 38.6 U/g d.s by co-culturing of *T*. *reesei* LM-UC4 and *Aspergillus phoenicis* QM 329, which was higher than the monocultures in SSF using sugarcane bagasse. There was also improved production of cellulase enzymes system in SSF of

sugarcane bagasse supplemented with soymeal by co-culturing of *T*. *reesei* LM-UC4E1 and *A*. *niger* (Gutierrez-Correa *et al*., 1999).

In summary, the production of plant cell wall degrading enzymes by fungi is a complex process that is dependent on getting the correct signal that triggers a cascade of transcriptional factors. The regulation of transcriptional factors is also affected by physiological parameters such as starvation, pH, temperature etc. (Walsh et al., 2012).

4.4.5. Effect of temperature and pH on the activity of the cellulase enzymes system

Temperature and pH are important factors affecting enzyme activity during the hydrolysis of cellulosic biomass. Therefore, it is imperative to determine the optimum conditions under which enzymes will function effectively. The optimum temperature and pH of the cellulase enzymes system activities were investigated in the ranges of 40 – 80 °C and pH 4.5 – 8.5. The extent to which the enzyme activity diminished at higher temperatures varied between the enzyme(s) produced by different microorganisms.

Trichoderma and *Aspergillus* species are mesophilic fungi and often their enzymes are optimally active at 25 to 50 °C (Vieille & Zeikus, 2001). *T*. *longibrachiatum* LMLSAUL 14-1 produced thermo-tolerant cellulases and β-glucosidases with optimum activity at 60 °C and endoglucanase activity at 50 °C, respectively. Thermo-tolerant is defined as the ability of an enzyme to maintain catalytic activity at high temperature for a short-period, whereas thermo-stability as the enzyme's ability to resist irreversible inactivation at high temperatures and maintain its activity over an extended period (Vieille & Zeikus, 2001). Both thermo-tolerant and thermo-stability of an enzyme are the result of co-existence and action of more than one factor such as protein ionic (slat-bridges) and hydrogen bonding, metals and cofactors (Hildén et al., 2009; Vieille & Zeikus, 2001). Similarly, Leghlimi *et al*. (2013) reported maximum activities of cellulases and endoglucanases produced by *T*. *longibrachiatum* (GHL) occurring at 60 °C and 55 °C, respectively.

T. *harzianum* LMLBP07 13-5 also produced more thermo-tolerant enzymes than other fungi with a maximum activity of endoglucanase and β-glucosidase at 70 °C.

Cellulases of *T*. *harzianum* was active at 40 °C and between 70 and 80 °C. *A. fumigatus* LMLPS 13-4 produced a thermo-tolerant cellulase and endoglucanase with maximum activity at 60 °C and β-glucosidase activity at 70 °C. The above results are comparable to a thermophilic β-glucosidase from a themophilic fungus *Myceliopthora heterothalica* F.2.1.4 which showed maximum activities at 65 °C in SSF and 70 °C in SmF (Silva *et al*., 2016). Another thermophilic fungus is *M*. *thermophile* which exhibited maximum cellulase activity at 65 °C (Matsakas *et al*., 2015). Moreover, another study reported that the β-glucosidase of *A*. *fumigatus* Z5 and the same β-glucosidase expressed in *Pichia pastoris* X33 showed optimum activity at 60 °C (Liu *et al*., 2012).

Grigorevski-Lima *et al*. (2009) reported thermophilic endoglucanases produced by *A*. *fumigatus* FBSPE05 with optimum activity at 65 °C. However, another study reported the optimum endoglucanases activity at moderate temperatures. Examples are Nurudeen *et al*. (2015) who reported the optimum temperature and pH for endoglucanase produced by *A*. *terreus* and *T*. *longibrachiatum* to be 50 °C. The *Trichoderma* and *Aspergillus* species used in the current study produced cellulase enzymes system which shared thermo-kinetic properties with the thermophilic (including hyperthermophilic) enzymes. The thermophilic enzymes are optimally active at a temperature between 60 and 125 °C (Vieille & Zeikus, 1996; Vieille *et al*., 1996).

The maximum activity of crude enzymes at high temperature is important for the biofuel industry as it may assist in disrupting the bonds between cellulosic biomass thereby making the sites available and accessible to other enzymes for hydrolysis to occur. Performing enzymatic hydrolysis at high temperatures allows higher substrate concentrations, short hydrolysis period, minimise contamination, lower viscosity of the slurry and improved mass transfer (Matsakas *et al*., 2015; Vieille & Zeikus, 2001).

The dependence of enzyme activity on the pH of the assay mixture is also influenced by the reaction temperature. At some reaction temperatures, the enzyme activity follows a bell-shaped trend. The increase in enzyme activity from low to high is due to protonation of functional groups of amino acids and co-factors involved in the catalytic reaction and the native three-dimensional protein structure of the enzyme

(Bisswanger, 2014). That means when the three-dimensional protein structure of the enzyme undergoes conformational changes in either extremely acidic or basic environment it leads to loss of the enzyme activity (Zeng *et al*., 2016).

The cellulase enzymes system produced by both *Trichoderma* and *Aspergillus species* showed increased activities as pH increased up to certain pH value. The increase of enzyme activities, particularly for cellulases and endoglucanases from *Trichoderma* species and *Aspergillus fumigatus* LMLPS 13-4 continued up to pH 6.5. At pH values higher than 6.5 activities of these enzymes decreased. β-glucosidase was the only enzyme which showed higher activity at pH 5.5 and the activity remained steady until pH 8.5. This suggests that the structural and functional conformation of β-glucosidase remained unchanged throughout pH 5.5 – 8.5.

There are studies reported on enzymes with higher activities at pH values below 4.5. For instance, Grigorevski-Lima *et al*. (2009) reported an endoglucanase from *A*. *fumigatus* FBSPE05 with maximum activity at pH 2.0, whereas, Leghlimi *et al*. (2013) reported highest endoglucanase activity at pH 4.0. In instances where more than one enzyme activity peak were observed such as in Fig. 4.13, 4.15 and 4.17 it could be an indication of the presence of more than one enzyme (i.e. isozymes/or isoforms) in the crude enzymes with different pH/or temperature optima values. In comparison to cellulases and endoglucanases, all the fungi produced β-glucosidase that showed 90% activity across a wide pH range, i.e. from 5.5 to 8.5. A thermostable β-glucosidase gene *bgl3* from *A*. *fumigatus* Z5 expressed in *P*. *pastoris* X33 and the native β-glucosidase of *A*. *fumigatus* Z5 was optimally active at pH 6.0 (Liu *et al*., 2012).

Myceliophthora heterothallica F.2.1.4 have been reported to produce β-glucosidase with maximum activity at pH 5 (Silva *et al*., 2016). The carbon source (substrate) also has an effect on the pH at which maximum activity can be attained. Okoye *et al*. (2013) reported maximum enzyme activity at pH 6 and pH 7 of cellulases produced by *A. fumigatus* when cultured on the inner part of corn cob and outer part of corn cob, respectively. In another study, Liu *et al*. (2011) also reported a maximum endoglucanase activity produced by *A*. *fumigatus* Z5 at pH 7.0. *A. fumigatus* M.7.1 has also been shown to exhibit maximum endoglucanase activity at pH 5.5 (Moretti *et al*., 2012).

4.4.6. The thermostability of cellulase enzymes system at selected temperatures

The stability of an enzyme is fundamental for any experimental design or production in biotechnological processes. Thermal stability of an enzyme refers to the degree to which the enzyme's secondary, tertiary and quaternary structures are affected by temperature changes in their surroundings. This effect is monitored by enzyme activity after exposure to various temperatures in the absence of substrate (Matsakas *et al*., 2015; Okoye *et al*., 2013). High enzyme activity and a three-dimensional structural stability are important in the hydrolysis of cellulosic biomass. Cellulase possessing the above properties will be effective in the hydrolysis of cellulose and the process utilising such cellulase would require the addition of a smaller amount of the enzymes.

All the cellulase enzymes system produced by *Trichoderma* and *Aspergillus* species were incubated at selected temperatures for 24 hours prior to the determination of the enzyme's kinetic stability. The enzyme's kinetics stability as stated by Vieille and Zeikus, (2001) is often expressed as its half-life ($t_{1/2}$) at a defined temperature. The half-life of an enzyme is the time after which the activity of the enzyme is reduced to 50% of the original activity at a defined temperature (Saqib *et al*., 2010).

The cellulases of *T*. *longibrachiatum* LMLSAUL 14-1 reached half-life after 24 hours at 40 °C and at higher temperature the enzyme became deactivated. Endoglucanase exhibited good thermostability between 40 and 50, retaining 70% of its original activity. However, beyond 50 °C the enzyme seemed to have entered into an irreversible inactive form with over 95% loss in activity. The decrease in activity was due to thermal inactivation/denaturation of enzymes caused by the breakdown of weak interactions holding the globular protein structure together. When the three-dimensional structure of enzymes is thermo-sensitive and it becomes destabilised at high temperature causing denaturation (irreversible state). The progression of denaturation depends both on the temperature and on time of exposure (Bisswanger, 2014). β-glucosidase from *T*. *longibrachiatum* LMLSAUL 14-1 was also stable at 40 °C and at temperature higher than 40 °C, the enzyme followed similar trend exhibited by endoglucanses. The thermal stability of the above enzymes was within the optimum temperature of the microorganism's growth limits. Leghlimi *et al*.

(2013) reported a *T*. *longibrachiatum* (GHL) thermostable endoglucanase at 70 °C, capable of retaining 80% of its original activity after 5 hours of pre-incubation. Similarly, a *T*. *harzianum* LMLBP07 13-5 produced a thermostable endoglucanase with a longer half-life as elaborated below.

Regarding the cellulase enzymes system of *T*. *harzianum* LMLBP07 13-5, thermal activation of cellulase and endoglucanase activity was observed at 40 and 50 °C. This activation effect (i.e. enhanced activity) could tentatively be due to; (a) mild heat activating the enzyme by destroying other proteins which are partially inhibitory to the enzyme. (b) and or because the stability tests for the enzymes was carried out under newly optimised assay conditions (60 °C and pH 6.5) versus the 50 °C and pH 5.0, generally accepted for mesophilic enzymes. Cellulases were unstable at a temperature higher than 50 °C. This observation confirms that most enzymes are stable at the optimum temperatures of the microorganism's growth range. An exception was the endoglucanases which remained stable from 40 to 70 °C, retaining activity beyond the enzymes' half-life. The enzymes were very sensitive to 80 °C. β-glucosidases were stable at 40 and 50 °C and higher temperatures led to irreversible inactivation of the enzyme. However, the β-glucosidase of *T*. *harzianum* LMLBP07 13-5 seemed to be more stable when compared to both *T*. *longibrachiatum* LMLSAUL 14-1 and *A*. *fumigatus* LMLPS 13-4. The β-glucosidases from *T*. *harzianum* LMLBP07 13-5 was stable more than another mesophilic β-glucosidase from *T*. *harzianum* IOC-3844, which exhibited high sensitivity to 50 ° and 60 °C, presenting half-life time of 4 hours and less than 1 hour, respectively (Castro *et al*., 2010).

Aspergillus fumigatus LMLPS13-4 cellulases were stable between 40 and 60 °C. The enzymes reached its half-life after 24 hours at 60 °C and the activity further diminished at higher temperatures. The cellulases of *A. fumigatus* LMLPS 13-4 shared some thermal properties with enzymes from the thermophile fungus *M*. *thermophila* which showed higher thermal stability at 60 °C (Matsakas *et al*., 2015). Regarding endoglucanases, there were also enhanced activity at 50 °C and the enzyme remained fairly stable at high temperatures having a half-life at 80 °C. Santos *et al*. (2016) explained such an increase in enzyme activity as a result of temperature-induced activation in which atoms acquired more energy and thus greater tendency to move. Similarly, Liu *et al*. (2011) reported thermal stability of *A*.

fumigatus Z5's crude enzyme (endoglucanase), which maintained more than 80% of its original activity after 20 hours of pre-incubation at temperature ranged from 40 – 60 °C. However, there was a gradual loss of enzyme activity, with less than 10% of activity retained at 80 °C and 90 °C as compared to endoglucanase of *A*. *fumigatus* LMLPS 13-4 at 80 °C.

Some studies use shorter time to determine the enzyme's thermostability. For instance, Adav *et al*. (2013) investigated the thermostability of endoglucanase from *A*. *fumigatus* LF9 at temperature ranging from 40 – 70 °C. After 30 minutes of pre-incubation, the enzyme was stable, retaining 100% activity between 40 – 50 °C. However, the enzyme gradually lost activity, retaining 60% at 70 °C. Such very short enzyme half-life could me misleading since complete and efficient hydrolysis (saccharification) process of lignocellulose biomass requires time in hours or days. Surprisingly, since *Aspergillus* is regarded as a β-glucosidase producer, β-glucosidase of *A*. *fumigatus* LMLPS 13-4 exhibited very poor thermostability; the enzyme could not retain 50% of its original activity for any of the temperature points used.

According to Li *et al.* (2011), thermophilic fungal cellulases are active in the pH range 4.0 – 7.0 and have high activity at 50 – 80 °C. In addition, thermophilic cellulases exhibit a remarkable thermal stability and are more stable at 60 °C than those from other fungi. The study by Castro *et al*. (2010) also reported the sensitivity of β-glucosidase to higher temperatures of 50°C and 60 °C. In that study, after 23 hours of pre-incubation of crude enzyme at 60 °C, cellulase lost over 80% of its activity. Furthermore, both β-glucosidase and endoglucanase completely lost their activities (Castro *et al*., 2010).

Other studies on thermostability have relied on shorter incubation period which complicates direct comparison of stability results. For instance, Santos *et al*. (2016) reported 100% of activity retained by cellulase produced by *A*. *niger* after 90 minutes of incubation at 60 °C while *Rhizopus sp*. at the same temperature retained 100% after approximately 4 hours of incubation. It is important to note that thermostability of enzyme is necessary for the process but it is not sufficient for thermo-activity. The findings of this study revealed that often there is a "trade-off" between activity and thermostability. Some of the enzymes which showed high thermo-activity at a

particular temperature could be less stable at the same temperature (Daniel & Danson, 2010). For a longer saccharification process, maximum activity of enzyme at high temperature alone is undesirable since the enzyme must maintain its activity for the duration of the process; hence enzyme stability is so crucial (Saqib *et al*., 2012).

4.4.7. The stability of cellulase enzymes complex at selected pH values

The pH stability of cellulase enzymes system produced by the fungi used in this study was also investigated. Both cellulases and β-glucosidases by *T*. *longibrachiatum* LMLSAUL 14-1 exhibited good stability in acidic conditions (pH 4.5 – 6.5). Most of the cellulase activity was lost at pH values above 6.5, while β-glucosidases remained fairly stability up to a pH 8.5, retaining 59% of its original activity. The endoglucanases of *T*. *longibrachiatum* LMLSAUL 14-1 was unstable throughout the pH ranges (4.5 – 8.5). On the contrary to this, cellulase of *T*. *harzianum* LMLBP07 was most stable in alkaline conditions (pH 7.5 – 8.5), whereas β-glucosidase was stable throughout the pH range with its 50% activity reached at pH 7.5. *T*. *harzianum* LMLBP07 produced a pH sensitive endoglucanase having 54% residual activity at pH 4.5 and pH above 4.5 led to gradual loss of enzyme activity.

In a study by Zeng *et al*. (2016) the authors reported a stable endoglucanase produced by *T*. *virens* that retained 85% of its original activity at pH 5.0 after 24 hours of pre-incubation. Cellulase produced by *A*. *fumigatus* LMLPS 13-4 has retained 53% activity at pH 5.5 after 24 hours of pre-incubation. This fungus produced stable endoglucanase and β-glucosidase at pH 4.5 – 8.5. However, endoglucanse was most stable at pH 4.5 – 5.5. Both endoglucanase genes (*egl2* and *egl3*) and β-glucosidase gene (*bgl3*) of *A*. *fumigatus* Z5 expressed in *P*. *pastoris* X33 (including native β-glucosidase) showed stability in the range 4 – 7 (Liu *et al*., 2012). According to Li *et al*. (2011), a systematic characterization of cellulase enzyme system is necessary to better understand the thermostability and evolutionary relationship between thermophilic and mesophilic cellulase enzymes system. Generally, the thermostability and optimal activity at high temperatures are inherent properties of thermophilic or hyperthermophilic enzymes (Vieille & Zeikus, 2001).

4.5. CONCLUSION

The production of cellulase enzymes system is influenced by various factors that include temperature, nature of the growth substrate (i.e. moisture, modification and source) and culturing technique/strategy (i.e. monoculture versus co-culture). Both *Trichoderma* and *Aspergillus* species are mesophilic fungi and have shown different temperature optima for the productions of cellulase enzymes system. The moisture content of the BPS also influenced the production of cellulase enzymes. For instance, each of the enzymes is produced at different optimal moisture of the substrate. The pretreatment of the BPS did not improve the overall production of cellulase enzymes, in the case of *T*. *longibrachiatum* LMLSAUL 14-1. However, mild alkaline and acidic pretreated BPS improved the production of β-glucosidase by *T*. *harzianum* LMLBP07 13-5 and *A*. *fumigatus* LMLPS 13-4, respectively. There is an improvement in cellulase enzyme system production by co-cultivation of *A*. *fumigatus* LMLPS 13-4 and *T*. *harzianum* LMLBP07 13-5.

An active and stable enzyme(s) at high temperature offers biotechnological benefits to the biofuel industry. All the fungi investigated, produced thermo-tolerant and acidophilic cellulases and endoglucanases, whilst β-glucosidases are both acidophilic and alkaliphilic. The cellulase enzymes system secreted by *T. harzianum* LMLBP07 13-5 is most stable, followed by *A*. *fumigatus* LMLPS 13-4 while the least stable cellulase enzymes were secreted by *T*. *longibrachiatum* LMLULSA 14-1. This study demonstrates banana pseudostem to be a useful agricultural waste for the production of cellulase enzyme system (cellulase, endoglucanase and β-glucosidase). These enzymes show desirable properties such as high thermo-activity and stability and will be assessed for effective saccharification in **Chapter 5**

CHAPTER 5

Hydrolysis and fermentation of banana pseudostem to produce ethanol

5.1. INTRODUCTION

The productions of first generation bioethanol using grains and vegetable oil for biodiesel are faced with challenges due to the competition with the food supply, particularly in developing countries (Demirbas, 2011). Due to sugar-rich feedstock's direct influences on food prices, it has become imperative for future biofuel expansion to be based on the use of lignocellulosic biomass, a second generation bioethanol (Cheng & Zhu, 2012). Because of the nature of cellulosic biomass structure in plants including herbaceous plants, the release of fermentable sugars poses a challenge. The process requires pretreatment; a pre-hydrolysis step that exposes cellulose fibers making it susceptible to enzymatic hydrolysis (breaking down of cellulose to fermentable sugars) before fermentation of the sugars yielded. The efficiency of pretreatment is influenced by the methods used and the nature and composition of lignocellulose biomass (Gabhane *et al*., 2014). Currently, the amount of cellulases required for saccharification and the low ethanol yield are key factors affecting the overall cost to produce bioethanol (Liu *et al*., 2016).

Lignocellulosic biomass is comprised of forestry, agro-industrial and food wastes and is renewable, available in excess and inexpensive (Maitan-Alfenas *et al*., 2015). The agro-industrial residues are obtained after the harvest and the processing of the product. The examples of the post-harvest residues are wood bark and banana pseudostem, whereas the processing residues include corncob, rice husk, saw dust etc. (Duque *et al*., 2015). Lignocellulosic mainly consist of sugar polymers (i.e. cellulose and hemicellulose) and lignin. The use of these polymers for the production of value added products such as biofuel, food additives, organic acids, enzymes, *etc*. can remediate environmental problems attributed to waste accumulation (Maitan-Alfenas *et al*., 2015).

The banana pseudostem, an example of agricultural waste, is an attractive non-edible cellulosic biomass for bioethanol production because of its high cellulose and moderate hemicellulose and low lignin content. Unlike wood based lignocellulosic biomass, the banana plant has a short life cycle, it requires 10 – 14 months to bear fruit depending on the geographic location and soil type (Chaurasia *et al*., 2017). The banana plant bears fruit once in its life cycle and for every cycle of banana production, wastes which are four times the harvested fruit are generated. Banana wastes range from rotten fruit, peels, fruit-bunch stem, leaves, pseudostem and rhizome (Abdullah *et al*., 2014). After the harvesting of the banana fruit, banana pseudostem become available in abundance as waste (60 – 80 t/ha), which is either left to rot at the local dumpsite or left to decompose at the plantation to serve as organic soil fertiliser (Meena *et al*., 2015; Li *et al*., 2010).

The conversion of banana pseudostem to reducing sugar has not been effective due to the high crystallinity of the banana fibres (Gabhane *et al*., 2014). The current study investigates the utilisation the banana pseudostem as a source of sugars that can be used in the production of bioethanol.

5.2. MATERIALS AND METHODS

5.2.1. Collection and preparation of banana pseudostem

Fresh banana pseudostem (BPS) were cut and collected from Alleesbeste farm located in Tzaneen, Limpopo province of South Africa. Details of the preparations are described in section 4.2.2.

5.2.2. Pretreatment of banana pseudostem

One hundred and fifty grams of ground BPS was suspended in each pretreatment solution; i.e. 3% NaOH (Filho *et al*., 2013); 5% H_2SO_4 (El-Zawawy *et al*., 2011; Gabhane *et al*., 2014; Lin *et al*., 2015) and H_2O, at a ratio of 1:10 solid: liquid. Further details on the pretreatment are described in section 4.2.3. Figure 5.1 below depicts BPS before (native biomass) and after the pretreatment.

Figure 5.1. Banana pseudostem before and after different pretreatments. **A** 5% H_2SO_4, **B** 3% NaOH, **C** Hot water and **D** Native (Untreated).

5.2.3. Analysis of the chemical composition of banana pseudostem

The chemical composition of the untreated, thermo-alkali, thermo-acid and liquid hot water (or hydrothermal) pretreated BPS samples were determined. The cellulose and lignin content was analysed by a reaction with sulphuric acid according to a standard method by TAPPI-T222 om-88 and the hemicellulose content was obtained as described in TAPPI1T19m-54 standards (Motaung & Anandjiwala, 2015).

5.2.4. Fourier transform infrared spectroscopy (FTIR) of banana pseudostem fibers

Fourier transform infrared spectroscopy of untreated, thermo-alkali, thermo-acid and hydrothermally pretreated BPS samples were acquired using Spectrum 100 FTIR (PerkinElmer, Waltham, MA, USA) equipped with an attenuated total reflection (ATR) accessory with a diamond/ZnSe crystal (64 scans. 4 cm^{-1} resolution, with wavenumber range 500-4000 cm^{-1}) according to Motaung & Anandjiwala, (2015).

5.2.5. Cellulase enzymes production and extraction

Three fungal species, namely *Trichoderma harzianum* LMLUL 13-5, *Trichoderma longibrachiatum* LMLUL 14-1 and *Aspergillus fumigatus* LMLUL 13-4 were used for production of cellulases in solid state fermentation (details refer to sections 4.2.4 and 4.2.5).

5.2.6. Enzymatic hydrolysis of pretreated banana pseudostem

Crude cellulase enzymes system from *T*. *longibrachiatum* LMLSAUL 14-1 and *T*. *harzianum* LMLBP07 13-5 cultures was used. The saccharification of alkaline (3% NaOH) and acid (5% H_2SO_4) pretreated BPS at 10 g, 12.5 g, and 15 g substrate loadings in 100 mL were carried out in triplicate at 50 °C, 150 rpm for 76 hours. The saccharification mixture contained crude enzyme (10 FPU/g of substrate loading, Gregg & Saddler, 1996), 0.005% sodium azide and buffered with 0.05 M Na-citrate at pH 5.0. Sampling was carried out according to Low *et al*. (2015). Two millimetres of the sample was placed in boiling water for 15 minutes and later centrifuged at 12470 x g for 10 minutes. The supernatant was stored at -20 °C until required for analysis. The released glucose was analysed using High Performance Liquid Chromatography (HPLC).

5.2.7. Bioethanol production using banana pseudostem hydrolysate

5.2.7.1. Inoculum preparation

Saccharomyces cerevisiae UL01 was inoculated into 50 ml of YPD (1% yeast extract; 2% peptone and 2% dextrose) medium and incubated at 30 °C with shaking at 150 rpm for 14 hours.

5.2.7.2. Hydrolysis of banana pseudostem

The hydrolysis of BPS was achieved using mild acid (5% H_2SO_4) and crude cellulases (final concentration of 10FPU/g) from *T*. *longibrachiatum* LMLSAUL 14-1. The acid pretreated BPS (pretreatment outlined in section 5.2.2) liquid fraction was separated from solid biomass and divided into two parts, namely centrifuged (clarified) at 3834 x g at 4 °C for 30 minutes and non-centrifuged (non-clarified) fraction.

After enzymatic hydrolysis of alkaline pretreated BPS (pretreatment conditions outlined in section 5.2.2), the slurry was centrifuged at 3834 x g at 4 °C for 30 minutes. The glucose content of the liquid fractions was determined using HPLC and later used in fermentation.

5.2.7.3. Fermentation of banana pseudostem hydrolysate

A 100 mL fraction of the fermentable sugars obtained through acid and enzymatic hydrolysis was supplemented with 0.2 g K_2HPO_4; 0.7 g KH_2PO_4; 0.1 g NH_4SO_4; and 0.15 g yeast extract following a modification of the procedure by Thakur *et al*. (2013). The pH of the BPS hydrolysate media was adjusted to 5.0 and autoclaved at 121 °C, 15 psi for 1 hour prior to inoculation. The BPS hydrolysate media was allowed to cool and thereafter inoculated to an initial OD_{600nm} of 0.4 at zero hour, t_0. The progression of fermentation was monitored by periodic sampling and the samples were filtered through non-sterile 0.22 µm filter membrane prior to glucose and ethanol analysis.

5.2.7.4. Co-fermentation by *Trichoderma longibrachiatum* LMLSAUL 14-1 and *Saccharomyces cerevisiae* UL01

Three grams of each of the pretreated BPS was suspended in 100 mL modified synthetic medium by Peixoto (2006) containing 0.2 g K_2HPO_4, 0.05 g KCl, 1 mg $FeSO_4.7H_2O$, 15 mg $MgSO_4.7H_2O$, 0.7 g KH_2PO_4, 0.1 g NH_4SO_4 and 0.15 g Yeast extract. The medium was inoculated with *T*. *longibrachiatum* LMLSAUL 14-1 and *S*. *cerevisiae* UL01 at a ratio of 1:3 (i.e. 1 ml of 1 x 10^6 cells/g substrate for *T. longibrachiatum* LMLSAUL 14-1 and 3 ml for *S*. *cerevisiae* UL01 of 1.8 OD_{600}) and incubated at 30 °C, with 150 rpm shaking for 91 hours. The experiment was carried out in triplicate and the progression of fermentation was monitored by sampling at 0, 20, 27, 44, 51, and 68 hours to quantify ethanol, residual sugar and cellulase activity.

5.2.7.5. Sugars content of banana pseudostem hydrolysate and fermented hydrolysate

The sugars in the banana pseudostem hydrolysate samples were analysed using HPLC on a Shimadzu Prominence 20 HPLC system equipped with Rezex RHM-monosaccharide H+ column (300 x 7.8 mm) fitted with 5 micron Rexez organic guard column (Phenomenex, USA). The detection of eluents was done by using a refractive index detector, RID 10A (Shimadzu, Kyoto, Japan). The column temperature was maintained at 85 °C and analytes were eluted using deionised water at a flow rate of 0.6 ml/minute. Peak detection and integration were done using LC Solutions software from Shimadzu (Kyoto, Japan) and peak heights were used

for calculations of sugar concentrations. Known concentrations of glucose, xylose and cellobiose were used as standards to determine the concentration of individual sugars in BPS hydrolysates.

The calculation of glucose yield as a percentage of cellulose conversion to glucose (% digestibility) by both acid and enzymatic hydrolysis was done according to the method by Dowe & McMillan, (2008).

$$\text{Glucose yield } (\%) = \frac{[\text{glucose}] + 1.053\,[\text{cellobiose}]}{1.111\,f\,[\text{biomass}]} \times 100 \qquad (1)$$

Where [glucose] is the glucose concentration minus any glucose present at the beginning of hydrolysis (g/L); [cellobiose] is the cellobiose concentration (g/L); [biomass] is dry biomass concentration at the beginning of the hydrolysis (g/L); f is cellulose fraction in dry biomass (g/L); 1.111 is cellulose conversion factor to glucose equivalent and 1.053 is cellobiose conversion factor to glucose equivalent.

5.2.7.6. Ethanol from fermented banana hydrolysate

Ethanol was analysed by capillary gas chromatography using a Shimadzu GC2010Plus equipped with auto injector AOC 20i (Shimadzu) and an AOC 20S (Shimadzu) auto sampler with a flame ionization detector (FID) and Zebron ZB wax Plus 30 M (Phenomenex, USA) column (30 m, 0.25 mm ID and film thickness of 0.25 µm). Nitrogen was used as the carrier gas at a flow rate of 17.6 mL/minute. The oven temperature was initially maintained at 40 °C for 1 minute then increased to 140 °C at a rate of 20 °C/min and further increased to 200 °C at a rate of 50 °C/min and maintained at this temperature for 3 minutes. The injection temperature was 200°C and the injection volume was 1 µL. A split injection mode with split ratio of 10 was applied. Ethanol was detected using a FID at 250 °C. Absolute ethanol was used for preparation of standard concentrations (v/v). Peaks detection and integration were done using GC Solutions software from Shimadzu (Kyoto, Japan). Peak heights were used to determine the unknown concentrations of ethanol in fermented BPS hydrolysate.

The conversion of cellulose to ethanol (%) was calculated based on the mass of the biomass using the formula below according to (Lu *et al*., 2012);

$$\text{Conversion of cellulose to ethanol } (\%) = \frac{[\text{EtOH}]}{(f * \text{biomass} * 1.111 * 0.51)} X\ 100 \quad (2)$$

Where [EtOH] is ethanol at the end of the fermentation minus any ethanol available in a medium (g/L) at time 0; *f* is cellulose fraction of dry biomass (g/g); Biomass is the dry biomass concentration at the beginning of the fermentation (g/L); 0.51 is the conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast; 1.111 is the conversion factor of cellulose to equivalent glucose.

5.2.8. Statistical analysis

All the experiments were done in triplicates. The data generated was statistically analysed by Two-way analysis of variance (ANOVA) test for saccharification experiments using MS Excel 2010. Differences were considered significant when probability value (p) was <0.05. The error bars in the graphs represent standard error, SE.

5.3. RESULTS

5.3.1. Chemical composition of untreated and pretreated banana pseudostem

Banana pseudostem is a clustered cylindrical aggregation of leaf stalk bases (Mukhopadhyay *et al*., 2008). BPS contains polymers such as, cellulose, hemicellulose, and lignin. Pretreatment introduce changes in the BPS, opening the structure to expose the carbohydrate polymers allowing improved enzyme access for improved enzymatic hydrolysis (saccharification). The amounts of the three polymers found in untreated and pretreated BPS are shown in Table 5.1. The pretreatment results showed an increase of cellulose, and a loss of hemicellulose as well as lignin. The loss of lignin is desirable in the production process of second generation bioethanol, since it has been suggested that lignin binds and limits the accessibility of cellulases to cellulose.

Table 5.1. Chemical composition of untreated and pretreated banana pseudostem**.**

BPS and pretreatment	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Untreated	24.47 ± 0.839	22.56 ± 1.658	14.14 ± 1.585
3% NaOH	52.32 ± 2.878	10.84 ± 1.591	8.68 ± 0.464
5% H_2SO_4	48.17 ± 0.351	9.88 ± 1.641	8.31 ± 1.688
Hot water	25.44 ± 0.314	15.02 ± 1.189	9.25 ± 0.072

NB: Standard deviation (SD) ± values of independent triplicates

Both thermo-alkaline and thermo-acid pretreatment solubilised over 50% of hemicellulose, also removing approximate 40% of the lignin and improved cellulose availability by 114% and 97% for alkaline and acid pretreatment, respectively. Hot water pretreatment of BPS resulted in 33% hemicellulose solubilised, 34% lignin removed and very little change in cellulose content. The percentages of these polymers can also vary from one cultivar to another, and the differences may also occur within the same cultivar due to geographic location.

5.3.2. Characterization of untreated and pretreated banana pseudostem

FTIR spectroscopy was used to study the chemical structural and conformational variations introduced by pretreatment of BPS. Lignocellulosic biomass contains many O-H bonds due to the presence of cellulose, hemicellulose and lignin. During FTIR analysis, the covalent bonds of the functional groups absorb a certain amount of energy from infrared radiation, causing the bonds to be stretched. After the absorption of energy, the O-H bonds are stretched resulting in the increase of the peak intensity indicated by either sharpness or broadness (Azizan *et al*., 2016).

The FTIR spectra of native (untreated) and pretreated BPS are shown in Fig. 5.2. The spectrum of the native BPS shows distinct peaks/bands at various wavenumbers with strong broad band occurring at 3363 cm^{-1}, 2931 cm^{-1}, 2794 cm^{-1}, 1619 cm^{-1}, 1263 cm^{-1}, 1010 cm^{-1} and multiple small peaks at 863 cm^{-1}, and 759 cm^{-1}, 725 cm^{-1} and 718 cm^{-1}. The spectra of all pretreated BPS fibers are similar to those of the native BPS and exhibit common peaks and values, which indicate the similarity in the materials, BPS. However, chemical modification through pretreatments had decreased the peaks intensities and some other peaks completely diminished. For instance, peak at wavenumber 2794 cm^{-1} diminished in

all the pretreated BPS samples, suggesting solubilisation of hemicellulose as evident in Table 5.1 above. There was a decrease of peak intensities in the pretreated BPS samples at wavenumber 3363 cm^{-1}, 2931cm^{-1}, 1619 cm^{-1} 1235 cm^{-1} and 1010 cm^{-1} indicating fewer functional groups such as O-H, C-H and C=C that are associated with stretching vibrations mainly in cellulose and hemicellulose or lignin.

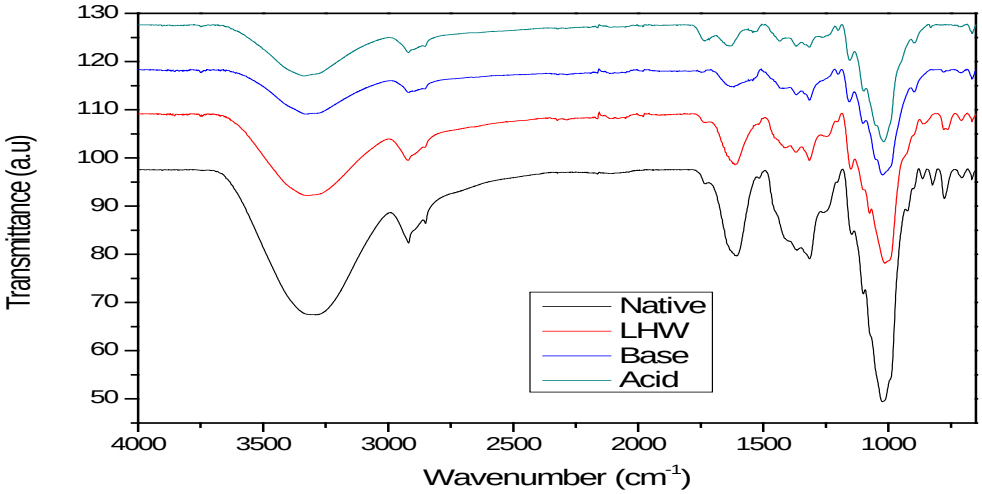

Figure 5.2. Figure 5.2: FTIR spectra of banana pseudostem. – Native (untreated); – LHW (Liquid hot water); – Base (3% NaOH) and – Acid (5% H_2SO_4).

5.3.3. Saccharification of banana pseudostem with *Trichoderma* species cellulase enzymes system

Based on the holocellulose amount as shown in Table 5.1, chemically pretreated (i.e. acid and alkaline) BPS were chosen for enzyme digestibility assessment (saccharification) using crude cellulase enzymes produced by *T. longibrachiatum* LMLSAUL 14-1 and *T. harzianum* LMLBP07 13-5 at a concentration of 10 FPU/g d.s. The hydrolysis of acid pretreated BPS by cellulase enzymes system produced by *T. harzianum* LMLBP07 13-5 released about 5.4 g/L of glucose at high substrate loading. The concentration of glucose increased proportionally with increasing of substrate loading during hydrolysis, Fig. 5.3. The time required to release maximum glucose from acid pretreated BPS varied between the solid substrate loadings.

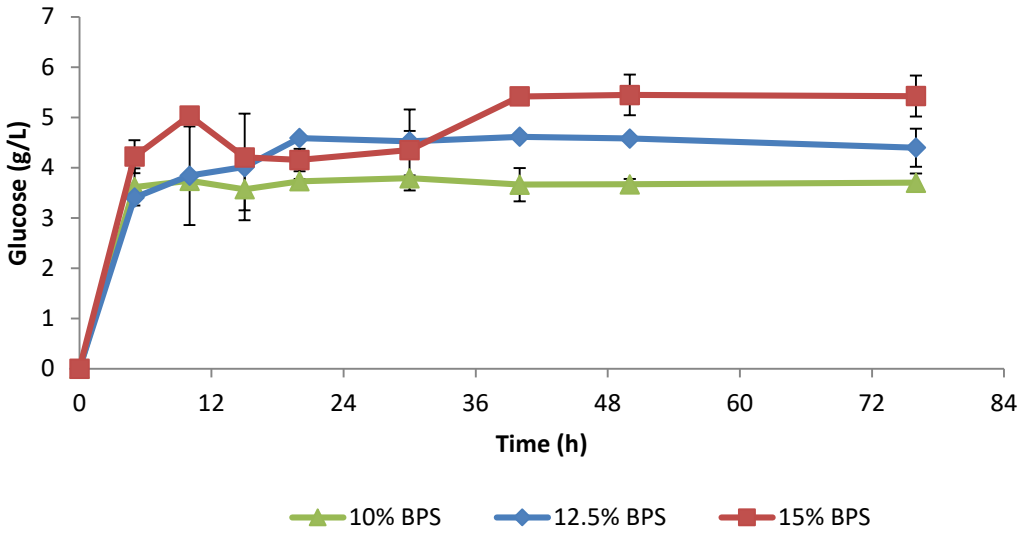

Figure 5.3. The time course of glucose production during hydrolysis of 5% H_2SO_4 pretreated banana pseudostem at different solid loadings by *T. harzianum* LMLBP07 13-5 cellulase enzymes system.

The hydrolysis of acid pretreated BPS by cellulase enzymes system produced by *T. longibrachiatum* LMLSAUL 14-1 is shown in Fig. 5.4. Most of the sugar was released within the first 5 to 10 hours of incubation and a further increase of hydrolysis time did not improve the glucose yield.

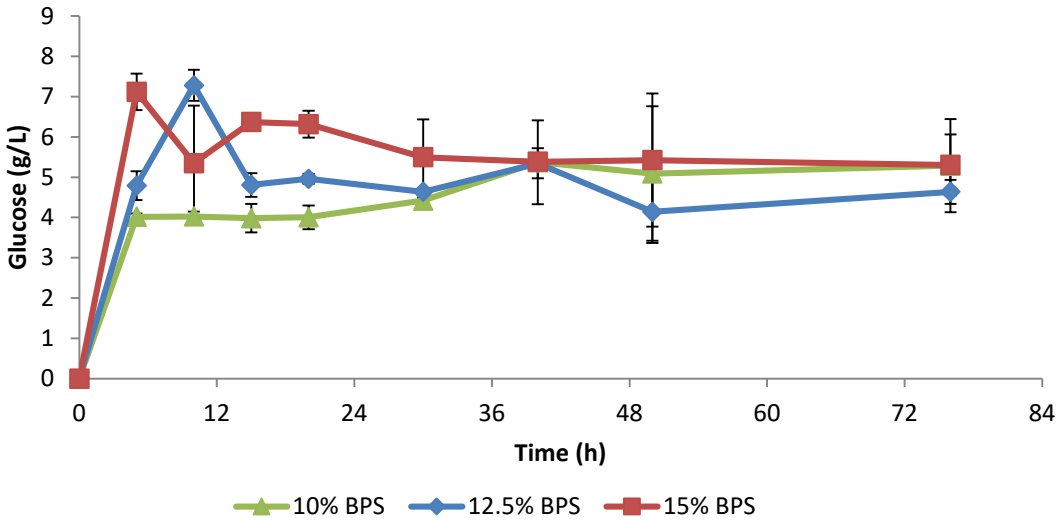

Figure 5.4. The time course of glucose production during hydrolysis of 5% H_2SO_4 pretreated banana pseudostem at different solid loadings by *T. longibrachiatum* LMLSAUL 14-1 cellulase enzymes system.

Generally, acid pretreated BPS exhibited recalcitrance towards cellulase enzymes system hydrolysis with the overall glucose released not reaching 10 g/L. There was a proportional increase in glucose with the biomass loading, Fig 5.3 and 5.4, however, glucose yield as a percentage of cellulose conversion to glucose decreased. The glucose yield ranged from 7.1 to 6.7 when using cellulase enzymes system obtained from *T. harzianum* LMLBP07 13-5 and 10.0 to 8.9 when using cellulase enzymes system obtained from *T. longibrachiatum* LMLSAUL 14-1, Table 5.2.

As opposed to acid pretreated BPS biomass, the alkaline pretreated BPS was more digestible by both *T. harzianum* LMLBP07 13-5 and *T. longibrachiatum* LMLSAUL 14-1 cellulase enzyme systems. The hydrolysis with *Trichoderma* species cellulases released high concentrations of glucose from alkaline pretreated BPS, significantly more than from the acid pretreated BPS. The cellulase enzymes system produced by *T. harzianum* LMLBP07 13-5 hydrolysed alkaline pretreated BPS and released glucose in the range of 13.9 to 20.1 g/L, Fig. 5.5. The higher glucose level was attained at the highest solid loading.

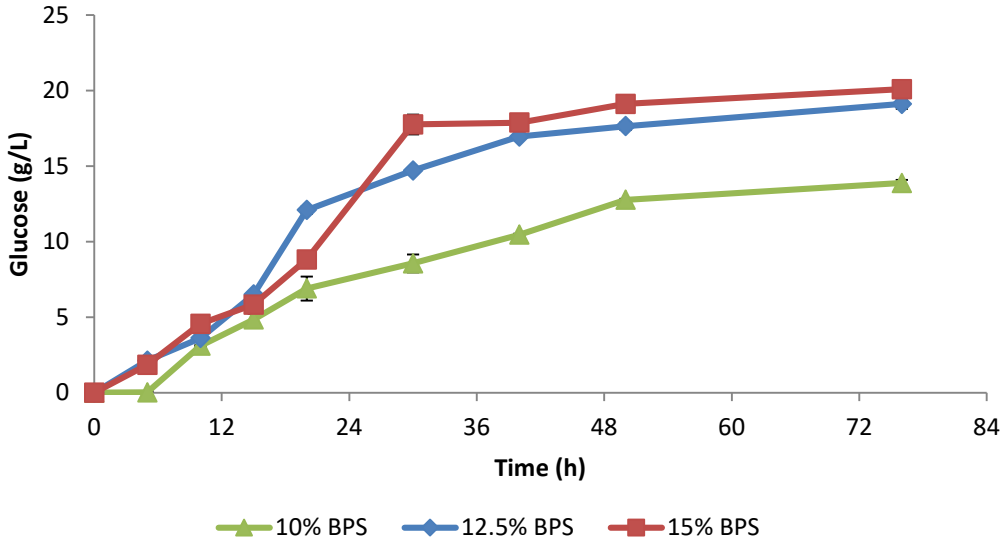

Figure 5.5. The time course of glucose production during hydrolysis of 3% NaOH pretreated banana pseudostem at different solid loadings by *T. harzianum* LMLBP07 13-5 cellulase enzymes system.

Table 5.2. Summary of enzymatic saccharification of acid pretreated banana pseudostem at varying solid loadings by crude enzyme produced by *Trichoderma harzianun* LMLBP07 13-5 and *Trichoderma longibrachiatum* LMLSAUL 14-1.

Organism	BPS biomass loading (% w/v)	Crude cellulases loading (FPU/g d.s)	Glucose produced (g/L)	Time (h)	Cellulose conversion to glucose (% w/w)
T. harzianum **LMLBP07 13-5**					
	10	10	3.8 (± 0.0565)	30	7.1 (± 0.1056)
	12.5	10	4.6 (± 0.0200)	40	6.9 (± 0.0299
	15	10	5.4 (± 0.4024)	40	6.7 (± 0.5013)
T. longibrachiatum **LMLSAUL 14-1**					
	10	10	5.4 (± 1.0413)	40	10 (± 1.9454)
	12.5	10	7.4 (± 0.3843)	10	11 (± 0.5745)
	15	10	7.1 (± 0.4510)	5	8.9 (± 0.5618)

NB: Standard deviation ± values of independent triplicates

The cellulase enzymes system produced by *T. longibrachiatum* LMLSAUL 14-1 was more effective in hydrolysing alkaline pretreated BPS than the *T. harzianum* LMLBP07 13-5, Fig. 5.6. The enzymatic hydrolysis released glucose in the range of 29.7 to 43.5 g/L. The maximum concentration of glucose released during the hydrolysis was at the highest solid loading.

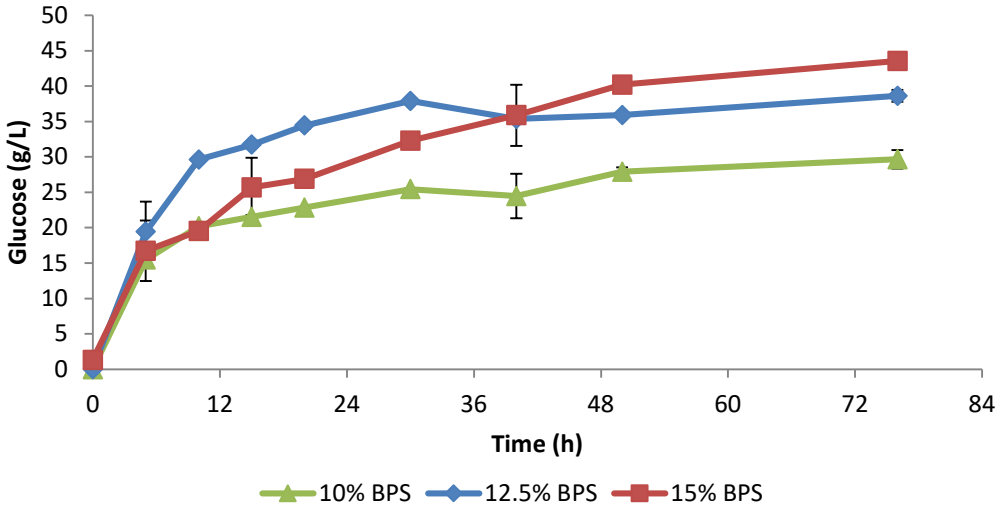

Figure 5.6. The time course of glucose production during hydrolysis of 3% NaOH pretreated banana pseudostem at different solid loadings by *T. longibrachiatum* LMLSAUL 14-1 cellulase enzymes system.

A significant improvement in the enzymatic conversion of alkaline pretreated BPS to glucose as evident when compared with acid pretreated BPS. The highest glucose yield of 26.3 and 53.2 was obtained at 12.5% BPS biomass loading by crude cellulase enzymes from *T. harzianum* LMLBP07 13-5 and *T. longibrachiatum* LMLSAUL 14-1, respectively. Table 5.4 shows that the digestibility of pretreated BPS biomass is higher than the acid pretreated biomass,

Table 5.3. Summary of enzymatic saccharification of alkaline pretreated banana pseudostem at varying solid loadings by crude enzyme produced by *Trichoderma harzianun* LMLBP07 13-5 and *Trichoderma longibrachiatum* LMLSAUL 14-1.

Organism	BPS biomass loading (% w/v)	Crude cellulases loading (FPU/g d.s)	Glucose produced (g/L)	Time (h)	Cellulose conversion to glucose (% w/w)
T. harzianum **LMLBP07 13-5**					
	10.0	10	13.9 (± 0.3131)	76	23.9 (± 0.5387)
	12.5	10	19.1 (± 0.2149)	76	26.3 (± 0.2957)
	15.0	10	20.1 (± 0.1628)	76	23.1 (± 0.1867)
T. longibrachiatum **LMLSAUL 14-1**					
	10.0	10	29.7 (± 1.3132)	76	51.4 (± 2.2592)
	12.5	10	38.6 (± 0.8465)	76	53.2 (± 1.1650)
	15.0	10	43.5 (± 0.7044)	76	49.9 (± 0.8079)

NB: Standard deviation ± values of independent triplicates

Based on the results of cellulosic BPS conversion to glucose as summarised in Table 5.2 and 5.3 above, the alkaline pretreatment of BPS and *T. longibrachiatum* LMLSAUL 14-1 cellulase enzymes system were chosen for further use in fermentation studies.

5.3.4. Separate hydrolysis and fermentation of banana pseudostem hydrolysate

After the hydrolysis of BPS by acid and cellulase enzymes system from *T. longibrachiatum* LMLSAUL 14-1, both acid pre-hydrolysates and enzymatic hydrolysate contained initial glucose amounts of 21 g/L and 74 g/L, respectively. The inoculum size was 5% of the fermentation medium resulting in OD_{600nm} of about 0.4 at the beginning of fermentation. The non-clarified acid pre-hydrolysate was not neutralised and the fermentation progressed for 48 hours period. At the end of fermentation, the residual glucose was 8.2 g/L (i.e. 61% of glucose been consumed) and the concentration of ethanol reached 8.9 g/L, Fig. 5.7 and cellulosic BPS conversion yield (ethanol yield) to ethanol was 64% of the theoretical maximum yield.

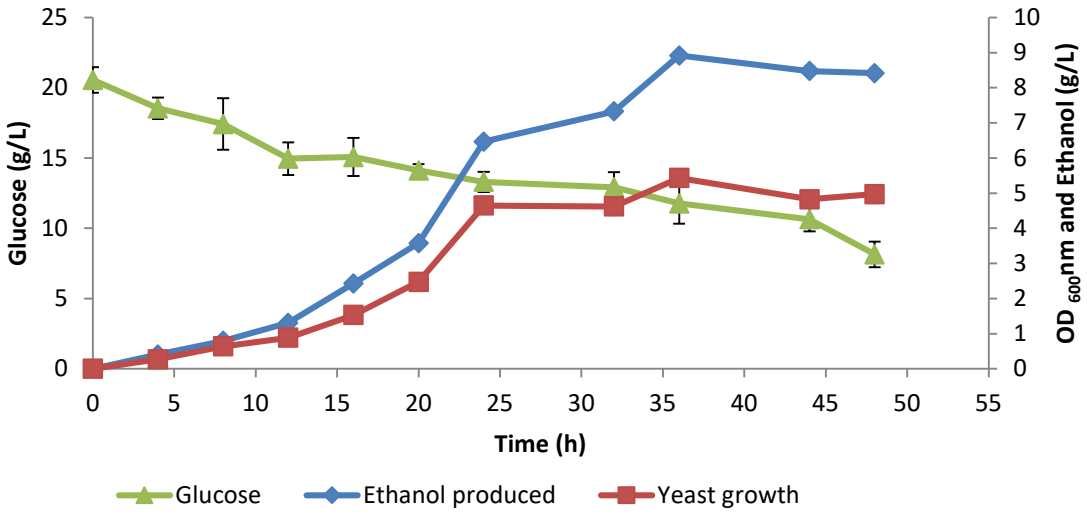

Figure 5.7. Time course of ethanol production and sugar consumption in non-clarified acid pre-hydrolysate through separate hydrolysis and fermentation (SHF).

For the centrifuged acid pre-hydrolysate, the *S. cerevisiae* consumed all the sugar within 16 hours with the ethanol concentration reaching 11.9 g/L and yeast growth of

0.9 OD_{600nm}. The fermentation was complete after 15 h as shown in Fig. 5.8 and the ethanol yield was 86% of the theoretical yield.

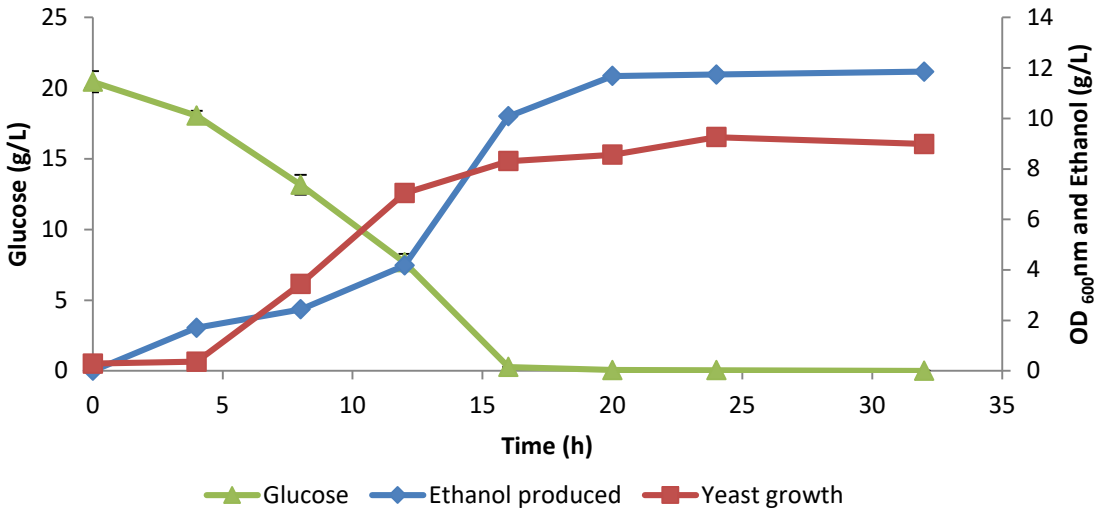

Figure 5.8. Time course of ethanol production and sugar consumption in clarified acid pre-hydrolysate through separate hydrolysis and fermentation (SHF).

While hydrolysate from partial acid hydrolysis of BPS showed high fermentability, enzymatic hydrolysis is preferred for its compatibility with the environmental laws and regulation regarding pollution and the absence of toxic substances produced (Keshwani & Cheng, 2009). For the enzymatic hydrolysate of BPS, the concentration of ethanol obtained at the end of fermentation was 17.6 g /L, which can be translated to 51% of glucose been consumed (residual glucose of 36.3 g/L), Fig. 5.9. The fermentation was sluggish and incomplete. The ethanol yield was 60% of the theoretical yield.

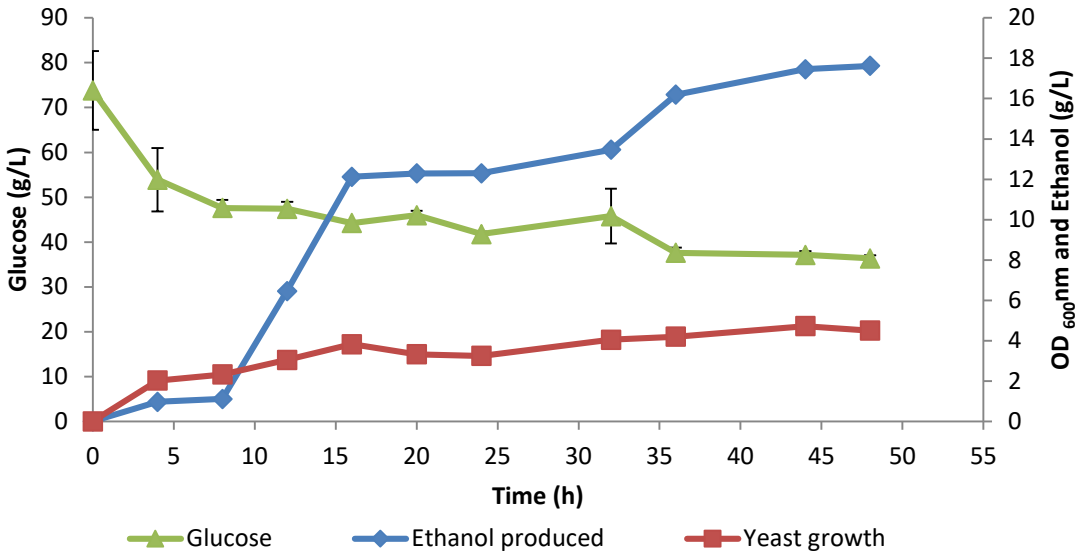

Figure 5.9. Time course of ethanol production and sugar consumption in *T. longibrachiatum* LMLSAUL 14-1 cellulase enzyme system hydrolysate through separate hydrolysis and fermentation (SHF).

5.3.1. The production of ethanol through co-culturing of *Saccharomyces cerevisiae* and *Trichoderma longibrachiatum* LMLSAUL 14-1

Another fermentation strategy often used in industry for the production of ethanol is co-culturing. In this study co-culturing of *S. cerevisiae* and *T. longibrachiatum* LMLSAUL 14-1 was investigated. Generally, *Trichoderma* and *S. cerevisiae* are the "workhorses" of the cellulase and ethanol industries. Co-culturing of the cellulolytic and fermentative microorganisms allows the secretion of cellulase, hydrolysis of cellulose biomass and fermentation of the resulting sugars to take place in a single vessel. Co-culturing fermentation used different compositional and structural forms of BPS biomass, such as untreated and alkaline pretreated biomass.

Both untreated and alkaline pretreated BPS biomass has shown to induce high levels of cellulase (FPU/g d.s) secretion in the current study (refer from section 4.3.1. to 4.3.4.). After 20 hours of cultivation, the cellulase activity in alkaline pretreated BPS medium was 0.77 FPU/mL. This cellulase activity increased to 1.77 FPU/mL at 68 hours of cultivation. Both glucose and cellobiose reached the highest level of 2.6 and 2.5 g/L, respectively from 3 g of BPS after 20 hours. The production of ethanol reached a maximum of 0.79 g/L at 27 hours and the amount of ethanol remained

steady until the end of fermentation at 68 hours, Fig. 5.10. The amount of glucose consumed was 30% (residual glucose of 1.8 g/L) and additional glucose resulted from hydrolysis of cellobiose (i.e. about 8% cellobiose been hydrolysed). The cellulose conversion using co-culturing was low, with a conversion to ethanol of only 8.9%, Table 5.4.

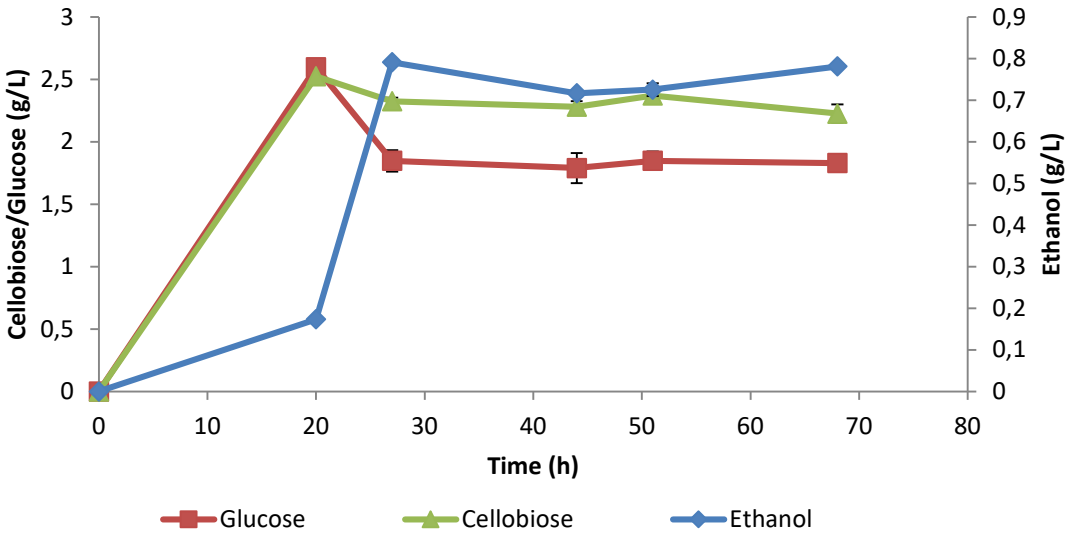

Figure 5.10. Time course of ethanol production, sugar production and consumption through co-culture of *T. longibrachiatum* LMLSAUL 14-1 and *S. cerevisiae* on alkaline pretreated banana pseudostem.

The fermentation of untreated BPS medium produced the highest cellulase activity of 3.1 FPU/g d.s at 44 hours and the activity decreased with fermentation time. The maximum glucose and cellobiose produced was 3.2 g/L and 4.4 g/L at 20 hours of cultivation. At the end of fermentation, the residual glucose and cellobiose was 1.5 g/L and 1.3 g/L, respectively. This means that at the end of fermentation about 70% of cellobiose was hydrolysed to glucose, an additional source of glucose for fermentation to ethanol, Fig. 5.11. The concentration of ethanol in the fermented hydrolysate (i.e. untreated BPS broth) was 1.7 g/L, 2-fold higher than fermented hydrolysate of alkaline BPS.

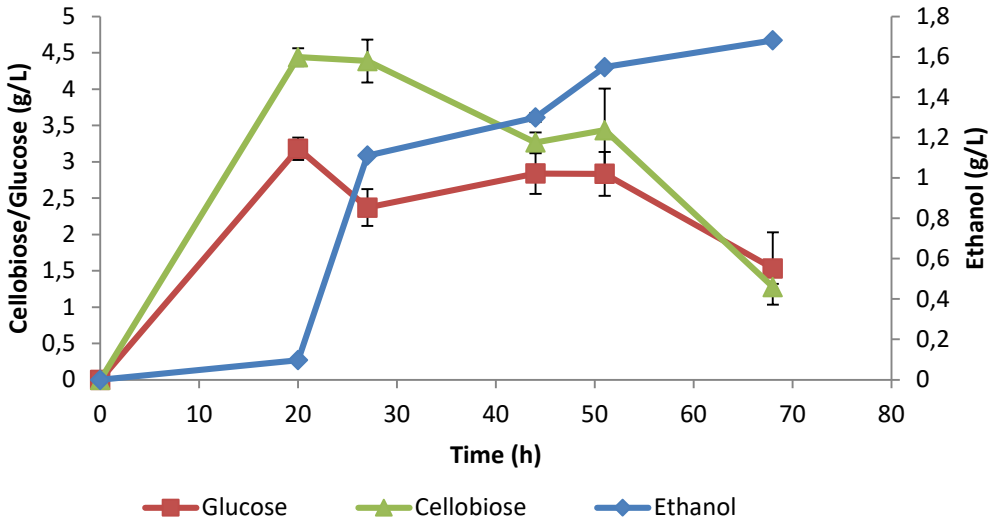

Figure 5.11. Time course of ethanol production, sugar production and consumption through co-culture of *T. longibrachiatum* LMLSAUL 14-1 and *S. cerevisiae* on native banana pseudostem.

Table 5.4. Comparison of different fermentation techniques used for ethanol production from banana pseudostem.

Fermentation type	Substrate	Hydrolysis reaction	Fermentation medium	Total initial glucose (g/L)	Glucose consumed (%)	Ethanol concentration (g/L)	Cellulosic conversation to ethanol (%) eq. 2
SHF	Native BPS	Mild – acid	Non-centrifuged pre-hydrolysate	20.1	61	8.9	64
SHF	Native BPS	Mild – acid	Centrifuged pre-hydrolysate	20.5	100	11.9	86
SHF	Alkaline BPS	Enzymatic	Hydrolysate	73.8	51	17.6	60
Co-fermentation	Alkaline BPS	Enzymatic	Peixoto (2006)	N.D	40	0.79	8.9
Co-fermentation	Native BPS	Enzymatic	Peixoto (2006)	N.D	Over 60 of available glucose	1.7	40.9

NB: N.D refers to non-detected glucose. Standard deviation ± values of independent triplicates

5.4. DISCUSSION

5.4.1. Chemical composition of untreated and pretreated banana pseudostem

The banana pseudostem (BPS) consisted of the following; outer sheath, a middle portion and core portion (i.e. pith). All the BPS portions were mixed and analysed for polymer content. The cellulose content of untreated BPS at 24.5%, Table 5.1 was comparable to 20.1% cellulose reported by Guerrero *et al*. (2018). However, this is lower compared with those reported by other studies. For instance, several authors reported between 30 – 44% cellulose, 15 - 30% hemicellulose and 6 – 12% lignin in untreated BPS (Souza *et al*., 2017; Li *et al*., 2016; Gabhane *et al*., 2014; Thakur *et al*., 2013; Li *et al*., 2010; Cordero *et al*., 2004). The discrepancies in chemical composition may be attributed to the cultivar of the banana plant and climatic (geographic) conditions. However, the pretreatment of BPS changed the chemical, physical and morphological structures of the biomass. This observation was evident as shown in Table 5.1 and FTIR results, Fig. 5.2. The results showed an increase in cellulose and partial loss of hemicellulose and lignin, particularly in chemically pretreated BPS more than hydrothermally (liquid hot water) pretreated BPS. The effect of acid and alkaline pretreatment on the reduction of hemicellulose and lignin in BPS are consistent with the findings of Souza *et al*. (2017).

The effect of alkaline pretreatment (3% NaOH) on BPS polymers as shown in Table 5.1 was in agreement with Low *et al*. (2015) reporting cellulose of up to 73.74 % and hemicellulose of 8.35% and lignin, 10.10% in alkaline (4% NaOH) pretreated BPS. These authors also observed that prolonged soaking time of BPS in alkaline solution and increased concentration of NaOH did not completely remove lignin. The loss of hemicellulose in NaOH pretreated biomass is thought to be the result of a peeling mechanism, which removes the terminal sugar molecules one at a time at the reducing end (Gupta, 2008).

The modification of the biomass by pretreatment removes the hydrogen bonding in the biomass network structure. The effects of pretreatment on BPS chemical composition contents were also compared with other agricultural residues. For example, the pretreatment of wheat straw by hydrothermal and steam-explosion also increased cellulose content and reduced hemicellulose content (Kristensen *et al*.,

2008; 2007). Zhang *et al*. (2013) reported about 58% cellulose, 28.85% hemicellulose and 17.75% lignin, which amounted to 19.53% increase of cellulose and 13.98% decrease of lignin content with a small increase of hemicellulose after alkaline pretreatment (1% NaOH) of wheat straw. The higher cellulose content and reduced hemicellulose and lignin content enhanced enzymatic saccharification. Motaung and Anandjiwala (2015) also observed an increase of cellulose while hemicellulose and lignin content decreased in chemically pretreated sugarcane bagasse. In addition, the authors also mentioned higher cellulose and hemicellulose content in acid pretreated sugarcane bagasse (SB) than in alkaline pretreated SB.

5.4.2. Characterization of untreated and pretreated banana pseudostem

FTIR spectroscopy was used to analyse the chemical structural and conformational variations introduced by pretreatment processes in banana pseudostem (BPS). The primary constituents of pseudostem are cellulose, hemicellulose and lignin. Generally, cellulose, hemicellulose and lignin consist of molecules containing functional groups such as O-H, C-O-C and O-CH_3 (El-Fels *et al*., 2015; Pereira *et al*., 2014). During FTIR analysis, the covalent bonds of the functional groups absorb a certain amount of energy from infrared radiation (IR) thereby causing the bonds to be stretched. After the absorption of energy, the O-H bonds are stretched resulting in the increase of the peak intensity. The peak intensity is indicated by either peak sharpness or broadness (Azizan *et al*., 2016). Analysis of FTIR spectra reveals the appearance or disappearance of characteristic bands at some functional groups or by showing the band(s) shifts in the case of reactions that involve structural changes (El-Fels *et al*., 2015).

The broad band (peak) occurring at wavenumber 3363 cm^{-1} in Fig. 5.2 has been associated with the intermolecular O-H stretching vibrations of cellulose, 2931 cm^{-1} with C-H stretching absorption/vibration from –CH_2 group of cellulose and hemicellulose (Gopinathan *et al*., 2017; Becker *et al*., 2013; Shah *et al*., 2013), 1619 cm^{-1} with C=C stretching of benzene ring and 1010 cm^{-1} with C-O-C stretching absorption in raw banana fibres (Becker *et al*., 2013). According to Faix *et al*. (1994), lignin compounds are characterized by the frequencies of the quaiacyl units, corresponding to wavenumber 1269 cm^{-1}, G-ring and C=O stretch at 1140 cm^{-1}. Both untreated and LHW pretreated BPS material showed smaller peaks at 863 cm^{-1} and

759 cm^{-1}. Peak between 800 cm^{-1} and 880 cm^{-1} are associated to CH in plane deformation (Cochet *et al*., 2001). Another peak at wavenumber 750 cm^{-1} signifies ArC-H out of plane deformation of lignin. The peaks at wavenumber 687 – 625 cm^{-1} are associated with the out of plane bending vibrations of intermolecular H-bonded O-H group and out of plane O-H bending (Waleed & El-zawawy, 2006).

In another study, FTIR analysis also revealed that dilute acid pretreatment of wheat straw alone was not sufficient for complete lignin removal, although complete removal of soluble lignin was attained as shown by slight appearance or disappearance of a peak associated with both lignin types (Dhabhai *et al*., 2013). Barreto *et al*. (2010) found that the vibration modes of functional groups of pretreated banana fibre did not show differences compared with the main bands of untreated banana fibre after alkaline solution in the concentration ranging from 0.25 – 1% (NaOH) at 60 – 70 °C for 6 hours. However, there were clear differences between the pretreated and untreated BPS biomass. The discrepancy between the observation reported by Barreto *et al*. (2010) and the finding in this study could be attributed to pretreatment conditions, as the current study used a higher percentage of NaOH (3% w/v) and temperature (121 °C) on BPS.

5.4.3. Saccharification of banana pseudostem with crude cellulase enzymes system produced by *Trichoderma* species

The sole purpose of saccharification (hydrolysis) stage was to release the sugars, mainly glucose (and xylose depending on the enzyme composition) which would be fermented in the subsequent stage. Enzymatic saccharification of lignocellulosic biomass is dependent on both the availability of cellulose or hemicellulose and the hydrolytic activity of the enzymes. In this study, the conditions for saccharification of BPS were 50 °C, with shaking speed of 150 rpm, final enzyme dosage of 10 FPU/g substrate and substrate loading ranged from 10 – 15%. Based on reported findings by Sun & Cheng, (2002) and Gregg & Saddler (1996),) found that cellulase dosage for effective hydrolysis could vary between 7 – 33 FPU/g substrate, depending on the type of substrate. Guerrero *et al*., 2018 achieved hydrolysis of banana waste with 15 FPU/g cellulase dosage at high solid loading (> 15% solid loading).

During the hydrolysis of BPS, there was a directly proportional increase of glucose released by crude cellulase enzyme complex from *T*. *harzianum* LMLBP07 13-5 and *T*. *longibrachiatum* LMLSAUL 14-1 with increasing of acid or alkaline pretreated BPS biomass loading. Concurrent to that effect, there was a decrease of cellulosic BPS conversion efficiency at high biomass loading. This observation was in agreement with the findings made by Guerrero *et al*. (2018) who observed that higher solids loadings led to higher glucose content and low cellulosic conversion efficiency. The decrease in cellulosic conversion efficiency to glucose is highly undesirable as it off-sets the significant advantages of working at high solid biomass concentration (Kristensen *et al*., 2009). The enzyme-substrate interaction becomes ineffective due to the higher viscosity of the slurry. Ghose (1992) found that high viscous slurry restricts the enzyme movement and also make the hydrolysis sites inaccessible.

It is imperative to select a suitable pretreatment method that increases the concentrations of fermentable sugar after enzymatic saccharification in order to improve the efficiency of cellulosic bioethanol production (Maurya *et al*., 2015). The saccharification of acid (5% H_2SO_4) pretreated BPS by crude cellulase enzyme system from both *Trichoderma* species was ineffective with total glucose of less than 8 g/L been produced. Based on the results indicated in Table 5.1, acid pretreated BPS contained about 58.0% of holocellulose (.i.e. cellulose + hemicellulose). The enzymatic release of the sugars from acid pretreated BPS was poor, perhaps as a result of indigestibility of cellulose fibres which presumably may be containing many crystalline regions on the cellulose surface. The above observation was in agreement with findings by Abraham *et al*. (2010), who found that Oxalic acid hydrolysis solubilised the amorphous regions of the cellulose leaving more crystalline regions in the cellulose fibres. An increase of acid (Oxalic acid) concentration up to 10% increased the crystalinity of the BPS, Jute stem and pineapple leaf fibre and concentration higher than 20% degraded the cellulose fibres (Abraham *et al*., 2010).

On the contrary to saccharification of acid pretreated BPS, enzymatic saccharification of alkaline pretreated BPS, which contained 63.0% of holocellulose, by crude cellulase enzyme system from *T*. *harzianum* LMLSABP07 13-5 and *T*. *longibrachiatum* LMLSAUL 14-1 accumulated 20 g/L and 44 g/L of glucose in the hydrolysate (slurry), respectively. On the contrary, to these findings enzymatic

hydrolysis subsequent to alkaline pretreatment of BPS yielded high glucose. Souza *et al*. (2017) reported high crystalinity of the alkaline pretreated BPS when compared to acid pretreated BPS, which led to poor enzymatic conversion of alkaline BPS cellulose fibres to reducing sugar (glucose). As a result, high reducing sugar (33.74 g/L) was obtained from acid pre-treated BPS compared to 19.4 g/L obtained from alkaline pretreated BPS (Souza *et al*., 2017). The high digestibility of acid pretreated BPS reported in the study by Souza *et al*. (2017; 2014) could be due to the low acid solution (i.e. 2% H_2SO_4) used when compared to the 5% H_2SO_4 used in this study.

According to El-Zawawy *et al*. (2011), the concentration of glucose in the hydrolysate was influenced by the type of pretreatment applied to the biomass and the type of hydrolysis. The authors utilised steam explosion pretreatment of banana plant waste followed by enzymatic hydrolysis of the plant waste by cellulases from *T*. *reesei* ATCC 26921, which released a higher concentration of glucose. Filho *et al*. (2013) reported an increase of fermentable sugars from 3% NaOH pretreated BPS by 8-fold and 23-fold after the hydrolysis of the biomass by acid and enzyme, respectively. Similarly, Thakur *et al*. (2013) also reported that alkaline (1 N NaOH) pretreated BPS and wheat straw (WS) produced reducing sugar, 15 g/L more than acid pretreated (1 N H_2SO_4), 10.5 g/L and 6.8 g/L for biological (Fungal) pretreated biomass. Chidi *et al*. (2015) also found that alkaline (2% NaOH) pretreatment of BPS combined with microwave irradiation (170 W, 10 minutes) yielded the highest concentration of reducing sugar for the production of bioethanol.

Alrumman (2016) reported that 4% substrate loading of alkaline pretreated date palm leaves had increased the production of sugar and saccharification percentage. Saccharification of pretreated (2N NaOH in autoclave) BPS by 2% dosage of commercial cellulase SQzyme CS (20, 000 U/g) yielded 29.8 g/L. These reported findings on enzymatic digestibility of alkaline pretreated lignocellulosic biomass are similar to the observations in the current study. Consenquently, Xu *et al*. (2016) inferred that alkaline pretreatment technology is a promising pretreatment method of lignocellulosic biomass with relatively low lignin because of improved enzymatic digestibility of the pretreated cellulosic biomass yielding high sugar concentrations. Recently, techno-economic and environmental studies conducted by Duque *et al*. (2015), listed banana pseudostem amongst other agricultural residues such as

sugarcane bagasse, corn cob and rice husk as potential feedstock for the production of bioethanol. Future work should focus on developing an efficient enzyme cocktail formulations that hydrolyse different lignocellulosic biomass to make cellulosic ethanol cost effective process (Guerrero *et al*., 2018).

5.4.4. Production of ethanol through separate hydrolysis and fermentation

In separate hydrolysis and fermentation (SHF) the glucose produced was 21 g/L and 74 g/L for acid pre-hydrolysate and enzymatic hydrolysate, respectively. The glucose levels reported in this study are higher than the levels obtained from 5 g pretreated banana pseudostem (15.3 g/L sugar) by Thakur *et al*. (2013).

The production of ethanol is strongly dependent on the growth of the fermenting organism. *Saccharomyces cerevisiae* is known to consume glucose rapidly during growth and tolerate high levels of ethanol produced. However, the poor growth of *S*. *cerevisiae* in the early stages of the fermentation of both non-clarified acid pre-hydrolysate and enzymatic hydrolysate led to the high level of glucose remaining at the end of fermentation. In non-clarified hydrolysate, the medium still contains particulate matter (PM) resulting from pretreatment, which might have been inhibitory to yeast thereby causing the fermentation to be sluggish and incomplete with ethanol of 9 g/L in 36 hours. In contrast, the fermentation of clarified acid pre-hydrolysate progressed fast and complete thereby producing 12 g/L of ethanol after 20 hours. A fast fermentation process for ethanol production is desirable since it results in improved ethanol productivity, which has a direct impact on the economics of the fermentation process and its commercial feasibility (Oberoi *et al*., 2011).

The sluggish and incomplete fermentation of BPS enzymatic hydrolysate yielded 18 g/L ethanol with 60% conversion of cellulosic components to ethanol and 0.01 g/L/h of ethanol productivity. Thakur *et al*. (2013) also produced ethanol from enzymatic hydrolysates of different pretreated banana pseudostem by *S*. *cerevisiae* NCIM 3570. The authors found that ethanol produced in the enzymatic hydrolysate of alkaline pretreated BPS reached a maximum of 3.8 g/L with an ethanol yield of 0.35 g/g, which were higher than the levels obtained from acid pretreated BPS (1.9 g/L and 0.20 g/g). Although enzymatic hydrolysate of fungal pretreated BPS also yielded low ethanol (2.0 g/L), the ethanol yield was higher (0.40 g/g) than fermented

hydrolysate from chemically pretreated BPS (Thakur *et al*., 2013). Kusmiyati and Sukmaningtyas (2018) reported ethanol of 4.32 g/L produced from alkaline (2 N NaOH) pretreated BPS at 10% solid loading in simultaneous saccharification and fermentation (SSF). A low level of < 3.5 g/L ethanol was produced from banana plant waste (El-Zawawy *et al*., 2011).

5.4.5. The production of ethanol through co-culturing of *Saccharomyces cerevisiae* UL01 and *Trichoderma longibrachiatum* LMLSAUL 14-1

The production of ethanol was carried out in cellulase – producing media (Peixoto, 2006) by co-culturing of *T*. *longibrachiatum* LMLSAUL 14-1 and *S*. *cerevisiae* on BPS as a substrate. The co-culturing approach enables direct conversion of plant biomass, e.g. BPS to ethanol in a single step process. This strategy is similar to simultaneous saccharification and fermentation (SSF) and also consolidated bioprocessing (CBP) in that enzyme production, saccharification and fermentation occur in a single vessel or tank. Banana pseudostem has been shown to induce cellulase production in the current study and the ability of BPS to induce and promote the production cellulase is well known (Bhavsar & Bhalerao, 2012; Shah *et al*., 2005).

In co-culture fermentation, the low production of bioethanol seemed to be the consequences of poor induction of cellulase production by the BPS biomass. This low production of bioethanol might have been exacerbated by the insufficient nutrients (nutrient competition amongst the co-cultured microorganisms), which affected the growth of *S*. *cerevisiae* leading to the accumulation of glucose. High glucose inhibits particularly β-glucosidase from efficient hydrolysis of cellobiose to glucose. The accumulation of cellobiose also inhibited exoglucanase and could be the result of insufficient of β-glucosidase in the fermentation medium. According to Chawla *et al*. (2009), the optimal design of the medium is imperative for the growth of microorganisms in co-culture and to also stimulate product formation. Essential nutrients that are required for the growth of microorganisms include carbon, nitrogen, phosphorus, sulphur, potassium and magnesium salts.

The biological conversion of cellulosic biomass in hydrolysis reaction requires enzymes that interact synergistically to liberate oligomers and monomers (such as

glucose, xylose etc.) which are required for the production of bioethanol and other chemical products (Moreira *et al*., 2012). During the production of cellulase by *T*. *longibrachiatum* LMLSAUL 14-1 in co-culturing, the consortium of enzymes presents facilitated *in-situ* hydrolysis (saccharification) of BPS biomass to release glucose which is converted to ethanol by *S*. *cerevisiae*. The co-culture technique becomes a potential bioprocess if there are microbial compatibility and limited substrate competition. Ping *et al*. (2011) has demonstrated the feasibility of ethanol production from cassava pulp without any pretreatment and any addition of enzyme by applying sequential co-culturing. At the end of co-culturing fermentation, the authors reported a concentration of 8.67 g/L ethanol.

Park *et al*. (2012) also co-cultured *Acremonium cellulolyticus* C1 and *S*. *cerevisiae* to produce ethanol with Solka-Floc (SF) as substrate. An ethanol concentration of 25.6 g/L was obtained from 150 g SF with the ethanol yield of 0.17 g/g substrate and ethanol productivity of 0.36 g/L/h. Duque *et al*. (2015) conducted techno-economic and environmental analysis of ethanol from ten agro-industrial residues. The results of the study showed the potential of sugarcane bagasse, banana pseudostem, corn cob and rice husk in the production of bioethanol. For industrial applications, yeast should exhibit the following fermentative properties such as ethanol yield (> 90.0% theoretical yield), ethanol tolerance (> 40.0 g/L), ethanol productivity (> 1.0 g/L.h) and resistance to hydrolysate inhibitors (Dien *et al*., 2003). The high cellulase loading (dosage) required for saccharification and the low ethanol yield are the main factors affecting the bioethanol production from lignocellulosic biomass (Liu *et al*., 2016).

5.5. CONCLUSION

Alkaline pretreated BPS resulted in higher holocellulose content and yielded high concentrations of glucose after enzymatic saccharification irrespective of the crude cellulase enzyme system evaluated. High glucose yield was achieved with crude cellulase enzyme system from *Trichoderma longibrachiatum* LMLSAUL 14-1 compared to hydrolysed BPS by *T*. *harzianum* LMLBP07 13-5. Also, an ethanol concentration of 12 g/L and 18 g/L was produced from clarified acid pre-hydrolysate of BPS and crude enzyme hydrolysate, respectively. Fermentation by means of co-culture also showed the potential to produce ethanol in a single step and the

possibility that under optimised conditions more ethanol can be produced. The overall production of cellulosic bioethanol was a relatively cheaper process because the cellulase enzyme system and ethanol were produced from the same cellulosic biomass.

CHAPTER 6

6.1. GENERAL DISCUSSION AND CONCLUSIONS

In order to produce ethanol from BPS, it is imperative to search for organisms capable of breaking down *Musa acuminata* and *Strelizia alba*. Avicel (microcrystalline cellulose) agar media is often used to screen for potential cellulase secreting fungi. In this study, the screening procedure yielded six potential fungal strains. Of these fungal strains, four are identified as *Aspergillus fumigatus*, Table 3.1 and remaining two were *Trichoderma longibrachiatum* and *Trichoderma harzianum*.

The microbial production of endoglucanse (endo 1,4-β-glucanase; EC 3.2.1.4), exoglucanase (exo 1,4-β-glucanase; EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) is a highly regulated, energy consuming process and these enzymes are induced in the presence of specific substrates and are inhibited in the presence of glucose (Amore *et al*., 2013). In particular, detailed information on the regulation of cellulase gene expression in *Trichoderma* and *Aspergillus* species is reviewed by (Amore *et al*., 2013). The production of cellulase enzyme system is achieved in submerged fermentation (SmF) with Avicel as an enzyme inducing substrate and also in solid state fermentation (SSF) with banana pseudostem (BPS) as a substrate.

Submerged fermentation with Avicel as substrate induced all the enzymes of the cellulase complex in all the fungi investigated. However, due to the cost of this substrate, an alternative, cheap and abundant BPS substrate is used in the production of cellulase enzyme system using SSF. In SSF, native (untreated) banana pseudostem supported the growth of *Trichoderma* and *Aspergillus* species and the substrate is able to induce the secretion of cellulase enzyme system that differed in proportions from one fungus to the other. Thus the amount of secreted cellulase is enzyme specific and also fungus specific. The influences of temperature, pH, moisture and nature of the substrate on each fungus possibly activate the regulatory gene systems involved in the biosynthesis of specific individual enzymes of cellulase system different from one fungus to another. The results also reveal that effective co-culturing requires the compatibility of the microorganisms involved in the production of these enzymes.

Trichoderma longibrachiatum LMLSAUL 14-1 produced acid-thermo tolerant cellulase enzymes system with good stability of both endoglucanase and β-glucosidase at 40 °C and in acidic conditions. However, β-glucosidase showed good activity at both acid and basic pH conditions. Cellulase enzyme system from *T*. *harzianum* LMLPB07 13-5 is also acid-thermo tolerant showing remarkable stabilities at 50 °C and basic pH conditions, retaining over 70% of original activities. Only endoglucanase from *T*. *harzianum* LMLBP07 13-5 is stable in acidic conditions. Another fungus, *A*. *fumigatus* LMLPS13-4 also produced acid-thermo tolerant cellulase enzymes system with stability at 40 °C and acidic conditions. Interestingly, all the fungi produced β-glucosidase with catalytic activity and stability in both acid and basic condition.

Cellulase enzymes system from *T*. *longibrachiatum* LMLSAUL 14-1 and *T*. *harzianum* LMLBP07 13-5 were further evaluated for their ability to hydrolyse pretreated BPS. The hydrolysis results show that the cellulase system from *T*. *longibrachiatum* LMLSAUL 14-1 is more effective in hydrolysing BPS yielding more glucose than cellulase system from *T*. *harzianum* LMLBP07 13-5. The hydrolysis of BPS biomass through enzymatic hydrolysis to produce fermentable sugars shows that pretreatment was essential to alter the chemical, physical and morphological characteristics of the biomass to enhance biomass digestibility. Generally, there is a poor BPS conversion efficiency to glucose as substrate loading increases. Hence, there is a need for highly active cellulase enzyme system for effective hydrolysis

The production of bioethanol from cellulosic biomass typically requires several steps: BPS biomass pretreatment, hydrolysis/or saccharification and fermentation (and including distillation). In this study, the production of bioethanol through separate hydrolysis and fermentation (SHF) of acid pre-hydrolysate, as well as enzymatic hydrolysate and co-culturing fermentation techniques, has been investigated. The production of bioethanol in separate hydrolysis and fermentation of non-clarified acid pre-hydrolysate is prolonged and incomplete. This undesirable effect on the fermentation could be attributed to the presence of inhibitory compounds and other debris in the pre-hydrolysate, which hindered yeast growth and consequently lead to low ethanol yields. However, when the acid pre-hydrolysate is clarified by centrifugation the fermentation is fast and completes in a short time. High yeast

growth and ethanol are attained in clarified BPS hydrolysate, suggesting that clarification is essential to remove plant debris and inhibitory compounds.

Enzymatic hydrolysate contained 3.3-fold more glucose than acid pre-hydrolysate. The fermentation of enzymatic hydrolysate (74 g/L glucose) is protracted and incomplete with about 50% of original glucose being consumed at the end of fermentation. The incomplete fermentation could be due to inadequate nutrients, and low inoculum size. Therefore, essential nutrients are required for fermenting yeast to convert all sugars to ethanol and other by-products.

In co-culture with *T*. *longibrachiatum* LMLSAUL 14-1 and *S*. *cerevisiae*, the alkaline pretreated BPS could not support good production of cellulase and ethanol. This was probably as a result of the accumulation of cellobiose and glucose in large quantities, which inhibited cellulase production and activity. Poor or low β-glucosidase would lead to high cellobiose and low glucose in the fermentation medium which would inhibit exo-glucanase activity and limit the amount the glucose available to support the growth of *S*. *cerevisiae* and ethanol production. On the other hand, the native BPS contains nutrients such as proteins and minerals (Ma, 2015; Ho *et al*., 2012), which supported the growth of both organisms allowing cellulase production and fermentation of glucose to ethanol at levels higher than in pretreated materials.

Therefore, in order to reduce the cost of cellulase, it is imperative to use cheap abundant material and the enzyme be able to hydrolse the cellulosic material at a low dosage (low enzyme units). The current study integrated the production of cellulase enzymes and ethanol by utilising the same cellulosic banana pseudostem in both processes, thereby reducing overall cellulosic ethanol production costs. Efficient cellulase enzyme system could make cellulosic bioethanol competitive with the current gasoline prices. Banana pseudostem is shown to be a potential lignocellulosic biomass for both cellulase enzyme system and bioethanol production. Further optimisation, particularly of the fermentation medium formulation and inoculum size could improve the yield of ethanol.

6.2. FUTURE WORK

Banana pseudostem is a good source of bioethanol production. It would be worthwhile to further investigate the characterization of the cellulases studied and further optimise the saccharification processes involved. Further investigation in the use of thermotholerant yeasts in the fermentation process would reduce costs and possibly improve the yileds.

CHAPTER 7

7.1. REFERENCES

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7.2. ACKNOWLEDGEMENT

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