PRODUCTION, CHARACTERIZATION AND EVALUATION OF FUNGAL CELLULASES FOR EFFECTIVE DIGESTION OF CELLULOSE

by

MOKATSE KHOMOTSO

[redacted]

RESEARCH DISSERTATION

Submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

in

MICROBIOLOGY

in the

FACULTY OF SCIENCE & AGRICULTURE

(School of Molecular & Life Sciences)

at the

UNIVERSITY OF LIMPOPO

SUPERVISOR: Prof I. Ncube

2013

DECLARATION

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

Mokatse K.M.P _____ Signature: _____

[redacted] Date: _____

DEDICATION

This work is dedicated to my parents, Emmanuel and Mary-Jane Mokatse who supported me throughout my graduate career. My caring siblings Tebogo and Solomon for always being there and for putting a smile on my face when I need it the most.

.

ACKNOWLEDGEMENTS

Firstly I would like to thank the Almighty for his blessings if it wasn't for him I wouldn't have done/completed my MSc but by his grace he kept me until today

I would like to express my sincere gratitude to the following people and Institutions.

- My supervisor Prof Ncube for his support, assistance and for taking very good care of me. The lab felt at home because of him and I am very thankful. His guidance contributed a lot to my work from the initial to the final level of my study.

- Dr Thembekile Ncube for her guidance and my labmates for making the lab a better place to be in.

- The support I received from staff members from the department of Biochemistry, Microbiology and Biotechnology is highly appreciated.

- To all my friends who were with me throughout my graduate career

- University of Limpopo Women's Academic Solidarity Association (ULWASA); the writing retreats contributed a lot, Ladies Thank you!

- Vlaamse Interuniversitaire Raad (VLIR-UOS) and National Research Foundation (NRF) for their financial support.

- Lastly my entire family for their love and support

ABSTRACT

The production of cellulase is a key factor in the hydrolysis of cellulosic materials and it is essential to make the process economically viable. Cellulases are the most studied multi-enzyme complex and comprise of endo-glucanases (EG), cellobiohydrolases (CBH) and β-glucosidases (BGL). The complete cellulase system; comprising CBH, EG and BGL components thus acts synergistically to convert crystalline cellulose to glucose. Cellulases are currently the third largest industrial enzyme worldwide. This is due to their wide applications in cotton processing, paper recycling, juice extraction, as detergent enzymes and additives in animal feed. In this study, production of cellulase by five fungal isolates (BTU 251-BTU 255) isolated from mushrooms, was investigated and optimised. Internal transcribed spacer regions (ITS1 and ITS4) were applied to identify the five fungal microorganisms. Isolates were identified as follows: BTU 251 as *Aspegillus niger*, BTU 253 as *Penicillium polonicum*, and BTU 255 as *Penicillium polonicum.* Cellulase was produced in shake flask cultures using Mandel's mineral solution medium and Avicel as a carbon source. Cellulase activity was tested using 3, 5-Dinitrosalicylic acid assay and zymography, *A. niger* BTU 251 showed five activity bands ranging from 25- 61 kDa had an average nkat of 7000. Cultures from BTU 252 were the least active with an average nkat/ml of 200 and one activity band of 25 kDa. *P. polonicum* BTU 253 showed three activity bands ranging between 45 and 60 kDa and had an average nkat/ml of 2200. BTU 254 showed five activity bands ranging from 22-116 kDa and had average nkat of 350. *P. polonicum* BTU 255 produced the highest cellulase activity of 8000 nkat/ml and with three activity bands estimated at 45-60 kDa on zymography. The optimal temperature for activity of the cellulases was between 55-70°C and enzymes were most active within a pH range of 4-6. Optimal pH for production of cellulases by *P. polonicum* BTU 255, *P. polonicum* BTU 253 and *A. niger* BTU 251 was 4 while optimal temperature for production of the cellulases was between 50-55°C. Total cellulase activity was determined using Whatman No.1 filter paper as a substrate and β- glucosidase production was determined in polyacrylamide gels using esculin as a substrate. In the hydrolysis of crystalline cellulose (Avicel), a combination of *A. niger* BTU 251 and *P. polonicum* BTU 255 (1:1), (1:9), (1:3), and (1:2) produced maximum glucose as follows: 1:1 (0.83g/L), 1:9 (10.4g/L), 1:3 (0.77g/L) and 1:2 (0.73g/L). Cellulases from *P. polonicum* BTU 255 were partially purified using affinity precipitation and analysed using MALDI-TOF/TOF. Peptide sequences of *P. polonicum* obtained from MALDI-TOF/TOF analysis

were aligned by multiple sequence alignment with *C. pingtungium*. Conserved regions were identified using BLAST anaylsis as sequences of cellobiohydrolases. More research is required in producing a variety of cellulases that are capable of hydrolysing crystalline cellulose, the current study contributes to possible provision of locally developed combinations of cellulases that can be used in the production of bioethanol.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

APS	ammonium persulfate
β	beta
BTU	biotechnology unit
$CaCl_2$	calcium chloride
CHCA	cyna- 4- hydroxycinamic acid
CMC	carboxymethylcellulose
°C	degrees centrigade
DNS	3, 5-Dinitrosalicylic acid
EDTA	ethylenediamine tetraacetic acid
Exo	outer
Endo	inner
HCL	hydrochloric acid
HPLC	high performance liquid chromatography
kDal	kilo Dalton
MALDI	matrix assisted laser desorption/ ionization
mL	millilitre
mg	milligram
min	minute
NaOH	sodium hydroxide
nkat	nano kat

%	percentage
PAGE	polyacrylamide gel electrophoresis
PDA	potato dextrose agar
PCR	polymerase chain reaction
pH	potential hydrogen
PMF	peptide mass fingerprinting
pNPG	p-nitrophenyl-β-D-glucopyranoside
pNPL	p-nitro phenyl-β-D-lactopyranoside
rpm	revolutions per minute
SDS	sodiumdodecyl sulphate
SSF	solid state fermentation
SmF	submerged fermentation
TCEP	triscarboxyethyl phosphine
TEMED	N,N,N',N'-tetramethyl-ethylendiamine
TFA	trifluoroacetic acid
TOF	time of flight
µl	microlitre
w/v	weight per volume
ZR	zymo research

CHAPTER 1

INTRODUCTION

1.1 Background information

Cellulases are a group of hydrolytic enzymes capable of hydrolyzing cellulose to glucose. The enzymatic process for the depolymerization of cellulose requires three types of enzymes: Endoglucanase, which hydrolyses the internal β-1,4 glucan chain of cellulose within amorphous regions and displays low hydrolytic activity toward crystalline cellulose; Exoglucanase, an exoacting cellobiohydrolase which removes cellobiose from the reducing and non- reducing end of cello-oligosaccharide and of crystalline amorphous and acid or alkali treated cellulose; Cellobiase or β-glucosidase which hydrolyses cellobiose to yield two molecules of glucose which completes the depolymerization of cellulose (Jahangeer *et al*., 2005). For complete cellulose hydrolysis the cellulase system works synergistically. Cellulases have potential in industries and are used in food, beverages, and textile, laundry, paper and pulp industries. Cellulolytic enzymes are produced by a number of microorganisms. Fungi and bacteria are the main agents of natural cellulose degradation in cthe environment. Fungi however are known to secrete cellulases in large amounts (Lederberg, 1992).

Use of fossil fuels has become a concern with regards to global climate change resulting from accelerated carbon emissions. Another concern is the unstable and uncertain petroleum supplies, as well as the rising cost of these fuels. These concerns have shifted global efforts to utilise renewable resources for the production of a greener energy replacement which can meet the high energy demand of the world. The depletion of fossil fuels requires a transition from non-renewable carbon sources to renewable bioresources such as lignocellulose. Lignocellulosic materials consist mainly of three polymers; cellulose, a homopolymer of glucose; hemicellulose, a heteropolymer of pentoses and hexoses and lignin, an amorphous of phenyl propanoid units (Deswal *et al*., 2011).

Lignocellulosic biomass, also known as plant biomass is a good potential resource for the production of biofuels because it is abundant and inexpensive. Agricultural residues such as leaves, stems, and stalks from sources such as corn fibre, corn stover, sugarcane bagasse, rice

hulls, woody crops, and forest residues are a source of lignocellulosic biomass which is renewable and unexploited. There are also multiple sources of lignocellulosic waste from industrial and agricultural processes, e.g. citrus peel waste, sawdust, paper pulp, industrial waste, municipal solid waste, and paper mill sludge (Maki *et al*., 2009).

Lignocellulosic biomass is renewable with great potential for bioconversion to bioproducts that have value (Maki *et al*., 2009). However, the biorefining process remains unworkable due to a lack of biocatalysts that can overcome costs such as cooling from high temperature, pumping of oxygen/stirring, and neutralization from acidic or basic pH. The extreme environmental resistance of bacteria permits screening and isolation of novel cellulases to help overcome these challenges. Rapid, efficient cellulase screening techniques, using cellulase assays and metagenomic libraries have become an important approach in obtaining novel cellulases. Cellulases with activities on soluble and crystalline cellulose have been isolated from strains of *Paenibacillus* and *Bacillus* and have shown to have high thermostability and activity over a wide pH spectrum whilst novel cellulases from strains like *Cellulomonas flavigena* and *Terendini bacterturnerae*, consist of multifunctional cellulases with broader substrate utilization (Maki *et al*., 2009).

The production of fuel ethanol from lignocellulosic biomass is becoming one of the most important technologies for production of renewable transportation fuels. Most of the fuel ethanol produced in the world is currently sourced from starchy biomass or sucrose substrates such as molasses or cane juice. Technology for ethanol production from non-food plant sources is being developed so that large-scale production will be a reality in the future (Jervis *et al*., 1997). The production of ethanol from lignocellulosic biomass involves the different steps of pretreatment, hydrolysis (saccharification) and ethanol recovery.

1.2 Motivation of study

Cellulases play a large market potential and important role in the bio-based and bioenergy industry and this provides a great motivation to develop better cellulase preparations for cellulose hydrolysis. However the lack of efficient combinations of cellulases still remains a limitation in the development of cellulose based bioenegy processes. It is therefore important to conduct more research on new affordable and efficient cellulases since enzyme cost is considered to be a major issue in the commercialisation of cellulose hydrolysis.

1.3 PURPOSE OF STUDY

1.3.1 Aim and Objectives

The main aim of this study is to produce and characterise fungal cellulases for effective digestion of cellulose.

In order to achieve the main aim of the study the objectives are as follows:

i. Evaluation of cellulase enzymes from fungal isolates
ii. Determination of cellulase activity by DNS assay
iii. Characterisation of cellulose activity by electrophoretic methods (zymograms)
iv. Determination of pH and thermal stability of the cellulases
v. Optimization of temperature and pH conditions for enzyme production
vi. Evaluation of combinations of the cellulases for degradation of celluloses.

1.3.2 Significance of the study

More novel cellulases are needed in the bio-based and bioenergy industry, the current study therefore is intended to characterise and evaluate different types of cellulases from fungal isolates for digestion of cellulose. This will provide possibility for locally developed combinations of cellulases that can be used in the production of bioethanol.

CHAPTER 2

LITERATURE REVIEW

2.1 Structure of cellulose

Cellulose is a linear polysaccharide polymer made up of glucose units, which are connected by a β-acetal linkage (Figure 1). The acetal linkage makes cellulose different from starch. This difference in acetal linkages results in indigestibility of cellulose by humans.

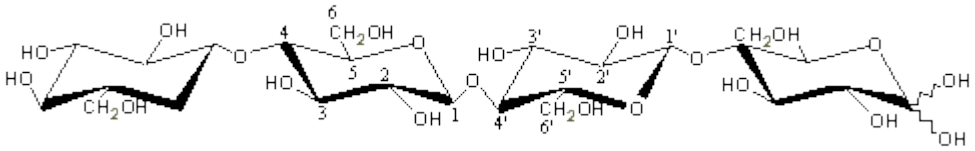

Figure 1: Structure of cellulose (http//www.elmhurst.edu/chm/vchembook/547)

The two chain ends on the cellulose structure are chemically different. One end has a D-glucopyranose unit in which the anomeric carbon atom is involved in a glycosidic linkage whereas the other end has a D-glucopyranose unit in which the anomeric carbon atom is free. This cyclic hemiacetal function is in an equilibrium in which a small proportion is an aldehyde giving rise to reducing properties at this end of the chain so that the cellulose chain has a chemical polarity. The key step in the utilization of cellulose is its hydrolysis into monomeric sugars and their eventual conversion into valuable chemicals and energy. The enzyme which is responsible for hydrolysis of cellulose is known as cellulase (Deswal *et al*., 2011).

Hemicelluloses are polysaccharides in plant cell walls that have β-(1→4)-linked backbones with an equatorial configuration. These include xyloglucans, xylans, mannans and glucomannans, and β-(1→3, 1→4)-glucan (Saha *et a*l., 2005, Scheller and Ulvskov, 2010). Hemicelluloses are the second most abundant polymer after cellulose and are located in the spaces between cellulose microfibrils in the primary and secondary walls. Hemicelluloses differ from celluloses in that they have side groups linked to the backbones, making this heteropolysaccharide less crystalline. Hemicelluloses also have a lower molecular weight than cellulose with 100-150 sugar residues per chain.

2.2 Cellulase enzyme system

Cellulase (EC 3.2.1.4) is an enzyme that breaks down cellulose; the carbohydrate that is the main part of the cell walls of plants (Lo *et al*., 2010). The cellulase enzyme complex breaks down cellulose to β-glucose (Saha and Bothast, 1999). Cellulases belong to a class of enzymes that are produced mainly by fungi and symbiotic bacteria in the ruminating chambers of herbivores and protozoans. Cellulases are also produced by other types of organisms such as plants and animals; however these types of cellulase enzymes differ structurally and mechanistically. There are two steps involved in cellulose degradation by microorganisms beginning with the preparatory pre-hydrolytic first step which involves an enzyme which swells or hydrates anhydrous glucose chains. The second step uses hydrolytic cellulase enzymes and β-glucosidase (cellobiase) (Abdullah, 2006). *Trichoderma reesei* has an extensively studied cellulase enzyme complex. This complex converts crystalline, amorphous, and chemically derived celluloses quantitatively to glucose. Cellulase derived from *Trichoderma longbrachiatum* is comprised of an enzyme complex consisting of three endoglucanases (EGI to EGIII) which converts cellulose to beta-dextrins and to D-glucose (Leghlimi *et al*., 2013).

Cellulase hydrolyzes the β-D-1, 4-glycosidic bonds of cellulose. Cellulase enzymes show activity during the ripening of some fruits, where their effects on cell walls results in the softening of the fruit. The vital characteristics of this cellulase complex are that the system is multi-enzymatic, at least three enzyme components are both physically and chemically distinct and also that all three components play essential roles in the hydrolysis of cellulose to glucose (Han *et al*., 1995).

2.3 Types of cellulases that form the cellulose enzyme system

There are three types of reactions that are catalyzed by cellulases. Unlike most enzymes, cellulase is a complex of enzymes that work synergistically to attack cellulose. The three enzymes act synergistically for the complete hydrolysis of cellulose into glucose. These reactions includes endo-cellulase, which breaks the internal bonds to disrupt the crystalline structure of cellulose; one that is involved in the hydrolysis of the individual cellulose fibers to break it into oligosaccharides called exo-cellulase; and another called β-glucosidase which is involved in hydrolysis of disaccharides and tetrasaccharides into glucose (Pason *et al*., 2006).

2.3.1 Endo-cellulases

Endoglucanases are often classified as endo-acting cellulases because they cleave β-1, 4-glycosidic bonds internally only and appear to have cleft-shaped open active sites (Figure 2). Endoglucanases are active on internal regions of cellulose and thus their activity can be assayed using soluble cellulose substrates such as carboxymethylcellulase (CMC). Sources of these cellulases include *Trichoderma reesei*, *Trichoderma viride* and *Aspergillus niger* (Dashtban *et al*., 2009).

2.3.2 Exo-cellulases

Exo-cellulase cleaves 2-4 units from the ends of the exposed chains produced by endo-cellulase resulting in tetrasaccharides or disaccharide such as cellobiose (Figure 2). There are two main types of exo-cellulases; cellobiohydrolases, that catalyzes the cellulolysis or hydrolysis of cellulose - one working progressively from the reducing end, and another working progressively from the non-reducing end of cellulose. Cellobiohydrolases (exoglucanases) were classified as exo-acting based on the fact that they all cleave β-1,4-glycosidic bonds from chain ends. In addition, those enzymes truly exo-acting often have a tunnel-shaped closed active site which retains a single glucan chain and prevents it from re-adhering to the cellulose crystal (Ghorai *et al*., 2010b). It was found that enzymes containing endocellulases only have little effect on native cellulose. However, those containing both endo and exocellulases will cause significant degradation on native cellulose thus the endo and exocellulases appear to work in a synergistic manner (Kostlev and Wilson, 2012).

2.3.3 Cellobiases

Cellobiase or β-glucosidase (Figure 2) hydrolyses the exo-cellulase product into monosaccharides. Cellobiase catalyzes the hydrolysis of β1→4 glycosidic bonds of the disaccharide cellobiose. Sources of cellobiases include *Aspergillus niger, Trichoderma viride* and *Saccharomyces cerevisae* (Chang *et al*., 2013). Cellobiase is responsible for removing the aglycone moiety from flavanoids and isoflavanoid glucosides, which are phenolic and phytoestrogen glucosides that occur in fruits, vegetables, tea, red wine and soybeans.

Detoxification of cassava, aroma enhancement and removing bitter compounds from citrus fruit juices or unripe olives are some other applications of cellobiase that are found in the food-processing industry (Khan *et al*., 2011).

2.3.4 Oxidative cellulases

Oxidative cellulases such as cellobiose dehydrogenase (acceptor) are enzymes that depolymerize cellulose by radical reactions. These enzymes belong to the family of oxidoreductases, specifically those acting on the CH-OH group of a donor with other acceptors. The name given to this enzyme class is cellobiose: acceptor 1-oxidoreductase. Other names for this enzyme class include cellobiose dehydrogenase, cellobioseoxidoreductase, *Phanerochaete chrysosporium* cellobiose (Ximenes *et al*., 2011) oxidoreductase, CBOR, cellobiose oxidase, cellobiose:oxygen 1-oxidoreductase, CDH, and cellobiose: (acceptor) 1-oxidoreductase. Cellobiose dehydrogenase can employ cofactor, FAD, but in most cases both a heme and a FAD group are required. Cellobiose dehydrogenase makes this enzyme one of the more complex extracellular oxidoreductases that is produced by wood degrading organisms (Ximenes *et al*., 2011).

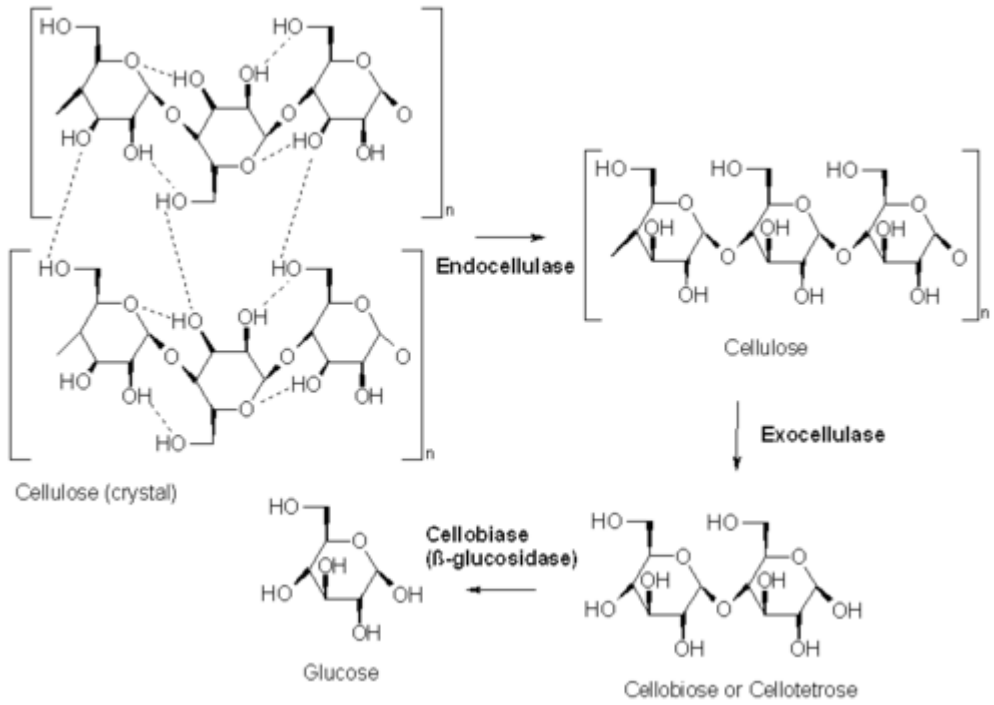

Figure 2: Breakdown of cellulose by cellulase enzymes comprising the cellulase enzyme system (www.wikepedia.org).

2.4 Applications of cellulases

2.4.1 Textile industry

A wide range of applications have made cellulase one of the most desirable enzymes. Cellulase enzymes could replace the pumice stones used by industry to produce 'stone-washed' denim garments. Stones can damage the clothes, particularly the hems and waistbands, and most manufacturers are now using cellulase enzyme (Hamlyn, 1997). Cellulase enzymes may also be applied in biopolishing, the removal of fuzz from the surface of cellulosic fibres which eliminates pilling making the fabrics smoother and cleaner-looking (Bhat, 2000).Using cellulases in the textile industry comes with a variety of advantages; the enzymes are easy to use, mild treatment conditions can be used e.g., pH and temperature, the enzymes are completely biodegradable, they save energy and chemicals also require less processing times (Ahuja *et al*., 2004).

2.4.2 Saccharification of cellulose

Cellulases are glycosyl hydrolases of varying structure. Most cellulases consist of three domains, a catalytic domain (the locus of hydrolysis), a cellulose binding domain (that anchors the whole enzyme onto the cellulose surface and orients the cellulose fiber towards the tunnel containing the active site) and a glycosylated flexible linker connecting the two other domains (providing sufficient spatial separation required between them, it allows processive motion and likely also plays a role in energy storage (Gilkes *et al*., 1997).

The relative activity of each enzyme in a given cellulase preparation is dependent on the source of the enzymes (Anderson, 2007). The saccharification properties of a given cellulase are usually studied on the basis of kinetic analyses. Kinetic studies are used to compare the cellulolytic capacity of enzyme systems from different sources, as well as the relative enzymatic susceptibility of different cellulosic substrates. Generally, the comparative studies include a measurement of the time course of saccharification over some initial reaction period as well as the quantification of the total extent of hydrolysis after a fixed, relatively long, reaction period (Gomez *et al*., 2010).

The degradation of cellulase mainly produces glucose, which can be fermented to produce ethanol. Therefore, cellulase presents a renewable energy resource for the production of an environmentally friendly transportation fuel (Du Plessis, 2008). Plant biomass regarded as wastes are biodegradable and can be converted into valuable products such as biofuels, chemicals, cheap energy sources for fermentation, improved animal feeds and human nutrients (Acharya *et al*., 2008).

2.4.3 Pulp and paper industry

Cellulases have been used in the pulp and paper industry for various purposes. A commercial cellulase preparation named Pergalase-A40, from *Trichoderma reesei* has been used by many paper mills around the world for the production of release papers and wood-containing printing papers (Freiermuth *et al*., 1994). Pere *et al*. (1996) and Rahkamo *et al*. (1996) investigated the effect of major cellulase components from *Trichoderma reesei* on the fibre properties of unbleached soft wood kraft and dissolving pulps in (Rahkamo *et al*., 1996).They found that the cellobiohydrolases had moderate effect on fibreviscosity; while endoglucanases, especially endoglucanase II, dramatically decreased the pulp viscosity even at a low concentration. Treatment of pulp with either cellobiohydrolase I and II had no effect on the development of pulp properties; whereas endoglucanase, especially endoglucanase II, improved the pulp beat ability, sheet density and other properties of the paper (Pere *et al*., 1996).

2.5 Sources of cellulase

Microorganisms such as bacteria, actinomycetes and fungi are known to degrade cellulose. Within the fungi group soft rot and white rot fungi have been studied while brown rots have not been studied extensively (Micky *et al*., 2011). Cellulolytic enzymes from soft rot and white rot fungi have been studied in organisms such as *Trichoderma viride* and *Phanerochaete chrysosporium*. *Trichoderma viride* produces a good amount of exoglucanases and endoglucanases but low levels of β-glucosidase. This is insufficient for the effective conversion of cellulose to glucose (Kanmani *et al*., 2009).

Fungi are generally known to secrete cellulases in large amounts. There are a variety of fungi that can produce cellulolytic enzymes under appropriate culture conditions including

Trichoderma, *Aspergillus*, *Penicillium* and *Fusarium*. *Trichoderma reesei* is one of the most efficient cellulase producers and has been well studied for the production of cellulolytic enzymes (Ishtiaq *et al*., 2010).

2.5.1 Fungal cellulases

Cellulases found in fungi play an important role in the breakdown of organic materials. A number of these cellulases are produced commercially (Gakilavani and Gopalakrishnam, 2009). Almin *et al*., (1975) reported five endo-β-1,4glucanases from *Chrysosporium lignorum*. Microorganisms of the genus *Trichoderma* produce large quantities of endo-ß-glucanase and exo-ß-glucanase, but only low levels of ß-glucosidase, while those of the genus *Aspergillus* produce relatively large quantities of endo-ß-glucanase and ß-glucosidase with low levels of exo-ß-glucanase. Synthesis of cellulases by fungi is considered to be induced by cellulose substrates, or more specifically by their water soluble short chain depolymerisation products such as cellobiose (Almin *et al*., 1975). Besides the fungus type, cellulase production is also influenced by media components; especially carbon and nitrogen sources and minerals, physical factors such as pH, temperature and moisture (Lynd *et al*., 2002). The fungal cellulase system has the ability to penetrate cellulosic substrate through hyphal extensions and do not produce stable high molecular weight complex.

2.5.2 Bacterial cellulases

Bacteria are a source for the production of industrial cellulase. Cellulolytic bacteria may be aerobes or anaerobes. Most of the bacteria produce mainly endoglucanases. Many thermophilic bacterial species produce cellulases that are stable and active at high temperature, resistant to proteolytic attack and are stable when subjected to mechanical and chemical denaturation. Cellulase production in bacteria, however is low compared to fungi (Linden and Shiang, 1991). The bacterial cellulase system has a complex system wherein the complexes are produced and tightly bound on the cell wall of cellulolytic bacteria to flexibly position the cellulase enzymes on the substrate for cellulose hydrolysis (Niranje, 2006).

2.6 Methods used for production of cellulase

2.6.1 Solid state fermentation

Solid-state fermentation (SSF) is defined as fermentation occurring in the absence or near-absence of free water employing either a natural support or an inert support as a solid material. The Solid substrate (matrix) must however contain enough moisture, which depends on the nature of the substrate, the amount of water absorbed could be one or several times more than its dry weight, which leads to high water activity (a_w) on the solid/gas interface in order to allow higher rate of biochemical process. SSF plays an important role in biotechnology or production of cellulases and has great potential for the bioconversion of plant biomass. Lignocellulose may be a good feedstock for the production of biofuels, enzymes and other biochemical products by SSF. Crop residues such as straw, corn by-products, bagasse etc. are suitable for this purpose since they are available in large quantities. Solid-state fermentation is simpler and less energy intensive (Tengerdy and Szakacs, 2003).

There are several aspects which should be considered in general for the development of any bioprocess in SSF. These include selection of a suitable microorganism and substrate, optimization of process parameters and isolation and purification of product. Theoretically, based on water activity, only fungi and yeast are suitable microorganisms for SSF (Toca-Herrera *et al*., 2007).

The risk of contamination is lower than in submerged fermentation because the hyphal growth mode of the filamentous fungi employed gives them an advantage over potential contaminating bacteria, which cannot easily access the cellulose inside the substrate in the absence of free water (Singhania *et al*., 2010).

The production of cellulase has always been studied in submerged culture processes but the high cost of enzyme production has hindered the industrial application of cellulose bioconversion. Solid state fermentation on the other hand is an attractive process to produce cellulase economically due to its lower operating expenses. Filamentous fungi can grow to a significant extent in the absence of free water. Considering that submerged free floating fungal growth is not natural, growth on and within solid substrates is fundamentally related to cell adhesion (Demir *et al*., 2012).

2.6.2 Submerged fermentation

Submerged fermentation (SmF) is defined as fermentation in the presence of excess water. Large scale enzyme producing facilities use SmF because of better monitoring capabilities and ease of handling. The use of filamentous fungi for the production of commercially important metabolites has increased over the years and the production of enzymes in SmF has been long established. Though bacteria and actinomycetes have been reported as cellulase producers, they produce very low products and thus do not make the technology economically feasible (Singhania *et al*., 2010). Most cellulases are produced by filamentous fungi under submerged fermentation and the production of cellulases is controlled by parameters such as cellulosic substrate, pH of medium, nutrient availability, inducer supplementation and fermentation temperature. Filamentous fungi such as *Trichoderma, Penicillium, Aspergillus* and *Humicola* can produce cellulases at different ranges of pH and temperature.

Media used for fermentation is very important since there is no general composition that can give optimum growth and cellulase production. Microbial cellulases are subject to induction and repression mechanisms and the process design and media formulation for cellulase production have to account for these aspects. Lactose is the only known cellulase inducer that is economically feasible in industrial fermentation media. Though some processes are batch, there have been attempts to produce cellulase in fed-batch with improved enzyme production, which helps to override the repression caused by accumulation of reducing sugars. Pure cellulose preparations have been used in the liquid cultures of cellulolytic microbes for production of the enzymes. Natural cellulosic materials, when used as the carbon source, gave poor enzyme yields (Hammad *et al*., 2010). The physiology of fungi is different on solid substrates than in liquid cultures.

2.7 Techniques and assays used for determination of cellulase activity

2.7.1 3, 5- Dinitrosalicylic acid assay

The 3, 5 Dinitrosalicylic acid (DNS) assay has been available since 1955 and is still very useful for the quantitative determination of reducing sugars (DeLong Frost, 2004). DNS IUPAC also known as 2-hydroxy-3; 5-dinitrobenzoic acid is an aromatic compound that

reacts with reducing sugars and other reducing molecules. It forms 3-amino-5-nitrosalicylic acid, which absorbs light mainly at 540nm (Lorenz, 1959). This assay was introduced as a method to detect reducing substances in urine and has since been widely used in various applications. It is mainly used in the assay of alpha-amylase; however, enzymatic methods use DNS due to their specificity. It can also be used for measuring amylases, mannases, pectinases and xyloglucanases. The DNS reaction is carried out by mixing and incubating a dilution of the enzyme preparation with a known amount of substrate at a buffered pH and set temperature (DeLong Frost, 2004).

2.7.2 Zymography

A Zymogram is a substrate containing polyacrylamide gel used for detection of hydrolytic enzymes. Zymograms were developed for the evaluation of matrix metalloproteinases. The zymographic technique has shown to be the most suitable technique for detecting isoenzymes of CMCase because it is only applicable to enzymes whose activity requires participation of subunits with similar molecular weights, all sequenced CMCase have been reported to encode only a single polypeptide gene (Khalili *et al*., 2011). It has been proven that zymography is highly useful for identification of extracted enzymes, rapid estimation of enzyme molecular weights and semiquantitative measurement of specific isoenzymes (Lacks and Springhorn, 1980). Overestimation of molecular masses of proteases has been reported using a zymogram, therefore zymograms need to be used carefully (Pagano, 1999).

2.7.3 Filter paper assay

The filter paper assay is usually used to detect the total cellulase activity by using filter paper as a substrate. Total cellulase activity resulting from the action of endoglucanase (EG) and predominantly exoglucanase (ExG) or both endo-and exoglucanase activities can be measunred using cotton fiber, Avicel (dyed or undyed) or filter paper. Filter paper Whatman No.1 has been the preferred substrate mainly because it is widely available and uniform, results are expressed as a measure of Filter Paper Activity (FPAse) (Silveira *et al*., 2012). The filter paper assay requires a fixed amount (2 mg) of glucose released from 50 mg sample of filter paper (3.6 % hydrolysis of substrate) which ensures both amorphous and crystalline fractions of the substrate are hydrolyzed. A series of enzyme dilution is required to achieve

the fixed degree of hydrolysis. Advantages of this assay is that (1) It is based on a widely available substrate, (2) it uses a substrate that is susceptible to cellulase, (3) it is based on a simple procedure (the removal of residual substrate is not necessary prior to addition of DNS reagent). The FPA is reproduced in most laboratories and has long been recognised for its complexity and susceptibility to operators (Zhang *et al*., 2006).

2.7.4 β-Glucosidase assay

The enzyme β-glucosidase catalyzes the hydrolysis of glycosidic linkages formed between the hemiacetal-OH group of a cyclic aldose or glucose and the –OH group of another compound sugar, amino-alcohol, aryl-alcohol or primary, secondary or tertiary alcohols. In other words, it catalyzes the hydrolysis of the soluble substrates, cellobiose and cello-oligosaccharides, with a low degree of polymerization, to cellobiose and glucose (Silveria *et al*., 2012).The hydrolytic and synthetic activities of cellobiase are important for certain biotechnological applications. The role of cellobiase in cellulose hydrolysis is significant because cellobiose is an inhibitor of both endo- and exo-glucanases, and must therefore be removed to allow complete saccharification of cellulose. This is done by increasing the rate of cellulose hydrolysis, making the reaction more economical for production of alcohol. β-glucosidase and cellobiohydrolase can be measured using p-nitrophenyl-β-D-glucopyranoside (pNPG) or p-nitro phenyl-β-D-lactopyranoside (pNPL) respectively (Ghorai *et al*., 2010a).

2.8 Affinity precipitation

Affinity precipitation is a novel separation procedure, it uses macroligands to first capture and then to remove the target. It exploits the specific interation between a biological ligand (e.g. substrate, coenzyme, hormone, antibody etc.) Or it’s synthetic analog and its complementary binding site on a protein. The protein binds to a specific ligand but the latter is free in solution rather than bound to an insoluble support. Ligand binding results in precipitation of the protein which may be separated by centrifugation. The pellet usually contains protein of interest and the ligand, whereas other components of the mixture remain in the supernatant allowing easy separation. The most common precipitation used to separate proteins is ammonium sulphate precipitation because it is simple and easy to use. It stabalises

most proteins in solution and helps remove lipid content of the sample. Ammonium sulphate precipitation removes about 50% and therefore reduces the load for subsequent chromatography. The disadvantage of protein separation by precipitation is that it lacks specificity (Ward and Sniatek, 2009).

2.9 Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS)

Matrix-Assisted Laser Desorption/Ionization (MALDI) is a very sensitive technique used in mass spectrometry. MALDI is used to analyse biomolecules such as proteins, peptides and sugars. In proteomics, MALDI is used for identification of proteins isolated through 1D or 2D SDS-PAGE. The type of mass spectrometer that is most widely used with MALDI is the Time of Flight Mass Spectrometer (TOF-MS) mainly due to its large mass range. The TOF procedure is suited to the MALDI ionization process since the pulsed laser does not work in a continuous operation but rather takes individual shots (Lewis *et al*., 2006). Proteins or peptides can be sequenced using MALDI-TOF-MS with a ladder sequencing technique, which consists of a time dependant concentration dependant chemical degradation from either the N- or C- terminus of the protein or peptide into fragments each of which differs by one amino acid residue. This type of analysis determines the masses of a series of peptides/proteins that are present in a single MALDI sample. The order in which the sequence occurs defines the sequence of amino acids in the original protein or peptide. The sequence of the peptide is deduced from its metastable ion decay mass spectrum (Moe *et al*., 2004).

For the identification of proteins MALDI-TOF is carried out by digesting the protein with trypsin (that cleaves at R-X, K-X except when X is a Pro). Peptide ions are then subjected to MALDI-TOF/TOF analysis, providing information that can be used to determine the sequence. The results from both types of analysis are combined and searched using algorithm eg. MASCOT (http:// www.matrixscience.com) against protein or translated DNA/EST database. The database is then theoretically digested with trypsin and the experimentally generated mass list is compared to the theoretically digested database. This method is limited for identification of proteins that are not in the database however is most suitable for identification of known purified proteins. It is not suitable for proteins < 15 kDa in size

because the matches are based on peptide masses and not sequence information and only able to suggest post-transitional modifications.

2.10 Computational Techniques used for Identification of Proteins

2.10.1 Multiple sequence alignment (MSA)

Protein sequence alignment is an important bioinformatics tool. It has important application in biological evolution analysis and protein structure prediction. The quality of sequence aligning results directly affects the accuracy of other researches. Therefore it is very important that quality results are obtained from multiple sequene alignment (MSA). Multiple sequence alignment is a sequence alignment for DNA, Protein and RNA. The multiple sequence alignment usually aligns sequences that have an evolutionary relationship by which they share a lineage and are from a common ancestor. From MSA, sequence homology can be inferred and phylogenetic analysis can be conducted to access the sequences shared evolutionary origins (Zhang *et al*., 2006).

Multiple alignment programs can be divided into two main categories methods aligning sequences over their entire length (global) and methods aligning regions of high similarity only (local). Multiple alignment programs focus on global methods exemplified by CLUSTAL W as they perform well in cases when all sequences are of similar lengths (Lassmann and Sonnahamer, 2002).

2.10.2 Basic Local Alignment Search Tool (BLAST)

BLAST is used for comparing primary biological sequence information such as the amino acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search is used to compare a query sequence to that of the database. BLAST is one of the most used bioinformatics programs because it addresses a fundamental problem and algorithm it uses is much faster than calculating an optimal alignment (http://blast.ncbi.n/mnih.gov).

BLAST requires a query sequence to search for, and a sequence to search against (also called the target sequence) or a sequence database containing multiple such sequences. BLAST will find sub-sequences in the database which are similar to sub-sequences in the query, usually

the query sequence is much smaller than the database, e. g., and the query may be one thousand nucleotides while the database is several billion nucleotides (Tatusova and Madden, 1999).

2.11 Biofuels

Biomass based fuels also known as biofuels are among the alternative fuels that are being considered. Biofuels mainly from lignocellulosic materials have been considered in that; lignocellulosics are abundant and evenly distributed geographically as compared to the fossil fuels, they generate low net greenhouse emissions, minimize the conflicts between land use for food and feed production as well as energy feedstock production and may provide employment for rural areas (Sarkar *et al*., 2006).

Biofuels are fuels produced from organic matter. Other types of fuel are derived from biological carbon fixation. Biofuels include fuels derived from biomass conversion, as well as solid biomass, liquid fuels and various biogases. Organic materials used for biofuel production include starch and sugary plants such as corn, wheat or sugar cane; oily plants such as rape seed, soya beans or jatropha; vegetable oils and animal fats; wood and straw; algae and organic waste and others (Demirbas, 2009).

Bioethanol production from lignocellulosic biomass is beginning to emerge due to recent advances in conversion technology. The idea of converting biomass-derived sugars to transportation biofuels was first proposed in the 1970s (Stephanopoulus, 2007).The main aim of biofuel research is to produce energy products such as alcohols mainly ethanol, also propanols and butanols as well as diesel, hydrogen and biogas from biological source (Elshahed, 2010).

There are two main techniques currently used in biofuel research aiming at alcohol production; direct and indirect fermentation (Elshahed, 2010). Direct fermentation depends on the conversion of various plant materials to biofuels, mainly ethanol. In general there are two processes involved: the degradation of starting plant material into fermentable sugars, and the conversion of sugar to alcohol.

Production of fuel ethanol from lignocellulosic biomass is becoming one of the most important technologies for production of renewable transportation fuels. Ethanol has a higher octane rating than gasoline and produces fewer emissions and is being recognized as an additive to gasoline. Most of the fuel ethanol produced in the world is currently from starchy biomass or sucrose (molasses or cane juice), but the technology for ethanol production from non-food plant sources is being developed so that large-scale production can be possible in the future (Gasparatos *et al*., 2011).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Microbial Strains

Microorganisms used in this experiment were initially isolated from mushrooms obtained from culture collection at the Biotechnology Unit, University of Limpopo (Turfloop campus) South Africa.

Identification of selected species was done by Inqaba Biotech Company Pretoria, South Africa.

3.1.2 Reagents

Sodium chloride, 3,5- Dinitrosalicylic acid, Ammonium phosphate, Magnesium sulphate, Carboxymethyl cellulose, Calcium chloride, Manganous sulphate, Ferrous sulphate, Zinc sulphate, Urea, Peptone, Yeast extract, Sodium phosphate (monobasic and dibasic) , Sodium hydroxide , Sodium citrate, Citric acid, Congo red, Sodiumdodecyl sulphate (SDS), N,N,N',N'- tetramethyl-ethylealamine (TEMED), Trizma base, Ammonium persulphate (APS), Agarose,Glucose, Melliziotose, Galactose, Lactose, Maltose, Fructose, Sodium acetate, *p*-nitrophenyl-β-D-glucopyronoside,Glycine, Dialysis tubing Cellulose membrane were obtained from Sigma-Aldrich Co. St Louis, MO, USA. Coomasie Brilliant blue R-250, Coomasie Brilliant Blue G-250 and Acrylamide were obtained from Fluka Biochemika. Ethanol, Hydrochloric acid, Isopropanol was obtained from Rochelle chemicals (Rochelle Chemical and Laboratory Equipment cc, Johannesburg, RSA) Protein molecular Weight marker was obtained from Fermentas (Ferments- Inqaba biotec, Pretoria, South Africa). Bromophenol blue was obtained from Amersham Life Science, (Pty) Ltd, SA. Whatman No.1 filter paper was from Macherey-Nagel GmbH &Co., (Duren), Germany.

3.1.3 Equipment

Equipment used in the study included a Speedy autoclave (vertical type) (Taiwan), Beckman Coulter Allegro X 22R centrifuge, Beckman Coulter DTX 880 Multimode Detector

microplate reader. Shimadzu LC High performance liquid chromatograph (HPLC) Prominence 20 System, BioRad Techware PS250-2 SDS-PAGE apparatus. Savant Speed Vac (SC110) Concentrator and Gel Dryer pump EC 353, Syngene Genesnap Imaging System (Thermoscientific, UK), Neubauer bright lined counting chamber, light microscope.

3.2 Methods

3.2.1Identification of fungal isolates

Five fungal isolates BTU 251, BTU 252, BTU 253, BTU 254 and BTU 255 were obtained from culture collection available at Biotechnology Unit of University of Limpopo. These isolates had been previously shown to produce cellulases. The fungal isolates were identified at Inqaba Biotech using Internal transcribed spacer (ITS) regions for rDNA, the procedure is as follows: the fungal isolates were added to a ZR bashing bead lysis tube and processed in a cell disrupter at maximum speed for 5 min; the ZR bashing bead lysis tube was centrifuged at 10 000 x g for 1 min. The supernatant (400 µl) was transferred to a zymo spin_IV filter in a collection tube and centrifuged using a microcentrifuge at 7000 rpm for 1 min. Fungal DNA binding buffer (1.2 ml) from the previous step was added to the filtrate in the collection tube, 800 µl of the mixture was transferred to a zymo- spin IIC column in a collection tube and centrifuged at 10 000 x g for 1 min, the flow through was discarded. DNA pre-wash buffer (200 µl) was added to the zymo spin IIC column centrifuged at 10 000 x g for 1 min and washed with 500 µl fungal DNA wash buffer. The column was transferred to a clean 1.5 ml microcentrifuge tube and 100 µl DNA elution buffer was added directly to the column matrix and centrifuged at 10 000 x g for 30 min to elute the DNA. The internal transcribed spacer (ITS) region of rDNA gene for each isolate was amplified using the Dream *T*aq (Thermo Scientific) with the ITS 1 TCC GTA GGT GAA CCT GCG G and ITS 4 TCC TCC GCT TAT TGA TAT GC as primers. PCR products were then sequenced on the ABI Big dye V3.1on a ABI 3500XL genetic analyzer (Life Technologies).

3.2.2 Harvesting of spores and culturing of fungi

Fungal spores from the five fungal isolates were grown on potato dextrose agar (PDA) and incubated at 30°C for 72 hrs. After 72 hrs of incubation the spores covering the agar medium were harvested by the addition of 10 ml of 1% (v/v) Tween 80 in sterile distilled water and

spores were gently brushed using a sterile swab. The spores were suspended in 100 µl sterile distilled water and were counted using a hemocytometer and stored at 4°C.

3.3 Fungal cellulase production

3.3.1 Cellulase production

Cellulase production was carried out in 250 ml Elernmeyer flasks each containing Mandels medium pH 4.2 that comprises of Na_2HPO_4 (15g), $CaCl_2$ (0.8g), $MgSO_4$.7H2O (1.2g), $MnSO_4$.7H2O (0.0016g), $FeSO_4$ (0.00271g), $ZnSO_4$ (0.0014g), Urea (3g), Peptone (0.75g), Yeast extract (0.3g) and Avicel (10g) in 200 ml media. To each flask 1 X 10^5 spores were added and incubated at 30°C, with shaking at 200 rpm and samples were collected every 24 hrs until maximum cellulase activity was reached. The culture was centrifuged at 3500 x g using a centrifuge (Beckman, USA) for 15 min and the supernatant was stored at -20°C until required for cellulase assay.

3.4 Cellulase assays

3.4.1. 3, 5-Dinitrosalicylic acid assay method for cellulase activity

Cellulase activity was measured using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1960), through the determination of amount of reducing sugars liberated from 1% carboxymethylcellulose (CMC) solubilized in 50 mM citrate buffer pH 5.0 (Bailey and Viikari, 1993). A combination of 100 µl enzyme and 900 µl of 1% CMC substrate solution were incubated at 50°C for 5 min. The reaction was stopped by addition of DNS solution and reaction mixture was boiled for 15 min, cooled in water for colour stabilization, and the optical density was measured at 540 nm. Cellulase activity was determined by using a calibration curve obtained from standard solutions of glucose. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of glucose per minute.

3.4.2 Filter paper assay for saccharifying cellulase

Total cellulase activity was determined using Whatman No.1 filter paper strip (50mg) as a substrate (Ghose, 1987). The assay mixture contained (1 ml of 0.05 M) sodium citrate buffer pH 4.8, Whatman No.1 filter paper strip (50mg) and 0.5 ml of diluted enzyme, at least two dilutions were made 5X and 10X dilution. The sample mixture was incubated at 50°C for 60 min. For the determination of total celluase activity (FPA_{tot}), 3ml of DNS solution was used to stop the reaction and the test tubes were boiled for 5 min, cooled and diluted using 20 ml of deionized water. The colour formed was measured against the blank at 540 nm. Glucose was used as a standard and total cellulase activity determined was defined by mg of glucose released x 0.185 (Ghose, 1987).

3.5 Factors affecting cellulase production

3.5.1 Effect of initial pH on cellulase production

The optimum initial pH for cellulase production by *Penicillium polonicum* and *Aspergillus niger* was determined using buffers ranging from pH 3 to 6 in Mandels media with either HCl or NaOH being used to adjust the initial pH. Cellulases were harvested every 24 hrs until maximum cellulase activity. Cellulase activity was determined using DNS assay as previously described (Section 3.4.1).

3.5.2 Effect of temperature on cellulase production

The optimum temperature for cellulases production by *Penicilium polonicum* and *Aspergillus niger* was determined using temperatures ranging from 35°C to 50°C. Cellulases were harvested every 24 hrs until maximum cellulase activity. Cellulase activity was determined using the DNS assay (Section 3.4.1).

3.5.3 Effect of temperature on enzyme activity

The optimum temperature of the enzyme for hydrolysis of carboxymethylcellulose (CMC) in 50 mM Citrate buffer (pH 5.0) by *Penicillium polonicum* and *Aspergillus niger* was determined by incubating the mixture of the enzyme (100µl) and 1% (w/v) CMC (900µl) for

5 min at different temperatures ranging from 25 to 90°C. The reaction was stopped by the addition of DNS solution and cellulase activity was determined.

3.5.4 Effect of pH on enzyme activity and stability

The optimum pH for cellulase activity was determined by incubating the mixture of the enzyme (100 µl) and 1% (w/v) CMC (900 µl) in the presence of appropriate buffers; 50 mM citrate buffer (pH 3, 4, 5 and 6), 50 mM sodium phosphate (pH 6, 7 and 8), 50 mM Tris–HCl (pH 8 and 9) and 50 mM glycine–NaOH (pH 9, 10 and 11). The reaction mixtures in various pH buffers were incubated for 5 min at 50°C after which the cellulase activity was assayed by DNS method (Section 3.4.1).

3.6 Electrophoretic methods

3.6.1 Zymography for cellulase

Enzyme extracts (20µl) were loaded on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Laemmli, 1970). Unstained Protein Molecular Weight Marker (Fermentars) was used for estimation of the molecular weights of the protein bands obtained. The cellulase zymograms were prepared using CMC substrate incorporated into the gel prior to polymerization to give a final concentration of 0.1% w/v (Ratanachomsri *et al*., 2002). After electrophoresis the gels were washed using sodium phosphate buffer pH 7.2/ 40% isopropanol (1:1) for 1 hr. Further washing was done using sodium phosphate buffer pH 7.2 for 1 hr to remove the isopropanol. The washed gels were placed in a 1:1 mixture of 50 mM sodium phosphate buffer/1 mM EDTA at 40°C for 30 min to renature the protein (Dutta *et al.*, 2008). The gel was then incubated at 50°C for 30 min for enzymatic degradation of the substrate. Congo red 0.1% (w/v) was used to stain the gels by incubation over 30 min followed by de-staining with 1 M sodium chloride for 20 min. The enzymatic reaction was stopped by immersing the gel in 0.5% (v/v) acetic acid (Ratanachomsri *et al*., 2002). The gels were documented using Synegene G-Box Image Capture (Synegene, USA).

3.6.2 Detection for β- glucosidase activity in polyacrylamide gels

Gels prepared as already described in (Section 3.6.1) were used for detection of β-glucosidase activity with Esculin as a substrate. After electrophoresis, the gel was soaked in 0.2 M sodium acetate buffer (pH 4) for 10 min at room temperature in order to exchange the buffer system. The gel was incubated in 0.2 M sodium acetate buffer containing 0.1% (w/v) Esculin and 0.03% w/v ferric chloride for 5 min at 50°C. During incubation, the black bands corresponding to the β-glucosidase appeared against a transparent background. The reaction was stopped by immersing the gel in a 10% (w/v) aqueous solution of glucose an inhibitor of β-glucosidase (Kwon *et al*., 1994).

3.7 Ammonium sulphate precipitation of the crude enzyme extracts

Concentration of the crude enzyme extracts was achieved by precipitating the protein from the supernatant by addition of ammonium sulphate to 80% saturation of the supernatant. The solution was stirred at 150 rpm and 4°C overnight. The precipitate was obtained by centrifuging the formed suspension at 3000 x g for 20 min at 4°C. The pellet was re-dissolved in (0.05M) acetate buffer pH 5 to give 10 times concentrated enzyme solution. The enzyme solution was dialysed against (0.05M) acetate buffer pH 5 at 4°C overnight. The dialysed enzyme solution was kept at -20°C and thawed when needed.

3.8 HPLC Analyses

Samples from Avicel digestion were filtered through a 0.45 µm millipore filter into a 2000 µl HPLC vial. A volume of 20 µl of the filtrate was injected into a Phenomenex RCM-Monosaccharide 300 X 7.00 mm column and eluted using deionised water at a flow rate of 0.6ml/min. The column was maintained at 85°C and the sugars were detected using a Shimadzu RID-10A refractive index (RI) detector (Saha *et al*., 2005).

3.9 Preparation of phosphoric acid cellulose (Walseth cellulose)

Avicel (10g) was suspended in concentrated phosphoric acid 88% (w/v) and left at 4°C for 1 hour with occasional stirring. The mixture was poured into 4 litres of ice cold water and left to stand for 30 min. The swollen Avicel was washed several times with cold water by

decantation. After washing with 1% (w/v) $NaHCO_3$ solution, the thick suspension of swollen cellulose was dialysed at 4°C against water. The suspension was blended using a waring blender for 60 sec to obtain a smooth textured phase (Wood, 1971).

3.9.1 Affinity precipitation of Cellulases

Acid-swollen cellulose (20 g) was added in a 100 ml Schott bottle containing 50 ml of the crude enzyme extract. The mixture was stirred for 12 hrs at room temperature. Cellulose was separated by centrifugation at 3500 x g for 10 min. The cellulose was washed 3 times using 50 mM acetate buffer pH 5, containing 10mM $CaCl_2$ and 1mM DTT to remove the unbound proteins. Bound proteins were eluted from cellulose by suspending 25 ml of 0.1 M cellobiose solution in distilled water. The suspension was stirred for 2hrs at room temperature before removing the cellulose by centrifugation. Eluates containing bound proteins were filtered using 0.2 µm Millipore filter to remove the fine cellulose particles. The eluates were concentrated using Amicon ultra centrifugal filter unit (Millipore, Ireland) with 10 kDa cutoff (Bassam *et al.*, 1995). SDS-PAGE was performed on the cellulase eluates.

3.9.2 Preparation of samples for protein sequencing

The stained SDS-gels that were previously prepared (Section 3.10.1) were placed on clean glass plates and selected bands were cut using a clean sterile blade. Each band was placed in a labeled microcentrifuge tube which had holes punched on the lid. The samples were then dried in a Savant Speed Vac (SC 110) concentrator (Holbrook, NY, USA) equipped with a Gel Dryer Pump EC 353 (E.C Apparatus Corp, Milford, MA, USA). The gel slices were dried for 3 hrs at a medium drying rate and kept in a desiccator prior tryptic digestion and mass spectrometry analysis.

3.10 Identification of Cellulases

3.10.1 In-gel digestion

All reagents were analytical grade or equivalent. Gel slices supplied were destained in a microcentrifuge tube with 200 mM NH_4HCO_3: Acetonitrile 50:50 until clear (Burdick & Jackson; Sigma). Samples were dehydrated and desiccated before reduction with 2 mM

triscarboxyethyl phosphine (TCEP; Fluka) in 25 mM NH_4HCO_3 for 15 minutes at room temperature with agitation. Excess TCEP was removed and the gel pieces again dehydrated. Cysteine residues were carbamidomethylated with 20 mM iodoacetamide (Sigma) in 25 mM NH_4HCO_3 for 30 minutes at room temperature in the dark. After carbamidomethylation the gel pieces were dehydrated and washed with 25 mM NH_4HCO_3 followed by another dehydration step. Proteins were digested by rehydrating the gel pieces in (20ng/µL) trypsin (Promega) solution and incubating at 37°C overnight. Peptides were extracted from the gel pieces once with 50 µ of 10.1% trifluoroacetic acid (TFA) (Sigma). The samples were dried down and 200µL water added and concentrated to less than 20 µL to remove residual NH_4HCO_3. The samples were dried and re-dissolved in 0.1% TFA. The samples were purified and concentrated using a C18 ZipTip according to the manufacturer's instructions. The purified samples were eluted with -cyano-4-hydroxycinamic acid (CHCA) at 5mg/mL in 50% acetonitrile/ H2O containing 0.1% trifluoroacetic acid (TFA) and spotted manually onto a MALDI target plate.

3.10.2 Mass spectrometry

MALDI MS was performed at Centre for Proteomic and Genomic Research using a 4800 MALDI TOF/TOF system (AB SCIEX) with instrument control through 4000 Series Explorer. Parent spectra were acquired in reflector positive mode at a laser intensity of 4000 arbitrary units using 600 laser shots per spectrum. The san range was *m/z* = 800 -4000. The grid voltage was set to 16kV. Spectra were internally calibrated using trypsin autolytic fragments. Fragmentation data was acquired in positive mode with a deceleration voltage of 1kV. The spectra were acquired with a laser intensity of 4500 arbitrary units and 1600 shots per spectrum.

3.10.3 Data Analysis

Database interrogation was performed with the Mascot algorithm using the MSDB database on a GPS workstation. Search parameters were as follows:

Species – All; Fungi
Enzyme – trypsin;
Maximum number of missed cleavages -1;
Fixed modifications – carbamidomethyl (C);

Variable modifications oxidation (M); deamination (N/Q); pyroglutamic acid

Precursor tolerance - 100 ppm.

Fragment tolerance – 0.2 da

3.10.4 Multiple sequence alignment (MSA) and Basic local search alignment tool (BLAST)

Peptide sequence of *P*enicillium *polonicum* was aligned using CLUSTAL W (www.ebi-uk/tools/msa/clustalw2) against peptide sequences of organisms, *Chaetomidium pingtungium*, *Thermoascus aurantiacus*, *Botryosphaeria rhodina* and *Trichophaea saccata* to identify regions of similarity that may be of functional or evolutionary relationship. The conserved peptide regions were compared to the known peptide sequences using BLAST (http://blast.ncbi.nlm.nih.gov) to identify the name of the proteins, their fuctions, active sites and their relationship.

CHAPTER 4

RESULTS

4.1 Time course for production of cellulases by five fungal isolates

Five fungal isolates (BTU 251, BTU 252, BTU 253, BTU 254 and BTU 255) obtained from Biotechnology Unit, University of Limpopo were used to produce cellulases. Production of the cellulases was perfomed in submerged fermentation using Avicel™ as a carbon source. *P. polonicum* BTU 255 was the best cellulase producer with an nkat of 8000, followed by *A. niger* BTU 251 with an nkat of 7000. BTU 252 was the least cellulase producer with an nkat of 200, BTU 254 had an nkat 600 of and *P. polonicum* BTU 253 had an nkat of 2200 (Figure 4).

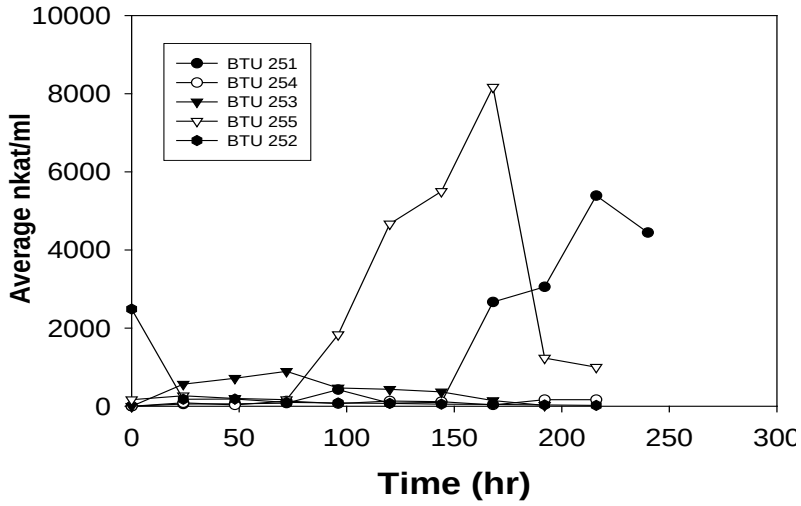

Figure 3: Time course for production of cellulases by five fungal isolates in submerged fermentation with Avicel ™ as a carbon source at 30°C. Results are expressed as average nkat/ml that gave the highest enzyme activities.

4.2 Zymogram and enzyme activity of cellulases in selected fungal isolates.

Zymography was performed on selected fungal isolates BTU 251, BTU 252, BTU 253, BTU 254 and BTU 255 which produced relatively higher cellulase activity over time, 0.1% CMC was used as an in-gel substrate. The clear bands against the dark background on the zymogram indicate cellulase activity (Figure 4, 5, 6 and 7). BTU 251 showed five cellulase activity bands of 61 kDa, 50 kDa, 42 kDa, 28 kDa and 25 kDa (Figure 4a) and had an average nkat of 7000 (Figure 4b). BTU 253 showed three cellulase active bands of 60 kDa, 47 kDa, 45 kDa (Figure 5a), BTU 254 showed five cellulase activity bands with different molecular weights of, 116 kDa, 66 kDa , 45 kDa, 33 kDa and 22 kDa (Figure 6a). BTU 254 had an nkat of 350 (Figure 6b), and BTU 255 also had three cellulase active bands of 45 kDa, 47 kDa and 60 kDa (Figure 7) respectively.

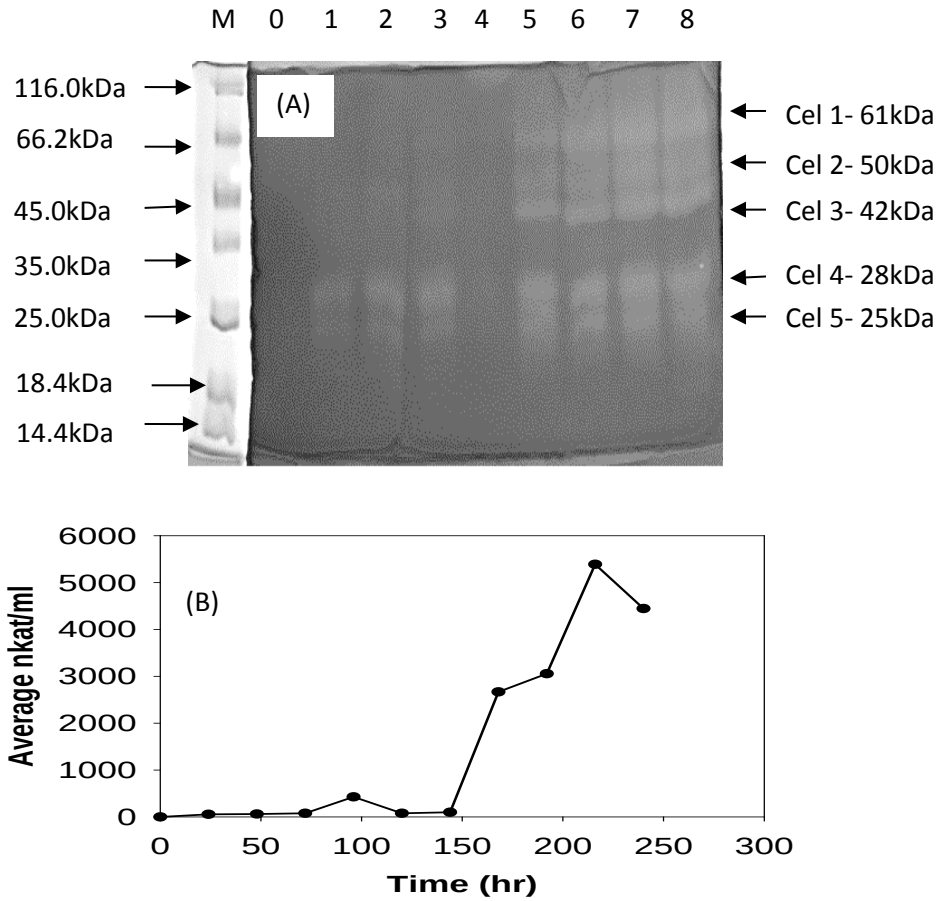

Figure 4: Zymogram (A) and time course of BTU 251 (B) showing cellulase activity over a period of 248 hrs using Avicel™ as a carbon source. Lane M: protein molecular weight marker, lanes 0-8: represent the number of days of fermentation (Day 0-8). The average nkat value represents the average of triplicate experiments.

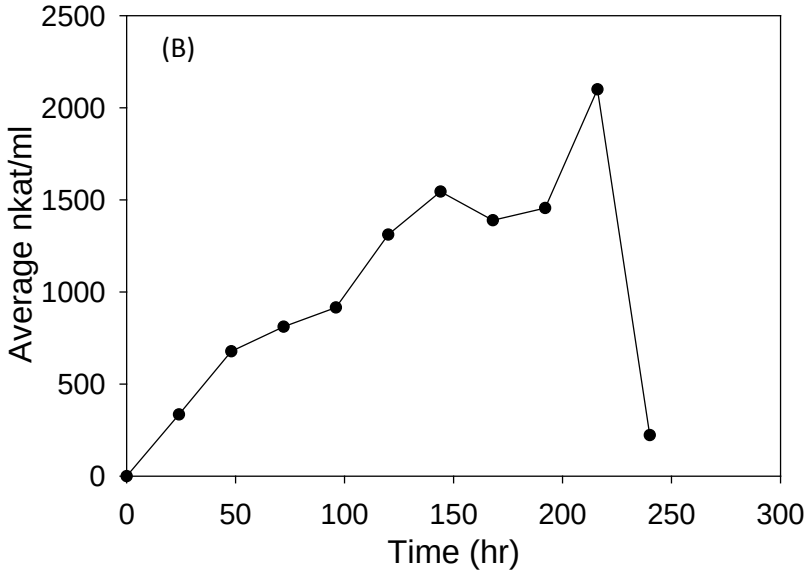

Figure 5: Zymogram (A) and time course (B) of BTU 253 showing cellulase activity over a period of 248 hrs using Avicel™ as a carbon source. Lane M: protein molecular weight marker, lanes 0-8: represent the number of days of fermentation (Day 0-8) using Avicel as a carbon source. The average nkat value represents the average of triplicate experiments.

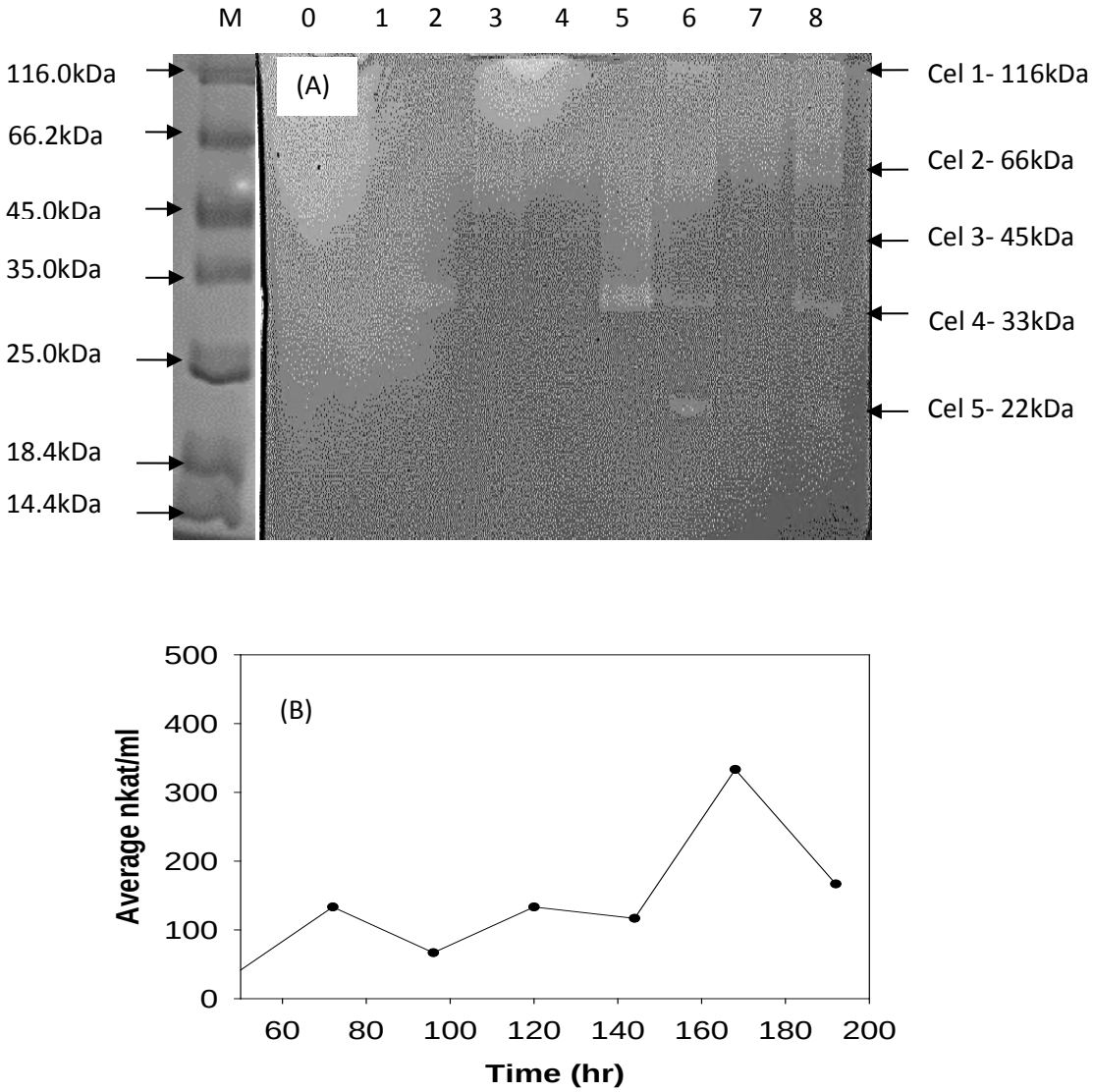

Figure 6: Zymogram (A) and time course (B) of BTU 254 showing cellulase activity over a period of 198 hrs using Avicel™ as a carbon source. Lane M: protein molecular weight marker, lanes 0-8: represent the number of days of fermentation (Day 0-8). The average nkat value represents the average of triplicate experiments.

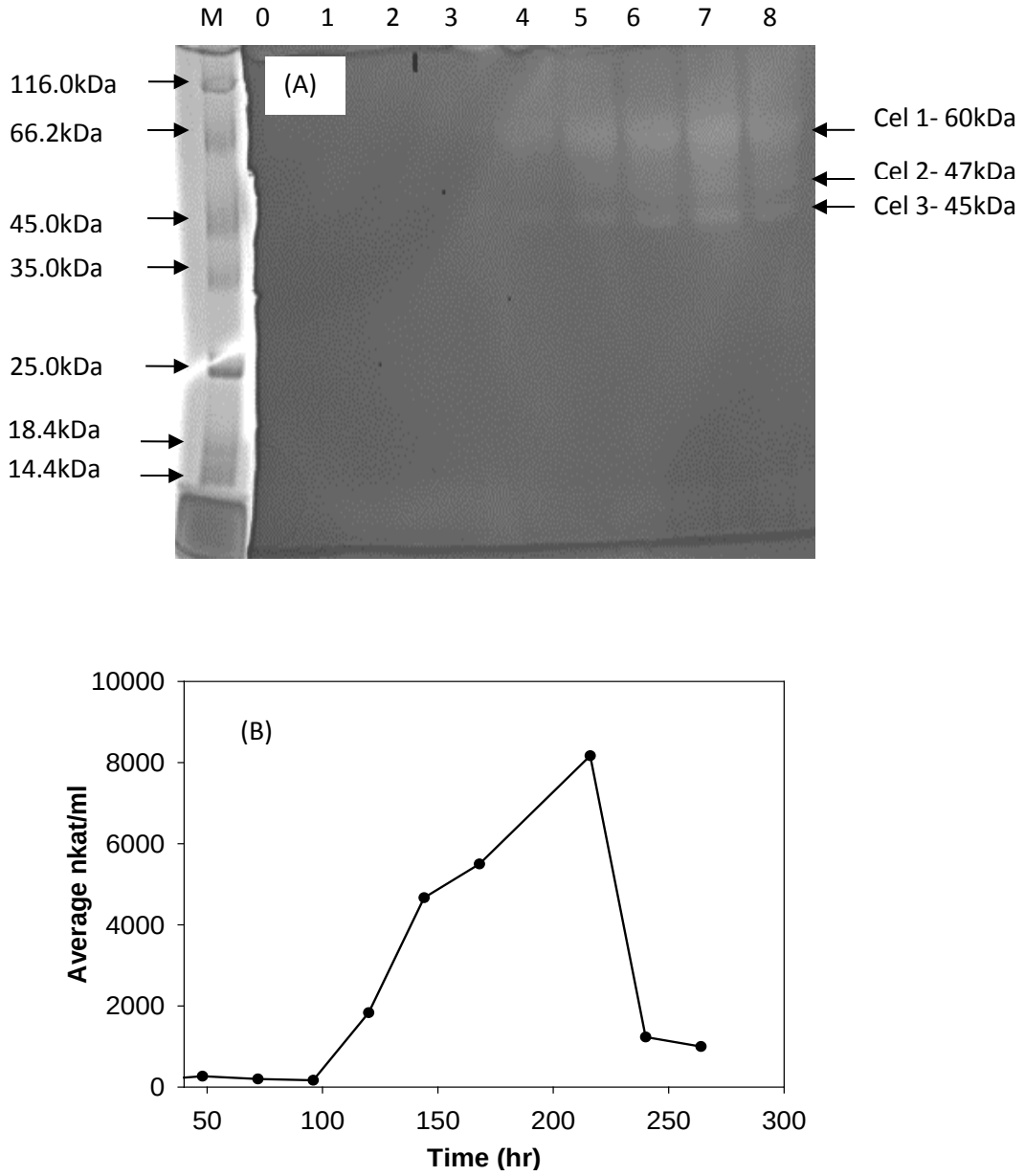

Figure 7: Zymogram (A) and time course (B) of BTU 255 showing cellulase activity over a period of 256 hrs using Avicel™ as a carbon source. Lane M: protein molecular weight marker, lanes 0-8: represent the number of production days (Day 0-8). The average nkat value represents the average of triplicate experiments.

4.3 Effect of temperature on activity of cellulases of the five fungal isolates

Optimum temperature for activity of the cellulases was determined at temperatures ranging between 40 and 70°C. Generally the optimum temperature for the cellulases was between 55 and 65°C (Figure 8). Activity of the cellulases was stable within a broad range (55 to 70°C) from the five fungal isolates. One enzymatic unit was defined as the amount of enzyme that released 1 µmol of glucose per minute. *A. niger* BTU 251, BTU 252, *P. polonicum* BTU 253, BTU 254 and *P. polonicum* BTU 255 were active at 65°C, 60°C and 55°C respectively.

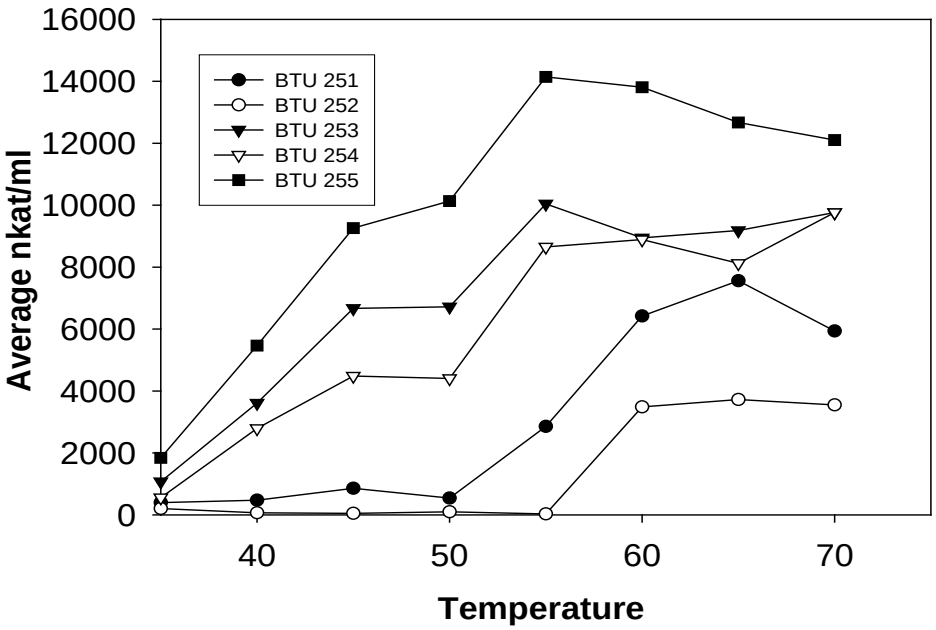

Figure 8: Optimum temperature for activities of cellulases produced by five fungal isolates (BTU 251-BTU 255) was tested at temperatures between 40 and 70°C in submerged fermentation. The average nkat value represents the average of triplicate experiments.

4.4 Effect of temperature on cellulase production by *A. niger* BTU 251, *P. polonicum* BTU 253 and *P. polonicum* BTU 255

A. niger BTU 251, *P. polonicum* BTU 253 and *P. polonicum* BTU 255 were produced on submerged fermentation at different temperatures to determine the effect that temperature has on production of cellulases. In shake flask culture maximum cellulase activity for *A. niger* BTU 251 was 58888nkat/ml achieved at 50°C (Figure 9) after 98 hrs, maximum cellulase activity for *P. polonicum* BTU 253 was 43827 nkat/ml achieved at 50°C (Figure 11) and maximum cellulase activity for *P. polonicum* BTU 255 was 58827 nkat/ml at 55°C (Figure 12) after 124 hrs. Results are expressed in Average nkat/ml.

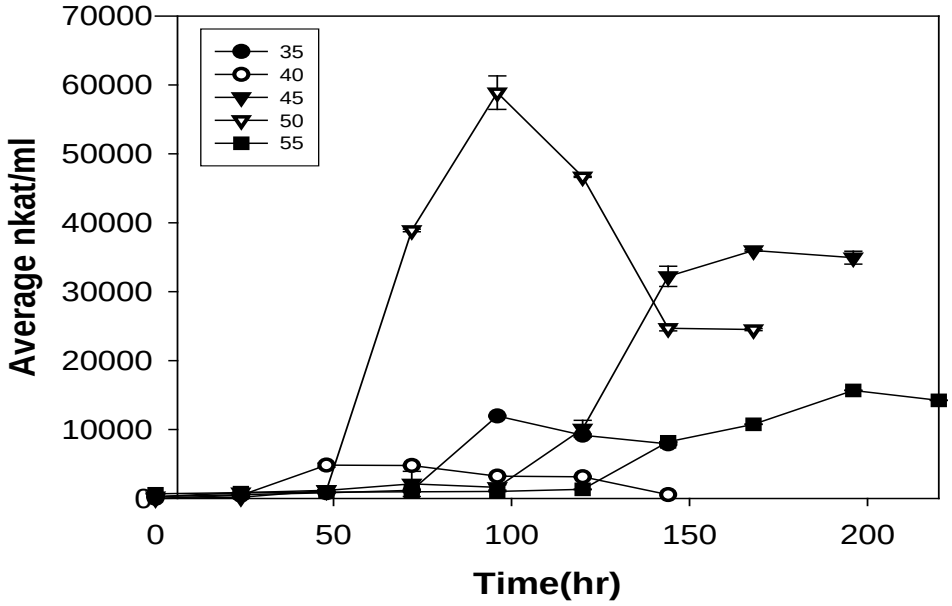

Figure 9: Optimum temperature for production of cellulase by *A. niger* BTU 251 at temperatures between 35 and 55°C in submerged fermentation using Avicel™ as a carbon source.

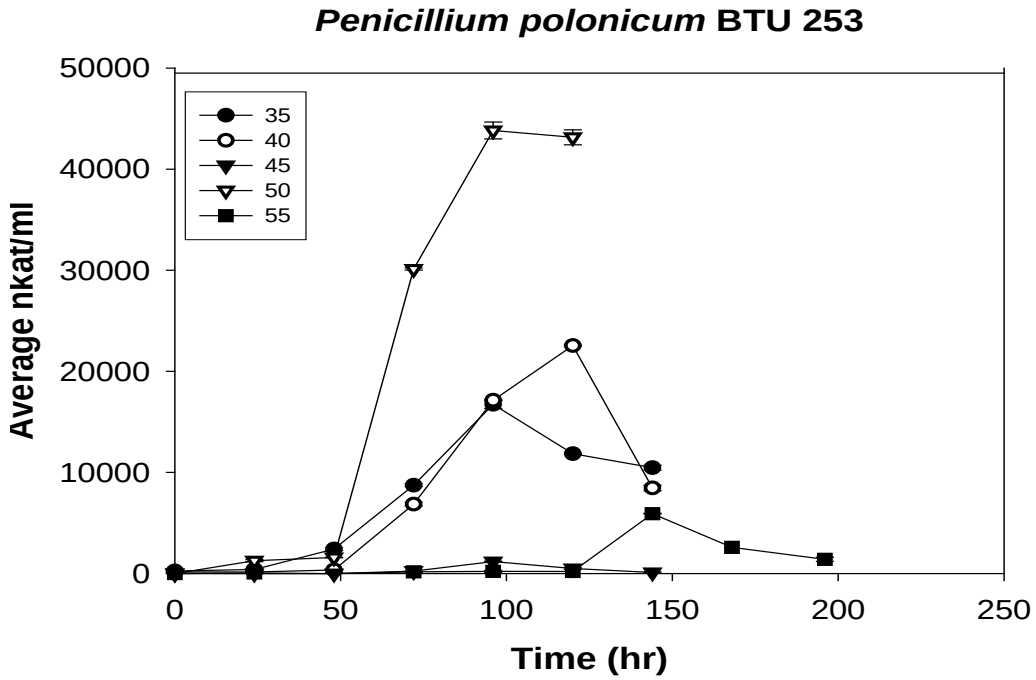

Figure 10: Optimum temperature for production of cellulase by *P. polonicum* BTU 253 at temperatures between 35-55°C in submerged fermentation using Avicel™ as a carbon source.

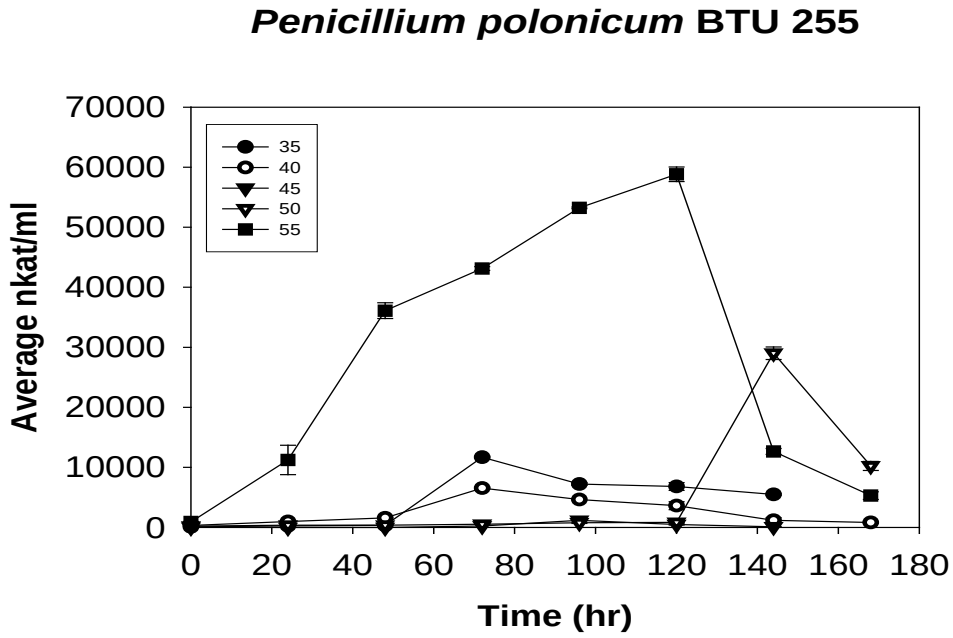

Figure 11: Optimum temperature for production of cellulase by *P. Polonicum* BTU 255 at temperatures between 35 and 55°C in submerged fermentation using Avicel as a carbon source.

4.5 Effect of pH on activity of cellulases from five selected fungal isolates

Optimum pH on activity of cellulase was determined at pH ranging between 4 and 8. The optimum pH for cellulolytic activity for *A. niger* BTU 251, BTU 252, *P. polonicum* BTU 253, BTU 254 and *P. polonicum* BTU 255 was between 4 and 6 (Figure 12). *A. niger* BTU 251 was most active with an average nkat of 1800 at pH 4, BTU 252 was most active at pH 4.5, *P. polonicum* BTU 253 and BTU 254 were active at pH 4 and *P. polonicum* BTU 255 was active at pH 6.The activity of the cellulases was more in the acidic range.

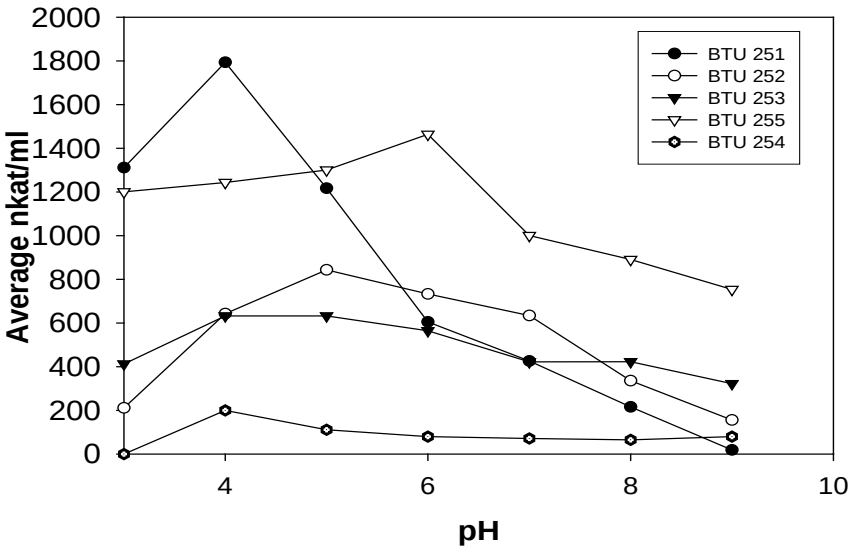

Figure 12: Optimum pH for activities of cellulases produced by five fungal isolates (BTU 251- BTU 255) at pH range between 4 and 8 in submerged fermentation. The average nkat value represents the average of triplicate experiments.

4.6 Effect of pH on cellulase production by *A. niger* BTU 251, *P. polonicum* BTU 253 and *P. polonicum* BTU 255

A. niger BTU 251, *P. polonicum* BTU 253 and *P. polonicum* BTU 255 were produced in submerged fermentation using Avicel™ as a carbon source to determine the effect that pH has on production of cellulases. In shake flask culture maximum cellulase for *A. niger* BTU 251 was 82160nkat/ml achieved at pH 4 (Figure 13) after 198 hrs of incubation at 50°C, maximum cellulase activity for *P. polonicum* BTU 253 was 259259 nkat/ml, achieved at pH 4 (Figure 14) after 216 hrs of incubation and maximum cellulase activity for *P. polonicum* BTU 255 was 81481 nkat/ml achieved at pH 4 (Figure 15) after 216 hrs.

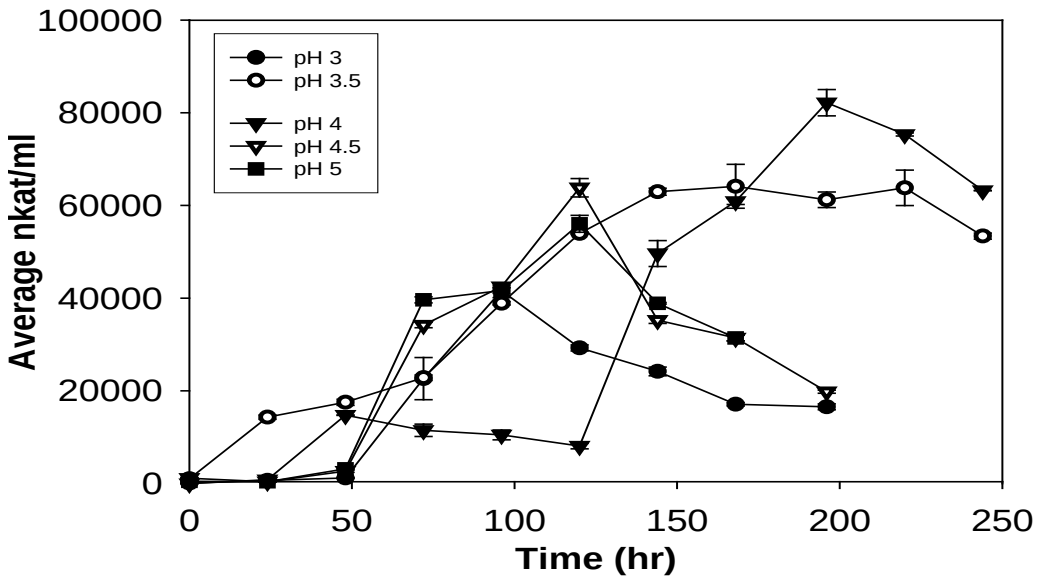

Figure 13: Optimum pH for activity of cellulase by *A. niger* BTU 251 at pH range between 4 and 8 in submerged fermentation using Avicel as a carbon source.

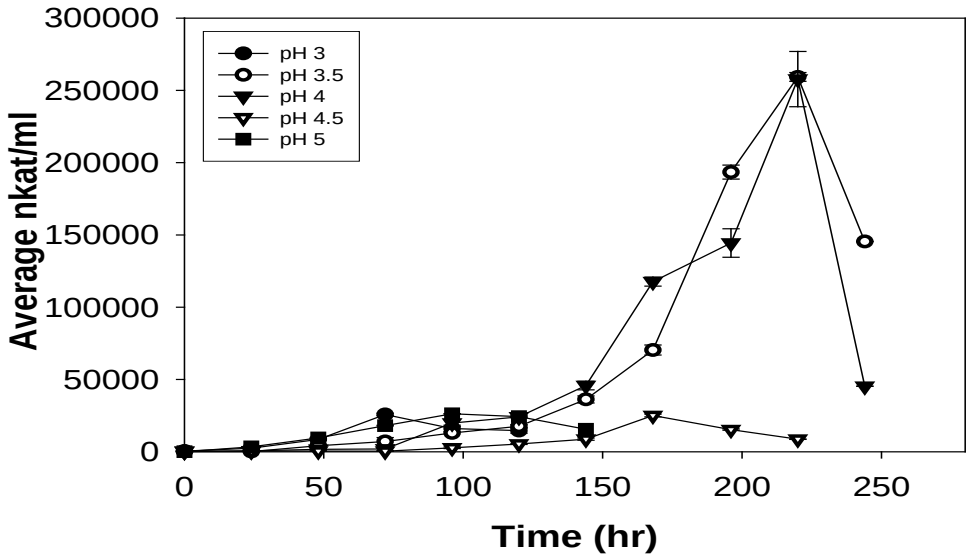

Figure 14: Effect of pH on production of cellulases by *P. polonicum* BTU 253 at pH range between 3 and 5 at 50°C for 244hrs in submerged fermentation.

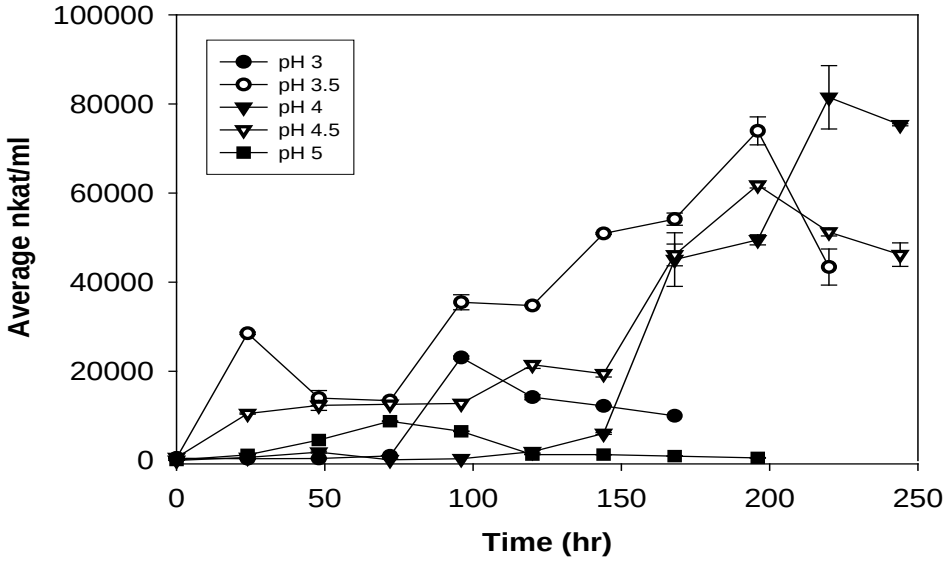

Figure 15: Effect of pH on production of cellulases by *P. Polonicum* BTU 255 at pH 3, 3.5, 4, 4.5 and 5 and 50°C for 244 hrs on submerged fermentation using Avicel as a carbon source.

4.7 Total activity of cellulases for fungal cellulases

Total activity cellulases for *A. niger* BTU 251, *P. polonicum* BTU 253 and *P. polonicum* BTU 255 was measured using filter paper assay, Whatman No.1 filter paper was used as a substrate. The assay carried out over 30 min and DNS was used to stop the reaction. *A. niger* BTU 251 produced (0.11FPU/ml), *P. polonicum* BTU 253 (0.046 FPU/ml) and *P. polonicum* BTU 255 (0.013 FPU/ml) of total cellulase activity.

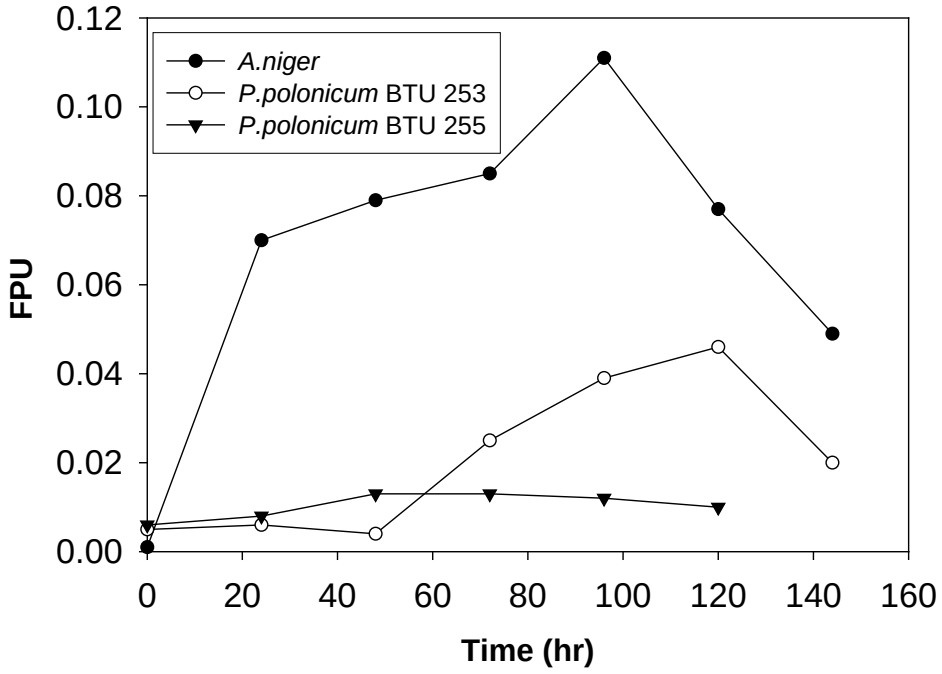

Figure 16: Total cellulase activity for *A. niger* BTU 251, *P. polonicum* BTU 253 and *P. polonicum* BTU 255 using filter paper as a substrate. The unit of FPU is based on the International Unit (IU). IU= 1 micromole min $^{-1}$ of glucose formed during the hydrolysis reaction. The average nkat value represents the average of triplicate experiments.

4.8 Hydrolysis of crystalline cellulose (Avicel) to glucose by *A. niger* BTU 251 and *P. polonicum* BTU 255.

A. niger BTU 251 and *P. polonicum* BTU 22 were used in the experiment to produce high yields glucose. Cellulases were digested on Avicel™ to determine their effect on glucose production. Glucose was produced by *A. niger* BTU 251 with a FPU value of 0.11 and *P. polonicum* BTU 255 with an FPU value of 0.013 Cellulases from *A. niger* BTU 251 and *P. polonicum* BTU 255 were mixed at different ratios (1:9), (1:3), (1:2) and (1:1). *A. niger* BTU 251 and *P. polonicum* BTU 255 produced a similar amount of glucose of 10.7 g/l and 10.3 g/l respectively.

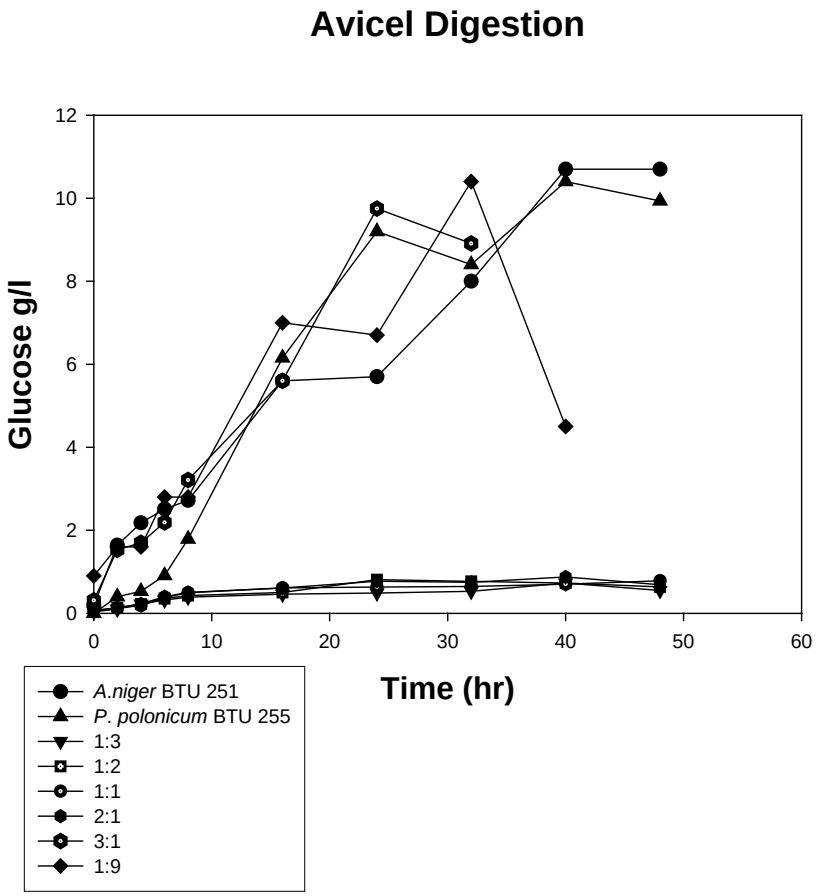

Figure 17: Hydrolysis of crystalline cellulose by Avicel using cellulases from *A. niger* BTU 251 and *P. polonicum* BTU 255 at different concentrations over 48 hrs. The average nkat value represents the average of triplicate experiments.

4.9 Purification of fungal cellulases from crude extract

4.9.1 Affinity precipitation

Cellulases from *P. Polonicum* BTU 255 and *A. niger* BTU 251 were purified by affinity precipitation wherein 20% (w/v) Avicel was used as an affinity matrix. Sodium dodecyl sulfate polyacrylamidegel electrophoresis (SDS-PAGE) was used for analysis of the purified cellulases. SDS-PAGE analysis showed that partial purification of cellulases was achieved as selected proteins bound to Avicel. Seven proteins of different molecular weights from *P. polonicum* BTU 255 and four proteins of different weights from *A. niger* BTU 251 bound to Avicel.

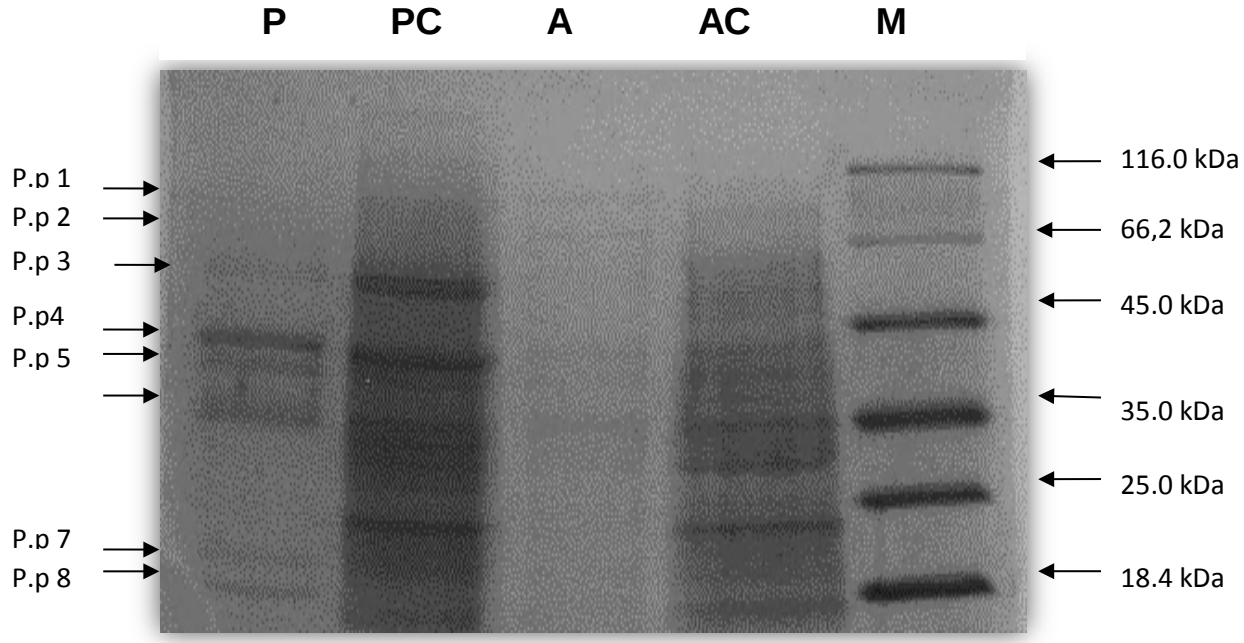

Figure 18: SDS-PAGE analysis of *P. polonicum* BTU 255 and *A. niger* BTU 251 culture supernatant and fractions from cellulose affinity-precipitation. Lane M- molecular weight marker, Lane P- *P. polonicum* BTU 255 purified fraction, Lane PC- *P. polonicum* BTU 255 culture supernatant; Lane A- *A. niger* BTU 251 purified fraction and AC-*A. niger* BTU 251 culture supernatant.

4.9.2 Zymography of partially purified cellulases

Cellulase zymography was performed for the crude and purified extracts from affinity precipitation with 0.1% CMC used as substrate. The clear bands against the dark background are an indication of cellulases in the particular fraction. *P. polonicum* BTU 255 (crude) showed four cellulase active bands estimated as 26, 38, 55 and 78 kDa and *P. polonicum* BTU 255 (purified) also showed four cellulase active bands 26, 38 53 and 78 kDa *A. niger* BTU 251 (crude) showed three cellulase active bands of 55, 38 and 26 kDa and *A. niger* BTU 251 (purified) showed two cellulase active bands that were similar to that of the crude (38 and 25 kDa). This is an indication that the cellulase enzymes were able to bind to the Avicel. *A. niger* BTU 251 (crude) showed three cellulase active bands while *A. niger* BTU 251 (purified) showed only two cellulase active bands that were similar to that of the crude.

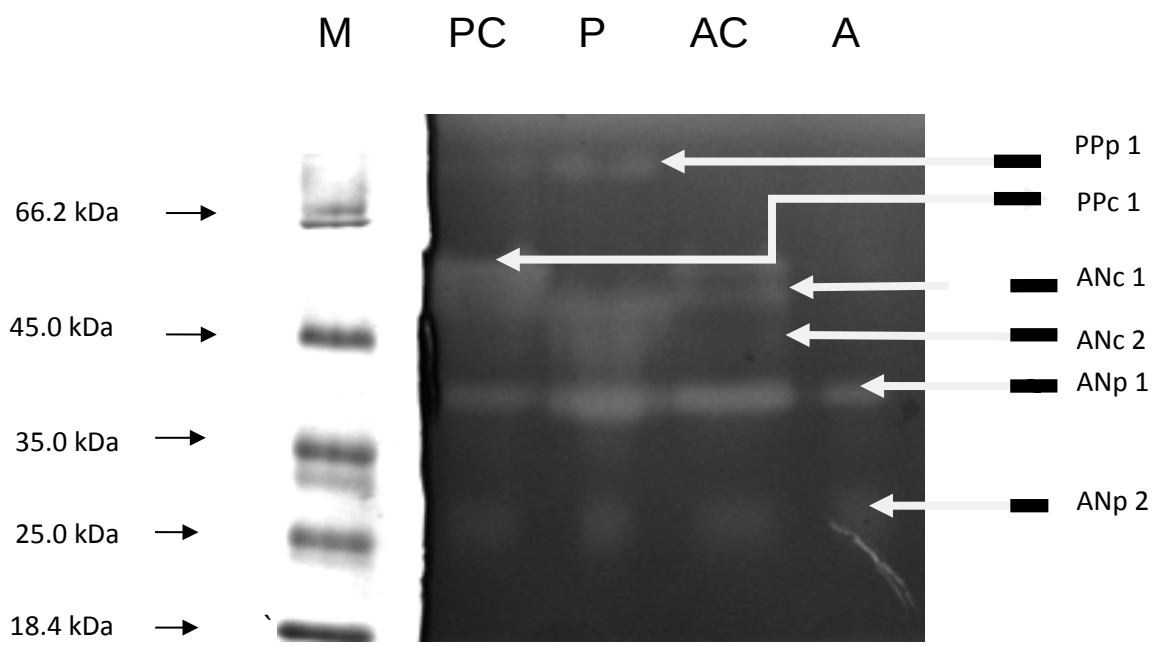

Figure 19: Zymography of *P. polonicum* BTU 255 and *A. niger* BTU 251 crude culture supernatant and fractions eluted during affinity precipitation. Lane M: molecular weight marker, Lane P- *P. polonicum* BTU 255 purified fraction, Lane PC- *P. polonicum* BTU 255culture supernatant; Lane A- *A. niger* BTU 251 purified fraction and AC- *A. niger* BTU 251 culture supernatant. PPp stands for *P. polonicum* (partially purified) and PPc stands for *P. polonicum* (crude). ANc stands for *A. niger* (crude) and ANp *A. niger* (partially purified)

4. 9.3 β- glucosidase Zymography

β- glucosidase activity was detected using polyacrylamide gels with $C_{15}H_{16}O_9$ (Esculin) as a substrate. The dark bands against the transparent background indicate β- glucosidase activity. *P. polonicum* (crude and purified) showed one β- glucosidase band of 44 kDa, *A. niger* (crude) did not show any β-glucosidase activity and *A. niger* (purified) showed one band of 44 kDa.

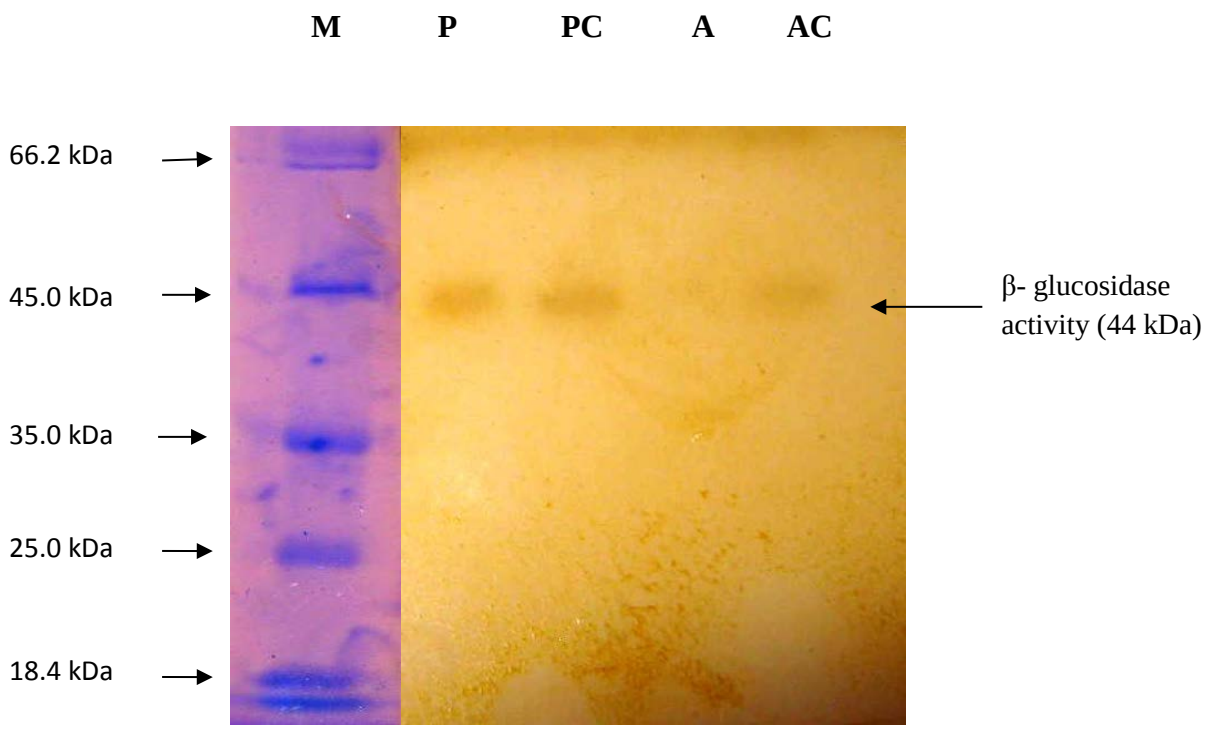

Figure 20: β- glucosidase zymogram for *P. polonicum* BTU 255 and *A. niger* BTU 251 crude culture supernatant and fractions eluted from cellulase affinity precipitation. Lane M represents: molecular weight marker, Lane P: *P. polonicum* BTU 255 purified fraction, Lane PC: *P. polonicum* BTU 255 culture supernatant, Lane A: *A. niger* BTU 251 purified fraction and AC: *A. niger* BTU 251 crude culture supernatant.

4.10 Protein Identification

The protein bands from *P. polonicum* BTU 255 were analysed using MALDI-TOF, the identification of a protein is dependent on the quality of the purified cellulase complex and the affinity purification. The peptide sequences of the gel bands indicated that the protein sequences were most similar to *Chaetomidium pingtungium* for gel bands P.p 4, P.p 5 and P.p 6 with a protein score of 198,122 and 163 respectively and a confidence level of 100 %.

Table 1: Proteins identified from eight gel samples. P. p 4- P. p 6 had the highest protein score. Protein score => 60.

Sample Identification Number as indicated on Gel	Peptide sequences	Protein Identified	Protein Score
P. p 4	FYVQNGKFYVQNGKVVIDANW RRFYVQNGKDDSTYEMFKLNF VTTSQQKTFYGPGMTVDTK SLFQDQNVFEKYGTGYCDSQC PRYGTGYCDSQCPRLYMMKD DSTYEMFKYGGTCDPDGCDF NSFRYGGTCDPDGCDFNSFR FTVVTQFITDDGTSSGTLK GTCDISSGVPADVEANH PDAYVVYSNIK	Sequence 55 from Patent WO03000941.-*Chaetomidium pingtungium.*	198
P. p 5	FYVQNGKFYVQNGKFYVQNG KVVIDANWRRFYVQNGKDDST YEMFKLNFVTTSQQKTFYGPG MTVDTKSLFQDQNVFEKSLFQ DQNVFEKYGTGYCDSQCPRY GTGYCDSQCPRYGGTCDPDG CDFNSFR	Sequence 55 from Patent WO03000941.-*Chaetomidium pingtungium.*	122
P. p 6	FYVQNGKFYVQNGKVVIDANW RRFYVQNGKDDSTYEMFKLNF VTTSQQKTFYGPGMTVDTKSL FQDQNVFEKSLFQDQNVFEKL YMMKDDSTYEMFKYGGTCDP DGCDFNSFRFTVVTQFITDDGT SSGTLKGTCDISSGVPADVEAN HPDAYVVYSNIK	Sequence 55 from Patent WO03000941.-*Chaetomidium pingtungium.*	163

4.11 Matrix assisted laser desorption ionisation time of flight analysis of partially purified cellulase bands from SDS-PAGE gels for *P. polonicum* BTU 255

Peptide sequences of *Chaetomidium pingtungium, Thermoascus aurantiacus, Botryosphaeria rhodina* and *Trichophaea saccata* were aligned with that of *Penicillium polonicum* BTU 255. The sequences were aligned using CLUSTAL W (www.ebi-uk/tools/msa/clustalw2) sequence alignment programme (Figure 21, 22, 23 24 and 25). Peptide sequences of *C. pingtungium* were similar to those of *T. saccata* with a protein score of 52**,** *T. aurantiacus* with a protein score of 50 and *B. rhodina* with a protein score of 49.The sequences of the organisms aligned well and most of the conserved regions were found to be cellobiohydrolases.

```
Chaetomidium      MLASTFSYRMYKTALILAALLGSGQAQQVGTSQAEVHPSMTWQSCTAGGSCTTNNGKVVI
Penicillium       ----------------------------------------FYVQNGKFYVQNGKVVI
                                                           . .*.  .:******

Chaetomidium      DANWRWVHKVGDYTNCYTGNTWDTTICPDDATCASNCALEGANYESTYGVTASGNSLRLN
Penicillium       DANWRRFYVQNGKD-----------------------------------DSTYEMFKLN
                  ***** .:  ..                                       :: : ::**

Chaetomidium      FVTTSQQKNIGSRLYMMKDDSTYEMFKLLNQEFTFDVDVSNLPCGLNGALYFVAMDAGGG
Penicillium       FVTTSQQK-----------------------------------------TFYGPGMTVDT
                  ********                                           :: .  : .

Chaetomidium      MSKYPTNKAGAKYGTGYCDSQCPRDLKFINGQANVEGWQPSSNDANAGTGNHGSCCAEMD
Penicillium       KSLFQDQNVFEKYGTGYCDSQCPR----------------------YGTGYCDSQCPRLY
                   * :  ::.  *************                        ***  .* *..:

Chaetomidium      IWEANSISTAFTPHPCDTPGQVMCTGDACGGTYSSDRYGGTCDPDGCDFNSFRQGNKTFY
Penicillium       MMKDDSTYEMFKYG---------GTCDPDGCDFNSFRYGGTCDPDGCDFNSFR-------
                  : : :*    *.            * *. *  :.* ****************

Chaetomidium      GPGMTVDTKSKFTVVTQFITDDGTSSGTLKEIKRFYVQNGKVIPNSESTWTGVSGNSITT
Penicillium       -----------FTVVTQFITDDGTSS-----------------------------------
                             ***************

Chaetomidium      EYCTAQKSLFQDQNVFEKHGGLEGMGAALAQGMVLVMSLWDDHSANMLWLDSNYPTTASS
Penicillium       ------------------------------------------------------------


Chaetomidium      TTPGVARGTCDISSGVPADVEANHPDAYVVYSNIKVGPIGSTFNSGGSNPGGGTTTTTTT
Penicillium       ---GTLKGTCDISSGVPADVEANHPDAYVVYSNIK-------------------------
                     *. :**************************

Chaetomidium      QPTTTTTTAGNPGGTGVAQHYGQCGGIGWTGPTTCASPYTCQKLNDYYSQCL
Penicillium       -----------------------------------------------------
```

Figure 21: CLUSTAL W sequence alignment of peptides obtained from tryptic digestion of *Penicillium polonicum* BTU 255 purified from affinity precipitation against the full sequence of cellobiohydrolase I from *Chaetomidium pingutigium* that was identified on MASCOT.

Figure 22: CLUSTAL W sequence alignment of peptides obtained from tryptic digestion of *Penicillium polonicum* BTU 251 purified from affinity precipitation against the full sequence of cellobiohydrolase I from *Thermoascus aurantiacus* that was identified on MASCOT.

Figure 23: CLUSTAL W sequence alignment of peptides obtained from tryptic digestion of *Penicillium polonicum* BTU 255 purified from affinity precipitation against the full sequence of a cellobiohydrolase I from *Trichophaea saccata* that was identified on MASCOT.

```
Botryosphaeria      MSLLLLLSSGAIALAQQVYVSADGPAQCTASQTYSATYASPTYAFSNFSFTQTETVRTAT
Penicillium         ------------------------------------------------------------

Botryosphaeria      SVKSAPVTTYAPPYASLSHLVPDLSTTTWGNWDPNATATATDTADPYGQAAWSALWEHAS
Penicillium         ------------------------------------------------------------

Botryosphaeria      LANFTFRGLYSTTVSPTPVPTSELVLPPPEYFTPQDCSYFPDDFMFGVAGSASQIEGAIA
Penicillium         ------------------------------------------------------------

Botryosphaeria      DEGRTPSLMEILISPSTGKPTNYVTNENYYLYKQDIERLAAMGVKYYSFTIPWSRILPFV
Penicillium         ----------------------FYVQNGKFYVQNGKVVIDANWRRFYVQNGKDDSTYEMFK
                                          ** * ::*: : .:   *     * .     *    *

Botryosphaeria      LEGTPLNQQGLDHYDDLINFVLEKGMQPTVTLIHFDTPLQFYGNNLSTAADPPLIGYTNG
Penicillium         LNFVTTSQQ--------KTFYGPGMTVDTKSLFQDQNVFEKYGTGYCDSQCPRYG-----
                    *: .. .**         .*        * :*:: :. :: **.. . :  *

Botryosphaeria      AYQNETFEDAFVNYGKIVMTHFADRVPVWFTFNEPLLYCDNGKSVNTVVKAHARLYHFYH
Penicillium         -----------------------------------TGYCDSQCPRLYMMKDDSTYEMFK-
                                                       ***.  .   ::* .:    *

Botryosphaeria      EEINGTGKVGIKFNDNFGVPRDPQDSSDVDAANHFNEFQLATFANPIFLGKDYPEAFKMT
Penicillium         ----------------YGGTCDP-------DGCDFNSFRYGGTCDP--DGCDFNSFRFTV
                                   :* . **        . .**.*: .  .:*   * *: .    .

Botryosphaeria      VPDYVPLSQEDLEYIGGTSDFLGIDPYTATVVSPPPDGIAVCAANTSDPLFPYCVEQSTL
Penicillium         VTQFITDDGTSSGTLKGTCDIS-------------------------------------
                    *.:::. .  .   : **.*:

Botryosphaeria      TSTGWNIGYRSQTYVYITPKYLRTYLSYLWNTFQHPVMITEFGYPVFGEADKEDLSDQLY
Penicillium         ----------------------------------------SGVPADVEAN---------
                                                            * *.  **:

Botryosphaeria      DLPRSYYYLSFMSEVLKAIWEDNVHVLGAFAWSFADNWEFGDYAQQFGIQVVNRTTQERY
Penicillium         -HPDAYVVYSNIK-----------------------------------------------
                      * :*   * :.

Botryosphaeria      YKKSFFDLVDFVAARTKS
Penicillium         ------------------
```

Figure 24: CLUSTAL W sequence alignment of peptides obtained from tryptic digestion of *Penicillium polonicum* BTU 255 purified from affinity precipitation against the full sequence of cellobiohydrolase I from *Botryosphaeria rhodina* that was identified on MASCOT.

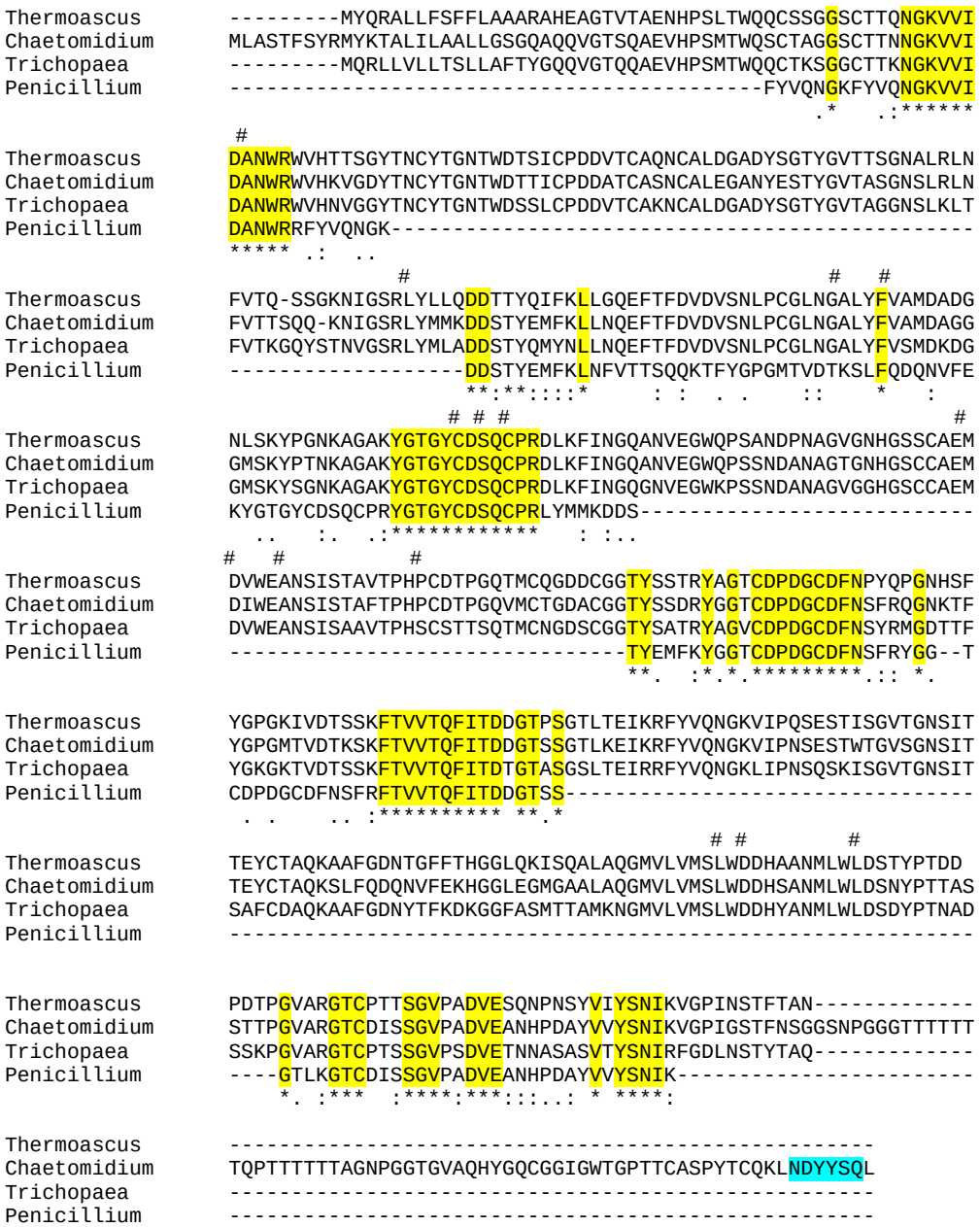

Figure 25: Clustal W sequence alignment of peptides obtained from tryptic digestion of *Penicillium polonicum* purified from affinity precipitation against the full sequence of cellobiohydrolases of *Thermoasus aurantiacus*, *Chaetomidium pingtungium* and *Trichophaea saccata* that was identified on MASCOT. The # represents the active sites on the conserved regions. NDYYSQ represents the carbohydrate binding domain.

CHAPTER 5

DISCUSSION

5.1 Time course for production of cellulases by five fungal isolates and zymography

Time course for production of cellulases by five fungal isolates was established in submerged fermentation for a period of 216 hrs. *P. polonicum* BTU 255 produced cellulases with high activity of 8000 nkat/ml achieved after 168 hrs and BTU 252 was the least cellulase producer of 900 nkat/ml. *P. polonicum* is a common mold occurring on dry-cured meat products and is able to produce verrucosidin, a potent neurotoxin (Andrade *et al*., 2005). Little research has been done on the production of cellulases from this organism. However the current study indicates that this new *P. polonicum* BTU 255 is able to produce highly active cellulases. *A. niger* BTU 251 produced cellulases with an nkat of 7000. *A. niger* is a well-known cellulase producer and in this study it was one of the organisms that produced highly active cellulases. It has also been a very important microbe in the field of biotechnology because many of the enzymes produced by *A. niger* such as citric acid, amylases, lipases, cellulases, xylanases and proteases, are considered GRAS (generally recognized as safe) (Yang *et al*., 2010). BTU 252 and BTU 253 produced cellulases with very low activity of 200 and 2200 nkat/ml respectively and this could be due to the substrate used, either it was not easily digestable by these organisms or due to the fact that it is not good cellulase inducer. Cellulase is an inducible enzyme and it is affected by the nature of substrate used for production therefore meaning that the production of cellulase for the utilization of cellulase is induced only in the presence of specific substrate (or product thereof) but suppressed when easily utilizable sugars such as glucose are available (Lynd *et al*., 2002). It was observed from the results that increase in incubation times led to a decrease in enzyme production. The decrease in enzyme concentration with incubation time may be due to protease activity because the enzymes are broken down by protease to polypeptides and hence a decrease in enzyme concentration.

From zymography *A. niger* BTU 251 showed five activity bands of 25, 28, 42, 50 and 61 kDa, *P. polonicum* BTU 253 showed three activity bands of 45, 47 and 60 kDa, BTU 254 showed five activity bands of 22, 33, 45, 66 and 116 kDa and *P. polonicum* BTU 255 showed three cellulase activity bands of 43, 46 and 60 kDa, the different molecular weights indicate that the five fungal isolates were capable of producing cellulases of different molecular masses. The molecular masses of the cellulases in the study range between 20 and 116 kDa. The reported molecular masses of endoglucanases from various microorganisms vary widely and are found to be in the range 11- 100 kDa however the most typical are 30- 60 kDa (Pol *et al*., 2012) which correspond to the molecular masses of *A. niger* BTU 251 (42, and 50 kDa), *P. polonicum* BTU 253 (45, 47 and 60 kDa), BTU 254 (33 and 45 kDa) and *P. polonicum* BTU 255 (45, 47 and 60 kDa). The production of cellulases with different molecular weights could suggest that they are different endoglucanases, this serves as an advantage because endoglucanases have been very useful in biotechnology for example they can be applied in biopolishing of cotton fabric, they act on the fabric , improving the processing of paper pulp, de-inking paper, enhancing the efficiency of laundry detergents and increasing the utilization efficiency of plant materials in animal feed manufacture (Kuhad *et al*., 2011).

It has been proven that zymography is highly useful for identification of extracted enzymes, rapid estimation of enzyme molecular weights and semiquantitative measurements of specific enzymes (Lacks and Springhorn, 1980). When comparing the zymogram with cellulase time course for production in this study, it is clear that the more intensive cellulase bands correspond with the higher cellulase activity indicating that zymographic analysis can also provide semiquantitative information about the cellulase isoenzymes. For *A. niger* BTU 251 as time increases the higher the intensity of cellulase bands and the more cellulases are being produced after the 4^{th} day, *P. polonicum* BTU 253 indicates that cellulases start to show and increase with time on the zymogram and this corresponds with the time course, BTU 254 shows an increase in the number of cellulase bands with time however time course shows a fluctuation of cellulase activity, *P. polonicum* BTU 255 shows an increase with cellulases after the 3^{rd} day which corresponds to the time course. The results of cellulase zymography support the hypothesis that measuring high cellulase activity can be associated with more intense cellulase bands on the zymographic gel (Khalili *et al*., 2011). The number of cellulase bands can also be associated with the high activity of cellulases, the more number of cellulase bands appear on the gel, the higher the activity (Figure 4, 5, 6 and 7) respectively.

5.2 Optimum temperature for cellulase activity and production

Optimum temperature for cellulases from five fungal isolates was between 55 and 65°C. Due to high temperatures the results showed that the activity of the cellulases decreased when the temperature increased above 65°C, the optimum temperature for activity does not mean that they are stable at these temperatures; however it means that high activity of the cellulases was observed. At temperatures lower than 55°C and higher than 65°C enzyme activity was low this could be due to that at higher temperatures the organisms use more energy for protection of the cell system against thermal inactivation whilst at lower temperatures than the optimum transport of nutrients into the organism are hindered.

For cellulase production, organisms *A. niger* BTU 251, *P. polonicum* BTU 253 and *P. polonicum* BTU 255 were tested at temperatures between 35 and 55°C. *A. niger* BTU 251 produced highest cellulase activity at 50°C after 96 hrs, *P. polonicum* BTU 253 produced highest cellulase activity at 50°C after 96 hrs and *P. polonicum* BTU 255 produced highest cellulase activity at 55°C after 120 hrs with an activity of 58827 nkat/ml. These organisms produced cellulases maximally at temperatures between 50°C and 55°C. (Juwaied *et al*., 2011) reported that *A. niger* produces cellulases best at 40°C however it was not the case for this strain. A number of researchers have reported different temperatures for maximum cellulase production either in flask or in fermentor studies using *Aspergillus* sp and *Trichorderma* sp for cellulase suggesting that production varies depending on variation of microorganism (Gautum *et al*., 2011). The behavior of *A. niger* BTU 251, *P. polonicum* BTU 253 and *P. polonicum* BTU 255 at high temperatures could suggest that they are thermophilic.

5.3 Optimization of pH for cellulase activity and production

The effect of pH on cellulase activity shown in Figure 14 was between pH 4 and 6. *A. niger* BTU 251 was most active at pH 4, Akiba *et al*., (2002) observed that the optimal pH for cellulase activity from *A. niger* was between 6 and 7. However, (McCleary and Glennie-holmes, 1985) reported that the optimal pH activity of *A. niger* cellulase was found to be between 4 and 4.5 (Acharya *et al*., 2008). The results from this study concur with McCleary and Glennie-Holmes (1985). However it is possible that the results differ from Akiba *et al*., because the fungal strains differ but belong within the same genus. *P. polonicum* BTU 253 was most active at pH 4, BTU 252 was most active at pH 4.5, *P. polonicum* BTU 255 was most active at pH 6. For most endoglucanases from *Penicillium* sp, the optimum pH lies in the range of pH 4-5. However, the reported optimum pH range is 4- 6.5 for endoglucanases from other microorganisms. Cellulases are active over a wide range of pH, there are limits to their tolerances since pH has an effect on the binding of the substrate to the enzyme, ionisation of the substrate, catalytic activity of the enzyme and the variation of the protein which usually happens at extreme pH. The pH range also suggests that the cellulases are or could be acidic which serves as an advantage because acidic cellulases have application in the non- ionic surfactant assisted acidic deinking of old news print and old magazines. The use of acidic cellulases during deinking is advantageous as it improves pulp freeness and repulping efficiency. The findings also suggest that the cellulases produced by *A. niger* BTU 251, *P. polonicum* BTU 253, *P. polonicum* BTU 255, BTU 252 and BTU 254 can be used in a combination to be highly effective on pure cellulose since they are active over a broad range. *A. niger* BTU 251, BTU 252 and *P. polonicum* BTU 253 are all active at pH 4 however their activity extends until pH 6 which makes them all a good combination.

Optimum pH for cellulase production of *A. niger* BTU 251, *P. polonicum* BTU 253 and *P. polonicum* BTU 255 was evaluated at pH 3-5. *A. niger* BTU 251 produced cellulases best at pH 4 with an activity of 82160 nkat/ml, *P. polonicum* BTU 253 resulted in the highest activity of 259259 nkat/ml at pH 4 and *P. polonicum* BTU 255 produced cellulases at pH 4 with an activity of 81481 nkat/ml. When comparing the values of pH and temperature, pH plays an important role in the production of the cellulases because it is able to produce cellulases with higher activity. From Figure 13 at pH 3.5 the pH trend is significantly high.

5.4 Total cellulase activity

Total cellulase activity for *A. niger* BTU 251, *P. polonicum* BTU 253 and *P. polonicum* BTU 255 was investigated. *A. niger* BTU 251 had an average total cellulase activity of 0.11 FPU/ml. *P. polonicum* BTU 255 had the lowest total cellulase activity of 0.013 FPU/ml. *A. niger* is a well-known cellulase producer that is capable of producing more endoglucanases and can also produce β- glucosidases, this could be the reason why it had a higher total cellulase activity as compared to *P. polonicum*. *Penicillium* sp are also well known cellulase producers and have shown to produce both endoglucanases and β-glucosidases. The filter paper assay was used to examine organisms that produce synergistic cellulases for complete hydrolysis of cellulose as shown in Figure 17, for complete cellulose degradation organisms that produce cellulases should produce three main types of enzymes: endo-1, 4-β-D-glucanases which have the ability to absorb anywhere along the cellulosic substrate, the exo-1, 4-β-D-glucanase or cellobiohydrolase which hydrolyse the polymer from its reducing or nonreducing ends releasing cellobiose and the enzyme β-glucosidase that catalyses the hydrolysis of the soluble substrates cellobiose and cello-oligosaccharides to glucose (Romero *et al*., 1999). From the results obtained total cellulase activity was not of required standard simply because the method used, Ghose's procedure is suitable for the determination of concentrated preparations and is not adequate for low titre cellulase concentrations found in culture supernatants of wild type microorganisms which often fall below the requirement leading to non-comparative activity reports. It has been shown previously that organisms that lack endoglucanases, exoglucanases and β-glucosidase have little effect on native cellulose, however those like *A. niger* and *P. polonicum* used in the current study will be able to cause significant degradation on native cellulose thus the exo and endocellulases appear to work in a synergistic manner.

5.5 Hydrolysis of crystalline cellulose to glucose

Hydrolysis of crystalline cellulose to glucose was investigated, cellulose hydrolysis with high yields of glucose requires the synergistic action of endo and exo-acting enzymes (Anderson and Alcin, 2008). Organisms *A. niger* BTU 251 and *P. polonicum* BTU 255 were investigated for hydrolysis of cellulose. *P. polonicum* BTU 253 was not used in this experiment because

from previous results (Figure 5) produced a low titer of endoglucanases. *A. niger* BTU 251 (0.11 FPU/ml) produced 10.7 g/L of glucose, *P. polonicum* BTU 255 (0.013 FPU/ml) produced 10.3 g/L of glucose, *A. niger* BTU 251 (1.14 FPU/ml) produced 10.4 g/L of glucose and *A. niger* (1.86 FPU/ml) produced 9.75 g/L of glucose. *A. niger* BTU 251 (0.11 FPU/ml) produced a similar amount of glucose to *P. polonicum* BTU 255 (0.013FPU/ml) this suggests these organisms may be used in combination for successful cellulose hydrolysis. The successful hydrolysis of cellulose from *P. polonicum* BTU 255 and *A. niger* BTU 251 can be an explanation that these cellulases contained carbohydrate binding domain (CBD) because the CBD serves to promote enzymatic hydrolysis of crystalline cellulose by increasing the concentration of fungal cellulases on cellulase surface and hence successful hydrolysis (Rosgaard, 2007). *Aspergillus* sp and *Penicillium* sp are known to be as some of the best cellulase producers (Khokhar *et al*., 2012). *Aspergillus* sp have shown to be best producers of β-glucosidase and they are very important in the production of glucose because β-glucosidases are responsible for the final step in cellulose degradation namely hydrolysis of cellulose derived cellobiose to glucose which could explain why *A. niger* produced a higher glucose concentration than *P. polonicum*. Organisms like *Trichoderma reesei* that is also known to be a good cellulose producer is not a good producer of β-glucosidases (BG1 and BG2) respectively which makes it a poor organism for the production of glucose (Rosgaard, 2007) since β-glucosidases are the most important when it comes to production of glucose.

There was a reduction in the rate of hydrolysis of Avicel after 48 hours and this may be as a result of the presence of enzymes such as glucose oxidase that catalyses the reaction between the glucose and oxygen to produce gluconolactone .

5.6 Purification and Identification of cellulases

Purification of cellulases produced from *A. niger* BTU 251 and *P. polonicum* BTU 255 was investigated using affinity precipitation. Affinity precipitation is a technology for the purification of proteins. From SDS-PAGE analysis there were few bands on the purified as compared to that of crude which is an explanation that the cellulases were able to produce a multi enzyme system though some were not able to bind with Avicel. Eight proteins of different molecular weights bound to Avicel from *P. polonicum* BTU 255 and four proteins of different molecular weights bound to Avicel from *A. niger* BTU 251.

From previous results (Figure 4-7) it is clear that *P. polonicum* BTU 255 and *A. niger* BTU 251 are able to produce endoglucanases because from zymography analysis, *A. niger* BTU 251 and *P. polonicum* BTU 255 produced a variety of endoglucanases and this could explain the fact that the carbohydrate binding domain (CBD) from endoglucanases (EGI) has a higher affinity for cellulose than that of β-glucosidases and hence resulting in good hydrolysis of the cellulose as in the previous results (Figure 17). Another fact could be that the presence of the binding domain leads to strong association of the entire enzyme with cellulose however most cellulolytic microorganisms produce many different cellulases, some of which lack CBDs either for natural reasons, as a result of proteolytic cleavage or due to the introduction of a stop codon after the catalytic domain hence cellulases are unable to bind to Avicel (Dashtban *et al*., 2009).

The protein compositions of the crude enzyme preparation and cellulose-binding proteins (purified extracts) analysed by zymography using CMC and Esculin as a substrate were successfully investigated. Data indicates that the crude and purified extracts produced both endoglucanases and β-glucosidases that bound to Avicel. *P. polonicum* BTU 255 (crude and purified) produced four endoglucanases, *A. niger* BTU 251 (crude) produced four endoglucanases and *A. niger* BTU 251 (purified) produced two endoglucanases the reason for *A. niger* BTU 251 (purified) producing only two endoglucanases could be that during purification the endoglucanases did not bind to Avicel. For β-glucosidase activity the results indicate that only few cellulases with β-glucosidase activity were present. The apparent molecular weight of the β-glucosidase from *P. polonicum* BTU 255 and *A. niger* BTU 251 was estimated to be 44 kDa. The theoretical molecular weight of β-glucosidases is between 75 and 117 kDa respectively (Chauve *et al*., 2010). However, the estimated molecular weight of *A. niger* BTU 251 and *P. polonicum* BTU 255 does not correspond to the theoretical weight of 75 kDa for β-glucosidases. This might suggest that the produced β-glucosidase may be different from the known β-glucosidases and further studies should be done on it since β-glucosidases are very important in biotechnology due to their applications in food detoxification, biomass conversion and flavour enhancement in wines and beverages.

Proteins obtained after SDS-PAGE were identified using Mass spectrometry and database searches (MASCOT and BLAST). MASCOT analysis of the peptide ion masses obtained

from MALDI-TOF/TOF analyses of the tryptic digests identified three gel bands from *P. polonicum* BTU 255 with an acceptable confidence level. The bands (P.p 4, P.p 5 and P.p 6) were positively identified as cellulases similar to *Chaetomidium pingtungium* cellobiohydolases. *C. pingtungium* is a fungus from the Chaetomiaciae family whilst *P. polonicum* is also a fungus that occurs in dry cured meats from the Trichocomaceae family. These two organisms are different because of their different family groups.

The cellulose peptide sequences of *Chaetomidium pingtungium*, *Trichophaea saccata*, *Thermoascus aurantiacus* and *Botryosphaeria rhodina* which were shown using BLAST analyses to be similar to *Penicillium polonicum* were aligned with the peptide sequence from *P. polonicum* BTU 255 using Clustal-W. Clustal-W alignment showed high similarity of the cellulases with a 59.62% overall match while sequence homology from individual alignments was 48.5% with *T. saccata*, 49.74% with *T. auranticus* and 6.88% with *B. rhodina*. Cellobiohydrolases are divided into two groups the Cellobiohydrolase I (CBHI) works from the reducing end and Cellobiohydrolase II (CBHII) works from the non-reducing end to produce tetrasaccharides or disaccharides, cellobiohydrolases are considered as very important in the breakdown of cellulose because in *T. reesei* CBH I makes up 60% and CBHII makes 20% of the total cellulolytic protein, these two enzymes can achieve complete cellulose hydrolysis even without the help of endoglucanase (Lynd *et al*., 2002).

C. pingtungium which showed to be closely similar to *P. polonicum, C. pingtugium* is a fungus within the Chaetomiaceae family and is thermophilic. *T. aurantiacus* is also a thermophilic fungus and can perform at the same level as commercially available enzymatic cocktail for biomass deconstruction, without strain development or genetic modifications (McCleodon *et al*., 2012). Therefore, *T. aurantiacus* provides an excellent platform to develop a thermophilic fungal system for enzyme production for the conversion of biomass to biofuels. *Trichophaea* is a genus of fungi in the family Pyronemataceae. It is a thermophilic fungus. *B. rhodina* is a fungal plant pathogen that causes cankers on many plant species; *B. rhodina* has the ability to produce cellulases. Thermophilic fungi have attracted increased interest due to their ability to secrete enzymes that deconstruct biomass at high temperatures. *P. polonicum* from the current study produced cellulases well at high temperatures.

From sequence alignment *P. polonicum* aligned with three peptides NGKVVIDANWR, CDPDGCDFNSFR and FTTVVTQFITDDGTSS which were found to be cellobiohydrolases

or cellulases from the glycoside hydrolase family-7, the glycoside hydrolase family-7 from *T. reesei* is one of the most studied with the ability to degrade highly crystalline cellulose, this serves as an explanation for the ability of the produced cellulases to be able to degrade highly crystalline cellulose since CBHs are instrumental in the hydrolysis of natural cellulose that contains highly ordered crystalline regions. These act from cellulose chain ends, releasing mainly cellobiose. *A. niger* proteins were not fully analysed on MALDI-TOF because *A. niger* produces well-known cellulases. However, *P. polonicum* is not a well-known cellulase producer and therefore research on its ability to produce cellulases is valuable and had to be conducted. The overall binding efficiency of the cellulases produced is much enhanced by the presence of the CBD and the enhanced binding clearly correlates with better activity towards the insoluble cellulose (Avicel).

CHAPTER 6

CONCLUSION

The current study demonstrated that five fungal isolates BTU 251, BTU 252, BTU 253, BTU 254 and BTU 255 are capable of producing a variety of endoglucanases since on zymography, endoglucanases with different molecular masses were produced. BTU 251, BTU 253 and BTU 255 were identified as *A. niger* and *P. polonicum* respectively. *A. niger* BTU 251 and *P. polonicum* BTU 255 are good hydrolysers of crystalline cellulose because in the current study *P. polonicum* BTU 255 and *A. niger* BTU 251 showed total cellulase activity of 0.11 FPU/ml and 0.013 FPU/ml respectively, when used in combination, glucose which can be used for the production of bioethanol, was produced to levels as high as 10.7 g/L. Cellulases from *A. niger* BTU 251 and *P. polonicum* BTU 255 indicated that they are capable of producing a β-glucosidase with an apparent molecular weight of 44 kDa. Proteins of *P. polonicum* BTU 255 that was partially purified by affinity precipitation and analysed using computational techniques were characterised as cellobiohydrolases. *P. polonicum* BTU 255 exhibits thermophilic behaviour because it was able to produce cellulases at temperatures higher than 50°C and also has a sequence that is related to the thermostable cellobiohydrolases obtained from similarity searches.

CHAPTER 7

RECOMMENDATIONS

To further characterise the cellulase enzymes future work that needs to be done include:

1. Further studies including selection of best substrate that is cost effective on *P. polonicum* BTU 255 should be conducted.
2. Characterising the β-glucosidase enzyme with apparent molecular weight of 44 kDa.
3. Use of cellulases from *P. polonicum* BTU 255 can be investigated on biomass and waste material such as waste paper to determine if they can saccharify these materials.

8. REFERENCES

ABDULLAH, A. 2006. Cellulase enzyme system. *Journal of Biochemistry,* 23**,** 1223- 1228.

ACHARYA, P. B., ACHARYA, D. K. & MODI, H. A. 2008. Optimisation for Cellulase Production by *Aspergillus Niger* using Saw Dust as a Substrate. *African Journal of Biotechnology,* 7**,** 4147-4142.

AHUJA, S. K., FERREIRA, G. M. & MOREIRA, A. R. 2004. Utilization of enzymes for environmental application. *Journal of Biochemistry* 24**,** 125-154.

ALMIN, K., ERIKSSON, K. & PETTERSSON, B. 1975. Extracellular enzyme system utilized by the Fungus *Sporotrichum pulverulentum* (*Chrysosporium lignorum*) for the Breakdown of Cellulose. 2. Activities of the Five Endo-1,4,b-glucanases towards Carboxymethyl-cellulose *European Journal of Biochemistry.,* 51**,** 207.

ANDERSON, N. 2007. *Enzymatic hydrolysis of cellulose.* PhD, Technical University of Denmark.

ANDERSON, W. F. & ALCIN, D. E. 2008. Structural and Chemical Properties of Grass Lignocelluloses Related to Conversion for Biofuels. *Journal of Microbiology,* 35**,** 355-366.

ANDRADE, M. J., RODRIGUEZ, M., SANCHEZ, B., ARANDA, E. & CARDABA, J. J. 2005. DNA typing methods for differentiation of yeasts related to dry cured meats products *International Journal of Food Microbiology,* 1.

BAILEY, M. J. & VIIKARI, L. 1993. Production of xylanases by *Aspergillus fumigates* and *Aspergillus oryzae* on xylan- based media. *World Journal of Microbiology and Biotechnolology,* **9** 80-84.

BHAT, M. K. 2000. Cellulases and related enzymes in biotechnology. *Biotechnology Advances,* 18**,** 355-383.

CHANG, J., HO, F., WU, Y., HOU, Y., HUANG, C., SHIH, M. & LI, W. 2013. Assembling a cellulase cocktail and cellodextrin transporter into a yeast host for CBP ethanol production. *Biotechnology for Biofuels,* 6**,** 1-13.

CHAUVE, C., MATHIS, H., HUC, D., CASANAVE, D., MONOT, F. & FERREIRA, N. L. 2010. Comparative kinetic analysis of two fungal B-glucosidases. *Biotechnology for Biofuels*.

DASHTBAN, M., SCHRAFT, H. & QIN, W. 2009. Fungal bioconversion of lignocellulosic residues, oppotunities and perspectives. *International Journal of Biological Sciences,* 5**,** 500-516.

DELONG FROST, L. 2004. Glucose assay revisited: experimental determnation of the glucose concentration in honey. *The Chemical Educator,* 9**,** 1-3.

DEMIR, H., GOGUS, N., TARI, C., HEERD, D. & LAHORE, M. 2012. Optimization of the process parameters for the utilization of orange peel to produce polygalacturonose by solid-state fermentation from *Aspergillus sojae* mutant strain. *Turkish Journal of Biology,* 36**,** 394-404.

DEMIRBAS, A. 2009. Political, economic and environmental impacts of biofuels. *A review: Applied Energy***,** 108-117.

DESWAL, D., KHASA, Y. P. & KUHAD, R. C. 2011. Optimization of cellulase production by a brown rot fungus *Fomitopsis sp.*RCK 2010 under solid state fermentation. *Bioresource Technology,* 102**,** 6065-6072.

DU PLESSIS, L. 2008. *Co- expression of cellulase genes in Sacchromyces cerevisiae for cellulose degradation.* Unpublished Master of Science Dissertation, University of Stelenbosch.

DUTTA, T., SAHOO, R., SENGUPTA, R., RAY, S. S., BHATTACHARJE, A. & GHOSH, S. 2008. Novel cellulases from extremophilic filamentous fungi *Peniillium citrium* : Production and characterisation *Journal of Industrial Microbial Biotechnology,* 35**,** 275-282.

ELSHAHED, M. E. 2010. Microbiological aspects of biofuel production: Current status and future directions. *Journal of advanced research***,** 103-111.

FREIERMUTH , B., GARRETT, M. & JOKINEN, O. 1994. The use of enzymes in the production of release papers *Paper Technolology,* 25**,** 21-30.

GAKILAVANI, R. & GOPALAKRISHNAM, D. 2009. Bio Tech Textiles - Future Trend. *Journal of Biotechnology***,** 23-44.

GASPARATOS, A., STORMBERG, P. & TAKEUCHI, K. 2011. Biofuels, ecosystem services and human wellbeing: Putting biofuels in the ecosystem services narrative. *Journal of Agriculture, Ecosystems and Environment,* 142**,** 111-128.

GAUTUM, S. P., BUNDELA, P. S. & PANDEY, A. K. 2011. Optimization for the Production of Cellulase Residue bt Two Novel Cellulolytic Fungi. *Biotechnology Research International,* 10**,** 1-8.

GHORAI, S., CHOWDHURY, S., PAL, S., BANIK, S. P., MAKHERJEE, S. & KHOWALA, S. 2010a. Enhanced Activity and Stability of Cellobiase (*B-*

glucosidase:EC 3.2.1.21) Produced in The Presence of 2-Deoxy-D-Glucose From The Fungus *Termitomyces clypeatus*. *Carbohydrate Research,* 345**,** 1015-1022.

GHORAI, S., CHOWDHURY, S., PAL, S., BANIK, S. P., MUKHERJEE, S. & KHOWALA, S. 2010b. Enhanced activity and stability of cellobiase ([beta]-glucosidase: EC 3.2.1.21) produced in the presence of 2-deoxy-d-glucose from the fungus *Termitomyces clypeatus*. *Carbohydrate Research,* 345**,** 1015-1022.

GHOSE, T. K. 1987. Measurement of cellulase activities. *Pure and Applied Chemistry,* 59**,** 257-268.

GILKES, N. R., KWAN, E., KILBURN, D. G., MILLER, R. C., ANTONY, R. & WARREN, J. 1997. Attack of carboxymethylcellulose at opposite ends by two cellobiohydrolases from cellulomonas fimi. *Journal of Biotechnology,* 57**,** 83-90.

GOMEZ, L. D., WHITEHEAD, C., BARAKATE, A., HALPIN, C. & MCQUEEN-MASON, S. J. 2010. Automated saccharification assay for determination of digestibility in plant materials. *Biotechnology for Biofuels,* 3**,** 1-12.

HAMLYN, P. F. 1997. Fungal Biotechnology. *British Mycological Society Newsletter,* 9**,** 147 - 152.

HAMMAD, A. I., ABO- STATE, M. A., SWELIM, M. & GANNAM, R. B. 2010. Enhanced production of cellulases by *Aspergillus* spp. isolated from agriculture wastes by solid state fermentation. *Journal of Agriculture and Environmental Science,* 8**,** 402-410.

HAN, S. J., YOO , Y. J. & KANG, H. S. 1995 Characterization of a bifunctional cellulase and its structural gene. The cell gene of Bacillus sp. D04 has exo- and endoglucanase activity. *Journal of Biological Sciences,* 260**,** 9-12.

ISHTIAQ, A., MUHAMMAD, A., Z. & HAFIZ MUHAMMAD, M. I. 2010. Bioprocessing of proximally analyzed wheat straw for enhanced cellulase production through process optimization with *Trichoderma viride* under SSF. *Journal of Biological and Life sciences,* 6**,** 164.

JAHANGEER, S., KHAN, N., SOHAIL, M., SHAHZAD, S. & AHMAD, A. 2005. Screening and characterization of fungal cellulases isolated from the native environmental source. *Journal of biotechnology,* 3**,** 739 - 748.

JERVIS, E. J., HAYNES, C. A. & KILBURN, D. G. 1997. Surface diffusion of cellulases and their isolated binding domains on cellulose. *Journal of Biochemistry,* 272**,** 24016-24023.

JUWAIED, A. A., AL-AMIERY, A. A. H., ABDUMUNIAN, Z. & ANAAM, U. 2011. Optimization of cellulase production by *Aspergillus niger* and *Tricoderma viride* using sugar cane waste. *Journal of yeast and fungal research,* 2**,** 19-23.

KANMANI, P., KARUPPASAMY, P., POTHIRAJ, C. & ARUL, V. 2009. Studies on lignocellulose biodegradation of coir waste in solid state fermentation using *Phanerocheate chrysosporium* and *Rhizopus stolonifer*. *African Journal of Biotechnology,* 8**,** 6880- 6887.

KHALILI, B., NOURBAKHSH, F., NILI, N., KHADENI, H. & SHARIFNABI, B. 2011. Diversity of soil cellulase isoenzymes is associated with soil cellulase kinetic and thermodynamic parameters. *Soil Biology and Biochemistry,* 43**,** 1639-1648.

KHAN, J. A., SINGH, S. C. & TIWARI, S. T. 2011. Production,partial purification and characterization of cellulases by Aspergillus niger using corncob as a substrate

International Journal of Science and Pharma Educational Research, 1**,** 9-17.

KHOKHAR, I., HAIDER, M. S., MUSHTAQ, S. & MUKHTAR, I. 2012. Isolation and screening of highly cellulolytic filamentous fungi. *Journal of applied science and environmental management,* 16**,** 223-226.

KOSTLEV, M. & WILSON, D. 2012. Synergistic interaction in cellulase hydrolysis. *A review: Biofuels,* 3**,** 61-70.

KUHAD, R. C., GUPTA, R. & SINGH, A. 2011. Microbial Cellulases and their Industrial Applications. *Review Article***,** 1-11.

KWON, K., LEE, J., KANG, H. G. & HAH, Y. C. 1994. Detection of B-glucosidase activity in polyacrylamide gels with esculin as substrate. *Journal for Applied and Environmental Microbiology,* 60**,** 4584-4586.

LACKS, S. A. & SPRINGHORN, S. S. 1980. Renaturation of enzymes after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. *Journal of Biology and Chemistry, 255***,** 7467-7473.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature,* 270**,** 680-685.

LASSMANN, T. & SONNAHAMER, E. L. L. 2002. Quality assessment of multiple alignment programs. *A review***,** 126-130.

LEDERBERG, J. 1992. Cellulases. *Encyclopaedia of Microbiology,* 1.

LEGHLIMI, H., MERAINI, Z., BOUKALFA-LESSER, H., CAPINET, E. & DUCHIRAN, F. 2013. Probuction and characterization of cellulolytic activities produced by *Trichoderma longibrachiatum*. *African Journal of biotechnology,* 12**,** 465-467.

LEWIS, K. J., WEI, J. & SIUZDAK, G. 2006. Matrix-assisted laser desorption/ Ionization mass spectrometry in peptide and protein analysis. *In:* MEYERS, R. A. (ed.) *Encyclopedia of Analytical Chemistry.* John Wiley and Sons Ltd.

LINDEN, J. C. & SHIANG, M. 1991. *Enzymes in biomass conversion,* Colorado, Departments of Agricultural and Chemical Engineering and of Microbiology, Colorado State University, Fort Collins, CO 80523.

LO, C.-M., ZHANG, Q., CALLOW, N. V. & JU, L.-K. 2010. Cellulase production by continuous culture of *Trichoderma reesei* Rut C30 using acid hydrolysate prepared to retain more oligosaccharides for induction. *Bioresource Technology,* 101**,** 717-723.

LORENZ, M. G. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Journal of Analytical Chemistry,* 3**,** 426-428.

LYND, L. R., WEIMER, P. J., ZYL, W. H. V. & PRETORIOUS, I. S. 2002. Microbial cellulose Utilization: Fundamentals and Biotechnology. *Microbiology and Molecular Biology Reviews,* 66**,** 506-577.

MAKI, M., LEUNG, K. T. & QIN, W. 2009. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass *International Journal of Biological Sciences* 5**,** 500-516

MCCLEARY, B. V. & GLENNIE-HOLMES, M. 1985. Enzymic quantification of (1>3)(1>4) B-Glucan in barley and malt. *Journal of the institute of brewing,* 91**,** 285-295.

MCCLEODON, S. D., BATTH, T., PETZOLD, C. J., ADAMS, P. D., SIMMONS, B. A. & SINGER, S. W. 2012. *Thermoascus aurantiacus* is a promising source of enzymes for biomass deconstruction under thermophilic conditions. *Biotechnology for Biofuels,* 5**,** 1-10.

MICKY, V., POMETTO III, A. L. & HANS VAN LEEUWEN, J. 2011. Evaluation of potential fungal species for the *in situ* simultaneous saccharification and fermentation (SSF) of cellulosic material. *Malaysian Journal of Microbiology,* 7**,** 129-138.

MILLER, G. L. 1960. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Journal of Analytical Biochemistry,* 31 426-428.

MOE, O. A., PATTON, W. A., KWON, Y. K. & KEDNEY, M. G. 2004. Ladder sequencing of a peptide using MALDI-TOF mass spectrometry. *Chem Educator,* 9**,** 272-275.

NIRANJE, A. 2006. *Screening diverse cellulase enzymes from the white rot fungus Phlebia gigantea for high quality and large scale applications.* Doctor of Philosophy, Royal Melbourne Institute of Technology.

PAGANO, M. 1999. Application of electrophoresis and related methods, such as western blotting zymography to the study of some proteins and enzyme. *Analytica Chimica Acta,* 383**,** 119-125.

PASON, P., KYU, K. L. & RATANAKHANOCHOI, K. 2006. *Paenibacillus curdlonolytic* strain B-6 xylanolytic-cellulolytic enzyme system that degrades insoluble polysaccharides. *Applied and Environmental Microbiology,* 72**,** 2483-2490.

PERE, J., PAAVALAIENEN, L., SIIKA- AHO, M., CHENG, Z. & VIIKARI, L. 1996. Potential use of enzymes in drainage control of non-wood pulps. *Proceedings of 3rd International Non-wood fibre pulping and paper making conference,* 2**,** 421-430.

POL, D., LAXMANA, R. S. & RAO, M. 2012. Purification and Biochemical Characterisation of Endonuclease from *Penicillium pinophilum* MS 20. *Indian Journal of Biochemistry and Biophysics,* 49**,** 89-194.

RAHKAMO, L., SIIKA-AHO, M., VEHVILÄINEN, M., DOLK, M., VIIKARI, L., NOUSIAINEN, P. & BUCHERT, J. 1996. Modification of hardwood dissolving pulp with purified *Trichoderma reesei*

cellulases. *Journal of Biochemistry,* 3**,** 153–163.

RATANACHOMSRI, U., SPRIPRANG, R., SORNLEK, W. M., BUABAN, V. C., TANAPONGPIPAT, S. & EURWILAICHITR, L. 2002. Thermostable xylanase from *Marismus sp*: Purification and characterization. *Journal of Biochemistry and Molecular Biology,* 39**,** 105-110.

ROMERO, M. D., AGUADO, J., GONZALEZ, L. & LADETO, M. 1999. Cellulase Production by *Neurospora crassa* on Wheat Straw. *Enzyme and Microbial Technology,* 25**,** 244-250.

ROSGAARD, L. 2007. *Enzymatic Hydrolysis of Pretreated Barley and Wheat Straw*. PHD, Technical University of Denmark.

SAHA, B. C. & BOTHAST, R. J. 1999. Lignocellulose Biodegradation and Applications in Biotechnology. *Journal of Industrial Microbial Biotechnology.,* 22**,** 633-636.

SAHA, B. C., ITEN, L. B., COTTA, M. A. & WU, Y. V. 2005. Dilute acid pretreament, enzymatic saccharification and fermentation of wheat straw to ethanol. *Process Biochemistry,* 40**,** 3693- 3700

SARKAR, N., GHOSH, S. K., BANNERJEE, S. & AIKAT, K. 2006. Bioethanol production from agricultural wastes. *A review***,** 19-27.

SCHELLER, H. V. & ULVSKOV, P. 2010. Hemicelluloses. *Annual Rev Plant Biology,* 61**,** 263-289.

SILVEIRA, M. H. L., RAU, R., PINTO DA SILVA BON, E. & ANDREAUS, J. 2012. A simple and fast method for the determination of endo- and exo-cellulase activity in cellulase preparations using filter paper. *Enzyme and microbial technology,* 1**,** 2-15.

SINGHANIA, R., SUKUMARAN, R., PATEL, A. K., LARROCHE, C. & PANDEY, A. 2010. Advancement and coparative profiles in the production technologies using solid state and submerged fermentation for microbial cellulases. *Journal of Microbiology,* 46**,** 541- 549.

STEPHANOPOULUS, G. 2007. Challenges in Engineering Microbes for Biofuels Production. *Journal of Science***,** 801.

TATUSOVA, T. A. & MADDEN, T. L. 1999. BLAST 2 sequences, a new tool for comparing protein and nucleotides sequences. *FEMS Microbiology Letters,* 174**,** 247-250.

TENGERDY, R. P. & SZAKACS, G. 2003. Bioconversion of lignocellulose in solid substrate fermentation. *Biochemical Engineering Journal,* 13**,** 169-179.

TOCA-HERRERA, J. L., OSMA, J. F. & COUTO, S. R. 2007. Potential of solid state fermentation for laccase production. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology,* 1**,** 391-400.

WARD, W. W. & SNIATEK, G. 2009. Protein Purification. *Current Analytical Chemistry,* 5**,** 1-21.

WOOD, T. M. 1971. The cellulase of *Fusarium solani* purification and specificity of the -(1-4)-glucanase and the -D-glucosidase components. *Journal of Biochemistry,Microbiology and Biotechnology,* 121**,** 353-362.

XIMENES, E., KIM, Y., MOSIER, N., DIEN, B. & LADISCH, M. 2011. Deactivation of cellulases by phenols. *Enzyme and Microbial Technology,* 48**,** 54-60.

YANG, B., DAI, Z. & WYMAN, C. E. 2010. Microbial enzyme systems for biomass conversion emerging paradigms. *A review:Biofuels,* 1**,** 323-341.

ZHANG, Y. P., HIMELL, M. E. & MIELENZ, J. R. 2006. Outlook for cellulase improvement:Screening and selection strategies. *Biotechnology Advances,* 24**,** 452-481.