A PROTEOMIC APPROACH FOR DISCOVERY OF MICROBIAL CELLULOLYTIC ENZYMES

by

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Research Dissertation

Submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

in

MICROBIOLOGY

in the

FACULTY OF SCIENCE AND AGRICULTURE

(School of Molecular and Life Sciences)

at the

UNIVERSITY OF LIMPOPO

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2020

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.

Matlala MS	14 December 2020
Surname, initials (Ms)	**Date**

DEDICATION

I dedicate this work to:

The memory of my supervisor, Prof I. Ncube, who always believed in my ability to be successful in the scientific field. You are gone but your belief in me has made this journey possible.

My grandmother, my parents (Tlou and Khomotjo Matlala) who stood by me throughout my academic career. My caring siblings (Phuti, Noko, Motlatjo and Tlou) for always being there and my beautiful daughter Oratilwe, your presence in this world has always motivated me to be the best.

ACKNOWLEDGEMENTS

I would like to thank the almighty for his blessings, strength, wisdom and courage He has given me throughout this study.

- My thanks and appreciation to the late Prof I. Ncube for persevering with me as my supervisor throughout the time it took me to complete this research and write the dissertation.
- I am grateful as well to Prof KLM Moganedi for coordinating and overseeing the administrative concerns that made it possible for me to submit this dissertation.
- I am grateful to Dr Thembekile Ncube for her generous time, knowledge and expertise to better my work.
- My gratitude and appreciation to MRs M.R Lekganyane for support, research assistance and technical guidance.
- My thanks must go also to Biotechnology Unit staff and postgraduate students for their assistance, advise and support. Thank you so much for making the lab a better place to be in.
- My gratitude and deep appreciation to Ms RR Lediga, your friendship, hospitality, knowledge and wisdom supported, enlightened and entertained me over the many years of our friendship.
- I am grateful too for the support and advise from all the BMBT (Biochemistry, Microbiology and Biotechnology) staff members and postgraduate students.
- I must acknowledge the many friends as well who assisted and supported my research.
- My gratitude to The National Research Foundation (NRF) for financial support.
- Lastly, my thanks go to my entire family for their love, support, patience and prayers. They have consistently helped me keep perspective on what is important in life.

ABSTRACT

Bioethanol production from lignocellulosic biomass is seen as an alternative source of energy. However, large-scale production of bioethanol from lignocellulosic biomass is still not feasible due to the high cost of cellulase and lack of cellulases with a high specific activity that can act on crystalline cellulose. The study aimed at screening for microbial cellulolytic enzymes using a proteomic approach. The objectives were to screen for microbial cellulases with a high specific activity and separate the cellulolytic enzymes using a combination of zymography and two-dimensional (2-D) gel electrophoresis followed by tryptic digestion, matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) and bioinformatics analysis. Fungal and bacterial isolates were cultured in M9 minimal and Mandel media for a period of 168 hours at 60°C and 30°C with cellobiose and Avicel as carbon sources. Microbial cells were separated from the supernatants through centrifugation and the crude enzymes from the cultures were used for the determination of cellulase activity, zymography, SDS-PAGE and two-dimensional gel electrophoresis. Five isolates, with lytic action on carbon sources studied were a bacterial strain, (BARK) and fungal strains (VCFF1, VCFF14, VCFF17 and VCFF18). Peak cellulase production by the isolates was found to be 3.8U/ml, 2.09U/ml, 3.38U/ml, 3.18U/ml and 1.95U/ml, respectively. Beta-glucosidase zymography resulted in a dark brown band and clear zones against a dark background for endoglucanase. Affinity precipitation of the VCFF17 isolate's crude enzyme resulted in seven glycoside hydrolases with a carbohydrate binding module (CBM). The presence of the CBM in the glycoside hydrolases produced by the VCFF17 confer the isolate's potential to be used in the hydrolysis of plant biomass for bioethanol production. Two-dimensional gel protein maps resulted in the separation and quantitative expression of different proteins by the microbial isolates. MALDI-TOF analysis and database search showed that the expressed proteins in this study closely relate to different glycoside hydrolases produced by other microbial species *(Hypocrea jecorina*, *Emericella nidulans*, *Trichoderma pseudokoningii* and *Trichoderma koningii*). BARK, VCFF1, VCFF14, VCFF17 and VCFF18 showed great potential as cellulolytic enzyme producers for bioethanol production. The BARK isolate exhibited the highest beta-glucosidase activity. The isolates studied may benefit the industry in reducing the costs associated with bioethanol production in consolidated bioprocessing system.

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LIST OF ABBREVIATIONS

NH_4CL	Ammonium chloride
NH_4HCO_3	Ammonium Carbonate
$(NH_4)_2SO_4$	Ammonium sulfate
$CaCL_2$	Calcium chloride
CO_2	Carbon dioxide
CMC	Carboxymethylcellulose
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicyclic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
$FeSO_4$	Ferric sulphate
G	Gram
HCCA	α-Cyano-4-hydroxycinnamic acid
pH	Potential of Hydrogen
IPG	Immobilized pH gradient
kDa	Kilodaltons
µl	Microliter
ml	Milliliter
M	Molarity
Umol	Micromoles
mg/ml	Milligram per milliliter
$MgSO_4$	Magnesium sulphate

MALDI-TOF MS	Matrix assisted laser desorption ionization time of flight mass spectrometry
MS	Mass spectrometry
Ng	Nanogram
L^{-1}	per liter
K_2HPO_4	Dipotassium phosphate
KH_2PO_4	Monopotassium phosphate
KCL	Potassium chloride
K_3FeCN_6	Potassium ferricyanide
Rpm	Revolutions per minute
NaCl	Sodium chloride
$Na_2HPO_4.7H_2O$	Sodium monohydrogen phosphate heptahydrate
Na_2HCO_3	Sodium bicarbonate
$Na_2S_2O_3$	Sodium thiosulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Xg	Times gravity
TCA	Trichloroacetic acid
Tris-CL	Hydroxymethyl aminomethane hydrochloride
w/v	Weight to volume

CHAPTER 1

INTRODUCTION

1.1. Background information

Currently, the world is mostly dependent on fossil and petroleum-based fuels for transportation, domestic and industrial applications. However, there are challenges relating to the use of these conventional fuels which include unstable fuel prices, non-renewable nature of fossil and petroleum-based fuels, and their contribution to greenhouse gas (GHG) emissions as well as political crisis which has led to rethinking of dependence on fossil fuels (Martins *et al*., 2018; Jiang *et al*., 2019; Wood and Roelich, 2019). Such crises destabilise the energy sector of both developed and developing nations (Ogbonna *et al*., 2001). The challenges associated with petroleum-based fuels have raised interest to use biomass as a renewable energy source (Jung *et al*., 2015). Food crops (plant starches, oils, and sugarcane) are currently being used as energy sources to produce first-generation biofuels (Ullah *et al*., 2015; Kumari and Singh, 2018; Raud *et al*., 2019). Though the first-generation biofuels show potential to reduce GHG emissions and balance the energy sector, the first-generation biofuels have several pitfalls with ongoing concerns for many, but not all, being that they contribute to the rising of food prices by competing with food crops. First-generation biofuels do not adhere to their claimed environmental benefits due to unsustainable production of biomass feedstock, acceleration of deforestation, negative impact on biodiversity and competition for scarce water resources in some regions (Zabed *et al*., 2016).

To address concerns and drawbacks related to the production of first-generation biofuels, research efforts have been focused on the production of biofuels from cheap, abundant and renewable agricultural, forest residues and non-food lignocellulosic feedstocks (Zhao and Xia, 2010; Lee *et al*., 2013; Bhatia *et al*., 2017). Non-food lignocellulosic biomass are made up of cellulose, hemicellulose, and lignin (Hosseini Koupaie *et al*., 2019). Lignocellulosic contents vary based on the biomass's physical properties and source. Cellulose and hemicellulose are strongly joined to lignin by covalent and hydrogen bonds. The greatly crossed nature of cellulose makes the lignocellulosic structure highly vigorous and resistant to

depolymerisation by carbohydrate-active enzymes from microorganisms to simple fermentable sugars (Limayem and Ricke, 2012). Bioethanol production from lignocellulosic biomass can be achieved through several bioprocesses such as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF) and consolidated bioprocessing (CBP). The steps involved in bioethanol production include pre-treatment, enzymatic saccharification, fermentation and product recovery (Den Haan *et al*., 2015). Despite the processes being known and documented, industrial production of bioethanol from lignocellulosic biomass is still not economically feasible because of a lack of microbial cellulases with high specific activity to act on crystalline cellulose and the high cost of cellulase production. In addition, the genes for commercialised hyper-producing enzymes are protected by intellectual property of multinational companies, hence, not available for the development of new strains. This problem leds to a need to screen for potentially novel microbial cellulolytic enzymes that can then be expressed in suitable vectors for bioethanol production through the consolidated bioprocessing system.

1.2. HYPOTHESIS

Proteomics can be applied in the discovery of cellulases.

1.3. MOTIVATION OF STUDY

Proteomic approaches were used in the current study to characterise and identify cellulase proteins encoded by active genes. This discovery-based approach has less inclination towards existing knowledge and may be a valuable source of new information for the discovery of new enzymes for process development. More importantly, proteomic techniques may identify novel genes and protein targets, besides giving information about known proteins and pathways. From a biotechnological dimension, data generated in this study may be used to identify cellulose targets for heterologous expression.

A bottom-up proteomic approach was used in this study whereby the isolated cellulases were separated by gel electrophoresis followed by proteolytic digested of individually separated proteins. The digested peptides were then analysed by mass

spectrometry for protein identification and peptide sequencing with a database search.

1.4. PURPOSE OF STUDY

1.4.1. Aim and Objectives

The study aimed at screening for potentially novel microbial cellulases using a proteomics approach.

To achieve the main aim of the study the objectives were as follows:

(i) To screen for microbial cellulases with a high specific activity that can act on crystalline cellulose.

(ii) To separate the cellulases using a combination of zymography and two-dimensional gel electrophoresis.

(iii) To obtain peptide sequence data of the enzymes using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight MS (MALDI-TOF-MS) and associated proteomics software and databases.

(iv) To mine related cellulases using National Center for Biotechnology Information (NCBI) database search tools such as BLAST.

(v) To carry out sequence alignments and establish cellulase protein sequence relationships using Clustal W.

1.4.2. Significance of the study

For industrial production of second-generation biofuels to be feasible, cellulases with high specific activity are needed. The current study, therefore, intended to develop a quick method for use in screening for cellulases and other enzymes. The success of this study may contribute towards reducing the costs associated with bioethanol production using cellulases with a high specific activity in consolidated bioprocessing systems.

CHAPTER 2

LITERATURE REVIEW

2.1. Biofuels

A biomass-based fuel (biofuel) is a fuel that is produced through anaerobic digestion and biological degradation of agricultural and animal biomass, rather than a fuel produced from the remaining of prehistoric plants and animal matter (fossil fuels), such as coal and petroleum (Naik *et al*., 2010). Biofuels can be produced directly from plants, or indirectly from agricultural, domestic, industrial and commercial wastes. Renewable biofuels are made from the conversion of atmospheric carbon dioxide into organic carbon compounds, and through the conversion of biomass. The biomass for biofuel production can be converted to convenient energy-containing substances through thermal, chemical, and biochemical conversion processes. The resulting fuel from conversion of biomass may be in either solid, liquid, or gaseous form (Samuel *et al*., 2013). The biomass can also be used directly for biofuels without modifications. Bioethanol is an alcohol produced through fermentative activity of some microorganisms on carbohydrate molecules found in starch, corn, sugarcane, or sweet (Mohr and Raman, 2013). Several species of trees and grasses are also under development as feedstocks for ethanol production (Khare *et al*., 2015).

Biofuels are classified into two groups, primary and secondary biofuels. The classification system is based on the type of biomass and its processing technology. Primary biofuels include gas, pellets fuelwood, and are utilised for cooking, heating and to produce electricity. Secondary biofuels include biogas, biodiesel, ethanol and are used in transport and industrial sectors (Saladini *et al*., 2016).

2.2. Classification of biofuels

Biofuels are classified according to the source of biomass and production technology (Figure 2.1.)

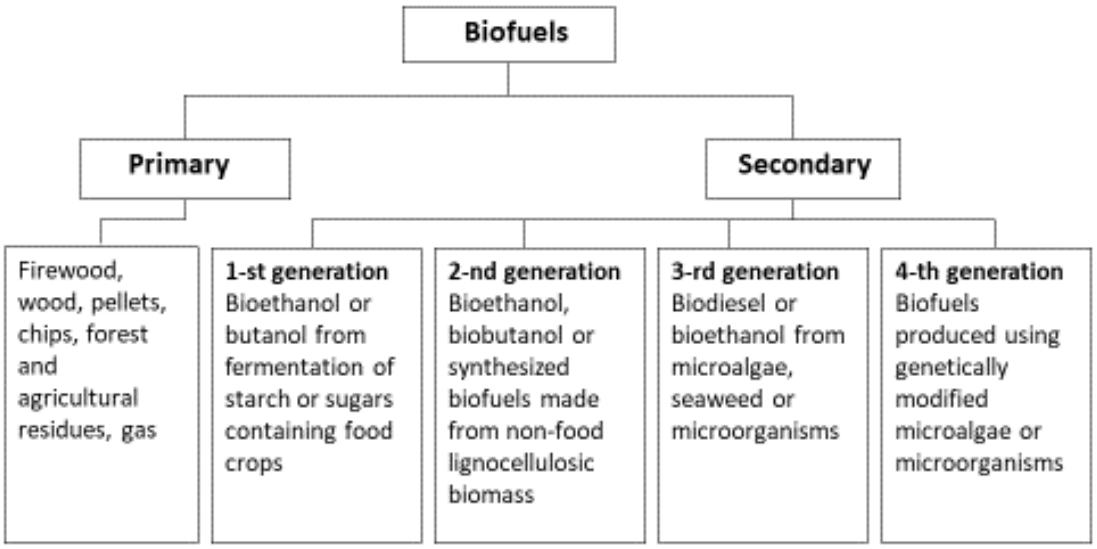

Figure 2.1: Classification of biofuels (Raud *et al*., 2019).

2.2.1. First-generation biofuels

The rapid consumption of natural energy resources in the industry and transport sectors accelerated energy dependency growth, emissions of greenhouse gases to the atmosphere and supply insecurities (Siqueira *et al*., 2008). One of the ways to deal with these problems was to move from natural energy resources (fossil energy) to biofuels.

A first-generation biofuel is defined by its ability to be mixed with conventional (fossil) fuels, burned, and distributed in combustion engines and throughout the existing infrastructure. First-generation biofuels are also characterised by their ability to run in Flexible Fuel or natural gas vehicles (Patel *et al*., 2016). The first-generation biofuels are commercially produced, with an annual production of 50 billion litres. Biogas produced from manure and other biomass materials through the anaerobic treatment is also commercial today. Although biogas is produced at an industrial scale, the volumes used for transportation are relatively small today (Winquist *et al*., 2019).

First-generation biofuels are produced from different kinds of raw materials with corn, wheat, sugarcane, soybean, rapeseed, and sunflower being the preferred feedstocks. The preferences of these feedstocks differ by regions depending on climatic factors (Balat, 2011). Brazil produces bioethanol from sugarcane while Europe and United States mainly use wheat, barley and corn starch. The use of sugarcane as a feedstock for biofuel has increased in the last decade with Brazil having the highest sugarcane yield constituting about 31% of total global bioethanol production. Most of the EU-25 countries produce biofuels using sugar beet crops as feedstocks (Rajaeifar *et al*., 2019).

Regardless of the available technology, there seems to be a lot of doubts when it comes to the production of first-generation biofuels. The doubts revolve around carbon balances and environmental impacts relating to the production and use of the first-generation biofuels (Pengilly *et al*., 2015). The major drawbacks of first-generation biofuels include the food-versus-fuel debate which addresses issues such as the competition of biofuels with food crops for arable land and resources (water and fertilizers), unstable biomass supply and destruction of biodiversity. It is perceived that an increase in the production of these biofuels is one of the major reasons for ever-increasing food prices (Ajanovic, 2011).

2.2.2. Second-generation biofuels

The problems associated with the utilisation of the first-generation biofuels created a need to search for an alternative feedstock that can be used for biofuels production. Therefore, the lignocellulosic feedstock is an alternative source of energy production due to its potential of providing novel biofuels ‘second-generation biofuels’ (Bhalla *et al*., 2013). The high levels of cellulose and hemicellulose found in lignocellulosic biomass give it the advantage of being used to produce biofuels (bioethanol) (Cheng *et al*., 2008). The production of bioethanol from lignocellulosic biomass involves three essential and interdependent steps namely, pre-treatment, saccharification which is achieved by lignocellulolytic enzymes and fermentation of simple sugars generated from saccharification (Kumar *et al*., 2008). The effectiveness of the pre-treatment and saccharification steps determines the viability and yield of the fuel and other platform chemicals (Taherzadeh and Karimi, 2007). Lignocellulosic feedstock refers largely to plant biomass. Plant biomass is one of the biological resources on

earth that is highly abundant and underutilised. Due to its high abundancy and less usage biomass from plants has potential to be used as a source of fuels and other value-added products (Chen *et al*., 2012). However, production of second-generation biofuels is non-commercial anywhere in the world and this is due to problems associated with the cost of raw material, high projected capital, and processing costs, although pilot and demonstration facilities have been developed (Erdei *et al*., 2013). There is hope that if production is implemented these second-generation biofuels hold potential to significantly reduce CO_2 emissions, not influence rising food prices and may also support better engine performance. At a commercial scale, the estimated cost of second-generation biofuels seems to be more comparable with average petrol, diesel, and holds potential to be the most cost-effective route to renewable, low carbon energy for road transport (Naik *et al*.,2010).

2.2.3. Third-generation biofuels

Another alternative source of energy is aquatic biomass such as algae and seaweed. Algal biomass has shown potential as a promising feedstock to produce biofuels due to its high lipid and carbohydrate contents (Rodionova *et al*., 2017). Algae are easily grown, require less land and have capabilities to uptake high atmospheric carbon dioxide thus, reducing its concentration in the atmosphere (Dragone *et al*., 2010; Azad *et al*., 2014). Although algal biomass has potential as a source to produce different biofuels (biohydrogen, biodiesel, bioethanol), industrial production of algal and fourth generation biofuels is still in its infancy due to expensive and underdeveloped production technologies (Scott *et al*., 2010; Raud *et al*., 2019).

2.2.4. Fourth-generation biofuels

Fourth-generation biofuels are produced through geo-synthesis, advanced biochemistry, low-temperature electrochemical process, and petroleum-hydro-processing. These techniques harvest atmospheric carbon to produce biofuels (Azad *et al*.,2015). Another way to produce fourth-generation biofuels is through the genetic modification of algae and microorganisms that have the capability to uptake atmospheric carbon dioxide. The above-mentioned methods help decrease the concentration of carbon dioxide in the environment (Lü *et al*. 2011; Cheng *et al*., 2011).

The burning of harvested organic matter has been used by mankind for years and it remains the primary source of energy for people in developing countries. Though it is the primary source of energy, the burning of organic matter is not user-friendly due to the release of harmful substances with serious health implications (Raud *et al*., 2019). Nevertheless, some technologies that can convert organic matter into more efficient fuels. One of the most abundant natural resource that can be converted into different biofuels is lignocellulosic biomass which include residues from agricultural and timber industry, food crops (corn, wheat, sugar), non-food crops (grasses and trees) and dedicated energy crops such as switchgrass (Thompson, 2010; Brosse *et al*., 2012). Due to its low cost and sustainable supply, lignocellulosic biomass has become the focus of research as a promising source of energy (Agbor *et al*., 2011)

2.3. Lignocellulose as a biofuel feedstock

Lignocellulose is a renewable and abundant plant biomass, such as waste from pulp and paper industry, coniferous and deciduous woods, and energy crops. The major components that make up the lignocellulosic material are cellulose 50 %, hemicellulose 25 %, and lignin 20-25 %. The compositions of the major components vary depending on the plant species, age, soil and climate conditions (Steffien *et al*., 2014). The remaining fraction of the plant biomass includes minor components such as oils, proteins, and ash (Peng *et al*., 2010). Cellulosic and hemicellulosic fractions make up the largest part of the lignocellulose and are the major sources of single fermentable sugar molecules used to produce biofuels (Kobayashi *et al*., 2016). Cellulose is linked to hemicelluloses, which in turn binds to lignin, and together these components contribute to the structural rigidity of plant tissues (Bhalla *et al*., 2013; Goncalves *et al*., 2013).

2.3.1. Cellulose

Cellulose is an unbranched glucose polymer made of D-glucose units linked by a 1,4-β-D-glucosidic bond as illustrated in Figure 2.2. Cellulose is the most abundant organic compound on earth, and the key substance in all plants' cell walls (Heredia *et al*., 1995). Plant cellulose is not easily degradable due to its insoluble nature and its highly ordered crystalline regions in which the cellulose chains are closely packed

therefore inaccessible to microbial and enzymatic attacks (Kan *et al*., 2017; Volynets *et al*., 2017). The highly crossed cellulose structure supports and contributes to the structural rigidity of the plant cell wall (Hong *et al*., 2009). Cotton is one of the purest natural forms of cellulose, and filter paper is used in laboratories as a source of partially pure cellulose. Cellulose is also a potential food source for herbivorous organisms, but human beings cannot digest it since they lack enzymes for its hydrolysis (Dorée, 1947). Due to its nature of being renewable and easily accessible, cellulose is considered a potentially valuable carbon source that can be utilised to produce value-added chemicals (Wang *et al*., 2010).

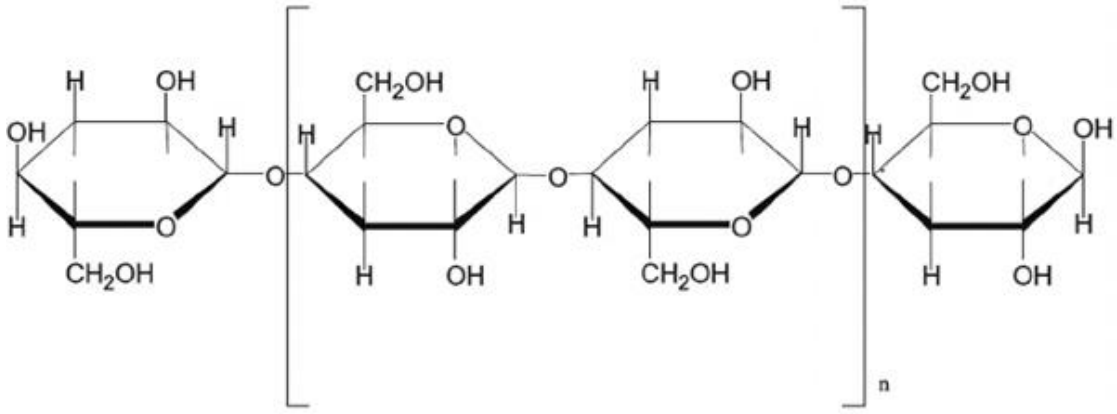

Figure 2.2: Cellulose structure (Dorée, 1947).

2.3.2. Hemicellulose

Hemicellulose is a branched polymer made of shorter chains of polysaccharides such as xylan, glucan, callose, glucoronoxylan, arabinoxylan, mannan, glucomannan and xyloglucan. These polysaccharides contain many different sugar monomers which include arabinose, xylose, galactose, rhamnose and mannose. Some of these sugars are fermented to produce ethanol (Yeoman *et al*., 2010). Hemicellulose is embedded in the cell walls of plants and it binds pectin to cellulose to form a network of cross-linked fibres. Hemicellulose is easily hydrolysed by dilute acid and base, or hemicellulose enzymes which include β-D-xylosidases and endo-β-1,4-xylanases that acts on the structural backbone as well as debranching enzymes like α-1-arabinofuranosidases, α-glucoronidases, and esterases (Klyosov, 1990).

2.3.3. Lignin

Lignin is a cross-linked racemic macromolecule consisting of three hydroxycinnamyl alcohol monomers and it is one of the most abundant organic polymers on earth. Lignin is found in wood and it is embedded in the cell wall between cellulose, hemicellulose, and pectin. The polymer is covalently linked to hemicellulose and therefore crosslinks different plant polysaccharides. Due to its crosslinking capacity, lignin offers mechanical strength to the plant cell wall (Goncalves *et al*., 2013). Lignin lacks the ability to solubilise in water and alcohol but is capable of solubilising in weakly alkaline solution and can be precipitated by acid. Lignin is hydrolysed by enzymes called ligninases (Wood and Garcia-Campayo, 1990).

2.4. Pre-treatment of lignocellulosic biomass

Pre-treatment of lignocellulosic biomass is the first and the most challenging step in bioethanol production. The method used for pre-treating biomass is very critical since it largely impacts the digestibility of cellulose and influences costs relating to enzyme loading, detoxification and waste treatment (Zhang and Cai, 2008; Vasconcellos *et al*., 2015). Pre-treatment step is the most expensive step in bioethanol processing, and it constitutes above 40% of the total processing cost (Rastogi *et al*., 2010). Lignocellulosic biomass is pre-treated before enzymatic hydrolysis to render microbial enzymes access to convert carbohydrate polymers such as cellulosic and hemicellulosic fractions into fermentable sugars (Mosier *et al*, 2005; Rodríguez-Zúñiga *et al*., 2014). The main goal of pre-treating the biomass is to eliminate the lignin fraction and minimise the crystalline nature of cellulose (Gupta and Verma, 2015).

The selection and use of proper treatment methods may lead to an increase in the concentration of single fermentable sugars thus increasing the efficiency of the entire pre-treatment process (Sindhu *et al*., 2016). An ideal pre-treatment method exposes the individual components of the lignocellulosic biomass to enzymatic hydrolysis with increased yields of single fermentable sugars. The pre-treatment method also reduces the need for size reduction of biomass, restrain the formation of inhibitory compounds and it should also reduce energy, capital and operational cost demands (Kalyani *et al*., 2013).

Several enzymes such as cellulases, xylanases, ligninases, and pectinases are required for complete hydrolysis of lignocellulosic biomass to fermentable sugars. Pre-treatment methods are categorised into three groups: physical, chemical and biological processes (Bhalla *et al*., 2013).

The following (Figure 2.3) is a schematic representation of the effect of pre-treatment on lignocellulosic biomass to its components: cellulose, hemicellulose, and lignin.

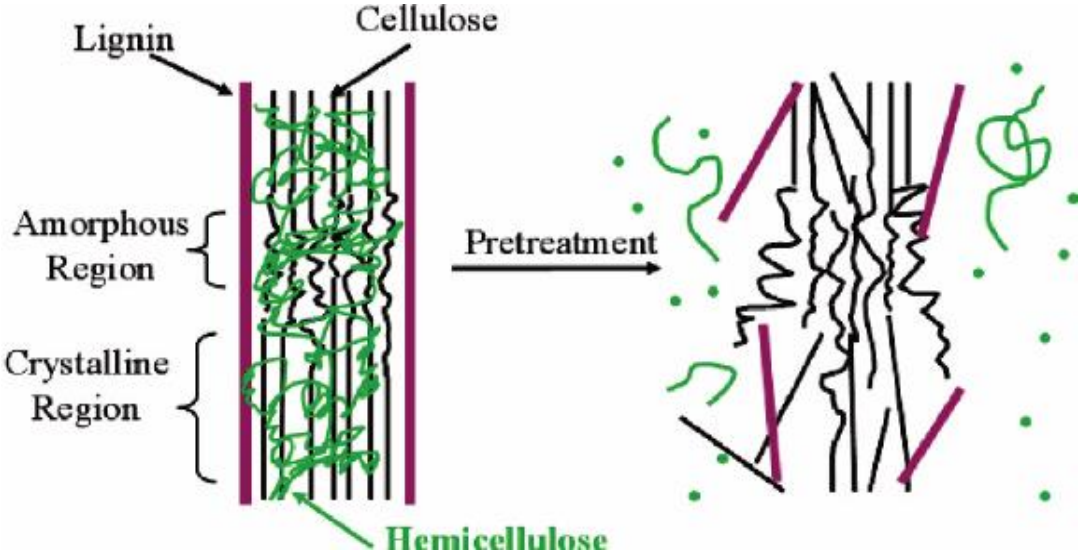

Figure 2.3: Effects of pre-treatment on lignocellulose (Mosier *et al*, 2005).

2.4.1. Physical pre-treatment of lignocellulosic biomass

Physical pre-treatment methods of lignocellulosic biomass include grinding, chipping and milling of lignocellulosic materials to reduce particle size, crystalline nature of cellulose and increase surface area. Steam explosion and hydro thermolysis are also used for the heat conversion of cellulose and hemicellulose into single fermentable sugar molecules (Balat, 2011; Steffien *et al*., 2014).

2.4.2. Chemical pre-treatment of lignocellulosic biomass

Chemical pre-treatment of lignocellulosic biomass involves the use of alkali and acid to hydrolyse lignocellulosic materials. Alkaline pre-treatment utilises chemical compounds such as ammonium hydroxide, calcium, sodium hydroxide and potassium to break down the lignocellulosic material. These chemicals lead to an increase in internal surface area by swelling the lignocellulosic material. The swelling

of the lignocellulosic material disrupts the lignin structure by separating it from carbohydrates and also lead to a decrease in cellulose crystallinity (Balat, 2011; Steffien *et al*., 2014).

Acid pre-treatment of lignocellulosic biomass is mostly achieved by the use of acidic compounds such as nitric, sulfuric or hydrochloric acids. The acidic compounds act by removing the hemicellulose components and thus exposing the cellulose structure to enzymatic hydrolysis. The major objective of acid pre-treatment is to obtain large quantities of fermentable sugars from the lignocellulosic material. Acid pre-treatment of the lignocellulosic materials can be carried out either under high temperature and low acid concentration or under low temperature and high acid concentration (Taherzadeh and Karimi, 2008; Steffien *et al*., 2014).

Chemical additives such as phenol, dioxane, ozone, organosolv, glycerol, or ethylene glycol are among cellulose solvents that can also be used to dissolve cellulose. These additives are highly active resulting in 90% cellulose conversion to glucose but not practically utilized due to their high cost and biomass specificity (Mosier et al., 2005).

2.4.3. Biological pre-treatment of lignocellulosic biomass

Biological pre-treatment of the lignocellulosic biomass relies on the hydrolytic activity of fungal species such as white, brown and soft-rot to solubilise hemicellulose and degrade lignin. Biological pre-treatment is environmentally friendly and requires low energy input. The treatment process can also be carried out under mild environmental conditions. Though it may be the preferred process for treating lignocellulosic material, the process has the disadvantage of low hydrolysis rate (Balat, 2011; Steffien *et al*., 2014). Biological treatment of lignocellulolytic biomass is carried out by microbial enzymes having the hydrolytic activity.

2.5. Microbial enzymes in the production of valuable products

Biological enzymes act as natural catalysts to speed up biochemical reactions in living systems. Biological enzymes are produced by plants, animals, and microorganisms. Though all living organisms produce enzymes, the industrial

enzyme sector is dominated by the microbial enzymes (Jisha *et al*., 2013). Microbial enzymes are mostly used in industry as biocatalysts, and that is due to the microorganisms' ability to grow fast and the relative consistency in supply. This means that industrial microbial enzyme production continuously occurs with great enzyme yield, without being affected by seasonal, agricultural, and other factors (Hernández-Martínez *et al*., 2011). Enzyme-assisted processing is green to the environment and result in no health and safety issues (Simpson *et al*., 2012). Most of the microbial enzymes that are currently used at an industrial level for production of valuable products are hydrolytic in action with the proteases remaining the dominant enzyme types. Enzymes also play a major role in biological research by assisting researchers to explain, understand and manipulate the structures and synthesis of proteins (Soares da Silva *et al*., 2018). Various carbohydrases including amylases and cellulases form the second largest group of enzyme types and are used mostly in starch, textile, detergent, and baking industries. High consumption of industrial enzymes is seen in technical industries such as detergent, starch, textile, and fuel (Kirk *et al*., 2002).

2.6. Cellulases

Cellulases are enzymes (EC 3.2.1.4) that catalyse the depolymerisation of cellulose by employing two different catalytic mechanisms namely the inverting and the retaining mechanisms. These enzymes belong to the glycoside hydrolase family (Tamboli *et al*.,2017; Legodi *et al*., 2019; www.cazy.org) and are important industrial enzymes with a wide range of applications (Kumar *et al*., 2012; Pradeep *et al*., 2012). In addition, cellulases act as sources of carbon in the biosphere thus facilitate the global carbon cycle (Nowak *et al*., 2005). Cellulases differ structurally and in their mode of action (Payne *et al*., 2015). The enzymes are produced by all plants, few animals and microorganisms ranging from bacteria, fungi, and actinomycetes. Fungi are considered the best cellulase producers due to their metabolic capacity of producing huge amounts of extracellular cellulose-degrading enzymes (Gragg *et al*., 2015). Most microbial cellulases have a carbohydrate-binding module which aid the enzymes in binding to the highly crossed linked cellulose structure and thus enhance cellulase activity (Bayer *et al*., 1998). Though cellulases catalyse the depolymerisation of cellulose, collaborative action of three interdependent cellulolytic

enzyme activities, namely (1,4-beta-D-glucan glucohydrolase [EC 3.2.1.4]), exoglucanase (1,4-beta-D-glucan cellobiohydrolase [EC 3.2.1.91]), and beta-glucosidase (beta-D-glucoside glucohydrolase, [EC3.2.1.21]) is required for complete and effective hydrolysis (Wang *et al*., 2010).

2.6.1. Exoglucanases

Beta-1,4-Exoglucanases or cellobiohydrolases (CBHs) function in the depolymerisation of cellulose. These hydrolytic enzymes (CBHs) facilitate the production of cellobiose, a disaccharide that can be converted to single fermentable glucose molecules by β-glucosidases (Kumar and Murthy, 2013). Exoglucanases form part of the glycoside hydrolase families (GH) 6, 7, 9 and 48 (Yeoman *et al*., 2010). These plant degrading enzymes cleave both the reducing and the non-reducing ends of cellulose chains generating glucose or cellobiose as products of cellulose hydrolysis (Hasunuma *et al*., 2013).

2.6.2. Endoglucanases

Endo-1,4-beta-D-glucanases work in synergy with the cellobiohydrolases in the cellulolytic deconstruction of cellulose. Endoglucanases hydrolyse the β-glycoside linkages of cellulose randomly, disrupting the highly ordered structure of the substrate (Hasunuma *et al*., 2013). The enzymes then cleave to the unstructured regions and progress to bind cellobiose units. The endoglucanases are found in the glycoside hydrolase families 5-9, 12, 44, 45, 48, 51, 61, and 74 (Yeoman *et al*., 2010).

2.6.3. Beta-glucosidases

The beta-glucosidase enzymes (glucosidases and cellodextrinases) play a major role in cellulolytic systems by facilitating the hydrolysis of β-glucosidic linkages in soluble cellodextrins and cellobiose liberating free glucose molecules (Zimbardi *et al*., 2013). The beta-glucosidases are termed the rate-limiting enzymes because they are responsible for the complete hydrolysis of cellulose molecules. In addition to producing fermentable glucose molecules from cellobiose, the beta-glucosidases also reduce cellobiose mediated repression on both endoglucanase and

cellobiohydrolase activities. Beta-glucosidases are grouped into three categories based on substrate specificity: aryl-beta-glucosidases, cellobiases, and broad-specificity beta-glucosidases. Aryl-beta-glucosidases act mostly on aryl-beta-glucosides, whereas cellobiases act only on cello-oligosaccharides (including cellobiose). Members of the broad-specificity beta-glucosidases, show significant activity on both aryl-beta-glucosides and cello-oligosaccharides. Due to significant hydrolytic activity on both substrate types, the broad-specificity beta-glucosidases are regarded as the most observed group in cellulolytic microbes. Beta-glucosidases are universal enzymes, produced by organisms representing all domains of life ranging from bacteria to highly evolved mammals (Yeoman *et al*., 2010).

The following diagram (Figure 2.4) represents the cellulose structure and different sites of cellulase hydrolysis.

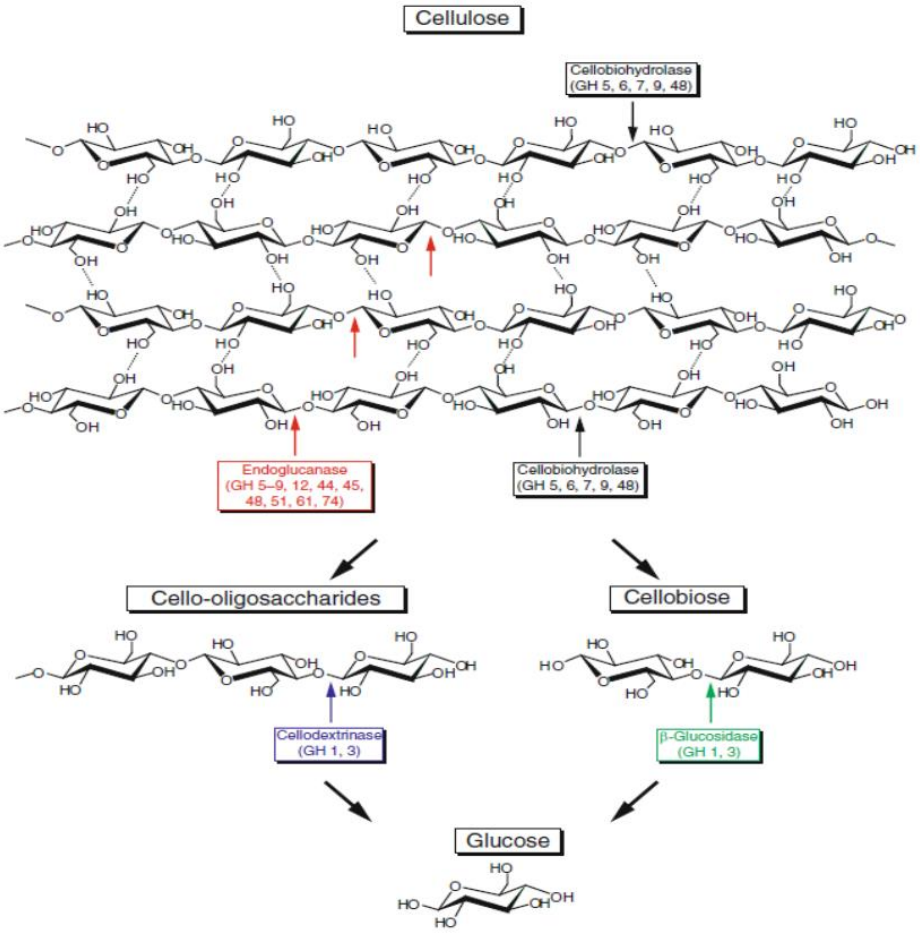

Figure 2.4: Cellulose degrading enzymes (Yeoman *et al*., 2010).

2.7. Activity assays for cellulases

Cellulase activity assays are carried out using both natural and synthetic substrates. Activity assays are conducted to determine the overall activity of cellulase and to also check for the activity of each individual principle cellulase (Dashtban *et al*., 2010; *Shuanggi et al*., 2011).

Many assay methods for detecting cellulase activity have been reported including, the FPA (filter paper) total cellulase activity, which is the principal method for determining total cellulase activity. The FPA method uses the 1x6 cm strip of Whatman no. 1 filter paper as substrate. The FPA assay employs 3,5-dinitrosalicylic acid (DNS), glucose oxidase (GOD) or high-performance liquid chromatography as reducing sugar detectors to measure cellulase activity (Dashtban *et al*., 2010).

The endoglucanase activity (carboxymethyl cellulase activity) method measures endoglucanase activity using carboxy methylcellulose (CMC), which is a cellulose derivative with a high degree of polymerisation. This method does not determine overall cellulase activity, it specifically determines the activity of the endoglucanase in the sample of interest. This method detects endoglucanase activity using the DNS reagent, which aid the entire assay process by measuring the total amount of reducing sugars present in the assay sample (Dashtban *et al*., 2010). Exoglucanase activity (Avicelase activity) method specifically assay for the activity of exoglucanase using crystalline cellulose Avicel as a substrate. This method also uses the DNS reagent to measure the total amount of reducing sugars (Dashtban *et al*., 2010). The beta-glucosidase activity method measures beta-glucosidase activity using cellobiose as the substrate. The amount of glucose released is measured by HPLC (*Shuanggi et al*., 2011). Alternatively, cellulase activities can be measured using synthetic substrates such as 4-nitrophenyl glucoside for determination of beta-glucosidase activity and Remazol brilliant blue (RBB)-dyed CMC/Avicel for determination of endo- and exoglucanase activities. These synthetic substrates are colorimetric. The change in colour of the reaction system due to the release of the RBB dye and 4-nitropheny can be measured spectrophotometrically at 595 nm for exo and endoglucanase and 410 nm for beta-glucosidase. Fluorometric and automated measurements of cellulase activity can also be utilised (Ng and Zeikus, 1980; 1983).

2.8. Cellulase production technologies

Solid-state (SSF) and submerged fermentation (SmF) processes have commonly been used to produce cellulases from microorganisms. Commercial production of cellulases is currently being achieved using filamentous fungi *T. reesei* and *A. niger* under the SmF. Although bacteria and actinomycetes are available, cellulase titres produced by these microorganisms are less making the technology economically unfeasible (Singhania *et al*., 2017). The SSF involves the fermentation of the substrate by microbes in the absence of water, the substrate is often moistened enough to support the microbial metabolism thus promoting the growth of the desired species (Singhania *et al*., 2009; Marin *et al*., 2019). SmF is currently used in large scale enzyme-producing facilities since this process is easy to monitor and handle. The process involves the fermentation of the substrate in an environment devoid of excess water. Though the SmF is the preferred process in enzyme production industries, filamentous fungi are found closely associated with their substrate in nature and for this reason, the SSF is the preferred process to use when studying microbial morphology and metabolic differentiation (Pandey, 2003). SmF also suffers from the reproducibility of cultivations due to the multicellular nature of filamentous fungi (Hansen *et al*., 2015). SSF process is highly cost-effective in the sense that it utilises low-cost agricultural residues as substrates and also has advantages of higher productivity, higher yield, lower catabolite repression, and low capital investment. Even though the SSF process is cost-effective, it is not utilised at the industrial level because of the lack of proper technology (Farinas, 2015).

2.9. Applications of cellulases

Microbial cellulases are used in various industries such as pulp and paper, textile, food biotechnology, detergents, animal feed, brewing, pharmaceuticals, agriculture and during the production of bioethanol (Kuhad *et al*., 2011).

2.9.1. Paper and pulp industry

The application of cellulases help improve drainage and beatability in the paper and pulp industry by avoiding mechanical pulping and refining that was employed in the last decade. Cellulases are also involved in the de-inking processes from wastepaper (Bhat, 2000). The enzymes are also used in detergents for cleaning, colour clarification and anti-redeposition of soil and stains (Kirk *et al*., 2002).

2.9.2. Textile industry

The cellulases are also used in the textile industry to refine denim-finishing and to soften cotton (Kirk *et al*, 2002). Traditionally, pumice stones were used to wash and desize jeans (Sreenath *et al*., 1996). Nowadays, cellulases are being used instead of pumice stones to treat denim garment and other cellulosic fabrics to look like aged denim fabrics through the process called biostoning (Kuhad *et al*, 2011). Cellulase effect their stoning activity by detaching small fibre ends on the cotton fabric, and loosen the dye, which is removed by mechanical abrasion during the wash cycle (Ibrahim *et al.,* 2011; Moreira *et al*, 2016).

2.9.3. Wine and beer industry

Beta-glucosidases improves the flavour and aroma of wine by modifying non-volatile glycosidic precursors found in wine. Besides aroma profiles, beta-glucosidases are also capable of degrading anthocyanins, which are compounds responsible for the colour of red wines, freeing sugar aglucone anthocyanidins. Anthocyanidins are less soluble than anthocyanin thus, being easily removed during filtration. The removal of anthocyanidins decreases the alteration in colour that would occur with juice and wine during the pasteurization process (Chakraborty *et al.,* 2016). Macerating enzymes such as complex cellulases, xylanases, and pectinases help improve the pressability, settling, and juice yields of grapes used for wine fermentation. All the

enzymes that are involved during winemaking improve clarification, filtration, stability, colour and wine quality (Bhat, 2000).

2.9.4. Food processing industry

Cellulases are used to improve tea aroma, which is an important factor that determines tea beverages (Chandini *et al*., 2011). Beta-glucosidase is mainly involved in the hydrolysis of β-glycoside releasing non-reducing β-glucose and aglycones, thus, increasing the aroma quality of many tea beverages (Su *et al*., 2010). Beta-glucosidase also hydrolyses isoflavone glycosides found in soybean thus, releasing aglucones genistein, diadzen, glycetein that have preventive and inhibitive effects on mammalian (e.g., prostate) cancer. Isoflavones also lower the risk of cardiovascular diseases and improves bone health. Beta-glucosidases are also involved in the detoxification of cassava and in the improvement of texture, flavour and aroma properties of fruits and vegetables (Soares *et al*., 2016; Singhania *et al*., 2017).

During the production of wheat bread microorganisms act on free sugars including pentoses and hexoses released by the action of the hydrolytic enzymes such as cellulases, pentosanases and xylanases (Martinez-Anaya *et al*., 1999). The presence of these hydrolytic enzymes during the production of wheat bread help improve bread quality by increasing porosity of crumb and specific volume (Haseborg and Himmelstein, 1998).

2.9.5. Detergent Industry

The use of cellulases together with protease and lipase improved the detergents industry. The cellulase cocktails function by modifying cellulose containing fibres thus increasing colour brightness, feel, depilling, and dirt removal from cotton. Among microbial cellullases, bacterial cellulases are the most utilised biocatalysts in detergents (Kuhad *et al*., 2011).

2.9.6. Animal Feed Industry

Most of the cereal's energy content is in the form of non-starch polysaccharide which cannot be digested by several animals. The indigestible nature of the polysaccharide

requires the addition of hydrolytic enzymes, such as cellulases and hemicellulases in cereals to aid in the breakdown of the polysaccharide cell walls to usable forms. (Kuhad *et al*., 2011). The addition of cellulases in the diet of broiler chickens reduced intestinal viscosity, improved weight and feed-conversion efficiency of chicks (Laudadio *et al*., 2014).

2.9.7. Recycling of Waste

Waste derived from agricultural and forest sectors consist of a large number of unused cellulose sources polluting the environment. To reduce this polluting effect, unutilised cellulosic wastes are being used to produce valuable products (carbon sources for fermentation, saccharides, enzymes, biochemicals, biofuels) and enrich animal feeds (Sukumaran and Pandey, 2005; Milala *et al*., 2005; Zhang *et al*., 2006; Kuhad *et al.*, 2016).

2.9.8. Agricultural Sector

Various enzyme cocktails including cellulases, hemicellulases, and pectinases are used in the agricultural sector to increase the yield of crops and decrease plant diseases. Plant diseases are controlled by the use of cellulases from saprophytic fungi. The fungal species effect their cellulolytic plant disease control activity by degrading the cell wall of plant pathogens. Seed germination, plant root system, growth and flowering are promoted by the agricultural use of established cellulolytic fungi, such as *Trichoderma*, *Gliocladium*, *Chaetomium*, and *Penicillium* species (Bailey and Lumsden, 1998).

2.9.9. Pharmaceutical industry

Beta-glucosidase is used in the health industry, where it catalyses transglycosylation reactions (Pal *et al*., 2010). During the transglycosylation reactions, one glycosyl residue is transferred from a glycoside group onto a receptor molecule to form a new glycosidic linkage, leading to the biosynthesis of polysaccharides. The products obtained from this process can be used in the pharmaceutical industry during the production of cosmetics, where the adoption of synthetic chemistry is sometimes not acceptable (Karnaouri *et al*., 2013).

Cellulases are used to treat phytobezoars found in the stomach of humans. Phytobezoars are caused by the accumulation of undigested vegetable fibres. In addition, cellulases also possess antitumor or antimicrobial agents and are being used to treat blood clots, herniated discs, and enzyme deficiencies. Cellulases remedy the human body of digestive problems such as “malabsorption” by acting as digestive aid. Human beings poorly digest cellulose fibre, supplementing human diet with a cellulase digestive enzyme, such as Digestin, may help with the breakdown of cellulosic fibre and also maintain the health state of human cells (Sharada *et al*., 2014).

2.9.10. Bioethanol production

The production of biofuels from non-food lignocellulosic biomass via the enzymatic route is by far the most investigated concept relating to the application of cellulases. Bioconversion of lignocellulosic biomass to useful bio-products require multi-step processes including pre-treatment, enzymatic hydrolysis of the polymers into fermentable monomers to produce valuable products (Sticklen, 2008). Bioethanol is one of the products obtained from the bioconversion of lignocellulosic material and is the most widely used biofuel for transportation worldwide (Balat and Balat, 2008). Commercial production of bioethanol is currently from food crops such as sugar cane, sugar beet, corn, barley, wheat, yam, potato, sweet sorghum and cassava. Brazil utilises sugar cane as the predominant feedstock for bioethanol while the European countries use sugar beet and wheat. In Canada and China, corn, wheat, and cassava are the predominant bioethanol feedstocks (Guo *et al*., 2015). The major drawback of bioethanol production is the availability of feed stock for the production. The production and yield of feed stock vary depending on season and geographic location (Balat, 2011).

Several microorganisms are reported to have the ability to produce ethanol. Traditionally production of bioethanol through simultaneous saccharification (SSF) and submerged fermentation processes is carried out by the yeast *Saccharomyces cerevisiae* (Mohd Azhar *et al*., 2017). Genetically modified strains including *Clostridium thermosaccharolyticum, C. thermohyrosulfuricum, Escherichia coli*, *Thermoanaerobacter mathranii, T. brockii T. ethanolicus* and *Zymomonas mobilis,* having capabilities to ferment both hexose and pentose sugars are being studied

(Balat, 2011; Javed *et al*., 2019). The consolidated bioprocessing system (CBP) is gaining interest for the economical cellulosic bioethanol production since it does not involve a dedicated process for deconstructing enzyme production. The CBP system involves enzyme production, fermentation, and biomass hydrolysis in a single fermenter and it is perceived to be a more cost-effective system as compared to other bioprocesses. The production of ethanol from lignocellulosic biomass may reduce crude oil consumption and environmental pollution, thus sustaining the environment (Hayes, 2009).

Figure 2.5 represents different bioprocesses for bioconversion of raw material into bioethanol.

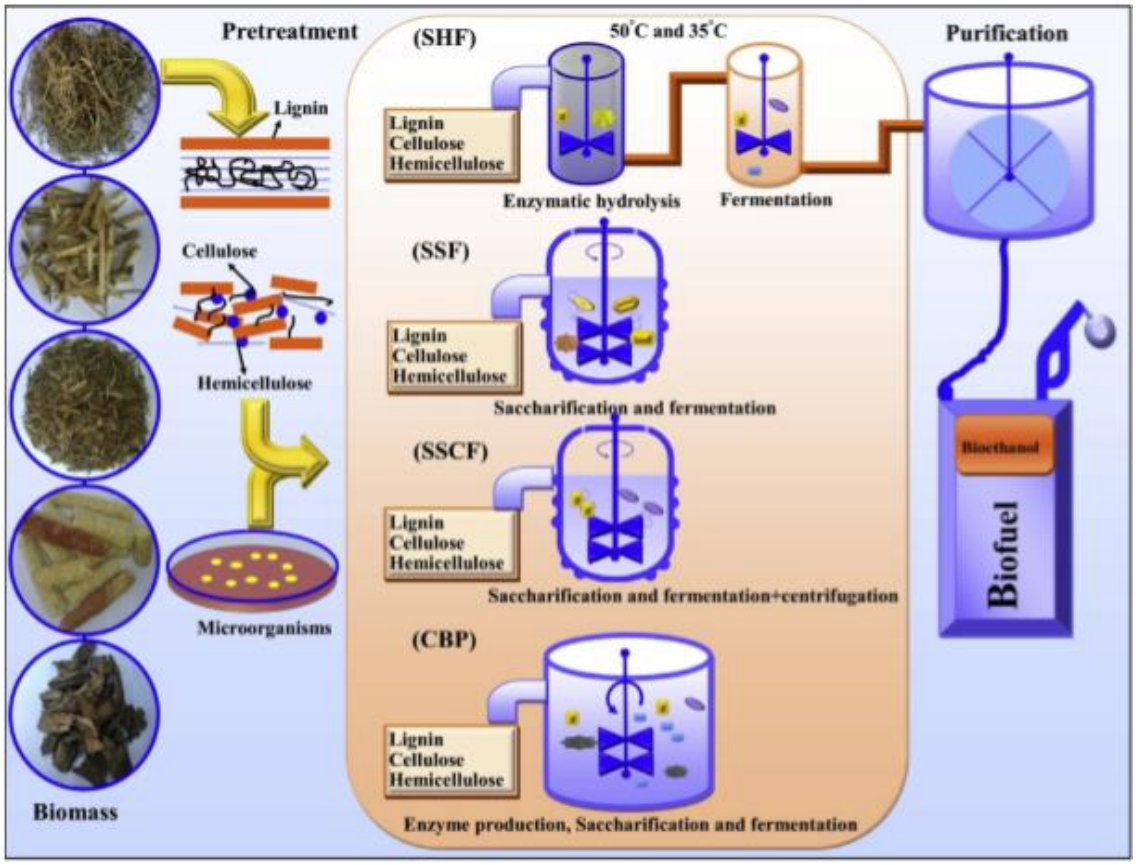

Figure 2.5: Integrated processes for the production of biofuels (Javed *et al*., 2019). Separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), consolidated bioprocessing (CBP).

2.10. Proteomic studies in the discovery of microbial enzymes

Proteins are essential organic compounds that control biological processes occurring in living organisms. The total set of proteins expressed by an organism is termed the proteome which was first coined by Wilkins *et al*., in 1995. Proteomics is a field of study that deals with proteins, temporal expression and determination of biological structures, functions, and interactions (Andersen and Mann, 2006). Most if not all protein studies require the use of analytical technology characterized by high selectivity, resolution, and sensitivity (Pomastowski and Buszewski, 2014)

Initially, proteomic studies were trialled using mainly two-dimensional (2-D) gel electrophoresis. However, nowadays most proteomic studies use two-dimensional gel electrophoresis plus mass spectrometry (MS) with electrospray ionization or Matrix Assisted Laser Desorption/Ionization (Aebersold, 2003). Mass spectrometry (MS) is an imperative high throughput proteomics technique. A mass spectrometer is an instrument capable of ionizing the sample, separating them based on their mass to charge ratio and recording the mass to charge ratio and relative intensities of ions to produce a mass spectrum under specified vacuum conditions (Aebersold and Mann, 2003).

MS allows for quick identification of peptides obtained from very small amounts but highly complex biological molecules. Protein profiling by MS is divided into bottom-up and top-down approaches. Bottom-up (peptide-based) proteomics relies on separation of protein mixtures, proteolytic digestion of separated proteins, peptide sequencing, and identification of the corresponding proteins via database sequence search engines. Whereas, top-down (protein-based) proteomics carry out high-resolution fragmentation and measurement of an intact protein molecule obtained by complex purification procedures. The bottom-up approach covers a broad proteome range with a high-throughput analysis but rarely identify and localise protein modifications. Conversely, ‘top-down’ approach covers greater yields of amino acid and is precise in identifying and localising protein modifications. Though the top-down approach identifies and localise protein modifications proteome coverage, sensitivity, and throughput are compromised (Chalupová *et al*., 2014).

Proteomic studies have unlocked doors in healthcare, agricultural and biological science studies. The proteomic approaches are used to develop drugs, produce recombinant therapeutic proteins, study prostate cancer, microbial communities and biological systems (Cao *et al*., 2019). In the present study, microbial proteomic analysis was conducted to characterise and identify cellulases encoded by active genes with the ultimate goal of generating protein profiles, comparing and detecting differences between them.

2.11. Bioinformatics in proteomic studies

Bioinformatics is a single scientific discipline that merged biology, computer science, and information technology. The bioinformatics field deals with the use of computers to collect, organise, analyse, manipulate, present and share biological data. The field targets biological molecules to solve problems. Bioinformatics has three sub-disciplines within it, which are the development of new algorithms and statistics, analysis and interpretation of biological molecules data, implementation and development of tools to enable efficient access, management, construction and curation of biological databases (www.iasri.res.in/ebook). The three sub-disciplines are interconnected and analyse large data set stored in different databases, enabling the discovery of new biological insights and providing a global perspective of the cell (Xiong, J. 2006).

2.9.1. Applications of bioinformatics in science, today

Bioinformatics has not only become essential for basic genomic and molecular biology research. The subject has applications in forensic DNA analysis, biomedical sciences (Molecular medicine, personalised medicine, preventative medicine, gene therapy and drug development); agricultural biotechnology (crop improvement, insect resistance, improve nutritional quality, development of drought-resistant varieties, veterinary science); climate change studies, bioweapon creation and microbial genome applications (genome assembly, re-sequencing, comparative analysis, evolutionary studies, antibiotic resistance and waste clean-up) (Nalluri *et al*., 2018).

CHAPTER 3

METHODOLOGY AND ANALYTICAL PROCEDURES

This project was a quantitative study, that undertook an observational experimental research design approach in order to validate the hypothesis.

3.1. Strain revival

Frozen fungal and bacterial cultures (100 µl) within the Biotechnology Unit, University of Limpopo, South Africa, were aseptically transferred to the 50 ml Erlenmeyer shake flasks containing 20 ml of sterile nutrient broth and malt extract (ME) agar plates. The submerged cultures (fungal isolates) were incubated at 30 °C for a period of 120 hours at 121 rpm while the culture plates (bacterial isolate) were incubated at 60 °C for a period of 120 hours under static conditions.

3.2. Inoculum and subculture preparation

Erlenmeyer shake flasks (250 ml), each containing 100 ml of modified Mandel (L^{-1}: 2g K_2HpO_4, 0.5g KCl, 0.005g $FeSO_4$, 0.15g $MgSO_4$. $7H_2O$, 7g KH_2pO_4, 1g $(NH_4)2SO_4$, 1 g yeast extract and 2% Avicel) and M9 (L^{-}1: 64g $Na_2HPO_4.7H_2O$, 15g KH_2PO_4, 2.5g NaCl, 5.0g NH_4Cl, 61.62g $MgSO_4$, 11.09g $CaCl_2$, 5 g tryptone, 5g yeast extract and 5% cellobiose) media were autoclaved and aseptically inoculated with a 1-x 10^6 fungal spores/ml and 1 ml of bacterial culture from the nutrient broth, respectively (Mandels *et al*.,1974; Song and Wei, 2010). The flasks were incubated at 30 °C (fungal isolates) and 60 °C (bacterial isolate) for 120 hours in an orbital shaker (New Brunswick Scientific, Excella E25) at 121 rpm. For sub-culturing and maintenance of cultures, fresh nutrient and ME agar plates were inoculated with microbial cultures and incubated at 30 °C (fungal isolates) and 60 °C (bacterial isolate) for 120 hours. The bacterial and fungal cultures were maintained on agar plates and kept at 4 °C. The fungal isolates were preserved in sterile distilled water, at room temperature. The bacterial isolate was preserved in a 50% glycerol solution, kept at -80 °C. All chemicals for media were obtained from Sigma-Aldrich Co, SA.

3.3. Production of cellulolytic enzymes in submerged fermentation

3.3.1. Production of endoglucanase from fungal isolates

Four fungal isolates (VCFF1, VCFF14, VCFF17, and VCFF18) were cultured in 500 ml Erlenmeyer flasks containing 200 ml Mandels media with 20 g Avicel as the sole carbon source. The culturing media was sterilised for 20 minutes at 121 °C. The flasks were allowed to cool down then inoculated with spore suspensions giving a final concentration of 1 x 10^6 spore/ml of media used. Incubation of the cultures was carried out at 30 °C for 168 hours in an incubator (New Brunswick Scientific, Excella E25) shaking at 121 rpm. Time-course analysis of the fungal endoglucanase production was done by aseptically withdrawing 1 ml of the culture into a sterile Eppendorf tube every 24 hours. Fungal cells were separated from the supernatant by centrifuging the cultures at 12470 x g for 15 minutes at 4 °C using a (Beckman Coulter) microfuge. For proteomic analysis, the crude enzyme was harvested before endoglucanase production, during endoglucanase peak production and after endoglucanase peak production times. The harvested crude enzyme was stored at -20 °C.

3.3.2. Production of beta-glucosidase from a bacterial strain

The bacterial isolate BARK was cultured in 500 ml Erlenmeyer flasks containing 200 ml of M9 salts with 5 g of cellobiose as a sole carbon source. The culture was incubated in a shaking incubator (New Brunswick Scientific, Excella E25), shaking at 121 rpm at 60°C for optimal growth of the bacterial isolate. For time-course analysis of the bacterium’s beta-glucosidase production, sampling was done after every 3 hours for 42 hours by withdrawing 1 ml of the sample and centrifuging at 12470 x g for 15 minutes using a (Beckman Coulter) microfuge. For proteomic analysis, the bacterial isolate was cultured, and the crude enzyme was harvested at 3 hours, 6 hours and 30 hours incubation time and stored at -20 °C.

3.4. Enzyme assays

3.4.1 Endoglucanase activity on carboxymethylcellulose (CMC)

The carboxymethylcellulase (CMCase) activity was measured by incubating 125 µl of culture supernatant, 125 µl of acetate buffer pH 5 with 250 µl of 1% CMC prepared in 0.1 M acetate buffer, pH 5 at 50°C for 30 minutes. The reducing sugars liberated were estimated using the 3,5-dinitrosalicylic acid (DNS) reagent whereby 1.5 ml DNS was added to the above 500 µl reaction mixture, boiled in capped glass tubes for 5 minutes, cooled and then the absorbance measured at 540 nm. Enzyme activity was defined as the amount of enzyme capable of releasing 1 µmol of glucose per ml per minute (Goldbeck *et al*., 2012).

3.4.2. Beta-glucosidase assay

The beta-glucosidase activity was determined by monitoring the release of p-nitrophenol using p-nitrophenol-β-D-glucopyranoside (pNPG) as a substrate in micro plate assay. The reaction mixtures contained 25 µl of the crude enzyme, 50 µl of sodium phosphate buffer pH 7 and 25 µl of 10 mM pNPG. The mixture was incubated at 50°C for 30 minutes and the reaction stopped by adding 100 µl of 0.1 M of Na_2HCO_3. The release of p-nitrophenol was monitored by reading absorbance at 405 nm in a 96-well microtiter plate using DTX 880 Multimode Detector, micro plate reader. One unit of beta-glucosidase activity corresponded to the amount of enzyme required to release 1 µmol of p-nitrophenol per minute under assay conditions (Okamoto *et al*., 2011).

3.5. Determination of reducing sugar by DNS method

Glucose standards ranging from 2 to 5 µmol/ml were prepared by dissolving 0.15 g of glucose in 0.05 M acetate buffer pH 5. A 100µl of each dilute standard was placed in test tubes, 0.9 ml acetate buffer was added to each test tube and mixed with the glucose standard. After mixing, a 1.5 ml DNS reagent was added to the mixture. The test tubes were placed in a boiling water for 15 minutes and then cooled to room temperature. Absorbance of the mixture was read at 540 nm. The concentration of the reducing sugars was estimated through the use of the glucose standard curve.

3.6. Preparation of phosphoric acid-swollen Avicel

Phosphoric acid-swollen cellulose was prepared from Avicel (Fluka, PH-101). An amount of 10g of Avicel was suspended in 100 ml of phosphoric acid. The solution was stirred for 1 hour at 4 °C. After stirring, the swelled mixture was washed four times with ice-cold water at room temperature. The residual phosphoric acid was neutralised two times with 1% (w/v) $NaHCO_3$ solution, followed by dialysis of the thick suspension of swollen cellulose against water at 4 °C. The cellulose suspension was homogenised with Waring blender (Wood, 1971).

3.7. Cellulose affinity chromatography

The binding affinities of carbohydrate binding modules to phosphoric acid-swollen Avicel were investigated by mixing 10g of swollen Avicel with 50 ml of the crude enzyme. The reagents were mixed in a 100 ml Sholt bottle by stirring for 2 hours at room temperature. The mixture was centrifuged at 903 x g for 10 minutes to separate cellulose. To remove the unbound proteins, cellulose was washed 4 times with a solution containing 0.05 M acetate buffer pH 5, 0.01 M $CaCl_2$ and 0.001 M DTT. A 0.1 M cellobiose solution was used to elute bound proteins from cellulose. Eluates were filtered using a 0.2 µm Millipore filter and concentrated using Amicon Ultra centrifugal filter unit (Millipore, Ireland) with a 10 kDa cut off.

3.8. Trichloroacetic acid-acetone precipitation of the crude enzyme

The crude enzyme from both the bacterial and fungal cultures was concentrated by precipitation. An amount of 10 ml of the crude supernatant was mixed with 1g of trichloroacetic acid (TCA) and 40 ml of ice-cold acetone. The mixture was incubated at -20 °C overnight followed by centrifugation at 1494 x g for 20 minutes at 4 °C to obtain the precipitate. The supernatant was discarded after centrifugation, and the pellet was re-dissolved in 100 µl of lysis buffer and stored at -20 °C for further use.

3.9. Protein quantification by the Bicinchoninic acid assay (BCA)

The BCA reagent was prepared by mixing 500 µl BCA solution and 10 µl copper (II) solution. Reactions were done in triplicate by mixing 10 µl of protein sample and 200 µl of BCA solution in a 96 well microtiter plate and then incubated in the dark at 37 °C for 30 minutes. The absorbance of the mixture was read at 595nm. For the estimation of the quantity of protein, 0.2 to 1 mg/ml Bovine Serum Albumin (dissolved in deionised water) was used as a standard. Equal amounts of protein concentration were loaded on SDS-PAGE for comparison based on the BCA assay results.

3.10. Electrophoretic separation of proteins

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was used for the separation of proteins in the crude enzyme of selected isolates. The isolates were selected based on their ability to break down Avicel and cellobiose (based on the endoglucanase and beta-glucosidase activities).

3.11. Detection of active beta-glucosidase and endoglucanase bands

Zymography was performed to detect active protein bands on a 12% SDS-PAGE gel. Beta-glucosidase in-gel activity assay was carried out by soaking an unstained SDS-PAGE gel with bacterial protein samples in 0.1 M sodium phosphate buffer, pH 7.0 and 40% isopropanol (1:1 proportion) for 10 minutes at room temperature. The gel was washed with 0.1 M sodium phosphate buffer, pH 7.0 followed by incubation with the same buffer containing 0.1% (w/v) esculin and 0.03% (w/v) ferric chloride for 5 minutes at 50°C. The reaction was stopped by immersing the gel in a 10% (w/v) aqueous solution of glucose (Kwon *et al*., 1994).

For endoglucanase in-gel activity, 0.1% (w/v) CMC was used as a substrate (Ratanachomsri *et al*., 2002). Two ml of 0.1 % CMC substituted deionised water during the preparation of the 12% resolving gel. Crude enzyme samples (10 µl) from the four fungal cultures were loaded onto the SDS-PAGE gels. Coomassie brilliant blue R250 was used to visualise the molecular weight marker while the other part of the gel was subjected to endoglucanase zymographic analysis. The gel was washed

for 1 hour with a 1:1 solution of 40% isopropanol and 0.05 M sodium phosphate buffer pH 7.2. Isopropanol was removed by further washing the gel for 1 hour with 0.05 M sodium phosphate buffer pH 7.2. Proteins were allowed to renature in a 1:1 mixture of 0.05 M sodium phosphate buffer and 0.001 M EDTA at 4 °C for 1 hour (Dutta *et al*., 2008). Enzymatic degradation of CMC was carried out for 1 hour at 50 °C. The gels were stained with 0.1% (w/v) Congo red for 30 minutes while shaking at 40 rpm. The bands were visualised by destaining the gel with 1 M sodium chloride for 20 minutes at 40 rpm. A 0.5% (w/v) acetic acid solution was used to stop the enzymatic reaction (Ratanachomsri *et al*., 2002). Active protein bands from the zymograms were manually excised using a sterile razor blade and stored at -80 ºC for further analysis (Ncube *et al*., 2012).

3.12. Isoelectric focusing of proteins

Twelve microliters of TCA-acetone precipitated crude enzyme samples from both the bacterial and fungal isolates were mixed with 132 µl of rehydration buffer [8 M urea, 2 M thiourea, 2% CHAPS, 2 M DTT, 6% ampholytes and a trace of bromophenol blue] for rehydration of an immobilised pH gradient 7 cm, 3-10 ReadyStrip™ (Bio-Rad) IPG strip prior to isoelectric focusing. The strips were rehydrated for 16 hours at room temperature. For the reduction of proteins, the focused IPG strip was equilibrated for 15 minutes with the equilibration buffer 1 (20 ml of 0.375 M Tris-HCL (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 2% w/v DTT) and 1.5% iodoacetamide substituted for the DTT in equilibration buffer 2 for alkylation of proteins, then placed on top of an SDS-PAGE gel. The equilibrations were performed in gentle shaking at 25°C for 20 minutes. After the equilibration steps, the strip was immobilised using a 0.5% agarose solution containing a trace amount of bromophenol blue. After separation based on molecular weight, the proteins were visualised by staining the gel with Coomassie Brilliant blue R250. The protein spots on the SDS-PAGE gel were manually cut with a sterile blade, transferred to 0.5 µl microfuge tubes and stored at -80°C for further analysis.

3.13. Sequencing and identification of proteins through matrix-assisted laser desorption ionization time-of-flight mass spectrometry

3.13.1. In-gel digestion of protein spots and bands

After PAGE electrophoresis, the excised protein spots and bands were washed with water for 10 minutes. The protein bands and spots were cut into 1 mm cubes and smaller gel pieces respectively then transferred to a 0.5 µl microfuge tube. The gel pieces were destained by adding approximately 30 µl of a freshly prepared 1:1 (v/v) mixture of $Na_2S_2O_3$ and $K_3[Fe(CN)_6]$ dissolved in water followed by incubation at room temperature for 30 minutes. The destaining solution was discarded after the incubation period. The gel pieces were washed with water and 50 mM NH_4HCO_3/acetonitrile (ACN) 1+1 (v/v) for 15 minutes. The wash solution was discarded, and the gel pieces were covered with enough acetonitrile. The gel pieces were left to shrink and stick together. The acetonitrile solution was discarded, and the gel pieces were dried in a vacuum centrifuge. The gel pieces were reduced and alkylated by swelling them in 100 µl of freshly prepared 10 mM dithiothreitol (DTT)/ 50 mM NH_4HCO_3 solution and incubated for 45 minutes at 56°C. The tubes were chilled to room temperature and excess liquid was discarded and replaced quickly with roughly the same volume of freshly prepared 55 mM iodoacetamide (IAA) in 50 mM NH_4HCO_3. The mixture was incubated for 30 minutes at room temperature in the dark. The iodoacetamide solution was removed and the gel pieces were washed with 50 mM NH_4HCO_3/acetonitrile (ACN) 1+1 (v/v), one or two changes each, 15 minutes per change. The solution was removed, and enough acetonitrile was added to cover the gel pieces. The gel pieces were left to shrink and dried down for 15 minutes at a high speed using a Savant Speed Vacuum SC110 centrifuge.

For in-gel digestion, a freshly prepared enzyme solution (25 mM NH_4HCO_3 with 5 ng/ µl of trypsin) was added to cover the gel pieces and incubated the mixture at 37 °C for 30 minutes. The excess enzyme solution was removed and enough 25 mM NH_4HCO_3 was added to keep the gel wet. The mixture was incubated at 37°C overnight. The peptides were extracted by adding the extraction buffer [30% ACN, 70% (0.1% Trifluoroacetic acid in water)] enough to cover the gel plugs completely. The extraction process was supported by ultrasonicating the gel mixture for a few minutes.

3.13.2. Peptide sequencing and identification

The tryptic peptides together with the HCCA matrix and the calibration standard were spotted onto the MALDI target plate and subjected to MS analysis, which was performed on a Bruker Ultraflextreme MALDI-TOF. All acquired spectra of samples were processed using protein Scape software in a default mode. The MASCOT search engine was used to search the respective protein database (NCBInr and SWISS-PROT) using MS spectral data for protein identification. Protein spots and bands with scores greater than 75 were considered statistically significant ($P<0.05$) and accepted.

CHAPTER 4

RESULTS

The following chapter focus on the proteomic analysis of the active proteins produced by both the bacterial and fungal isolates. The proteins were subjected to a battery of characterization studies which include functional characteristics, evidence of purity and structural studies (molecular mass, isoelectric points, amino acids composition, amino acids sequencing, and identification). The emphasis is based on the cellulolytic enzymes produced using cellobiose and Avicel as carbon sources.

4.1. Time course analysis for beta-glucosidase production by the bacterial isolate

The bacterial isolate BARK, which was previously collected from the hot geyser in the Zambezi Valley, Zimbabwe was cultured in M9 minimal medium for beta-glucosidase production using 5 % cellobiose as a carbon source. The highest enzyme production was found to be 3.8.U/ml after 30 hours of the incubation period.

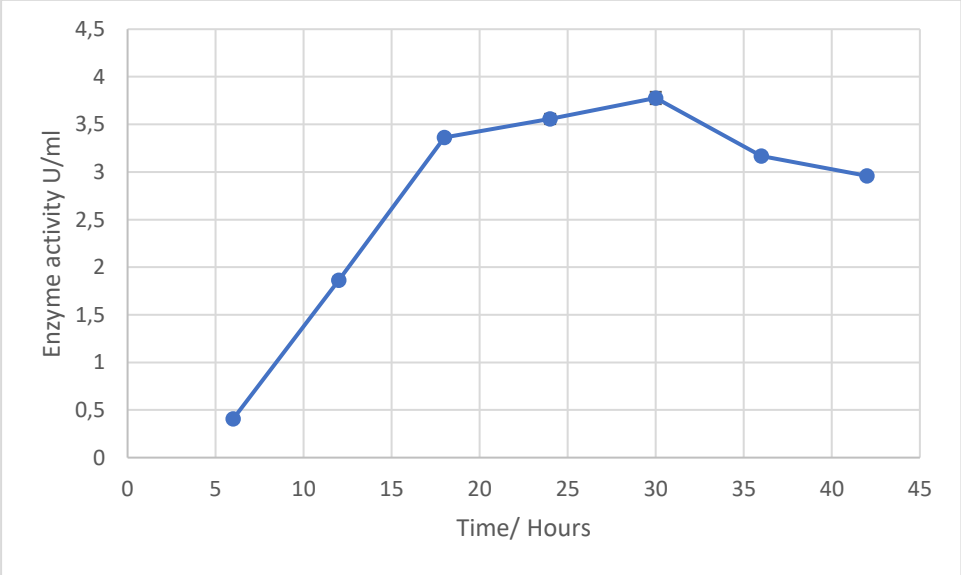

Figure 4.1: Beta-glucosidase activity from the bacterial isolate BARK at 60°C over 45 hours of incubation in M9 minimal medium.

4.2. SDS-PAGE analysis of the proteins produced by the bacterial isolate

After incubation of the bacterial isolate in a cellobiose containing medium for 45 hours at 60 °C, the secreted proteins were separated on an SDS-PAGE gel. The gel analysis focused on the 3-, 6- and 30-hour samples for comparison of the bacterial isolate BARK proteins produced during the incubation period. Figure 4.2 shows that proteins were expressed with either increased or decreased protein bands intensity over time. The intensity of protein bands relates to the quantitative expression of proteins by the microbial isolate. It can be observed that the intensity of bands increased after 3 hours, and this is due to cellobiose hydrolysis.

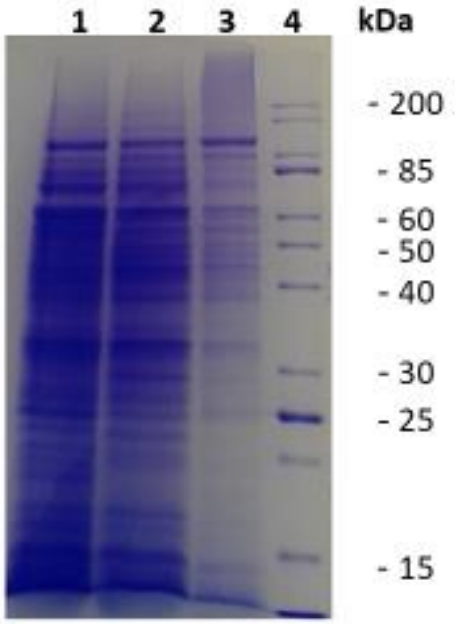

Figure 4.2: SDS-PAGE analysis of the bacterial isolate BARK crude enzyme after 30 hours of incubation. Lane 1: 30-hour sample, Lane 2: 6-hour sample, Lane 3: 3-hour sample and Lane 4: molecular weight marker.

4.3. Beta-glucosidase zymography

In-gel activity assay was performed to detect active beta-glucosidase from the bacterial isolate BARK crude sample, using esculin as a substrate. Upon hydrolysis of esculin by an active beta-glucosidase, there was the development of a brown band from a 30-hour sample with a molecular size of approximately 45 kDa (Figure 4.3).

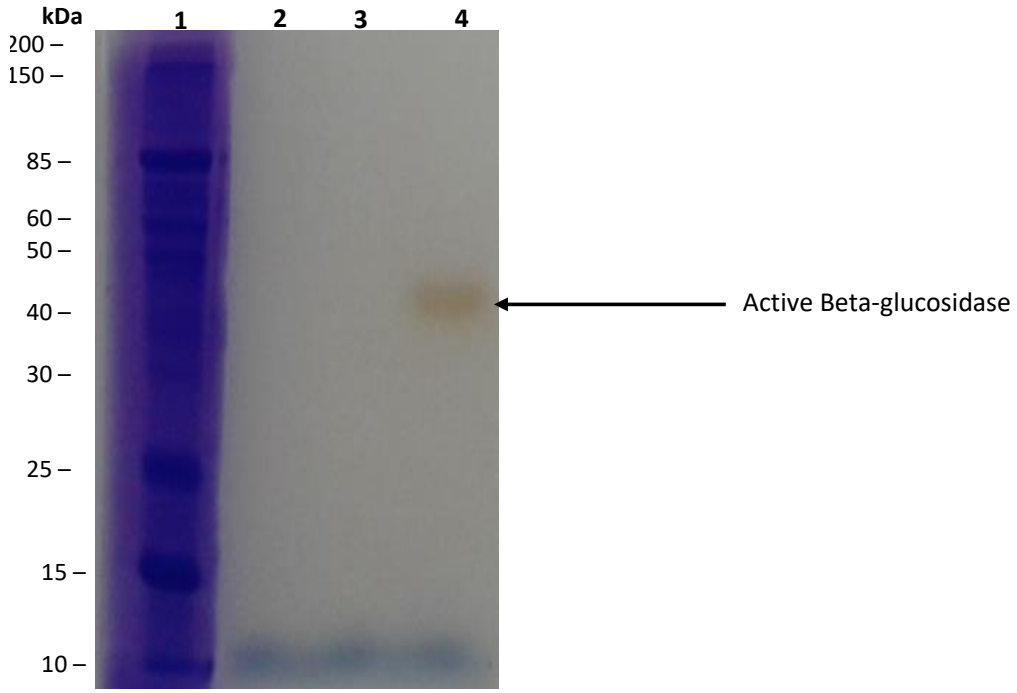

Figure 4.3: Zymogram for betaglucosidase obtained from the supernatant of the bacterial isolate BARK. Lane 1: Molecular weight marker, Lane 2: 3-hour sample, Lane 3: 6-hour sample and Lane 4: 30-hour sample.

4.4. Two-Dimensional electrophoretic gels analysis of the bacterial isolate proteins

The different proteins expressed by the bacterial isolate BARK after 3, 6 and 30 hours of incubation were first separated based on their isoelectric point (pIs) in one dimension, and then, according to their molecular weights in a second dimension. Figure 4.4 shows that the concentration of the expressed proteins by the bacterial isolate BARK increased as the incubation period increased, and their pI values ranged between units of 7 and 10.

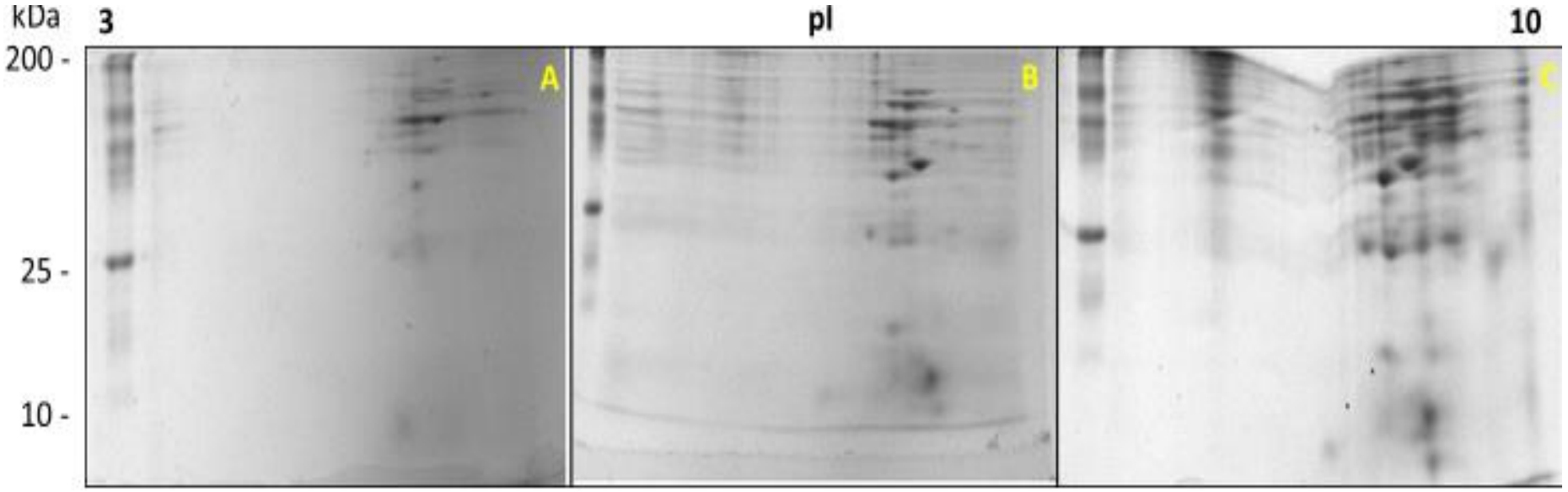

Figure 4.4: Two-dimensional electrophoresis gel analysis of the proteins expressed by the bacterial BARK isolate after 30 hours of the incubation period. A: 3-hour sample, B: 6-hour sample C:30-hour sample, 3 and 10: pI values.

4.5. Time course analysis for endoglucanase production by the fungal isolates

The fungal isolates (VCFF1, VCFF14, VCFF17, and VCFF18) previously isolated from Victoria Falls rain forest, Zimbabwe were cultured on Mandels medium for endoglucanase production using 20 % Avicel as a carbon source for 7 days at 30°C. Based on Figure 4.5, the VCFF1 isolate had its highest activity of 2.09 U/ml after 5 days, VCFF14 isolate had an activity of 3.38 U/ml after 4 days while the VCFF17 isolate had the highest activity of 3.18 U/ml after 6 days and VCFF18 isolate had the highest activity of 1.95 U/ml after 5 days of incubation.

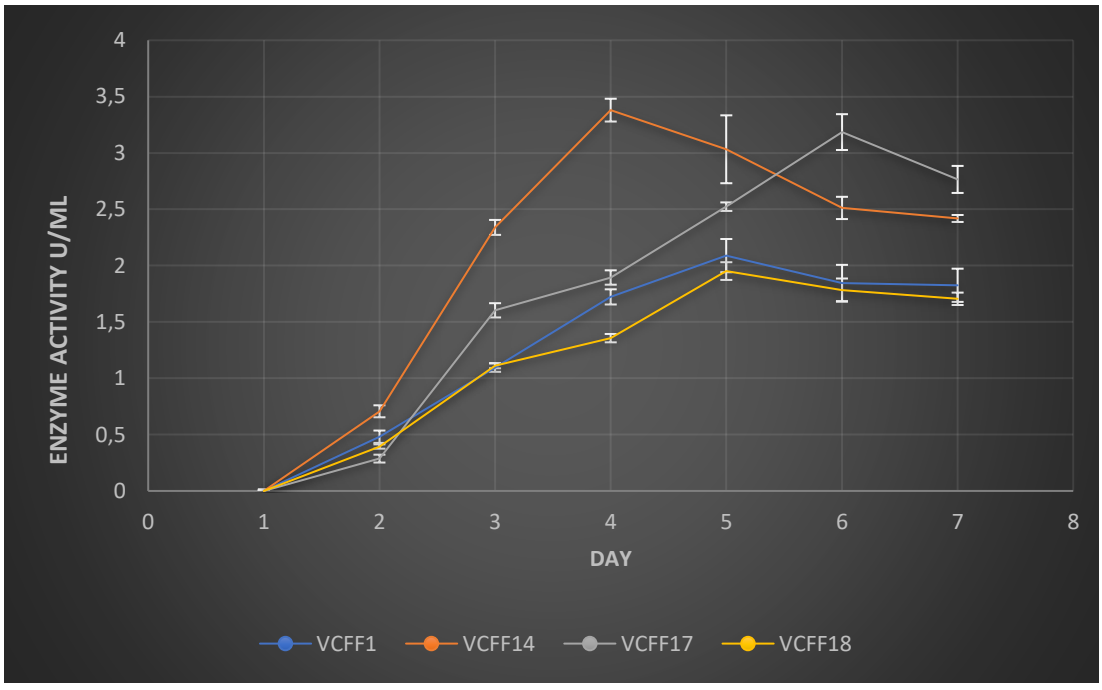

Figure 4.5: Endoglucanase activity from the fungal isolates at 30 °C over 7 days of incubation.

4.6. SDS-PAGE analysis of the proteins produced by the different fungal isolates

After incubation of the fungal isolates in an Avicel containing medium for 7 days at 30 °C, the secreted proteins were separated on an SDS-PAGE gel. The gel analysis focused on peak endoglucanase production samples from the four fungal isolates. Figure 4.6 shows protein expressed by the fungal isolates with molecular weights ranging from 11-160 kDa. The proteins were expressed differently based on the isolate's metabolic capabilities.

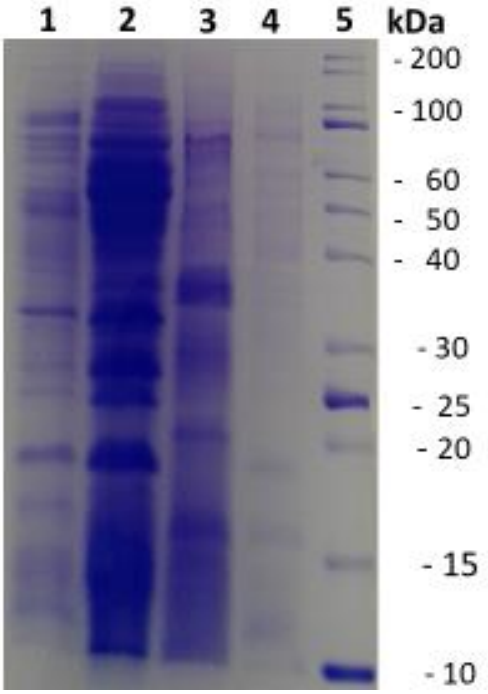

Figure 4.6: SDS-PAGE analysis of the peak endoglucanase production samples from four fungal isolates. Lane 1: VCFF1 peak sample, Lane 2: VCFF14 peak sample, Lane 3: VCFF17 peak sample, Lane 4: VCFF18 peak sample and Lane 5, molecular weight marker.

4.7. Endoglucanase zymography

Zymography of different molecular weight proteins resolved by SDS-PAGE that were active against carboxymethylcellulose (CMC) as a substrate. The lytic action of the endoglucanases produced by the fungal isolates on an SDS-PAGE gel is shown by the clear zones against a dark background (Figure 4.7 B).

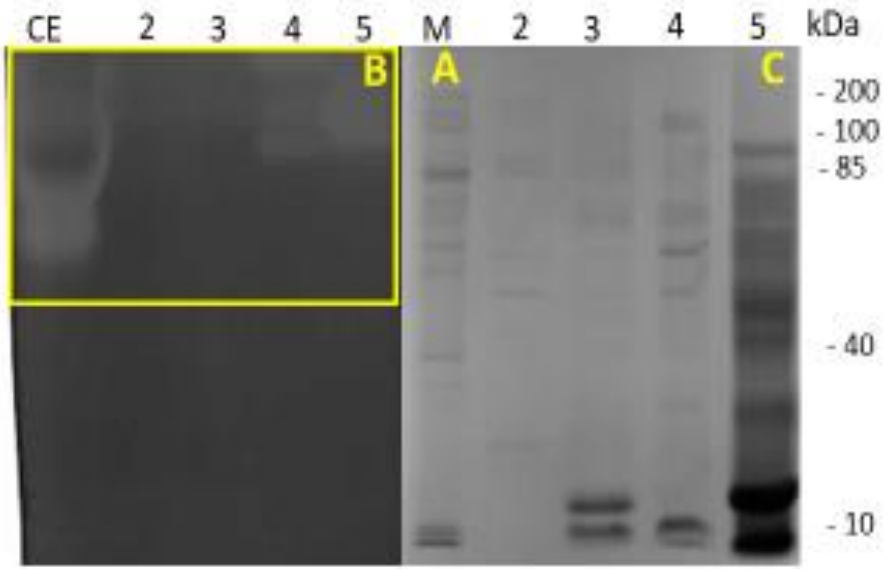

Figure 4.7: Zymography for endoglucanase and SDS-PAGE analysis from the supernatants of fungal isolates at peak enzyme production. A: molecular weight marker, B: zymogram analysis, C: SDS-PAGE of fungal isolates, CE: commercial enzyme, L2: VCFF1 sample, L3: VCFF14 sample, L4: VCFF17 sample, L5: VCFF18 sample.

4.8. Two-dimensional electrophoresis gels of the proteins expressed by the fungal isolates

The different proteins expressed by VCFF1 isolate at 24, 96 and 168 hours of incubation period in Avicel containing media were first separated based on their isoelectric point (pIs) in one dimension, and then, according to their molecular weights in a second dimension. Figure 4.8. shows that the concentration of the expressed proteins increased as the incubation period increased. The molecular sizes of the proteins ranged from 10 to 100 kDa with pI values of between 3 and 7.

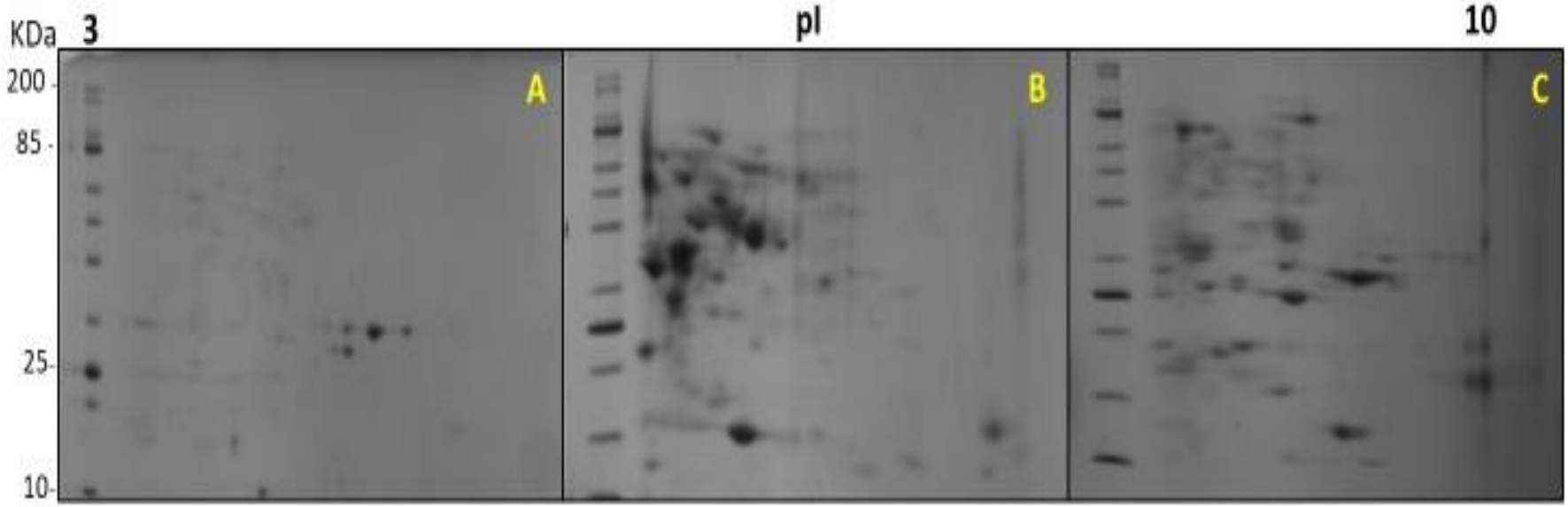

Figure 4.8: Two-dimensional electrophoresis gel analysis of the proteins expressed by the VCFF1 isolate after 168 hours of the incubation period. A: 24-hour sample, B: 120-hour sample, C: 168-hour sample, 3 and 10: pI values.

Figure 4.9 shows that the concentration of the different proteins expressed by the VCFF14 isolate at 24, 96 and 168 hours increased over time with molecular weights and pI values ranging from 10 to 100 kDa and units of between 3 and 6 respectively.

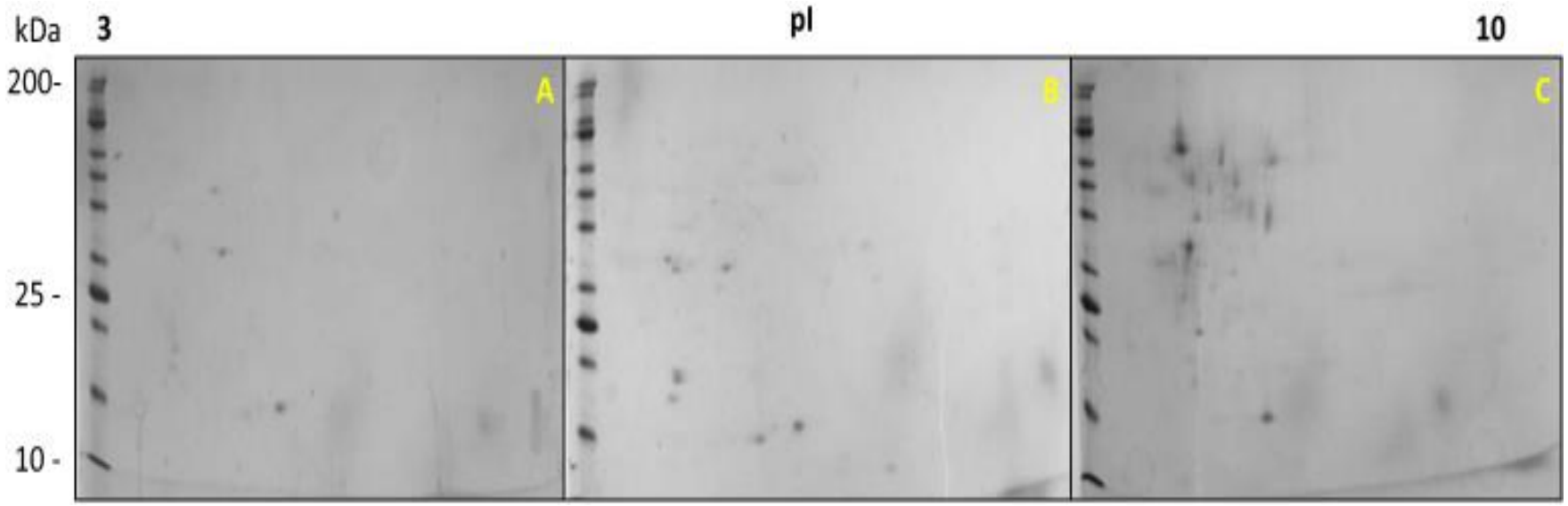

Figure 4.9: Two-dimensional electrophoresis gel analysis of the proteins expressed by the VCFF14 isolate after 168 hours of the incubation period. A: 24-hour sample, B: 96-hour sample, C: 168-hour sample, 3 and 10: pI values.

Figure 4.10 demonstrate the expression of proteins by VCFF17 isolate at 24, 144 and 168 hours of the incubation period. The protein concentration and quantity increased over time with varying molecular weights of between 10 to 150 kDa and pI values of between 3 and 6.

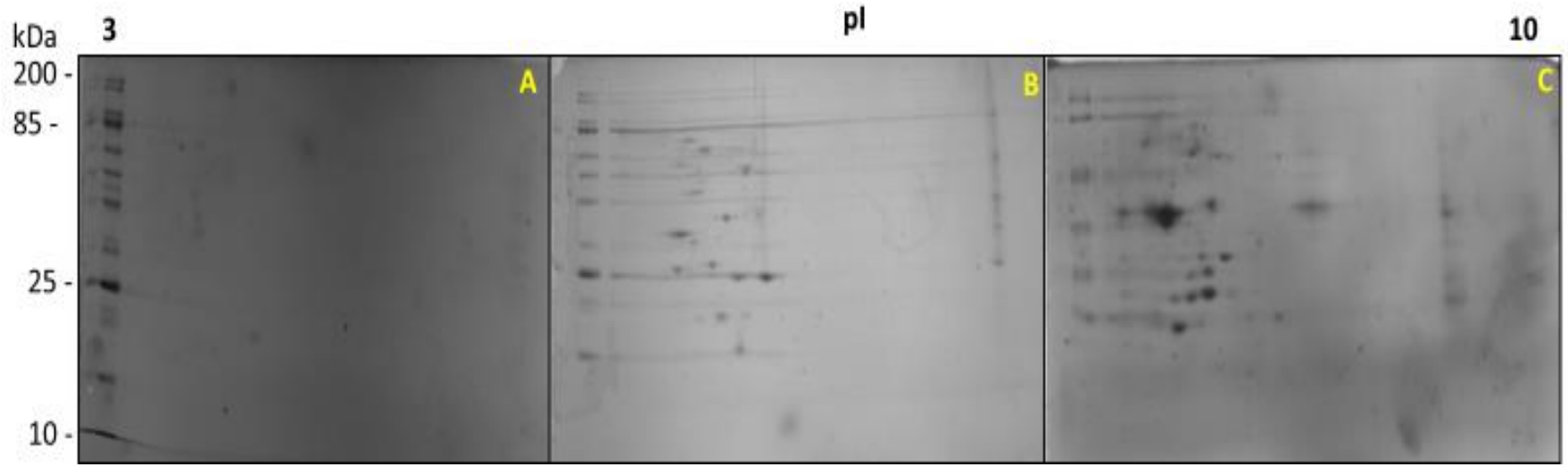

Figure 4.10: Two-dimensional electrophoresis gel analysis of proteins expressed by the VCFF17 isolate after 168 hours of the incubation period. A: 24-hour sample, B: 144-hour sample, C: 168-hour sample, 3 and 10: pI values.

The same trend was observed (Figure 4.11) in the expression of proteins by VCFF18 isolate at 24, 120 and 168 hours of the incubation period. The protein concentration and quantity increased over time with varying molecular weights of between 15 to 150 kDa and pI values of between 3 and 7.

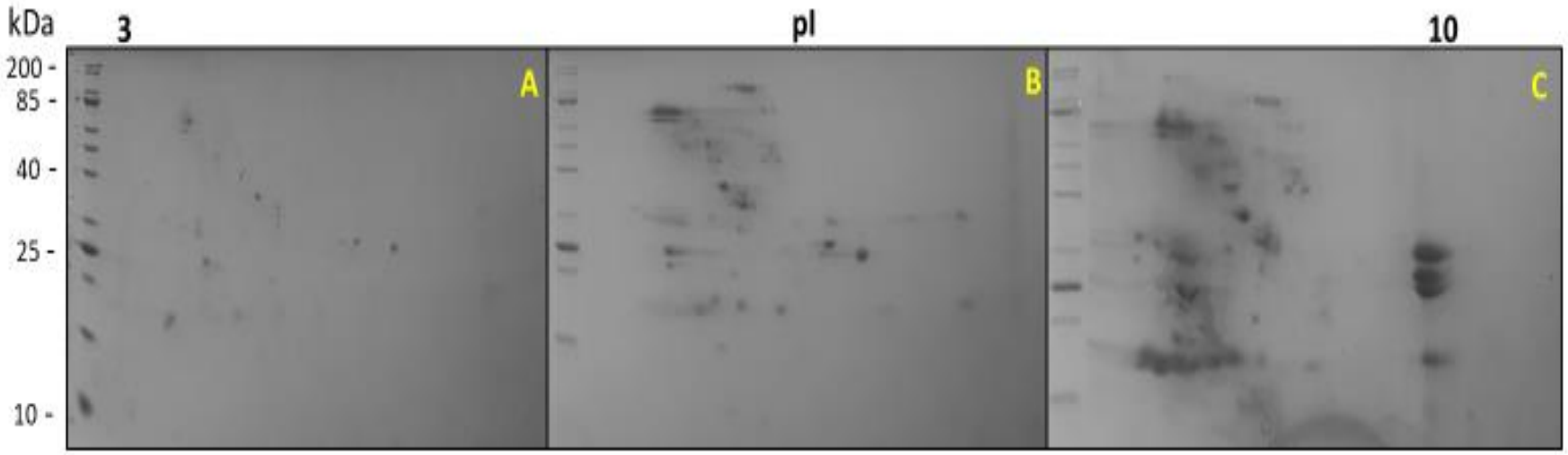

Figure 4.11: Two-dimensional electrophoresis gel analysis of proteins expressed by the VCFF18 isolate after 168 hours of the incubation period. A: 24-hour sample, B: 120-hour sample, C: 168-hour sample, 3 and 10: pI values.

4.9. SDS-PAGE analysis of the phosphoric acid-swollen Avicel purified cellulases from the fungal isolates

The cellulases expressed by the fungal isolates (VCFF1's 120 hours sample, VCFF14's 96 hours sample, VCFF's 144 hours sample and VCFF18's 120 hours sample) which were bound to the swollen Avicel were separated on an SDS-PAGE gel, visualised by Coomassie blue staining, excised and subjected to in-gel digestion by an endopeptidase (trypsin). Purification of the VCFF17 sample led to the development of seven distinct cellulase bands of varying molecular sizes on an SDS-PAGE. The absence of bands in lanes 2, 3 and 5 indicate that the fungal isolates (VCFF 1, VCFF14 and VCFF18) produce cellulases that lack the carbohydrate binding domain.

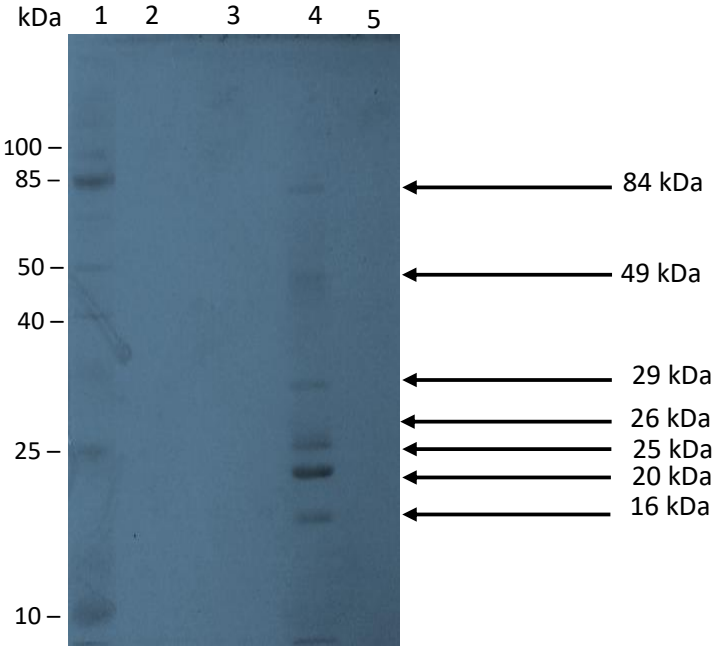

Figure 4.12: SDS-PAGE analysis of the phosphoric acid-swollen Avicel purified fungal cellulases. Lane 1: molecular weight marker, Lane 2: VCFF1 sample, Lane 3: VCFF14 sample, Lane 4: VCFF17 sample, Lane 5: VCFF18 sample.

4.10. Matrix assisted laser desorption ionisation time of flight analysis of phosphoric acid-swollen Avicel purified cellulase bands from SDS-PAGE gel for VCFF17 isolate

Peptide sequences from VCFF17 isolate's five digested bands were successfully aligned with peptide sequences of *Hypocrea jecorina*, *Trichoderma pseudokoningii*, and *Trichoderma koningii*. The sequences were aligned and identified using MASCOT and assisting sequence alignment programmes (Table 1). The peptide sequences were found to be closely related to those of glucoamylase and cel6a from *Hypocrea jecorina* with protein scores of 88.55 and 237.88, endoxylanase from *Trichoderma pseudokoningii* with a protein score of 294.11, and endoglucanase from *Trichoderma koningii* with a protein score of 268.26. Most of the conserved regions were found to belong to the glycoside hydrolase families.

Table 1: List of secreted proteins identified from VCFF17 swollen-Avicel purified sample using MASCOT and database searches.

Prot No.[a]	Accession[b]	Protein name	Scor[c]	Seq Cov (%) [d]	No. of pep[e]	MW (kDa)	Family name[f]	pI
84 kDa	gi|261825113	Glucoamylase	88.55	4.20	2	64	Glycoside hydrolase family 15	4.85
26 kDa	gi|443428055	Cel6a	237.88	10.70	4	38.80	Glycoside hydrolase family 6 and 7	4.92
25 kDa	gi|165906534	Endoxylanase	294.11	15.80	5	39	Glycoside hydrolase family 10	5.87
20 and 16 kDa	gi|21842121	Endoglucanase	268.26	14.50	3	25.30	Glycoside hydrolase family 12	5.42

[a] Protein number assigned in Mascot software.

[b] Protein accession numbers obtained from the NCBInr database search against sequences of fungal species.

[c] Protein score generated by Mascot software relating to the confidence of protein identification.

[d] Percent coverage is determined by the number of amino acids of sequenced peptides against the total length of the protein.

[e] Sequenced peptide refers to the number of peptides that were sequenced and gave rise to protein identity.

[f] Family name as predicted by NCBInr database.

4.11. CLUSTAL O sequence alignment of peptides obtained from tryptic digestion of VCFF17 isolate bands purified from affinity precipitation against the full sequence of glycoside hydrolases that were identified on MASCOT

Full sequences of *Hypocrea jecorina, Trichoderma pseudokoningii and Trichoderma koningii* were aligned with peptide sequences of VCFF17 isolate (figure 4.13, 4.14, 4.15 and 4.16). The conserved regions were found to be glycoside hydrolases.

```
VCFF17          ------------------------------------------------------------
Hypocrea        SVDDFISTETPIALNNLLCNVGPDGCRAFGTSAGAVIASPSTIDPDYYYMWTRDSALVFK

VCFF17          ------------------------------------------------------------
Hypocrea        NLIDRFTETYDAGLQRRIEQYITAQVTLQGLSNPSGSLADGSGLGEPKFELTLKPFTGNW

VCFF17          ------------------------------------------------------------
Hypocrea        GRPQRDGPALRAIALIGYSKWLINNNYQSTVSNVIWPIVRNDLNYVAQYWNQTGFDLWEE

VCFF17          ------------------------------------------------------------
Hypocrea        VNGSSFFTVANQHRALVEGATLAATLGQSGSAYSSVAPQVLCFLQRFWVSSGGYVDSNIN

VCFF17          ------------------------------------------------------------
Hypocrea        TNEGRTGKDVNSVLTSIHTFDPNLGCDAGTFQPCSDKALSNLKVVVDSFRSIYGVNKGIP

VCFF17          ------------------------------------------------------------
Hypocrea        AGAAVAIGRYAEDVYYNGNPWYLATFAAAEQLYDAIYVWKKTGSITVTATSLAFFQELVP

VCFF17          ------------------RFTETYDAGL--QRRKYVPADGSLAEQFDRN-----------
Hypocrea        GVTAGTYSSSSSTFTNIINAVSTYADGFLSEAAKYVPADGSLAEQFDRNSGTPLSALHLT
                                  . ..**  *:  :  ****************

VCFF17          ------------------------------------------------------------
Hypocrea        WSYASFLTATARRAGIVPPSWANSSASTIPSTCSGASVVGSYSRPTATSFPPSQTPKPGV

VCFF17          ------------------------------------------------------------
Hypocrea        PSGTPYTPLPCATPTSVAVTFHELVSTQFGQTVKVAGNAAALGNWSTSAAVALDAVNYAD

VCFF17          ------------------------------------------------------------
Hypocrea        NHPLWIGTVNLEAGDVVEYKYINVGQDGSVTWESDPNHTYTVPAVACVTQVVKEDTWQS
```

Figure 4.13: CLUSTAL O (https://www.ebi.ac.uk/Tools/msa/clustalo/) sequence alignment of peptides obtained from tryptic digestion of VCFF17 isolate (figure 4.12, 84 kDa band) against the full sequence of glucoamylase from *Hypocrea jecorina*.

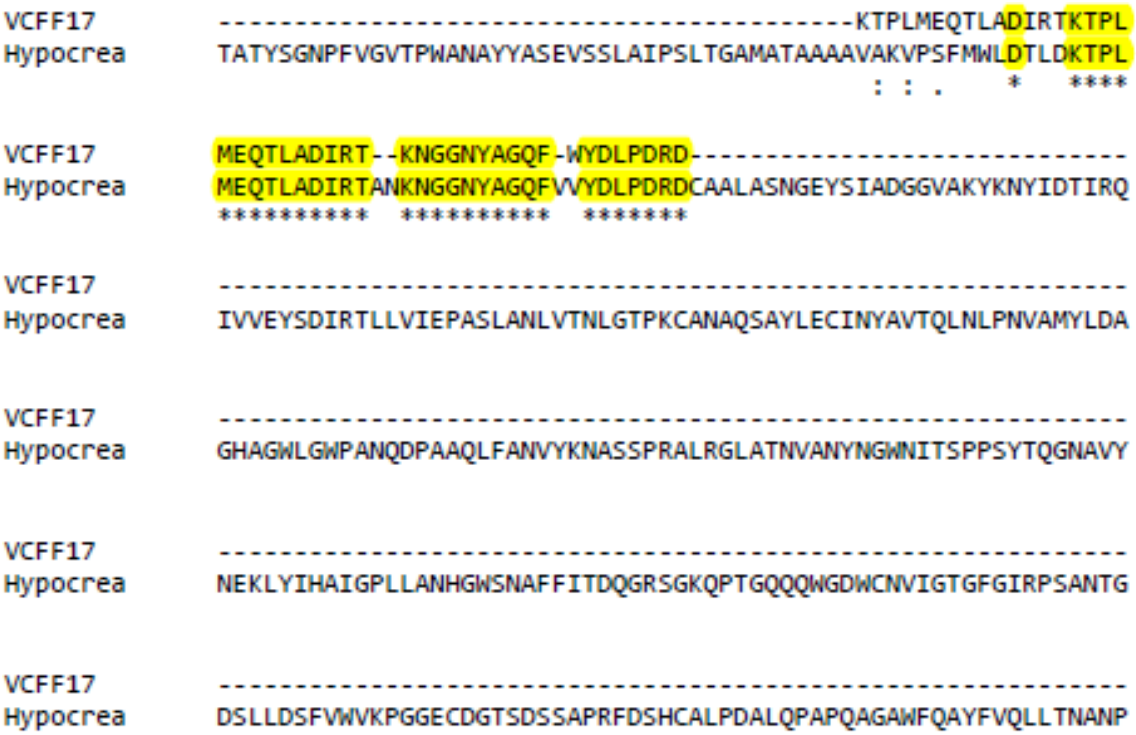

Figure 4.14: CLUSTAL O (https://www.ebi.ac.uk/Tools/msa/clustalo/) sequence alignment of peptides obtained from tryptic digestion of VCFF17 isolate (figure 4.12, 26 kDa band) against the full sequence of Cel6a from *Hypocrea jecorina*.

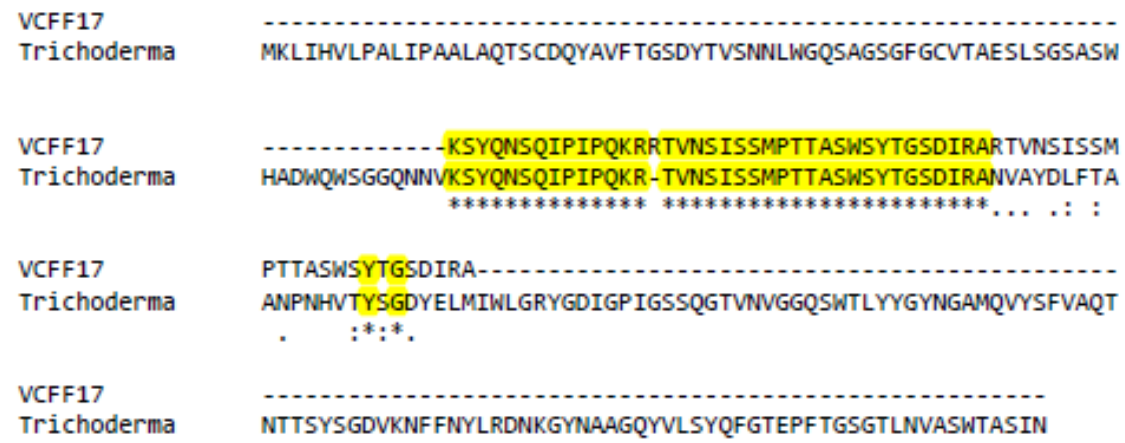

Figure 4.15: CLUSTAL O (https://www.ebi.ac.uk/Tools/msa/clustalo/) sequence alignment of peptides obtained from tryptic digestion of VCFF17 isolate (figure 4.12, 20 kDa band) against the full sequence of endoglucanase from *Trichoderma koningii*.

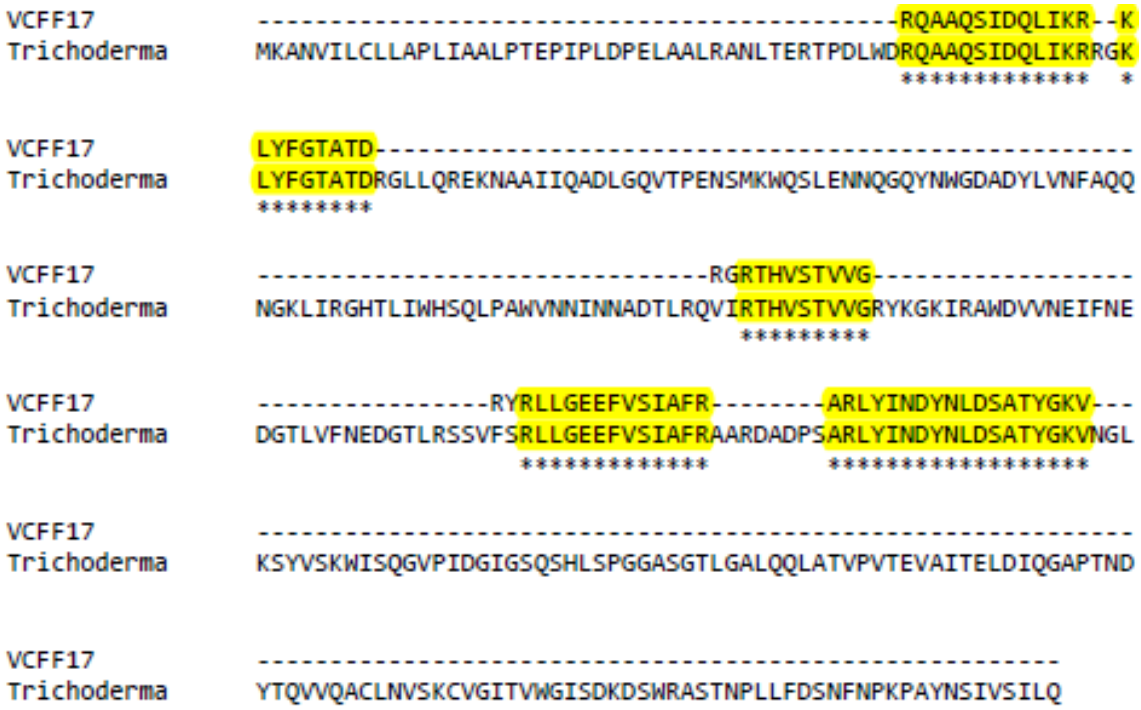

Figure 4.16: CLUSTAL O (https://www.ebi.ac.uk/Tools/msa/clustalo/) sequence alignment of peptides obtained from tryptic digestion of VCFF17 isolate (figure 4.12, 25 kDa band) against the full sequence of endoxylanase from *Trichoderma pseudokoningii*.

CHAPTER 5

DISCUSSION

Environmental sustainability has taken a centre stage in human life, all over the world. The conventional sources of energy (fossil) are non-renewable and have detrimental environmental impact, hence the need to move from conventional to non-conventional renewable energy sources (biofuels) to satisfy the world's energy demands (Srivastava *et al*.,2018). The high abundance and ubiquitous nature of lignocellulosic biomass opened paths for the biomass to be used for benefit of mankind. Bioethanol from lignocellulosic biomass through the enzymatic route has the potential to replace gasoline in petroleum (Vaishnav *et al*., 2018). Even though a lot of research has and is still being done on the production of bioethanol from lignocellulosic biomass, economic feasibility remains a question and this is due to the lack of novel cellulases with high specific activity acting on crystalline cellulose, and the high cost of cellulase production. Hence it is imperative to screen for potentially novel microbial cellulolytic enzymes for commercial viability using different approaches such as proteomics.

Five isolates namely VCFF1, VCFF14, VCFF17, VCFF18, and bacterial isolate BARK were screened for cellulase activity using Avicel and cellobiose as carbon sources through the submerged fermentation for 168 hours at 30°C and 60°C. The enzyme activity was confirmed using endoglucanase and betaglucosidase assays. VCFF14 produced endoglucanase with the highest activity of 3.38 U/ml after 96 hours of incubation period and VCFF18 was the least endoglucanase producer with the enzyme activity of 1.95 U/ml after 120 hours of incubation time. Vaishnav *et al.,* (2018) stated that fungi are the most potent cellulase producers, using an established cellulase producer *Trichoderma reesei* as an example, Vaishnav *et al*., 2018 observed that even though the fungus produces cellulases maximally, the amount of beta-glucosidases produced is inadequate for effective hydrolysis of cellulose to fermentable glucose molecules, which are further fermented for bioethanol production. In this context, the present study opted to produce beta-glucosidase using a bacterial isolate BARK which resulted in beta-glucosidase activity of 3.8 U/ml after 30 hours of incubation period in a cellobiose containing medium.

The beta-glucosidase amount (3.8 U/ml) produced in this study is high compared to 1.95 U/ml produced by the fungus *Penicillium* sp using carboxymethylcellulose as a substrate (Prasanna *et al.,* 2016), 0.9 U/ml by *Trichoderma reesei* using microcrystalline cellulose as a substrate and 0.6 U/ml achieved by *Trichoderma harzianum* IOC 3844 cultured on partially delignified cellulignin from sugarcane bagasse (Passos *et al*.,2018). The results show the potential of the beta-glucosidase from bacterial isolate BARK to be used in cellulase cocktails for biomass hydrolysis during bioethanol production.

In addition to the culturing conditions, the substrate used also plays a major role for the production of cellulases. A good substrate is easily digestible by microorganisms and has the potential to induce cellulase production ((Pandey *et al*., 2016; Li *et al*., 2019). It is observed from the cellulase production profiles that an increase in incubation time leads to a decrease in enzyme production and this might be due to the proteolytic activity of proteases produced by the microorganism during growth, change in pH, decrease in substrate concentration and accumulation of inhibiting products (*Zhang et al*., 2019).

In-gel activity for beta-glucosidase and endoglucanase was estimated using esculin and CMC zymogram assays. The in-gel activity assays were performed to validate the enzyme activity assays and to prove the presence of the enzymes of interest in the crude samples. Figure 4.3 represents a beta-glucosidase zymogram obtained from the supernatant of the bacterial isolate BARK, out of three analysed samples only the 30-hour sample showed an activity band of approximately 45 kDa in size that appeared brown against a clear background. The brown colour developed due to the reaction of esculetin and ferric ion. Esculetin is a product of the hydrolytic activity of betaglucosidase on esculin. The 3- and 6-hour samples showed no lytic action on esculin and this might be due to the bacterial isolate still adjusting and adapting to the culturing conditions at that time. The bacterial isolate might have been synthesising the necessary enzymes (beta-glucosidase) required for the breakdown of the substrate (cellobiose). Figure 4.7 represents endoglucanase zymogram obtained from the supernatants of the four fungal isolates, the hydrolytic sites are shown by the presence of clear zones against a dark background on an SDS-PAGE.

The clear zones appear due to the hydrolytic activity of endoglucanases on carboxymethylcellulose. VCFF1 and VCFF14 showed two activity bands of approximately 120 and 160 kDa in sizes. VCFF17 and 18 showed three activity bands of approximately 85, 120 and 160 kDa. The fungal isolates produced cellulases of different molecular masses which ranged from 85-160 kDa. Literature reports molecular masses of beta-glucosidases from various microorganisms range from 45-250 kDa (Bala and Singh,2018), whereas the molecular masses of endoglucanases are reported to vary from 11-250 kDa (Santa-Rosa *et al*., 2018). The sizes of the cellulases produced by both the fungal and bacterial isolates in the present study are within the reported range of previously isolated and studied cellulases from various microorganisms.

Production of various forms of cellulases by the isolates is advantageous because their different biochemical properties can support higher degradation of the complex plant biomass. The different masses also suggest that the cellulases may not be of the same families and might differ with their substrate specificities, conferring the enzymes an advantage to be applied in various bioprocesses: textile, pulp and paper, detergents and in animal feed industries (Kumar *et al*., 2018; Sankarraj and Nallathambi, 2018). The endoglucanase zymogram results (Figure 4.7) are in line with previous studies that stated that many filamentous fungi produce multiple endoglucanases with different molecular masses (Santa-Rosa *et al*., 2018). The concentration of an enzyme in a sample and assay conditions play an important role during an in-gel activity assay. If the concentration is low, and conditions are not optimal, the activity of an enzyme will not be visible on a gel. On the other hand, indistinctive bands or smear-like appearance will be visible if the concentration of an enzyme is high. In this context, optimal conditions are of high importance during enzyme activity assays.

To assess if the produced cellulases by the fungal isolates had the carbohydrate-binding modules, affinity precipitation analysis was done using phosphoric acid swollen-Avicel. The endoglucanase peak production time samples from the fungal isolates (VCFF1's 120 hours sample, VCFF14's 96 hours sample, VCFF17's 144 hours sample, and VCFF18's 120 hours sample) were concentrated and purified using the swollen Avicel. Seven distinct bands appeared only in the lane that

contained the VCFF17 sample. This shows that VCFF17 isolate could produce different glycoside hydrolases with a carbohydrate-binding module. The presence of the carbohydrate-binding module in the produced cellulases by the VCFF17 isolate gives the isolate the potential to be used for hydrolysis of lignocellulosic biomass for the production of bioethanol and aid the isolate to display greater activity in the hydrolysis of crystalline cellulose. The molecular sizes of the produced glycoside hydrolases obtained from the VCFF17 isolate ranged from 16- 84 kDa. The phosphoric acid swollen effect during affinity precipitation decreased the microcrystalline cellulose (Avicel) structure and increased the number and size of micropores, generating surface area for cellulases to bind and act.

The combination of electrophoretic separation and mass spectrometric analysis is considered to be a very powerful tool for protein analysis (Lakshmi *et al*., 2018). The differences in protein maps between three related samples from each isolate were assessed using a bottom-up proteomic approach that involved initial protein recovery, concentration, purification, electrophoretic separation, endopeptidase digestion, peptide sequencing, and identification. Protein concentrations of between 66-280 µg from each isolate were analysed by two-dimensional (2-D) gel electrophoresis. The 2-D electrophoretic gel analysis revealed the quantitative variation of several protein spots that were increased in the after-peak endoglucanase and beta-glucosidase production time samples. The differences in protein expression between the three related samples from each isolate might be due to the differences in the metabolic capabilities and growth patterns of the isolates. Based on the 2-D gel electrophoresis, it can be said that most of the bacterial isolate BARK proteins' optimum pH is at neutral to basic pH since the isoelectric point (pI) values of most of its predominantly expressed proteins were found to be between units of 7 and 10, whereas the pH of the proteins from the fungal isolates lie more towards the acidic side since their pI values ranged between units of 3 and 6.

pH is an important factor for the stability of enzymes and needs to be controlled for the effective functioning of an enzyme if it is too high or too low it generally leads to loss of activity for most enzymes. The optimum pH value varies greatly from one enzyme to another as shown by the 2-D electrophoresis protein maps (figure 4.4,

4.8, 4.9, 4.10 and 4.11). The differences in optimum pH values of enzyme activity depend on the microbial species, enzyme purity, buffers and substrates (Carvalho and Orlanda, 2017).

Two-Dimensional gel electrophoresis is a protein expression profiling technique capable of separating thousands of proteins in a single gel (Goez *et al*., 2018). This technique has the advantage of separating protein isoforms that differ by a pI of 0.001 pH units and identify specific chemical structures or modifications present in the protein of interest. Since proteins separated by this technique are nearly pure, they can be excised and subjected to tryptic digestion for sequencing and identification by mass spectrometry (Lohnes *et al*., 2016). Though is a very sensitive technique with high resolving power and a preferred method for carrying out proteomic analysis studies, the technique requires a lot of optimisation (Jorrin-Novo *et al*., 2018).

MALDI-TOF analysis identified five protein bands from swollen-Avicel affinity precipitation to be different glycoside hydrolases (Table 1) with an acceptable confidence level of 100%. MASCOT and other related database searches (SwissProt and NCBInr) positively identified the bands labelled as 84 kDa, 26 kDa, 25 kDa, 20 kDa and 16 kDa (Figure 4.12). The identified bands, 84 kDa was found to be related to glucoamylase family 15 from *Hypocrea jecorina* (teleomorph *Trichoderma reesei*) by two peptides with a score of 88.55. This shows that VCFF 17 isolate may be used in starch-based bioethanol production. The 26 kDa band from VCFF17 isolate was found to be closely related to cel6a (NCBInr) with four peptides match and a score of 237.88 from *H. jecorina*. Cel6a is an important cellobiobydrolase (CBHs) that plays an important role in the biodegradation of lignocellulose. CBHs are divided into two types, CBH I and CBH II mostly belonging to glycoside hydrolase families 6 and 7. CBHs catalyse the hydrolysis of the non-reducing end β-1,4-glucosidic linkages in cellulose to release cellobiose (Gao *et al*., 2011).

The band with a molecular size of 25 kDa from VCFF17 isolate was found to be related to endoxylanase with four peptides match and a score of 294.11 from *Trichoderma pseudokoningii* and similar to endo-1,4-β-xylanase with one peptide match and a score of 58.32 from *Emericella nidulans* and closely related to glycoside hydrolase family 10 with three matching peptides and a score of 138.50 from *T.*

reesei (results not shown). Endoxylanases are cellulolytic enzymes that break down the heterogeneous polysaccharide xylan in plant tissues (Knott *et al*., 2014).

The 20 and 16 kDa bands obtained from VCFF17 isolate were found to closely relate to endoglucanase with a score of 268.26 from *Trichoderma koningii*. *T. koningii* is one of the filamentous fungi known to be best in the production of cellulolytic enzymes (Wang *et al*., 2013). Endoglucanases forms part of the cellulolytic enzyme complex. The enzyme plays a significant role during plant biomass degradation by acting on the internal β-1,4-glycosidic bonds at amorphous regions. The pI values of the related glycoside hydrolases from MASCOT search ranged between 4-6 with molecular size ranges of between 25-64 kDa. The pI values are in line with the values of this study, further confirming that most fungal species produce enzymes that function optimally at low acidic pH values (Singhania *et al*., 2017). Glycoside hydrolases from VCFF17 isolate show potential to be used in the hydrolysis of plant biomass for bioethanol production.

CHAPTER 6

CONCLUSION

The current study shows that proteomic approaches may be used to screen for cellulolytic and other enzymes from a microorganism. Zymography, affinity precipitation, and gel electrophoresis analysis resulted in the separation of different quantitatively expressed cellulolytic and other enzymes from three related microbial samples. The bacterial isolate BARK and four fungal isolates VCFF1, VCFF14, VCFF17, and VCFF18 have great potential as cellulolytic enzyme producers in bioethanol production. BARK isolate presented the highest beta-glucosidase activity of 3.8 U/ml while VCFF14 presents the highest endoglucanase activity of 3.38 U/ml. MALDI-TOF sequencing and peptides identification indicated that the expressed cellulolytic enzymes closely relate to different glycoside hydrolases expressed by other microorganisms. The ability to produce different glycoside hydrolases by the five studied microbial isolates, confer the isolates a potential to be considered in bioethanol production from lignocellulolytic biomass and starch-based industries. The use of the cellulolytic enzymes produced by the fungal isolates (VCFF1, VCFF14, VCFF17 and VCFF18) supplemented with the betaglucosidase from the bacterial isolate BARK may result in efficient hydrolysis of the cellulose structure to fermentable glucose molecules. The isolates from this study may be of benefit to the industry in reducing the costs associated with bioethanol production through the consolidated bioprocessing system.

RECOMMENDATIONS

1. Identification of the microbial isolates.
2. MALDI-TOF sequencing and identification of protein spots from the 2-D electrophoresis
3. Further studies of the isolates cellulolytic enzymes using native cellulose rather than artificial cellulosic substrates.
4. Heterologous expression of the cellulase genes in a suitable microorganism for bioethanol production through the consolidated bioprocessing system.

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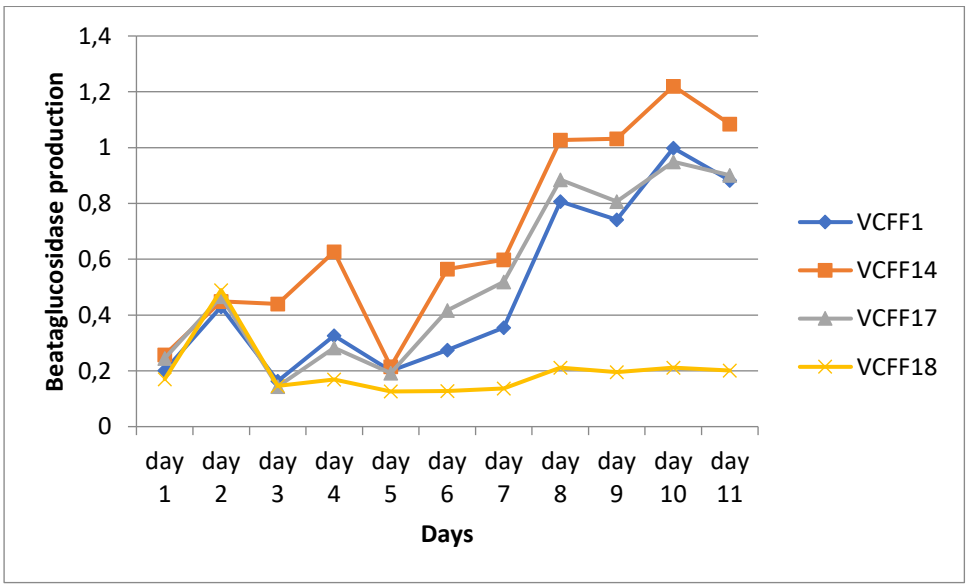
APPENDICES

Appendix 1

Beta-glucosidase production curve by the fungal isolates (VCFF1, 14, 17 and 18).



Appendix 1-Figure 1: beta-glucosidase activity by four fungal isolates at 30°C over 11 days of incubation.

Matrix assisted laser desorption ionisation time of flight analysis of phosphoric acid-swollen Avicel purified cellulase bands from SDS-PAGE gel

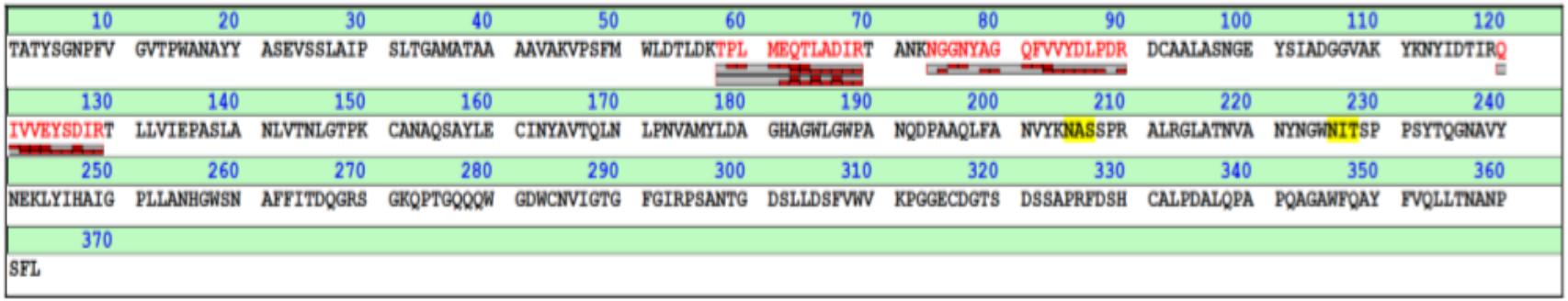
Protein 1:	Chain A, Glycoside Hydrolase Family 15 Glucoamylase From Hypocrea Jecorina		
Accession:	gi|261825113	**Score:**	88.55
Database:	NCBInr	**MW [kDa]:**	64.00
Seq. Coverage [%]:	4.20 %	**pI:**	4.85
		No. of Peptides:	2

10	20	30	40	50	60	70	80	90	100	110	120
SVDDFISTET	PIALNNLLCN	VGPDGCRAFG	TSAGAVIASP	STIDPDYYYM	WTRDSALVFK	NLIDRFTETY	DAGLQRRIEQ	YITAQVTLQG	LSNPSGSLAD	GSGLGEPKFE	LTLKPFTGNW
130	**140**	**150**	**160**	**170**	**180**	**190**	**200**	**210**	**220**	**230**	**240**
GRPQRDGPAL	RAIALIGYSK	WLINNNYQST	VSNVIWPIVR	NDLNYVAQYW	NQTGFDLWEE	VNGSSFFTVA	NQHRALVEGA	TLAATLGQSG	SAYSSVAPQV	LCFLQRFWVS	SGGYVDSNIN
250	**260**	**270**	**280**	**290**	**300**	**310**	**320**	**330**	**340**	**350**	**360**
TNEGRTGKDV	NSVLTSIHTF	DPNLGCDAGT	FQPCSDKALS	NLKVVVDSFR	SIYGVNKGIP	AGAAVAIGRY	AEDVYYNGNP	WYLATFAAAE	QLYDAIYVWK	KTGSITVTAT	SLAFFQELVP
370	**380**	**390**	**400**	**410**	**420**	**430**	**440**	**450**	**460**	**470**	**480**
GVTAGTYSSS	SSTFTNIINA	VSTYADGFLS	EAAKYVPADG	SLAEQFDRNS	GTPLSALHLT	WSYASFLTAT	ARRAGIVPPS	WANSSASTIP	STCSGASVVG	SYSRPTATSF	PPSQTPKPGV
490	**500**	**510**	**520**	**530**	**540**	**550**	**560**	**570**	**580**	**590**	**600**
PSGTPYTPLP	CATPTSVAVT	FHELVSTQFG	QTVKVAGNAA	ALGNWSTSAA	VALDAVNYAD	NHPLWIGTVN	LEAGDVVEYK	YINVGQDGSV	TWESDPNHTY	TVPAVACVTQ	VVKEDTWQS

Cmpd.	No. of Cmpds.	m/z meas.	Δ m/z [ppm]	z	Rt [min]	Score	Site [%]	P	Range	Sequence	Modification	Type
	1	1300.5984	-14.16	1		33.62		0	66-76	R.FTETYDAGLQR.R		CID
	1	1567.7286	-6.42	1		54.93		0	395-408	K.YVPADGSLAEQFDR.N		CID

Appendix 1- Figure 2: Sequence alignment of peptides obtained from tryptic digestion of VCFF17's 84 kDa band against the full sequence of glucoamylase from *Hypocrea jecorina* that was identified by MASCOT.

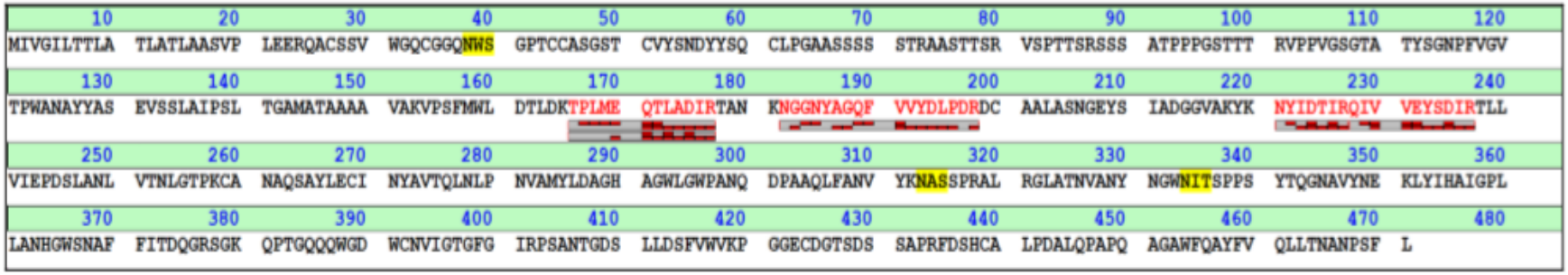
Protein 1:	Chain A, Hypocrea Jecorina Cel6a D221a Mutant Soaked With 6-chloro-4- Methylumbelliferyl-beta-cellobioside		
Accession:	gi|443428055	**Score:**	237.88
Database:	NCBInr	**MW [kDa]:**	38.80
Seq. Coverage [%]:	10.70 %	**pI:**	4.92
		No. of Peptides:	4
Modification(s):	Oxidation, Deamidated		



Cmpd.	No. of Cmpds.	m/z meas.	Δ m/z [ppm]	z	Rt [min]	Score	Site [%]	P	Range	Sequence	Modification	Type
	1	1387.6807	-31.92	1		36.89		0	58-69	K.TPLMEQTLADIR.T		CID
	1	1403.6751	-31.91	1		81.80		0	58-69	K.TPLMEQTLADIR.T	Oxidation: 4	CID
	1	1885.8069	-34.25	1		71.97	99.76	0	74-90	K.NGGNYAGQFVVYDLPDR.D	Deamidated: 8	CID
	1	1221.6117	-29.21	1		47.22		0	120-129	R.QIVVEYSDIR.T		CID

Appendix 1- Figure 3: Sequence alignment of peptides obtained from tryptic digestion of VCFF17's 26 kDa band against the full sequence of Cel6a from *Hypocrea jecorina* that was identified by MASCOT

Protein 1:	Exoglucanase 2 OS=Hypocrea jecorina GN=cbh2 PE=1 SV=1		
Accession:	GUX2_HYPJE	**Score:**	256.55
Database:	SwissProt	**MW [kDa]:**	49.60
Seq. Coverage [%]:	9.80 %	**pI:**	5.11
		No. of Peptides:	5
Modification(s):	Oxidation, Deamidated		



Cmpd.	No. of Cmpds.	m/z meas.	Δ m/z [ppm]	z	Rt [min]	Score	Site [%]	P	Range	Sequence	Modification	Type
	1	1387.6807	-31.92	1		36.89		0	166-177	K.TPLMEQTLADIR.T		CID
	1	1403.6751	-31.91	1		81.80		0	166-177	K.TPLMEQTLADIR.T	Oxidation: 4	CID
	1	1885.8069	-34.25	1		71.97	99.76	0	182-198	K.NGGNYAGQFVVYDLPDR.D	Deamidated: 8	CID
	1	894.4335	-38.52	1		18.67		0	221-227	K.NYIDTIR.Q		CID
	1	1221.6117	-29.21	1		47.22		0	228-237	R.QIVVEYSDIR.T		CID

Appendix 1- Figure 4: Sequence alignment of peptides obtained from tryptic digestion of VCFF17's 26 kDa band against the full sequence of Exoglucanase from *Hypocrea jecorina* that was identified by MASCOT and SWISS-PROT database.

Protein 1:	endoxylanase [Trichoderma pseudokoningii]		
Accession:	gi|165906534	**Score:**	294.11
Database:	NCBInr	**MW [kDa]:**	39.00
Seq. Coverage [%]:	15.80 %	**pI:**	5.87
		No. of Peptides:	5
Modification(s):	Gln->pyro-Glu		

```
        10         20         30         40         50         60         70         80         90        100        110        120
MKANVILCLL APLIAALPTE PIPLDPELAA LRANLTERTP DLWDRQAAQS IDQLIKRRGK LYFGTATDRG LLQREKNAAI IQADLGQVTP ENSMKWQSLE NNQGQYNWGD ADYLVNFAQQ
       130        140        150        160        170        180        190        200        210        220        230        240
NGKLIRGHTL IWHSQLPAWV NNINNADTLR QVIRTHVSTV VGRYKGKIRA WDVVNEIFNE DGTLVFNEDG TLRSSVFSRL LGEEFVSIAF RAARDADPSA RLYINDYNLD SATYGKVNGL
       250        260        270        280        290        300        310        320        330        340        350        360
KSYVSKWISQ GVPIDGIGSQ SHLSPGGASG TLGALQQLAT VPVTEVAITE LDIQGAPTND YTQVVQACLN VSKCVGITVW GISDKDSWRA STNPLLFDSN FNPKPAYNSI VSILQ
```

Cmpd.	No. of Cmpds.	m/z meas.	Δ m/z [ppm]	z	Rt [min]	Score	Site [%]	P	Range	Sequence	Modification	Type
	1	1197.6318	-13.01	1		60.86		0	46-56	R.QAAQSIDQLIK.R	Gln->pyro-Glu: 1	CID
	1	1043.5039	-11.23	1		45.29		0	61-69	K.LYFGTATDR.G		CID
	1	955.5244	-7.86	1		22.63		0	155-163	R.THVSTVVGR.Y		CID
	1	1380.7417	-7.60	1		66.16		0	200-211	R.LLGEEFVSIAFR.A		CID
	1	1749.7965	-20.88	1		99.17		0	222-236	R.LYINDYNLDSATYGK.V		CID

Appendix 1- Figure 5: Sequence alignment of peptides obtained from tryptic digestion of VCFF17's 25 kDa band against the full sequence of endoxylanase from *Trichoderma pseudokoningii* that was identified by MASCOT and NCBInr.

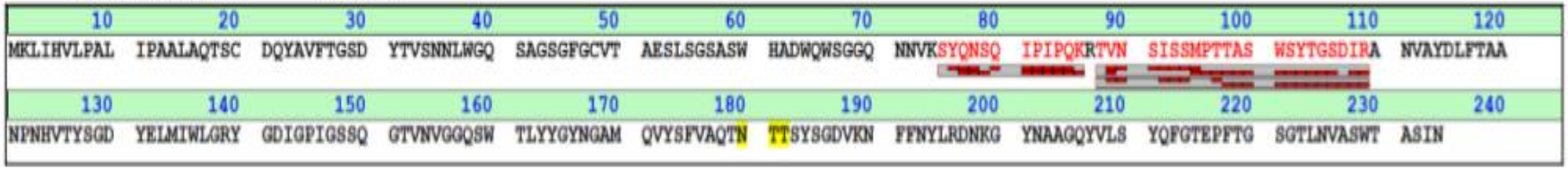
Protein 1:	Endo-1,4-beta-xylanase C OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) GN=xlnC PE=1 SV=1		
Accession:	XYNC_EMENI	**Score:**	74.14
Database:	SwissProt	**MW [kDa]:**	35.40
Seq. Coverage [%]:	4.60 %	**pI:**	5.02
		No. of Peptides:	1

10	20	30	40	50	60	70	80	90	100	110	120
MVHLKTLAGS	AVFASLATAA	VLPRQSASLN	DLFVAAGKSY	FGTCSDQALL	QNSQNEAIVA	SQFGVITPEN	SMKWDALEPS	QGNFGWSGAD	YLVDYATQHN	KKVRGHTLVW	HSQLPSWVSS
130	**140**	**150**	**160**	**170**	**180**	**190**	**200**	**210**	**220**	**230**	**240**
IGDANTLRSV	MTNHINEVVG	RYKGKIMHWD	VVNEIFNEDG	TFRNSVFYNL	LGEDFVRIAF	ETARAADPDA	KLYINDYNLD	SASYAKTQAM	ASYVKKWLAE	GVPIDGIGSQ	AHYSSSHWSS
250	**260**	**270**	**280**	**290**	**300**	**310**	**320**	**330**			
TEAAGALSSL	ANTGVSEVAI	TELDIAGAAS	SDYLNLLNAC	LNEQKCVGIT	VWGVSDKDSW	RASDSPLLFD	GNYQPKDAYN	AIVNALS			

Cmpd.	No. of Cmpds.	m/z meas.	Δ m/z [ppm]	z	Rt [min]	Score	Site [%]	P	Range	Sequence	Modification	Type
	1	1749.7965	-20.88	1		74.14		0	192-206	K.LYINDYNLDSASYAK.T		CID

Appendix 1- Figure 6: Sequence alignment of peptides obtained from tryptic digestion of VCFF17's 25 kDa band against the full sequence of endo-1,4-beta-xylanase from *Emericella nidulans* that was identified by MASCOT and SWISS-PROT database.

Protein 1:	endoglucanase [Trichoderma koningii]		
Accession:	gi|21842121	**Score:**	268.26
Database:	NCBInr	**MW [kDa]:**	25.30
Seq. Coverage [%]:	14.50 %	**pI:**	5.42
		No. of Peptides:	3
Modification(s):	Oxidation		



Cmpd.	No. of Cmpds.	m/z meas.	Δ m/z [ppm]	z	Rt [min]	Score	Site [%]	P	Range	Sequence	Modification	Type
	1	1402.6796	-37.69	1		52.88		0	75-86	K.SYQNSQIPIPQK.R		CID
	1	2377.0159	-34.40	1		72.58		0	88-109	R.TVNSISSMPTTASWSYTGSDIR.A	Oxidation: 8	CID
	1	2361.0264	-32.32	1		98.71		0	88-109	R.TVNSISSMPTTASWSYTGSDIR.A		CID

Appendix 1- Figure 7: Sequence alignment of peptides obtained from tryptic digestion of VCFF17's 20 and 16 kDa bands against the full sequence of endoglucanase from *Trichoderma koningii* that was identified by MASCOT and NCBInr database.

Appendix 2

M9 Minimal media preparation

M9 salts

$Na_2HPO_4.7H_2O$	64 g
KH_2PO_4	15 g
NaCl	2.5 g
NH_4Cl	5.0 g

Dissolve the M9 salts in distilled water to a final volume of 1000 ml.

Preparation of 1 M $MgSO_4$ (dissolve 61.62 g of $MgSO_4$ in 1 litre of distilled water).

Preparation of 1 M $CaCl_2$ (dissolve 11.09 g of $CaCl_2$ in 0.1 litre of distilled water).

Add 5 g/l of tryptone

Add 5 g/l of yeast extract

To a total of 750 ml sterile distilled water, add 200 ml of M9 salts, 2 ml of 1 M magnesium sulphate, 0.1 ml of 1 M calcium chloride and 40 ml of 5% Cellobiose, then add distilled water to a final volume of 1000 ml.

Preparation of 10Mm *p*NPG

Mr = 301, 25 g/mol

m = C×V×Mr

= 0.01 M × 0.01 L× 301, 25 g/mol

= 0.030125 g

Dissolve 0.030 g of *p*NPG in 10 ml of distilled water.

Malt Extract agar

Malt extract	20g
Agar	15g
Distilled water	make up to 1000 ml

Mix and autoclave for 15 min. Add 0.02g/l chloramphenicol. Pour out on plates or universal bottles for slants.

Preparation of 0.05 M Sodium Acetate buffer pH 5

Prepare 0.1M Sodium Acetate Buffer pH 5

Solution A: 0. 2M Acetic acid (11.55ml/l)

Solution B: (16.4g/l) Sodium Acetate

Mix 14.8 ml Sol A

35.2 ml Sol B

Dilute to 100ml in water.

DNS reagent

Dinitrosalicylic acid	40g
Phenol	8g
Sodium sulfate	2g
Potassium sodium tartarate	800g

Dissolve in 2 liters of 2% (w/v) NaOH and then make up to 4 liters with distilled water.

Preparation of SDS-PAGE gels for electrophoresis

	12% resolving gel	Stacking gel	Sealing gel
Water	3.35 ml	2 ml	0 ml
1.5M Tris pH 8.8	2.5 ml	0 ml	0 ml
0.5M Tris pH 6.8	0ml	1.25 ml	0 ml
Acrylamide/bis (30% T, 2.7% C)	4 ml	0.65 ml	1 ml
10% SDS	100 µl	50 µl	50 µl
10% Ammonium persulphate	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl

Overlay agarose solution

Prepare one bottle containing 50 ml of 0.5% low melting point agarose in 25Mm Tris, 192 Mm glycine, 0.1% SDS and a trace of bromophenol blue.

Preparation of 1.5 M Tris HCl pH 8.8

Dissolve 18.2 g Tris base in 80 ml water. Adjust pH to 8.8 with HCl and add water to a final volume of 100 ml

Preparation of 0.5 M Tris HCl pH 6.8

Dissolve 6.1 g in 80 ml water and adjust pH to 6.8 with HCl. Add water to a final volume of 100 ml. Store at 4 °C.

Preparation of 10% SDS

Dissolve 10 g sodium dodecyl phosphate in 60 ml of water. When dissolved add to water to a final volume of 100 ml.

Stock Sample buffer

Water	4.8 ml
0.5 M Tris-HCl	1.2 ml
10% SDS	2.0 ml
Glycerol	1.0 ml
0.5% bromophenol Blue (w/v) water	0.5 ml

(0.06 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.025% Bromophenol blue).

Store at room temperature.

Electrode buffer (5 times concentrated)

15 g Tris base, 72 g glycine, 5 g SDS/ liter electrode buffer. Mix the reagents and confirm that the pH is near 8.3.

(0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS, pH 8.3).

To use 5x concentrate, dilute with four parts water.

Acrylamide concentrate (30% T, 2.7% C)

Dissolve 29.2 g of acrylamide and 0.8 g of bisacrylamide in 70 ml of deionized water. When the acrylamide is completely dissolve, add water to a final volume of 100 ml. filter the solution under vacuum and store at 4°C in a dark bottle.

10% ammonium persulfate (APS)

dissolve 100 mg APS in 1 ml of water. Make the APS solution fresh daily.

0.1% Congo red

Weigh out 0.1 g Congo red in 100 ml of distilled water.

Coomassie Brilliant Blue R-250 dye stain

Prepare 0.1% Coomassie Brilliant Blue-R-250 (w/v) in 40% (v/v) methanol, 10 % acetic acid (v/v). Filter the staining solution after the dye has dissolved. Store at room temperature.