Bioprospecting for Cellulase Enzymes of Soil-borne Bacteria for Possible Application into Bioethanol Production

By

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Declaration

I Keokeditswe Raven Motsewabangwe, declare that the research dissertation submitted to the Department of Biological Sciences at the North-West University, Mafikeng Campus, for the Bachelor of Science Masters in Biology degree has never been submitted at the university or at any other institution elsewhere. This is my own work and all the sources used or quoted have been indicated and acknowledged.

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Signature: ____________________________ Date: _______________________

Dedications

This work is dedicated to my parents, Loeto and Naome Motsewabangwe who have shown patience and support to my academic endeavors. My siblings Ivan and Tsholofelo Motsewabangwe for cheering me on through the years. All my close friends for the constant support and finally to my late friend, Tshepang Masemola, the dream still continues my friend.

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Abstract

Rising crude oil prices and strict emission standards have prompted a global demand for alternative fuels that are able to reduce the consumption of fossil resources, improve energy security and mitigate climate change. Bioethanol produced from vastly abundant lignocellulosic biomass has been found to be the ideal fuel for such an endeavor. Cellulases have been found to play an important role in producing fermentable sugars from the degradation of lignocellulose for bioethanol production. Although many industrial cellulases have been extracted from a variety of fungal species, researchers have in recent years begun paying attention to various bacterial species that have the ability to produce cellulases. This is due to their ability to produce multi-enzyme complexes, resistance to extreme conditions and rapid growth. In this study, soil samples within lake ecosystems of the Ngaka Modiri Molema District were investigated for the presence of cellulolytic bacteria with the potential to produce cellulases that could one day be utilized in the production of bioethanol. To determine this, cellulose activity was taken as an important parameter to determine the performance and efficiency of the bacterial cellulases. A total of 8 bacterial isolates identified as LD04 (*Bacillus cereus*), LD08 (*Bacillus thuringiensis*), LD09 (*Brevibacterium halotolerans*), LD11 (*Bacillus cereus*), LD19 (*Bacillus anthracis*), LD25 (*Bacillus tequilensis*) and LD28 (*Bacillus subtilis*) were found to have favourable cellulolytic potential. A DNS assay with CMC as sole carbon source showed that isolate LD25 and LD28 had the highest enzyme activity at 0.125 UI/ml and 0.167 UI/ml respectively and were selected for further analysis, 0.1UI/ml was selected as the minimum favourable enzyme activity. A substrate-based enzymatic assay was employed to determine the ability of bacterial isolates to produce endoglucanase (CMC), exoglucanase (Avicel) and β-glucosidase (cellobiose). LD25 and LD28 produced high volumes of reducing sugars during the degradation of CMC followed by cellobiose (0.081 UI/ml and 0.14UI/ml respectively), indicating that the isolates had the ability to produce endoglucanase and β-

glucosidase. The isolates showed minimal degradation of avicel as a carbon source indicating the possible inability to produce the exoglucanase enzyme. Partial purification of cellulose enzyme showed the highest protein yield and activity at 60% ammonium sulphate saturation. Optimization studies indicated that the highest enzyme activity for isolate LD25 was 0.125 UI/ml and 0.167 UI/ml for LD28, these activities occurred after a 24 hour incubation period, during the log phase of bacterial growth. Enzymatic activity peaked at pH 4 for isolate LD25 with an activity of 0.132 UI/ml whereas the activity for isolate LD28 peaked at pH 5 with an activity of 0.167 UI/ml. The enzymatic activity of LD25 and LD28 was greater when incubated at 50°C and 60°C yielding activities of 0.125 UI/ml and 0.195 UI/ml respectively, indicating that enzymes from both isolates were thermostable. Peptone was found to be the ideal nitrogen source to stimulate high enzyme activity among both isolates.. In- silico cloning showed that the celluloytic enzyme activity of *Bacillus subtilis* (LD28) was attributed to the presence of the CelDr endoglucanase gene. The Cel Dr gene was successfully cloned into a pGEM-T vector to yield a pGEM-CelDr recombinant. The cellulolytic enzyme activity of *Bacillus tequilensis* (LD25) was found to be attributed to the presence of the 1,3-1,4-β-glucanase gene. Successful cloning of this gene was accomplished through insersin of the gene fragment into a pGEM-T vector to yiend the recombinant, pGEM-E-b-Glu. *B. tequilensis* (LD 25) and *B. subtilis* (LD28) have been shown to be competitive cellulase producers even though they do not fully exhibit the synergistic tri-enzyme complex required for the complete degradation of lignocellulosic biomass. The study does, however, contribute to a pool of knowledge concerning the possible usage of soil borne cellulolytic bacteria from the Ngaka Modiri Molema District in the production of bioethanol or other emerging enzyme industries.

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List of Definitions

16S rDNA:	A component of the 30S small subunit of prokaryotic ribosomes used in reconstructing phylogenies.
Bioethanol:	Ethanol (alcohol) derived from the fermentation of plant starches.
Biofuel:	A renewable type of energy derived from organic matter such as plant residues.
Biomass:	Plant matter derived from agricultural and or agro-industrial activity with possible applications in the production of biofuels.
Cellulase:	An enzyme having the capability to degrade cellulose into simple disaccharides or glucose.
Cellulose:	Constituent of plant cell walls consisting of long chains of glucose monomers.
Hemicelluloses:	A polysaccharide constituent of the plant cell wall having a similar structure to cellulose.
Lignin:	Amorphous polymer complex bound to cellulose fibers, providing rigidity to plant cell walls.
Lignocellulose:	A complex of cellulose, hemicellulose and lignin present in plant cell walls.

List of Abbreviations

$(NH_4)_2SO_4$: Ammonium Sulphate

CAZy: Carbohydrate- Active Enzyme Database

CBM: Cellulolytic Binding Molecule

CMC: Carboxyl methylcellulose

DNA: Deoxyribonucleic acid

DNS: Dinitrosalicylic acid

EAI: Enzyme Activity Index

EU: European Union

GH: Glycoside Hydrolase

GHG: Greenhouse Gases

K_2HPO_4: Dipotassium Hydrogen Phosphate

KNO_3: Potassium Sulphate

$MgSO_4.7H_2O$: Magnesium Sulphate Heptahydrate

MM: Minimal Media

MUC: Methylumbelliferyl-β-cellobiose

MUG: Methylumbelliferyl-β-D-glucuronide

$NaNO_3$: Sodium Sulphate

NCBI: National Centre for Biotechnology Information

NH_4Cl: Ammonium Chloride

PCR: Polymerase Chain Reaction

TAE: Tris-acetate EDTA

USA: United States of America

β : Beta

Chapter 1

1. Introduction

The large increase in the world's population and global prosperity has resulted in a substantial increase in energy demand which has expanded 2.4 fold from 5000 million tons equivalent in 1971 to 11700 million tons in 2010. This has led to the prediction that fossil fuel demand will continue to expand, and the continued supply of these fuels to equal demand in a stable manner will become an increasingly important challenge (Matsuo *et al*., 2013, Heeres *et al*., 2014).

Depleting oil reserves have been recognized as the main challenge to energy supply in the next decade (Festel *et al*., 2014). Furthermore, clear scientific evidence shows that emissions of greenhouse gases such as carbon dioxide, methane, and nitrous oxide arising from the fossil fuel combustion are disturbing the earth's climate (Cherubini, 2010). Such evidence is supported by the progress made in climate science over the past 20 years which has improved our forecasting capabilities. It can therefore be confidently stated that the earth will warm up during the next centuries as a result of human fossil fuel use (Zecca and Chiari, 2010).

The use of renewable energy has been shown to be an excellent way to minimize climate change. One of the renewable energy alternatives is the utilization of energy from biomass. Biomass can be used as a solid fuel, or converted into liquid or gaseous forms for the production of electric power, heat or chemical fuels (Mizsey and Racz, 2010). Bioethanol is currently considered as an important chemical renewable fuel to potentially replace fossil derived fuels (Kang *et al*., 2014). The annual global production of this fuel has increased from 50 million liters in 2007 to 100 million liters in 2012 (Kang *et al*., 2014). For the production of bioethanol, lignocellulosic biomass constitutes a feedstock that does not compete with food production and animal feed, which in turn leads to environmental stability (Limayem and Ricke, 2012). However; because of its recalcitrant nature, lignocellulosic-based feedstocks require intensive

labour through pretreatment as well as high capital costs for processing, making it potentially economically non-viable. It is for this reason that enzymatic decomposition of this feedstock needs to be taken into account as it presents a cost effective alternative to feedstock hydrolysis (Limayem and Ricke, 2012).

The production of cellulases has become a major factor for the hydrolysis of cellulose in bioethanol production. This hydrolysis involves the synergistic action of a tri-enzyme cellulase complex composed of endoglucanase, exoglucanase and β-glucosidase that breaks down crystalline cellulose into fermentable sugars (Tang *et al*., 2010).

Both fungi and bacteria have been extensively exploited for their ability to produce a wide variety of cellulases but more emphasis was placed on fungal cellulases due to their ability to produce large amounts of cellulases that are easily extracted and purified (Maki *et al*., 2009). In recent years, isolation, screening, and selection techniques have favoured the discovery of several novel cellulose-producing bacteria. Cellulases from *Paenibacillus*, *Bacillus*, and *Cellulomonas* species have been found to display high thermostability and broad pH spectrum and affinity to soluble crystalline cellulose, a trait that could make the production of lignocellulose derived bioethanol an attainable exercise (Maki *et al*., 2009).

1.1 Problem Statement

Owing to the rising crude oil price and stricter emission standards, the demand for alternative fuels is growing. Fuels that are able to mitigate climate change, improve energy security, save natural resources and reduce the consumption of fossil resources are increasingly being promoted by various governments around the world (Mizsey and Racz, 2010, Festel *et al*., 2014).

The perfect candidate for production of such fuels is lignocellulosic biomass derived from agricultural and agro-industrial residues such as wood chips; corn stover, sugar cane, rice and wheat straw which contain cellulose and hemicelluloses produced at approximately 7.2×10^{10} and 6×10^{10} tons per annum respectively. Such materials provide a potentially vast source of renewable energy for fuel production that is not tied to food crops or in limited supply (Gupta *et al*., 2014). However, no commercially efficient cellulase enzyme complex has been produced and the high cost of enzyme production limits its industrial use in the production of soluble sugars. Such factors limit the possibility of utilizing cellulases in the production of bioethanol which could provide a good substitute for gasoline in internal combustion engines (El-Hadi *et al*., 2014).

1.2 Objectives

1. To prospect for bacterial species exhibiting cellulolytic potential from Mahikeng soil samples.
2. To identify the bacterial isolates having the best cellulolytic potential.
3. To determine the enzymatic activity of bacterial isolates on different substrates to determine their suitability for use at industrial levels.
4. To optimize the cellulolytic activity of isolates at varying physiological parameters.

1.3 Aims

The research aims at exploring the local soil environment for the presence of bacterial consortiums that have the ability to produce the cellulase enzymes endoglucanase, exoglucanase and β-glucosidase that could potentially be used to degrade lignocellulosic biomass in bioethanol production.

Chapter 2

2. Literature Review

For over two centuries, fossil fuels have been used as the major energy source in the world. Fuels such as petroleum oil, coal and natural gas have been shown to contribute up to 80% of the total primary energy sources in the world today. Unfortunately, global population increase as well as prosperity has resulted in an increase in energy demand thus resulting in an increase in the need to acquire fossil fuels. This demand has expanded 2.4 fold from 5000 million tons of oil equivalent in 1971 to 11700 million tons by 2010. Such extensive fossil fuel use is contributing to emission problems such as global warming as well as international conflicts related to energy security (Cherubini, 2010, Song *et al*., 2015). Depleting oil reserves have now been recognized as the main challenge to energy supply within the next decade (Festel *et al*., 2014). This occurrence has led to the prediction that fossil fuel demand will continue to expand, and the even distribution of these fuels to a point of demand in a stable manner will become an increasingly important challenge (Matsuo *et al*., 2013, Heeres *et al*., 2014).

Even with a high demand for fossil fuels, clear scientific evidence through the years has shown that the combustion of fossil fuels has resulted in the emission of greenhouse gases such as carbon dioxide, methane and nitrous oxides which are affecting the earth's climate (Cherubini, 2010). Progress made in climate sciences over two decades has improved forecasting capabilities and it can confidently be stated that the earth will continue to warm up during the next few centuries as a result of human fossil fuel usage (Zecca and Chiari, 2010).

Although energy security is becoming an increasing concern, climate change has been recognized as one of the greatest challenges the global community faces today with the anthropogenic emission of greenhouse gases (GHG) being the main culprit (Timilsina *et al*., 2011). One way of mitigating GHG emissions is to reduce the combustion of fossil fuels or

substituting fossil fuels with non-fossil energy resources (Timilsiina *et al.* ,2011). The use of renewable energy (biofuels) has been shown to be an excellent way to minimize climate change.

2.1 Biofuels

The term biofuel is any liquid or gaseous fuels produced predominantly from biomass (plant matter such as trees, grasses, agricultural crops or other biological materials) (Demirbas, 2008, Mizsey and Racz, 2010). Biomass technologies use this waste plant matter to produce energy having lower levels of greenhouse gas emissions than their fossil counterparts (Demirbas, 2008). This biomass can be used as a solid fuel, or converted into liquid or gaseous forms for the production of electric power, heat or chemical fuels (Mizsey and Racz, 2010). A variety of fuels can be produced from biomass resources, including but not limited to liquid fuels such as ethanol, methanol, biodiesel, Fischer-Tropsch diesel and gaseous fuels such as hydrogen and methane (Demirbas, 2008).

The demand for biofuels is on the rise and will continue to grow through to the year 2020 as predicted by Mordor Intelligence (2015). Mordor Intelligence also points out that this rapid expansion is changing the global dynamics of food, agricultural and energy markets in a big way. The USA, Brazil, and the European Union have come out as mass producers of ethanol and biodiesel thus making them key drivers for the demand of biofuels coupled with government policies such as the Kyoto Protocol that have coaxed producers into finding ways to increase biofuel production (Sarkar *et al.*, 2012, Mordor Intelligence, 2015).

2.1.1 Biofuels in Africa

Growing interests in biofuels in many African countries can be explained by factors such as high crude oil prices, fluctuations in price due to geopolitical uncertainties, local and global environmental impacts of fossil fuels such as climate change, opportunities for job creation,

new research and technological advancements, economic development and the need to increase access to energy services (Amigun *et al*., 2011). Amigun *et al*. (2011) also points out that although Africa possesses 9.9% of the world's proven petroleum oil reserves with a 12% contribution to global oil production, the uneven distribution of energy resources results in the majority (42 countries) of African countries becoming energy importers, importing petroleum products at costs that place a heavy economic burden while reducing energy security and independence.

In response to the increase in promotion of biofuels, several African countries are making efforts to introduce biofuels specific policies (Amigun *et al*., 2011). Government policies implemented in Malawi to reduce the volume of imported fossil fuels have created favorable conditions for the production of ethanol resulting in the country continuously producing ethanol and blending it with gasoline (10% blend) since 1982 (Amigun *et al*., 2011, Jumbe *et al*., 2009).

In 2004, Mauritius started producing and shipping bioethanol to the E.U with plans to export up to 30 million tons per year (Amigun *et al*., 2011). Amigun *et al*. (2011) also identifies other commercial ethanol plants existing in countries such as Mozambique, Tanzania, Zambia, Kenya, Angola, Swaziland, Egypt, Ethiopia, and Uganda.

South Africa is the leading country on the African continent to develop advanced technologies for large-scale production of biofuels but there are currently no large-scale biodiesel plants on the continent and the market is characterized by several small and medium scale producers (Jumbe *et al*., 2009).

2.1.2 Areas of Concern in Biofuel Production

The global interest in biofuel production has raised widespread debate concerning biofuel trade among developed and developing countries. The debate has focused mainly on areas of "food versus

fuels". There exists great concern that the rising demand for biofuels will result in an increase in the price of food commodities. Although there exists enough agricultural land to meet the food and fuel demand, starvation among the poor and middle class will not occur as a result of the lack of food but as a result of the lack of buying power (Sachs, 2007, Hall *et al*., 2009).

The release of greenhouse gasses from land use in the establishment of feedstock farms for biofuel production has also been identified as a concern in biofuel production. Although biofuels are considered to be a self-sustaining fuel alternative, it is estimated that the time that will be required to overcome the carbon debt incurred through greenhouse gas emissions will be between 100-1000 years (Kim *et al*., 2009).

It is important to note that even though biofuels have the ability to reduce greenhouse gas emissions by more than 30% compared to traditional fossil fuels, production of these fuels have greater aggregate environmental costs (Scharlemann and Laurance, 2008).

2.2 Bioethanol

Ethanol is a colourless, biodegradable and non-toxic alcohol having the chemical formula C_2H_5OH and structural formula shown in Fig 2.1. This alcohol can be manufactured through chemical processes or through the fermentation of sugars to produce bioethanol (Zeiger, 2015).

At present, bioethanol, is the most promising alternative to conventional fuels because of its desirable characteristics such as high octane value with energy density and good combustion efficiency and it is currently the cleanest renewable alternative fuel. Compared to gasoline, ethanol's high octane number means that the need for octane enhancing additives is limited (Gupta *et al*., 2014). In addition, the combustion of ethanol releases oxygen as a byproduct, a characteristic essential for reducing the emission of greenhouse gases such as carbon monoxide, nitrogen oxides and non- combusted hydrocarbons and other volatile organic compounds into the environment (Gupta *et al*., 2014).

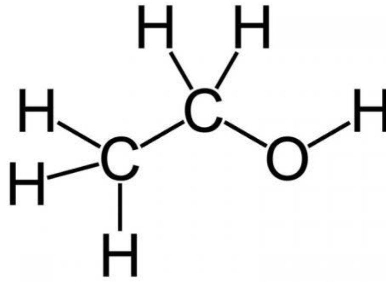

Fig 2.1 Structural formula of bioethanol/ethanol (Zeiger, 2015).

The global market for bioethanol has entered a phase of rapid growth. Many countries around the world have shifted their focus to such a renewable resource for power production as a response to depleting crude oil reserves. Although production cost of bioethanol is greater as compared to that of fossil fuels, world bioethanol production has enjoyed an increase in production from 31 billion liters in 2001 to 39 billion liters in 2006 with Brazil and the United States of America accounting for 62% of the global production within that period. With the continued rapid growth in production, outputs of this alcohol were expected to increase well over 100 billion liters by 2015 (Sarkar *et al*., 2012).

The current large scale production of bioethanol is mainly dependent on sucrose from crops such as sugarcane and starch from corn; however, these feedstocks are undesirable due to their food and feed value (Sarkar *et al*., 2012). Lignocellulosic biomass is an ideal raw material for the production of bioenergy from sources such as plant residues because plant cell walls from which the lignocelluloses are derived are the world's most abundant biomass. In addition, fuel production does not compete with food production (Kim and Dale, 2004). Kim and Dale (2004) have estimated that 442 billion liters of bioethanol can be produced from lignocellulosic biomass and that crop residues and wasted crops can produce 491 billion liters of ethanol annually. Unfortunately, due to their complex structures, it is very difficult to convert lignocellulose into biofuel and also the production cost of fuel from this biomass is very high

due to capital costs of pretreatment and production and/or purchase of cellulases (Maehara *et al*., 2013).

2.3 Lignocellulosic Biomass

Lignocellulosic biomass primarily consists of three types of polymers, which are cellulose, hemicellulose and lignin as shown in Fig 2.2. The carbohydrate components (cellulose and hemicellulose) are fermentable after hydrolysis making the biomass a suitable substrate for bioenergy production (Zheng *et al*., 2014). Table 2.1 outlines the distribution of polymers within lignocellulosic biomass from agricultural waste that has potential as feedstock for bioenergy production.

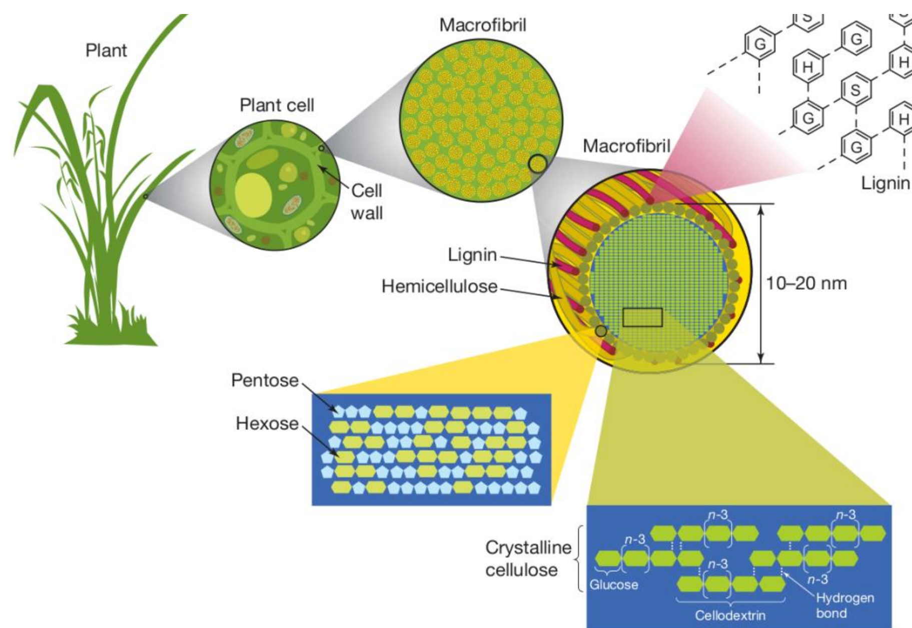

Fig 2.2: The structure of lignocellulosic biomass with indication of cellulose, hemicellulose, and lignin (Rodríguez, 2013).

Table 2.1: Contents of cellulose, hemicellulose and lignin found within lignocellulosic biomass from agricultural waste.

Lignocellulosic Material	Cellulose %	Hemicellulose %	Lignin %
Hardwoods stems	40–55	24–40	18–25
Softwood stems	45–50	25–35	25–35
Corn cobs	45	35	15
Grasses	25–40	35–50	10–30
Wheat straw	30	50	15
Leaves	15–20	80–85	0
Switch grass	45	31.4	12.0

Sourced from (Sun and Cheng, 2002)

2.3.1 Cellulose

Cellulose is the main cell wall polysaccharide of plants. It is entangled within lignin and hemicellulose carbohydrate polymers and it is a water insoluble polymer composed of repeated units of β-D- glucopyranose interlinked by β-1, 4 glycosidic bonds as illustrated in Fig 2.3. Cellulose molecules have different orientations throughout the structure which results in different levels of crystallinity. These molecules consist of amorphous (low crystallinity) as well as a crystalline (high crystallinity) regions measured by the crystalline index. The higher the crystalline index, the more difficult the biodegradation of the cellulose becomes (Nayebyazdi *et al*., 2012, Zheng *et al*., 2014)

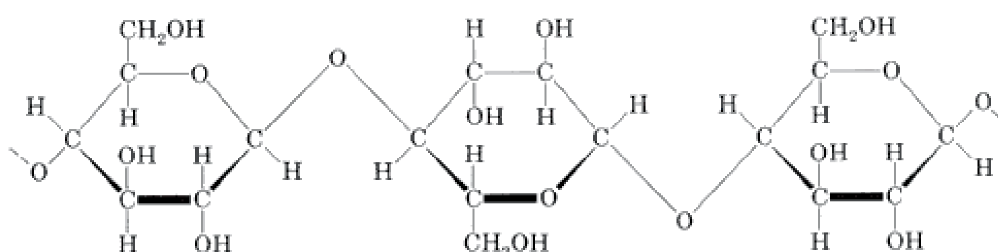

Fig 2.3: Structural formula of cellulose molecule indicating repeated units of β-D-glucopyranose interlinked by β-1, 4 glycosidic bonds (Senese, 2015).

2.3.2 Hemicellulose

In comparison to cellulose, hemicelluloses are more amorphous, random and branched heterogenic polysaccharides of various pentoses (xylose and arabinose), hexoses (glucose, galactose, mannose, and or rhamnose) and acids (glucuronic acid, methyl glucuronic acid and galacturonic acid) as indicated in Fig 2.4. Short branched chains of hemicelluloses help build a network with cellulose micro fibrils and interact with lignin, rendering the cellulose-hemicellulose matrix extremely rigid. The amorphous and branched properties make hemicelluloses highly susceptible to biological, thermal, and chemical hydrolysis of their monomeric compounds (Irmak *et al*., 2013, Zheng *et al*., 2014).

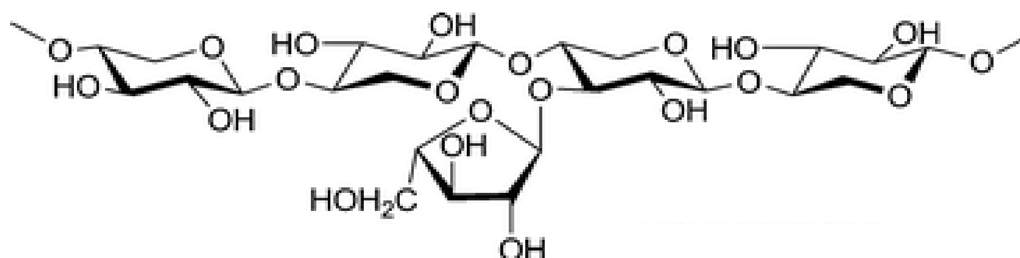

Fig 2.4: Structural formula of hemicellulose having random and branched heterogenic polysaccharides of various pentoses, hexoses and acids (Dhepe and Sahu, 2010).

2.3.3 Lignin

Lignin is described by Irmak *et al*. (2013) and Zheng *et al*. (2014) as the second most abundant organic compound in nature and it is a large and complex aromatic and hydrophobic amorphous heteropolymer constructed from phenyl propane units. The units include coniferyl alcohol and sinapyl alcohol with hydroxyl, methyl, and carbonyl functional groups, as shown in Fig 2.5 Lignin possesses binding properties that enable cross-linking between cellulose and hemicellulose to form rigid three-dimensional structures of the cell wall. Its ability to dissolve in water at high temperatures of about 180ºC, neutral pH or acidic/alkaline conditions makes it the most recalcitrant component of the cell plant wall (Romano *et al*., 2013).

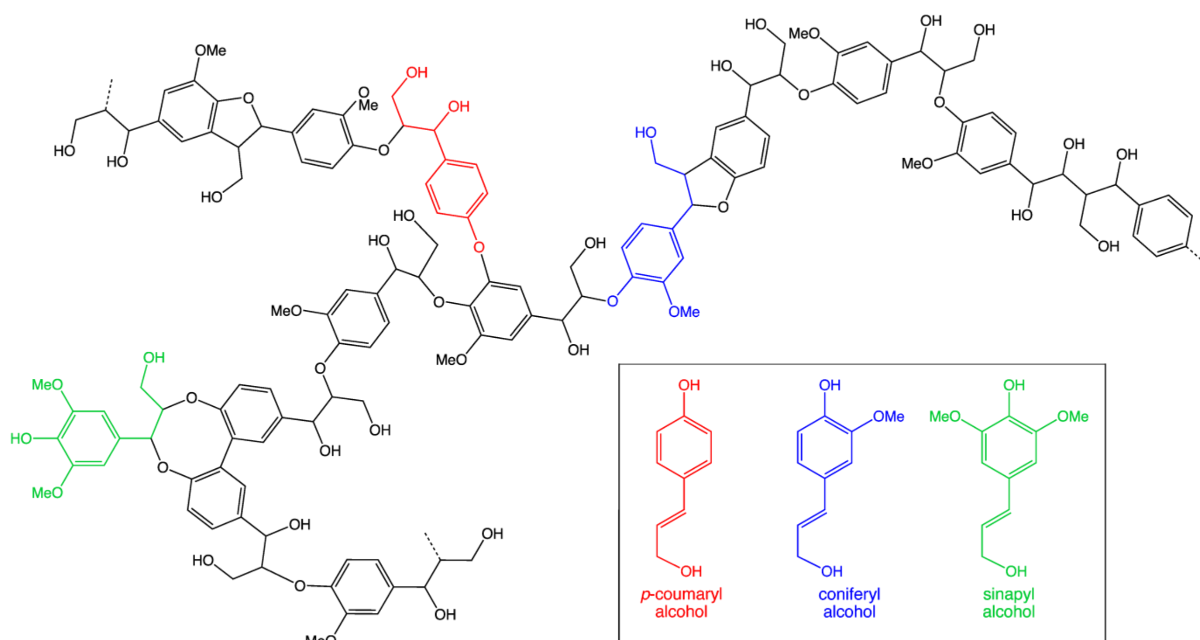

Fig 2.5: Structural formula of lignin with coniferyl alcohol and sinapyl alcohol with hydroxyl, methyl and carbonyl functional groups(Rodríguez, 2013).

2.4 Pretreatment of Lignocellulosic Biomass for Enzymatic Pretreatment

Many physiochemical, structural and compositional factors hinder the hydrolysis of cellulose present in biomass into sugars and other organic compounds that can be converted into biofuels (Kumar *et al*., 2009). It is for this reason that pretreatment processes for increasing the enzymatic digestibility of hemicellulose have been a key step in commercialized production of bioethanol (Zhao *et al*., 2009).

2.4.1 Physical Pretreatment

In this procedure, waste materials are reduced in size by a combination of chipping, grinding and milling to reduce the crystallinity of cellulose. The size of materials after chipping are usually 10-100 mm in size. Milling and grinding further reduces the size of materials to 0.2 to 2 mm. Vibratory ball milling has been found to be more effective in breaking down cellulose crystallinity and improving digestibility of biomass (Sun and Cheng, 2002, Brodeur *et al*., 2011)

2.4.2 Physiochemical Pretreatment

Steam-explosion pretreatment uses both physical and chemical techniques in order to break down the structure of lignocellulose. This hydrothermal method subjects materials to high pressures and temperatures (190°C - 270°C) for short periods of time (1-10 minutes) after which they are rapidly depressurized, disrupting the structure of lignocellulosic fibrils. Disruption of the fibrils increases accessibility of the cellulose to enzymes during hydrolysis. Similar physiochemical procedures include liquid hot water (LHW) treatment and ammonia fiber explosion (AFEX) which uses liquid and anhydrous ammonia under high pressure and moderate temperatures to create disruptions in lignin-carbohydrate linkage, hemicellulose hydrolysis and ammonolysis of glucuronic cross-linked bonds and partial decrystallization of the cellulose structure (Brodeur *et al*., 2011).

2.4.3 Chemical Pretreatment

Chemical pretreatment improves biodegradability of cellulose by removing lignin and hemicelluloses and to decrease the degree of polymerization and crystallinity of the cellulosic component of lignocelluloses. Chemicals ranging from oxidizing agents, alkali, acids and salts can be used to degrade lignin, hemicelluloses, and cellulose from lignocellulosic wastes. Organic acids such as oxalic acids, acetylsalicylic acids, and salicylic acids can be used as catalysts with organic or aqueous organic solvent mixtures used to break internal lignin in hemicellulose bonds (Behera *et al*., 2014).

2.4.4 Biological Pretreatment

Biological pretreatment is mostly associated with the action of fungi that are capable of producing enzymes that degrade lignin, hemicellulose and polyphenols present in the biomass. The potential advantages over physical/chemical pretreatments include substrate reaction specificity, low energy requirements, no generation of toxic compounds, and high yield of

desired products. However, treatment is a slow process that requires control of growth conditions and large space to perform hydrolysis. In addition, most ligninolytic microorganisms solubilize/consume not only lignin but also hemicellulose and cellulose. The degradation of lignocellulosic biomass is achieved by specific groups of cellulolytic microorganism that are able to produce composite enzymes such as cellulases, hemicellulases, and pectinases that work together in the degradation of cellulose into its associated cell wall components (Romano *et al*., 2013).

2.5 Cellulases and Catalytic Mechanism of Cellulases

Three principal cellulases synergically that confer the complete hydrolysis of cellulose are shown in Fig 2.6. These cellulases are endoglucanase (endo-1, 4-β-D-glucan-4-glucanohydrolases) which hydrolyze β-1,4glucosidic linkages at any region within the cellulose, producing long chains of oligomers with non-reducing ends. Exoglucanases (exo-1, 4-β-D-glucan-4-cellobiohydrolases) cleave the long-chain oligosaccharides generated by endoglucanase action into short chain oligosaccharides. The final action is executed by β-glucosidases that hydrolyze liberated cellobiose into glucose (Viikari *et al*., 2012, Romano *et al*., 2013).

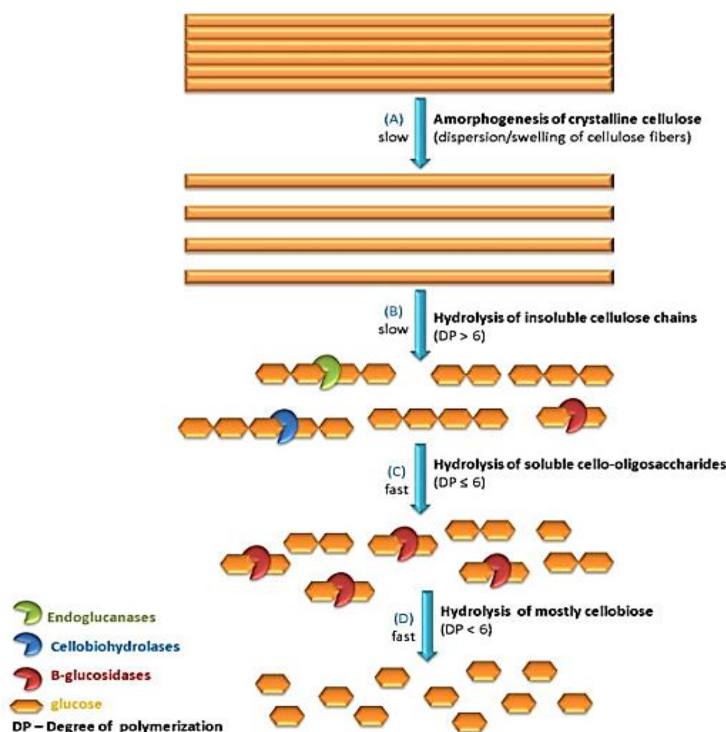

Fig 2.6: Catalytic mechanism of cellulases on crystalline cellulose molecule (Arantes and Saddler, 2010).

Cellulose degrading enzymes are further grouped into glycoside hydrolase (GH) families. According to the Carbohydrate – Active Enzymes (CAZy) database, endoglucanases are found in the GH families 5-8, 12, 16, 44, 45, 48, 51, 64, 71, 74, 81, 87, 124 and 128. Exoglucanases or cellobiohydrolases are found in GH families 5-7 and 48 while β- glucosidases are found in families 1, 3, 4, 17, 30 and 166 (Juturu and Wu, 2014).

2.6 Structure of Cellulases

Catalytic modules of cellulases have been classified into numerous families depending on their amino acid sequences and crystal structures. These cellulases contain non-catalytic carbohydrate bonding molecules along with other modules whose function is either known or unknown. The modules can be either located at the C-terminus or the N- terminus of the catalytic module (Zhang and Zhang, 2013).

Cellulolytic microorganisms have evolved two strategies for utilizing their cellulases: discrete non-complexed cellulases and complexed cellulases.

2.6.1 Discrete Non-complexed Cellulases

Cellulolytic filamentous fungi and actinomycetes bacteria have the ability to penetrate cellulosic substrates through hyphal penetration. This ability enables these organisms to present their cellulose systems in confined cavities within the cellulosic particle. The enzymes in these systems do not form stable, high molecular weight complexes and are thus termed "non-complexes" (Lynd *et al.*, 2002).

2.6.2 Complexed Cellulases

Anaerobic bacteria lack the ability to effectively penetrate cellulosic materials. It is, for this reason, that alternative mechanisms for degrading cellulose (while also gaining access to hydrolysis products in the presence of microbial competition) were developed. These microorganisms developed complexed cellulase systems called cellulosomes. Cellulosomes are protuberances that are produced on the cell wall of cellulolytic bacteria when growing on cellulosic materials. Although these complexes are firmly bound to the bacterial cell wall, they are flexible enough to also bind tightly to microcrystalline cellulose. Binding of the complex to the substrate allows for enzyme activity at close proximity to the bacterial cell, enabling optimum synergism between cellulases presented on the cellulosomes and bacterial cell by minimizing the distance between hydrolysis products and bacterial cell (Lynd *et al.*, 2002).

2.7 Cellulose Degrading Microorganisms

Ethanol producing processes employ cellulose producing microbes for the depolymerization of cellulose. These enzymes are produced by a number of microbes including fungi, yeasts, and bacteria. The classical tri-enzyme synergistic cellulase system is also used by fungal and

bacterial microbes and its stability needs to be maintained, for high cellulolytic activity within industrial and uncontrolled natural environment (Lee, 1997)

Cellulolytic enzymes expressed by a wide variety of microorganisms in nature are usually produced by saprophytic microorganisms growing on dead and decaying organic matter. Cellulolytic microorganisms are isolated from soil samples obtained from forests, nature reserves, hot water springs, compost, sewage, animal manure and bovine rumen (Juturu and Wu, 2014). Microbes that that have been found to have the ability to continue producing high volumes of enzymes within commercial/industrial environments, resulting in complete depolymerization of cellulose to give high concentrations of sugars (80 g/L) while being resistant to these industrial processes have been brought into industrial applications (Juturu and Wu, 2014).

Fungi belonging to the genus *Trichoderma*, specifically species such as *T. reesei* and *T. viride* are able to secrete large amounts of endoglucanase and cellobiohydrolase enzymes; however, the amount of β-glucosidases secreted by these species are very low and this may lead to the buildup of cellobiose and incomplete cellulose hydrolysis when cellulolytic extracts produced by fungi are used in the saccharification process (Falkoski *et al*., 2013). Other important cellulase producing fungal species that have been identified include *Penicillium* and *Aspergillus*. Although these species are shown to be able to secrete large amounts of β-glucosidase, the total cellulase activity found in their enzymatic extracts is relatively low (Falkoski *et al*., 2013).

Bacteria have been considered as robust and versatile enzyme producers because of their high growth rate, stability at extreme conditions and presence of multi-enzyme complexes (Balasubramanian and Simões, 2014). In bacteria, cellulases are present as aggregated structures attached to the cells. Out of the 1495 bacterial genomes listed in the Carbohydrate-

Active Enzyme (CAZy) database, only 575 genomes have been found to contain at least a single enzyme involved in cellulose hydrolysis. These enzymes are synthesized and secreted by bacteria to hydrolyze polymeric structures of cellulose to be utilized as food substrates (Morrell-Falvey *et al*., 2015). Although the bacteria that exhibit cellulolytic activity amount to just under 40% within the CAZy database, they can further be categorized into 4 groups due to the presence of the cellulose coding gene with their genome, which are (1) saprophytes that do not synthesize cellulase, (2) cellulase synthesizing saprophytes, (3) cellulase synthesizing non- saprophytes, (4) neither saprophytic nor cellulase producers (Juturu and Wu, 2014). Bacterial cellulases have been reported in bacteria such as *Bacillus amyloliquefaciens*, *Bacillus sp* D04, *Bacillus brevis*, *Bacillus pumilus*, *Bacillus sphaericus*, *Streptomycetes thermoveolaceous* and *Clostridium thermocellum* (Balasubramanian and Simões, 2014).

2.8 Enzyme Hydrolysis of Cellulose to Produce Bioethanol

Cellulase enzymes provide a key opportunity for achieving the tremendous benefits of biomass utilization in the long term because of their high glucose yields and possible opportunities to apply modern biotechnological tools to reduce costs (Himmel *et al*., 1999). Tong *et al*. (2012) describe the production of ethanol from cellulosic biomass in the following steps

<u>Pretreatment</u>

In this process, cellulose is made more susceptible to enzyme degradation. This is done by partially removing lignin and hemicellulose to release cellulose embedded within the plant cell wall (Tong *et al*., 2012).

Enzyme hydrolysis

Polysaccharides attained from pretreatment are converted into simple sugars through cellulolytic synergy between endoglucanase, exoglucanase, and β-glucosidase. The tri-enzyme hydrolysis involves cleavage of long chain cellulose into short 4-5 glucose chain oligosaccharides, the breakdown of these fragments into shorter molecules composed of 2 glucose molecules and finally conversion of these molecules into fermentable single glucose molecules (Tong *et al*., 2012).

Fermentation

Sugars obtained from enzyme hydrolysis are converted into fuel ethanol by bacteria and yeasts within bioreactors. The fermentation step usually follows enzyme hydrolysis as a separate step known as "separate hydrolysis and fermentation" (SFH) however; to obtain higher ethanol yields, the two steps are combined to form the "simultaneous saccharification and fermentation" (SSF) process *(Tong et al., 2012)*.

Distillation

Ethanol is recovered from the fermentation broth by taking advantage of the low boiling point of ethanol (78°C). Through this step, ethanol vapors are separated from the liquid media while lignin residues along with unreacted cellulose, hemicellulose, ash, enzymes and remaining microorganisms end up at the bottom of the distillation column (Tong *et al*., 2012)

2.9 Cellulases in Other Industries

Cellulases are currently the third largest industrial enzyme worldwide by monetary value because of their applications in industries such as starch processing, alcohol fermentation, malting, brewing, extraction of fruit and vegetable juices, pulp and paper industries as well as

the textile industry (Lee, 1997). However, with its introduction into ethanol or fermentation industries, it will become the largest industrial enzyme produced (Wilson, 2011).

2.9.1 Wine and Brewery

In wine production, enzymes play an important role by improving colour extraction, skin macerating, must clarification, filtration, wine quality and stability. Beta-glucosidases improve the aroma of wines by modifying glycosylated precursors. Macerating enzymes are also responsible for improving pressability, settling and grape juice yields for fermentation. Beer brewing is based on the action of enzymes activated during malting, β-glucosidases are responsible for the hydrolysis of seed reserves while endoglucanases and exoglucanases are responsible for a maximum reduction in the degree of polymerization of wort viscosity (Kuhad *et al*., 2011).

2.9.2 Textiles

Acidic cellulases improve the softness and water absorbance properties of fibers. They are responsible for strongly reducing the formation of pills (small balls of fibers appearing on the surface of fabrics) while providing cleaner surface structures with less fuzz. Cellulase combinations rich in endoglucanases are best suited for bio-polishing and enhancing fabric looks, feel and color without the need for chemical coating. Cellulases are also responsible for creating smooth and glossy appearance in fibers while improving color brightness through an environmentally friendly environment (Kuhad *et al*., 2011)

2.9.3 Food Processing

Cellulases have a wide range of applications within the food biotechnology industry. The production of fruit and vegetable juices require improved methods of extraction, clarification, and stability. Cellulases have important applications as part of macerating enzyme complexes used for extraction and clarification of juices for increased yield. These enzymes increase yield

performance without the need for capital investment. Texture, flavor and aroma properties of juices are improved by reducing excessive bitterness of citrus fruits through infusion with enzymes such as pectinases and β-glucosidases (Kuhad *et al*., 2011)

2.9.4 Pulp Industry

Mixtures of cellulases (endoglucanases and hemicellulases) have been used for biomodification of fiber properties with the aim of improving drainage and beatability in paper mills before and after beating of the pulp. Cellulases alone or in combination with xylanases are beneficial for deinking of different types of paper wastes. Most applications use cellulases and hemicellulases for the release of ink from the fiber surface by partial hydrolysis of carbohydrate molecules (Kuhad *et al*., 2011).

2.10 Future Prospects for Cellulase Technology

Isolation, screening, and selection techniques have favored the discovery of several novel cellulase producing bacteria from a wide variety of environments, which has has resulted in the creation of new routes for the exploration of improved biorefining technologies and industries (Maki *et al*., 2009).

Biotechnology companies, Gencore International and Novozymes Biotech have reported the development of technology that has reduced the cellulase cost for cellulose to ethanol process from US $5.40 per gallon of ethanol to approximately 20 cents per gallon of ethanol in which two main strategies were used: (1) An economical improvement in production of cellulase to reduce cost per gram (g) of enzyme by process and strain enhancement and (2) an improvement in cellulase enzyme performance to reduce the amount of enzyme (g) for achieving equivalent hydrolysis by cocktails and substrate improvements (Zhang *et al*., 2006). Although these claims have not been widely accepted, they have provided a glimpse into the potential of making cellulase technologies more efficient with regard to cost and enzyme performance.

Chapter 3

3. Materials and Methods

3.1 Soil Sampling

Samples were collected from Mahikeng within the Ngaka Modiri Molema District South Africa. The sample sites were the Modimola Dam, Lotlamoreng Dam and Cookes Lake which were all located within a 10 km radius of the Mahikeng CBD. The lake ecosystems were selected because they provided adequate moisture to aid in plant degradation as well as plant growth. Collection was conducted from random locations around the lake so that the majority of bacterial communities from the ecosystem were represented. Surface soil of 0-20 cm depth was collected. The soil collected was uninfluenced by agronomic activity, human or animal contamination. Soil samples were then refrigerated at -20°C within one hour of collection to halt metabolic processes until examinations were conducted.

3.2 Cellulolytic Bacteria Isolation

3.2.1 Serial Dilution and Spread Plating

Cellulolytic bacteria were isolated from soil by using the serial dilution technique modified by using a physiological solution containing 1% K_2SO_4, 0.03% $MgSO_4.7H_2O$, 0.25% $(NH_4)_2SO_4$, and 0.2% gelatin as a dilution matrix. Dilutions were incubated at 37°C in a shaker incubator for 48 hours at 140 rpm to stimulate the growth of cellulolytic bacteria.

3.3 Screening for Cellulolytic Activity

To screen for cellulolytic organisms, 100 µl of the dilution mixture was plated onto minimal agar plates containing 0.2% K_2SO_4, 0.03% $MgSO_4.7H_2O$, 0.25% $(NH_4)_2SO_4$, 1% agar, 0.2% gelatine and 0.04% trypan blue dye. Plates were then incubated at 37°C for 24 hours. Cellulolytic activity was indicated by discoloration of trypan blue dye (clear zones) around bacterial colonies (Apun *et al*., 2000, Irfan *et al*., 2012).

3.4 Enzyme Activity Index

To identify bacterial colonies having the best cellulolytic activity, an enzyme activity index was employed. The diameter of each bacterial isolate having a zone of discoloration along with the diameter of the area of discoloration were measured as seen in Fig 3.1 and the following equation was used to determine the EAI:

$$\text{EAI} = \frac{DIAMETER\ OF\ CLEARZONE + DIAMETER\ OF\ COLONY}{DIAMETER\ OF\ COLONY}$$

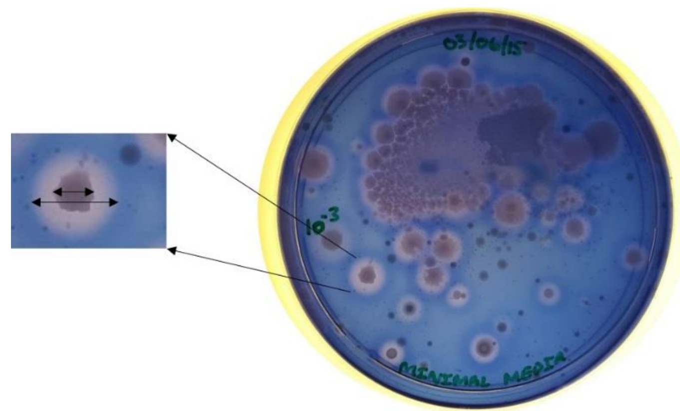

Fig 3.1: Measurements taken to determine the enzyme activity index, the diameter of the bacterial colony as well as diameter of the zone of trypan blue discoloration

A cutoff value above 2.4 was used as an indicator of maximum cellulolytic enzyme production. Isolates showing a superior EAI were then taken for further analysis (Kaur and Arora, 2012, Romano *et al*., 2013).

3.5 Enzyme Activity Assay

The submerged fermentation process was used to determine the enzyme activity of each bacterial isolate. Isolates were inoculated into minimal broth identical to that used for serial dilution, containing 0.2% K_2HPO_4, 0.03% $MgSO_4.7H_2O$, 1% peptone, 0.25% $(NH_4)_2SO_4$ and 1% CMC. The inoculated medium was incubated in a shaker incubator at 150 rpm for 48 hours.

After the incubation period, the broth medium was centrifuged at 15000 rpm at 4°C for 15 minutes. The resulting supernatant was collected as crude enzyme and the remaining cells were

discarded. Dintrosalicylic acid was used to detect the presence of reducing sugars from the degradation of CMC. DNS reagent was prepared by dissolving 10 g of Dintrosalicylic acid salt and 1.98 g of NaOH in 141.6 ml of distilled water over a hot plat at 80°C. To this solution, 30.6 g of potassium tartrate, 0.03 g of phenol crystals and 0.83 g of potassium metasulphate was added to yield 150 ml of regent (Adney and Baker, 1996, Irfan *et al.*, 2012). Sodium citrate phosphate buffer was used as the reaction matrix.

The sodium citrate stock solution was prepared by dissolving 7.3 g of sodium citrate in 250 ml of distilled water, while sodium phosphate was prepared by dissolving 7.1 g sodium phosphate in 250 ml distilled water. From the stock solutions, 48.5 ml of sodium citrate solution was added to 51.5 ml of sodium phosphate solution to prepare the buffer, 1% CMC was added to the buffer , the pH was adjusted to 5 using 32% HCl solution.

To determine the enzyme activity, 340 µl of supernatant was added to 340 µl of phosphate-citrate buffer in a 1500 µl Eppendorf tube, the mixture was incubated at 50°C for 60 minutes using a heating block. After the incubation period, 740 µl of DNS reagent was added to the mixture and incubated at 100°C for 10 minutes. After the incubation period, the reaction mixture was placed in an ice bath to halt the reaction. The reaction mixture was then decanted into a 2 ml cuvette and liberated sugars were determined by measuring absorbance of the mixture at 540 nm using the Thermo Spectronic Helios Epsilon UV-visible spectrophotometer (Adney and Baker, 1996).

The enzyme activity was calculated with the use of a standard glucose curve. Anhydrous glucose powder was diluted in distilled water to prepare 0.4 mg/ml, 0.5 mg/ml, 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml, 0.9 mg/ml and 1 mg/ml solutions. The solutions were subjected to the DNS assay according to methods above. Absorbance readings were used to plot a linear graph against glucose concentration. The enzyme activity of isolates was extrapolated from the graph.

3.8 Bacterial Identification

3.8.1 Morphological and Biochemical Characterization

The cell morphology of the bacterial isolates having cellulase activity was determined based on culture configuration, margin, elevation, and color. Some 24-hour pure cultures were assessed for these characteristics on minimal media agar plates that contained 0.2% K_2HPO_4, 0.03% $MgSO_4.7H_2O$, 1% peptone, 0.25% $(NH_4)_2SO_4$ and 1% CMC.

3.8.1.1 Gram Staining

Gram's staining technique was applied to the samples following basic laboratory procedures indicated by Madigan *et al*. (2004).

3.8.1.2 Catalase Test

This test is designed for detection of the enzyme catalase. This enzyme is responsible for preventing hydrogen peroxide accumulation within bacterial cells during aerobic metabolism. A bacterial smear was prepared on the slide, and a drop of H_2O_2 (Merck) was added onto the smear. A positive test was indicated by the formation of effervescence. The absence of this effervescence indicated a negative test (Taylor and Achanzar, 1972).

3.8.1.3 Oxidase Test

An oxidase kit, cytochrome oxidase reagent was used to carry out the test. A sterile wire loop was used to transfer a colony onto a Whatman filter paper and a drop of oxidase reagent was added to it. The results were observed after 30 seconds. Formation of a purple colour indicated a positive test, while no colour change; indicated an oxidase negative organism (Manero and Blanch, 1999).

3.8.1.4 Citrate Utility Test

Citrate utilization test determines the ability of cultivated soil bacteria to convert citrate into oxaloacetate. In the citrate medium; citrate is the only carbon source available to the bacteria. To perform the test, 23.3 g of Simmons Citrate Agar (Merck) was dissolved in 1000 ml of distilled water. The medium was boiled to dissolve. About 10 ml was dispensed into test tubes. The tubes containing the agar solution were autoclaved at 121°C for 15 minutes. The tubes were then placed as slants and allowed to cool down. The media were inoculated by streaking with bacterial isolates on the slants' surfaces using a sterile wire loop. The test tubes were capped and incubated at 37°C for 24 hours. The results were read and recorded based on the colour change, from green to blue. If bacteria cannot utilize citrate as a carbon source then it will not grow on the medium and if they can utilize citrate as a source of carbon; then the media will turn bright blue (Madigan *et al*., 2004)

3.8.1.5 Motility Test

In this test, 30 g of nutrient agar was suspended in 1000 ml distilled water and allowed to dissolve by boiling. The medium was poured into test tubes and autoclaved at 121°C for 15 minutes. After cooling, soft agar was stab inoculated with bacteria tested and incubated for 24-48 hours at 37°C. A positive result was indicated by diffuse or cloudy growth mostly at the top and bottom of the stab and the negative result was detected by growth in distinct zone directly along the stab (Manero and Blanch, 1999).

3.8.1.6 Starch Hydrolysis Test

The purpose of the test was to establish whether the microbe could utilize starch, a complex carbohydrate made from glucose as a source of carbon and energy for growth. Some 21g of starch agar was added to 1000 ml distilled water, allowed to dissolve by boiling and autoclaved at 121°C for 15 minutes. The medium was allowed to cool and was poured on petri dish plates.

An inoculum from a pure culture was streaked on the starch agar plates and incubated at 35°C for 24 hours. Iodine was then added to flood the bacterial growth. The presence of clear halos surrounding colonies was an indication of a positive test (Madigan *et al.*, 2004)

3.8.2 Molecular Identification

3.8.2.1 Bacterial DNA Extraction

Bacterial isolates were sub-cultured for 24 hours before DNA extraction. Extraction was done using the Zymo Research Microbe DNA MiniPrep according to the manufacturers instruction guide. About 100 mg (wet weight) of bacterial isolate was inserted into a ZR Bashing Bead™Lysis Tube that contained 750 µl Lysis solution. The tube was secured onto a Distributor Genie™ bead beater fitted with a 2 ml tube holder and processed at maximum speed for 5 minutes. The Bashing Bead™Lysis tube was then centrifuged in a Tomos MultiStar 21 microcentrifuge at 10000 rpm for 1 minute. Some 400 µl of the supernatant was transferred to a Zymo-Spin™ IV Spin Filter in a collection tube and centrifuged at 7000 rpm for 1 minute. After centrifuging, 1200 µl of soil DNA Binding Buffer was added to the filtrate in the collection tube. About 800 µl of the resulting mixture was transferred to a Zymo-Spin™ IIC column in a collection tube and centrifuged at 10000 rpm for 1 minute. The flow through was discarded and the process was repeated using the remaining 800 µl from the previous step. To the Zymo-Spin™ IIC column in a new collection tube, 200 µl of DNA Pre-Wash Buffer was added and the tube was centrifuged at 10000 rpm for 1 minute, 500 µl of Soil DNA Wash Buffer was added to the Zymo-Spin™ IIC column and again centrifuged at 10000 rpm for 1 minute. The column was then transferred to a clean 1.5 ml micro centrifuge tube and 100 µl of DNA Elution Buffer was added directly to the column matrix and centrifuged at 10000 rpm for 30 seconds to elute the DNA.

3.8.2.2 16S rDNA Gene Amplification

The 16S rDNA gene was amplified from genomic DNA obtained from bacterial cultures by PCR with previously described fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') (Weisburg *et al*., 1991). PCR was performed in a total volume of 50 µl containing 30-50 ng DNA, 100 mM each primer, 0.05 U/µl *Taq* DNA polymerase, 4 mM $MgCl_2$, and 0.4 mM of each dNTP. The amplification reaction was performed with a BioRad C1000 Touch Thermal Cycler. The thermal cycling condition used were an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 1 minute and extension at 72°C for 2 minutes, followed by a final extension at 72°C for 5 minutes. The PCR amplicons were analysed by electrophoresis on 1% (w/v) agarose gel. The gel containing ethidium bromide (10µg/ml) was viewed under a BioRad Universal Hood III Gel Doc Imager to confirm the expected size of the PCR products. The Sequencing of the purified PCR products were conducted at facilities of Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa.

Nucleotide sequences were analysed and edited using BioEdit software (Hall, 1999). The obtained sequences were compared to sequences in the NCBI GenBank database with the Basic Alignment Search Tool (BLAST) (Altschul *et al*., 1990). Multiple alignments of the sequences were carried out by Mafft program 7.050 (Katoh, 2013) against corresponding nucleotide sequences retrieved from GenBank.

3.8.3 Phylogenetic Analysis

Phylogenetic and molecular evolutionary analyses were conducted using the software MEGA version 5.2.2 (Tamura *et al*., 2011). Evolutionary distance matrices were generated as described by Jukes and Cantor (1969) and a phylogenetic tree was inferred by neighbour-joining method (Saitou and Nei, 1987). Tree topologies were evaluated by bootstrap analysis

based on 1000 resampling of the neighbour-joining data set (Felsenstein, 1985). Manipulation and tree editing were carried out using Tree View (Page, 1996).

3.6 Substrate Based Enzyme Assay

In order to determine the production of endoglucanase, exoglucanase, and β-glucosidase by bacterial isolates, CMC, avicel, and cellobiose were used as enzyme stimulating substrates respectively.

Submerged fermentation medium consisting of 1% peptone, 0.2% K_2HPO_4, 0.03 $MgSO_4.7H_2O$, 0.25% $(NH_4)_2SO_4$ dissolved in distilled water was used. The medium was inoculated with bacterial isolates having the best cellulolytic enzyme activity. The inoculated medium was incubated at 37°C for 48 hours and enzyme activity assay was carried out as per instructions above (Romano *et al*., 2012; Gupta *et al*., 2014). Fig 3.2 illustrates how inoculation media were supplemented with different carbon substrates for the stimulation of different cellulose enzymes.

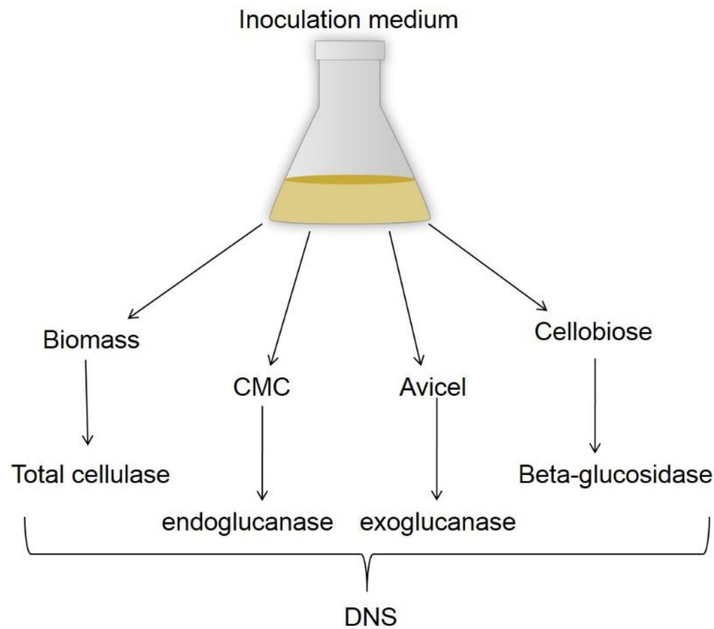

Fig 3.2: Inoculation medium modified with different carbon substrates to stimulate endoglucanase, exoglucanase and beta-glucosidase enzyme production subjected to DNS assay.

3.6 Optimization of Enzyme Activity

3.6.1 Optimization by Incubation Period

The optimum incubation period was determined by inoculating bacterial isolates into minimal media broth consisting of 1% peptone, 0.2% K_2HPO_4, 0.03 $MgSO_4.7H_2O$, 0.25% $(NH_4)_2SO_4$. The inoculum was incubated at 37°C for a total of 120 hours and the cellulose activity was determined by the DNS method at 24 hour intervals.

3.6.2 Temperature Optimization

The effect of temperature on enzymatic activity was tested by performing the DNS assay with slight modifications. The phosphate-citrate CMC buffer and crude enzyme mixtures were incubated at 20°C, 30°C, 40°C, 50°C, 60°C, 70°C and 80°C for 1 hour before DNS reagent was added. The assay was carried out according to instructions above.

3.6.3 pH Optimization

The optimum pH for bacterial enzyme activity was determined by performing a DNS assay with appropriate buffers having different pH levels. The buffers mentioned below were prepared according to protocols by Gomori (1955).

Preparation of Glycine-HCl buffers

Glycine-HCl buffer was used to measure the enzyme activity at pH 2, 3 and 4.

In order to prepare a buffer at pH 2, 50 ml of 0.2 M glycine solution was added to 44 ml of 0.2 M HCl solution in a volumetric flask, the solution was then diluted to 200 ml using distilled water.

To prepare a buffer at pH 3, a volume of 50 ml of 0.2 M glycine solution was added to 11.4 ml of 0.2 M HCl solution in a volumetric flask, the solution was then diluted to 200 ml using distilled water.

To prepare a buffer at pH 4, some 50 ml of 0.2 M glycine solution was added to 3.6 ml of 0.2 M HCl solution in a volumetric flask, the solution was then diluted to 200 ml using distilled water.

Preparation of phosphate-citrate buffers

Phosphate-citrate buffer was used to measure enzyme activity at pH 5, 6, 7 and 8

To prepare a buffer at pH 5, some 24.3 ml of 0.2 M solution of sodium citrate solution was added to 25.7 ml of sodium phosphate in a volumetric flask, the solution was diluted to 100 ml using distilled water.

To prepare a buffer at pH 6, 17.9 ml of 0.2 M solution of sodium citrate solution was added to 32.1 ml of sodium phosphate in a volumetric flask, the solution was diluted to 100 ml using distilled water.

To prepare a buffer at pH 7, 6.5 ml of 0.2 M solution of sodium citrate solution was added to 43.6 ml of sodium phosphate in a volumetric flask, the solution was diluted to 100 ml using distilled water.

To prepare a buffer at pH 8, 6.5 ml of 0.2 M solution of sodium citrate solution was added to 43.6 ml of sodium phosphate in a volumetric flask, the pH was adjusted to 8 using 0.2 M NaOH solution, the solution was diluted to 100 ml using distilled water.

Preparation of glycine-NaOH buffers

Glycine-NaOH buffer was used to measure enzyme activity at pH 9 and 10

To prepare a buffer at pH 9, 50 ml of 0.2 M glycine solution was added to 8.8 ml NaOH solution in a volumetric flask, the solution was diluted to 200 ml using distilled water.

To prepare a buffer at pH 10, 50 ml of 0.2 M glycine solution was added to 32 ml NaOH solution in a volumetric flask, the solution was diluted to 200 ml using distilled water.

To all buffers, 1% CMC was added as a carbon substrate to stimulate cellulase activity.

3.6.4 Nitrogen Substrate Optimization

The effect of variation in nitrogen source within the inoculation minimal medium on enzyme activity was tested using $(NH_4)_2SO_4$, KNO_3, yeast extract, $NaNO_3$, urea and NH_4Cl as alternative nitrogen based minerals. The inoculation medium was prepared using the ingredients listed above (1% peptone, 0.2% $MgSO_4$.7H2O, 1% CMC) with addition of 0.25% of the selected nitrogen mineral. A standard DNS assay was performed to determine the enzymatic activity of bacterial isolates.

3.7 Partial Purification of Enzyme

Crude enzyme was partially purified using the ammonium sulphate precipitation technique. About 50 ml of minimal medium broth containing 1% peptone, 0.2% K_2HPO_4, 0.03% $MgSO_4.7H_20$, 0.25% $(NH_4)_2SO_4$ and 1% CMC was prepared. The medium was inoculated with bacterial colonies from isolates having the best cellulolytic activity. The inoculum was incubated at 37℃ in a shaking incubator at 140 rpm for 48 hours. After the incubation period, the broth medium was centrifuged at 10 000 rpm for 10 minutes at 4℃. The supernatant was taken as crude enzyme and the bacterial pellet was discarded.

To the 50 ml of crude enzyme supernatant, ammonium sulphate was added to bring the solution to 40% saturation based on a standard ammonium sulphate precipitate nomogram. The supernatant was incubated at 4℃ in a shaking incubator for 24 hours at 140 rpm. After the incubation period, the supernatant was centrifuged at 10 000 rpm for 5 minutes. The precipitate was collected and dissolved in phosphate-citrate buffer at pH 5 and dialyzed in a cellulose dialysis membrane submerged in phosphate-citrate buffer. This was then incubated in a shaking incubator at 4℃ at 80 rpm for 12 hours. The dialyzed protein was then tested for enzyme activity(Okoye *et al.*, 2013, Anuradha Jabasingh *et al.*, 2014).

3.8 In-silico Restriction Cloning

A GenBank nucleotide search was conducted to obtain the nucleotide sequences coding for endoglucanase genes specific for the bacterial isolates *B. subtilis* and *B. tequilensis*. The endoglucanase genes CelDr and 1,3-1,4-β-glucanase were identified and their nucleotide sequences were obtained from the GenBank nucleotide database as genes exhibited by *B. subtilis* and *B. tequilensis* respectively (Li *et al.*, 2008, Wang *et al.*, 2014) . In-silico PCR was conducted using the primers DR-up: 5′-GCG GGATCCATGAAACGGTCAATCTC-3′ and DR-down: 5′-TGCGGCCGCCTAATTTGGTTCTG-3′ for detection of the CelDr gene as well as bglt-f (5′-CGCGGATCCATGAAACGAGTGTTGCTAATTCTT-3′ and bglt-r (5′-

CGCTCGAGTTATTTTTTTGTATAGCGCACCCA-3′ for the detection of 1,3-1,4-β-glucanase (Li *et al*., 2008, Wang *et al*., 2014).

In-silico gene cloning was further conducted to clone both the CelDr and 1,3-1,4-β-glucanase genes into a pGEM-T vector. The pGEM-T vector map was used to determine appropriate restriction enzymes for the insertion of foreign DNA.

Chapter 4

4. Results

4.1 Isolation of Cellulolytic Bacteria

A total of 31 isolates showing cellulolytic potential were isolated from soil samples which were serially diluted onto minimal medium agar with CMC as a sole carbon source and trypan blue dye as an indicator for cellulolytic activity. The EAI of all isolates was determined and 7 isolates that displayed an index greater or equal to 2.4 are shown in Fig 4.1. Isolate LD25 showed the highest index followed by isolate LD28 as illustrated in Fig 4.2. The high enzyme activity index observed in isolate LD25 is as a result of a relatively small colony size compared to its zone of decolouration.

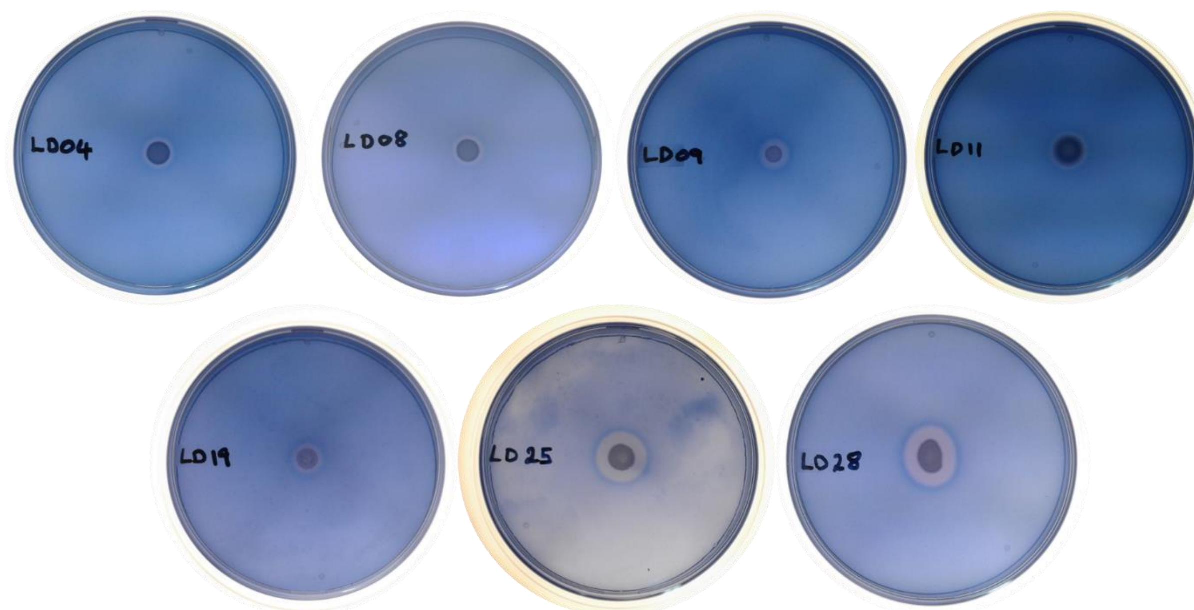

Fig 4.1. Bacterial growth on minimal media agar plate supplemented with trypan blue dye. Bacteria showing cellulase activity are characterized by decolouration of trypan blue around the bacterial colony.

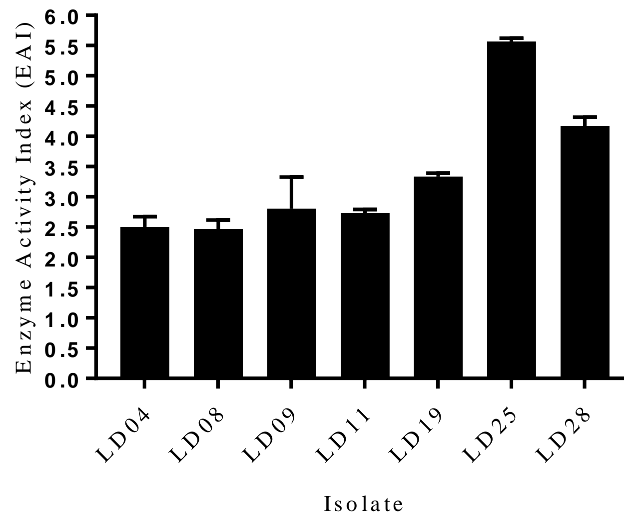

Fig 4.2: Enzyme Activity Index of bacterial isolates, characterized by the relation between the diameter of the bacterial colony and the diameter of the trypan blue dye zone of decolouration.

4.2 Bacterial Identification

Table 4.1 Cultural characterisation of isolates

Isolate	Configuration	Margin	Elevation	colour
LD04	concentric	smooth	raised and flat	opaque
LD08	round	wavy	Convex	cream
LD09	round	smooth	Convex	pale yellow
LD11	concentric	irregular	Raised	white
LD19	filamentous	lobate	Flat	white
LD25	concentric	smooth	Flat	opaque
LD28	Irregular wrinkled	wavy	Raised and flat	Pale brown

Table 4.1 displays morphological characteristics exhibited by the cellulolytic isolates. Their biochemical characteristics are also shown in Table 4.2

Table 4.2 Biochemical Characterization of Isolates

Isolate	Gram stain	Cell Shape	Oxidase test	Catalase test	Motility test	Starch hydrolysis	Citrate test
LD04	+	rod	-	+	+	+	+
LD08	+	rod	+	+	+	+	+
LD09	+	rod	-	+	+	+	+
LD11	+	rod	-	+	+	+	+
LD19	+	rod	+	+	+	+	-
LD25	+	rod	+	+	+	+	+
LD28	+	rod	+	+	+	+	+

Results obtained from the 16S rDNA PCR amplification are shown in Fig. 4.3. Table 4.3 contains results obtained from the GenBank nucleotide sequence analysis obtained from BLAST. The BLAST search showed that all isolates belonged to the *Bacillus* genus with similarities ranging from 98% to 100%. Figure 4.4 represents Neighbour-joining tree of the bacterial isolates and representative species based on partial 16S rDNA gene sequences. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 40% are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

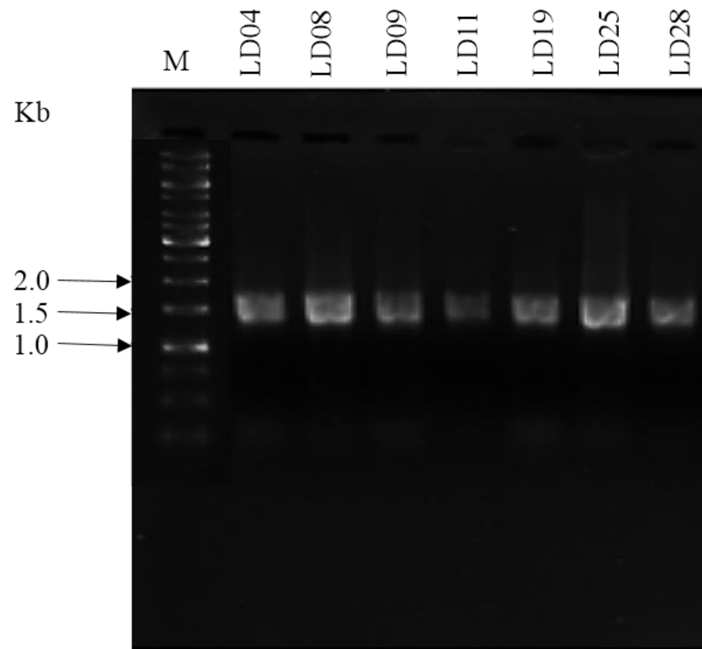

Figure 4.3: Agarose gel electrophoresis of 16S rDNA PCR amplification products of isolates having cellulolytic activity. With 'M' indicating 1 Kb marker.

Table 4.3 GenBank Nucleotide sequence analysis

Isolate Number	Sequence Length	Description	Assigned accession number	% similarity	E-value
LD04	1410	*Bacillus cereus*	KX355793	100	0.0
LD08	1398	*Bacillus thuringiensis*	KX355795	100	0.0
LD09	1401	*Brevibacterium halotolerans*	KX355796	100	0.0
LD11	1409	*Bacillus cereus*	KX355797	100	0.0
LD19	1274	*Bacillus anthracis*	KX355798	98	0.0
LD25	1279	*Bacillus tequilensis*	KX355794	100	0.0
LD28	1344	*Bacillus subtilis*	KX355799	100	0.0

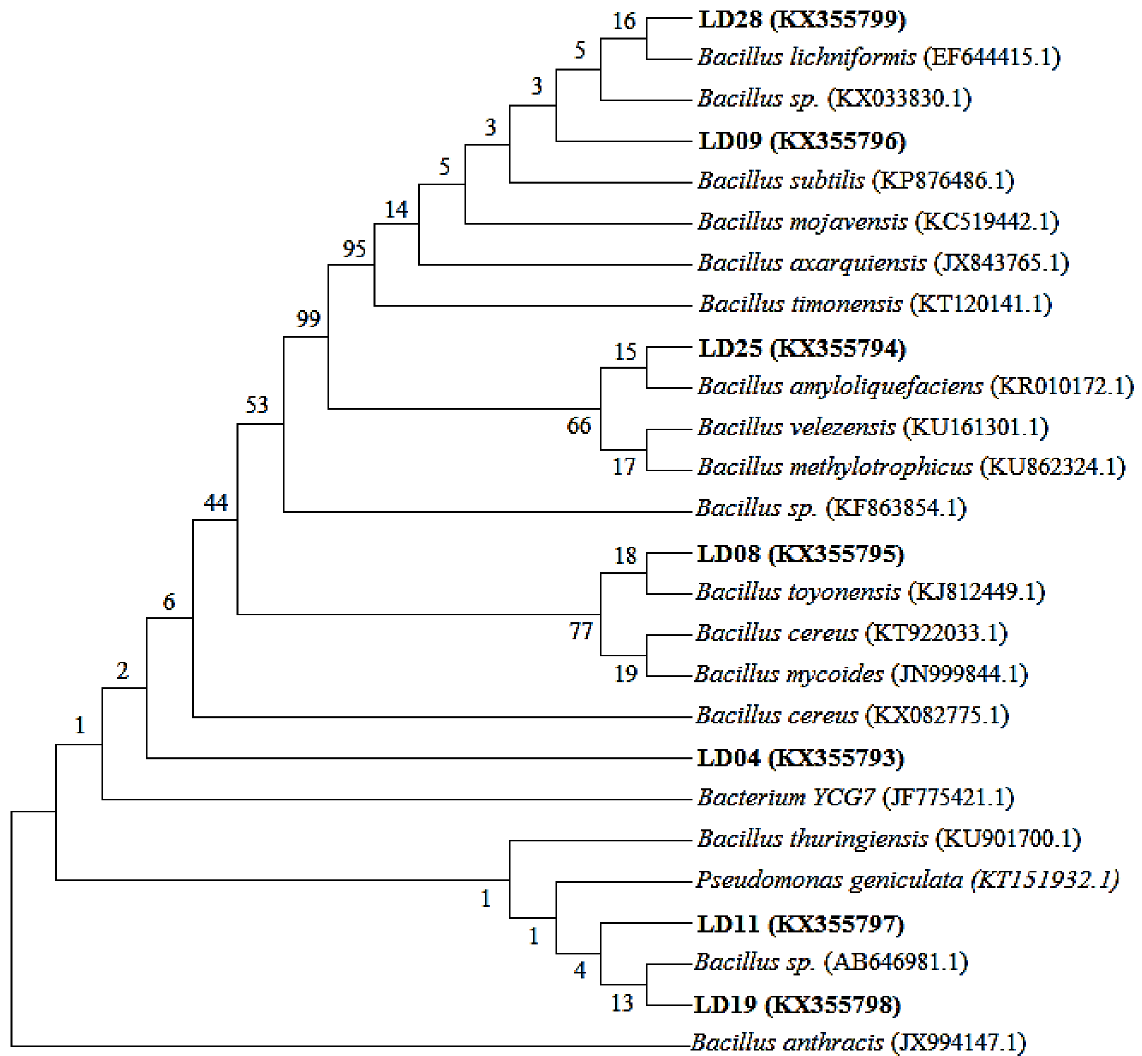

Fig 4.4: A neighbour-joining phylogenetic tree from 16S rDNA gene sequences of bacterial isolates showing evolutionary relationship between bacterial isolates and existing GenBank nucleotide sequences. The evolutionary history was inferred using the neighbour-Joining method with the bootstrap consensus tree inferred from 1000 replicates taken to represent the evolutionary history of the data.

4.3 Screening for Cellulase Activity

The enzyme activity assay performed on the 7 isolates having the highest EAI revealed that isolate LD25 and LD28 had the highest enzyme activity with an enzyme activity of 0.125 UI/ ml and 0.167 UI/ml respectively shown in Fig 4.5. The 2 isolates with the highest cellulolytic activity were selected for further analysis.

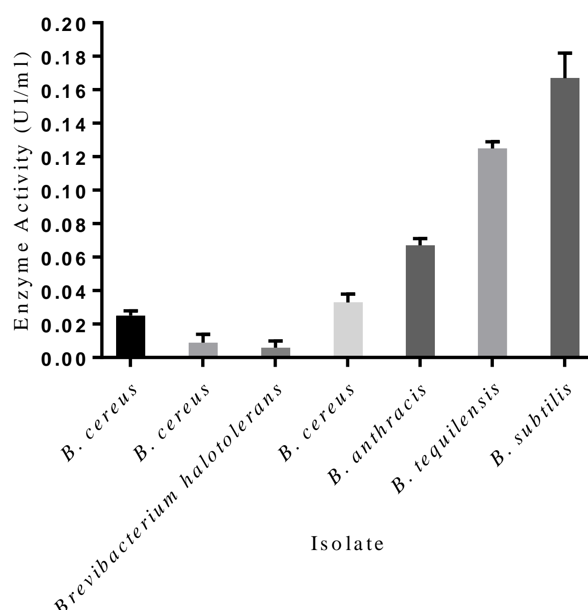

Fig 4.5: The cellulolytic activity of bacterial isolates after a 48 hours incubation period in minimal submerged fermentation medium with error bars indicating standard errors.

4.4 Substrate-Based Enzyme Assay

Fig 4.6 indicates that both isolates displayed high total cellulase production, a consequence of the synergic action of all cellulases in the degradation of maize bagasse. Isolate LD28 showed the highest production of enzyme with an activity of 0.234 U/ml compared to LD25 at 0.197 U/ml. The isolates displayed the ability to produce endoglucanase through utilization of CMC as a carbon source, LD28 showed the highest endoglucanase activity at 0.167 U/ml with LD25 having an activity of 0.125 U/ml. Both isolated produced exoglucanase at relatively low

amounts compared to the other enzymes, growth on avicel media produced activities of 0.01 and 0.019 U/ml from isolates LD25 and LD28 respectively. Production of β-glucosidase was confirmed with both isolates yielding activities of 0.081 and 0.142 U/ml respectively.

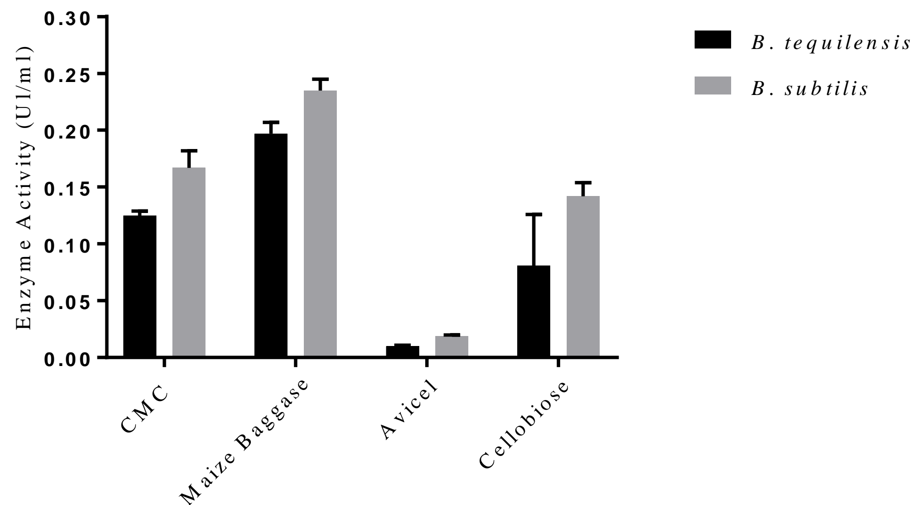

Fig 4.6: Enzyme activity of isolate *B. tequilensis* and *B. subtilis* on different carbon substrates stimulating total cellulase, endoglucanase, exoglucanase and beta-glucosidase production with bars indicating standard error.

4.5 Enzymatic Assay of Partially Purified Enzyme

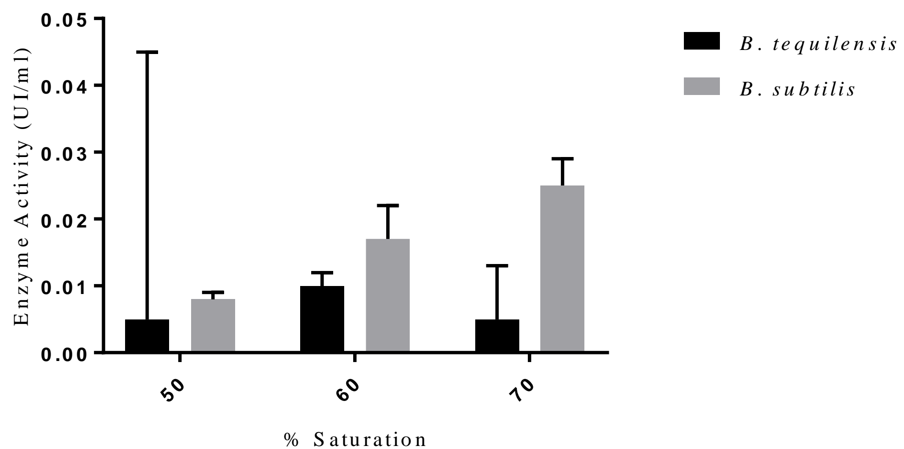

Fig 4.7. Enzyme activity of partially purified enzymes from *B. tequilensis* and *B. subtilis* with bars indicating standard error.

4.6 Optimization of Cellulolytic Activity

4.6.1 Optimization by Incubation Period

Figure 4.8 Illustrates that the isolates showed rapid growth within the first 24 hours of incubation, it was also within this period that the isolates produced the highest cellulase activity with isolate *B. subtilis* (LD28) having a maximum activity of 0.167 UI/ml and *B. tequilensis* (LD25) with an activity of 0.125 UI/ml. The activity of both isolates was shown to decrease with an increase in incubation period.

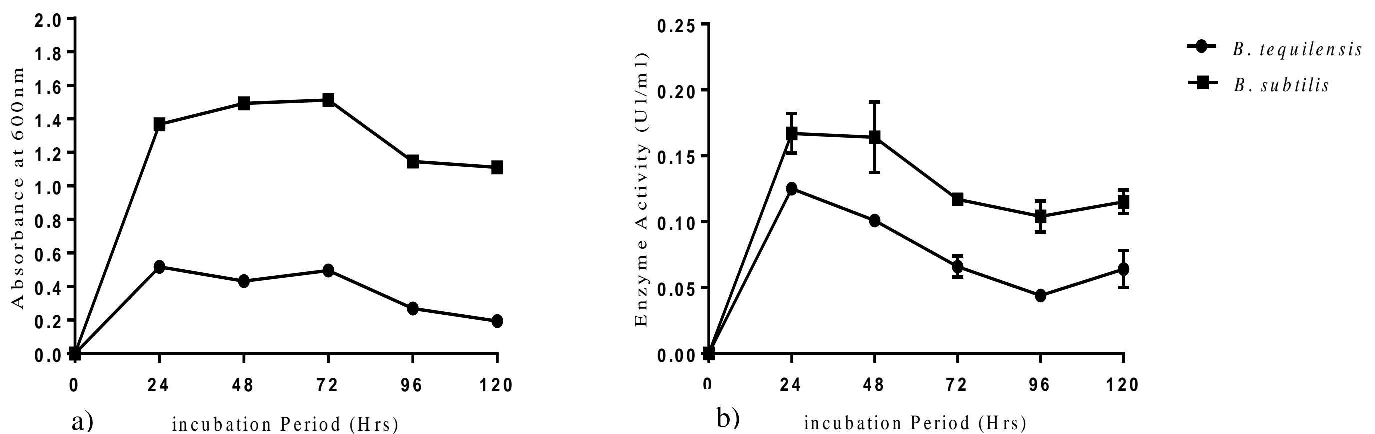

Fig 4.8: Effect of incubation period on enzymatic activity where a) represents the bacterial growth curve of bacterial isolates and b) represents the cellulolytic activity of each isolate at given time period with error bars showing statistical variation in mean values (standard error)

4.6.2 pH Optimization

Figure 4.9 indicates that isolate LD25 showed optimum enzyme activity at pH 4 while LD28 displayed activity at a slightly higher pH of 5. These results indicate an affinity to acidic conditions for both isolates for the stimulation of cellulase production.

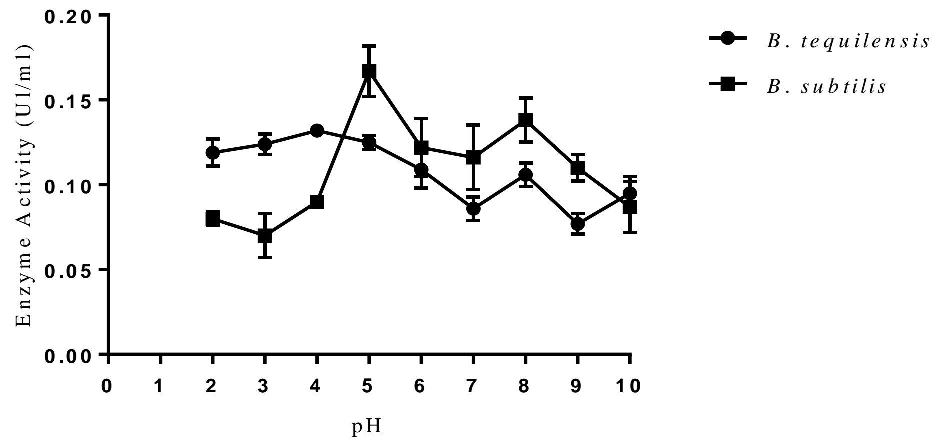

Fig 4.9: Effect of varying pH on cellulase activity *B. tequilensis* and *B. subtilis* with standard errors indicated by error bars.

4.6.3 Temperature Optimization

The enzyme activity for bacterial *B. tequilensis* and *B. subtilis* was tested at temperatures of 20°C, 30°C,40°C, 50°C, 60°C, 70°C and 80°C. Figure 4.10 shows that highest cellulase activity was recorded at 50°C for *B. subtilis* and at 60°C for *B. tequilensis*, with the activity of both isolates decreasing with an increase in temperature post the point of optimal temperature.

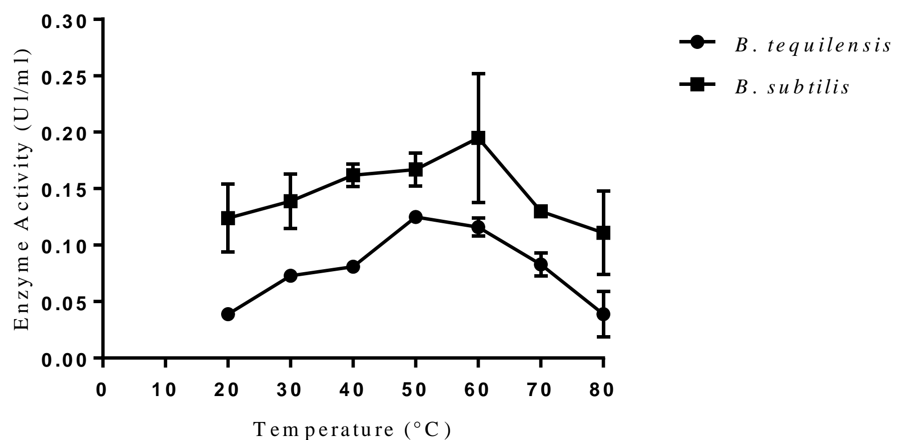

Fig 4.10: Effect of varying temperature on the enzymatic activity of *B. tequilensis* and *B. subtilis* with standard errors indicated by error bars.

4.6.4 Nitrogen Source Optimization

As a key nutrient in bacterial growth and health, bacterial isolates were assayed for their ability to produce cellulases in response to the introduction of different nitrogen substrates (potassium nitrate, yeast extract, sodium nitrate, urea, ammonium nitrate and peptone).Figure 4.11 shows that isolate LD28 responded significantly better to the peptone compared to LD25, showing an activity of 0.167 U/ml and 0.125 U/ml respectively. Isolate LD25 showed the least enzyme activity in the presence of urea as a nitrogen source while isolate LD28 showed its activity to be susceptible to sodium nitrate.

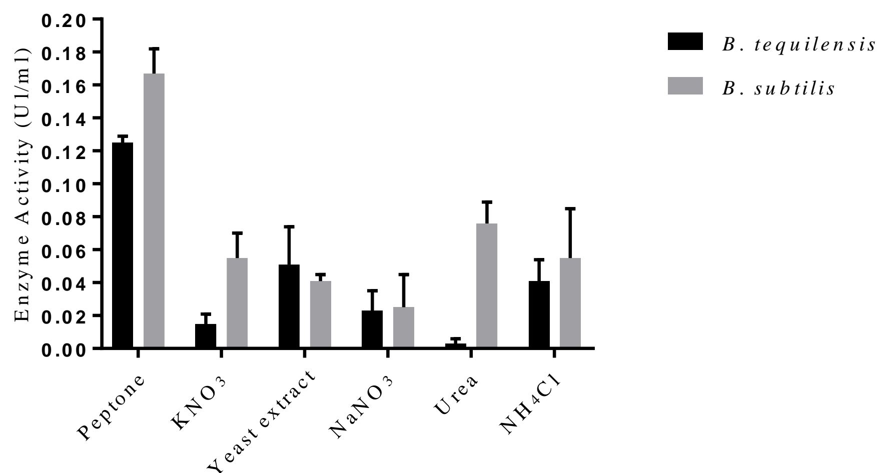

Fig 4.11: Effect of different nitrogen sources on the enzymatic activity of *B. tequilensis* and *B. subtilis* with standard errors indicated by error bars.

4.7 In-silico Restriction Cloning

Figure 4.12 shows the successful cloning of the CelDr endoglucanase gene consisting of 1527 bp into the pGEM-T vector at sites characterised by BsaAI and BpmI restriction sites to yield the recombinant pGEM-CelDr. The endoglucanase gene 1,3-1,4-β-glucanase consisting of 276 bp was also cloned into the pGEM-T vector at sites characterised by BtgZI and SpeI restriction enzymes to yield the recombinant, pGEM-E-b-Glu.

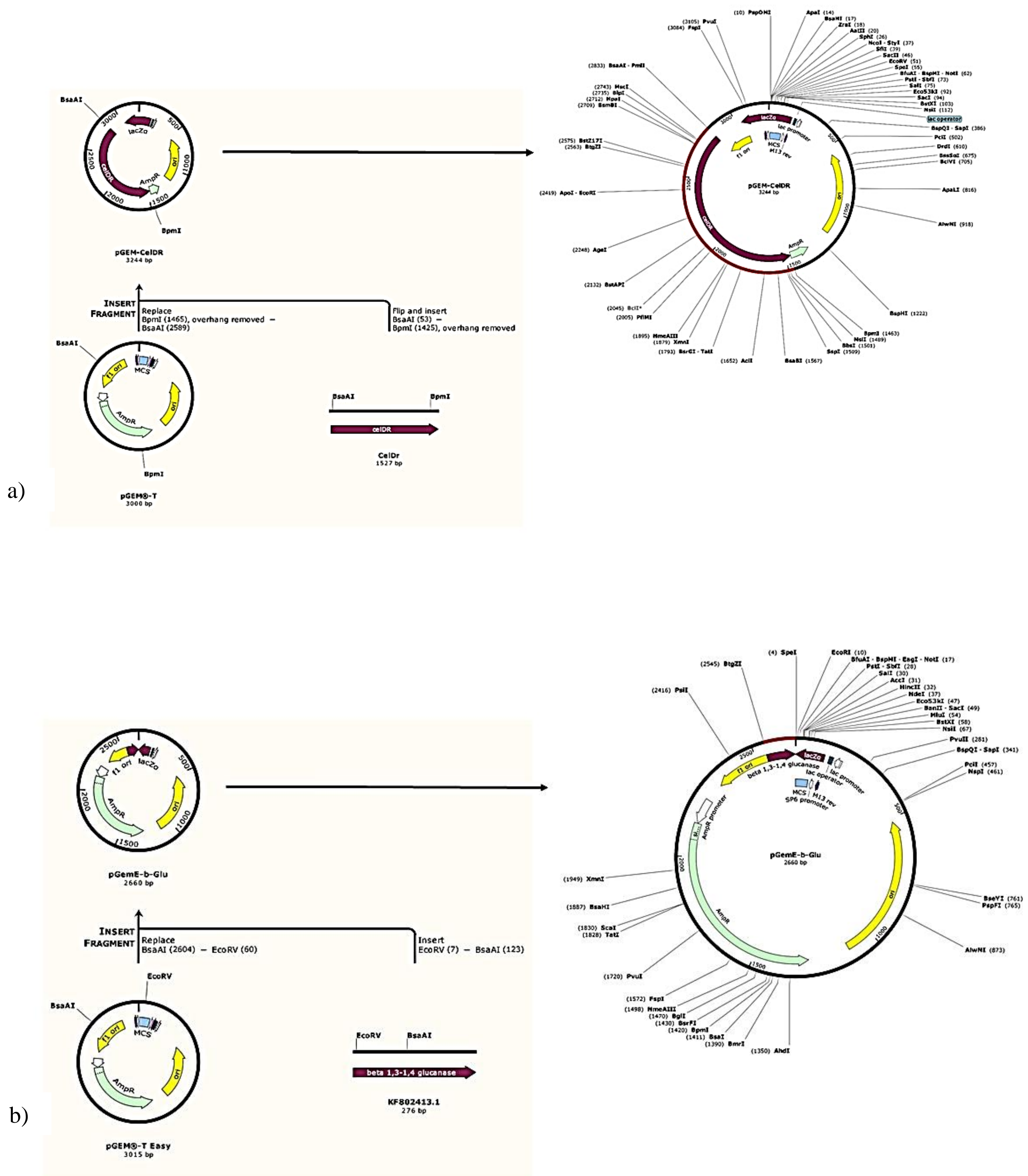

Fig 4.12: Cloning maps of vectors with insertion of gene of interest where a) represents insertion of CelDr gene into the pGEM-T vector to yield pGEM-CelDr recombinant and b) represents insertion of the 1,3-1,4-β-glucanase gene into the pGEM-T vector to yield pGemE-b-Glu recombinant.

Chapter 5

5. Discussion

5.1 Screening Isolation and of Cellulolytic Bacteria

An essential step in the global carbon cycle is the hydrolysis of plant matter consisting predominantly of cellulose, the most abundant source of carbon within the terrestrial environment (Wilson, 2011). Cellulolytic bacteria in soil play a major role in this process, they are responsible for providing carbon sources within the soil which in turn improve soil fertility and sustain the nutrient balance of the soil ecosystem through decomposition of cellulosic remains (Yang *et al*., 2014).

A total of 6 bacterial species having cellulolytic activity were isolated from soil samples, these isolates were identified as LD04/ LD11 (*B. cereus*), LD08 (*B. thuringiensis*), LD09 (*Brevibacterium halotolerans*), LD19 (*B. anthracis*), LD25 (*B. tequilensis*) and LD28 (*B. subtilis*). All isolates were selected based on a calculated enzyme activity index 2.4 and above. The endoglucanase enzyme activity assay performed on all isolates revealed that isolate LD25 (*B. tequilensis*) and LD28 (*B. subtilis*) produced the highest enzyme activity at 0.125 UI/ml and 0.167 UI/ml respectively, for this reason, the isolates were subjected to further analysis.

The DNS assay, a widely used method recommended by the International Union of Pure and Applied Chemistry (IUPAC) established in 1982 was employed in the study. In this method, cellulase activity was determined from the enzyme concentration required to produce a certain amount of reducing sugar in 1 minute. The amount of sugar produced was determined spectrophotometrically under the assumption that all sugars produced are glucose (Hu *et al*., 2009).

Fungal species have been found to produce abundant cellulases that are easily extracted with most of them finding great commercial importance (Liang *et al*., 2014). In recent years, researchers have begun paying attention to various bacteria that have the ability to produce cellulases, this is in response to their ability to grow fast, produce multi-enzyme complexes and resistance to extreme conditions. Isolated bacteria have included those belonging to genera such as *Clostridium, Cellulomonas, Cellulosimicrobium, Thermomonospora, Bacillus, Ruminococcus, Erwinia, Bacteriodes, Acetovibrio, Streptomyces, Microbispora, Fibrobacter,* and *Paenibacillus* (Liang *et al*., 2014).

There are a number of forms of cellulose that are used to assay for cellulases, however, in this study CMC was chosen as the ideal form of cellulose due to its solubility and stimulation of endoglucanase production without the requirement of CBMs for hydrolysis (Wilson, 2011). The activity of cellulases was selected as an important parameter in this study since it characterises the performance and efficiency of the cellulase.

According to Liang *et al*. (2014), several studies have been carried out to investigate the cellulolytic activity of aerobic bacteria. A maximum activity of 0.48 U/ml has been recorded from *Acinetobacter anitratus* while activities of 0.02 and 0.058 U/ml have been determined from *Brevibacillus sp. and Geobacillus sp.* respectively.

The phylogenetic evaluation in this study showed that *B. subtilis* (LD28) had close evolutionary similarities to *B. licheniformis, B. mojavensis, B. axarquiensis and B. timonensis*. *B. tequilensis* (LD25) showed evolutionary similarities to *B. amyloliquefaciens, B. velezensis* and *B. methylotrophicus.* Althouth these evolutionary relatives are not prominent cellulase producers, previous studies show that relatives possess cellulases that have activities that are higher or close to those of isolates obtained in the current study, for example, Lee *et al*. (2008) found that *B. amyloliquefaciens* effectively hydrolysed rice hull and other cellulosic waste materials

with a CMCase activity that varied between 0.528 UI/ml and 0.132 UI/ml. Similarly, *B. methylotrophicus* was found to have a cellulase activity up to 0.230 UI/ml and *B. licheniformis* with an activity of 0.045 UI/ml (Singh *et al*., 2014, Chantarasiri, 2014).

With relation to isolates obtained in this study, Sharada *et al*. (2013) reported that *B. subtilis* produces a considerable amount of cellulase activity and it is for this reason that this bacterial species is utilized in the saccharification of wheat straw, rice straw and sugar cane bagasse. Similarly (Deka *et al*., 2011) found that *B. subtilis* produced an enzyme activity of 0.25 UI/ml, an amount slightly higher than that found in the present study but within a similar range of activity. The cellulolytic potential of *B. tequilensis* is not intensely studied, however, a study by Khianngam *et al*. (2014) isolated the bacterial species from palm meal samples having an endoglucanase activity of 0.05 Ul/ml.

Several cellulolytic anaerobic bacteria secrete large amounts of cellulases as tightly packed clusters of multi-enzyme complexes called cellulosomes which are difficult to disrupt without the loss of cellulase activity. *Bacillus* species specifically lack a complete cellulase system and it is for this reason that endoglucanase activity on CMC is a predominant observation when studying these species (Mawadza *et al*., 2000).

It has been established that 3 types of enzymes are employed by a cellulose system for the complete degradation of cellulose to glucose. These enzymes are endoglucanases, exoglucanases and β-glucosidases all of which are stimulated by CMC, avicel and cellobiose degradation respectively (Niranjane *et al*., 2007). *B. tequilensis* (LD25) and *B. subtilis* (LD28) were subjected to submerged fermentation where maize bagasse, avicel and cellobiose were utilized as sole carbon sources. These carbon substrates were used to stimulate total cellulase, exoglucanase and β-glucosidases production by the isolates.

B. subtilis produced the highest cellulase activities of 0.167 UI/ml with CMC as sole carbon source, 0.019 UI/ml in avicel, 0.142 UI/ml in cellobiose and 0.235 UI/ml in maize bagasse. *B. tequilensis* produced activities of 0.125 UI/ml in CMC, 0.010 UI/ml in avicel, 0.081 UI/ml in cellobiose and 0.197 UI/ml in maize bagasse. These results indicate that to some degree, isolates were able to produce synergic enzymes towards the hydrolysis of crystalline cellulose substrates. These findings prove that not all *Bacillus* species lack a complete cellulase system.

5.2 Optimization of Enzyme Activity

Environmental conditions have widely been identified as key drivers of vital processes in living organisms, such conditions include geochemical and physical variables such as pH, nitrogen and carbon availability, temperature, pressure, incubation period and radiation. The development of adaptive responses to these stress factors has allowed microorganisms to synthesize particular types of enzymes that provide resistance to harsh environmental conditions, cellulase yields in this regard have been shown to depend greatly on these factors (Gautam *et al*., 2011, Soares Jr *et al*., 2012).

5.2.1 Optimization of Incubation Period

The relation between bacterial growth and enzyme production was determined. Both *B. subtilis* and *B. tequilensis* produced maximum cellulase activity after 24 hours of incubation. According to the growth curve, 24 hours signaled the logarithmic phase of bacterial growth a period of rapid growth and enzyme production for the hydrolysis of the available carbon substrate to further facilitate growth (Maier, 2000). It is for this reason that further analysis of the dynamics of cellulases produced by these isolates was carried out after 24 hours of incubation.

The stationary phase of bacterial growth for both isolates occurred between 48 hours of incubation and 72 hours. It was during this period that there was a significant decrease in

cellulase activity among the isolates. The decrease in cellulase activity is associated with a decrease or termination of net growth and it is at this point that the carbon substrate is depleted (Maier, 2000).

5.2.2 pH Optimization

In this study, the optimal pH for cellulase activity was found to be at pH 4 and pH 5 for *B. tequilensis* and *B. subtilis,* from the point of optimum pH, there was a gradual decrease in enzyme activity with an increase in pH. The primary cellulase enzyme produced by both *B. subtilis* and *B. tequilensis* was found to be endoglucanase. According to Immanuel *et al*. (2006) , the endoglucanase enzyme is most active at pH ranging between 4 and 7. An increase in pH gradually denatures the enzyme resulting in a decrease in enzyme activity, a trend that has been observed with most bacterial species having cellulolytic activity (Immanuel *et al*., 2006, Das *et al*., 2011).

A great number of studies have shown that the optimal pH for cellulolytic activity of bacteria ranges between pH 5 and pH 8. Liang *et al*. (2014) found that *Paenibacillus terrae* ME27-1 had optimal cellulolytic activity at pH 8, while *B. subtilis* isolated by Fagade and Bamigboye (2012) showed optimal activity at pH 7 (Fagade and Bamigboye, 2012). Differences in optimal pH for *B. subtilis* obtained in this study and that obtained by Fagade and Bamigboye (2012) can be attributed to genetic adaptations to unique environments from which the bacteria were isolated, these adaptations may be stimulated by nutrient compositions within the soil as well as the overall climate of the region.

5.2.3 Temperature Optimization

Optimal temperatures for *B. subtilis* and *B. tequilensis* were at 50°C and 60°C respectively, similar to the trend with optimal pH, an increase in temperature resulted in a gradual decrease in enzyme activity. Both isolates, however, sustained competitive enzyme activity up to 70°C,

proof of the thermostability of cellulase produced by these isolates. An increase in temperature resultd in an overall decrease in enzyme activity due to denaturing of the cellulase enzyme, conversely, a decrease in temperature resulted in inactivation of the enzyme (Immanuel *et al.*, 2006). Andreaus *et al*. (1999) explains that the decrease in cellulase activity is due to deactivation of the catalytic domain of the enzyme system, a key component which plays a major role in the binding of the enzyme onto the cellulose substrate.

Most industrial processes are carried out at relatively high temperature and there is, therefore, a need for thermostable enzymes. Cellulases produced by bacteria have been found to be more effective catalysts, this is attributed to their ability to remain active in the presence of hydrolyzed materials and are constitutively produced compared to fungal cellulases that are inducible in nature (Acharya and Chaudhary, 2012). Ladeira *et al*. (2015) reported that thermophilic *Bacillus* were able to produce large amounts of cellulose components with the ability to degrade CMC and avicel when grown at temperatures up to 50°C. Kiio *et al*. (2016) also noted that the optimum temperature for crude enzyme activity was higher than that of the predetermined optimum temperature for growth of the isolate, a trend that was observed within this study. Such a findings suggest that within industrial processes requiring high temperatures, it would be advantageous to use extracellularly extracted enzymes rather than the organism itself (Kiio *et al*., 2016)

5.2.4 Optimization of Nitrogen Sources

Nitrogen is a key nutrient substance in microbial growth and thus having a direct influence on the breakdown of organic matter such as cellulose (Kashem *et al*., 2004). In a study by Sethi *et al*. (2013), ammonium sulphate was found to be the best nitrogen source for cellulase production for *Bacillus sp* and *Pseudomonas sp.* In this study, peptone was found to be the best nitrogen source for stimulation of cellulase activity. *B. subtilis (*LD28) showed the lowest

activity in the presence of $NaNO_3$ whereas the presence of urea resulted in a substantial decrease in the activity of *B. tequilensis* (LD25). The substantial decrease in enzyme activity with the presence of urea could be attributed to its property as a protein denaturant. Urea has been found to break intermolecular bonds that are responsible for the tertiary structure of enzyme molecules (Abuin *et al*., 2005). Both isolates did, however, show affinity to yeast extract as a carbon source. Observation of previous studies has shown that different bacterial, as well as fungal cellulolytic species respond differently to different nitrogen substrates. While a certain nitrogen substrate may enhance cellulase activity for a certain species, it may inhibit the activity of another. Among tested substrates, peptone, yeast extract, NH_4NO_3 and beef extract have consistently shown to be ideal cellulase activity enhancers (Kachlishvili *et al*., 2006, Gautam *et al*., 2011, El-Hadi *et al*., 2014).

5.3 In-silico Restriction Cloning

The endoglucanase gene CelDR unique to *B.subtilis* was cloned into a pGEM-T vector using the restriction enzymes BamHI and NotI by Li *et al*. (2008). In contrast, In-silico simulation conducted in this study utilized the restriction sites BsaAI and BpmI to clone the 1527 bp long gene into a pGEM-T vector. Similar finding were observed with regard to cloning of the 1,3-1,4-β-glucanase. Wang *et al*. (2014) showed that cloning of the 1,3-1,4-β-glucanase gene found in *B.tequilensis* into a pGEM-T vector can be achieved by utilizing the restriction enzymes BamHI and XhoI a deviation from simulation results that showed that BsaAI and EcoRV were the most favored restriction sites.

The knowledge of genetic information has proven in recent years to be important in biotechnological research and improvements in sequence technology. It is through the knowledge of genomic data that novel proteins having biotechnological importance have been identified (Passier and Doevendans, 2004). In-silico cloning has been proven as a useful tool

in the examination of plasmid maps for the identification of restriction enzymes which may be used to clone genes of interest into suitable vectors (Elkins, 2011). In-silico cloning also allows for rapid and cost effective screening of cloned products (Baghban-Kohnehrouz and Nayeri, 2016)

Through recent research, a great number of genetically modified cellulolytic bacteria have been produced through gene cloning. Among the three prominent cellulases involved in cellulose hydrolysis, genes encoding for endoglucanase activity have been at the forefront of research aimed at producing the perfect cellulolytic microorganism. Amore *et al*. (2012) identified and expressed the endoglucanase gene CelStrep obtained from *Streptomyces sp* using NdeI and Hind III restriction enzymes into an *E.coli* host. Analysis of the gene showed that the open reading frame of this gene encoded 379 amino acid residues. Ibrahim *et al*. (2015) cloned and expressed the endoglucanase CelB and CelC from *Pectobacterium carotovorum* using the restriction enzymes, XhoI and SmaI for CelB and EcoRI and Bgl II for CelC. Both CelB and CelC genes had lengths of 795 bp and 1105 bp encoding 266 and 369 amino acids respectively.

Biofuels obtained from cellulolytic hydrolysis of biomass continue to be an attractive alternative to conventional fuels, however, producing these fuels at competitive levels still remains a mammoth task.

Chapter 6

7. Conclusions

A total of 6 bacterial species were isolated in this study. All isolates displayed the ability to hydrolyze the soluble cellulose substrate CMC. Hydrolysis of this substrate demonstrated the ability of these bacterial species to produce the cellulolytic enzyme endoglucanase. The enzyme activity assay on the bacterial species revealed that isolate LD25 and LD28 showed the highest enzyme activity during the hydrolysis of the carbon substrate CMC at 0.125 UI/ml and 0.167 UI/ml respectively. The isolates were identified as *B. tequilensis* and *B. subtilis* respectively and were then further evaluated. When these isolates were inoculated into medium containing avicel and cellobiose, it was found that the isolates possessed the cellulolytic enzymes exoglucanase and β-glucosidase.

Optimization studies showed that both isolates produced maximum cellulase activity after 24 hours of incubation with optimum pH for cellulase production determined between pH 4 and pH 7. Cellulases from both isolates showed tolerance to moderately high temperatures with activities above the 0.100 UI/ml threshold between 50°C and 80°C.

Partial purification of cellulase enzyme resulted in a substantial decrease in enzyme activity however an increase in the ammonium sulphate saturation resulted in an increase in enzyme activity.

Peptone was shown to be an optimal nitrogen source in the enhancement of cellulase activity of *B. tequilensis* and *B. subtilis* with urea and sodium nitrate having detrimental effects on the cellulase activity of the isolates.

The discovery of new celluloytic microorganisms have aided in providing hope for the successful production of a “super” cellulase producer, however, it is through the application of biotechnological interventions such as the designing of genetically modified cellulolytic

microorganisms that the timeframes for producing cellulosic biofuels will be significantly accelerated in no distant future.

The study has thus shown that soils of the Ngaka Modiri Molema District do harbor some bacterial species that can possibly play a major role in the production of renewable energy in the form of ethanol through the saccharification of cellulose-rich biomass.

Recommendations

Molecular analysis of cellulases obtained from bacterial isolates needs to be conducted to further categorize them as either endoglucanase, exoglucanase or β-glucosidase. Methods to assist in this regard include but not limited to:

- Enzyme purification by chromatography.
- SDS-PAGE Zymography to determine enzyme molecular weights.

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