ESTABLISHING A MICROBIAL CO-CULTURE FOR PRODUCTION OF CELLULASE USING BANANA (*Musa paradisiaca*) PSEUDOSTEM

By

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[redacted]

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DECLARATION OF INDEPENDENT WORK

I, Mulaudzi M.L (student number: [redacted]), declare that the dissertation hereby submitted to the University of Limpopo, for the degree of Master of Science in Microbiology has not previously been submitted by me for a degree at this or any other University; that this is my work in design and in execution, and that all material contained herein has been duly acknowledged.

…… ……. ……

Signature **Date**

DEDICATION

I dedicate this work to my parents, Thivhusiwi Barnath and Itani Cynthia Mulaudzi who supported me throughout my graduate career. My caring siblings Muano and Mutondwa for always being there and for putting a smile on my face when I need it the most.

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ABSTRACT

In nature, saccharification is done by a variety of microorganisms, secreting a variety of cellulase in addition to other proteins. Co-culturing enables the production of more efficient enzyme preparations that would mimic the natural decomposition of lignocelluloses. During the decay of banana (*Musa paradisiaca*) pseudostem, a potential feedstock for second-generation biofuels, there may be a number of microorganisms producing cellulolytic enzymes, and other factors, which in combination might decompose the lignocelluloses more efficiently. The aim of the study was to establish a microbial co-culture for the production of highly active cellulase preparations. Banana pseudostems (BPS) and microbial samples from decaying banana pseudostems were collected in the Mopani District Allesbeste Nursery, Limpopo Province, South Africa. Fungi and bacteria were isolated using CMC agar plates. The best cellulase producing fungi and bacteria were tested for cellulase activity in monocultures and in various combinations (fungi-fungi, fungi-bacteria, bacteria-bacteria, fungi-live bacterial cells and fungi-dead bacterial cells) in submerged fermentation, using Avicel™ as a carbon source. Solid-state fermentation was also performed using banana pseudostem as a carbon source. Zymography was done in studying the variety of cellulase in the secretions from co-cultures/ mixed cultures. Identification of the bacterial and fungal isolates from decomposing banana pseudostems was also done using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) or DNA sequencing. A mixed culture of fungi in combination with dead bacterial cells was the best combination to produce higher levels of endoglucosidase and β-glucosidase activities in both submerged fermentation and solid-state fermentation. During SmF, endoglucosidase was (0.229 after 144 h) and β-glucosidase (4.519 after 96 h) activities and SSF, endoglucosidase (12.793 after 48 h) and β-glucosidase (37.45 after 144 h). Endoglucosidase zymography showed that monocultures and co-cultures produced four active bands for endoglucanase, except for the monoculture *Trichoderma longibrachiatum* 1B that produced a faint or unclear band. The current study demonstrated that three fungal strains namely, *T longibrachiatum* 1B, *Aspergillus fumigatus* 5A, and *Aspergillus flavus* 2A and one bacterial strain *Enterobacter asburiae* 1 are capable of producing a variety of endoglucanases. It seems that a combination of fungi with dead cells could significantly improve endoglucosidase and

β-glucosidase activities. The use of *A. fumigatus* in mixed cultures is highly recommended in order to produce high levels of β-glucosidases, no matter the combination used.

ABBREVIATIONS

BPs- Banana pseudostem

CD-Catalytic domain

CBM- Cellulose-binding module

CMCase- Carboxymethyl cellulase

Da- Daltons

DNS- Dinitrosalicylic acid

EC- Enzyme Commission

EDTA- Ethylenediaminetetraacetic acid

EG- Endoglucanase

Fig- Figure

FPA-Filter paper assay

FPU- Filter paper unit

GHG-Greenhouse gas

HCCA- Hydroxycinnamic acid

IUPAC- International Union of Pure and Applied Chemistry

h - Hours

LF- Liquid fermentation

M- Molar

MALDI-TOF- Matrix-assisted laser desorption/ionization

Min- Minutes

MK- Monacolin K

mL- Millilitre

mM- Millimolar

MS- Mass Spectrometry

MT- Million Tons

OD - Optical Density

PAGE- Polyacrylamide gel electrophoresis

*p*NPG- *p*-nitrophenyl- β-D glucopyranoside

RA -Relative activity

rpm -Revolution per minute

SDS-Sodium dodecyl sulphate

SISA- Simple Interactive Statistical Analysis

SmF- Submerged fermentation

spp- Species

SSF - Solid-State Fermentation

µl - Microliter

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CHAPTER 1
INTRODUCTION

1.1. Study rationale

Ethanol-based biofuel is an important alternative fuel for vehicles. Energy security and greenhouse gas (GHG) emission concerns can be reduced by the use of ethanol-based biofuel (Zou *et al*., 2016). Currently, products from corn grain and sugarcane syrup are dominated by first-generation. However, due to potential negative impacts on food security, there is a general resistance to the expansion of the production of first-generation ethanol. As a result, lignocellulosic biomass has been identified as a promising feedstock for sustainable production of second-generation biofuels (Fang *et al.,* 2016).

Banana, also known as *Musa paradisiaca,* is a fast-growing crop. Approximately, 125 million tons (MT) of banana is produced in the world (Sharma and Tewari, 2016). The cultivation of banana covers about ten per cent (10%) of the total fruit area (297,860 ha) globally with 535 000 tons of total production (Ja'afaru, 2013). Banana pseudostem (BPS) is one of the most abundantly available agricultural fruit waste product in tropical and subtropical regions (Ingale *et al*., 2014). The pseudostem biomass produced after harvesting is approximately 60-80 t/ha. The biomass is often burnt, while some are dumped on the roadsides. These activities have a detrimental effect on the environment. The lignocellulosic content of banana pseudostem comprises cellulose (55%), hemicelluloses (20%) and lignin (10%) with a good mechanical property (Li *et al*., 2010). The availability of banana pseudostem (BPS) and the lignocellulose content makes it a viable option for the production of ethanol (Idrees *et al*., 2013). Therefore, bioconversion of BPS to fermentable sugars, followed by fermentation to ethanol, is considered an attractive route for the production of low-cost ethanol (Chen *et al*., 2009).

This cellulose content in BPS is greater than that in wheat straw (30%), grasses (25%-40%) and olive waste (31.4%), but at a similar level to pinewood (46.4%). These lignocellulosic biomass materials are important substrates for bacteria and

some fungi that produce cellulolytic and ligninolytic enzymes used in a number of applications in industrial processes (Dashban *et al.,* 2009).

In nature, saccharification of lignocellulose is done by a variety of microorganisms secreting a variety of cellulase in addition to other proteins. The co-culture of microorganisms that secrete cellulase could enable the production of more efficient enzyme preparations that would mimic the natural decomposition of lignocelluloses (Goswami *et al.,* 2016). It has been observed that mixed cultures and combination of microorganisms are beneficial due to the advanced harvest of enzymes (i.e. higher production of enzyme) and providing an extended length of time of enzymes (Abdel-Rahman *et al*., 2016). A co-culture of cellulolytic microorganisms originating from the decomposing pseudostem may be advantageous in the hydrolysis of BPS since the combination would be well adapted to this lignocellulosic material and co-exist symbiotically on this natural solid substrate in nature.

1.2. Research problem

Cellulose is the most abundant biopolymer produced by plants. Natural cellulose degradation is mainly microbiological, involving the production of cellulase by bacteria, fungi and protozoa. There is insufficient saccharification of lignocellulose biomass by the use of enzymes from one microorganism (Cheng & Zhu, 2012; Alessi *et al.,* 2018).

In nature, enzymatic degradation of lignocellulose is more efficient compared to *in vivo* bioprocess situations. In nature, the degradation is carried out by enzymes from a combination of microorganisms or microbial communities, including fungi and bacteria, whereas most processes that are developed involve enzymes from a few microorganisms used. In addition to that, it is probable that these microbial communities secrete other proteins that aid the process of degradation of lignocellulose (Cortes-Tolalpa *et al.,* 2017). During the decay of the banana (*Musa paradisiaca*) pseudostem, a potential feedstock for second-generation biofuels, there could be a number of microorganisms that are producing cellulolytic enzymes and other contributing factors.

1.2.1. Aim

The aim of this study was to establish a microbial co-culture for the production of highly active cellulase preparations using BPS as substrate.

1.2.2. Objectives

The objectives were to:

i. Use a variety of culture media to isolate cellulose-degrading microorganisms from decomposing BPS.
ii. Identify the isolates from decomposing BPS using MALDI-TOF.
iii. Select the microorganisms producing enzymes with high cellulase activity.
iv. Optimise production of cellulase by co-cultures of the isolated microorganisms producing high levels of cellulase.
v. Use zymography in studying the variety of cellulase in the secretions from co-cultures.

1.3. Hypothesis

Co-culture of microorganisms improves insufficient saccharification of lignocellulose in the production of biofuel.

1.4. Significance of the study

The findings from the current study may inform sufficient enzyme production from an inexpensive fermentation process. Optimisation of the production of cellulase by co-cultures of microorganisms may cause the production of high enzyme activity levels of cellulase. The current study will also contribute to scientific knowledge on the use of banana pseudostem in the production of bioethanol. A co-culture of cellulolytic microorganisms originating from the decomposing pseudostem may be advantageous, since the combination of the microorganisms may be well adapted to the saccharification of the lignocellulosic material. In nature, the isolated microorganisms co-exist symbiotically on the banana pseudostems in order to decompose it. It is possible that the production of lignocellulolytic enzymes will be induced.

Since more efficient cellulase enzymes are required in the Bioenergy industry, the current study intends to establish a stable microbial community (co-culture) for the production of cellulase using banana pseudostems. This will potentially provide a profitable cellulase technology aimed at high biofuel production.

CHAPTER 2
LITERATURE REVIEW

2.1. Cellulase

Cellulase is a category of hydrolytic enzymes hydrolysing β-1, 4-glucosidic bonds in cellulose, that is a dominant component in the cell wall of plants. The cellulase system comprises of three highly specific enzymes, namely: the exoglucanases, the endoglucanases and β-glucosidases (Silva *et al*., 2018). These enzymes play a major role in converting native cellulose to glucose (Cheng & Timilsina, 2010). The hydrolysis of the joint groups of the three enzymes completes the conversion of cellulose into glucose as shown in Figure 2.1(Cheng & Timilsina, 2010). Different microorganisms produce cellulases, such as fungi, symbiotic bacteria and protozoans. The most abundant and widely studied cellulase-systems are of fungal origin. *Trichoderma*, a softwood rotting fungus is the most studied cellulase-system producer (Kuhad, 2011).

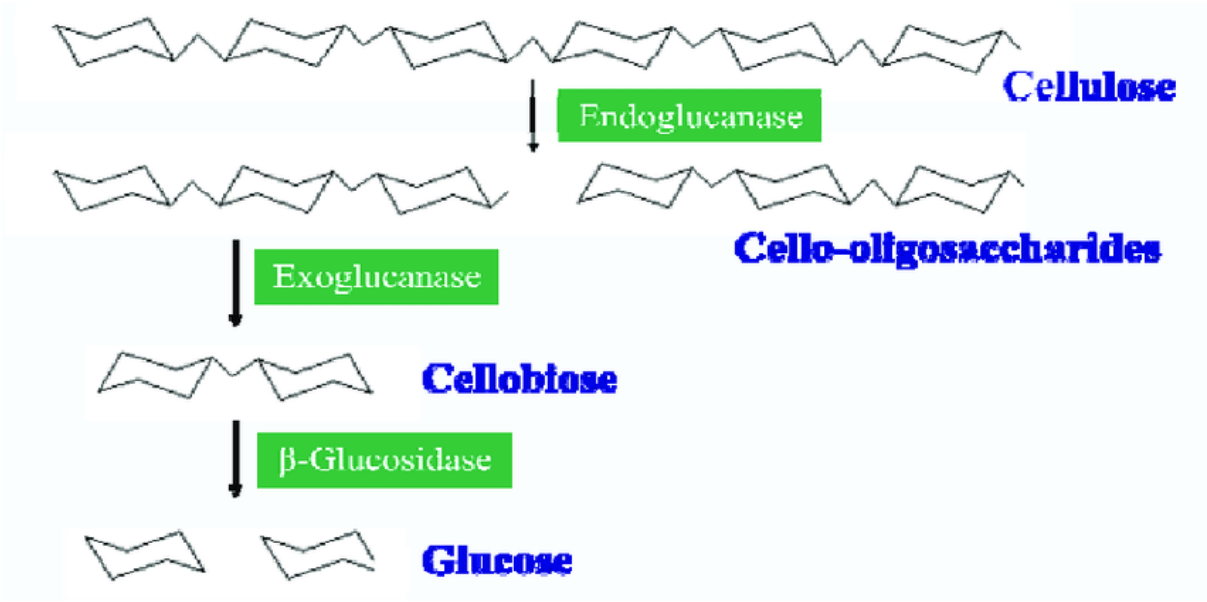


Figure 2.1: Enzymatic hydrolysis of cellulose to glucose ((Cheng & Timilsina, 2010).

2.1.1 Endoglucanases or Glucanohydrolases (EC 3.2.1.4)

Endoglucanases (EG, EC 3.2.1.4), also called CMCase, are enzymes that split β-1, 4-glycosidic bonds internally, generating new ends. The 3, 5-dinitrosalicylic acid

(DNS) method is used to quantify or determine the reducing sugars liberated by these endoglucanases as an indicator of the hydrolytic activity of an enzyme (Scapin *et al.,* 2017). Endoglucanases in internal regions of cellulose are active and soluble cellulose substrates, such as carboxymethyl cellulose (CMC) (Dashtban *et al*., 2010). Endoglucanases are used for different goals in the pulp and paper industry. They facilitate fermentation of biomass into biofuels, which are used in the textile industry and in laundry detergents (Dashtban *et al*., 2010).

2.1.2. Exoglucanases

These enzymes poorly degrade crystalline and cello-oligosaccharides but are highly active on amorphous and swollen cellulose. They cleave 2-4 units from the ends of the exposed chains formed by endocellulase, bringing out tetrasaccharides or disaccharides, such as cellobiose (Ghorai *et al*., 2010). Exoglucanases are divided into two types. The first type (CBH I) progressively works from the non-reducing end of cellulose, while the second type (CBH II) performs progressively from the reducing end. These enzymes possess a burrow active area and also retains a single glucan (Ghorai *et al*., 2010). However, the endo and exocellulases appear to perform in a synergistic manner. Consequential degradation in natural cellulose occurs when both endo and exocellulases are present (Kostylev & Wilson, 2012).

2.1.3. Cellobiases (β-glucosidases)

Cellobiases also called β-glucosidase, do not participate directly in the degradation of cellulose, but participate in saccharification; these enzymes are grouped into two types (BGL BGL II and I). The hydrolysis of the glycosidic bonds is catalysed by cellobiases in a beta-D-glucosides terminal of non-reducing residues, oligosaccharides, and glucose is released (Singhania *et al.,* 2012). There is very little literature about beta-glucosidase interaction with their substrates, particularly with aglycone moiety. The synthetic substrates like pNPG (para nitrophenyl beta-D-glucopyranoside) or methyl umbelliferyl beta-D-glucosidase (MUG) are used to measure β-glucosidase activities (Singhania *et al.,* 2012). Cellobiases are obtained from different sources, including *Aspergillus niger*, *Trichoderma viride* together with *Saccharomyces cerevisiae* (Chang *et al*., 2013). These enzymes are extracellular,

intercellular or cell membrane-bound. The phenolic and phytoestrogen glucosides are removed by cellobiases. These occur in red wine, fruits, vegetables, tea, and soybeans (Kaur & Joshi, 2015). Detoxification of cassava, aroma improvement and eradication of bitter mixtures from citrus fruit juices are some additional implementations of cellobiases. Most β-glucosidases are inactive towards these and other polymeric substances such as Avicel™, filter paper and cotton (Chang *et al*., 2013).

2.2. Sources of Cellulase

Degradation of cellulose is caused by a number of microorganisms such as bacteria, fungi and actinomycetes. Inducible enzymes synthesized by these microorganisms on a cellulosic material during their growth are known as cellulase. A large diversity of these microorganisms can be either anaerobic, aerobic, thermophilic or mesophilic (Kuhad *et al*., 2011). Bacteria within genera *Clostridium, Bacillus, Cellulomonas* and *Thermomonospora* are the most studied bacterial cellulase producers (Kuhad *et al*., 2011).

2.2.1. Bacterial and fungal cellulase enzymes

Most bacteria produce mainly endoglucanases. These cellulase produced are active and stable at high temperatures and when subjected to both chemical and mechanical denaturation. Structurally bacterial cellulase systems and cellulosomes are complicated in comparison with cellulase produced by fungi known to be simple (Martinez *et al.,* 2005). Cellulase enzymes produced by fungi typically have a cellulose-binding module (CBM) and a catalytic domain (CD) with the catalytic domain at the N-terminal linked by a short polylinker region. Fungi play a dominant role in lignocellulose conversion. Due to their high enzyme activities, fungi produce a majority of these laboratory and commercial cellulase enzymes (Hong *et al*., 2015).

The bacterial cellulolytic enzymes are not directly comparable to those of fungi. Fungi are better producers of these enzymes, hence the origin of bacteria cellulase are less studied. Microorganisms from the genome such as *Bacillus, Acinetobacter, Cellulomonas* and *Clostridium* are known to produce bacterial cellulolytic enzymes. Bacteria from rumen degrade structural components of cell walls and are therefore

characterized as producers of cellulase enzymes (Chander *et al*., 2016). In their study, Lokapirnasari *et al*. (2015), reported on bovine rumen fluid waste of Surabaya Abattoir, Indonesia, bacteria *Enterobacter cloacae,* which was isolated and produced endoglucosidase (0.09 U/ml) and β- glucosidase (0.10 U/ml). In one study done by Rezaei *et al.* (2009), *E. cloacae* were first demonstrated to accomplish electricity generation and to result in the degradation of cellulose. *Enterobacter* was also able to produce endo-1, 4-β-D-glucanase activity and cellulose degradation.

Enterobacter asburiae is a motile (peritrichous flagella), facultatively anaerobic, which belongs to the *Enterobacteriaceae* family. It is a Gram-negative, non-spore forming, and rod-shaped bacterium. It is a new species in the *Enterobacter* genus that was formerly known as the Enteric Group 17. A variety of clinical and environmental specimen are also used to isolate its strains, hence also known to be an opportunistic pathogen (Mardaneh & Dallal, 2016).

High amounts of cellulase are also known to be produced by fungi. Fungi, such as *Trichoderma*, *Penicillium*, *Fusarium* and *Aspergillus* produce cellulase under appropriate cultural conditions. The production yield of cellulase in Fungi such as *Trichoderma species*, *Aspergillus sp* and *Penicillium sp* is higher compared to that in bacterial cellulase which is low (Ahmed & Bibi, 2018). *Trichoderma reesei* produces very low amounts of β-glucosidase in comparison with *Aspergillus niger,* but efficient endo- and exo-glucanases (Ahmed & Bibi, 2018).

Higher fungi and their enzymes, spores or metabolites, are adjusted well for growing on solid wet substrates where growth is encouraged by the form of dispersed mycelia. This enhances the interaction between the cells and the substrate; hence, enzyme production increases (Hong *et al*., 2015).

2.2.2. Co-culture cellulase enzymes

In order to obtain high cellulose material degradation, the synergistic effect of all three cellulase enzymes has to be achieved (Chen, 2011; Sharada *et al*. (2013). Most researchers suggest that mixed culturing between two strains and β-glucosidase supplementation from *Aspergillus* improved the total cellulase enzyme activity of *Trichoderma* (Abu-Bakar *et al.,* 2010). In a study conducted by Sharada *et al*. (2013), a mixed culture of fungi was found to result into a higher product yield and

growth rate in poor nutritional residue and to strengthen the protection of the culture against contamination. In another study done by Abu-Bakar *et al.* (2010) mixed culture of fungal strains, protease, was produced which might explain the low cellulase enzyme activity obtained in a mixed culture system compared to pure cultures. Competition amongst strains for available carbon sources may also contribute to low cellulase enzyme activity (Abu-Bakar *et al.,* 2010).

Improvement of cellulase production through solid-state fermentation (SSF) is developed using several strategies. Co-culturing of *Aspergillus* and *Trichoderma* was one of those strategies (Gupta and Madamwar, 1997). In a study done by Duenas *et al.* (1995), it was reported that activity of cellulase obtained during a co-culture of ***T. reesei*** LM-UC4 and *Aspergillus phoenicis* QM329 was 3 and 6 times higher than that in corresponding monocultures with sugarcane bagasse as substrate.

Maheshwari *et al.* (1994) also reported similar results and observed that a mixed culture of ***A. niger*** and ***T. reesei*** and optimal cellulase production on paper mill sludge was achieved. Another study was done by Taher *et al.* (2017) clearly highlighted a significant higher cellulase activity, especially for CMC-ase during a co-culture of *T. reesei* and *A. niger*. Moreover, these activities are higher than the amount obtained from the respective monocultures, suggesting that improvement of the enzyme production was caused by synergistic effects of the two strains.

Other studies on the advantages of using co-culture during the production of industrial enzymes were reported (Taha, 2015). One of these studies focused on the use of monocultures to produce pectinases (Torimiro, N, & Raphael, O, 2013). A mixed culture or co-culturing was found to be more advantageous due to a higher production yield than in found in using monocultures, (i.e. higher enzyme production) and it also provided an extended shelf-life product (Rathnan & Balasaravanan, 2014). The co-culture is a potential bioprocess if there are no cross-interactions among microorganisms, and each microorganism metabolising its substrate is unaffected by the presence of other microorganisms (Bader *et al.,* 2010).

During SSF, co-culturing of fungi improves the secretion of cellulolytic enzymes, as they co-exist symbiotically on a natural solid substrate (Gupta & Madamwar, 1997). More efficient enzyme mixtures for industrial processes are produced by co-cultures in comparison with monocultures (Gupta & Madamwar, 1997; Yoon *et al*., 2014). For

lignocellulosic biomass to be efficiently hydrolysed into reducing sugars, the preparation of enzymes necessitates balanced activities of all the constituents of cellulase, for example, endo-1, 4-glucanases, exo-1, 4-glucanase, and β-glucosidase along with xylanases (Brijwani *et al*., 2010; Yoon *et al*., 2014).

2.3. The application of cellulase

Cellulase is an enzyme of industrial interest and plays a major role in cellulose hydrolysis. Cellulase enzymes are used in the laundry detergent industry, bioethanol production, in textile industries, food and feed industry and in the pulp and paper industry (Srivastava *et al*., 2015).

2.3.1. Textile industry

Cellulase enzymes are used to improve the appearance of cellulose-based textiles and their softness. These enzymes could substitute pumice stones to make 'stonewashed' denim garments (Lee *et al*., 2011). With repeated washing, most cotton or cotton-blend garments begin to dull and become fluffy. Replacement of conventional pumice stones by cellulase enzymes could reduce human labour and fibre damage on clothes (Moubasher *et al*., 2018). Microorganisms, such as *Trichoderma* and *Humicola* produce cellulase used for bio-stoning of jeans. During bio-polishing, these enzymes hydrolyse small protrusions of fibres from cotton clothes and thus remove the fussiness of the fibres from the surface to make the clothes smooth and glossy in appearance (Lee *et al*., 2011, Saravanan and Prakash, 2017).

Cellulase enzymes improve the dye absorbance of fibres, remove excess dye, and can potentially be applied as an additive in laundry detergents to improve fabric brightness and softness (Lee *et al*., 2011). The advantages of cellulase enzymes in the textile industry include minor usage conditions e.g., temperature, pH, and the fact those cellulases are not complicated to use. Cellulase is completely degradable and leads to saving on electricity costs, water, chemicals and processing time (Mojsov, 2011).

2.3.2. Paper and pulp industries

Refining and grinding of woody raw materials are mechanical processes that yield a pulp that contains a great content of fines, substance and toughness. In contrary, cellulase used in biochemical pulping give rise to energy-saving (20% - 40%) throughout purifying and enhancements in hand-sheet strength properties (Kuhad *et al*., 2011). Moreover, it provides environmental-friendly processes, by reducing the use of harmful chemicals. For example, these enzymes enhanced the drainability of the pulp (by 80%) and no energy consumption changes were observed (Kubicek *et al.,* 2009). In a study done by Mansfield *et al.* (2012), it was observed that treatment with cellulase decreases the defibrillation, hence reducing the fibre coarseness. Employing mixtures of cellulase and hemicellulase during enzymatic deinking of waste enhances the quality and brightness of the recycled paper (Mayeli and Talaeipour, 2010; Ibarra *et al*., 2012). These enzymes play a major role in the paper and pulp industries as well as bio-bleaching, improving the drainage of paper mills wherein clogged fibre residues are dissolved (Kuhad *et al*., 2011). The recent paper and pulp industries use cellulase with an intention to increase production rate and improve the complete performance.

2.3.3. Biofuel Production

An application of cellulase currently studied is biofuel production using lignocellulosic waste. Although cellulosic residues are available, there is a foremost disadvantage of biodegradation and cost-effectiveness in the bioconversion of lignocellulosic wastes. The lignocellulosic materials can be converted by cellulase enzymes into fermentable sugars such as glucose and maltose (Hafid *et al*., 2017). They can also be used as substrates to produce bioethanol and other products. The conversion of lignocellulosic material into bioethanol requires a multistep process (Zabed *et al*., 2016). During the process of pre-treatment, hemicellulose and lignin fractions are improved for further processing. At a temperature of 50 °C, the residues are hydrolysed to produce fermentable sugars and finally, microorganisms were employed to convert cellulosic wastes into alcohol (Zabed *et al*., 2016).

2.4. Fermentation methods used for the production of cellulase

Biological conversion of complex substrates into simple compounds by various microorganisms like bacteria and fungi is known as fermentation technique. Fermentation is classified into Solid-state Fermentation (SSF) and Submerged Fermentation (SmF)/Liquid Fermentation (LF), characterized based on the type of substrate used during fermentation. These techniques are developed in relation to different parameters used, such as microorganisms, substrates and environmental parameters used during fermentation.

2.4.1. Submerged Fermentation

The production of biomolecules in which enzymes and other reactive compounds are submerged in a liquid such as alcohol, oil or a nutrient broth is also known as submerged fermentation (Subramaniyam & Vimala, 2012). The microorganisms, that require higher moisture content for growth and other metabolic pathways such as bacteria are very suitable for this fermentation technique (Subramaniyam & Vimala, 2012). One of the advantages of this technique is that the purification of the products is simpler. This technique is mainly used during the extraction of secondary metabolites that need to be used in liquid form (Kokila, & Mrudula, 2010).

2.4.2. Solid-state Fermentation

The process that utilises solid substrates, like bran, bagasse and paper pulp is known as solid-state fermentation (SSF) (Pandey & Ramachandran, 2005). During this process, microorganisms are able to cultivate in a background lacking free water, or with very little content of free water. The solid substrate (matrix) must contain enough moisture in order to allow optimal growth to occur (Pandey & Ramachandran, 2005).

The production of enzymes in SSF offers several advantages over SmF. The major benefit of utilizing agro-industrial substrates is that nutrient-rich waste materials can be easily recycled as substrates. During the SSF technique, the substrates are used very gradually and progressively, causing the same substrate to be used for

prolonged durations during that fermentation. Consequently, the release of nutrients is also controlled (Farinas, 2015).

Some of the advantages of using SSF include low production costs, high productivity and a low risk of contamination due to the inability of many organisms to grow at a low water activity (Singhania *et al*., 2010). An inadequate number of microorganisms, mostly yeasts, fungi and some bacteria can carry out SSF fermentation in the presence of low moisture content (Babu & Satyanarayana, 1996). It does not require complex machinery, equipment and control systems. Nevertheless, SSF has some limitations such as high heterogeneity, which makes it difficult to focus on one category of hydrolytic process, and thus lead to poor trials of modelling. It is also labour intensive and lacks uniformity in the substrate, a characteristic that is the most significant problem of SSF (Ghosh & Ray, 2011).

An indication of a 10-fold reduction in the production cost when SSF was implemented in the production was reported by other studies, in comparison with that in the production of cellulase in SmFand SSF systems (Zhang and Zhang, 2013). Previous studies showed that SSF exhibits a greater advantage than SmF in the cultivation of *Monascus purpureus* wherein there were high concentrations of glycerol into Monacolin K (MK) production (Lu and Yu, 2013; Zhang and Zhang, 2013). Similar studies observed a high concentration of products, and lower catabolic repression during the application of SSF processes (Hölker and Lenz, 2005).

Generally, factors that affect microbial growth for the production of enzymes during SSF comprise the assortment of a suitable microorganism and substrate, optimization of process parameters, isolation, and refining of the manufactured goods (Toca-Herrera *et al*., 2007).

2.5. Assays and Techniques used to determine cellulase activity

2.5.1. 3, 5- Dinitrosalicylic acid assay

Since the year 1955, 3, 5 Dinitrosalicylic acids (DNS) assay is still very useful for the quantitative determination of reducing sugars. (Delong Frost, 2004). The 2-hydroxyl-3, 5-dinitrobenzoic acid is known as the DNS IUPAC. These react with reducing sugars and other reducing molecules and It forms 3-amino-5-nitrosalicylic acid,

which absorbs light mainly at 540 nm (Lorenz, 1959). This assay has been widely used in various applications and introduced to detect reducing substances in the urine. Due to their specificity, enzymatic methods use DNS. Measurements of xyloglucanases, mannases, amylases, and pectinases also used DNS method. A DNS reaction is carried out by mixing a dilution of the enzyme preparation with a known substrate incubated at a set temperature, with a buffered pH (Delong Frost, 2004).

2.5.2. Filter paper (Total cellulase activity)

The International Union of Pure and Applied Chemistry (IUPAC) established a determination of the total cellulase activity using Whatman No. 1 filter paper as the substrate for filter paper assay (FPA) (Zhang *et al.,* 2006, Batool *et al.,* 2015). The filter paper Whatman No. 1 is more preferred than cotton fibre and Avicel (dyed or undyed) because it is uniform and widely available (Silveira *et al*., 2012). IUPAC's method main objective is that cellulase must be diluted until the amount of product plotted against cellulase concentration is reasonably linear. A fixed amount (2 mg) of glucose released from a 50 mg filter paper (1 × 6 cm) is required for the assay. To achieve a fixed degree of hydrolysis a sequence of cellulase dilution solutions are required (Ghose, 1987; Yu *et al*., 2016). The advantages of this assay are: (1) it utilizes a substrate receptive to cellulase and (2) removal of residuals is not necessary while adding the DNS reagent (Murad & Azzaz, 2010).

2.5.3. β-Glucosidase activity

The glucosidic linkages formed between the –OH group of another compound sugar and a hemiacetal-OH group of a cyclic aldose or glucose, aryl-alcohol and amino-alcohol are catalysed by β-glucosidase. The hydrolysis of substrates that are soluble, cellobiose and cello-oligosaccharides have a little degree of polymerization, to cellobiose and glucose (Silveria *et al*., 2012). The synthetic and hydrolytic activities of these enzymes are significant in a number of applications in biotechnology. Cellobiose is an inhibitor of both Exo- and endoglucanases, hence play a major role in cellulose hydrolysis (Silveria *et al*., 2012). These should be removed to complete saccharification of cellulose. It is achieved by increasing the speed of hydrolysis of

cellulose, making the reaction more economical for the production of alcohol. The p-nitrophenyl-β-D-glucopyranoside (pNPG) or p-nitrophenyl-β-D-lactopyranoside (pNPL) respectively can be used to measure cellobiohydrolase and β-glucosidase (Ghorai *et al*., 2010).

2.6. Matrix-assisted laser desorption/ionization

In the year 1996 Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry took the lead in microorganism's identification. The advantages of this technique are high sensitivity, short analysis time, intact cell measurements, having a wide m/z range, compatibility with different analysers, as well as the possibility of automation. MALDI ionization process has a few boundaries and difficulties such as suppression effects, sweet spots as well as selective ionisation (Kudina *et al.,* 2016).

The mass of proteins and peptides can be determined by a sensitive technique called MALDI. The identified protein masses play a role in proteomics. The time of flight (TOF) analyser, which measures the time it takes for molecules to travel at a fixed distance, is also attached to MALDI. A short laser pulse together with nitrogen gas, are used to ionise molecules in the MALDI ionization technique (Hail *et al.,* 2004; Welker & Moore 2011). MALDI-TOF MS has been increasingly studied and applied in the last few years, for the identification and typing of microorganisms (Welker & Moore, 2011).

Bacteria can be identified by MALDI-TOF through the determination of small proteins and the exact molecular mass of peptides. This technique uses mass to charge ratio (m/z) of a bioanalyte (Mellmann *et al*., 2008, Barreiro *et al*., 2010). The advantage of this method is that it takes a very short time to analyse bacterial extracts. Spectra are recorded in a linear model, within a mass range of 2000 to 20000 Daltons (Da) (Coombes *et al.,* 2005). Bacterial identification is based on spectral fingerprints with each microorganism with its own fingerprint using MALDI-TOF MS. Comparing the microbial sample mass fingerprint with a database containing reference mass fingerprints is the first step for species identification during this process. The SARAMIS (AnagnosTec/bioMérieux) and MALDI Biotyper (Bruker Daltonics) are the two main software's that are currently used during the analysis in the laboratory. The MALDI Biotyper software was developed by Bruker for use on its own instrument.

The database, together with the generated numerical value (score) based on the likeness between the stored datasets are compared with the collected spectra using these software (Rahi and Schouche, 2016). A good species-level identification is considered at a score greater than 2.0 and identification species-level is said to be excellent at scores above 2.3. The score less than 1.7 (no significant likeness) and those within 1.7 and 2.0 are reliable (Seng *et al.,* 2009; Cherkaoui *et al.,* 2010).

2.7. Polyacrylamide gel electrophoresis and Zymography

Protein separation according to their charge, electrophoretic mobility based on the length of a polypeptide chain is a technique known as Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The protein sample is applied with SDS to linearize proteins and to cause a negative charge to linearized proteins. The fractionation by approximate size during electrophoresis results when SDS binds to the polypeptide chain cause an even distribution of charge per unit mass that occurs in most proteins (Rath *et al*., 2009).

Substrates are co-polymerized with the polyacrylamide gel during zymography, which is based on SDS-PAGE, and the activity of an enzyme, which is, studied *in situ* using electrophoresis. Mammalian varieties have been analysed using this technique. This includes microbial enzymes, a histone acetyltransferase, lipase, elastase, plant, and α-L-arabinofuranosidase including, matrix metalloproteases, endoglucanase, phytase, poly (ADP-ribose) glycohydrolase, and DNase and ribonucleases assortment. The advantages of zymography over conventional enzyme activities are that it permits activities of an enzyme to be assigned on polypeptides with specific physical characteristics, like molecular weight or isoelectric point. Studying variety of enzymes (like in biological samples), posttranslational modification of a specific enzyme (for example, glycosylation), the heterogeneity of enzyme isoforms, to analyse enzyme activity within various fractions protein simultaneously is also possible using this technique (Scadden *et al.,* 2001, Leonard *et al*., 2019).

2.8. Factors affecting cellulase production

Cellulose digestion is affected by pH, temperature, agitation speed, the concentration of cellulose and the microorganism used. These factors play a major role in lignocellulose or other carbon sources cellulose degradation and the amount of cellulase produced.

2.8.1. Temperature

Cellulase producing microorganisms grow very well at a wide range of temperatures. Most cellulase-producing microorganisms have their own optimum temperature for cellulase production. In SSF temperature affects the growth of microorganisms, spore germination and cellulase production (Cunha *et al*., 2012, Liu *et al*., 2014). Assareh *et al.* (2012) reported temperatures ranging from 40 to 60 °C *Geobacillus* sp T1 grew well and produced high levels of CMCase activity but at temperatures less than 40 °C no growth observed. The optimum temperature for cellulase production by *Geobacillus sp*. T1 was 50 °C amongst the tested temperatures. Waghmare *et al.* (2014) studied at different temperatures (50–90 ºC) for enzyme production using *Klebsiella* sp. PRW-1. A constant pH of 5.0, at a temperature of 50 ºC *Klebsiella* sp. PRW-1 showed higher production of cellulase in the presence of all cellulosic substrates.

A study done by Shahriarinour *et al.* (2011) reported that incubation temperature played a major role in cellulase production using *A. terreus*. It was observed that *A. terreus* grew very well over a temperature range of 24 °C to 32 °C. The temperature at 28 °C obtained the highest β-glucosidase, FPase and CMCase activities. The production of cellulase was reduced by about 40 to 50% at higher temperatures (36 °C and 40 °C), as compared to that obtained at the optimal temperature (28 °C).

2.8.2. pH

The medium pH plays a significant part through inducing morphological changes in microbes and during enzyme production. The optimal pH varies between enzymes and different microorganisms. The activity of the enzymes is strongly affected by pH because the active sites within the enzymes depend on the ionic species present to

maintain conformations to binding to the substrates (Maciel *et al*., 2014). Maheswari *et al.* (1993) reported the medium set at a pH ranging from 5 – 6 with *Trichoderma viride* strains produced or achieved maximum cellulase production. Zhang *et al.* (2017) reported an influence of pH on cellulase production by *A. niger* under SSF at pH 4.6 in the culture.

The pH was also a key factor that affects the production of cellulase enzymes from *T. harzianum* under solid-state fermentation and plate screening medium according to the study done by Shafique *et al.* (2009). Acidic media are mostly preferred by the fungi, whereas the bacteria prefer a neutral to alkaline pH. Waghmare *et al*. (2014) studied different enzymes such as β-glucosidase, exoglucanase, glucoamylase, and endoglucanase and xylanase activity using *Klebsiella sp*. PRW-1 with the effect of different pH values (2–10). The optimum pH was found to be 5 at 50 ºC and in the presence of all cellulosic substrates. The endoglucanase enzyme produced by *Klebsiella* sp.PRW-1 was found to be less stable and the residual enzyme activity was only between 20-30% than its initial activity after incubation at 50 ºC for 1 h.

Shahriarinour *et al.* (2011) found at an initial culture pH ranging from 5 to 6 times the cellulase produced by *A. terreus* was detected. At pH 5.5 all, the components of cellulase (FPase, CMCase and β-glucosidase) obtained the highest activities. During the fermentation process, when the consumption of cellulose by the fungi reduced, the culture pH was reduced to an acid pH. The fungal mycelium absorbed the ammonium ions hence pH reduced in the culture medium (Shahriarinour *et al.* 2011). The pH of the cultures then increased after cellulose was completely consumed. This possibly occurred during fermentation early stages in the culture medium due to the consumption of organic acids.

2.8.3. Effect of shaking speed

The production of an enzyme in submerged fermentation systems is affected by a crucial factor known to be agitation speed. Therefore, in order to obtain maximal enzyme production, it is necessary to determine the optimal agitation speed (Shahriarinour *et al*., 2011). In a study done by Shahriarinour *et al.* (2011), cellulase production increased with increasing shaking speed. It was explained that increased dissolved oxygen in the culture also increases with the shaking speed. This is

essential for uniform distribution of the medium contents such as nutrients and catabolites and cell membrane components. Growth of *A. terreus* (4.90 g L^{-1}) was about 3 times higher in cultures agitated at 200 rpm as compared to those obtained in static culture (1.40 g L^{-1}). Darah *et al.* (2011) also reported an improved tannase activity at 200 rpm instead of 150 rpm by *A. niger* FETL FT3.

2.9. Literature review Summary

The cellulase system comprises of three highly specific enzymes, namely: the exoglucanases, the endoglucanases and β-glucosidases (Silva *et al*., 2018). Degradation of cellulose is caused by a number of microorganisms such as bacteria, fungi and actinomycetes. In order to obtain high cellulose material degradation, the synergistic effect of all three cellulase enzymes has to be achieved (Chen, 2011; Sharada *et al*. (2013). Cellulase is an enzyme of industrial interest and plays a major role in cellulose hydrolysis. Cellulase enzymes are used in the laundry detergent industry, bioethanol production, in textile industries, food and feed industry and in the pulp and paper industry (Srivastava *et al*., 2015).

The SSF advantages over SmF include low production costs, high productivity and a low risk of contamination due to the inability of many organisms to grow at a low water activity (Singhania *et al*., 2010). Cellulose digestion is affected by pH, temperature, agitation speed, the concentration of cellulose and the microorganism used. The advantages of zymography over conventional enzyme activities are that it permits activities of an enzyme to be assigned on polypeptides with specific physical characteristics, like molecular weight or isoelectric point. In this study enzyme activities 3, 5-Dinitrosalicylic acid assay, Filter paper assay, and β-Glucosidase activity were used and Zymography was used to confirm the presence of different cellulase in both monocultures, co-cultures and mixed cultures.

CHAPTER 3
MATERIAL AND METHODS

3.1. Ethical clearance

This study does not require any ethical clearance. All the biological wastes were disposed of safely according to national legislative requirements.

3.2. Study site and sample collection

Banana pseudostems (BPS) and microbial samples from decaying banana pseudostems were collected in the Mopani District (Figure 3.1) which is located in the Limpopo Province of South Africa. The District is composed of five local municipalities namely Greater Giyani; Greater Letaba; Greater Tzaneen; Phalaborwa and Maruleng. This study focused specifically on the Tzaneen Municipality. Decomposing BPS were collected from the Allesbeste Nursery (23.8020° S, 30.1216° E) in Tzaneen, Limpopo Province, South Africa and transported back to the University of Limpopo. The BPS was kept at 4 °C in the cold room until use.

In preparation for use, the BPS was cut into approximately 10 cm pieces in width and dried over 7 days in an oven at 60 °C. The dried-out BPS were powdered into a fine-textured powder and stored in a sealed container until use at room temperature.

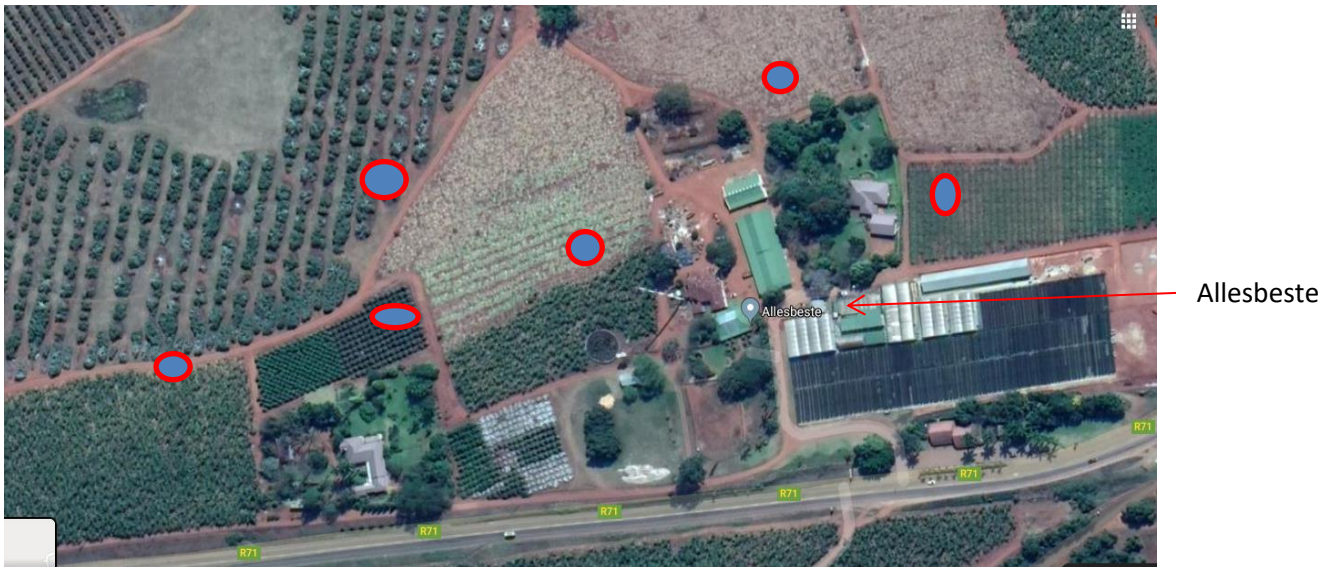


Figure 3.1: Indicated position from where banana pseudostems and microbial samples were collected in the Mopani district municipality (Tzaneen municipality) (Google maps)

Figure 3.2: Collection of microbial samples from a decaying banana pseudostem.

3.3. Equipment

In the current study, different equipment's were used. This included Beckman Coulter DTX 880, Multimode Detector microplate reader, a Waring Commercial blender, Speedy Autoclave (Taiwan), United Wire test Sieve (stainless steel) Neubauer bright lined counting chamber, light microscope. Beckman Coulter Allegro X 22R centrifuge, General purpose UV/vis spectrophotometer, Beckman Coulter DU 720, Savant Speed Vac (SC 110) Concentrator and Gel Dryer pump EC 353, Syngene Gene snap Imaging System (Thermo Scientific, UK), Bio-Rad Tec ware PS 250-2 SDS-PAGE apparatus.

3.4. Reagents

Sodium chloride (NaCl), 3,5-Dinitrosalicylic acid (DNS), ammonium phosphate($(NH_4)_2SO_4$), sodium citrate ($Na_2C_6H_5O_7$), magnesium sulphate ($MgSO_4$), potassium hydrogen phosphate (K_2HPO_4), carboxymethyl cellulose (CMC), ammonium chloride ($(NH)_4Cl$), Congo red, calcium chloride (dehydrate), ferrous sulphate ($FeSO_4$), yeast extract, bacteriological agar, tryptone, potassium

dihydrogen phosphate (KH_2PO_4), ethanol, chloramphenicol, cycloheximide, hydrochloric acid (HCl), Macherey-Nagel GmbH &Co supplied Whatman No.1 filter paper, (Duren), Germany. Sigma-Aldrich Co. St Louis, MO, USA supplied Coomasie blue R250. Sodium acetate, Glycine, p-nitrophenyl-β-D-glucopyronoside and Fermenters (Ferments-Inqaba Biotech, Pretoria, South Africa) supplied Protein molecular weight marker Trizame base, Sodium dodecyl sulphate (SDS), Acrylamide, bisacrylamide, Bromophenol blue, glycerol, ammonium persulfate (APS), glycine, sodium phosphate, isopropanol, esculin, ferric chloride, glucose, EDTA, and acetic acid.

3.5. Isolation and screening of microorganisms from the decaying banana pseudostems

3.5.1. Isolation of microbes from decaying banana pseudostems

Culture media consisting of 2 g/l K_2HPO_4, 0.5 g/l KCl, 0.005 g/l $FeSO_4$, 0.073 g/l $MgSO_4$, 7 g/l KH_2PO_4, 1 g/l $(NH_4)_2SO_4$, 1 g/l Yeast extract, 12 g/l Bacteriological agar and 20 g/l powdered BPS were prepared and sterilized at 121 °C and used for 15 minutes. The culture media were divided into two portions of 300 ml each. In one of the portions, 6 ml of a 0.5 mg/ml cycloheximide solution (to inhibit fungal growth) was added and in the other portion, 3.5 ml of a 20 µg/ml chloramphenicol (to inhibit bacterial growth) was added (Moubasher *et al.,* 2018). The two portions of the media were poured into Petri dishes. Microbial samples collected from five decomposing banana pseudostems were suspended in sterile distilled water and about 50 µl of each suspended microbial sample was spread on agar plates of both portions of prepared Mendel media and incubated for 48 hours at 30 °C. Colonies were streaked on the same media until pure colonies were obtained.

3.5.2. Screening of cellulase producing fungal Isolates

Fungal isolates obtained from the isolation process were screened for cellulose degradation on agar plates containing 2 g/l K_2HPO_4, 7 g/l K_2HPO_4, 0.073 g/l $MgSO_4.7H_2HO$, 0.5 g/l KCl, 1 g/l $(NH_4)_2SO_4$, 1 g/l yeast extract, 0.005 g/l $FeSO_4$, 5 g/l carboxymethylcellulose (CMC) and 12 g/l bacteriological agar at pH 5.5 and incubated at 30 °C for 120 h. After the incubation period, plates were flooded with

sterilized 0.1% Congo red for 15 min and then de-stained using sterilized 1 M NaCl for 30 min. The colonies that showed a clearing zone around their periphery colony were selected as cellulase producing fungi (Gupta *et al.,* 2012).

Agar blocks (1 cm × 1 cm) from plates of cellulase producing fungi were inoculated into Erlenmeyer flasks of 250 ml containing 100 ml of broth containing Mendel salts at pH 6.5 (2 g/l K_2HPO_4, 7 g/l K_2HPO_4, 0.073 g/l $MgSO_4.7H_2HO$, 0.5 g/l KCl, 1 g/l $(NH_4)_2SO_4$, 1 g/l yeast extract, 0.005 g/l $FeSO_4$) and 20 g/l Avicel™ as a carbon source. The flasks were incubated at 30 °C at 200 rpm with shaking for 240 h. Samples were collected every 24 h. The samples were micro-centrifuged at 13000 x g using a microcentrifuge (Beckman, USA) for 10 min and the supernatants were stored at -20 °C until the cellulase assays (Filter paper, endoglucosidase and β-glucosidase assays) were performed.

3.5.3. Screening of cellulase producing bacterial isolates

Cellulase producing bacterial isolates obtained from the isolation process was screened for cellulase on agar plates containing M9 salts. The media containing M9 salts were prepared by dissolving the following salts in deionized water to a final volume of 1 litre: 5 g CMC 20 ml 1 M $CaCl_2$, 0.1 ml with the addition of 42.5 g $NaHPO_4$, 15 g KH_2PO_4, 2.5 g NaCl, 5 g NH_4Cl. 50 ml of M9 salts, 2 ml 1M $MgSO_4$, 0.1 ml 1M $CaCl_2$, 10 g tryptone, 0.9 g yeast extract, and 12 g bacteriological agar. The previously isolated bacteria were streaked on the agar plates and incubated for 120 h at 37 °C. Thereafter, the plates were flooded with sterilized 0.1% Congo red for 15 min, then distained using sterilized 1 M NaCl for 30 min. The colonies showing clearing zones around their peripheries were selected as cellulase producing bacteria (Sethi *et al.,* 2013).

3.5.4. Identification of microorganisms using MALDI-TOF

The bacteria previously isolated from the CMC plates shown in section 3.5.2 were grown onto nutrient agar plates containing 28 g/l and incubated at 37 °C for 24 h. The formic acid extraction method was done as follows: a single colony of bacteria was washed with 300 µl deionised water and 900 µl of absolute ethanol in a 2 ml Eppendorf tube. About 70% formic acid and pure acetonitrile were added in a 1:1

(v/v) ratio to the pellet of bacteria and the concoction was vortexed for 30s. The supernatant, obtained after microcentrifugation for 2 min at 13,000 rpm, was transferred into a new tube. For all microorganisms tested, 1 µl of the extracted cells and homogeneous cell smears were added on a 384-well stainless steel MALDI target plate spot sites. Target plate was air-dried for about 10 min. 1 µl of matrix solvent mixture was added to the spots and allowed to dry. A duplicate of each sample was spotted and air-dried. Subjection to MALDI-TOF MS analysis was done after the MALDI plate was inserted into the mass spectrometer. The nitrogen laser (337 nm) was used to operate the MALDI instruments in the linear and positive ion mode. The resulting mass spectra were queried against a Bruker Daltonics database of bacteria (Wunschel *et.al.,* 2005).

A rotator was used to shake “overhead” 15 ml tubes inoculated with 750 µl of isolated fungal spores (1 $\times 10^6$ spores/ml) with 5 ml Mendel media composition as shown in section 3.5.1 and incubated at 30 °C for 24 h. The tubes were placed on the bench for 10 min for the filamentous fungi to form a sediment at the bottom of the tubes. Afterwards, 1.5 ml sediments were transferred into Eppendorf tubes. Microcentrifugation was done for 2 min at 13,000 rpm and the supernatant was removed, followed by the formic acid extraction method as indicated earlier. The resulting mass spectra were queried against a Bruker Daltonics database of fungi.

The fungal isolates, which the MALDI-TOF could not identify, were identified at Inqaba Biotech using the following protocol. Genomic DNA was extracted from the cultures received using the Quick-DNA™ fungal/bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005. The ITS aimed part was increased by utilizing OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) with primers ITS1 and ITS4. The PCR products were carried on a gel extracted with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The extracted fragments were sequenced in a forward and reverse direction (Nimagen, BrillantDye™ Terminator Cycle Sequencing Kit V3.1, and BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue NO. D4050). The fragments purified were analysed on the ABI 3500xl Genetic Analyser (Applied Biosystems, Thermofisher Scientific) for every sample for each reaction. CLC Bio Main Workbench v7.6 was utilized to analyse the sequences generated by

the ABI 3500XL Genetic Analyser and was then compared using BLAST search (NCBI) (Altschul *et al.,* 1997; White *et al.,* 1990).

3.5.5. Co-culture of fungi

The best three fungal strains with high cellulase activities (section 3.5.2) were co-cultured in different combinations. One agar block (1 cm × 1 cm) of each cellulase producing fungus was inoculated into Erlenmeyer flasks of 250 ml comprising 100 ml of broth containing Mendel salts (2 g/l K_2HPO_4, 7 g/l K_2HPO_4, 0.073 g/l $MgSO_4.7H_2HO$, 0.5 g/l KCl, 1 g/l $(NH_4)_2SO_4$, 1 g/l yeast extract, 0.005 g/l $FeSO_4$) and 20 g/l Avicel™ as the carbon source at pH 6.5 and incubated at 30 °C for 5 days at 200 rpm with shaking and samples were collected every 24 h. The samples were centrifuged for 10 min at 13000 x g with a microcentrifuge (Beckman, USA) and the supernatants were stored at -20 °C until cellulase assays are done.

3.5.6. Co-culture of bacterial strains

Selected bacterial strains able to produce cellulase were inoculated into Erlenmeyer flasks of 250 ml containing 0.6 g nutrient broth in 100 ml dH2O at pH 7 and incubated for 16 h at 37 °C and 200 rpm. The bacteria were inoculated with a starting OD of 0.6 for all flasks.

Monocultures of bacterial strains (500 µl) and a co-culture of different combinations of the isolated bacterial strains were grown in Erlenmeyer flasks of 250 ml with 100 ml consisting of 7 g/l K_2HPO_4; 0.1 g/l $MgSO_4$; 2 g/l KH_2PO_4; 1 g/l yeast extract; 0.5 g/l sodium citrate, 1 g/l yeast extract; 1 g/l $(NH_4)_2SO_4$; 0.073 g/l $MgSO_4 \cdot 7H_2O$; 0.005 g/l $FeSO_4$, 1 g/l yeast extract and 20 g/l Avicel™. The flasks were incubated for 240 h at 37 °C for 120 h at 200 rpm with shaking and samples were collected every 24 h. The samples were centrifuged for 10 min at 13000 x g with a microcentrifuge (Beckman, USA) and the supernatants were stored at -20 °C until cellulase assays (endoglucosidase and β-glucosidase) was required. The experiments were done in triplicates.

These experiments were done to determine the effect of co-culturing bacteria compared to the single strains of the isolated bacteria during cellulase production in submerged culture.

3.5.7. Co-culturing of bacteria in combination with fungi

Selected bacterial strains were inoculated into 250 ml Erlenmeyer flasks with 0.6 g nutrient broth in 100 ml of distilled H2O at pH 7 for 16 h of incubation. Various combinations of bacteria and fungi were grown in media consisting of 42.5 g $NaHPO_4$, 15 g KH_2PO_4, 2.5 g NaCl, 5 g NH_4Cl, 61.62 g 1M $MgSO_4$, 11.09 g 1M $CaCl_2$, 10 g tryptone, 1 g yeast extract, 0.005 g $FeSO_4$ and 20 g Avicel in a litre of distilled H2O at pH 6.5. Agar blocks (1 cm x 1 cm) of each of the cellulase-producing fungi were inoculated into 100 ml of the medium. Fusarium, 500 µl of the bacterial culture (OD of 0.6) was added and incubated at 35 °C, at 200 rpm with shaking for 10 days. One-millilitre samples were collected after every 24 h. The determination of cellulase activities was done using the 3, 5-Dinitrosalicylic acid assay method, filter paper assay and p-nitrophenyl-β-D glucopyranoside (pNPG) method described in section 3.9. The experiments were done in triplicates.

3.6. Preparation of fungi for solid-state fermentation

Agar blocks (1 cm x 1 cm) from plates of cellulase producing fungi were inoculated on CMC agar plates as shown in section 3.5.1 and incubated at 30 °C for 96 h. Harvesting of spores was carried out from the surface of solid media by pouring 10 ml of sterile distilled water with 1% Tween-80 onto fungal plates. The spores were gently brushed using a sterile swab for about 2 min. The spores were then poured into 50 ml tubes and used as stock for spore solution. A diminutive amount of the stock was diluted serially and spore counts were taken using a haemocytometer (Superior Marienfeld, Germany).

The haemocytometer and the coverslip were cleaned thoroughly in water. It was then rinsed in alcohol and wiped with a tissue paper. The coverslip was placed on the slide exactly over the depression in the counting chamber. 1 ml of spore suspension was drawn using a pipette and expelled into the depression below the coverslip. After 3-5 min, spore count was taken under phase contrast at 300-400

times magnification and adjusted to 106 spores/ml. For each dilution, three replicates were maintained. The numbers of spores/ml were calculated. The 50 ml tubes were then stored at 4 °C until use (Rice, 1939).

3.7. Solid-state fermentation using a co-culture

Preparation of live bacteria inoculum was prepared by transferring bacterial colonies from the CMC plates (section 3.5.1) into 100 ml nutrient broth in 250 ml Erlenmeyer flasks. The flasks were incubated at 37 °C, 120 rpm for 16 h. The cell concentration was adjusted to a starting OD of 0.6. The dead bacterial inoculums were prepared as follows: the cells grown after 16 h were transferred into 50 ml Eppendorf tubes and centrifuged at 200 rpm. The supernatants were discarded and the pellets adjusted to 0.5 g.

Solid-state fermentation was implemented in 250 ml Erlenmeyer flasks by weighing 3 g of the banana pseudostem powder as a carbon source (section 3.2). The substrate was sterilized by autoclaving it at 121 °C for 15 min and thereafter 15 ml of a nutrient mixture of Mendel salts (1 g K_2HPO_4, 0.25 g KCl, 0.04 g $FeSO_4$, 0.04 g $MgSO_4$, 3.5 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$, 0.5 g yeast extract at pH 6.5 in 500 ml distilled H20) was added. The flasks were inoculated with 750 µl of isolated fungal spores (1 $\times 10^6$ spores/ml) obtained from the three fungal isolates (section 3.7), 500 µl of live bacterial isolates and 0.5 g of dead bacterial isolates. The final moisture content of the medium was adjusted to approximately 77 %. Fermentation was done in an incubator at 30 °C for 10 days (240 h) (Ramanjaneyulu and Reddy, 2016). Solid-state fermentation was only done for the best fungal and bacterial isolates in various combinations. Ten flasks were prepared for every combination. A flask was removed daily and sampled for crude enzyme analysis. The crude enzyme extraction was carried out by the addition of 15 ml of 0.1 M citrate buffer (pH 6.8) and stirring for 30 min at 130 rpm. The fermentation mixtures were filtered through cheesecloth and subsequently centrifuged at 13000 rpm using a microcentrifuge (Beckman, USA) for 10 min (Dhillon, 2012). The supernatants were collected and used as crude enzymes and cellulase assays (endoglucosidase and β-glucosidase assays) were done in triplicates.

3.8. Determination of enzyme activity

3.8.1. Endoglucanase activity on carboxymethylcellulose

The CMCase activity was measured by incubating 25 µl of culture supernatant, 25 µl of 0.1 M sodium citrate pH 4.8 and 25 µl of 1% CMC prepared in 0.1 M sodium citrate buffer pH 4.8 at 50 ˚C for 30 min. The 3.5-DNS method was utilized to estimate the liberated reducing sugars, whereby 30 µl of DNS reagent was added to the mixture with 15-µl reaction mixture, 9 ml glass tubes were capped and boiled for 5 min. The solution was cooled by adding 200 µl of deionized water and the absorbance was measured at 540 nm. Glucose was used as the standard to determine the reduced sugars. The assay was done in triplicates. Under the assay conditions, the amount of enzyme that released 1 µmol of glucose equivalents per minute was defined by one unit of enzyme activity (Goldbeck, 2012).

3.8.2. Filter paper assay (Total cellulase activity)

Total cellulase activity was measured by incubating 25 µl of culture supernatant, 50 µl of 0.1 M sodium citrate buffer pH 4.8, with one stripe of Whatman No.1 0.5 x 3 cm. The 3.5-DNS method was used to estimate the liberated reducing sugars, whereby 150 µl of DNS reagent was added to the reaction mixture, 9 ml glass tubes were capped and boiled for 5 min and proceeding as described for the CMC assay (section 3.8.1). The assay was done in triplicates. Under the assay conditions, the amount of enzyme that released 1 µmol of glucose equivalents per minute was defined by one filter paper unit (FPU) unit of enzyme activity (Ghose, 1987).

3.8.3. β-glucosidase activity

The *p*-nitrophenyl- β-D glucopyranoside (*p*NPG) was used as a substrate through a 96 well microtiter plate method as described by (Parry *et al.,* 2001) during the β-glucosidase assay. A reaction mixture of 100 µl containing 50 µl of an enzyme, 25 µl of 10 M pNPG as a substrate and 50 µl of 0.1 M acetate buffer pH 5.0, was incubated at 50 ºC for 15 min and 100 µl of ($NaCO_3$) buffer 0.2 M, pH 10.8 was added to discontinued the reaction. The absorption of the developed yellow colour was read at 405 nm using a Beckman Coulter DTX 880 Multimode Detector

microplate reader. The assay was done in triplicates. The *p*-nitrophenyl (*p*NP) was used as a standard to quantify the amount of p-nitrophenol released. Under the assay conditions, the amount of enzyme required to release 1 µmole of PNP per minute was expressed by one unit of β-glucosidase activity (Ghose, 1987).

3.9. Electrophoretic methods

3.9.1. Zymography for cellulase

12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was used and 15 µl was loaded (Laemmli, 1970). Molecular weights of each protein bands obtained were estimated by the unstained Protein Molecular Weight Marker (Fermenters). CMC substrate incorporated into the gel prior to polymerization was used to prepare cellulase zymograms with 0.1% (w/v) to give a final concentration (Ratanachomsri *et al*., 2006). Coomasie blue was used to stain the molecular weight maker cut after electrophoresis, while the other part of the gel was washed with a combination of 40% isopropanol and sodium phosphate buffer pH 7.2 (1:1) for 1 h. Sodium phosphate buffer, pH 7.2 was used for further washing, to remove the isopropanol for a period of 1 h. The washed gels were placed in a mixture of 1 mM EDTA with 50 mM sodium phosphate buffer (1:1) for 1 h at 4 °C in order to renature the protein (Dutta *et al*., 2008). Enzymatic degradation of the substrate was achieved when the gel was further incubated for 1 h at 50 °C. 0.1% (w/v). Congo red was used to stain the gels shaking at 40 rpm for 30 min. 1 M sodium chloride was used to remove excess Congo red by shaking at 40 rpm for 20 min. The gel was immersed in 0.5% (w/v) acetic acid to stop the enzymatic reaction. Syngene G-Box Image Capture (Syngene, USA) was used to document or capture zymogram together with the molecular weight marker gel piece.

3.9.2. Detection for β-glucosidase activity on polyacrylamide gels

12% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) was used and 15 µl enzyme extracts were loaded (Laemmli, 1970). Molecular weights of each protein band obtained were estimated by the use of a stained Protein Molecular Weight Marker (Fermenters). β-glucosidase zymography was done as described by Grudkowska *et al.,* (2013), whereby 0.1% esculin was used as a substrate. After

electrophoresis, the gels were washed with 40% isopropanol in 0.1 M sodium citrate buffer pH 4.8 for 30 min at room temperature. 0.1 M sodium citrate buffer pH 4.8 was used to further wash the gel at room temperature to remove isopropanol for 10 min. A glass bowl that contained 0.1 M citrate buffers with 0.1% esculin (w/v) and 0. 03% (w/v) ferric chloride was further incubated at 50 °C together with the washed gel for 5 min. The black bands are equivalent to the β-glucosidase developed against a translucent background during incubation. Immersing the gel in a 0.5% (w/v) glucose aqueous solution as an inhibitor of β-glucosidase activity stopped the reaction.

3.10. Statistical analyses

Statistical analysis and graphs were reformed using Microsoft Excel® 2013. Data were analysed for statistical significance by the student's t-test simple interactive statistical analysis (SISA).

CHAPTER 4

RESULTS

4.1. Isolation and screening of cellulase-producing microbial isolates

Seven fungal isolates and six bacterial isolates were isolated using CMC as the carbon source (Fig 4.1). MALDI-TOF analysis was used to identify the isolated cellulose producing bacteria and fungi from banana pseudostem waste (Table 4.1). The identification of the bacteria and fungi were based on spectral fingerprints from the MALDI-TOF for each microorganism compared to that found in the database created for MALDI-TOF by Bruker. However, not all the microbes are represented on the database and therefore some of the fungal isolates could not be identified. These fungal isolates were sent for rDNA sequencing (Table 4.1).

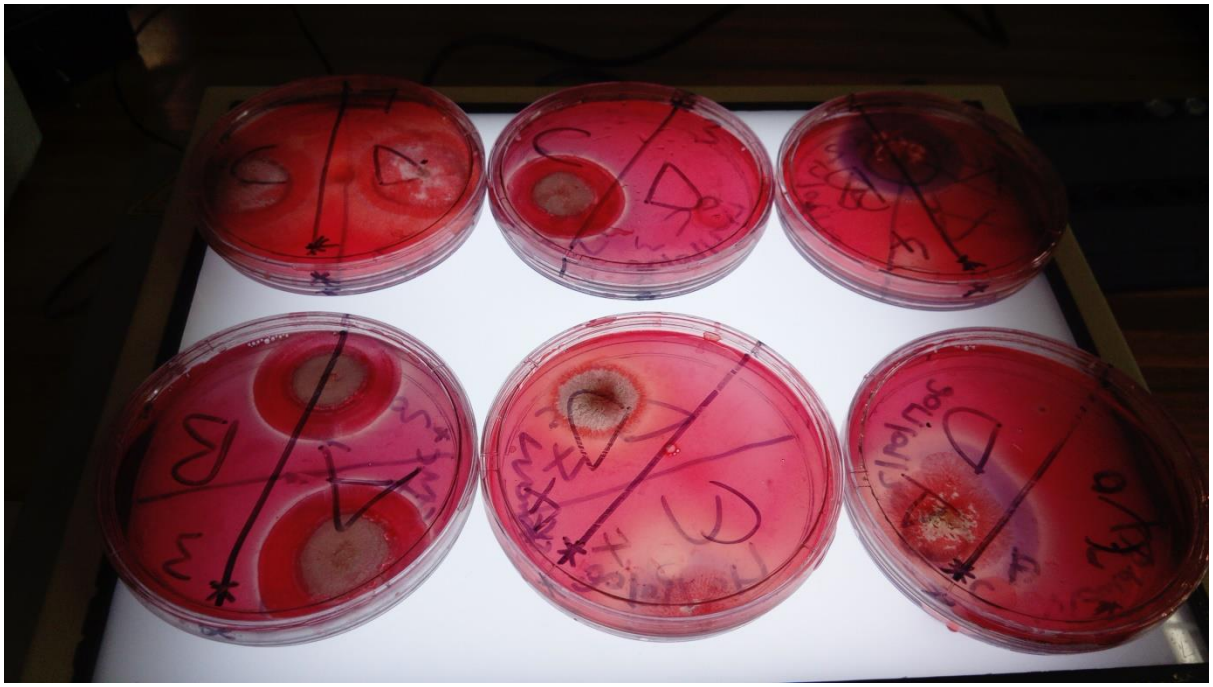


Figure 4.1: Cellulase activity of different fungal isolates on CMC agar plates.

Table 4.1: Isolated microbes identified using MALDI-TOF and rDNA sequencing.

Isolate no	Species	MALDI scores	% Similarity (ITS region)
1	*Enterobacter asburiae*	1.982	N/A
2	*Enterobacter aerogenes*	1.892	N/A
3	*Klebsiella pneumoniae*	1.816	N/A
4	*Enterobacter cloacae*	2.047	N/A
5	*Enterobacter radicincitans*	1.204	N/A
6	*Klebsiella varicon*	1.605	N/A
7	*Enteroaggresive Escherichia coli-EAST-1*	2.093	N/A
1B	*Trichoderma longibrachiatum*	N/A	99
2A	*Aspergillus flavus*	N/A	99
5A	*Aspergillus fumigatus*	N/A	100

Seven fungal isolates (1B, 2A, 3C, 4C, 5A, 6A and 7A) obtained from decaying banana pseudostems that tested positive on CMC plates were further investigated for cellulase activity with Avicel as carbon source (Fig 4.2). *Aspergillus fumigatus* 5A was the best producer of endoglucosidase after 144 h (0.436 U/ml) and the second-best producer of exoglucosidase (0.957 FPU/ml) after 144 h. *Trichoderma longibrachiatum* 1B was the second-best producer of endoglucosidase (0.327 U/ml) after 120 h and the best producer of exoglucosidase (1.174 FPU/ml) after 120 h. *Aspergillus flavus* 2A produced the third-highest exoglucosidase and endoglucanase activity (0.04 FPU/ml) and (0.179 U/ml) after 168 h and 192 h, respectively. *Aspergillus fumigatus* 5A produced the highest β-glucosidase activity (0.139 U/ml) after 120 h.

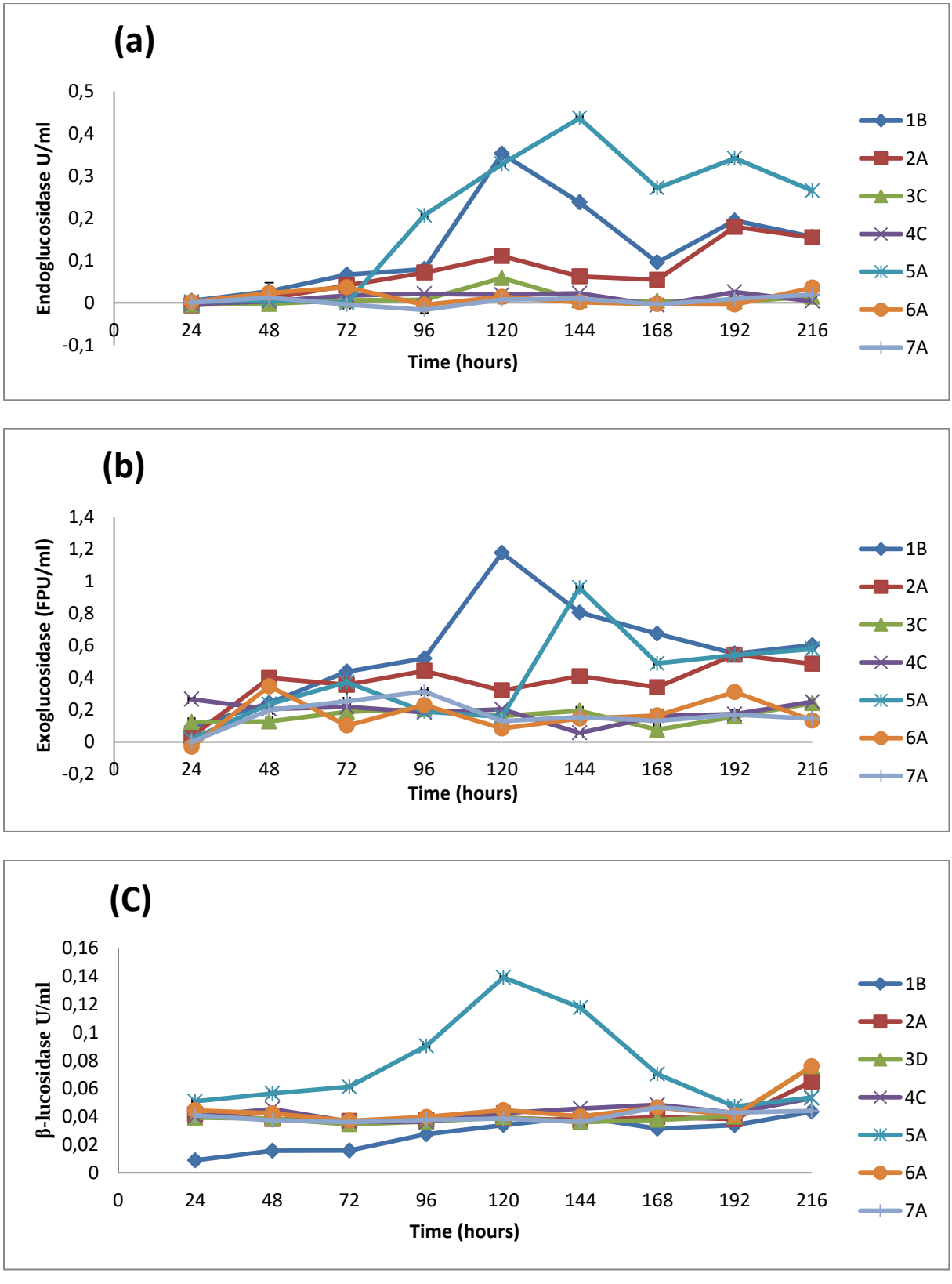


Figure 4.2: Cellulase activity for seven fungal isolates obtained from banana pseudostem with Avicel as carbon source in submerged culture (Endoglucosidase (a) Exoglucosidase (FPU- filter paper unit) (b) and (c) β-glucosidase activities).

4.2. Co-culture of isolated fungal strains with high cellulase activity

The fungal strains *A. fumigatus* 5A, *T. longibrachiatum* 1B and *A. flavus* 2A with high cellulase activities were studied in different combinations to determine any improvement in cellulase production. The combinations consisted of all three fungal strains combined, followed by combining two fungal strains in different combinations. The results are depicted in Fig 4.3. Co-culturing the 3 different fungal strains together (*A. fumigatus* 5A, *T. longibrachiatum* 1B and *A. flavus* 2A **(5A1B2A)** produced the highest endoglucosidase activity (0.187 U/ml after 168 h) but performed poorly in terms of exoglucosidase (0.26 FPU/ml after 168 h) and β-glucosidase activities. A co-culture of *A. fumigatus* 5A and *T. longibrachiatum* 1B **(5A1B)** was the best producer of β-glucosidase (4.31 U/ml after 72 h) and the second-best combination in exoglucosidase production (0.6 FPU/ml after 96 hours) and the best producer of β-glucosidase (4.317 U/ml after 72 h). *A. fumigatus* 5A and *A. flavus* 2A **(5A2A)** was the best producer of exoglucosidase (0.949 FPU/ml after 192 h), and the second-best producer of endoglucosidase (0.086 U/ml after 192 h), but performed poorly in terms of β-glucosidase. The combination of *T. longibrachiatum* 1B and *A. flavus* 2A **(1B2A)** was the third-best producer of both endoglucosidase (0.056 U/ml after 120 h) and exoglucosidase (0.32 FPU/ml after 120 h), but a poor producer of β-glucosidase activity.

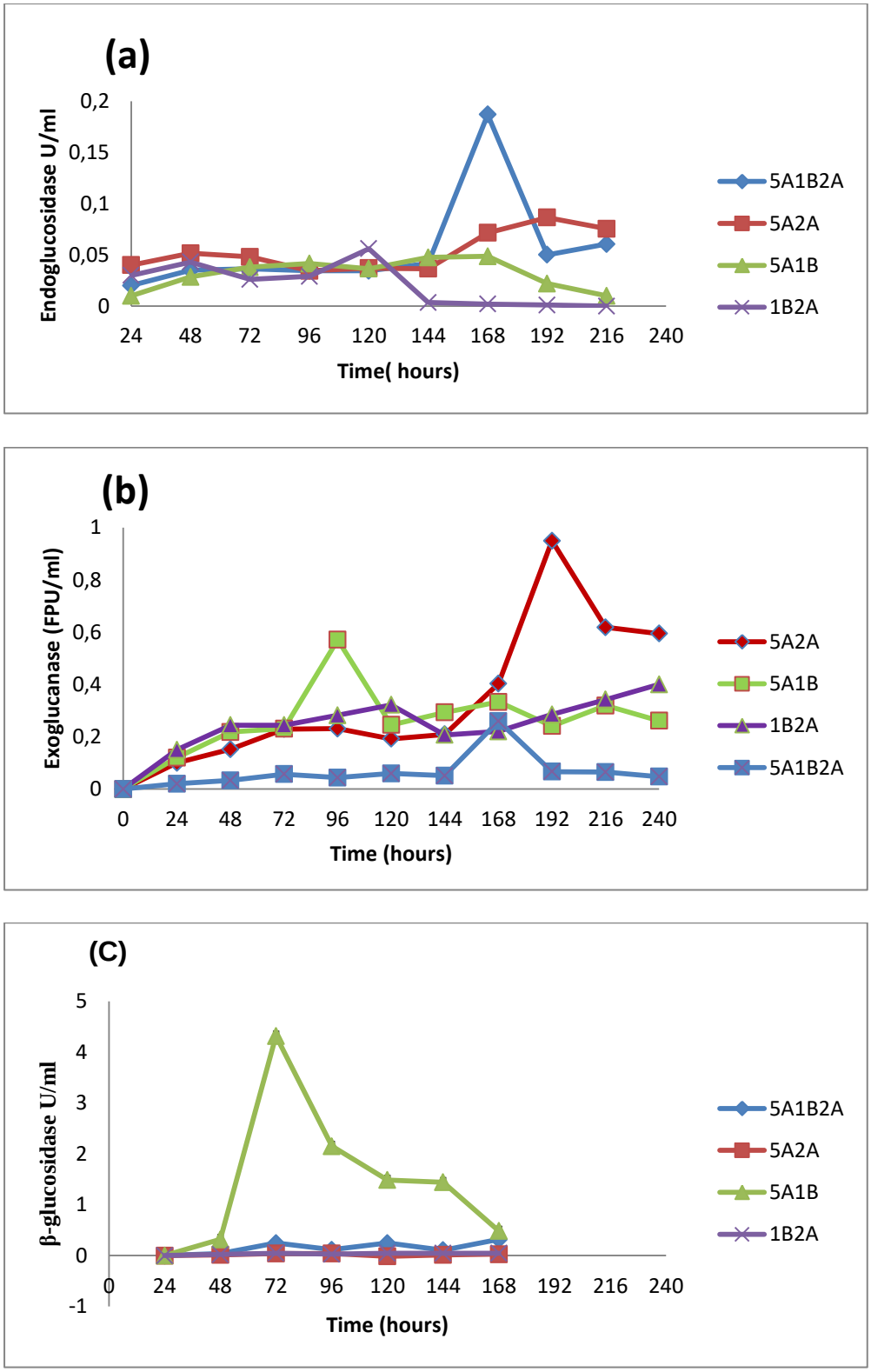


Figure 4.3: Co-culture of isolated fungal strains producing Endoglucosidase (a), Exoglucosidase (b) and β-glucosidase (c) over a period of 240 h in submerged fermentation using Avicel™ as a carbon source (1B - *T. longibrachiatum* 1B; 2A - *A. flavus* 2A; 5A - *A. fumigatus* 5A).

4.3. Co-culture of bacterial strains for cellulase production

Two bacterial strains, *Enterobacter asburiae* 1 and *Enterobacter cloacae* 4 were studied further since they indicated the optimum growth on plates with CMC. Single bacterial strains and a co-culture of different combinations of the isolated bacterial strains were grown in submerged media using M9 salts, Mendel's medium and Avicel™ as a carbon source at 35 °C for a maximum of 10 days (240 h) for cellulase production. This fermentation with the selected bacteria was done to determine the effect of co-culturing bacteria against the single strains of the bacteria during cellulase production in submerged culture (Fig 4.4). The various chemical products with a variety of sugars as carbon sources during industrial production have been produced by using *Escherichia coli* as a host. However, endogenous cellulose degradation neither enzymes nor secrets heterologous cellulase are produced by *Escherichia coli* for its poor secretory capacity.

Enteroaggregative *Escherichia coli* EAST-1 (EAEC) isolated from chicken intestines were used to observe the efficacy in these experiments for endoglucosidase and β-glucosidase activities. With an enzyme, the activity of 0.254 U/ml noted for endoglucosidase after 96 h and 1.5 U/ml for β-glucosidase after 72 h. *E. coli* (EAEC) was further co-cultured with *Enterobacter asburiae* 1 and *Enterobacter cloacae* 4 to evaluate the effect of using *E. coli* EAST-1 with other microorganisms. Amongst the single bacteria, isolated, *E. asburiae* 1 seemed to produce a higher endoglucosidase activity of 0.074 U/ml after 124 h (Fig 4.4a).

Comparing the single strain of *E. asburiae* 1 with *E. cloacae* 4 to the co-cultures (1 + 4), *E. asburiae* 1 seemed to be the second-best producer of endoglucosidase (0.073 U/ml after 120 h) and the lowest activity for β-glucosidase (0.97 after 48 h). Strain *E. cloacae* 4 produced a similar endoglucosidase activity (0.057 U/ml after 120 h) than the co-culture (1 + 4), but *E. asburiae* 1 with *E. cloacae* 4 (1 + 4) produced the highest β-glucosidase (1.46 U/ml after 48 h) compared to *E. cloacae* 4 (0.959 U/ml after 120 h). The best combination for the bacterial strains was *E. asburiae* 1 combined with *E. cloacae* 4 and *E. coli* (1 + 4 + *E. coli*) for endoglucosidase activity and *E. asburiae* 1 with *E. cloacae* 4 (1+4) for β-glucosidase activity (1.459 U/ml after 48 h). The combination of *E. asburiae* 1 and *E. cloacae* 4 with *E. coli* (EAEC) produced the lowest endoglucosidase and β-glucosidase activities (approximately 0.95 U/ml after 48 h and 120 respectively).

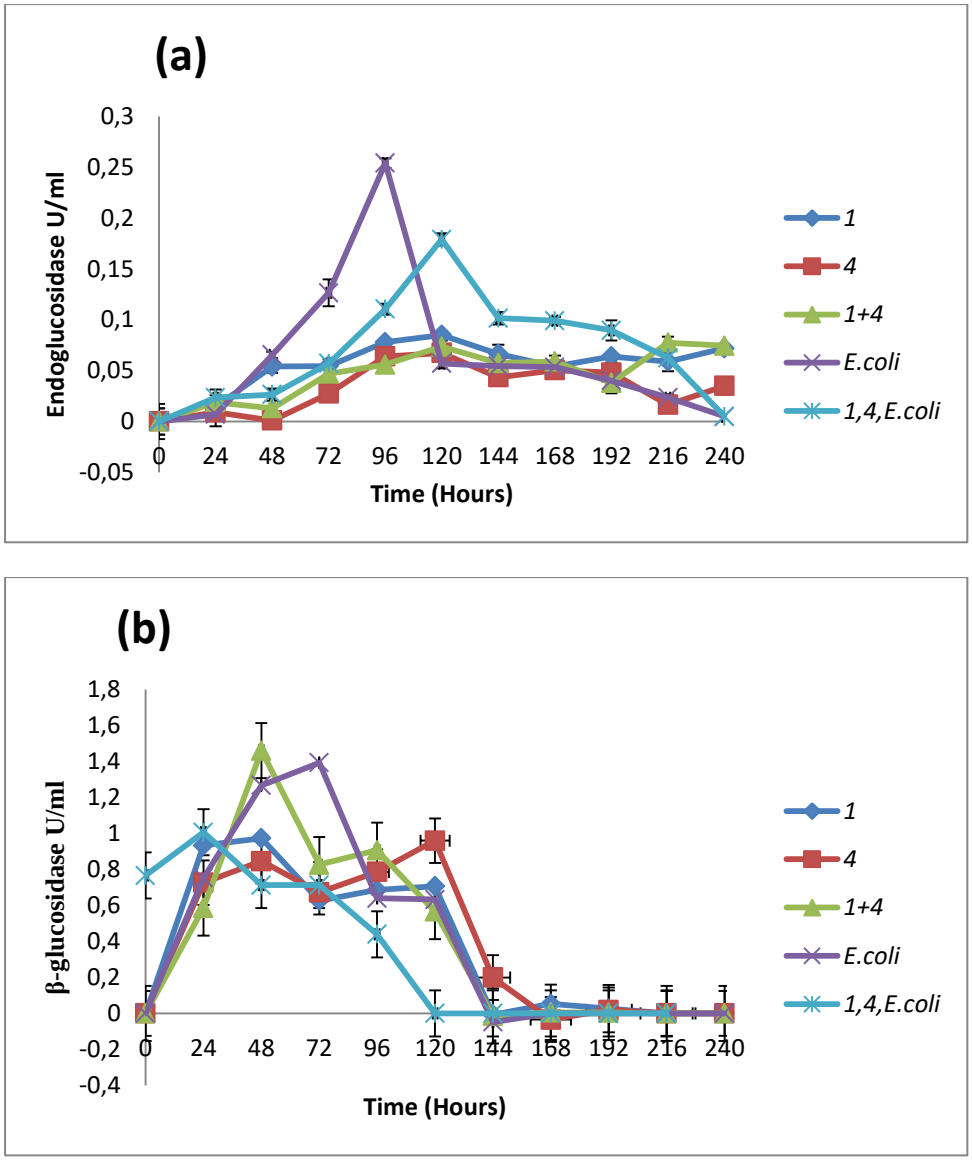


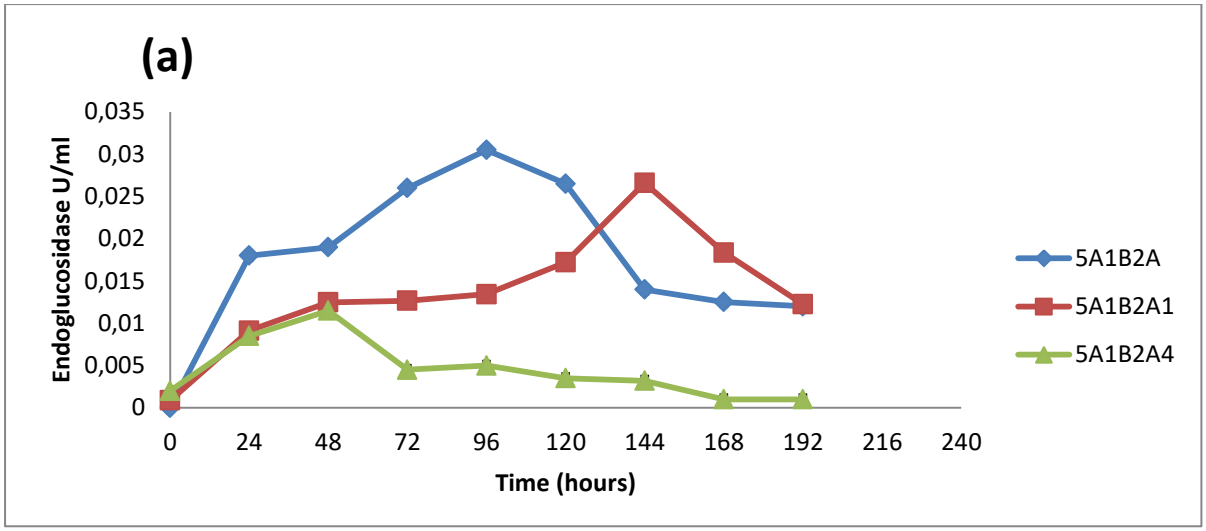
Figure 4.4: Cellulase activity of the co-culture and single strains of selected isolated bacteria in submerged fermentation using Avicel™ as a carbon source. (a) Endoglucosidase and (b) β-glucosidase. (1 - *Enterobacter asburiae* 1; 4 - *E. cloacae* 4; *E. coli* - *Escherichia coli* EAST-1).

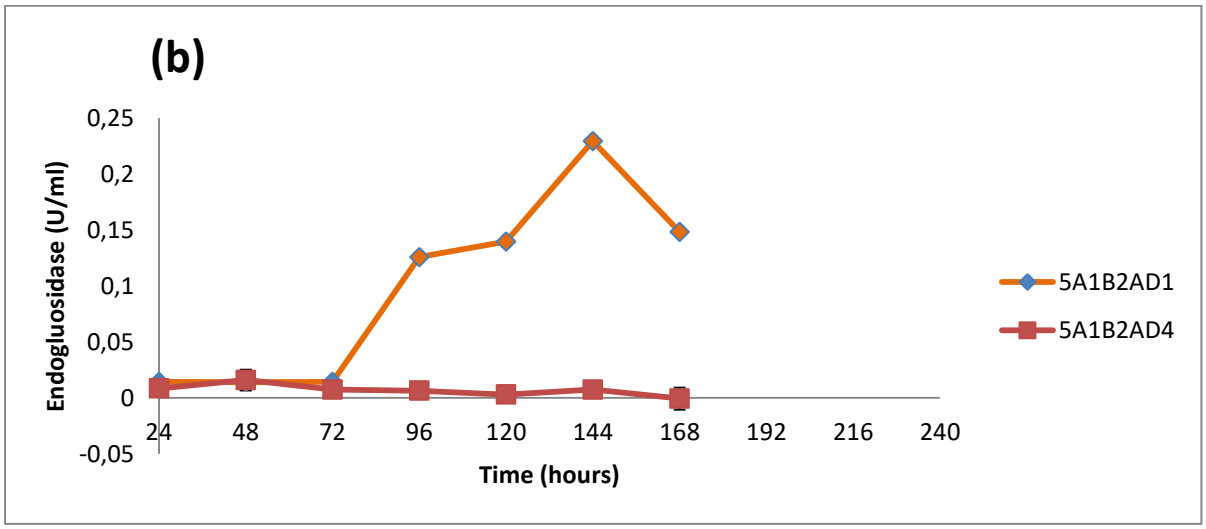
4.4. Mixed culture of fungal isolates and bacterial isolates

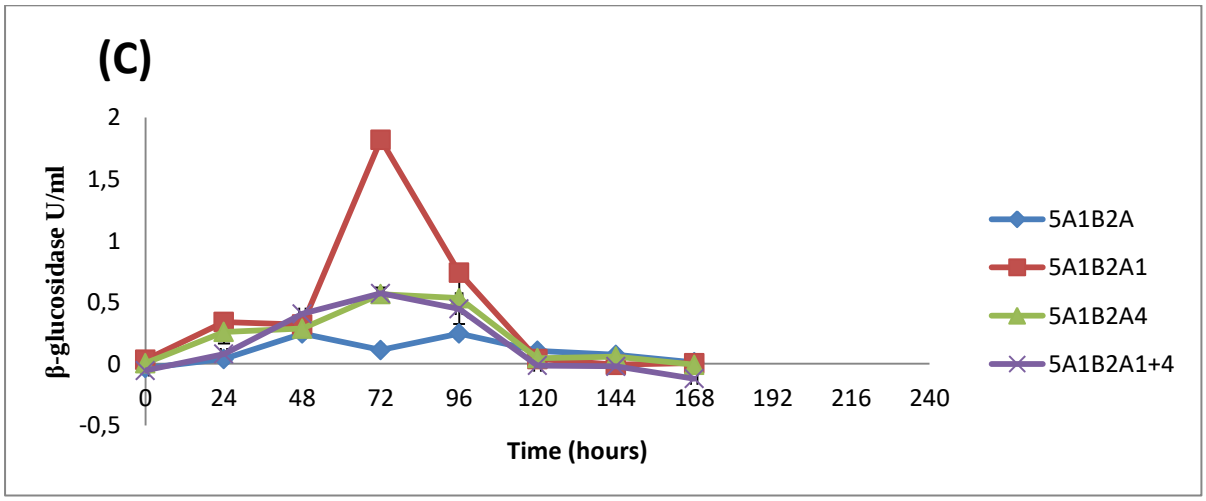
A mixed culture of different microorganisms that include the isolated fungi and bacteria were grown together in media (M9 salts together with Avicel™ at 35 °C), which could be favourable for both microorganisms. A mixed culture study was done to further improve cellulase production.

A co-culture of the fungi *A. fumigatus* 5A, *T. longibrachiatum* 1B and *A. flavus* 2A (**5A1B2A**) were grown in submerged media with M9 salts, Mendel's medium and Avicel™ as a carbon source. The cellulase-producing bacteria *E. asburiae* 1 and *E. cloacae* 4 were added to the fungal co-culture in different combinations (**5A1B2A1, 5A1B2A4, 5A1B2A1+4**) as well as dead *E. asburiae* (indicated as D1) and *E. cloacae* (indicated as D4) **(5A1B2AD1** and **5A1B2AD4)** at 35 °C for 216 h. Only endoglucosidase and β–glucosidase activities were determined for these combinations in submerged fermentation (SmF) (Fig 4.5).

The co-cultures of the fungal isolates with the dead *E. asburiae* D1 **(5A1B2AD1**) produced a higher endoglucosidase activity (0.256 U/ml) after 144 h and β-glucosidase activity (4.519 U/ml) after 96 h compared to the co-culture of fungi with live bacteria **5A1B2A1** endoglucosidase activity (0.027 U/ml) after 144 h, β-glucosidase (1.819 U/ml) and **5A1B2A4** endoglucosidase activity (0.011 U/ml) after 48 h and β-glucosidase (0.573) after 72 h (Fig. 4.5). Mixed cultures of fungi and live bacteria (**5A1B2A1** and **5A1B2A**) showed approximately the same levels of production for endoglucosidase activity (0.0265 U/ml and 0.03 U/ml, respectively) after 144 h and 96 h, respectively (Fig 4.5a). Generally, mixed cultures of fungal and bacterial strains (particularly, 1 and D1) improved cellulase production in submerged cultures.


(a)
Endoglucosidase U/ml
0,035
0,03
0,025
0,02
0,015
0,01
0,005
0
0
24
48
72
96
120
144
168
192
216
240
Time (hours)
5A1B2A
5A1B2A1
5A1B2A4


(b)
Endogluosidase (U/ml)
0,25
0,2
0,15
0,1
0,05
0
-0,05
24
48
72
96
120
144
168
192
216
240
Time (hours)
5A1B2AD1
5A1B2AD4


(C)
β-glucosidase U/ml
2
1,5
1
0,5
0
-0,5
0
24
48
72
96
120
144
168
192
216
240
Time (hours)
5A1B2A
5A1B2A1
5A1B2A4
5A1B2A1+4

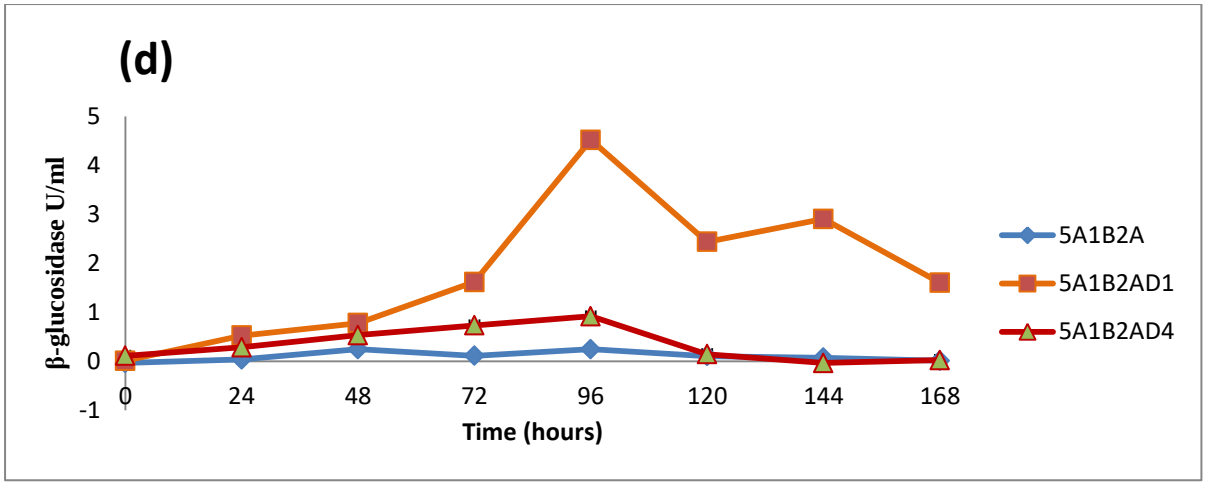


Figure 4.5: Mixed cultures of three fungal strains with live bacterial strains (a and c) and dead bacterial strains (b and d) producing Endoglucosidase (a and b) and β-glucosidase (c and d) over 240 hours in submerged fermentation using Avicel as a carbon source (1B - *T. longibrachiatum* 1B; 2A - *A. flavus* 2A; 5A - *A. fumigatus* 5A; 1 - *Enterobacter asburiae* 1; 4 - *E. cloacae* 4; D- dead strain).

4.5. Mixed culture of three fungal isolates with dead *Enteroaggregative Escherichia coli* (EAEC) at different masses (0.5 g, 1 g, and 2 g)

A mixed culture of *Trichoderma longibrachiatum* 1B, *Aspergillus flavus* 2A, *Aspergillus fumigatus* 5A and EAEC of different masses was used to observe if there could be an increase in both endoglucosidase and β-glucosidase activities. A combination of these microorganisms was grown in a submerged medium using M9 salts, Mendel's medium and Avicel™ as a carbon source at 35 °C for a maximum of 10 days (240 h) for cellulase production.

It was observed that a mixed culture with the lowest mass (0.5 g) of dead *E. coli* EAEC produces the highest endoglucosidase activity (0.047 U/ml) after 168 h (Fig 4.6) and none of the mixed fungal and EAEC cultures produced β-glucosidase. Therefore, these results suggest that the increase of EAEC mass decreases endoglucosidase and β–glucosidase production in submerged medium.

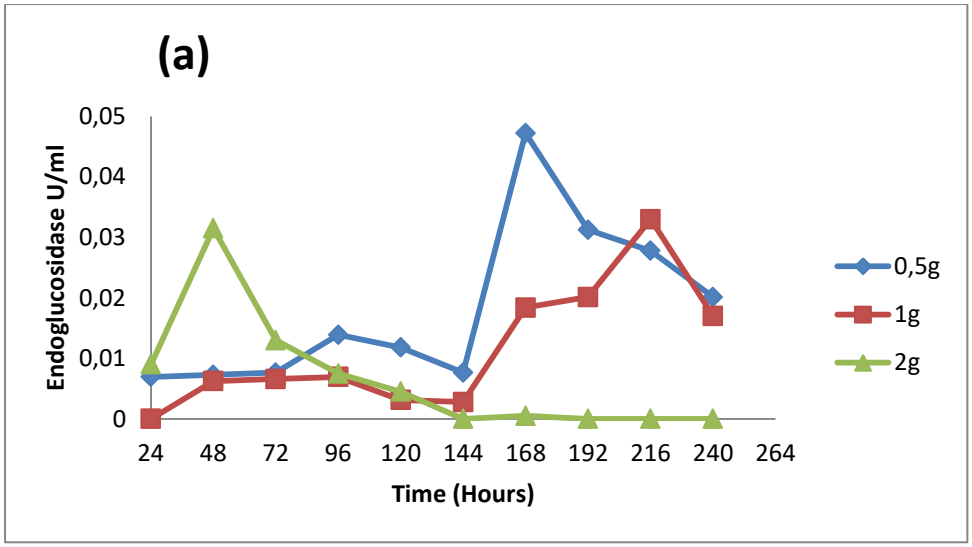


Figure 4.6: A Mixed culture of three fungal isolates with dead *Enteroaggregative Escherichia coli* EAST-1 at different masses.

4.6. Production of cellulase by isolated fungal strains in Solid State Fermentation

The fungal strains *T. longibrachiatum* 1B, *A. flavus* 2A and *A. fumigatus* 5A were investigated for cellulase production (endoglucosidase and β-glucosidase) in solid-state fermentation, as monocultures and as a combination (**5A1B2A).** The fungi were grown on banana pseudostem, as a carbon source at 30 °C for a maximum of 10 days (240 h) to test for cellulase production. The fungal isolates hydrolysed the banana pseudostem in the solid-state fermentation producing varying amounts of cellulase (Fig 4.7).

It was observed that *T. longibrachiatum* 1B was the best producer of both endoglucosidase (6.7 U/ml after 72 h) and β-glucosidase (37 U/ml after 216 h), while *Aspergillus flavus* 2A was the worst producer of endoglucosidase activity (2.5 U/ml after 72 h) and β-glucosidase (28 U/ml after 168 h). The combination of fungal strains 5A1B2A was the third-best producer of endoglucosidase (4.13 U/ml after 120 h) and β-glucosidase (32.83 U/ml after 96 h).

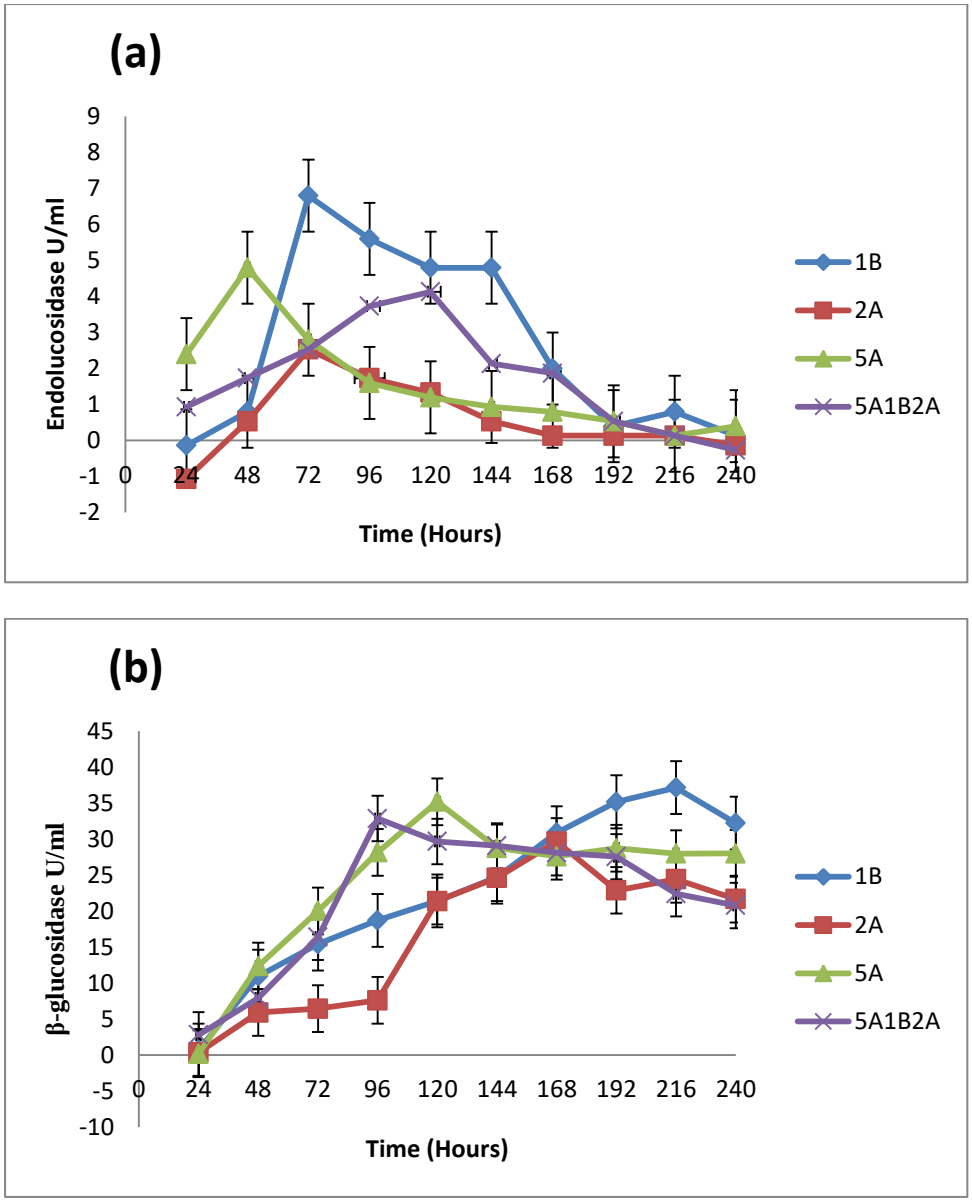


Figure 4.7: Monocultures and co-culture of fungal strains producing (a) Endoglucosidase and (b) β-glucosidase grown in solid-state Fermentation with the banana pseudostem as carbon source (1B - *T. longibrachiatum* 1B; 2A - *A. flavus* 2A; 5A - *A. fumigatus* 5A).

4.7. A mixed culture of three fungal strains with bacterial strains in solid-state fermentation

A mixed culture of the three fungal strains *T. longibrachiatum* 1B, A. *flavus* 2A and *A. fumigatus* 5A were combined with either live *E. asburiae* 1 (**5A1B2A1**) or dead cells of *E. asburiae* 1 (**5A1B2AD1**) to produce cellulase in solid-state fermentation with banana pseudostem as carbon source.

These mixed cultures were able to hydrolyse banana pseudostem in order to produce endoglucosidase and β-glucosidase (Fig 4.8). It was observed that the combination **5A1B2AD1** (dead) was the best producer of endoglucosidase activity (12.7 U/ml after 48 h) and β-glucosidase activity (37.5 U/ml after 144 h). *Enterobacter asburiae* 1 produced the lowest endoglucosidase activity (1.6 U/ml after 48 h) and β-glucosidase activity (9.9 U/ml after 192 h).

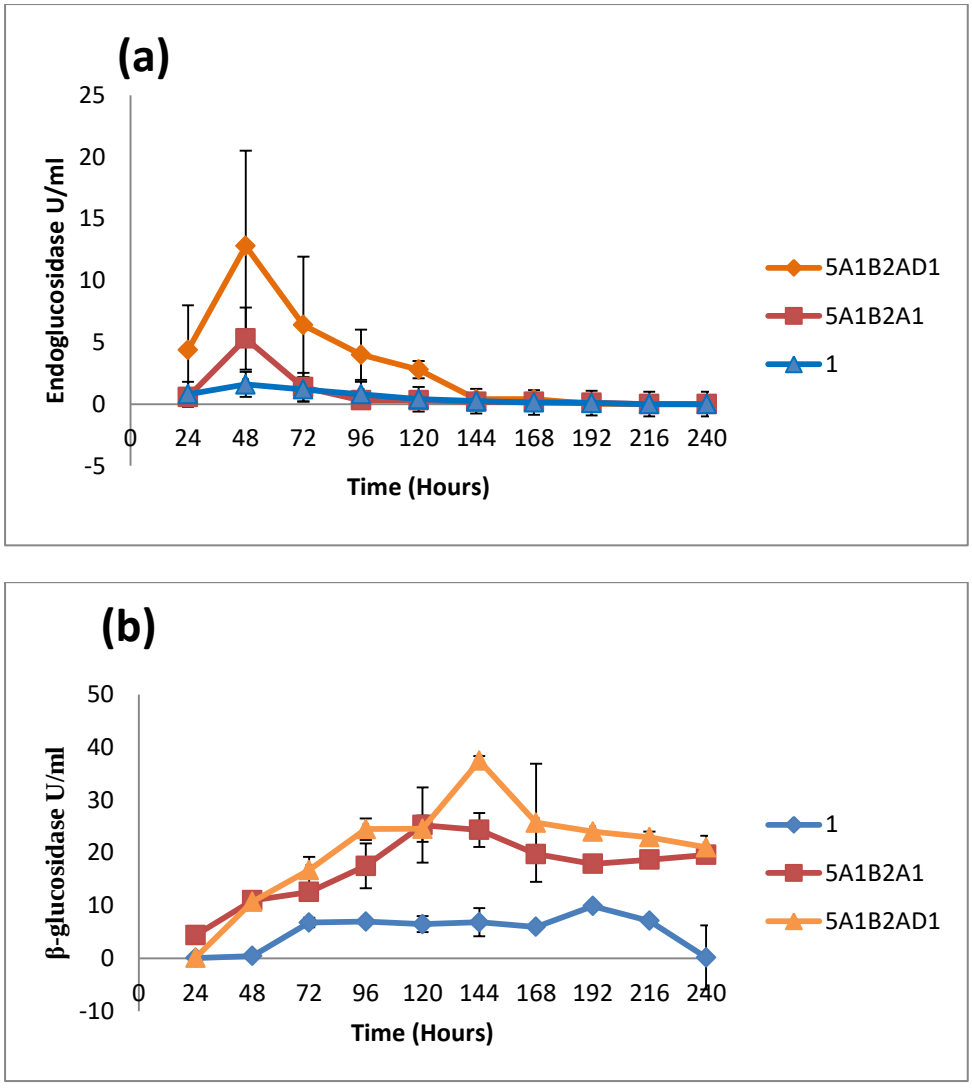


Figure 4.8: A mixed culture of three fungal strains with live and dead bacterial strains of *E. asburiae* 1 producing Endoglucosidase (a) and β-glucosidase (b) over a period of 240 hours in solid-state fermentation with banana pseudostem as carbon source (1B - *T. longibrachiatum* 1B; 2A - *A. flavus* 2A; 5A - *A. fumigatus* 5A; 1 - *Enterobacter asburiae* 1, D – dead cells of *Enterobacter asburiae* 1).

4.8. Endoglucosidase zymography

Zymography of the supernatant was performed on the selected fungal strains *T. longibrachiatum* 1B, *A. flavus* 2A, *A. fumigatus* 5A, the three fungal strains in combination (**5A1B2A**), the three fungal strains in combination with live *E. asburiae* 1 (**5A1B2A1**) and in combination with dead *E. asburiae* D1 (**5A1B2AD1**) and the monoculture of *E. asburiae* 1. These microorganisms produced relatively higher cellulase activity over time. Cellulase zymography was done in order to assess the diversity of cellulase in comparison to the molecular marker with 0.1% CMC as a substrate. The endoglucosidase zymogram activity is indicated by clear bands against the dark background (Fig 4.9). All the strains (monoculture or mixed) showed estimated bands at 150 kDa,100 kDa, 85 kDa and 60 kDa cellulase activity with the exception of *T. longibrachiatum* 1B where just one band with 100 kDa fibres cellulase activity band was detected.

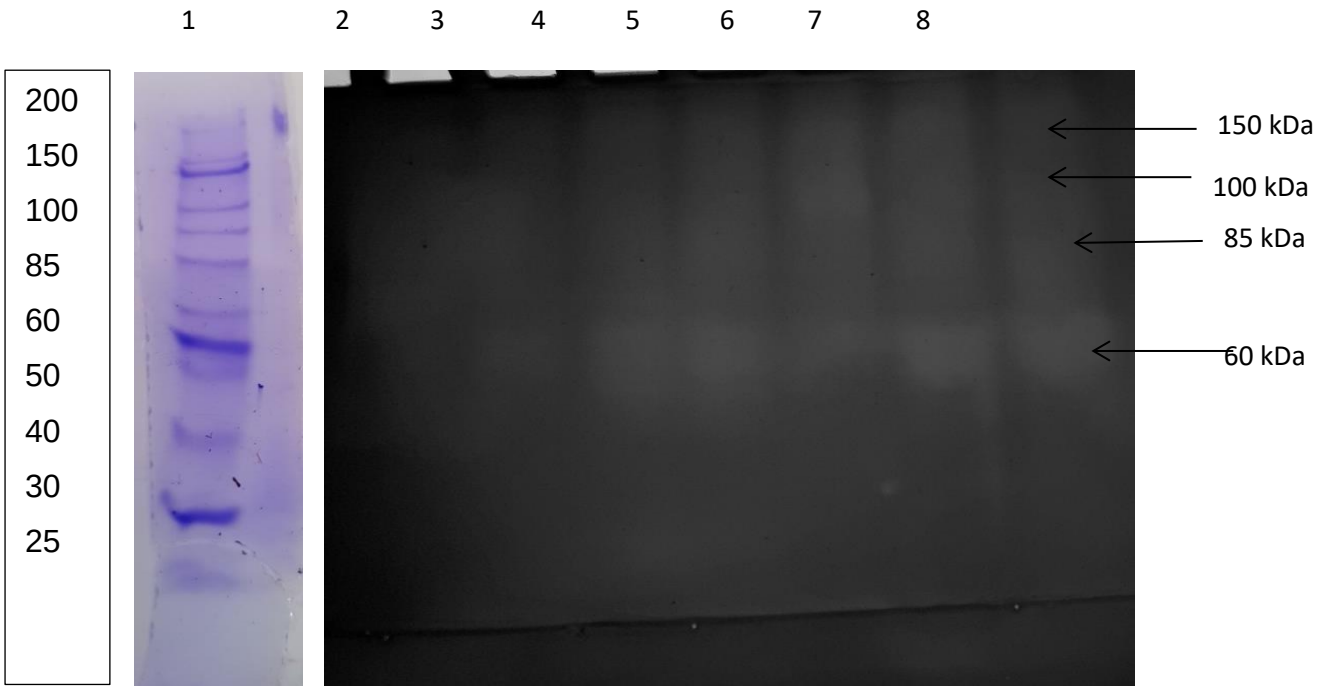


Figure 4.9: Zymography of the culture supernatant showing active endoglucosidase (Lane 1: Marker, Lane 2: *T. longibrachiatum* 1B. Lane 3: *A. flavus* 2A, Lane 4: *T. longibrachiatum* 5A, Lane 5: *A. fumigatus*, *T. longibrachiatum*, *A. flavus* 5A1B2A, Lane 6: *E. asburiae* 1 Lane 7: *A. fumigatus*, *T. longibrachiatum*, *A. flavus*, *E. asburiae* 1 5A1B2A1. Lane 8: *A. fumigatus*, *T. longibrachiatum*, *A. flavus*, *E. asburiae* D1 5A1B2AD1).

4.9. β-glucosidase zymography

Polyacrylamide gels were used to detect β-glucosidase activity using esculin as a substrate from the supernatant with same monocultures and mixed cultures as indicated in section 4.8. The β-glucosidase activity is indicated by the dark bands against the translucent background. It was observed that *T. longibrachiatum* 1B and *A. fumigatus* 5A produced two β-glucosidase activity bands of 40 kDa and 60 kDa. The rest of the microbial strains showed a single band for the β-glucosidase activity of 60 kDa (Fig 4.10).

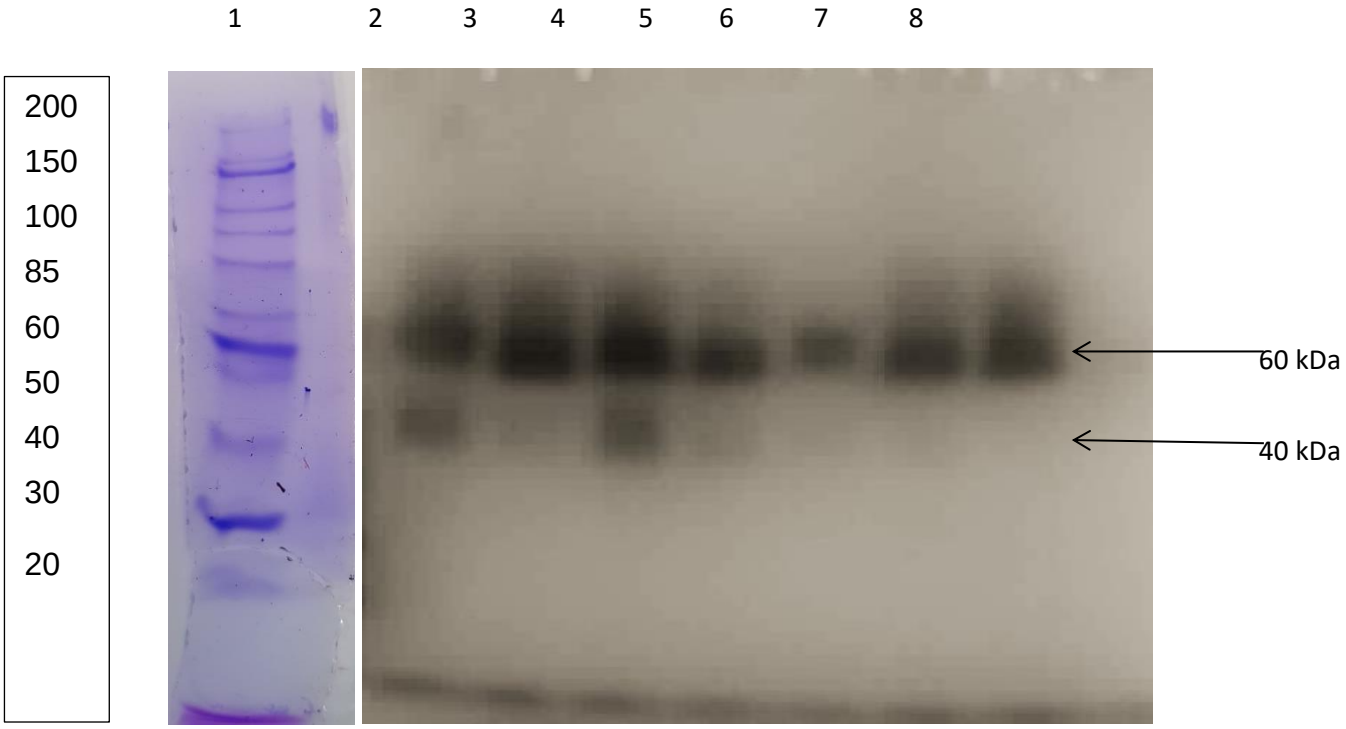


Figure 4.10: β-glucosidase zymography of the culture supernatant showing active enzyme activity (Lane 1: Marker, Lane 2: *T. longibrachiatum* 1B. Lane 3: *A. flavus* 2A Lane 4: *A. fumigatus* 5A, Lane 5: *A. fumigatus, T. longibrachiatum*, *A. flavus* 5A1B2A, Lane 6: *E. asburiae* 1, Lane 7: *A. fumigatus*, *T. longibrachiatum*, *A. flavus*, *E. asburiae* 1 5A1B2A1. Lane 8: *A. fumigatus*, *T. longibrachiatum*, *A. flavus*, *E. asburiae* D1 5A1B2AD1).

CHAPTER 5

DISCUSSION

5.1. Isolation and screening of cellulase producing fungal and bacterial isolates

Seven bacterial and seven fungal isolates, able to hydrolyse cellulose, were obtained from the decaying banana pseudostems. These fungal isolates were primarily screened on CMC agar plates and are a good indicator of cellulolytic activity (Gupta *et al*., 2015). Bacterial isolates were identified by MALDI-TOF (*Enterobacter asburiae* 1, *Enterobacter aerogenes* 2, *Klebsiella pneumoniae 3*, *Enterobacter cloacae* 4, *Enterobacter radicincitans* 5, *and Klebsiella varicon* 6, *Enteroaggresive Escherichia coli*- EAST-1) whereas; the fungal isolates could not be identified using this technique. This could be that essential spectra to reference with are not present in the database (Lau *et al*., 2013). Gautier *et al*. (2014) also reported five fungal isolates that could not be identified with MALDI-TOF and was subsequently identified using DNA sequencing.

The secondary screening was done for the fungal isolates in submerged media using Avicel™ as a carbon source at 30 °C for a maximum period of 240 h (Fig 4.2). The activity of the cellulase enzyme increased with incubation time until a maximum activity was reached. Further incubation resulted in decreased enzyme activity. The fungal strains with the best cellulase activities were *Trichoderma longibrachiatum* 1B (Exo and endoglucanase), *Aspergillus fumigatus* 5A (Exo- and endoglucanase and β-glucosidase) and *Aspergillus flavus* 2A (Exo and endoglucanase). *A. fumigatus* are known producers of endoglucosidase, exoglucosidase and β-glucosidase (Srivastava *et al*., 2015). *Trichoderma* strains can produce a high amount of endo and exo-glucanase activities including *T. longibrachiatum* but do not excrete a sufficient amount of β-glucosidase like the *Aspergillus* species (Oberoi *et al*., 2012; Priyanka *et al*., 2017).

The present study showed that *A. fumigatus* 5A produced a higher endoglucosidase (0.436 U/ml after 144 h) and β-glucosidase (0.139 U/ml after 120 h) activities when compared to the study done by Neseeb *et al*. (2015) where 0.025 U/ml were noted for endoglucosidase and 0.044 U/ml for β-glucosidase by *A. fumigatus*. Sarkar,

(2014) however, reported *A. fumigatus* with a higher endoglucosidase activity (2.31 U/ml) compared to the current study. However, the exoglucosidase activity measured in the present study was higher than the 0.261 U/ml obtained by Sarkar, (2014). Dutt and Kumar (2012) also reported *A. flavus* with a higher endoglucosidase (5.1 U/ml) and the least exoglucosidase (0.5 FPA/ml) activities compared to the current study. The lower β-glucosidase observed in this study for *T. longibrachiatum* is commonly associated with *Trichoderma* species (Stenberg *et al*., 1997, Ryu and Mandels 1980).

5.2. Co-culture of fungal strains with high cellulase activity

In nature, microbial communities live in co-existence to utilise complex substrates. Co-cultivation of microbes appears to be very beneficial because of their synergistic expression of metabolic pathways of all microorganisms involved in the co-culture (Chimtong *et al*., 2014). The co-culture of *A. fumigatus* 5A with *T. longibrachiatum* 1B and *A. flavus* 2A (5A+1B+2A) produced the highest enzyme activity for endoglucanase, but not when compared to the monocultures. The combination of *A. fumigatus* 5A and *T. longibrachiatum* 1B produced a 4.138 fold increase in β-glucosidase activity (4.317 U/ml after 72 h) when compared with the monocultures. The present study is in agreement with the study done by Hernandez, (2018) where the co-culture between *A. niger* and *Fusarium oxysporum* increased the production of β-glucosidase with respect to *A. niger* monocultures. Similarly, Hu *et al.* (2011) reported that the highest β-galactosidase, β-glucosidase, α-cellobiohydrolase, and laccase activities were found for *A. oryzae* during co-culture with other fungal strains, specifically with *Phanerochaete chrysosporium* and the highest β-xylosidase activity was achieved when *A. niger* was co-cultivated with *P. chrysosporium* on wheat bran. This is the first report where these combinations were used where β-glucosidase activity increased when these combinations were used.

5.3. Bacterial strains with their co-cultures for cellulase production

Bacteria can also synthesize cellulose, with higher purity and hydrophilicity than plant cellulose (Menendez *et al.,* 2015). The *Escherichia coli* are a Gram-negative bacterium. The various chemical products with a variety of sugars as carbon sources during industrial production have been produced by using this strain as a host. However, endogenous cellulose degradation enzymes nor secrets heterologous cellulase are produced by *Escherichia coli* for its poor secretory capacity (Gao *et al.,* 2015). The study by Pang *et al*. (2017) reported on the first *E. coli* strain isolated on bovine rumen to produce endoglucosidase, exoglucosidase and beta-glucanases. The current study also used *Enteroaggressive Escherichia coli* East-1 (EAEC) to see if a pathogenic *E. coli* strain known to produce enterotoxins and cytotoxins could cause other bacterial strains in combination to produce secondary metabolites, which could be produced during their stressed environments. This is the first report on this strain. These combinations were done to improve cellulase activity. *Escherichia coli* East-1 produced a maximum endoglucosidase activity of 0.253 U/ml (after 96 h) and β-glucosidase activity of 1.396 U/ml (after 72 h) (Fig 4.4). This is the first study to use *Escherichia coli* East-1 for cellulase production and is a good producer of endoglucosidase and β-glucosidase. This study further co-cultured *E. coli* with *Enterobacter asburiae* 1 and *Enterobacter cloacae* 4 (*E. coli* + 1+4), with an increase in both endoglucosidase activities (0.179 U/ml after 120 h) and β-glucosidase activity (1.01 U/ml after 24 h) shown in Fig 4.3. However, *E. asburiae* and *E. cloacae* (1+4) produced the highest β-glucosidase and *E. coli* East-1 the most endoglucosidase activities.

This study was the first report of a co-culture of *Enterobacter asburiae* 1 and *E. cloacae* 4. *E. asburiae* is a new species in the *Enterobacter* genus that was formally the Enteric Group 17. *E. asburiae* strains were isolated from a variety of clinical and environmental specimens and it is known as an opportunistic pathogen (Mardaneh and Dallal, 2016). No studies were done on *E. asburiae* as cellulolytic bacteria and limited information is available on *E. cloacae*. In the study done by Lokapirnasari *et al.* (2015) *E. cloacae* were grown on Luria Bertani (LB) and produced endoglucosidase (0.09 U/ml) and β-glucosidase (0.10 U/ml) with similar results obtained during this study with Avicel as carbon source. The co-culture of *E. asburiae* 1 with *E. cloacae* 4 produced the highest β-glucosidase activity (1.459 U/ml

after 48 h) for the bacterial strains. This is also the first report where the co-culture of these strains improved β-glucosidase activity.

5.4. Mixed culture of fungal strains and bacterial strains

Mixed cultures of *A. fumigatus* 5A with *T. longibrachiatum* 1B and *A. flavus* 2A with *E. asburiae* 1 or *E. cloacae* 4 were investigated for an improvement in cellulase activity. There was an increase in β-glucosidase activity (1.82 U/ml) when the three fungal strains were used with *E. asburiae* 1 (Fig 4.5c). No literature on mixed cultures of bacteria and fungi with β-glucosidase activities was found. This is the first report. Few studies are done on mixed cultures of bacteria and fungi. A study done by Kato *et al*. (2004) reported improvement of cellulose degradation efficiency by mixed culture of *Clostridium thernocellium* and *Methanobacterium thermoautophicum* compared to the monocultures of these strains. The fungal–bacterial combination may lead to microorganisms competing for the same substrates. Due to the stressed environment, bacteria may produce endotoxins, which may cause fungi to produce secondary metabolites; hence, cellulase activity is improved (Johnston *et al.,* 2016).

Interestingly, an improvement in both endoglucanase and β-glucosidase activities (1.79 U/ml after 48 h and 37.45 U/ml after 144 h, respectively) were noted when the three fungal strains were used in combination with dead cells of *E. asburiae* 1 (Fig 4.5). Fungi are known to decompose dead organic matter. They obtain nutrients from dead plants and animal matter, by absorbing soluble organic compounds. The presence of the dead bacterial cells could have served as a nutrient source for the fungi, hence, increasing the cellulase activity (Takano *et al*., 2017; Carmona-Fontaine and Xavier, 2012).

5.5. Mixed culture of three fungal isolates with *Enteroaggregative Escherichia coli* (EAEC) at different masses (0.5 g, 1 g, and 2 g)

The co-culture of the three fungal strains **(5A1B2A)** with different masses of *Enteroaggresive E. coli* East-1 bacterial cells were investigated for improvement in endoglucanase and β-glucosidase activity. It was shown that an increase in dead bacterial cells decreased the endoglucosidase activity with no β-glucosidase

detected (Fig 4.6). This is the first report to investigate fungal strains mixed with dead cells of *E. coli* strains.

5.6. Production of cellulase by fungal strains in solid-state fermentation

The current study showed that *T. longibrachiatum* 1B, *A. fumigatus* 5A and *A. flavus* 2A exhibited a higher cellulase activity during solid-state fermentation (SSF)(Fig 4.7) compared to submerged fermentation (SmF)(Fig 4.2) A study was done by Saqib *et al*., (2010) also indicated an increase in enzyme activity during SSF. Kokila and Mrudula (2010) also reported that *A. niger* produced a higher endoglucosidase and exoglucosidase activity during SSF (8.89 and 3.56 U/ml) than during SmF (3.29 and 2.3 U/ml). Neseeb *et al*. (2015) also reported *A. fumigatus* during SSF produced higher endoglucosidase and β-glucosidase activities (1.26 U/ml and 1.556 U/ml, respectively) than SmF (0.025 U/ml and 0.044 U/ml, respectively).

More cellulase activity was produced in SSF because the natural habitat of most filamentous fungi compared to SmF was mimicked. During SSF morphology of fungi is of a mycelia form with aerial and substrate penetration hyphae are produced when fungi are grown on a solid-state (Te Biesebeke *et al*., 2002). During submerged fermentation, most filamentous fungi produce spherical pellets and the difference in morphology is observed while compared to SSF, which also gives a good explanation of high production of an enzyme in SSF (Papagianni, 2004).

Trichoderma longibrachiatum 1B was the best producer of both endoglucosidase (6.7 U/ml after 72 h) and β-glucosidase (37 U/ml after 216 h). The combination of the three fungal strains did not produce more enzyme activity compared to the monocultures during SSF. This is in contrast with Rathman and Balasaravan (2014), who reported that the co-culture of *Trichoderma sp,* Aspergillus *sp* and *Penicillium sp* produced higher activities of endoglucosidase, exoglucosidase and β-glucosidase compared to the monocultures. The advantage of mixing three strains was more pronounced in SSF because the colonization of the substrate was accomplished better in symbiotic association (Rathma and Balasaravan 2014).

Neagu *et al*. (2012) indicated that solid-state fermentation had higher efficiency compared to that of submerged fermentation using wheat brain waste as a carbon source. Filamentous fungi produce more enzymes during SSF compared to SmF,

because of familiar conditions to their natural habitats. In the presence of plant polysaccharides, microorganisms like *A. niger* species are able to produce an extensive range of cellulase enzymes to efficiently degrade the biomass (Cunha *et al.,* 2012). Filamentous mycelia were predominant in the SSF, which resulted in higher endoglucosidase and β-glucosidase activities than in SmF. SSF mimics the natural environment to which fungi are adapted to and occurs in the unavailability of free water, which then causes a higher cellulase production (Kumar *et al.,* 2011). Low catabolite repression is another factor that is in favour of SSF, which appears to be limiting the production of an enzyme in SmF (Kumar *et al.,* 2011).

The present study indicated utilizing lignocellulose biomass (banana pseudostem) reduced the time for endoglucosidase production by monocultures and co-cultures during solid-state fermentation compared to the results obtained during submerged fermentation (Fig 4.2 – 4.7). During SSF, most fungi are able to produce mycelia and an invasive growth is observed; hence, in SmF due to more availability of water, they are unable to attach their mycelia properly on the carbon source (Yu *et al.,* 2014).

5.7. Mixed culture of three fungal strains with bacterial strains in solid-state fermentation

It was observed that the combination *A. fumigatus* 5A*, T. longibrachiatum* 1B*, A. flavus* 2A with dead *Enterobacter asburiae* 1(5A1B2AD1) was the best producer of endoglucosidase activity (12.7 U/ml after 48 h) and β-glucosidase activity (37.5 U/ml after 144 h) (Fig 4.8). This is the first study to report on a mixed culture of live cells with dead cells. Lah *et al*., 2012 reported that cellulase activity determined in SSF with a mixed culture of bacteria and fungi was higher than in monocultures. This might be due to the possible interaction between fungi and bacteria that synergistically reduce cellulose resulting in high endoglucosidase and βglucosidase activities (Ashish & Deepak, 2005).

5.8. Endoglucosidase and β-glucosidase zymography

The compositions of protein in crude enzyme were analysed by zymography. β-glucosidases were studied using CMC and Esculin as substrate. Endoglucanases and β-glucosidases bound to Avicel were produced by these crude extracts. The monocultures and co-cultures produced four active bands for endoglucanase, except for the monoculture *T. longibrachiatum* 1B that produced a faint or unclear band. A faint band may show that the enzyme did not bind to the Avicel or degradation occurred.

A study was done by Delabona *et al*. (2012) on *A. fumigatus* showed 3 bands for endoglucosidase and Soni *et al*. (2010) reported four bands in the presence of corn cob. Grigorevski-Lima *et al*. (2009) showed 6 endoglucosidase bands in the presence of sugarcane as a carbon source. The differential expression observed of cellulase can be endorsed to structural heterogeneity of the cellulosic substrate during cultivation as well as the variances in culture conditions (Saqib *et al*., 2010). The several forms (bands) of endoglucosidase have also been described in other *Aspergillus sp* (Larrondo *et al*., 2003; Liu *et al*., 2003).

T longibrachiatum 1B and *A. fumigatus* 5A produced two β-glucosidases one with 40 kDa and the other with 60 kDa (Fig 4.10). Theoretically, β-glucosidases molecular weight is between 75 and 117 kDa, in that order (Chauve *et al.,* 2010). Similarly, a study was done by Pachauri *et al.* (2017) also showed a novel isolate of *Trichoderma longibrachiatum* with the molecular mass of the enzyme to be 67 ± 1 kDa. In contrast, Kumar *et al*. (2016) reported one band for β-glucosidase in *A. flavus.* All the other monocultures and co-cultures produced only one 60 kDa band for β-glucosidase. This could be due to the degradation or partially production of the enzyme due to a synergetic effect within the mixtures.

CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1. Conclusions

The current study demonstrated that three fungal strains *T longibrachiatum* 1B, A. fumigatus 5A, A. flavus 2A, and one bacterial strain *Enterobacter asburiae* 1 are capable of producing a variety of endoglucanases. It seems that a combination of fungi with dead cells could significantly improve endoglucosidase and β-glucosidases activities. The use of *A. fumigatus in* mixed cultures is highly recommended in order to produce high amounts of β-glucosidases, regardless of the combination used. It also shows that SSF which mimic the natural decomposition of lignocellulose was the best condition to use in order to achieve a higher cellulase activity using co-culture/ mixed culture because in nature sufficient saccharification of lignocellulose is done by a variety of microorganisms (fungi, bacteria and dead cells of these microbes); hence increase cellulase activity.

6.2. Recommendation

- ✓ Further studies on co-culture of fungi on other substrates could improve the production of cellulolytic enzymes.
- ✓ The use of fungal co-cultures with bacteria should be investigated further. This could improve the production of cellulolytic enzymes on various lignocellulolytic waste materials.
- ✓ More studies should be done on co-cultures and mixed cultures for zymography analysis.

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