PRODUCTION OF ENZYMES FROM UNDERUTILISED CROPS USING BIOLOGICAL PRETREATMENT

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ABSTRACT

A rapidly growing demand for food, feed, fuel and fibre has put a strain on vital resources, leading to increased concern of energy security. This, along with threats of global warming has attracted research into renewable energy sources and the development of new technologies for biofuel and biochemical production. The use of lignocellulosic material for the production of sustainable and cost-effective value-added products could offer a solution, with underutilised crops playing an important role as the raw material used in the biorefining process. However, enzymatic hydrolysis of the lignocellulosic material is one of the major barriers to an economically viable process, preventing its widespread application.

The aim of this research was to investigate the feasibility of using solid state fermentation (SSF) of underutilised crops for the generation of cellulase and glucoamylase enzymes and fermentable sugars, providing the basis for a biorefining process for converting the crops to bioethanol and/or biochemicals.

Several underutilised crops were investigated – Bambara, Leucaena, Napier grass, Nipa palm, Oil palm fronds, and Sago hampas. Characterisation of the crops was performed to determine their basic composition. The crops were screened to investigate which crop(s) had the highest potential as a substrate for fungal cellulase production during SSF and submerged fermentation (SmF). Two fungi, *Aspergillus niger* and *Trichoderma reesei,* were used, exploring different fermentation conditions to optimise the process. The use of *A. niger* during SSF resulted in the highest cellulase activity overall. Under baseline conditions (addition of deionised water to 80% (w/v) moisture content (MC)), the cellulase activity after five days of incubation ranged from 1.08 ± 0.06 FPU/g to 17.17 ± 0.44 FPU/g, with the use of Napier resulting in the highest

activity. This activity was increased significantly with the addition of starch (0.0070 g/g), yeast extract (YE, 0.0175 g/g) and minerals. When these nutrients were added to Napier, the cellulase activity increased to as high as 31.02 ± 1.01 FPU/g.

Since Sago hampas contained over 50% (w/w) starch, it was also investigated as a substrate for the production of fungal glucoamylases and fermentable sugars, using the fungus *A. awamori*. Glucoamylases could not be detected in SSF recovered fungal filtrate, although glucose was being produced. A SSF with washing cycles was designed to recover the glucose, examining several parameters including nutrients added, length between washing cycle and washing solution used. The highest glucose was obtained from a daily washing cycle, with the use of 40.0 x 10^6 spores/g and the addition of YE (0.0175 g/g) and minerals to 80% (w/v) MC to the Sago hampas. This resulted in the conversion of 46.53 % of the available starch into glucose after six days of incubation. This was compared with the initial continuous SSF over 21 days of incubation which gave 10.11% conversion of starch.

The processes explored in this work could enable the creation of novel biorefining processes, using on-site produced cellulase enzymes to hydrolyse underutilised crops to a sugar-rich hydrolysate. This, as well as the sugar-rich filtrate produced with the Sago hampas, could then be used in the production of biofuels and/or biochemicals.

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LIST OF ABBREVIATIONS AND SYMBOLS

α	Alpha
AD	Anaerobic digestion
A. awamori	*Aspergillus awamori*
A. niger	*Aspergillus niger*
β	Beta
CFF	Crops for the Future
CO_2e	Carbon dioxide equivalent
CBH-I	Cellobiohydrolase I
CBH-II	Cellobiohydrolase II
DP	Degree of polymerisation
DNS	3,5-dinitrosalicylic acid
FAO	Food and Agricultural Organisation of the United Nations
FE	Fungal extract
GHG	Greenhouse gases
GOPOD	Glucose oxidase peroxidase
HGA	Homogalacturonan
HMF	5-hydroxymethyl furfural
HPLC	High performance liquid chromatography
LiP	Lignin peroxidases
MSI	Mineral solution I
MSII	Mineral solution II
MC	Moisture content
RG-I	Rhamnogalacturonan I
RG-II	Rhamnogalacturonan II
rpm	Revolutions per minute
SD	Standard deviation
SmF	Submerged fermentation
spores/g	Fungal spore concentration per gram substrate
SSF	Solid state fermentation
T. reesei	*Trichoderma reesei*
VP	Versatile peroxidases
YE	Yeast extract

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CHAPTER 1 INTRODUCTION

1.1 ENERGY CRISIS AND ENVIRONMENTAL CONCERNS

It is reported that the global population is projected to increase from around 7 billion people today to just over 9 billion people by the year 2050 (United Nations, 2015). This population growth, as well as the expansion of the industrial sector and changes in people's lifestyle, has resulted in a rapidly growing demand for food, feed, fuel and fibre. Global primary energy consumption continues to rise, with consumption reaching 13,148 million tonnes oil equivalent in 2015, an increase of 1% from 2014 data (BPstats, 2016).

Much of this energy is derived from fossil fuels (petroleum, coal and natural gas), which provided 86.0% of total energy consumption in 2015 when split by fuel source (BPstats, 2016). Conversion of fossil fuels results in the emission of greenhouse gases (GHG), such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), into the atmosphere, leading to climate changes. In order to reduce the negative effects from GHG emissions, the Committee on Climate Change (2008) has estimated that we need to reduce global GHG emissions by 50% by 2050; this is equivalent to "reductions of global GHG emissions between 20-24 billion tonnes CO_2 equivalent (CO_2e) in 2050, with further reductions to 8-10 billion tonnes by 2100". Furthermore, energy security due to threats of potential energy shortages is also a major concern for many countries.

In order to continue to meet these growing energy demands, yet reduce GHG emissions, research into the use of renewable energy sources and the development of new biosynthetic technologies that will help reduce dependence on fossil fuels is key. Although there was an increase of 15% over 2014 data, currently less than 10% of global primary energy

consumed comes from renewable energy, with only 2.8% coming from wind, solar, geothermal, biomass and waste (BPstats, 2016). Nevertheless, biomass has the potential to contribute to future energy, as it can be used for the production of bioenergy, which can be harnessed in several ways, such as in the production of associated co-products like feed and chemicals or as a fuel in the transport sector (Food and Agriculture Organisation of the United Nations (FAO), 2008a; FAO, 2008b).

1.2 PROBLEMS ASSOCIATED WITH BIOMASS CONVERSION

When talking about the use of biomass for bioenergy production, this is often used to mean biomass from plant material (Biomass Energy Centre, 2011; Crops for the Future (CFF) Research, 2011a). Although the use of biomass for the commercial production of first generation biofuels is prevalent, most of the biomass used comes from commercially grown food crops. Currently, agricultural production relies on fewer and fewer crops. Throughout history, 7000 species have been used in agriculture; however, today only 120 crop species contribute 90% of total calories consumed (CFF Research, 2011a).

There are many crops and plants that are underutilised which could play an important role in the production of sustainable and cost-effective renewable energy, both economically and environmentally. Although these underutilised crops are often overlooked they could provide new opportunities, in terms of food for increasing populations, as well as being used for the production of biofuels and biochemicals. Since these crops usually grow in hostile environments they can be grown in areas where large-scale commercial crops do not perform well. Alternately, they can be grown alongside some monocultures by intercropping, helping to reduce soil erosion and degradation, improving sustainability, and creating alternate sources of income to reduce agricultural risk. Also, such areas

are often inhabited by poorer communities or these crops are already subsistently farmed by such communities. Therefore, the development of these crops will benefit local communities by developing local industries and create an income for poorer people.

However, even if underutilised crops were to be used in the production of biofuels and biochemicals, the use of non-food crops or the waste from food crops would need to be used in order to avoid the 'food versus fuel' conflict. This poses a challenge as the plant biomass from non-food crops and waste from food crops typically consists mostly of lignocellulosic material which cannot easily be processed. Much research has been performed using lignocellulosic material to produce bioenergy; however, the production cost is a large hurdle to overcome (Bon & Ferrara, 2007; Sarkar *et al*., 2012). This high cost of production is partly due to the low efficacy on the pretreatment step and the lack of cost-effective enzymes used to hydrolyse the pretreated biomass into usable sugars (Kumar *et al*., 2009).

There are several different types of pretreatment methods, including physical, chemical, physiochemical, and biological, or a combination of these methods (Hendricks & Zeeman, 2009; Talebnia *et al*., 2010). Recently biological pretreatment has received attention as it has low operational costs and low energy inputs, due to the use of low temperatures, pressure, water and chemicals; and it also has a wide adaptability on various biomass materials (Dashtban *et al*., 2009). However, biological conversion processes are not yet well established and further research needs to be performed to make it commercially viable. Currently, the operation time is long, the enzyme productivity is low and the impact of this step on following hydrolysis and fermentation steps is still unclear (Dashtban *et al*., 2009).

1.3 AIMS AND OBJECTIVES OF THE THESIS

This project aimed to explore the potential for a biorefining process for the production of a fermentable sugar solution that could be used for the synthesis of value added products (biofuels and biochemicals) from lignocellulosic material, using underutilised crops as the biomass source. Several crops were used in this project, including Bambara, Leucaena, Napier, Nipa palm, Oil palm and Sago palm.

The main focus of the project was to investigate the possibility of using a short-term biological process as an efficient way of producing fungal hydrolytic enzymes (cellulases and glucoamylases), which could then be used onsite for the production of fermentable sugars to be used for biofuel or biochemical production. In order to do this, soft-rot fungi were applied to the underutilised crops during solid state fermentation (SSF) and submerged fermentation (SmF). Since the enzymes needed for hydrolysis during biofuel or biochemical production are costly and need to be replaced often, on-site production of enzymes via a biological process could significantly reduce costs and therefore improve the economic viability of biofuel or biochemical production from non-food crop and food crop waste sources.

Within this framework, the objectives of this work were as follows:

1. Characterisation of underutilised crops –
    - To perform compositional analysis of selected underutilised crops.
2. Production of fungal cellulases –
    - To screen and identify the most suitable underutilised crops to be used as substrates for the production of fungal cellulases via solid state fermentation (SSF) and submerged fermentation (SmF), using soft-rot fungi.

- To optimise SSF and SmF conditions for maximum cellulase production, using the crop that showed the greatest potential.

3. Production of fungal glucoamylases and sugar-rich solution –
    - To investigate the potential of the use of Sago hampas (as it contained over 50% (w/w) starch) as a substrate for the production of fungal glucoamylases via SSF and SmF, using soft-rot fungi.
    - To propose a novel biological pretreatment to produce a fermentable sugar-rich solution from Sago hampas hydrolysis via SSF with washing process.
    - To optimise the conditions for glucose production during SSF with washing of Sago hampas.

1.4 STRUCTURE OF THE THESIS

Chapter 1 gives an overview of the project, an explanation of the current problems and some suggestions for solutions to these problems. This is followed by a general literature review in Chapter 2, and a description of the materials and methods used within this research is given in Chapter 3.

The work presented in Chapter 4 shows analysis of the composition of the underutilised crops. Contents analysed included moisture, ash, lignin, protein, lipid, starch, cellulose and hemicellulose content, and elemental analysis.

In Chapter 5, the underutilised crops were used as a biomass substrate for the production of fungal cellulases via solid state fermentation, using soft-rot fungi *Aspergillus niger* and *Trichoderma reesei*. The crops were first screened to determine which crop(s) showed the most potential as a substrate. Several SSF conditions were then optimised using the crops which showed the most potential. Conditions investigated included fungus

used, addition of starch, addition of nitrogen source and/or minerals, incubation period, and culturing of microorganisms on different feed stocks. All the underutilised crops were then screened again under the optimised conditions to determine the effect on cellulase production. The production of fungal cellulases via submerged fermentation was also investigated.

In Chapter 6, Sago hampas was used as a substrate for the production of fungal glucoamylases and glucose via solid state fermentation using the soft-rot fungus, *A. awamori*. Optimisation of conditions for increased production were investigated, including addition of nutrients, culturing of microorganisms on different feed stocks, washing techniques, incubation period, and extraction techniques. The production of fungal glucoamylases via submerged fermentation was also investigated.

A general discussion, along with conclusions of this work and suggestions for future studies are presented in Chapter 7.

CHAPTER 2 LITERATURE REVIEW

2.1 PLANT CELL WALL POLYMERS

The majority of plant biomass constitutes lignocellulosic material, which makes up the plant cell walls. Plant cell walls are important features of plant cells that perform a number of essential functions for the plant. They provide structure and shape to the many different cells types of the plant. Plant cell walls also allow plants to withstand large 'pulling' and 'pushing' forces, for example, the plant moving in the wind, whilst providing sufficient support to withstand the forces of gravity (Burton *et al*., 2010). Plant cell walls play key roles in growth regulation, differentiation of cells, movement of water and nutrients through the plant cells, intercellular adhesion and communication and plant defences (Cosgrove, 2005). As an alternative to starch, plant cell wall polysaccharides can also be a source of metabolisable energy for some plants. For example, galactomannans (hemicellulose) in fenugreek seeds, xyloglucans (hemicellulose) in tamarind seeds and pectins in lupin seeds (Burton *et al*., 2010).

The plant cell wall is able to provide these various functional requirements due to its composition. The primary cell walls of higher plants are composed of mainly carbohydrates (up to 90% of the dry weight) and the remainder as structural glycoproteins (2 – 10%), esters (less than 2%), minerals and enzymes (O'Neill & York, 2003). The carbohydrate portion is made up of cellulose fibrils that are embedded in a gel-like matrix of other complex polysaccharides. These polysaccharides can be divided into two classes – hemicelluloses and pectins. Plant cell walls also contain many proteins and glycoproteins (Keegstra, 2010). Hemicelluloses bind to the cellulose which, together with cellulose, forms a robust network that gives structural support to the plant. Pectins are probably the most complex polysaccharides and have several functions. Pectins form hydrated gels

that form part of the gel-like matrix of the cell wall that allows for deposition and extension of the cellulose-hemicellulose network (Willats *et al*., 2001). They are involved in the control of the porosity and thickness of the cell wall, as well as acting as a "glue" to hold cells together by forming an adhesive layer known as the middle lamella. Pectins are also very important in plant-defence responses, as pectins bound by receptor-like kinases can initiate a range of cellular changes in response to a pathogen attack (Cosgrove, 2005; Cantu *et al*., 2008).

As the cells of higher plants stop growing, their walls thicken as they take on a structural role, 'locking' the cell into a final shape. The cellulose fibrils become laminated and lignin, a large polyphenolic molecule is produced. Lignin is impermeable, restricting the passage of small molecules and gives structural support by making the wall resistant to compressive ('pushing') forces (Burton *et al*., 2010).

There are two general types of plant cell walls based on two things - the structure and amounts of hemicelluloses, and the relative amounts of pectins present in the wall. Cellulose accounts for about 30% of plant primary cell walls in both types. Type I walls contain around 20-30% hemicelluloses (typically xyloglucan and/or glucomannan) and 20-30% pectin. Type II walls contain around 40-50% hemicelluloses (typically arabinoxylan) and around 10% pectin. Type I walls are found in all gymnosperms, dicotyledons and non-graminaceous monocotyledons and type II walls are found in the Poales order (Poaceae family) (eg. barley and rice) (Carpita, 1996; Nishiyama, 2009; Scheller & Ulvskov, 2010; Vanholme *et al*., 2010).

2.1.1 Cellulose

Cellulose is a common component found in most plants, several marine animals, and to a lesser extent in bacteria, fungi, algae and invertebrates. Cellulose is one of the most abundant materials, and is the most common renewable organic polymer produced on earth (Poletto *et al*., 2013). In general, cellulose is a tough, water-insoluble, fibrous substance that plays an important part in plant cell wall structure (Habibi *et al*., 2010). Cellulose is insoluble in water and most organic solvents; however, it can be chemically broken down into glucose by concentrated acids and high temperature (Tabet & Aziz, 2013).

Cellulose is an unbranched homopolysaccharide of anhydro-β-D-glucose molecules with a high molecular weight. These anhydro-β-D-glucose rings are covalently bonded between the C4 of one glucose ring and the oxygen bonded to C1 of the adjacent glucose ring (Figure 2.1). This bond is known as a β-1,4 glucosidic bond. Due to the angle of the bond, each glucose molecule added to the chain is rotated 180°, forming a long straight chain (Moon *et al*., 2011; Tabet & Aziz, 2013). The repeat unit of cellulose is cellobiose – a dimer of two anhydro-D-glucose rings, with a formula $(C_6H_{10}O_5)_n$. The overall degree of polymerisation, *n*, is dependent on the source material of the cellulose, and can range up to 20,000 (Collinson & Thielemans, 2010; Moon *et al*., 2011). Each chain has directional chemical asymmetry as it has a non-reducing functional group (hydroxyl group) at one end of the chain and a reducing functional group (hemiacetal unit) at the other end, as shown in Figure 2.1 (Habibi *et al*., 2010).

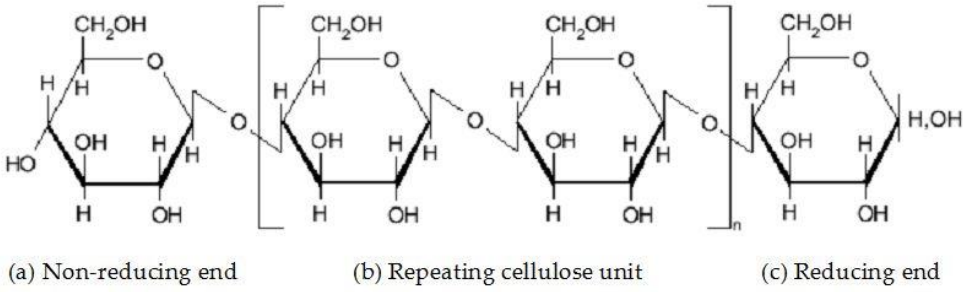


Figure 2.1 Schematic diagram of cellulose (Cave & Walker , 1994).

The hydroxyl groups of each glucose ring are able to form hydrogen bonds with the oxygens of the adjacent glucose rings (intra-chain hydrogen bonding), as shown by the dotted lines in the diagram on the right in Figure 2.2. These interactions stabilise the β-1,4 glucosidic linkages and results in the linear configuration of the cellulose chains (Moon *et al*., 2011).

Cellulose is synthesized and assembled in the plasma membrane within a rosette-shaped plasma membrane complex having a diameter of 30 nm. Once the cellulose polymers have been synthesized, approximately 36 molecules are brought together to form elementary fibrils (Habibi *et al*., 2010). Many non-covalent complexes – formed through hydrophobic interactions (Van der Waals) and intermolecular (inter-chain) hydrogen bonds between the hydroxyl groups and oxygens of adjacent molecules – promote the stacking of multiple cellulose chains to form the fibrils (diagram on left in Figure 2.2) (Moon *et al*., 2011). These elementary fibrils can then aggregate into larger microfibrils, and then further into macroscopic cellulose fibres (Habibi *et al*., 2010). The larger microfibrils range between 5 – 50 nm in diameter and several micrometres in length (Moon *et al*., 2011). The intra- and inter-chain hydrogen bonding network results in cellulose being a relatively stable polymer, with high axial stiffness that is the main reinforcement phase in plants (Poletto *et al*., 2013).

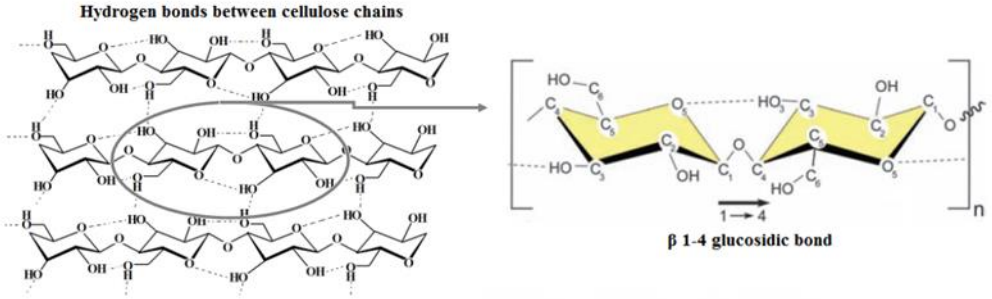


Figure 2.2 Molecular structure of several cellulose chains, showing the inter-chain and intra-chain hydrogen bonding (dotted lines) (Left) and the molecular structure of a cellulose repeating unit, showing the β-1,4 glucosidic linkage and intra-chain hydrogen bonding (dotted lines) (Right) (Poletto *et al*., 2013).

Within the cellulose fibrils there are two different regions – crystalline and amorphous regions – and the transition between these regions is gradual. The crystalline regions, which make up about two-thirds of the cellulose in the cell wall, have a highly ordered structure and are strongly connected by hydrogen bonding. The amorphous regions, however, have no definite arrangement (Tabet & Aziz, 2013). This crystalline alignment, corresponding to the location of hydrogen bonds between and within strands, results in four different allomorphs for cellulose – cellulose I (I_α and I_β), cellulose II, cellulose III (III_I and III_{II}) and cellulose IV (IV_I and IV_{II}) (Collinson & Thielemans, 2010).

Although the fundamental chemical structure of cellulose is almost identical between plants, there is a lot of variation in the degree of polymerisation, molecular orientation of cellulose, the hydrogen-bonding network and also in the degree of crystallinity. These factors result in different properties of the cellulose and will ultimately have an influence on the digestibility of the cellulose (Burton *et al*., 2010; Habibi *et al*., 2010).

2.1.2 Hemicellulose

Similar to cellulose, hemicellulose is primarily found in plant cell walls. It is a complex carbohydrate that interacts with the cellulose polymers, serving

as a connection between the cellulose and the lignin and therefore providing rigidity to the plant cell wall (Nishiyama, 2009; Scheller & Ulvskov, 2010). The hemicelluloses can form hydrogen bonds with the surface of the cellulose fibrils, they can penetrate the fibril disrupting the crystalline shape, or they can anchor the cellulose fibrils in the plant cell wall by spanning between cellulose fibrils and locking them in (Burton *et al*., 2010). Hemicelluloses are not soluble in hot water or chelating agents, but are soluble in aqueous alkali (O'Neill & York, 2003; Tabet & Aziz, 2013).

All hemicelluloses are synthesized in the Golgi apparatus and then deposited to the cell wall surface by vesicles (Cosgrove, 2005). Hemicelluloses are quite small macromolecules compared to cellulose as they are usually made of only 150 – 200 monomer units (Tabet & Aziz, 2013). Unlike cellulose, hemicellulose is a heteropolymeric compound as it is made up of different building blocks and often has many branched side chains, making it unable to form a crystalline structure. As a result, it is easily broken down by using either chemicals or enzymes.

The building blocks used to make hemicelluloses include pentose sugars, hexose sugars and sugar acids. Pentose sugars include D-xylose, L-arabinose, L-fucose and L-rhamnose; hexose sugars include D-glucose D-galactose, and D-mannose; and sugar acids include D-glucuronic acid, D-galacturonic acid, and 4-O-methyl-glucuronic acid (O'Neill & York, 2003; Guimarães, 2012). These sugars are combined in different sequences to form many different hemicellulose structures, as exemplified in Figure 2.3.

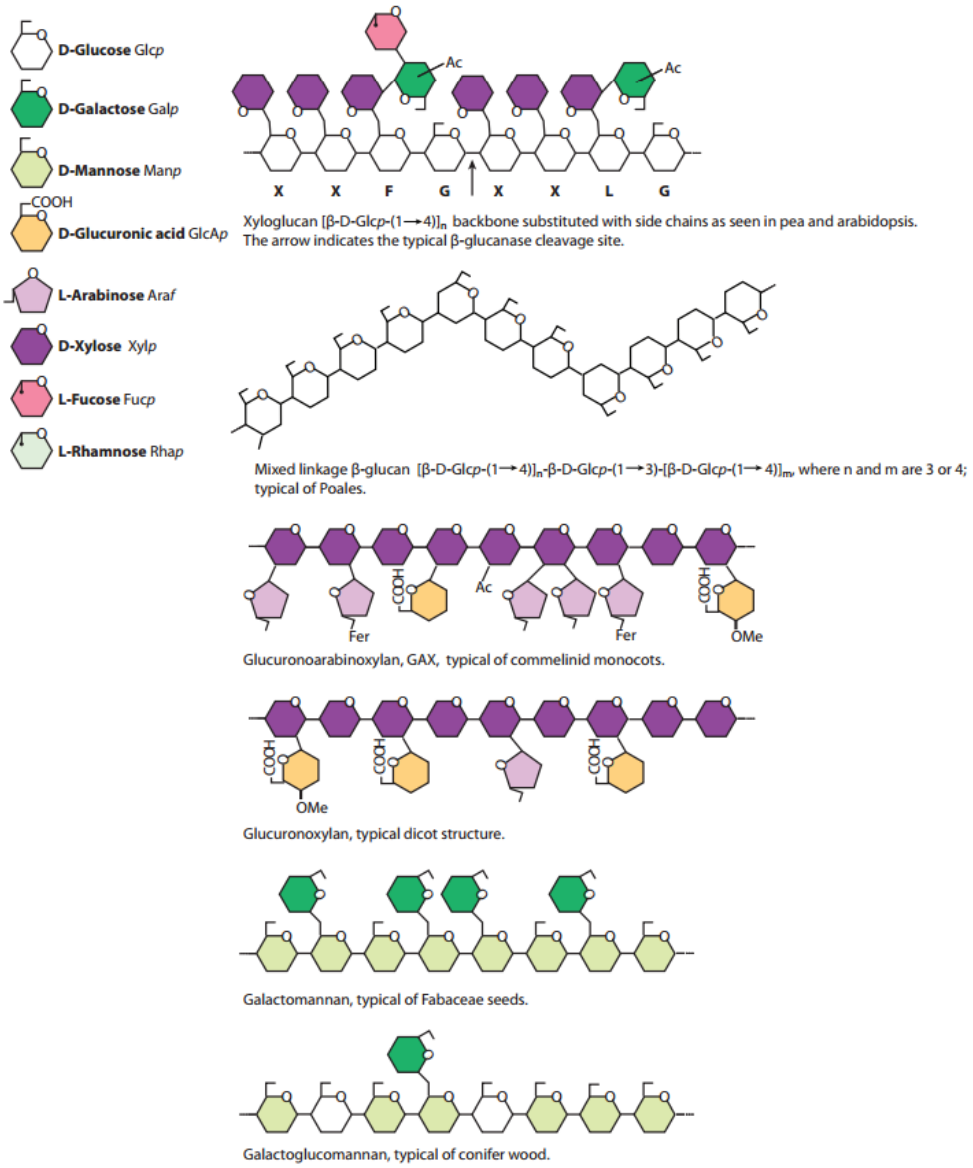


Figure 2.3 Schematic diagram of different types of hemicelluloses (Scheller & Ulvskov, 2010).

Hemicelluloses include arabinogalactan, xyloglucan, xylans (arabinoxylan, glucuronoarabinoxylan, glucuronoxylan), and mannans (galactomannan, galactoglucomannan, glucomannan). All hemicelluloses (except arabinogalactan) are structurally similar to cellulose as they have a backbone of (1,4)-linked β-D-pyranosyl residues such as xylose, glucose and mannose (O'Neill & York, 2003).

Xyloglucan and arabinoxylan are two of the most abundant hemicelluloses. Xyloglucan polysaccharides, which have a β-(1,4)-linked glucose backbone

with xylose linked side chains on 3 out of 4 glucose residues, are predominant in type I plants. The major hemicelluloses found in type II plants are xylans, which are comprised of a β-(1,4)-linked β-D-xylose backbone. Pure xylans are rare, and most have a xylose backbone with a number of side chains coming off it, particularly arabinose – forming arabinoxylans. Other examples of hemicelluloses are galactomannans and mixed-linked glucans, both of which are high in type 2 plants (Cosgrove, 2005; Caffall & Mohnen, 2009; Burton *et al*., 2010).

2.1.3 Pectins

Pectin is a structurally complex polysaccharide which encompasses a range of polysaccharides rich in galacturonic acid. These polysaccharides are a major component of primary cell walls of all land plants and perform several roles within and between cells (Willats *et al*., 2001). As a group they contribute to the mechanical strength and stiffness of the cell wall, and help with adhesion and porosity of the plant. They also help in many plant processes such as intercellular signalling (Caffall & Mohnen, 2009; Burton *et al*., 2010).

Pectic polysaccharides account for about one-third of all primary cell wall macromolecules of type I plants (dicotyledons) and nongraminaceous monocotyledons; however, it only makes up about 10% of plant walls of the order Poales (family Poaceae) and related orders (Leonetti *et al*., 2007; Caffall & Mohnen, 2009). Pectin is the only plant polysaccharide that is mostly restricted to primary cell walls, and its presence is greatly reduced or even absent in the secondary cell walls of plants (Willats *et al*., 2001).

Pectins are synthesized in the Golgi apparatus and are deposited to the cell wall surface by vesicles (Cosgrove, 2005). Three pectic polysaccharide

groups have been isolated from primary cells walls – homogalacturonan (HGA), substituted galacturonans and rhamnogalacturonans (O'Neill & York, 2003). HGA, rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) are thought to occur in all primary cell walls (Willats *et al*., 2001) and a simplified diagram of their composition is shown in Figure 2.4. HGA and RG-I occur in larger amounts in the cell wall matrix, with RG-II, as well as xylogalacturonan, arabinan, arabinogalactan I, being present in smaller amounts (Cosgrove, 2005).

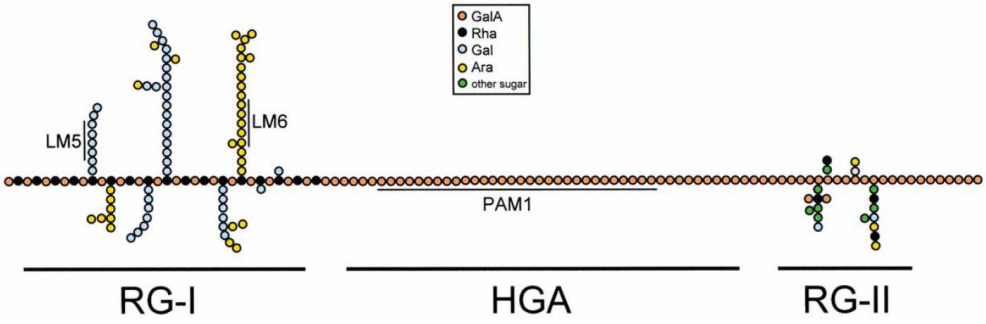


Figure 2.4 Simplified schematic diagram of the three main pectic polysaccharides - homogalacturonan (HGA), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II) (Willats *et al*., 2001)

HGA is a linear homopolymer. It consists of long chains of 1,4-linked α-D-galacturonic acid residues, with variable amounts of the residues methyl-esterified at the C_6 carboxyl position (Leonetti *et al*., 2007). When the HGA is deposited in the cell wall matrix, the methyl ester groups are removed, freeing the carboxyl groups on the galacturonic acid residues. This allows the HGA to be cross-linked by calcium, creating a calcium-linked gel structure which helps increase the firmness of the wall (Willats *et al*., 2001).

RG-I is a heterogeneous polymer which consists of a backbone of alternating rhamnose/galacturonic acid monomers (Caffall & Mohnen, 2009; Burton *et al*., 2010). About 20-80% of the rhamnose residues are substituted with side chains of varying arrangements and lengths (anywhere from a single glycosyl residue to 50 or more). Common

features of the side chains include polymeric residues of galactose (1,4-β-D-galactosyl residues) and arabinose (1,5-α-L-arabinosyl residues) (Willats *et al*., 2001).

RG-II is a highly complex structure. It consists of long chains of 1,4-linked α-D-galacturonic acid residues (same backbone as HGA). Within a stretch of seven to nine galacturonic residues there are four well-defined side chains, made up of 12 different glycosyl residues (Caffall & Mohnen, 2009).

2.1.4 Lignin

After cellulose, lignin is the second most abundant polymer in nature and is also present in the cell wall. During development of the secondary plant cell wall, the matrix becomes lignified. This serves to strengthen the cell wall by giving it structural support. Lignin is hydrophobic and is impermeable to water. Therefore, it acts as a water sealant and plays an important part in the control of water transport through the cell wall. Lignin also helps the plant resist microbial attack by impeding the penetration of microbial enzymes (Boerjan *et al*., 2003; Collinson & Thielemans, 2010; Vanholme *et al*., 2010).

Lignin is a heterogeneous aromatic polymer that is 3-dimensional and amorphous in structure. It is formed from 4-hydroxyphenyl propanoid building blocks. These building blocks are derived from three primary precursors (monolignol monomers) – p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Boerjan *et al*., 2003; Vanholme *et al*., 2010). These precursors consist of the same aromatic ring with a three-carbon side chain, a hydroxyl group off the aromatic ring, plus two different R-groups off the aromatic ring as shown in Figure 2.5.

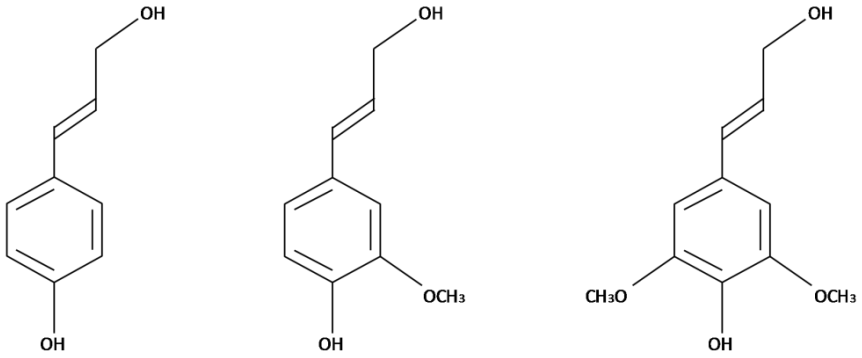


Figure 2.5 Primary precursors of lignin (from left to right): p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. Adapted from Collinson and Thielemans (2010).

In the lignin polymer, these precursors are synthesized into three types of subunits – p-hydroxyphenyl (H-type), guaiacyl (G-type), and syringyl (S-type) units. Lignins are divided into two major classes – guaiacyl lignins and guaiacyl-syringyl lignins. Guaiacyl lignins only contain p-hydroxyphenyl units (no methoxy ($-OCH_3$) groups on the aromatic ring from p-coumaryl alcohol) and guaiacyl units (one $-OCH_3$ group on the aromatic ring from coniferyl alcohol). Guaiacyl-syringyl lignins contain p-hydroxyphenyl units and guaiacyl units, as well as syringyl units (two $-OCH_3$ groups on the aromatic ring from sinapyl alcohol) (Collinson & Thielemans, 2010).

The composition of the lignin varies between species, and can also vary between tissue types within a plant. For example, most gymnosperm cell walls contain guaiacyl lignins; whereas angiosperms and herbaceous plant cell walls contain guaiacyl-syringyl lignins (Boerjan *et al*., 2003; Vanholme *et al*., 2010).

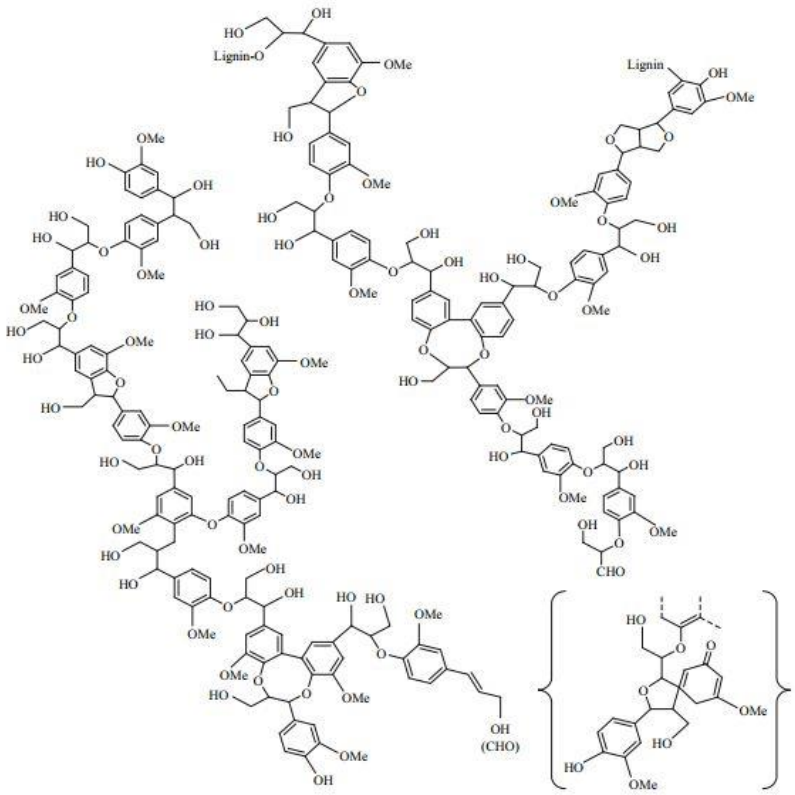


Figure 2.6 Structural model of a section of lignin (Qiu & Chen, 2006).

The dehydrogenative polymerisation of the precursors is initiated by enzymes and results in bonds with high stability. There is no single repeating bond structure between the subunits of the lignin polymer, but there is a random arrangement of at least 10 different types of bonds. Some of these bonds include "biphenyl carbon–carbon linkages between aromatic carbons, alkyl–aryl carbon–carbon linkages between an aliphatic and aromatic carbon, and hydrolysis-resistant ether linkages", with the most common bond being the β-aryl ether (β-O-4) bond (Lankinen, 2004; Collinson & Thielemans, 2010).

Lignin is very hard to digest or separate from cellulose/hemicellulose for several reasons. Lignin is a very unique compound in that it has no repeating bonds between the subunits. This makes it very hard to break down enzymatically (Lankinen, 2004). Lignin is also able to bond to the cellulose-hemicellulose network. Since cellulose is hydrophilic and lignin is

mainly hydrophobic, compatibility is achieved through hemicelluloses as they have both hydrophobic and hydrophilic regions (Collinson & Thielemans, 2010). This cross-linking strengthens the cells wall and protects the relatively easily degradable cellulose from attack; however, it makes it very difficult to access the cellulose and hemicellulose during enzymatic digestion (Guimarães, 2012).

2.2 DIGESTION OF LIGNOCELLULOSIC MATERIAL

The majority of plant biomass constitutes lignocellulosic material, which makes up the plant cell walls. Plant biomass has been used for centuries as a source of food and is used in many industries to make products, such as pulp and paper, or renewable chemicals. However, these processes often result in the accumulation of plant biomass waste, especially agro-industrial residues, and this can cause serious ecological problems. This biomass waste is often composed of lignocellulosic material.

The polymers of the cell wall can be broken down naturally by the plants themselves using various enzymes. However, these polymers can also be broken down by microbial enzymes to produce saccharides for energy. Therefore, these carbohydrate-rich materials are of economic value as they can be used for various biotechnological processes, such as biofuel production, and thus have attracted the attention of many researchers and industrial sectors.

2.2.1 Cellulases

For the complete breakdown of cellulose, an enzymatic complex (known as cellulases) is required. Cellulases are modular enzymes belonging to the broad group of glycoside hydrolases, which catalyse the hydrolysis of oligosaccharides and polysaccharides. Over 100 glycoside hydrolase families have been described. This complex consists of three enzymes –

endo-1,4-β-D-glucanases (carboxymethyl cellulases), exo-1,4-β-D-glucanases (cellobiohydrolases) and 1,4-β-glucosidases (cellobiases) (Guimarães, 2012). These three groups work together to break down cellulose, by creating new hydrolysis sites for each other, as shown in Figure 2.7. They also work on the products produced by each other so that these products do not accumulate and inhibit enzyme activity (Kumar *et al*., 2008).

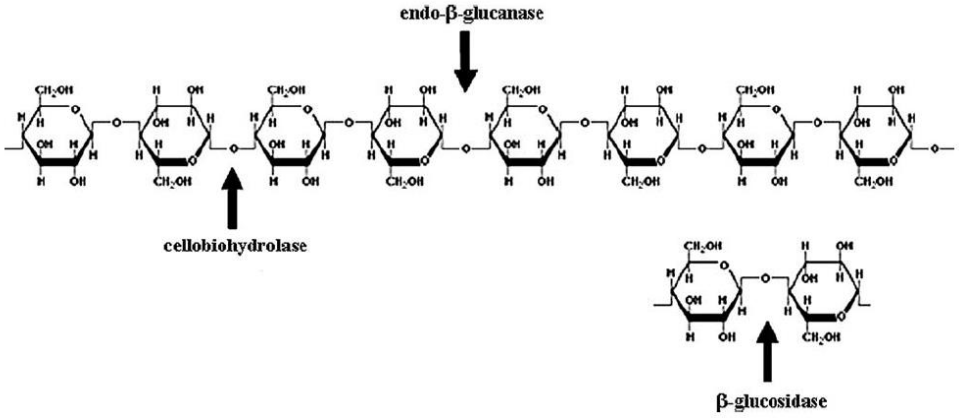


Figure 2.7 Molecular structure of cellulose and action sites for three cellulase enzymes (endoglucanase, exoglucanase and β-glucosidase) (Kumar *et al*., 2008).

2.2.1.1 Endo-1,4-β-glucanases (EC 3.2.1.4)

Endo-1,4-β-D-glucanases, also known as carboxymethyl cellulases, degrade cellulose by randomly cleaving the internal β-1,4-D-glycosidic linkages of the cellulose chain. Preferably, these enzymes attack the low crystallinity regions (amorphous regions) of the cellulose, making it more accessible for cellobiohydrolases by creating free chain ends (Xie *et al*., 2007; Kumar *et al*., 2008; Guimarães, 2012).

2.2.1.2 Exo-1,4-β-glucanases (EC 3.2.1.91)

Exo-1,4-β-D-glucanases, also known as (CBH), attack the crystalline regions of cellulose. They degrade the cellulose by hydrolysing the cellobiose units from the free chain ends created by the endoglucanases.

There are two main types of cellobiohydrolases, each one functioning on different ends of the chain – CBH-I works on the reducing end and CBH-II works on the non-reducing end. However, cellobiose, the product of this hydrolysis process, inhibits the activity of cellobiohydrolases (Xie *et al*., 2007; Kumar *et al*., 2008).

2.2.1.3 β-glucosidases (EC 3.2.1.21)

β-glucosidases, also known as cellobiases, break down cello-oligosaccharides and cellobiose (produced by exoglucanases) into D-glucose monomers (Xie *et al*., 2007; Kumar *et al*., 2008). This is an important step in reducing the inhibition of cellobiose on exoglucanases because an increase in cellobiose inhibits the activity of exoglucanases. However, the β-glucosidases activity is in turn competitively inhibited by the production/presence of glucose (Guimarães, 2012).

2.2.2 Hemicellulases

As mentioned previously, hemicellulose is made up of different building blocks - pentose sugars, hexose sugars and sugar acids. Theses sugars are combined in different sequences to form many different hemicellulose structures. Therefore, the degradation of hemicelluloses poses a challenge in that several different enzymes are required to work synergistically in order to effectively degrade hemicellulose.

Like cellulases, hemicellulases are also members of the broad group of glycoside hydrolases and can be of the endo- or exo- types. Hemicellulases can be divided into two main types – enzymes that hydrolyse the backbone of the main-chain and enzymes that degrade the sidechain substituents or short-end products (Kumar *et al*., 2008; Mafe *et al*., 2014). The action sites of several of these enzymes are shown in Figure 2.8.

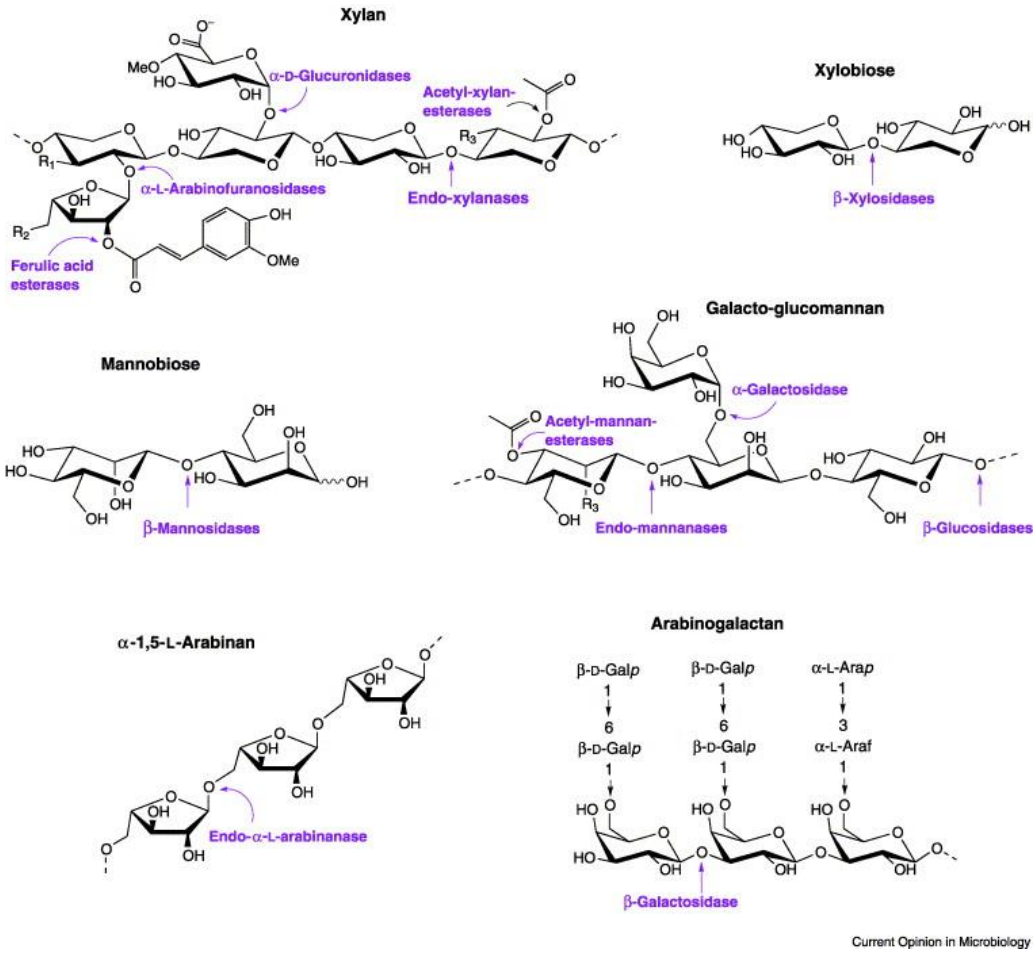


Figure 2.8 Structural components found in hemicelluloses and the action sites of some enzymes involved in their degradation (Shallom & Shoham, 2003).

The following enzymes work on xylans (xylan, glucuronoarabinoxylan, glucuronoxylan, arabinoxylan) and xylo-oligosaccharides:

2.2.2.1 Endo-1,4-β-xylanases (EC.3.2.1.8)

Endo-1,4-β-xylanases (also known as 1,4-β-D-xylan xylanohydrolases) catalyse the hydrolysis of the internal β-1,4 xylosidic linkages of xylans, which have a backbone of β-D-xylose monomers (Figure 2.9). This reaction releases β-D-xylopyranosyl oligomers, reducing the degree of polymerisation (Dashtban *et al*., 2009; Mafe *et al*., 2014). This is considered one of the most important hemicellulases as xylans are the major hemicellulose found in type II plants (Poales order), which are of agricultural and economic significance (Guimarães, 2012).

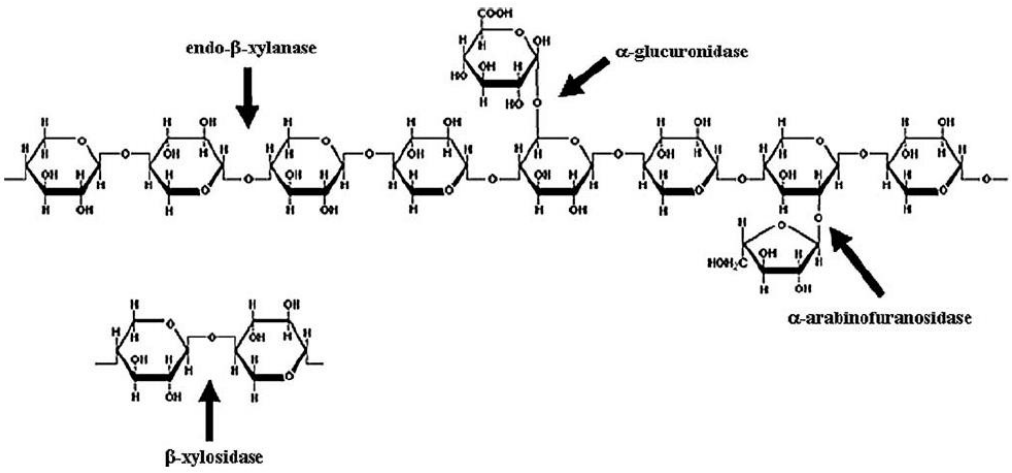


Figure 2.9 Action sites of some enzymes involved in degradation of xylans (Kumar *et al*., 2008).

2.2.2.2 Exo-1,4-β-D-xylosidases (EC.3.2.1.37)

Exo-1,4-β-D-xylosidases (also known as 1,4-β-D-xylan xylohydrolases) catalyse the hydrolysis of xylo-oligosaccharides and xylobiose (a dimer of xylose) produced from the endo-1,4-β-xylanase reactions, producing β-D-xylopyranosyl residues (Figure 2.9) (Dashtban *et al*., 2009; Mafe *et al*., 2014). The enzyme specifically bonds to the non-reducing ends of the short xylo-oligomers.

2.2.2.3 Exo-α-L-arabinofuranosidases (EC 3.2.1.55)

Exo-α-L-arabinofuranosidases work on the arabinofuranosyl sidechain residues in arabinoxylan and arabinan by hydrolysing the α-1,2 and α-1,3 linkages α-1,5 linkages respectively, as shown in Figure 2.9 (Mafe *et al*., 2014).

2.2.2.4 α-D-glucuronidases (3.2.1.139)

α-D-glucuronidases work on the glucuronic sidechains in xylan by hydrolysing the α-1,2 glycosidic linkages as shown in Figure 2.9. This reaction produces glucuronic acid which, along with glucose, represses the activity of α-D-glucuronidases.

Additional enzymes, such as acetyl xylan esterase (EC 3.1.1.72) and ferulic acid esterase (EC 3.1.1.73) are required to remove side-chain substituents that may be attached to the xylan structure. As a result, this creates more sites for subsequent enzymatic hydrolysis reactions (Moreira & Filho, 2008).

The following enzymes work on mannans (glucomannan, galactomannan, galactoglucomannan):

2.2.2.5 Endo-1,4-β-mannanases (EC 3.2.1.78)

Endo-1,4-β-mannanases (1,4-β-D-mannan mannohydrolases) randomly cleave the β-1,4 linkages found in mannan backbones to produce mannobiose (dimer of mannose) or manno-oligomers (Shallom & Shoham, 2003). The degradation of mannans is greatly affected by the pattern and number of side chains on the mannan backbone (Moreira & Filho, 2008).

2.2.2.6 Exo-1,4-β-mannosidases (EC 3.2.1.25)

Exo-1,4-β-mannosidases (1,4-β-D-mannopyranoside hydrolases) break down mannobiose and manno-oligomers produced by the reaction of endo-β-1,4-mannases, producing mannose (Shallom & Shoham, 2003). This enzyme works at the non-reducing end of the oligosaccharides (Moreira & Filho, 2008).

2.2.2.7 β-glucosidases (EC 3.2.1.21)

β-glucosidases (1,4-β-D-glucoside glucohydrolases) works at the non-reducing ends of the oligosaccharides released by β-mannanases. It hydrolyses 1,4-β-D-glucopyranose, releasing D-glucopyranose (Moreira & Filho, 2008).

Additional enzymes, such as α-galactosidase (1,6-α-D-galactoside galactohydrolase; EC 3.2.1.22) and acetyl mannan esterase (EC 3.1.1.6) are required to remove side-chain substituents that may be attached to the mannan structure. Thus, this creates more sites for subsequent enzymatic hydrolysis reactions (Moreira & Filho, 2008).

2.2.2.8 Endo-β-1,4-galactanases (EC 3.2.1.89)

Endo-β-1,4-galactanase (also known as endo-1,4-β-galactosidase) hydrolyses the 1,4-β-galactosidic linkages found specifically in type I arabinogalactans (Mafe *et al*., 2014).

2.2.2.9 Endo-α-1,5-arabinanases (EC 3.2.1.99).

These enzymes remove L-arabinose residues from arabinan polymers by cleaving α-1,5 linkages (Mafe *et al*., 2014).

2.2.3 Ligninases

Ligninolytic enzymes are oxidative enzymes that can be divided into two main families – phenol oxidases and peroxidases. The main enzymes involved in lignin degradation are laccases (phenol oxidase family), manganese peroxidases (MnPs) (peroxidase family), and lignin peroxidases (LiPs) (peroxidase family). All three enzymes work by oxidising phenolic compounds and aromatic amines of the lignin structure (Guimarães, 2012; Mafe *et al*., 2014). A new group of peroxidases, known as versatile peroxidases (VPs), are able to oxidise phenolic and Mn^{2+} compounds, as well as nonphenolic compounds. In addition to these enzymes, hydrogen peroxide producing enzymes, such as aryl alcohol oxidase and glyoxal oxidase, are also considered to belong to the ligninolytic system (Lankinen, 2004). The simplified reactions of laccases, MnPs and LiPs are shown in Figure 2.10 and Figure 2.11.

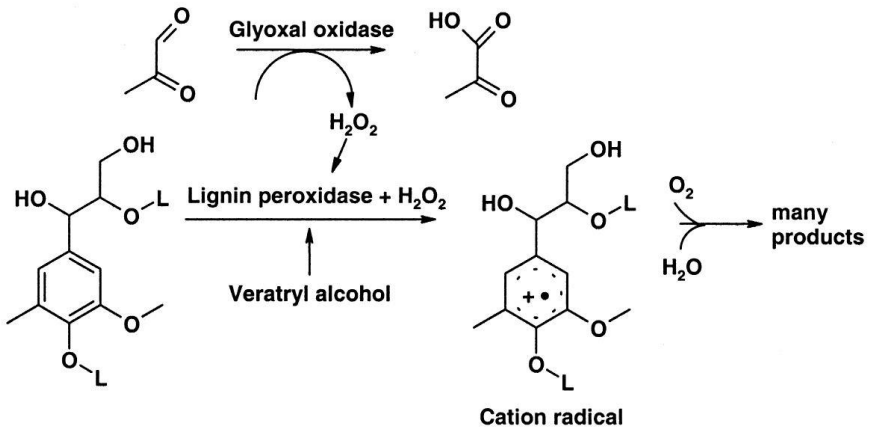


Figure 2.10 Simplified reaction of enzymes involved in lignin degradation – lignin peroxidase (LiP) and glyoxal oxidase (Hatakka, 2001).

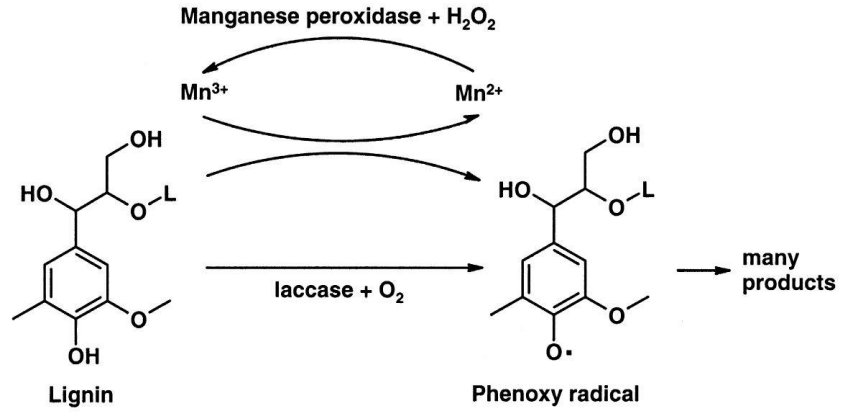


Figure 2.11 Simplified reaction of enzymes involved in lignin degradation – manganese peroxidase (MnP) and laccase (Hatakka, 2001).

2.2.3.1 Laccases (EC 1.10.3.2)

Laccases are copper-containing phenol oxidases that require the presence of oxygen (O_2) as a co-factor. Laccases oxidise phenolic compounds into phenoxyl radicals, which degrade spontaneously. During lignin degradation by fungi, laccases are usually the first enzymes secreted into the surrounding media (Hatakka, 2001; Lankinen, 2004).

2.2.3.2 Manganese peroxidases (EC 1.11.1.13)

Manganese peroxidases (MnPs) are heme-containing enzymes that require the presence of hydrogen peroxide (H_2O_2) as a co-factor. MnPs oxidise Mn(II) to Mn(III) in the presence of H_2O_2. Mn(III) in turn oxidises phenolic

compounds to phenoxyl radicals which then degrade spontaneously (Hatakka, 2001; Collinson & Thielemans, 2010).

2.2.3.3 Lignin peroxidases (1.11.1.14)

Similar to MnPs, lignin peroxidases (LiPs) are also heme-containing enzymes that require the presence of hydrogen peroxide (H_2O_2) as a co-factor. LiPs catalyse the oxidation of nonphenolic aromatic compounds by cleavage of C-C bonds and ether (C-O-C) bonds. The aromatic rings are oxidised, by removal of one electron, to cation radicals which can be further broken down chemically (Hatakka, 2001; Collinson & Thielemans, 2010).

2.3 STARCH

2.3.1 Sources and utilisation of starch

Starch is produced by plants as a carbohydrate source, and is stored in a variety of granular forms and dimensions. Starch can be found in seeds, fruit, roots, tubers, pollen, tubes, stem-pith, and leaves. The size of the starch granules depends on the botanical source and can vary from 0.5 μm up to 175 μm (Zobel, 1988). Starch granules are synthesized on a daily basis inside plant plastids and are used as an energy source for respiration during periods where photosynthesis does not occur. However, starch is also found in amyloplasts in the seeds, roots and tubers of plants, and the starch accumulates in these organelles as water-insoluble starch granules for long-term storage (El-Fallal *et al*., 2012).

Starch is one of the main energy reserves in nature and represents a staple food in most human diets; starch is also processed into a variety of different food products, such as glucose syrups and fructose (El-Fallal *et al*., 2012). Starch also has many non-food uses. For example, it is used in

the textile industry as an adhesive or as a gum, and in the papermaking industry. It is also used as a feedstock for the production of many products, such as enzymes, biofuels (bioethanol and biogas), biochemicals (succinic acid, polylactic acid) to name a few (Laycock & Halley, 2014). The main industrial sources of starch come from tapioca, potato, maize and wheat.

2.3.2 Starch structure

Starch is a naturally occurring blend of two homopolysaccharides – amylose, which is mainly linear, and amylopectin, which is highly branched and variable. Anhydro-D-glucose is the main building block of both polymers. Amylose is a linear chain with α-1,4 glucosidic linkages, and a degree of polymerisation ranging up to about 25,000 (Preiss, 2004). Amylopectin also has α-1,4 glucosidic linkages, but forms branches with α-1,6 glucosidic linkages, and the number of glucosyl units ranges between 10^5 and 10^9 (Collinson & Thielemans, 2010). The rate of branching in amylopectin is approximately 4–5% (every 20 units) and there are various forms of amylopectin (Cheetham & Tao, 1998). The chemical structure of these two molecules is shown in Figure 2.12.

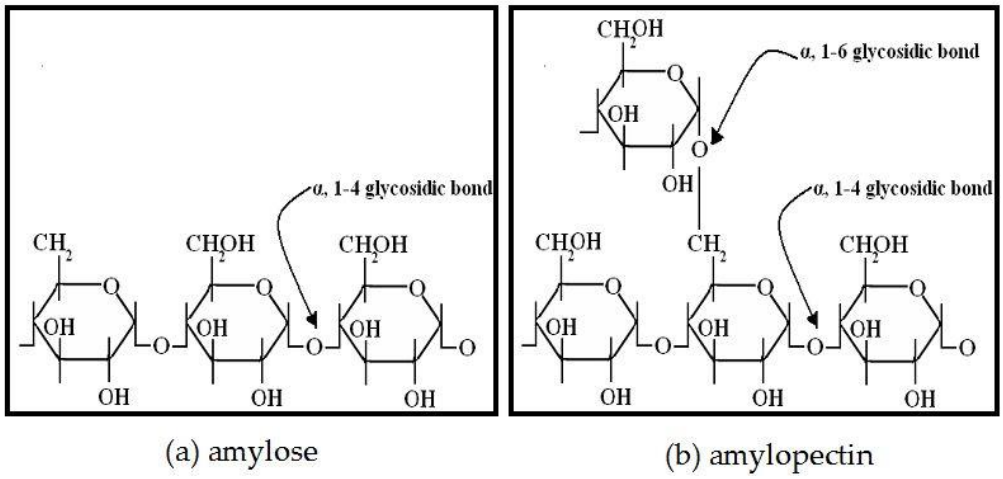


Figure 2.12 Chemical structure of starch components – amylose and amylopectin (El-Fallal *et al.*, 2012).

The α-1,4 linkage in the amylose molecule results in a gradual, natural twist in the chain. This is in contrast to the β-1,4 linkage of cellulose which results in each adjacent residue being rotated 180 °C, producing a flat ribbon (Zobel, 1988) (Figure 2.13).

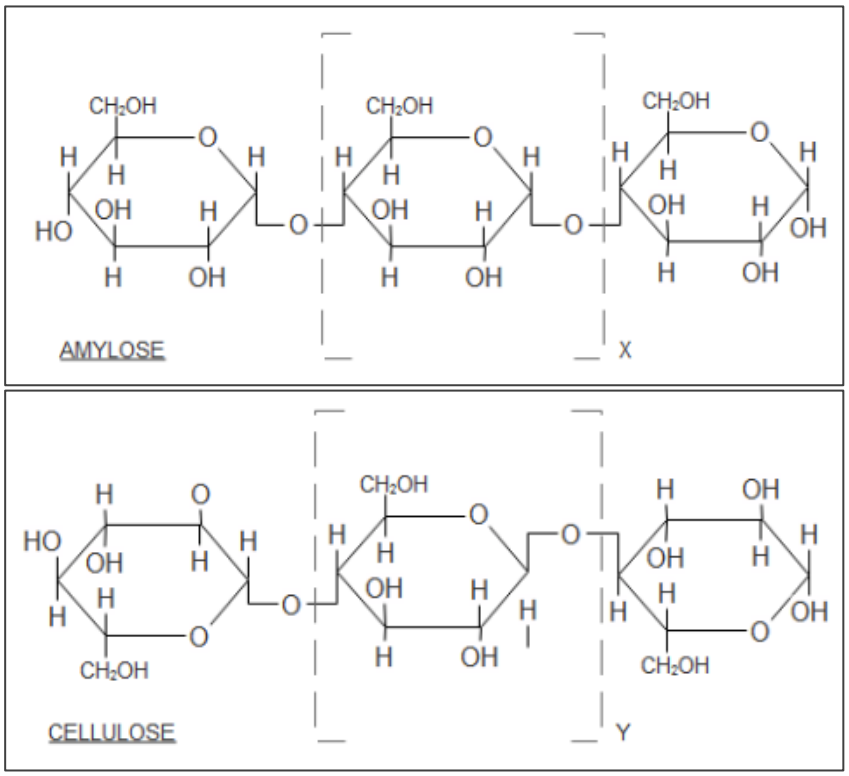


Figure 2.13 Schematic diagram of amylose (α-1,4) and cellulose (β-1,4) glucose polymers. Adapted from Zobel (1988).

The ratio of amylose to amylopectin found in starch granules is dependent on the species itself, but it usually varies from 1:4 to 1:2 in standard starches. 'Amylose-rich' starches are high in amylose and 'waxy' starches are high in amylopectin (Collinson & Thielemans, 2010). The interaction of these two chains gives the starch granules alternating crystalline and amorphous regions due to the branched amylopectin structure. The variation in the amylopectin chains (extent and length of branching) and the varying amylose-amylopectin ratios results in several crystalline polymorphic forms (Guimarães, 2012). Type A is found in cereals, Type B is found mainly in tubers as well as in maize starches, and Type C (a

combination of both type A and type B) is found in legumes, roots and fruits (Collinson & Thielemans, 2010; Guimarães, 2012).

2.3.3 Starch gelatinisation

Amylopectin is soluble in water, but amylose is not. Therefore, when starch granules are placed in cold water they are relatively insoluble. However, when heated in excess water, the starch granules undergo physical changes known as gelatinisation, and these changes are irreversible. Although many techniques have been employed in an attempt to understand gelatinisation and several models proposed, no universally accepted explanation has been found. During the gelatinisation process water penetrates the amorphous regions of the granules, causing substantial swelling of the granules and disruption of the semi-crystalline structure. As a result, this causes the amylose to progressively leech out, making the starch more soluble and generally more viscous (Donald, 2004).

The gelatinisation temperature is different for each species as it is dependent on the composition and crystallinity of the starch, and the fat or protein content. It is also dependent on a number of operating conditions, such as the starch-water ratio, the salt or sugar concentration, and the pH. Zobel (1988) measured the gelatinisation temperatures for a range of type A (oats, rye, wheat, sorghum, waxy maize, amongst others), type B (amylomaize, canna, potato), and type C (sweet potato, horse chestnut, tapioca) starches and found the temperatures ranged from 60–89 °C.

2.3.4 Digestion of starch by amylolytic enzymes

All living organisms produce amylases; however, they vary in specificity, activity level and requirements from species to species, and few organisms produce a complete set of enzymes which are capable of degrading starch

effectively. Starch, being a large, water-insoluble polymer, cannot enter into cells. Therefore, starch hydrolysis occurs extracellularly and amylases are either found bound to cell membranes or are secreted into the medium around cell membranes (El-Fallal *et al*., 2012).

Thus, similar to cellulose, an enzymatic complex is required for the complete breakdown of starch due to its complexity. This enzymatic complex consists of four main groups – endoamylases, exoamylases, debranching enzymes, and transferases – which all work together to hydrolyse starch.

2.3.4.1 Endoamylases

Endoamylases, such as α-amylase (EC 3.2.1.1), degrade starch by catalysing the cleavage of arbitrary internal α-1,4-glycosidic linkages between the glucose molecules of both amylose and amylopectin chain (Figure 2.14). Oligosaccharides of varying lengths are released from the chains (Butler *et al*., 2004).

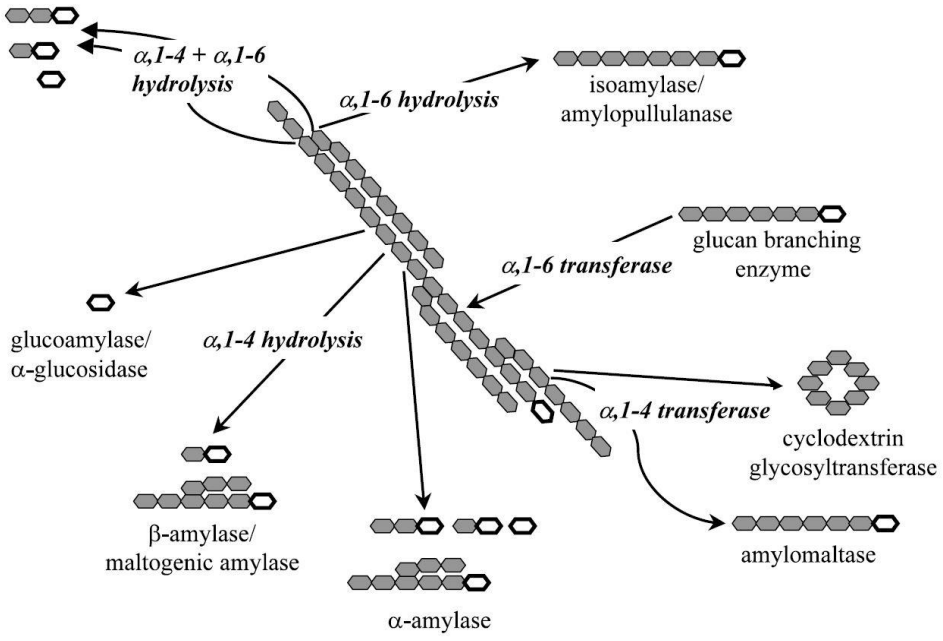


Figure 2.14 Different enzymes involved in the degradation of starch. The open end structure represents the reducing ends of a starch molecule (van der Maarel *et al*., 2002).

2.3.4.2 Exoamylases

Exoamylases work by hydrolysing the external glucose residues of the amylose and amylopectin chains to release glucose, maltose (dimer of glucose) and β-limiting dextrin. Three main exoamylases are: (i) β-amylases (EC 3.2.1.2); (ii) glucoamylase (amyloglucosidase; EC 3.2.1.3), and (iii) α-glucosidase (EC 3.2.1.20) (El-Fallal *et al*., 2012; Guimarães, 2012).

2.3.4.2.1 β-amylases (EC 3.2.1.2)

β-amylases work specifically on the α-1,4 glycosidic bonds, producing maltose and β-limiting dextrin (Figure 2.14).

2.3.4.2.2 Glucoamylases (EC 3.2.1.3) & α-Glucosidases (EC 3.2.1.20)

Glucoamylases, as well as α-glucosidases, hydrolyse both the α-1,4 and α-1,6 glycosidic bonds producing glucose (Figure 2.14). However, these two enzymes differ in their preference of substrate length - glucoamylases work better on long-chain polysaccharides, whereas α-glucosidases work best on short malto-oligosaccharides (van der Maarel *et al*., 2002) (Figure 2.14).

2.3.4.3 Debranching enzymes

Debranching enzymes exclusively hydrolyse the α-1,6 glycosidic bonds. Isoamylases (EC 3.2.1.68) can only degrade amylopectin. Pullulanase type 1 (EC 3.2.1.41) can degrade both amylopectin and pullulan (Guimarães, 2012). The action site of both types of debranching enzymes is shown in Figure 2.14. Since both act on amylopectin and not amylose, they leave long linear polysaccharides (van der Maarel *et al*., 2002).

2.3.4.4 Transferases

Several enzymes are classified in this group – (i) amylomaltases (EC 2.4.1.25), (ii) cyclodextrin glycosyltransferases (EC 2.4.1.19), and (iii) branching enzymes (EC 2.4.1.18). The action sites of these enzymes are shown in Figure 2.14. These enzymes work by cleaving an α-1,4 glycosidic bond of one molecule and transferring part of this molecule to another place (glycosidic acceptor), producing a new glycosidic bond.

Amylomaltases and cyclodextrin glycosyltransferases have similar reaction mechanisms, forming new α-1,4 glycosidic bonds; however, amylomaltases produce a linear product, whereas cyclodextrin glycosyltransferases produce a cyclic product with 6–8 glucose residues. Branching enzymes have a different reaction mechanism and form new α-1,6 (rather than α-1,4) glycosidic bonds (van der Maarel *et al*., 2002; El-Fallal *et al*., 2012).

2.4 BIOFUEL & BIOCHEMICAL PRODUCTION PROCESS

In order to maximise the production of biofuels or biochemicals, the use of the entire crop, including the lignocellulosic material, is important. Also if a food crop is used, it is important that the edible part (usually the part high in starch/sugars) of the crop is not used for the production of bioenergy. Therefore, the non-edible part, which is generally high in lignocellulosic material, should be used for the production of bioenergy and biochemicals. However, the lignocellulosic material is recalcitrant to breakdown and often the enzymes that are used to hydrolyse the carbohydrates into usable sugars cannot reach these due to the rigidity of the cell wall, and the insoluble nature of the lignin. Therefore, this poses a processing challenge in the production of the biofuels and biochemicals, and an efficient method for breaking this material down, known as a pretreatment process, may be

necessary. The bioethanol and biochemical process from lignocellulosic biomass consists of four steps – pretreatment, hydrolysis, fermentation, and distillation.

2.4.1 Pretreatment

The main goals of the pretreatment process should be the separation/solubilisation of the lignin, cellulose and hemicellulose fractions allowing easier access for the breakdown of the carbohydrates, either directly or by enzymes (Figure 2.15). As well as this objective, focus should also be placed on limiting/avoiding the loss of sugars and the formation of inhibitory products, and minimising energy demands and costs of the process (Sanchez & Cardona, 2008; Sarkar *et al*., 2012).

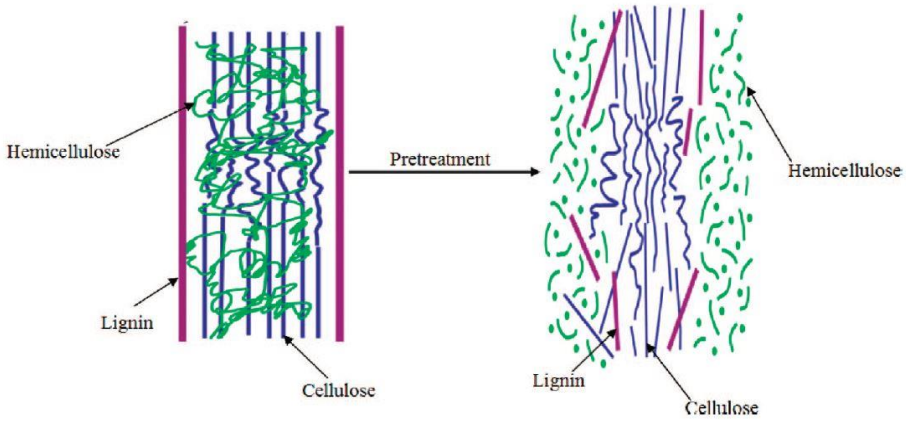


Figure 2.15 Schematic diagram showing the role of pretreatment methods (Kumar *et al*., 2009).

The pretreatment process can be separated into several categories - physical, chemical, physiochemical, and biological methods; as well as a combination of these. The effectiveness of the individual pretreatment is dependent on both composition of the biomass substrate and the pretreatment operating conditions. Table 2.1 shows a list of the pretreatment methods most commonly used and their possible effects.

Table 2.1 Some pretreatment methods used to degrade lignocellulosic material and their possible effects (DP - degree of polymerisation; SA – surface area). Adapted from Talebnia *et al.* (2010) and Verardi *et al.* (2012).

Pretreatment	Examples	Advantages	Disadvantages
Physical pretreatment:			
Mechanical	- Milling - Grinding - Chipping	- Increase in pore size and SA - Decrease in DP and crystallinity of cellulose - No inhibitors produced	- High energy consumption
Physiochemical pretreatment:			
Hydrothermal	- Steam explosion - Liquid hot water	- Increase in pore size and SA - Partial degradation of hemicellulose - Transformation of lignin - Fast; inexpensive	- Acid catalysts may be required for high lignin biomass - Production of inhibitors - Incomplete disruption of lignin-carbohydrate matrix
With chemicals	- Ammonia fibre explosion (AFEX)	- Increases accessible SA - Partial degradation of hemicellulose - Transformation of lignin - Low production of inhibitors - Short process time	- Expensive - Ammonia recovery required - Hazardous - Not suitable on high lignin biomass
Chemical pretreatment:			
Acid hydrolysis	- Dilute acid - Concentrate acid	- Increase in porosity and surface area due to swelling - Decrease in DP and crystallinity - Degradation of hemicellulose - Lignin structure altered	- Production of inhibitors - Acid recovery required with concentrated acid - Corrosion resistant equipment required
Alkaline hydrolysis	- NaOH - Lime	- Increases accessible SA due to swelling - Decrease in DP and crystallinity - Degradation of hemicellulose - Removal of lignin - Low production of inhibitors	- Slow reaction process - Residual salts in biomass
Organosolv	- Formic acid - Methanol	- Degradation of hemicellulose - Lignin degradation - Possible to dissolve different biomass	- Expensive - Solvent recovery required
Biological pretreatment:			
Fungal treatment	- White-rot - Brown-rot - Soft-rot	- Increase in pore size and SA - Degradation of hemicellulose - Degradation of lignin	- Takes a long time - Low hydrolysis rate - Loss of cellulose -

2.4.1.1 Physical pretreatment

Physical pretreatment is essentially processes that increase the surface area of the biomass, by reducing the size or disrupting the structure. For example, mechanical pretreatment reduces the size of the biomass material by milling, chipping or grinding the material. Although a reduction in particle size via mechanical pretreatment has been shown to increase glucose and xylose hydrolysis yields in several biomass materials, it is not used on a commercial scale. This is because there is high energy consumption associated with mechanical pretreatment and high capital and operating costs of the equipment required (Mafe *et al*., 2014).

2.4.1.2 Physiochemical pretreatment

Physiochemical pretreatment methods usually occur at high temperatures and pressure (McCann & Carpita, 2008). Steam explosion is one of the most commonly used methods for lignocellulosic biomass. During the process, the biomass is exposed to high-pressure saturated steam (temperatures of 160 – 260 °C, corresponding pressure of 690 – 4830 kPa) for a period of time (several seconds – few minutes) before the pressure is suddenly reduced to atmospheric pressure. This change in pressure causes the materials to undergo an explosive decompression (Kumar *et al*., 2009). The process results in the transformation of lignin and degradation of hemicellulose, resulting in more efficient cellulose hydrolysis. Sometimes H_2SO_4 or CO_2 is added to the process to decrease the process time and temperature. This decreases the number of inhibitors produced and degrades the hemicellulose completely.

Ammonia fibre explosion (AFEX) pretreatment combines liquid ammonia with steam explosion. Although this process is fast, it can be hazardous and the recovery of ammonia is essential to make it cost-effective due to

the high cost of ammonia. The process can't be used with biomass of high lignin content and there is only solubilisation of a very small fraction of the solid material, particularly hemicellulose (Sarkar *et al*., 2012).

2.4.1.3 Chemical pretreatment

Several chemicals can be used during chemical pretreatment including acid, alkali, ammonia, organic solvent (organosolv), CO_2 and other chemicals.

Acid pretreatment is considered one of the most important pretreatment techniques, and uses either dilute acid (usually between 0.2 – 5.0 % w/w) or concentrated acid, at temperatures ranging from 130 – 210 °C. Common acids used include sulphuric acid, hydrochloric acid, nitric acid and phosphoric acid (Cardona *et al*., 2009). The acid causes the hemicelluloses to degrade to xylose and other sugars, increasing the cellulose hydrolysis significantly. However, the acid can continue to degrade the xylose into furfurals and 5-hydroxymethyl furfural (HMF), growth inhibitors of microorganisms, which can affect processes like fermentation. Therefore, materials usually need to be detoxified after pretreatment. Concentrated acids are also toxic and corrosive and require corrosion resistant equipment, which makes the process expensive. Additionally, recovery of concentrated acid is essential to make it cost-effective (Kumar *et al*., 2009). Dilute acid pretreatment has been used to degrade various lignocellulosic feedstocks, such as hardwoods, softwoods, wheat straw, sugarcane bagasse, rice straw and corncobs (Mafe *et al*., 2014).

Alkaline pretreatment dissolves both lignin and hemicellulose and causes swelling of the cellulose, decreasing the crystallinity. However, the effectiveness of the pretreatment depends on the lignin content of the

biomass. Hydroxides, usually sodium, potassium, calcium and ammonium, are used in the process (Sarkar *et al*., 2012). Alkaline pretreatment is usually carried out at room temperature, but take hours to days for completion, unlike acid pretreatment. In comparison with acid pretreatment, alkaline pretreatment results in the production of fewer inhibitors, but this is due to the fact that it causes less sugar degradation (Kumar *et al*., 2009). Alkaline pretreatment has been used to degrade various lignocellulosic feedstocks, such as Oil palm empty fruit bunches, rice hulls, sorghum straw and barley hulls (Mafe *et al*., 2014).

2.4.1.4 Biological pretreatment

Although much research has been done using physical, chemical and physiochemical pretreatment procedures, these can incur high capital costs, are energy intensive and a large waste stream is generated which is not environmentally friendly. Therefore, an alternative approach is the use of biological pretreatment to break down the matter into a nutrient rich medium that can be further fermented or anaerobically digested. As well as being a relatively safe and environmentally benign process, biological pretreatment is carried out under mild conditions and so does not generate toxic compounds such as furfural and HMF (Kumar *et al*., 2009).

There are many organisms that can break the lignocellulosic material down naturally, primarily saprophytic fungi and some bacteria, using a cocktail of digestive enzymes, including cellulases and xylanases. Commercial versions of these enzymes are frequently derived from plant-decaying fungi and can be used in experimental procedures. However, the enzymes currently used are slow and unstable, often making the process expensive. Also, the correct proportion of the enzymes required needs to be

determined (de Vries & Visser, 2001). Therefore, the need to find cheap and efficient enzymes to maximise yields is necessary.

Studies have shown that lignin and carbohydrate polymers can both be removed using biological pretreatment (Taniguchi *et al*., 2005; Shi *et al*., 2008). Although many species, such as cellulolytic bacteria, white-rot fungi, brown-rot fungi, and soft-rot fungi, have been investigated for this process, biological pretreatment is still a relatively new research topic and needs further studying.

2.4.1.4.1 White-rot, brown-rot and soft-rot fungi

White-rot fungi can effectively degrade all components of the lignocellulosic material; while only some can target predominantly the lignin component. White-rot fungi have been used in several biological pretreatments on a range of lignocellulosic materials (Okano *et al*., 2005; Lee *et al*., 2007; Shi *et al*., 2008; Yang *et al*., 2011; Zeng *et al*., 2012). However, although white-rot fungi produce many lignocellulolytic enzymes, they require a long residence time of pretreatment and consume a large amount of the cellulose; therefore, they are not a favoured choice for production at an industrial scale.

Brown-rot fungi can only partially break down the lignin component as they lack ligninolytic enzymes, but they can break down the cellulose and hemicellulose components of lignocellulosic material.

Soft-rot fungi generally break down the surface layer polysaccharides of lignocellulosic material, and although they do not generally attack the lignin, soft-rot fungi can produce some lignin modifying enzymes (Couturier & Berrin, 2013; Janusz *et al*., 2013).

2.4.2 Hydrolysis

Polysaccharides can be broken down into their respective monosaccharides by either chemical hydrolysis or enzymatic hydrolysis.

2.4.2.1 Chemical hydrolysis

Chemical hydrolysis is usually performed using acids, mainly hydrochloric acid and sulphuric acid. The acid attacks the amorphous regions of the cellulose by disrupting the hydrogen bonding between the cellulose fibrils, and it disrupts the ester and ether bonding in hemicellulose to release the respective sugars (Verardi *et al*., 2012). Many factors affect sugar yields from acid hydrolysis, including the particle size of the substrate and any pretreatment, the concentration of the acid, temperature, retention time, and mixing rate of the process.

Acid hydrolysis is advantageous over enzymatic hydrolysis as it has a much faster retention time (usually about two hours). However, the acid used in the process can be very corrosive on equipment used and therefore corrosion resistant equipment needs to be used, increasing the capital and operational costs. The process also releases many acid residues which can inhibit yeast cells in the fermentation stage, resulting in a lower ethanol production rate (Verardi *et al*., 2012).

2.4.2.2 Enzymatic hydrolysis

Enzymatic hydrolysis is performed using cellulases, hemicellulases and ligninolytic enzymes, or usually a combination of the enzymes. During the process, the enzymes degrade the lignocellulosic material into its respective fermentable sugars.

The conditions used for enzymatic hydrolysis are mild; therefore, no inhibitors, which could affect fermentation, are generated. Since no acids

are used in the process, the use of expensive corrosion-resistant equipment and the removal of acid residues downstream are not required. Due to these factors, enzymatic hydrolysis is often favoured over acid hydrolysis for the digestion of biomass. However, enzyme hydrolysis does require a pretreatment – usually steam explosion or hot water – and this can generate inhibitors. Also, the enzymes used are costly, and can often account for one-third of the cost of ethanol production. Low rates of hydrolysis are often obtained as the correct pretreatment and enzymes combination is necessary, as well as conditions for enzyme hydrolysis (Binder & Raines, 2010).

2.4.3 Production of biofuels and biochemicals

Fermentable sugars are a valuable commodity as they can be easily converted into value added products, such as biofuels and biochemicals.

2.4.3.1 Bioethanol and biochemical production via fermentation

The production of bioethanol occurs when sugars are fermented, under anaerobic conditions into ethanol (Gibson *et al*., 2007; Goh *et al*., 2010). This is a well-established process and has been used in the production of many products, namely alcoholic beverages. The sugars produced during the hydrolysis stage are used as the starting substrate and can be fermented by various microorganisms, including yeast and bacteria. Some ideal conditions for fermentation are a medium pH of 6.0 – 6.5, incubation temperature of 24–28 °C, and fermentation/growth rate substrate concentration of 2.0–2.5 % (Gibson *et al*., 2007). However, these conditions are seldom met resulting in yeast stress during fermentation that needs to be limited as much as possible, as this can have a negative effect on the bioethanol/biochemical production.

Several microorganisms have been used in the fermentation process, such as *Saccharomyces cerevisiae*, *Escherichia coli*, *Zymomonas mobilis*, *Pachysolen tannophilus*, *Candida shehatae*, *Pichia stipitis*, *Candida brassicae*, *Mucor indicus.* Among these microorganisms, the most common used in ethanol production from hexose sugars are the yeast, *S. cerevisiae,* and the bacterium, *Z. mobilis.* However, since *S. cerevisiae* cannot utilise pentose sugars, such as xylose, it cannot utilise many sugars from the degradation of hemicelluloses. Attempts have been made to genetically modify *S. cerevisiae* to use the hexose sugars; however, other organisms, like *Pichia* and *Candida* species, have been investigated as they are able to utilise these pentose sugars. Unfortunately, their ethanol production rates are reported as being much lower (Sarkar *et al*., 2012).

A similar fermentation process can also be used to produce biochemicals, such as succinic acid, using different yeast strains. Some research has been performed using a metabolically modified yeast strain of *S. cerevisiae* in order to produce succinic acid (Raab *et al*., 2010; Raab & Lang, 2011). Biochemicals can also be produced using bacteria, such as *Actinobacillus succinogenes*, which produced succinic acid when it fermented wheat flour hydrolysate (Du *et al*., 2007). Succinic acid is a valuable chemical as it is used in many processes and products, such as detergents, pharmaceuticals, foods and surfactants (Zeikus *et al*., 1999).

2.5 ENZYME PRODUCTION VIA FUNGAL SOLID STATE FERMENTATION OR SUBMERGED FERMENTATION

In 2014, the industrial enzyme market was valued at US $4.2 billion, with the majority of enzymes used being hydrolytic enzymes. These enzymes are used in the food and beverage industry, cleaning agent industry and in the animal feed industry, and in the production of biofuels and

biochemicals (MarketsandMarkets, 2015). The use of lignocellulosic enzymes shows potential in all these industries.

Solid state fermentation (SSF) and submerged fermentation (SmF) can be used for the production of a wide range of fungal hydrolytic enzymes, such as cellulases and glucoamylases, and these enzymes have been produced using a variety of biomass substrates. SSF utilises solid substrates in the absence of free water, on which the microorganisms are able to grow, whereas SmF is performed in large tanks utilising free flowing liquid substances (Guimarães, 2012; Subramaniyam & Vimala, 2012).

Although SmF is the preferred method for enzyme generation since it is easier to control the culture conditions (pH, temperature, aeration) for maximum fungal growth and enzyme production, SSF does have many advantages over SmF – such as low energy inputs, operational costs and water consumption, reduction in bacterial and yeast contamination problems, easier downstream processing, and generation of a protein-rich by-product (Moreira & Filho, 2008; Guimarães, 2012). However, many conditions can affect the enzyme yield, including factors such as moisture content, substrate, nitrogen source and media used, transfer of gases, temperature and pH, and therefore these need to be researched in order to optimise enzyme yields (Bon & Ferrara, 2007; Kumar *et al*., 2008).

The use of agro-industrial residues such as Sago hampas, wheat straw, and sugar cane bagasse, could be used as alternative sources of carbon for the production of enzymes. However, the initial conversion of lignocellulosic biomass to sugars is a key problem and the production of more efficient, stable and cost-effective enzymes would help lower the overall costs of lignocellulosic conversion.

2.6 USE OF UNDERUTILISED CROPS AS SUBSTRATES

There is no specific definition for an underutilised crop, however, the term can be used "as a broad reference to alternative options that have so far not received significant research or development attention" (CFF Research, 2011a). Thus, very little is known about them, in terms of their compositional analysis and market potential. Using this definition there are a large number of underutilised crops that could, or are, being grown for food or energy production. This section will consider only those that have been utilised in this thesis.

2.6.1 Bambara nut (*Vigna subterranea*n)

Bambara is an annual legume, with a well-developed tap-root system, and the pods are harvested by pulling the plant out of the soil (Mkandawire, 2007). The nuts are an important source of protein in diets of poorer communities, particularly in African communities (Baryeh, 2001; Mkandawire, 2007; Hillocks *et al*., 2011). The remaining biomass (leaves, stems, and roots) is used for animal feed, medicinal purposes, or added back into the soil to increased soil fertility (Mkandawire, 2007; Hillocks *et al*., 2011). Bambara is a choice crop in poorer African communities for its high-protein nuts, but also because it is resistant to drought and high temperatures, puts very little demand on the soil and can be grown on marginal soils (Baryeh, 2001; Mkandawire, 2007).

2.6.2 Leucaena (*Leucaena leucocephala*)

Leucaena is a thornless, leguminous hardwood tree, which grows to between 7–20 m tall. It is mainly used as a fodder crop for grazing animals but it is also constant forage for honey bees (Rout *et al*., 1999). It has a very fast growth rate, is able to grow on steep slopes and in marginal areas and is immune to floods, drought, fire and strong winds. As a result,

it is used as a firebreak and windbreak, planted to prevent soil erosion, or is used to provide shelter and/or support to other crops, such as cocoa, coffee and tea. It can however, be invasive due to its very high growth rate (Feria *et al*., 2011). Leucaena is one of the major sources for paper pulp and construction material and is also used in reforestation programmes in the tropics due to its high growth rate (Rout *et al*., 1999). It has a lignocellulosic composition of around 38–41% cellulose, 19–21% hemicellulose and 24% lignin (Lopez *et al*., 2010; Feria *et al*., 2011).

2.6.3 Napier grass (*Pennisetum purpureum*)

Napier grass grows very quickly in robust bamboo-like clumps up to 3 m tall (Farrell *et al*., 2002). It has no nutritional value for humans, and is grown mainly as an energy crop. However, it is also used for animal food and serves as a firebreak or windbreak due to its size. Napier is also used as a trap 'pull' crop around the perimeter of maize or sorghum fields in the push-pull technology for controlling pests, mainly stemborers (Farrell *et al*., 2002; Khan *et al*., 2007). The Napier grass protects the maize by emitting chemicals to attract the stemborers towards itself, and then it secretes a sticky substance which physically traps the pests.

Napier grass has low water and nutrient requirements for growth. It can be grown on land not suitable for food crops, and has a high biomass production rate (it can be harvested about 4–6 times/year). Research shows it can obtain 50–150 tonnes green matter/ha/year, depending on climate, soil conditions and inputs (Farrell *et al*., 2002; Flores *et al*., 2012). It is made up of around 36–46% cellulose, 33% hemicellulose and 20–30% lignin (Reddy *et al*., 2012; de Araujo Morandim-Giannetti *et al*., 2013).

2.6.4 Nipa palm (*Nypa Fruticans*)

Nipa is often called the 'mangrove palm' as it grows very well in mangrove environments. It also grows in brackish water environments, along coastlines and in semi-liquid mud areas of rivers (Neri, 1994). Thus, it can be grown in areas not suitable for many crops, and needs very little fertilisation, herbicides or irrigations (Hamilton & Murphy, 1988). Currently there are many uses for the sugar-rich sap, which is stored in the tree truck and extracted by cutting off the flowers and draining it. About 98,600 – 141,000 L/ha can be collected per year (Tamunaidu *et al*., 2011), and it is used for animal feed in the dry seasons and for human consumption as treacle, or easily fermented to alcohol or vinegar (Tsuji *et al*., 2011). There is very little waste generated during this process, no machinery needed, and the sap can be extracted continuously (Hamilton & Murphy, 1988; Tamunaidu *et al*., 2011). The fronds from the Nipa palm are currently used for roof thatching, or for making umbrellas and several other products (Neri, 1994).

2.6.5 Sago palm (*Metroxylon sagu*)

The Sago palm is an important crop, socio-economically, in South East Asia. It grows well in humid, tropical lowlands and can reach about 25 m tall. It takes about 8–12 years to reach maturity; at this point it flowers and then dies shortly afterwards (Abd-aziz, 2002). Just before flowering the plant converts its stored nutrients to starch and stores it in its trunk (Abd-aziz, 2002). This is extracted and can be processed into many products, such as glucose, syrups, chemicals for the pharmaceutical industry, paper industry and many others (Flach, 1983). Sago 'hampas' which is the fibrous residue left behind after most of the starch has been washed out, still contains some starch granules trapped inside a lignocellulosic matrix (Vickineswary *et al*., 1994). This can be used as

animal feed, compost for mushrooms, as well as several other processes (Singhal *et al*., 2007). Sago palm trees thrive in most soil conditions, such as saline soils, acidic peat soils, swamps (Flach & Schuilling, 1989), and promote a stable agroforestry system, with about 25 tonnes/ha of starch produced per year in Malaysia (Singhal *et al*., 2007).

2.6.6 Oil palm fronds (*Elaeis guineensis*)

Oil palm is not an underutilised crop, as it is commercially grown in several tropical countries for the production of palm oil. However, the oil palm fronds, which are considered biomass waste, were used in this research.

Most global palm oil production comes from Indonesia and Malaysia. Oil palm is the highest yielding oil crop, and is currently the most widely used vegetable oil (Sumathi *et al*., 2008). Recently, the creation of oil palm monocultures has gained much negative press and there have been some efforts focused to increase the sustainability chain – economically, socially and environmentally – of the oil palm industry (Wilcove & Koh, 2010; Malaysia GSIAC, 2011). Palm oil derivatives are used as ingredients in a wide range of food products, such as margarines, frying oils, breads, biscuits, gravy granules, and in many non-food products, such as shampoos, beauty products, candles and detergents.

The waste remaining after the oil extraction process includes palm oil mill effluent and biomass waste, which consists of the fronds and the empty fruit bunches. In Malaysia, agricultural waste accounts for much of its biomass waste production, with about 90% of this biomass waste coming from the oil palm industry (Goh *et al*., 2010; Crops for the Future (CFF) Research, 2011b; Malaysia Palm Oil Council (MPOC), 2012). The biomass waste produced during oil extraction can be used to produce various value added products, such as bioethanol and biogas (Sumathi *et al*., 2008).

CHAPTER 3 MATERIALS AND METHODS

3.1 PLANT MATERIAL

The underutilised crops, and the parts of these used in this study are shown in Table 3.1. All crops were obtained through Crops for the Future (CFF) based at the University of Nottingham Malaysia Campus, Semenyih, Selangor, Malaysia. Before being shipped to the University of Nottingham Sutton Bonington Campus, UK, they were air dried for longer than 10 days.

Table 3.1 Crops used during this research.

Common name	Latin name	Part of the crop used
Bambara nut	Vigna subterranean	Leaves, branches, roots
Leucaena	Leucaena leucocephala	Leaves, small branches
Napier grass	Pennisetum purpureum	Leaves or stems
Nipa palm	Nypa Fruticans	Fronds (leaves)
Oil palm	Elaeis guineensis	Fronds (leaves)
Sago palm	Metroxylon sagu	Sago hampas*

*waste after the processing of Sago palm for starch extraction.

Although oil palm is not an underutilised crop, the fronds were considered in this research as they are biomass waste from the palm oil industry.

3.2 PREPARATION AND STORAGE OF SAMPLES

3.2.1 Knife milling

All samples were oven dried overnight at 70 °C and then knife-milled using a Laboratory Mill (Pulverisette 19, Fritsch, Germany) and passed through a sieve with a mesh size of 2.0 mm. A portion of this sample was then further processed by knife-milling with the same machine to pass through a sieve with a mesh size of 0.5 mm. All samples were stored in an air tight container at 4 °C until use.

3.2.2 Ball milling

A portion of the sample (passed through a sieve with a mesh size of 0.5 mm) was ball-milled in a Planetary Mill (Pulverisette 5, Fritsch, Germany) at 250 rpm with 2 min milling time, followed by 2 min pause time, for a total time of 20 min (total grinding time of 10 min). Each 80 mL stainless steel milling pot was filled with 30 steel grinding balls (10 mm diameter) and 5 g of sample. All samples were collected and stored in an air tight container at 4°C until use in composition analysis.

3.3 CHEMICALS

All chemicals were obtained from Sigma-Aldrich, MP Biomedicals, Scientific Laboratory Supplies, VWR International, Fisher Scientific, Merck, Oxoid, and Acros Organics, and were of analytical grade, unless otherwise stated.

3.4 MEDIA SOLUTIONS

Several solutions were used when carrying out solid state fermentations and submerged fermentations. These additional nutrients included starch, yeast extract (YE), and/or mineral solutions. Two different mineral solutions were used, MSI and MSII, and their composition is shown in Table 3.2 and Table 3.3 respectively. To each solution 10 mL trace elements solution (Table 3.4) was added and then made up to 1.0 L using deionised water.

Table 3.2 Composition of mineral solution I (MSI), with the addition of 10 mL trace elements solution and then made up to 1.0 L with deionised water.

Mass (g)	Substance
1.0	Ammonium sulphate ($(NH_4)_2SO_4$)
0.5	Potassium dihydrogen phosphate (KH_2PO_4)
0.5	Dipotassium hydrogen phosphate (K_2HPO_4)
0.2	Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$)

Table 3.3 Composition of mineral solution II (MSII), with the addition of 10 mL trace elements solution and then made up to 1.0 L with deionised water.

Mass (g)	Substance
26.0	Potassium chloride (KCl)
26.0	Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$)
76.0	Potassium dihydrogen phosphate (KH_2PO_4)

Table 3.4 Composition of trace elements solution, made up to 1.0 L using deionised water.

Mass (g)	Substance
0.8	Copper (II) sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$)
0.8	Ferric chloride ($FeCl_3$)
0.8	Manganese (II) sulphate monohydrate ($MnSO_4 \cdot H_2O$)
0.8	Sodium molybdate dihydrate ($NaMoO_4 \cdot 2H_2O$)
2.0	Zinc sulphate ($ZnSO_4$)

3.5 MICROORGANISMS

3.5.1 Microorganisms

Three fungi, *Aspergillus niger* N402, *Trichoderma reesei*, and *Aspergillus awamori* were used in this research. *A. niger* N402 and *T. reesei* were obtained from the School of Life Sciences, University of Nottingham, UK. *A. awamori* was obtained from Professor Colin Webb, School of Chemical Engineering and Analytical Science, University of Manchester, UK.

3.5.2 Growth of microorganisms

A. niger and *T. reesei* were cultured based on procedures described by Delmas *et al.* (2012). Potato dextrose agar (3.9% w/v, as instructed on bottle; microbiology grade) was used as the culture medium.

A. awamori was cultured based on procedures described by Koutinas *et al*. (2001). The culture medium used contained 2.0% (w/v) agar powder (microbiology grade) + 5.0% (w/v) Sago hampas + 0.5% (w/v) yeast extract (YE) + 0.002% (v/v) silicone anti-foam (molecular biology grade) using MSI to make into a solution.

Based on initial experimentation performed, these culture mediums proved best for the culturing of fungal spores. Unless stated, these culture mediums were used for the production of fungal spores for all experiments performed.

The agar solutions were autoclaved at 121 °C and 100 kPa (15 psi) above atmospheric pressure for 15 min. After autoclaving, the solution was immediately distributed into sterile Petri dishes under sterile conditions and allowed to cool. Once the agar was set 0.05 mL of the corresponding fungal suspension was added to the agar in each Petri dish and spread around the agar. All spore cultures were incubated in a dark, static incubator set at 28 °C for up to 7 d and then stored at 4 °C until required.

3.5.3 Preparation of spore suspension

The cultured spores were used for inoculation of the underutilised crops during solid state fermentation (SSF) and submerged fermentation (SmF) procedures. When needed, 10 mL sterile 0.01% (v/v) Tween 80 solution was added, under sterile conditions, to a Petri dish containing spores. The Petri dish was gently swirled for a few minutes, releasing the spores into the solution. This spore suspension was then transferred to a sterile centrifuge tube ready for use in SSF or SmF procedures; or the suspension could be prepared for long term preservation of the strain. The spore concentration (spores/mL) was determined by sampling an aliquot of the spore suspension for spore counting using a haemocytometer and microscope.

3.5.4 Long-term storage of microorganisms

For long team storage of spores, the spore suspension and 50% (v/v) sterilised glycerol were added in a 1:1 ratio, using sterile pipette tips, to 1.2 mL sterile cryogenic vials and preserved at -80 °C.

3.6 SUBSTRATE COMPOSITIONAL ANALYSIS

3.6.1 Moisture content

The moisture content of the biomass samples was determined by the standard biomass analytical method "Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples" provided by the National Renewable Energy Laboratory (NREL), prepared for publication by Sluiter *et al*. (2008a).

3.6.1.1 Preparation of samples

Aluminium weighing dishes were pre-dried before being used. The dishes were labelled and placed in a drying oven set to 105 °C for a minimum of 4 h, and then cooled in a desiccator. Once cool they were weighed and the mass recorded to the nearest 0.1 mg. A 5 g sample (knife-milled to pass through a 0.5 mm sieve) of each substrate was weighed out into the appropriately labelled aluminium dish, and the mass noted to the nearest 0.1 mg. All samples were placed into the drying oven set at 105 °C and dried for a minimum of 4 h. The samples were removed and cooled to room temperature in a desiccator before being weighed. The mass was recorded and the samples were placed back into the oven and dried to constant mass.

3.6.1.2 Analysis of samples

The percent moisture content was calculated using Equation 3.1.

Equation 3.1

$$\%\ Moisture\ content = \left[\frac{(Mass_{sample\ as\ received} - Mass_{dry\ sample})}{Mass_{sample\ as\ received}}\right] x\ 100$$

$$where:\ \ Mass_{dry\ sample} = Mass_{dry\ foil\ plus\ dry\ sample} - Mass_{dry\ foil}$$

3.6.2 Ash content

The amount of inorganic material (ash) in the biomass samples was determined by the standard biomass analytical method "Determination of Ash in Biomass" provided by the National Renewable Energy Laboratory (NREL), prepared for publication by Sluiter *et al*. (2008b).

3.6.2.1 Preparation of samples

Ashing crucibles were labelled, dried to constant mass and the mass recorded to the nearest 0.1 mg. A 2 g sample of each substrate (knife-milled to pass through a 0.5 mm sieve) was weighed out into the appropriately labelled crucible and the mass recorded to the nearest 0.1 mg. All samples were placed into the muffle furnace set at 575 ± 25 °C for 24 h, and then cooled for 1 h in a desiccator. After 1 h they were weighed and the mass recorded to the nearest 0.1 mg. The crucibles were placed back in the furnace and the samples were dried to constant mass.

3.6.2.2 Analysis of samples

The percent ash was calculated using Equation 3.2, and all results were reported relative to the 105 °C oven dry weight of the sample.

Equation 3.2

$$\%\ Ash = \left(\frac{Mass_{crucible\ plus\ ash} - Mass_{crucible}}{ODW_{sample}}\right) x\ 100$$

where:

$$ODW_{sample} = \frac{Mass_{air\ dried\ sample}\ x\ (100 - \%\ moisture\ content)}{100}$$

3.6.3 Elemental analysis

A Flash 2000 Organic Elemental Analyser (Thermo Scientific, USA) was used to determine the carbon, hydrogen, nitrogen and sulphur contents of the underutilised crop samples. The advanced combustion method has

been adopted by the Association of Official Analytical Chemists (AOAC), official method 972.43 (2006). A 5 mg sample (ball milled) was sealed in a tin capsule and the exact mass of the sample was recorded. The samples were combusted at approximately 1800 °C for a few seconds, converting the substances into elemental gases. These gases were then reduced, before being separated by a chromatographic column and detected by a highly sensitive thermal conductivity detector. Quantification was achieved with Thermo Scientific Eager Xperience software using high purity standards (Thermo Scientific, 2008).

3.6.4 Protein content

The protein content of the crop samples was measured by a combustion measurement performed according to AOAC official method 992.23 (2005a). The combustion method performed was that of the elemental analysis and the percent protein was calculated using Equation 3.3. A factor of 6.25 was used to convert nitrogen content into protein content as the average nitrogen content of proteins has been found to be about 16 % (1/0.16 = 6.25).

Equation 3.3

$$\% \, Protein = \% \, Nitrogen \; x \; 6.25$$

3.6.5 Starch content

Starch analysis was performed using the Megazyme total starch assay procedure (amyloglucosidase/α-amylase method), using the kit provided with the procedure (Megazyme, 2014). This method has been adopted by the AOAC (official method 996.11).

3.6.5.1 Preparation of samples

100 mg of sample (knife-milled to pass through a 0.5 mm sieve) was weighed into a centrifuge tube and the exact mass recorded. To each tube 5 mL 80% (v/v) aqueous ethanol was added and then incubated in an 85 °C water bath for 5 min. The contents of the tubes were mixed on a vortex stirrer, before adding an additional 5 mL 80% (v/v) aqueous ethanol. The tubes were then centrifuged at 1,800 xg for 10 min and the supernatant discarded. The pellet was re-suspended in 10 mL 80% (v/v) aqueous ethanol and mixed on a vortex mixer. The tubes were centrifuged again at 1,800 xg for 10 min and the supernatant carefully discarded.

A magnetic stirrer bar was added to each test tube and the tubes were placed in an ice/water bath over a magnetic stirrer. To each tube, 2.0 mL of 2 M potassium hydroxide (KOH) was added and vigorously stirred for 20 min. After the resistant starch was dissolved, 8.0 mL 1.2 M Na-acetate buffer, pH 3.8 was added to each tube, still stirring on magnetic stirrer. Immediately after, 0.1 mL thermostable α-amylase (1,600 U/mL on Ceralpha reagent at pH 5.0 and 40 °C; supplied in kit) and 0.1 mL amyloglucosidase (3,300 U/mL on soluble starch; supplied in kit) were added to each tube, and the contents mixed well. The tubes were placed in a 50 °C water bath for 30 min, with intermittent mixing on a vortex mixer throughout the incubation period.

After incubation the samples were processed in either one of two ways, depending on the percent starch present in the samples.

<u>STARCH CONTENT >10%</u>

All tubes were removed from the incubator and the entire contents quantitatively transferred to a 100 mL volumetric flask. A wash bottle containing deionised water was used to rinse the tube contents thoroughly

and the volume adjusted to 100 mL. The contents were mixed thoroughly and then an aliquot of this solution was centrifuged at 1,800 xg for 10 min. The supernatant was used for further analysis, with the final volume for calculations being 100 mL.

STARCH CONTENT 1-10%

All tubes were removed from the incubator, the contents mixed thoroughly and transferred to centrifuge tubes with no dilution. The tubes were then centrifuged at 1,800 xg for 10 min. The supernatant was used for further analysis, with the final volume used for calculations being 10.4 mL.

3.6.5.2 Measurement of glucose using GOPOD Reagent

Duplicate aliquots of 0.1 mL supernatant were transferred to labelled glass test tubes. To each test tube (including the reagent blank and glucose control tubes), 3.0 mL Glucose Determination Reagent (GOPOD Reagent) was added, and incubated in a 50 °C water bath for 20 min. GOPOD Reagent (supplied in kit) contained GOPOD Reagent enzymes (glucose oxidase, peroxidase and 4-aminoantipyrine), dissolved in GOPOD Reagent buffer (1 M potassium phosphate buffer, pH 7.4, 0.22 M ρ-hydroxybenzoic acid, and 0.4% (w/w) sodium azide).

All the tubes were removed from the water bath and the contents mixed using a vortex stirrer. The absorbance of all samples and standard solutions was measured using a spectrophotometer set at a wavelength of 510 nm. The reagent blank was used to zero the spectrophotometer before taking readings for the glucose controls and samples, ensuring the absorbance readings were in the range of 0.1 – 1.0 A.

3.6.5.3 Maize standard, reagent blank and glucose standards

Maize standard samples were prepared when the substrate samples were prepared, following the same procedure for the substrate samples.

The reagent blank and glucose control tubes were only prepared when the GOPOD Reagent was ready to be added to the sample tubes. The reagent blank tube contained 0.1 mL deionised water and 3.0 mL GOPOD Reagent in a glass test tube. The glucose control tubes (prepared in quadruplicate) contained 0.1 mL glucose standard (supplied in kit; 100 µg/0.1 mL) and 3.0 mL GOPOD Reagent in a glass test tube.

3.6.5.4 Analysis of samples

The starch concentration of the substrates was calculated using Equation 3.4 and Equation 3.5.

Equation 3.4

$$Starch\ \%\ w/w\ (as\ is) = \Delta A \times F \times \frac{FV}{0.1} \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180}$$

$$= \Delta A \times \frac{F}{W} \times FV \times 0.9$$

$where$:

$\Delta A =$ $Absorbance\ read\ against\ the\ reagent\ blank\ at\ 510\ nm$

$F =$ $\frac{100\ (\mu g\ of\ D-glucose)}{absorbance\ for\ 100\ \mu g\ of\ glucose}$ $(conversion\ from\ absorbance\ to\ \mu g)$

$FV =$ $Final\ volume\ (ie.equals\ 100\ mL\ or\ 10.4\ mL)$

$\frac{1}{1000} =$ $Conversion\ from\ \mu g\ to\ mg$

$\frac{100}{W} =$ $Factor\ to\ express\ ""starch""\ as\ a\ percentage\ of\ flour\ weight$

W = $Weight\ in\ mg$ ("as is" $basis$)$of\ the\ flour\ analysed$

$\frac{162}{180} =$ $Adjustment\ from\ free\ glucose\ to\ anhydro\ glucose\ (as\ occurs\ in\ starch)$

Equation 3.5

$$Starch\ \%\ w/w\ (dry\ wt.basis) = Starch\ \%\ w/w\ (as\ is) \times \left[\frac{100}{(100 - moisture\ content\ (\%\ w/w))}\right]$$

3.6.6 Total sugar content

Total sugar analysis was performed by a total acid hydrolysis method used by Saeman, Bubl & Harris (1945).

3.6.6.1 Preparation of samples

All starch was removed from the samples before the total acid hydrolysis was performed. To each test tube, containing 0.2 g of sample (knife-milled to pass through a 0.5 mm sieve), 0.4 mL 80% (v/v) aqueous ethanol was added and mixed. Immediately, 4 mL dimethyl sulphoxide (DMSO) was added and the contents of each tube mixed using a vortex mixer. All tubes were placed in a vigorously boiling water bath for 5 min. The tubes were then centrifuged at 4,696 xg for 10 min and the supernatant was removed. This process was repeated several times until all the starch had been removed. Once complete, the remaining substrate was transferred to an aluminium foil tray and dried overnight in an oven set at 105 °C. Once dry, the substrate was used for the total acid hydrolysis procedure.

For the total acid hydrolysis, 30 mg of each substrate was weighed into 50 mL Teflon cap tubes (Pyrex, UK) and 1.0 mL 12 M sulphuric acid was added. All tubes were incubated in a 37 °C water bath for 1 h. The tubes were then removed and 11.0 mL deionised water was added to each. The tubes were then placed in a 100 °C water bath for 2 h. Once complete, the tubes were removed and the hydrolysate was allowed to cool to room temperature. All samples were filtered using 0.2 µm pore size Whatman GD/X syringe filters (GF/C 25 mm filter diameter; Whatman International Ltd., UK), and diluted 1:100, ready for sugar analysis.

3.6.6.2 Preparation of sugar standard solution

A standard 'sugar stock solution' containing arabinose, galactose, glucose, and xylose was prepared and then diluted several times to obtain various solutions of known concentrations. To make the sugar stock solution, exactly 2.000 g of each sugar was weighed, dissolved in deionised water and then made up to 1.0 L. The sugar stock solution, of concentration 2 g/L, was serially diluted to final concentrations as shown in Table 3.5.

Table 3.5 Sugar standard solution preparation.

Dilution	Volume source (L)	Source	Volume deionised water (L)	Final conc. (g/L)
Dilution #1	0.500	Stock (2 g/L)	Up to 1.0 L	1.000
Dilution #2	0.500	Dilution #1	Up to 1.0 L	0.500
Dilution #3	0.500	Dilution #2	Up to 1.0 L	0.250
Dilution #4	0.500	Dilution #3	Up to 1.0 L	0.125

All sugar standard samples were filtered using 0.2 µm pore size Whatman GD/X syringe filters (GF/C 25 mm filter diameter; Whatman International Ltd., UK), and diluted 1:100 ready for sugar analysis.

3.6.6.3 Analysis of samples

The sugar content of the samples was quantified by high performance liquid chromatography (HPLC). The monosaccharides, arabinose, glucose, fructose, and xylose, were analysed using Dionex ICS-3000 Reagent-Free™ Ion Chromatography equipped with Dionex ICS-3000 system, electrochemical detection using ED 40 and computer controller. The CarboPac™ PA 20 column (3 x 150 mm; Dionex, USA) was used, with a column temperature of 30 °C. The solution used for the mobile phase was 10 mM NaOH, with a flow rate of 0.5 mL/min and an injection volume of 10 µL.

The peak area and retention time of each sugar in the standard sugar solution was recorded. The peak area for each sugar dilution was plotted

against the sugar concentration to draw a standard curve. The concentration of each sugar in the substrates was calculated by interpolation on the standard curve for each sugar.

3.6.7 Lignin content

Lignin analysis was performed using the acetyl bromide method (Fukushima & Hatfield, 2001).

3.6.7.1 Preparation of samples

100 mg of each substrate (knife-milled to pass through a 0.5 mm sieve) was weighed into a 50 mL Teflon cap tube (Pyrex, UK) and dissolved in 4.0 mL 25% (v/v) acetyl bromide in glacial acetic acid. All samples were incubated in a 50 °C water bath for 2 h, and then allowed to cool to room temperature. Once cool, the samples were diluted to 16 mL using glacial acetic acid (12 mL added) and centrifuged at 3,000 xg for 15 min to sediment.

From each sample, 0.5 mL of supernatant was transferred to a new tube and the following reagents were added: 2.5 mL glacial acetic acid, 1.5 mL 0.3 M NaOH, and 0.5 mL 0.5 M hydroxylamine HCl (laboratory grade). Finally, the total volume in each tube was made up to 10 mL with glacial acetic acid, as shown in Table 3.6.

Table 3.6 Reagents added to sample tubes after incubation.

Reagent	Volume (mL)
Supernatant	0.5
Glacial acetic acid	2.5
0.3 M NaOH	1.5
0.5 M hydroxylamine HCl	0.5
Sub-total	5.0
Glacial acetic acid to add	5.0
Total	10.0

The absorbance of all samples and standard solutions was measured using a spectrophotometer set at a wavelength of 280 nm. A quartz cuvette was

used for analysis in the spectrophotometer to avoid UV interference, and all analysis was performed in a fume hood. The reagent blank was used to zero the spectrophotometer before taking readings for the standard solutions and samples.

3.6.7.2 Reagent blank and lignin standard solutions

The reagent blank was prepared by mixing 0.2 mL dioxane with 0.5 mL 25 % acetyl bromide in glacial acetic acid. The tube was incubated at 50 °C for 30 min (added 1.5 h through incubation period of samples), and then allowed to cool down to room temperature.

A lignin standard stock solution was prepared by dissolving 10 mg of isolated lignin in 6.0 mL 80% (v/v) dioxane in a 50 mL Teflon cap tube (Pyrex, UK). The standard stock solution was diluted by pipetting 0.2 mL, 0.3 mL, 0.4 mL, 0.5 mL and 0.6 mL into 50 mL Teflon cap tubes (Pyrex, UK), each containing 0.5 mL 25% (v/v) acetyl bromide in glacial acetic acid. To prepare the reagent blank, 0.2 mL dioxane was mixed with 0.5 mL 25% (v/v) acetyl bromide in glacial acetic acid. All tubes were mixed thoroughly and incubated at 50 °C for 30 min (added 1.5 h through incubation period of samples), and then allowed to cool down to room temperature.

After cooling, 2.5 mL glacial acetic acid, 1.5 mL 0.3 M NaOH and 0.5 mL 0.5 M hydroxylamine HCl (laboratory grade) were added to each tube. Finally, the final volume in each tube was made up to 10 mL with glacial acetic acid. The total amounts of reagents added to each tube are shown in Table 3.7.

Table 3.7 Reagents added to standard lignin solutions.

Reagent	Volume added to each stock solution dilution (mL)				
Stock solution	0.2	0.3	0.4	0.5	0.6
25% acetyl bromide	0.5	0.5	0.5	0.5	0.5
Glacial acetic acid	2.5	2.5	2.5	2.5	2.5
0.3 M NaOH	1.5	1.5	1.5	1.5	1.5
0.5 M hydroxylamine HCl	0.5	0.5	0.5	0.5	0.5
Glacial acetic acid to add	4.8	4.7	4.6	4.5	4.4

3.6.7.3 Analysis of samples

The absorbance readings of the standard lignin solutions were plotted against their lignin concentrations to obtain a standard curve. The lignin concentration of the substrates was calculated by interpolation on the standard curve for lignin. The percent lignin was obtained using Equation 3.6.

Equation 3.6

$$Lignin\ (\%) = Lignin\ concentration \times \left(\frac{16\ mL}{0.5\ mL}\right) \times \left(\frac{1}{0.100g\ material}\right)$$

3.6.8 Lipid content

Following the AOAC official method 945.16 (AOAC, 2005b) lipid content of the substrates was analysed using a Soxtherm Fat Analyser (Gerhardt®) using petroleum ether as a solvent.

3.6.8.1 Preparation of samples

Up to 2 g of substrate (ball milled) was weighed onto an 11 cm qualitative grade filter paper, and the exact mass recorded to 0.1 mg. The filter paper was loosely folded to enclose the sample, and labelled with a pencil. Each filter paper was placed inside an extraction thimble, gently pushing it to the bottom of the thimble, and a plug of cotton wool placed on top.

Three anti-bump stones were added to each of the six glass extraction flasks. The mass of the flasks plus the stones was recorded, along with the number on the flask. The flasks, which had been oven-dried and cooled in

a desiccator before addition of the anti-bump stones, were returned to the desiccator until use.

3.6.8.2 Extraction of lipid using Gerhardt Soxtherm Fat Analyser

The Soxtherm hotplates were heated to 150 °C and the water flow was checked, ensuing it was 2.5 L/min or greater. Six glass flasks containing the anti-bump stones were placed into the large aluminium rack and 155 mL petroleum ether (40-60° fraction; laboratory grade) was added to each in a fume hood. The corresponding thimble, containing the substrate samples, was added to each glass flask, pushing firmly into place and ensuring the green "O" rings at the bottom of the flask were present and properly seated. The aluminium rack with the glass flasks was installed on the Soxtherm and the lipid extraction started when the hotplates had reached 150 °C.

Once all the solvent in the flasks had disappeared, the flasks were removed from the Soxtherm. The thimbles were removed from the flasks and the flasks were placed in an oven set at 100 – 103 °C for 1 h. The flasks were then transferred to a desiccator to cool to room temperature. Once cool, the flasks (containing the anti-bump stones and fat extract) were weighed and the mass recorded.

3.6.8.3 Analysis of samples

The mass of Soxhlet fat present (extract) was quantified as the weight of the glass flask after extraction and solvent recovery. The percent Soxhlet fat present in the substrates was calculated using Equation 3.7.

Equation 3.7

$$Soxhlet\ fat\ (\%) = \left(\frac{Mass\ of\ extract\ (g)}{Mass\ of\ sample\ (g)}\right) \times 100$$

3.7 SOLID STATE FERMENTATION

3.7.1 Preparation of substrates

For each condition and incubation period tested, 6 g substrate (knife-milled to pass through a 2.0 mm sieve, except Sago hampas which was used as is) was weighed and placed into a Duran bottle. A moisture content (MC) of 80% (w/v) was obtained with the addition of deionised water. Depending on the substrate used and its initial MC, this corresponded to a solid to liquid ratio ranging from 1:3.25 to 1:3.50 (w/v). The contents of the Duran bottles were autoclaved at 121 °C and 100 kPa (15 psi) above atmospheric pressure for 15 min, and then allowed to cool.

If required, the addition of nutrients – which included starch (0.0070 g/g substrate), yeast extract (YE; 0.0175 g/g substrate) and/or mineral solutions (MSI or MSII) – were added to the substrates before autoclaving. Either MSI or MSII was added instead of deionised water to adjust to 80% (w/v) MC.

3.7.2 Addition of spore suspensions and incubation

For cellulase production either *A. niger* N402 or *T. reesei* were used. For both fungi a spore concentration of 1.0×10^6 spores/g of dry substrate was added to each Duran bottle. For amylase production either *A. awamori* or *A. niger* N402 were used. For *A. awamori* a spore concentration of 4.0×10^6 spores/g of dry substrate was added to each bottle. For *A. niger* N402 a spore concentration of 1.0×10^6 spores/g of dry substrate was added to each bottle. The inoculum was distributed evenly throughout the substrate using a sterilised spatula, and then distributed evenly between three Petri dishes. After the addition of the spore suspension, all petri dishes were incubated in a static incubator at 28 °C for up to 7 d.

3.7.3 Extraction of fungal enzymes

3.7.3.1 Extraction for cellulase analysis

Extraction of fungal enzymes was performed using a method described by Pensupa *et al.* (2013). At the end of the incubation period, the fermented mash from each Petri dish was transferred into a blender. For each 1 g of fermented mash added, 15 mL of 0.05 M Na-citrate buffer, pH 4.8 was added to the blender. The mixture was blended for 10 seconds on 'high' power, and then transferred to a beaker and stirred at 300 rpm on a magnetic stirrer, at 4 °C for 30 min. The mechanical force of the blitzing helped break apart the fungal spores, allowing the enzymes to be released into the buffer solution during stirring, improving enzyme extraction.

The mixture was then transferred to centrifuge tubes and centrifuged at 4,696 xg for 10 min. The clear supernatant (fungal extract) was removed and analysed for enzyme production (described in Section 3.9). The remaining biomass (fungal and undigested substrate) was oven-dried at 70 °C overnight and then stored at 4 °C for future use/analysis.

3.7.3.2 Extraction for glucoamylase analysis

Extraction of fungal enzymes was performed using a modified method described by Du *et al.* (2008). At the end of the incubation period, the fermented mash from each Petri dish was transferred into a beaker and 6.0 mL 0.2 M Na-acetate buffer, pH 4.5 was added per gram of substrate (not deionised water as done by Du *et al*. (2008)). The mixture was stirred at 300 rpm on a magnetic stirrer, at 4 °C (not room temperature as done by Du *et al.* (2008)) for 60 min, extracting the enzymes from the spores.

The mixture was then transferred to centrifuge tubes and centrifuged at 4,696 xg for 10 min. The clear supernatant (fungal extract) was removed

and analysed for glucoamylase and glucose production (described in Section 3.9). The remaining biomass (fungal and undigested substrate) was oven-dried at 70 °C overnight and then stored at 4 °C for future use and/or analysis.

3.8 SUBMERGED FERMENTATION

3.8.1 Preparation of substrates

For each condition and incubation time tested, 6 g substrate (knife-milled to pass through a 2.0 mm sieve, except Sago hampas which was used as is) was weighed and placed into a 250 mL glass conical flask. A moisture content of 80% (w/v) was obtained with the addition of deionised water. The contents of the flasks were autoclaved at 121 °C and 100 kPa (15 psi) above atmospheric pressure for 15 min, and then allowed to cool. Afterwards, autoclaved deionised water was added to the flasks to adjust the total volume of liquid added to 100 mL (6% w/v), and the pH of the mixture was adjusted.

If required, the addition of nutrients – which included starch (0.0070 g/g substrate), yeast extract (YE; 0.0175 g/g substrate) and/or mineral solutions (MSI or MSII) – were added to the conical flasks before autoclaving. When MSI or MSII were added, the amount added was equal to the total amount added in the SSF procedure, and the remaining volume (up to 100 mL working volume) was achieved using deionised water.

3.8.2 Addition of spore suspensions and incubation

For cellulase production either *A. niger* N402 or *T. reesei* were used. For both fungi a spore concentration of 1.0×10^6 spores/g of dry substrate was added to each flask. For amylase production either *A. awamori* or *A.*

niger N402 were used. For *A. awamori* a spore concentration of 4.0 x 10^6 spores/g of dry substrate was added to each bottle. For *A. niger* N402 a spore concentration of 1.0 x 10^6 spores/g of dry substrate was added to each bottle. After the addition of the spore suspension all flasks were recapped and incubated in a shaking incubator, set at 250 rpm and 28 °C, for up to 7 d.

3.8.3 Enzyme extraction

At the end of the incubation period, the mixture in each conical flask was transferred to 50 mL centrifuge tubes and centrifuged at 4,696 xg for 10 min. The clear supernatant (fungal extract) was removed and analysed for enzyme and glucose production (described in Section 3.9). The remaining biomass (fungal and undigested substrate) was oven-dried at 70 °C overnight and then stored at 4 °C for future use/analysis.

3.9 ENZYME AND GLUCOSE ASSAYS

3.9.1 Cellulase assay (filter paper units)

Filter paper cellulase activity was analysed according to National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedure for cellulase activity, prepared for publication by Adney & Baker (1996). The substrate used was a 50 mg Whatman No. 1 filter paper strip.

3.9.1.1 Preparation of samples

The sample tubes were prepared by adding a rolled filter paper strip and 1.0 mL 0.05 M Na-citrate buffer, pH 4.8 to each test tube, ensuring the filter paper was saturated. The tubes were then placed in a 50 °C water bath for 5 min to equilibrate the contents of the tubes. After 5 min, 0.5 mL of the corresponding enzyme extract was added to the sample tubes. The

reaction mixtures were incubated at 50 °C for exactly 60 min and at the end of the incubation period the tubes were removed from the water bath.

The released reducing sugar (as glucose) was measured using the dinitrosalicylic (DNS) method (Miller, 1959). The enzyme reaction was terminated by adding 3.0 mL 3,5-Dinitrosalicylic acid (DNS) solution to each test tube, mixing well. The tubes were then placed in a boiling water bath for exactly 5 min and then transferred to an ice-water bath until cooled. The contents of each test tube were mixed using a vortex mixer and the absorbance was read using a spectrophotometer set at a wavelength of 540 nm. The reagent blank was used to zero the spectrophotometer before taking readings for the standards and samples, ensuring all absorbance readings were in the range of 0.1 – 1.0 A.

3.9.1.2 Preparation of reagent blank and controls

All substrate control, reagent blank and experimental control tubes were prepared at the same time as the experimental tubes. The reagent blank tube was prepared by adding 1.5 mL 0.05 M Na-citrate buffer to a glass test tube. The substrate control tube was prepared by adding a rolled filter paper strip to a glass test tube plus 1.5 mL 0.05 M Na-citrate buffer, pH 4.8, ensuring the filter paper was saturated. The experimental control tubes were prepared by adding 1.0 mL 0.05 M Na-citrate buffer, pH 4.8 to each tube, plus the addition of 0.5 mL enzyme extract after the 5 min equilibration period.

3.9.1.3 Preparation of glucose standards

Glucose standards were prepared from a 10.0 mg/mL working stock solution of anhydrous glucose as shown in Table 3.8. Glucose standard test tubes were prepared by adding 0.5 mL of each of the below glucose

dilutions to 1.0 mL 0.05 M Na-citrate buffer, pH 4.8, incubating the tubes along with control and sample tubes.

Table 3.8 Preparation of glucose standard solutions.

Addition working stock solution (mL)	Addition 0.05M Na-citrate buffer, pH 4.8 (mL)	Final concentration (mg/mL)	Final concentration (mg/0.5 mL)
1.0	0.0	10.00	5.00
1.0	0.5	6.70	3.35
1.0	1.0	5.00	2.50
1.0	2.0	3.30	1.65
1.0	4.0	2.00	1.00

3.9.1.4 Analysis of samples

The absorbance readings of the standard glucose solutions were plotted against their concentrations to obtain a standard curve. The glucose concentration of the substrates was calculated by interpolation on the standard curve, after subtraction of the enzyme and substrate controls.

The cellulase activity (U/mL) was then calculated and converted to U/g of dry weight substrate using Equation 3.8 and Equation 3.9 (Adney & Baker, 1996).

Equation 3.8

$$Cellulase\ activity\ (FP\ U/mL) = \left(\frac{0.37}{Concentration\ of\ enzyme\ that\ releases\ 2.0\ mg\ glucose}\right)$$

$where$:

$$0.37\ \mu mol/minute - mL = \left(\frac{(2.0\ mg\ gluocse/0.18016\ mg\ glucose/\mu mol)}{(0.5\ mL\ enzyme\ x\ 60\ minutes)}\right)$$

Equation 3.9

$$Cellulase\ activity\ (FPU/g) = \left(\frac{Cellulase\ activity\ (U/mL)\ x\ Total\ volume\ of\ fungal\ extract\ (mL)}{Dry\ weight\ of\ substrate\ used\ in\ SSF\ (g)}\right)$$

3.9.2 Carboxymethyl cellulase (endo-β-1,4-glucanase) assay

Carboxymethyl cellulase (endo-β-1,4-glucanase) was determined by the method of the International Union of Pure and Applied Chemistry, prepared for publication by Ghose (1987). The substrate solution used was

2% (w/v) carboxymethyl cellulose (CMC), dissolved in 0.05 M Na-citrate buffer, pH 4.8.

3.9.2.1 Preparation of samples

The sample tubes were prepared by adding 0.5 mL substrate solution to each glass test tube. The tubes were then placed in a 50 °C water bath for 5 min to equilibrate the contents of the tubes. After 5 min, 0.5 mL of the corresponding enzyme extract was added to the sample tubes. The contents of the tubes were mixed well and incubated at 50 °C for 30 min, and then removed from the water bath.

The released reducing sugar (as glucose) was measured using the dinitrosalicylic (DNS) method (Miller, 1959). The enzyme reaction was terminated by adding 3.0 mL 3,5-Dinitrosalicylic acid (DNS) solution to each test tube, mixing well. The tubes were then placed in a boiling water bath for exactly 5 min and then transferred to an ice-water bath until cooled. The contents of each test tube were mixed using a vortex mixer and the absorbance was read using a spectrophotometer set at a wavelength of 540 nm. The reagent blank was used to zero the spectrophotometer before taking readings for the standards and samples, ensuring all absorbance readings were in the range of 0.1 – 1.0 A.

3.9.2.2 Preparation of reagent blank and controls

All substrate control, reagent blank and experimental control tubes were prepared at the same time as the experimental tubes. The reagent blank was prepared by adding 1.0 mL 0.05 M Na-citrate buffer, pH 4.8 to a glass test tube. The substrate control tube was prepared by adding 0.5 mL substrate solution and 0.5 mL 0.05 M Na-citrate buffer, pH 4.8 to a glass test tube. The experimental control tubes were prepared by adding 0.5 mL

0.05 M Na-citrate buffer, pH 4.8 to a glass test tube, plus the addition of 0.5 mL enzyme extract after the 5 min equilibration period.

3.9.2.3 Preparation of glucose standards

Glucose standards were prepared from a 2.0 mg/mL working stock solution of anhydrous glucose as shown in Table 3.9.

Table 3.9 Preparation of glucose standard solutions.

Addition working stock solution (mL)	Addition 0.05M Na-citrate buffer, pH 4.8 (mL)	Final concentration (mg/mL)	Final concentration (mg/0.5 mL)
1.0	0.0	2.00	1.00
1.0	0.5	1.33	0.67
1.0	1.0	1.00	0.50
1.0	3.0	0.50	0.25

Glucose standard test tubes were prepared by adding 0.5 mL of each of the above glucose dilutions to 0.5 mL 0.05 M Na-citrate buffer, pH 4.8, incubating the tubes along with the control and experimental tubes.

3.9.2.4 Analysis of samples

The absorbance readings of the standard glucose solutions were plotted against their known concentrations to obtain a standard curve. The glucose concentration of the substrates was calculated by interpolation on the standard curve, after subtraction of the enzyme and substrate controls. One unit (U) of enzyme was defined as the amount of enzyme required to release 1 µmol of glucose from carboxymethyl cellulose per min, where 1 µmol of glucose is equal to 0.18 mg glucose. The activity (U/mL) was calculated and then converted to U/g of dry weight substrate using Equation 3.10 and Equation 3.11 (Ghose, 1987).

Equation 3.10

$$Endoglucanase\ activity\ (U/mL) = \left(\frac{0.185}{Concentration\ of\ enzyme\ that\ releases\ 0.5\ mg\ glucose}\right)$$

$where$:

$$0.185\ \mu mol/minute - mL = \left(\frac{(0.5\ mg\ gluocse/0.18016\ mg\ glucose/\mu mol)}{(0.5\ mL\ enzyme\ x\ 30\ minutes)}\right)$$

Equation 3.11

$$Endoglucanase\ activity\ (U/g) = \left(\frac{Activity\ (U/mL)\ x\ Total\ volume\ of\ fungal\ extract\ (mL)}{Dry\ weight\ of\ substrate\ used\ in\ SSF\ (g)}\right)$$

3.9.3 Avicelase (exo-1,4-β-glucanase) assay

Avicelase (exo-1,4-β-glucanase) activity was determined by a modified method of the International Union of Pure and Applied Chemistry, prepared for publication by Ghose (1987). The substrate used was 1% (w/v) Avicel, dissolved in 0.05 M Na-citrate buffer, pH 4.8.

3.9.3.1 Preparation of samples

The sample tubes were prepared by adding 0.5 mL substrate solution to each glass test tube. The tubes were placed in a 50 °C water bath for 5 min to equilibrate the contents of the tubes. After 5 min, 0.5 mL of the corresponding enzyme extract was added to the sample tubes. The contents of the tubes were mixed well and incubated at 50 °C for 30 min, and then removed from the water bath.

The released reducing sugar (as glucose) was measured using the dinitrosalicylic (DNS) method (Miller, 1959). The enzyme reaction was terminated by adding 3.0 mL 3,5-Dinitrosalicylic acid (DNS) solution to each test tube, mixing well. The tubes were then placed in a vigorously boiling water bath for exactly 5 min and then transferred to an ice-water bath until cooled. The contents of each test tube were mixed using a vortex mixer and the absorbance was read using a spectrophotometer at a

wavelength of 540 nm. The reagent blank was used to zero the spectrophotometer before taking readings for the standards and samples, ensuring all absorbance readings were in the range of 0.1 – 1.0 A.

3.9.3.2 Preparation of reagent blank and controls

All substrate control, reagent blank and experimental control tubes were prepared at the same time as the experimental tubes. The reagent blank was prepared by adding 1.0 mL 0.05 M Na-citrate buffer, pH 4.8 to a glass test tube. The substrate control tube was prepared by adding 0.5 mL substrate solution and 0.5 mL 0.05 M Na-citrate buffer, pH 4.8 to a glass test tube. The experimental control tubes were prepared by adding 0.5 mL 0.05 M Na-citrate buffer, pH 4.8 to a glass test tube, along with 0.5 mL enzyme extract after the equilibration period.

3.9.3.3 Preparation of glucose standards

Glucose standards were from a 2.0 mg/mL working stock solution of anhydrous glucose as shown in Table 3.10.

Table 3.10 Preparation of glucose standard solutions.

Addition working stock solution (mL)	Addition 0.05M Na-citrate buffer, pH 4.8 (mL)	Final concentration (mg/mL)	Final concentration (mg/0.5 mL)
1.0	0.0	2.00	1.00
1.0	0.5	1.33	0.67
1.0	1.0	1.00	0.50
1.0	3.0	0.50	0.25

Glucose standard test tubes were prepared by adding 0.5 mL of each of the above glucose dilutions to 0.5 mL 0.05 M Na-citrate buffer, pH 4.8, incubating the tubes along with the control and experimental tubes.

3.9.3.4 Analysis of samples

The absorbance readings of the standard glucose solutions were plotted against their known concentrations to obtain a standard curve. The glucose concentration of the substrates was calculated by interpolation on the standard curve, after subtraction of the enzyme and substrate controls. One unit of enzyme was defined as the amount of enzyme required to release 1 µmol of glucose from Avicel per min, where 1 µmol of glucose is equal to 0.18 mg glucose. The activity (U/mL) was calculated and then converted to U/g of dry weight substrate using Equation 3.12 and Equation 3.13 (Ghose, 1987).

Equation 3.12

$$Exoglucanase\ activity\ (U/mL) = \left(\frac{0.185}{Concentration\ of\ enzyme\ that\ releases\ 0.5\ mg\ glucose}\right)$$

$where$:

$$0.185\ \mu mol/minute - mL = \left(\frac{(0.5\ mg\ gluocse/0.18016\ mg\ glucose/\mu mol)}{(0.5\ mL\ enzyme\ x\ 30\ minutes)}\right)$$

Equation 3.13

$$Exoglucanase\ activity\ (U/g) = \left(\frac{Activity\ (U/mL)\ x\ Total\ volume\ of\ fungal\ extract\ (mL)}{Dry\ weight\ of\ substrate\ used\ in\ SSF\ (g)}\right)$$

3.9.4 β-glucosidase assay

β-glucosidase activity was determined by the method of Herr (1979). The substrate solution used was 2 mM ρ-nitrophenol-β-glucoside (ρNPG), dissolved in 0.05 M Na-acetate buffer, pH 5.0.

3.9.4.1 Preparation of samples

The samples tubes were prepared by adding 1.0 mL ρNPG substrate solution to each glass test tube. The tubes were placed in a 50 °C water bath for 5 min to equilibrate the contents of the tubes. After 5 min, 0.1 mL of the corresponding enzyme extract was added to the sample tubes. The

contents of the test tubes were mixed well and placed back in the 50 °C water bath for exactly 5 min. At the end of the incubation period, the tubes were removed from the water bath. The enzyme reaction was terminated by adding 2.0 mL 1.0 M sodium carbonate (Na_2CO_3) to each test tube and the contents mixed using a vortex mixer.

The absorbance of the samples was read using a spectrophotometer set at a wavelength of 405 nm. The reagent blank was used to zero the spectrophotometer before taking readings for the standard solutions and samples. The absorbance readings of the standard ρ-nitrophenol solutions were plotted against their known concentrations to obtain a standard curve.

3.9.4.2 Preparation of reagent blank and controls

All substrate control, reagent blank and experimental control tubes were prepared at the same time as the experimental tubes. The reagent blank was prepared by adding 1.1 mL 0.05 M Na-acetate buffer, pH 5.0 to a glass test tube. The substrate control tube was prepared by adding 1.0 mL substrate solution and 0.1 mL 0.05 M Na-acetate buffer, pH 5.0 to a glass test tube. The experimental control tubes were prepared by adding 1.0 mL 0.05 M Na-acetate buffer, pH 5.0 to a glass test tube, plus the addition of 0.1 mL enzyme extract after the equilibration period.

3.9.4.3 Preparation of ρ-nitrophenol standards

Standards of ρ-nitrophenol were prepared from a working stock solution of 2 mg/mL ρ-nitrophenol as shown in Table 3.11. Test tubes of ρ-nitrophenol standards were prepared by adding 0.1 mL of each of the below ρ-nitrophenol dilutions to 1.0 mL 0.05 M Na-acetate buffer, pH 5.0, incubating along with the control and experimental tubes.

Table 3.11 Preparation of ρ-nitrophenol standard solutions.

Addition working stock solution (mL)	Addition 0.05M Na-acetate buffer, pH 5.0 (mL)	Final concentration (mg/mL)	Final concentration (mg/0.1 mL)
1.0	0.0	2.00	0.20
1.0	1.0	1.00	0.10
1.0	2.0	0.66	0.066
1.0	3.0	0.50	0.05
1.0	7.0	0.25	0.025

3.9.4.4 Analysis of samples

The ρ-nitrophenol concentration of the substrates was calculated by interpolation on the standard curve, after subtraction of the enzyme and substrate controls. One unit of enzyme was defined as the amount of enzyme required to release 1 μmol of ρ-nitrophenol from ρ-nitrophenol-β-glucoside per min, where 1 μmol of ρ-nitrophenol is equal to 0.139 mg ρ-nitrophenol. The activity (U/mL) was calculated and then converted to U/g of dry weight substrate using Equation 3.14.

Equation 3.14

$$\beta - glucosidase\ activity\ (U/g) = \left(\frac{Activity\ (U/mL) x\ Total\ volume\ of\ fungal\ extract\ (mL)}{Dry\ weight\ of\ substrate\ used\ in\ SSF\ (g)}\right)$$

3.9.5 Glucoamylase assay

Glucoamylase activity was assayed using a modified method described by Koutinas *et al*. (2001), with the modifications being the assay time and the gelatinisation of the starch suspension. The substrate solution used was 2% (w/v) soluble starch suspension, dissolved in 0.2 M Na-acetate buffer, pH 4.5. The suspension was placed on a magnetic hot plate, set at 85 °C and 300 rpm, for 20 min to gelatinise the starch.

3.9.5.1 Preparation of samples

The sample tubes were prepared by adding 0.5 mL substrate solution to each test tube. The tubes, and the enzyme solution, were then placed in a 60 °C water bath for 5 min to equilibrate the contents of the tubes. After 5 min, 0.5 mL of the corresponding enzyme extract was added to the sample test tube. The contents of the test tubes were mixed well and incubated at 60 °C for up to 60 min. At the end of the incubation period, the tubes were removed from the water bath.

The released reducing sugar (as glucose) was measured using the dinitrosalicylic (DNS) method (Miller, 1959). The enzyme reaction was terminated by adding 3.0 mL 3,5-dinitrosalicylic acid (DNS) solution to each test tube, mixing well. The tubes were then placed in a vigorously boiling water bath for exactly 5 min and then transferred to an ice-water bath until cooled. The contents of each tube were mixed using a vortex mixer and the absorbance was read using a spectrophotometer at a wavelength of 540 nm. The reagent blank was used to zero the spectrophotometer before taking readings for the standards and samples, ensuring all absorbance readings were in the range of 0.1 – 1.0 A.

3.9.5.2 Preparation of reagent blank and controls

All substrate control, reagent blank and experimental control tubes were prepared at the same time as the experimental tubes. The reagent blank was prepared by adding 1.0 mL 0.2 M Na-acetate buffer, pH 4.5 to a glass test tube. The substrate control tube was prepared by adding 0.5 mL substrate solution and 0.5 mL 0.2 M Na-acetate buffer, pH 4.5 to a glass test tube. The experimental control tubes were prepared by adding 0.5 mL 0.2 M Na-acetate buffer, pH 4.5 to a glass test tube, plus the addition of 0.5 mL enzyme extract after the equilibration period.

3.9.5.3 Preparation of glucose standards

Glucose standards were prepared from a 10 mg/mL working stock solution of anhydrous glucose as shown in Table 3.12.

Table 3.12 Preparation of glucose standard solutions.

Addition working stock solution (mL)	Addition 0.2M Na-acetate buffer, pH 4.5 (mL)	Final concentration (mg/mL)	Final concentration (mg/0.5 mL)
1.0	0.0	10.00	5.00
1.0	0.5	6.70	3.35
1.0	1.0	5.00	2.50
1.0	2.0	3.30	1.65
1.0	4.0	2.00	1.00

Glucose standard test tubes were prepared by adding 0.5 mL of each of the above glucose dilutions to 0.5 mL 0.2 M Na-acetate buffer, pH 4.5, incubating the tubes along with the control and experimental tubes.

3.9.5.4 Analysis of samples

The absorbance readings of the standard glucose solutions were plotted against their known concentrations to obtain a standard curve. The glucose concentration of the substrates was calculated by interpolation on the standard curve, after subtraction of the enzyme and substrate controls. One unit (U) of glucoamylase was defined as the amount of enzyme required to generate 1 mg of reducing sugars equivalent to glucose per min under the assay conditions. The activity (U/mL) was calculated and then converted to U/g of dry weight substrate using Equation 3.15.

Equation 3.15

$$Glucoamylase\ activity\ (U/g) = \left(\frac{Activity\ (U/mL)\ x\ Total\ volume\ of\ fungal\ extract\ (mL)}{Dry\ weight\ of\ substrate\ used\ in\ SSF\ (g)}\right)$$

3.10 STATISTICAL ANALYSIS

All samples were performed at least in triplicate, and analysis was performed using Microsoft Excel, 2016. Statistical analysis was performed using IBM SPSS Statistics, version 24. One-way ANOVA test and Tukey Post Hoc multiple comparisons tests or two-way ANOVA tests were performed to compare the mean values, using a confidence interval of $p<0.05$ to designate a significant difference.

CHAPTER 4 COMPOSITIONAL ANALYSIS

4.1 INTRODUCTION

In this chapter, characterisation of the underutilised crops was investigated to determine the basic composition of these substrates. In order to understand what products could potentially be produced from using these materials, we need to know what's in them, in particular the starch, hemicellulose, cellulose and lignin content. Since not much information can be found in the literature, basic information on the properties of these crops is important information.

The crops used in this research were chosen after discussions with the sponsors of this research, Crops for the Future (CFF), and are shown in Table 3.1. The characteristics measured included moisture, elemental (carbon, hydrogen, sulphur, nitrogen), ash, protein, starch, total sugar, lignin and lipid contents. The methodologies for analysing these characteristics are described in Chapter 3. All characteristics were calculated on a dry weight basis.

4.2 RESULTS

4.2.1 Moisture content

The moisture content was determined by the standard biomass analytical method "Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples" provided by the National Renewable Energy Laboratory (NREL), prepared for publication by Sluiter *et al*. (2008a), as described in Section 3.6.1.

The moisture content of the crops ranged between 10 – 16 % w/w. Nipa fronds had the lowest moisture content (10.19 ± 0.62 % w/w) and

Leucaena had the highest moisture content (16.18 ± 0.14 % w/w), as shown in Figure 4.1.

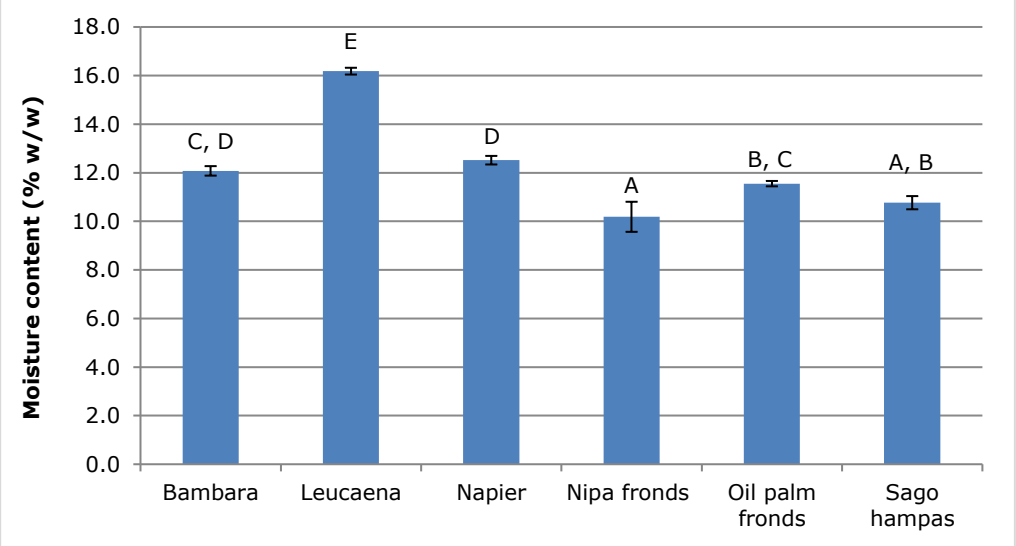


Figure 4.1 Moisture content for samples of underutilised crops. The results are the mean + SD (n=3). Bars with different letters are significantly different (p<0.05).

4.2.2 Ash content

The amount of inorganic material (ash) in the biomass samples was determined by the standard biomass analytical method "Determination of Ash in Biomass" provided by the National Renewable Energy Laboratory (NREL), prepared for publication by Sluiter *et al*. (2008b), as described in Section 3.6.2.

The ash content of the crops ranged between 23 – 130 mg/g substrate, giving a total percentage ranging from 2 – 13 % of the dry weight value, as shown in Figure 4.2. There were significant differences between all six crops. Bambara (12.85 ± 0.15 % w/w) and Leucaena (13.07 ± 0.16 % w/w) had the highest ash contents and these were similar (p=0.352); all other substrates had ash contents that were significantly different (p<0.05) with Sago hampas having the lowest ash content (2.33 ± 0.02 % w/w).

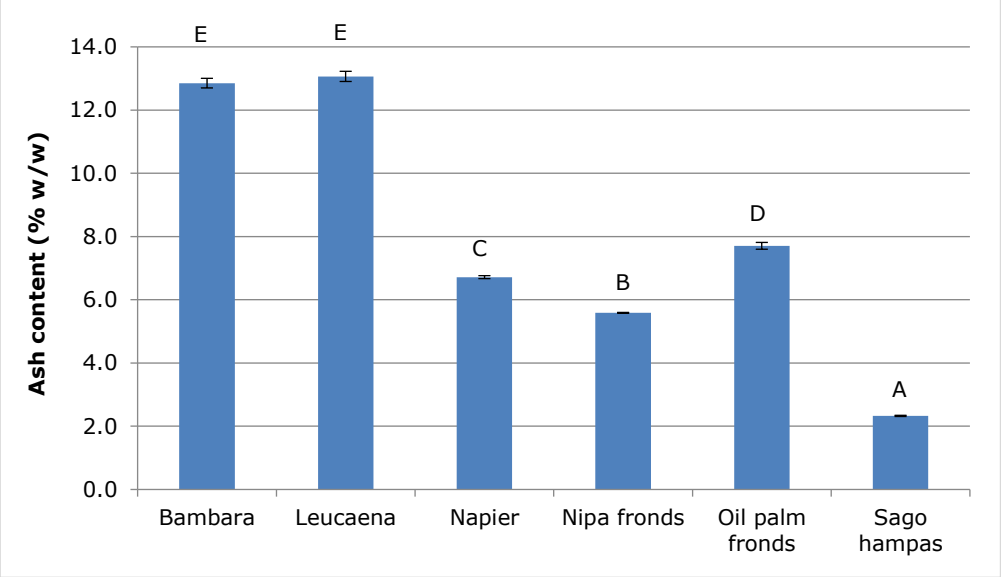


Figure 4.2 Ash content (dry weight basis) for samples of underutilised crops. The results are the mean + SD (n=3). Bars with different letters are significantly different ($p<0.05$).

4.2.3 Elemental content

The elemental analysis of the biomass samples was determined by the advanced combustion method, adopted by the Association of Official Analytical Chemists (official method 972.43 (2006)), using a Flash 2000 Organic Elemental Analyser (Thermo Scientific, USA), as described in Section 3.6.3.

4.2.3.1 Nitrogen, carbon, hydrogen and sulphur content

The total elemental content of nitrogen, carbon, hydrogen and sulphur in the crops ranged between 51 - 62 % of the dry weight value. Sago hampas (51.76 ± 0.24 % w/w) and Bambara (52.86 ± 0.31 % w/w) had the lowest total elemental content and these were statistically similar ($p=0.212$). Napier (54.26 ± 0.32 % w/w) was statistically similar to Bambara ($p=0.092$), but not to Sago hampas. Oil palm fronds (54.90 ± 0.39 % w/w) was statistically similar to Napier ($p=0.654$), but not to Bambara or Sago hampas. Leucaena had the highest content (61.92 ±

0.06 % w/w) and this was statistically different from Nipa fronds (57.57 ± 0.30 % w/w), which had the second highest content ($p<0.05$).

The nitrogen content of the crops ranged from 0 – 5 % (dry weight basis), with Sago hampas having the lowest nitrogen content (0.19 ± 0.00 % w/w) and Leucaena having the highest nitrogen content (4.90 ± 0.01 % w/w), as shown in Figure 4.3. There were significant differences between all the six crops ($p<0.05$). Napier (1.35 ± 0.01 % w/w) and Nipa fronds (1.49 ± 0.11 % w/w) had nitrogen contents that were similar ($p=0.323$); all other crops had nitrogen contents that were significantly different ($p<0.05$).

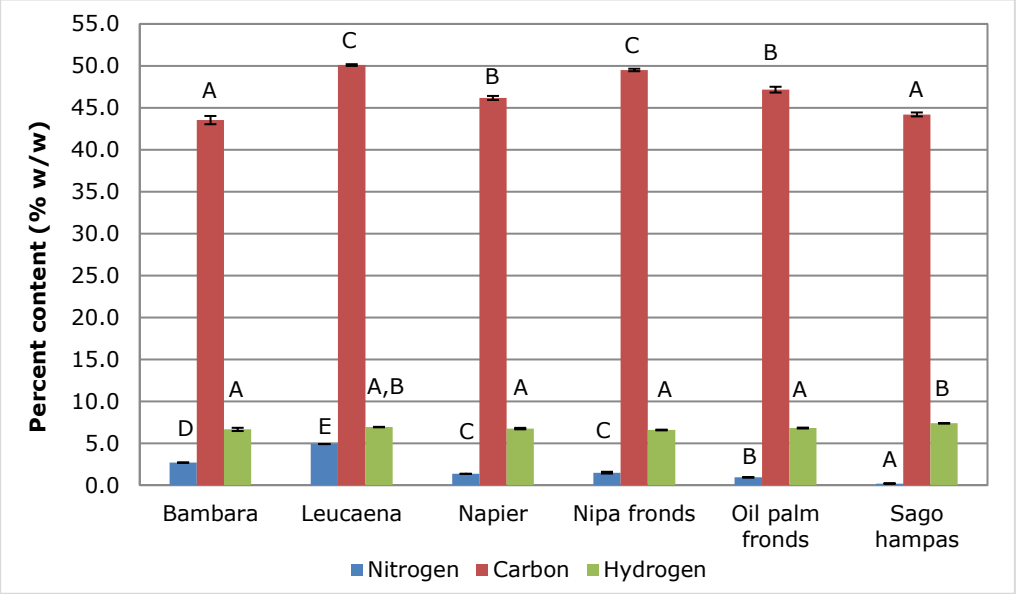


Figure 4.3 Nitrogen, carbon, hydrogen and sulphur present in samples of underutilised crops. The results are the mean + SD (n=3). Bars with different letters are significantly different ($p<0.05$).

The carbon content of the crops ranged from 43 – 50 % (dry weight basis) and there were statistically significant differences between the six crops ($p<0.05$) (Figure 4.3). Bambara had the lowest carbon content (43.53 ± 0.49 % w/w), and this was similar to Sago hampas (44.21 ± 0.23 % w/w), ($p=0605$). Leucaena had the highest carbon content (50.10 ± 0.10 % w/w) and this was similar to Nipa fronds (49.51 ± 0.15 % w/w),

(p=0.710). Napier (46.17 ± 0.24 % w/w) and Oil palm fronds (47.16 ± 0.34 % w/w) had similar carbon contents (p=0.283).

The hydrogen content of the crops ranged from 6.5 – 7.4 % of the dry weight value (Figure 4.3) with Nipa fronds having the lowest hydrogen content (6.57 ± 0.04 % w/w) and Sago hampas having the highest hydrogen content (7.37 ± 0.01 % w/w). Sago hampas had a hydrogen content that was significantly different from all the other crops ($p<0.05$), except Leucaena which had a hydrogen content of 6.92 ± 0.03 % w/w (p=0.086). All the other crops, including Leucaena, had hydrogen contents that were similar (p=0.206).

The sulphur was undetectable for all the underutilised crops analysed (data not shown).

4.2.4 Protein content

The protein content of the crop samples was measured by a combustion measurement performed according to AOAC official method 992.23 (AOAC, 2005a). The combustion method performed was that carried out for the elemental analysis and the protein content was calculated using the N x 6.25 conversion factor, as described in Section 3.6.4.

Since the nitrogen content of the crops was used to calculate the protein content, this followed a similar trend to the nitrogen content of the crops. The protein content ranged from 1 – 31 % (dry weight basis), with Sago hampas having the lowest protein content (1.17 ± 0.01 % w/w) and Leucaena having the highest protein content (30.62 ± 0.08 % w/w), as shown in Figure 4.4. There were significant differences between all the six crops ($p<0.05$). Napier (8.41 ± 0.07 % w/w) and Nipa fronds (9.30 ± 0.66 % w/w) had protein contents that were similar (p=0.323); all other substrates had protein contents that were significantly different ($p<0.05$).

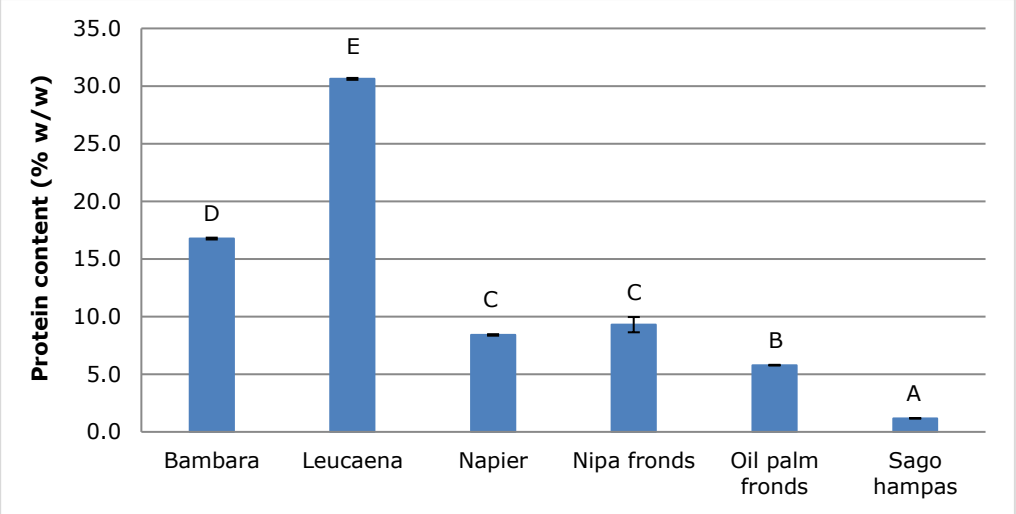


Figure 4.4 Protein content of samples of underutilised crops. The results are the mean + SD (n=3). Bars with different letters are significantly different ($p<0.05$).

4.2.5 Starch content

The starch content was analysed using the Megazyme total starch assay procedure (amyloglucosidase/α-amylase method), as described in 3.6.5.

Sago hampas (not shown in Figure 4.5) had a very high starch content of 51.91 ± 3.23 % w/w and this was significantly higher than that from all the other crops ($p<0.05$). A much smaller amount of starch (0 – 3 % of the dry weight value) was found in Bambara, Leucaena, Napier, Nipa fronds and Oil palm fronds (Figure 4.5), with significant differences between the five crops. Nipa fronds (0.38 ± 0.06 % w/w), Leucaena (0.54 ± 0.13 % w/w) and Oil palm fronds (0.56 ± 0.24 % w/w) had very little starch and these were similar ($p=0.987$). Bambara (2.66 ± 0.67 % w/w) and Napier (2.67 ± 0.11 % w/w) had slightly higher starch contents and these were similar ($p=1.000$).

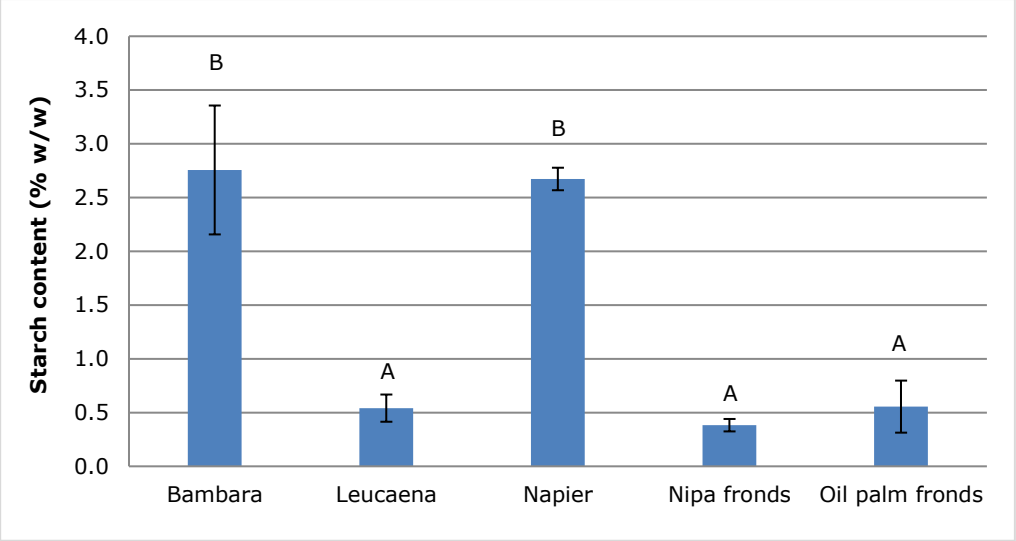


Figure 4.5 Starch content in samples of underutilised crops. The results are the mean + SD (n=3). Bars with different letters are significantly different; Sago hampas (not shown on graph) was significantly different ($p<0.05$).

4.2.6 Total sugar content

Total sugar analysis was performed using a total acid hydrolysis method outlined by Saeman, Bubl & Harris (1945), and quantified by high performance liquid chromatography (HPLC), as described in Section 3.6.6.

The total sugar content of the crops ranged from 113 – 628 mg/g substrate (dry weight basis), with Leucaena having the lowest total sugar content (113.3 ± 11.9 mg/g substrate) and non-starchy Sago hampas having the highest total sugar content (628.0 ± 29.0 mg/g substrate). There were significant differences between all the crops. Nipa fronds (274.5 ± 19.9 mg/g substrate) and Bambara (322.4 ± 18.5 mg/g substrate) had total sugar contents that were similar ($p=0.234$); all the other crops were significantly different ($p<0.05$).

The arabinose content of the crops ranged from 0 – 32 mg/g substrate of the dry weight value. Arabinose was found in only Napier (26.3 ± 1.3 mg/g substrate) and starchy Sago hampas (32.3 ± 1.9 mg/g substrate), and these two were significantly different ($p<0.05$) (Figure 4.6).

The glucose content of the crops varied from 84 – 451 mg/g substrate of the dry weight value, and there were statistically significant differences between all the crops ($p<0.05$), as shown in Figure 4.6. Leucaena had the lowest glucose concentration (84.9 ± 9.4 mg/g substrate). Starchy Sago hampas (450.7 ± 6.3 mg/g substrate) and non-starchy Sago hampas (415.5 ± 18.8 mg/g substrate) had the highest glucose concentration and these were similar ($p=0.131$). Oil palm fronds (279.6 ± 40.1 mg/g substrate), Bambara (254.0 ± 12.6 mg/g substrate) and Napier (256.4 ± 23.4 mg/g substrate) all had similar glucose concentrations ($p=0.485$).

The xylose content of the crops varied from 28 – 214 mg/g substrate of the dry weight value, and there were statistically significant differences between all the crops ($p<0.05$), as shown in Figure 4.6. Leucaena had the lowest xylose concentration (28.3 ± 3.7 mg/g substrate); non-starchy Sago hampas (213.5 ± 11.1 mg/g substrate) and Napier (198.1 ± 8.4 mg/g substrate) had the highest xylose concentration and these were similar ($p=0.391$). Sago hampas starchy (66.0 ± 1.4 mg/g substrate), Bambara (68.4 ± 6.0 mg/g substrate) and Nipa fronds (81.5 ± 6.1 mg/g substrate) all had similar xylose concentrations ($p=0.385$).

There was no galactose detected in any of the underutilised crops; however, this could mean that there was either little or no galactose present or that the galactose had been degraded during the total acid hydrolysis.

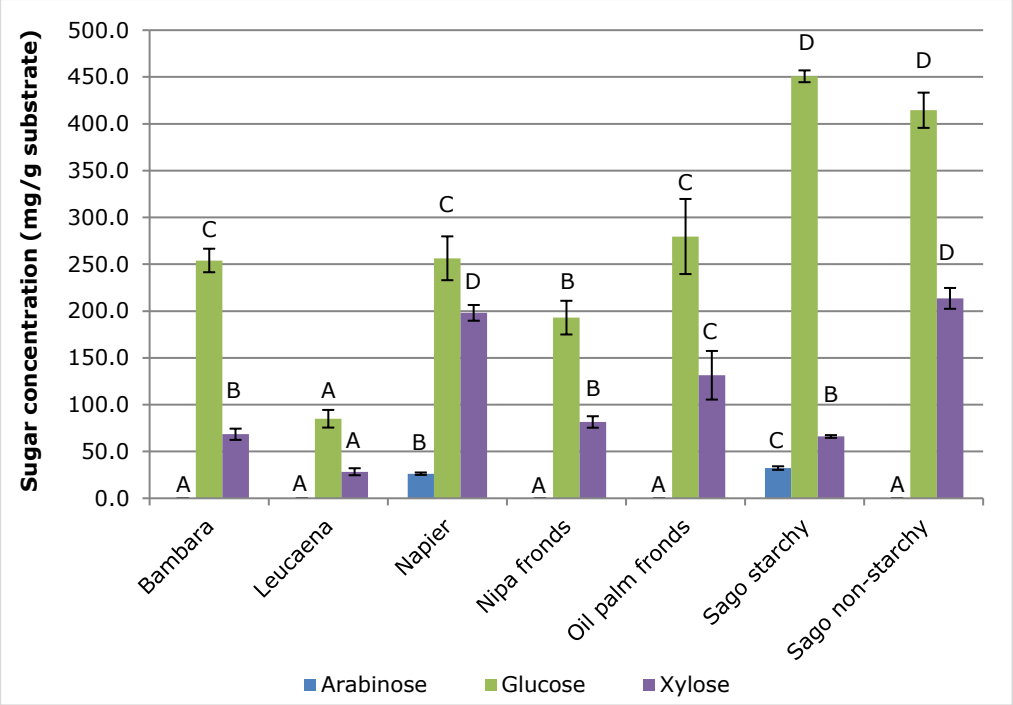


Figure 4.6 Sugar concentration for samples of underutilised crops. The results are the mean + SD (n=5). Bars with different letters are significantly different ($p<0.05$).

The mole percent for the sugar content of the underutilised crops is shown in Figure 4.7, and there were statistically significant differences for arabinose, glucose and xylose between the underutilised crops. The arabinose mole percent ranged from 0 – 6.81 %, with no arabinose detected in Bambara, Leucaena, Nipa fronds, Oil palm fronds and non-starchy Sago hampas. Napier (6.01 ± 0.44 %) and starchy Sago hampas (6.81 ± 0.32 %) were significantly different ($p<0.05$). The glucose mole percent of the crops ranged from 48 – 79 %. There were significant differences between all the six crops ($p<0.05$), with Napier (48.71 ± 1.59 %) having the lowest mole percent and starchy Sago hampas (79.26 ± 0.24 %) having the highest mole percent. The xylose mole percent for the crops ranged from 13 – 45 %, and there were significant differences between all the six crops ($p<0.05$). Starchy Sago hampas (13.93 ± 0.44 % w/w) had the lowest mole percent and Napier (45.27 ± 1.26 % w/w) had the highest mole percent. Nipa fronds (33.69 ± 2.48 % w/w) and Oil

palm fronds (35.93 ± 1.77 % w/w) had similar xylose mole percent (p=0.308); Oil palm fronds and non-starchy Sago hampas (38.20 ± 0.63 % w/w) had similar xylose mole percent (p=0.296).

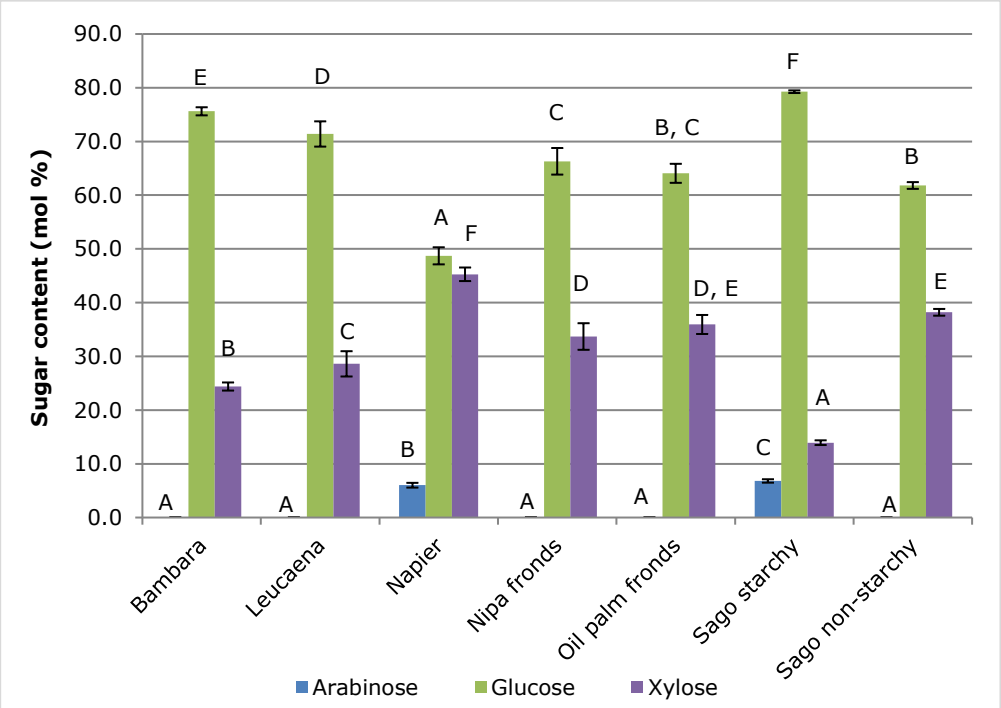


Figure 4.7 Sugar content for samples of underutilised crops. The results are the mean + SD (n=5). Bars with different letters are significantly different (p<0.05).

In general, the glucose-xylose ratio for the underutilised crops ranged from roughly 6:1 to 1:1. Bambara and Leucaena (roughly 3:1), had similar glucose-xylose ratios, as did Nipa fronds and Oil palm fronds (2:1). Napier had a 1:1 glucose-xylose ratio. Starchy Sago hampas (6:1) and non-starchy Sago hampas (1.6:1) had very different ratios.

4.2.7 Lignin content

The lignin content of the biomass samples was performed using the acetyl bromide method (Fukushima & Hatfield, 2001), as described in Section 3.6.7.

The lignin content of the six crops ranged between 0 – 30 % of the dry weight value (Figure 4.8), with significant statistical differences between

all the six crops ($p<0.05$). Nipa fronds (30.16 ± 4.11 % w/w) and Oil palm fronds (25.64 ± 1.06 % w/w) had the highest lignin contents and these were similar ($p=0.166$); Sago hampas had either no lignin present or an extremely small amount of lignin present that was hard to measure using the acetyl bromide method.

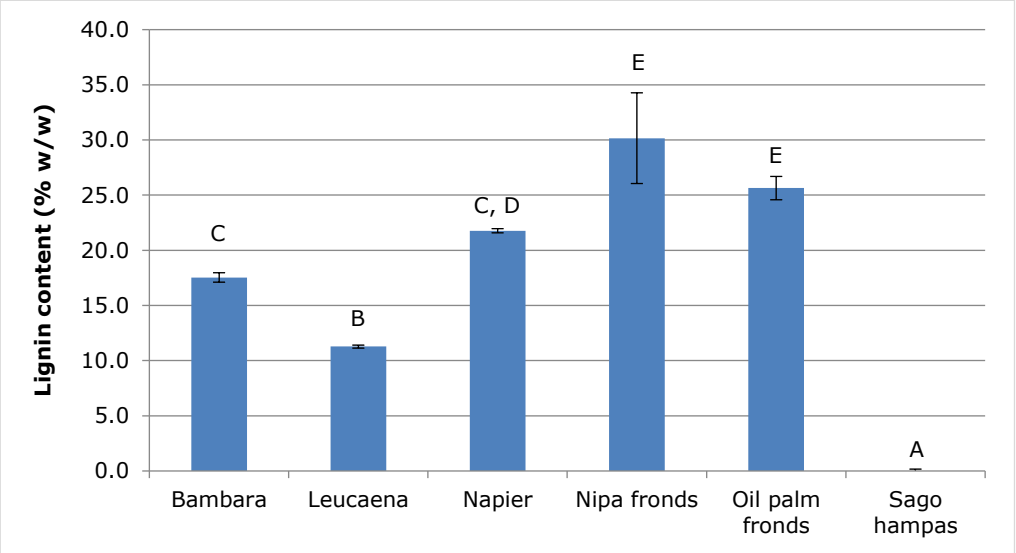


Figure 4.8 Lignin content (dry weight basis) for samples of underutilised crops. The results are the mean + SD (n=3). Bars with different letters are significantly different ($p<0.05$).

4.2.8 Lipid content

The lipid content of the biomass samples was determined by the AOAC official method 945.16 (AOAC, 2005b) using a Soxtherm Fat Analyser (Gerhardt®) using petroleum ether as a solvent, as described in Section 3.6.8.

The lipid content of the underutilised crop samples ranged from 0.3 - 31 mg/g substrate, accounting for up to 3.1 % of the total dry weight of the sample (Figure 4.9). There were significant differences between all the six crops ($p<0.05$), with Leucaena having the highest lipid content (3.09 ± 0.05 % w/w) and Sago hampas having an extremely small amount of lipid (0.03 ± 0.01 % w/w).

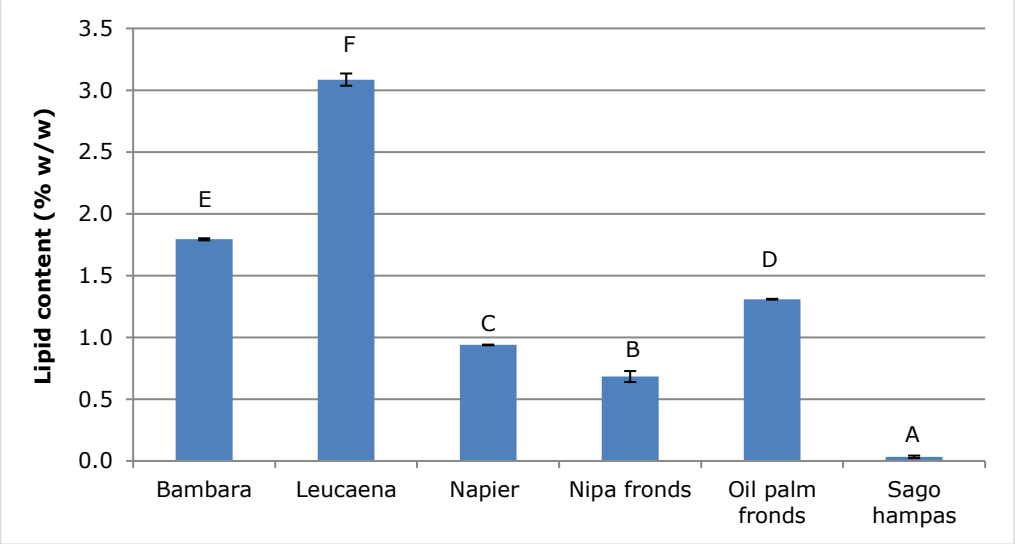


Figure 4.9 Lipid content (dry weight basis) in samples of underutilised crops. The results are the mean + SD (n=3). Bars with different letters are significantly different ($p<0.05$).

4.2.9 Mass balance

The mass balance for the crops ranged from 69.92 – 110.34 % w/w (dry weight basis), as shown in Table 4.1. Leucaena had the lowest total, accounting for only 69.92 ± 1.75 % (w/w) of the total dry weight value, with protein accounting for much of this at 30.62 ± 0.08 % (w/w). Sago hampas had the highest total, accounting for 110.34 ± 4.15 % (w/w) of the total value. Based on the analyses performed, polysaccharides (starch, cellulose, hemicellulose) accounted for 106.81 % (w/w) of this total.

Table 4.1 Mass balance for underutilised crops analysed.

Sample	Percent (% w/w, dry weight basis)						
	Ash	Protein	Starch	Lignin	Lipid	Total sugar	Total
Bambara	12.85	16.77	2.76	17.54	1.79	32.24	83.95
Leucaena	13.07	30.62	0.54	11.28	3.09	11.33	69.92
Napier	6.72	8.41	2.67	21.77	0.94	48.07	88.58
Nipa fronds	5.59	9.30	0.38	30.16	0.68	27.45	73.56
Oil palm fronds	7.71	5.79	0.56	25.64	1.31	41.10	82.10
Sago hampas	2.33	1.17	51.91	0.00	0.03	54.90	110.34

Leucaena may have been under reported due to the low lignin content and the low cellulose and hemicellulose content (total sugar). The low sugar content could have been due to the acid hydrolysis conditions not being suitable for the hydrolysis of the cellulose/hemicellulose in the biomass, or the biomass could have contained polymers made up of other sugar monomers not detected in this method. For the Sago hampas, potentially not all the starch was removed before performing the total acid hydrolysis. This could have resulted in a higher value than actually present for the cellulose and hemicellulose, leading to over-reporting on the mass balance. In general, there may have been other components that were not measured in all the underutilised crops. For example, waxes may not have been fully accounted for and therefore not reported in the mass balance calculations.

CHAPTER 5 PRODUCTION OF CELLULASES

5.1 INTRODUCTION

Biological pretreatment of cellulosic biomass has been used as an alternative to physical, chemical and physiochemical pretreatment methods as it is usually operated under milder conditions, requiring less energy input and producing less inhibitors and a more environmentally friendly waste stream. However, it requires a long retention time and this can result in the loss of sugars used for fungal growth, making it unfavourable compared with the other methods.

During bioethanol or biochemical production, the cost of enzymes for the enzymatic hydrolysis step is one of the major barriers to an economically viable process. Therefore, the on-site production of enzymes is an attractive option, especially if they are produced using cheap carbohydrate sources, such as agricultural waste. For example, wheat straw, corn stover, potato waste and sugarcane bagasse have all been used as substrates.

The production of enzymes can be carried out in both solid state fermentation and submerged fermentation. Although submerged fermentation is the preferred method, because it is easier to control the culture conditions for maximum enzyme production, solid state fermentation has many advantages – such as low energy inputs, water consumption and operational cost, easier downstream processing, and is closer to the fungus' natural environment. However, many conditions can affect the enzyme yield and therefore lignocellulosic conversion, including factors such as moisture content, substrate, nitrogen source and ratio to carbon, depth of culture, transfer of gases, temperature and pH (Bon & Ferrara, 2007; Kumar *et al*., 2008).

As a result, many different fungal strains, as well as the effect of different conditions on cellulase production have previously been investigated. For example, a broad range of moisture contents, ranging from around 50% - 95%, have been examined as well as incubation periods ranging from 7 d – 150 d (Xu *et al*., 2010; Zeng *et al*., 2011). Aeration, addition of various nutrients, pH, and temperature have also been investigated.

In this chapter, several underutilised crops were investigated for use as substrates for the production of cellulases during biological pretreatment by two fungal strains. The underutilised crops were initially screened to determine which crop(s) had the highest potential as a substrate for fungal cellulase production during solid state fermentation (SSF) and submerged fermentation (SmF) procedures. The two different fungi used were *Aspergillus niger* and *Trichoderma reesei*. These fungal strains have been shown to have high cellulase production (Mrudula & Murugammal, 2011); *T. reesei* can produce high levels of endoglucanases and exoglucanases, and *A. niger* can produce high levels of β-glucosidase and endoglucanases (Verardi *et al*., 2012). Therefore, these strains were chosen to determine whether they were effective strains for these specific substrates. Other factors examined in this chapter include incubation period, addition of starch, addition of a nitrogen source (as yeast extract (YE)), as well as the addition of different mineral elements.

Several conditions were not optimised as these had been previously optimised in the lab by other PhD students, Dr Nattha Pensupa and Dr Jwan Abdullah Al-Dabbagh. A crop moisture content (MC) of 80% (w/v) was used for all SSF experiments, an incubation temperature of 28 °C was used for all experiments, both SSF and SmF, and a rate of 300 rpm was used for all SmF incubations. If added, the quantity of starch, yeast extract and minerals added remained the same. For the addition of

minerals, two different mineral solutions were examined, mineral solution I (MSI) and mineral solution II (MSII). The composition of these nutrients is described in Section 3.4. The addition of these nutrients and/or mineral solutions was based on results reported by Pensupa *et al*. (2013), which showed that the addition of starch, YE and mineral solution increased cellulolytic activity by *A. niger* N402 when cultured on wheat straw. These conditions were investigated to determine if they were also optimum for the crops used in this research.

5.2 RESULTS

5.2.1 Growth of fungus on underutilised crops

The underutilised crops were first screened to determine if the microorganisms, *A. niger* and *T. reesei*, could utilise the crops as substrates and grow on them during the solid state fermentation process.

The fungi were grown on PDA for 7 days and the spores were collected and used in the SSF process. The moisture content of the underutilised crops was adjusted to 80% (w/v) with the addition of deionised water and no extra nutrients were added. The SSF process commenced with the addition of fungal spores, as described in Section 3.7, and the growth of the fungus was observed over the 5 d incubation period. These conditions were designated as the baseline conditions.

The initial moisture content of the crops was used to calculate the solid-to-liquid ratio required to obtain the 80% (w/v) MC required for the SSF process (Table 5.1).

Table 5.1 Initial moisture content and final moisture content of substrates for SSF process.

	Initial moisture content (% w/v)	Solid : liquid ratio (w/v)	Final moisture content (% w/v)
Bambara	12.08 ± 0.20	1 : 3.50	80.46
Leucaena	16.18 ± 0.14	1 : 3.25	80.28
Napier	12.52 ± 0.18	1 : 3.50	80.56
Nipa fronds	10.19 ± 0.62	1 : 3.50	80.04
Oil palm fronds	11.55 ± 0.11	1 : 3.50	80.34
Sago hampas	10.77 ± 0.27	1 : 3.50	80.17

The growth of *T. reesei* and *A. niger* mycelium on the underutilised crops over the 5 d incubation period was non-uniform. Spore formation could be seen after 24 h incubation and the number of spores increased over the culture period; however, the rate of growth varied between the crops. Figure 5.1 shows the growth of *A. niger* on Sago hampas and the growth of *T. reesei* on Oil palm fronds after 5 d incubation. In both cases, the fungus had completely spread over the substrate within the 5 d period. Based on these observations it was concluded that the fungi were able to utilise all the crops as substrates and grew well under the solid state fermentation conditions (80% (w/v) MC, 28 °C in a dark, static incubator).

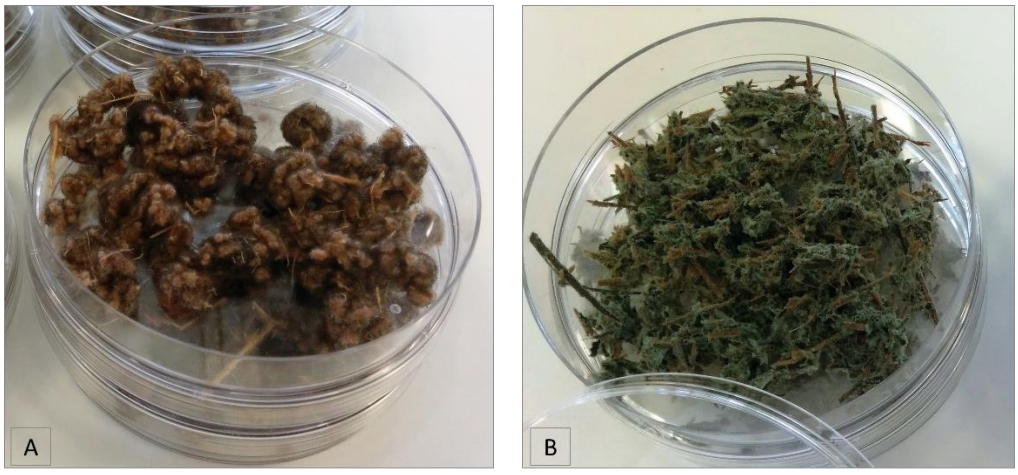


Figure 5.1 Growth of fungi on underutilised crops during solid state fermentation (28 °C in a static incubator for 5 d; substrates at 80% (w/v) MC). A: growth of *A. niger* on Sago hampas; B: growth of *T. reesei* on Oil palm fronds.

5.2.2 Initial screening of crops, using solid state fermentation

Once it was determined that the fungi could utilise the underutilised crops as substrates, the crops were then used as substrates for the production of cellulases. The crops were screened to determine whether fungal cellulases could be produced and if so, the level of activity that could be obtained for each crop during the SSF process.

During the initial screening the microorganism *A. niger* was used, and the fungal cellulase activity under the baseline conditions (spores grown on PDA; substrates at 80% (w/v) MC with no additional nutrients; 5 d incubation period) was investigated. The SSF process commenced with the addition of *A. niger* spores and the samples were analysed after the 5 d incubation period, as described in Section 3.7.

Figure 5.2 shows the production of cellulases by *A. niger* during the SSF process, under the baseline conditions. The fungal cellulase activity ranged from 1 – 17 FPU/g substrate (dry weight basis) and there were statistically significant differences between the crops ($p<0.05$).

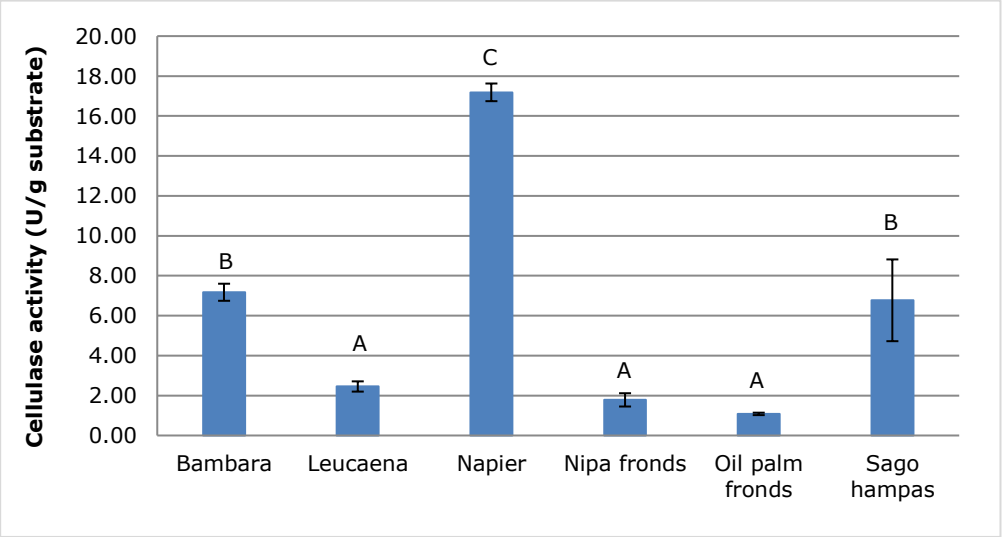


Figure 5.2 Cellulase activity recovered during SSF, using underutilised crops as substrates (fermentation by *A. niger* at 28 °C in a static incubator for 5 d incubation; substrates at 80% (w/v) MC). The results are the mean + SD (n=3). Bars with different letters are significantly different ($p<0.05$).

The highest cellulase activity was obtained using Napier as a substrate (17.18 ± 0.44 FPU/g substrate dry weight), and this was significantly different from all other crops ($p<0.05$). Using Bambara as a substrate resulted in the second highest cellulase activity (7.17 ± 0.43 FPU/g substrate), and this was statistically similar to Sago hampas (6.77 ± 2.05 FPU/g substrate), ($p=0.997$). The lowest cellulase activity was reported with the use of Oil palm fronds (1.08 ± 0.06 FPU/g substrate) and this was similar to both Nipa fronds (1.78 ± 0.33 FPU/g substrate) and Leucaena (2.45 ± 0.26 FPU/g substrate), ($p=0.646$).

Based on these results, the use of all the crops as substrates resulted in cellulase production; however, there was a large range of activity with very little activity for some crops (Nipa, Leucaena and Oil palm fronds) and a much higher activity for others (Napier). Based on these results, Napier seemed to be more suited as a substrate for the production of cellulases by *A. niger* under the designated SSF conditions.

5.2.3 Optimisation of SSF conditions

It has been reported that there are many factors which can affect cellulase activity production during the SSF process. Some of these factors include the microorganism used, composition of the substrate, temperature, pH, moisture content, addition of nutrients and the incubation time. In this investigation, the incubation period and the addition of nutrients were studied to determine their effect on the cellulase activity during the SSF process. Several different nutrients were added and these included the addition of starch (0.0070 g/g substrate), a nitrogen source (in the form of yeast extract (0.0175 g/g substrate)), and inorganic minerals (in the form of a mineral solution, MSI).

During the initial screening of the crops, the use of Napier grass as a substrate resulted in the highest cellulase activity. Therefore, it was used as the choice crop for the optimisation of conditions for the SSF process. The nutrients were added to the substrates before they were autoclaved. The MSI was added instead of deionised water to obtain an 80% (w/v) MC of the substrates. The SSF process commenced with the addition of *A. niger* spores (grown on PDA) and samples were taken at 1, 3, 5 and 7 d incubation periods, as described in Section 3.7.

In the first round of optimisation the effect of the addition of starch, YE, and MSI on cellulase production was compared to the baseline conditions (addition of no nutrients). Additionally, starch, YE and MSI were added separately to the Napier to determine their individual effect on the production of cellulases. These results are shown in Figure 5.3.

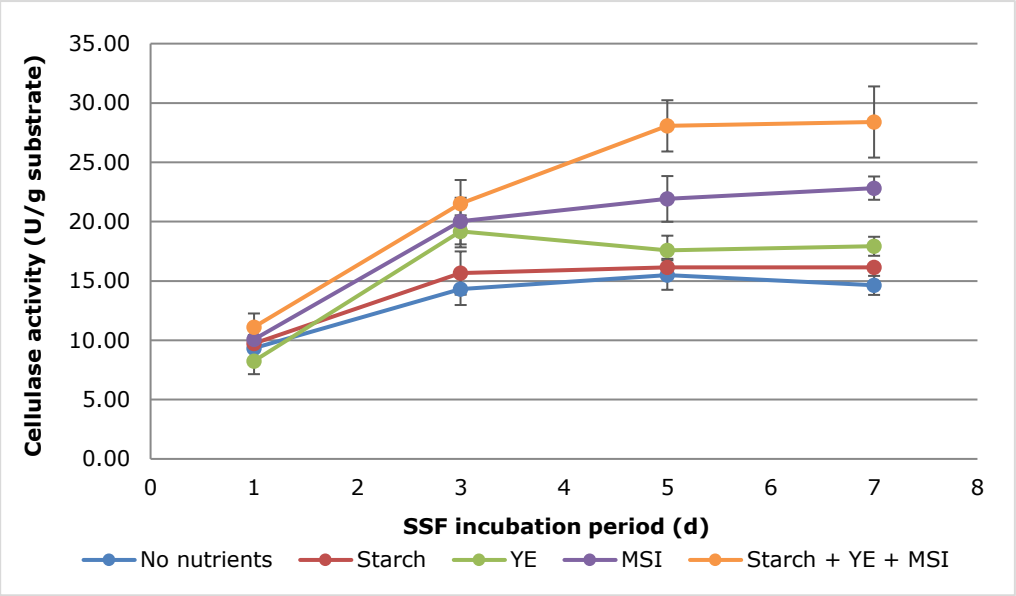


Figure 5.3 Effect of different nutrients on cellulase production by *A. niger* during SSF process (28 °C in a static incubator for up to 7 d incubation), compared to baseline conditions (no nutrients; addition of deionised water to 80% (w/v) MC). Nutrients added to the Napier included 0.0070 g/g starch, 0.0175 g/g YE and/or MSI to 80% (w/v) MC. The results are the mean + SD of each data set (n=3).

The initial (day 1) cellulase activity for all the conditions ranged between 8 – 11 FPU/g substrate and there was no significant difference between the

conditions at this point (p=0.868). Over the 7 d incubation period the cellulase activity increased for all the conditions; however, the production rate varied between the conditions. All four experiments with the addition of nutrients increased the cellulase activity when compared with the baseline conditions; however, not all increases were significant.

The cellulase activity for the baseline conditions (addition of no nutrients) increased slightly between 1 d (9.33 ± 0.55 FPU/g substrate) and 3 d (14.32 ± 2.79 FPU/g substrate), before plateauing. There were no significant differences between the cellulase activities for the 3, 5 and 7 d incubation periods (p=1.000). The cellulase activity after 5 d incubation (15.49 ± 1.07 FPU/g) was similar to the activity obtained in the previous experiment for Napier on day 5 under similar conditions (17.18 ± 0.44 FPU/g).

The rate of increase in cellulase activity was greatest with the addition of all three nutrients (starch, YE and MSI). This increased between the 1 d incubation period (11.11 ± 1.15 FPU/g substrate) and the 5 d incubation period (28.07 ± 2.16 FPU/g substrate), and there were significant differences between the 1 d, 3 d and 5 d cellulase activities ($p<0.05$). After 5 d, the cellulase activity began to plateau, resulting in a similar cellulase activity after 7 d incubation (28.39 ± 3.00 FPU/g substrate) (p=1.000). These values corresponded to an increase in cellulase activity of 19.1% (1 d), 50.3% (3 d), 81.2% (5 d) and 94.2% (7 d) when compared with the cellulase activities for the baseline conditions on the respective days.

When comparing the activity with the other experiments, the 3 d cellulase activity (21.52 ± 1.99 FPU/g substrate) was statistically different from the 3 d baseline conditions (p=0.002) and the 3 d addition of starch experiment (p=0.023); but it was similar to the cellulase activity for the 3

d MSI experiment (20.05 ± 1.96 FPU/g substrate; p=1.000) and the 3 d YE experiment (19.18 ± 1.35 FPU/g substrate; p=0.985). The 5 d and 7 d cellulase activities were significantly different from the cellulase activities for all the other experiments on the respective days ($p<0.05$).

The experiments with the addition of either starch, YE or MSI followed a similar trend to the baseline conditions - an increase in cellulase activity between 1 d and 3 d incubation periods, before beginning to plateau. The addition of MSI to the Napier resulted in the second highest cellulase activity by *A. niger*. This increased from 10.07 ± 0.06 FPU/g on day 1 to 20.05 ± 1.96 FPU/g substrate on day 3, before plateauing to 22.82 ± 0.98 FPU/g substrate on day 7. Besides the 1 d cellulase activity, all the other cellulase activities were significantly different from those for the baseline conditions on the respective days (p=0.024, p=0.006, p=0.0001 respectively). When compared to the addition of starch experiments, the cellulase activity was similar on day 3 (p=0.225) but significantly different after 5 d and 7 d incubation periods (p=0.022, p=0.003 respectively). However, the 3 d, 5 d and 7 d cellulase activities were similar to the cellulase activities for the addition of YE experiments on the respective days (p=1.000, p=0.238, p=0.103 respectively).

The experiment with the addition of starch reached a cellulase activity of 16.14 ± 0.72 FPU/g substrate (5 d). However, the cellulase activity for each incubation period was statistically similar to the cellulase activity for the baseline conditions for each respective day (p=1.000 for each incubation period). The same was true for the experiment with the addition of YE, which reached a cellulase activity of 19.18 ± 1.35 FPU/g substrate (3 d). This was statistically similar to the 3 d baseline cellulase activity (p=0.110). The cellulase activities for the 5 d YE and 7 d YE experiments

were also similar to the baseline conditions on the respective days (p=0.992; p=0.694 respectively).

Although the addition of only starch or YE separately did not significantly increase the cellulase activities from the baseline conditions, they did have an effect on the cellulase activity when added together with the MSI. This is inferred as the addition of all three nutrients resulted in a significantly higher cellulase activity when compared with the effect of the addition of MSI only. In order to determine if the addition of two out of the three nutrients would have an effect on the cellulase production a second round of optimisation experiments were run.

During these optimisation experiments the addition of starch, YE and MSI as well as the baseline condition experiments were redone. Additionally, three more experiments were run with the addition of only two out of the three nutrients (starch and YE; starch and MSI; YE and MSI). All experiments were incubated for 1, 3, 5, and 7 d. These results are shown in Figure 5.4.

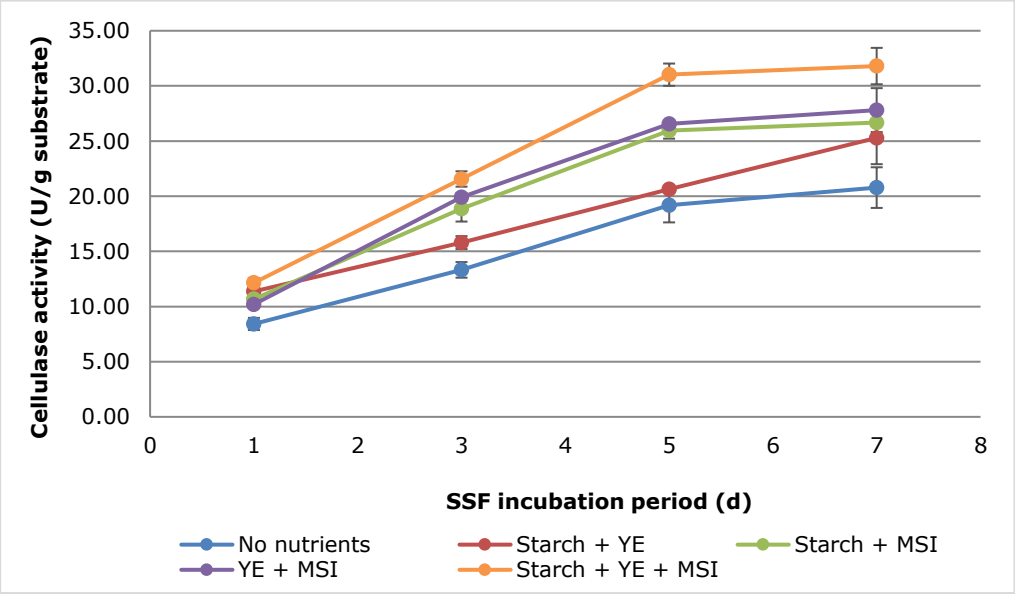


Figure 5.4 Effect of different nutrients on cellulase production by *A. niger* during SSF (28 °C in a static incubator for up to 7 d), compared to baseline conditions (no nutrients; addition of deionised water to 80% (w/v) MC). Nutrients added to the Napier included 0.0070 g/g starch, 0.0175 g/g YE and/or MSI to 80% (w/v) MC. The results are the mean + SD (n=3).

The initial (1 d) cellulase activity for all the conditions ranged between 8 – 12 FPU/g substrate and there was no significant difference between the conditions at this point ($p=0.161$). As with the first round of optimisation, the cellulase activity increased over the 7 d incubation period for all the conditions; however, the production rate varied between the conditions.

The cellulase activity for the baseline conditions (addition of no nutrients) increased between day 1 (8.42 ± 0.55 FPU/g substrate) and day 5 (19.18 ± 1.55 FPU/g substrate), before beginning to plateau. There were significant differences in cellulase activity for 1 d, 3 d and 5 d incubation periods ($p<0.05$); however, there were no differences between the 5 d and 7 d cellulase activities ($p=0.996$). The cellulase activity after 5 d incubation was slightly higher than the activity obtained in the previous optimisation experiment (16.14 ± 0.72 FPU/g). The addition of nutrients increased the cellulase activity when compared with the baseline conditions; however, not all increases were statistically significant.

The addition of starch and YE to Napier resulted in a cellulase activity that increased over the 7 d incubation period from 11.37 ± 0.50 FPU/g substrate (1 d) to 25.29 ± 2.39 FPU/g substrate (7 d), with statistical differences between all four incubation periods ($p<0.05$). When compared with the baseline conditions, the cellulase activity was similar on all the incubation days, except day 7 ($p=0.031$).

The addition of starch and MSI and the addition of YE and MSI followed similar cellulase activity trends. For the starch plus MSI experiment, the activity increased from 10.68 ± 0.71 FPU/g substrate (1 d) to 25.94 ± 0.72 FPU/g substrate (5 d), before beginning to plateau to 26.68 ± 1.34 FPU/g substrate (7 d). For the YE plus MSI experiment, the activity increased from 10.20 ± 0.31 FPU/g substrate (1 d) to 26.55 ± 0.32 FPU/g substrate (5 d), before beginning to plateau (27.81 ± 1.99 FPU/g

substrate on day 7). There were no differences between the results obtained for the two different experiments on the respective days ($p=1.000$ for all four incubation periods). When compared with the baseline conditions, the cellulase activities for both experiments were significantly different on all the incubation days ($p<0.05$).

The addition of all three nutrients (starch, YE and MSI) resulted in the highest cellulase production over the 7 d incubation period. The activity increased from 12.15 ± 0.43 FPU/g substrate (1 d) to 31.02 ± 1.01 FPU/g substrate (5 d), and the cellulase activities for all three incubation periods (1 d, 3 d and 5 d) were significantly different ($p<0.05$). The activity then began to plateau, reaching a final cellulase activity level of 31.80 ± 1.65 FPU/g (day 7) and this was similar to the 5 d cellulase activity ($p=1.000$). These values corresponded to an increase in cellulase activity of 44.3% (1 d), 61.9% (3 d), 61.7% (5 d) and 53.0% (7 d) when compared with the cellulase activities for the baseline conditions on the respective days. When comparing the cellulase activity to the other experiments, the 3 d activity was similar to the 3 d cellulase activity for both the addition of starch and MSI and the addition of YE and MSI experiments ($p=0.680$, $p=0.994$ respectively). However, the 5 d cellulase activity was significantly different from the cellulase activities for all the other 5 d experiments ($p<0.05$). The addition of YE and MSI gave the second highest cellulase activity on this day (26.55 ± 0.32 FPU/g), and the addition of all three nutrients resulted in a cellulase activity that was 16.8% higher (31.02 ± 1.01 FPU/g) than this activity. The 7 d cellulase activity was significantly different from all the other experiments, except the experiment with the addition of YE and MSI ($p=0.097$); the cellulase activity was still 14.4% higher for the addition of all three nutrients when compared to the addition of only YE and MSI.

Based on these results, it was determined that the addition of all three nutrients (starch, YE and MSI) resulted in the highest cellulase production. For these conditions an incubation period of 5 d was chosen as the optimal, as the rate of cellulase production started to decline between day 5 and day 7. These conditions were designated the optimised conditions for the production of cellulase during the SSF process.

5.2.4 Effect of growth media on fungal cellulase activity

In all the previous experiments, *Aspergillus niger* had been grown on PDA and the spores were then used in the solid state fermentations. In this investigation, the fungus was grown on different growth media to determine if the growth media would have an effect on the cellulase activity obtained after 5 d SSF process. Since Napier had resulted in the best cellulase activity, it was used in this investigation.

A. niger was grown on either PDA or on one of two different agar suspensions – (i) agar (2% w/v) containing Napier (5% w/v) or (ii) agar (2% w/v) containing Napier (5% w/v), starch (0.2% w/v), YE (0.5% w/v) and MSI (instead of deionised water). After 7 d growth, the spores were removed and used in the SSF process. The optimised SSF conditions (addition of 0.0070 g/g starch, 0.0175 g/g YE and MSI to the Napier; incubation for 5 d at 28 °C in static incubator) were used for all experiments and the cellulase activity was analysed after 5 d incubation.

The effect of the growth media on cellulase activity is shown in Figure 5.5. The fungal spores grown on PDA resulted in the highest cellulase activity during the SSF process (26.12 ± 0.11 FPU/g substrate), and this was significantly different from the cellulase activity for spores grown on agar ($p<0.05$). The spores grown on the two different agar suspensions had lower cellulase activities (22 - 23 FPU/g substrate) than the spores grown

on PDA and there was no difference in activity between the two groups (p=0.118).

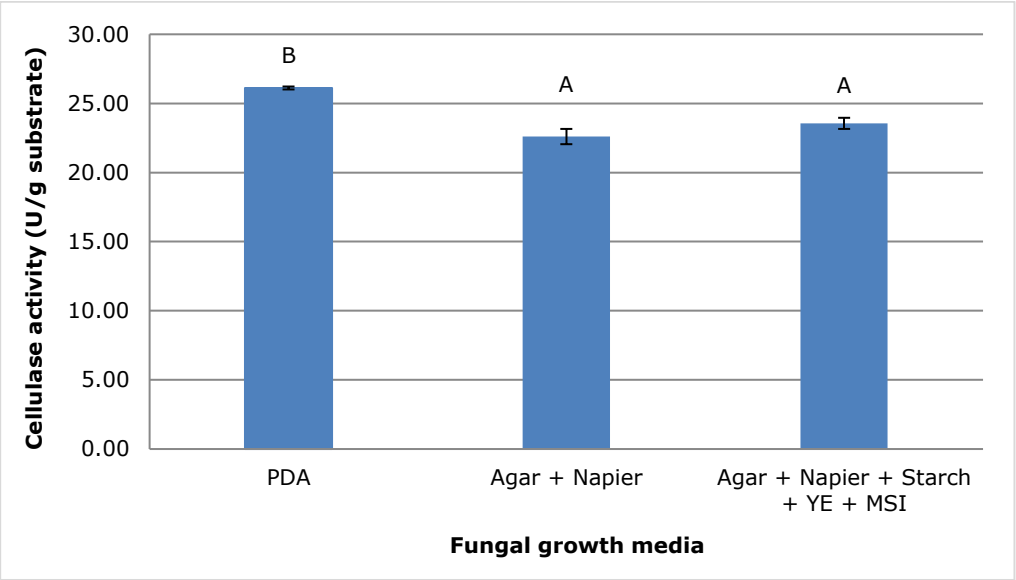


Figure 5.5 Effect of fungal growth media on cellulase production by *Aspergillus niger* during SSF under optimised conditions (Napier with the addition of 0.0070 g/g starch, 0.0175 g/g YE and MSI to 80% (w/v) MC; 5 d static incubation at 28 °C). The results are the mean + SD (n=3). Bars with different letters are significantly different (p<0.05).

Based on these results the *A. niger* grown on PDA resulted in the highest cellulase activity during the 5 d SSF process and this was designated the optimised fungal growth conditions for fungal cellulase production with *A. niger*. However, the cellulase activity was not compared on either 1 d or 3 d incubation periods and this may have resulted in different results.

5.2.5 Production of individual cellulase enzymes during SSF

The fungal extracts obtained in the previous section (Section 5.2.4) were further examined to determine the production of the individual cellulase enzymes – endoglucanase, exoglucanase, and β-glucosidase – during the optimised SSF process.

As previously mentioned, *A. niger* was grown on either PDA or agar (2% w/v) containing Napier (5% w/v), starch (0.2% w/v), YE (0.5% w/v) and MSI (instead of deionised water). After 7 d growth, the spores were

removed and used in the SSF process. The optimised SSF conditions (addition of 0.0070 g/g starch, 0.0175 g/g YE and MSI to 80% (w/v) to the Napier; incubation for 5 d at 28 °C in static incubator) were used for the experiments and the endoglucanase, exoglucanase and β-glucosidase activity was analysed after 5 d incubation. The activity of the individual enzymes is shown in Figure 5.6.

There were significant differences between the activities of all three cellulase enzymes ($p<0.05$), with β-glucosidase having the highest activity and exoglucanase having the lowest activity. However, there were no significant differences between the activities of each enzyme when comparing the fungal growth media used ($p=0.629$).

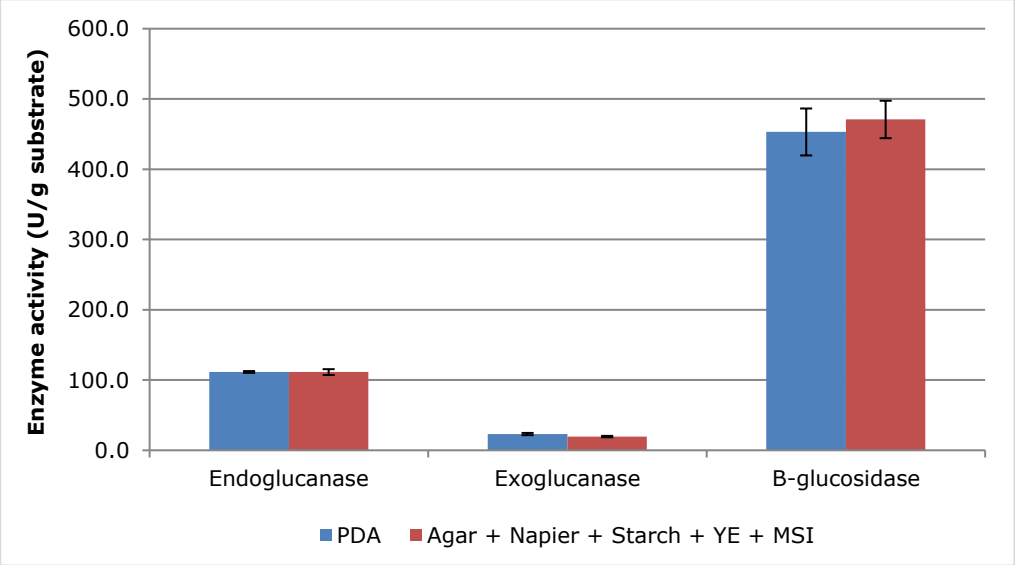


Figure 5.6 Production of individual cellulase enzymes (endoglucanase, exoglucanase and β-glucosidase) by *A. niger* during SSF under optimised conditions (Napier with the addition of 0.0070 g/g starch, 0.0175 g/g YE and MSI to 80% (w/v) MC; 5 d static incubation at 28 °C), when *A. niger* spores grown on different growth media (PDA or Agar + Napier + starch +YE + MSI). The results are the mean + SD (n=3).

The endoglucanase activity was 111.44 ± 1.33 U/g substrate for the spores grown on PDA, and 111.32 ± 4.13 U/g substrate for the spores grown on agar, and there was no significant difference in activity between the two groups ($p=0.970$). The exoglucanase activity was 23.11 ± 1.58

U/g substrate for the spores grown on PDA, and 19.51 ± 0.98 U/g substrate for the spores grown on agar, and there was no significant difference in activity between the two groups (p=0.052). The β-glucosidase activity was 463.05 ± 33.41 U/g substrate for the spores grown on PDA, and 470.83 ± 26.65 U/g substrate for the spores grown on agar, and there was no significant difference in activity between the two groups (p=0.558).

Based on these results, the fungal growth media had no effect on the individual enzyme activities. However, there was a significant difference in the individual enzyme production levels by *A. niger*. *A. niger* was able to produce more β-glucosidase than endoglucanase or exoglucanase.

5.2.6 Screening of crops under optimised SSF conditions

During the optimisation of the SSF conditions using Napier, it was determined that the addition of all three nutrients (starch, YE and MSI) and a 5 d incubation period was optimal for cellulase production. However, these conditions were optimised using Napier as a substrate. From the previous chapter, it was determined that the composition of the underutilised crops varied from one to another. Since the composition of the substrate can affect the cellulase activity, the crops were screened again under the optimised conditions for Napier. This was done to determine whether the new conditions would have a similar effect on cellulase production for the other underutilised crops.

The optimised conditions (addition of starch, YE and MSI to 80% (w/v) MC) were compared to the baseline conditions (addition of deionised water to 80% (w/v) MC). The SSF process commenced with the addition of *A. niger* spores (grown on PDA) and samples were taken at 5 d incubation period, as described in Section 3.7.

Figure 5.7A shows the fungal cellulase activity for the underutilised crops, under the optimised conditions. The cellulase activity ranged from 3 – 24 FPU/g substrate (dry weight basis) and there were statistically significant differences between the crops ($p<0.05$). The highest cellulase activity was obtained using Napier as a substrate (23.64 ± 0.59 FPU/g substrate dry weight), and this was statistically different from Bambara, which resulted in the second highest cellulase activity (14.64 ± 0.58 FPU/g substrate) ($p=0.0004$). Using Leucaena as a substrate resulted in the lowest cellulase activity (3.31 ± 0.32 FPU/g substrate) and this was statistically similar to Sago hampas (3.67 ± 0.68 FPU/g substrate), Oil palm fronds (6.49 ± 0.06 FPU/g substrate) and Nipa fronds (7.23 ± 3.32 FPU/g substrate), ($p=0.138$).

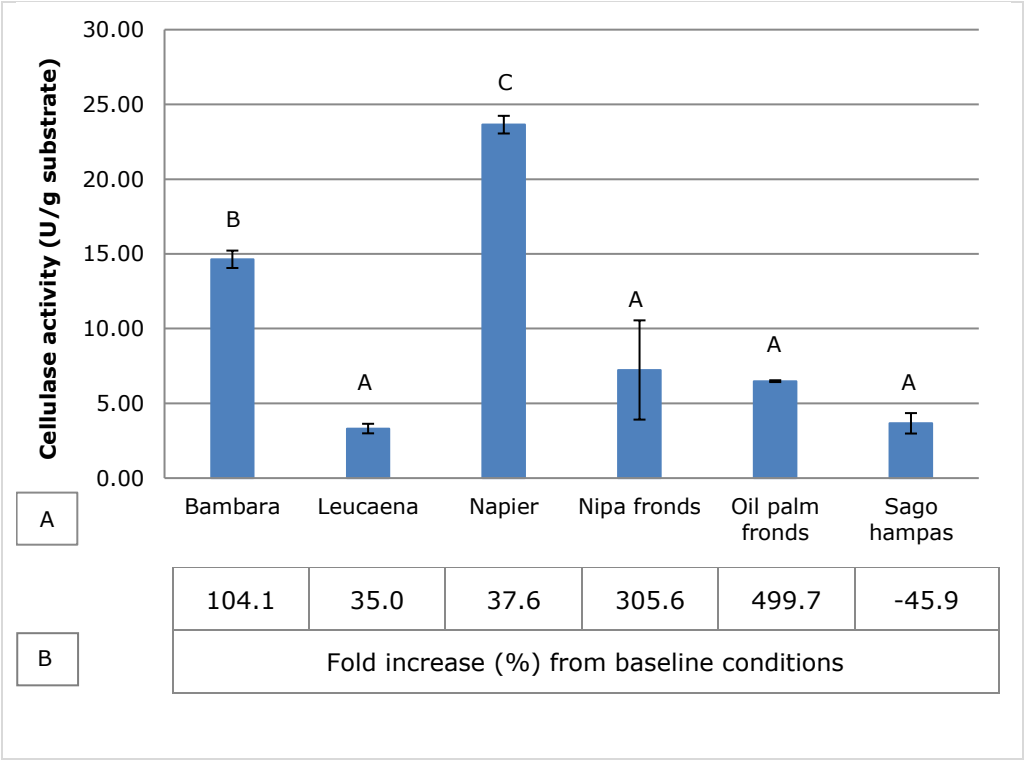


Figure 5.7 Comparison of cellulase production by *A. niger* using underutilised crops under optimised SSF conditions (addition of 0.0070 g/g starch, 0.0175 g/g YE and MSI to 80% (w/v) MC); SSF at 28 °C in a static incubator for 5 d incubation (A); and percent increase, compared with cellulase activity for the baseline conditions (B). The results are the mean + SD (n=3). Bars with different letters are significantly different ($p<0.05$).

When comparing the optimised SSF conditions with the baseline SSF conditions for the underutilised crops there were mixed results, with the cellulase activity either increasing or decreasing, as shown in Figure 5.7B. When compared to baseline conditions, the addition of nutrients to Napier, Bambara, Oil palm fronds, and Nipa fronds all resulted in a significant increase in fungal cellulase activity, with the greatest impact occurring with Oil palm fronds. The cellulase activity increased from 1.08 ± 0.06 FPU/g to 6.49 ± 0.06 FPU/g substrate (499.7%), and this was significantly different ($p=0.006$). However, using Napier as a substrate resulted in the highest cellulase activity (23.64 ± 0.59 FPU/g substrate). This had increased by 37.6% with the addition of nutrients, compared to the baseline conditions (17.18 ± 0.44 FPU/g substrate), and this increase was statistically significant ($p=0.0007$). The addition of nutrients to Bambara also resulted in an increased cellulase activity (104.1%) that was significantly different from the baseline conditions ($p=0.0001$). However, the addition of nutrients to Leucaena and Sago hampas resulted in a slight increase in cellulase activity and a decrease in activity respectively, although there were no statistically significant differences from the baseline conditions in either case ($p=0.999$, $p=0.329$ respectively).

The mixed results obtained under the optimised SSF conditions suggests that different conditions are necessary for different substrates used, and therefore different optimisation experiments would potentially need to be performed for each substrate.

5.2.7 Comparison of MSI & MSII, with *Aspergillus niger*

A second mineral solution (MSII) was also investigated at the same time to determine if this solution would have an impact on the cellulase activity of *A. niger* when using the underutilised crops as substrates. Conditions

optimised for Napier were used in this screening process, with the addition of starch (0.0070 g/g substrate) and YE (0.0175 g/g substrate) plus either MSI or MSII to 80% (w/v) MC. The mineral solutions were added to the substrates instead of adding deionised water, as described in Section 3.7. The SSF process was started with the addition of *A. niger* spores and the samples were removed and analysed after 5 d incubation.

Figure 5.8A shows the effect of MSII on cellulase production. The fungal cellulase activity ranged from 0 – 20 FPU/g substrate (dry weight basis) and there were statistically significant differences between the crops ($p<0.05$). The use of Napier as a substrate resulted in the highest cellulase activity (19.44 ± 0.06 FPU/g substrate) and this was statistically different from Bambara, which resulted in the second highest cellulase activity (11.62 ± 1.74 FPU/g substrate) ($p=0.00001$). The use of Nipa fronds as a substrate resulted in the lowest cellulase activity (0.22 ± 0.00 FPU/g substrate), and this was similar to Sago hampas (1.03 ± 0.89 FPU/g substrate) and Leucaena (2.50 ± 0.32 FPU/g substrate), ($p=0.166$).

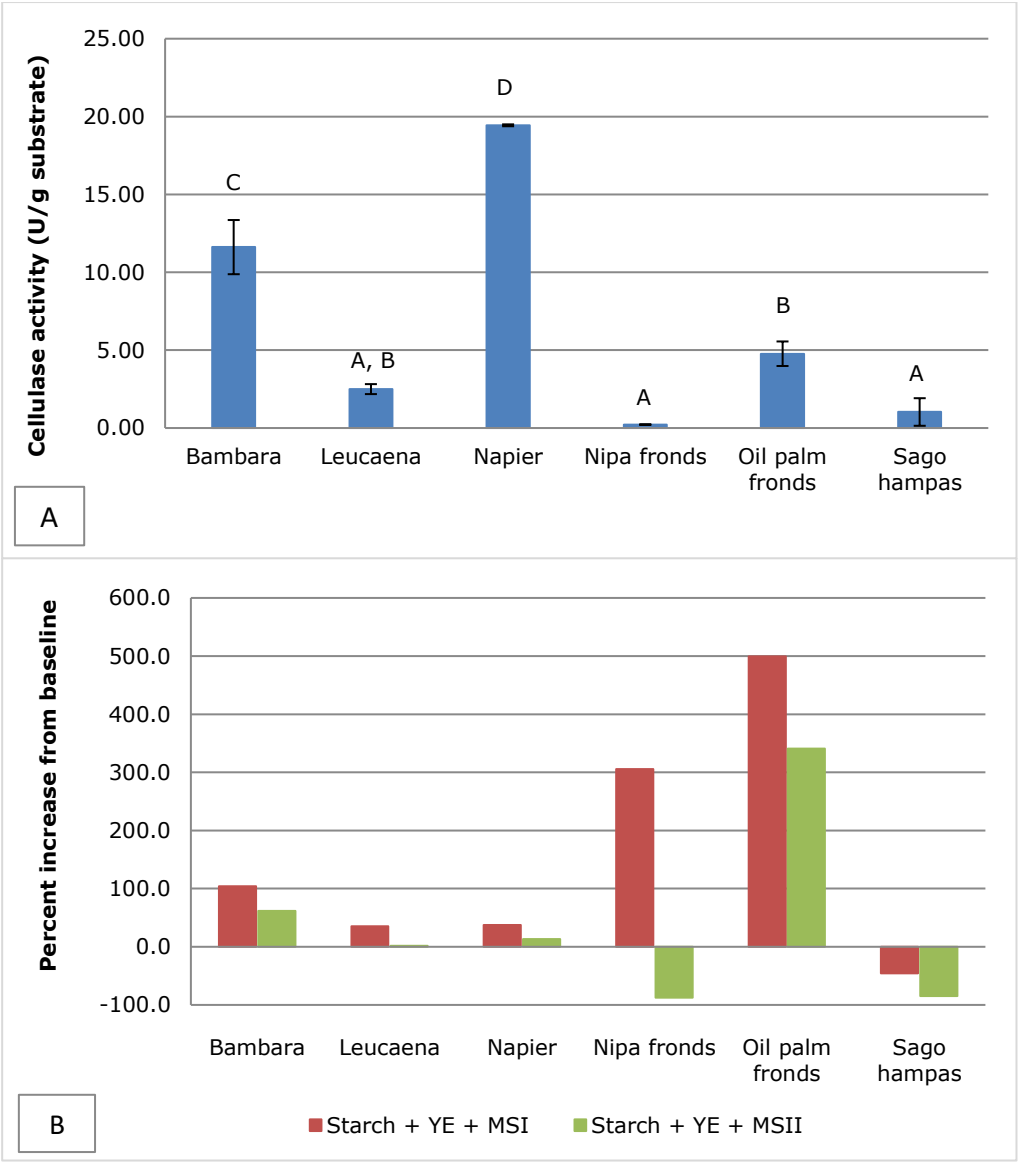


Figure 5.8 (A) Effect of MSII on fungal cellulase production during SSF (substrates at 80% (w/v) MC with the addition of 0.0070 g/g starch, 0.0175 g/g YE and MSII); (B) Comparison of addition of MSI and MSII to baseline conditions (addition of no nutrients). Fermentation by *A. niger* at 28 °C in a static incubator for 5 d incubation. The results are the mean + SD (n=3). Bars with different letters are significantly different ($p<0.05$).

When comparing the addition of MSI and MSII to the baseline conditions (regardless of crop), overall there was a significant difference in cellulase activity with the addition of MSI ($p<0.05$), but there was no significant difference in the cellulase activity with the addition of MSII ($p=0.159$). When looking at each crop, the addition of MSII did not have a significant effect on the cellulase activity in Leucaena ($p=1.000$), Napier ($p=0.350$) and Nipa fronds ($p=0.815$), but it did have an effect on the cellulase

activity in Bambara, Oil palm fronds and Sago hampas ($p<0.05$). The addition of MSII resulted in a significant increase in cellulase activity in Bambara (61.97%) and Oil palm fronds (341.04%), but in a significant decrease in activity in Sago hampas (-84.79%), (Figure 5.8B).

The addition of MSI resulted in a higher cellulase activity for all the underutilised crops, when compared to the addition of MSII, as shown in Figure 5.8B. Overall, the highest cellulase activity was achieved using Napier as a substrate, with the addition of MSI. The addition of MSII to Napier resulted in a cellulase activity that was slightly higher (13.12%) than the baseline conditions (7.17 ± 0.43 FPU/g substrate), although these were statistically similar ($p=0.820$). The addition of MSI to Napier resulted in a higher cellulase activity (23.64 ± 0.59 FPU/g substrate, which was a 37.61% increase when compared with the baseline conditions ($p=0.000118$), and a 21.64% increase when compared with the addition of MSII ($p=0.040$).

Based on the results it was determined that MSI had more of an impact on the enzyme activity than MSII, when using *A. niger*. Therefore, it was determined that the addition of MSI would be used, as this resulted in a higher enzyme activity than the addition of MSII.

5.2.8 Comparison of *Trichoderma reesei* and *Aspergillus niger*

The underutilised crops were also screened using a different fungus, *Trichoderma reesei*, to compare the cellulase production under the same conditions as with *Aspergillus niger.* Starch powder (0.0070 g/g), YE (0.0175 g/g) and either MSI or MSII to 80% (w/v) MC were added to each substrate. The SSF process commenced with the addition of either *T. reesei* or *A. niger* spores and the samples were analysed after 5 d incubation period.

Figure 5.9 compares the effect of the addition of starch, YE and MSI to the underutilised crop on cellulase production by *T. reesei* and *A. niger* during SSF. Under these conditions very low cellulase production by *T reesei* was observed for all the underutilised crops (less than 4 FPU/g substrate). Oil palm fronds (3.10 ± 0.06 FPU/g) and Napier (3.05 ± 0.22 FPU/g) resulted in the highest cellulase activity and these were similar (p=1.000), as shown in Figure 5.9A.

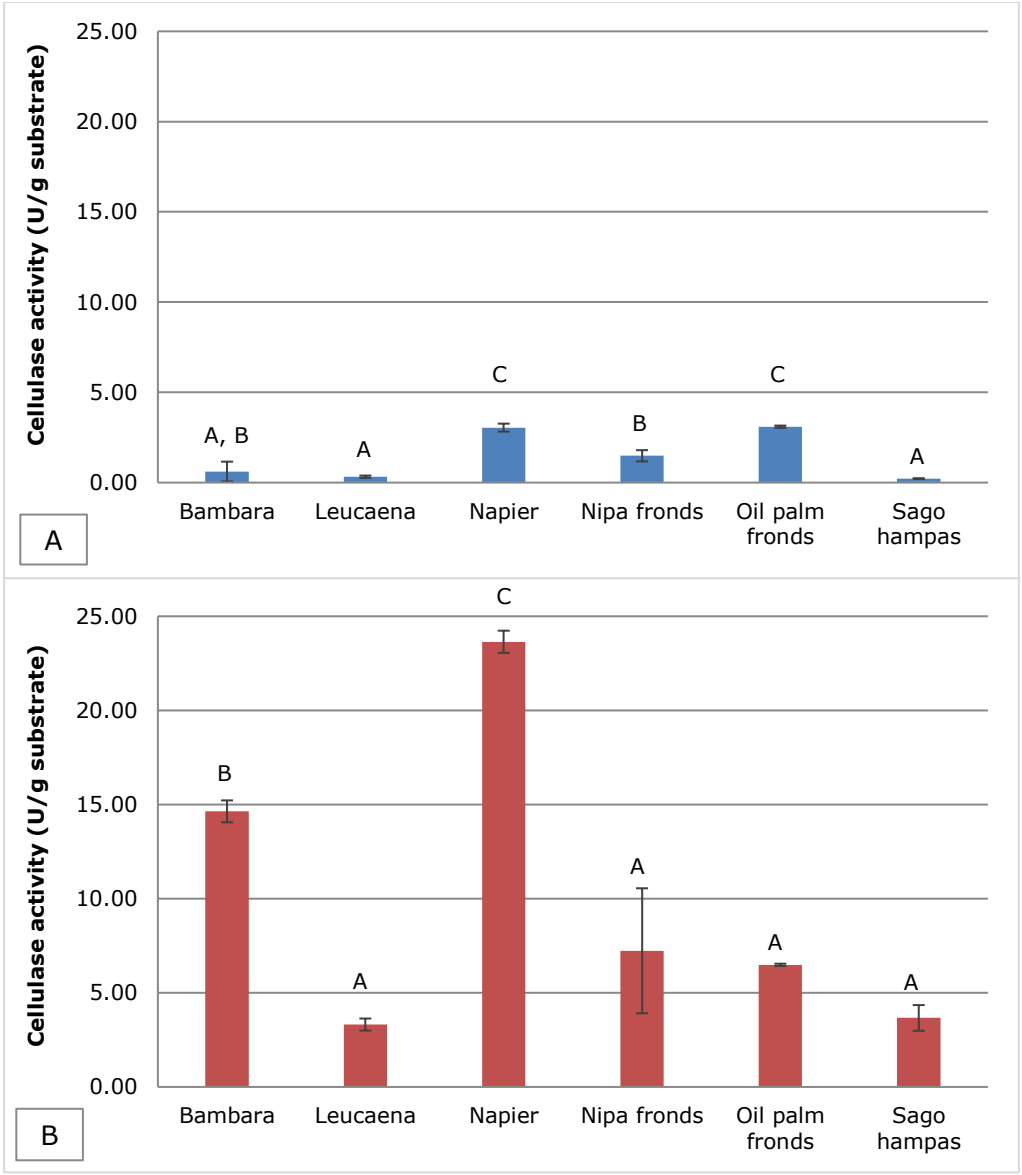


Figure 5.9 (A) Cellulase production by *Trichoderma reesei* during 5 d SSF under optimal conditions (addition of 0.0070 g/g starch, 0.0175 g/g YE and MSI, to 80% (w/v) MC); and (B) cellulase production by *Aspergillus niger* during 5 d SSF under the same conditions. The results are the mean + SD (n=3). Bars with different letters are significantly different (p<0.05).

When compared with the cellulase production by *A. niger*, the *T. reesei* was significantly outperformed by the *A. niger* under these conditions ($p<0.05$). As shown in Figure 5.9B, the cellulase activity for the *A. niger* ranged from 3 – 24 FPU/g substrate. The lowest activity achieved by *A. niger* was 3.31 ± 0.32 FPU/g using Nipa fronds as a substrate, and this activity was higher than the cellulase production achieved by *T. reesei* using any of the crops. The use of Napier as a substrate resulted in the highest cellulase activity by *A. niger* (23.64 ± 0.59 FPU/g substrate) and this was 676.47% higher than the cellulase activity achieved by *T. reesei* using Napier (3.05 ± 0.22 FPU/g substrate).

Since the addition of MSI had resulted in very low cellulase activity by *T. reesei*, the addition of MSII was investigated and compared with the results for the cellulase production by *T.* reesei with the addition of MSI, as shown in Figure 5.10.

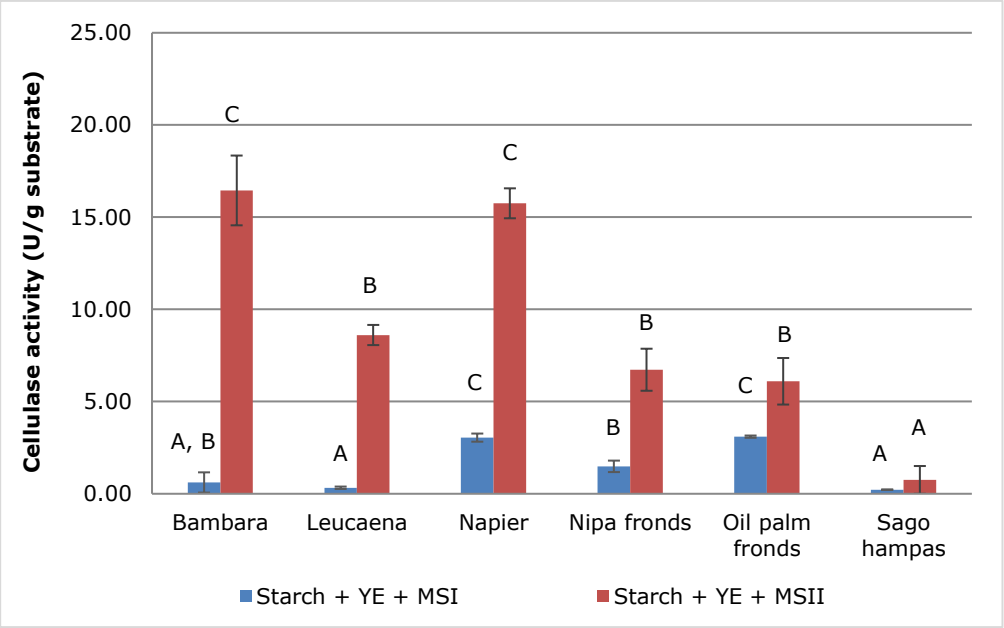


Figure 5.10 Impact of MSI and MSII on cellulase production by *Trichoderma reesei* during SSF under optimised conditions (substrate at 80% (w/v) MC with the addition of 0.0070 g/g starch, 0.0175 g/g YE and either MSI or MSII; Incubation at 28 °C for 5 d). The results are the mean + SD (n=3). Bars with different letters, within the same data set, are significantly different ($p<0.05$).

When MSII was used, the cellulase activity by *T. reesei* was higher for all the crops except Sago hampas, which had a similar activity as that with the addition of MSI. The activity ranged from 0.75 – 17 FPU/g substrate and there were significant differences between all the crops ($p<0.05$). The use of Bambara and Napier as substrates resulted in the highest cellulase activities (16.45 ± 1.89 FPU/g and 15.75 ± 0.81 FPU/g, respectively) and these were statistically similar ($p=0.989$). Leucaena (8.61 ± 0.55 FPU/g), Nipa fronds (6.73 ± 1.14 FPU/g and Oil palm fronds (6.10 ± 1.26 FPU/g) had similar activities ($p=0.316$).

When comparing MSI versus MSII, the addition of MSII to the substrates resulted in a significantly higher cellulase activity for Bambara (2,600%), Leucaena (2,500%), Napier (400%), Nipa fronds (350%) and Oil palm fronds (100%), ($p<0.05$). The cellulase activity with the addition of MSII to Sago hampas was 200% higher than the activity with the addition of MSI to Sago, however, these values were similar ($p=0.999$).

Overall the cellulase production by *T. reesei* was much better with the addition of MSII to the underutilised crops, rather than with the addition of MSI. Since *T. reesei* performed better with the addition of MSII and *A. niger* performed better with the addition of MSI, these two conditions were compared to determine which resulted in the highest cellulase activity Figure 5.11).

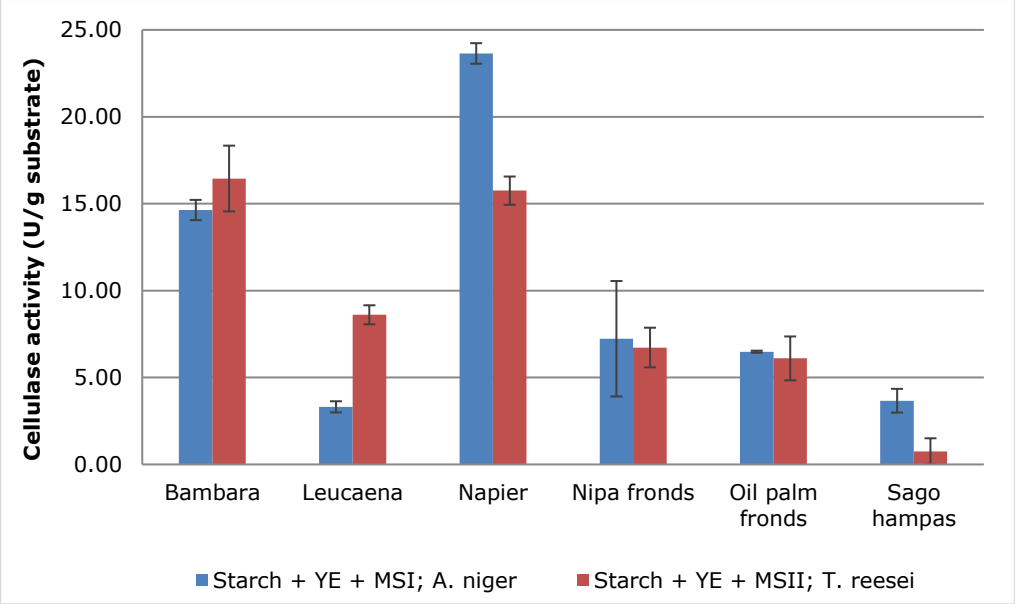


Figure 5.11 Comparison of cellulase production by *Aspergillus niger* during SSF under optimised conditions (substrate at 80% (w/v) MC with the addition of 0.0070 g/g starch, 0.0175 g/g YE and MSI) and cellulase production by *Trichoderma reesei* during SSF under optimised conditions (substrate at 80% (w/v) MC with the addition of 0.0070 g/g starch, 0.0175 g/g YE and MSII). The results are the mean + SD (n=3).

Significantly different results were obtained for the two conditions when using Napier and Leucaena as a substrate. When comparing the cellulase activity using Napier, the use of MSI and *A. niger* resulted in a higher cellulase activity (23.64 ± 0.59 FPU/g) than the use of MSII and *T. reesei* (15.75 ± 0.81 FPU/g), and these were significantly different ($p=1.67 \times 10^{-7}$). However, the opposite was achieved using Leucaena, with the use of MSII and *T. reesei* resulting in a higher cellulase activity (8.61 ± 0.55 FPU/g), than the use of MSI and *A. niger* (3.31 ± 0.32 FPU/g), (p= 0.001).

When using Bambara as a substrate, the use of MSII and *T. reesei* resulted in a slightly higher cellulase activity (16.45 ± 1.89 FPU/g) than the use of MSI and *A. niger* (14.64 ± 0.58 FPU/g); however, these two results were similar (p=0.979). There were also no significant differences when using Oil palm fronds or Nipa fronds as a substrate (p=1.000 for both).

Overall, the use of Napier as a substrate, with the addition of starch (0.0070 g/g), YE (0.0175 g/g) and MSI and *A. niger* spores resulted in the

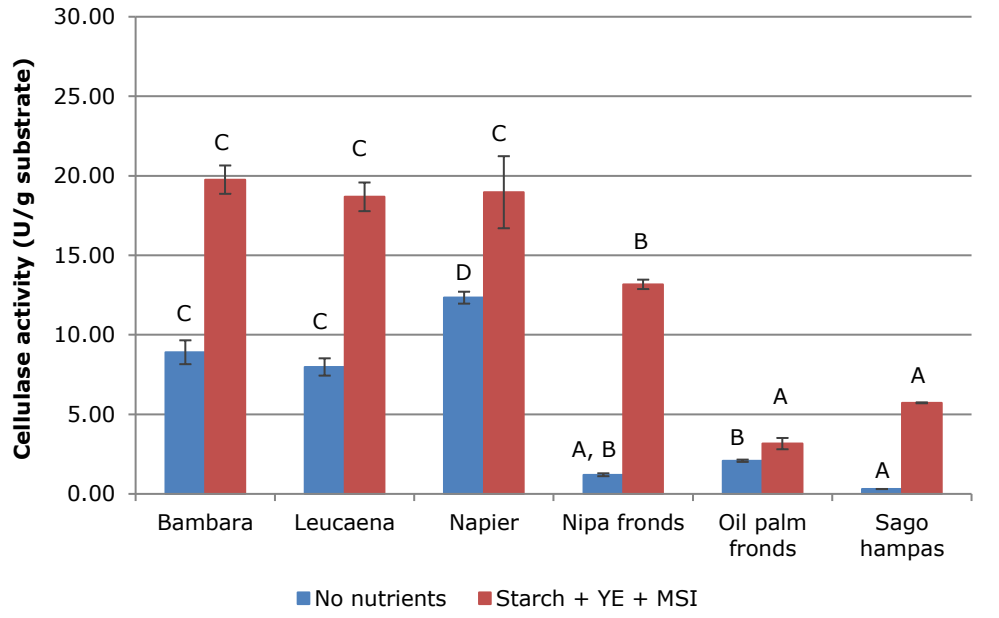
highest cellulase activity after the 5 d SSF incubation period and this was significantly different from the cellulase activity for the different conditions for all the underutilised corps ($p<0.05$).

5.2.9 Screening of crops, using submerged fermentation

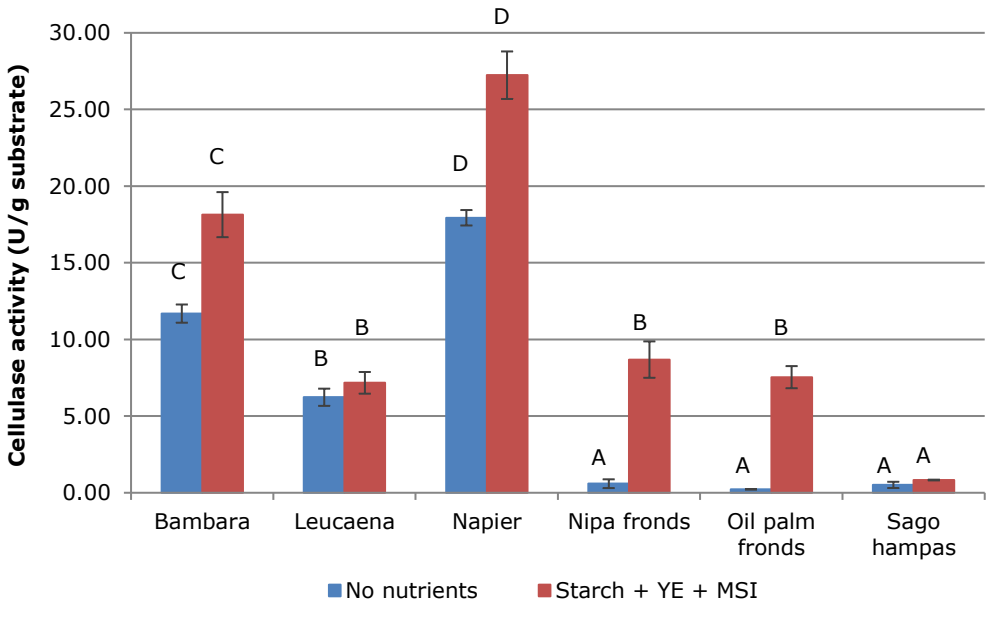
The underutilised crops were screened to determine whether fungal cellulases could be produced during submerged fermentation and if so, the level of activity that could be obtained for each crop. The crops were screened under the baseline conditions as well as the optimised conditions that were used during the SSF process, using the microorganism, *A. niger*.

The optimised conditions (addition of 0.0070 g/g starch, 0.0175 g/g YE and MSI) were compared to the baseline conditions (addition of deionised water) during both SmF and SSF procedures. Both processes commenced with the addition of *A. niger* spores (grown on PDA) and samples were taken after 5 d incubation period, as described in Sections 3.7 and 3.8.

Figure 5.12 shows the results for the cellulase activity for the two different conditions examined for both SmF (Figure 5.12A) and SSF (Figure 5.12B) procedures.



	121.9	134.0	53.8	994.7	51.8	1775.0
A	Fold increase (%) from baseline conditions					



	55.2	15.1	51.8	1367.0	3270.0	62.0
B	Fold increase (%) from baseline conditions					

Figure 5.12 Cellulase production by *A. niger* under optimised conditions (addition of starch, YE and MSI) and baseline conditions (no nutrients) during SmF (A) and SSF (B). The results are the mean + SD (n=3). Bars with different letters, within the same data set, are significantly different ($p<0.05$).

Under the baseline conditions, the fungal cellulase activity during SmF procedure ranged from 0 – 12 FPU/g substrate (dry weight basis); and there were statistical differences between the crops ($p<0.05$) (Figure 5.12A). The highest cellulase activity was obtained using Napier as a substrate (12.34 ± 0.37 FPU/g substrate), and this was significantly different from all other crops ($p<0.05$).

The addition of nutrients (starch, YE and MSI) to the crops resulted in a significant increase in the cellulase activity during the SmF procedure for all crops ($p<0.05$) except Oil palm fronds ($p=0.967$). Under these optimised conditions, the cellulase activity during the SmF procedure ranged from 2 – 19 FPU/g substrate; and there were statistical differences between the crops ($p<0.05$) (Figure 5.12A). The highest cellulase activity was obtained using Bambara as a substrate (19.76 ± 0.89 FPU/g substrate); representing an increase of 121.9 %, when compared with the baseline conditions. However, this activity level was similar to Leucaena (18.68 ± 0.90 FPU/g substrate; 134.0 % increase when compared to the baseline conditions, $p=0.967$) and Napier (18.97 ± 2.27 FPU/g substrate; 53.8 % increase when compared to the baseline conditions, $p=0.997$). The lowest cellulase activity was reported with the use of oil palm fronds (3.16 ± 0.35 FPU/g substrate) and this was similar to Sago hampas (5.73 ± 0.03 FPU/g substrate), ($p=0.128$).

As with previous experiments, the addition of nutrients to the crops significantly increased cellulase activity during the SSF procedure for all crops ($p<0.05$), except Leucaena and Sago hampas ($p=0.998$ and $p=1.000$, respectively). The cellulase activities obtained during the SSF procedure for the optimised and baseline conditions were comparable with the cellulase activities obtained for previous SSF experiments under the same conditions (see Sections 5.2.2 and 5.2.6).

The comparison of the SmF results (Figure 5.12A) with the SSF results (Figure 5.12B) under optimised conditions indicates mixed results as to which procedure worked best and was dependent on the crop. Looking at the three highest performing crops from the SmF procedure (Bambara, Leucaena and Napier), the cellulase activity using Bambara as a substrate was similar in both procedures ($p=0.937$); the cellulase activity using Leucaena was significantly higher in the SmF procedure ($p<0.05$); and the cellulase activity using Napier was significantly higher in the SSF procedure ($p<0.05$).

5.3 SUMMARY

Overall, the highest cellulase activity was achieved using Napier as a substrate during SSF under optimised conditions (27.24 ± 1.55 FPU/g substrate), and this was significantly higher than any other activities achieved during SSF or SmF procedures ($p<0.05$). This cellulase activity was 37.9% higher than the highest cellulase activity achieved during SmF under the same conditions – using Bambara as a substrate (19.76 ± 0.89 FPU/g substrate), ($p<0.05$). The cellulase extracted using Napier as a substrate would ultimately need to be further analysed further, investigating whether it could be used to effectively degrade Napier into fermentable sugar to be used for bioethanol production.

Although very little cellulase activity was achieved using Sago hampas as a substrate the fungus grew very well on the substrate. This suggests that the fungus was hydrolysing something other than cellulose to produce glucose for growth, potentially starch. Based on this observation the Sago hampas was further investigated to determine if it could be used as a substrate for glucoamylase production, rather than cellulase production.

CHAPTER 6 PRODUCTION OF GLUCOAMYLASES & GLUCOSE FROM SAGO HAMPAS

6.1 INTRODUCTION

The Sago palm is an important crop, socio-economically, in South East Asia. The starch, stored in the trunk, is extracted and processed into many products, such as glucose, syrups, and chemicals for the pharmaceutical and paper industries. During this extraction process a waste stream, known as Sago hampas, is produced. Although the process is designed to extract starch, this fibrous residue left over still contains some starch granules trapped inside a lignocellulosic matrix. Currently, this is used as animal feed, compost for mushrooms, as well as several other processes.

Potentially, the Sago hampas could be used as a source for the production of biofuels and/or biochemicals, from either the starch or the lignocellulosic material, in a biorefining process. However, the enzymes needed to hydrolyse the starch into fermentable sugars are produced by other bio-industries, increasing the biofuel production costs. Therefore, the on-site production of enzymes is an attractive option, especially if they are produced using the Sago hampas, which is a cheap carbohydrate source. These enzymes can be produced via solid state fermentation, and then extracted to be used in the hydrolysis step. The fermentable sugars produced can then be fermented into bioethanol and/or biochemicals, providing value added products whilst eliminating some of the Sago hampas waste from the starch extraction process.

Based on the compositional analysis performed there was still just over 50% w/w (dry weight basis) starch present in the Sago hampas used in this research. Although very little cellulase activity was achieved during the SSF (Section 5.2.2 and Section 5.2.6) or SmF (Section 5.2.9)

processes when using the Sago hampas as a substrate, the fungus grew well suggesting that it was probably hydrolysing the starch, rather than the lignocellulosic material of the Sago hampas. Therefore, in this chapter the Sago hampas was investigated to determine if it could be used as a substrate during solid state fermentation (SSF) to produce glucoamylases in the first stage of the biorefining process. The fungus, *Aspergillus awamori*, was chosen for the SSF process as it is known to be a good producer of a wide variety of enzymes, namely glucoamylases, pectinases, proteases, lipases, phosphatases, cellulases and xylanases (Koutinas *et al*., 2004). The Sago hampas was also used as a substrate to determine how much fermentable sugars could be produced during the solid state fermentation process using the *A. awamori*, and whether this amount was enough to make it a viable process for production of fermentable sugars for the fermentation step.

The SSF conditions optimised in the previous chapter (addition of YE and MSI) were used in this work. The composition of these nutrients is described in Section 3.4. The methodologies for the SSF process, glucose analysis and glucoamylase analysis are described in Sections 3.7, 3.6.6 and 3.9.5, respectively.

6.2 RESULTS

6.2.1 Free sugars in Sago hampas

The compositional analysis of the Sago hampas was repeated to determine whether there was any free glucose and/or maltodextrins present in the sample as described in Section 3.6.5. Two slightly different procedures were followed at the beginning of the experiment. For the removal of the glucose and maltodextrins (measurement of starch only), the procedure described in Section 3.6.5 was followed. To determine if any glucose

and/or maltodextrins were present, the same procedure was followed; however, the initial two steps involving the addition of 80% (v/v) ethanol (designed to remove free glucose and maltodextrins) were skipped and instead only 0.2 mL 80% (v/v) ethanol was added before adding the magnetic stir bar and KOH.

As seen in Figure 6.1, the starch content (without the removal of D-glucose and/or maltodextrins) was slightly higher (52.83 ± 3.68 % w/w dry weight basis) than the starch content alone (50.88 ± 1.96 % w/w dry weight basis). However, there was no significant difference between these two values (p=0.345), indicating that whilst there may have been some free D-glucose and/or maltodextrins present this was not significant. These values are comparable to previous measurements (Section 4.2.5) of the starch content of the Sago hampas (51.91 ± 3.23 % w/w).

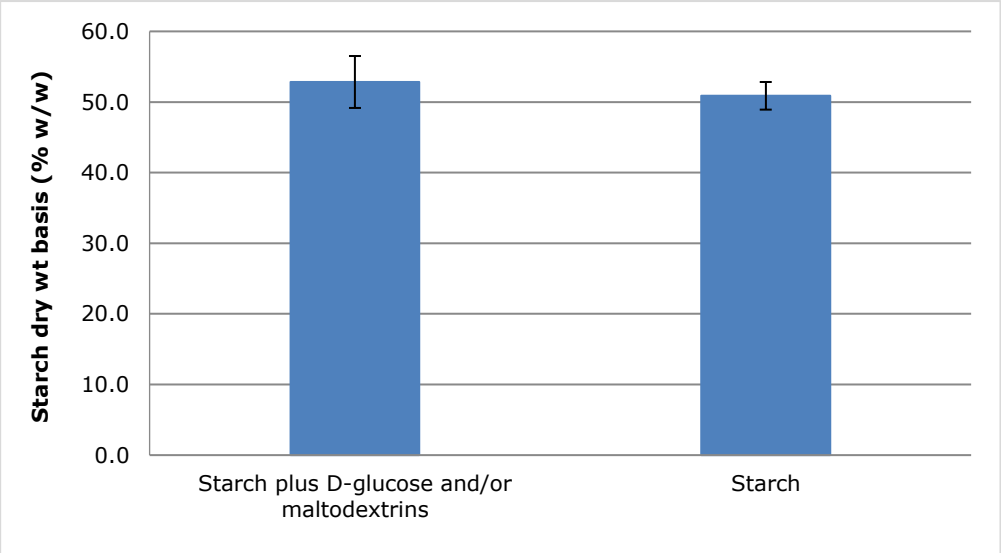


Figure 6.1 Starch content, as well as D-glucose and/or maltodextrin content, in samples of Sago hampas. Values are means + SD (n=3).

6.2.2 Growth of *Aspergillus awamori* on different growth media

Before determining if the Sago hampas could be used as a substrate for the production of enzymes and/or fermentable sugars during SSF, the *Aspergillus awamori* was grown on different growth media, to determine

which conditions were best for spore culturing. This was done as the culturing of the spores was based on procedures described by Koutinas *et al*. (2001), who used whole wheat flour as a substrate for culturing. Since this experiment used Sago hampas as a substrate, the culturing of the *A. awamori* spores was repeated, investigating four different growth media.

The compositions of the different growth media are shown in Table 6.1. The growth media were autoclaved and then inoculated with *A. awamori* spores, as described in Section 3.5.2, and incubated for 7 days in a dark, static incubator at 28 °C.

Table 6.1 Media conditions used for growth of *A. awamori* spores.

Experiment	Media conditions for spore growth			
	Addition of 2% (w/v) agar	Addition of 5% (w/v) Sago hampas	Addition of 0.5% (w/v) YE	Solution used to make up to volume
A	Yes	Yes	No	Deionised water
B	Yes	Yes	Yes	Deionised water
C	Yes	Yes	No	MSI
D	yes	yes	Yes	MSI

Figure 6.2 shows the spore growth after the incubation period on the different growth media conditions. The *A. awamori* spores grew best on the growth media containing YE and MSI, and grew the least on the growth media containing just the Sago hampas. The growth media containing either YE or MSI had around the same amount of spore growth as one another. When compared to the media containing only Sago hampas they showed more spore growth; however, they did not show as much growth as the media containing both YE and MSI. Based on these observations, the optimal growth conditions for the *A. awamori* spore culturing included 2% (w/v) agar + 5% (w/v) Sago hampas + 0.5% (w/v) YE + MSI to make up to solution, and this growth medium was used for all further experiments.

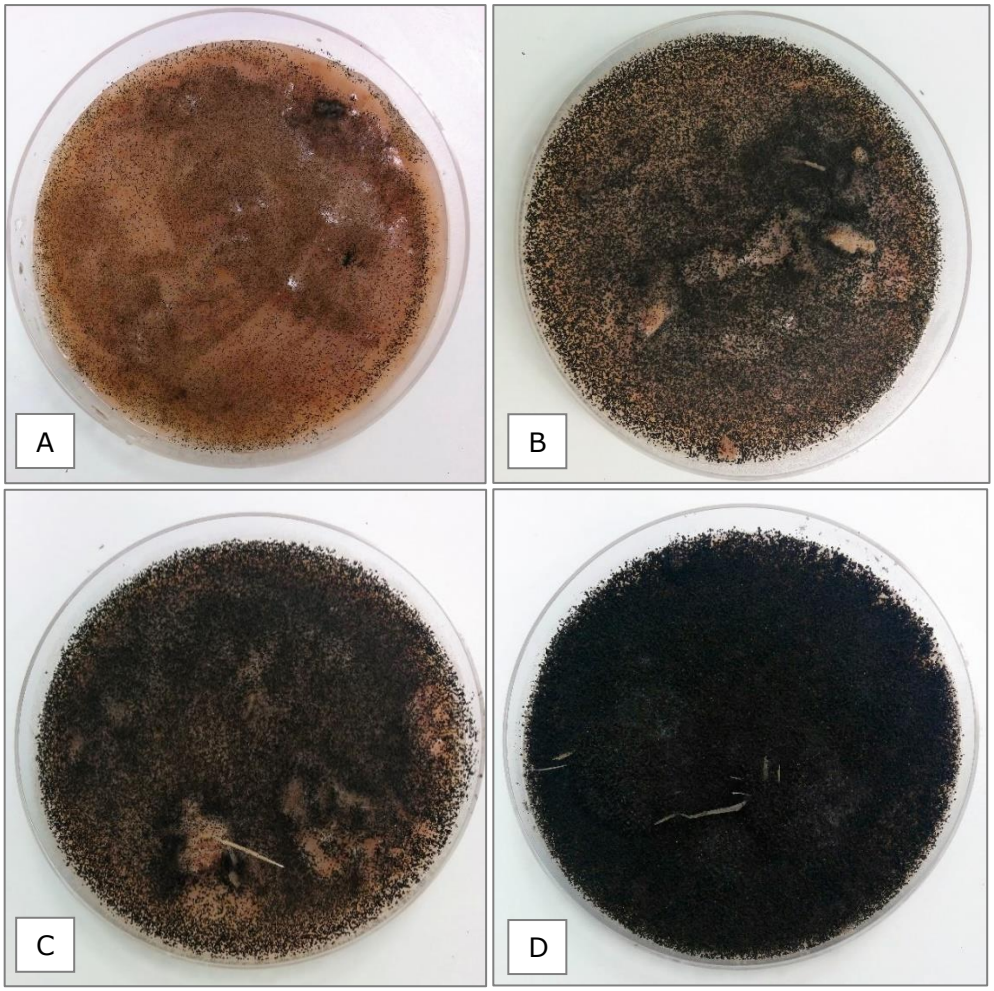


Figure 6.2 Growth of *Aspergillus awamori* spores on different growth media. All media contained 2% (w/v) agar + 5% (w/v) Sago hampas plus (A) deionised water, (B) 0.5% (w/v) YE + deionised water, (C) MSI, (D) 0.5% (w/v) YE + MSI. All samples were incubated for 7 d at 28 °C in a dark static incubator.

6.2.3 Glucoamylase activity recovered from SSF process

After the optimal growing conditions for the *A. awamori* spores were determined, the spores were used in a solid state fermentation to investigate whether the *A. awamori* could produce glucoamylase using Sago hampas as a substrate.

To prepare the Sago hampas, 0.0175 g/g YE and MSI (up to 80% (w/v) MC) were added and autoclaved. The SSF process commenced with the addition of *A. awamori* spores and the samples were analysed after a 5 d incubation period, as described in Section 3.7. The glucoamylase activity of

the recovered fungal filtrate was assayed using the original method described by Koutinas *et al*. (2001), and not the modified method as described in Section 3.9.5. In this investigation, the same substrate solution was used (2% (w/v) soluble starch suspension, dissolved in 0.2 M Na-acetate buffer, pH 4.5); however, the starch suspension was not gelatinised. Also, the duration of the assay was measured at times 0, 5, and 10 min, as described by Koutinas *et al*. (2001). The rest of the procedure was followed as described in Section 3.9.5.

Figure 6.3 shows the activity of glucoamylase activity recovered in the fungal filtrate after the SSF incubation period. The concentration of glucose did not increase over the 10 min glucoamylase assay period, and stayed around 2.0 mg/mL (p=0.507).

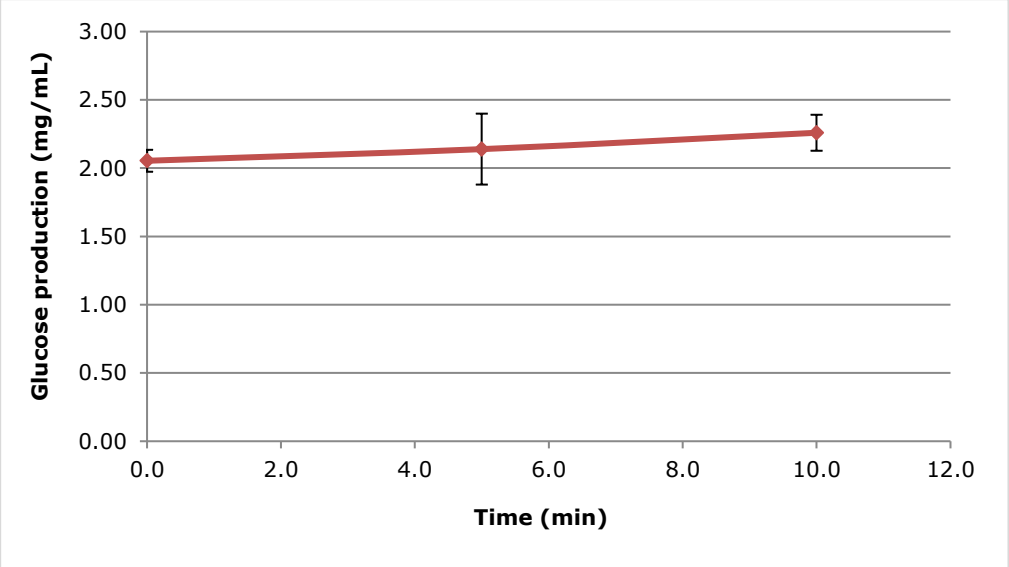


Figure 6.3 Glucoamylase activity of fungal filtrate. The fungal filtrate was collected after 5 d incubation and glucoamylase activity was determined as glucose production from non-gelatinised soluble starch. The assay was carried out at pH 4.5 and 60 °C for up to 30 min. The results are the mean + SD (n=3).

This indicated that no or little glucoamylase activity was detected in the recovered SSF fungal filtrate, as the starch was not broken down into glucose during the assay period. This could have been due either to there being no or little enzyme being produced by the fungus during the SSF

incubation period, or that the glucoamylase assay was not working correctly.

6.2.4 Glucoamylase activity using commercial glucoamylase

To determine whether the glucoamylase assay was working correctly the assay was performed using a commercial glucoamylase. Several trials were carried out to first determine if the assay worked using the commercial enzyme and then to determine the optimal conditions (substrate preparation procedure, dilution, time, pH, and temperature) for the glucoamylase assay.

6.2.4.1 Gelatinised starch versus non-gelatinised starch solution

The glucoamylase assay was originally performed using non-gelatinised soluble starch. In the first experiment, the impact of gelatinisation was tested. The substrate solution used was 2% (w/v) soluble commercial corn based starch suspension, dissolved in 0.2 M Na-acetate buffer, pH 4.5. For gelatinisation, the suspension was placed on a magnetic hot plate set at 85 °C and 300 rpm, for 20 min. The length of the assay was also modified, with measurements being taken at 0, 10, 30 and 60 min. The assay was carried out at 37 °C following the remainder of the procedure as described in Section 3.9.5.

Figure 6.4 shows the production of glucose using the commercial glucoamylase (300 U/mL, Sigma-Aldrich, UK), at a 200-fold dilution. Extremely little glucose was produced over the 60 min assay period when using the non-gelatinised starch (maximum yield = 0.58 ± 0.12 mg/mL) ($p=0.166$). However, the use of gelatinised starch showed a rapid increase in glucose within the first 10 min after which the activity plateaued, reaching a maximum of 21.32 ± 0.58 mg/mL.

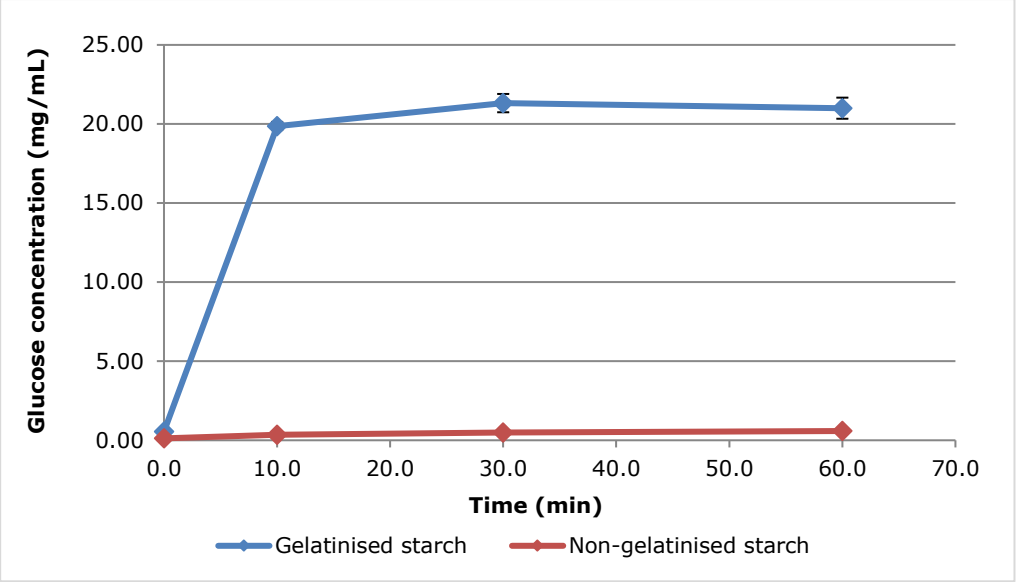


Figure 6.4 Effect of starch gelatinisation on the glucoamylase assay. A commercial glucoamylase (200-fold dilution) was assayed using either gelatinised or non-gelatinised soluble starch. The assay was carried out at pH 4.5 and 37 °C for up to 60 min. The results are the mean + SD (n=3).

Using a conversion factor of 1.11 (theoretical conversion of glucose from starch), it was calculated that the maximum glucose that could be produced during the assay experiment was 22.0 mg. Therefore, the amount of glucose yielded with the gelatinised starch reached almost maximal within 10 min. Since extremely little glucose was present at time 0, the glucose produced was due to the starch being broken down by the commercial glucoamylase. This showed that the enzyme assay worked with gelatinised starch, but it did not work with non-gelatinised starch.

6.2.4.2 Optimisation of enzyme concentration and assay time

In the previous experiment the enzyme concentration of the commercial glucoamylase was too high, as the reaction reached completion within 10 min. Therefore, the next experiment was set up to determine the optimal enzyme concentration required for the assay, as well as to determine the assay time to be used for further experiments.

Several different enzyme concentrations were investigated and prepared as shown in Table 6.2.

Table 6.2 Preparation of commercial glucoamylase (AMG) dilutions.

	AMG stock added (mL)*	Buffer added (mL)*	Total volume (mL)	Final AMG conc. (U/mL)
200-fold dilution	0.10	19.90	20.00	1.50
1000-fold dilution	0.10	99.90	100.00	0.30
2000-fold dilution	0.10	199.90	200.00	0.15
3000-fold dilution	0.10	299.90	300.00	0.10
4000-fold dilution	0.10	399.90	400.00	0.08

*AMG stock – 300 U/mL; Buffer used was 0.2 M Na-acetate buffer, pH 4.5

Once the dilutions had been prepared, 0.5 mL of each was used for the glucoamylase assay. The glucoamylase assay was performed using gelatinised starch as the substrate, and measurements were taken at 0, 10, 30 and 60 min. The assay was carried out at 37 °C and pH 4.5, following the remainder of the procedure as described in Section 3.9.5.

Figure 6.5 shows the production of glucose over time during the glucoamylase assay for the 200-fold and 4000-fold enzyme dilutions.

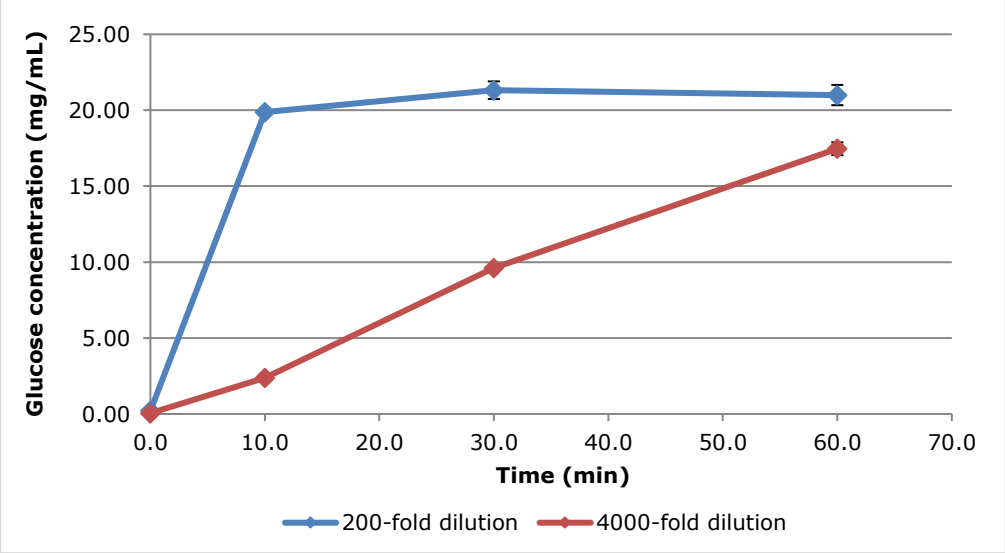


Figure 6.5 Production of glucose from gelatinised starch by commercial glucoamylase at different concentrations. Stock glucoamylase had an activity of 300 U/mL. The assay was carried out at pH 4.5 and 37 °C for up to 60 min. The results are the mean + SD (n=3).

As previously noted the reaction for the 200-fold enzyme reached completion within the first 10 min, yielding a glucose concentration of 19.87 ± 0.26 mg/mL before beginning to plateau. However, the reaction for the 4000-fold enzyme was much slower, with the increase in glucose being more gradual over the 60 min assay period. The glucose concentration had reached 17.46 ± 0.42 mg/mL at the end of the 60 min period; however, it had not begun to plateau.

Figure 6.6 shows a linear line of best fit drawn through the glucose production values over time for the glucoamylase at 4000-fold dilution.

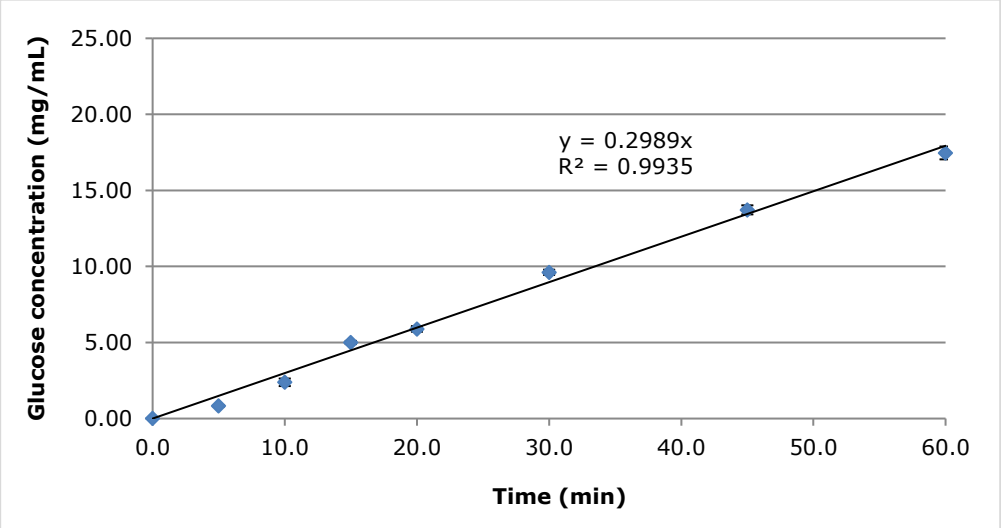


Figure 6.6 Production of glucose over time due to activity of commercial glucoamylase (1-4000 dilution) on gelatinised starch. The assay was carried out at pH 4.5 and37 °C for up to 60 min. The results are the mean + SD (n=3).

An R^2 value of 0.9935 was obtained indicating that the production of glucose over time followed a linear pattern. Therefore, the rate of change of glucose over time was the same, and so this enzyme dilution was chosen for further experiments. An assay time of 30 min was also chosen for further experiments, as at this time point it was estimated that just under 50% of the starch had been broken down.

6.2.4.3 Optimisation of pH

Based on the literature, the optimal pH for glucoamylase is around pH 4.5. However, to determine if this was optimal for our assay, 11 different pH levels were investigated, ranging from pH 3.0 – pH 8.0, with an incremental increase of 0.5. The assay was performed using gelatinised starch suspension and the 4000-fold enzyme dilution. In order to obtain the correct pH level, both the gelatinised starch suspension and the enzyme dilution were made with 0.2 M Na-acetate buffer, adjusted to the corresponding pH. For example, for the pH 3.0 value, the 2% (w/v) starch suspension was made using 0.2 M Na-acetate buffer, pH 3.0 and the enzyme dilution was made by taking 2.5 µL enzyme stock (300 U/mL) and making it up to 10 mL solution with 0.2 M Na-acetate buffer, pH 3.0. This was done for all the pH levels. The starch suspensions were then gelatinised and the enzyme dilutions were left for 10 min before being placed in the water bath for 5 min to adjust to temperature.

Once the enzyme dilutions and starch suspensions were made, 0.5 mL of each was used for the glucoamylase assay. The assay was carried out at 37 °C for 30 min, following the remainder of the procedure as described in Section 3.9.5, and the results are shown in Figure 6.7.

The optimal pH for the glucoamylase activity was determined to be between pH 3.5 and pH 5.5. Based on this observation, a pH of 4.5 was chosen as the pH to be used for the assay.

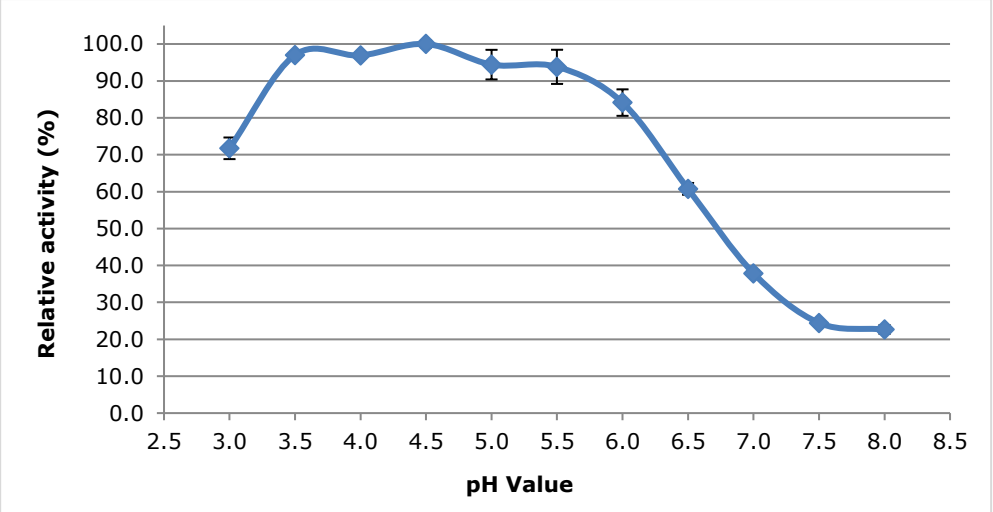


Figure 6.7 Effect of pH on activity of commercial glucoamylase (4000-fold dilution). The assay was carried out on gelatinised soluble starch at 37 °C for 30 min. Results are the mean (n=3).

6.2.4.4 Optimisation of temperature

Finally, the optimal temperature for the assay was investigated. Several different temperatures were investigated, ranging from 37 – 100 °C. The assay was performed using gelatinised starch suspension and the 4000-fold enzyme dilution at pH 4.5. Once the enzyme dilutions and starch suspensions were made, they were added to the water bath of the corresponding temperature and left for 5 min to acclimate to that temperature. The assay was then started and was carried out for 30 min, following the remainder of the procedure as described in Section 3.9.5.

Figure 6.8 shows the relative activity of glucoamylase activity at the different temperatures. The optimal temperature for the glucoamylase activity was determined to be 60 °C. At temperatures higher than 60 °C, the glucoamylase activity dropped rapidly, with a relative activity of 36.4% at 70 °C. Temperatures higher than 80 °C showed no glucoamylase activity.

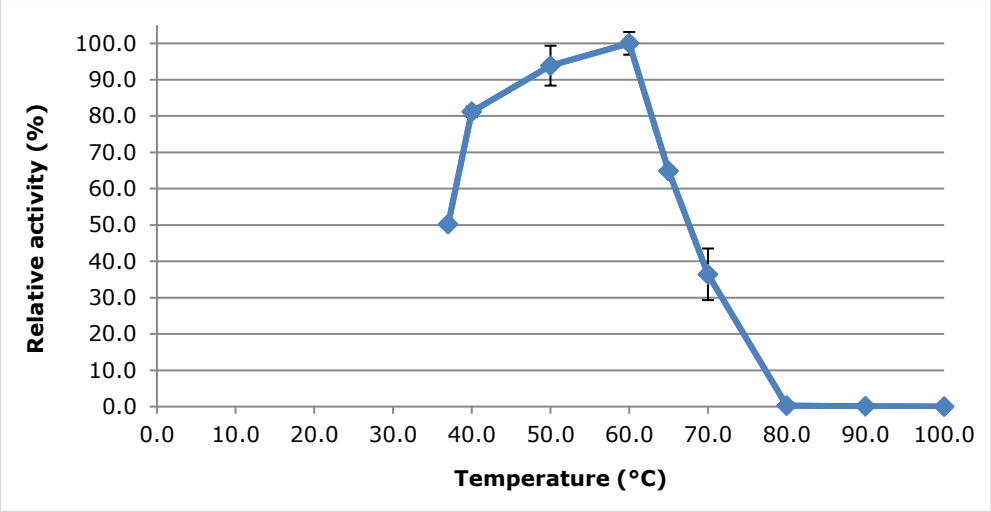


Figure 6.8 Effect of temperature on activity of commercial glucoamylase (4000-fold dilution). The assay was carried out on gelatinised soluble starch at pH 4.5 for 30 min. Results are the mean (n=3).

6.2.5 Glucoamylase analysis of fungal filtrate revisited

Based on the results obtained for the commercial glucoamylase, it was determined that the original glucoamylase assay did work and that the optimal conditions for the assay included the use of gelatinised starch at pH 4.5 and 60 °C. Based on this information, the assay was repeated using the recovered SSF fungal filtrate, to determine glucoamylase activity. To obtain the fungal filtrate, the Sago hampas substrate was prepared with the addition of yeast extract (YE; 0.0175 g/g substrate) and MSI up to 80% (w/v) MC. The SSF process commenced with the addition of *A. awamori* spores and the samples were analysed after a 5 d incubation period, as described in Section 3.7. The glucoamylase activity was assayed using the optimised conditions, with the method being described in Section 3.9.5.

Figure 6.9 shows the glucoamylase activity of the fungal filtrate produced by *A. awamori* during the SSF process. The glucose concentration was recorded at 0, 10 and 30 min. At the beginning of the assay, there was very little glucose present (0.048 ± 0.127 mg/mL), and there was no

significant change in glucose concentration over time (maximum glucose reached 0.489 ± 0.216 mg/mL), (p=0.161).

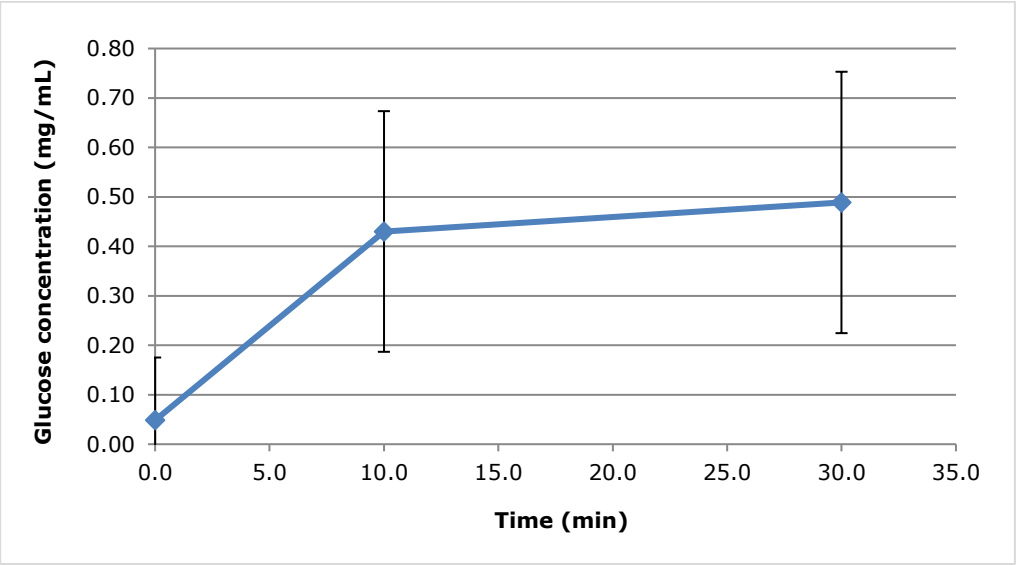


Figure 6.9 Glucoamylase activity of fungal filtrate. The fungal filtrate was collected after 5 d incubation and glucoamylase activity was determined as glucose production from gelatinised soluble starch. The assay was carried out at pH 4.5 and 60 °C for up to 30 min. The results are the mean + SD (n=3).

Although the glucoamylase activity of the fungal filtrate was low, it was noted that the glucose concentration of the fungal filtrate itself was relatively high. The experimental control (which contained 0.5 mL fungal filtrate + 0.5 mL 0.2 M Na-acetate buffer, pH 4.5) gave a value 7.839 mg/mL glucose. Therefore, most of the glucose present in the experimental tube was from the fungal filtrate, and was present at the beginning of the assay.

6.2.5.1 SSF with inoculated versus non-inoculated substrate

From the results of the previous experiment it was determined that no or little glucoamylase activity could be detected in the fungal filtrate. However, there were high levels of sugar present in the fungal filtrate at the beginning of the assay (end of SSF process). This high level of sugars present in the fungal filtrate could be due to several reasons; either the

fungus could have broken down the Sago starch during the SSF process, or the sugars present could have been a result of the autoclaving process before the SSF process. To determine the reason for the presence of sugars, two different SSF experiments were set up.

The substrate used for both experiments was Sago hampas + 0.0175 g/g YE + MSI to 80% (w/v) MC. Two substrate samples were autoclaved and then *A. awamori* spores added to one of these; the second one was not inoculated with spores. Both experiments were then incubated for 5 d at the same time, as described in Section 3.7. The extraction of the fungal filtrate and performance of the glucoamylase assay were carried out the same way for both experiments, as described in Sections 3.7 and 3.9.5, respectively.

Over the 30 min glucoamylase assay period, there was little change in the glucose concentration for both SSF experiments (data not shown). This indicated that there was no enzyme or little enzyme activity detected, as the gelatinised starch was not broken down into glucose. However, as found in the previous experiment, the glucose concentration of the enzyme control tube (the fungal filtrate) was much higher, and there was a significant difference between the glucose concentrations of the two enzyme control tubes, as shown in Table 6.3 ($p=8.554 \times 10^{-10}$).

Table 6.3 Glucose concentration of enzyme control tubes (0.5 mL SSF fungal filtrate + 0.5 mL 0.2 M Na-acetate buffer, pH 4.5) for inoculated and non-inoculated substrates. Both experiments were incubated for 5 d at 28 °C. The glucoamylase assay was carried out on gelatinised starch at pH 4.5 and 60 °C for 30 min (n=3).

Experiment	Glucose concentration (mg/mL)
No spores added	0.262 ± 0.003
Spores added	10.398 ± 0.049

The glucose concentration of the fungal filtrate from the experiment that was not inoculated was much lower compared with the fungal filtrate from the inoculated experiment, indicating that the glucose present was not due

to the autoclaving process, but must have been produced afterwards, presumably from fungal activity.

6.2.5.2 Determining if fungal filtrate was inhibiting glucoamylase activity

Since the glucose present in the fungal filtrate was from the SSF process, it was assumed the fungus must have been producing some enzymes in order to break down the Sago starch into glucose during the SSF process. However, no or very little glucoamylase activity was detected. This may have been due to several reasons – (i) either the background level of sugars (reducing groups) in the solution was saturating the solution, or (ii) there was no or little glucoamylase activity at the time. This second reason could have been due to either no enzyme being present or the enzyme was present but the sugars in the solution were inhibiting the enzyme.

In order to determine if there were factors in the fungal filtrate that were inhibiting glucoamylase activity, assays were carried out using the following enzyme solutions: (i) D5 SSF fungal filtrate, (ii) commercial enzyme (4000-fold dilution), (iii) D5 SSF fungal filtrate + commercial enzyme (4000-fold dilution). The glucoamylase assay was carried out on the three enzyme solutions as described in Section 3.9.5. For the enzyme solution containing a mixture of the D5 SSF fungal filtrate + the commercial enzyme (2000-fold dilution), the enzyme solution was made by mixing the two enzyme solutions in a 1:1 ratio. To carry out the glucoamylase assay, 0.5 mL of this solution was added to the test tubes.

Table 6.4 shows the glucoamylase activity after the 30 min assay for the different enzyme solutions. All three solutions had a glucose concentration close to 0 mg/mL at the beginning of the assay. The glucose concentration for the D5 SSF fungal filtrate did not change over the assay period. However, the glucose concentration for the commercial enzyme, as well as

the D5 SSF fungal filtrate + commercial enzyme did change significantly over the 30 min period ($p<0.05$). The glucose concentration increased to 8.363 ± 0.531 mg/mL for the commercial enzyme, and increased to 6.975 ± 0.664 mg/mL for the D5 SSF fungal filtrate + commercial enzyme.

Table 6.4 Glucoamylase activity for three enzyme solutions – (i) D5 SSF fungal filtrate, (ii) Commercial enzyme (4000-fold dilution), (iii) D5 SSF fungal filtrate + Commercial enzyme (4000-fold dilution). Glucoamylase activity was determined as glucose production from gelatinised soluble starch. The assay was carried out at pH 4.5 and 60 °C for 30 min (n=3).

Experiment	Glucose concentration at 30 min (mg/mL)
Fungal filtrate	0.003 ± 0.019
Commercial enzyme	8.363 ± 0.531
Fungal filtrate + Commercial enzyme	6.975 ± 0.664

This implied that the fungal filtrate does not contain any factors that inhibit the commercial enzyme. However, the fact that the commercial enzyme plus fungal filtrate gave a slightly lower result than the commercial enzyme implies that the enzyme assay is indeed becoming "saturated" with glucose potentially due to the high background level of sugar in the filtrate.

A glucoamylase assay was also carried out on the D5 fungal filtrate following dialysis to remove the sugars. Whilst the enzyme control in this instance showed a much lower glucose concentration, the assay itself failed to detect any glucoamylase activity (data not shown).

6.2.5.3 Glucoamylase activity during the SSF

The experiments described above have shown that there was little or no glucoamylase activity present in a fungal filtrate after 5 d incubation but that there was an appreciable amount of free reducing sugars being produced. To determine if the enzyme could be detected earlier in the SSF process, the glucoamylase assay was repeated on fungal filtrates recovered from shorter SSF incubation periods.

To do this three fungal SSF experiments were set up with varying incubation periods. The Sago hampas (with the addition of 0.0175 g/g YE and MSI to 80% (w/v) MC) was inoculated with *A. awamori spores* and then incubated for either 6 h, 24 h or 5 d at 28 °C. The enzymes (fungal filtrate) were extracted as described in Section 3.7, and the glucoamylase assay was carried out as described in Section 3.9.5.

Over the 30 min glucoamylase assay period, there was little change in the glucose concentration for all the SSF experiments (data not shown). This indicated that there was no enzyme or little enzyme activity detected, as the gelatinised starch was not hydrolysed. However, similar to previous experiments, there was a significant difference between the glucose concentrations of the enzyme control tubes. As shown in Table 6.5, the glucose concentration increased significantly over the three time periods from 0.414 ± 0.002 mg/mL (6 h SSF filtrate), to 12.096 ± 0.024 mg/mL by D5 ($p<0.05$).

Table 6.5 Glucose concentration of enzyme control tubes (0.5 mL SSF fungal filtrate + 0.5 mL 0.2 M Na-acetate buffer, pH 4.5). 6 h, 24h h, D5 fungal SSF were carried out for 6 h, 1 d and 5 d, respectively (n=3).

Experiment	Glucose concentration (mg/mL)
6 h SSF fungal filtrate	0.414 ± 0.002
24 h SSF fungal filtrate	1.891 ± 0.006
D5 SSF fungal filtrate	12.096 ± 0.024

From these results, it was concluded that there were sugars already present at 6 h and this increased over the 5 d incubation period. Therefore, the fungal enzymes must have been hydrolysing the starch; however, there was still no glucoamylase activity detected.

6.2.6 Production of glucose during SSF process

Since glucoamylase activity could not be detected in the fungal filtrate, the production of glucoamylases for extraction and concentration was not viable. However, glucose was being produced during the SSF process, and

it was assumed this was due to the breakdown of Sago starch by the fungal enzymes. Therefore, the focus of the research was shifted to the production and extraction of glucose directly during the SSF process.

In the first instance, the digestibility of the starch in the Saga hampas was determined. This was done by performing an enzymatic hydrolysis of the Sago hampas, using a commercial glucoamylase (300 U/mL, Sigma-Aldrich, UK). The amount of Sago hampas used was 6 g and this was made up to 21 mL with 0.2 M Na-acetate buffer, pH 4.5 and autoclaved as for the SSF experiments. The mash was then made up to 10% (w/v) Sago hampas by adding a further 39 mL autoclaved buffer (totalling 60 mL). The commercial enzyme was added and the experiment was placed in a shaking incubator set at 60 °C and 150 rpm for up to 24 h. The enzyme concentration used was 0.25 U/g Sago hampas (addition of 5.0 µL) and samples were taken over 24 h. The sugar composition for all samples was quantified by HPLC and prepared as described for the previous SSF experiments. The glucose produced over the 24 h hydrolysis period is shown in Figure 6.10.

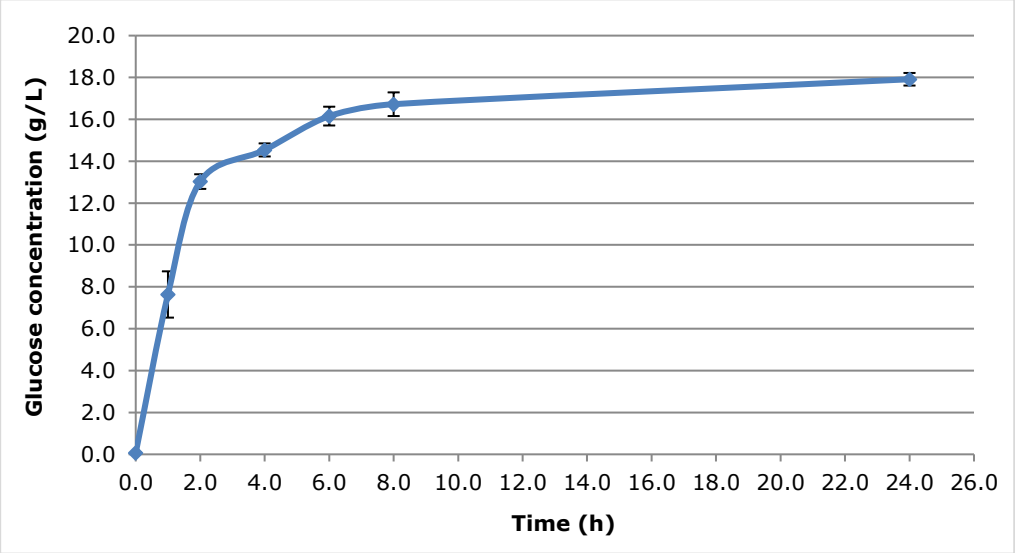


Figure 6.10 Production of glucose using a commercial glucoamylase (stock – 300 U/mL) at 0.25 U/g Sago hampas. 6 g Sago hampas, made up to 10% (w/v), was used as substrate; hydrolysis performed in shaking incubator at 60 °C and 150 rpm. The results are the mean + SD (n=3).

A glucose concentration of 17.92 ± 0.30 g/L was reached within the 24 h period, with the fastest rate of production occurring within the first 2 h (13.03 ± 0.35 g/L). The represented a 31.72% conversion of glucose after the 24 h period (0.179 ± 0.003 g/g Sago).

This experiment demonstrated that the Sago hampas starch, as prepared for the SSF experiments, could be readily digested by the commercial enzyme. Thus indigestibility of the starch would not be a factor in determining the efficiency of glucose production. In order to assess the efficiency of glucose production from the Sago hampas during SSF, three experiments were set up – one continuous SSF, and two SSF experiments with washing. The substrate preparation, addition of fungal spores, incubation, and extraction of the fungal filtrate were carried out the same for all three experiments, as described in Section 3.7. The fungal filtrate for all samples were filtered using 0.2 µm pore size Whatman GD/X syringe filters (GF/C 25 mm filter diameter; Whatman International Ltd., UK), and diluted 1:100. The samples were quantified by high performance liquid chromatography (HPLC), as described in Section 3.6.6.3. Sugar standards were prepared, as described in Section 3.6.6.2, and analysed with samples. The remaining pellet (fungus and undigested biomass) was oven dried overnight, and then weighed and analysed for starch content, as described in Section 3.6.5.

In the continuous SSF experiment, the total time that the inoculated mash was incubated for was 21 d. However, enough Sago was prepared to take 17 time points at the following times - 0 h, 3 h, 6 h, 8 h, 12 h, 1 d, 1.5 d, 2 d, 3 d, 4 d, 5 d, 6 d, 7 d, 10 d, 14 d, 19 d and 21 d. At each time point, three Petri dishes containing inoculated mash (3 x 2 g Sago hampas each) were removed from incubation and the fungal filtrate extracted and

analysed for sugars. The pellet remaining after the extraction was oven dried overnight, weighed and analysed for starch.

In the SSF with washing experiments, two sets of petri dishes with mash were set up. These were incubated under the same conditions as the continuous SSF, but plates from one set were washed (to recover the fungal filtrate) every 3 days and the other set every 5 days. After washing, the mash was left to continue incubation. For the D3 SSF washing experiment there were six sampling points giving a total 18 d incubation, and for the D5 SSF washing experiment there were four sampling points giving a total 20 d incubation. After the last extraction, the remaining pellets were oven dried overnight, weighed and analysed for starch.

To set up each washing experiment, 3 x 6 g Sago hampas samples were prepared and inoculated with fungal spores. Each 6 g sample of inoculated mash was distributed equally amongst three Petri dishes and incubated together for either 3 d or 5 d. For the D3 SSF washing experiment, the Petri dishes (three sets of three dishes) were removed after the 3 d incubation period. Each 6 g sample of inoculated mash was mixed in a blender with 36 mL 0.5 M Na-acetate buffer, pH 4.5 (6 mL/g Sago) and the fungal filtrate recovered. The pellet remaining after centrifugation (and removal of the filtrate) was redistributed amongst three Petri dishes and incubated for another 3 d before repeating the washing and incubation cycle. The same procedure was carried out for the D5 SSF washing experiment; however, the samples were incubated for 5 d before being removed, the filtrate extracted and then the pellet incubated for another 5 d before repeating the extraction process.

The glucose concentration in the fungal filtrate for the continuous SSF experiment is shown in Figure 6.11. Over the 21-day incubation period, the glucose concentration in the recovered fungal filtrates increased,

reaching 0.077 ± 0.006 g, and this increase was significant ($p<0.05$). The trend of glucose production generally slowed, resulting in it beginning to plateau around day 12. However, there were oscillations around this general trend. For example, a similar glucose concentration was recovered in the day 14 fungal filtrate (0.081 ± 0.002 g), ($p=0.786$); but the glucose concentration in the D19 fungal filtrate was much lower at 0.059 ± 0.006 g ($p<0.05$). This might indicate that the fungus was breaking down the starch, releasing glucose into the solution. However, at times the rate of consumption was greater than the rate of production (resulting in the glucose concentration of the solution decreasing). The concentrations of arabinose, galactose and xylose were measured for all incubation time points; however, the concentrations of these sugars in the recovered fungal filtrates was negligible (data not shown).

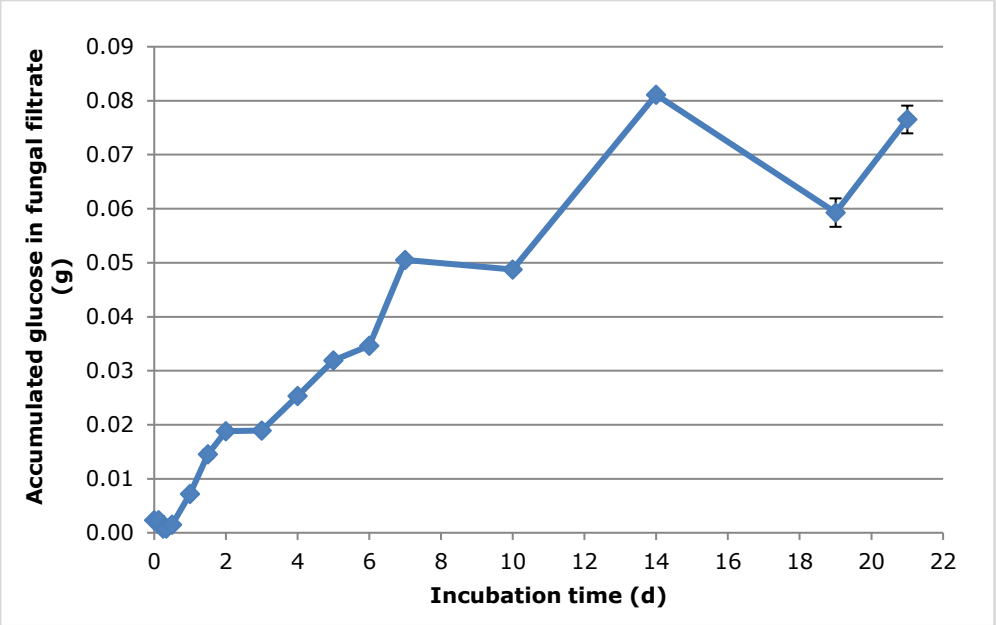


Figure 6.11 Glucose recovered in continuous SSF fungal filtrates over 21 d incubation period. SSF carried out using *A. awamori* on Sago hampas (with addition of 0.0175 g/g YE and MSI to 80% (w/v) MC), at 28 °C in a static incubator for up to 21 d. The results are mean + SD (n=3).

During the 21 d SSF incubation period there was an initial increase in the mass of the pellet after 1 d incubation and then a significant decrease over the next 20 d to 1.05 ± 0.02 g (52.33 ± 1.25 % (w/w initial mass)

($p<0.05$). It was interesting to note that the mass of the 0 d dried pellet was 1.27 ± 0.01 g (63.67 ± 0.47 % w/w initial mass), meaning around 36% of the total mass (just over 2 g) was lost during the autoclaving, extraction and drying process. The initial mass of starch added was 1.02 ± 0.03 g, and this calculation was based on previous determination of the starch content of Sago hampas (50.88 ± 1.96 % w/w dry weight basis). The starch content of the pellet after the autoclaving, extraction and drying process was 0.71 ± 0.03 g. This corresponded to 69.88 ± 2.04 % (w/w initial starch) starch remaining before the SSF began. During the 21 d SSF incubation period, the starch content of the pellet gradually decreased to 0.36 ± 0.02 g by day 21, corresponding to 35.06 ± 1.94 % (w/w initial starch) starch remaining, and this loss was significant ($p<0.05$). Based on this information, 0.35 g of starch was used up during the SSF process, indicating that the fungus was breaking the starch down into glucose during the SSF.

The glucose recovered in the fungal filtrate for the D3 and D5 washing experiments is shown in Figure 6.12. For the D3 washing experiment, the inoculated mash was washed every 3 days, for a total of 6 washes (18 d incubation), and for the D5 washing experiment, the inoculated mash was washed every 5 days, for a total of 4 washes (20 d incubation). The glucose recovered after each wash was similar for both washing experiments (Figure 6.12A), with the highest glucose being recovered after wash 2 (7.76 ± 0.06 g/L for D3 washing and 7.00 ± 0.25 g/L for D5 washing). Overall, a small amount of glucose was recovered after wash 1, followed by a spike in wash 2, and then a steady decrease in glucose recovered after subsequent washes.

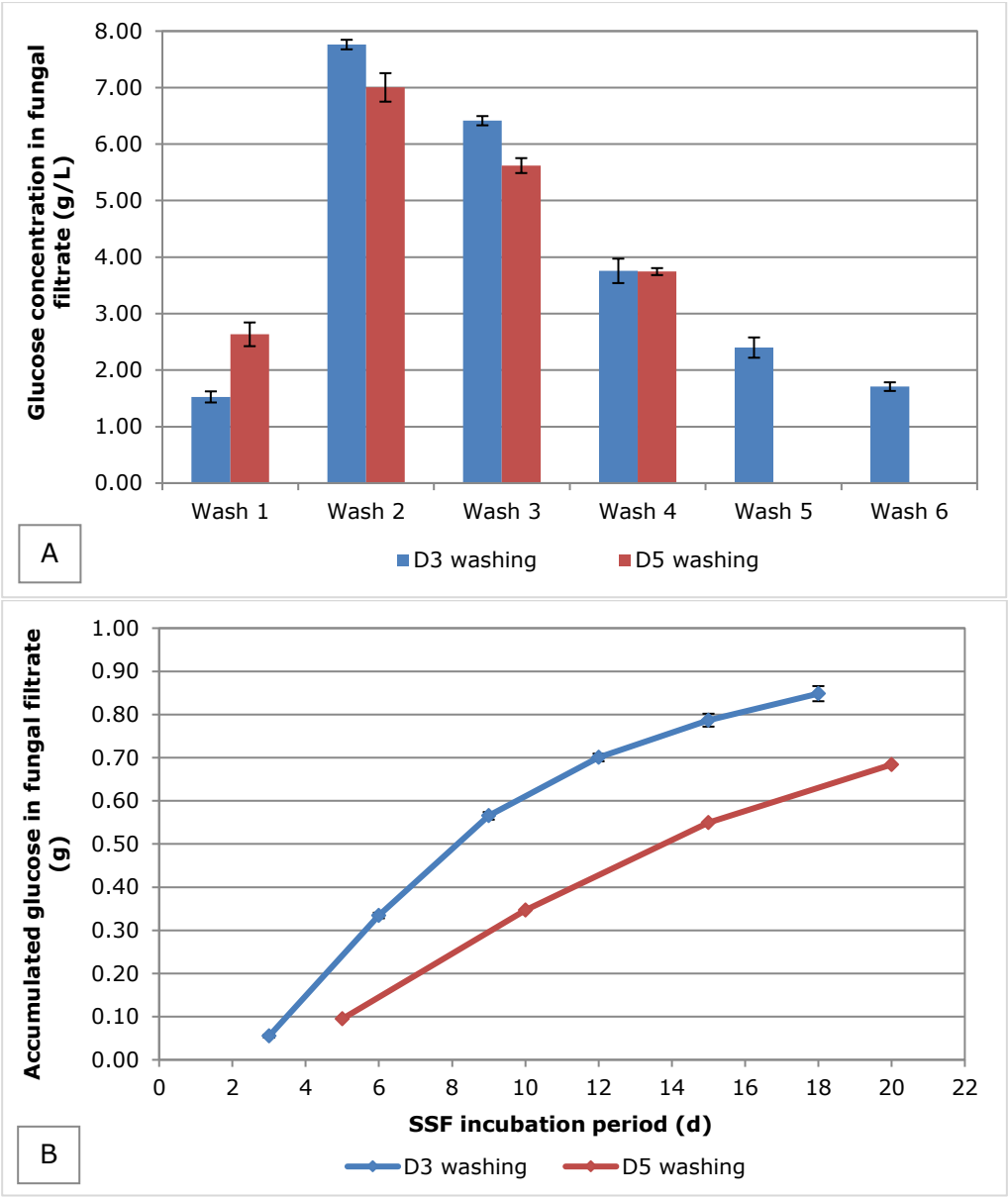


Figure 6.12 Glucose recovery from washed SSF experiments. (A) Glucose recovered in SSF fungal filtrates and (B) Accumulation of glucose in fungal filtrate for D3 and D5 washing experiments. SSF carried out using *A. awamori* on Sago hampas (with addition of 0.0175 g/g YE and MSI to 80% (w/v) MC), at 28 °C in a static incubator for up to 20 d incubation. The results are mean + SD (n=3).

Figure 6.12B shows the accumulation of glucose recovered from the fungal filtrates over the SSF incubation period. For the D3 washing experiment, a total of 0.848 ± 0.018 g glucose was recovered over the 18 d SSF. For the D5 washing experiment, a total of 0.684 ± 0.016 g glucose was recovered over the 20 d SSF, and these were significantly different (p=0.001). Although a similar amount of glucose was produced after each wash for

both the D3 and D5 washing experiments, since the D3 washing experiment was washed every 3 d, an extra two washes were collected within the same time period as the D5 washing experiment. This resulted in the accumulation of more glucose in a similar period of time. When comparing the continuous SSF experiment with the washing experiment, the total glucose recovered from the washing experiments was significantly greater than the glucose recovered from the 21 d continuous SSF experiment (0.077 ± 0.0.05 g) ($p<0.05$).

To confirm that the continuous SSF experiment was performing similar to the washing experiments, the glucose recovered from the time points which were under the same conditions were compared. The fungal filtrate from day 3 of the continuous SSF (1.58 ± 0.08 g/L) was compared with the first wash of the D3 washing experiment (1.53 ± 0.10 g/L) and found to be similar ($p=0.594$). The same was done with the fungal filtrate from day 5 of the continuous SSF (2.66 ± 0.07 g/L) and the fungal filtrate from the first wash of the D5 washing experiment (2.63 ± 0.21 g/L) which were again similar ($p=0.872$). These results indicate that the fungal spores in the continuous SSF were performing the same as the fungal spores in the washing experiments. Therefore, it can be concluded that the higher glucose recovered from the washing experiments, was due to the washing of the inoculated mash throughout the SSF process.

During the SSF process there was a decrease in the starch content of the pellet for both washing experiments. The starch remaining after the 20 d D5 washing experiment (16.76 ± 1.40 % (w/w initial starch)) was slightly higher than the starch remaining after the 18 d D3 washing experiment (13.96 ± 1.72 % (w/w initial starch)). These values were similar ($p=0.295$); however, both values were significantly lower than the starch

remaining after the continuous SSF experiment (35.06 ± 1.94 % (w/w initial starch), ($p<0.05$).

Overall, the washing experiments produced higher levels of glucose and resulted in a greater utilisation of starch. To determine which experiment was most efficient at starch conversion, the actual glucose produced was compared with the theoretical (maximum) glucose production that could have been achieved for each experiment, and these results are summarised in Table 6.6. The continuous SSF had the least efficient conversion of glucose at 10.11%. The SSF with washing experiments were much more efficient at converting starch to glucose, with the D3 SSF washing experiment being the most efficient at producing glucose (29.10%).

Table 6.6 Mass balance showing starch consumed and glucose produced during the three different experiments, and the efficiency of the conversion.

	SSF with D3 Washes			SSF with D5 Washes			Continuous SSF		
	In	Out	Net	In	Out	Net	In	Out	Net
Sago (g)	6.00	-		6.00	-		2.00	-	
Starch (g)	3.05	0.43	-2.63	3.05	0.51	-2.54	1.02	0.36	-0.66
Theoretical glucose produced (g)	-	2.92	2.92	-	2.82	2.82	-	0.73	0.73
Actual glucose produced (g)	-	0.85	0.85	-	0.68	0.68	0.002	0.077	0.074
Efficiency (%)	-	-	29.10	-	-	24.25	-	-	10.11

Although the washing experiments resulted in a higher production of glucose compared with the breakdown of starch, the efficiency was just below 30%, meaning some starch and/or glucose was lost in the process. This could have been due to either consumption of glucose by the fungus, or the starch could have been lost in the fungal filtrate during washing before it was converted to glucose. To determine how much, if any, starch was lost in the washing process, the fungal filtrates from wash 1 of both washing experiments were treated with commercial glucoamylase (300

U/mL, Sigma-Aldrich, UK) to determine if any sugars were produced. The glucoamylase assay was performed using the fungal filtrate as the substrate (0.5 mL), and the commercial enzyme was diluted 4000-fold. The assay was carried out at 60 °C and pH 4.5 for 30 min as described in Section 3.9.5.

The starch recovered in the fungal filtrate from wash 1 was 1.790 ± 0.098 and 1.031 ± 0.043% of the total in the mash for D3 and D5 washing, respectively. The starch lost from the D3 washing experiment was significantly more than the starch lost from the D5 washing experiment ($p=0.001$). Although only a minimal amount of starch was lost from wash 1, the starch lost from washes 2 – 6 were not analysed and so this may have increased or decreased. If starch was lost during these washes, the total starch lost during the SSF process could become significant and have a significant effect on the glucose production and efficiency of the process.

6.2.6.1 Optimisation of SSF with washing for the production of glucose

The previous experiment determined that SSF with washing resulted in a higher accumulation of glucose when compared with the continuous SSF. It was also shown that the timing of the wash cycle period was important, as the D3 washing resulted in a higher yield of glucose than the D5 washing. Therefore, the washing experiments were repeated, looking at washing cycles shorter than the 3 d cycle. The washing solution used to extract the filtrate was also examined, using either buffer (0.2 M Na-acetate, pH 4.5) or 0.0175 g/g YE + MSI to 80% (w/v) MC.

The substrate preparation, addition of fungal spores, incubation, and extraction of the fungal filtrate were carried out the same for all experiments, as described in Section 3.7. The sugar composition of the fungal filtrates for all samples was quantified by HPLC, as described in

Section 3.6.6.3. The remaining pellet was oven dried overnight, and then weighed and analysed for starch content, as described in Section 3.6.5. To set up each washing experiment, 6 g Sago hampas samples were prepared and inoculated with fungal spores (in triplicate). Seven experiments were set up and were all incubated for 6 d, with periodic washing for the different experiments.

Four different washing experiments were set up looking at different washing cycles. The inoculated mash was either washed (with buffer) every day (D1 washing), every second day (D2 washing), every third day (D3 washing), or after three days and then every day there-after (D3,D1 washing). The D3 washing experiment was performed as a comparison to the D3 washing experiments run previously. Three more experiments were set up to look at the effect of wash solution on the glucose production. These were washed (with YE + MSI) every day (D1 washing), every second day (D2 washing), or after three days and then every day there-after (D3,D1 washing).

The glucose concentrations in the recovered fungal filtrate for the seven different experiments is shown in Figure 6.13. When comparing the wash solution used (buffer or YE + MSI), the glucose recovered after wash 1 was similar (between 1.5 – 2.0 g/L), regardless of wash solution ($p=0.066$, $p=0.133$, $p=1.000$ for D1, D2 and D3,D1 washing experiments respectively). However, for subsequent washes, the experiments washed with buffer solution resulted in a significantly higher glucose concentration recovered in the filtrate for all experiments ($p<0.05$).

Overall, very little glucose was produced over the 6 d SSF for the three experiments washed with YE+MSI. The highest glucose concentration was recovered after wash 1 and this was followed by a decrease in glucose production in subsequent washes, as seen in Figure 6.13.

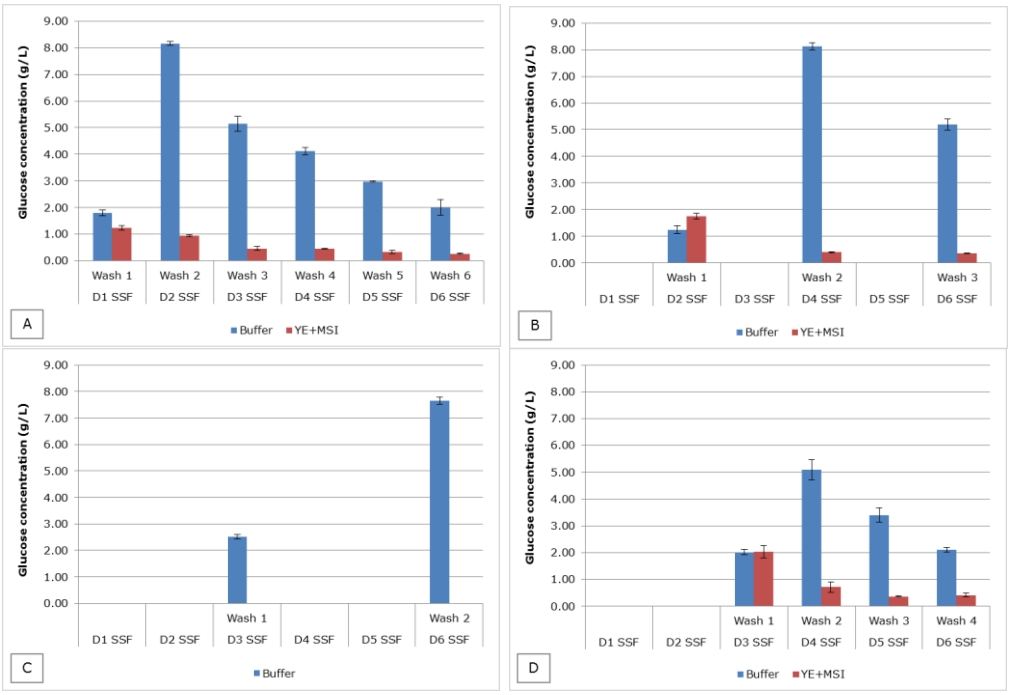


Figure 6.13 Effect of washing cycle and wash buffer on glucose production. SSF was carried out using *A. awamori* on Sago hampas (with the addition of 0.0175 g/g YE and MSI to 80% (w/v) MC on day 0), at 28 °C in a static incubator for up to 6 d. The glucose recovered in SSF fungal filtrates was measured for (A) D1 washing, (B) D2 washing, (C) D3 washing, (D) D3,D1 washing experiments, respectively. The fungal filtrate was extracted using either 0.2 M Na-acetate, pH 4.5 buffer or 0.5% (w/v) YE + MSI. The results are the mean + SD (n=3).

Comparing the results for the experiments washed with buffer, the glucose recovered in the fungal filtrates after each wash followed a similar trend, regardless of wash cycle (Figure 6.13). Overall, a small amount of glucose was recovered after wash 1, followed by a spike in wash 2, and then a steady decrease in glucose recovered after subsequent washes. The glucose recovered after each wash for the D1, D2 and D3 washing experiments was similar (around 2 g/L, 8 g/L and 5 g/L recovered after washes 1, 2 and 3 respectively). However, although the D3,D1 washing experiment followed the same trend, it produced slightly less glucose in wash 2 (5.09 ± 0.38 g/L), wash 3 (3.41 ± 0.26 g/L) and wash 4 (2.12 ± 0.08 g/L), and these were significantly different ($p<0.05$).

Figure 6.14 shows the accumulation of glucose recovered from the fungal filtrates over the SSF incubation period, when washed with buffer solution.

The glucose accumulated for each wash cycle was significantly different from one another, with the D1 washing experiment resulting in the highest glucose accumulation (a total of 0.885 ± 0.022 g glucose recovered from the 6 washes over the 6 d SSF incubation period), ($p<0.05$). Although a similar amount of glucose was produced after each wash for the D1, D2 and D3 washing experiments, since the D1 washing experiment was washed every day, at least an extra three washes were performed within the same time period. This resulted in the accumulation of more glucose in a similar period of time.

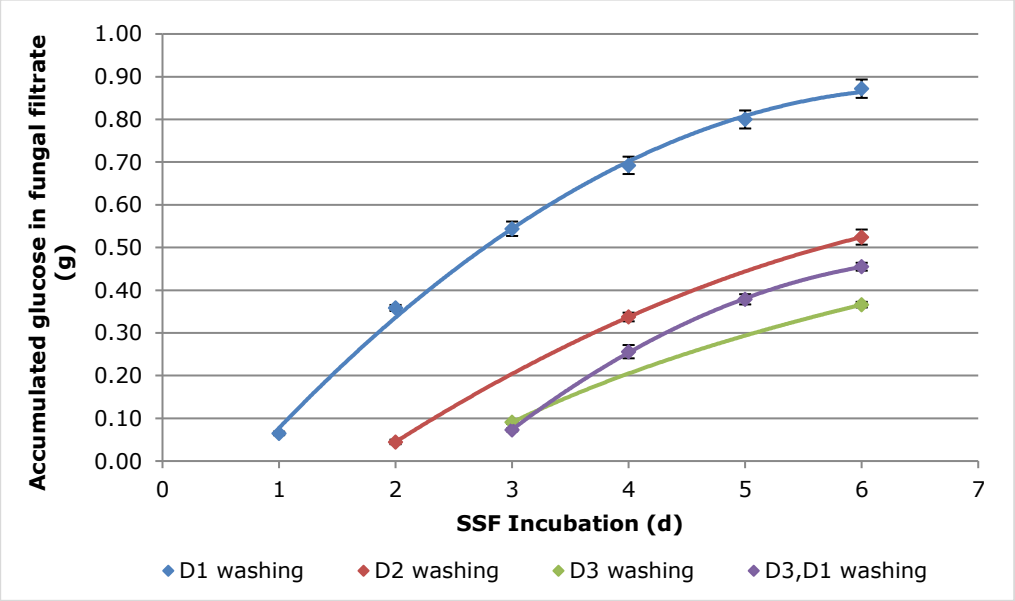


Figure 6.14 Accumulation of glucose in fungal filtrate for D1, D2, D3, and D3,D1 washing experiments. SSF carried out using *A. awamori* on Sago hampas (with addition of 0.0175 g/g YE and MSI to 80% (w/v) MC on day 0), at 28 °C in a static incubator for up to 6 d incubation. Filtrates were collected by washing with 0.2 M Na-acetate buffer, pH 4.5. The results are the mean + SD (n=3).

At the beginning of the SSF, 6 g of Sago hampas was used (in triplicate) for all experiments. After the 6 d incubation the mass of the pellets for all experiments had decreased to between 1.88 – 2.83 g (31 – 47 % w/w initial dry mass), with the greatest decrease being in the D1 washing experiment and the least decrease being in the D3 washing experiment. The percent pellet remaining for the YE+MSI wash and buffer wash within

each wash cycle was similar (p=0.904, p=0.864 and p=0.165 for the D1, D2 and D3,D1 washing experiments respectively).

However, when comparing the percent starch remaining between the washes, the experiments that were washed with YE + MSI had significantly less starch remaining than the experiments washed with buffer ($p<0.05$), even though the pellet remaining was similar (Figure 6.15).

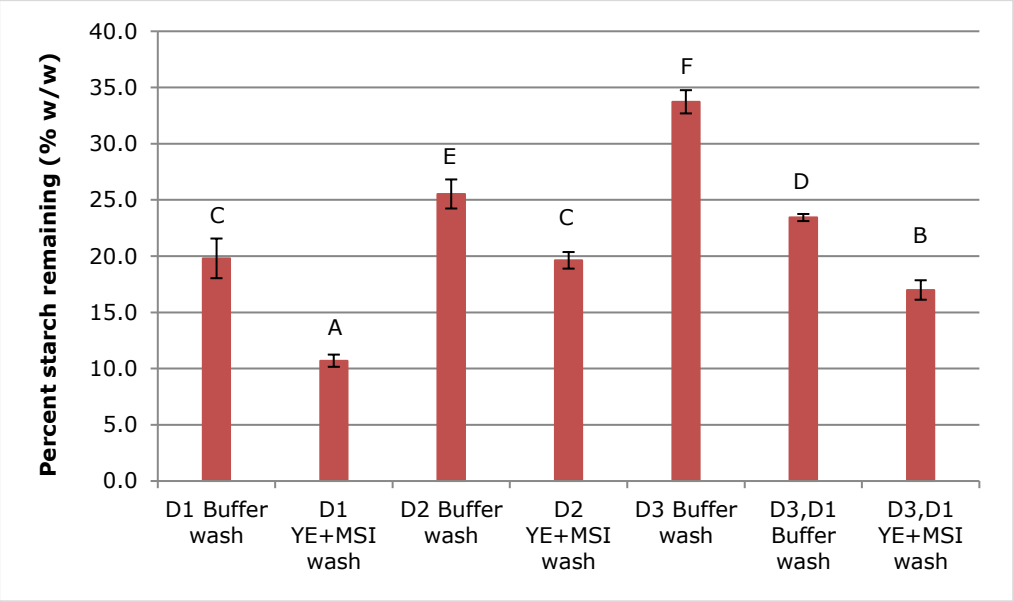


Figure 6.15 Percent starch (based on 50.88% starch in Sago hampas) remaining at end of SSF incubation. SSF carried out using *A. awamori* on Sago hampas (with addition of 0.0175 g/g YE and MSI to 80% (w/v) MC on day 0), at 28 °C in a static incubator for up to 21 d. The results are the mean + SD (n=3). Bars with different letters, within the same data set, are significantly different (p<0.05).

For example, the starch remaining in the D1 washing experiment when washed with buffer was 19.80 ± 1.76 % (w/w initial starch) compared with 10.70 ± 0.54 % (w/w initial starch) when washed with YE + MSI ($p=6.962 \times 10^{-13}$). The experiments washed with YE + MSI resulted in very little accumulation of glucose in the extracted solutions over the 6 d period; however, these results indicate that the starch was being hydrolysed. Therefore, the fungus must have been consuming much more of the glucose than was being consumed in the buffer washes. This suggests that

the YE + MSI washing solution contained a lot of nutrients that the fungus could make use of and capitalise on growth.

When looking at the experiments washed with buffer, the starch remaining after each experiment was significantly different ($p<0.05$), with the D1 washing experiment resulting in the lowest starch remaining (0.60 ± 0.05 g, 19.80 ± 1.76 % (w/w)) and the D3 washing experiment resulting in the highest starch remaining (1.03 ± 0.03 g, 33.73 ± 1.03 % (w/w)). The D1 washing experiment had also been washed the most times, and the D3 washing experiment washed the least.

To determine which experiment (washed with buffer) was most efficient at starch conversion, the actual glucose produced was compared with the theoretical (maximum) glucose production that could have been achieved for each experiment, and these results are summarised in Table 6.7. The D1 washing experiment was the most efficient at converting starch to glucose, with an efficiency of 32.14%. This was followed by the D2 washing experiment (20.78 %) and then the D3 washing and D3,D1 washing experiments were the least efficient (16.31 % and 16.18% respectively).

Table 6.7 Mass balance showing starch consumed and glucose produced during the four different experiments, and the efficiency of the conversion.

	D1 washing			D2 washing			D3 washing			D3,D1 washing		
	In	**Out**	**Net**	**In**	**Out**	**Net**	**In**	**Out**	**Net**	**In**	**Out**	**Net**
Sago (g)	6.00	-		6.00	-		6.00	-		6.00	-	
Starch (g)	3.05	0.60	-2.45	3.05	0.78	-2.27	3.05	1.03	-2.02	3.05	0.52	-2.53
Theoretical glucose (g)	-	2.72	2.72	-	2.52	2.52	-	2.25	2.25	-	2.81	2.81
Actual glucose (g)	-	0.87	0.87	-	0.52	0.52	-	0.37	0.37	-	0.46	0.46
Efficiency (%)	-	-	32.1	-	-	20.8	-	-	16.3	-	-	16.2

Overall the D1 washing experiment resulted in the highest accumulation of glucose in this experiment. This was compared with the highest achieving

experiment from the previous experiment, which was the D3 washing experiment (over 18 d incubation). The glucose accumulated after each of the 6 washes was similar in both ($p=0.136$) and the mass of pellet remaining at the end of each experiment was similar ($p=0.640$). However, the starch remaining at the end of the D1 washing experiment (19.80 ± 1.76 % (w/w)) was significantly higher than the starch remaining at the end of the D3 washing experiment (13.96 ± 1.70 % (w/w) ($p=0.00034$). This resulted in a slightly higher conversion of starch for the D1 washing experiment (32.14%) compared with 29.14% for the D3 washing experiment. Also, the D1 washing experiment achieved this level of glucose in 6 d versus 18 d for the D3 washing experiment.

Since the performance of the fungus was similar in each experiment, any improvement in the glucose production in Experiment 2 is likely a result of the wash cycle or wash solution, and not due to the fungus performing better. Based on this information it was concluded that the decrease in wash cycle from 3 d to 1 d was better as the 1 d wash cycle achieved the same glucose concentration in one-third of the time, and consumed less starch.

6.2.6.2 Optimisation of nutrient composition

The previous experiment determined that shorter washing cycles performed best in terms of glucose production, and that washing with buffer solution was better than washing with YE + MSI. In this set of experiments four different experiments were run, changing either the nutrients added to the Sago hampas before autoclaving or the concentration of fungal spores added at the start of the SSF, as shown in Table 6.8. These were set up as in the previous experiment. All experiments were incubated for 6 d, with 1 d washing. The fungal extract

was used instead of YE, and was obtained from the previous experiment (the dried pellet remaining from the D1 washing was crushed up and used in this experiment).

Table 6.8 Nutrients and fungal spore concentration added for four different SSF experiments, all carried out for 6 d with washing every day.

Experiment	Nutrients added before autoclaving	Fungal spore concentration (spores/g Sago)
Sago + water	Water to 80% (w/v) MC	4.0×10^6
Sago + YE + MSI (Control)	0.0175 g/g YE + MSI to 80% (w/v) MC	4.0×10^6
Sago + FE + MSI	0.0175 g/g fungal extract + MSI to 80% (w/v) MC	4.0×10^6
40 x 10^6 spores/g	0.0175 g/g YE + MSI to 80% (w/v) MC	40.0×10^6

The glucose concentration of the fungal filtrates for the four different experiments is shown in Figure 6.16A. Very little glucose was produced when no nutrients were added to the Sago hampas (Sago + water experiment), indicating that the addition of YE and MSI had an impact on glucose production by the fungus. The glucose recovered after each wash for the control experiment (addition of Sago + YE + MSI) followed a similar trend to that seen in previous experiments, with a similar amount of glucose being recovered after each wash.

The glucose recovered from the experiment with the addition of fungal extract (FE) to the Sago hampas (instead of YE), followed a similar pattern to the control; however, more glucose was recovered after each wash, with significant differences occurring in washes 1, 3, 4 and 6 ($p<0.05$). The same was true for the experiment with the addition of 40.0×10^6 spores/g. This resulted in a significantly higher glucose recovery after each wash (except wash 2) when compared with the control experiment ($p<0.05$). When compared with the Sago + FE + MSI experiment, a similar amount of glucose was recovered from washes 1 ($p=1.000$) and 2

(p=0.996); however, after this, a significantly higher glucose was recovered from the 40.0 x 10^6 spores/g experiment (p<0.05).

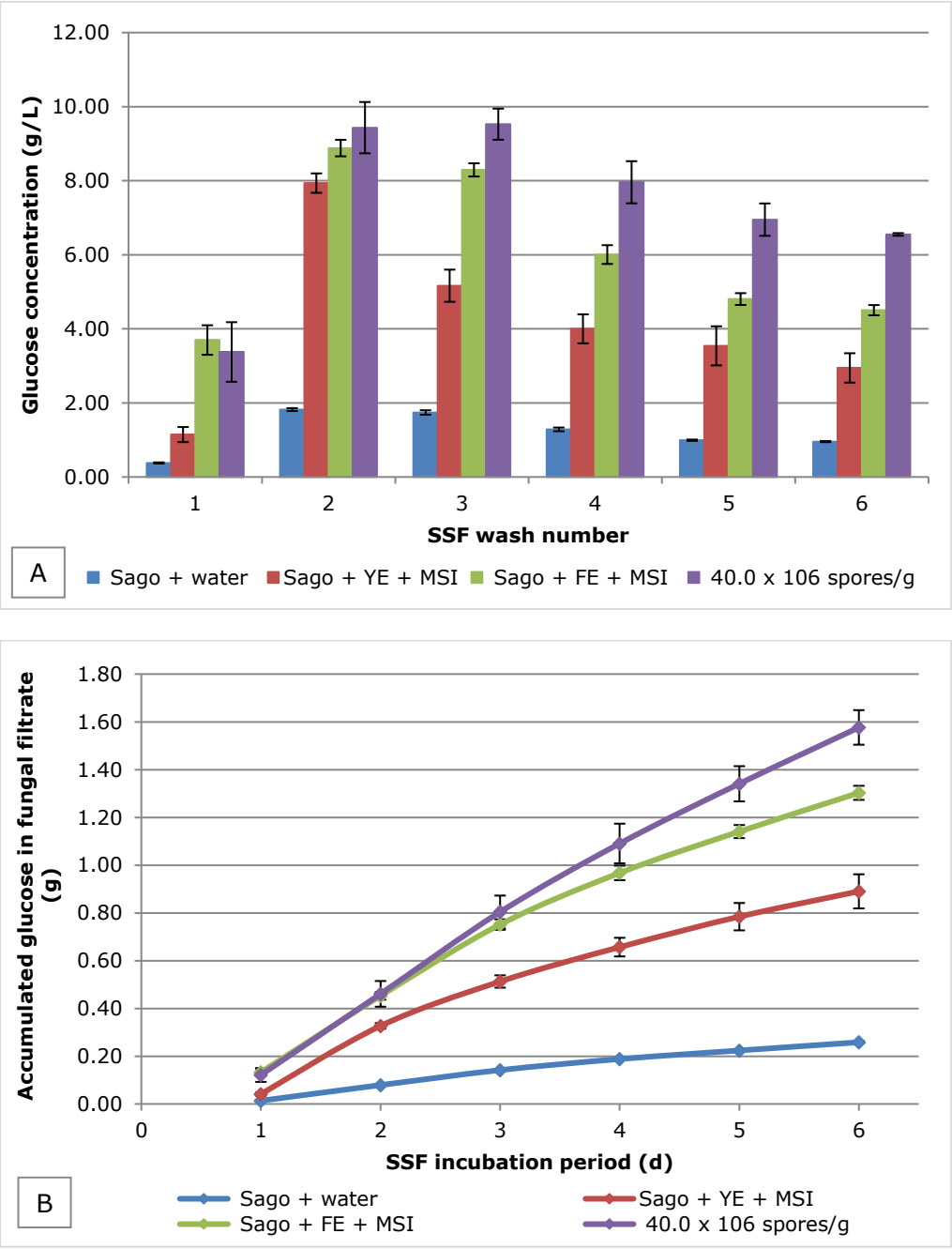


Figure 6.16 Effect of nutrients and fungal spore loading on glucose production. (A) Glucose recovered in fungal filtrates after each wash and (B) Accumulation of glucose over SSF. SSF carried out using *A. awamori* on Sago hampas, at 28 °C in a static incubator for up to 6 d incubation. Nutrients and spores added on day 0 – (i) Sago + water (addition of deionised water to 80% (w/v) MC); (ii) Sago + YE + MSI (addition of 0.0175 g/g YE and MSI to 80% (w/v) MC); (iii) Sago + FE + MSI (addition of 0.0175 g/g fungal extract and MSI to 80% (w/v) MC); (iv) 40.0 x 10^6 spores/g (addition of 0.0175 g/g YE and MSI to 80% (w/v) MC, plus 40.0 x 10^6 spores/g). For all other experiments 4.0 x 10^6 spores/g added. Filtrates were collected by washing with 0.2 M Na-acetate buffer, pH 4.5. The results are the mean + SD (n=3).

The accumulation of glucose from the fungal filtrates over the SSF incubation period was significantly different for all washing experiments ($p<0.05$), as shown in Figure 6.16B. The experiment with the addition of 40.0 x 10^6 spores/g resulted in the highest glucose accumulation with a total of 1.577 ± 0.072 g glucose being recovered from the 6 washes over the 6 d SSF incubation period ($p=0.000006$ when compared with control). The experiment with the addition of FE + MSI resulted in the next highest glucose accumulation with a total of 1.303 ± 0.029 g glucose being recovered over the 6 d SSF incubation period ($p=0.000249$ when compared with control). The efficiency of glucose conversion for the experiment with the addition of 40.0 x 10^6 spores/g was 46.53 % and the efficiency for the experiment with the addition of FE + MSI was 38.44%. The starch content remaining after the SSF incubation period was not calculated and so this could not be used when calculating the glucose conversion efficiency. If some starch was remaining in the pellet at the end of the washing process, the efficiency of glucose conversion would increase. However, even without taking this into account, both these values were still much higher than the efficiency achieved for the D1 washing experiment in the previous experiment (32.14%). This indicates that the addition of FE is better for glucose production than YE, and the addition of more fungal spores results in a higher glucose production.

6.2.7 Production of glucose during submerged fermentation

The Sago hampas was used as a substrate in submerged fermentation to compare the production of glucose with that of the SSF experiments. The same amount of Sago hampas was used as was used in the SSF experiments (6 g). Two different experiments were run, changing the nutrients added to the Sago hampas during preparation. Either 21 mL 0.2 M Na-acetate buffer, pH 4.5 was added to the Sago hampas (Sago +

Buffer experiment), or 0.105 g YE (0.0175 g/g Sago) + 21 mL MSI, pH 4.5 was added to the Sago hampas (Sago + YE + MSI experiment). All samples were autoclaved, as with the SSF experiments, and were then made up to 100 mL (6% w/v Sago) with a further 79 mL autoclaved buffer. MSI was not used for the Sago + YE + MSI experiment so as to keep the amount of MSI added the same as the amount added in the SSF experiments (21 mL). The fungal spores used for the inoculation came from the same batch used in the last of the SSF washing experiments and were incubated at the same time. Incubation was carried out in a shaking incubator set at 28 °C and 150 rpm for 6 d. Samples were taken daily as described in Section 3.8, and were prepared for HPLC as described previously.

The glucose produced in the two different experiments is shown in Figure 6.17. The Sago + Buffer experiment showed very little glucose production over the 6 d incubation period (highest glucose concentration was 0.279 ± 0.046 g/L after 6 d incubation), (p=0.887).

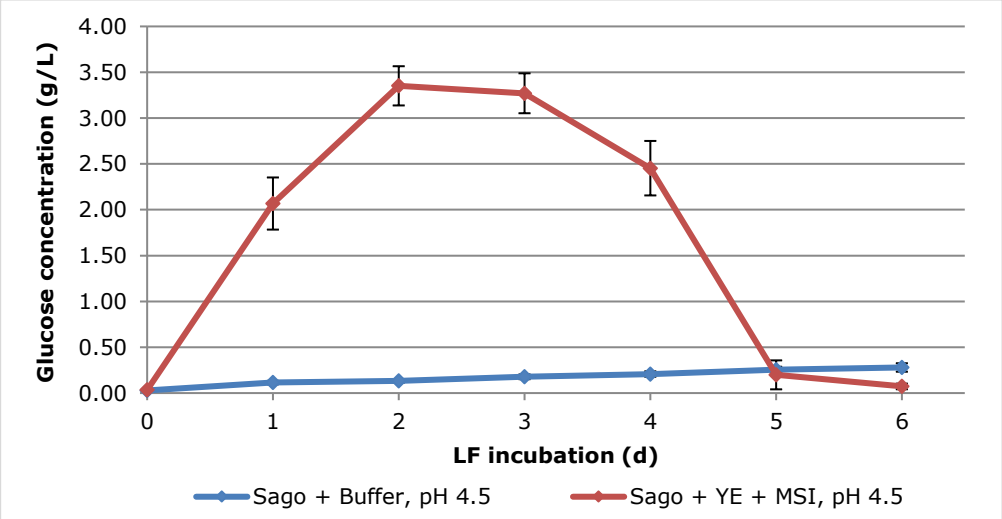


Figure 6.17 Glucose production from Sago hampas substrate by *A. awamori* during submerged fermentation (SmF). Sago hampas prepared with addition of 0.2 M Na-acetate buffer, pH 4.5 to 80% (w/v) MC or prepared with addition of 0.0175 g/g YE and MSI to 80% (w/v) MC; SSF carried out at 28 °C in a shaking incubator at 150 rpm for up to 6 d incubation. The results are the mean + SD (n=3).

However, the glucose production for the Sago + YE + MSI experiment increased significantly to around 3.35 g/L on days 2 and 3 (representing 8.39% conversion yield), but then began to decrease on subsequent time points, reaching close to zero by day 6. This indicates that the fungus was breaking down the starch into glucose during the SmF process. Initially (0 – 2 d) the rate of production was faster than the rate of consumption by the fungus. However, after day 2, the growth rate of the fungus was much faster, dictating how quickly the substrate was being used up. The starch could have been depleted by day 5 and day 6. Overall, however, the glucose production during SmF was not as efficient as the glucose production from SSF.

6.3 SUMMARY

No glucoamylases could be detected in the recovered fungal filtrate during the glucoamylase assay; however, reducing sugars were detected and the level of reducing sugars present increased with incubation period. Since no glucoamylases activity was detected, the focus of the research shifted to the production and extraction of glucose directly during the SSF process. The washing experiments resulted in the accumulation of much higher glucose levels than the continuous fermentation experiments. Therefore, the removal of glucose from the SSF medium was critical for the accumulation of high glucose levels as it prevented product inhibition, catabolite repression and/or consumption of glucose by the fungus. In this research the use of SSF proved better than the use of SmF. Overall the highest glucose recovery was obtained under a one-day washing cycle, with the use of 40.0 x 10^6 spores/g and the addition of nutrients. This resulted in 43.79 ± 2.01 g/L glucose being accumulated over the six day SSF, giving a 46.5% saccharification yield.

CHAPTER 7 DISCUSSION, CONCLUSIONS & FURTURE WORK

7.1 DISCUSSION

The main focus of this research was to investigate the possibility of using a short-term biological process as an efficient way of producing fungal hydrolytic enzymes, which could then be used as a platform for the production of value-added products in a biorefining process. Several underutilised crops were investigated, including Bambara, Leucaena, Napier grass, Nipa palm and Sago hampas. This project had three main areas of research – (i) characterisation of the underutilised crops, (ii) production of cellulases from the underutilised crops, and (iii) production of glucoamylases and a sugar-rich solution from Sago hampas – and the results obtained from these three areas are discussed below.

7.1.1 Characterisation of underutilised crops

Since not much information could be found on the basic composition of the underutilised crops, the first objective of this research was characterisation of the crops (refer to Table 4.1 for results). Based on the results obtained, all the crops had a very low/negligible lipid content (Figure 4.9) and all crops, except Sago hampas, had a very low/negligible starch content (Figure 4.5). There were significant variations in the ash content of the crops (Figure 4.2); and, these variations could have been due to the crop part analysed.

Napier, Nipa fronds and oil palm fronds all had similar ash contents and for all these crops only the leaves/fronds were analysed. Huge variations have been reported in the literature for the ash content of oil palm fronds, with the ash content obtained in this research falling within this range (Hong *et al*., 2012; Yuliansyah & Hirajima, 2012). The ash content obtained for

Napier was also similar to results reported in the literature (de Morais *et al*., 2009; Mohammed *et al*., 2015). The ash content for Bambara and Leucaena were similar and significantly higher than the other crops. For these crops, the leaves, as well as the branches, were analysed, suggesting that branches may have a higher ash content. Since samples of these crops were a mixture of the leaves and branches (and roots in the case of Bambara) no data could be found in the literature for ash content of samples consisting of a mixture of plant tissues. The ash content for Sago hampas was significantly lower than all the other crops. This was likely because Sago hampas is the waste remaining after the starch extraction from the Sago.

Other components analysed included protein, lignin and cellulose/hemicellulose contents. Since these relate directly to cellulase and amylase production, they will be discussed later in the discussion where applicable.

7.1.2 Production of cellulases

Once the crops had been characterised, the next objective was to investigate whether the underutilised crops could be used as substrates for the production of cellulase enzymes during SSF and SmF. The initial screening of the crops under baseline conditions (addition of deionised water to 80% w/v MC) with *A. niger*, suggested that all the crops could potentially be used as substrates for the production of cellulases via SSF. Cellulases were also produced during SmF with all the underutilised crops, although a higher production was achieved during SSF, suggesting that SSF was better suited for cellulase production. Several other reports have also obtained better results for cellulase production using SSF when compared with SmF (Mrudula & Murugammal, 2011; Reddy *et al*., 2015).

Although all the crops could be used as substrates for the production of cellulases, a large range in enzyme activities was achieved, with the use of Napier resulting in the highest activity and the use of oil palm fronds resulting in the lowest activity (Figure 5.2). According to the literature, a range of cellulase activities (0.3 – 84 U/g) from *A. niger* during SSF have been recorded for various substrates, such as rice hulls, wheat bran, wheat straw, and sugarcane bagasse (Kim *et al*., 1997; Lee *et al*., 2011; Mrudula & Murugammal, 2011; Bansal *et al*., 2012; Reddy *et al*., 2015). Although different media and operating conditions were used, many researchers performed comparable tests where several crops were tested under the same conditions and different cellulase activities were achieved. This highlights the fact that the substrate used has an effect on the enzyme activity achieved, and this could be due to the composition of the crops themselves.

The use of Sago hampas resulted in low cellulase activity, even though *A. niger* was able to grow well on the Sago hampas. The Sago hampas had a very different composition when compared to the other crops screened. The ash and protein contents were much lower, and no lignin could be detected with the methods used; however, it had a significantly higher starch content. The composition of Sago hampas has been analysed by several other researchers, all reporting similar ranges as in this research for the protein, lipid, ash and lignin contents (Abd-aziz, 2002; Kumoro *et al*., 2008; Linggang *et al*., 2012; Awg-Adeni *et al*., 2013; Vincent *et al*., 2015). However, a large range in values for both the starch content and the hemicellulose and cellulose contents were reported by the various researchers. The starch content of the Sago hampas can vary greatly since Sago hampas is the waste product remaining from the starch extraction process. As a result, the starch content of the Sago hampas will vary

between processing facilities depending on the sophistication of the methods used and the quality of the extraction process (Kamal *et al*., 2007).

Although the starch content observed in this research fell within the range reported by other researchers, the cellulose and hemicellulose content observed (Figure 4.7) was quite high compared with other reports. However, variation in the values obtained could be due to differences in the methods used for analysis. Nevertheless, although the Sago hampas contained a large amount of hemicellulose and cellulose, much of the dry weight of the Sago hampas was starch. As a result, the *A. niger* was probably preferably utilising the starch, as it is more readily hydrolysed.

Very little starch was present in any of the other crops, and so the fungus was forced to hydrolyse the cellulose and hemicellulose into usable sugars for its growth, producing cellulases and hemicellulases. The production of cellulases is complex and is affected not only by the presence of cellulose, but also by the structure of the cellulose, as well as the interactions between the cellulose and the hemicellulose and lignin fractions. Although the fundamental chemical structure of cellulose is almost identical between the crops, there is a lot of variation in the degree of polymerisation, the molecular orientation of the cellulose, the hydrogen-bonding network and in the degree of crystallinity (Burton *et al*., 2010; Habibi *et al*., 2010). Ultimately, these factors result in different properties of the cellulose and as a result will affect the ability of the fungus to digest it. Furthermore, the hemicellulose fraction serves as a connection between the cellulose and the lignin, and therefore, the way the hemicellulose interacts with the cellulose via hydrogen bonding can affect the digestibility of the cellulose and as a result the cellulase activity. Finally, the presence of lignin, which is very hard to digest or separate from the cellulose/hemicellulose, makes

it very difficult to access the cellulose. Since the amount present, as well as the composition of the lignin may vary between species, the interactions with the hemicellulose/cellulose fractions will also vary and this will affect the cellulase activity of the various crops.

This explanation may help explain the large range in cellulase activities recorded for the other crops screened. Of all the crops analysed, Napier contained the highest cellulose/hemicellulose content, followed by oil palm fronds and then Bambara. However, although the oil palm fronds had a high cellulose/hemicellulose content, it also contained a much higher lignin content than both Napier and Bambara. When compared with values reported in the literature, similar values were obtained for the lignin contents, but the cellulose/hemicellulose values obtained in this research were slightly lower for both Napier and oil palm fronds; no information could be found for Bambara (Hong *et al*., 2012; Reddy *et al*., 2012; Yuliansyah & Hirajima, 2012; de Araujo Morandim-Giannetti *et al*., 2013; Mohammed *et al*., 2015).

A similar pattern was seen for Nipa fronds and this also resulted in a very low cellulase activity similar to that of oil palm fronds. The lignin content obtained in this research was similar to that reported in the literature; however, the hemicellulose/cellulose content was much lower (27% reported in this research; 55 – 62% reported in the literature) (Jahan *et al*., 2006; Phaiboonsilpa & Tamunaidu, 2011; Tamunaidu & Saka, 2011). This much lower content could be a reason why the mass balance achieved for Nipa fronds in this research was only 74%.

Nevertheless, the increased lignin content of both Nipa and oil palm fronds, or a combination of this increased lignin with the factors listed above, could have affected the digestibility of these crops, resulting in either the Napier being easier to digest or the oil palm fronds and Nipa

fronds being harder to digest, ultimately leading to differences in cellulase activities. Alternately, the oil palm fronds and Nipa fronds could lack other nutrients that are required for growth and enzyme production in the fungus.

Besides the substrate used, several other factors can also have an effect on the production of enzymes. Such operating parameters include moisture content, temperature, pH, addition of nutrients, and fungus used; and optimisation of these parameters will influence the enzyme activity achieved (Shanmugam *et al*., 2008). Therefore, once it was established that cellulases could be produced using the underutilised crops, the most suitable crop for cellulase production (Napier in this case) was used for further optimisation, looking at the effect of fungus used, incubation period, and addition of nutrients on cellulase activity. Based on previous optimisation work performed by fellow PhD student, Dr Nattha Pensupa, some conditions, such as moisture content, pH and temperature, were kept constant. However, a slightly lower moisture content was used than that used by Pensupa *et al.* (2013), and this was kept constant at 80 % w/v. While Pensupa *et al*. (2013) found a moisture content of 88.8 % w/v to be best for cellulase production using *A. niger* on wheat straw, many other researchers found lower moisture contents to be better performing (Lee *et al*., 2011; Bansal *et al*., 2012; Narra *et al*., 2012; Delabona *et al*., 2013). Although SSF is operated under low moisture conditions, if the moisture content is too low, this can result in inefficient heat exchange, oxygen transfer and dispersal of nutrients throughout the culture (Pandey, 2003; Shahrim *et al*., 2008). These conditions ultimately affect the fungal growth and therefore reduce the enzyme activity. On the other hand, if the moisture content is too high the fungal growth and enzyme production can also be negatively affected as the increased water could block spaces in

the material structure, resulting in reduced diffusion of gases (oxygen) and solutes (Satyanarayana & Johri, 2005; Shahrim *et al*., 2008). The method of blending designed by Pensupa *et al*. (2013) was also used during enzyme extraction, as they showed that blending wheat straw mash before mixing resulted in a 71% increase in the cellulase activity, compared with just mixing on a magnetic stirrer.

During the optimisation process, the incubation period was examined to determine if this had an effect on cellulase production. A broad range of incubation periods have previously been studied, ranging from 7 – 150 days (Xu *et al*., 2010; Zeng *et al*., 2011). However, in this research a relatively short incubation period was chosen as this is preferred commercially since it is more cost effective. Using the Napier as a substrate, the optimum incubation period for cellulase production was five days, after which the production plateaued. This slowing of cellulase production could have been due to reduction, and potentially depletion, of the cellulose content, or the nutrients added could have been depleted by the fungus (Singh *et al*., 2011). Alternately, since the cellulose was being broken down into glucose, the accumulation of glucose could have inhibited the synthesis of cellulases due to catabolite repression (Hanif *et al*., 2004). Another potential reason, as described by Couri *et al*. (2000), could be the production of protease enzymes by the fungus, which would hydrolyse the cellulases, leading to reduced activity.

In order to grow during the fermentation process (solid-state or submerged), the fungus not only requires a carbon source, but also a nitrogen source and the necessary minerals/trace elements needed for growth. The nitrogen source and the composition of the minerals, as well as the concentrations needed for both, will be dependent on the carbon source used, in this case the underutilised crops, as well as on the

microorganism used. A number of different nitrogen sources, both organic and inorganic, could be used and their effects on cellulase activity have been studied by various researchers (Hanif *et al*., 2004; Kachlishvili *et al*., 2006; Maeda *et al*., 2010; Azzaz *et al*., 2012). Several reports have also suggested that the addition of starch to the fermentation medium improved fungal growth and induced the expression of cellulolytic enzymes, which would result in increased enzyme activity (Liang *et al*., 2012; Inoue *et al*., 2013; Khokhari *et al*., 2013).

When starch, yeast extract (as a nitrogen source) and minerals were added to Napier, there was a significant increase in the cellulase activity by *A. niger*, when compared with the baseline conditions, corresponding to an increase in cellulase activity of up to 81.2%. Although the minerals content of Napier was not analysed, the protein content of Napier was only 8%, and this was similar to results reported in the literature (Reddy *et al*., 2012; Mohammed *et al*., 2015). Therefore, it would be expected that the addition of protein to the Napier would result in an increase in cellulase activity. However, it was found that the addition of only starch or YE separately did not significantly increase the cellulase activities from the baseline conditions in this research (Figure 5.3). Although, when they were added together with MSI, they did have a positive effect on the cellulase activity. This was inferred as the addition of all three nutrients resulted in a significantly higher cellulase activity when compared with the effect of the addition of MSI only. Additionally, when comparing the addition of two of the three nutrients, the addition of just starch and YE did not significantly increase the cellulase activity from baseline conditions (Figure 5.4). This was in contrast to results reported by Pensupa *et al*. (2013) who found that the addition of starch and YE to wheat straw significantly increased the cellulase activity by *A. niger* to 18.6 ± 1.64 U/g after five

days of incubation, when compared with baseline conditions (9.515 ± 0.54 U/g) and this may be due to the different substrates being employed in each case. However, although the addition of starch and YE did not significantly increase the cellulase activity in this research, the addition of all three nutrients resulted in a significantly higher cellulase activity than the experiments with just the addition of two of the three nutrients.

Although the optimisation process had resulted in a significant increase in the cellulase activity achieved using Napier as a substrate, it was uncertain whether the same impact would occur with the other underutilised crops. When screened again under the optimised conditions, a significant increase in the cellulase activity was achieved for all the crops, except Leucaena and Sago, which showed no significant changes. These results indicate that the addition of starch, YE and minerals was important for the production of cellulases by *A. niger* in most of the crops. However, although significant increases were achieved, a range in cellulase activity was still seen. As previously mentioned, the cellulase production under the baseline conditions for oil palm fronds and Nipa fronds was the lowest, and this could have been due to structure of, and interactions between, the cellulose, hemicellulose and lignin fractions, or due to a lack of nutrients. The addition of nutrients to these crops did result in a significant increase in cellulase production, indicating that nutrients could have been lacking in these crops. Since the protein content of both crops was rather low, with similar values being reported in the literature, this could make sense (Tamunaidu & Saka, 2011; Hong *et al*., 2012). However, the cellulase activity achieved under the optimised conditions was still much higher for Napier, indicating that Napier could still be better suited to digestion by *A. niger* than oil palm fronds and Nipa fronds. Another factor that could be considered is that the lower cellulase activity could be due to different

nutrients being needed for these crops, since conditions were optimised using Napier.

The cellulase activity obtained with the use of Leucaena under baseline conditions was very low (around 2.5 FPU/g) and the addition of nutrients to Leucaena had no effect on the cellulase activity. Since the addition of nutrients had a positive effect on cellulase activity in all the other crops (except Sago hampas as it was assumed that the fungus was utilising the starch), one would assume the same would be true for Leucaena. However, this may not have been the case for a couple reasons. The protein content of Leucaena was significantly higher than all other crops and very high in general at 30% (w/w), and this corresponded with data from the literature (Ghosh & Bandyopadhyay, 2007; Xuan *et al*., 2013). This high protein content would mean that the fungus already had a supply of some nutrients for growth and enzyme production, resulting in little difference between the cellulase activity obtained under baseline conditions and optimum conditions. However, although this high level of protein may explain why there were no differences in cellulase activity, it does not explain why the cellulase activity was very low in general.

Leucaena is used as animal fodder as it has a rich palatable protein in the leaves. However, high levels of mimosine (non-protein amino acid) limits its use as this can be toxic to animals (Ghosh & Bandyopadhyay, 2007). Mimosine is found in all parts of the Leucaena plant; however, is greatest in the leaves (Xuan *et al*., 2006). In the plant, mimosine is used as an allelochemical, preventing the growth of other plants (Xuan *et al*., 2013). Furthermore, several studies have also shown that mimosine negatively affects the growth of certain bacteria and fungi (Murugesan & Radha, 1994; Anitha *et al*., 2005; Xuan *et al*., 2013). Therefore, the low cellulase activity obtained with the use of Leucaena during SSF could be due to the

composition of the cellulose, hemicellulose and lignin fractions, or it could be because the mimosine was inhibiting the growth of the fungus, resulting in a low cellulase activity. During SmF, it was interesting to note however that the cellulase activity achieved using Leucaena increased significantly with the addition of nutrients, achieving an activity similar to that of Bambara and Napier (which achieved the highest cellulase activity).

The results discussed so far showed that cellulase activity could be achieved with the various underutilised crops when using *A. niger* as the microorganism. When screened using the microorganism *Trichoderma reesei,* it was found that cellulase activity was also achieved using all the crops as substrates. However, as seen with the *A. niger,* there were differences in the cellulase activities achieved. When comparing the two fungi, A*. niger* generally performed better than *T. reesei*, resulting in higher cellulase activities. Similar results were found by Lee, Darah and Ibrahim (2011), who reported that the cellulase production by *A. niger* was 35.3% higher compared to *T. reesei* when incubated on palm kernel cake and sugarcane bagasse at 1:1 (w/w) ratio over five to six days SSF incubation.

However, what was more interesting was that the mineral solutions used (MSI or MSII) had a different effect on cellulase activity, depending on the fungus used, suggesting the fungi require different minerals and potentially in different quantities. The use of MSI resulted in better results with *A. niger* and MSII resulted in better results with *T. reesei.* Many researchers have investigated the effects of different minerals on cellulase production and have also reported significant effects; although, depending on the strain and substrate used, these have varied (Muniswaran *et al*., 1994; Andrade *et al*., 2011; Lee *et al*., 2011; dos Reis *et al*., 2015).

The use of the two mineral solutions had the greatest impact on *T. reesei*, with the addition of MSI to the underutilised crops significantly hampering cellulase production. However, since *T. reesei* performed well with the addition of MSII, the differences in the composition of MSI and MSII (see Section 3.4 for composition) could indicate a few things about the minerals required by *T. reesei* for cellulase production. The concentration of the salts added to each mineral solution varied greatly, with MSII containing much higher concentrations than MSI. This could indicate that *T. reesei* required, or could tolerate, a much higher salt concentration in general. In terms of the actual minerals added, there were differences between MSI and MSII. Potassium chloride was missing from MSI. Since potassium ions were added from the addition of potassium dihydrogen phosphate, this could suggest that *T. reesei* required chloride ions for enzyme production. On the other hand, as reported by Sinegani and Emtiazi (2006), the presence of the ammonium ions in MSI could have inhibited cellulase production in *T. reesei*. Finally, the lack of cellulase activity could also be due to insufficient magnesium ions being present, as the concentration was reduced from 26.0 g magnesium sulphate heptahydrate in MSII to only 0.2 g in MSI. It has been shown that the absence of magnesium can result in slow growth and a lack of cellulase production; however, at higher concentrations, magnesium can show some inhibitory effect on production (Mandels & Reese, 1957). The low levels of magnesium ions in MSI could have resulted in the slow growth and lack of cellulase production in *T. reesei*; however, the higher concentrations did not appear to inhibit cellulase production. On the other hand, the higher concentrations in MSII could have inhibited cellulase production in *A. niger,* and several other reports also indicated that higher concentrations of magnesium ions impaired cellulase activity (Gautam *et al*., 2010; dos Reis *et al*., 2015).

Overall, the underutilised crop that resulted in the highest cellulase activity was Napier grass. With the addition of starch, YE and minerals to the Napier grass, the best activity was achieved during SSF using *A. niger* spores (up to 31 FPU/g). This was higher than activities achieved using *T. reesei* during SSF or activities achieved using *A. niger* during SmF. These results are comparable with other cellulase activities reported in the literature, and better than cellulase activities reported by Pensupa *et al*. (2013), who achieved 24.0 ± 1.76 FPU/g using *A. niger* on wheat straw under optimised conditions. Therefore, the results obtained in this research show promise for the biological production of cellulases and further optimisation could potentially achieve higher activity levels. Furthermore, the use of these cellulase enzymes for the on-site hydrolysis of Napier into fermentable sugars could be possible.

7.1.3 Production of glucoamylases and fermentable sugars

Another main objective of this research was to investigate whether the Sago hampas could be used as a substrate for the production of glucoamylase enzymes during SSF, using *Aspergillus awamori.* The Sago hampas was chosen since it had a high starch content. However, glucoamylase activity could not be detected in the fungal filtrate recovered from the SSF, even though reducing sugars were present in the fungal filtrate. Based on experiments performed, these sugars were produced during the SSF process and not during the autoclaving or extraction process, and it was assumed they were a result of the fungus hydrolysing the starch.

Although no glucoamylase activity was recorded in this research, glucoamylase activity by *A. awamori* during SSF has been reported in the literature, using a range of substrates such as wheat straw, wheat bran, corn cobs, sugarcane bagasse, mustard cake, and potato starch. The

glucoamylase activity recorded ranged from 2 – 24 U/mL or 21 – 64 U/g after two to four days of incubation, depending on values used for reporting (Bertolin *et al*., 2003; Du *et al*., 2008; Pavezzi *et al*., 2008; Ali *et al*., 2014). Glucoamylase activity has also been reported using Sago hampas as a substrate during SSF and various fungi (*Trichoderma* sp. KUPM0001, *Myceliophthora thermophila* and *Chalara* sp.), with activity ranging from 2 – 11 U/mL after two to four days of incubation (Vikineswary *et al*., 1996; Shahrim *et al*., 2008).

No reports could be found using Sago hampas as a substrate and *A. awamori* as the fungus in SSF. However, since glucoamylase activity was detected when *A. awamori* was grown on various other substrates, and when Sago hampas was used as a substrate with other microorganisms, it was assumed that *A. awamori* would be able to produce glucoamylases when incubated on Sago hampas. Therefore, the lack of glucoamylase activity in this research was puzzling and several potential reasons for this lack of activity were investigated – including determination of whether the glucoamylase assay was working correctly, whether the background level of sugars in the glucoamylase assay solution was saturating the solution, whether the enzyme was being deactivated by high sugars in the fungal filtrate, and finally whether glucoamylase activity could be detected earlier in the SSF process.

Since the assay procedure was found to be adequate and was not saturated with reducing sugars, the lack of glucoamylase activity could have been due to two possibilities. The level of reducing sugars in the fungal filtrate could have been so high that even when diluted in the assay this still resulted in product inhibition of the enzyme. However, this was not the case as no glucoamylase activity was observed in early SSF filtrates (with very low sugar levels), or after dialysis to remove the sugar.

Even though no glucoamylase activity was detected earlier in the SSF process, the concentration of sugars in the fungal filtrate continued to increase with incubation time. Therefore, the most likely explanation for the lack of glucoamylase activity is that the levels of glucose in the SSF were sufficient to repress the synthesis of the glucoamylase in the first place. The fungus was thus able to keep a very tight control on the synthesis, and the enzyme never accumulated to levels high enough to be detected by the assay.

Why other research groups have managed to get glucoamylase activity in similar experiments is difficult to explain. Where other fungi have been used this may be that these have a less effective repression system and make higher levels of the enzyme even in the presence of glucose. Since no glucoamylases activity could be detected, the focus of the research shifted to the production and extraction of glucose directly during the SSF process.

To do this, the Sago hampas was used as a substrate during SSF with *A. awamori*, and the results showed that the production and extraction of glucose directly from the SSF process was possible. A continuous SSF over 21 days of incubation showed that the highest glucose achievable was 0.080 ± 0.0002 g/g starch. A novel process where the mash was periodically washed throughout the incubation period resulted in a significant increase in the glucose accumulated during the SSF process (0.278 ± 0.006 g/g starch). Furthermore, this resulted in a greater utilisation of starch, and a more efficient conversion of starch to glucose (29.1 % yield for SSF with washing versus only 10.1 % yield for continuous SSF).

The significant increase in glucose recovered from the washing experiments versus the continuous SSF experiment could be explained by

either product inhibition or catabolite repression of the enzymes involved in starch mobilisation. In the continuous SSF experiments, the glucose concentration in the fungal filtrate increased with time up to a plateau at around day 12, with oscillations around this general trend. A similar trend has been shown by other researchers who reported that glucoamylase activity is fungal growth-related and fluctuates during SSF (Vikineswary *et al*., 1996; Sivaramakrishnan *et al*., 2007). This fluctuation in enzyme activity could be due to enzyme inhibition by the reducing sugars produced during hydrolysis, or it is also possible that this is due to catabolite repression. The significantly higher glucose concentration recovered from the SSF with washing experiments would then simply arise from the continuous removal of the sugars, thus negating any product inhibition or catabolite repression effects (Kumoro *et al*., 2008). Furthermore, removal of the glucose from the culture medium not only meant that the fungus was unable to consume the glucose; but it also meant the food source for the fungus was removed, forcing it to continue hydrolysing the starch into glucose to meet its energy demands.

What was interesting from these initial washing experiments was that the most efficient conversion of starch to glucose was seen with the SSF experiments with one-day washing, as these experiments achieved the same amount of accumulated glucose, but had better starch utilisation. In further optimisation experiments, two findings were worth noting – nutrients (composition and timing of addition) and spore concentration used had a significant effect on the glucose accumulated during the SSF.

As previously reported, the composition and the concentration of minerals and nitrogen added to the culture medium is important, and can affect both amylolytic enzyme production and glucose consumption (Mandels & Reese, 1957; Bertolin *et al*., 2003; Sinegani & Emtiazi, 2006; Gautam *et*

al., 2010; dos Reis *et al*., 2015). Since SSF is performed in the absence of free water, even minor variations in the C/P ratio and C/N ratio can affect fungal growth and enzyme production. A lack of minerals can result in slow growth and lack of enzyme production. On the other hand, ample nutrients can result in faster growth and enzyme production, which leads to a higher glucose production, but also a higher glucose consumption.

Most experiments performed in this research used the addition of YE as a nitrogen source and this appeared to be beneficial. Several reports have shown conflicting views on the effect of YE on microbial growth and enzyme production. Sivaramakrishnan *et al*. (2007) reported that the addition of nitrogen sources, either in organic form (such as YE) or in inorganic form (such as ammonium sulphate), actually resulted in lower production of amylolytic enzymes, which in turn would result in lower glucose production. In their research (performed on wheat bran using *A. oryzae*), the addition of YE resulted in the lowest enzyme production. On the other hand, Hamilton, Kelly and Fogarty (1999) reported that the addition of YE to starch or lactose resulted in the best production of starch degrading enzymes by *Bacillus* sp. IMD 435. These conflicting results suggest that the effect of nutrients (in this case nitrogen source) is quite complex and is probably affected by both the substrate used as well as the microorganism.

In this research, the addition of YE had a positive effect on glucose production by *A. awamori* from the Sago hampas, and therefore probably enzyme production. However, optimisation of the nitrogen source used showed that the addition of fungal extract (FE) instead of YE resulted in a significantly higher glucose production thus FE may be more suitable, as a nitrogen source for the *A. awamori,* than YE. The fungal extract consisted of dried pellet remaining from previous washing experiments, which was

milled and added in the same quantities as the YE. The FE therefore, may have contained some residual nutrients and starch that the fungus could utilise. Alternately, the fungus in the previous experiments had assimilated the nutrients added (YE and MSI) into more usable nutrients for itself, making the nutrients in the FE more readily available for the fungus and in a form that is specific to the metabolic needs of *A. awamori*. Overall, these results show that the source of nutrients added to the Sago hampas is important and further optimisation of nutrients could potentially result in additional increases in glucose recovered.

The spore concentration used for inoculation also had a significant effect on the glucose accumulated and the addition of a spore concentration ten-times greater achieved a significant increase in glucose accumulated. One might predict that at the higher spore concentration the initial biomass would be greater. Thus, since amylolytic enzyme production is growth related, the larger initial biomass would result in a higher enzyme activity and therefore higher glucose production. However, this could also lead to increased glucose utilisation. Eventually one might also predict that the fungal masses in each case could converge as nutrient depletion occurred for the higher spore loading and the lower spore loading was catching up. However, in these experiments this did not occur in the incubation period examined. As a result, the higher spore loading resulted in an increase in glucose recovery and did not show any detrimental effects to this with time.

Although the addition of FE (instead of YE) and the addition of increased spores resulted in a higher overall glucose accumulation, it was observed that the glucose produced between washes followed a similar pattern for all experiments performed, regardless of optimisation (day, nutrients or spore concentration). For all buffer washed experiments, the glucose

recovered was low on day one, maximal on days two and three, and then it declined in subsequent washes.

The small amount of glucose recovered from wash one was likely due to the fungus needing time to 'acclimatise' to the substrate and turn on the genes encoding starch degrading enzymes. The production of the starch hydrolysing enzymes not only requires the absence of inhibitors, but it also requires the presence of inducers (Ghosh *et al*., 1990; Sivaramakrishnan *et al*., 2007). This process of enzyme production and starch degradation takes some time; delaying the production of glucose. After the fungus had become acclimatised to its substrate, it most likely entered a phase of rapid growth and enzyme production, resulting in a huge increase in glucose recovered. One would assume that the starch degrading enzymes were still present in the culture medium, and were still active. Assuming this, the starch would be hydrolysed to glucose more quickly, resulting in a higher concentration of glucose recovered. Furthermore, since the glucose was suddenly removed from the culture medium (due to washing in wash one), the fungus needed to produce glucose quickly to meet its higher glucose demands, resulting in a higher accumulation of glucose in wash two.

After washes two and three, some inhibition of growth occurred and this could have been caused by several factors, such as substrate depletion, nutrient depletion or lack of other nutrients, lack of oxygen, or a decrease in spore viability. At the end of all the washing experiments there was still some starch remaining. However, during hydrolysis of the starch, the more easily digested starch would be broken down first, leaving the less digestible starch (Polakovic & Bryjak, 2004). As a result, this could take longer to hydrolyse, which may explain why the glucose levels decreased with subsequent washes. Nutrient depletion or a lack of other nutrients

could also have resulted in a decrease in glucose production as it could cause a decrease in the production of enzymes or in fungal growth. Potentially, reduced gas volume, as well as reduced gaseous diffusion, due to less viscosity and structure of the Sago hampas, could have resulted in low oxygen transfer. Oxygen transfer during SSF is a major concern and for aerobic fungi, like *A. awamori*, depletion of oxygen can limit growth and cause a decrease in metabolite production (Rahardjo *et al*., 2005; Sivaramakrishnan *et al*., 2007). Finally, the fungal biomass could have been decreasing with each wash, leading to a decrease in the glucose recovered with each wash. In order to get a better understanding of what was happening between washes, the starch, protein and nutrient levels, as well as the glucose and other reducing sugar concentrations, would need to be analysed after each wash. The glucosamine levels could also be analysed and used as an indication of biomass concentration, since it has been shown to have a direct correlation with fungal cell growth (Hsieh *et al*., 2007).

Overall, the highest starch saccharification yield achieved with the SSF with one-day washing was 46.53 %, and this could potentially be increased with further optimisation. In the literature, no reports could be found using a similar washing approach for glucose production. Most researchers reported the use of Sago hampas as a substrate for the production of glucose using commercial enzymes. However, this research obtained similar conversion yields as some of these reports using the washing approach. For example, Awg-Adeni *et al.* (2013) reported a 52.72 % yield through a three cycle saccharification process using the commercial enzyme, dextrozyme (Novozyme, Denmark), which was a mixture of glucoamylase and pullulanase, suggesting a high degree of saccharification.

Furthermore, from their findings, Awg-Adeni *et al.* (2013) reported that the hydrolysate collected during all stages of Sago hampas starch hydrolysis was composed of a mixture of glucose, dextrin, maltose and maltotriose, with around 85 – 90% being glucose. This mixture of substances could have been due to partial hydrolysis of the starch or it could have been due to the fact that the hydrolysis of malto-oligosaccharides to glucose is reversible. Therefore, if the glucose concentrations in the culture medium are high this can occur, leading to an equilibrium constant with a certain concentration of malto-oligosaccharides (Polakovic & Bryjak, 2004; Kumoro *et al*., 2008). Since only the glucose concentration was measured in this research and not the total reducing sugars concentration, it is hard to know exactly how many intermediary substances had not yet been converted to glucose, and if this would influence the overall glucose accumulated.

The fact that the glucose conversion yields achieved so far in this research are also comparable with yields achieved using commercial enzymes is promising as the use of commercial enzymes can be expensive. Although commercial amylases are cheaper and potentially have a higher efficacy than commercial cellulases, the ability to produce fermentable sugars directly from the Sago hampas using fungi could be advantageous.

A few reports were found where Sago hampas was used as a substrate for the production of fungal enzymes and/or reducing sugars. Linggang *et al*. (2012) reported the production of crude cellulases from *A. fumigatus* UPM2 using Sago hampas as a substrate. They then used the crude cellulases in the hydrolysis of 5 % (w/v) Sago hampas, producing 20.77 g/L reducing sugars, resulting in an overall hydrolysis percentage of 73%. Shahrim *et al*. (2008) reported glucoamylase activity using *Trichoderma* sp. KUPM0001, and obtained a maximum reducing sugar concentration of 46

g/L after a five day SSF. This corresponded to 0.66 g reducing sugars/g starch. In this research, using the optimised washing method, a total of 44 g/L glucose was recovered and this corresponded to 0.52 g glucose/g starch. Although only the glucose was measured in this research, and not the total reducing sugars as done in the reports above, the glucose concentrations achieved so far in this research are still comparable with other research performed.

Feasibility of using the fermentable sugars in a fermentation to produce bioethanol or biochemicals would need to be assessed. However, if the glucose recovered from the washing procedure were fermented to bioethanol, theoretically 21.99 g/L ethanol could be produced (2.2 % w/v). Various bioethanol conversion yields have been reported, ranging up to 93% (Kumoro *et al*., 2008; Awg-Adeni *et al*., 2013; Vincent *et al*., 2015). Since no pre-treatments (other than autoclaving) were used in this research, it is assumed that very few inhibitors would be present in the fermentation medium and therefore very high glucose to bioethanol conversion yields could be achieved in this research.

7.2 CONCLUSIONS

In this study a short-term SSF using soft-rot fungi was developed for the production of cellulases and a glucose-rich hydrolysate, using underutilised crops as substrates. Several underutilised crops were investigated, including Bambara, Leucaena, Napier grass, Nipa palm, Oil palm, and Sago hampas.

It was shown that all crops tested could be used as substrates for the production of cellulases during SSF and SmF, although a large range in cellulase activities was observed. The highest cellulase activity was achieved using *A. niger* in a five day SSF with the addition of nutrients to

the crops. The use of Napier as a substrate resulted in the highest cellulase activity, reaching 31.02 ± 1.01 FPU/g after five days of incubation.

Sago hampas was further studied to determine if it could be used as a substrate during SSF for the production of glucoamylases, using the soft-rot fungus *A. awamori.* No glucoamylases could be detected in the recovered fungal filtrate during the glucoamylase assay; however, reducing sugars could be detected and this increased with incubation period. Since no glucoamylases activity was detected, the focus of the research shifted to the production and extraction of glucose directly during the SSF process. It was found that the highest glucose recovery was obtained under a one-day washing cycle, with the use of 40.0 x 10^6 spores/g and the addition of nutrients. This resulted in 43.79 ± 2.01 g/L glucose being accumulated over the six day SSF, giving a 46.5% saccharification yield.

Pre-treatments shown in this work will enable the creation of novel biorefining processes, using the cellulase enzymes on-site to hydrolyse the underutilised crops to a sugar-rich hydrolysate. This, as well as the sugar-rich filtrate produced with the Sago hampas, could then be used in the production of biofuels and/or biochemicals.

7.3 FUTURE WORK

The cellulases produced by *Aspergillus niger* during the five day SSF incubation, using Napier grass as a substrate were in quantities comparable with literature results obtained for other crops. For future work the feasibility of using these enzymes for onsite hydrolysis of Napier grass to a fermentable-sugar rich solution could be investigated to determine the efficacy of such enzymes in biomass conversion. Optimisation of the hydrolysis step, including enzyme and substrate loading rates, time,

temperature and pH, would be required to ensure maximum sugar production. The sugar production from hydrolysis of untreated Napier (besides autoclaving for sterilisation) could be compared with Napier that has been modified using other pre-treatments, such as acid-soaking. The sugar-rich hydrolysate produced from the hydrolysis of the Napier grass (both untreated and treated) could be fermented into bioethanol or biochemicals to determine the conversion efficiency of fermentable sugars.

The use of SSF for the production of cellulases could be further optimised, looking at several aspects. Firstly, *A. niger* could be co-cultured with *T. reesei* during SSF as the level of individual cellulase enzymes in the recovered filtrate can affect the total cellulase activity, which will ultimately affect the hydrolysis step. Since *A. niger* is known to produce high levels of β-glucosidases and endoglucanases, but low levels of exoglucanases, while *T. reesei* produces high amounts of endoglucanases and exoglucanases but a lower level of β-glucosidase (Verardi *et al*., 2012), combining the spores during SSF, could potentially result in a better cellulase activity obtained.

This research showed that the addition of nutrients increased cellulase production significantly. However, the use of YE can be expensive and varies in form, therefore finding cheaper alternates for this could be investigated. One possibility could be the use of fungal extract (FE) obtained from pellets of previous SSF experiments, as this resulted in a significant increase in glucose production over the addition of YE in the Sago washing experiments. Also the effect of initial moisture content of the Napier could be examined as this can greatly affect cellulase activity.

The glucose-rich solution recovered from the SSF washing experiments using Sago hampas could be further used in a fermentation to produce bioethanol or biochemicals, with optimisation of the process being

performed. To improve glucose recovered from the SSF, further optimisation of the SSF washing experiments could be performed, such as looking at the use of fungal extract with the addition of a higher spore concentration. Since it was discovered that FE performed better as a nitrogen source than YE, it would be interesting to determine if the addition of FE to the Sago hampas as well as the addition of a higher spore concentration would result in a further accumulation of glucose. Furthermore, it would be beneficial to determine the ideal spore concentration required for maximal glucose accumulation. Although increased spore concentration leads to increased glucose accumulation, at some point the culture can become over-crowded, leading to competition for space, oxygen and nutrients which can have a negative effect on microbial activity and ultimately glucose produced. Knowing the concentration at which this occurs would be important. The addition of new spores and/or nutrients later in the SSF (such as after wash three when glucose recovery began to decline) could also be investigated to determine if these are possible causes for the drop in glucose production. Extraction methods used for the recovery of the glucose could be analysed to determine if further improvements could be made to the amount of glucose recovered from the washes.

Although glucoamylases could not be detected in the recovered filtrate, they were being produced, since the starch was being broken down. This was probably due to the fact the enzymes were being turned on and off. Therefore, genetically engineering the fungus to keep the enzymes always turned on could result in higher production of amylases, and therefore higher glucose yields achieved during the washing cycles. Furthermore, strategies to reduce the consumption of glucose by the fungus could be investigated, as this probably reduced the glucose yields achieved.

Finally, the pellet that was remaining after the SSF washing experiments, which contained a much lower starch concentration, could be analysed for cellulose and hemicellulose and then either re-used as a substrate for the SSF using *A. niger* to determine if cellulases could be produced. Alternately, the cellulases produced using Napier grass during the SSF with *A. niger* could be used to hydrolyse the remaining Sago hampas into fermentable sugars, increasing the yield of glucose achieved.

CHAPTER 8 REFERENCES

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