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The Development of a Biorefining Strategy for the Conversion of Wheat Straw and Sorghum Bran to Value Added Products

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B.Sc., M.Sc.

A thesis submitted to the University of Huddersfield in partial fulfilment of the requirements for the degree of Doctor of Philosophy

June, 2019

Copyright statement

Abstract

Renewable energy, such as biofuel has been highlighted as a future fuel that could replace fossil fuels. The conflict between biofuel and food security has encouraged the research on the conversion of lignocellulosic biomass into biofuels. Although lignocellulosic biomass is abundant, the presence of lignin and the cost of enzymes have caused several major issues in regards to the commercialization of lignocellulosic biofuels.

The aim of this study was to investigate the feasibility of using wheat straw and sorghum bran to produce value added products, such as enzymes and bioethanol. The utilization of wheat straw for cellulase production and the subsequent hydrolysis was investigated. Six fungal strains (*Aspergillus niger* N402*, Aspergillus niger* N403, *Aspergillus niger* CKB, *Trichoderma reesei* R32, *Trichoderma reesei* R33 or *Rhizomucor variabilis* RS) were investigated using both solid-state fermentation (SSF) and submerged fermentation (SmF).

In SSF, cellulase production increased from 3.2±0.05 FPU/g to 8.1±0.3 FPU/g (Filter Paper Unit) when wheat straw was modified using alkali treatment. The addition of starch improved the cellulase production with a cellulase activity of 23.14±0.09 FPU/g being obtained when 0.04% starch was added. The inoculum and reactor size also affected cellulase production. *A. niger* N402 with an inoculation ratio of $1x10^7$ spores/g resulted in the highest cellulase activity of 55.93 FPU/g and 30.43 FPU/g in SSF using Petri Dish and 250 mL shake flask, respectively.

The optimisation of cellulase production using a newly isolated fungal strain, *R. variabilis* (RS) was performed in both submerged fermentation (SmF) and solid-state fermentation (SSF). The impact of various parameters, including pH, mineral addition, nitrogen source, temperature, and substrate concentration, was investigated for SmF and incubation time, pH, temperature, inoculation size, moisture content, and nitrogen source were investigated for SSF. An optimum fermentation condition was determined to be: pH 6.5, 0.03% tryptone and fermentation for 3 days for SmF, a cellulase activity of 18.44 FPU/g was obtained. Similarly, an optimum fermentation condition for SSF was determined to be: pH 7, 28°C, inoculation size of $1×10^7$ spores per g substrate, 0.03% tryptone and

fermentation for 5 days. The cellulase activity was 30.19 FPU/g. Response Surface Methodology (RSM) was used to further optimize cellulase activity in SmF and SSF. This approach resulted in cellulase activity of 23.81 FPU/g for SmF and 24.80 FPU/g for SSF. Two rounds of physical mutagenesis of RS strain were carried out using UV lights and microwave heat. A mutant strain MW15-03 was obtained, which showed 21.6% higher cellulase production capacity in comparison with the parent strain.

Sorghum bran, a starch rich food processing waste, was investigated for the production of glucoamylase in SmF and SSF. The fermentation parameters, such as cultivation time, substrate concentration, pH, aeration rate, inoculation ratio, temperature, nitrogen source, and mineral addition were investigated for SmF. The glucoamylase activity was improved from 1.90 U/mL in an initial test to 19.26 U/mL at 10% substrate concentration, pH 6, fermentation volume 200 mL in 500 mL shaking flask and fermentation of 3 days. RSM was used to further optimize glucoamylase activity in SmF and glucoamylase activity of 59.03 U/mL was achieved at the following conditions: substrate concentration 8%, pH 6, yeast extract concentration 5 g/L and fermentation volume 100 mL in 250 mL shaking flasks. Larger scale production of glucoamylase enzyme in 2 L bioreactors under the optimum condition resulted in 21.67 U/mL of glucoamylase activity at 72 hours of fermentation, while further increasing sorghum bran concentration to 12.5% gave an improved glucoamylase activity of 37.55 U/mL at 115 hours of the fermentation.

The crude glucoamylase solution was used for the enzymatic hydrolysis of the sorghum bran. A sorghum bran hydrolysis carried out at 200 rpm, 55°C for 48 hours at a substrate loading ratio of 80 g/L resulted in 11.74 g/L glucose, which was comparable to that obtained using a commercial enzyme (12.72 g/L). Larger scale sorghum bran hydrolysis in 2 L bioreactors with crude glucoamylase enzyme resulted in a glucose concentration of 38.7 g/L from 200 g/L sorghum bran.

Wheat straw hydrolysate, sorghum processing wastewater and sorghum bran hydrolysate were used as substrates for the production of bioethanol. The addition of minerals accelerated the rate of yeast fermentation. Marine yeast strain *W. anomalus* M15 resulted in a very high ethanol yield of 49.79%. Upto 19.3 g/L bioethanol was obtained. Autoclaved wheat straw at 121°C for 15 minutes gave the highest ethanol yield of 16.95% using the marine yeast *W. anomalus* M15.

Acknowledgements

All thanks to the Almighty God who has blessed me with this opportunity to fulfil my dream. My gratitude also goes to the University of Huddersfield for the scholarship to undertake this programme.

I thank my supervisor Dr Chenyu Du for his numerous support. I will also want to thank my co-supervisor, Professor Andrew Laws, for his assistance. My appreciation also goes to all the technicians in the School of Applied Sciences. I also appreciate the support from colleagues and friends.

I would like to thank my wonderful in-laws, particularly for their support during the death of my mother-in-law in March 2019. May her soul rest in perfect peace. To my loving parents Elder and Mrs Oyegoke Oyeleke. I would not have been able to accomplish this without your financial support and encouragement. Thank you for your entire help mummy for babysitting, prayers to mention few, you are the best; and thanks Daddy for all the sacrifices from the beginning. Adura mi nipe ejeun omo pe, ninu inu didun ati Alafia pelu emi gigun Amin. My siblings as well thank you all. Ape fun ra wa loruko Jesu.

Lastly, I would like to thank my husband for his love, support and encouragement. Ade teminikan Adebayo Makanjuola, you are the best. To my baby girl Anjolaoluwa, I appreciate your putting up with it all. Mummy thanks and love you.

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List of abbreviations

AACC – American Association for Cereal Chemists

AFEX – Ammoniun Fibre Explosion

BG – β-Glucosidase

CAZ – Carbohydrate Active Enzyme

CAZy – Active Enzymes

CBH – Cellobiohydrolase

CSF – Calculate Severity Factor

DDGs – Distillers Dried Grains

DNSA – Dinitrosalicyclic Acid

EBP – Electron Beam Processing

EFC – Electronic Flow Control

FID – Flame Ionization Detector

FLW – Food Loss and Waste

FPU – Filter Paper Unit

FSD – Food Security Department

GA – Glucoamylase

GHG – Greenhouse Gases

HPAEC-PAD – High Performance Anion Exchange Chromatograph

LMEs – Lignin Modifying Enzymes

MTWS – Microwave Treated Wheat Straw

MWR – Microwave Radiation

OD – Optical Density

PDA – Potato Dextrose Agar

RSM – Response Surface Methodology

SEM – Scanning Electron Microscope

SmF – Submerged Fermentation

SSF – Solid-state Fermentation

SWW – Sorghum Waste Water

WWCM – Wet Weight Cell Mass

YE – Yeast Extract

YEPD/YPD – Yeast Extract Peptone Dextrose

1 Introduction

This chapter provides an overview of this thesis, starting with the current global energy shortage and environmental concern arising from fossil fuel energy usage and the need for sustainability. Then it gives information about renewable energy as a potential alternative way to reduce the environmental issues. It also covers the current problems of bioenergy and the structure of this project.

1.1 Global energy shortage and environmental concern

The global total energy consumption is strongly influenced by increasing population (7.69 billion in 2019 from worldometers) and by industrial expansion (Huang & Fu, 2013). The population in developing countries is projected to increase rapidly to 8 billion, while the population of developed countries will either be stable or will increase by around 1 billion by 2050 (Saito, 2010). There has been an estimated increase of about 56% in energy consumption in the near future by many specialists, international agencies, institutes and organisations with the current (2013 figures) total world energy consumption (IEA) as 3.89×10^{20} Joules. Currently, 80% of world energy consumption is provided by fossil fuel such as coal, crude oil and natural gas (Saito, 2010). An increase in energy consumption and the recent global warming caused by CO_2 emissions raised the need for a sustainable approach to energy generation and usage (Huang & Fu, 2013; Oluwakemi, Mafe, Roberts, & Du, 2014), has drawn the world attention to a pervasive, renewable and environmental friendly energy (Dai & Liu, 2012; Saito, 2010). Alternative sources of energy are required to replace fossil fuel with biomass highlighted as the only sustainable source of organic carbon and its use for the production of fuels and chemicals (Huang & Fu, 2013).

Various considerations and measures for the mitigation of climate change are expected in various sectors such as energy supply, transport and its infrastructure, residential and commercial buildings, industry, agriculture, forestry and waste management (Saito, 2010). Exploiting a renewable, sustainable and environmentally friendly energy is of high priority.

1.2 Renewable energy

Renewable energy is defined as energy obtained from natural resources that can be constantly replenished and are classified as primary, domestic and clean or inexhaustible energy resources *e.g* solar energy, wind energy, biomass energy, geothermal energy etc. (Bilgen, Kaygusuz, & Sari, 2004; Dincer, 2001; Rathore & Panwar, 2007). They are often referred to as alternative sources of energy. The provision of sustainable and clean energy has been the recent focus of renewable energy in the world with the goal of global decarbonisation (Mohammed, Mustafa, Basir, & Mokhtar, 2013). Sustainable development is the major challenge faced by both developed and developing countries for the provision of essential services to humanity by preserving the environment, in order to achieve economic and social development (Mohammed et al., 2013).

Fossil fuels have a significant adverse impact on the environment. According to Farad, Saffar-Avval, and Sinaki (2008), its use has resulted in increased health risks and a threat to global climate change. Although, the world is moving towards sustainable production methods, waste minimization, reduced air pollution from vehicles and reduction of greenhouse gas emission (R. E. H. Sims, 2003). The recent energy crisis over depletion of fossil fuel to meet the world energy demand has generated a resurgence in promoting renewable alternatives to meet the world's growing energy needs (Horst & Hovorka, 2009; Youm, Sarr, Sall, & Kane, 2000).

Renewable energy (biomass) has several advantages over fossil fuels, such as reducing carbon emissions, increasing agricultural output value, and reducing the cost of disposing municipal waste by employing technology converting this waste into biogas production. The conversion of lignocellulosic biomass into biofuel is forecast to play an important role in the near future.

1.3 Current challenges with bioenergy

The first generation of biofuel has been well developed. However, using food material as feedstock for bioethanol production has been criticised in the area of food security. Therefore, researchers have been investigating the development of an economically feasible second generation of biofuel production in order to resolve the

dispute with first generation biofuel, with the usage of inedible materials from lignocellulosic biomass.

The main challenge with bioethanol is the cost of production, as the cost of production is due to the complexity of the biomass and issues related to feedstock supply cost. Several processing steps are required for the conversion of biomass to liquid transportation fuel such as; pre-treatment, hydrolysis, microbial fermentation, and fuel separation (Balan, 2014). The pre-treatment process is designed to disrupt the cross-links of the hemicellulose-lignin complex. Effective pre-treatments increase the rate of enzyme hydrolysis and significantly decrease the amount of enzymes needed to convert biomass into fermentable sugars, which can be utilized by microorganisms (Balan, 2014). As a pre-treatment normally produces inhibitory compounds that often affect the subsequent fermentation process (see Figure 1.1), the detoxification of these inhibitory compounds is required, which also adds to the total production cost.

Currently, it is estimated that the conversion of lignocellulosic biomass to biofuels is costlier than its crude oil counterparts. However, the cost of bioethanol production could be reduced economically through several approaches. Firstly, the use of land waste biomass like wheat straw and sorghum bran. Secondly, operating cost can be reduced by using established pre-treatment techniques that could result in little or no inhibitory compound production and optimising the process. Thirdly, the cost of enzyme for the hydrolysis process can be reduced through on-site enzyme production. Lastly, developing a biorefinery strategy that fully utilises the whole substrate for the production of a range of value added products.

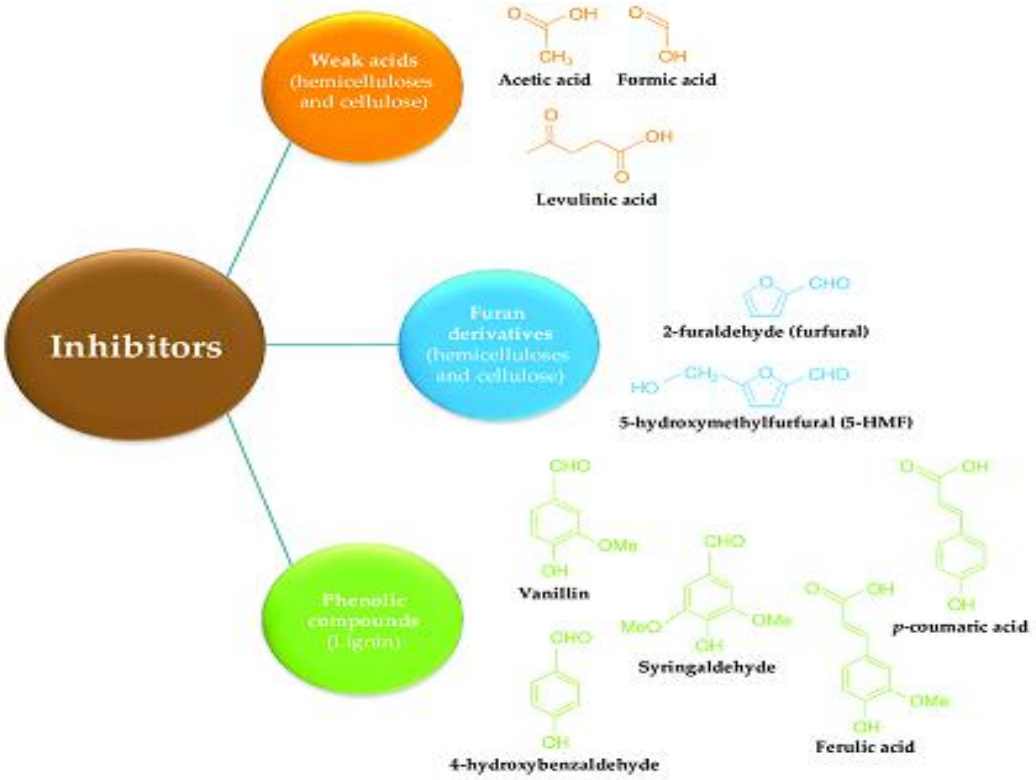


Figure 1.1 Common inhibitory compounds present in lignocellulosic pre-treated materials, indicating main sources of formation (Fillat et al. (2017).

1.4 Structure of the thesis

Following the introduction chapter, an overview of the literature review related to biofuel, its current challenges, process development and the objectives of this research are presented in Chapter 2. Chapter 3 details the experimental materials and methodology. The results chapters then follows, starting with Chapter 4, where initial trial of several fungi for cellulase production using wheat straw as substrate led the selection of a novel fungus for further research. The enzymatic hydrolysis of wheat straw with crude cellulase enzyme solution was also study in this chapter. Chapter 5 presents the investigation of several parameters for the production of cellulase with the selected novel fungus strain. The optimisation of fermentation as well as the strain mutation for its ability to produce cellulase was investigated. Chapter 6 presents the development of a biorefinery process using sorghum bran for glucoamylase production and the hydrolysis of sorghum bran to produce a sugar rich hydrolysate. Chapter 7 contains a study on yeast fermentation for ethanol production. Finally, conclusions and future works are discussed in Chapter

8.Renewable energy (biofuel) through lignocellulosic materials is essential in order to reduce carbon emission; increase agriculture output value and reduces the cost of disposing municipal waste by converting this waste into value added products. The utilization of lignocellulosic materials as a renewable energy currently as its challenges. If this challenges can be reduce or eliminated the environmental issues can be resolved thereby resulting in an economic and social development with the provision of a clean energy.

2 Literature review

Biofuels is an important renewable fuel, which is capable of replacing fossil fuels. Replacement of fossil fuels with biofuels has been shown to contribute to a greener environment by reducing air pollution caused by the burning of fossil fuels.

This chapter introduced the general background in bioethanol production and its main challenges, especially in the processing of raw materials and enzymatic hydrolysis. It ends with the exploitation of enzyme production from wheat straw and sorghum bran for bioethanol and biochemical production.

2.1 Biofuel

Biofuels have been used since the pre-recorded history with the use of fire as a source of heat and for cooking by the burning of wood and other materials (Arsuf & Sussman, 1983; Russel, 2003). Liquid biofuels came into existence as liquid oil was used as light source in the home. Biofuels such as olive and whale oils were derived from plants and animals, until they were largely replaced by kerosene (Russel, 2003).

2.1.1 First generation of biofuel

The first generation of biofuel uses mainly food based materials such as grains, sugar cane and vegetable oils as the starting materials (Babu, Thapliyal, & Patel, 2014; Mohr & Raman, 2013) for the synthesis of bioethanol and biodiesel. Table 2.1 briefly lists a few examples of the technologies used for the production of first generation biofuels. However, biofuels have been criticised due to the concerns of global food security and other social, environmental, economic and ethical challenges (Lin & Luque, 2014; Mohr & Raman, 2013). These criticisms have restricted the expansion of biofuels. Therefore, the use of lignocellulosic biomass and non-editable oil was explored for the production of biofuels.

2.1.2 The second generation of bioethanol

Biofuels, which have been termed second-generation, have emerged. For their production use inedible biomass such as agricultural residues, residues from forestry, dedicated biomass crops and woody biomass for their production. However, a few technical and economic challenges have been identified, which are major constraints preventing full commercial deployment of these biofuels. These include the energy requirement for pre-treatment, the cost of enzyme as well as the reduction of inhibitors in the hydrolysate. However, these biofuels are a promising option when considering future sustainability criteria (Sims, Mabee, Saddler, & Taylor, 2010). These challenges will be discussed in the following chapters 2.2.1 and 2.2.2.

2.1.3 Lignocellulosic Biomass

Lignocellulosic biomass is considered as the most abundantly available raw material on earth for the production of bioethanol. It is composed of carbohydrate (cellulose, hemicellulose), and lignin (aromatic polymers). These carbohydrate polymers contain different sugar monomers (hexose and pentose sugars) and they are tightly bound to lignin. Lignocellulosic biomass can be categorised into waste biomass and energy crops. Lignocellulosic materials are highlighted as the most promising feedstock of natural and renewable resources essential to the functioning of modern industrial societies (Anwar, Gulfraz, & Irshad, 2014).

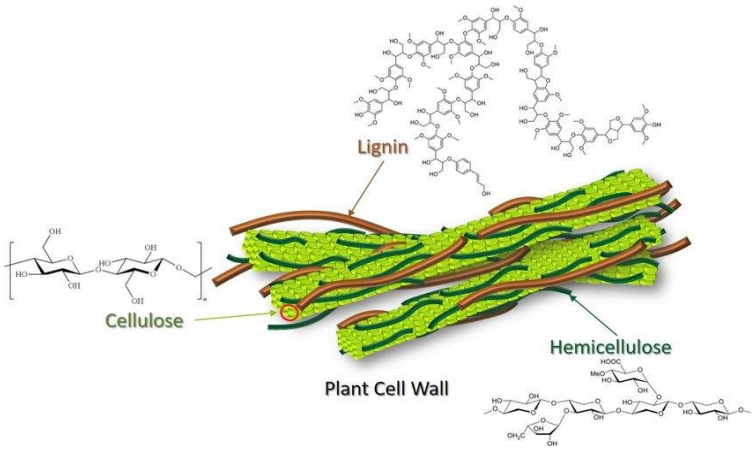


Figure 2.1 Lignocellulosic biomass structure (Jensen, Rodriguez Guerrero, Karatzos, Olofsson, & Iversen, 2017)

Table 2.1 Technologies used for first generation of biofuels production

Biofuel type	Specific name	Feedstock	Conversion technologies
Biodiesel	Biodiesel from energy crops: methyl and ethyl esters of fatty acids.	Oil crops (soybean, rapeseed, palm, etc.)	Cold and warm pressing extraction, purification, transesterification
	Biodiesel from waste	Waste, cooking/frying oil	Hydrogenation
Bioethanol	Conventional ethanol	Sugar beet, sugarcane	Direct fermentation of juice
	Starchy ethanol	Corn, wheat and other grains	Enzymatic hydrolysis, fermentation.

2.1.4 Lignocellulose Structure

The lignocellulose is principally made up of two different polymeric carbohydrates, which are cellulose, hemicellulose and lignin (an aromatic polymers). The complex structure of lignocellulosic biomass makes its bioconversion into bioethanol and other biochemicals a complex and challenging process.

Cellulose

Cellulose is a poly disperse linear 1,4-β-glucan (see Figure 2.1). The global production of cellulose was estimated around 1.5 trillion tons per year and it can be considered an almost inexhaustible source of raw material (Chang, 2014). Cellulose is the substance that makes up most of the plant's cell walls and it isthe most abundant organic polymer on Earth (Dieter, Brigitte, Hans-Peter, & Andrews, 2005). It is described as a complex carbohydrate with the formula $(C_6H_{10}O_5)_n$. Plants make

use of glucose to make cellulose by linking many glucose units together to form long chains. These glucose units are bound together by β (1,4) D-glucose linkages. These long chains make cellulose insoluble in water and many organic solvents. Cellulose is tasteless, odourless, hydrophilic, chiral and biodegradable. It can be broken down into its glucose units by treating with concentrated acids at high temperature (Stephan & Michael, 2011). Cellulose is mainly used for foodstuff, coatings, pharmaceuticals but recently research is focusing on the catalytic conversion of cellulose to fuels and chemicals, and the modification of functional materials with cellulose derivatives (Chang, 2014).

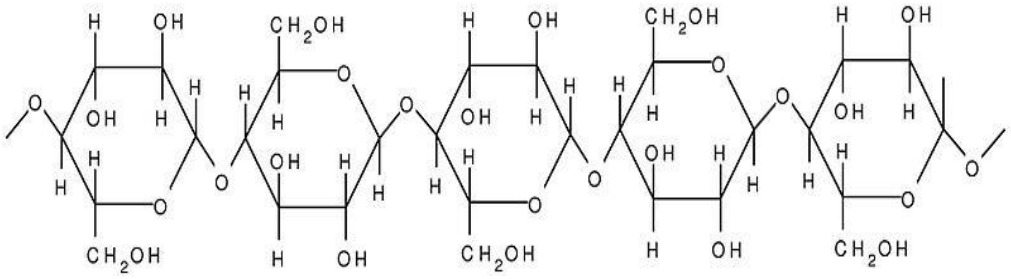


Figure 2.2 Structure of cellulose Hemicellulose

Hemicellulose is heterogeneous class of lignocellulose biomass (Figure 2.2), which consists of pentoses (β-D-xylose, α-L-arabinose); hexoses (β-D-mannose, β-D-glucose, α-D-glucose, and α-D-galactose) and uronic acids (α-D-glucuronic, α-D-4-o-methylgalacturonic and α-D-galacturonic acids), α-L- rhamnose and α-L-fructose are usually present in small amounts. The most important hemicellulose is xylan. Xylan is also the most abundant hemicellulose constituting about 20-30% of the biomass of hardwoods and herbaceous plants (Ebringerova, Hromadkova, & Heinze, 2005).

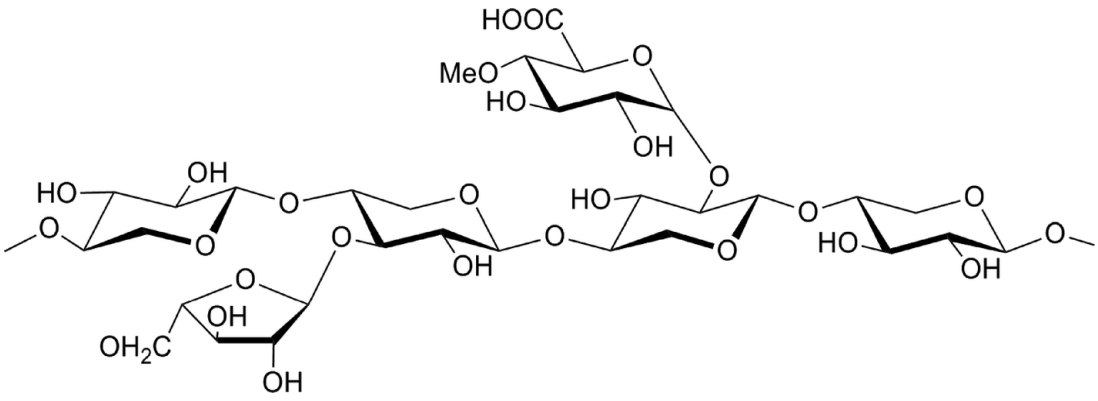


Figure 2.3 Structure of xylan-type hemicellulose

Lignin

Lignin is a major component of cell wall and the third most abundant biopolymer (Eudes, Liang, Mitra, & Loque, 2014). It is an aromatic polymer made up of three dimensional amorphous polymers, consisting of methoxylated phenylpropane structures (Chakar & Ragauskas, 2004). Lignin when bound to cellulose and hemicellulose, gives strength and rigidity to plants (Ritter, 2008). This complex makes the removal of lignin and other components of lignocellulosic biomass highly resistant to chemical and biological hydrolysis, which contributes to the high cost of lignocellulosic sugar production (Boerjan, Ralph, & Baucher, 2003; Zakzeski, Bruijnincx, Jongerius, & Weckhuysen, 2010). Thus, there is an increased need to develop efficient processes for lignin and other biomass components decomposition for the production of renewable energy and chemicals.

Lignin has non repeating bonds between subunits (see Figure 2.3) (Lankinen, 2004) It is a non-soluble compound and in plant cell wall, lignin can be bound to cellulose with either a hydrogen bond or an ether bond. This crosslinking strengthens the cell wall, making lignin more difficult to degrade than cellulose and hemicellulose (Harmsen, Huijgen, Bermudez Lopez, & Bakker, 2010).

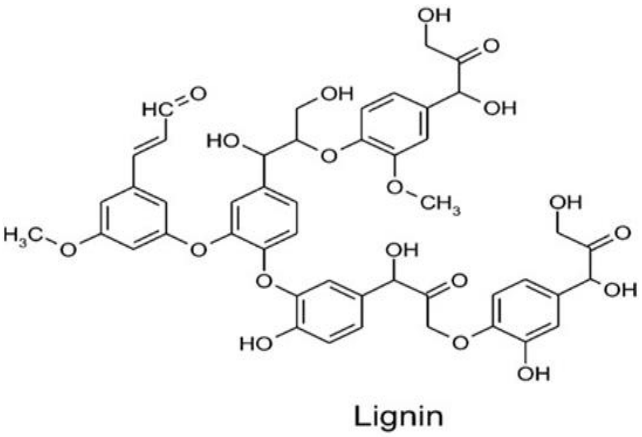


Figure 2.4 Structure of Lignin (Chhabra, 2014)

2.2 The second generation of bioethanol production processes

Bioethanol production process from lignocellulosic biomass consists of mainly five steps, which are (i) pre-treatment (ii) cellulose hydrolysis (iii) detoxification (iv) fermentation and (v) distillation.

2.2.1 Pre-treatment

Pre-treatment is an essential step for the biochemical conversion of lignocellulosic biomass into bioethanol. It is required to alter the structure of cellulosic biomass to expose cellulose to enzyme during enzymatic conversion of carbohydrate polymers into fermentable sugars (Kazi et al., 2010). The alteration of biomass from pre-treatment enables enzymatic hydrolysis of cellulose and hemicellulose to be achieved more rapidly and with greater yield (Harmsen et al., 2010). Pre-treatment aims at the removal of hemicellulose and lignin structure around the cellulose by softening the biomass (Hamelinck, van-Hooijdonk, & Faaji, 2005; Sun & Cheng, 2002), increasing the surface area and porosity, reducing the crystallinity of cellulose and modification of lignin structure (Harmsen et al., 2010) thus making cellulose more accessible during enzymatic hydrolysis. Pre-treatment processes are primarily thermo-chemically catalysed while the conversion reaction is usually aided by cellulase enzymes secreted by suitable microorganisms (Babu et al., 2014).

Effective and economical pre-treatment process requires avoiding hemicellulose and cellulose destruction as well as the formation of inhibitors (Oluwakemi et al., 2014). The hemicellulose can be converted into soluble sugars mainly xylose (Aden et al., 2002; Kazi et al., 2010). Pre-treatment techniques applied (Figure 2.4) include physical (e.g size reduction, steaming/boiling, ultrasonification, and popping), chemical (e.g acids, bases, salts, and solvents), physicochemical (e.g liquid hot water and ammonium fibre explosion or AFEX), and biological (e.g white-rot/brown rot fungi and bacteria) and several combinations to fractionate the lignocellulose into its components (Bensah & Mensah, 2013).

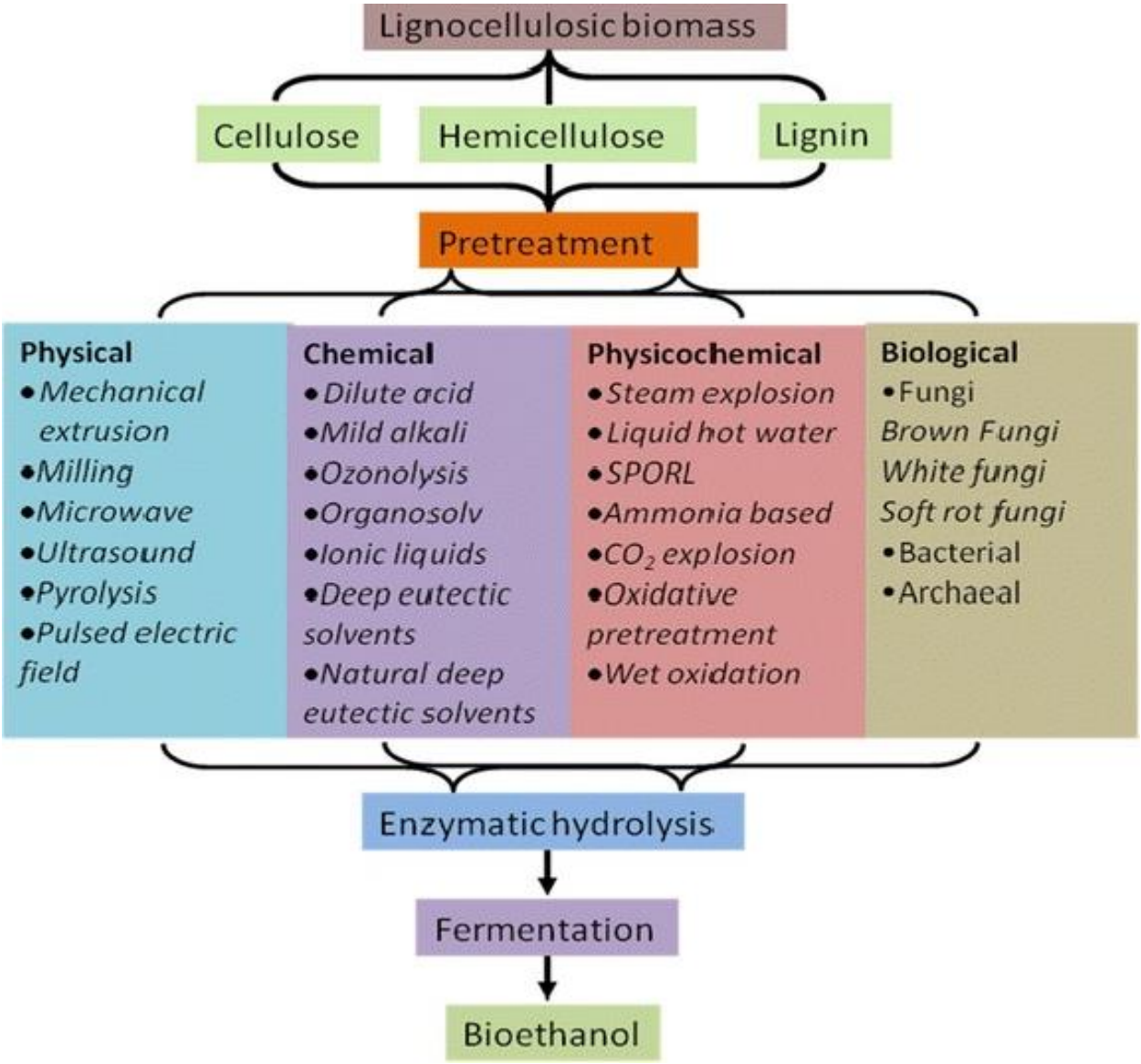


Figure 2.5 Overview of different pre-treatment processes (Kumar & Sharma, 2017)

2.2.1.1 Physical pre-treatment

Physical or mechanical pre-treatment is the processing of lignocellulosic biomass into a small size by increasing the accessible surface area and pore size of lignocelluloses, decreasing the crystallinity and degree of polymerization of the cellulose in lignocelluloses. Physical pre-treatment includes using methods such as milling, grinding etc (Babu et al., 2014) to increase enzymatic digestibility of lignocellulosic waste materials.

The effect of milling as a pre-treatment is the most studied using ball milling, hammer milling, disk milling, two-roll milling, colloid milling, vibratory ball milling etc. Size reduction is one of the most effective methods for increasing the enzymatic

accessibility of lignocelluloses but most of the physical methods employed for lignocelluloses size reduction are still not economically feasible due to high-energy requirement and costly equipment (Behera, Arora, Nandhagopal, & Kumar, 2014).

The electric and magnetic field of microwaves apply forces that rapidly change in orientation at a rate of 2.4 x 10^9 times per second (Galema, 1997). Microwave radiation (MWR) accelerates biological, chemical and physical processes due to heat and extensive collision brought about by the vibration of polar molecules and ion movement (Sridar, 1998). Microwave heating also accelerates cellulose dissolution in ionic liquids (Zhu et al., 2006). Moretti et al. (2016) studied the effect of microwave irradiation as a pre-treatment process on sugarcane bagasse and straw. The result showed that sugar yield of pre-treated bagasse and straw were improved up to 1.4 times and 78% respectively when compared with untreated bagasse and straw. Currently, MWR is carried out at the lab scale, as the equipment is very small and it is difficult to apply to potential industrial projects, thus it is not one of the most promising pre-treatment methods.

The use of electron beam processing (EBP) for pre-treatment has also been reported to increase the conversion yield of cellulose to glucose in sugarcane bagasse from 8 - 12% (Duarte et al., 2012). Thermal treatment after EBP was reported to have increased the cellulose yield after enzymatic hydrolysis up to 71.55% with complete hydrolysis of hemicelluloses (Duarte et al., 2012).

2.2.1.2 Chemical pre-treatment

Chemical pre-treatment has been investigated extensively in delignification of cellulosic materials. Chemicals ranging from oxidizing agents, alkali, acids and salts can be used to degrade lignin, hemicellulose and cellulose from lignocellulosic wastes (Behera et al., 2014). Common chemical pre-treatment techniques includes acid and alkali pre-treatment. Moreover, oxidation agents, ionic liquids and organic solvents have been applied prior to hydrolysis.

Acid pre-treatment

Acid pre-treatment can result in the improvement of lignocellulosic biomass to release fermentable sugars during enzymatic hydrolysis. Acid pre-treatments are mainly used for hardwoods, grasses and agricultural residues due to their effect in

improving cellulose hydrolysis as they attack the polysaccharides. Acids, such as sulphuric acid, hydrochloric acid, nitric acid and phosphoric acid, are widely used for acid pre-treatment, especially sulphuric acid (Keskin, Nalakath Abubackar, Arslan, & Azbar, 2019).

This process can be grouped into two categories based on acid concentration and temperature used:

1) Concentrated acid pre-treatment

2) Dilute acid pre-treatment.

Concentrated acid pre-treatment requires strong acid such as sulphuric (65-86% w/v), hydrochloric (41%), or phosphoric (85%) acids at low temperatures (30-60˚C) and pressures for treating biomass (Bensah & Mensah, 2013). Goshadrou, Karimi, and Taherzadeh (2011) reported an increased in glucose yield of sweet sorghum bagasse by 26.4% after treatment with 85% (v/v) phosphoric acid at 50ºC for 30 min compared to untreated sweet sorghum bagasse. The advantage of concentrated acid pre-treatment is that it can be applied to all kinds of feedstock, however, its major disadvantages include; its toxic effect, corrosion and hazardous effect. Thus, the process requires reactors that are resistant to corrosion, processes to recycle the acid reagent are needed thus making the pre-treatment process expensive. Furthermore, acid pre-treatment results in the production of various inhibitors like acetic acid, furfural and 5-hydroxymethylfurfural, which at high enough concentrations can inhibit microbial growth.

Dilute acid pre-treatment has been one of the most effective methods applied for treating biomass. Low concentration of acid (0.2-2.5% w/w) in combination with high temperature from 120˚C to 210˚C and pressure held for a short period (in seconds or minutes) are used, which is thus suitable for a continuous process (Bensah & Mensah, 2013). When compared with concentrated acid, dilute acid has less corrosion problems and generates fewer inhibitors such as hydroxymethylfurfural, formic acid and levulinic acid. Dilute sulphuric acid is majorly regarded as the most economic and efficient pre-treatment to commercialise lignocellulosic ethanol (Chovau, Degrauwe, & Van der Bruggen, 2013). A dilute acid pre-treatment on municipal solid waste (carrot and potato peelings, grass, newspaper, and crap paper) with dilute acid (H_2SO_4, HNO_3, and HCl) showed that glucose yield of pre-

treated substrates depended more on acid concentration and enzyme loading than reaction temperature (Li, Antizar-Ladislao, & Khraisheh, 2007). Lee and Jeffries (2011) investigated the different catalytic properties of sulphuric acid and organic acids (oxalic and maleic acids) on the degradation of corncob pellet biomass compounds over a range of calculated severity factor (CSF) at 170˚C for 18 min. Their results showed that glucose and xylose concentrations increased with oxalic and sulphuric acid pre-treatments as the CSF increased with maleic acid giving the highest value. In another work on maple wood, oxalic pre-treatment resulted in 87.4% total sugar yield compared to dilute sulphuric and hydrochloric acids of 73.8% sugar yield (Zhang, Kumar, & Wyman, 2013). Dilute H_3PO_4 was applied on potato peels resulting in 82.5% sugar yield (Lenihan et al., 2010) and its application to bamboo and corncob resulted in high sugar yield of 22.65 g/L at 170˚C for 45 min (Hong, Xue, Weng, & Guo, 2012) and 140˚C for 10 min (Satimanont, Luengnaruemitchai, & Wongkasemjit, 2012) respectively. Dilute sulphuric acid (0.25-0.7 wt% in the reactor) pre-treatment of rice straw at temperatures ranging from152 to 166˚C for 10 min was reported to have led to an increase in glucose yield from 37% in native rice straw to 51.9% in the pre-treated rice straw (Kapoor et al., 2017). Dilute acid pre-treatment has been studied on various types of lignocellulosic biomass as shown in Table 2.2 the main disadvantages of dilute acid pre-treatment, despite its wide attention from researchers, is that the process requires special corrosion resistant reactors, which are expensive when compared to other chemical (dilute alkali) and physicochemical methods.

Alkaline pre-treatment

Alkaline pre-treatment of lignocellulosic biomass digests the lignin matrix making cellulose and hemicellulose accessible for enzymatic hydrolysis. Alkaline pre-treatment utilises lower temperatures and pressures than other pre-treatments methods while alkaline conditions cause less sugar degradation when compared with acid processes (Lee & Shah, 2013). For an alkaline pre-treatment, hydroxides of sodium, potassium, calcium and ammonium are mostly used. Lignin removal by alkaline pre-treatment increases enzyme effectiveness by eliminating non-productive absorption sites and by increasing access to cellulose and hemicellulose.

Pre-treatment with sodium hydroxide (NaOH) has been extensively studied for bioconversion of lignocellulosics. Sodium hydroxide has been found to be very

effective in increasing the digestibility of hardwood and agricultural residue with low lignin content (Bali, Meng, Deneff, Sun, & Ragauskas, 2014). An alkaline pre-treatment with sodium hydroxide has been shown to work at reduced temperatures and exhibits a remarkable delignification capacity relative to its severity (Bali et al., 2014). The effect of dilute NaOH was investigated on corn stover with concentration range of 0.1 to 1.0 N and the results showed that enzymatic hydrolysis of corn stover was increased five times when compared with the control with best performance observed with 0.3 N NaOH (Li et al., 2004).

Lime has also been extensively studied as a pre-treatment due to its ability to remove lignin, which improves the enzymes' effectiveness because it eliminates non-productive adsorption sites (acetyl groups) and increases access to cellulose and hemicellulose (Bali et al., 2014) as well as its low cost, safety in handling, availability in many countries, and ease of recovery (Bensah & Mensah, 2013). Lime pre-treatment was investigated on sugarcane bagasse at 90˚C for 90 h and the results showed that lignin was selectively removed at low carbohydrate losses at lime loading rate of 0.4 g/g bagasse (Fuentes, Rabelo, Filho, & Costa, 2011). Its application to corn stover (Kaar & Holtzapple, 2000), switch grass (Garlock et al., 2011), and sugarcane bagasse (Rabelo, Filho, & Costa, 2008) resulted in high conversion of carbohydrate to simple sugars. However, it requires a longer reaction time and large volumes of water for pre-treatment when compared with NaOH under similar conditions (Bensah & Mensah, 2013).All alkaline pre-treatment types have these conditions in common: they increase the digestibility of the lignocellulosics, which is achieved by either changing the complex lignin–hemicellulose network or by increasing lignin removal. Some examples of different alkaline pre-treatment studies with various types of lignocellulosic biomass are shown in Table 2.3.

Table 2.2 Examples of dilute acid pre-treatment on different feedstock

Substrate	Acid	Acid concentration (%)	Temperature (˚C)	Time (minutes)	Reference
Rice straw	Sulphuric acid	0.25 – 0.7	152	10	Kapoor et al. (2017)
Oil palm trunk	Sulphuric acid	1 - 3	160 - 180	20 - 40	Noparat, Prasertsan, O-Thong, and Pan (2015)
Rice straw	Sulphuric acid	0.5	120	60	Kshirsagarab, Waghmareb, Lonic, Patila, and Govindwar (2015)
P. aquatic (Harding grass)	Sulphuric acid	1 – 2	110 - 130	30 - 60	Karapatsia, Pappas, Penloglou, Kotrotsiou, and Kiparissides (2017)
Sorghum stalk	Sulphuric acid	0.5 – 4	120 - 200	15 - 60	Akanksha et al. (2014)
Corn stover	Phosphoric acid	0.5	180	15	Avci, Saha, Dien, Kennedy, and Cotta (2013)

Table 2.3 Alkali pre-treatment on different feedstock

Substrate	Alkali	Alkali concentration (%)	Temperature (˚C)	Time (minutes)	Reference
Sweet sorghum bagasse	NaOH	12	0	180	(Goshadrou et al., 2011)
Switch grass	AFEX		150	30	(Garlock et al., 2011)
Barley straw	NaOH	1	40 - 60	20	(Iroba, Tabil, Dumonceaux, & Baik, 2013)
Switch grass	NaOH	0.2	100 - 160	30	(Karp et al., 2015)
Wheat straw	NaOH & $Ca(OH)_2$		80	39	(Jaisamut, Paulová, Patáková, Rychtera, & Melzoch, 2013)
Sawdust	NaOH	3 – 10	60	30 - 120	(Trevorah & Othman, 2015)
Corn stover	NaOH	50	20 - 200	10 - 25	(Chen, Stevens, Zhu, Holmes, & Xu, 2013)

2.2.1.3 Physicochemical pre-treatment

This pre-treatment combines both the physical and chemical processes in dissolving hemicellulose and alterating lignin structure by providing accessibility of the cellulose for the hydrolytic enzyme. This includes pre-treatment methods such as steam explosion, liquid hot water, ammonia fiber explosion, wet oxidation, CO_2 explosion etc. These pre-treatment depends on process conditions and solvents used which affects the physical and chemical structures of the biomass.

Steam explosion

Steam explosion has been used as a pre-treatment of various biomass feedstocks at a high-saturated pressure steam (5 – 50 atm) and temperature of 160 – 260˚C for a short duration of time (1 – 10 minutes). The steam explosion causes individual fibres to separate and the cell wall structure to be disrupted (Kumar & Satyanarayana, 2009). The disruption of the fibrils increases the accessibility of the cellulose for enzyme hydrolysis (Brodeur et al., 2011). The effectiveness of steam explosion correlates with biomass particle size, which is a major contributing factor of the process. Relatively large particle sizes have been reported to yield maximum sugar concentrations as decreasing particle sizes require further mechanical processing of the raw material thus, leading to an increase in production cost (Brodeur et al., 2011).

Banoth, Sunkar, Tondamanati, and Bhukya (2017) have studied the effect of steam explosion on rice straw. Their results showed that pre-treating rice straw at 170˚C for 10 minute reduces the hemicellulose at about 50% with 61.1% holocellulose recovery and lignin content of rice straw with little or no change in carbohydrate contents. Pre-treating rice straw by steam explosion resulted in a better enzymatic hydrolysis, which produced more sugars than the raw substrate (Banoth et al., 2017).

Steam explosion efficiencies are affected by retention time, temperature, size of biomass, moisture content, pre-treatment severity and pressure difference of the explosion (Pielhop, Amgarten, von Rohr, & Studer, 2016; Zhang et al., 2008). Oliveira et al. (2013) investigated the effect of temperature (180 to 200˚C) during steam explosion on sugarcane straw. The results show that even under the lowest pre-treatment temperature, a major part of the hemicellulose (67.1%) was solubilized

and a maximum of 92.7% glucose concentration was reached, which indicated an increase in hemicellulose solubilisation as the temperature increased. The effect of steam explosion (11, 19 and 31 bar) on spruce wood chips was studied systematically at temperatures of 184, 210 and 235˚C for 5 minutes by Pielhop et al. (2016). Their results showed that pre-treatment at 184 and 210˚C with or without explosion had no influence on digestibility and hydrolysis yields were low. The steam pre-treatment at 235˚C led to a visible defibration. Steam explosion is considered effective for the pre-treatment of agricultural residues and hardwoods but less effective for softwoods (Pielhop et al., 2016).

Liquid hot water/Hydrothermal (Autohydrolysis)

Liquid hot water pre-treatment is one of the most promising and effective methods for the recovery of hemicelluloses in the liquid stream. Liquid hot water pre-treatment involves the use of water at elevated temperatures and high pressures in order to promote disintegration and separation of lignocellulosic matrix. Temperature ranges from 160˚C to 240˚C over a length of time (usually a few minutes to an hour) are used with no external chemical addition (Brodeur et al., 2011). The main aim of this process is to solubilize hemicellulose completely and to form lower concentration of inhibitors.

Li et al. (2017) investigated the effect of liquid hot water pre-treatment on poplar. The pre-treatment resulted in lignin content reduction from 23.7% in untreated poplar to 21.3% in the liquid hot water pre-treated poplar solid which indicated the non-effectiveness of liquid hot water in lignin removal from biomass. Corncobs pre-treated with liquid hot water at temperature range of 140 – 180˚C and residence time of 5 – 20 minutes were investigated by Imman, Laosiripojana, and Champreda (2018). Their results showed that liquid hot water resulted in substantial solubilisation and hydrolysis of hemicelluloses in the feedstock to monomeric sugars. Maximal yield of 58.8% of pentose was recovered from the biomass (corncob) pre-treated at 160˚C for 10 minutes. Longer operation times resulted in lower sugar yields which might be due to degradation of sugars to dehydrated products (Imman et al., 2018).

Ammonia fiber explosion

Ammonia fiber explosion (AFEX) is a process similar to steam explosion pre-treatment process. Liquid anhydrous ammonia is used as a catalyst in this process

under high pressures (100 to 400 psi) and moderate temperatures (60 - 100˚C). The pre-treatment condition is held from either a short reduced residence time of 5 to 10 minutes to a moderate residence time of 30 minutes depending on the degree of saturation needed for the biomass. Although the degree of disruption to biomass structure depends on the temperature which has an effect on the rapidness of the ammonia vaporization (Brodeur et al., 2011). The hemicellulose fraction in the biomass is converted to smaller oligomers and the bonds between lignin and carbohydrate are broken down leading to an increase in cellulose accessibility (Kumar, Barrett, Delwiche, & Stroeve, 2009). AFEX pre-treatment on switch grass was investigated (Alizadeh, Teymouri, Gilbert, & Dale, 2005) which revealed that AFEX had an optimal pre-treatment effect at 90ºC for 5 minutes residence time resulting in 93% glucose conversion compared with 16% glucose conversion in untreated switch grass. Studies on AFEX has also been done on corn stover (Teymouri, Laureano-Pérez, Alizadeh, & Dale, 2004), leading to 2.3 times increase in ethanol yield when compared with untreated corn stover, switch grass harvested in different seasons (Bals, Rogers, Jin, Balan, & Dale, 2010), and *Miscanthus* (Murnen et al., 2007; Yu et al., 2014).

The advantages of AFEX include lower moisture content, lower formation of sugar degradation products, 100% recovery of solid material and the ability for ammonia to lessen lignin's effect on enzymatic hydrolysis. The disadvantages are the costs due to recycling and the chemicals used in the process (Brodeur et al., 2011).

2.2.1.4 Biological pre-treatment

Biological pre-treatment mostly involves the action of fungi that are capable of producing enzymes, which degrade lignin, hemicelluloses and polyphenols present in biomass. In biological pre-treatment process of lignocelluloses, biomass-converting enzymes degrade hemicellulose and lignin and increase the accessibility of cellulose for hydrolysis into simple sugars, which can be fermented by microorganisms into valuable products. Biological pre-treatments are carried out under mild conditions and are very cost effective, relatively safe, with low-energy requirement and environmentally friendly when compared to chemical or physicochemical pre-treatment (Narayanaswamy, Dheeran, Verma, & Kumar, 2013; Saritha, Arora, & Lata, 2012). In addition, the biological pre-treatment produces low

concentrations of toxic compounds such as furfural and hydroxymethylfurfural (HMF), (Narayanaswamy et al., 2013) which have an inhibitory effect on yeast fermentation process. The main drawback is the duration of the pre-treatment period which takes several weeks (Rouches, Zhou, Steyer, & Carrere, 2016).

Many species of ligninolytic microorganisms have been investigated using direct microorganisms in biological pre-treatment, such as, white-rot fungi, soft-rot fungi, and brown-rot fungi, and bacteria, which degrade lignin through the action of lignin-degrading enzymes such as peroxidase and laccases (Saritha, Arora, & Lata, 2012). Each of these microorganisms has their own specific biological approach to break down biomass structure.

White rot fungi

White-rot fungi are microorganisms capable of complete mineralization of both the lignin and the polysaccharide components of plant. They are identified as the best delignifying organisms (Rouches et al., 2016) as white rot fungi can improve hydrolysis and subsequent sugar yield. Zhi and Wang (2014) have investigated the effect of white rot fungal pre-treatment using *P. chrysosporium* on wheat straw at 30˚C under solid-state fermentation. They found that after 12 days of pre-treatment, about 28.5% of lignin had been removed and further microscopic structure observation showed that the lignocellulose structure was disrupted after fungal pre-treatment.

Li and Zhang (2014) also studied the biological pre-treatment using *P. chrysosporium* and *T. versicolor* strains on cotton stalks. They reported that after 5 days pre-treating the cotton stalk with *P. chrysosporium* showed the strongest degrading capacity of lignocellulose with total degrading ratio of 16.14%. White rot fungi *P. ostreatus* was used for the pre-treatment of switch grass by Li (2013) at different storage time which showed that *P. ostreatus* selectively consumed the lignin with glucose fraction ranging from 1.7% to 7.8% than the control throughout storage time. Salvachúa et al. (2011) reported that pre-treating wheat straw for 21 days with *P. chrysosporium* revealed no lignin degradation. However, there was a 35% degradation of cellulose and 70% of degradation of hemicellulose with glucose yield of 69% and 66% after 21 days pre-treatment with *P. subvermispora* and *I lacteus* respectively.

Paddy straw pre-treated with *Trametes hirsuta* was reported to enhance carbohydrate content by 11.1% within 10 days of incubation (Saritha, Arora, & Nain, 2012). Deconstruction of lignin and decomposition of main linkages between hemicellulose and lignin was reported by Yang, Ma, Yu, Zhang, and Chen (2011). Their results showed that corn stover pre-treated with white rot fungus *Echinodontium taxodii* 2538 could contribute to the improvement of pyrolysis at low temperature.

Although white-rot fungi are promising for effective lignocellulosic biomass pre-treatment, due to long residence time of pre-treatment, it is not favourite as a choice for industrial scale production.

Soft rot fungi

Soft-rot fungi are mainly found in wet environment on wood and have been identified from *Deuteromycotina* or *Ascomycotina* (Madadi & Abbas, 2017). These fungi have been reported to decrease lignin in woody plants more than herbaceous crops. Soft rot fungi degrade wood components very slowly when compared to white-rot and brown-rot fungi (Kang, Li, Fan, & Chang, 2013). Most extensively studied soft-rot fungi are the members of the genera *Trichoderma*, *Humicola* and *Penicillium*. Ray, Leak, Spanu, and Murphy (2010) studied the biological pre-treatment efficacy using different fungal strains on pinus radiate sapwood. They reported that pre-treated biomass with *Chaetomium globosum* ATCC 6205 at 25˚C for 20 days, resulted in a 10% weight loss with no improvement in glucose yield.

Brown rot fungi

Brown-rot fungi principally degrades cellulose and hemicellulose faster than lignin breaking down the polymeric structures of their molecules (Madadi & Abbas, 2017). Moreover, when compared with other fungi and bacteria, the way of digestibility of plant cell wall by brown-rot fungi is entirely different, because the reduction mechanism is non-enzymatic and lacks exoglucanases. Brown-rot fungi predominantly grow on herbaceous crops rather than woody plants. Amongst the brown-rot fungi, *Serpula lacrymans* and *Gloephyllum trabeum* are found to destruct the structure of woody plants without difficulty (Madadi & Abbas, 2017).

Biological pre-treatment of wheat straw using *G. trabeum* has been investigated by Hermosilla et al. (2018). It was found that *G. trabeum* shows a fast degradation of

26.4% hemicellulose in the first 10 days with preferential degradation of hemicellulose over cellulose after 40 days reaching 37.6% and 13.2% respectively with no quantifiable lignin degradation. Monrroy, Ortega, Ramírez, Baeza, and Freer (2011) investigated *G. trabeum* (ATCC 11539) and *L. sulphureus* (ATCC 52600) for the treatment of wood chips (*P. radiate* and *E. globulus*). The wood chips showed a higher biodegradation of hemicellulose 31% and 24% respectively after eight weeks of biotreatment with *G.trabeum*.

Bacteria

Many bacteria have been screened for lignin degradation such as *Novosphingobium* sp., *C. basilensis* and *Comamonas* sp. Bacteria degrade lignin firstly through depolymerisation of extracellular lignin then degradation of intracellular lignin (Zhuo et al., 2018). There are just a few reports on bacterial pre-treatment directly applied to biomass.

In conclusion, all the pre-treatment technologies employed to either remove or reduce the recalcitrant property of lignocellulosic biomass are prone to different challenges. Biological pre-treatment is seen as the most effective in reducing the cost of pre-treatment (energy requirement, equipment) and environmental hazards (chemical recovery) associated with other pre-treatment technologies. However, the long fermentation period involved in biological pre-treatment is the major limitation to its acceptance commercially. In order to overcome this challenge, it is important to discover novel microorganisms that could reduce the fermentation period with the desired result after undergoing biological pre-treatment. The advantages and disadvantages of each pre-treatment method are listed in Table 2.4

Table 2.4 The advantages and disadvantages of each pre-treatment methods

Pre-treatment	Advantages	Disadvavtages
Physical pre-treatment	No production of inhibitors Increased surface area	High energy requirement & High cost of maintenance
Physicochemical pre-treatment	Increased enzyme accessibility	Risk of producing inhibitors, High heat demand Effective only up to a certain temperature
Chemical pre-treatment	Solubilizes hemicellulose Removes lignin Modifies lignocellulosic structures	High cost of acids, Corrosion problem Risk of producing inhibitors High alkali concentration in reactor High cost of chemical recovery after pre-treatment
Biological pre-treatment	Reduced formation of inhibitory substances Minimization of applied chemicals and energy input Degrades lignin Solubilizes hemicellulose & Lower costs for waste water	Pre-treatment is limited by the rate of microbial growth. Long pre-treatment duration

2.2.2 Biomass hydrolysis

The hydrolysis step involves the conversion of the exposed cellulose from lignocellulosic biomass into glucose (Balat & Bala, 2008). The two major methods used are acid hydrolysis (diluted acid and concentrated acid) and enzymatic hydrolysis (cellulase enzymes). The conversion of lignocelluloses into fermentable sugars for fuel production is preferentially performed by enzymatic hydrolysis of polysaccharides (Yang, Zhang, Zuo, Men, & Tian, 2011). In the following section, the literature survey focuses on the enzymatic hydrolysis.

2.2.2.1 Acid hydrolysis

Acid hydrolysis is widely used for the hydrolysis of lignocellulosic biomass. Lignocellulosic biomass can be hydrolysed using sulphuric acid to produce xylose, arabinose, glucose and acetic acid. The hydrolysis process is operated under two different conditions; (1) the use of concentrated acid at a lower temperature and (2) the use of dilute acid at a higher temperature.

Acid hydrolysis has been studied using different types of feedstock for producing sugar, such as potato skin (Lenihan et al., 2010), corn stover (Binder & Raines, 2010), wheat straw (Guerra-Rodríguez, Portilla-Rivera, Jarquín-Enríquez, Ramírez, & Vázquez, 2012; Ji, Shen, & Wen, 2015) and *Miscanthus* (Chung, Charmot, Olatunji-Ojo, Durkin, & Katz, 2014). High hydrolysis yield of cellulose of up to 90% of the theoretical glucose yield has been reported when concentrated acid (10-30%) is used for acid hydrolysis (Verardi, De Bari, Ricca, & Calabrò, 2012). It has the advantage of penetrating lignin without any preliminary biomass pre-treatment.

The hydrolysis of lignocellulosic biomass using acid has several disadvantages such as corrosion of equipment, formation of toxic compounds such as, furfural, hydroxylmethylfurfural, acetic acid, formic acid, levulinic acid etc. These compounds are potential inhibitors and negatively affect the fermentation process (Verardi et al., 2012). The removal of these compounds before fermentation increases the production cost. However, enzymatic hydrolysis process could be used to replace acid hydrolysis, since it is economical and environmental friendly.

2.2.2.2 Enzymatic hydrolysis

Enzymatic hydrolysis is an effective method for the conversion of lignocellulosic biomass into fermentable sugars. Cellulose structural features and the mode of enzyme action influence the efficiency of enzymatic hydrolysis. Enzymatic hydrolysis has less formation of undesirable by products, less acid waste, does not require corrosion resistant equipment, characteristics that make it more desirable over acid hydrolysis.

Lignocellulosic material is degraded during enzymatic hydrolysis using lignocellulolytic enzymes. Cellulosic enzymes normally comprise of cellulase, hemicellulase and ligninolytic enzymes.

2.2.3 Cellulosic enzymes

2.2.3.1 Cellulase

Enzymatic hydrolysis of lignocellulosic biomass involves the use of cellulase as the primary enzyme for bioethanol production.The high cost of enzyme was estimated as high as 40% of the total cost of bioethanol by Spano (1978). With recent technologies and advances in research, the cost of cellulase has been significantly reduced. Although the cost of cellulase enzyme is still high for commercialization of bioethanol (around 10%) (Saravanan, Muthuvelayudham, Rajesh Kannan, & Viruthagiri, 2012) in order to compete with fossil fuel.

Cellulase is synthesized by a large diversity of microorganisms like fungi and bacteria during their growth on cellulosic materials. These microorganisms can be aerobic, anaerobic, mesophilic or thermophilic of which *Aspergillus* is the most extensively studied cellulase producer (Kuhad, Gupta, & Singh, 2011a). Cellulase breaks down cellulose molecules into monosaccharides such as glucose, shorter polysaccharides and oligosaccharides.

Cellulase consists of at least three groups of enzymes (endoglucanase, exoglucanase, and β-Glucosidase). Cellulase has been available commercially for over 30 years (Kuhad, Gupta, & Singh, 2011b) and it has found different applications in various industries such as the pulp and paper industry, textile industry, bioethanol industry, wine and breweries industries, food processing industry, animal feed

industry, agricultural industry, olive oil extraction, carotenoid extraction, detergent industry and waste management (Karmakar & Ray, 2011).

Endoglucanase or Endo-1,4-β-D-glucanase, EG

This enzyme randomly cleaves β-1,4-bonds of cellulose chains, creating new chain ends. Archaea, bacteria, fungi, plants, and animals with different catalytic modules produce different endoglucanases. However, some endoglucanases can act possessively based on their ability to hydrolyse crystalline cellulose and generate the major products as cellobiose or longer cellodextrins (Cohen, Suzuki, & Hammel, 2005; Li & Wilson, 2008; Mejia-Castilo, Hidalgo-Lara, Brieba, & Ortega-Lopez, 2008; Parsiegla, Reverbel, Tardif, Driguez, & Haser, 2008; Yoon, Cha, Kim, & Kim, 2008).

Exoglucanase also known as cellobiohydrolase, CBH

Exoglucanase acts in a possessive manner (cling tightly) on the reducing or non-reducing ends of cellulose polysaccharide chains, liberating either cellobiose or glucose as major products. Exoglucanases can effectively work on micro-crystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure (Teeri, 1997). Cellobiohydrolase (CBH) is the most-studied exoglucanase with different CBHs produced by many bacteria and fungi.

β-Glucosidases or cellobiases, BG

Cellobiase or β-Glucosidase (BG) hydrolyses soluble cellodextrins and cellobiose to glucose. The activity of BG on insoluble cellulose is negligible. BGs degrade cellobiose, which is a known inhibitor of CBH and endoglucanase, into individual monosaccharides. Various archaea, bacteria, fungi, plants, and animals, with different catalytic modules, produce different BGs. It is reported that aerobic fungi produce extracellular BGs, and anaerobic bacteria keep their BGs in cytoplasm (Yang, El-Ensashy, & Thongchul, 2013). BGs have a pocket-shaped active site, which allows them to bind the non-reducing glucose unit and clip glucose off from cellobiose or cellodextrin.

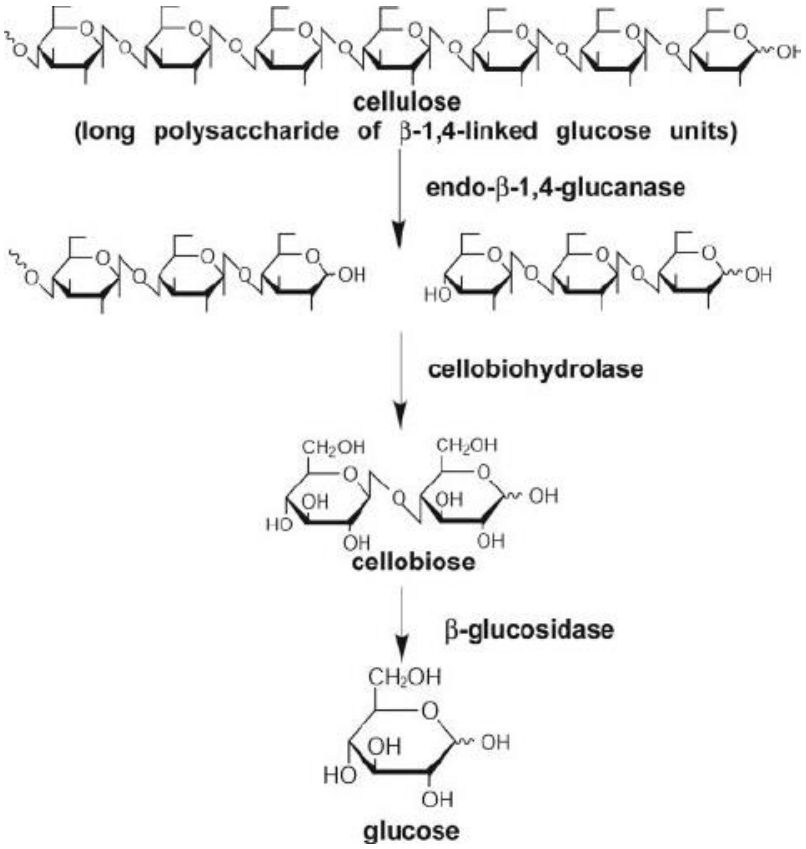


Figure 2.6 Cellulose degradation pathway (Xie et al., 2007)

2.2.3.1.1 Cellulase Enzyme Production through Fermentation

The production of cellulase involves the growth of microorganisms (fungi or bacterial) on cellulosic materials (Gamarra, Villena, & Gutiérrez-Correa, 2010; Saravanan et al., 2012; Schuster, Dunn-Coleman, Frivad, & van Dijck, 2002). It can also be produced from several plants. However, a large number of microorganisms is capable of degrading cellulose while few of these microorganisms were reported in producing significant quantities of enzymes that could completely hydrolyse cellulose (Amore et al., 2015). Fungi are considered as the main cellulase-producing microorganisms (Amore et al., 2015; Wen, Liao, & Chen, 2005; Yang et al., 2011) though a few bacteria and actinomycetes have been recently reported to yield cellulase (Amore et al., 2015; Kuhad et al., 2011a). Table 2.5 shows the representative cellulase-producing microorganisms.

Cellulase can be produced through solid-state fermentation (SSF) and submerged fermentation (SmF). Nearly all companies have chosen submerged fermentation in order to produce relatively low cost cellulase because they were able to produce

more than 100 g of crude cellulase (weight) per litre of broth (Zhang & Zhang, 2013). Most enzyme companies, like Novozymes, Genencor, Iogen, etc produce cellulase commercially using *Trichoderma sp* and *Aspergillus sp* or their derivative strains and during the past years, these companies have claimed a 20 – 30 fold reduction in cellulase production costs to 20 – 30 cents per gallon of cellulosic ethanol (Himmel & Bayer, 2009).

SSF is the fermentation process that involves solid substrate, which is carried out in the absence or near absence of free water. The credibility of SSF has increased in the past few years in biotech industries, in relation to its potential applications in cellulase production and has become an attractive alternative to submerged fermentation (Singhania, Sukumaran, & Pandey, 2007). The production cost of cellulase can be reduced by a multifaceted approach, which includes the use of cheap lignocellulose substrate in SSF (Singhania et al. (2007). The major challenges faced by SSF include the microorganisms and substrate selection, optimum process parameters and purification of the end product. Although, fungi and yeast are considered suitable microorganisms for SSF, bacteria have been considered unsuitable whereas, Arai et al. (2006); Sabu, Augur, Swati, and Pandey (2006) claims that it can be manipulated and adapted for SSF. Table 2.6lists recent studies using SSF for the cellulase production.

Table 2.5 Representative cellulase-producing microorganisms

	Microorganism		Microorganism
Fungi	*Acremonium cellulolyticus*	Bacteria	*Clostridium thermocellum*
	Aspergillus acculeatus		*Ruminococcus albus*
	Aspergillus fumigatus		*Streptomyces sp.*
	Aspergillus niger		
	Fusarium solani		
	Irpex lacteus		
	Penicillium funmiculosum		
	Phanerochaete	Actinomycetes	*Streptomyces sp.*
	Chrysosporium		*Thermoactinomyces sp.*
	Schizophyllum commune		*Thermomonospora curvata*
	Sclerotium rolfsii		
	Sporotrichum cellulophilum		
	Talaromyces emersonii		
	Thielavia terrestris		
	Trichoderma koningii		
	Trichoderma reesei		
	Trichoderma viride		

Table 2.6 Recent SSF studies for cellulase production

Substrate	**Conditions**	**Enzyme activity**	**References**
Wheat bran	Potato dextrose agar plate. Temp. 30˚C, Duration: 5days, SSF	Cellulase activity	Singhania et al. (2007)
Sugar cane bagasse and spruce wood	Temp. 30˚C at 150rpm Duration: 11days, SSF	Endoglucanase activity. Conversion of other lignocellulose derived compounds such as acetic acid, furan, aldehydes and phenolic compounds.	Alriksson et al. (2009)
Cow dungs		4210 U/g	Ponnuswamy Vijayaraghavan, Arun, Vincent, Arasu, and Al-Dhabi (2016)
Microcrystalline cellulose	Glucose agar plate Temp. 35˚C and shaking frequency 200 rpm. Duration: 96 hours, SSF	4.88 FPU/mL cellulase activity	Bendig and Weuster-Botz (2013)

Microcrystalline cellulose	Glucose agar plate. pH 4.5. Temp. 25˚C at 1200rpm. Duration: 96 hours. Mandels medium, Fed-batch fermentation	Cellulase activity 2.09 FPU/mL	Bendig and Weuster-Botz (2012)
	Potato dextrose agar plate. pH 4.5. Temp. 30˚C at 3000 rpm for 10mins. Duration 12-15 days and sub-cultured every three months.	Fpase 1.21 U/mL CMcase 29.8IU/mL Xylanase 21.5 IU/mL. β-glucosidase 0.06 IU/mL	Adsul, Bastawde, Varma, and Gokhale (2007)
Rice straw and sugarcane bagasse	PDA and YEPD agar slants respectively. pH 4 .8. Temp. 30˚C at 10,000rpm for 10 mins. Duration: 72 hours. SSF	CMcase 299.55 U/gDs CMcase 14.98 U/mL Total cellulase 22.8 FPU/gDs Total cellulase 1.14 U/mL	Sukumaran, Singhania, Mathew, and Pandey (2008)

SmF is traditionally used for enzyme production from microorganisms for a long period. The fermentation process in SmF involves the free flowing of liquid substrate. Compared to SSF, media sterilization, purification, recovery of the end products and the control of process parameters such as pH, temperature, oxygen transfer and aeration can be carried out easily (Suriya, Bharathiraja, Krishnan, Manivasagan, & Kim, 2016). *Trichorderma reesei* is considered as one of the most important cellulase producer in submerged fermentation and has been widely used in industries such as food, feed and biorefinery. Faheina Jr et al. (2015) investigated strategies to increase cellulase production in SmF using fungi isolated from the Brazilian biome. Their result shows that *Trichoderma* sp CMIAT 041 gave the highest activity after 72 hours of fermentation (49.0 FPU/L). Cellulase production was also investigated by Mrudula and Murugammal (2011) under SmF with *Aspergillus niger* using 5 substrates. The result shows that coir waste gave the maximum cellulase production of 0.51 U/mL when fermented with *A. niger* under SmF.

The production of cellulase in SmF by *T. reesei* and *A. niger* has been extensively studied. However, there is no report on the production of cellulase in SmF by *Rhizomucor variabils*.

2.2.3.2 Hemicellulase

Hemicellulases are similar to endoglucanes and act on the hemicellulose polymer. The complex composition and structure of hemicellulose require multiple enzymes to break down the polymer into simple sugars.

Xylanase

Xylanases are glycosidases (O-glycoside hydrolases, EC 3.2.1.x) which randomly cleave the β-1,4 backbone of the complex plant cell wall polysaccharide xylan (Figure 2.6). They are a widespread group of enzymes, involved in the production of xylose. Xylose is a primary carbon source for cell metabolism and in plant cell infection by plant pathogens. Xylanases are produced by a plethora of organisms including bacteria, algae, fungi, protozoa, gastropods, and arthropods (Prade, 1996). According to the recently updated Carbohydrate Active enzymes (CAZy) database (Cantarel et al., 2009), xylanase activity can be affected by the presence of proteinaceous inhibitors in cereals (rye, barley, maize, rice, durum, and bread wheat).

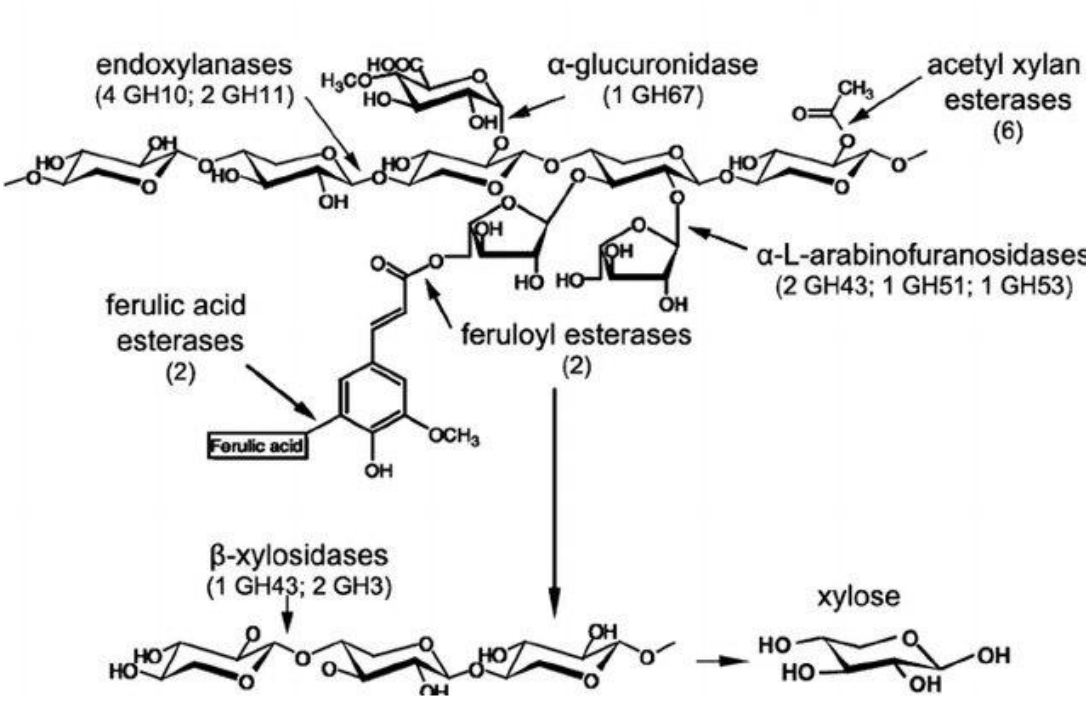


Figure 2.7 Hemicellulase degrading enzymes (mainly xylan) (Sun, Tian, Diamond, & Glass, 2012)

2.2.3.3 Lignin-modifyng enzymes (LMEs) or ligninase

Lignin is a complex phenolic heteropolymer, which degrades at a much slower rate compared to cellulosic and noncellulosic polysaccharides and proteins. LMEs are produced by fungi, bacteria that catalyse lignin break down. LMEs include peroxidases, such as lignin peroxidase, manganese peroxidase, and laccases or phenol oxidase.

2.2.4 Detoxification of lignocellulosic hydrolysate

Lignocellulosic hydrolysate consists of different concentration of inhibitors depending on the raw material composition used in the process, and the severity and type of pre-treatment employed. The formation of inhibitors during hydrolysis are divided into the following major groups: (a) furans such as furfural and 5-hydroxymethylfurfural, phenolic compounds; (b) weak organic acids (levulinic, formic and acetic acid), and (c) heavy metal ions. The presence of inhibitors in the hydrolysates has negative effect on the microorganisms used for fermentation therefore, detoxification of the hydrolysate is often employed to reduce the concentrations of inhibitors.

Detoxification involves the application of different types of treatments of the hydrolysates such as physical (evaporation, membrane medated detoxification), chemical (neutralization, calcium hydroxide over liming, activated charcoal treatment), and biological detoxification (enzymatic mediated using laccase, lignin peroxide) (Chandel, da Silva, & Singh, 2011), which have shown to improve the fermentability of strongly inhibitory lignocellulosic hydrolysates (Alriksson, Sjöde, Nilvebrant, & Jönsson, 2006). Sugarcane bagasse and Norwegain spruce (*Picea abies*) hydrolysates were treated with sodium borohydride by Cavka and Jönsson (2013) and the results showed improved fermentability of the hydrolysates compared to the untreated hydrolysates. Morozova and Semyonov (2016) used activated sludge for detoxification of *Miscanthus* and spruce hydrolysates. The result showed that activated sludge treatment resulted in the removal of 98% and 99% of 5-hydroxymethylfurfural and furfural from the hydrolysate respectively.

2.2.5 Bioethanol fermentation

Bioethanol fermentation involves the conversion of hexose or pentose sugars (glucose, fructose and sucrose) into ethanol and carbon dioxide by alcoholic fermentation microorganisms. Many microorganisms, including bacteria and yeasts, are widely used for the production of ethanol as fermentation product from carbohydrates. The most commonly employed microorganism is *S. cerevisiae* for ethanol production due to its high ethanol production rate, hardness over a wide range of low pH and high ethanol tolerance. The theoretical maximum yield of both hexoses and pentoses is respectively 0.511 kg ethanol and 0.489 kg CO_2 per kg sugar (Alriksson et al., 2009; Chovau et al., 2013).

2.2.6 Distillation

Distillation is used for liquid purification and for separating mixtures of liquids into individual components (Babu et al., 2014). After fermentation of lignocellulosic biomass, the yeast cells are removed by centrifugation and distillation process is used to separate all ethanol from the liquid based on differences in volatiles of mixture components for ethanol to be usable as a fuel. The solid residue fraction is called Distiller's dried grains with soluble (DDGS), which could be used to produce cellulase for enzymatic hydrolysis or other various applications (Alriksson et al., 2009). Figure 2.7 below shows the basic steps employed in bioethanol production.

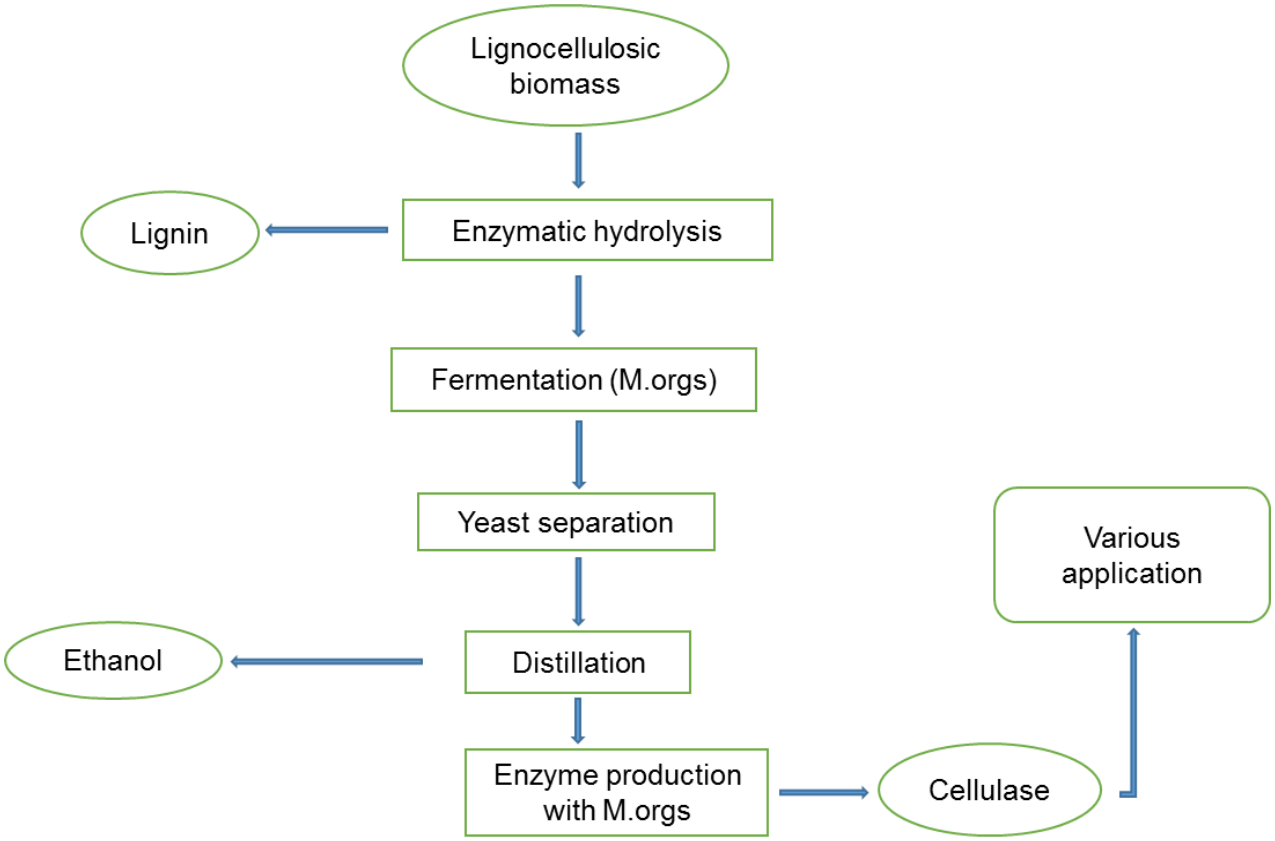


Figure 2.8 Schematic flowchart for bioethanol production

2.2.7 Challenges in bioethanol production

Lignocellulosic biomass has been a promising feedstock as an alternative sustainable energy for the production of second-generation biofuels. Significant progress has been made to overcome technical and economic challenges of second-generation biofuel (Sims, Mabee, Saddler, & Taylor, 2010). The production cost of lignocellulosic bioethanol is still a major constraint in the replacement of fossil fuel with bioethanol. The challenges are related to biomass feedstock supply to meet commercial scale plant, pre-treatment cost, operating technologies and operating time (Sims et al., 2010).

Furthermore, the cost of enzyme production for the enhancement of effective cellulose hydrolysis into fermentable sugar is also very high. Pre-treatment of lignocellulosic biomass and the cost of enzymes are the major limitation highlighted for the commercialisation of lignocellulosic bioethanol production (Wi et al., 2015). The challenge could be to minimize if the cost of processing lignocellulosic biomass into bioethanol could be reduced i.e. pre-treatment step and the cost of commercial

enzyme used during hydrolysis. The cost of commercial enzymes used in hydrolysis step can be reduced by generating on-site enzymes.

2.3 Biorefining process to convert lignocellulose to bioethanol

According to IEA Bioenergy Task 42 “Bioerefinery is the sustainable processing of biomass into a spectrum of marketable products and energy” (IEA, 2007). The petrol, fine chemicals, polymers, fibres and plastics are produced from fossil fuel refinery. The depletion of fossil fuel and global energy shortage is the driving force for the development of a biorefinery process to replace fossil fuel with biomass for the production of bioethanol and biochemical such as succinic acid, itaconic acid, lactic acid and 1,3-propanediol acid.

2.3.1 Bioethanol production

Ethanol has become the largest biofuel produced worldwide (Olson, Sparling, & Lynd, 2015) and it is considered as the next generation transportation fuel. Currently, ethanol is produced from corn and sugar cane through fermentation process. Lignocellulosic biomass as a feedstock for ethanol production is seen as the next solution step towards expanding ethanol production capacity significantly (Xiu, Zhang, & Shahbazi, 2011). The production of ethanol from renewable resources is expected to reduce dependence on petroleum as an alternative fuel because it is from a renewable source , less toxic and its by-products are less toxic than by-products formed from fossil fuel (Vohra, Manwar, Manmode, Padgilwar, & Patil, 2014). The world total ethanol production in 2017 according to the Renewable fuel Association is around 27.05 billions of gallons and the US is the largest producer of ethanol.

Ethanol can be produced as a major fermentation product from carbohydrate from various agricultural residues such as corn stover, sugarcane bagasse, wheat straw, barley straw, and rice straw, processing by-products (corn fiber and rice hulls) and energy crops (switchgrass and *Miscanthus*), using several microorganisms such as bacteria and yeasts (Saini, Saini, & Tewari, 2015). Industrial ethanol fermentation is currently carried out using the yeast strain *S. cerevisiae* due to its low pH and high ethanol tolerance. In addition, the bacterium *Zymomonas mobilis* has been reported

to have a higher specific ethanol productivity and yield from glucose and sucrose (Yang, Liu, & Zhang, 2007).

The leaves of energy cane were used for ethanol production by Shields and Boopathy (2011) using the *Klebsiella oxytoca* ATCC strain 68831 and a higher ethanol yield of 6995 mg/L was reported to be produced on the 6th day of fermentation.

2.4 Wheat straw as a second generation lignocellulosic ethanol feedstock

Wheat (*Triticum aestivum L*.) was originally produced in Western and Eastern Asia nearly 10,000 years ago. Wheat has been a staple food crop in Europe, West Asia and North Africa for 8,000 years. Recently, this crop has grown on more land areas in over 115 nations around the world with annual global production of dry wheat estimated to be over 650 Tg in 2008 (Talebnia, Karakashev, & Angelidaki, 2010b). Wheat is utilised as food, feed, seed as well as waste, which is estimated at about 850 Tg annually. With large quantities of wheat straw (Figure 2.8) left on field after harvesting, it could be ploughed back into soil or burned. Burning of wheat straw as a disposal method has been challenged as it resulted in air pollution and concern over health effect.

The full removal of wheat straw is still of high interest despite its utilisation in feed production industry, pulping and packaging industry, furniture manufacturing. In order to reduce the environmental impact of wheat straw disposal and fossil fuel, wheat straw has been identified as a potential biomass source for the production of monomeric sugars for second-generation bioethanol due to its complex composition of cellulose, hemicellulose and lignin. The cellulose and hemicellulose fraction of wheat straw could be hydrolysed into simple sugars such as glucose, xylose and arabinose, which could then be converted to biofuels such as bioethanol and methane (Zheng et al., 2018).

The main advantages of utilizing wheat straw as a substrate for second generation biofuel which is considered as a major source of renewable energy and as lignocellulosic biomass. Wheat straw is inexpensive and abundant, hence, has a great potential for biofuel production. Although due to its recalcitrant character, pre-treatment is required, thus increasing the cost of ethanol production.

Figure 2.9 Wheat straw

Wheat straw has been investigated under numerous pre-treatment methods for bioethanol production (Akanksha et al., 2014; Alizadeh et al., 2005; Xuewei Yang et al., 2011). Novy, Longus, and Nidetzky (2015) employed steam explosion for the pre-treatment of wheat straw and their result showed that 22 g/L ethanol was produced within 50 h of fermentation. These pre-treatment technologies for biomass required energy intensive process due to corrosive resistant reactor needed, the treatment of wastewater and the recovery of chemicals have resulted in high investment cost. In addition, the cost of enzyme for hydrolysis is a major variable cost for ethanol production from wheat straw and other lignocellulosic biomass. In order to produce bioethanol that could compete with fossil fuel, the energy consumption required for the pre-treatment of lignocellulosic biomass and the cost of enzyme for hydrolysis should be reduced. Therefore, biological pre-treatment is seen as an alternative route to reduce energy consumption and production cost for the production of bioethanol from lignocellulosic biomass due to its low capital cost, low energy consumption and mild environmental conditions. However, the long retention time is the main limitation of biological pre-treatment compared to other technologies. Therefore, the continuous study of microorganisms for their ability to treat plant material quickly and efficiently is of necessity in order to reduce the cost of bioethanol production through pre-treatment of lignocellulosic biomass.

2.5 Sorghum

Sorghum (*Sorghum bicolor*) is a cereal plant (Figure 2.9) of the grass family *Gramineae,* subfamily *Panicoideae* and the tribe *Andropogoneae* (the tribe of big blue stem and sugar cane) its origin is Africa (Fuller, 2014). Sorghum is an important cereal crop in Nigeria with several cultivars grown in the Savannah and Sahelian region of Northern Nigeria covering above 45% of the total land for cereal production in the country (Nasidi, Akunna, Deeni, Blackwood, & Walker, 2010). Sorghum is the 5th most important crop cereal in the world according to Beta, Chisi, and Monyo (2004); (Waniska, Rooney, & McDonough, 2004) in terms of its acreage and production. Nigeria is the 2nd largest producer in the world with over 9.2 million tonnes per annum and it accounts for 71% of sorghum production in West Africa (Nasidi et al., 2010). Sorghum is valued in the hot and arid region because of its resistance to drought and heat. It is the main source of food grain in Africa, Asia and China. It is grown in the Southern part of USA and nearly all the cultivated sorghum grain in the USA is used for livestock feed (Fuller, 2014). Only 1% of the total production of sorghum is grown in Europe (France, Italy, Spain and some South Eastern countries) and it is extensively cultivated in Russia and Ukraine. Sorghum is a main crop for food, fodder and alcoholic and non-alcoholic beverage production especially in African countries as well as for biofuels production.

The sorghum kernel is a caryopsis with the pericarp being completely fused with the endosperm (Figure 2.10). Sorghum grains have extremely hard endosperm and the pericarp is brittle when compared to wheat (Umwungerimwiza, 2015). The endosperm is the largest component of the sorghum kernel and contains the starch granules and protein bodies.

Figure 2.10 Sorghum crops in field. (Kelly, 2017)

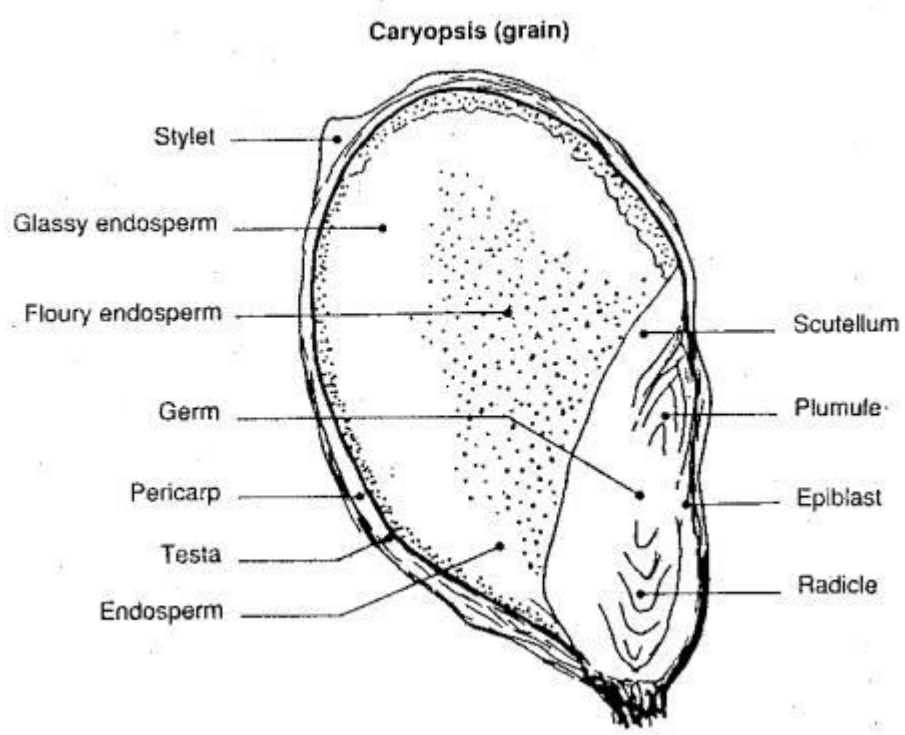


Figure 2.11 Structure of sorghum grain (after Sautier and O'Deye, 1989)

2.5.1 Sorghum milling

Sorghum milling involves the removal of the fibrous and the highly coloured pericarp with the testa layers to reduce the grain to flour. Efficient mechanical dehulling of

sorghum has been the major challenge of processing sorghum. This has become an area where most sorghum milling research has been concentrated (Muller, 1970).
The presence of polyphenols, the grain structure and the strong interaction between the starch and protein in the kernel prove difficulty in the milling of sorghum (Donley, 2013; Zhu, 2014). Sorghum milling processes are categorised into traditional (Figure 2.11) and modern. The traditional processing includes machete, knives, mortar and pestle, grinding stones, and beating stick while the modern processing are threshers, dryers, dehuller, milling machines etc. Sorghum can be processed by either dry or wet milling.

In Nigeria, sorghum is dehulled, milled and sieved (dry milling process) to obtain three flour fractions with different particle sizes using Buhler mill (Adeyemi, 1983). Sorghum grains are cleaned manually, conditioned, tempered and debranned in grain hulliers to remove the outermost fibrous layer. This process also removes the germ. The grits are cleaned and milled to desired particle size using a hammer mill (Olatunji, Koleoso, & Oniwande, 1992). The two common milling methods found in Nigeria and Ghana are the stone mill and the mortar and pestle mill, which were also used in Egypt 2500B.C. The modern stone mill consists of a baseplate, often fluted and a roller (Muller, 1970).

Wet milling process for the production of sorghum starch on an industrial scale involves cleaning, steeping and milling. The starch is washed and recovered from the slurry before drying. Various milling and steeping conditions are used to increase the yield and quality of starch with the aid of enzymes or sonication (Donley, 2013).

Currently, sorghum has been milled using Buhler Experimental Mill, a Great Western Gyratory Sieve and Quadrumat Brabender Sr Experimental Mill. Another milling method was employed by processing decorticated sorghum in a process using hammer mill, a Great Western Gyratory Sieve and an Alpine Pin Mill. The milling processes lead to the same particle size distribution but the milling procedure that used the Buhler and Quadrumat mill produces the highest amount of damaged starch in the flour while the hammer mill and pin mill recorded the lowest (Donley, 2013).

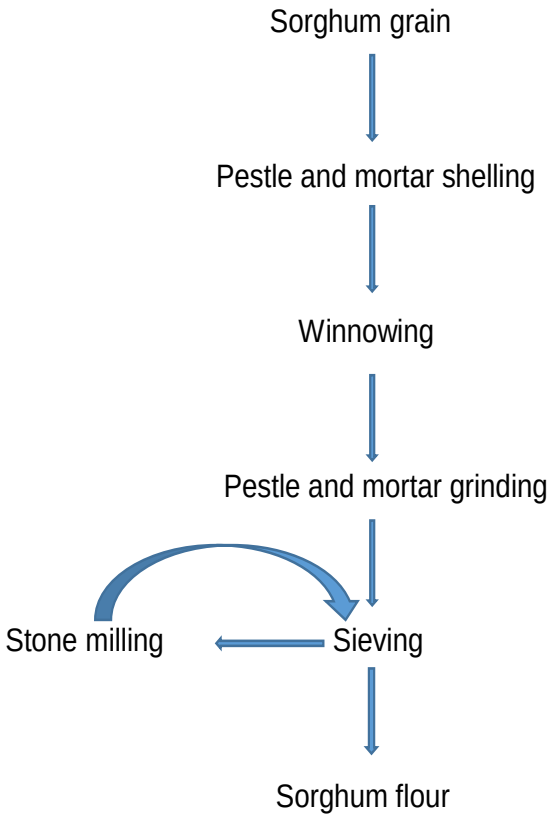


Figure 2.12 Schematic flow diagram of traditional milling of sorghum kernel into flour

The various technologies used to dehull sorghum are categorised and include roller milling equipment and peeling rolls, rice dehulling equipment, abrasive type dehuller and attrition type dehuller. The last two categories have been specifically developed for sorghum and similar grains.

Although sorghum starch has been produced on domestic and industrial scale by wet milling process, while on the laboratory scale, various milling and steeping conditions with the aid of enzymes or sonication are employed to improve the yield and the quality of resulting starch. The starch obtained through this process has coloured pigment from the pericarp and testa. In some Nigerian native foods, this colour is desirable, while it is reported by Dunford (2012) that the colouration is undesirable industrially and several techniques, such as bleaching and low cost abbreviated milling, are employed to remove the coloured pigment. However, the current sorghum milling processes are not efficient, leaving high amount of starch un-recovered in the Sorghum bran.

2.5.2 Sorghum bran for bioethanol production

Sorghum has a variety of industrial applications mainly in the animal feed sector, alcohol distilleries and starch industries. It is also used in the production of bio-industrial product like bioethanol, glucose and it also serves as source material for isolation of starch in scarcity of maize (Shewale & Pandit, 2011). Recently, sorghum has gained an interest as a new generation bioenergy crop due to its wider adaptability to varied agroclimatic conditions. Sorghum flour has also been used as a filler and extender in petroleum based adhesives. Sorghum kafirin has a great potential as biodegradable packaging materials and biopolymer production. Sorghum wax, a by-product of wet milling and ethanol production has a potential as a source of bioplastic films and coatings for foods due to its hydrophobicity (Qi, Li, Sun, & Wang, 2016).

Sorghum is being considered as an energy crop due to its merit of substituting fossil fuel with ethanol. It overcomes the challenges of first generation of biofuel, as ethanol can be produced using sorghum stalks as it contain several carbohydrates such as glucose and sucrose in its juice. In addition, sorghum bagasse also contain cellulose and hemicellulose. Thus, the fermentation of sweet sorghum biomass into bioethanol has a higher maximum theoretical yield than just glucose (0.51 g ethanol /g glucose). In addition, the grain is not in high demand in the global food market and thus has little impact on food prices and food security (Nahar, 2011).

Sorghum whole grains and stalk have been employed in ethanol and enzyme production, while the bran have been employed in biochemical production such as itaconic and succinic acid (Ahmed El-Imam, 2017) because they contain residual fermentable starch. There are no reports found on glucoamylase enzyme and bioethanol production from sorghum bran (Figure 2.12). It is thus a biomass material with yet untapped potential in this area.

Figure 2.13 Sorghum bran

2.6 Glucoamylase production

Glucoamylase is an important enzyme for starch hydrolysis due to its catalytic effect in releasing glucose from the non-reducing ends of starch (Pardeep Kumar & Satyanarayana, 2009). Glucoamylases are industrially important hydrolytic enzymes of biotechnological significance, which are used in food and pharmaceutical industries (Joshi, Pandey, & Sandhu, 1999) mainly for the production of glucose syrup, high fructose corn syrup, and alcohol.

Traditionally, filamentous fungi have produced glucoamylase, although a diverse group of microorganisms have been used to produce glucoamylase since they secrete large quantities of the enzyme extracellularly. *A. niger* and *Rhizopus oryzae* are mostly used for its commercial production (Norouzian, Scharer, & Young, 2006). The industry's preference for glucoamylase from these fungi stems from high enzyme activity at neutral pH, as well as the thermal stability.

The production of glucoamylase by fermentation for various substrates has been reported including wheat bran, green gram bran, black gram bran, corn flour, barley flour, maize bran, rice bran, rice flakes and food waste (Izmirliogiu & Demirci, 2016). Media composition and growth conditions influence glucoamylase production. Maltose and cassava flour have been reported as glucoamylase inducers, while fructose slows down its production. At low concentrations, glucose has also been reported as an inhibitor for the production of glucoamylase while some nitrogen sources such as yeast extract, ammonium sulfate, ammonium nitrate, urea, meat extract and peptone have found their application in glucoamylase production

(Pardeep Kumar & Satyanarayana, 2009; Pandey, Selvakumar, & Lakshmikuttyamma, 1994). Different fermentation procedures have also been studied for glucoamylase production under SSF and SmF.

According to Izmirliogiu and Demirci (2016), there was a substantial increase in glucoamylase and glucose production via the strain selection of *Aspergillus* and medium optimization using industrial waste potato mash and the study suggests an inexpensive medium composition for glucoamylase production. Negi and Banerjee (2009) reported an optimum increase in glucoamylase production under SSF at 37°C for 4 days using wheat bran as substrate using *A. awamori*. Another study by Zambare (2010) showed a 24% increase in glucoamylase activity through optimization of SSF media and parameters by *A. oryzae* using rice husk, wheat bran, rice bran, cotton seed powder, corn steep solid, bagasse powder, coconut oil cake and groundnut oil cake as a substrate.

The exclusive production of this enzyme (glucoamylase) was achieved by *Aspergillus niger* according to Selvakumar, Ashakumary, and Pandey (1998); Wang, Bai, & Liang, (2006), *A. oryzae* (te Biesebeke et al., 2005*)*, and *A. terreus* (Berka, Dunn-Coleman, & Ward, 1992) in enzyme industry. These strains were reported to produce substantial amount of glucoamylase in submerged (Berka et al., 1992) and solid-state fermentation (Alazard & Raimbault, 1981).

There are no reports on the production of glucoamylase using sorghum bran as substrate therefore this study has focus on glucoamylase production by *A. awamori* on SSF and SmF of sorghum bran underutilised waste material in Nigeria.

Table 2.2.7 Recent studies on glucoamylase production

Substrate used	Fungi used	Fermentation type	Enzyme yield	References
Waste bread & cakes, cafeteria waste, fruits, vegetables & potatoes	*A. awamori*	SSF/6days	108.47 U/gds	Uçkun Kiran, Trzcinski, Ng, and Liu (2014)
Pastry waste & mixed food culture	*A. awamori*	SSF/10days	76.1±6.1 U/mL	Lam, Pleissner, and Lin (2013)
Wheat bran with salt solution	*A. awamori*	SSF/4days	9157 U/gds	Negi and Banerjee (2009)
Rice bran	*A. awamori, niger, terreus, tamarii*	SmF	264.5 U/gds	Abdalwahab, Ibrahim, and Dawood (2012)
Babassu cake (kernel residue)	*A. awamori*	SSF/4days		López, Lázaro, Castilho, Freire, and Castro (2013)
cassava, potato, sorghum, maize & yam starch	*A. niger, S. cerevisae*	SmF/3days		Abu, Ado, and James (2005)
Babassu cake, castor seed, sunflower & canola cakes	*A. awamori, A. wenti & P. verrucosum*	SSF/4days	glucoamylase 29.8 U/g	Machado de Castro, Carvalho Alves, Freire, and Castilho (2010)
Kitchen waste/ Wheat bran	*A. niger*	SSF in aluminium plate/ 5days	GA 1838 U/g within 96 hours	Xiao, Qun, Ying, and Hong (2009)
Wheat bran	*A. niger*	SSF/4days	GA 1.345±0.009 IU/mL/min	Imran et al. (2010)

2.7 Problem statement

The global drive towards sustainable development with the exploitation of renewable energy (lignocellulosic biomass) to replace fossil fuel has been investigated. Although it has the potential to reduce environmental hazards caused by fossil fuel, the production cost of lignocellulosic bioethanol is still a major constraint in the replacement of fossil fuel with bioethanol. Pre-treatment and the cost of enzymes are the major limitations highlighted for the commercialisation of bioethanol production. Further investigation is required to reduce the cost of bioethanol production in terms of pre-treatment employed and the on-site production of enzyme to replace commercial enzyme. However, there will be a considerable climate benefit when fossil resources are replaced with renewable biomass in the production of value added products.

2.8 Objectives of this project

The objectives of this project were to investigate potential solutions to the challenges with bioenergy as stated in section 1.3.

The main aim was to assess the potential use of wheat straw and sorghum bran (food waste from sorghum grain) in the production of on-site crude enzyme via solid-state fermentation and submerged fermentation. Explore the conversion of sugar rich hydrolysate obtained from the hydrolysis of wheat straw and sorghum bran for bioethanol production via yeast fermentation.

The main experimental objectives of this research were

- To assess the potential use of wheat straw and sorghum bran for the production of on-site crude enzyme.
- To assess the suitability of the produced on-site crude enzyme for the hydrolysis of wheat straw and sorghum bran into a fermentable sugar.
- To explore the conversion of sugar rich hydrolysate for bioethanol production.

The sub-objectives of this research are:

- To determine the viability of *Rhizomucor variabilis* for the production of crude cellulase enzyme from wheat straw.
- To determine cellulase production from other biomass materials (*Miscanthus*, waste cloth and willow) by *Rhizomucor variabilis*.
- To assess the suitability of microwave pre-treated wheat straw for cellulase enzyme production.
- To identify optimum conditions for the production of cellulase enzyme in SSF and SmF.
- To assess the ability of mutant strain *Rhizomucor variabilis* for its ability to produce cellulase in a plate.
- To carry out enzymatic hydrolysis of wheat straw in order to obtain sugar-rich hydrolysate.
- To explore the application of wheat straw hydrolysate for the production of bioethanol.
- To assess the suitability of sorghum bran for the production of crude glucoamylase enzyme using *Aspergillus awamori*.
- To identify optimum conditions for the production of glucoamylase enzyme in SSF and SmF.
- To scale up sorghum bran enzymatic hydrolysis and attain stable yields for yeast fermentation.
- To explore the application of sorghum wastewater for the production of bioethanol during yeast fermentation.
- To assess whether various strains of yeast can use the sugar rich hydrolysates from sorghum bran to produce ethanol.

3 Material and Methods

The general materials and methods employed in this study are described in this chapter. All reagents used were analytical grade and were purchased from Fisher Scientific (Loughborough, UK) or Sigma Aldrich (Lillington, UK).

3.1 Wheat straw

Wheat straw (*Triticum aestivum L*.) was obtained from the University of Nottingham Farm (Sutton Bonington, UK). A Knife-miller was used to reduce the size of the air-dried wheat straw and passed through a 2 mm screen sieve (Fritsch, Idar-Oberstein, Germany). The wheat straw was collected and stored in an airtight bag and placed in a cool, dry place until use.

3.2 Sorghum bran

Sorghum (*Sorghum bicolor*) which is a variety of red sorghum was purchased from a local market in Ikorodu, Lagos State, Nigeria. The sorghum was subjected to three different milling processes using smart peanut butter maker (wet milling), blender (wet milling) and knife-miller dry milling. For wet milling, sorghum was steeped in water (2:5 w/v) for 3 days at room temperature and was wet milled using either a smart peanut butter maker or a blender. The milled biomass was sieved with muslin cloth to remove the starch component from the slurry and the remaining component of sorghum bran (consisting of the outer layers of the cereal grain and residual starch) was dried in an oven at 60˚C for 3 days. Figure 3.1 shows the operation procedures.

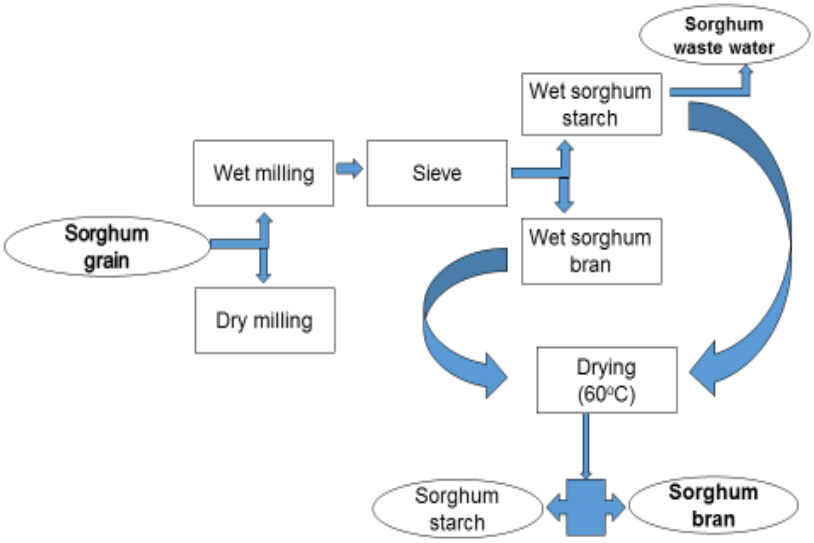


Figure 3.1 Sorghum milling process

The dry milling of sorghum was carried out using the same process described in chapter 3.1 for wheat straw.

3.3 Microorganisms used for cellulase and glucoamylase production

Aspergillus niger (N402), and *Trichoderma reesei* (R32) were obtained from the University of Nottingham, UK and *Aspergillus niger* (N403) and *Trichoderma reesei* (R33) were obtained from Hong Kong City University, *Aspergillus awamori* was obtained from from Manchester University while *Rhizomucor variabilis* (RS), and *Aspergillus niger* (CKB) were obtained from Tsinghua University, in China. Table 3.1 shows the strain list used for cellulase production.

The strains were cultured on Potato Dextrose Agar (PDA) plate and were incubated at 28˚C for 72 hours. The colonies were sub-cultured on PDA slants and incubated under the same conditions and were preserved in a refrigerator at 4˚C for short-term storage till further use.

For long-term strain preservation, 50 mL of glycerol and 50 mL of deionised water were mixed and autoclaved at 121˚C for 15 minutes and allowed to cool. 1 mL of 50% glycerol and 500 µL of fungal spore suspensionspore suspension were added into a cryogenic vial and kept at -80°C.

Table 3.1 List of strains and fermentation type used for cellulase production

Full name	Abbreviation used in this study	Origin	Used in Solid fermentation	Used in submerged fermentation
Aspergillus niger	N402	Nottingham	Yes	-
Aspergillus niger	N403	Hong Kong	Yes	-
Aspergillus niger	CKB	China	Yes	Yes
Trichoderma reesei	R32	Nottingham	Yes	-
Trichoderma reesei	R33	Hong Kong	Yes	-
Rhizomucor variabilis	*R. variabilis* RS	China	Yes	Yes

3.3.1 Suspension preparation

The strains were cultured for 3 days on PDA Petri Dishes at 28˚C in a static incubator. 10 mL of autoclaved 0.1% (w/v) tween 80 solution were added into the cultured plate to form a fungal spore suspensionspore suspension. The spores were detached using a sterile spatula and the spore suspensionspore suspension was collected in a sterile tube.

An aliquot of fungal spore suspension was sampled for spore counting using a haemocytometer under an optical 3D Microscope (Keyence, VHX 2000).

3.4 Media preparation for wheat straw solid-state fermentation

The wheat straw was firstly modified using the alkali soak method. 60 g of milled wheat straw was soaked in 600 mL of 1% NaOH at room temperature for 24 hours and washed with distilled water until the pH was neutral. The modified wheat straw

was dried in the oven at 60˚C until no further weight loss was observed. 6 g of wheat straw were put into a 500 mL Duran bottle. Moisture content was adjusted by adding 45 mL distilled water to achieve to water to wheat ratio of 7.5 to 1 (w/w). The medium was autoclaved at 121°C for 15 minutes and was cooled to ambient temperature before inoculation.

In the investigation of the impact of starch, 0.1-0.5% (w/w) starch was added. In the investigation of the impact of minerals, the following mineral solution was prepared: K_2HPO_4- 5 g/L, NH_4NO_3- 3 g/L, $(NH_4)_2HPO_4$- 3 g/L, $MgSO_4$- 0.24 g/L & NaCl- 0.5 g/L. Then, the mineral solution was used to replace the distilled water in the adjustment of the moisture content before autoclave.

3.4.1 Scanning Electron Microscope (SEM)

The scanning electron microscope (SEM) was used to investigate the effect of alkali pre-treatment on the structure of the modified wheat straw.

3.4.1.1 Sample Preparation

The samples (modified and non-modified wheat straw) were placed on a metal sampler with sticky conducting tape and was fixed into a mount stem to the mounting platform. The sample was coated in gold using a Hummer 6.2 sputter deposition system at sputter deposition rate of about 20 Angstroms per minute. Sputter deposition was carried out for about 1 minute to have enough metal to conduct the SEM electrons and in order to prevent any alteration in the topography of the sample.

3.4.1.2 Sample Loading

The coated sample was mounted on the SEM sample mount and vented for loading. The stigmation and focus were used to sharpen and increase the magnification.

3.4.2 Solid-state fungal fermentation

The solid-state fermentation was carried out in Petri Dishes. The fermentation was started by adding different amount of spore concentrations (e.g. $1x10^6$ spores/g and $5x10^6$ spores/g). After adding spores, the mesh was mixed and approximately 2 g of the biomass were separated into a Petri Dish. The inoculated Petri Dishes were

incubated at 28˚C in a static incubator. Samples were taken for analysis every 24 hours. All experiments were carried out in triplicate where three Petri Dishes were taken out for analysis every 24 hours and are been analysed separately.

3.5 Wheat straw submerged fermentation

Submerged fermentation was carried out for the cellulase production in shake flasks and in a 2-L fermenter (electrolab FerMac 360) for larger scale enzyme production.

3.5.1 Submerged fermentation in shake flask for cellulase production

Alkali modified wheat straw was autoclaved with mineral solution 10g/L (w/v) at 121˚C for 15 minutes. *R. variabilis* RS spore suspension was added at inoculation ratio of 1×10^7 spores/g and submerged fermentation was carried out in a 250 mL Duran bottle in a shaking incubator (SciQuip Incu-Shake FL24-1R) at 160 rpm and temperature of 28˚C. Samples were taken for 7 days to analyse the cellulase profile. The samples obtained are analysed individually in triplicate.

3.5.2 Larger scale cellulase production in 2 L fermenter

For the investigation of fungal biomass production, the following experiments were carried out. The fungi were cultured in a 2-L fermenter electrolab FerMac 360 (Figure 3.5) using glucose (20 g/L) and yeast extract (10 g/L), autoclaved at 121˚C for 15 minutes as the fermenter media. 5 mL of fungal spore suspensionspore suspension (spore concentration $1x10^7$ spores/mL) and 1 mL of autoclave silicon antifoam solution (10% w/w) were added to the fermenter medium. The fermentation was carried out at 28˚C, pH at 6.0, 300 rpm and air aerated at 1.0 L/min. 20 mL of sample were taken three times a day and kept in the fridge and were analysed for wet biomass weight (as described in chapter 3.5.6).

For the cellulase production, 10 g/L wheat straw with the addition of the mineral solution (as described in chapter 3.4) were autoclaved at 121˚C for 15 minutes and allowed to cool before inoculation with different fungal strains. The fermentation was carried out at 28˚C, pH at 6.0, 300 rpm and aerated at 1.0 L/min. 20 mL of sample were taken and kept in the fridge and were analysed in triplicate for cellulase activity.

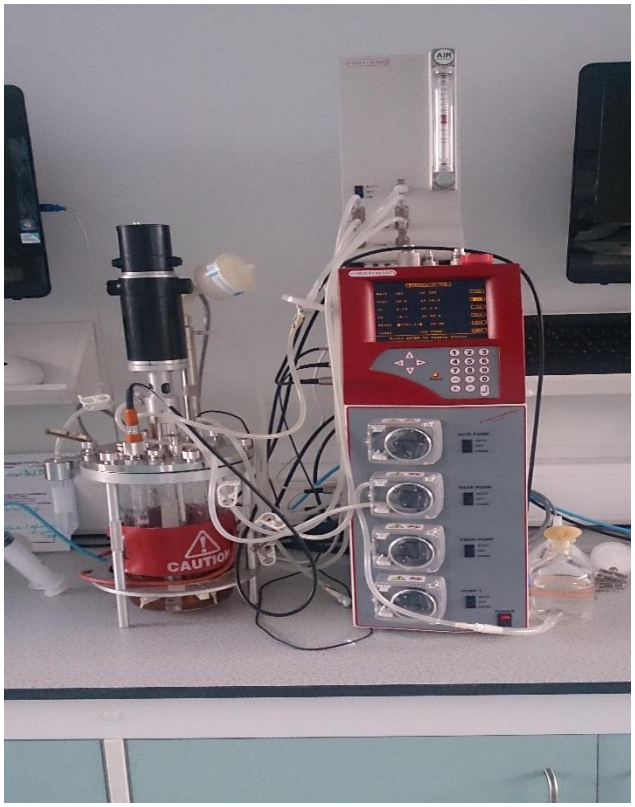


Figure 3.2 2-L electrolab FerMac 360 fermenter

3.5.3 Estimation of wet weight cell mass (WWCM)

The fungi wet weight was used as a measurement of the fungal growth. Wet cell mass was determined by filtering 20 mL of the cultured broth through pre-weighed Whatman filter paper No. 1 until no water passes through the filter paper. The wet cell mass was weighed and calculated as g/mL by subtracting the initial weight of the filter paper from the final weight.

3.6 Enzyme extraction

The fermented wheat straw was transferred into a blender (cookworks glass blender) and 30 mL of 0.05 M citric acid buffer pH 4.8 were added per Petri Dish. The mixture was blended for 10 seconds. The mixture was transferred into a separate beaker and it was stirred using a magnetic stirrer at 300 rpm for 20 minutes in an ice water bath. The mixture was centrifuged at 5,000 g for 5 minutes using mini spin

Eppendorf centrifuge. The clear supernatant (fungal extract) was used as the crude enzyme. The fungal extract was kept at 4˚C until used.

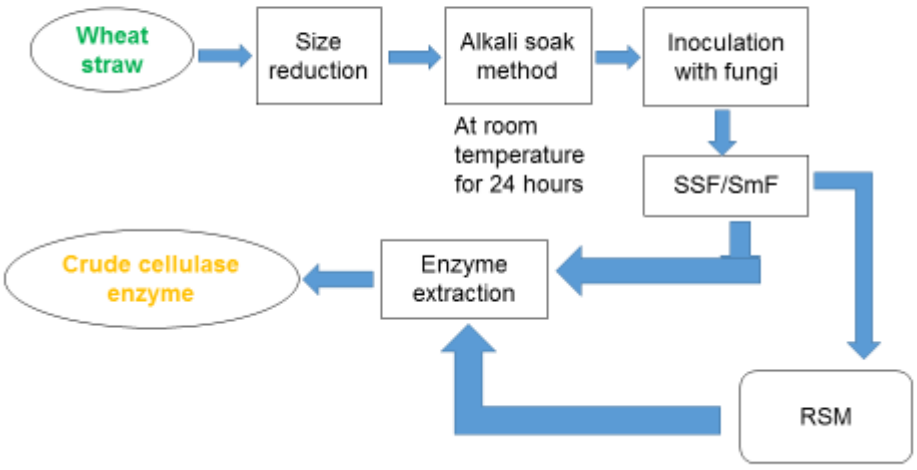


Figure 3.3 Schematic diagram of cellulase production from wheat straw under SSF and SmF

3.7 Cellulase activity assays

3.7.1 Filter paper activity

Cellulase activity was determined as filter paper units (FPU) according to NREL Laboratory Analytical Procedure (Adney & Baker, 1996). Briefly, 1 mL of 0.05 M citric acid buffer, and 0.5 mL of fungal extract were mixed in a test tube containing one Whatman No. 1 filter paper stripe (1.0cm x 6.0cm). The solution mixtures were incubated at 50˚C for 60 minutes. 3 mL of Dinitrosalicylic (DNS) was added to the mixture to terminate the enzyme reaction immediately, and the mixture was boiled for 5 minutes in a vigorous boiling water. After boiling, it was transferred to a cold ice-water bath. The cooled mixture was colorimetric measured at 540 nm (UV-Vis Spectrophotometer). The procedures for preparing the citric acid buffer and DNS solution were described in Adney & Baker (1996). The filter paper activity (U/mL) was calculated using the following equation (Adney & Baker, 1996).

$$\textit{Filter paper activity, } \frac{U}{mL} = \frac{0.37}{\textit{concentration of enzyme that release } 2.0mg \textit{ glucose}} \quad \text{Equation 3.1}$$

Where 0.37 is a constant from equation 3.1

Then it was converted to U per gram of dry weight wheat straw using the following equation.

$$Filter\ paper\ activity\ \frac{U}{g} = \frac{Filter\ paper\ activity\ \left(\frac{u}{mL}\right)\ X\ Total\ volume\ of\ the\ fungal\ extract\ (mL)}{Dry\ weight\ of\ the\ wheat\ straw\ used\ in\ SSF\ (g)}$$

Equation 3.2

3.8 Glucosamine analysis

Glucosamine is a monomer of chitin and chitosan. It has been used as an indirect method for measuring fungal growth during fermentation. Glucosamine content was determined by a colorimetric method (Elson & Morgan, 1993).

3.8.1 Reagent preparation

- 4% (v/v) acetyl acetone reagent

4 mL of acetyl acetone was added into 100 mL of 1.25 N Na_2CO_3

- Ehrlich reagent

1.6 g of N-N dimethyl-p-aminobenzaldehyde was added to 60 mL solution containing 50:50 (v/v) of absolute ethanol: concentrated HCl.

3.8.2 Sample preparation

0.5 g dry weight of sample was hydrolysed in 2mL of concentrated sulphuric acid (98%) at room temperature for 24 hours. The mixture was diluted to 1 N sulphuric acid solution (18.3-time dilution by volume) then autoclaved at 121˚C for 15 minutes. Then it was neutralized with NaOH to pH 7 and further diluted with water to final volume of 100mL.

3.8.3 Glucosamine measurement

The glucosamine concentration was analysed based on the method reported by Sakurai, Lee, and Shiota (1977). The glucosamine was determined as follows: 1 mL of the above sample solution was transferred into a test tube. 1 mL acetyl acetone

reagent (4% (v/v) acetyl acetone in 1.25 N Na_2CO_3) was added then incubated at 100˚C for 20 minutes. After cooling to room temperature, 6 mL of absolute ethanol were added and then mixed gently. 1 mL of Ehrlich reagent was added. The mix was incubated at 65˚C for 10 minutes and the absorbance value was determined at 530 nm (UV-Vis Spectrophotometer).

3.9 Strain mutation

The RS fungal strain was mutated using the following conditions:

(1) Ultraviolet light (UV) only

(2) Microwave treatment only

(3) Combined Ultraviolet light and Microwave.

for 30 seconds at the distance of 20 cm above the plate, or heated in a microwave at 700 W for 10, 15 and 20 seconds or a combination of both microwave and UV at 10 & 30 seconds, 15 & 30 seconds and 20 & 30 seconds respectively after inoculation. The treated RS strains were cultured on PDA Petri Dishes for 48 hours at 28˚C. Then the single colonies were streaked on the cellulase selection Agar Plate in order to determine their ability to produce cellulase.

3.9.1 Cellulase selection agar plate

The cellulase selection agar medium consisted of 15 g/L carboxymethyl cellulose (CMC-Na), 5 g/L sodium chloride (NaCl), 0.2 g/L magnesium sulfate ($MgSO_4$), 1.0 g/L potassium phosphate monobasic (KH_2PO_4), 18 g/L Agar, 10 g/L peptone, and 5 g/L yeast extract and was adjusted to pH 7.2.

The medium was autoclaved at 121˚C for 15 minutes and was poured into Petri Dishes. The *R. variabilis* RS strain was stroked on the Petri Dish at three different places and incubated at 28˚C for 1 day. Cellulase producing mutant strains were screened by staining using 1 g/L Congo red for 20 minutes and washed with 1 mol/L NaCl.

The zone and strain diameter were measured with a ruler in cm to determine their ability after mutation for cellulase enzyme production. The mutant strain showing

largest zone of decolourization after staining was selected as positive cellulolytic *R. variabilis* RS mutant strain.

3.10 Total Starch Content of Sorghum Bran

The total starch content of the three sorghum bran samples were determined according to AACC Method 76-13.01 (Megazyme, 2014). 0.1 g of dried sorghum bran was weighed into a glass test tube in duplicate and 0.2 mL of aqueous ethanol (80% v/v) were added to wet the sample and aid dispersion. The mixture was stirred on a vortex mixer for 30 seconds and 3 mL of thermostable α-amylase was added immediately. The test tubes were then incubated in a boiling water bath for 6 minutes. Then, 0.1 mL of amyloglucosidase was added to the test tube, stirred on a vortex mixer for 30 seconds and incubated at 50˚C for 30 minutes. The entire content was transferred into 100 mL volumetric flask and it was adjusted to volume with distilled water. An aliquot of the solution was centrifuged at 3,000 g for 10 minutes and the clear, undiluted filtrate was used for the following assay. Triplicate aliquots (0.1 mL) were transferred into glass test tubes and 3 mL of GOPOD Reagent was added to each test tube and incubated at 50˚C for 20 minutes. The absorbance of the sample and the D-glucose control were read at 510 nm against the reagent blank.

D-glucose control consists of 0.1 mL of D-glucose standard solution (1 mg/mL) and 3 mL of GOPOD Reagent while reagent blank solution consist of 0.1 mL of water and 3 mL of GOPOD Reagent.

The total starch was calculated using this formula;

$$\textit{Starch \%} = \frac{\Delta A \times F \times FV}{0.1} \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180} \qquad \text{Equation 3.3}$$

$$\%\ \text{Starch} = \frac{\Delta A \times F}{W} \times FV \times 0.9 \qquad \text{Equation 3.4}$$

Where:

ΔA = absorbance (reaction) read against the blank.

$$F = \frac{100(\mu g\ of\ D\text{-}glucose)}{Absorbance\ for\ 100\mu g\ of\ glucose\ (conversion\ from\ absorbance\ to\ \mu g)} \qquad \text{Equation 3.5}$$

(conversion from absorbance to µg)

FV = Final volume (equals 100 mL)

0.1 = Volume of sample analysed

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

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

W = The weight in mg of the sample analysed

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

3.11 Sorghum bran solid-state fermentation

Spore concentrations of $1x10^7$ spores/g were used for solid-state fermentation of the substrate (6 g of sorghum bran). The added spores were mixed using a spatula and approximately 2 g of the biomass were separated into a Petri Dish. The inoculated Petri-dishes were incubated at 28˚C in a static incubator. Samples were taken for analysis every 24 hours. All experiments were carried out in triplicate. Three Petri Dishes were taken out for analysis every 24 hours and are been analysed separately.

3.12 Sorghum bran submerged fermentation

Submerged fermentation was carried out for the production of glucoamylase. It was carried out in shake flasks for preliminary assessment and also in 2-L fermenters (electrolab FerMac 360) for larger scale enzyme production.

3.12.1 Submerged Fermentation in Shake Flask

The sorghum bran concentration used in the fermentation was 4% (w/v). A 250 mL shaking flask was used in most experiments with a working volume of 100 mL unless otherwise specified. Several drops of silicon antifoam (0.002% v/v) were added to the complex medium in order to prevent foaming. Unless specified, no other nutrients or chemicals were added into the fermentation media. The media were sterilised at 121˚C for 15 minutes and allowed to cool down before adding *A.*

awamori at an inoculation ratio of $1x10^7$ spores/g. The mixture was fermented under submerged fermentation in a shaking incubator (SciQuip Incu-Shake FL24-1R) at 28˚C and 200 rpm. Glucoamylase production profile was investigated using different conditions like different time profile (up to 15 days), pH range (3 - 7), temperature (26 - 30), substrate concentration (2 – 10 w/v), aeration ratio (50 – 250 mL in 500 mL bottle), yeast extract (0 – 10 g/L), minerals and different inoculation ratio. The variation of fermentation parameters is described in Chapter 6.2 along with the experimental schedules. All SmFs were carried out in duplicate. The samples obtained are analysed individually in duplicate.

3.12.2 Submerged fermentation for glucoamylase production in 2 L fermenter

Larger scale glucoamylase production was carried out using the best condition obtained from the response surface methodology result in a working volume of 1000 mL.

The fungi strain of *A. awamori* was cultured in 250 mL shaking flask containing 50 mL inoculation medium. The inoculation medium contains 2 g of glucose and 0.5 g of yeast extract. The fermentation was carried out at 28˚C, 200 rpm for 3 days. The fermentation medium contains 10% sorghum bran, 2.5% yeast extract, 200 mL deionised water in a 500 mL shake flask, which were autoclaved separately. 800 mL deionised water in the 2 L bench fermenter was autoclaved using a large autoclave at 121˚C for 20 minutes. 1 mL of sterilised silicon antifoam (0.02% v/v) was added to the fermenter media. The fermentation was carried out at 28˚C, 500 rpm and an aeration rate at 1.0 L/min. The pH was controlled to 6.0 by adding 2 M NaOH solution or 2 M HCl solution. 10 mL of sample were taken and centrifuged at 3,000 g for 5 minutes. The supernatant was considered as the crude enzyme, which was analysed for glucoamylase activity as described in chapter 3.13.

3.13 Glucoamylase Enzyme

Glucoamylase activity was measured using Bernfeld method (Bernfeld, 1955). A reaction mixture containing 0.9 mL of 0.05 mM citrate buffer (pH5), 1.0 mL starch solution (1%, w/v) and 0.1 mL of crude enzyme was incubated at 50˚C for 20 minutes. The released reducing sugars were measured by adding 3 mL of 3, 5-

dinitrosalicyclic acid (DNSA) reagent according to Miller (1959) to the incubated mixture. The reaction mixture was heated in a vigorously boiling water for 5 minutes and was allowed to cool. Absorbance was measured at 540 nm using pure glucose as a standard.

Glucoamylase activity unit (U) was expressed as the amount of enzyme releasing one µmole of glucose equivalent per minute under assay condition and enzyme activity was express in terms of units per mL (U/mL).

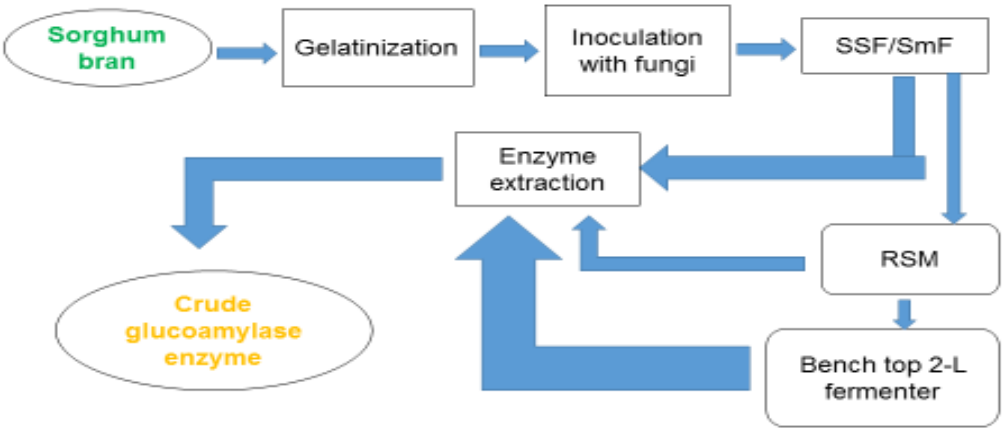


Figure 3.4 Schematic diagram of glucoamylase production from sorghum bran

3.14 Fermentation improvement using response surface methodology

Response surface methodology (RSM) was used to explore the relationships between different variables with the aim of optimising cellulase and glucoamylase enzyme using the Design Expert Software version 11.

RSM for the optimisation of cellulase activity in SSF and SmF was carried out using the central composite design under four numeric factors. In SmF, the four numeric factors of substrate concentration, tryptone concentration, pH and temperature with 30 runs of experiment were carried out with 6 runs in central points. Design runs are shown in Table 5.2. In SSF, the four numeric factors of moisture content, tryptone concentration, pH and inoculation rate with 30 runs of experiment were carried out with 6 central points. Design runs are shown in Table 5.3.

For glucoamylase activity, the RSM was carried out using central composite design in SmF under four numeric factors of pH, substrate concentration, aeration ratio and yeast extract concentration with 30 runs of experiment were carried out with the 6 runs in central points the samples were prepared and incubated at 28˚C for 3 days. See design runs in Table 6.5. In SSF, four numeric factors of moisture content, temperature, pH and yeast extract concentration with 30 runs of experiment were carried out with 6 central points. Design runs are shown in Table 6.6.

3.15 Enzymatic Hydrolysis

Both enzymatic hydrolysis of modified wheat straw using cellulase and enzymatic hydrolysis of sorghum bran using glucoamylase were carried out.

3.15.1 Enzymatic Hydrolysis of Modified Wheat Straw

The hydrolysis of alkali soaked modified wheat straw consisted 0.1 g of biomass, 3.5 mL of citrate buffer, 2.0 mL of fungal enzyme (cellulase at a ratio of 22 FPU/g). The hydrolysis of microwave modified wheat straw consisted 0.1 g of biomass, 3.5 mL of citrate buffer, 2.0 mL. crude enzyme (1:30 D1 enzyme at a ratio of 3.56 FPU/g). The hydrolysis of four different biomass samples consisted 0.1g of substrate, 3.5 mL of citrate buffer (pH 4.8), 2.0 mL of crude enzyme solution. The combination of biomass and crude enzyme is shown in Table 4.2. The experiment was carried out in duplicate and samples obtained from each duplicate were analysed separately in duplicate as well.

The mixture was heated in a water bath at 50˚C for 10 minutes and was transferred into a shaking incubator (SciQuip Incu-Shake MIDI) agitating at 200 rpm at temperature of 50˚C for 72 hours. 1 mL of each samples was taken at 0, 24 and 72 hours and stored at -20˚C until analysis.

The samples were centrifuged at 10,000 g for 3 minutes and supernatants were collected and filtered through 0.2 µm syringe filter into an auto vial sample prior to High Performance Anion-Exchange Chromatograph (HPAEC-PAD) analysis.

3.15.2 Enzymatic Hydrolysis of Sorghum Bran

The hydrolysis of sorghum bran was carried out by gelatinizing a mixture of 4 g sorghum bran in 50 mL of deionised water in a boiling water bath at 100˚C for 20 minutes in a 250 mL conical flask. The agitation was carried out using a glass rod to mix for a half minute in every 5 minutes. After gelatinisation, the substrate was cooled to 55˚C and various amounts of crude glucoamylase enzyme solution or commercial enzymes (glucoamylase and α-amylase from Megazyme@) were added into the reactor. The hydrolysis was carried out in a shaking incubator (SciQuip Incu-Shake MIDI), 200 rpm at 55˚C for 48 hours. The experiment was carried out in duplicate and samples obtained from each duplicate were analysed separately in duplicate as well.

The hydrolysed samples were filtered through a 0.2 µm syringe filter into an auto vial sampler for HPAEC-PAD analysis. Other hydrolysis conditions used are given in detail in chapter 6.5.

3.16 Hydrolysate Analysis

3.16.1 Sugar Standard Solution Preparation

Mixed sugar standard solution containing analytical grade glucose, galactose, mannose, xylose and fructose was prepared in different concentrations to get a 7-point calibration curve. The following concentrations were used: 5, 10, 20, 40, 60, 80 and 100 ppm.

3.16.2 Sugar analysis

The amounts of sugars were quantified by HPAEC-PAD. The sample or standard was transferred into a 1.5 mL agilent auto sampler vial. The monosaccharides were analysed using Dionex ICS-3000 Reagent-FreeTM Ion Chromatography equipped with Dionex ICS-3000 system, electrochemical detection using ED 1 and computer controller. The CarboPacTM PA 20 column (3 x 150 mm/; Dionex, USA) was used and the mobile phase was 10 mM NaoH with a flow rate of 0.3 mL/min. The injection volume was 25 µL and the column temperature was 30˚C. The sample analysis was completed in 30 minutes.

Sugar concentration of samples was calculated by interpolation on the standard curve.

Saccharification yield was calculated as:

$$Saccharification\ yield\ (\%) = \frac{Sugar\ released\ (g)\ X\ Volume\ X\ Dilution\ factor}{Initial\ carbohydratye\ content\ (g) X\ Sample\ mass} X\ 100$$ Equation 3.6

3.17 Ethanol fermentation

The purpose of this experiment was to examine the ethanol production from different yeast strains using sorghum bran hydrolysate as the substrate. Yeast peptone dextrose (YPD) medium was used as a control.

3.17.1 Media

Yeast peptone dextrose (YPD) broth was prepared by dissolving 20 g of bacto peptone, 10 g of yeast extract and 20 g of glucose in distilled water and the total volume was adjusted to 1000 mL and was autoclaved at 121˚C for 15 minutes.

The glucose concentration of the hydrolysate obtained from the enzymatic hydrolysis was measured by HPAEC-PAD. The hydrolysate was centrifuged using Allegra X-12 centrifuge at 4000 g for 10 minutes at 20˚C. The cream hydrolysate solution was decanted and stored at 4˚C until use.

3.17.2 Ethanol fermentation microorganisms

Saccharomyces cerevisiae NCYC 2592 was obtained from University of Nottingham, *Candida membranifaciens* M2 was isolated from a mussel from Skegness, UK, *Wickerhamomyces anomalus* M15 was isolated from a dried seaweed sample from Skegness, UK by Darren Greetham in the group and *Saccharomyces cerevisiae* AZ65 was isolated from seawater in Egypt by Abdelrahman Saleh zaky. All the yeast strains were stored at 4˚C and used as a working stock in further experiments.

3.17.3 Inoculum development

5 mL of yeast strain was added to 200 mL of YPD media in a 250 mL Duran flask. The fermentation was carried out at 28˚C for 3 days at 200 rpm. The cultured yeast strains were centrifuged with 50 mL centrifuge tube using the Allegra X-12 centrifuge at 4000 g for 5 minutes at 20˚C. The supernatant was discarded and the cream yeast was made up to 5 mL with distilled water and mixed vigorously with vortex mixer. The optical density of inoculum was measured at OD_{600} using Jenway visible spectrophotometer. This suspension was used as inoculum, after dilution.

3.17.4 Bioethanol fermentation process

Bioethanol fermentation was carried out in a mini fermenter vessel (Figure 3.6). 100 mL of the sterilised hydrolysate was aseptically transferred to pre-sterilised mini fermenter vessels containing magnetic stirrers. The inoculation ratio was $1x10^7$ cells/mL. The bottles were capped with sterile butyl plugs and covered with metal crimp caps with a 5.5 mm hole. The metal caps were held in place with a handheld vial crimper to ensure micro-aerobic conditions. A hypodermic needle was pushed through the butyl plug to enable the release of the carbon dioxide produced in the sealed system, through the slit in the rubber tubing. The mini fermentation vessels were then placed on a magnetic stirrer at 180 rpm and incubated at room temperature.

The fermentation vessels were weighed at regular intervals and the weight loss from carbon dioxide formation was recorded until constant weight was obtained and the experiment was terminated. The fermentation samples were collected and centrifuged (Allegra X-12) at 4000 g for 5 minutes and the supernatant was filtered through 0.25 µm Agilent syringe filter and kept at 4˚C until when analysed. The experiment was carried out in duplicate and samples obtained from each duplicate were analysed separately in duplicate as well.

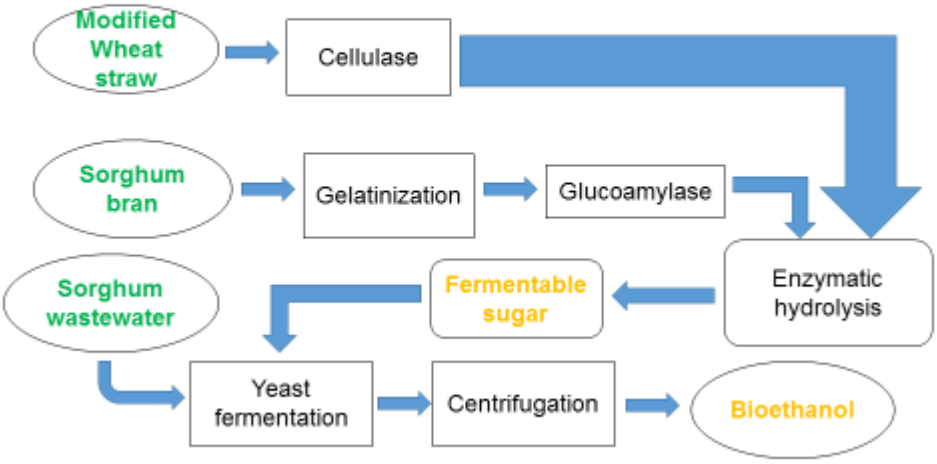


Figure 3.5 Schematic diagram of bioethanol production from wheat straw, sorghum bran and sorghum waste water

Figure 3.6 Mini fermenter vessels for bioethanol production process

3.17.5 Ethanol measurement using gas chromatography

Ethanol concentration was analysed by gas chromatography (Varian CP 3900) using PP20 column with a flame ionization detector (FID), fuel electronic flow control (EFC) of carrier and detector gases, compass CDS workstation. The column temperature was maintained at 80˚C and the carrier gases were nitrogen at 60 psi, hydrogen at 40 psi, air at 60 psi and make up of flow at 30 mL/min, 30 mL/min and 300 mL/min

respectively. The run time was 5.0 minutes as ethanol has a retention time of about 2.3 minutes. The injector temperature was at 200˚C and the detector temperature was at 280˚C. The flow rate was at 1.0 mL/min, split ratio 1/20, and the sample quantity of 1µL.

3.18 Statistical Analysis

The Microsoft excel (2013) was used to calculate the results obtained from all the experiments such as the standard deviation. Either a bar graph or scatter with straight line graph were plotted with error bars indicating the standard deviation. SPSS (statistics 22.0) was used to determine if there were any significant difference using the one way analysis of variance (ANOVA) at 95% confidence levels.

4 Cellulase Production in Solid-state and Submerged State Fungal Fermentation

In this chapter, various strategies have been investigated to improve the cellulase production using wheat straw as the substrate via SSF and SmF. The wheat straw was firstly subjected to different modification to improve accessibility of microorganisms. The modified wheat straw was inoculated with different fungal strains, including *Aspergillus niger, Trichoderma reesei and Rhizomucor variabilis*. The fungal extract was subsequently used as a crude cellulase solution for the hydrolysis of wheat straw.

4.1 Cellulase production in solid-state fermentation

Solid-state fungal fermentation was firstly investigated for the production of cellulase, using wheat straw as the substrate. The detailed operation procedure is described in chapter 3.4.

4.1.1 Impact of Alkali Soaking Modification on Cellulase Production

Alkali soaking modification was carried out using 1% NaOH at room temperature for 24 hours. Talebnia et al. (2010b) also reported an alkali pre-treatment method using calcium hydroxide on bagasse and wheat straw at a lower temperature range of 50 - 65˚C for 24 hours. Alkali pre-treatments were considered effective in a study carried out by Chang, Nagwani, and Holtzapple (1998). The modified and non-modified wheat straw microstructures were compared using the scanning electron microscope (SEM) (Figure 4.1 and Figure 4.2).

Figure 4.1 shows the bands of lignin in a cyclic form binding the cellulose in the non-modified wheat straw highlighted with a red circle. Figure 4.2 shows that the bands line of lignin were destroyed with the aid of alkali soaking reflecting a clearer microstructure of the cellulose in the wheat straw. This finding indicated that alkali modification of wheat straw after 24 hours of soaking at room temperature also had an effective role in the disruption of the cellulosic material to enhance enzymatic digestibility in order to achieve higher sugar yields.

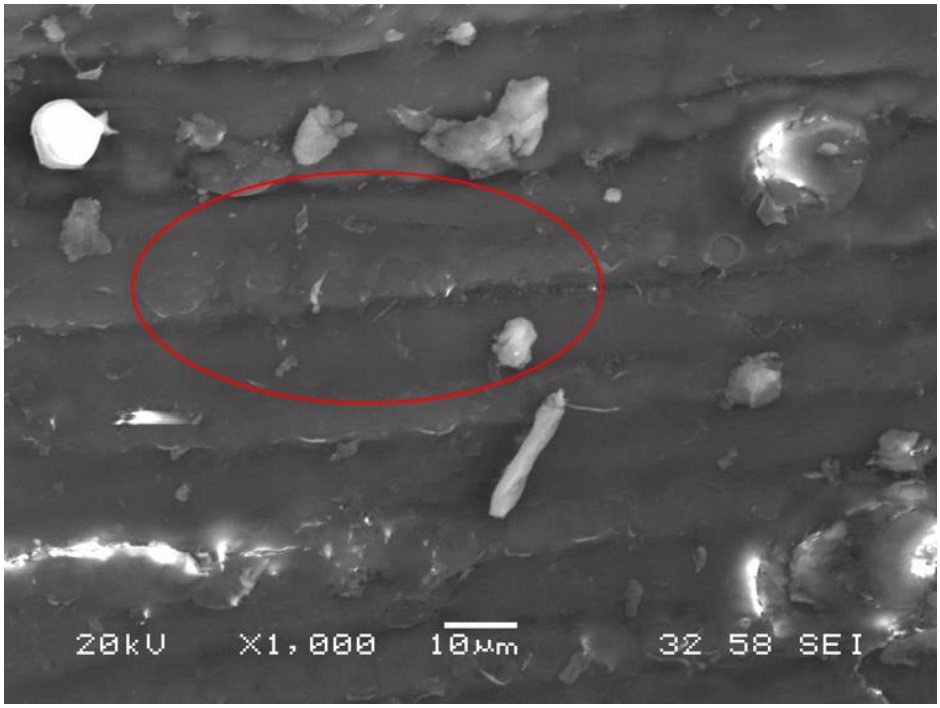


Figure 4.1 Non-modified wheat straw

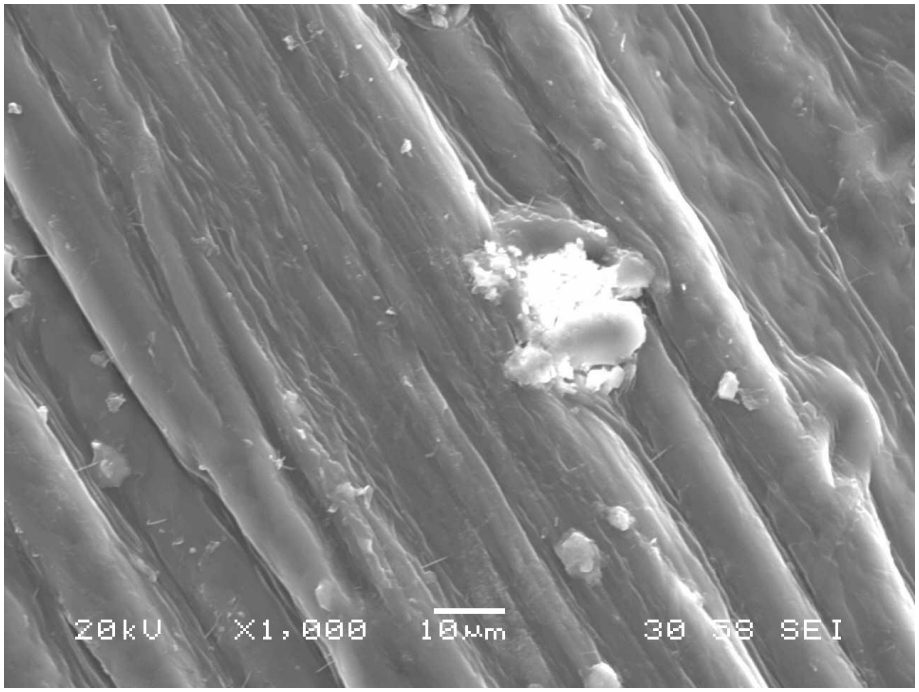


Figure 4.2 Modified wheat straw

The impact of alkali soak modification on wheat straw using *A. niger* using SSF for cellulase production was examined. The modified wheat straw showed an increase in cellulase production than the non-modified wheat straw (Figure 4.3). This supports the results of Gamarra et al. (2010); Saha, Iten, Cotta, and Wu (2005); Smuga-Kogut et al. (2015); Talebnia, Karakashev, and Angelidaki (2010a) who showed that

modification of wheat straw improved the accessibility of cellulose for effective enzymatic depolymerisation and other downstream processes.

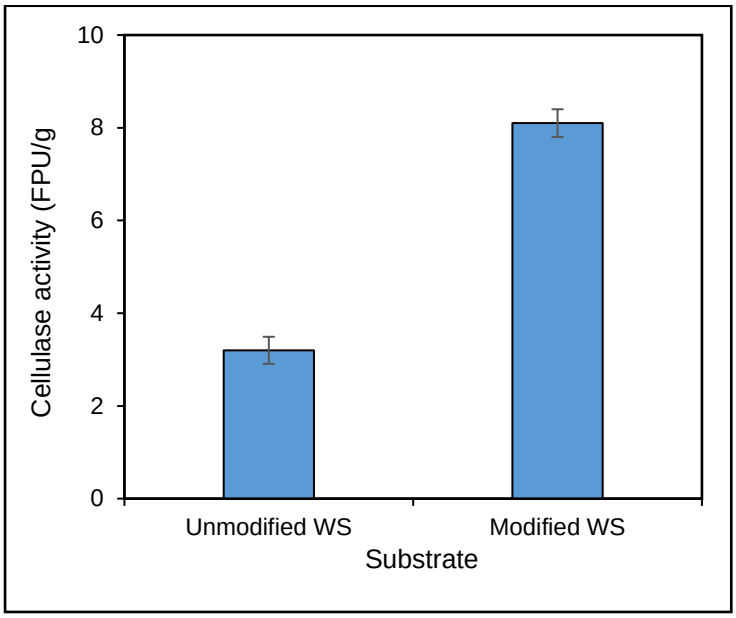


Figure 4.3 Impact of alkali soak modification on wheat straw (WS). Error bars show the standard deviation of three replicates. Moisture content (7.5:1 v/w), solid-state fermentation, 24 hours, *A. niger* N402.

4.1.2 The Impact of Starch Addition on Cellulase Production

The impact of starch addition on cellulase activities was investigated using alkali modified wheat straw at a water to wheat ratio of 7.5:1 (v/w) in SSF with the addition of starch at concentrations from 0 to 0.5% (w/w). The experiments were carried out in triplicate for 24 hours at 28˚C. The impact of starch addition on cellulase production had already been investigated using acid modified wheat straw by Pensupa, Jin, Kokoiski, Archer, and Du (2013) but the best concentration of starch was not clear. Therefore, in this study, a whole range of starch addition with a small step increase was investigated. As shown in Figure 4.4, an increase in cellulase activity correlated with an increase in starch concentration. This is probably due to the increased fungal growth during SSF that leads to an increase in cellulase activity. Liang et al. (2012) also reported an increase in cellulase production with starch addition in SSF using *Aspergillus sp* on rice grass. Table 4.1 below shows the increase in cellulase activity with addition of starch on lignocellulosic feedstocks.

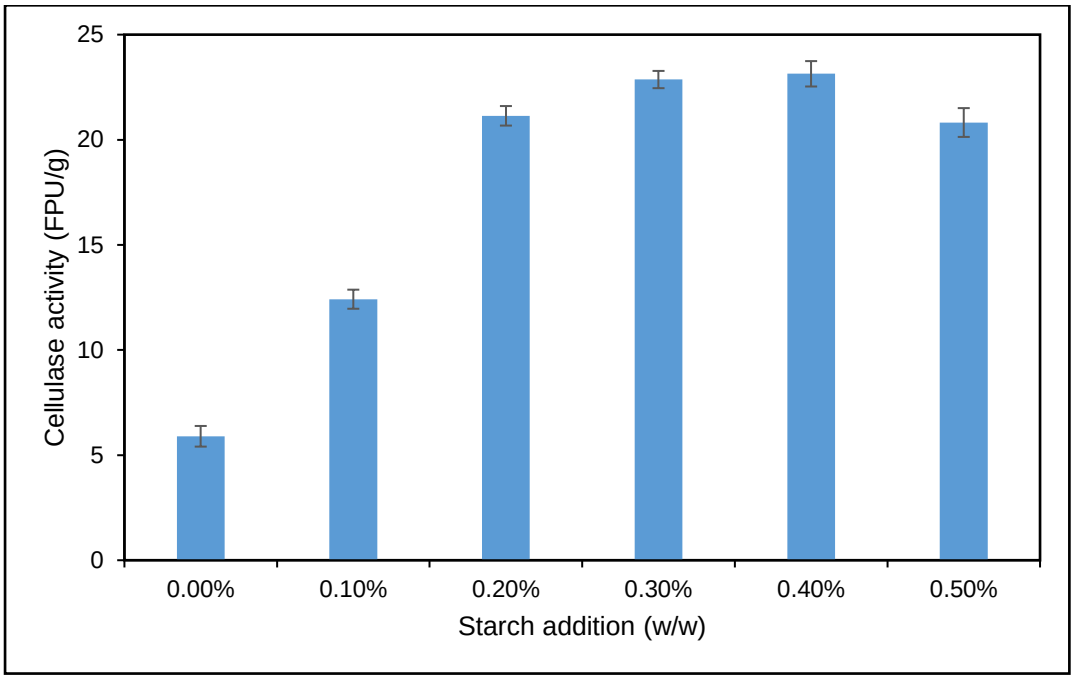


Figure 4.4 Impact of starch on cellulase activity using *A. niger* N402. Moisture content (7.5:1 v/w), solid-state fermentation, 24 hours. Error bars show the standard deviation of three replicates.

Table 4.1 Impact of starch addition on cellulase production from lignocellulosic feedstocks

			Cellulase production		
Strain	Substrate	Culture condition	without starch	with starch	Reference
A. niger N402	Wheat straw	89.5% moisture content, 28˚C, 24 hours	8.1 FPU/g	23.14 FPU/g	This study
A. niger N402	Wheat straw	85.1% - 91.1 % moisture content, 28˚C, 7 days	5.57 U/g	Reported to increase cellulase production and shortens culture time	Pensupa et al. (2013)
Aspergillus sp	Rice grass	75% moisture content, initial pH 6, 30˚C, 5 days, SSF	0.55 FPU/g	0.87 FPU/g	Liang et al. (2012)

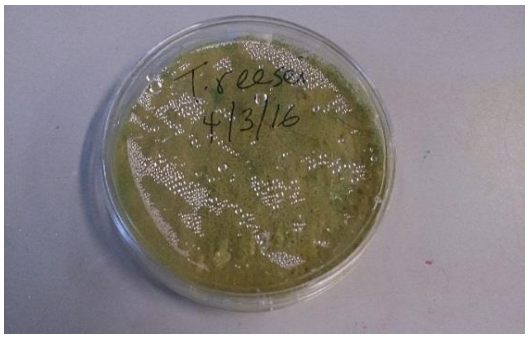
4.1.3 Cellulase Production using different fungi strains

Several fungal strains were selected for evaluation of cellulase production in SSF. The selection of these strains was based on their performance in previous publications. A newly isolated cellulase producing strain was also investigated in order to discover specific features of interest for cellulase production (e.g the ability to generate high cellulase production).

Two fungal strains *Aspergillus nig*er N402 and *Trichoderma reesei* R32 (Figure 4.5) obtained from the University of Nottingham. These were investigated initially for cellulase production using SSF and then compared with the fungal strains

Aspergillus niger N403 and *Trichoderma reesei* R33 which were obtained from City University in Hong Kong.

(A) *A. niger* N402



(B) *T. reesei* R32

Figure 4.5 Fungal growth on PDA plates. A, *A. niger* N402 B, *T. reesei* R32

As an increase in cellulase production by the addition of starch was observed in Figure 4.4, 0.2% (w/w) starch addition was selected in the subsequent experiments to examine the cellulase production from the four fungal strains. There was no significant increase in cellulase activity when over 0.2% (w/w) of starch addition was used up to 0.4% (w/w). Starch was added alongside with the fungal strains of ~10 million spores/g substrate on the alkali modified wheat straw and the cellulase production was examined under SSF at 28˚C, 7.5:1 (v/w) moisture content cultured for 5 days. All the experiments were carried out in triplicate. The details of the four fungal strains used in this study are described in chapter 3.3.

Figure 4.6 shows an increase in cellulase activity as the culture time increased for all fungal strains. In day 1, *A. niger* N402 had the highest cellulase activity (7.0 FPU/g) while in days 3 and 5 *T. reesei* R32 had the highest cellulase activity (14.0 & 20.0 FPU/g) respectively. In addition, the three strains, (*A. niger* N402, *A. niger* N403 and *T. reesei* R33) produced similar cellulase activity on day 5. The increase in cellulase activity correlated with the increase in fungi growth as the culture time increased

resulting in the fungi strain penetrating the wheat straw more and producing cellulase for the consumption of cellulose component in the wheat straw.

T. reesei R32 which initially had a slightly slow cellulase generating power (in day one) when compared with *T. reesei* R33 and *A. niger* N402. This may be due to long lag phase delaying the fungal propagation in the first day. However, *T. reesei* R32 produced the highest cellulase activities of (20 FPU/g) amongst the four fungal strains on day 5.

The highest cellulase activity obtained with *T. reesei* corresponds to literature values when compared with other fungal strains. *T. reesei* has been noted as a superior cellulase source for industrial applications which could reduce the cost of cellulase production by up to 40% due to its exceptional potential for various enzymes and proteins production (Keshavarz & Khalesi, 2016).

The cellulase activities for the fungal strains was significantly different after 5 days when compared with cellulase activities on days 1 and 3 respectively at 95% confidence level, while there was no significant different in cellulase activity obtained from day 1 and 3.

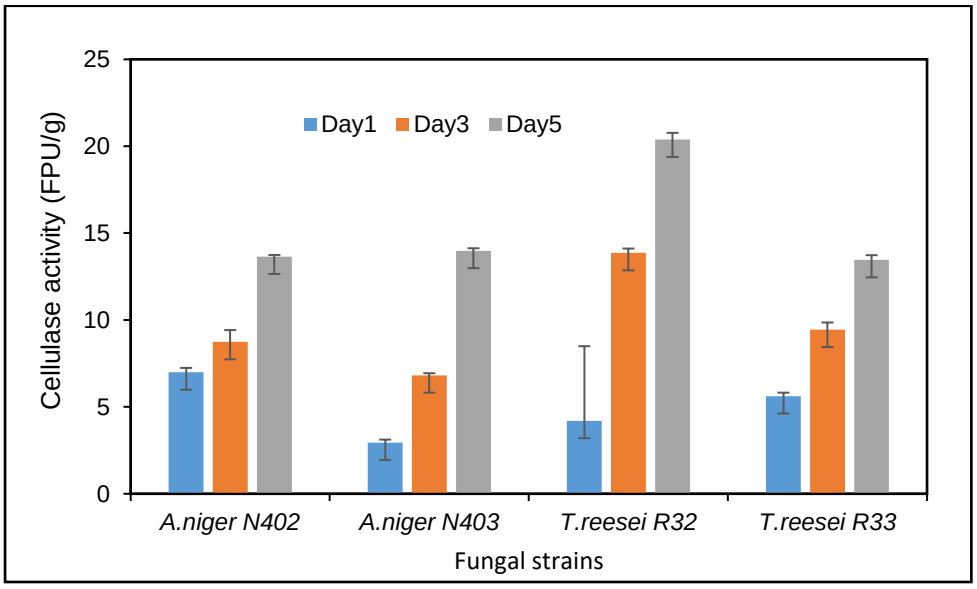


Figure 4.6 Cellulase production using four different fungal strains with 0.2% starch addition at 28˚C and moisture content of 89.5%

4.1.4 Impact of Inoculation Size and Reactor Size on Cellulase Production

Two additional fungal strains *Aspergillus niger* (CKB) and *Rhizomucor variabilis* (RS) which were obtained from Tsinghua University in China, were used to compare with *A. niger* N402 with the aim of examining the cellulase producing ability.

Firstly, the impact of inoculation size on the cellulase production was investigated as well as the reactor size.

The inoculation size of 5 million ($5x10^6$) spores/g and 10 million ($1x10^7$) spores/g were examined for *A. niger* N402, *A. niger* CKB and *R. variabilis* RS for cellulase production using petri dish (SSF) and shake flask (SSF as well) respectively.

The fermentation was used to assess the potential of the strains with different inoculation size for cellulase production and different culture time (day 1, 3 and 5) at 28˚C and moisture content of 89.5% (7.5:1 mL/g). There was a noticeable increase in cellulase activity in terms of the inoculation size simultaneously with the culture time.

The results obtained in Figure 4.7 revealed that the cellulase activities increase with inoculation size as the culture time progressed except for *R. variabilis* RS*. A. niger* N402 has the highest cellulase activities (55.93 FPU/g) among the fungal strains, which was obtained using an inoculation size of $1x10^7$ spores/g.

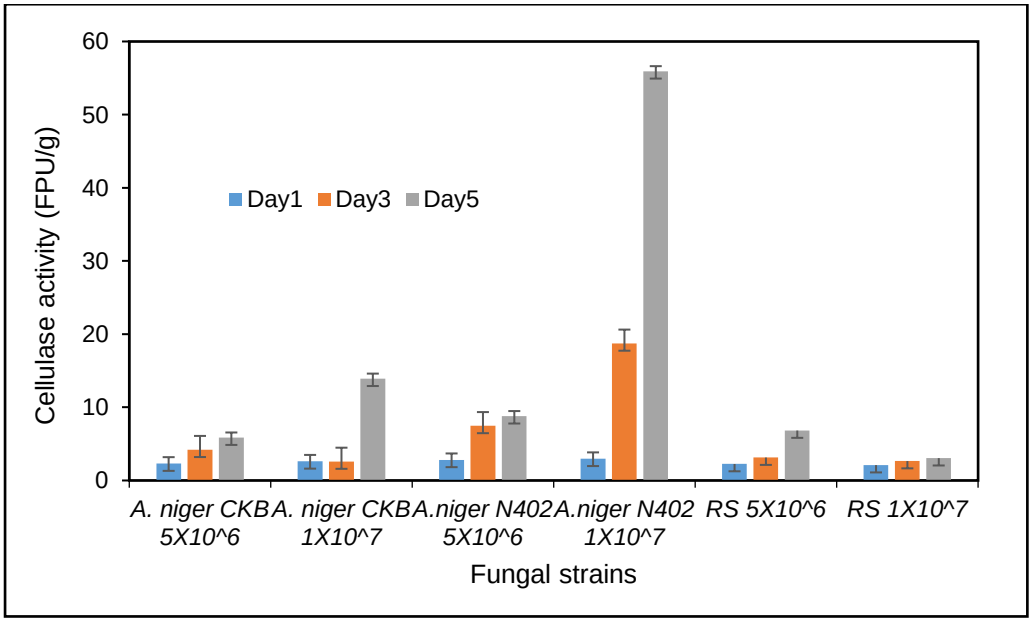


Figure 4.7 Impact of inoculation size in SSF in Petri Dish using various strains with different inoculation size at 28˚C and moisture content of 89.5%

In order to investigate the impact of reactor size on cellulase production, the above experiment was repeated using 250 mL shake flask. 5 days of cultivation was selected due the higher cellulase production obtained in the previously mentioned Petri Dish experiment.

Fungal strains (*A. niger* N402, *A. niger* CKB and *R. variabilis* RS) had an increase in cellulase activity as inoculation size increased (Figure 4.8). An increase in cellulase activities with inoculation size was similar to the results observed using a Petri Dish for *A. niger* N402 and *A. niger* CKB except for the *R. variabilis* RS strain which had a higher cellulase activity with lower inoculation size when Petri Dish was used (Figure 4.7). In SSF, using *R. variabilis* RS in a shake flask a higher cellulase activity of 3.31 FPU/g was obtained with a higher inoculation size, Petri Dish reactor size with lower inoculation size had a significant higher cellulase activity (6.83 FPU/g) for *R. variabilis* RS than the shake flask reactor size. *A. niger* N402 had the highest cellulase activities of 30.43 FPU/g among the fungal strains, using an inoculation size of $1x10^7$ spores/g but the cellulase obtained was not as high as the result obtained in Figure 4.7 (55.93 FPU/g) while *A. niger* CKB fungal strains displayed higher cellulase activities in Figure 4.8 than 4.7.

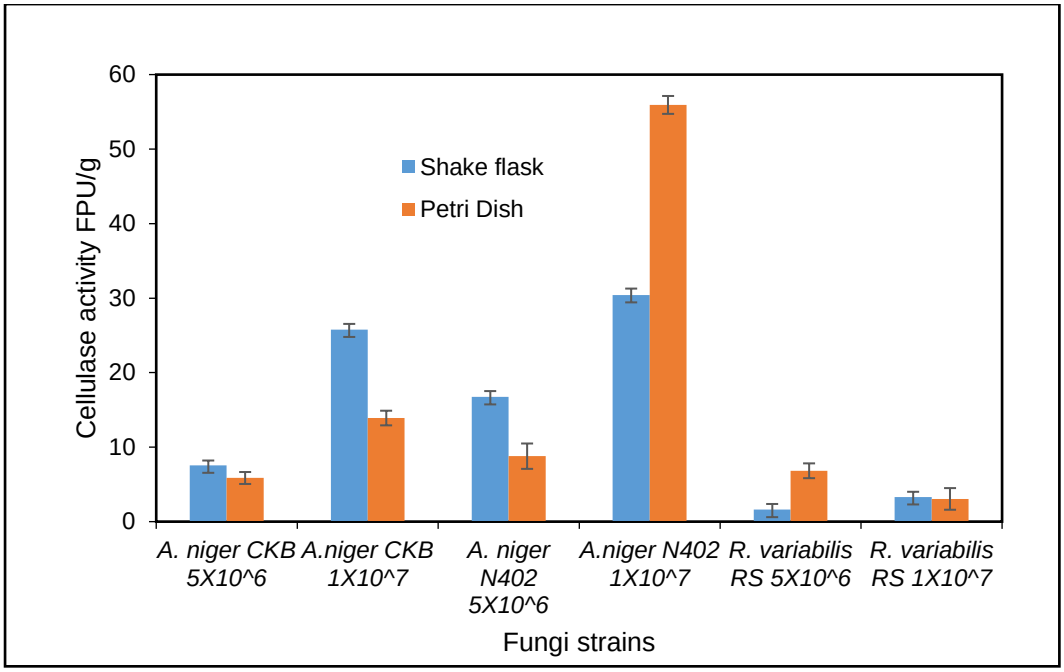


Figure 4.8 Cellulase production in SSF for 5 days in shake flasks and Petri Dishes using various strains with different inoculation size at 28˚C and moisture content of 89.5%.

An increase in inoculation size resulted in an increase in cellulase production for all the fungal strains used in this assay. Further increase in inoculation size was not carried out as excessive increase in inoculation size has been reported to decrease cellulase production by *A. niger.* This findings was similar to other fungi strains of *Trichoderma viride,* and *T. harzianum* with up to 5% - 10% increase in inoculation size led to decrease in cellulase production (Azzaz, Murad, Kholif, Hanfy, & Abdel Gawad, 2012) . The decrease in cellulase production with an increase in inoculation size might be as a result of clumping of cells which could have reduced sugar and oxygen uptake rate and enzyme release (Azzaz et al., 2012; Omojasola, Jilani, & Ibiyemi, 2008). From the data obtained with an inoculation size of 10 million spores/g using a 250 mL shake flask reactor size; these conditions were selected for further studies using *A. niger* CKB while Petri Dish was selected with an inoculation size of 5 million spores/g using *R. variabilis* RS.

4.1.5 The Relationship between Glucosamine and Cellulase Activities

Glucosamine was used to measure fungal growth during fermentation and the result obtained was used to compare fungal growth with cellulase activity.

Fungal growth was initially observed at different starch concentration using *A. niger* N402 and results revealed a slight increase in fungal growth with an increase in cellulase activities obtained as starch concentration increased up to 0.4% (w/w).

A glucosamine concentration time course was determined at 0.2% starch addition for the different fungal strains over different culture times (day 1, 3 and 5). An increase in fungal growth and cellulase activities was observed for all fungal strains as the culture time increased (Figure 4.9, 4.10, 4.11 & 4.12). Glucosamine has been reported to have a positive correlation with fungal cell growth (Pensupa et al., 2013).

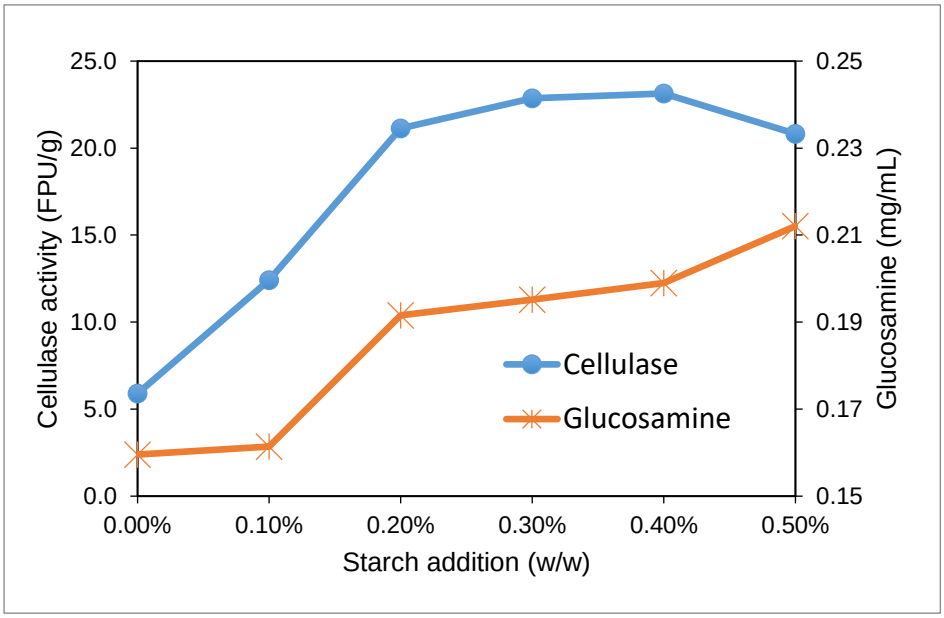


Figure 4.9 Relationship between glucosamine and cellulase at different starch concentrations *A. niger* N402.

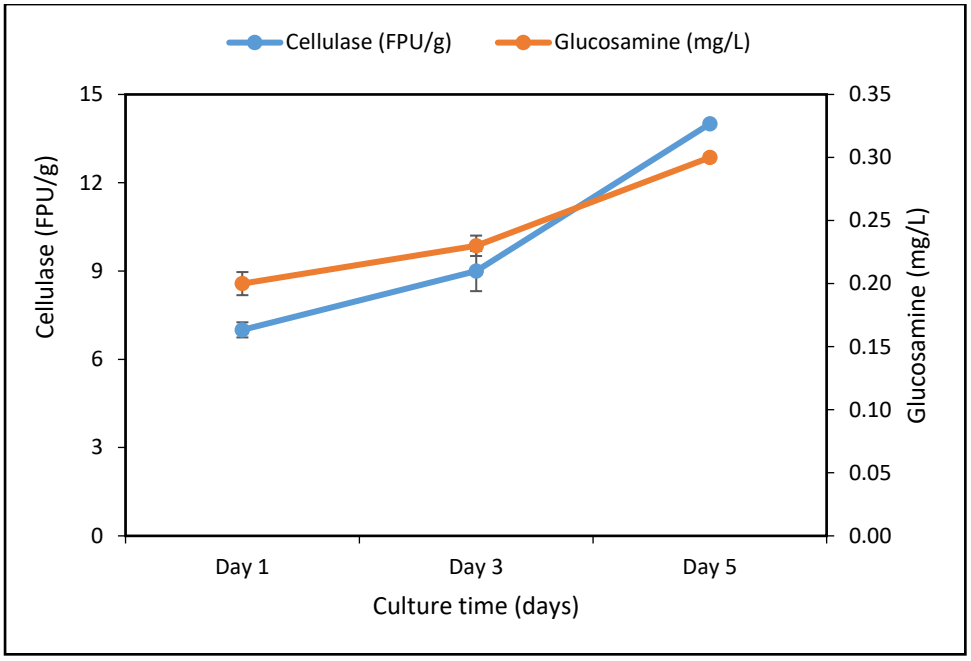


Figure 4.10 Relationship between glucosamine and cellulase in *A. niger* N402

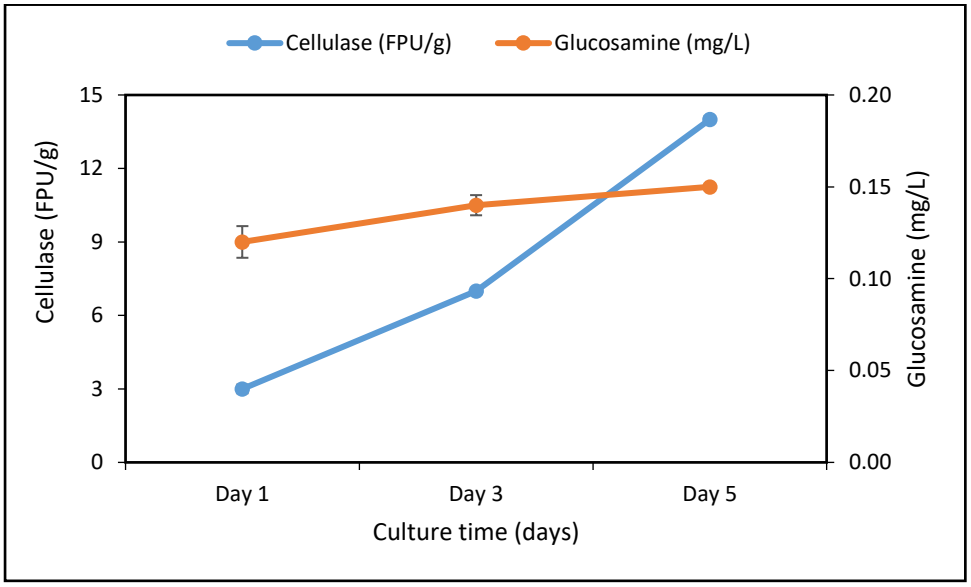


Figure 4.11 Relationship between glucosamine and cellulase in *A. niger* N403

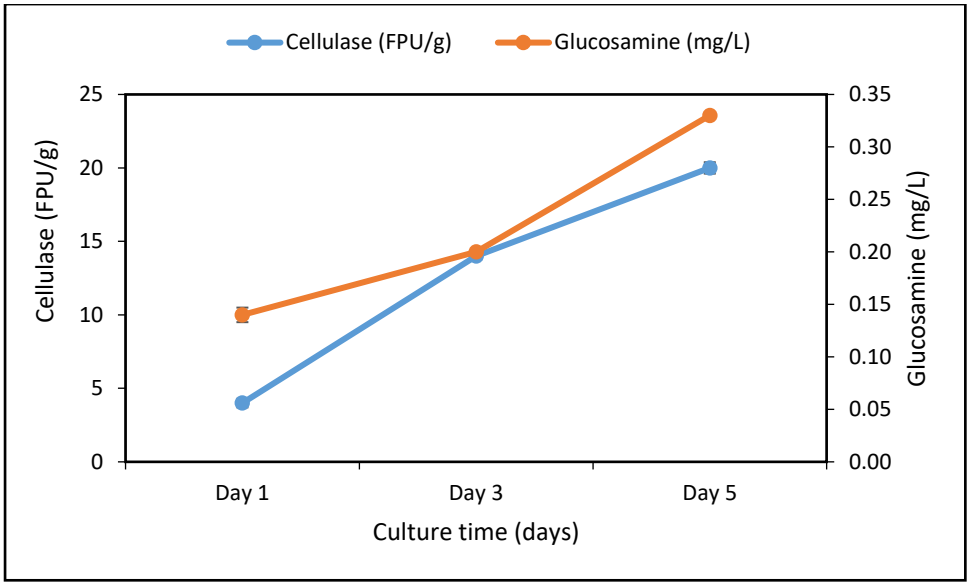


Figure 4.12 Relationship between glucosamine and cellulase in *T. reesei* R32

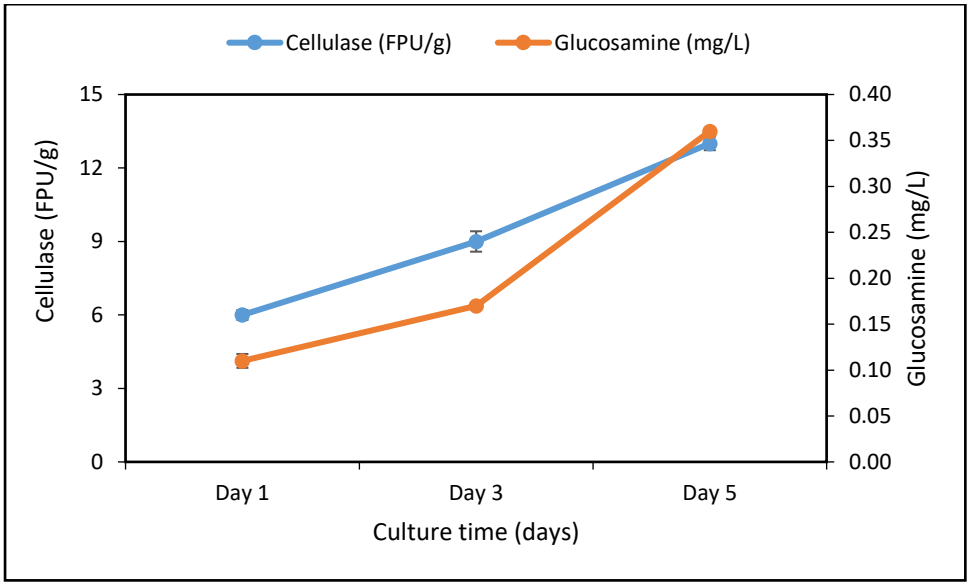


Figure 4.13 Relationship between glucosamine and cellulase in *T. reesei* R33

4.1.6 The impact of mineral addition on cellulase production from different fungi

To further improve cellulase production, minerals (K_2HPO_4 2.5 g/L, NH_4NO_3 1.5 g/L, (KH_2PO_4 1.5 g/L, $MgSO_4$ 0.12 g/L and NaCl 0.25 g/L) were added to the modified wheat straw, prior to SSF, using different inoculation size. The experiments were carried out using 250 mL shake flask to improve cellulase production since using

shake flask resulted in significant cellulase production. The experiment was carried out for 5 days at 28˚C and at moisture content of 7.5:1 (v/w).

Cellulase activities were higher for all fermentations, which had mineral addition (Figure 4.15). For fermentations using *A. niger* CKB and *A. niger* N402 lower inoculation size resulted in better cellulase activities while an increase in cellulase activity was obtained with *R. variabilis* RS at higher inoculation size (Figure 4.14). However, N402 with lower inoculation size and mineral addition resulted in the highest cellulase activity (51.56±0.05 FPU/g)

The decrease in cellulase activities with higher inoculation size using the *A. niger* strains could be as a result of clumping of cells more faster, as mineral addition shortens the fungal lag phase which could have reduced sugar and oxygen uptake rate and enzyme release (Azzaz et al., 2012; Omojasola et al., 2008). Whereas, the addition of mineral improves the release of enzyme with the *R. variabilis* RS fungal strain.

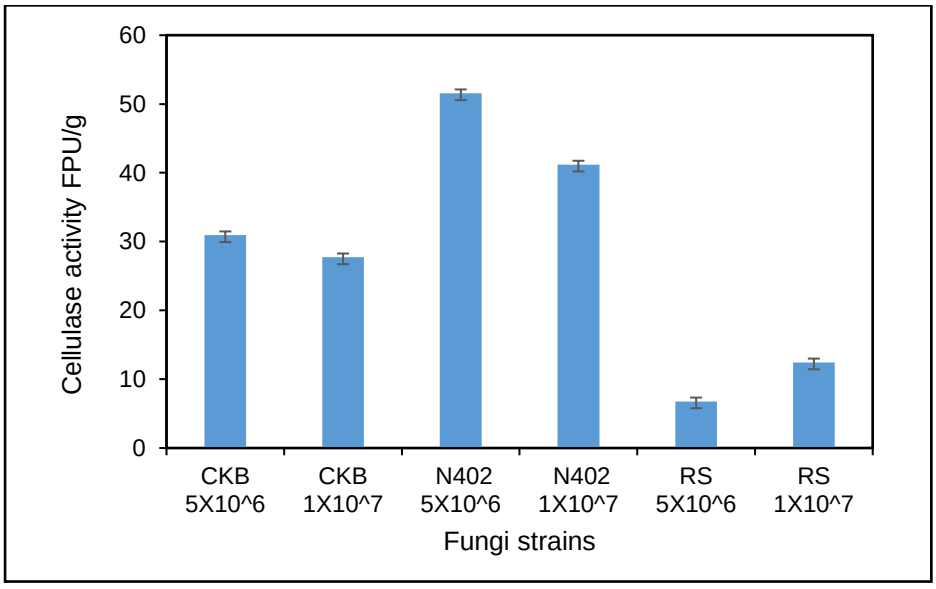


Figure 4.14 Cellulase production in SSF using 250 mL shake flask with the addition of minerals for 5 days at 28˚C and at a moisture content of 7.5:1 (v/w). The error bars are expressed as standard deviation.

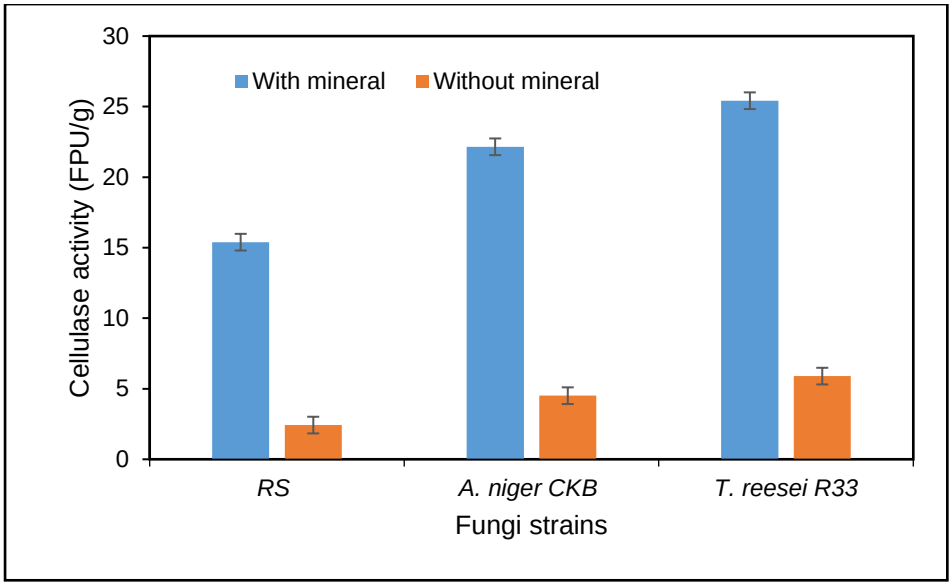


Figure 4.15 Cellulase activity of fungi strains with and without minerals in SSF at 28˚C and at a moisture content of 7.5:1 (v/w) for 5 days.

The preliminary experiment conducted earlier in this chapter with *R. variabilis* RS showed that cellulase production could be significantly increased using simple optimisation. This was in comparison with *A. niger* CKB and *T. reesei*, indicating that *R. variabilis* RS strain has the potential to be used for the production of cellulase enzyme. Therefore, the *R. varaibils* RS strain was selected for further optimisation in subsequent studies (chapter 5). *R. variabilis* RS was chosen because the fungal strain is a newly isolated strain, produced cellulase and could be further optimised as previous fungal strains (*A. niger* and *T. reesei*) have been explored by different researcher for the production of cellulase enzyme with different carbon sources.

4.1.7 The impact of microwave treated wheat straw (MTWS) on cellulase production

Wheat straw was treated using a microwave at a solid to liquid ratio of either 1:20 (w/v) or 1:30 (w/v). The solid fraction of the MTWS was inoculated with *A. niger* N402, under SSF for 5 days at 28˚C, in order to identify the impact of MTWS on cellulase production. MTWS (1:30) produced a high cellulase activity on day 1 (4.39±0.07 FPU/g) of fermentation, which decreased after 3 days and 5 days (Figure 4.16). When MTWS (1:20) was used as substrate, there was a consistent increase in cellulase activity (Figure 4.16). The highest cellulase activity obtained in this test was

4.39±0.07 FPU/g, which was obtained when MTWS 1:30 was used as substrate. The result showed that MTWS 1:30 was a relatively better substrate for cellulase production than MTWS 1:20.

However, the cellulase activity obtained in the above study was low in comparison with previous studies in chapter 4.1.1 when alkali modified wheat straw was used as substrate.

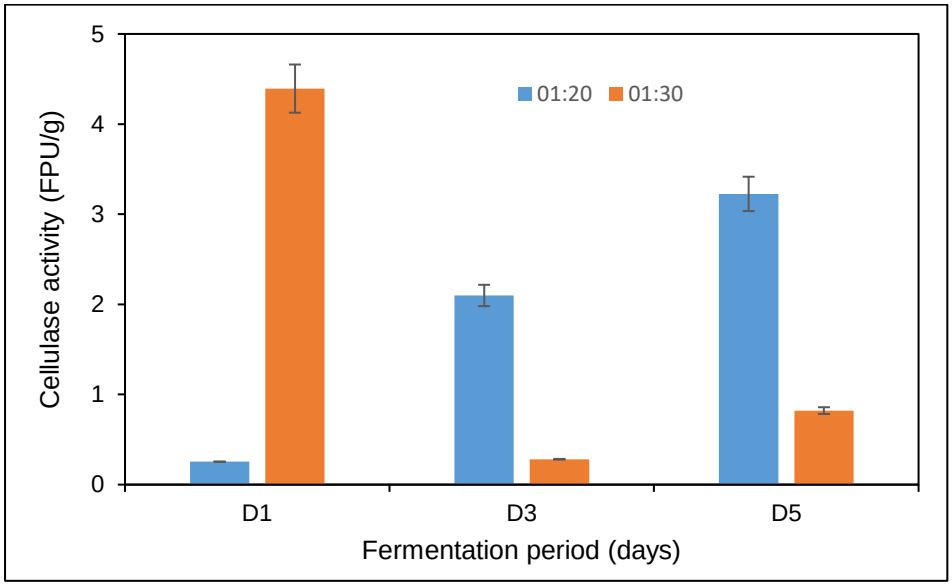


Figure 4.16 Cellulase activity of MTWS solid part in a SSF over 5 days in Petri-Dish using *A. niger* N402 at 28˚C * D1, D3 and D5 represent Day1, Day2 and Day5 respectively.

In order to improve cellulase production, an inoculum obtained from a submerged fermentation was used. The MTWS solid part was inoculated with *A. niger* liquid broth from SmF of the fungus. Then SSF fermentation was carried out for 5 days to determine the impact of inoculation method on cellulase production. The assay showed a higher cellulase activity for the MTWS solid part (1:20 and 1:30) 12.84 FPU/g and 14.10 FPU/g respectively (Figure 4.17). The cellulase activity was significantly higher than what was obtained in Figure 4.16. As this cellulase production was still lower than that obtained in the SSF using alkali modified wheat straw, the usage of MTWS was not continued. However, the method of using fungal liquid broth from SmF instead of fungal spores showed an attractive improvement of cellulase activity. This approach is worth further investigation.

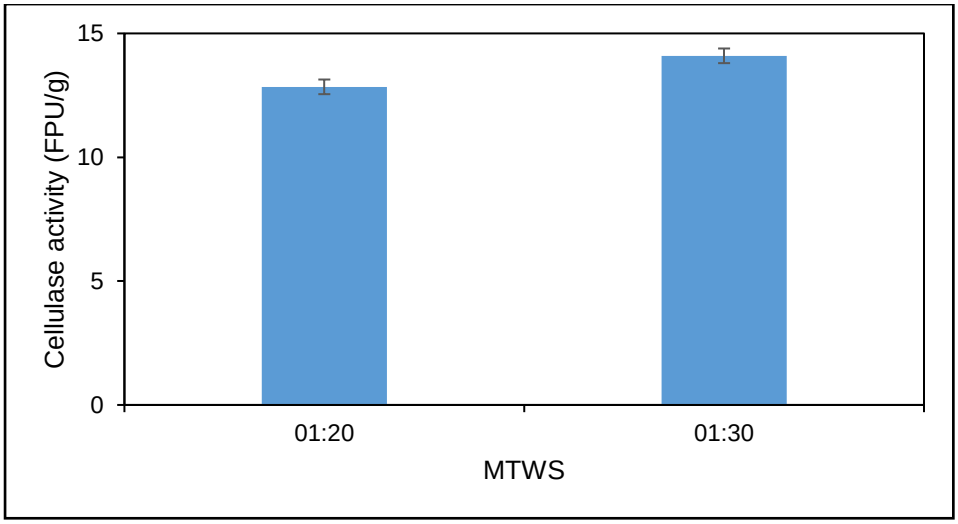


Figure 4.17 Cellulase activity of MTWS solid part in a SSF using *A. niger* N402 liquid broth as inoculum at 28˚C for 5 days.

4.1.8 The impact of different substrates on cellulase production

In order to investigate the impact of substrate on the cellulase production, a comparison experiment was carried out using different biomass materials namely: wheat straw, *Miscanthus*, willow and waste cloth (40%/60% cotton/polyester) via SSF with the addition of minerals. *A. niger* CKB with an inoculation of 10 million spores/g was used and SSF was carried out for 5 days at 28˚C using Petri-Dish. As expected, different substrates induced different amounts of cellulase formation. The SSF assays revealed that *Miscanthus* had the highest cellulase activity of 11.49 FPU/g (Figure 4.18). The cellulase activities for SSF of wheat straw, willow and waste cloth were 8.74 FPU/g, 6.81 FPU/g and 6.12 FPU/g, respectively (Figure 4.18).

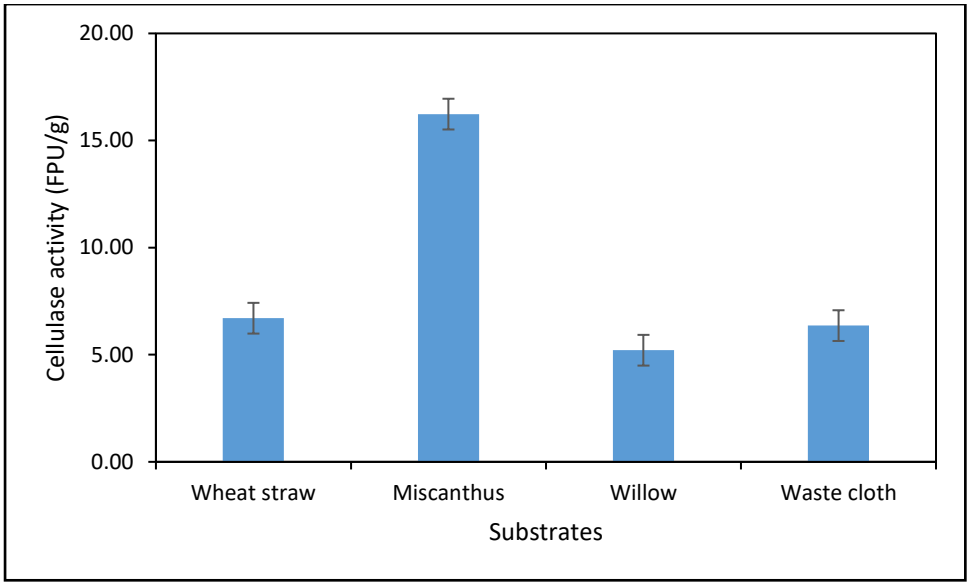


Figure 4.18 Average cellulase activities from SSF of *A. niger* CKB using different biomass materials at 28˚C for 5 days.

Table 4.2 Experimental trials of cellulase activity from SSF of *A. niger* CKB using different biomass materials

Biomass	**Cellulase activity (FPU/g)**		
	Trial 1	Trial 2	Trial 3
Wheat straw	6.12 ± 0.02^{a}	6.02 ± 0.38^{a}	5.94 ± 0.48^{a}
Miscanthus	18.01 ± 0.06^{b}	17.42 ± 0.07^{b}	18.01 ± 0.04^{b}
Willow	4.86 ± 0.02^{a}	4.79 ± 0.01^{a}	4.38 ± 0.02^{a}
Waste cloth	6.47 ± 0.11^{a}	6.53 ± 0.09^{a}	6.32 ± 0.33^{a}

The ± represent the standard deviation and the value with similar superscript in the same trial has no significant difference.

In order to confirm the result obtained in the above study, three repeated experiments were carried out as shown in Table 4.2. In general, use of *Miscanthus* correlated with the highest production of cellulase, which was confirmed by replication of the experiments. Willow resulted in the lowest cellulase production. However, the cellulase activity of *Miscanthus* was significantly different from other biomass materials, at the 95% confidence level (Table 4.1).

4.2 Cellulase production in submerged fermentation

Cellulase production in submerged fermentation was investigated in 2 L bench top fermenters using alkali soaked modified wheat straw.

4.2.1 Fungal Growth Curve

In a trial experiment, fungal strains *A. niger* CKB and RS were used. The strain RS was selected due to its novelty (newly isolated) and strain *A. niger* CKB was selected for comparison reasons. The growth curve of *A. niger* CKB and RS was firstly determined. For *A. niger*, the wet fungal biomass concentration (wet weight) showed that there was no detectable fungal growth after 24 hours (Figure 4.19). However, there was a noticeable increase in fungi growth between 32 and 52 hours of incubation (0.25 g/20 mL to 2.42 g/20 mL). This experiment was unfortunately stopped after 52 hours due to the closure of the laboratory for disinfectant purpose.

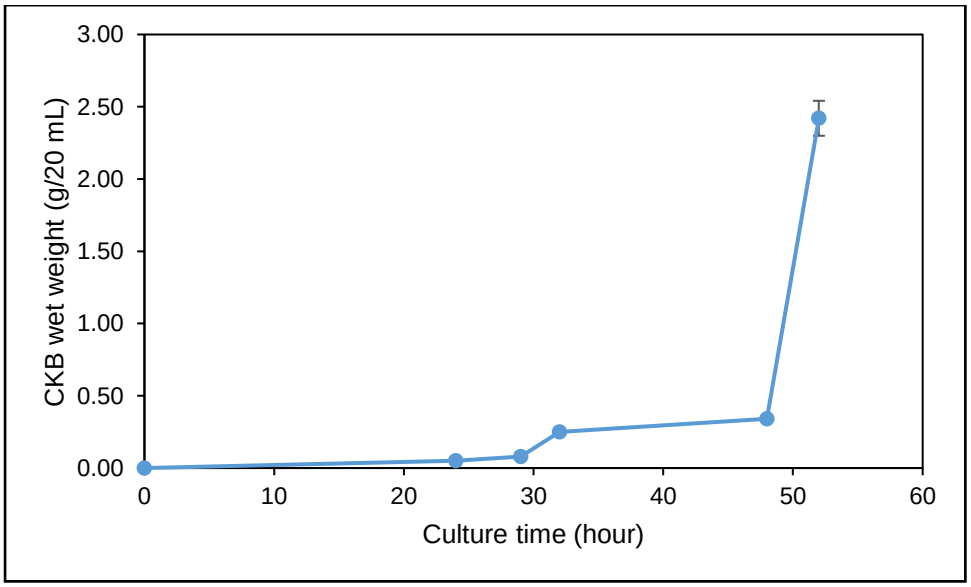


Figure 4.19: CKB fungal wet weight, at temperature of 28˚C, agitation at 300 rpm and an initial pH 6

In a repeat fermentation, the cellulase activity of *A. niger* CKB on alkali modified wheat straw was monitored in a 2 L fermenter for 168 hours. Cellulase activity of 0.15 FPU/g was detected at the start of the fermentation. Along with the fermentation, there was a general increase in cellulase activities until the end of the

fermentation at 168 hours (Figure 4.20). The highest cellulase activity obtained in this study was 0.98 FPU/g.

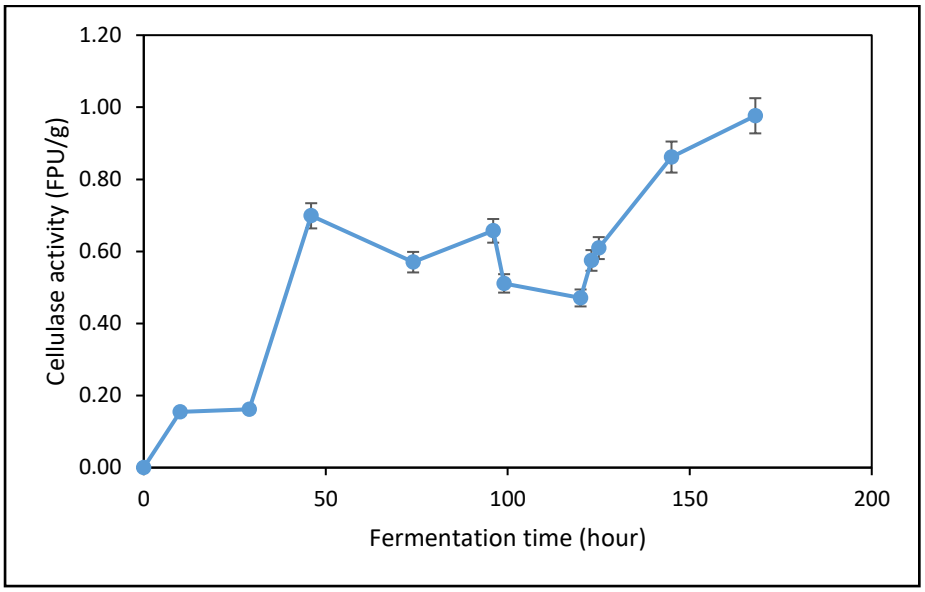


Figure 4.20 Cellulase activity of *A. niger* CKB in SmF, under the fermentation conditions, of 28˚C, 300 rpm and pH 6.

The cellulase production in SmF using *R. variabilis* RS strain was also determined in 2 L fermenters at 28˚C, 300 rpm and an initial pH of 6. The result obtained in Figure 4.21 shows the cellulase activity in SmF over 146 hours. The cellulase activity of fermentation with wheat straw addition increased significantly up to 52 hours (1.03 FPU/g to 7.07 FPU/g). *R. variabilis* RS synthesises cellulase in order to hydrolyze cellulose from the wheat straw to provide carbon source for its growth during the fermentation period. There was a decline in cellulase activity after 52 hours, which indicated that there was a reduction in cellulose content and nutrient supplement in the media.

For *R. variabilis* RS, the wet fungal biomass concentration (wet weight) revealed that there was no detectable fungi growth during the first 6 hours (Table 4.3). However, there was a noticeable increase in fungi growth between 24 and 96 hours (4.88 g/20 mL to 5.42 g/20 mL). The fungal wet weight has a correlation with the cellulase activity obtained in Figure 4.21. Although with an increase in fungi wet weight, an increase in cellulase activity was also observed while the cellulase activity declined

as the fungi entered into its death phase. Detailed experiment on the effect of pH on cellulase activity are discussed in chapter 5.1.3.

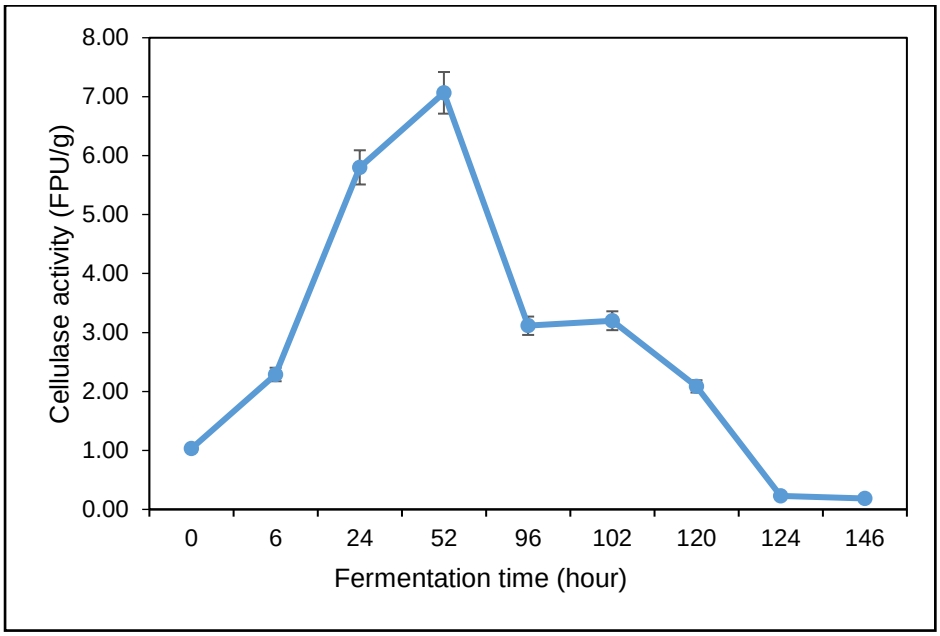


Figure 4.21 Cellulase activity of R. variabilis RS in submerged fermentation at 28˚C, 300 rpm and initial pH 6.

Table 4.3 Effect of pH on *R. variabilis* RS wet weight with time

Culture time (hour)	Wet weight (g)	pH
0	1.66	5.92
6	1.78	5.98
24	4.88	6.12
52	4.90	6.34
76	5.32	6.50
96	5.42	6.54
102	4.65	6.61
120	4.25	7.01
124	4.23	7.15
146	3.70	7.61

4.3 Enzymatic Hydrolysis

4.3.1 Enzymatic hydrolysis of different substrate with different corresponding crude enzyme

In chapter 4.1.8, four different substrates were used for cellulase enzyme production. These enzymes were then used for the enzymatic hydrolysis of different substrates to investigate the impact of cellulase. The substrates used were: wheat straw, willow, *Miscanthus* and waste cloth. The hydrolysis was carried out at in a 50˚C shaking incubator, at 200 rpm, for 24 hours. Table 4.4 shows the schedule of the enzymatic hydrolysis experiment.

Table 4.4 Enzymatic hydrolysis of different substrate mixed with each corresponding enzyme

	Enzyme			
Substrate	Wheat straw	*Miscanthus*	Willow	Waste cloth
Wheat straw	AA	BA	CA	DA
Miscanthus	AB	BB	CB	DB
Willow	AC	BC	CC	DC
Waste cloth	AD	BD	CD	DD

The first letter in the column represents:

A: Wheat straw derived cellulase enzyme solution

B: *Miscanthus* derived cellulase enzyme solution

C: Willow derived cellulase enzyme solution

D: Waste cloth derived cellulase enzyme solution

Reducing sugar analysis was conducted by HPAEC-PAD to identify the sugars present in the different hydrolysates of each substrate. Only glucose was detected which was then used for the calculation of the total sugar obtained. Higher glucose

concentrations were obtained in experiments using modified wheat straw derived cellulosic enzyme solution for the hydrolysis on the biomass materials.

The results of the saccharification yields (Figure 4.22) from these substrates was compared with published data. An increase in hydrolysis yield was obtained with alkali extrusion wheat straw biomass (Coimbra et al., 2016), aspen biomass pre-treated with 2 % aqueous NH_4OH (Jagtap et al., 2013) and sugarcane bagasse pre-treated with bisulfite (Liu, Lan, Li, Gao, & Zhang, 2017). Lignin is one of the main components of plant cell walls known to inhibit enzymatic hydrolysis efficiency by binding cellulose and hemicelluloses (Alvira, Tomás-Pejó, Ballesteros, & Negro, 2010; Kristensen, Thygesen, Felby, Jørgensen, & Elder, 2008; Xu & Huang, 2014).

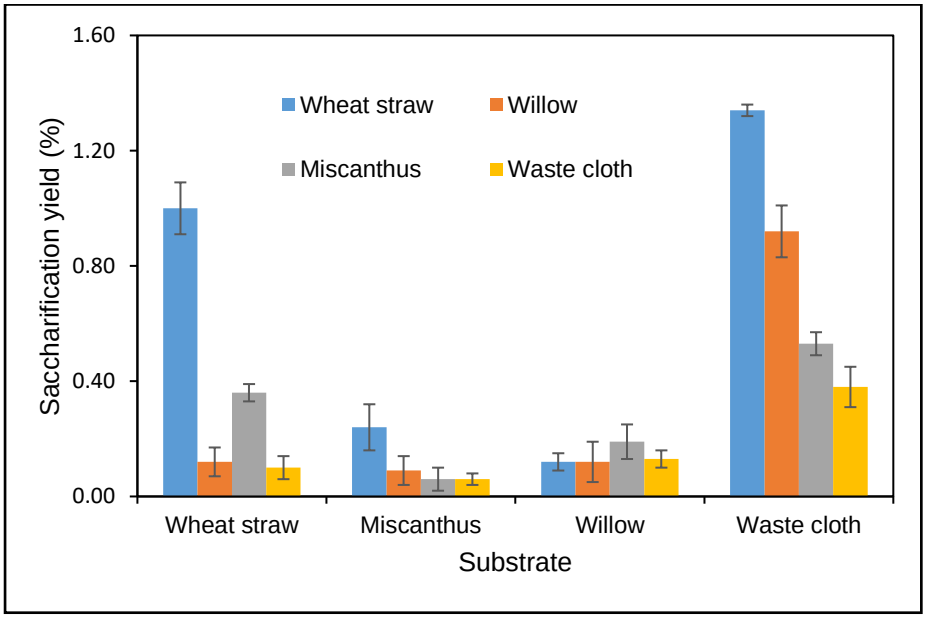


Figure 4.22 Saccharification yield (%) of different substrates with their corresponding enzymes

* The legend represent the corresponding enzyme

4.3.2 Enzymatic hydrolysis of autoclave wheat straw with/without fungal growth by using commercial enzyme

A commercial cellulolytic enzyme was used in enzymatic hydrolysis of biological modified autoclaved wheat straw with *R. variabilis* RS fungal growth and autoclaved wheat straw. This was to investigate the effect of *R. variabilis* RS fungal strain on the recalcitrant nature of wheat straw, which was compared with acid and alkali modified

wheat straw. The details of all modification/pre-treatment conditions of wheat straw used before enzymatic hydrolysis are given in Table 4.5.

An experiment was carried out to investigate the effect of pre-treatment on substrate characteristics for glucose production during enzymatic hydrolysis using commercial cellulase CTech from Novozyme. Fermented wheat straw with *R. variabilis* RS, autoclaved wheat straw, acid pre-treated wheat straw and non-modified wheat straw were used in this experiment. The enzymatic hydrolysis was assessed for 72 hours (Figure 4.23).

After 72 hours of hydrolysis, the glucose concentrations in the hydrolysis experiments using both acid modified wheat straw (AWS) and alkali fermented wheat straw (alkali FWS) for 1 and 3 days, were approximately 8 g/L. Alkali non-fermented wheat straw (alkali NFMWS) resulted in the highest glucose concentration of 15.69 g/L after 72 hours of hydrolysis. Non-modified wheat straw (RWS) has no glucose detected while autoclaved raw wheat straw (ARWS) gave a significant amount of glucose concentration of 9.11 g/L after 72 hours of hydrolysis. This is in agreement with a different report (Amin et al., 2017) that the breakdown of lignin during pre-treatment is essential for effective conversion of cellulose in lignocellulosic biomass into fermentable sugars (glucose).

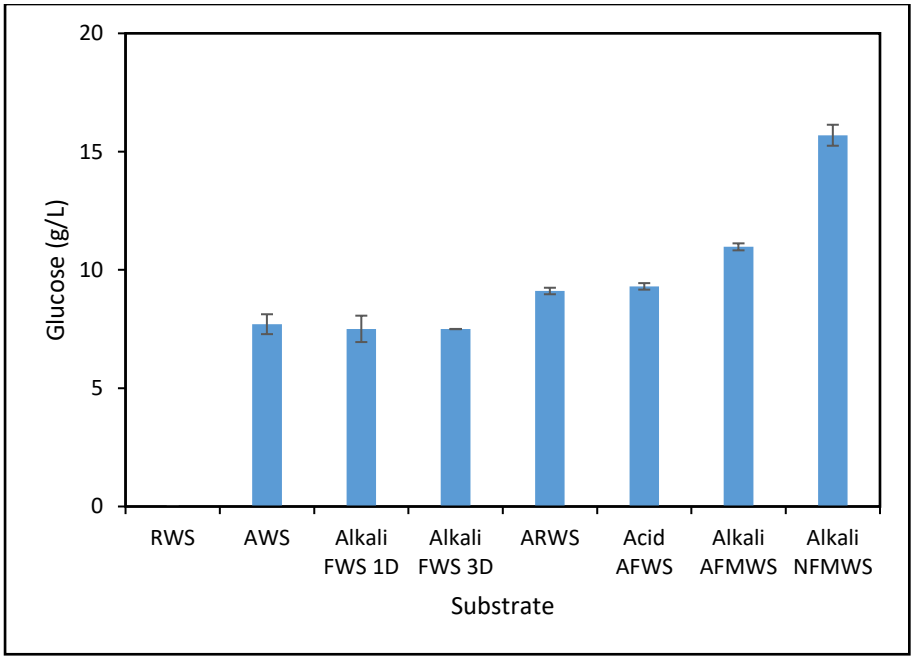


Figure 4.23 Enzymatic hydrolysis of raw, acid modified, autoclaved and alkali modified wheat straw for 72 hours, 200 rpm.

Further experiments looked at the impact of fermentation prior to hydrolysis of non-modified wheat straw and acid modified wheat straw for improved cellulose breakdown into sugar. The results showed that fermentation had a positive effect on non-modified wheat straw and acid modified wheat straw (Figure 4.24) when compared with the result obtained in Figure 4.23.

Hydrolysis using fermented acid modified wheat straw for 1 day produced higher amount of glucose (13.81 g/L) from 7.71 g/L obtained without fermentation prior to hydrolysis (Figure 4.23). However, fermented non-modified wheat straw showed a trend of decreasing presence of glucose profile after 5 days of fermentation when hydrolysed for 72 hours. The increase in glucose after hydrolysis of fermented non-modified wheat straw could be due to the swelling properties of the straw as it absorbs water thereby exposing the cellulose fraction of the wheat straw to enzymatic hydrolysis. The decrease in glucose obtained after 5 days of fermentation of the non-modified wheat straw might be due to the evaporation of water from the wheat straw, which could have resulted in the shrinking of the wheat straw back to its initial property thus making the cellulose not completely hydrolysed.

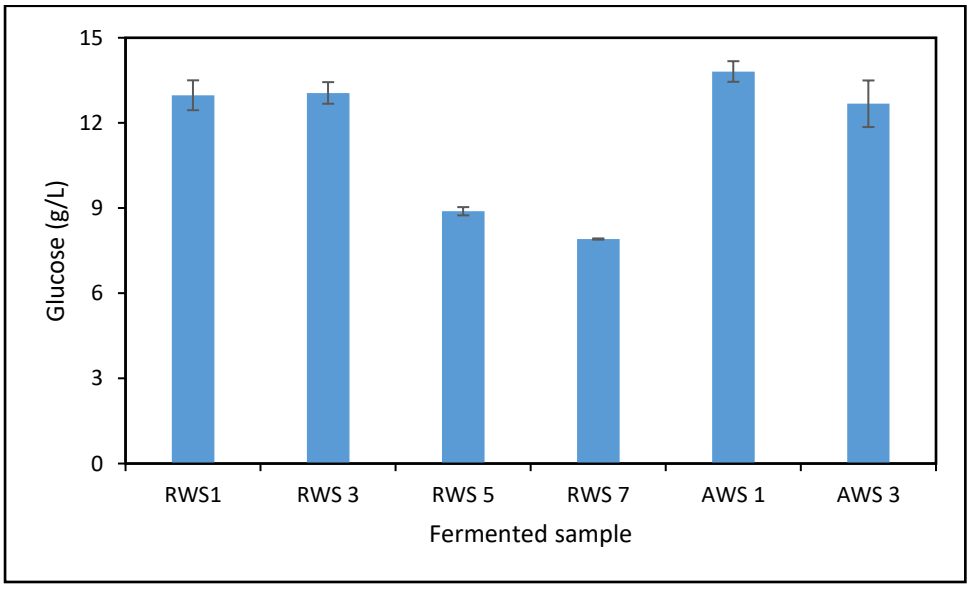


Figure 4.24 The effect of different fermentation days on non-modified wheat straw (RWS) and acid modified wheat straw (AWS) in enzymatic hydrolysis at 200 rpm.

Table 4.5 List of modification/pre-treatment condition of wheat straw before enzymatic hydrolysis

Wheat straw used	Code	other modification condition	Fermentation period (days)
Raw wheat straw	RWS	None	0, 1, 3, 5 and 7
Raw wheat straw	ARWS	Autoclaved at 121˚C for 15 minutes	3
Acid modified wheat straw	AWS	None	0, 1 and 3
Acid modified wheat straw	Acid AFWS	Autoclaved at 121˚C for 15 minutes	3
Alkali modified wheat straw	Alkali AFMWS	Autoclaved at 121˚C for 15 minutes	3
Alkali modified wheat straw	Alkali FWS	None	1 and 3
Alkali modified wheat straw	Alkali NFMWS	None	0

4.4 Summary

Various parameters were examined in SSF and SmF for the production of cellulase enzyme using six different fungal strains from different sources. The fungal strains were either inoculated on alkali soaked modified wheat straw, MTWS solid part (1:20 and 1:30), or different biomass materials. The impact of different fermentation conditions were assessed by determining cellulase production.

Modifying wheat straw by alkali soaking, significantly improved cellulase production from 3.2±0.05 FPU/g to 8.1±0.3 FPU/g. The addition of up to 0.4% (w/w) starch also enhanced cellulase accumulation when *A. niger* N402 was used but no significant increase when over 0.2% starch was used. The addition of 0.2% starch also resulted in an increase in cellulase production among the different fungal strains used with *T. reesei* R32 producing the highest amount of cellulase under this condition. In comparison with other strains, *A. niger* N402 had the highest cellulase production.

The cellulase activities using an inoculation size of $1x10^7$ spore/g in Petri Dish was 55.93 FPU/g, shake flask without mineral 30.42 FPU/g and shake flask with mineral addition 51.56 FPU/g, respectively. Alkali modified wheat straw yielded a higher cellulase activity than MTWS (1:20 and 1:30). The novel fungal strain of *R. variabilis* RS shows a great potential for cellulase production when compared with other known fungi.

Cell growth as determined by glucosamine concentration had a positive relationship with cellulase accumulation, indicating that an increased fungal growth is a key factor leading to increased cellulase production. The novel strain of *R. variabilis* RS demonstrated higher potential in cellulase production and was selected for further investigation for the optimisation of cellulase production and on biological pre-treatment of wheat straw. The enzymatic hydrolysis of acid, alkali modified and autoclaved wheat straw showed that a sugar rich stream is obtainable while alkali non-fermented modified wheat straw resulted in the highest glucose concentration (20.92 g/L) after 72 hours of hydrolysis.

5 Cellulase production by *Rhizomucor variabilis* (RS)

Based on the data generated by initial experiments (chapter 4.1.4 - 4.1.6)**,** *R. variabilis* RS fungal strain was selected for cellulase production. This strain was chosen because it was newly isolated and as the ability to produce cellulase enzyme.

5.1 Submerged fermentation using *Rhizomucor variabilis* (RS)

The cellulase production by *R. variabilis* RS in submerged fermentation was investigated. The substrate used in this section was alkali soak modified wheat straw unless otherwise specified. In order to optimise the cellulase activity using *R. variabilis* RS, various fermentation parameters, such as fermentation time, pH, temperature and medium composition were investigated independently in submerged fermentation to determine their impacts on biomass wet weight and cellulase activity.

5.1.1 Fermentation profile

The fermentation profile of cellulase production in SmF using *R. variabilis* RS strain was determined over 7 days at 28˚C, 200 rpm and with no addition of minerals. Cellulase activity was measured over the time-period of the fermentation and there was an increase in cellulase activity for the first 3 days of the assay (Figure 5.1). It reached the highest activity of 9.33 FPU/g on the third day; after this time, point there was a decline in cellulase activity afterward. The decline in cellulase activity could be due to fungal autolysis or depletion in nutrients in the medium that resulted in fungal physiology stress resulting in the inactivation of secretory machinery of the enzymes (Nochur, Roberts, & Demain, 1993).

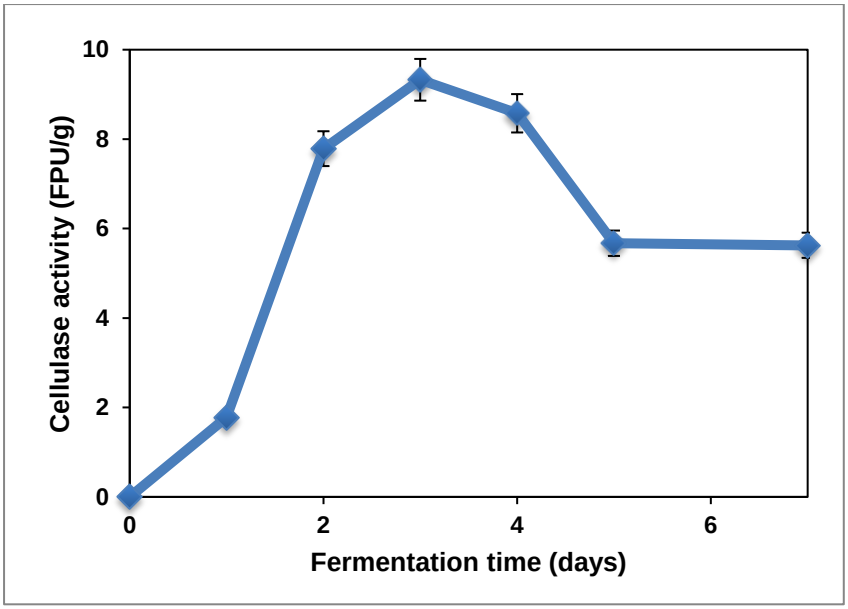


Figure 5.1 Cellulase production profile (FPU/g) during a submerged fermentation using *R. variabilis* RS at 28˚C, 200 rpm and initial pH 6.

5.1.2 Impact of glucose concentration on biomass production

In order to improve cellulase production, biomass production was examined. The impact of glucose concentration on biomass-wet weight over 9 days was determined. The *R. variabilis* RS strain was grown in 200 mL of 500 mL shake flask at 28˚C in a shaking incubator at 200 rpm, with different glucose concentration (20 – 120 g/L) serving as carbon source. Samples were taken daily to determine biomass wet weight, as described in Chapter 3.5.6.

Assays with 20-60 g/L glucose revealed that there was an increase in biomass-wet weight for the duration of the experiment (9 days) (Figure 5.2). There was little increase in biomass-wet weight observed in assays with 80 g/L and experiments with 100-120 g/L were identical with no growth observed.

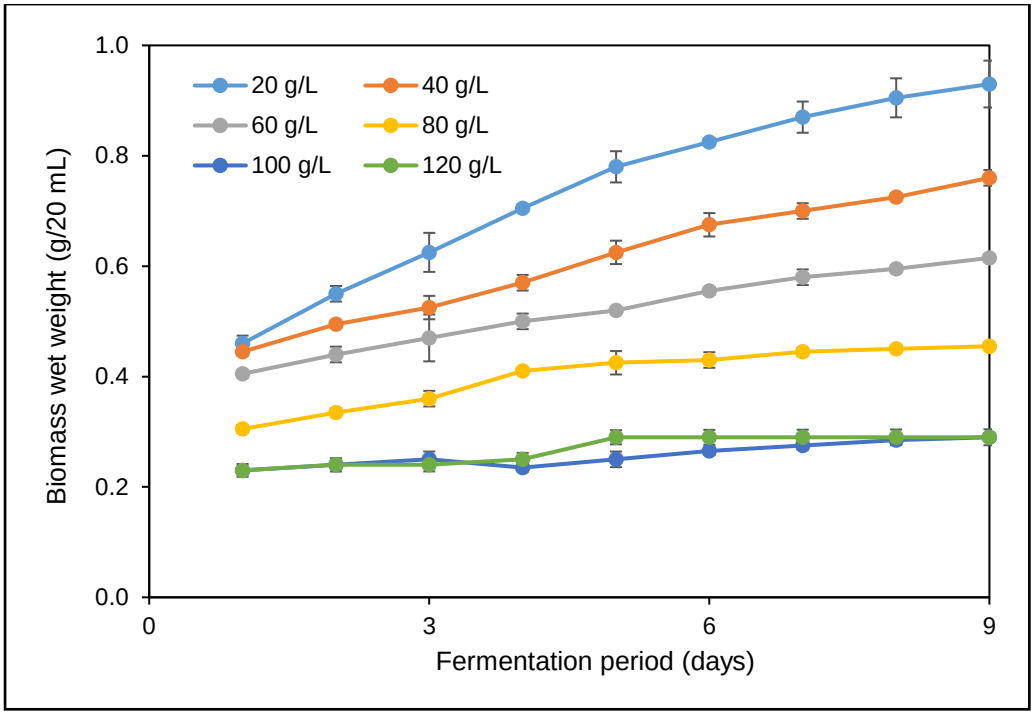


Figure 5.2: Biomass wet weight with high glucose concentration from 20 mL samples. The legends are the glucose concentration (g/L) at 28˚C, 200 rpm

The result revealed that 20 g/L of glucose was the best in the sugar concentrations investigated in this assay for maximum biomass production (Figure 5.2). The results indicated that a lower glucose concentration benefited fungal growth, while a high glucose concentration inhibited *R. variabilis* RS growth. Therefore, in order to obtain better biomass wet weight, a lower glucose concentration range was used for biomass production. The result obtained from Figure 5.3 shows that there was no biomass growth when glucose was not added to the media. This was similar to the results obtained at high glucose concentration of 100 to 120 g/L; at those conditions, the fungi was stuck at its lag phase adjusting to its environment. The highest biomass wet weight was recorded on day 3 when 5 g/L glucose was used before a decline in wet weight from day 4. There is no significant difference at 95% confidence level in biomass wet weight with glucose concentration of the same subset.

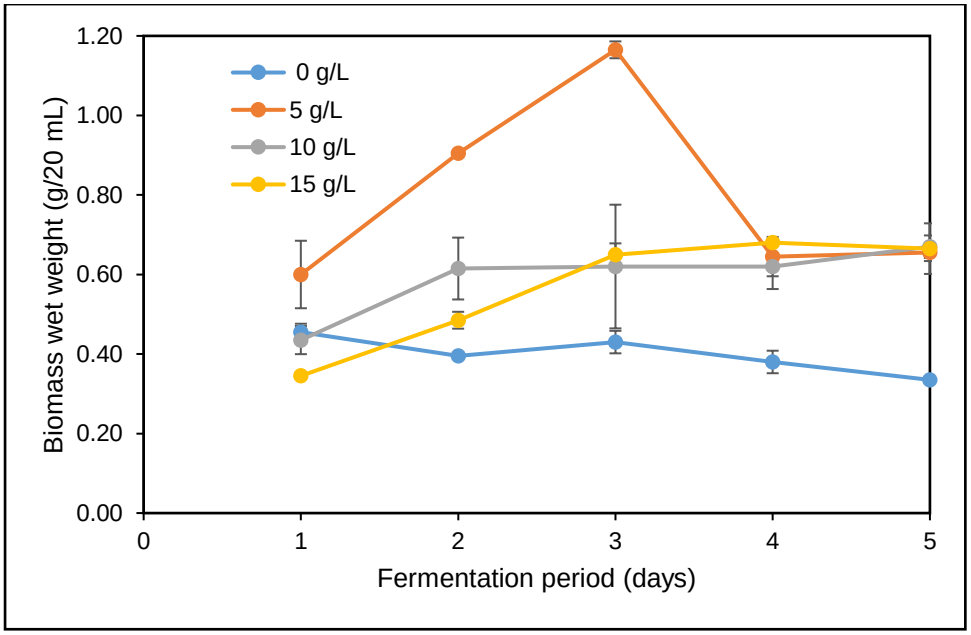


Figure 5.3 Biomass wet weight from low glucose concentration, from 20 mL samples. The legend is the glucose concentration (g/L).

An increase in biomass weight was reported by Greenman, Holland, and Cunliffe (1981) in fungal fermentation using *P. avidum*, *P. granulosum* and *P. acnes*, when glucose concentration was increased up to 0.3% – 0.4% (w/v, ~ 3-4 g/L). A constant or a slight decrease in biomass weight was obtained with further increase in glucose concentration. Although there was no significant difference at 95% confidence level when 5, 10 and 15 g/L and when 0, 10 and 15 g/L glucose concentration were used but there was a significant difference in biomass wet weight with no glucose concentration and when 5 g/L of glucose concentration was added to the medium.

The utilization of glucose by *R. variabilis* RS as carbon source from the different glucose concentration showed that the increased biomass wet weight obtained at lower glucose concentration indicated that higher glucose concentration might lead to the production of a higher concentration of organic acid, which may inhibit further fungal growth.

5.1.3 Impact of pH on cellulase activity and biomass production

The impact of pH on cellulase enzyme and biomass wet weight production using modified wheat straw was investigated. Different initial pH ranging from 4.0 to 7.0 were selected. The pH of the medium was adjusted by addition of either 1.0 M HCl or 1.0 M NaOH. The fermentation was carried out in SmF mode (Figure 5.4).

The impact of pH on cellulase enzyme production was carried out in a 250 mL shake flask with 100 mL working volume for 3 days; 3 days was chosen due to a decline in cellulase activity observed after 3 days of fermentation (Figure 5.1). This was done to determine an optimal pH for cellulase enzyme production using RS fungal strain.

Assay revealed that pH 6.5 resulted in the highest cellulase activity of 11.43 FPU/g (Figure 5.4) which was slightly higher than the highest cellulase activity obtained without initial pH control (Figure 5.1). The initial pH of 5.0 has the lowest cellulase activity around 8.75 FPU/g on day 3 of fermentation. The difference in cellulase activity could be because some enzymes were affected by the changes in pH.

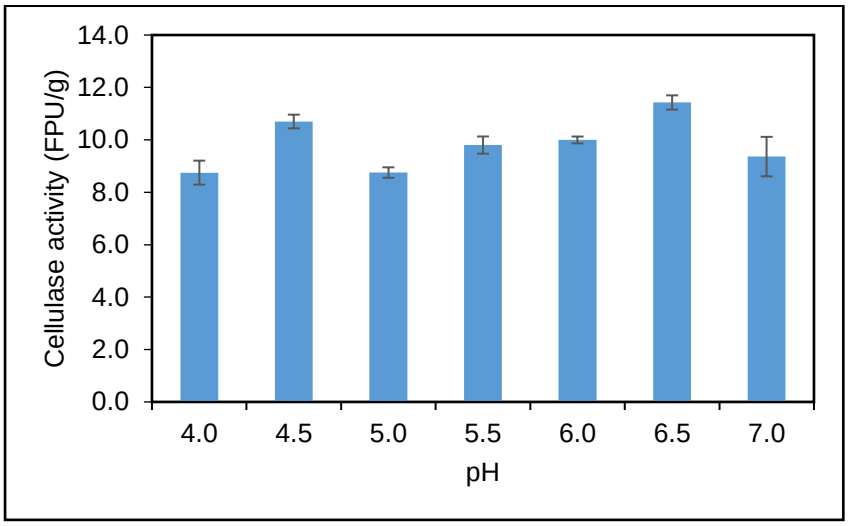


Figure 5.4 Impact of pH on cellulase activity using 1% modified wheat straw, distilled water, temperature at 28 ˚C, 250 mL shake flask, 200 rpm and culture time of 3 days.

The change of pH in a submerged fermentation using *R. variabilis* RS strain was monitored, and the data was correlated to changes in biomass-wet weight. The

experiment was carried out in a 2 L bench top fermenter. The initial pH of the medium was 5.92 and was not adjusted during the fermentation.

An increase in biomass-wet weight was observed during the first 24 hours (from 1.78 to 4.88 g/20mL). During this period, pH increased from 5.92 to 6.12 (Figure 5.5). After the first 24 hours, there was a continuous increase in biomass-wet weight and pH until the 96th hour of fermentation. After the 96th hour there was a decrease in biomass-wet weight (Figure 5.5); concurrently there was an increase in pH to over 7. The biomass wet weight profile correlated with the results obtained from the shake flask experiments (Figure 5.4), which denoted that the highest fungi growth occurred between the pH 6.5 (5.32 g/20mL) and 6.54 (5.42 g/20mL) while the highest cellulase activity was observed at pH 6.5 (11.43 FPU/g).

Control of pH is important for the optimisation of cellulase activity during a submerged fermentation of wheat straw using *R. variabilis* RS fungal strain. The result showed that pH control could improve the cellulase activity from 9.33 FPU/g obtained with no pH control to 11.43 FPU/g obtained from controlling pH at 6.5 (Figure 5.4). The optimum pH for cellulase enzyme production by *R. variabilis* RS was supported by the findings of Gautam et al. (2011) who reported optimum pHs for cellulase activity for *A. niger* and *Trichoderma* sp at 6.5.

Figure 5.6 shows the fungal wet weight profile has a correlation with the cellulase activity obtained with an increase in fungi wet weight, an increase in cellulase activity was observed while the cellulase activity declined as the fungi entered into its death phase.

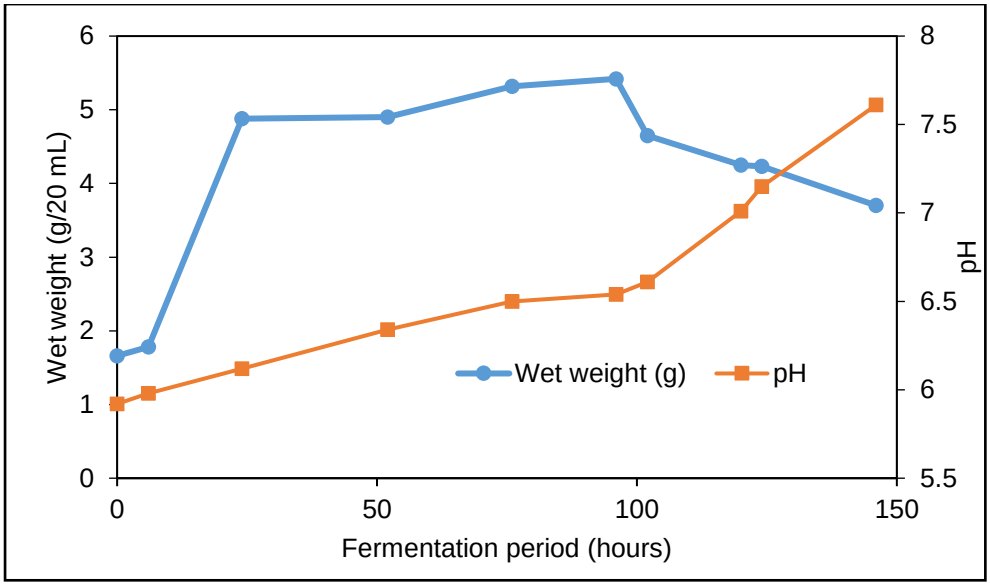


Figure 5.5 Biomass wet weight and pH profiles of *R. variabilis* RS in SmF, using 2 L bench top fermenter 28˚C, initial pH 5.92, agitation 300 rpm

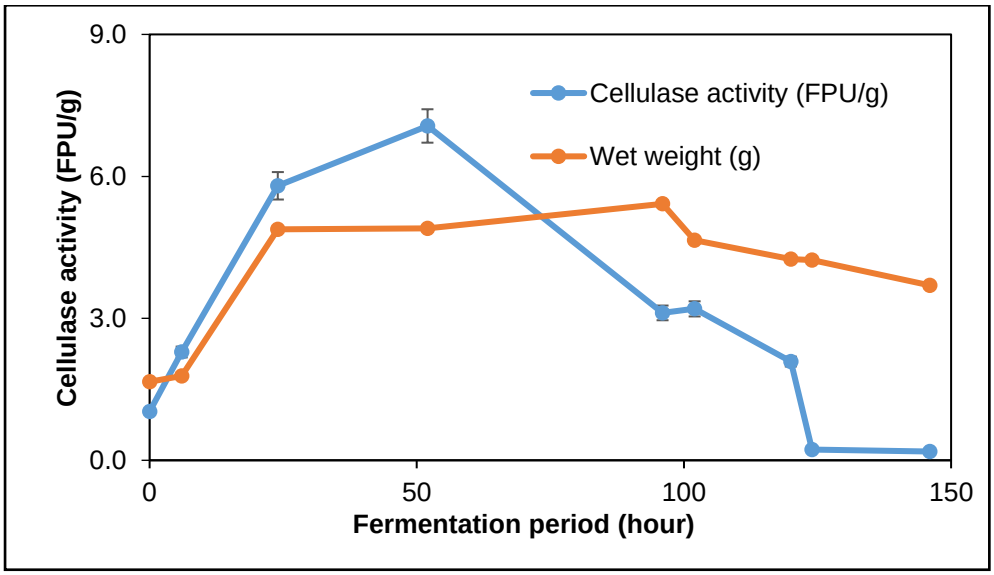


Figure 5.6 A correlated graph of cellulase activity and wet weight of *R. variabilis* RS in 2 L bench top fermenter 28˚C, initial pH 5.92, agitation 300 rpm.

5.1.4 Impact of mineral addition on cellulase activity and biomass production

Mineral medium (Table 5.1) was firstly designed based on literature (Pensupa et al., 2013); Bencerz et al., 2016; Yang et al., 2015), and the designed mineral solution

was named medium OM. A five-day culture using *R. variabilis* RS fungal strain with the designed mineral medium was carried out at 28˚C with 250 mL shake flask and compared with mineral medium from the selected literatures above.

Table 5.1 The composition of various mineral solutions used

Mineral medium	Composition	Reference
A	Glucose 10 g/L, YE 5 g/L, $(NH_4)2SO_4$ 1 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, $MgSO_4$ 0.2 g/L	Pensupa et al. (2013)
B	Glucose 10 g/L, Urea 4 g/L, KH_2PO_4 6 g/L, $MgSO_4.7H_2O$ 1 g/L, $FeCl_3.4H_2O$ 10 mg/L	Bancerz et al. (2016)
C	Glucose 5 g/L, YE 10 g/L, KH_2PO_4 1 g/L, $MgSO_4.7H_2O$ 0.3 g/L, $CaCl_3$ 0.3 g/L	Yang, Xiong, Yang, Yan, and Jiang (2015)
OM	Glucose 10 g/L, YE 10 g/L, KH_2PO_4 1 g/L, $MgSO_4.7H_2O$ 0.5 g/L, $FeCl_3.4H_2O$ 0.01 g/L, $CaCl_3$ 0.3 g/L	Designed in this study

The result obtained as shown in Figure 5.7 and Figure 5.8, showed that most samples at day 2 had the highest cellulase activitiy and wet weight biomass concentration. Mineral medium B and OM gave the highest cellulase activity on day 2 of the fermentation (19.07 FPU/g & 11.44 FPU/g) respectively (Figure 5.7). Day 2 of fermentation gave the highest biomass wet weight for all the mineral solution used (Figure 5.8). The result obtained showed that addition of different mineral solution improved cellulase activity and biomass wet weight was not directly proportional. Addition of mineral B and OM improved the cellulase activity from 9.33 FPU/g (Figure 5.1) to 19.07 and 11.44 FPU/g respectively, after the fermentation had run for 2 days (Figure 5.7). Statistical assessment using one way ANOVA showed that the cellulase activity result was significantly different under 95% confidence level when mineral medium was added.

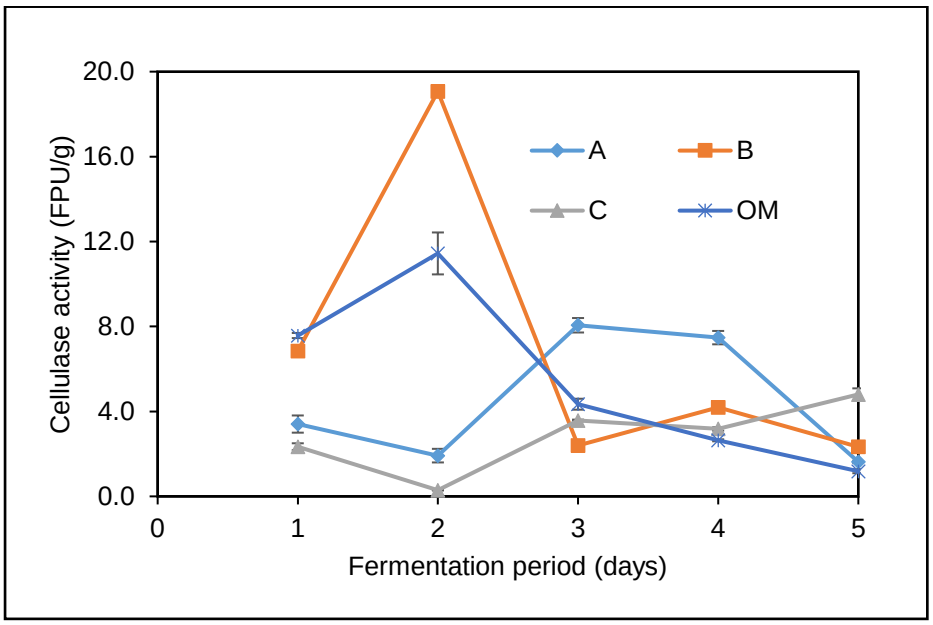


Figure 5.7 Impact of different mineral media on cellulase activity in SmF, shake flasks, 250 mL bottles, 28˚C, and 200 rpm. The legend represents the mineral media listed in Table 5.1.

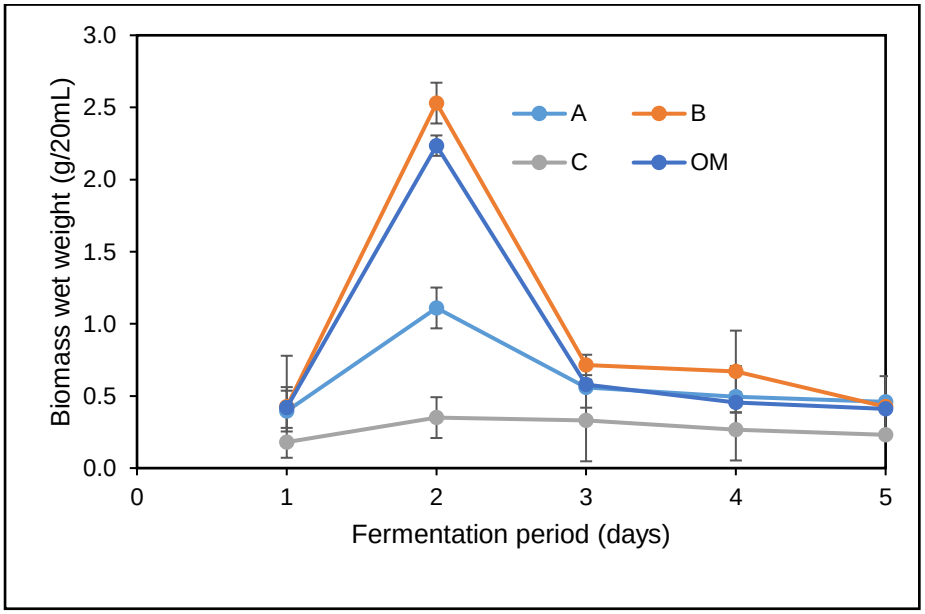


Figure 5.8 Impact of different mineral media on biomass wet weight on SmF shake flasks, 250 mL bottles, 28˚C, and 200 rpm. The legend represents the mineral media listed in Table 5.1.

5.1.5 Impact of nitrogen source on cellulase activity

Nitrogen sources play an important role in the growth of organisms and enzyme production and they are also used as the secondary energy sources by organisms. The effect of different nitrogen sources on the production of cellulase enzyme in SmF by *R. variabilis* RS was investigated. The nitrogen sources tested were ammonium sulphate $(NH_4)_2SO_4$, tryptone, sodium nitrate $NaNO_3$, urea and ammonium chloride NH_4Cl (all at 2 g/L).

Results revealed that the addition of nitrogen source has a positive effect on cellulase activity (Figure 5.9). An increase in cellulase activity from 8.21 to 12.09 FPU/g was observed with $(NH_4)_2SO_4$ as the fermentation progressed. The addition of tryptone led to the highest cellulase activity in day 3 (18.44 FPU/g) and a decline in cellulase activity was observed on day 5 of fermentation. A decline in cellulase activity was observed when urea and NH_4Cl was added, as the fermentation time progressed (7.94 to 5.20 FPU/g & 15.30 to 9.45 FPU/g) respectively. Although a higher cellulase activity was obtained with NH_4Cl on day 1 of fermentation, addition of tryptone has a more positive impact on cellulase activity than addition of any other nitrogen source used for RS fungal strain for cellulase enzyme production. Statistically, addition of tryptone was significantly different on cellulase activity when compared with addition of $NaNO_3$ and urea at 95% confidence level. Addition of urea, $(NH_4)_2SO_4$ and NH_4Cl were also significantly different from $NaNO_3$, however, there was no significant differences when $NaNO_3$, Urea and $(NH_4)_2SO_4$ were used as nitrogen source.

Tryptone was the best nitrogen source, which gave the highest yield of cellulase enzyme under SmF. It was reported by Elsebaay, Shoukry, Hassan, and Hany (2018) that organic nitrogen sources were better induces than inorganic ones for cellulase production from two *Pleurotus* mushroom species. Sodium nitrate ($NaNO_3$) has also been reported to produce lesser quantity of cellulase enzyme from *A. niger* by Gautam et al. (2011) which indicate similar trend with the result obtained in Figure 5.9.

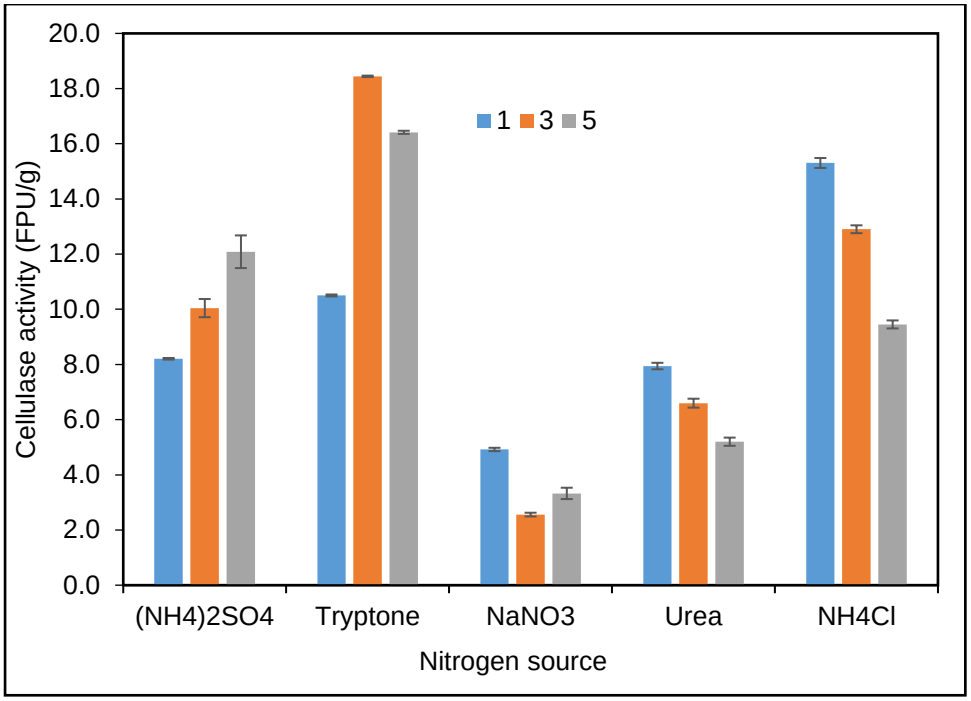


Figure 5.9 Impact of nitrogen source on cellulase activity, no minerals, shake flasks, 250 mL bottles, 28˚C, and 200 rpm using *R. variabilis* RS. The legend represents the fermentation days.

5.1.6 Impact of temperature on cellulase activity and biomass production

The effect of temperature on cellulase activity was determined by incubating *R. variabilis* RS in a 250 mL shake flask at a range of temperatures (26, 28, 30 and 32˚C), for 3 days. Temperature is one of the factors that affect enzyme production and stability, it also affects fungal growth as well.

The results obtained at different temperatures showed that the optimal temperature for cellulase activity produced by *R. variabilis* RS (14.37 FPU/g) was 26˚C (Figure 5.10). A decline in cellulase activity was observed as temperature increased. Different temperatures had been employed for cellulase enzyme production using different fungi strains, which suggests that the optimal temperature for cellulase production also depends on the strain variation of the microorganism (Gautam et al., 2011).

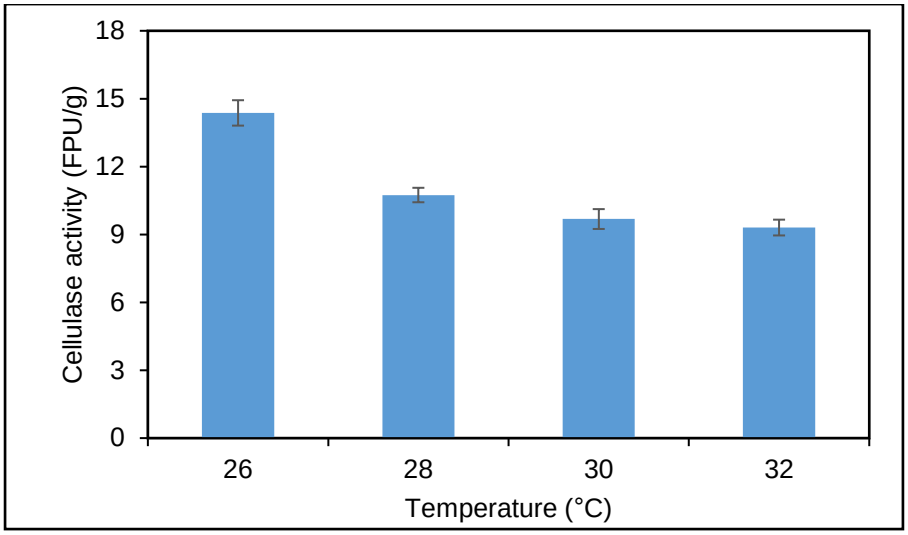


Figure 5.10 Impact of temperature on cellulase activity for 3 days in shake flasks, 250 mL bottles and 200 rpm.

The optimum biomass wet weight was obtained on day 4 with temperature at 26˚C, 28˚C, 32˚C (0.63, 0.62, 0.55 g/20mL) respectively while 30˚C was on day 5 (0.56 g/20mL) as shown in Figure 5.11. The biomass wet weight for 26˚C and 32˚C was similar on the 4th and 5th day of fermentation (0.56 g/20mL). The impact of temperature on biomass wet weight has no significant difference. Although the cellulase activity obtained shows that 26˚C was higher as shown in Figure 5.10.

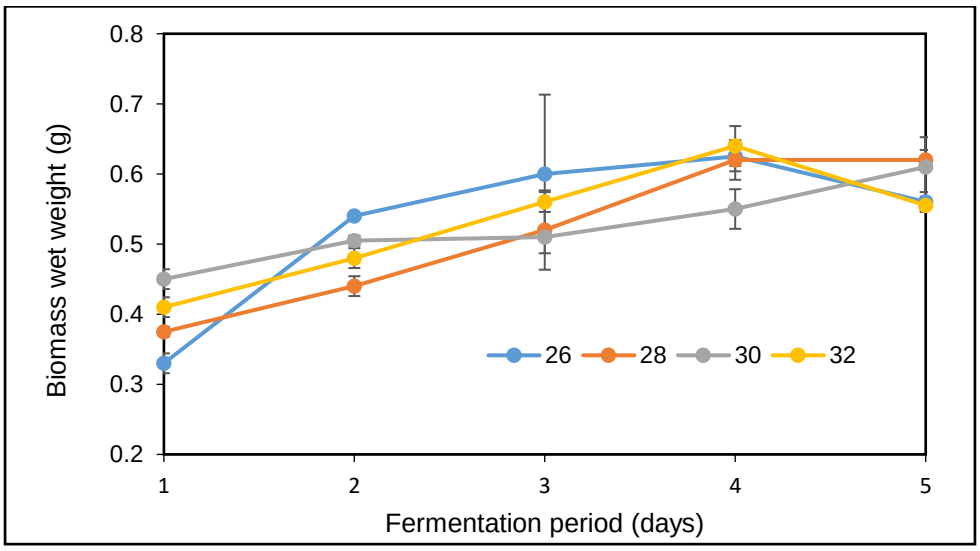


Figure 5.11 Impact of temperature on biomass wet weight in shake flasks, 250 mL bottles and 200 rpm. The legend represents the temperature (˚C).

5.1.7 Impact of substrate concentration on cellulase activity

Modified wheat straw was used to analyse the effect of substrate concentration on cellulase enzyme production by *R. variabilis* RS fungal strain. Substrate concentration of 2%, 6% and 12% was found to be optimized for maximum cellulase activity of 0.90 FPU/g, 1.29 FPU/g and 1.86 FPU/g on day 3 of fermentation respectively (Figure 5.12). Substrate concentration of 10% gave the optimum cellulase activity of 4.09 FPU/g on day 3 of fermentation. The substrate was very sticky, therefore, 12% was used instead of the 14% planned.

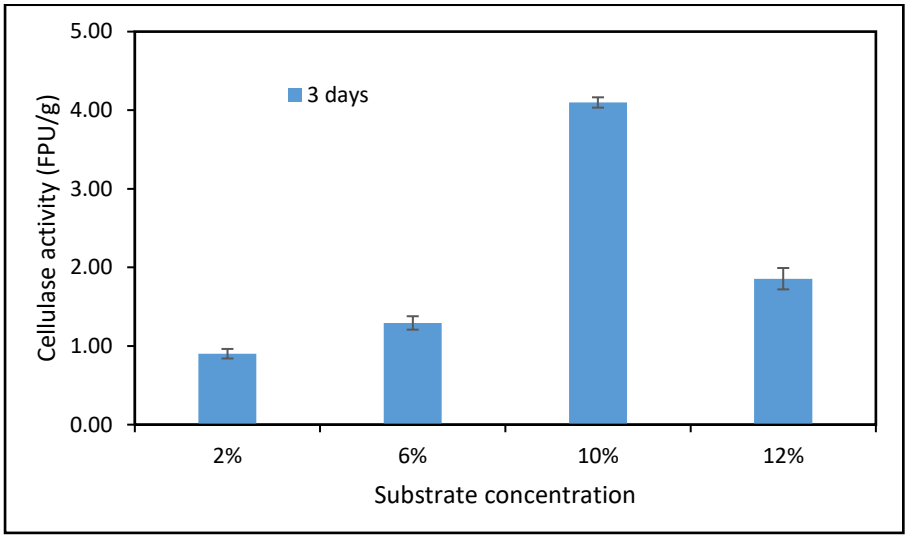


Figure 5.12 Impact of substrate concentration on cellulase activity in SmF, shake flasks, 250 mL bottles of 100 mL working volume, 28 ˚C, and 200 rpm for 3 days.

5.1.8 RSM for cellulase enzyme production in SmF

RSM was performed to optimise cellulase enzyme production from RS under four numeric factors (chapter 3.13). The four numeric factors were set in horizontal level and the design of each factor was as listed in Table 5.2. The specific condition of each run was as listed in Appendix i.

Table 5.2 Central composition design of factors on SmF for cellulase enzyme production

Numeric factor	Unit	Low value	High value	-alpha	+alpha
Substrate concentration	%	8	12	6	14
Tryptone	g	0.02	0.04	0.01	0.05
pH		6.0	7.0	5.5	7.5
Temperature	˚C	24	28	22	30

The cellulase activity obtained was in range of 0.99 – 23.81 FPU/g. The run standard order 3 (8% substrate concentration, 0.04% tryptone, pH 6 and 24˚C) gave the highest cellulase activity of 23.81 FPU/g (Figure 5.13). The model was identified as insignificant under ANOVA. According to the coefficient of each factor, the order of importance was “Temperature > Tryptone > pH > Substrate concentration”.

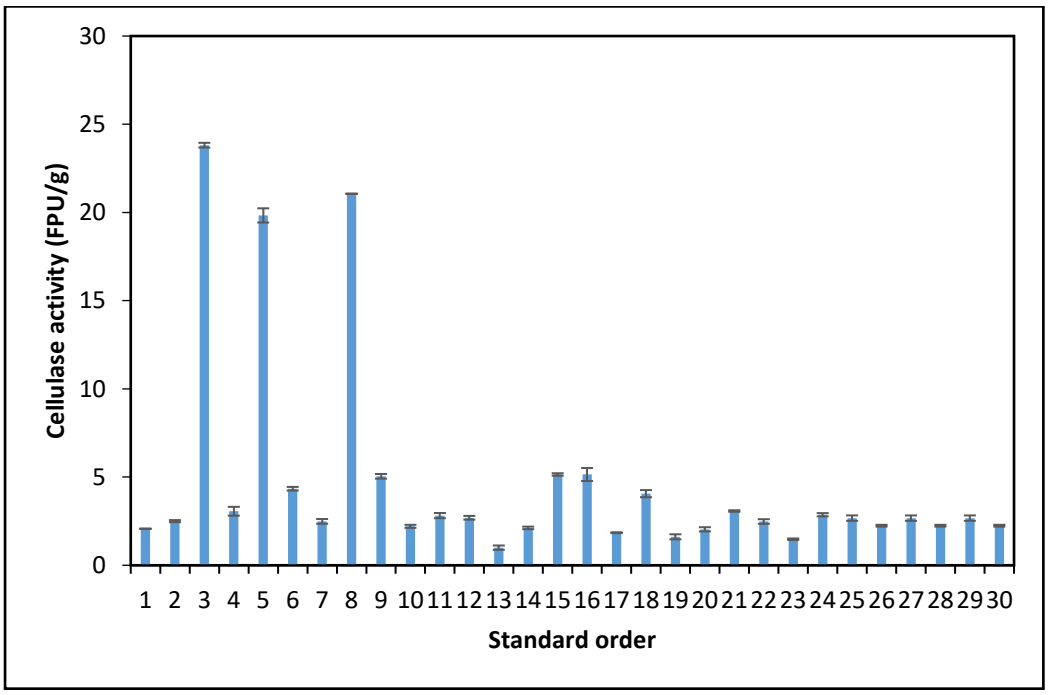


Figure 5.13 Cellulase enzyme production optimisation in SmF using 250 mL shake flask for 3 days. Central points (standard order of 25 to 30) substrate concentration 10%, Tryptone concentration 0.3g, pH 6.5, and temperature 26˚C.

The response surface plot shows the effect of the parameters used in optimisation of cellulase and their mutual effect on cellulase activity under RSM were examined using a 3D graph (Figure 5.14 – Figure 5.16). As the substrate concentration and pH increased, pH up to pH 7 had little effect on cellulase activity optimisation, while there was no positive effect on cellulase production as substrate concentration increased. The effect of substrate concentration, tryptone and their mutual effect on the production of cellulase resulted in an increased cellulase activity as tryptone concentration was increased with substrate concentration having no effect on cellulase activity. However, the combined effect of substrate concentration and temperature had a positive effect on cellulase activity at the starting point, while an increase in temperature resulted in a low cellulase activity.

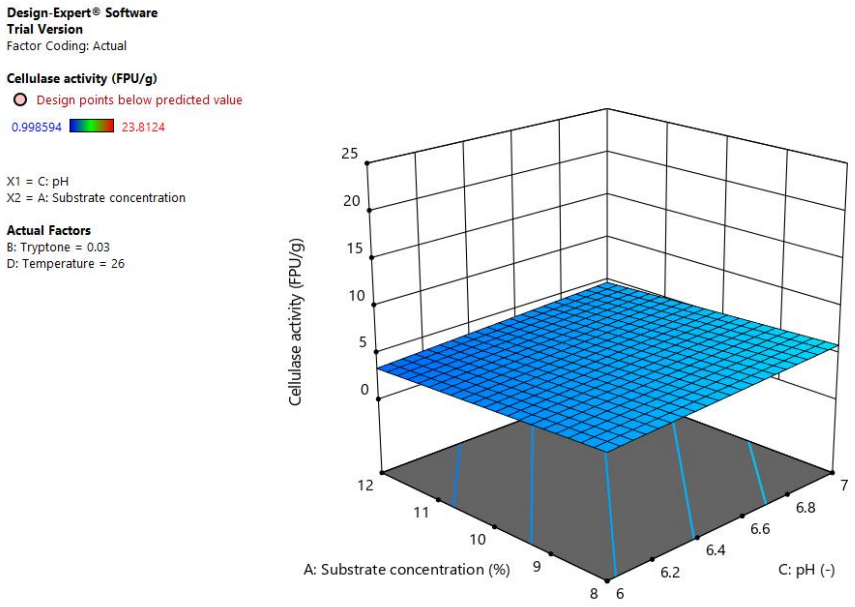


Figure 5.14 Response surface plot showing the effect on substrate concentration, pH and their mutual effect on the production of cellulase (FPU/g).

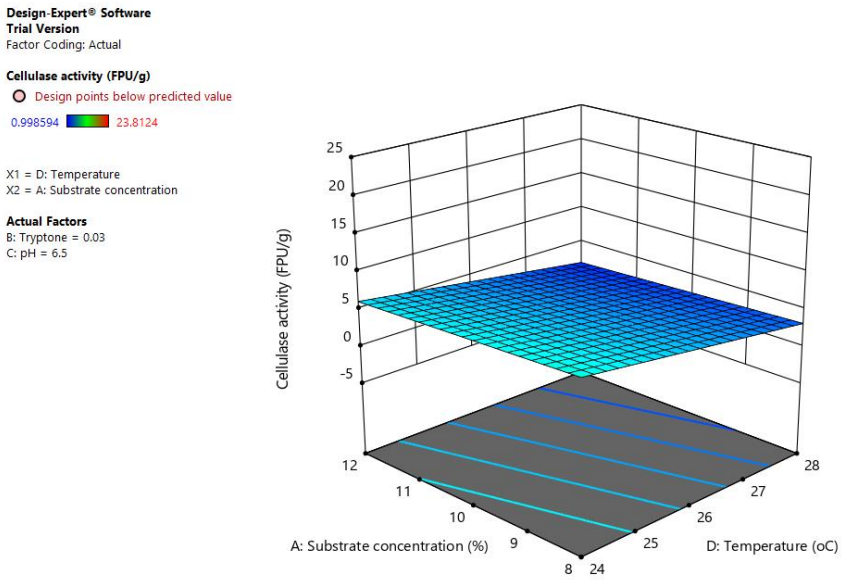


Figure 5.15 Response surface plot showing the effect on substrate concentration, temperature and their mutual effect on the production of cellulase (FPU/g).

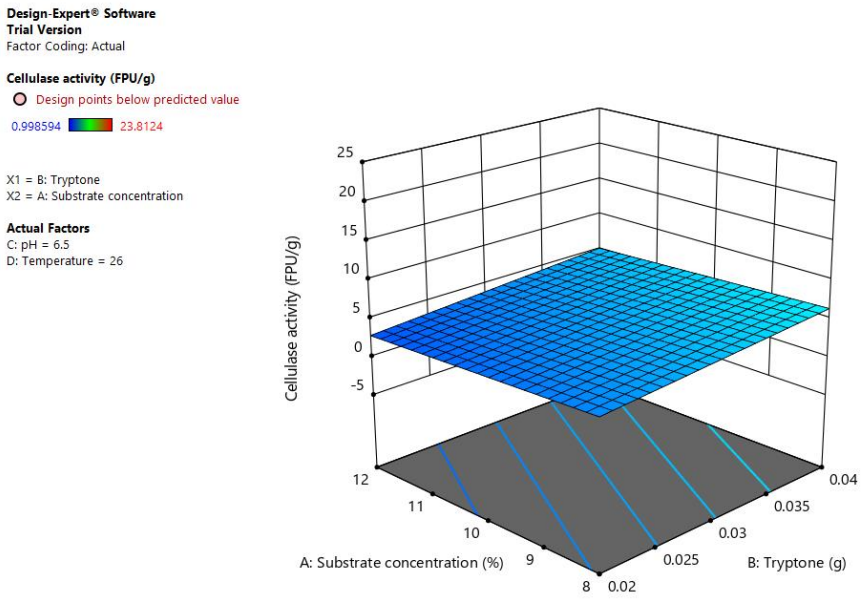


Figure 5.16 Response surface plot showing the effect on substrate concentration, tryptone and their mutual effect on the production of cellulase (FPU/g).

5.2 Optimisation of cellulase activity production in solid-state fermentation from *Rhizomucor variabilis* (RS)

The cellulase production by RS under solid-state fermentation of alkali soak modified wheat straw was investigated. In order to optimise the cellulase activity from RS, various operation parameters such as incubation period, pH, temperature, inoculation rate, moisture content and nitrogen source were conducted in SSF to determine their impacts on cellulase activity for optimum enzyme production.

5.2.1 Determination of incubation time on cellulase production by solid-state fermentation of *R. variabilis* RS

The effect of incubation time on cellulase production by SSF using *R. variabilis* RS was investigated for 7 days. The addition of mineral (K_2HPO_4 2.5 g/L, NH_4NO_3 1.5 g/L, (KH_2PO_4 1.5 g/L, $MgSO_4$ 0.12 g/L and NaCl 0.25 g/L) to the substrate was performed due to an increase in enzyme production obtained with addition of mineral solution observed in previous SmFs. Cellulase activity was measured on days 1, 3, 5 and 7 respectively. Result obtained in Figure 5.17 showed that an increase in cellulase activity with optimum cellulase activity of 19.83 FPU/g was obtained until day 5. A decline in cellulase activity was observed as incubation time increased, which might be due to depletion of nutrients in the medium. The result obtained shows an improved cellulase obtained when compared to when no mineral was added in SSF in Figure 4.15 (2.43 FPU/g).

Gautam et al. (2011) reported optimum incubation period of 3 to 5 days for enzyme production with *A. niger* and *Trichoderma* sp. Cellulase production by *A. flavus* AT-2 and *A. niger* AT-3 were reported by Dutt and Kumar (2012) to have attained maximum enzyme production on the 5th day. The results obtained from literature corresponded to the result obtained in Figure 5.17 with optimum enzyme production from *R. variabils* RS on the 5th day. Cellulases are part of the primary metabolites, which are produced during the exponential phase of growth and on the onset of the death phase, the enzyme secretion starts decreasing (Dutt & Kumar, 2012).

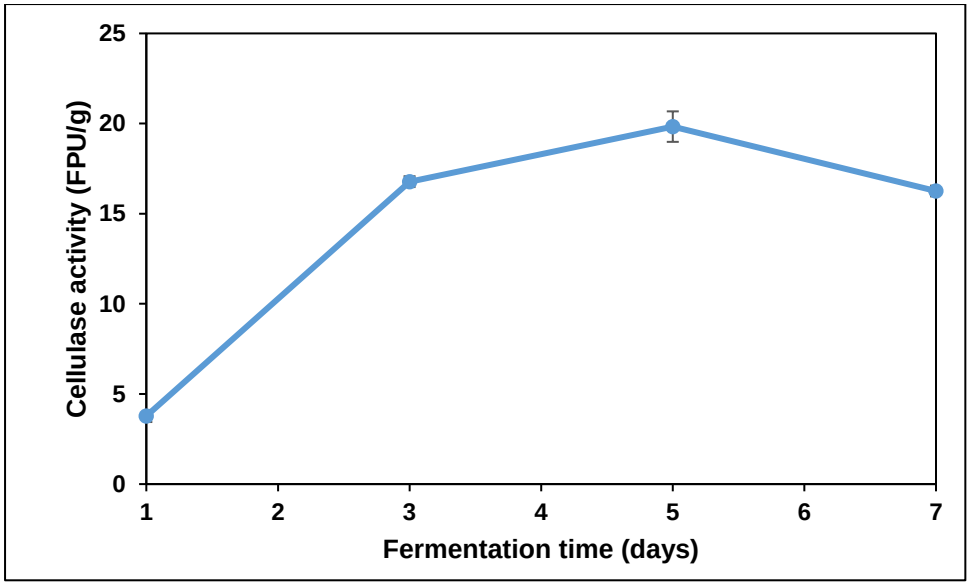


Figure 5.17 Fermentation profile of cellulase activity under SSF in Petri-Dishes over 7 days at 28°C.

5.2.2 Impact of pH on cellulase production

The impact of starting pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5 & 7.0) on cellulase activity by *R. variabilis* RS was investigated over 5 days at 28˚C and 85% moisture content. Results revealed that cellulase activity was highest in pH 6.0 – 7.0 (Figure 5.18). The optimal pH for cellulase production in SSF by RS was found to be 7.0 (10.01 FPU/g) without mineral addition. This suggested that cellulase produced by RS was able to maintain enzyme activity stability at a wide range of pH values during a SSF. The variation of pH below the optimum level might have resulted in enzyme denaturation, which reduced the enzyme synthesis ability.

Different optimum pH had been reported for various fungi employed for cellulase production. Optimum pH was reported between pH 4 to 6 by Maurya, Singh, Pratap, and Maurya (2012) for cellulase production with *T. reesei* NCIM 992. El-Sersy, Abd-Elnaby, Gehan, Ibrahim, and Nabil (2010) reported that *S. ruber* showed a high enzyme activity at a broad range of pH values 5.5 to 7 with an optimal pH of 6. They also reported about 50% decrease in enzyme production at pH 9.

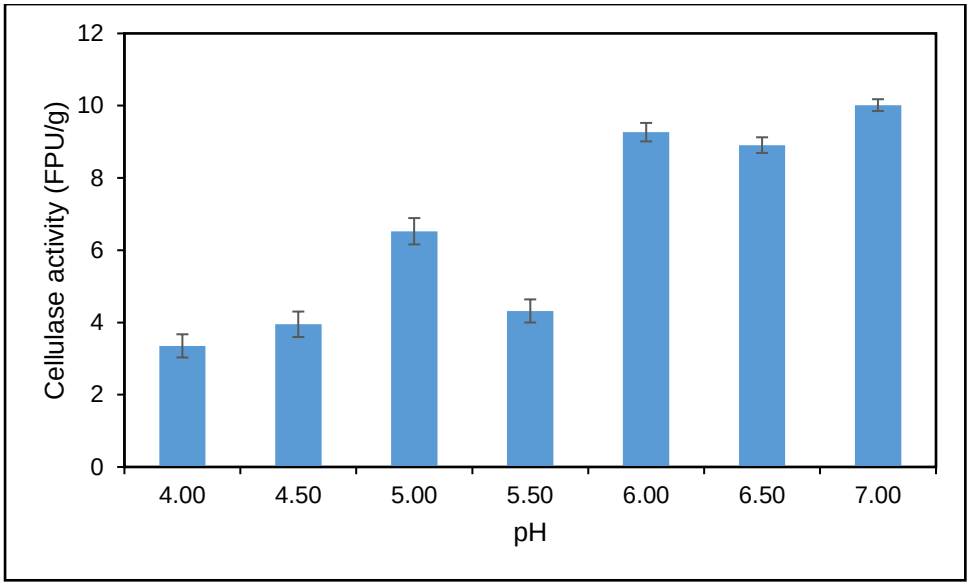


Figure 5.18 Impact of pH on cellulase activity under SSF in Petri-Dishes over 5 days of fermentation at 28°C.

5.2.3 Impact of temperature on cellulase production

The impact of temperature on cellulase production by *R. variabilis* RS under SSF was investigated. The cellulase activity was determined after inoculation for 5 days at different culture temperatures (26, 28, & 30˚C) respectively. Results showed that the cellulase activity slightly increased from 26˚C – 30˚C. The maximum cellulase activity of *R. variabilis* RS was recorded at 28˚C with celluase activity of 12.06 FPU/g (Figure 5.19). There is no significant difference with the results obtained.

The decrease in cellulase activity at lower temperature might be due to lower transport of substrate across the cells (Dutt & Kumar, 2012). Different temperatures for maximum cellulase activity have been reported in either flask or fermenter using *Aspergillus* sp and *Trichoderma* sp with the suggestion that the optimal temperature for cellulase production depends on the strain variation of the microorganism (Lu, Li, & Wu, 2003; Sakamoto, Hayashi, Moriyama, Arai, & Murao, 1982).

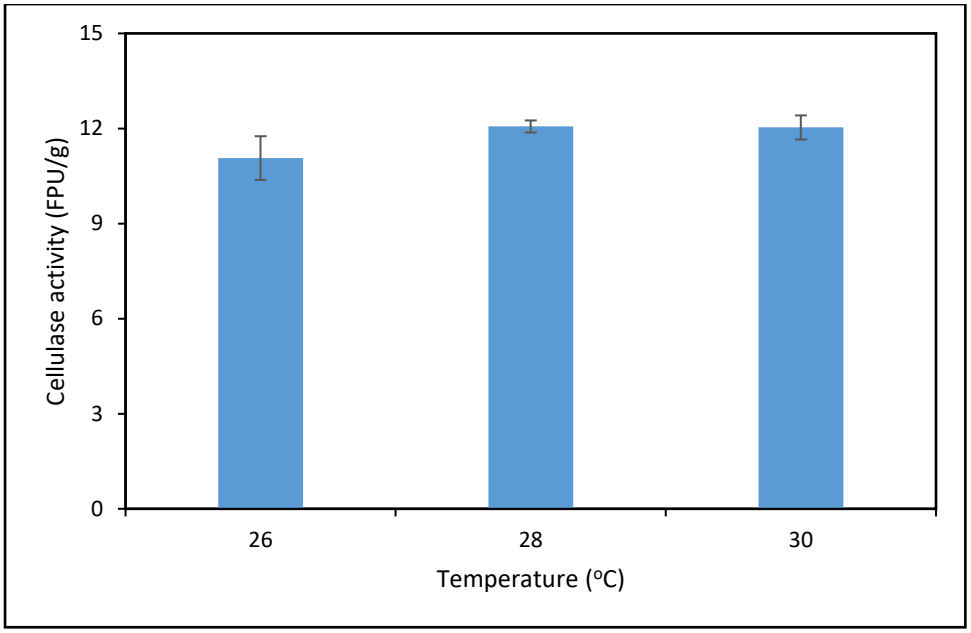


Figure 5.19 Impact of temperature on cellulase activity under SSF in Petri-Dishes over 5 days of fermentation at 28°C.

5.2.4 Impact of inoculation size on cellulase production

The effect of inoculum size on cellulase production by *R. variabilis* RS was examined using spore inoculations of $5x10^6$ & $1x10^7$ spores/g dry weights of wheat straw. The maximum cellulase activity (12.44 FPU/g) was obtained in 5 days of fermentation using $1x10^7$ spores/g (Figure 5.20). The inoculation size was not increased in this experiment as it was covered in the RSM. Furthermore, literatures showed that extra high inoculation size resulted in significant decrease in recovered cellulase activity (Abdullah, Greetham, Pensupa, Tucker, & Du, 2016; Mrudula & Murugammal, 2011). The decrease in cellulase activity with an increase in inoculation size could be due to the creation of anaerobic conditions or nutritional imbalance because of clumping of the cell due to more rapid growth of the microorganisms (Abdullah et al., 2016).

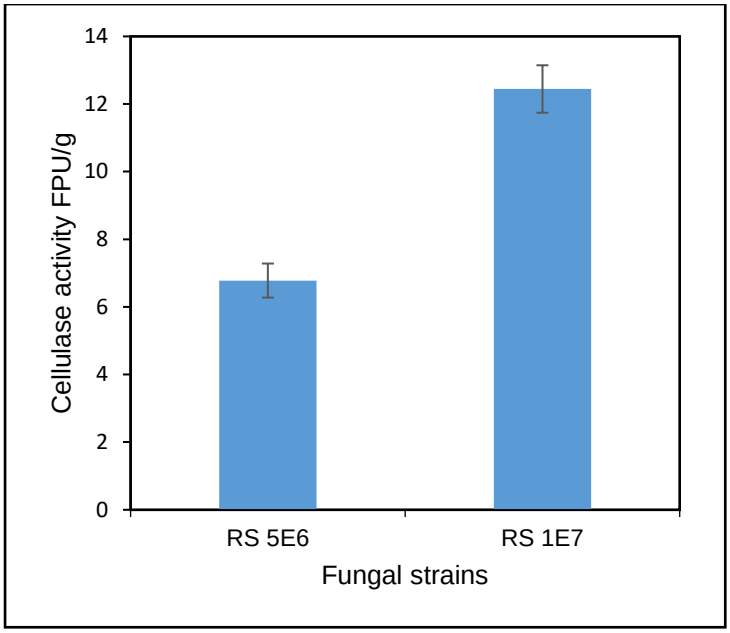


Figure *5.20* Impact of inoculation rate *on cellulase activity in SSF for 5 days Petri-Dishes at 28°C.** 5E6 means $5x10^6$ spores/g; 1E7 means $1x10^7$ spores/g

5.2.5 Impact of moisture content on cellulase production

The impact of moisture content (60, 65, 70, 75, 80 and 85%) on cellulase production by *R. variabilis* RS was investigated under SSF. Alkali modified wheat straw was inoculated with *R. variabilis* RS spores at a concentration of $1x10^7$ spores/g and left to ferment for 1, 3 and 5 days respectively. As the moisture content increased, the cellulase activity also increased as the fermentation progressed with 70 and 80% moisture content. There was a decrease in cellulase activity on day 5 with 65 and 85% moisture content while 60 and 75% moisture content had a decrease in cellulase activity on day 3 with slight increase in cellulase activity on day 5. The optimum cellulase activity of 2.76 FPU/g was obtained on day 5 with 80% moisture content as shown in Figure 5.21.

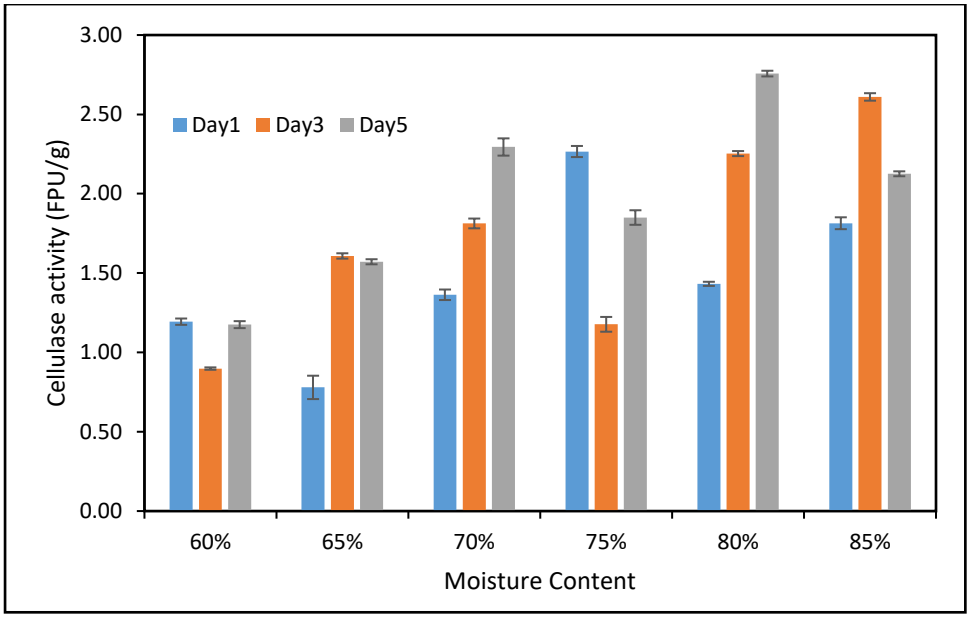


Figure 5.21 Impact of moisture content on cellulase activity under SSF in Petri-Dishes at 28°C.

Moisture content is generally considered as one of the most important factors which influences growth, oxygen transfer, nutrient accessibility and secretion of enzyme in SSF process efficiency (Abdullah et al., 2016; Mrudula & Murugammal, 2011; Pirota et al., 2016). High moisture promotes fungal growth, nutrient transportation and enzyme activities, thus limiting transfer of oxygen (Mrudula & Murugammal, 2011). Pirota et al. (2016) have evaluated the effect of initial moisture content on cellulase production by *A. oryzae* using SSF. Their result showed that 70% initial moisture content favoured cellulase enzyme production. Abdullah et al. (2016) investigated the impact of moisture content on cellulase production by both *T.reesei* and *A. niger* using municipal solid waste as substrate under SSF. Their results showed that 60% moisture content resulted in the highest cellulase activity after 5 days of fermentation with *T. reesei*.

The difference in optimum moisture content for cellulase production from different literatures could be due to the different substrates used and the moisture tolerance of the fungal strains employed. From the result obtained in Figure 5.21, lower moisture content resulted in lower cellulase activity. This is in accordance with Lonsane, Ghidyal, Budiatman, and Ramakrishna (1985) findings that lower moisture content causes reduction in the solubility of substrate nutrients while higher moisture levels can cause a reduction in enzyme yield because of steric hindrance of the

fungal growth strain thus reducing the solid matrix porosity and interfering oxygen transfer.

5.2.6 Impact of nitrogen source on cellulase enzyme production

Different nitrogen sources such as urea, ammonium chloride, ammonium sulphate, tryptone, yeast extract (YE) and sodium nitrate were incorporated in the medium at a 0.3% (w/v). The medium was incubated with *R. variabilis* RS for 5 days in SSF to determine the impact of the nitrogen sources on cellulase production. As shown in Figure 5.22, the results showed that organic nitrogen sources of tryptone and yeast extract were better choice for *R. variabilis* RS for cellulase production. The highest cellulase activity of 30.19 FPU/g was obtained in assays with addition of tryptone. Tryptone and yeast extract had the highest impact on cellulase activity in SSF when compared with other nitrogen sources used. There was no obvious difference among different inorganic nitrogen sources on enzyme production by *R. variabilis* RS. This result indicates that the selectivity of *R. variabilis* RS to inorganic nitrogen is not high although they had an impact in improving cellulase production compared to the results obtained without addition of nitrogen. Sakthivel, Karthikeyan, Jayaveny, and Palani (2010) reported that ammonium sulphate and sodium nitrate did not induce any enzyme production when *Cornybacterium lipophiloflavum* bacterium was incubated for 96 hours.

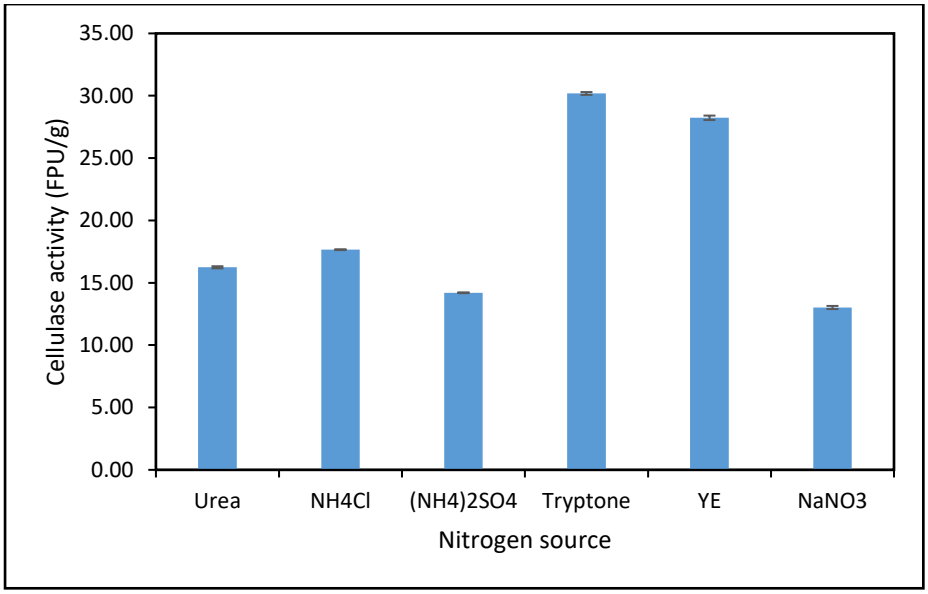


Figure 5.22 Impact of nitrogen source on cellulase activity under SSF in Petri-Dishes for 5 days at 28°C.

5.2.7 RSM for cellulase enzyme production in SSF

RSM was performed to optimise cellulase enzyme production from *R. variabilis* RS under four numeric factors in SSF. The four numeric factors were set in horizontal level and the design of each factor was as listed in Table 5.3. The specific conditions of each run was as listed in Appendix ii.

Table 5.3 Central composition design of factors on SSF for cellulase enzyme production

Numeric factor	**Unit**	**Low value**	**High value**	**-alpha**	**+alpha**
Moisture content	%	75	85	70	90
Tryptone	g	0.02	0.04	0.01	0.05
pH		5.5	6.5	5.0	7.0
Inoculation rate	Spores/g	$5x10^6$	$1x10^7$	$2.5x10^6$	$1.25x10^7$

The cellulase activity was in the range of 2.07 – 24.80 FPU/g. The run standard order 25 (80% moisture content, 0.03% tryptone, pH 6, inoculation rate of 7.5×10^6) gave the highest cellulase activity of 24.80 FPU/g (Figure 5.23). The model was identified as significant under ANOVA with the six central points (standard run 25 to 30) having the highest cellulase activity range. According to the coefficient of each factor, the order of importance should be “Moisture content > Tryptone > Inoculation rate > pH”

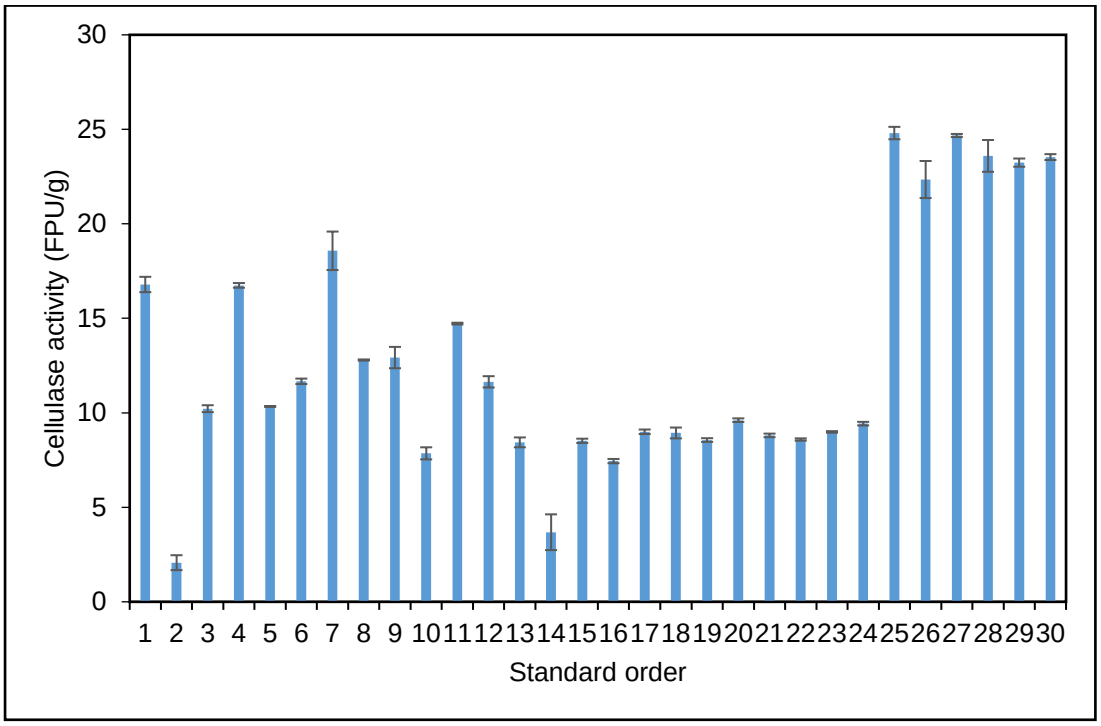


Figure 5.23 Cellulase enzyme production optimisation in SSF. Central points (standard order of 25 to 30) moisture content 80%, tryptone concentration 0.03g, pH 6, and inoculation rate 7.5×10^6 spores/g.

The combined effect of four selected factors on cellulase optimisation was determined using the response surface 3D graph (Figure 5.24 – Figure 5.29). At low concentration of tryptone and moisture concentrations, cellulase activity was low’ with an increase in cellulase activity observed as tryptone concentration and moisture content increased. At a higher concentration of tryptone (over 0.03g) and moisture content (above 80%) resulted in a decrease in cellulase activity (Figure

5.24). The combination of pH and moisture content on cellulase activity resulted in an increase in cellulase activity as moisture content increased, while there was less impact on cellulase activity as the pH was increased (Figure 5.25).

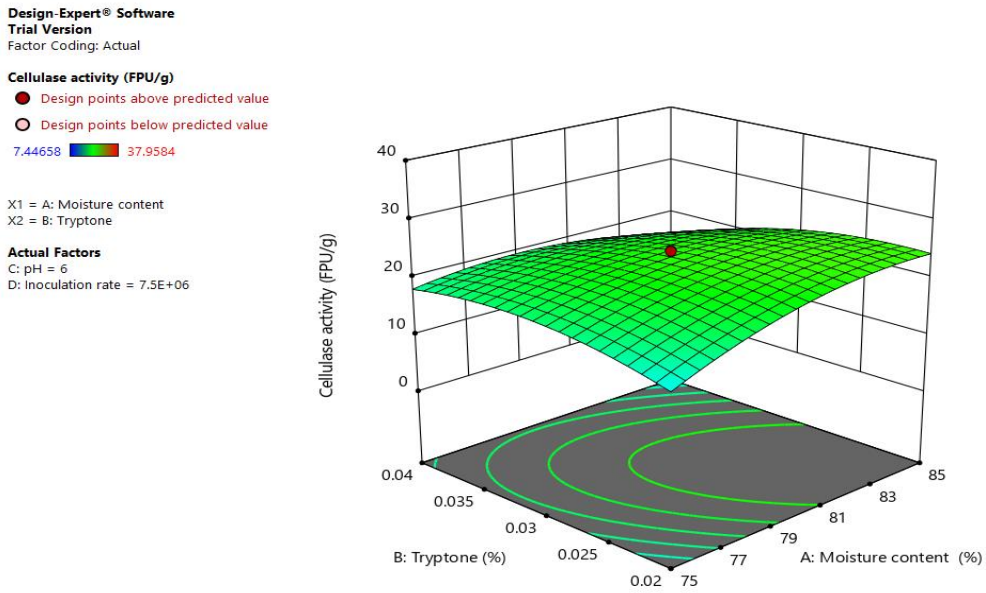


Figure 5.24 Response surface plot showing the effect on tryptone concentration, moisture content and their mutual effect on the production of cellulase activity (FPU/g).

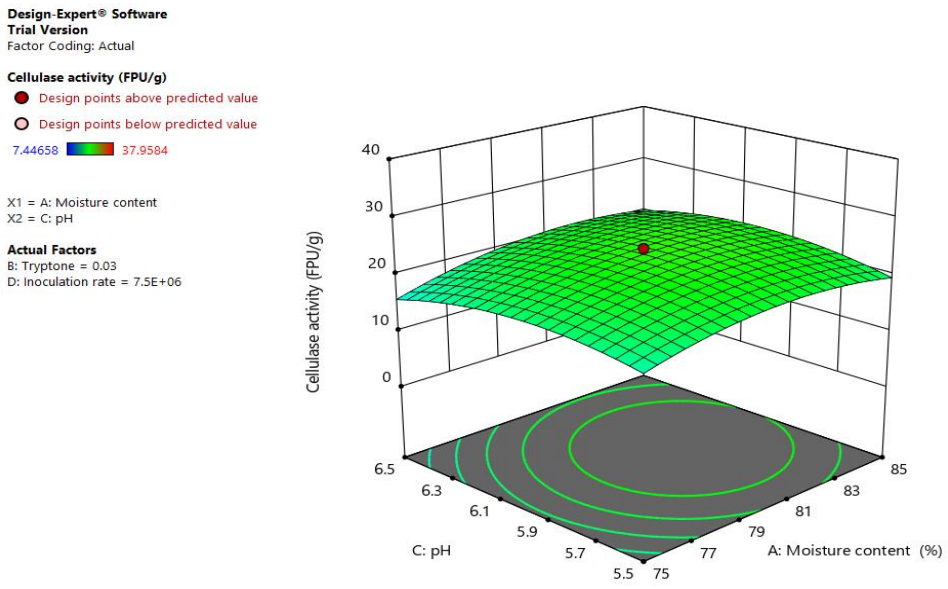


Figure 5.25 Response surface plot showing the effect on pH, moisure content and their mutual effect on the production of cellulase activity (FPU/g).

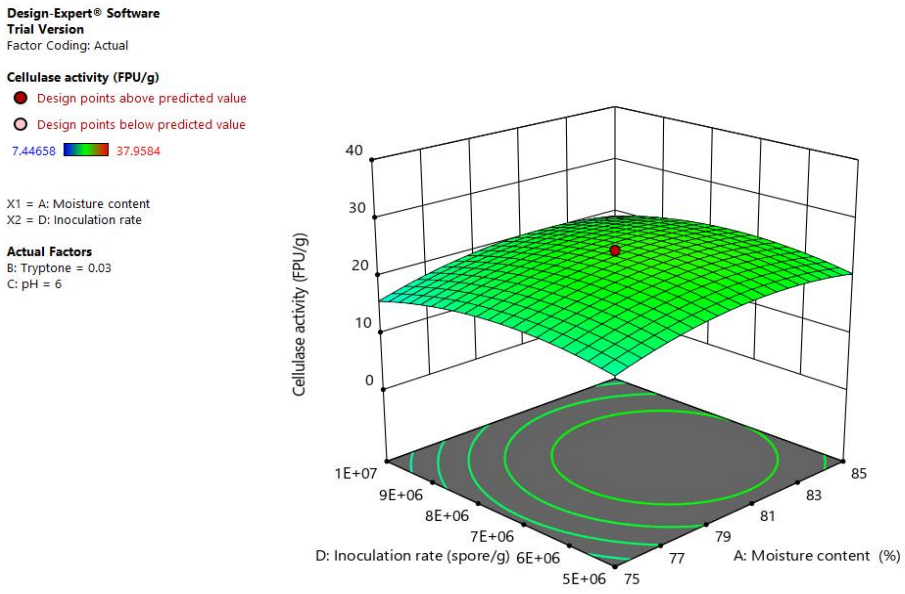


Figure 5.26 Response surface plot showing the effect on inoculaton rate, moisture content and their mutual effect on the production of cellulase activity (FPU/g).

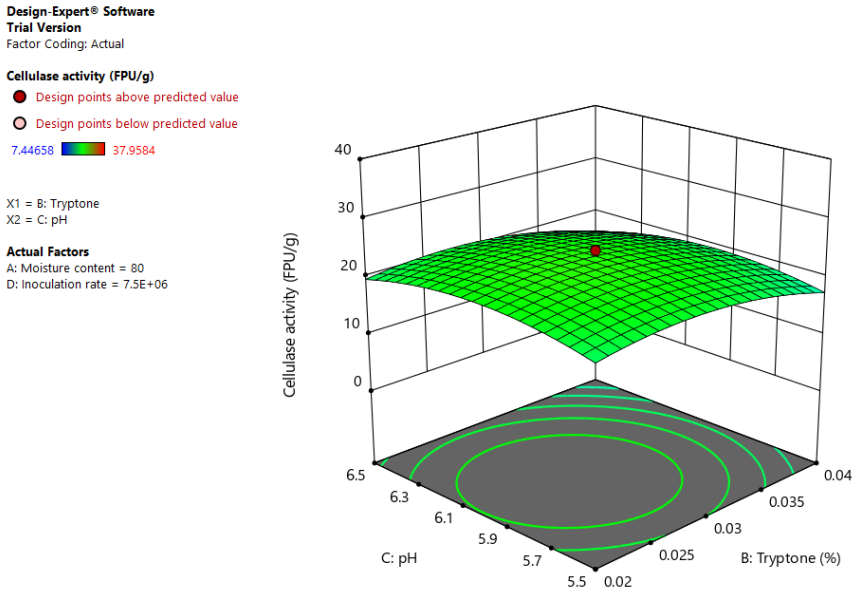


Figure 5.27 Response surface plot showing the effect on pH, tryptone concentration and their mutual effect on the production of cellulase activity (FPU/g).

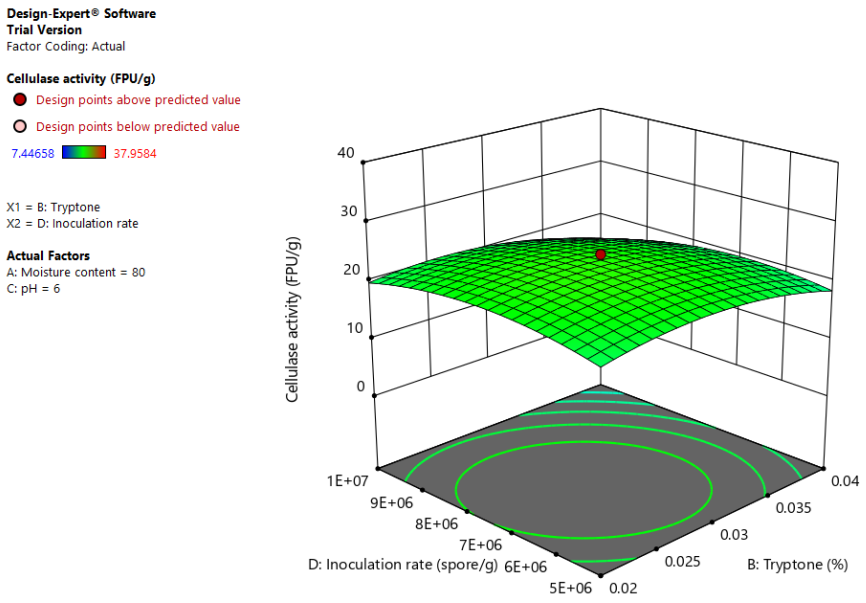


Figure 5.28 Response surface plot showing the effect on inoculation rate, tryptone concentration and their mutual effect on the production of cellulase activity (FPU/g).

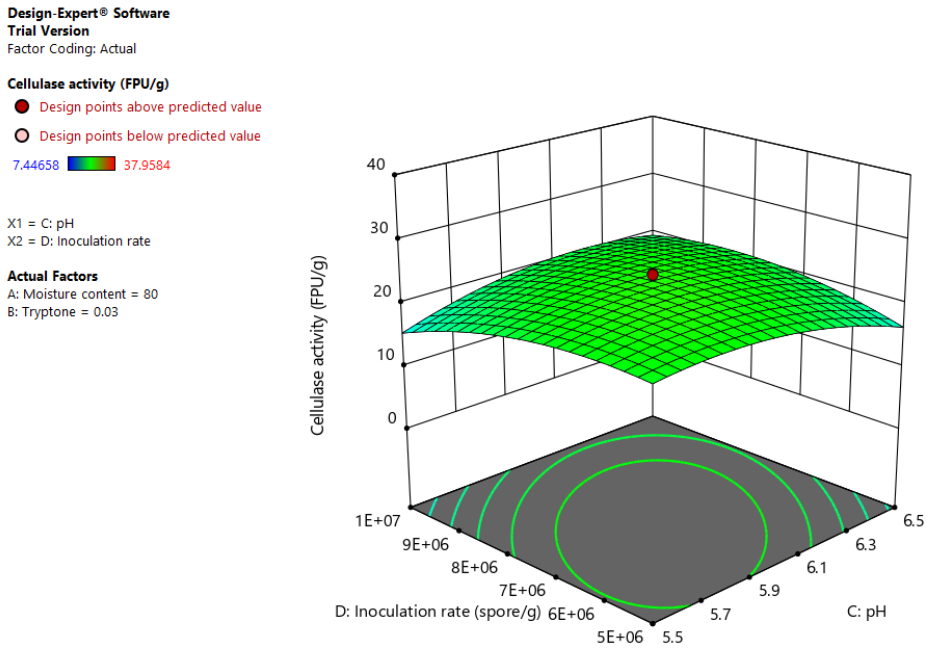


Figure 5.29 Response surface plot showing the effect on inoculaton rate, pH and their mutual effect on the production of cellulase activity (FPU/g).

5.3 Strain mutation and screening on cellulase production

A mutation is a change that occurs in DNA sequence because of environmental factors such as radiation by ultraviolet (UV) light. The strain mutation of *R. variabilis* RS fungal strain was investigated to determine if any alteration in its DNA through exposure to microwave (MW) and UV could improve cellulase enzyme production.

5.3.1 Impact of microwave, ultraviolet light and combination of microwave and ultraviolet light on the ability of RS to produce cellulase in a plate

The ability of *R. variabilis* RS fungal strain mutation to produce cellulase on a plate was analysed. The original and mutant *R. variabilis* RS fungal strains were first cultured on a PDA plate for 3 days to observe their surviving ratio. The *R. variabilis* RS strain mutation was carried out by exposure to MW at 800 watts for 10, 15 and 20 seconds, UV for 30 seconds and combination of MW & UV at 10 & 30 seconds and 20 & 30 seconds respectively (Figure 5.30). There was no growth on the PDA plate of RS strain exposed to MW at 20 seconds and MW & UV at 20 & 30 seconds respectively (Figure 5.31). This could be due to complete destruction of the *R. variabilis* RS spores at extreme high radiation heat from the microwave.

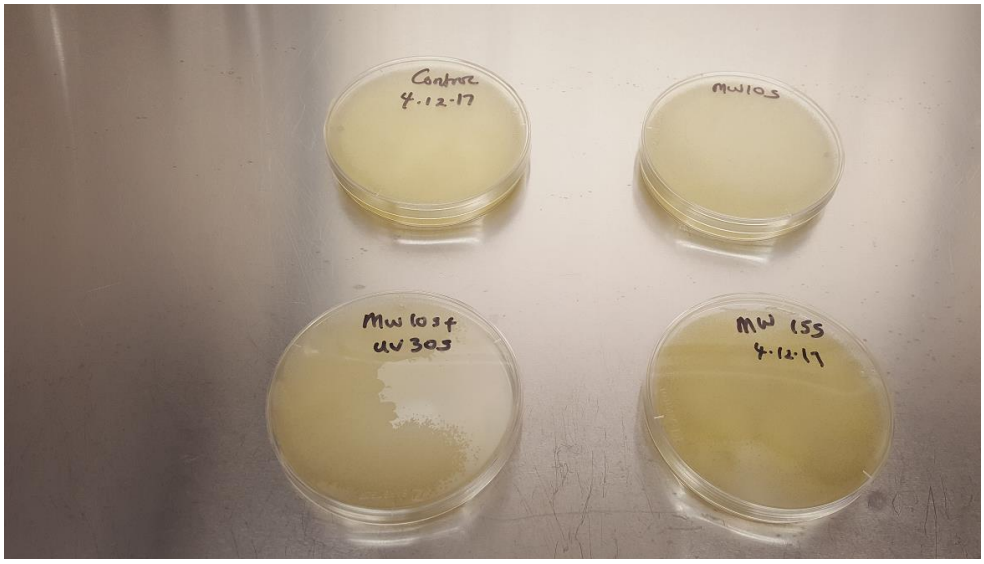


Figure 5.30: Cultured mutated *R. variabilis* RS strain on PDA plates for 3 days at 28˚C

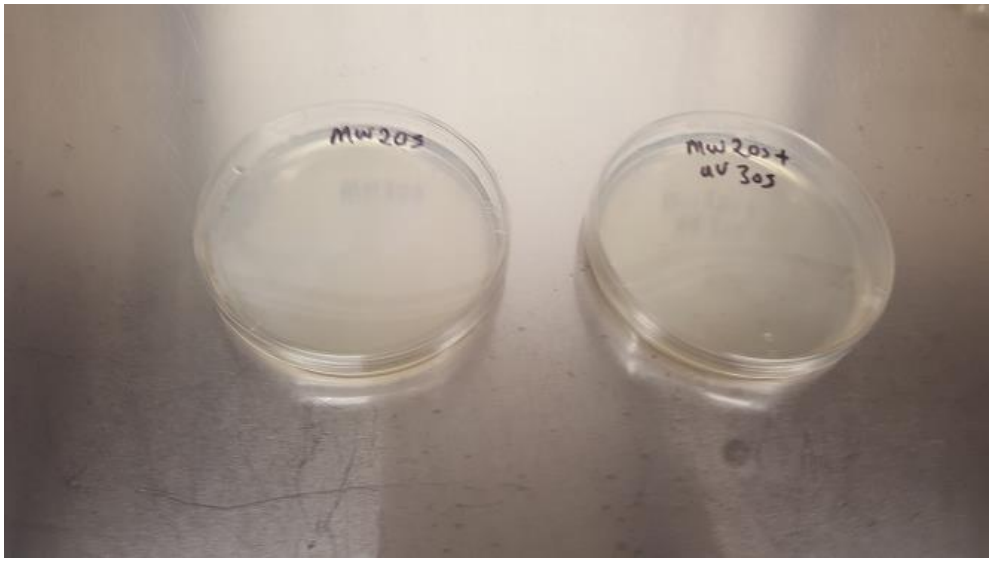


Figure 5.31 Mutated *R. variabilis* RS strain cultured on PDA plates for 3 days at 28˚C

The cultured mutated *R. variabilis* RS strains were analysed for their ability to produce cellulase on an agar plate (chapter 3.9.1). *R. variabilis* RS mutated strains were transferred on three different points on separate agar plates and were incubated at 28˚C for 3 days. The mutant *R. variabilis* RS strains were screened by the Congo red staining method. The Congo red was washed off with NaCl solution (1M) (Figure 5.32 – Figure 5.35). The stained zone on the PDA plate surrounding the colony and the colony diameters were measured (chapter 3.9.1), and the ratios were calculated in order to identify the best mutant strain for cellulase production.

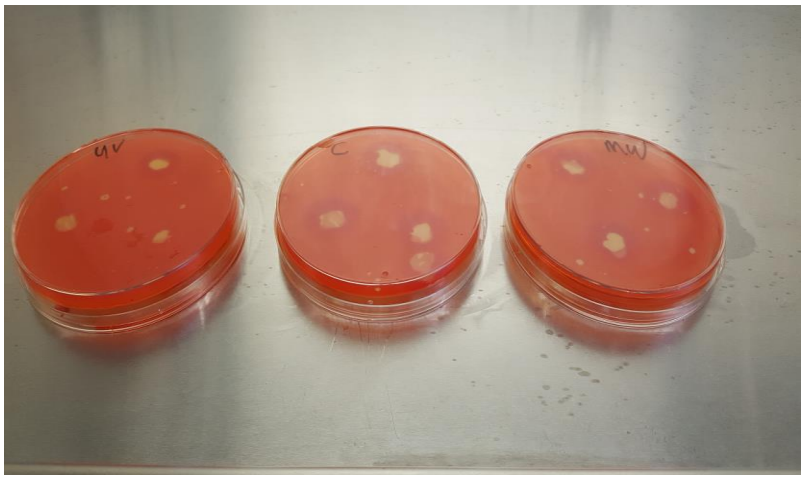


Figure 5.32 Mutant *R. variabilis* RS strains stained by Congo red on PDA (UV only (L), control (C) and MW (R))

Figure 5.33 Mutant *R. variabilis* RS strains stained by Congo red on PDA. The *R. variabilis* RS strains were exposed to microwave heat for 15 seconds

Figure 5.34 Mutant *R. variabilis* RS strains stained by Congo red on PDA. The *R. variabilis* RS strains were exposed to microwave heat for 10 seconds and then ultraviolet light for 30 seconds

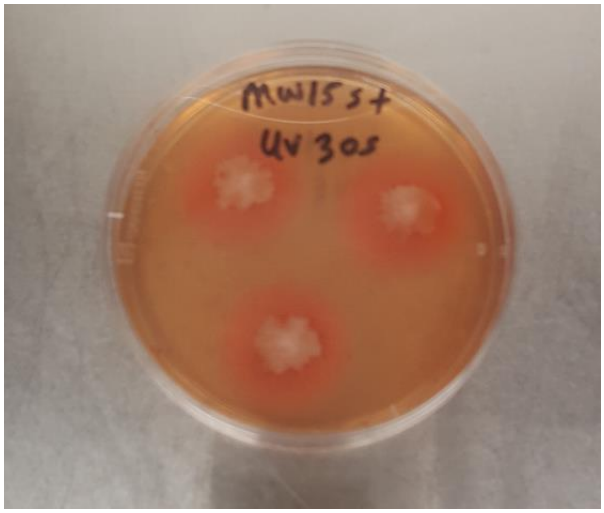


Figure 5.35 Mutant *R. variabilis* RS strains stained by Congo red on PDA. The *R . variabilis* RS strains were exposed to microwave for 15 seconds and then ultraviolet light for 30 seconds.

From the result obtained in Table 5.4, the *R. variabilis* RS mutant strain from MW 15 seconds gave the highest stained zone to colony diameter ratio, which showed its high tendency to produce cellulase. The *R. variabilis* RS mutant strain from MW 15 seconds and MW 15 seconds + UV 10 seconds were selected due to their high stained colony zone to colony diameter ratios for further optimisation for cellulase production.

The selected *R. variabilis* RS mutant strains (MW 15 seconds and MW 15 seconds + UV 10 seconds) from Table 5.4 with asterisk were re-examined for cellulase production. The selected mutant strains were renamed for easy identification. MW 15 secs was renamed in this order, as they were asterisk to MW15-01, MW15-02 and MW15-03 while MW 15 secs + UV 10 secs was renamed as they were asterisk to MWUV-01 and MWUV-02. This was carried out in order to further narrow down the best *R. variabilis* RS mutant strain with the ability to produce high amounts of cellulase. The results were shown in Table 5.5.

As shown in Table 5.5, the results revealed that R. variabilis RS mutant strain of MW15-03 gave the highest stained zone to colony diameter ratio, indicating its higher tendency to produce cellulase than other R. variabilis RS mutant strains in PDA plate.

Table 5.4 The stained zone and colony diameter of the parent and mutant *R. variabilis* RS strains.

R. variabilis RS strain	Zone diameter (cm)	Colony diameter (cm)	Zone to colony diameter ratio
Control	3.0	1.1	2.72
	3.1	1.0	3.1
	3.2	1.1	2.91
MW 10 secs	3.0	1.2	2.5
	3.1	1.0	3.1
	3.4	1.9	1.79
MW 10 secs + UV 30 secs	3.3	1.3	2.54
	2.9	1.0	2.9
	3.1	1.3	2.38
MW 15 secs	3.5 *	0.8	4.38
	3.2 *	0.6	5.33
	3.0 *	0.9	3.33
MW 15 secs + UV 10 secs	3.1*	0.9	3.44
	2.9 *	1.2	4.42
	3.1	1.0	3.1

Table 5.5 The stained zone and colony diameter of the parent and selected mutant *R. variabilis* RS strains.

Selected mutant strain	Zone diameter (cm)	Colony diameter (cm)	Zone to colony diameter ratio
Control	1.9	0.4	4.75
	2.1	0.6	3.50
	1.8	0.3	6.00
MWUV-01	2.6	0.6	4.33
	2.8	0.4	7.00
	2.3	0.6	3.83
MWUV-02	3.0	0.6	5.00
	2.6	0.5	5.20
	2.6	0.5	5.20
MW15-01	1.2	0.2	6.00
	1.6	0.5	3.20
	0.9	0.5	1.80
MW15-02	2.5	0.4	6.25
	2.5	0.5	5.00
	2.1	0.4	5.25
MW15-03	2.9	0.3	9.67
	2.4	0.3	8.00
	3.0	0.4	7.50

5.3.2 The cellulase production of *R. variabilis* RS mutant

The selected *R. variabilis* RS mutant strains from Table 5.5 were cultured on alkali modified wheat straw for 5 days at 28˚C in SSF and were compared with control RS strain. The result obtained in Figure 5.36 shows that the *R. variabilis* RS mutant strain of MW15-03 resulted in a higher cellulase activity than other *R. variabilis* RS mutant strains, although it was slightly lower than the control. This result confirmed the higher tendency of MW15-03 to produce cellulase.

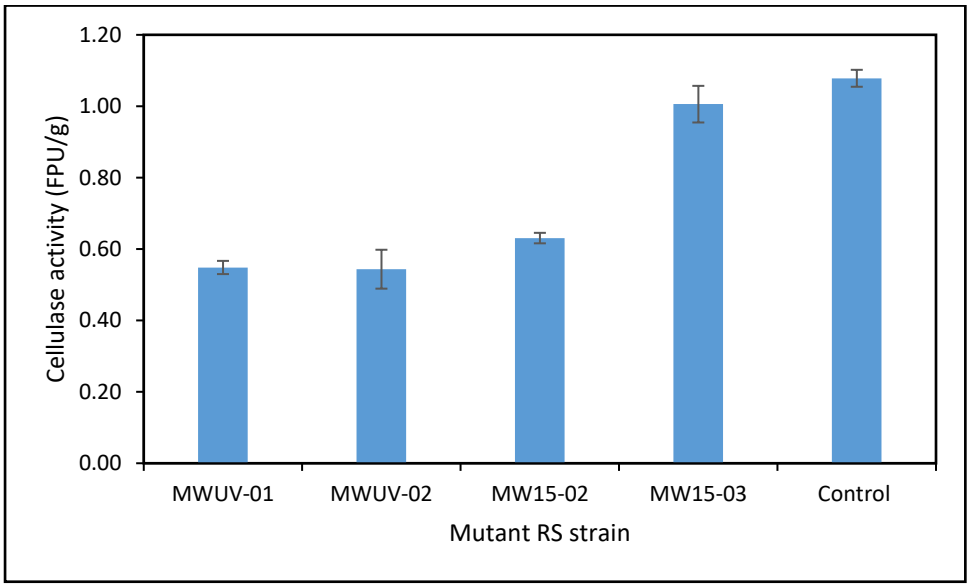


Figure 5.36 Cellulase activity from selected *R. variabilis* RS mutant strain in SSF for 5 days at 28˚C

5.3.3 Further mutation using *R. variabilis* RS MW15-03 as the starting strain

The MW15-03 *R. variabilis* RS mutant strain was selected from Figure 5.36 due to having similar cellulase activity to the control strain. MW15-03 was mutated by exposing to microwave radiation for another 15 seconds. The second round mutant strains were cultured on PDA plate to examine it ability to produce cellulase (Figure 5.37).

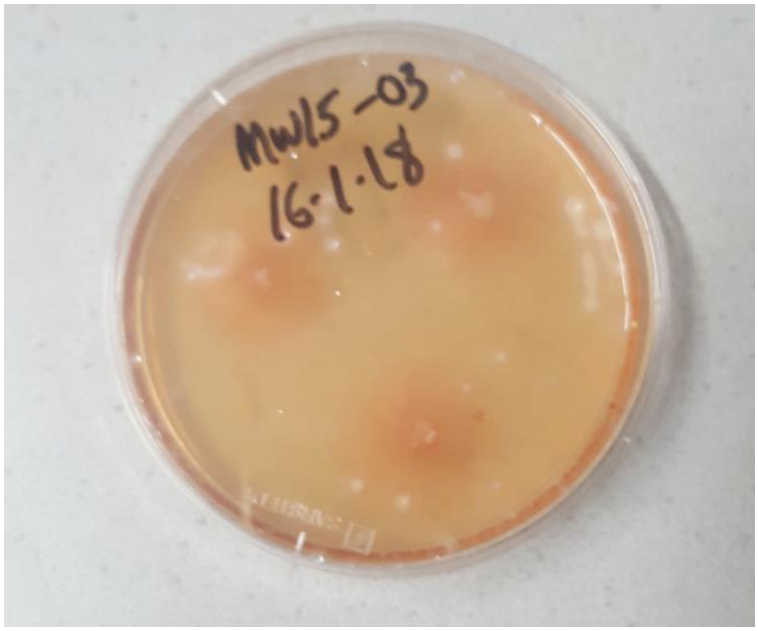


Figure 5.37 The second round of mutant *R. variabilis* RS strains stained by Congo red on PDA plate.

The second round mutant strain was compared with the original *R. variabilis* RS strain as control for cellulase production in SSF in a Petri Dish at 28˚C for 5 days. The second round mutant strain gave a higher cellulase activity (1.97 FPU/g) than the control as shown in Figure 5.38 with 21.6% increase in cellulase.

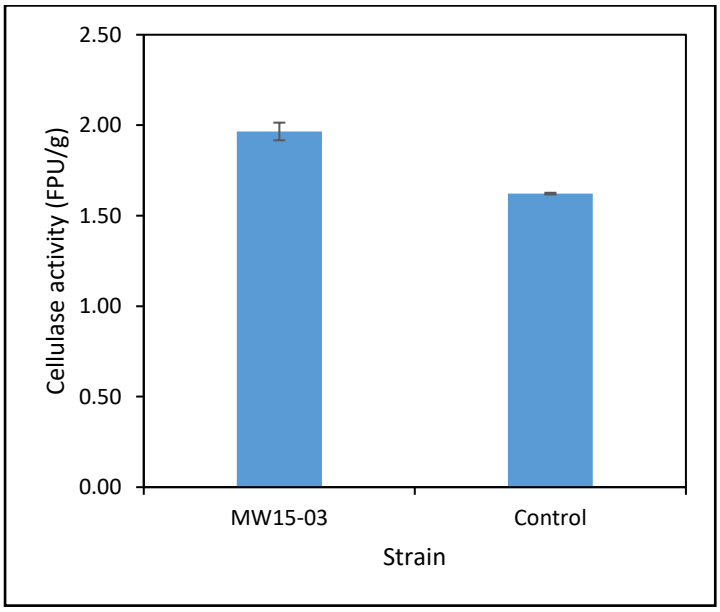


Figure 5.38 Cellulase activity of optimised mutant strain compared with control in SSF for 5 days at 28˚C

5.4 Summary

The impact of various fermentation operation parameters were examined in SmF and SSF for the optimisation of cellulase production from *R. variabilis* RS fungal strain using alkali modified wheat straw.

The initial fermentation profile for cellulase production in SmF resulted in cellulase activity 9.33 FPU/g on day 3, while the addition of a lower glucose concentration of 0 to 15 g/L, 5 g/L of glucose concentration resulted in a biomass-wet weight of 1.17 g/20mL on day 3 of fermentation. At pH 6.5 on day 3 of the fermentation, cellulase activity of 11.43 FPU/g was obtained, while biomass wet weight of 5.42 g/20 mL was obtained at pH 6.54 within 3 to 4 days of fermentation. The addition of mineral solution B (Glucose 10 g/L, Urea 4 g/L, KH_2O_4 6 g/L, $MgSO_4.7H_2O$ 1 g/L, $FeCl_3.4H_2O$ 10 mg/L), cellulase activity and biomass wet weight of 19.07 FPU/g and 2.53g/20mL were obtained on day 2 of fermentation respectively. The addition of tryptone as a nitrogen source gave the cellulase activity of 18.44 FPU/g on day 3 of fermentation when compared to other nitrogen source. In terms of the impact of substrate concentration (2, 6, 10 & 12%), 10% substrate concentration gave the cellulase activity of 4.09 FPU/g on day 3 of fermentation. The RSM experiments showed that the four factors model (temperature, tryptone, pH and substrate concentration) had no significant difference under ANOVA with standard order 3 (8% substrate concentration, 0.04% tryptone, pH 6 and temperature 24˚C) having the highest cellulase activity of 23.81 FPU/g.

In SSF, cellulase activity of 19.83 FPU/g was obtained on day 5 of fermentation with the addition of mineral. Cellulase activity of 10.01 FPU/g was obtained with pH7, while on day 5 of the fermentation using $1x10^7$ spores/g and 80% moisture content, cellulase activity of 12.44 FPU/g and 2.76 FPU/g were obtained respectively. The addition of tryptone as a nitrogen source resulted in the cellulase activity of 30.19 FPU/g than other nitrogen source used to improve cellulase activity in SSF. The RSM four model (moisture content, tryptone, inoculation rate and pH) was identified as significant under ANOVA with the central point (80% moisture content, 0.03% tryptone, pH 6 and inoculation rate 7.5×10^6) having the highest cellulase activity of 24.80 FPU/g.

The second round mutant *R. variabilis* RS strain in microwave for 15 seconds shows the highest ability to produce cellulase on a plate. It also resulted in the highest cellulase activity (1.97 FPU/g) when compared with the parent *R. variabilis* RS strain (control) resulting in 21.6% increasement in cellulase.

6 Glucoamylase production using *A. awamori* with sorghum bran

Glucoamylase is an important enzyme for starch hydrolysis due to its catalytic effect to release glucose from the non-reducing ends of starch (Pardeep Kumar & Satyanarayana, 2009). Glucoamylases are industrially important hydrolytic enzymes of biotechnological significance, which are currently used in food and pharmaceutical industries (Joshi et al., 1999) mainly for the production of glucose syrup, high fructose corn syrup, and alcohol.

Traditionally, filamentous fungi have produced glucoamylase, although a diverse group of microorganisms have been use to produce glucoamylase since they secrete large quantities of the enzyme extracellularly. *A. niger* and *Rhizopus oryzae* are principally used for its commercial production (Norouzian, Akbarzadeh, Scharer, & Young, 2006). The industry's preference for glucoamylase from these fungi is due to its high enzyme activity at neutral pH as well as thermal stability.

The production of glucoamylase by fermentation for various substrates has been reported including wheat bran, green gram bran, black gram bran, corn flour, barley flour, maize bran, rice bran, rice flakes and food waste (Izmirliogiu & Demirci, 2016). Media composition and growth conditions were reported to influence glucoamylase production while maltose and cassava flour have been reported as glucoamylase inducers. At low concentrations, glucose has been reported as an inhibitor for the production of glucoamylase while some nitrogen sources such as yeast extract, ammonium sulphate, ammonium nitrate, urea, meat extract and peptone have been used to promote glucoamylase production (Carina Pavezzi, Gomes, & Da Silva, 2008; Kumar & Satyanarayana, 2007; Pandey et al., 1994). Different fermentation procedures have also been studied for glucoamylase production under SSF and SmF.

According to Izmirliogiu and Demirci (2016), there was a substantial increase in glucoamylase and glucose production via the strain selection of *Aspergillus* and medium optimization using industrial waste potato mash. The study suggested an inexpensive medium composition for glucoamylase production. Negi and Banerjee (2009) reported a suitable condition for glucoamylase production under SSF at 37˚C for 4 days using wheat bran as a substrate and using *A. awamori*. Another study

carried out by Zambare (2010) showed a 24% increase in glucoamylase activity through optimization of SSF media and parameters by *A. oryzae* using rice husk, wheat bran, rice bran, cotton seed powder, corn steep solid, bagasse powder, coconut oil cake and groundnut oil cake as substrate.

There is no report on the production of glucoamylase using sorghum bran as substrate. Therefore, in this chapter, the production of glucoamylase enzyme (under SmF and SSF) from sorghum bran was investigated under different conditions. The aims were to study the feasibility of using fungi strain (*A. awamori*) for the production of a high glucoamylase concentration and to produce a sugar rich hydrolysate from sorghum bran, which can be fermented for the production of bioethanol. The milling efficiency for the separation of sorghum bran from its kernel has been assessed as well.

6.1 Sorghum bran characterisation

The structure of sorghum grain has a vital impact on its milling efficiency as the hardness and composition vary between sorghum grains (Wall & Blessin, 1969). The sorghum grain consists of the pericarp, the germ and the endosperm. The grain is made up of 82% endosperm, 10% germ and 8% bran (Wall & Blessin, 1969).

Although, in theory all the bran is cellulose and hemicellulose, appreciable quantities of starch are deposited in the mesocarp tissue of this fraction. Sorghum grain composition varies because of factors including the nature of hybrid, soil and climate conditions and manner of crop management. Due to the difficulties of the extraction of starch from the sorghum grain, large proportion of starch has been left over with sorghum bran. This starch can be and should be recovered to improve the values of the sorghum crop economy.

In this section, the impact of three milling methods (1) wet milling using peanut butter maker (2) wet milling using blender and (3) dry milling using knife mill were studied to determine the impact of milling process on sorghum bran composition, primarily the starch content in sorghum bran. The peanut butter maker was used as it as similar working mechanisms with the grinding mill used in Nigeria (detailed in Chapter 2.5).

6.1.1 The starch content in sorghum bran

Three milling processes peanut butter maker, blender and knife mill including both wet milling (peanut maker, blender) and dry milling (knife mill) were examined to identify the most effective milling process to obtain sorghum bran from sorghum kernel. The conditions used are illustrated in Table 6.1.

Table 6.1 Comparison of milling conditions for sorghum bran

Equipment used	Milling conditions
Peanut butter maker	Steeping (3 days), wet milling
Blender	Steeping (3 days) , wet milling
Knife mill	Dry milling

Three batches of sorghum bran were obtained from the milling processes (Table 6.1). The total starch composition of sorghum bran was analysed on the three batches of dried sorghum bran. The results obtained in Figure 6.1 revealed that extremely high starch (81.93%) was retained in the bran obtained from knife mill in dry milling of sorghum kernel. According to FAO (2017), the endosperm is the largest part of the kernel with 94% starch. The dry milling of sorghum kernel using a knife mill was not effective for the separation of the bran from the kernel, as a large proportion of starch (81.93%) was retained after milling. This indicated that using the knife mill with 2 mm screen sieve cannot separate bran from the milling resulted powder.

The wet milling using peanut butter and blender were more effective than the knife milling as the total starch in the sorghum bran was relatively low (16.35% and 12.95%, respectively). When compared with published data, dry milling had been employed in Nigeria for the separation of sorghum bran from sorghum kernel using either a Buhler mill (Adeyemi, 1983) or a hammer mill (Olatunji et al., 1992). This resulted in about 24% total starch in the sorghum bran while various milling and steeping conditions are used for wet milling with the aid of enzymes or sonication (Donley, 2013). 30% total starch of sorghum bran was reported by Corredor, Bean,

and Wang (2007) from a wet milling process. The total starch obtained from dry milling in literature was different from this research, which may be due to the different milling mechanism of the equipment used, and the other milling process used following Adeyemi (1983); Olatunji et al. (1992) were not available in the lab.

The extraction of starch from sorghum kernel after wet milling depends on the size of the sieve used and the effectiveness of the sieving methods, which was the main cause for the variation in total starch obtained from wet milling (Sorghum bran starch content from different milling process are given in Table 6.2). The waste recovered after washing the wet mill slurry is referred to as the “sorghum bran” which is regarded as food waste. Souilah, Boudries, Djabali, Belhadi, and Nadjemi (2015) reported that 90% to 99.55% total starch in sorghum starch was isolated when treated with lactic acid and sulfur dioxide before wet milling, which gives a lower total starch in the sorghum bran.

Table 6.2 Sorghum bran starch content from different milling process

Sorghum species	Milling processing	Starch content in sorghum bran (w/w)	Reference
Red sorghum	A tangential abrasive dehulling device	30%	Corredor et al. (2007)
Red sorghum	Buhler mill/hammer mill	24%	(Adeyemi, 1983; Olatunji et al., 1992)
Red sorghum	Peanut butter maker	16.35%	This study
Red sorghum	Blender	12.95%	This study
Red sorghum	Knife mill	81.93%	This study

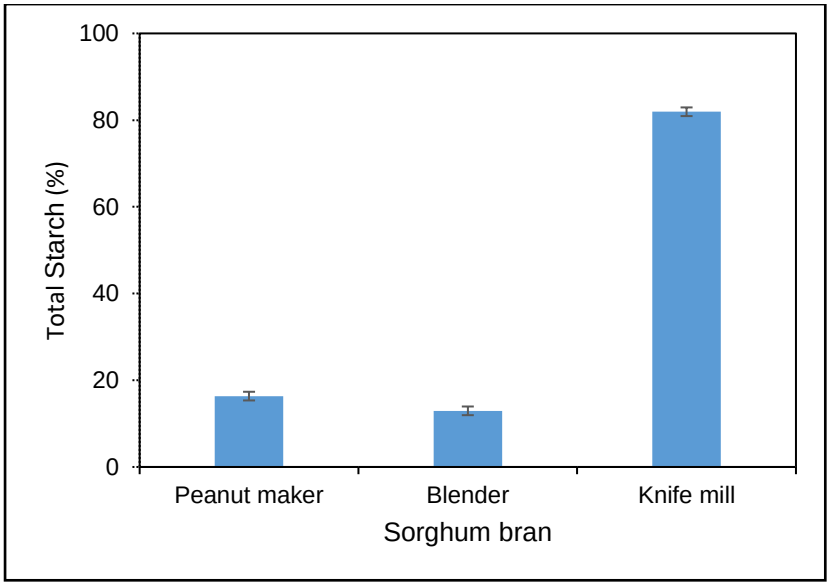


Figure 6.1 Total starch content of sorghum bran obtained from different milling methods

6.1.2 Mass balance

Mass balance is an application of conversion of mass to the analysis of physical systems by accounting for material entering and leaving a system. It is also used to infer food loss and waste (FLW) by measuring inputs and output alongside changes in weight of food during processing.

Mass balance calculation is used to quantify FLW where reliable measurement or approximation is not possible. In this chapter, the mass balance calculation was used to evaluate the accuracy of starch content analysis.

Mass balance calculation conversion balance is

$$C\ (\%) = \left(\frac{Ao}{Ai}\right) 100 \quad \text{..... } \textit{Equation 6.1}$$

C: Conversion factor

Ai: Amount of the process input material

Ao: Amount of the output yielded by the internal process

The total mass balance of sorghum kernel wet milling was determined for the process efficiency in terms of starch and sorghum bran recovery from sorghum kernel (detailed process in chapter 2.5) are given in Table 6.3.

The mass balance calculation conversion for sorghum kernel input and the output of sorghum bran and sorghum starch using equation 6.1;

Mass balance calculation conversion for sorghum bran output;

C (%) = (122.94/250)*100

C (%) = 49.18

Mass balance calculation conversion for sorghum starch output;

C (%) = (53.03/250)*100

C (%) = 21.21

Total mass balance calculation conversion

C (%) = (175.97/250)*100

C (%) = 70.39

The mass balance shows that the sorghum bran had the largest recovery output of 49.18% when compared with the sorghum starch of 21.12%. The mass balance indicated that the waste portion obtained from sorghum kernel is higher than the starch portion. The total mass balance showed that a high percentage of the input had been recovered at output (70.39%). The loss of 29.61% could have resulted from the accumulation of some of the milled sorghum kernel stocked in the milling equipment and some losses could have resulted from spillage during sieving as well as when decanting water from the settled starch after 24 hours. Although sorghum bran is used for animal feed, a large percentage of it is disposed of as food waste. The disposal of food waste contributes to environmental pollution (Soil, water and air pollution) and this occurs when they become contaminated with hazardous materials. This not only contributes to the creation of greenhouse gas effects when they are burn but can also cause significant harm to marine and wildlife.

Table 6.3 Sorghum kernel output milling process

	Sorghum kernel (g)	Water (mL)
Start input for steeping	250	500
After steeping	287.37	370
During milling	287.37	300
Sieving		1700
Wet weight of SB	214.28	
Decanted water from starch before drying		1320
Wet weight of sorghum starch	169.82	
Dry weight of sorghum bran	122.94	
Dry weight of sorghum starch	53.03	
Water loss from drying		208.13

Apart from this, it can also have adverse health effects on humans as over the years it has been responsible for the spreading of several diseases (like cholera, dysentery etc) and in some cases even death. Improper waste disposal can also interfere with the food supply as plant growth is impaired reducing the amount of food produced.

In order to reduce or eliminate these issues, the utilisation of sorghum crop residues, which generate 2-3 million metric tons annually (Nasidi, Agu, Deeni, & Walker, 2016) in which sorghum bran constitutes around 1.55 million tons a year in Nigeria, was investigated for the production of glucoamylase enzyme and its suitability as a feedstock for bioethanol production.

6.2 Glucoamylase Production via Submerged Fermentation

Sorghum bran derived as a waste from sorghum grain consists of cellulose, hemicellulose and starch, which can be either used as animal feed, burned or left to decay on the land. This makes the sorghum bran a suitable raw material for

fermentative production of high value chemicals e.g bioethanol and enzymes e.g glucoamylase. In this section, submerged fungal fermentation was investigated.

6.2.1 Impact of cultivation time on glucoamylase production

Dried sorghum bran obtained from wet milled sorghum using a peanut butter maker was used to determine the production profile of glucoamylase in a submerged fermentation. This experiment was carried out to investigate the utilisation of sorghum bran for the production of glucoamylase enzyme.

The glucoamylase production was initially examined for 24 hours to test the feasibility of using sorghum bran for glucoamylase production. There was an increase in glucoamylase activity during the 24 hours of SmF from 0.10 U/mL to 0.44 U/mL (Figure 6.2). However, a higher glucoamylase activity was desired for the enzymatic hydrolysis of sorghum bran. Therefore, a time profile analysis for glucoamylase was conducted for 5 days. A further increase in glucoamylase activity was observed as the fermentation progressed (Figure 6.3) to at least 5 days.

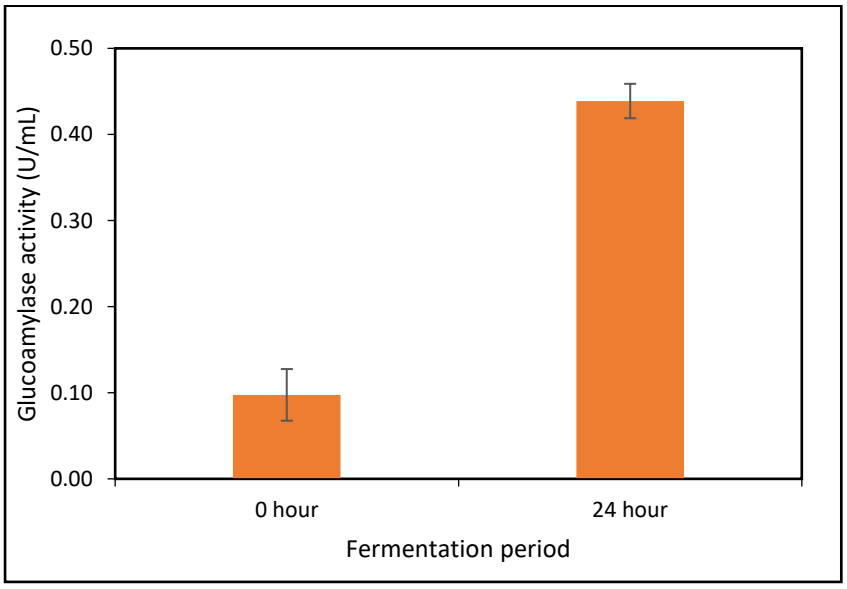


Figure 6.2 Glucoamylase activity for 24 hours in SmF at 4% substrate concentration, 200 rpm and 28˚C using 250 mL shake flask bottle.

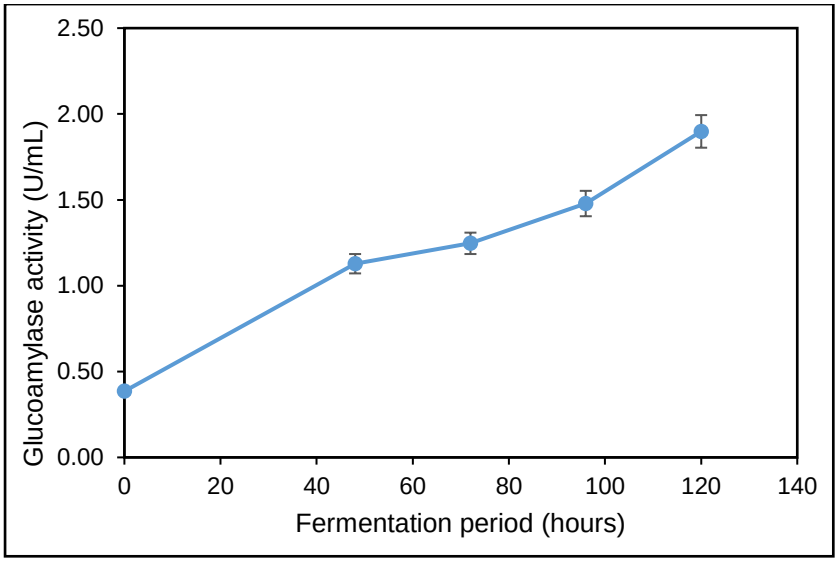


Figure 6.3 Glucoamylase production profile for 5 days in SmF at 4% substrate concentration, 200 rpm and 28˚C using 250 mL shake flask bottle.

Due to continuous increase in glucoamylase activity observed after 5 days; glucoamylase production profile was further determined for 15 days. An increase in glucoamylase activity was obtained as the incubation period was elongated and a decrease in glucoamylase activity was observed after 5 days (0.57 U/mL) (Figure 6.4). The glucoamylase activity profile in Figure 6.3 shows a steady increase in glucoamylase activity up to 5 days with a similar result (1.25 U/mL) obtained from Figure 6.3 and Figure 6.4 respectively for 3 days. The rapid decrease in enzyme production was probably due to depletion in nutrients, accumulation of waste product, cell death, formation of other by-products in the fermentation medium, denaturation of enzyme due to interaction with other compounds in the fermentation medium and catabolite repression (Krishna & Muthusamy, 1996).

The result obtained from the two graphs (Figure 6.3 and 6.4) with the similarity observed after 3 days showed that an increase in glucoamylase activity could only be obtained up to 5 days by *A. awamori* before a decline in glucoamylase activity. Maximum amylase enzyme production was reported by Saleem and Ebrahim (2014) after 6 days by *A. niger* and *R. stolonifer* fungi with an increase in length of incubation period while further incubation resulted in decreased enzyme production. Kim, Kim, Bai, and Ahn (2011); Uguru, Akinayanju, and Sani (2011) reported maximum production of α-amylase by *A. niger* after 6 and 5 days respectively.

Chimata, sasidhar, and Challa (2010) reported an optimum α-amylase production from *Aspergillus* MK07 after 120 hours while Erda and Taskin (2010) found maximum amylase production by *Penicillium expansum* after 6 days of incubation. The trends from literature shows similarity with the results obtained from Figure 6.3 and Figure 6.4 respectively.

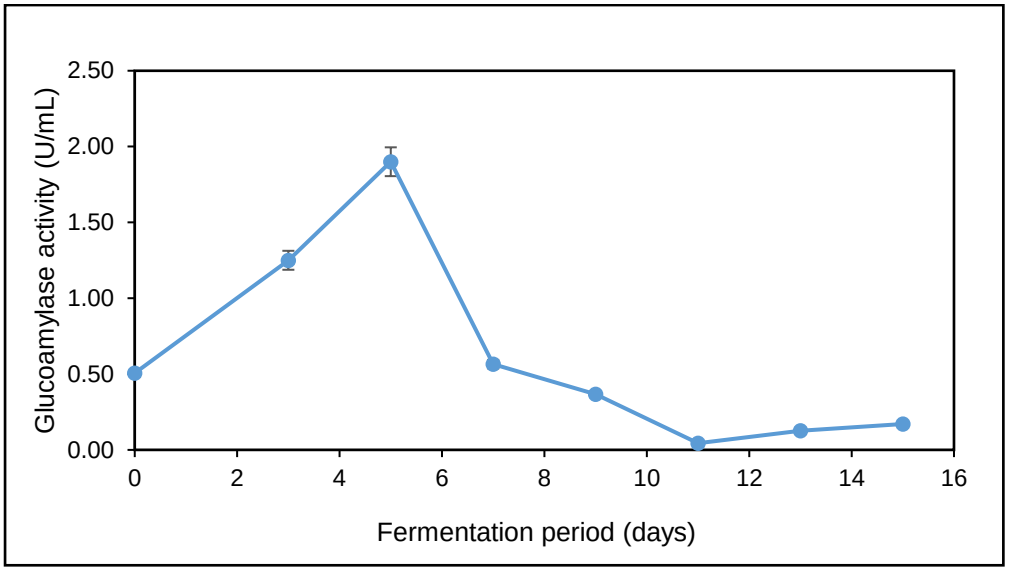


Figure *6.4* Glucoamylase activity of sorghum bran f*or 15 days in SmF at 4% substrate concentration, 200 rpm and 28˚C using 250 mL shake flask bottle.*

6.2.2 Impact of Substrate Concentration on Glucoamylase Production

To improve glucoamylase production, the effect of sorghum bran concentration (2%, 4%, 6%, 8%, and 10%) was examined. The utilisation of sorghum grain starch (SGS) 6% was also studied as a comparison. The result shows an increase in glucoamylase activity as the substrate concentration increases with 10% substrate concentration having the highest glucoamylase activity of 12.58 U/mL (Figure 6.5). SGS 6% gave similar glucoamylase activity (6.37 U/mL) when 8% sorghum bran was used for glucoamylase production (6.15 U/mL).

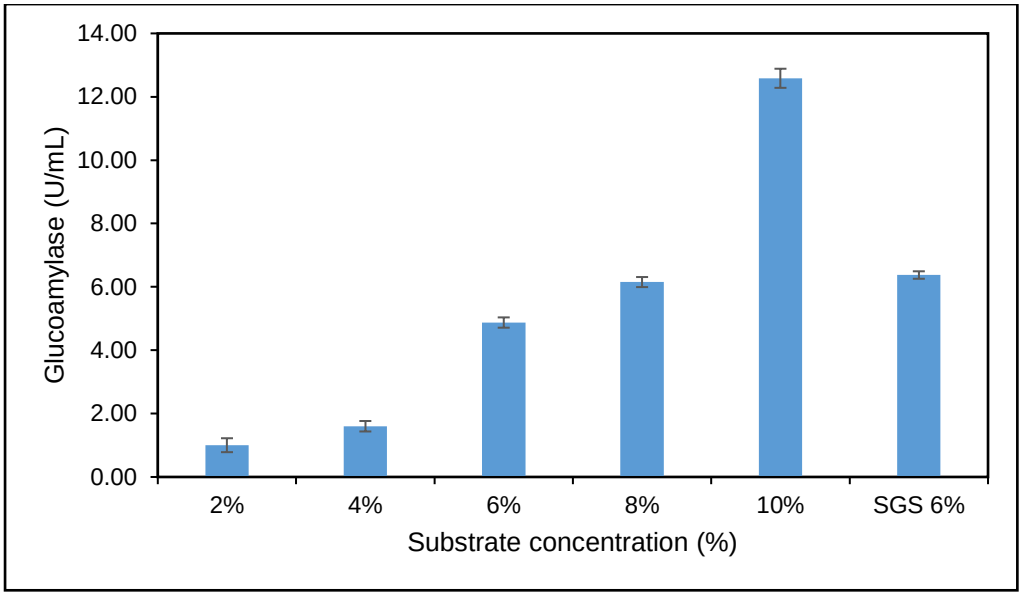


Figure 6.5 Glucoamylase profile for substrate concentration, sampling day (5 days) in SmF, 200 rpm and 28˚C using 250 mL shake flask bottle

The result obtained shows that sorghum bran concentration affected glucoamylase production and the desirable result was obtained from 10% concentration (12.58 U/mL).

It is expected that further improving the substrate concentration may possibly improve the glucoamylase production. However, this was not done in this study mainly due to the following reasons. 1): mixing becomes difficult at high substrate concentration due to the gelatinised starch, which thickens the medium from the release of amylose. 2) a multi parameter optimisation experiments using response surface method was planned with an increased substrate concentration in chapter 6.2.9 for optimum enzyme production. 3) High substrate concentration may be economically disadvantageous, due to low product to substrate yield and 4) in order to maximise the available substrate for further experimental plans.

6.2.3 Impact of pH on glucoamylase production

pH has a massive impact on enzyme production as it is a factor that determines enzymes stability and enzymes are affected by changes in pH because at extremely high or low pH values there is a complete loss of activity for most enzymes.

The effect of pH on glucoamylase production using sorghum bran at different initial pH (3 – 8) of fermentation medium was investigated in SmF (Figure 6.6). A gradual increase in enzyme production was observed at pH 3.0, 4.0, 5.0 and 6.0 for 3 days, peak enzyme production obtained was 8.66 U/mL, 16.86 U/mL, 16.53 U/mL, and 19.26 U/mL, respectively, before a decline in enzyme activity was observed. At pH 7, the peak enzyme activity was obtained at day 2 (11.87 U/mL) before a decline was observed and that was a noticeable increase in enzyme activity again at day 5 (10.43 U/mL). There was a slow increase in glucoamylase activity at pH 8 for 4 days before a peak enzyme activity was observed at day 5 (8.45 U/mL) then a further decline in enzyme activity was obtained after day 5 of the fermentation (Figure 6.6).

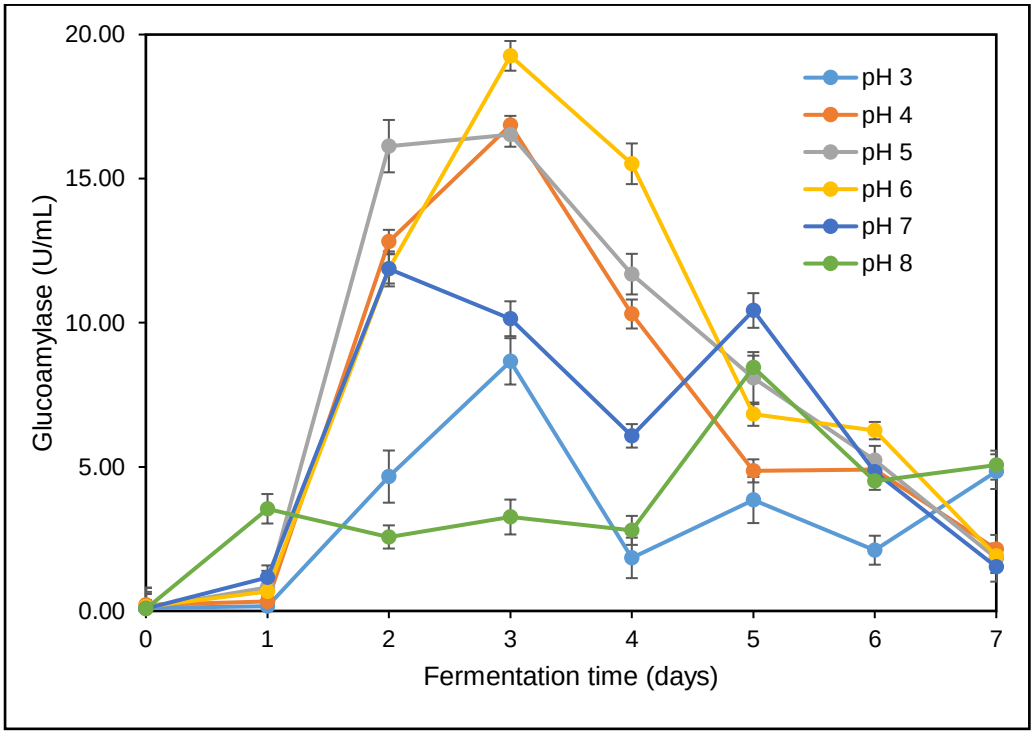


Figure 6.6 Effect of pH on glucoamylase production over 7 days in SmF in SmF at 4% substrate concentration, 200 rpm and 28˚C using 250 mL shake flask bottle.

The optimum glucoamylase enzyme production was obtained at pH 6 (19.26 U/mL) on the third day of the fermentation. The result obtained showed that glucoamylase enzyme production by *A. awamori* produced a higher enzyme activity close to neutral pH and its production is highly affected at extremely acidic and alkaline pH as further increase in pH reduced enzyme production.

A higher amylase synthesis was reported at pH 8 by *Bacillus sp* as well as from *Bacillus cereus* at an alkaline pH under SSF (Vijayaraghavan, Kalaiyarasi, & Vincent, 2015), which indicates alkaliphilic nature of the strains, most research reported the best pH for amylase production was around 6 (Alva et al., 2007; Khan & Yadav, 2011; Saleem & Ebrahim, 2014; Singh, Kumar, & Kumar, 2009). In this study, the result obtained in Figure 6.6 showed a maximum glucoamylase concentration at pH 6, which correlates with published data using different substrates and microorganisms.

The results from different literature and this research indicated that different substrate and fungi had a different optimum pH for different enzyme production as any increase or decrease in H+ ion concentration has a significant effect on the growth of mycelium as well as the enzyme excretion (Gupta, Gupta, Modi, & Yadava, 2008). Therefore, when pH was optimised, the cultivation period required for the highest glucoamylase production time shifted thus reducing the fermentation time in this case (Figure 6.6).

6.2.4 Impact of aeration rate on glucoamylase production

Aeration is the process by which air circulates through, mixed with or dissolved in the medium. Provision of an aeration system that can maintain a high dissolved oxygen level is a general requirement for a bioreactor for aerobic fermentation (Abdullah, Ul-Haq, & Javid, 2011). The impact of aeration rate was determined for GA production using sorghum bran in SmF.

The impact of different aeration ratio with the addition of minerals was carried out to increase GA enzyme activity in a 500 mL shake flask. In previous experiments, the submerged fermentation was carried out using 250 mL shake flasks with a liquid loading amount of 100 mL. There was no much increase in GA production when the aeration ratio of 50/500 mL and 100/500 mL were used for GA production. There was an increase in GA activity and a peak increase was obtained at day 3 when 150/500 mL and 200/500 mL (11.05 U/mL and 12.74 U/mL) was used respectively, while a peak increase in GA was obtained at day 4 when aeration ratio of 250/500 mL (11.90 U/mL) was used.

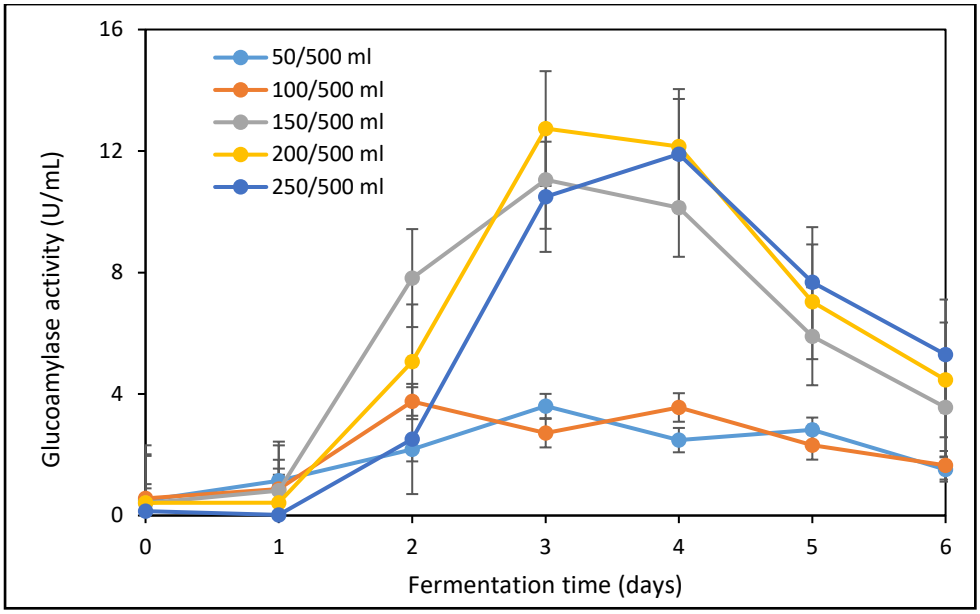


Figure 6.7 Effect of aeration rate on glucoamylase production in SmF at 4% substrate concentration, 200 rpm and 28˚C using 250 mL shake flask bottle.

A maximal GA production was obtained when the aeration rate was decreased with optimum GA activity at aeration rate of 200/500 mL at day 3 (12.74 U/mL) (Figure 6.7). The aeration rate (AR) is an important factor influencing enzyme production as insufficient aeration can lead to anaerobic conditions due to lack of oxygen while excessive aeration can slow down the production process via heat and water loss.

The anaerobic condition available to microorganism had a great impact on its physiology and metabolism. At low level of air supply the productivity of enzyme was greatly inhibited in the fermentation medium with little titre of enzyme activity. While higher aeration rates have some detrimental effects on the growth of microorganism and subsequently certain enzyme production in the bioprocess (Ionita et al., 2001). The optimal AR was found to depend on the composition of the raw materials and ventilation methods (Ionita et al., 2001).

6.2.5 Impact of inoculation ratio on glucoamylase production

Shafique, Bajwa, and Shafique (2009) reported a direct effect of inoculum on the growth of microorganisms and enzyme production. Different inoculation ratio of 1 – 20 million spores/g (Figure 6.8) were tested for enzyme production in a 250 mL

shake flask. The inoculation ratio of 20 million spores/g gave the optimum enzyme activity on day 1 in a shake flask. The different inoculation ratio used for the GA production did not follow a designated pattern as the fermentation days progressed. Although the inoculation ratio of 5 million spores/g has a steady increase in GA production with optimum GA production on day 4 (4.57 U/mL) before enzyme production decreased.

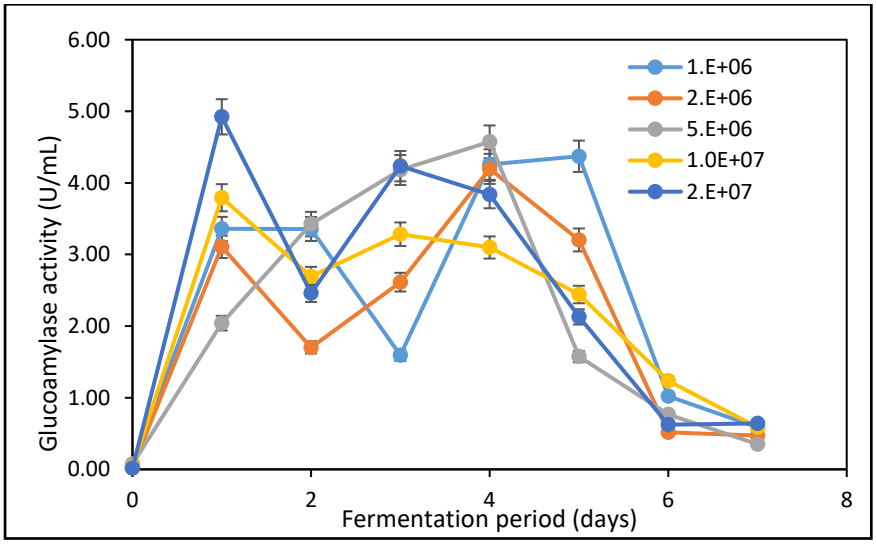


Figure 6.8 Effect of inoculation ratio on glucoamylase in SmF at 4% substrate concentration, 200 rpm and 28˚C using 250 mL shake flask bottle.

The decrease of GA production at later stage of culture may be due to the over growth of *A. awamori* producing anaerobic conditions during the fermentation which consumed majority of the substrate for growth and metabolic processes thus reducing enzyme production (Figure 6.8).

The impact of inoculation ratio was not significant. Therefore, it was excluded from the optimisation factor for GA production using response surface method in chapter 6.2.9.

6.2.6 Impact of temperature on glucoamylase production

Temperature has an effect on enzyme stability as many enzymes are adversely affected by high temperatures although in some cases the rate enzyme catalysed reaction increases, as the temperature increases.

When the fermentation began, enzyme production was steady; however, there was a sharp difference in the effect of temperature on the enzyme production after 3 days (Figure 6.9). Assays at 28˚C had the most significant GA activity after 4 days (10.83 U/mL), before a decrease in GA activity on day 5.

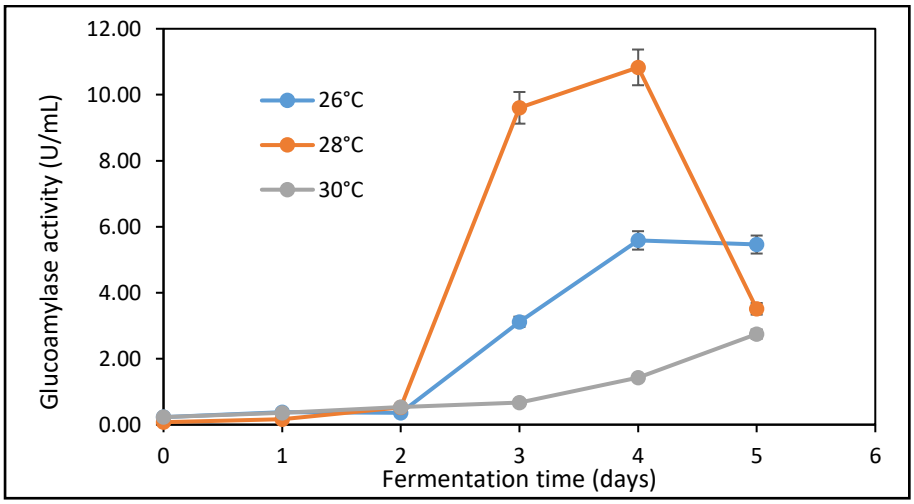


Figure 6.9 Effect of temperature on glucoamylase in SmF at 4% substrate concentration, 200 rpm using 250 mL shake flask bottle.

At 30˚C, the GA enzyme activity showed a slow gradual increase in GA activity up to day 5, while at 26˚C the GA enzyme activity had the same pattern with 28˚C. Maximum amylase production by *A. niger* and *R. stolonifera* was achieved at 30˚C with considerable amount of enzyme obtained at 25 and 35˚C, respectively (Simair et al., 2017). Khan and Yadav (2011) reported an optimum α-amylase at 28˚C by *Aspergillus niger* while Gupta et al. (2008); Haqh, Albdullah, and Shah (2002) found the optimum temperature for amylase production by *A. niger* at 30˚C.

The different optimal temperature for enzyme production may be because the reaction rate increased with temperature to a maximal level before a decline is

observed with further increase in temperature due to enzymes susceptibility to become denatured above optimum temperature as shown in Figure 6.9.

The optimum temperature of 28˚C was selected as a constant variable for the optimisation of GA as other analysed temperature had no significant impact on the GA production.

6.2.7 Impact of nitrogen source on glucoamylase production

Yeast extract (YE) is used as a nitrogen source and nutrient in bacterial culture media. YE contains abundant vitamins, minerals and amino acids, which are necessary for cell growth and enzyme synthesis in the cultivation of many microorganisms.

The addition of yeast extract was carried out with the aim to further increase GA enzyme production and the result obtained from Figure 6.10 shows the impact yeast extract addition had on GA activity from the initial fermentation time when compared to when no yeast extract was added (0 g/L). At day 0, there was GA activities detected as roughly a linear relationship to the substrate YE concentration. To determine whether the detected GA activities were true values, the background absorbent values of YE alone was analysed as shown in Appendix iii. With the increase of YE concentration, the OD_{540} increased. This indicated the GA value at the beginning of the fermentations was due to the effect of YE addition. The effect of YE was diminished as fermentation progressed.

The optimum GA activity was obtained after 3 days of fermentation (13.03 U/mL and 10.80 U/mL) with 2.5 g/L and 5.0 g/L yeast extract addition respectively.

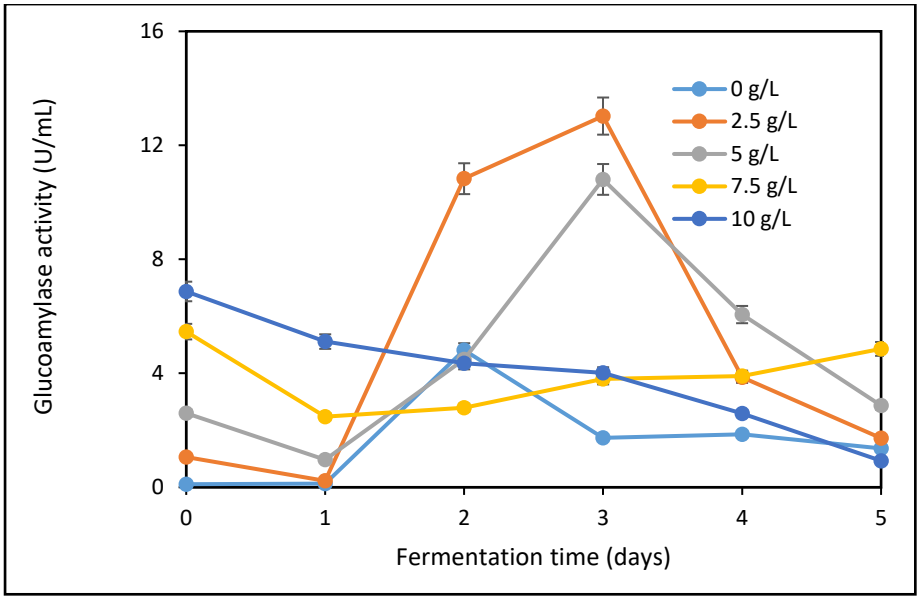


Figure 6.10 Effect of yeast extract as nitrogen source on glucoamylase production in SmF at 4% substrate concentration, 200 rpm and 28˚C using 250 mL shake flask bottle.

Yeast extract as a nitrogen source was found in this study as advantageous to the growth of the culture and the enhancement of GA production. Although fermentation time and yeast extract concentration could affect cell growth and GA production as timing and strength of yeast extract addition was reported to have adverse effect on the cell growth and polyvinyl alcohol-degrading enzymes (Li, Liao, Zhang, Du, & Chen, 2011).

6.2.8 Impact of minerals on glucoamylase production

Further to the addition of yeast extract, the production of GA was examined with the use of mineral solution for an increase in GA activity. The mineral solution used is the same as reported in chapter 4.1.6.

At the beginning of submerged fermentation study, the impact of mineral addition was investigated. Adding mineral increased the glucoamylase activity to 3.60 U/mL and reduced the fermentation time to three days when compared to the optimum GA activity of 1.89 U/mL at day 5 of fermentation when no mineral was used (Figure 6.11). This shows that mineral addition has a positive effect on the production of

glucoamylase time profile from sorghum bran by increasing the glucoamylase enzyme activity production and decreasing the fermentation time.

The mineral solutions from Table 6.4 were further used to determine the best mineral composition for the optimum production of GA using different compositions from literature and designed composition.

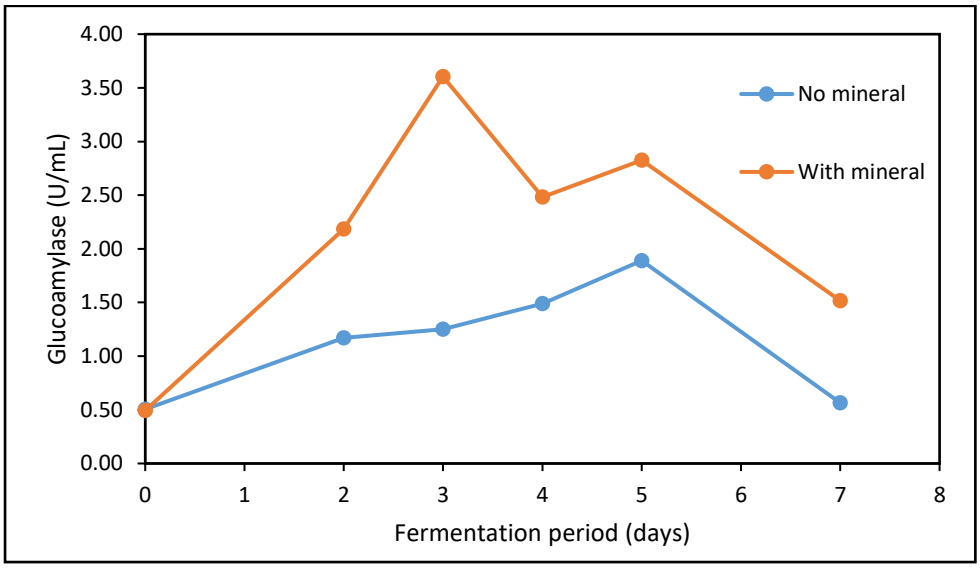


Figure 6.11 Effect of mix mineral solution addition on glucoamylase production in SmF at 4% substrate concentration, 200 rpm and 28˚C using 250 mL shake flask bottle.

Table 6.4 Mineral composition used for glucoamylase production

Mineral used	Composition	Reference
A	Glucose 10 g/L, YE 5 g/L, $(NH_4)_2SO_4$ 1 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, $MgSO_4$ 0.2 g/L	Pensupa et al. (2013)
B	Glucose 10 g/L, Urea 4 g/L, KH_2PO_4 6 g/L, $MgSO_4.7H_2o$ 1 g/L, $FeCl_3.4H_2O$ 10 mg/L	Bancerz et al. (2016)
C	Glucose 5 g/L, YE 10 g/L, KH_2PO_4 1 g/L, $MgSO_4.7H_2O$ 0.3 g/L, $CaCl_3$ 0.3 g/L	Yang et al. (2015)
D	Glucose 10 g/L, YE 10 g/L, KH_2PO_4 1 g/L, $MgSO_4.7H_2O$ 0.5 g/L	Designed in this study
E	Glucose 10 g/L, YE 10 g/L, KH_2PO_4 1 g/L, $MgSO_4.7H_2O$ 0.5 g/L, $FeCl_3.4H_2O$ 0.01 g/L, $CaCl_3$ 0.3 g/L	Designed in this study
FC	Deionised water	
FSW	Sea water	

The result revealed a high glucoamylase production at the initial start of fermentation, which is a reflection of the glucose addition to the mineral solution in mineral A-E except for mineral C, which has a lower glucose concentration, FC and FSW with no glucose concentration (Figure 6.12). The GA activity was reduced as the fermentation period progressed, which may be due to the utilization of glucose by the fungal strain for propagation. An increase in GA activity was seen on day 3 of fermentation due to the fungal release of enzyme externally after complete utilization of glucose and a further decline in GA activity was obtained from day 4, which may be due to lack of nutrients in the medium. The designed mineral E shows a higher GA activity on day 3 (4.65 U/mL) while the sea water (FSW) gave a higher GA activity of 1.50 U/mL on day 1 than the fresh water (FC), which has the optimum GA activity of 1.39 U/mL on day 2. Mineral composition C gave the highest GA activity of 5.03 U/mL on day 3.

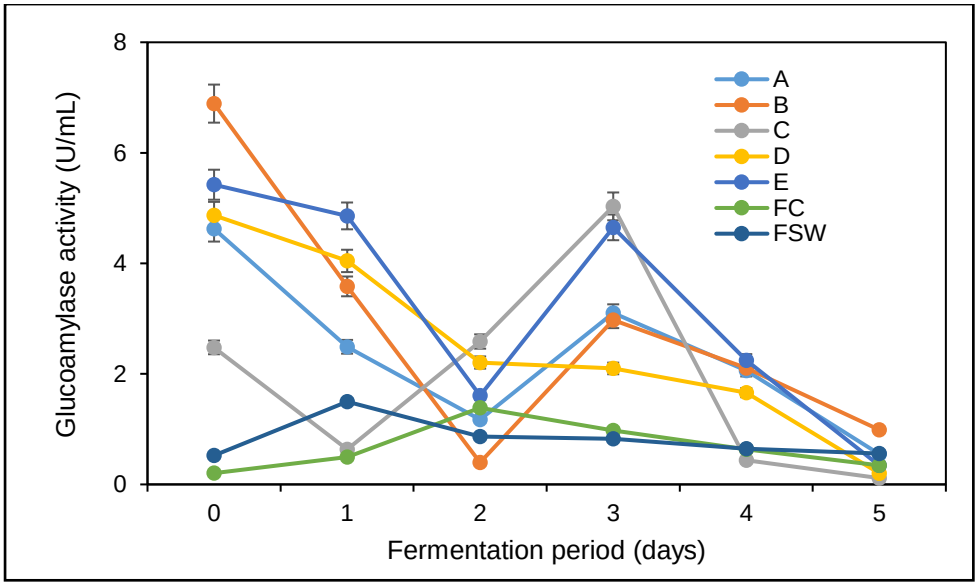


Figure 6.12 Effect of mineral addition on glucoamylase in SmF

6.2.9 RSM for glucoamylase enzyme production in SmF

RSM was performed to optimise GA activity under four numeric factors. Four numeric factors were set in horizontal level. The design of each factors was as listed in Table 6.5. The specific condition of each run was as listed in Appendix iv.

Table 6.5 Central composition design of factors on SmF for glucoamylase production

Numeric factor	Unit	Low value	High value	-alpha	+alpha
Substrate concentration	%	6	10	4	12
pH	-	5.5	6.5	5	7
Yeast extract	g/L	2.5	7.5	0	10
Aeration rate	mL	75	125	50	150

The GA activity was in the range of 0.18 – 59.03 U/mL. The run standard order 24 (substrate concentration 8 g/L, pH 6, yeast extract 5 g/L and aeration rate 150 mL/250 mL bottle) gave the highest GA activity of 59.03 U/mL (Figure 6.14). The model was identified as significant under ANOVA. According to the coefficient of

each factor, the order of importance should be “Substrate concentration > Aeration rate > Yeast extract > pH”.

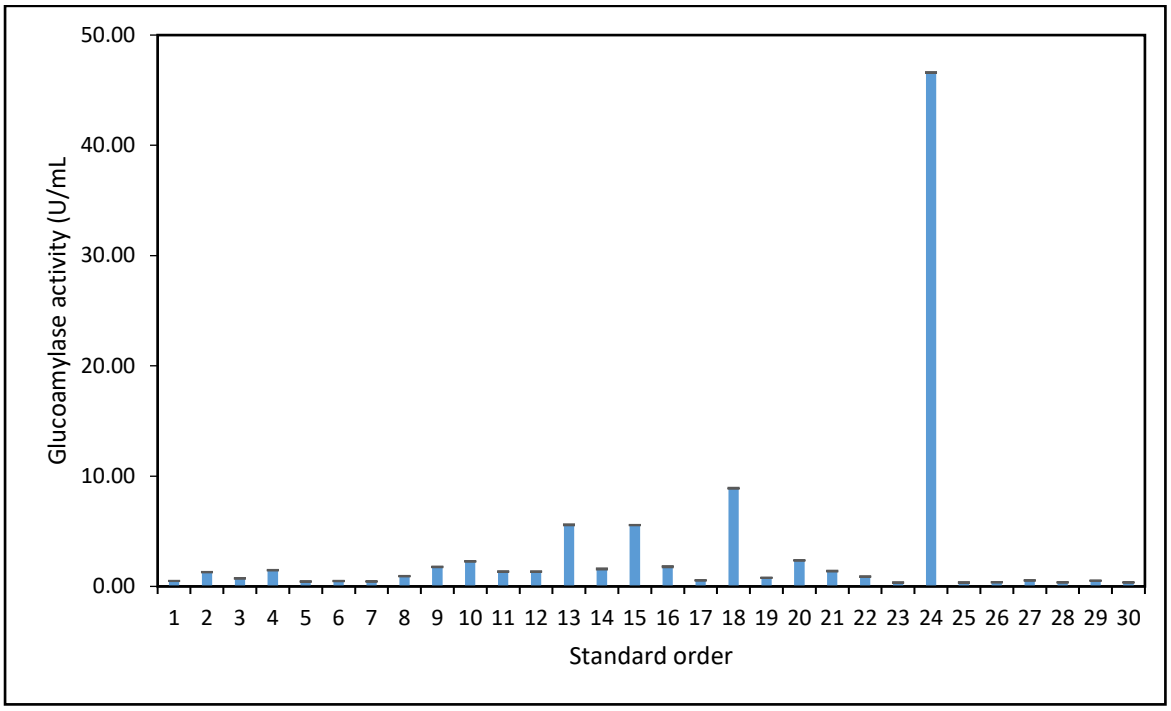


Figure 6.13 Glucoamylase optimisation first trial in SmF

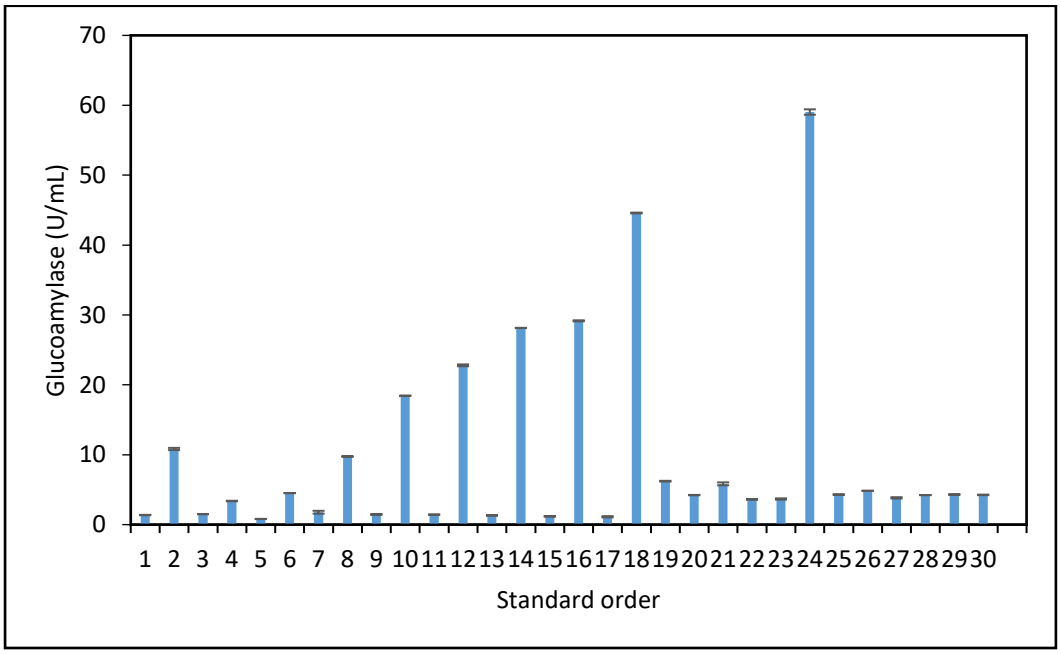


Figure 6.14 Glucoamylase production optimisation in SmF. Central points (standard order of 25 to 30) substrate concentration 8%, pH 6, yeast extract concentration 5 g/L and aeration rate 100 mL

The results obtained in Figure 6.14 shows similar trend with the results obtained in Figure 6.13. The best GA activity were obtained with standard order 24 (8% substrate concentration, pH 6, 5 g/L yeast extract, and 150 mL aeration rate. Figure 6.14 gave a better visuals for the result obtained than Figure 6.13. Therefore, Figure 6.14 was used for further result discussion in this section.

According to the result in Figure 6.14, the GA activity obtained in 6 central points were constant within the range of 3.83 – 4.84 U/mL. The model was significant and was used to work out the effect of each factor on GA activity.

3D response surface plots graphically represents the relationship between independent variable (X and Z) and dependent variable (Y), which was generated by the design expert model software (Figures 6.15 – 6.20). An increase in substrate concentration and aeration rate resulted in an increase in glucoamylase activity when compared with other factors selected such as pH and yeast extract for glucoamylase optimisation. pH and yeast extract has no effect on the optimisation of glucoamylase activity when their mutual effect was considered together (Figure 6.18) and when compared with other factors separately (Figure 6.15, 6.16, 6.19 and 6.20).

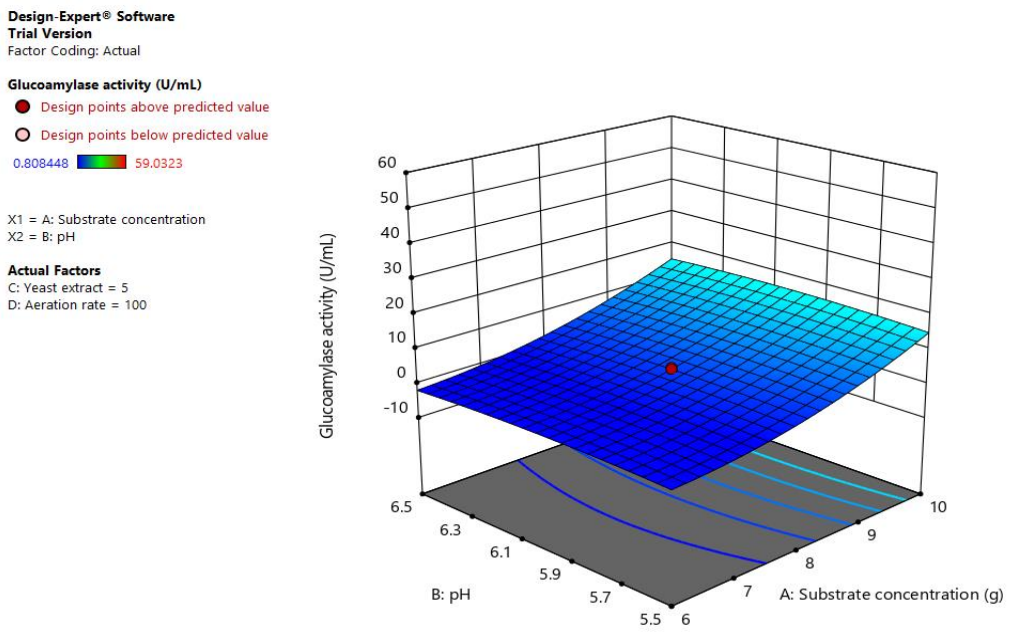


Figure 6.15 Response surface plot showing the effect on pH, substrate concentration and their mutual effect on the production of GA activity (U/mL).

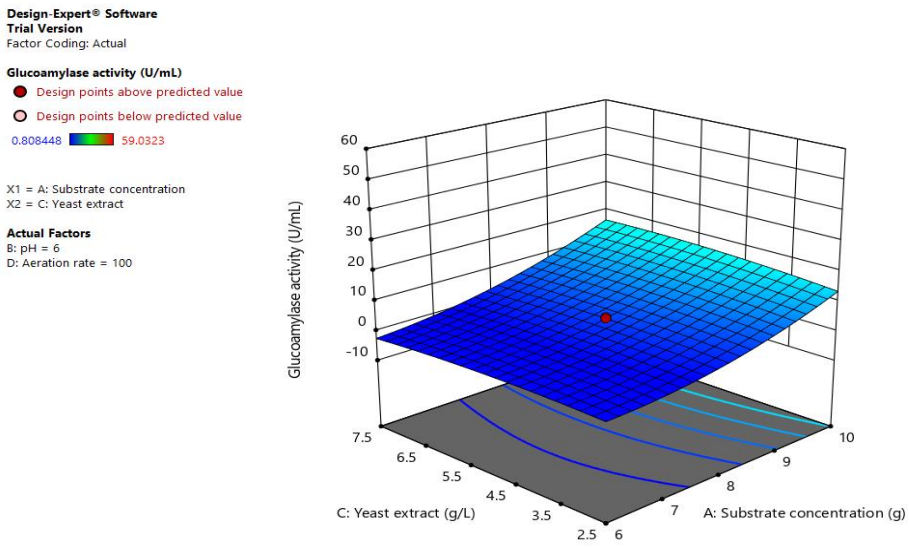


Figure 6.16 Response surface plot showing the effect on yeast extract concentration, substrate concentration and their mutual effect on the production of GA activity (U/mL).

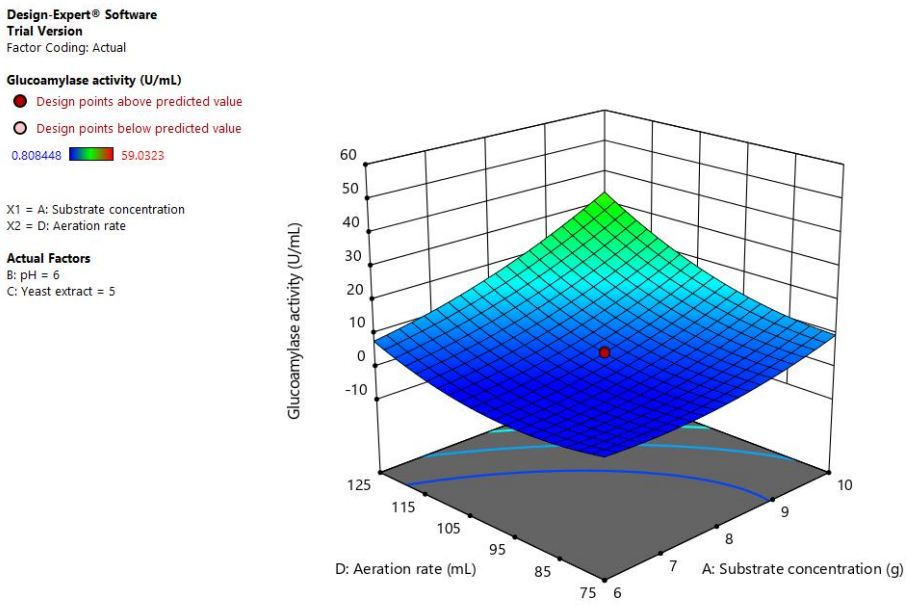


Figure 6.17 Response surface plot showing the effect on aeration rate, substrate concentration and their mutual effect on the production of GA activity (U/mL).

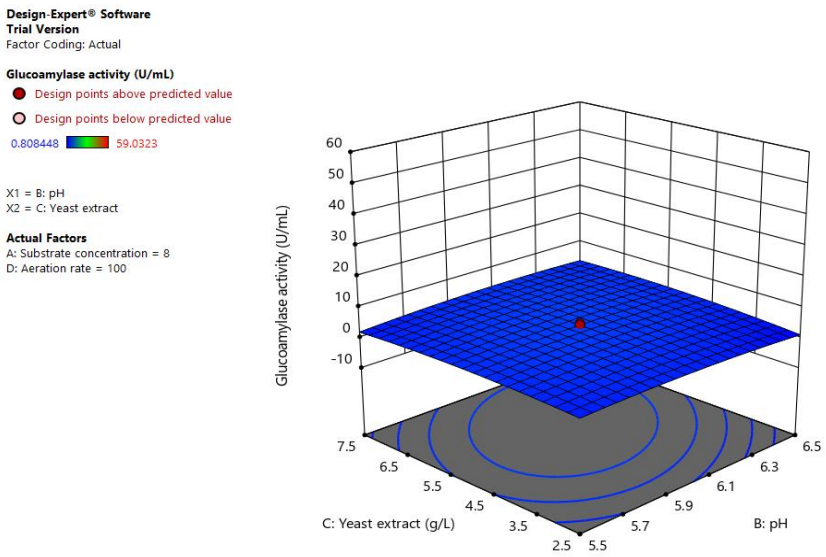


Figure 6.18 Response surface plot showing the effect on yeast extract concentration, pH and their mutual effect on the production of GA activity (U/mL).

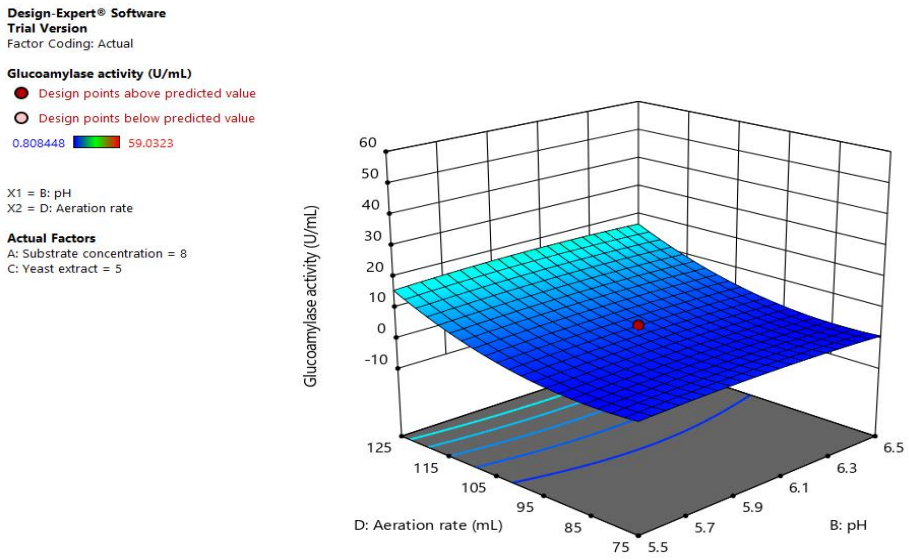


Figure 6.19 Response surface plot showing the effect on aeration rate, pH and their mutual effect on the production of GA activity (U/mL).

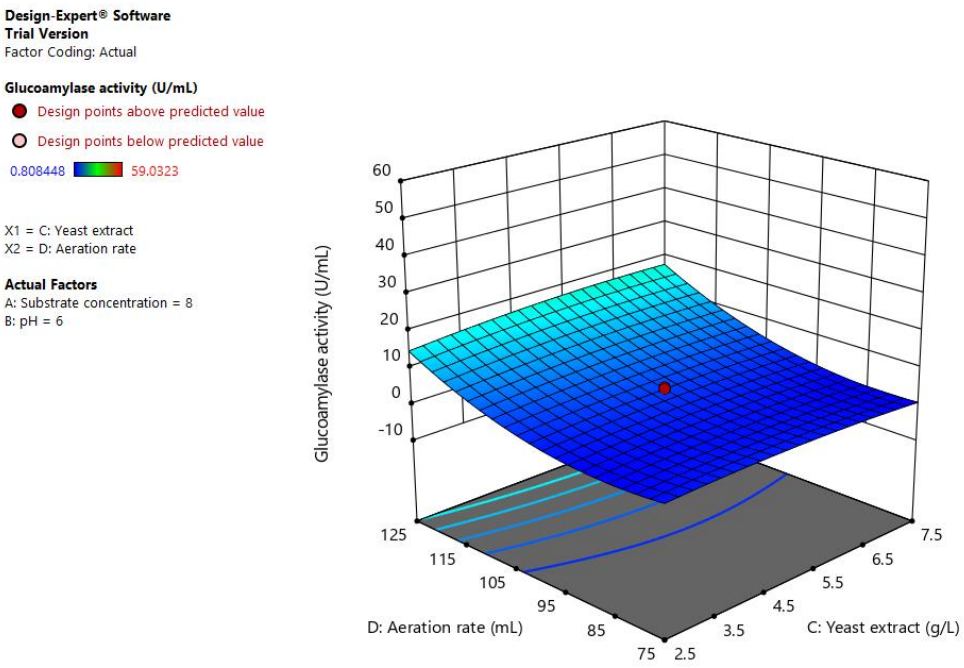


Figure 6.20 Response surface plot showing the effect on aeration rate, yeast extract concentration and their mutual effect on the production of GA activity (U/mL).

The contour plots above (Figure 6.15 – 6.20) showed the effect of four different factors on GA production. Substrate concentration and aeration rate had a positive effect on GA activity. An increase in GA activity was observed from the plots as substrate concentration and aeration rate increased. pH and yeast extract has no significant effect on GA activity with no effect observed with an increase in pH and yeast extract (Figure 6.17). The maximum GA activity of 59.03 U/mL was obtained using substrate concentration of 8%, pH 6, YE 5.0 g/L and aeration rate of 150 mL on the 3rd day of fermentation.

Although during fermentation, the nutrient concentration, pH and physical structure of the raw material changed continuously, all these parameters were reported to have an effect on microbial growth and enzyme production (Kiran et al., 2014).

From the graphs above, the substrate concentration shows a positive effect on glucoamylase activity. The results were used for bench top scale glucoamylase production in chapter 6.4.1.

6.3 Glucoamylase production via solid-state fermentation

Glucoamylase hydrolyses α-1,4 and α-1,6 linkages of glucose to produce glucose monomers as the sole end product from starch and other polymers. Although glucoamylase has been produced traditionally by SmF, recently, SSF has been increasingly applied for the production of glucoamylase enzyme.

SSF uses less complicated equipment, requires lower capital, reduced energy requirement, uses less water and produces lower waste water. Agro-industrial wastes are generally considered as the best substrates for the SSF processes and enzyme production (Ellaiah, Adinarayana, Bhavani, Padmaja, & Srinivasula, 2002).

Glucoamylase production has been reported by SSF using rice flake manufacturing waste products as substrate (Hema et al., 2006), rice bran, wheat bran and paddy husk (Shruti et al., 2013), waste bread, waste cake, cafeteria waste (Kiran et al., 2014) using different fungal strains and optimisation process as described in literature review chapter 2.6.

In this chapter, glucoamylase production has been studied under SSF with fungus *Aspergillus awamori* using sorghum bran as the growth substrate.

6.3.1 Impact of moisture content on glucoamylase production

The moisture content of the medium changes during fermentation due to evaporation and metabolic activities (Baysal, Uyar, & Aytekin, 2003). Thus optimum moisture level of the substrate is of importance for optimum enzyme production.

Sorghum bran was inoculated with *A. awamori* under SSF for 5 days with moisture content within the range of 50% to 90% to investigate the impact of moisture on glucoamylase production. The result (Figure 6.21) showed that 70% moisture content produced the highest glucoamylase activity (96.65 U/mL) on day 1 of fermentation. A peak enzyme activity was obtained on day 1 of incubation as well in SSF of sorghum bran when 80% and 90% moisture content were used before a decrease in enzyme activity was seen as the cultivaton period progressed. While an increase in enzyme activity was obtained from 50% and 60% moisture content of SSF of sorghum bran as the incubation period progressed (Figure 6.21).

The steady increase in GA activity with lower moisture content could be as a result of lower solubility of nutrients in lower initial moisture content while higher initial moisture contents resulted in decreased porosity and gas exchange which may have resulted in the decrease in GA activity as the initial moisture content increases.

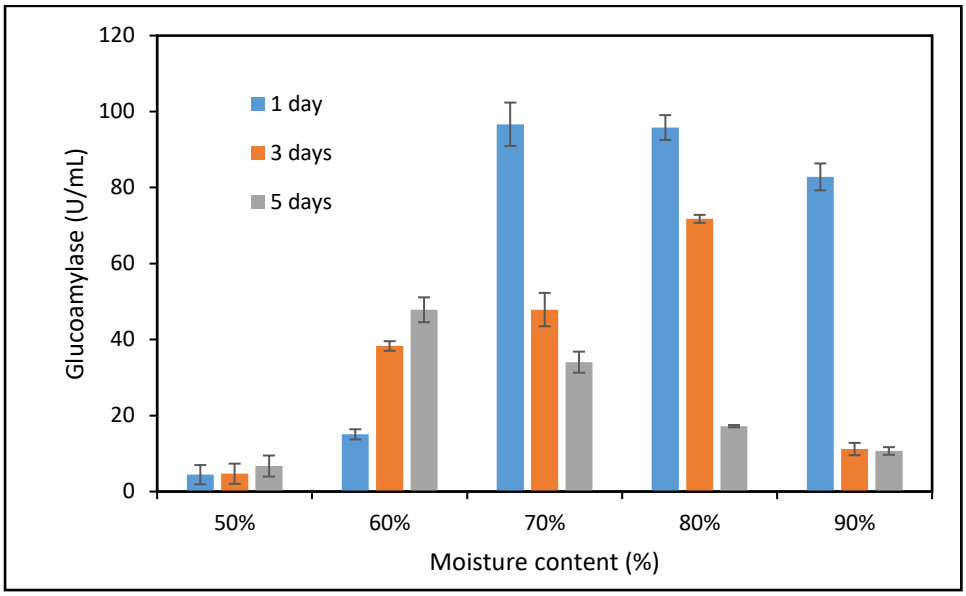


Figure 6.21 Impact of moisture content on glucoamylase production in SSF using Petri-Dish, 2 g of substrate at 28˚C.

6.3.2 Time profile

The result obtained from Figure 6.21 was further explored to establish a time profile for GA production under SSF using 70% moisture content. The result obtained (Figure 6.22) was similar in trend to that obtained in Figure 6.20, which indicated a higher GA activity (34.14 U/L) from sorghum bran on day 1 of the fermentation.

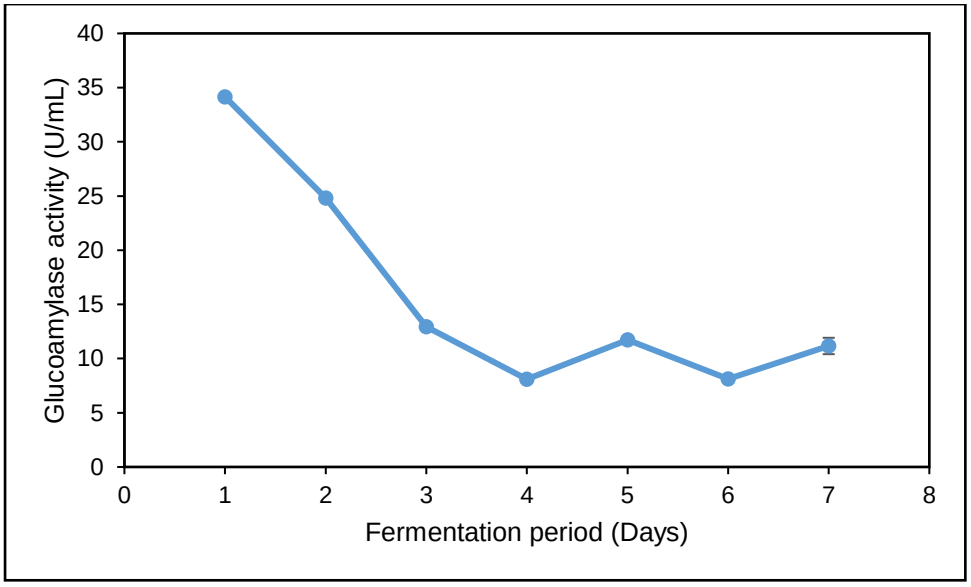


Figure 6.22 GA activity in assays with a 70% moisture content over time (days) using Petri-Dish, 2 g of substrate at 28˚C.

6.3.3 RSM for glucoamylase enzyme production in SSF

Fewer preliminary experiments were done on SSF for glucoamylase production therefore different conditions were computed from various experimental conditions from literature such as the best pH, fermentation period and temperature with 70% moisture content. These conditions were used as the central points for the RSM optimisation of GA in SSF.

Four numeric factors were set in horizontal level. The design of each factor was as listed in Table 6.6. The specific condition of each run was as listed in Appendix v.

Table 6.6 Central composition design of factors on SSF for glucoamylase production

Numeric factor	Unit	Low value	High value	-alpha	+alpha
Moisture content	%	60	80	50	90
Temperature	˚C	28	32	26	34
pH		5.5	6.5	5.0	7.0
Yeast extract	g/L	1.5	3.5	0.5	4.5

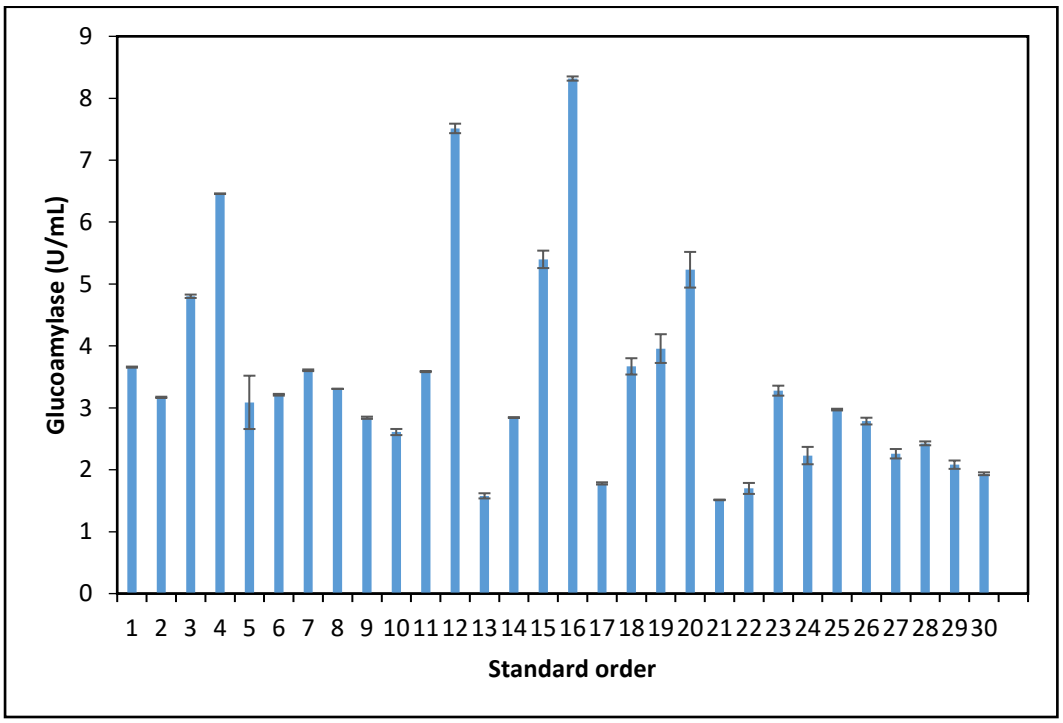


Figure 6.23 RSM of glucoamylase production via SSF for 5 days. Central points (standard order of 25 to 30) moisture content 80%, temperature 32˚C, pH 6.5, and yeast extract concentration 3.5%.

The 3D response surface plots show the relationship between three variable (X, Z and Y) as shown in Figures 6.24 – 6.29. An increase in moisture content and

temperature resulted in an increase in glucoamylase activity when compared with other factors selected such as pH and yeast extract for glucoamylase optimisation. pH and yeast extract has no effect on the optimisation of glucoamylase activity when their mutual effect was considered together (Figure 6.29) and when compared with other factors separately (Figure 6.25, 6.26, 6.27 and 6.28).

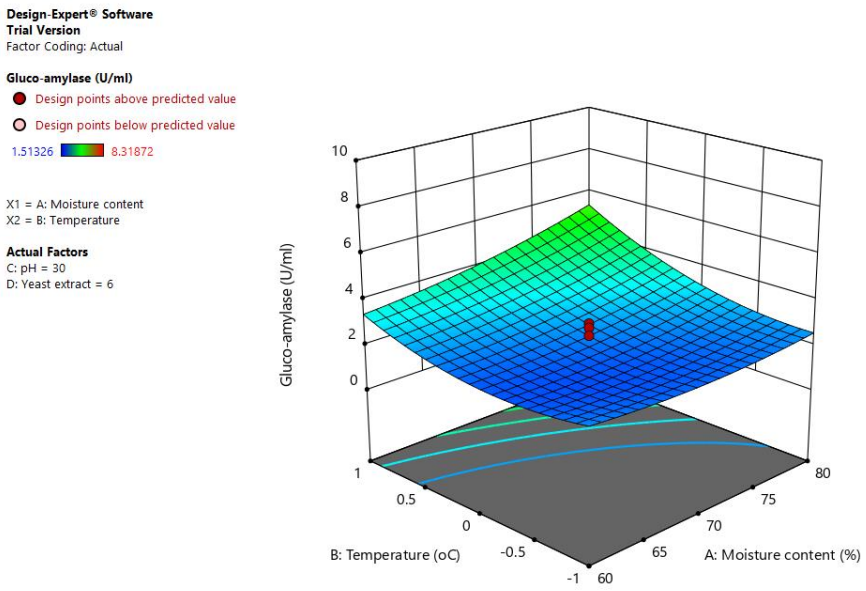


Figure 6.24 Response surface plot showing the effect on temperature (°C), moisture content (%) and their mutual effect on the production of GA activity (U/mL)

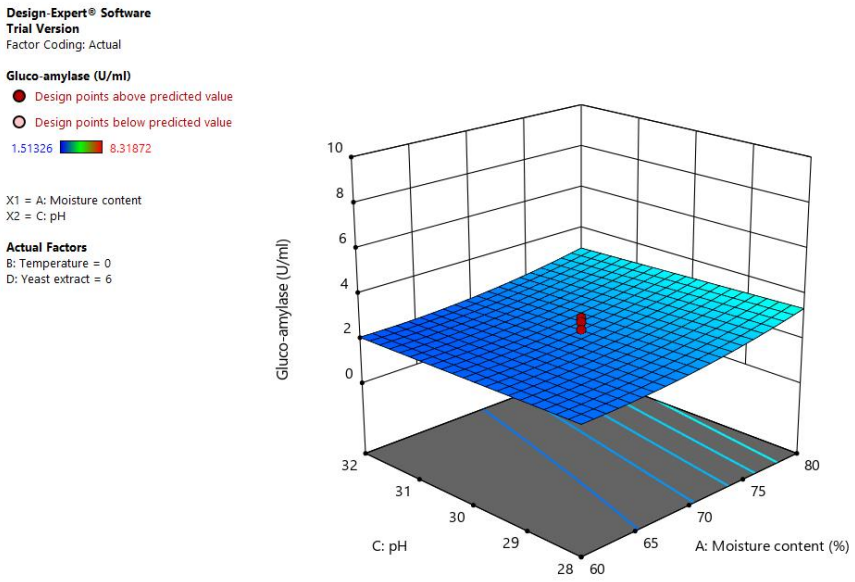


Figure 6.25 Response surface plot showing the effect on pH, moisture content (%) and their mutual effect on the production of GA activity (U/mL)

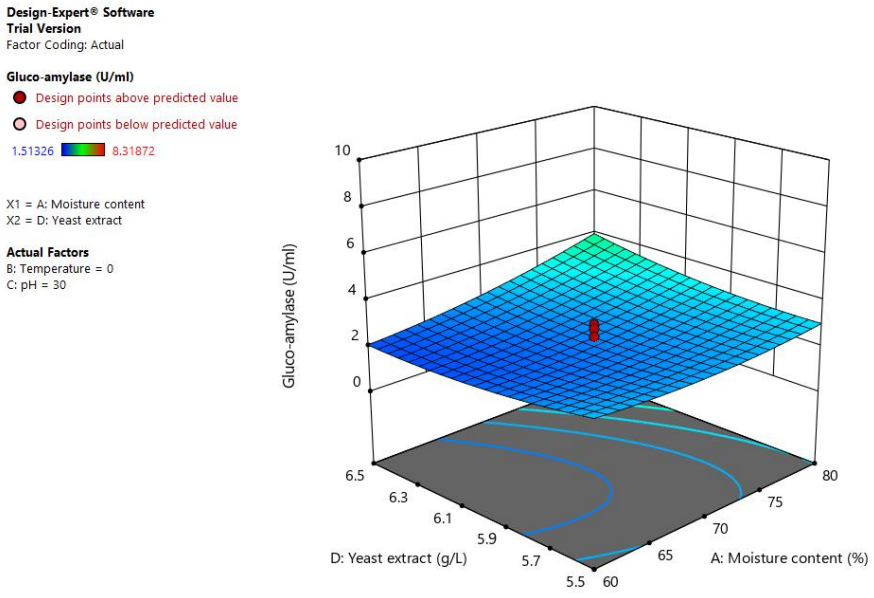


Figure 6.26 Response surface plot showing the effect on yeast extract (g/L), moisture content and their mutual effect on the production of GA activity (U/mL)

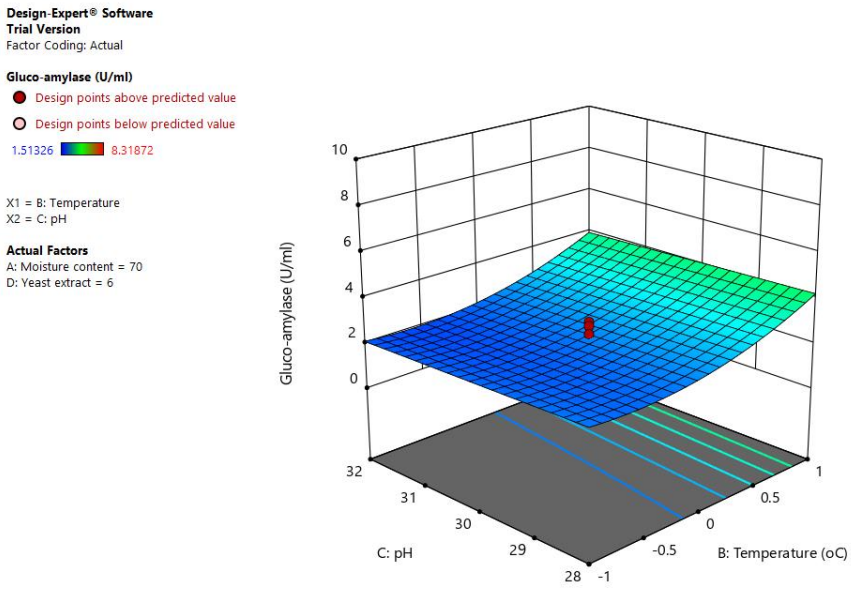


Figure 6.27 Response surface plot showing the effect on pH, Temperature (˚C) and their mutual effect on the production of GA activity (U/mL)

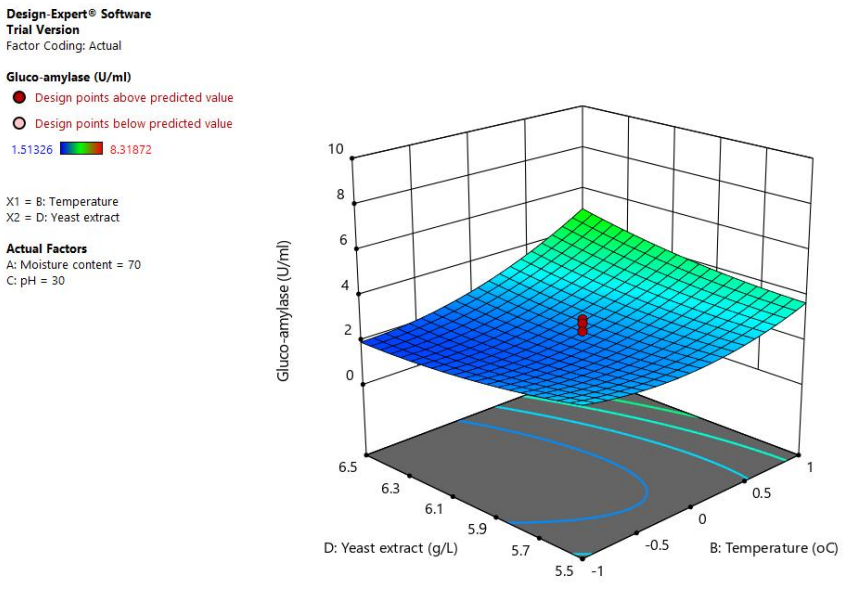


Figure 6.28 Response surface plot showing the effect on Yeast extract (g/L), Temperature (˚C) and their mutual effect on the production of GA activity (U/mL)

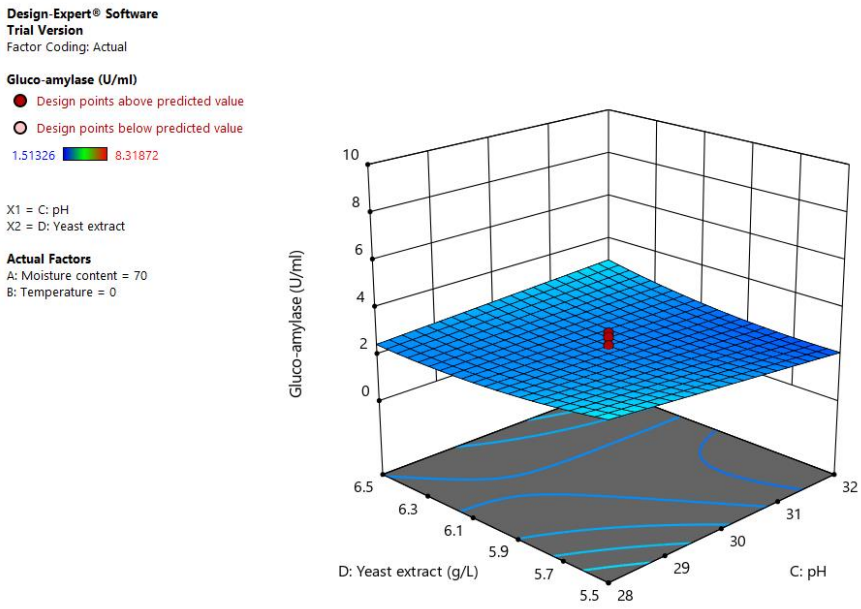


Figure 6.29 Response surface plot showing the effect on Yeast extract (g/L), pH and their mutual effect on the production of GA activity (U/mL).

6.4 Fermentation using 2 L bench fermenter for glucoamylase production in SmF

A 2 L fermenter was used for larger scale production of glucoamylase enzyme using the best condition (standard order 24) obtained from the RSM (Figure 6.13). The aim was to produce a large volume of enzyme solution for sorghum bran hydrolysis with enzyme activity ≥ 20.6 U/mL.

6.4.1 Scale up of gluco-amyalse production in SmF fermentation

The scale up was compared using sorghum bran and sorghum starch at 10% and 6% substrate concentrations, respectively. The medium was autoclaved separately with yeast extract and mineral addition. The fermentation was carried out at agitation speed of 500 rpm, controlled pH at 6, air flow of 1.0 mL/min. The result obtained showed a higher glucoamylase activity after 72 hours of the fermentation (21.67 U/mL) using sorghum bran, which corresponded with the optimum fermentation period, obtained in Chapter 6.2 while sorghum starch has the optimum glucoamylase activity of 11 U/mL at 51 hours of fermentation before a decline in enzyme activity

was obtained. The result obtained from Figure 6.30 using the best condition from Figure 6.13 resulted in a slightly higher enzyme activity (from 20.60 U/mL to 21.67 U/mL). This could be a result of different fermentation vessel and the method of agitation employed. The lower GA activity of sorghum starch could be due to lack of other lignocellulosic component (cellulose and hemicellulose).

The scale up was repeated in four batches using sorghum bran at 10% substrate concentration at agitation speed of 500 rpm, controlled pH at 6, air flow of 1.0 mL/min in a 2 L bench top fermenter. The result obtained in Figure 6.31 shows a similar trend of glucoamylase activity in the range of 20.69 U/mL to 23.53 U/mL with the GA activity obtained in Figure 6.30. There is no significant different among the different batches which shows that the experiment can be replicated.

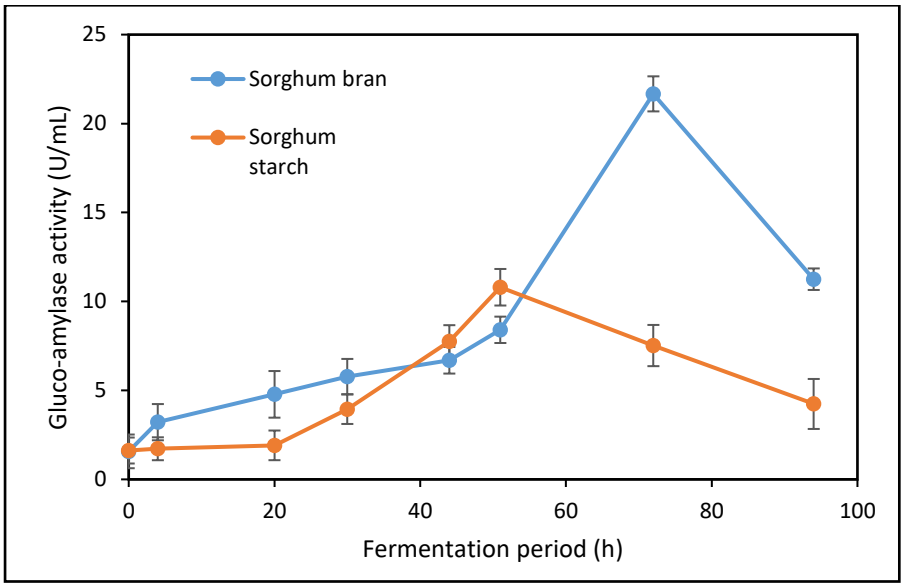


Figure 6.30 Glucoamylase production fermentation scale up from 10% sorghum bran and 6% sorghum starch using 2 L bench fermenter at 500 rpm, pH 6 and air flow of 1.0 mL/min.

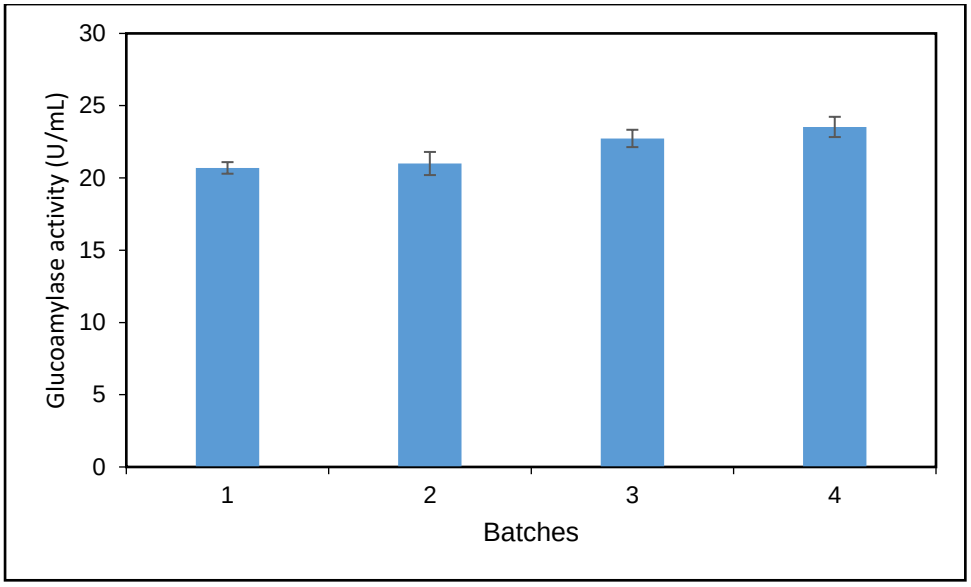


Figure 6.31 Batches of glucoamylase production from 10% sorghum bran in a 2 L bench top fermenter at 500 rpm, pH 6 and air flow of 1.0 mL/min.

Results revealed that the concentration of substrate had positive impact on glucoamylase production (Figure 6.5) . The substrate concentration was increased from 10% to 12.5% and 15% with other fermentation conditions maintained in Figure 6.32. A decrease in glucoamylase activity was firstly observed in both conditions. An increase in glucoamylase activity was obtained from 48 hours of fermentation in both conditions. A further increase in glucoamylase activity was obtained in 12.5% substrate concentration of sorghum bran as the fermentation period increased with optimal enzyme activity of 37.55 U/mL after 115 hours of fermentation. A decline in enzyme activity was obtained due to foaming in the fermenter resulting from fungal autolysis. 15% sorghum bran resulted in a lower glucoamylase production throughout the fermentation period. This could be due to mixing difficulty experienced, thereby resulting in a low product to substrate yield, which justifies why the experiment in chapter 6.2.2 was not carried out above 10% substrate concentration.

Higher stirring speeds above 500 rpm to facilitate the mixing of medium when 15% substrate concentration was used probably resulted in mechanical and oxidative stress, excessive foaming, disruption and physiological disturbance of cells, while lower stirring speed seemed to limit oxygen levels along with the lacking of

homogeneous suspension of the fermentation medium and breaking of the clumps of cells.

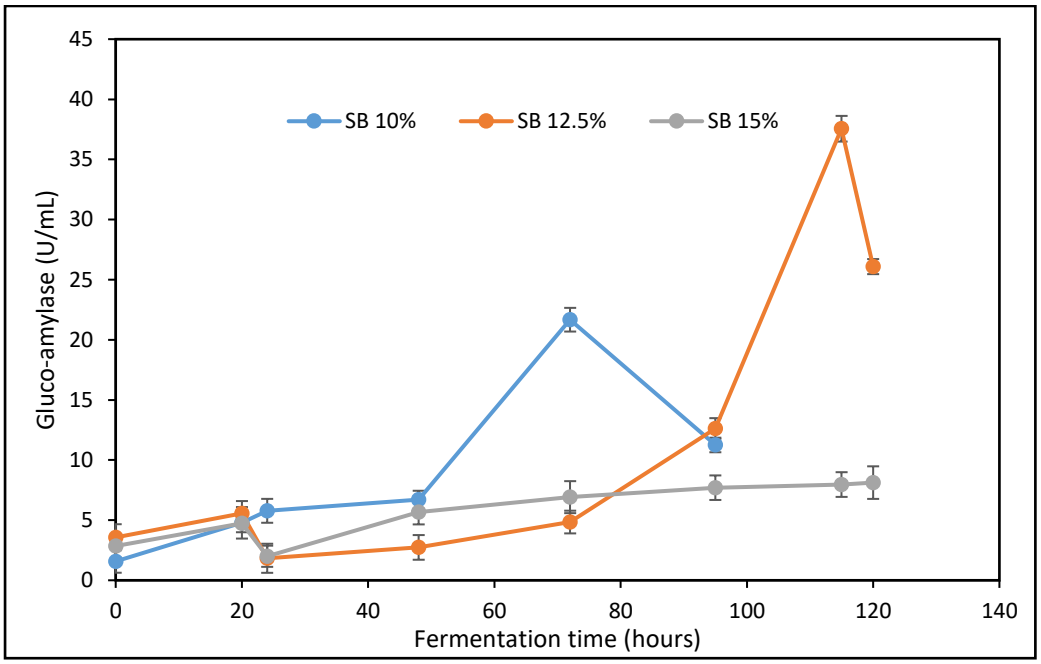


Figure 6.32 Larger scale glucoamylase production from sorghum bran using 2 L bench fermenter at 500 rpm, pH 6 and air flow of 1.0 mL/min.

Further experiment was not carried out due to time limitation.

6.5 Hydrolysis of sorghum bran

Enzymatic hydrolysis was carried out by mixing sorghum bran with deionised water to form a bran slurry. The bran slurry was subjected to hot water pre-treatment for gelatinisation. The yield at different enzyme and substrate loading rate using the crude glucoamylase enzyme was investigated.

6.5.1 Impact of enzyme loading rate on sorghum bran hydrolysis

Table 6.7 and Table 6.8 show the reducing sugar content in the sorghum bran hydrolysates and the saccharification yields of sorghum bran after addition of varied concentrations of fresh crude glucoamylase enzyme from *A. awamori*. An analysis was conducted by HPAEC-PAD to identify the sugars present in the hydrolysates.

However, in the three hydrolysates of sorghum bran only one distinctive peak was detected. The major sugar in the hydrolysates was glucose. As can be seen in Table 6.7, the sorghum bran hydrolysate with 10 mL of glucoamylase solution had the higher saccharification yield (69.76%) after 48 hours of hydrolysis. The addition of glucoamylase in the hydrolysates had a positive effect on the sugar content of sorghum bran after hydrolysis when compared with the control. The extent of reaction indicated that sorghum bran had high susceptibility for hydrolysis to glucose with higher result obtained with 10 mL of glucoamylase after 48 hours of hydrolysis (2.79 g/L in Table 6.7). However, there was slight difference with the result obtained with 10 mL and 15 mL of glucoamylase, respectively.

Table 6.7 Sugar contents from sorghum bran hydrolysate using varied concentration of crude glucoamylase enzyme

Time (hour)	**Control (g/L)**	**10 mL Glucoamylase (g/L)**	**15 mL Glucoamylase (g/L)**
0 hour	0.04 ± 0.07	0.63 ± 0.17	0.86 ± 0.33
24 hours	0.03 ± 0.07	0.95 ± 0.09	0.98 ± 0.73
48 hours	0.03± 0.02	2.79 ± 0.33	2.76 ± 0.54

* The values ± were standard deviations. All experimental assay was performed in triplicate.

Table 6.8 Saccharification yield of sorghum bran hydrolysate from different enzyme loading rate

Time (hour)	**Control (%)**	**Hydrolysis using 10 mL Glucoamylase (%)**	**Hydrolysis using 15 mLGlucoamylase (%)**
0 hour	0.90	15.76	21.40
24 hours	0.85	23.79	24.60
48 hours	0.83	69.76	69.00

6.5.2 Time profile of enzyme loading rate on sorghum bran hydrolysis

Different enzyme loading rate (10 U/mL – 50 U/mL) was used for the hydrolysis of 2 g of sorghum bran for 72 hours at 55˚C and 200 rpm. Samples were taken every 24 hours and analysed for released sugar.

The results revealed a steady increase in released sugar with higher enzyme loading rate and as the hydrolysis time progressed (Figure 6.33). There was no significant difference with the amount of sugar released at 72 hours for enzyme loading rate of 10, 20, 30 and 40 U/mL but there was a significant increase in the sugar released at 72 hours of hydrolysis when 50 U/mL of crude glucoamylase enzyme was used.

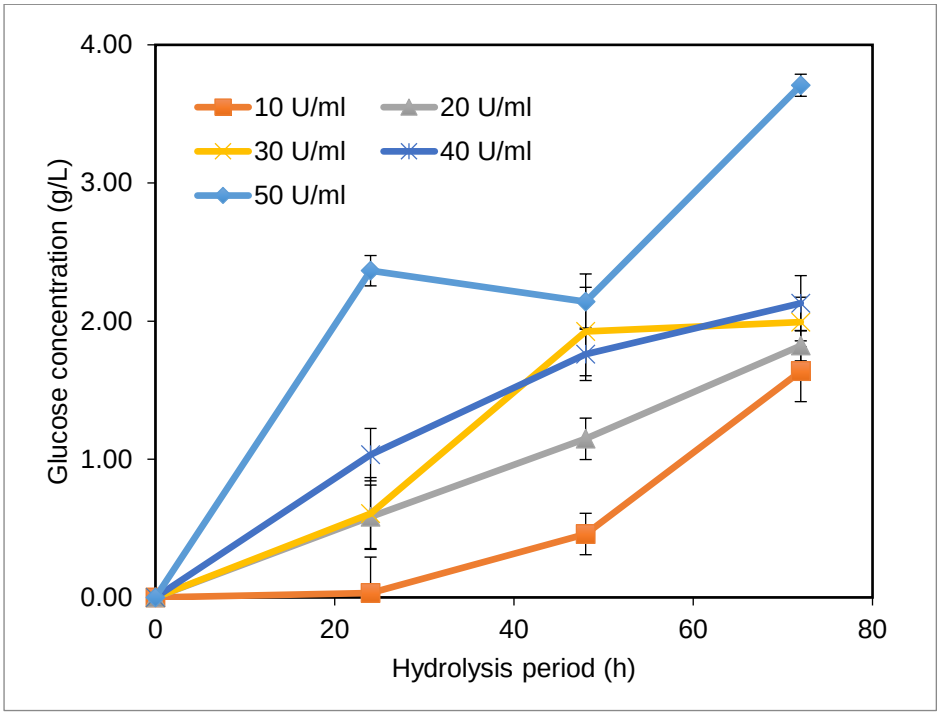


Figure 6.33 Impact of enzyme loading rate on sorghum bran hydrolysis at 200 rpm, 55˚C for 72 hours.

The saccharification yield increased as enzyme loading rate increased with hydrolysis time. The enzyme loading rate of 50 U/mL had the highest saccharification yield of 83.27% at 72 hours of hydrolysis (Table 6.9).

Table 6.9 Saccharification yield (%) of enzyme loading rate on sorghum bran hydrolysis

	10 U/mL	20 U/mL	30 U/mL	40 U/mL	50 U/mL
0	29.65	59.52	60.36	60.19	60.59
24	60.36	63.09	64.07	66.51	75.05
48	62.97	66.55	72.13	70.96	73.69
72	70.18	70.67	72.55	73.21	83.27

At an increased enzyme loading rate of 10 mL /g of 20 U/mL and 20 mL/g of 20 U/mL for sorghum bran hydrolysis for 72 hours using 2 g of substrate per 100 Ml, the optimum sugar concentration released was achieved with 10 mL/g of 20 U/mL enzyme loading rate. An increase in enzyme loading rate resulted in a decreased sugar released from the hydrolysis of sorghum bran with 10 mL/g of 20 U/mL enzyme loading after 72 hours of fermentation. The optimum sugar released (30.31 g/L) was obtained with enzyme loading rate A (10 mL/g of 20 U/mL enzyme solution) from Figure 6.34 at 48 hours of hydrolysis.

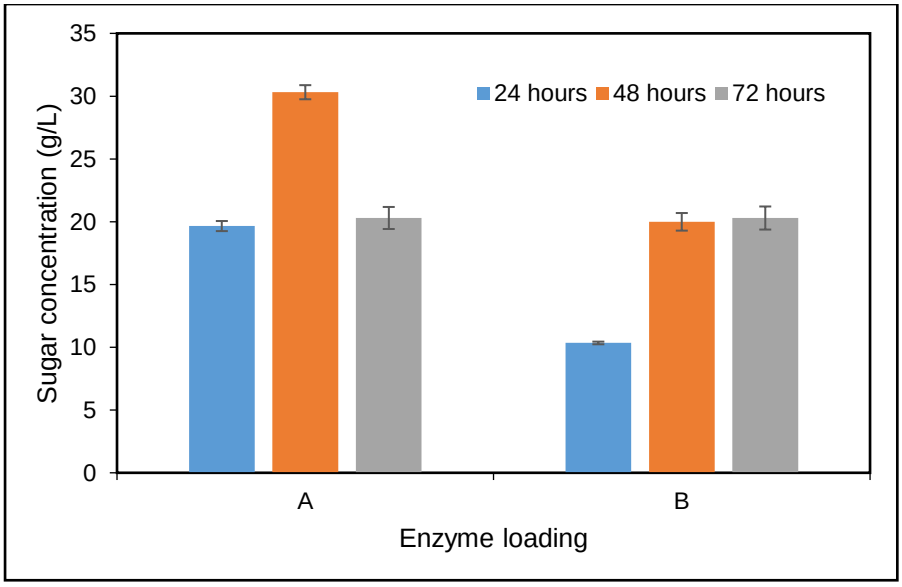


Figure 6.34 Effect of increased enzyme loading rate on sorghum bran hydrolysis at 200 rpm, 55˚C for 72 hours.

A: 10 mL /g of 20 U/mL of crude glucoamylase enzyme

B: 20 mL/g of 20 U/mL of crude glucoamylase enzyme.

6.5.3 Impact of different substrate loading rate on sorghum bran hydrolysis

The impact of different substrate loading rate was conducted using the enzyme-loading rate of 50 U/mL from Figure 6.33 for 72 hours of hydrolysis. From the result obtained in Figure 6.35, the sugar concentration increased with yield as the substrate loading increases with time.

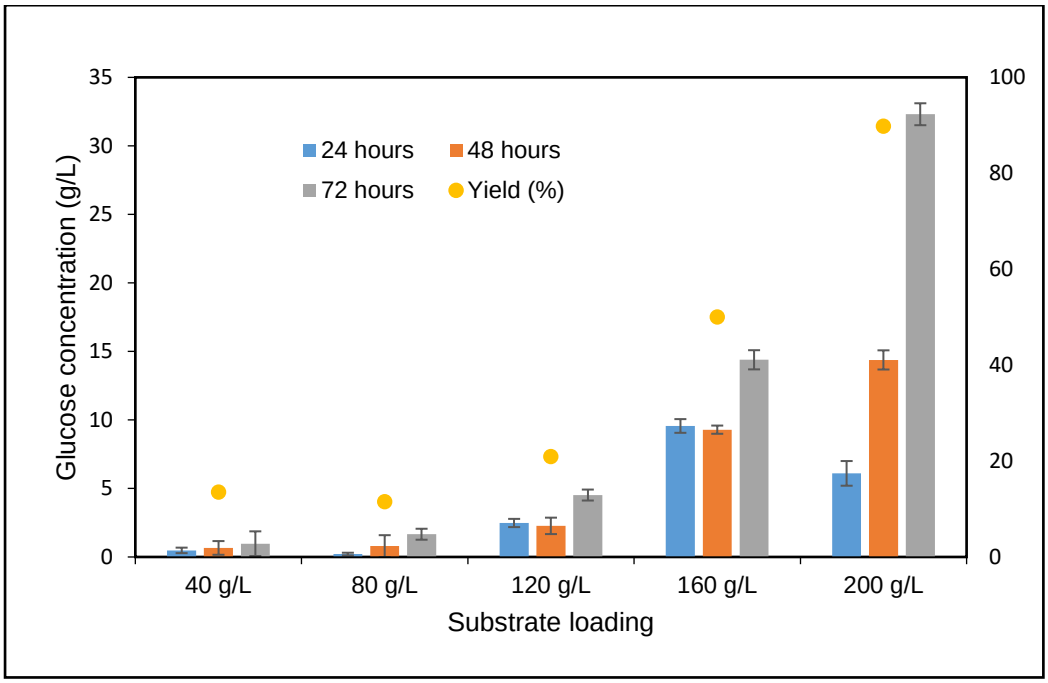


Figure 6.35 Impact of different substrate loading rate on sorghum bran hydrolysis at 200 rpm, 55˚C for 72 hours.

6.5.4 Comparison of commercial enzyme and crude home enzyme for sorghum bran hydrolysis

The commercial enzyme (glucoamylase and alpha-amylase) from megazyme starch kit was used to hydrolyse sorghum bran for 5 days and was compared with the crude glucoamylase enzyme (HE) for effective hydrolysis profile. The substrate loading rate was 4 g in 50 mL, equivalent to 80 g/L.The commercial enzyme (CE) shows an increase in sugar released as the hydrolysis time progressed with optimum sugar

released of 12.72 g/L obtained after hydrolysis on the 4th day before a decline in sugar after the 5th day. The crude home enzyme shows a progressive increase in sugar released up to day 5 of hydrolysis (11.74 g/L) (Figure 6.36).

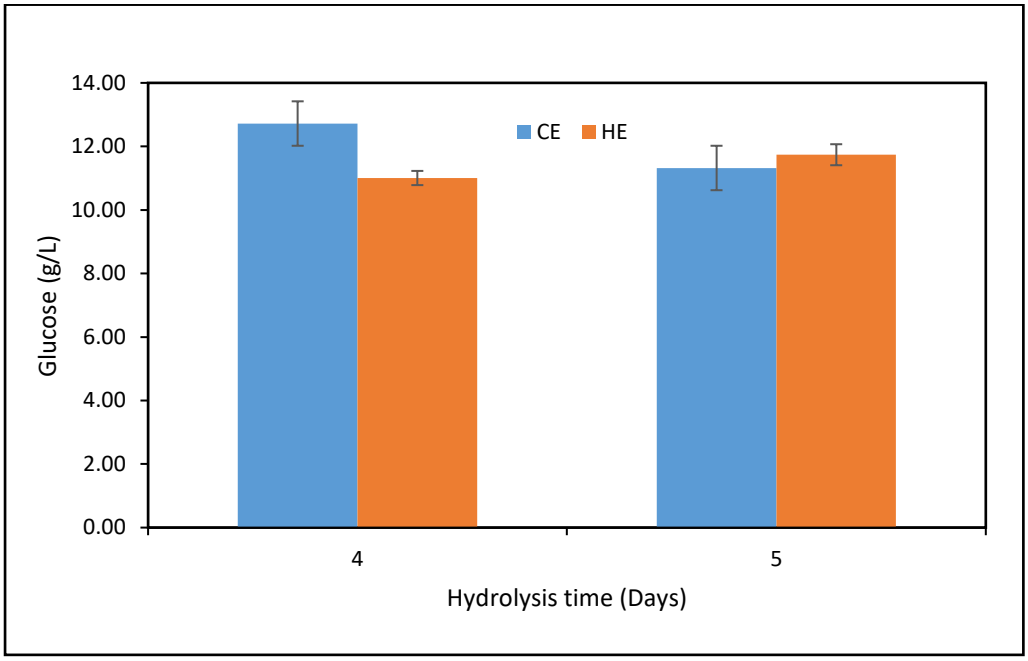


Figure 6.36 Comparison of commercial enzyme (alpha-amylase) with crude home enzyme (glucoamylase) at 200 rpm, 55˚C.

6.5.5 Larger scale sorghum bran hydrolysis

The larger scale sorghum bran hydrolysis was carried out in a 2 L bench top fermenter. The sorghum bran hydrolysis was carried out using 160 g of substrate gelatinised in 400 mL of 0.05M of citric acid buffer. 400 mL of 20 U/mL of enzyme solution was added. The hydrolysis was carried out at 55˚C, 500 rpm for 48 hours. The result obtained in Figure 6.37 shows that the average glucose concentration obtained after sorghum bran hydrolysis was 38.7 g/L. The theoretical glucose concentration was 38.7 g/L based on the starch content of 16.35% in the red sorghum bran. The glucose concentration shows that hydrolysed sorghum bran can be used for the production of value added products, which is demonstrated in chapter 7 for ethanol production.

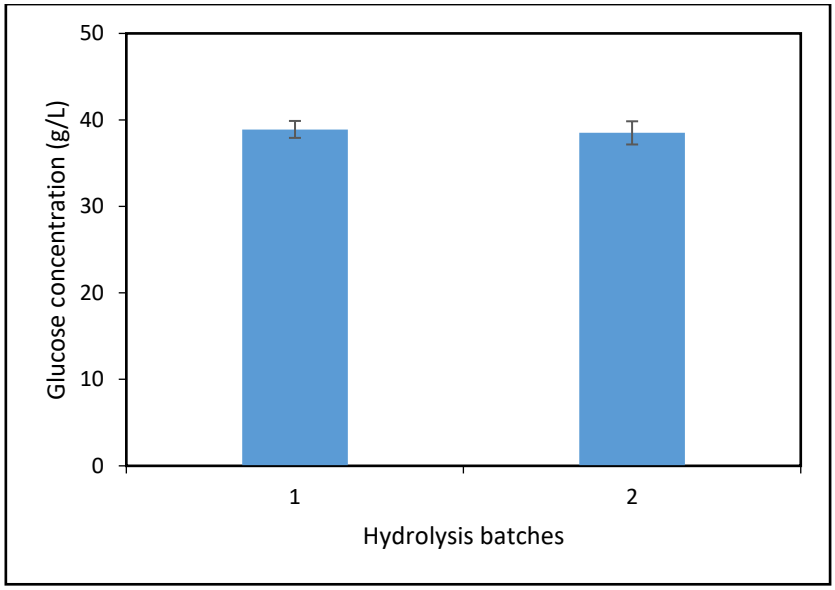


Figure 6.37 Glucose concentration of larger scale sorghum bran hydrolysis in 2-L bench top fermenter.

6.6 Summary

Three different milling processes (peanut butter maker, blender and knife mill) under two milling conditions were examined for the separation of sorghum bran from its kernel. Knife mill resulted in 81.93% of total starch while blender and peanut butter maker resulted in 12.95% and 16.35% total starch in the sorghum bran respectively. The mass balance shows that the sorghum bran had the largest recovery output of 49.18% while sorghum starch as 21.12%. Wet milling process of sorghum grain proved to be more effective than dry milling employed in separating sorghum bran from its kernel. The impact of various parameters under different fermentation conditions were assessed on glucoamylase production in SSF and SmF using *A. awamori*.

In SmF for GA production, the initial fermentation of sorghum bran for 24 hours shows the feasibility for GA production from sorghum bran. The time profile shows that optimum GA activity was obtained on day 5 of fermentation at 1.90 U/mL. 10% substrate concentration resulted in the highest GA activity of 12.58 U/mL on day 5 of fermentation. The impact of pH on GA showed that pH 6 gave the optimum GA activity of 19.26 U/mL on day 3 of fermentation. Aeration rate of 200 mL working volume in 500 mL shake flask (200/500mL) gave the maximum GA activity of 12.74

U/mL on day 3 of fermentation. The different inoculation ratios used for GA production followed no designated pattern except for the inoculation ratio of 5 million spores/g with optimum GA activity of 4.57 U/mL on day 4 of fermentation. Temperature of 28˚C had the most significant effect in GA activity with optimal GA activity of 10.83 U/mL on day 4 of fermentation. 2.5 g/L of yeast extract addition gave the highest GA activity of 13.03 U/mL on day 3 of fermentation. The addition of mineral increased the optimum GA activity from 1.89 U/mL on day 5 to 3.60 U/mL on day 3 of fermentation. Mineral composition C (glucose 5 g/L, YE 10 g/L, KH_2PO_4 1g/L, $MgSO_4.7H_2O$ 0.3 g/L and $CaCl_3$ 0.3 g/L) gave the highest GA activity of 5.03 U/mL on day 3 of the fermentation. The RSM four model factors (substrate concentration, aeration rate, yeast extract and pH) were identified as significant under ANOVA with the run standard order 24 (10% substrate concentration, pH 6, 5 g/L YE and 150 mL aeration rate) having the highest GA activity of 59.03 U/mL.

In SSF for GA production, limited preliminary experiments were conducted. Moisture content of 70% gave the optimum GA activity of 96.65 U/mL on day 1 of fermentation. The highest GA activity of 34.14 U/mL was obtained on day 1 of fermentation with 70% moisture content under 7 days' time profile. The RSM four model factors (moisture content, temperature, pH and yeast extract) were identified as significant under ANOVA. The standard order 16 (80% moisture content, 32˚C, pH 6.5 and 3.5 g/L YE.) gave the highest GA activity of 8.32 U/mL.

The larger scale GA production in SmF in 2 L bench top fermenter gave an optimum GA activity of 21.67 U/mL at 72 hours of fermentation (3 days) from 10% sorghum bran concentration. At a higher sorghum bran concentration of 12.5% an optimum GA activity of 37.55 U/mL was obtained at 115 hours of fermentation whereas, an increase in sorghum starch concentration resulted in a decrease in GA activity.

The enzymatic hydrolysis of sorghum bran using crude GA enzyme resulted in a higher glucose concentration with an increase in GA enzyme used for 72 hours of hydrolysis. A further increase of enzyme loading rate of 20 mL/g of 20 U/mL of crude GA enzyme resulted in a decrease glucose concentration from the hydrolysed sorghum bran. The glucose concentration increased with yield as the substrate loading increased with time. Commercial enzyme shows a slightly higher glucose concentration than the crude enzyme (home enzyme) used for sorghum bran

hydrolysis. The larger scale sorghum bran hydrolysis resulted in a glucose concentration of 38.7 g/L. This shows that a sugar rich stream is obtainable from hydrolysed sorghum bran for the production of value added products.

7 Ethanol fermentation from sorghum waste water and sorghum bran hydrolysate

As described in the literature review, bioethanol is considered as the next generation transportation fuel that could be generated from sustainable raw materials. Chapter 6 described various processes in optimising the production of glucoamylase enzyme in order to generate a sorghum bran hydrolysate. In this chapter, the utilisation of sorghum wastewater (SWW), sorghum bran hydrolysate and wheat straw hydrolysate as a fermentation medium for the production of biochemical/biofuel was explored. In this thesis, the fermentative production of bioethanol was selected as an example.

The obtained sorghum bran hydrolysate (as described in chapter 6.5.2) was centrifuged to separate the solid biomass from the liquid fraction. The liquid fraction of the sorghum bran hydrolysate and the sorghum wastewater (obtained from the sorghum starch producing process, as shown in Figure 3.1) were autoclaved for sterilization purposes. The ethanol fermentation experiments were carried out using three marine yeast strains: *Candida membranifaciens* M2, *Saccharomyces cerevisiae* AZ69, and *Wickerhamomyces anomalus* M15 and one terrestrial yeast: *Saccharomyces cerevisiae* NCYC 2592 (details in chapter 3.17.2). The ethanol fermentation from both sorghum waste water and sorghum bran hydrolysate was performed as described in chapter 3.17.4 and weight loss was monitored throughout the fermentation process and ethanol concentration were determined when no further weight loss was observed.

7.1 Yeast fermentation of sorghum waste water in mini fermenters

The yeast strain *Saccharomyces cerevisiae* AZ69 fermentation was carried out using YPD medium (control) and sorghum wastewater with and without the addition of yeast extract in a mini fermenter vessel. It is considered that sorghum wastewater contains starch and soluble sugars, which could be used for ethanol production. CO_2 was produced as a by-product during ethanol fermentation under facultative anaerobic conditions. Powell, Quain, and Smart (2003) proposed that sugar utilisation was linearly related to the weight loss of the fermentation system due to conversion of the sugars into CO_2; therefore, the weight loss could be used as a

fermentation progress indicator. The most notable weight loss occurred after incubation for 43 - 49 hours (Figure 7.1). The increase in weight loss indicated that the yeast cell metabolised the sugars in the SWW and YPD medium and converted it into ethanol and CO_2. It was strange that that there was no significant weight loss in SWW with yeast extract addition throughout the fermentation period (Figure 7.1).

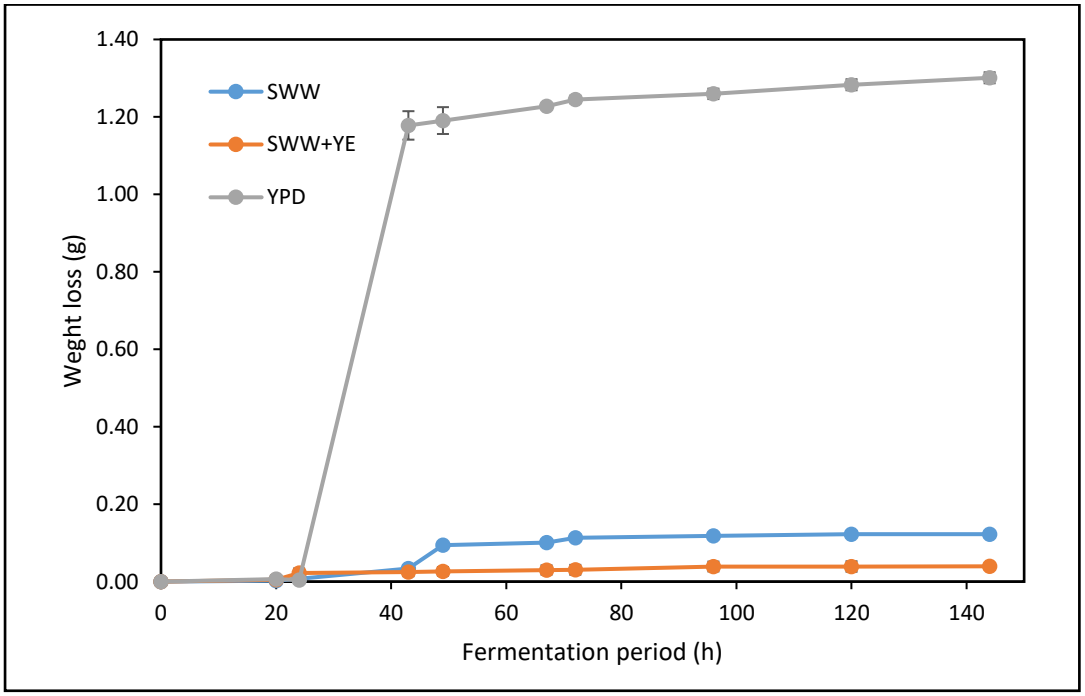


Figure 7.1 The weight loss of yeast fermentations using sorghum wastewater (SWW) at 180 rpm at room temperature.

Weight loss decreased when yeast extract was added to SWW, although studies have shown that addition of nutrients such as yeast extract, peptone and ammonium sulphate boost ethanol production (Duhan, Kumar, & Sunil Kumar Tanwar, 2013). Duhan et al. (2013) reported an increase in ethanol production from 6.55 to 7.11% and 6.83 to 7.58% when yeast extract (1.0 to 2.0 g/L) and peptone (0.5 – 1.5 g/L) were added to potato flour hydrolysate respectively. Laopaiboon, Nuanpeng, Srinophakun, Klanrit, and Laopaiboon (2009) also reported an increase in ethanol production efficiency when 3.0 g/L of yeast extract was added to sweet sorghum juice. According to literature, yeast extract addition at different concentration had a positive effect in improving ethanol production from different substrates. In this study,

the addition of yeast extract resulted in low ethanol production when SWW was used.

Gas chromatography was used to determine the ethanol concentration of SWW, SWW + YE and YPD, at the end of the yeast fermentation. The YPD fermented medium gave 8.43 g/L of ethanol concentration, while SWW and SWW+YE had no ethanol concentration detected. The results obtained from the weight loss experiment (Figure 7.1) showed that there was no significant weight loss in the yeast fermentation of SWW and SWW+YE, which correlated to no ethanol being produced.

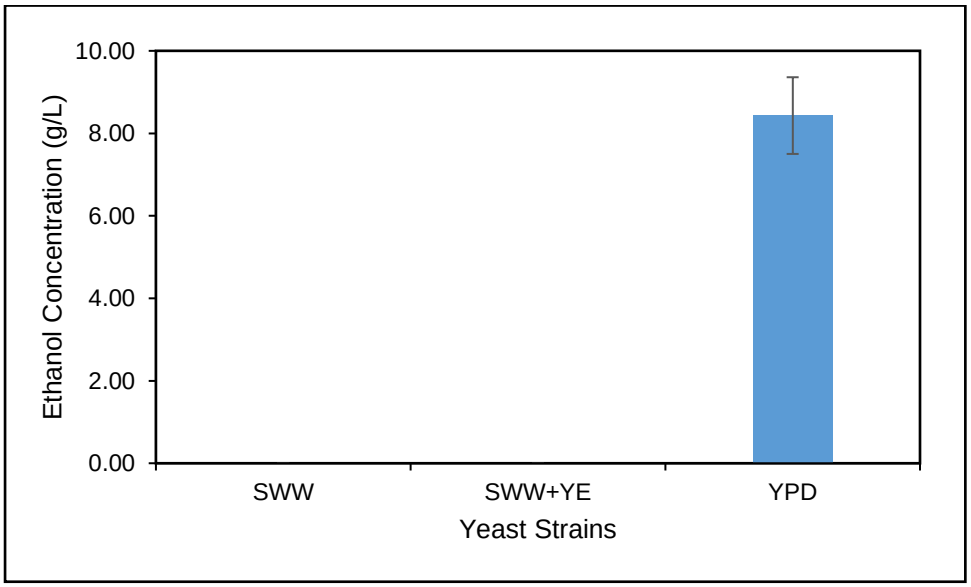


Figure 7.2 Ethanol concentration from sorghum wastewater and YPD

The effect of addition of selected minerals (chapter 4.1.6) to the medium to improve yeast performance was decided (Figure 7.3). The result obtained showed that there was significant weight loss after 49 hours. After this time point, the fermentation went into stationary phase for all assays (Figure 7.3), indicating that the sugars in the media could be depleted by then. With the addition of mineral, the weight loss from SWW without yeast extract was higher than that of SWW with yeast extract. This confirmed that addition of yeast extract to sorghum wastewater had a negative effect on yeast growth.

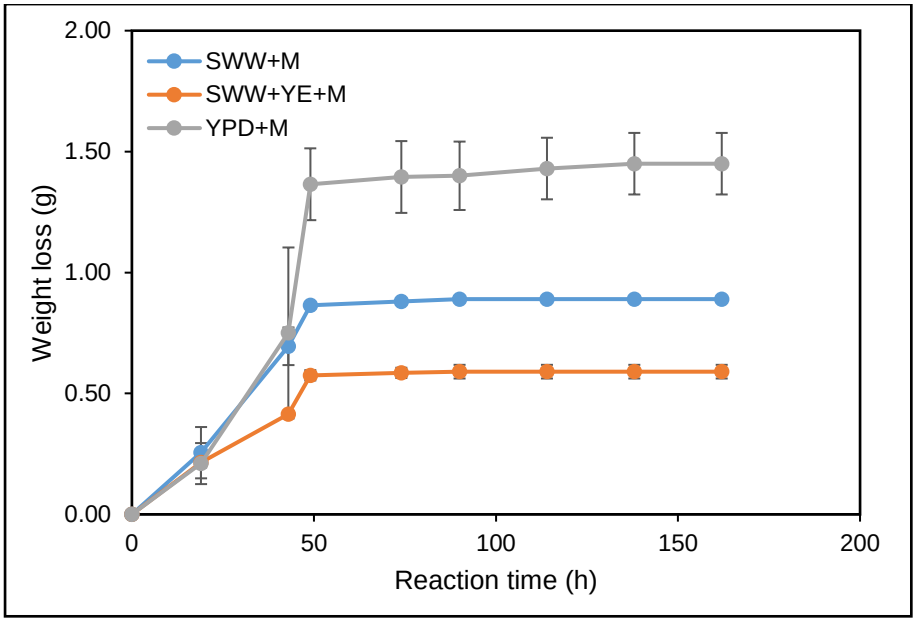


Figure 7.3 The effect of mineral addition on fermentation performance of *S. cerevisiae* AZ69 on sorghum wastewater without yeast extract (blue line), sorghum wastewater with yeast extract (orange line) and glucose synthetic media YPD (grey line).

7.2 Yeast fermentation of sorghum bran hydrolysate in mini fermenters

The sorghum bran hydrolysate obtained from the hydrolysis of sorghum bran (chapter 6.5.5) consisted of 38.7 g/L glucose. This hydrolysate was used for ethanol production by yeast fermentation in 0.1 L mini fermenters.

Four yeast strains were inoculated into sorghum bran hydrolysate medium and weight loss was monitored as described previously. Results revealed that there were weight loss was observed for all fermentations in the first 19 hours. After 40 hours there was very little further weight loss observed for all the strains assayed in this experiment (Figure 7.4), indicating that all the available sugars in the media were consumed.

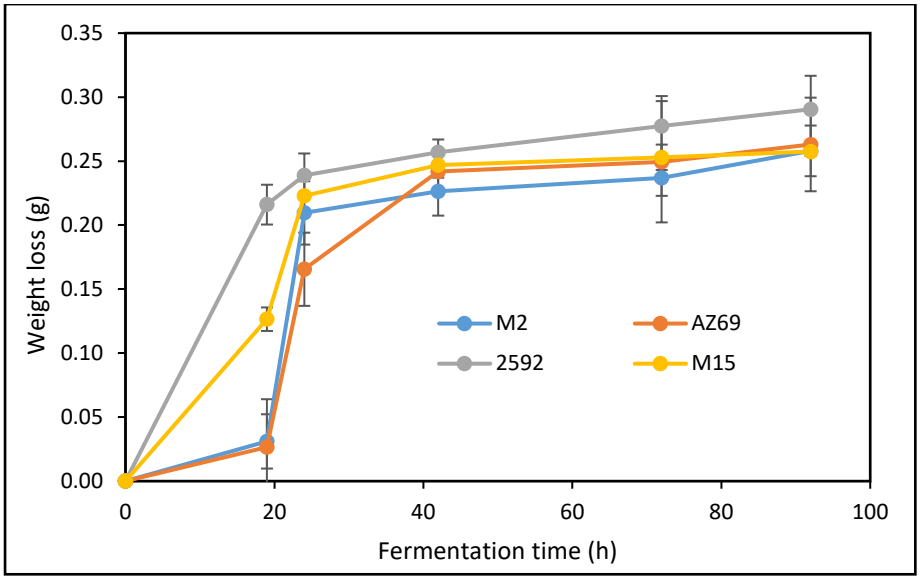


Figure 7.4 Fermentation performances of four yeast strains on sorghum bran hydrolysate in terms of weight loss analogous to CO_2 production

7.3 Ethanol concentration and yield

The natural diversity of the four yeasts was exploited in order to find the yeasts with good ethanol yield for sorghum bran hydrolysate. As shown in Figure 7.5 and Table 7.1, the terrestrial yeast *S. cerevisiae* NCYC 2592 showed a better glucose to ethanol conversion efficiency than other yeast strains. The marine yeast *Wickerhamomyces anomalus* M15 performed better than *S. cerevisiae* AZ65 and *Candida membranifaciens* M2. The highest ethanol production was 19.88 g/L obtained from sorghum bran hydrolysate by *Saccharomyces cerevisiae* NCYC2592 that equals 51.34% of actual ethanol yield. Ruyters et al. (2015) reported that *W. anomalus* have a comparable ethanol yield with *S. cerevisiae* having the highest ethanol yield in fermentation experiments while other non-*Saccharomyces* yeasts yielded lower ethanol amounts. The ethanol yield obtained in Table 7.1 is near the maximum theoretical value except for the yeast strain of NCYC2592, which exceeded the theoretical value. The yeast strain of M15 has the maximum ethanol yield expected near the maximum theoretical ethanol yield from the glucose concentration in the sorghum bran hydrolysate. Another experiment was carried out with the yeast strain of NCYC2592 and M15 to ascertain the result obtained in Figure 7.7.

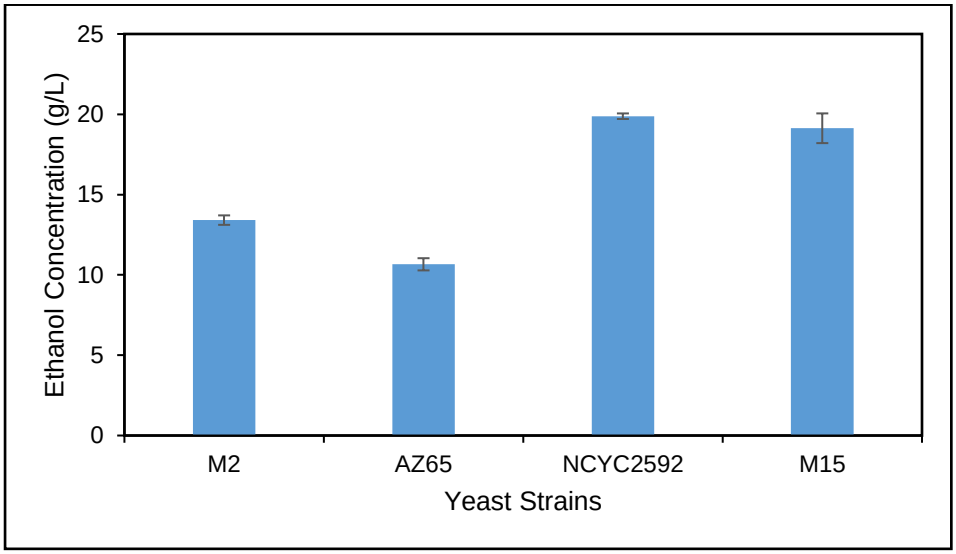


Figure 7.5 Ethanol concentration from sorghum bran hydrolysate using four yeast strains

Table 7.1 Ethanol concentration and actual ethanol yield obtained from yeast fermentations using sorghum bran hydrolysate.

Strain	**Ethanol (g/L)**	**Yield (%)**
***Candida membranifaciens* M2**	13.41±0.29	34.65
***Saccharomyces cerevisiae* AZ65**	10.66±0.38	27.55
***Saccharomyces cerevisiae* NCYC2592**	19.88±0.17	51.34
***Wickerhamomyces anomalus* M15**	19.13±0.92	49.43

From the previous experiment, M15 showed the highest ethanol production within the marine yeast strains used in this study. Therefore, it was taken for further exploration for ethanol production. In a repeat experiment, the yeast fermentation in a 0.1 L mini fermenter using sorghum bran hydrolysate by *W. anomalus* M15 and *Saccharomyces cerevisiae* NCYC2592 was carried out at room temperature, 180 rpm until no further weight loss was recorded as shown in Figure 7.6. The M15

marine strain gave the highest ethanol production of 19.27 g/L at 96 hours of fermentation and 49.79% actual ethanol yield (Table 7.2). The ethanol result obtained in Figure 7.7 shows that the maximum ethanol yield was achieved with the yeast strain of M15. The repeated experiment shows that the result obtained with NCYC2592 in Table 7.3 might have some impurities that influences the ethanol yield above the maximum theoretical value expected.

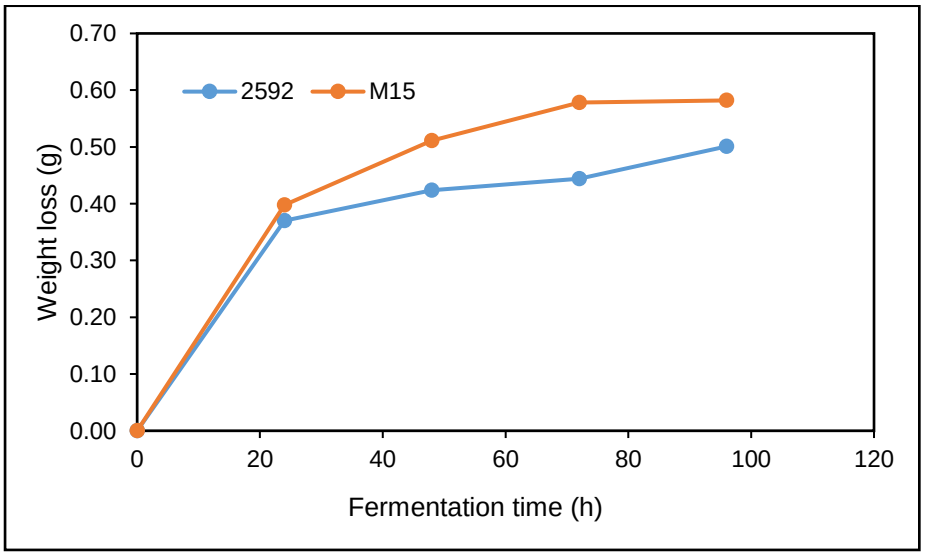


Figure 7.6 Fermentation performances of two selected yeast strains on sorghum bran hydrolysate in term of weight loss due to CO_2 production

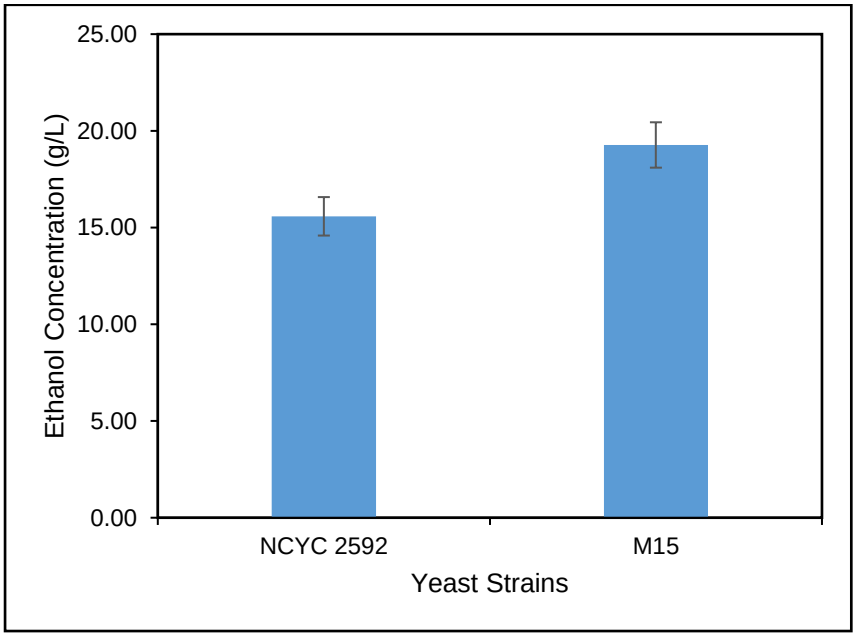


Figure 7.7 Ethanol concentrations from sorghum bran hydrolysate using two selected yeast strains

Table 7.2 Ethanol concentration and actual ethanol yield obtained from yeast fermentation of NCYC 2592 and M15 using sorghum bran hydrolysate

Strain	Ethanol (g/L)	Yield (%)
NCYC 2592	15.58±0.99	40.23
M15	19.27±1.17	49.79

7.4 Ethanol fermentation from modified wheat straw hydrolysate

The yeast strain of *W. anomalus* M15 was cultured on various wheat straw hydrolysate media (Figure 7.8) and the fermentation profiles in terms of weight loss were monitored (Figure 7.9). The glucose concentration of the modified wheat straw hydrolysate was determined as shown in Table 7.3.

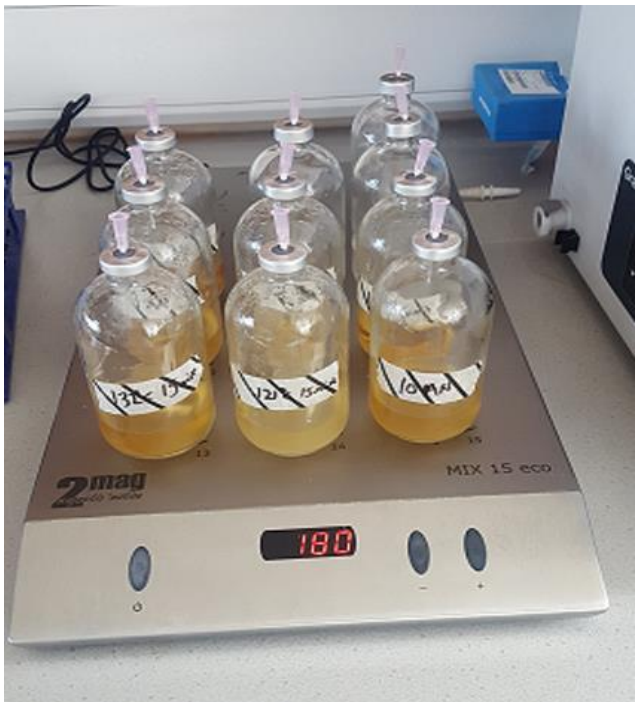


Figure 7.8 Yeast fermentation of wheat straw hydrolysate using mini fermenter vessel at room temperature, 180 rpm

Table 7.3 Glucose concentration of hydrolysed wheat straw for 72 hours using commercial cellulase enzyme (novozyme)

Modified wheat straw conditions	**Modifield wheat straw code**	**Glucose concentration (g/L)**
Fermented wheat straw by *Aspergillus niger* for 10 days	10AN	5.17
Autoclaved wheat straw at 121˚C for 15mins	121˚C 15mins	6.37
Autoclaved wheat straw at 121˚C for 15min and fermented by *Aspergillus niger* for 1 day	1AN 121˚C 15min	5.58
Autoclaved wheat straw at 121˚C for 30mins	121˚C 30mins	6.01
Modified wheat straw at 132˚C for 15mins	132˚C 15mins	5.29
Fermented wheat straw by *Rhizomucor variabilis* for 7 days	7RS	6.01
Modified wheat straw at 100˚C for 60mins	100˚C 60mins	6.13

The modified wheat straw hydrolysate produced by autoclaving at 132˚C for 15 minutes had a significant weight loss within 19 hours of fermentation when compared with other hydrolysates from modified wheat straw. Over the time-course of the fermentation modified wheat straw of 7RS resulted in the highest weight loss (Figure 7.9).

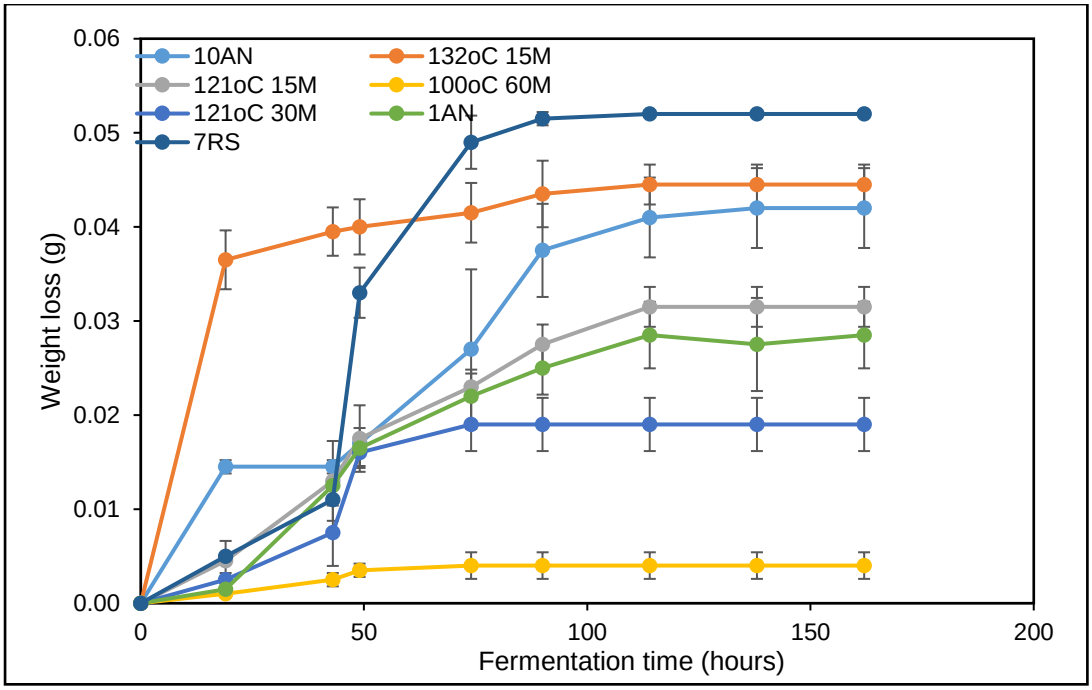


Figure 7.9 Fermentation performance of *W. anomalus* M15 on different modified wheat straw hydrolysates in terms of weight loss due to CO_2 production

Although the hydrolysate of modified wheat straw of 7RS resulted in the highest weight loss during the yeast fermentation, the hydrolysate of modified wheat straw at 121˚C for 15 minutes resulted in the highest ethanol production of 5.99 g/L (Figure 7.10) which equals 16.95% of the glucose to ethanol actual yield (Table 7.4). The result obtained shows that different modification methods had an effect on the recalcitrance of wheat straw for effective hydrolysis of cellulose to glucose, which can be readily fermented into ethanol.

The glucose concentration obtained in Table 7.3 is high in proportion to the amount of wheat straw used for the hydrolysis. The ethanol yield in Figure 7.10 shows that the yeast has fermented nearly all the glucose concentration in the hydrolysate to ethanol. The maximum ethanol yield has been obtained from the different pre-treatment methods employed. There is significant difference in the ethanol yield obtained from the different pre-treatment methods.

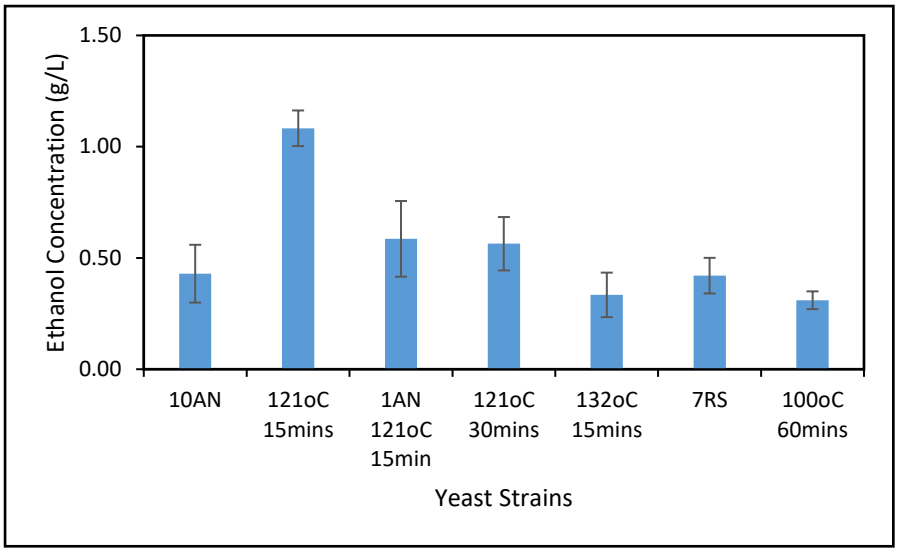


Figure 7.10 Ethanol concentration from modified wheat straw hydrolysate using W. anomalus M15

Table 7.4 Ethanol concentration and actual ethanol yield obtained from yeast fermentation using modified wheat straw hydrolysate.

Strain	**Maximum weight loss (g)**	**Ethanol (g/L)**	**Yield (%)**
Fermented wheat straw by *Aspergillus niger* for 10 days	0.042	0.43±0.13	8.31
Autoclaved wheat straw at 121˚C for 15mins	0.045	1.08±0.08	16.95
Autoclaved wheat straw at 121˚C for 15min and fermented by *Aspergillus niger* for 1 day	0.032	0.59±0.17	10.57
Autoclaved wheat straw at 121˚C for 30mins	0.004	0.56±0.12	9.32
Modified wheat straw at 132˚C for 15mins	0.019	0.33±0.10	6.24
Fermented wheat straw by *Rhizomucor variabilis* for 7 days	0.029	0.42±0.08	6.99
Modified wheat straw at 100˚C for 60mins	0.052	0.31±0.04	5.06

7.5 Summary

Yeast fermentation of sorghum wastewater, sorghum bran hydrolysate and wheat straw hydrolysate from various modification methods were investigated for bioethanol production.

The addition of yeast extract to sorghum wastewater resulted in a low ethanol production while the addition of mineral accelerated the rate of yeast fermentation in sorghum wastewater. Three marine yeasts and one terrestrial yeast were used for bioethanol fermentation of sorghum bran hydrolysate. The terrestrial yeast strain *S. cerevisiae* NCYC 2592 resulted in a higher ethanol yield of 51.34% while marine yeast strain of *W. anomalus* M15 gave an ethanol yield of 49.43%. In a further experiment, *W. anomalus* M15 resulted in a higher actual ethanol yield of 49.79%.

In fermentations using various wheat straw hydrolysates, autoclaved wheat straw at 121˚C for 15 minutes gave the highest actual ethanol yield of 16.95% using marine yeast of *W. anomalus* M15. These results indicated that it was possible to produce bioethanol using either sorghum bran hydrolysate or wheat straw hydrolysate.

8 Conclusions and future work

8.1 Conclusions

In this study, a fungal fermentation based strategy using various paraments such as; temperature, pH, different nitrogen sources, mineral addition etc and optimisation of these parameters with RSM had been developed for converting wheat straw and sorghum bran for the production of crude cellulase and glucoamylase respectively. The enzymes were then used for the production of a glucose rich hydrolysate. Then wheat straw and sorghum bran hydrolysate were subsequently used for ethanol production.

Firstly, the feasibility of using an alkali soak modified wheat straw under SSF and SmF using fungal strains of *A. niger*, *T. reesei* and *R. variabilis* RS for cellulosic enzyme production were investigated. Compared with *T. reesei* and *R. variabilis* RS, *A. niger* produced a higher cellulase in solid-state fermentation.

R. variabilis RS was selected as the main microorganism for cellulase production, as it was a novel isolated fungal strain that produced a decent amount of cellulase under preliminary experiment. The *R. variabilis* RS fungal strain produced maximum cellulase activity on day 3 of fermentation under various fermentation conditions in SmF. The optimisation of SmF condition for cellulase production by *R. variabilis* RS under RSM resulted in an optimum cellulase activity of 23.81 FPU/g at 8% substrate concentration, 0.4g tryptone concentration, pH6, and temperature 24˚C. A suitable cellulase activity was obtained on day 5 of fermentation in SSF under various fermentation conditions. The optimisation of SSF condition for the cellulase production by *R. variabilis* RS using RSM resulted in an optimum cellulase activity of 24.80 FPU/g at 80% moisture content, 0.03g tryptone concentration, pH6, and 7.5×10^6 inoculation rate. The mutant *R. variabilis* RS strain in microwave for 15 seconds resulted in a higher cellulase activity when compared to other *R. variabilis* RS mutant strains and non-mutant *R. variabilis* RS strain.

Secondly, the impact of three different milling processes was examined for the separation of sorghum starch from sorghum kernel. The milling process using blender gave the lowest total starch content in the sorghum bran, but the peanut butter maker process was selected as it has similar operating technique to the abrasive milling method used in Nigeria. The impact of various fermentation

parameters, such as pH, temperature, nitrogen source etc were investigated on GA production in SmF using *A. awamori*. The peak GA activity was obtained on day 3 of fermentation under most fermentation conditions. The optimisation in SmF using RSM gave an optimum GA activity of 59.03 U/mL. In SSF, the optimisation of GA activity gave an optimum activity of 8.32 U/mL of GA. The larger scale production of GA in SmF gave a GA activity of 21.67 U/mL after 72 hours of fermentation.

Enzymatic hydrolysis of alkali modified non-fermented wheat straw resulted in the highest glucose concentration of 15.69 g/L after 72 hours of hydrolysis. The enzymatic hydrolysis of sorghum bran gave a glucose-rich hydrolysate, containing upto 38.7 g/L glucose, which was used for yeast fermentation for ethanol production.

In the last step, the wheat straw and sorghum bran hydrolysate were explored for the production of bioethanol. Prior to utilising sorghum bran hydrolysate, a glucose based semi-synthetic medium (YPD) and a sorghum wastewater derived medium were used for the investigation of ethanol synthesis using *S. cerevisiae* AZ69 yeast strain. The YPD medium resulted in a higher yield of ethanol. Marine and terrestrial yeast strains were examined for ethanol production using sorghum bran hydrolysate. *S. cerevisiae* NCYC 2592 resulted in an ethanol yield of 51.34%. In confirmation of ethanol production from sorghum bran hydrolysate, marine yeast of *W. anomalus* M15 gave the highest actual ethanol yield of 49.79%. Ethanol fermentation from hydrolysates of different substrate derived from different modification methods all produced ethanol using *W. anomalus* M15. The hydrolysate from autoclaved wheat straw at 121˚C for 15 minutes resulted in the highest ethanol yield of 16.95%.

The study shows the biorefining strategy developed by producing cellulase from wheat straw using novel fungi of *Rhizomucor variabilis* RS and glucoamylase from sorghum bran using *Aspergillus awamori* on site, which could reduced depency on commercial enzyme thereby reducing the cost associated with enzyme for the overall production of bioethanol. The glucose-rich hydrolysate obtained from both sorghum bran and wheat straw showed that they can be fermented for the production of value added products, such as bioethanol. The utilization of these substrates for the production of value added products could help eliminate or reduce part of the global environmental challenges.

8.2 Future work

This study has provided an insight for future work related to the possibility of commercial production of cellulase enzyme from *Rhizomucor variabilis*, glucoamylase enzyme from sorghum bran and the use of sorghum bran hydrolysates in the production of value added products. The following future work could be carried out to improve this study:

- The use of fungal liquid broth for SSF instead of fungal spores is worth further investigation.
- The mutant strain of *Rhizomucor variabilis* need further investigation under different parameters and optimisation process for cellulase production.
- The optimisation of enzymatic hydrolysis from crude cellulase obtained from *Rhizomucor variabilis* needs to be investigated.
- The reduction in steeping time before sorghum wet milling needs further investigation in order to reduce process time.
- Further work is required for the scale up production of glucoamylase from sorghum bran for optimum enzyme activity.
- The enzymatic hydrolysis of sorghum bran needs further investigation and optimum conditions determined for possible increase in glucose rich hydrolysate.
- Sorghum wastewater needs further investigation for the production of value added products as it contains substantial amount of starch.
- Ethanol fermentation from sorghum bran hydrolysate could be performed in detail to determine its potential for improvement.
- Sorghum bran hydrolysate has the potential to be used for further exploitation for the production of value added products.
- Optimisation of biological pre-treated wheat straw by *Rhizomucor variabilis* needs further investigation and optimisation for ethanol production.

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Appendix

Appendix i: Specific condition run for RSM of cellulase activity in SmF

		Factor 1	Factor 2	Factor 3	Factor 4	Response 1
Std	Run	A:Substrate concentration	B:Tryptone	C:pH	D:Temperature	Cellulase activity
		%	g		˚C	FPU/g
17	1	6	0.03	6.5	26	2.0710
5	2	8	0.02	7	24	2.5013
3	3	8	0.04	6	24	23.8124
19	4	10	0.01	6.5	26	3.0614
15	5	8	0.04	7	28	19.8303
8	6	12	0.04	7	24	4.3387
18	7	14	0.03	6.5	26	2.4876
21	8	10	0.03	5.5	26	21.0597
12	9	12	0.04	6	28	5.0422
29	10	10	0.03	6.5	26	2.2076
20	11	10	0.05	6.5	26	2.8155
7	12	8	0.04	7	24	2.6926
26	13	10	0.03	6.5	26	0.9986
22	14	10	0.03	7.5	26	2.1188
11	15	8	0.04	6	28	5.1447
13	16	8	0.02	7	28	5.1447
23	17	10	0.03	6.5	22	1.8456
2	18	12	0.02	6	24	4.0587
25	19	10	0.03	6.5	26	1.6133
27	20	10	0.03	6.5	26	2.0368
16	21	12	0.04	7	28	3.0751
6	22	12	0.02	7	24	2.4808

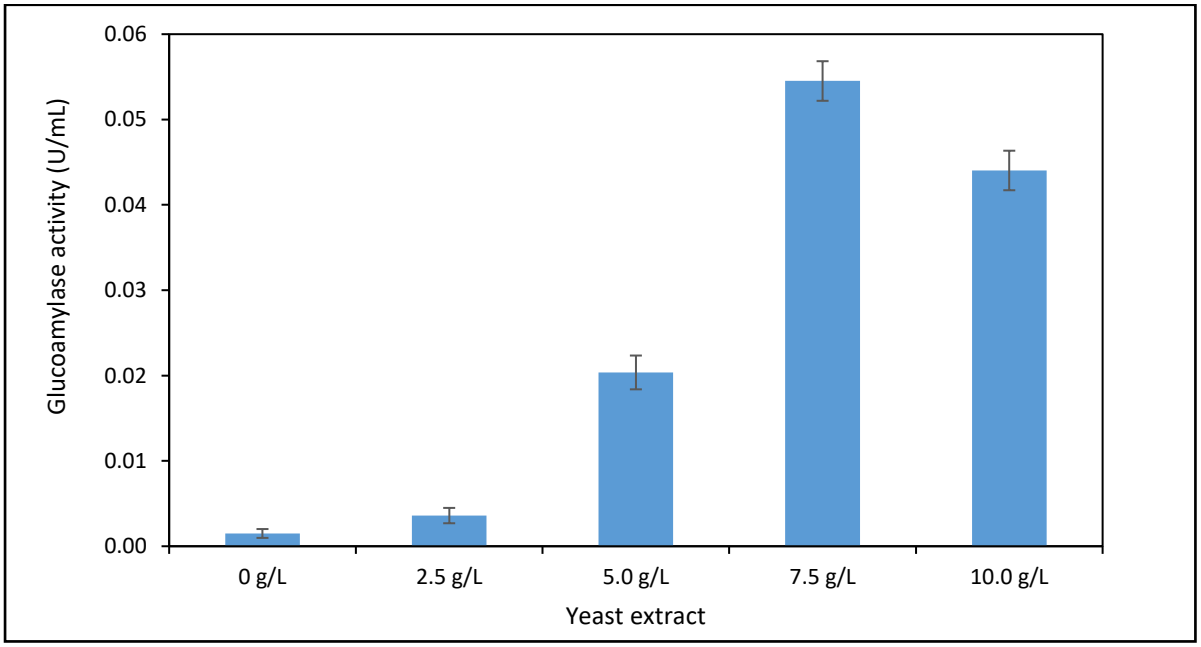
14	23	12	0.02	7	28	1.4767
30	24	10	0.03	6.5	26	2.8633
4	25	12	0.04	6	24	2.6721
24	26	10	0.03	6.5	30	2.2417
10	27	12	0.02	6	28	2.6721
28	28	10	0.03	6.5	26	2.2417
9	29	8	0.02	6	28	2.6721
1	30	8	0.02	6	24	2.2417

Appendix ii: Specific condition run for RSM of cellulase activity in SFF

		Factor 1	Factor 2	Factor 3	Factor 4	Response 1
Std	Run	A:Moisture content	B:Tryptone	C:pH	D:Inoculation rate	Cellulase activity
		%	g		spore/g	FPU/g
19	1	80	0.01	6	7.5E+06	8.55995
10	2	85	0.02	5.5	1E+07	7.85641
17	3	70	0.03	6	7.5E+06	8.9971
6	4	85	0.02	6.5	5E+06	11.6678
28	5	80	0.03	6	7.5E+06	23.5939
8	6	85	0.04	6.5	5E+06	12.7949
29	7	80	0.03	6	7.5E+06	23.2387
14	8	85	0.02	6.5	1E+07	37.9584
7	9	75	0.04	6.5	5E+06	18.5734
11	10	75	0.04	5.5	1E+07	14.7211
2	11	85	0.02	5.5	5E+06	32.7194
22	12	80	0.03	7	7.5E+06	8.58727
21	13	80	0.03	5	7.5E+06	8.80585
30	14	80	0.03	6	7.5E+06	23.5324
13	15	75	0.02	6.5	1E+07	8.437
25	16	80	0.03	6	7.5E+06	24.8029
5	17	75	0.02	6.5	5E+06	10.3359
23	18	80	0.03	6	2.5E+06	8.99027
27	19	80	0.03	6	7.5E+06	24.6731
9	20	75	0.02	5.5	1E+07	12.9246
1	21	75	0.02	5.5	5E+06	16.7907
16	22	85	0.04	6.5	1E+07	7.44658
4	23	85	0.04	5.5	5E+06	16.7429

15	24	75	0.04	6.5	1E+07	8.51896
18	25	90	0.03	6	7.5E+06	8.93562
3	26	75	0.04	5.5	5E+06	10.2198
20	27	80	0.05	6	7.5E+06	9.61184
26	28	80	0.03	6	7.5E+06	22.3439
12	29	85	0.04	5.5	1E+07	11.6405
24	30	80	0.03	6	1.25E+07	9.42742

Appendix iii: Absorbent value of yeast extract



Appendix iv: Specific condition run for RSM of glucoamylase activity in SmF

		Factor 1	Factor 2	Factor 3	Factor 4	Response 1
Std	Run	A:Substrate concentration	B:pH	C:Yeast extract	D:Aeration rate	Glucoamylase activity
		g		g/L	mL	U/mL
2	1	10	5.5	2.5	75	10.8219
24	2	8	6	5	150	59.0323
4	3	10	6.5	2.5	75	3.37847
27	4	8	6	5	100	3.82777
25	5	8	6	5	100	4.29804
8	6	10	6.5	7.5	75	9.7496
28	7	8	6	5	100	4.22316
15	8	6	6.5	7.5	125	1.17987
1	9	6	5.5	2.5	75	1.36858
11	10	6	6.5	2.5	125	1.42549
29	11	8	6	5	100	4.30403
21	12	8	6	0	100	5.83167
13	13	6	5.5	7.5	125	1.31466
10	14	10	5.5	2.5	125	18.4361
6	15	10	5.5	7.5	75	4.51071
7	16	6	6.5	7.5	75	1.76996
30	17	8	6	5	100	4.25311
20	18	8	7	5	100	4.22016
26	19	8	6	5	100	4.83721
18	20	12	6	5	100	44.5856
22	21	8	6	10	100	3.60312
17	22	4	6	5	100	1.11997

23	23	8	6	5	50	3.66303
9	24	6	5.5	2.5	125	1.44946
12	25	10	6.5	2.5	125	22.7914
14	26	10	5.5	7.5	125	28.1381
5	27	6	5.5	7.5	75	0.808448
19	28	8	5	5	100	6.21507
3	29	6	6.5	2.5	75	1.50038
16	30	10	6.5	7.5	125	29.1565

Appendix v: Specific condition run for RSM of glucoamylase activity in SFF

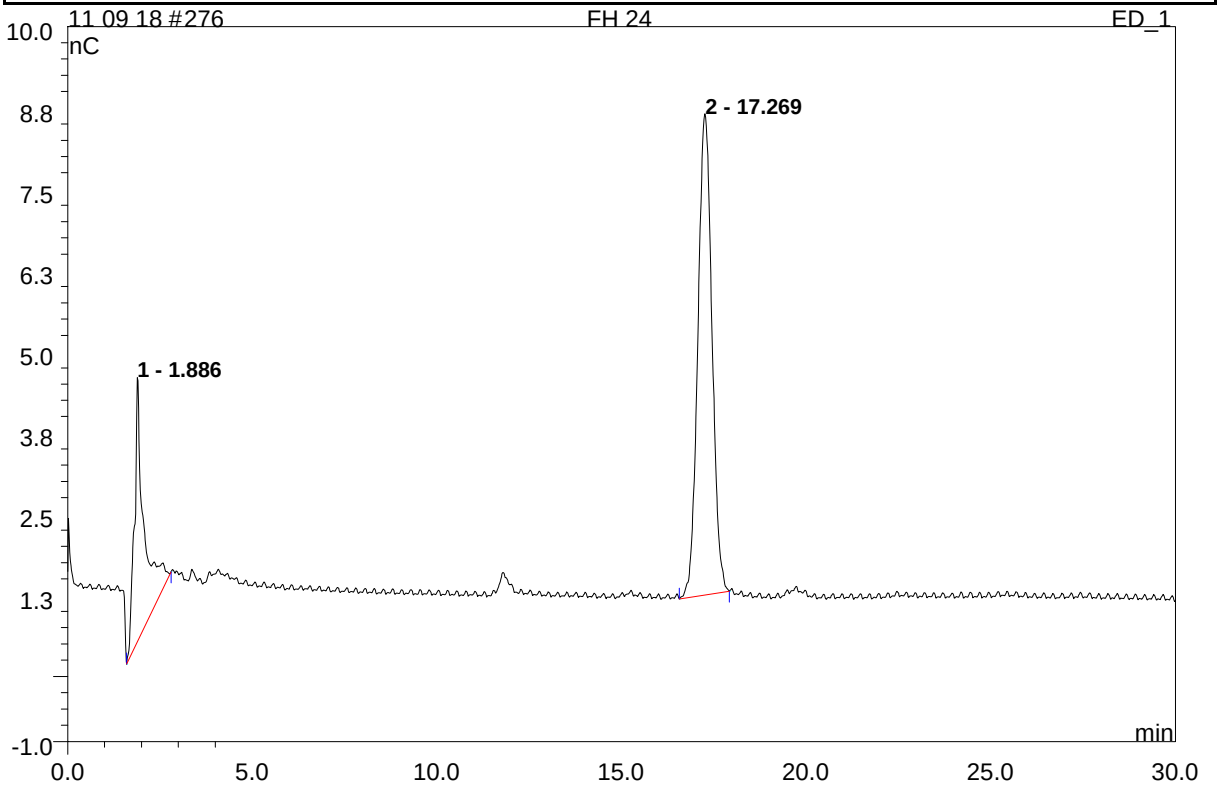
		Factor 1	Factor 2	Factor 3	Factor 4	Response 1
Std	Run	A:moisture content	B:Temperature	C:pH	D:Yeast extract	Glucoamylase activity
		%	˚C		g/L	U/mL
1	19	60	28	5.5	1.5	3.66
2	14	80	28	5.5	1.5	3.17
3	24	60	32	5.5	1.5	4.80
4	3	80	32	5.5	1.5	6.46
5	8	60	28	6.5	1.5	3.09
6	6	80	28	6.5	1.5	3.21
7	11	60	32	6.5	1.5	3.61
8	27	80	32	6.5	1.5	3.31
9	12	60	28	5.5	3.5	2.84
10	17	80	28	5.5	3.5	2.61
11	15	60	32	5.5	3.5	3.59
12	16	80	32	5.5	3.5	7.51
13	26	60	28	6.5	3.5	1.58
14	4	80	28	6.5	3.5	2.84
15	25	60	32	6.5	3.5	5.40
16	23	80	32	6.5	3.5	8.32
17	5	50	30	6	2.5	1.78
18	1	90	30	6	2.5	3.67
19	18	70	26	6	2.5	3.96
20	13	70	34	6	2.5	5.23
21	7	70	30	5	2.5	1.51
22	21	70	30	7	2.5	1.70

23	2	70	30	6	0.5	3.28
24	28	70	30	6	4.5	2.23
25	10	70	30	6	2.5	2.97
26	30	70	30	6	2.5	2.79
27	9	70	30	6	2.5	2.26
28	31	70	30	6	2.5	2.43
29	22	70	30	6	2.5	2.08
30	29	70	30	6	2.5	1.94

Appendix vi: Sorghum bran hydrolysis chromatograph

276 FH 24

Sample Name:	**FH 24**	*Injection Volume:*	**20.0**
Vial Number:	**12**	*Channel:*	**ED_1**
Sample Type:	**unknown**	*Wavelength:*	**n.a.**
Control Program:	**Test Carbo PA20 Col 10mM**	*Bandwidth:*	**n.a.**
Quantif. Method:	**default**	*Dilution Factor:*	**1.0000**
Recording Time:	**21/9/2018 14:24**	*Sample Weight:*	**1.0000**
Run Time (min):	**30.00**	*Sample Amount:*	**1.0000**



Appendix vii: List of publication/conferences/seminar

- Makanjuola, O.; Greetham, D.; Zou, X.; Du, C. (2019). The Development of a Sorghum Bran-Based Biorefining Process to Convert Sorghum Bran into Value Added Products. *Foods*, *8*, 279.
- Greetham, D., Saleh Zaky, A., Makanjuola, O., & Du, C. (2018). A brief review on bioethanol production using marine biomass, marine microorganism and seawater. Current opinion in Green and Sustainable Chemistry. 14:53-59.
- Speaker at the 2nd International Conference on Biofuel and Bioenergy. Paris, France. 27 – 28th of March 2019.
- Speaker at the 2nd World Energy Congress on Renewable Energy and Resources. Boston, Massachusetts, USA. August 27 – 28, 2018.
- Poster presentation at 'Biorefinery and bioprocessing' Research seminar, University of Huddersfield, July 2018.
- Speaker on 'Utilization of newly isolated novel microorganisms for efficient conversion of waste biomass to value-added products' Tsinghua University, Beijing China. One-day seminar, June 2016.
- University of Huddersfield Inaugural PGR Conference, Huddersfield, November 2015.