BIOETHANOL PRODUCTION FROM PRETREATED SUGARCANE BAGASSE UNDER OPTIMISED CONDITIONS USING SELECTED FUNGI

BY

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A THESIS IN THE DEPARTMENT OF MICROBIOLOGY SUBMITTED TO THE FACAULTY OF SCIENCE IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF DOCTORATE DEGREE (MICROBIOLOGY) OF THE UNIVERSITY OF IBADAN, NIGERIA.

February, 2021

ABSTRACT

Sugarcane Bagasse (SB) is a major waste of the sugar industry and constitutes disposal problem in the environment. The bagasse is known to contain cellulose and hemicellulose which can be converted to bioethanol. However, the recalcitrant nature of plant biomass demands optimal pretreatment method to make sugar components available for enzymatic depolymerisation. Therefore, this study was designed to optimally pretreat SB and to identify appropriate fungi for enhanced bioethanol yield.

Fungi (moulds and yeasts) were isolated from SB collected from a sugar industry dumpsite using pour-plate method. Standard methods were used to screen organisms (10^5 CFU/ml) for their ability to produce cellulases and hemicellulases. Selected isolates were identified using molecular techniques. Yeasts were further screened based on their ability to convert pentose and hexose sugars to bioethanol using different nitrogen sources to select the appropriate yeast. Yeast tolerance to temperature, acetic acid, ethanol and furfural was determined using turbidimetry. Optimisation of pretreatment of SB at different concentrations of potassium hydroxide (KOH), temperature and treatment time was determined using Response Surface Methodology (RSM). Pretreated SB was hydrolysed using selected moulds, while a commercial hemicellulase mixture served as control. Fermentation of pretreated SB hydrolysate with selected yeasts using Separate Hydrolysis and Fermentation (SHF) as well as Simultaneous Saccharification and Fermentation (SSF) of pretreated SB were also carried out. Bioethanol yield was determined; and data were subjected to descriptive statistics.

A total of 120 yeasts and 21 moulds were isolated. *Aspergillus niger* XY was the highest enzyme producer for endoglucanase (60.34±0.72 U/ml), beta-glucosidase (14.29±0.02 U/ml) and xylanase (82.67±0.65 U/ml). Eleven yeasts grew on both glucose and xylose and were identified as *Pichia kudriavzevii* (7), *Saccharomyces cerevisiae* (1), and *Candida tropicalis* (3). All yeasts converted glucose to ethanol but only *C. tropicalis* Y5 converted xylose to ethanol (4.83 g/l) with urea as the best nitrogen source. *Pichia kudriavzevii* Y2, *C. tropicalis* Y5 and *S. cerevisiae* Y10 tolerated temperatures up to 48 ^{o}C and 17.5% ethanol. *Pichia kudriavzevii* Y2 and *S. cerevisiae* Y10 adapted up to 6 g/l acetic acid with 49% and 45% growth while *C. tropicalis* Y5 adapted to 7 g/l acetic acid with 34% growth

after 48 hours of incubation. The isolates were able to adapt to 3 g/l furfural concentration with percentage growth of 53%, 47% and 46% for *P. kudriavzevii* Y2, *C. tropicalis* Y5 and *S. cerevisiae* Y10, respectively. Optimum pretreatment conditions were: 150 mg/g bagasse (KOH), 86 ^{o}C and 120 minutes. Hydrolysis with hemicellulase yielded reducing sugars of 600 mg/g bagasse within 20 hours while hydrolysis with *A. niger* XY took a longer time (12 days) and yielded 18.8 mg/g bagasse. Bioethanol yield using SHF and SSF were 19 g/l and 30 g/l, respectively.

Alkaline pretreatment followed by enzymatic hydrolysis gave a higher yield of total reducing sugars. *Candida tropicalis* Y5 converted both pentose and hexose to bioethanol and showed good prospect for its use in commercial fermentation of sugarcane bagasse.

Keywords: Bioethanol, Sugarcane bagasse, Potassium hydroxide, Stress tolerance, *Candida tropicalis* Y5

ACKNOWLEDGEMENTS

CERTIFICATION

I certify that this work was carried out by **ADEBARE JOHNSON ADELEKE** under my supervision in the Department of Microbiology, University of Ibadan, Nigeria.

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CHAPTER ONE

INTRODUCTION

In recent times, there has been a global awareness on the increasing challenges of environmental instabilities as well as a surge in energy demands and a constantly depleting reserve of currently used energy sources. This has resulted in the conscious scientific efforts towards finding alternative/complementary and sustainable energy sources which include solar, hydroelectric, wind, and biomass based energy forms. With respect to biomass-based energy substrates, Pauly and Keegstra (2008) took a constructive look at the potential energy substrates available in nature and stated that plant biomass stands as the most available natural form of renewable resource accounting for an approximate $150-170 \times 10^9$ tons annual generation. In general, lignocellulosic biomass is obtained from agricultural residues, energy crops, forest residues, and municipal cellulosic wastes (Parisuthan *et al*., 2014). It has been a viable base for bioalcohols production, especially second-generation bioethanol which are considered more economically viable compared to the first generation (food crop based). This is because of their inherent advantage over first generation as the first generation biofuel sources trigger food/fuel competition leading to increase in food prices.

According to Rubin (2008), lignocellulosic biomass chemically possess about 50-80% carbohydrates (in complex C5 and C6 sugars) typically making it quite challenging to bioconvert lignocelluloses to alcohols. Jouzani and Taherzadeh (2015) also affirmed that lignin content and the crystalline conformity of cellulose lead to difficulty in biomass dissolution thus requiring pretreatment to ease into the complex structure thereby paving way for inner molecular accessibility to enzymatic hydrolysis. This thus means that the conversion of lignocellulosic biomass to bioethanol involves a multi-step procedure of pretreatment, catalytic hydrolysis and fermentation which can become an ultimately cost

laden process (Kumagai *et al*., 2014). According to Jouzani and Taherzadeh (2015), the enzymatic cleavage and hydrolysis of lignocelluloses is about the most costly part of the process. Combinatorial enzymatic activities of hemicellulases, and cellulases are essential in this regard to breakdown the substrate into fermentable sugars (Lynd *et al*., 2002). More so, with a variability in occurrence of lignocelluloses within diverse environments, and the consequent variability in their composition over time, it is important to come up with procedures that can pretreat, saccharify and ferment these substrates using microorganisms with a good level of substrate versatility (Balat and Balat, 2009; Parisuthan *et al*., 2014).

There is also an upsurge of interest in 'second generation bioethanol' which are products from lignocellulosic biomasses. This is so because of the potential they pose on the reduction of burden on food crops/plants which were hitherto used for bioethanol production with first generation systems (Dionisi *et al*., 2015). A variety of freely available lignocellulosic matter for example – organic components of municipal solid wastes, forest plant residues and agro wastes constitute an infinite amount of resource valid for bioethanol production (Bohlmann, 2006). Sugarcane bagasse is a major agrowaste of the sugar industry which is readily available as a raw material for bioethanol production. However, the technical and economic hindrances which still exist in bioethanol production have to be adequately addressed. Cheap and highly efficient technologies as well as appropriate microbial strains must be made available in order to achieve a sustainable production of bioethanol.

1.1   Statement of Problem

Environmental challenges associated with over-dependence of fossil fuels, a surge in energy demands due to ever increasing world population and the gradual depletion of the currently used energy sources have been issues of global concern in recent times (Shide *et al.*, 2004; Banerjee *et al.*, 2010; Cherubini and Stromman, 2011). The situation has called for development of renewable energy sources which are more environmentaly friendly. Sugarcane bagasse is a major waste of the sugar industry and constitutes disposal problem in the environment. The bagasse is known to contain cellulose and hemicellulose which can be converted to bioethanol (Canilha *et al*., 2012). However, the recalcitrant nature of

plant biomass demands optimal pretreatment method to make the polysaccharides available for enzymatic depolymerisation. Also, successful conversion of pretreated plant biomass to bioethanol would greatly depends on the discovery of organisms with special abilities which can efficiently ferment a variety of sugars to bioethanol (Canilha *et al*., 2012.

1.2   Justification

Ethanol comes from a plant matter which is a sustainable resource and release of toxic compounds like carbonmonoxide is low when ethanol undergoes combustion. Therefore, bioethanol production in substantial quantities will help to curb over-dependence on fossil fuels and the negative effect of combustion of fossil fuels on the environment. Use of sugarcane bagasse as feedstock for bioethanol production would help to provide a safer and cleaner way of waste disposal. Wild microorganisms isolated from natural environments may contain metabolic abilities of industrial importance.

1.3   Aim and Objectives

1.3.1   Aim

The aim of this study was to optimally pre-treat sugarcane bagasse and to select appropriate fungi (moulds and yeasts) for enhanced bioethanol yield.

1.3.2   Specific objectives

1. To isolate, screen and identify cellulose and hemicellulose degrading moulds as well as yeasts capable of fermenting hexose and pentose.

2. To study the fermentative profile of the yeasts isolated.

3. To pre-treat and hydrolyse sugarcane bagasse to release maximum quantities of reducing sugars.

4. To ferment sugarcane bagasse hydrolysate to ethanol using single and a consortium of yeasts for maximum efficiency.

CHAPTER TWO

LITERATURE REVIEW

2.1   Biorefinery concept and development of biofuel

Growing global populations together with an ever changing life style has led to the continued changes in energy demand and pattern of use. The present mainstay for global energy production in form of fuels and commodity chemicals to drive turbines and industries is the crude oil feedstock. Currently, its rapid depletion and blatant misuse as well as overuse has led to pressures on industrial and societal based energy demands and emissions of greenhouse gases like nitrous oxide, carbon dioxide and methane as a consequence of combustion. All these have serious consequences in tilting the balance of climate and causing a global form of insecurity and unrest (Cherubini & Stromman, 2011). Following these circumstances, there have been calls for finding options of low cost and renewable energy to reduce the dependence on petroleum based fuel as well as reducing greenhouse gas emissions. Of all the renewable energy sources available to man, biomass stands as the most readily available, diverse and largest with potential to fill the gap of providing a good range of value added bioproducts (Cherubini, 2010).

Recently, an upsurge in biofuel research experimenting new technologies and bioconversion routes has led to production of bioethanol, biodiesel, biobutanol and other biologically linked products. Much like the conventional oil refinery system, the process of conversion of biomasses to biofuels needs the functionality of a biorefinery. A biorefinery can be defined as a facility that incorporates the modification of biomass to produce fuels, direct thermal and electrical energy, and value-added biochemicals from biomass feedstocks (Amidon and Liu, 2009). Cherubini *et al*. (2011) posited that biorefinery system is a good approach for achieving multifunctional process of multiple streams of energy generation. In the same light, it is also capable of maximizing the use of the biomass feedstock and reducing wastes (Thomsen, 2005). A biorefinery combines biomass conversion using designed systems to produce fuels and

other chemicals in a concept fairly similar to that of petroleum refineries as both systems produce multiple products and by products. The ability to produce multiple products has situated biorefineries at an advantaged point as they make use of different biomass feedstocks leading to a highly variable quantity of intermediates. In fact, a biorefinery can produce several low and high valued fuels as well as generate direct electrical energy alongside bringing about reduction in greenhouse gas release (Balat, 2011).

Suhag and Sharma (2015) described biorefinery concept by linking in a schematic diagram (Figure 2.1a) the feedstock in terms of biomass as well as the products and by products with the biorefinery system as the central focus. The choice of certain feedstock to be used in the biorefinery process is dependent on the degree of its availability as well as its versatility in production of by-products (Mabee, *et al*., 2005).

In this regard, there are vast arrays of potential feedstock sources such as starch/sugar laden crops, lignocellulosic biomass and photosynthetic organisms (Figure 2.1b). Typifying the biofuel generated by the biorefining process is based on the nature of feedstock used as the starting material. According to Lyko *et al*. (2009), it is explained that the bioconversion of biomasses can be regarded as 1st or 2nd generation systems. The explanations stated that the food based biomasses led to the 1st generation biofuels after biorefining, while the inedible biomasses like lignocelluloses along with inedible photosynthetic organisms like algae are sources of 2nd generation biofuels. The second generation biofuels were developed wholly in response to the need to disentangle the food demand of man from his energy demand (Parisuthan *et al*., 2014). A consequence of this is a great increase in the research works geared towards processing of lignocellulosic materials particularly cheap and available agro-wastes, agro-residues and forest residues which are not edible.
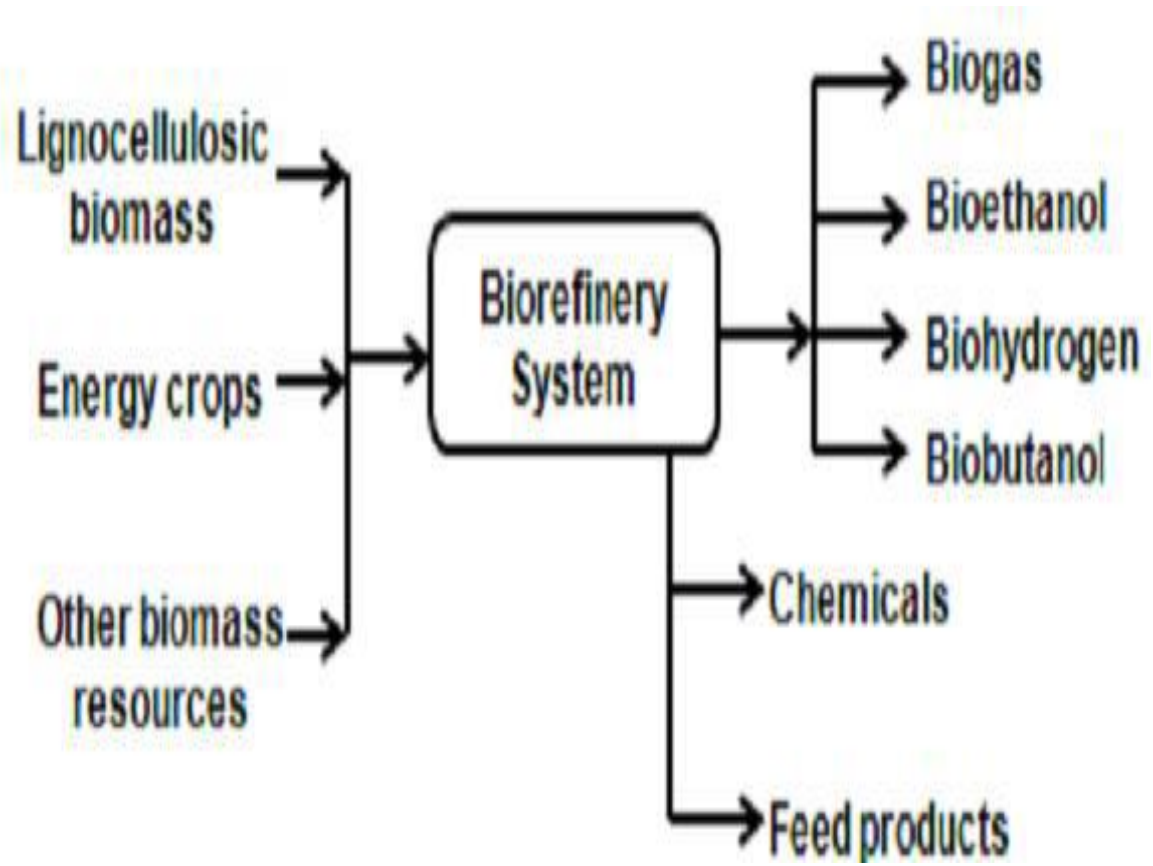
Figure 2.1a: Biorefinery system (Suhag and Sharma, 2015)
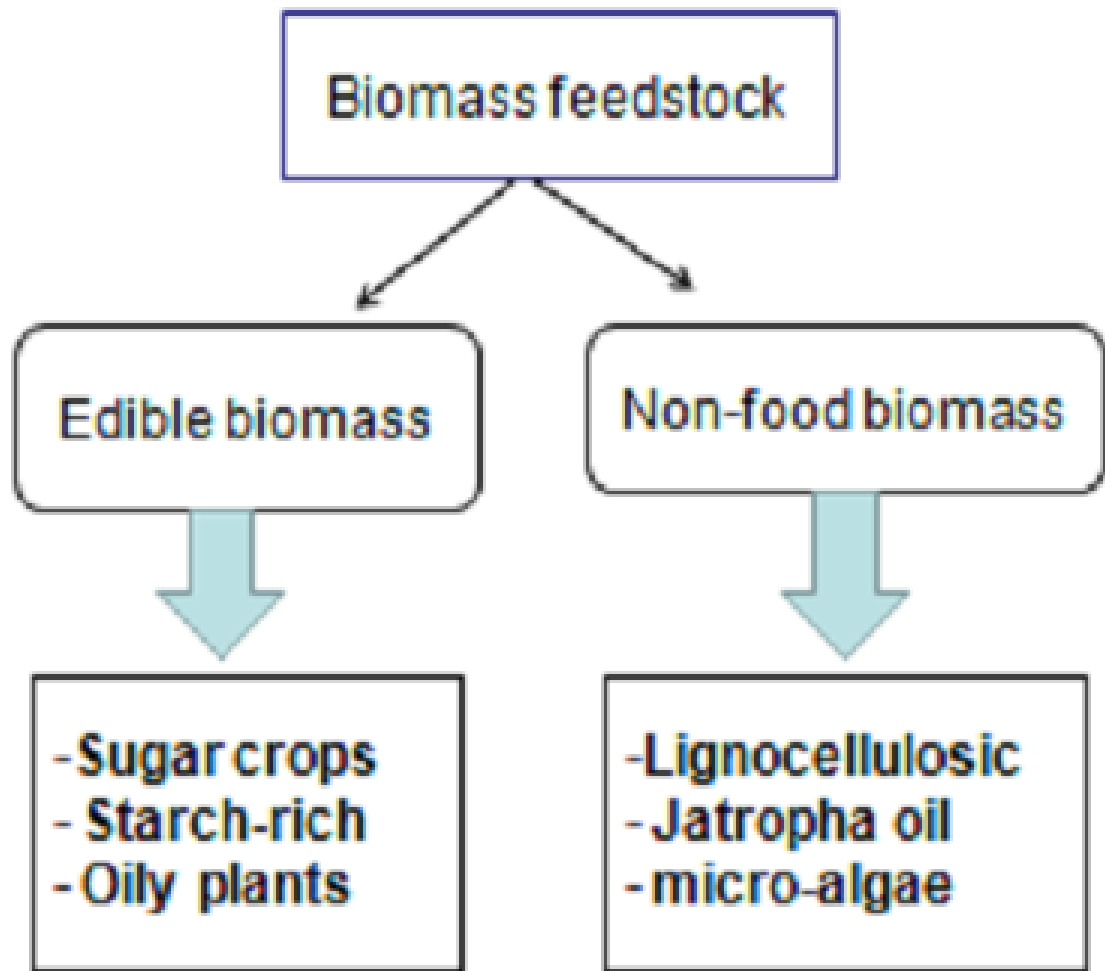
Figure 2.1b: Biorefineryfeedstock (Suhag and Sharma, 2015)

In the view of Carriquiry *et al.* (2011), lignocellulosic biomass available on the planet, can occur in three main forms: (a) forest residues (b) agro-based residue (e.g sugar cane bagasse) (c) herbaceous/woody lignocellulosic biomass forms. It is worthy of note that agro and forestry based residues as well as paper wastes are becoming more and more choice biomasses for renewable energy (Zhang *et al.*, 2010). Comparatively assessing lignocellulosic biomass with other feedstock, they have the advantage of : (a) help in curbing competition for land and water use for food cultivation (b) require relatively less input for growth (c) increase biomass per square feet of land (Schmer *et al.*, 2008).

Being described as the most abundant biomass on earth, lignocellulosic matter can significantly substitute fossil fuels through biorefinery approach of providing liquid fuels and biochemical by-products. A number of lignocellulosic feedstock has been documented based on research. Examples are sugarcane bagasse, wheat straw, switch grass and many others. Of all the types of lignocellulosic feedstock from wastes that have been applied, sugarcane bagasse appears to be the most appropriate in tropical countries because of its composition and its wide availability.

When compared with first generation food based biomass, the second generation lignocellulosic feedstocks are regarded as recalcitrant and have high degree of hold on structural sugars thus limiting the amount of biofuel produced (Takara and Khana, 2011). Even though lignocellulosic biomasses are tough feedstock due to the strength of chemical bonding between the cellulose, hemicelluloses and lignin parts, it still has great potentials in the energy circles and can have a projected yield of about 442 billion dollars in a year (Bohlmann, 2006). Many lignocellulosic biorefinery concepts are based on the deliberate bioconversion systems where the lignocelluloses matter are transformed into liquid fuels, lignin-based bioproducts, and other viable by-products and extractives by the aid of enzymatic and fermentation processes. In all these, biotransformation/bioconversion of lignocellulosic biomass matter has continued to garner a profound amount of interests globally owing to their large scale availability, comparatively low cost and massive abundance in nature.

2.2 Chemistry and composition of lignocellulosic biomass

The main components of lignocellulosics are lignin, cellulose and hemicelluloses fractions. A brief x-ray at the composition of each of them is given below. Figure 2.2 also shows the general structure of lignocellulosic plant matter.

2.2.1 Lignin

Lignin, a phenolic polymer consists of phenylpropanoid subunits (Vasquez *et al.*, 2007). It provides water-proofing, microbe-proofing, and mechanical reinforcing of plant cell wall structures, making it very important in plant physiology, and relatively the most important component of the plant that confers the unique architecture. As a result of its phenolic structure, it is very resistant to digestion by enzymes, thereby making it important to subject it to a pretreatment procedure before it is hydrolysed by enzymes. As earlier stated, the lignin part is an aromatic polymer with a carbon-carbon linkage holding its monomer units. According to Jefferies (1994), the structural units of lignin are p-coumaryl alcohol, sinapyl alcohol and coniferyl alcohol. Lignin itself has a molecular weight which ranges between 600 and 1000 kilo Daltons, which is considerably a large size (Kirk and Farell, 1987).

2.2.2 Cellulose

The cellulose component is also a polysaccharide structure comprising of glucose units, and possesses a molecular weight of >500 kDa (Kraemer, 1938). It is composed of microfibrils that came about due to linkage of glucose molecules by β-1,4, bonds. The conglomeration of microfibrils lead to the formation of linear semi-crystalline structures, with the linearity allowing the strong bond formation between microfbrils. Because of the crystalline nature, there is a conferment of hydrolytic resistance as a result of lack of water within the structure – hydrolases are prevented from acting on the beta bonds in this form (Gomez *et al.,* 2008).

2.2.3 Hemicellulose

This is a polymer with monomeric units of a number of different sugars which include but not limited to arabinose, xylose, galactose and manose, crosslinked together in form of glycans. There is a binding format between hemicelluloses and cellulose

microfibrils by the method of hydrogen bonding, thus forming a protection preventing microfibril-microfibril contact and building a firm framework. The major form of hemicellulose found within primary cell walls is xyloglucan while xylans and arabinoxylans are the major ones in secondary cellwall which are more dominat in plant biomass. As hemicelluloses comprise of 20-50% lignocellulosic polysaccharides, it is thus a main factor to consider during production of liquid biofuels (Gomez *et al*., 2008, Vasquez *et al*., 2007).

As hemicelluloses comprise of carbohydrate monomers like xylan, mannose, glucose and arabinose, the ratio of occurrence of these sugars differs from plant matter to plant matter, and the molecular weights of hemicelluloses are usually smaller than that of celluloses (Perez *et al*., 2002). In bioethanol processing, hydrolysing the hemicellulose part provides the non-glucose sugars needed for bioconversion into ethanol, thus making hemicelluoses very important in 2nd generation biofuels. The hydrolysis of hemicelluloses is also relatively easier than cellulose, posing less concerns, hence the use of mixed cultures of cellulolytic organisms in the fermentation systems will be efficient (Lynd *et al.*, 2002).
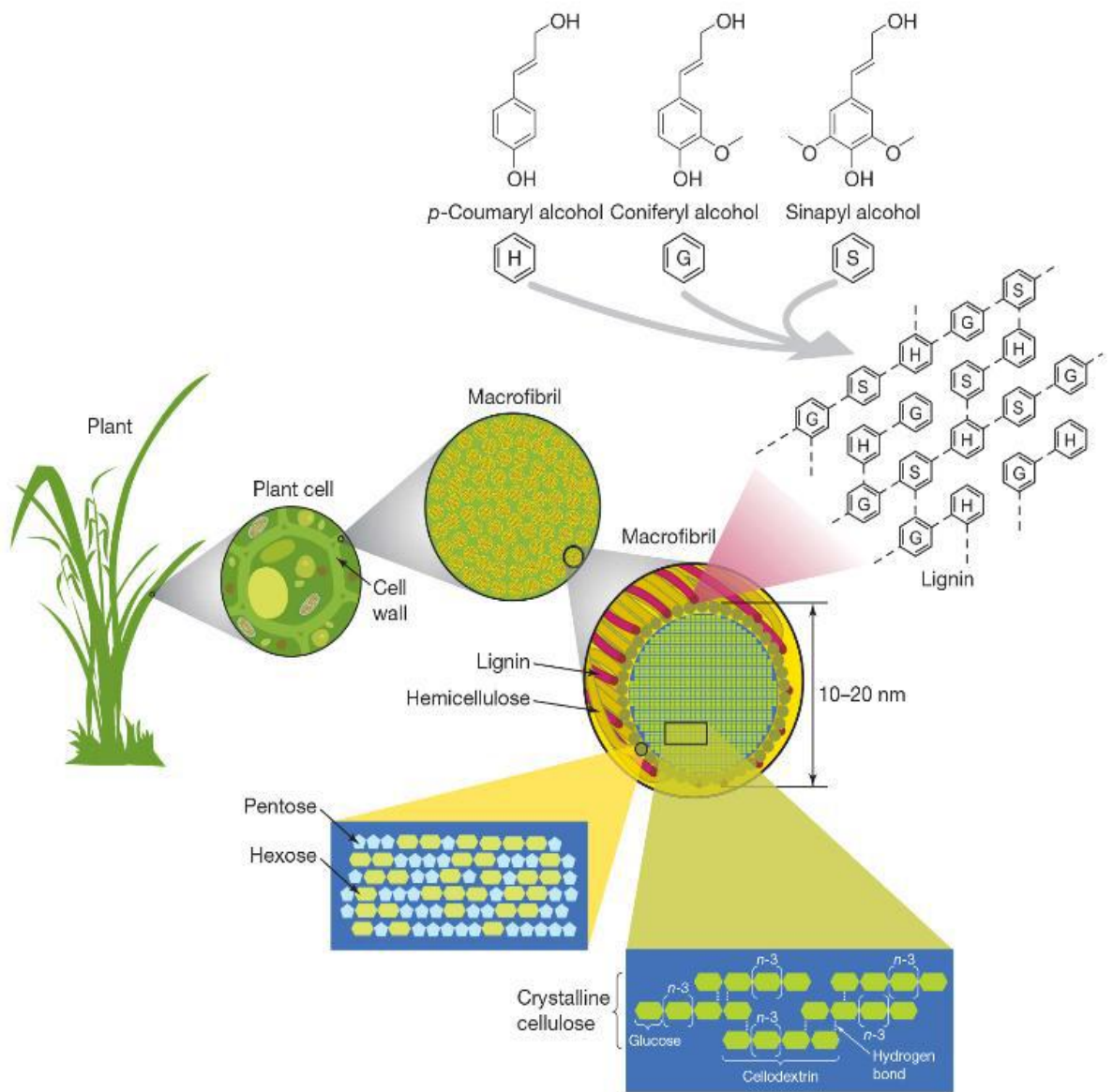
Figure 2.2: Generalised Structure of lignocellulosic biomass containing cellulose, hemicellulose and lignin (Rubin, 2008).

2.3   Pretreatment technologies for bioethanol production

Pretreatment of lignocellulosic biomass is a vital step during its bioconversion to bioethanol. The other steps that follow include enzymatic hydrolysis, fermentation and recovery of target bioethanol (Balat, 2011). Pretreatment is aimed at improving the surface area of activity on the substrate and its porosity, cellulose crystallinity, and disruption of cellulose heterogeneity (Talebnia *et al*., 2010). Pretreatment which is highly important in the bioethanol production process, helps in breaking apart the biomass structure and increasing the efficacy of permeability for enzymes and enzymatic hydrolysis (Singh *et al.*, 2015). Subsequent reaction with hydrolytic enzyme then promotes the fermentative bioconversion into bio-ethanol (Hasunuma *et al.,* 2013).

A brief insight into the main classes of pre-treatment methods is given below:

2.3.1   Physical pretreatment

This involves the direct physical action on the structure of the lignocellulosic features. A good example is milling. Milling can be described as a mechanical pretreatment method that reduces the particle size of the substrate and subsequently the crystallinity of the cellulose content (Sun and Cheng, 2002). Milling does not require chemicals; and the process does not generate inhibitors. Therefore, it is considered an environmentally safe technique (da Silva *et al*., 2010). A disadvantage of the milling process is that it requires large amounts of power to drive it. Another method is known as pyrolysis. Pyrolysis involves the use of very high temperature (> 300 ^{o}C). With this, there is high efficiency of cellulose degradation (Kumar *et al.*, 2009). Glucose obtained after pyrolysis can then be converted to ethanol (Acikalin *et al*., 2012). The efficiency and yield the pyrolysis process can attain is dependent on temperature, heating rate, flow rate, feedstock size, feedstock lignocellulosic ratio and reactor type (Agblevor *et al*., 1995). A third physical method rapidly in use is the application of microwaves on the biomass. This method is regarded as an alternative to conventional heating technique. During microwave pretreatment, direct interaction between the substrate and electromagnetic rays ensures generation of high heat energy efficiency. There is short reaction time and very low amount of inhibitors generated when biomass is treated (Binod *et al.*, 2012)

2.3.2 Physico-chemical Pretreatment

This class of pretreatment technique involves methods that combine physical as well as chemical techniques. One physic-chemical pretreatment technique is hydrothermal pretreatment. This is a thermochemistry based physicochemical procedure in which the biomass is subjected to steam. The lignocellulosic material is exposed to heat in form of steam and generates very low environmental concerns. A reactor is used in this process and a temperature of between 160 ^{o}C and 240 ^{o}C is targeted for optimum efficiency (Agbor *et al*., 2011; Chornet and Overend, 1991). A pressure of between 0.7 and 4.8 MPa is also applied (Agbor *et al*., 2011). The effectiveness of an increased saccharification process can be achieved by steam explosion. Another technique is ammonia fiber explosion (AFEX) technique, which combines liquid ammonia and steam explosion. The idea is to apply an alkaline thermal treatment method on lignocellulosic biomass under high pressure immediately followed by fast pressure release (Kumar *et al.,* 2009). AFEX technique is advantageous with respect to efficient lignin removal from the biomass with least amount of inhibitors. This technique is also short in execution time and has a relatively simple handling process. When AFEX is applied, the structure of the biomass changes with respect to the water holding capacity which results in a higher digestibility rate (Kumar *et al*., 2009). Factors that affect AFEX are temperature, pressure, moisture content, and ammonia loading (Bals *et al.,* 2011).

A third physico-chemical pretreatment method is CO_2 explosion. This involves the thermal application of CO_2 on the biomass with the targeted formation of carbonic acid, thus increasing hydrolysis in the process. This is so because, CO_2 molecules have a comparable size with water, and with an increase in pressure, the gas effectively penetrates the surface of the biomass and improves hydrolysis of lignocellulosic crystalline structures (Sun and Cheng, 2002; Kumar *et al.*, 2009). This method has some level of difficulty in handling. However, it leads to environmentally friendly and safe products (Canilha *et al*., 2012). Application of hot water on the biomass as a pretreatment method is also physicochemical in nature, especially when applied under high pressure. The use of hot water in this case involves a minimum contact time of 15 minutes and a temperature of 200-230 ^{o}C leading to the dissolution of 40-60% of total biomass and removal of hemicelluloses fractions (Taherzadeh and Karimi, 2008).

2.3.3 Chemical pretreatment

Chemical pretreatment employs direct use of chemicals alone with no physical force exerted. The application of acids for hydrolysis is a major method used, and can be classified as one of the oldest pretreatment methods. The concentrations of acid usually used are high, enabling the hydrolytic release of components of the biomass (Alvira *et al*., 2010).

Common acids usually used are sulphuric acid, phosphoric acid, nitric acid, oxalic acid and hydrochloric acid (Rodriguez-Chong *et al.*, 2004; Laopaiboon *et al.*, 2010). Temperature within which the hydrolysis performs best is usually between 120-180 ^{o}C and the treatment time ranges between 15-60 minutes (Alvira *et al*., 2010). In managing this pretreatment process, it is noteworthy that there is a likelihood of corrosion of equipment or reactor vessel which consequently adds to the cost of maintenance. Also, there is the possibility of formation of other byproducts that have the potentials of inhibiting microbial fermentation, examples of such by-products can be furans, carboxylic acids, formic levulinic, phenolic compounds and so on, thus warranting a detoxification step (Alvira *et al*., 2010; Palmqvist and Han-Hagerdal, 2000). Acid treatment is however advantageous because of its activity at low and medium temperatures driving down production costs by some magnitude (Girio *et al*., 2010).

Similar to acid treatment, alkaline pretreatment is also applied to delignify the biomass and solubilise hemicelluloses. This form of pretreatment usually employs calcium hydroxide, Sodium hydroxide, and ammonia hydroxide. The akalines act by saponifying ester bonds existing in-between intramolecular spaces of xylan hemicelluloses (Zheng *et al.*, 2009; Sun and Cheng, 2002). Effectiveness of alkaline pretreatment is dependent on the substrate involved and the lignocellulosic ratio within the substrate. This pretreatment method works best with agricultural residues with low or moderate level of lignin (Zheng *et al.*, 2009).

Oxidative delignification is another process of chemical pretreatment. The cellulose is made to chemically swell improving enzymatic saccharification in the process. The basic concept of this process is that degradation of lignin is achieved in the presence of an enzyme catalyst; peroxidase enzyme (Sun and Cheng, 2002). This method has been used to treat corn stover, rice straw, barley straw, sugar cane bagasse and so on

(Banerjee *et al.*, 2011). Ozonolysis is another method of chemical pretreatment. In this case, lignin and hemicelluloses are degraded using ozone gas. The gas functions based on its powerful antioxidant properties. Pretreatment using ozone gas leads to effective removal of lignin as the reaction is performed at ambient temperatures (Vidal and Molinier, 1988). However, the hitch is that enormous quantities of ozone are required thus increasing the running cost of the system. However, ozone gas can be easily decomposed with the aid of a catalytic bed or by applying elevated temperatures thus helping to reduce pollution (Kumar *et al.*, 2009).

The use of organosolv has been described as the most promising pretreatment technique for lignocellulosic biomass (Hage *et al.,* 2009). In this type of pretreatment, strong inorganic acids are used to catalyse the system enhancing the splitting of lignin-lignin bonds within the substrate (Holtzapple and Humphery 1984). With the removal of lignin there is an increased area and volume of the substrate matter thus increasing the accessibility to enzymes (Vidal and Molinier, 1988). Chemicals like NaOH or Na_2SO_3 are used to neutralize the hydrolysate, generating few wastes in the process (Taherzadeh and Karimi, 2008). Wet oxidation procedure can also be applied in this form of pretreatment using sodium carbonate as catalyst in the presence of oxygen. In this method, delignification occurs with increase in aliphatic acids, and it is one of the most expensive pretreatment techniques (Carvalhiero *et al.,* 2008). The advantage of the method however, is that there is no generation of furfural and other unwanted compounds from the system (Bjerre *et al.*, 1996).

2.3.4 Biological pretreatment

A major alternative to chemical hydrolysis and pretreatment techniques is biological pretreatment technique. Generally, microorganisms capable of degrading wood for instance brown rot fungi, white rot fungi, soft rot fungi and some bacteria are applied in the biological pretreatment technique (Zheng *et al.*, 2009). Most effective in this technique are the white rot fungi which are well known to secret lignolytic enzymes like laccase and peroxidases. The other fungi like brown rot fungi attack mainly cellulose (Mtui, 2009). Biological pretreatment is termed very environmentally friendly and low energy requirement, however, it can be argued that it poses the disadvantage of low efficiency, long execution time, strain specific activity, and stringency in growth requirement. However, researchers have devised means of

combining biological pretreatment with other treatments to boost its efficiency and relevance as a technique (Hamelinck *et al.*, 2005).

2.3.5 Advancement in Pretreatment technologies

With an estimated 18-20% of total biofuel production estimated to come from lignocellulosic materials, the efficiency in meeting up with this estimate is most typified by optimizing pretreatment mechanisms. Pretretament steps are carried out as a means of overcoming the barriers of physical structure and composition that hinder full catalytic accessibility to the fermentable sugars within the lignocellulosic matrix (Yang and Wyman, 2008). It has been reported that during pretreatment of different types, a general result of cross-linked matrix dissolution in hemicelluloses and lignin parts as well as hydrogen bond disruption in cellulose parts bring about increase in accessibility of hydrolytic enzymes to the substrate (Taherzadeh and Karimi, 2008; Li *et al*., 2010).

As stated above, several pretreatment methods have been developed. However, it is safe to say that none of the pretreatment techniques or methods can be recommended for all biomass types. This is because of their inherent drawbacks limiting application. In response, different combination systems have been developed to serve as a form of complementation for each of the techniques involved, and this has led to increased yields, reduced inhibitory activities, lesser processing times so far. Some of such pretreatment combinations include alkaline-acid (Lu *et al*., 2009), combination of organosolv and biological pretreatments as reported by Monrroy *et al*. (2010), combination of biological pretreatment and dilute acid as reported by Zhang *et al*. (2007), dilute acid pretreatments with microwave support (Chen *et al*., 2011), biological pretreatment with steam explosion as reported by Taniguchi *et al*. (2010), ionic liquids-ultrasonic pretreatments (Ninomiya *et al*., 2010) and dilute acid with steam explosion (Chen *et al*., 2011). Developing new pretreatment mechanisms and technologies will help to bring down bioethanol production cost in the near future. It can be submitted that it is important to comprehend the concepts of single or combination of pretreatment technologies and the make up of biomass feedstock at one's disposal for adequate biotransformation to biofuels.

2.4 Saccharification and fermentation

2.4.1 Separate hydrolysis and fermentation (SHF)

To optimize the efficiency of the bioalcohol production process, a system of saccahrification and fermentation are usually adopted. Separate hydrolysis and fermentation (SHF) has been adopted in many early production systems for bioethanol from lignocellulosic biomass. In this case, the hydrolysis/saccharification and the fermentation of sugars available from saccharification are separately performed one before the other. This method has however been stated to be time consuming; raising high cost concerns which currently mitigate against its full viability. The method of separate hydrolysis and fermentation however is regarded as advantageous in some quarters because of the fact that optimum conditions of fermentation and hydrolysis can be targeted separately in different chambers independent of each other. This however goes with another disadvantage as there is the possibility of the production of sugars causing a form of inhibition of cellulose activity (Ishola *et al*., 2013). To overcome some of these disadvantages, it is important to consider the simultaneous application of both systems in a cost effective value.

2.4.2 Simultaneous saccharification and fermentation (SSF)

Simultaneous saccharification and fermentation (SSF) describes the strategy which involves the combination of both saccharification and fermentation systems as a method of increasing the cellulose bioconversion to ethanol. In this case, the hydrolytic systems and fermentation of sugar products of hydrolysis take place within the same system. Strategically, there is maximized enzyme consumption which is depicted by the fact that the levels of sugars produced do not accumulate enough to inhibit fermentative systems of important microorganisms in feed back mechanisms (Brethauer and Wyman, 2010). Wingren *et al*. (2003) reported that the amount of ethanol generated from the SSF system is more than that of SHF, and the SSF technology has been applied in the treatment and fermentation of different energy lignocellulosic crops, with proven levels of high efficiency. A report by South *et al*. (1993) earlier stated the development of continuous conversion system using a blend of *Saccharomyces cerevisiae* and cellulases in a form of bioconversion of pretreated hardwood flour to ethanol. Another report by Fan *et al.* (2003) showed the development of a system of semi-continuous style conversion based on SSF for the

efficient transformation of paper sludge into ethanol. Results obtained explained the achievement of a mean conversion of 92% and a production quantity of 42 g/l of ethanol as 82 g/l of cellulose combined with enzymes was applied. Subsequently, there was an SSF module that was run in a fed-batch system targeted to surmount the retardation brought about by large quantity of sugar on *S. cerevisiae* during enzymatic hydrolysis of cassava (Moshi *et al*., 2014). Kumagai *et al*. (2014) established that the SSF procedure led to ethanol synthesis using from *Hinoki cypress* and Eucalyptus after a process of steam treatment and wet-disk milling. The SSF system was also applied in the production of bioethanol from the algae *Saccharina japonica* that had a composition of 55% laminarin and mannitol. The system in this case achieved 6.65 g/l quantity of ethanol and an efficient output of over 67% extrapolating from the available glucan in the pretreated *S. japonica*. In all these though, there stands a major demerit of the SSF system which is that the optimum temperature range that cellulases function best - between 45 and 60 ^{o}C, is higher than the temperature for microbial systems to function appropriately, thus hindering the bioprocess mechanisms in some extent (Brethauer and Wyman, 2010; Kumagai *et al.,* 2014).

2.4.3   Non – Isothermal simultaneous saccharification and fermentation (NSSF)

Based on the shortcomings of the SSF system, which has to do with the varying temperature requirements for optimum functioning between the enzymes and the microorganisms, the design of a non-thermal system has showed to be an augmentation. The NSSF system was designed to bridge that gap; it involves performing the enzymatic hydrolysis at temperatures below the optimum significantly affecting enzyme activity and subsequently resulting in increased enzyme consumption (Taherzadeh and Karimi, 2008). In practice, NSSF has been applied in the synthesis of bioethanol from several biomass systems from different biomass sources. Spen wood, paper sludge, soft wood spruce and hardwood oak have all been made to undergo NSSF producing bioethanol at the end (Kadar *et al.,* 2004).

2.4.4   Simultaneous saccharification, filtration, and fermentation (SSFF)

Ishola *et al.* (2013) developed a technology referred to as the Simultaneous Saccharification, Filtration and Fermentation (SSFF) for converting lignocellulosic matter to bioethanol. In explanation of the process, there is an exposure of pretreated lignocellulosic slurry to enzymatic activity and reactor based hydrolysis. As this is

carried out, the sugar laden suspension is pumped across a flow membrane in a continuous system unto the fermentation system. There is a back-pumping of fermented liquids into the vessel for hydrolysis with a simultaneous perfusion through the designed vessel/container after which it is forced to flow back into the hydrolysis container. A strain of *S. cerevisiae* was applied because of its settling activity (ability to flocculate) making the kinetics of fluid pumping and flow possible. Ethanol yield was also high (85%) and because of the flocculating ability of the strain used, up to 5 cultivations were possible using the same initial inoculum, making it possible to reuse the fermenting organisms more than once (Ishola *et al.* 2013).

2.4.4. Simultaneous saccharification and co – fermentation (SSCF)

While carrying out bioethanol production the concentration of alchohol is also quite as important as the alchohol yield generated as well; this is so because the cost of final distillation is reduced in the case of higher concentration, thus increasing the efficiency of the bioprocess (Sassner *et al.*, 2008). A shortfall of the SSF system is represented by the focus of fermentation on just the hexoses without catering for the pentose sugars use. Glucose concentrations within the fermentation system negatively affect the uptake of xylose especially when yeast like *S. cerevisiae* is used, thus the glucose concentrations should be maintained at low levels so as to enable the organism to efficiently consume xylose. Co–fermentation of glucose and xylose is influenced positively by reduced quantity of glucose or a rise in xylose to glucose ratio (Meiamder *et al.*, 1999). When fermenting lignocellulosic matter to ethanol, as hexoses are important to be obtained, it is also important to remember the pentose parts due to the high xylan content of lignocellulosics. In this regard, the co-fermentation of both types of sugars has been a subject of activity using the SSCF co-fermentation system. SSCF is similar to the SSF, but differs in the regard of simultaneous saccharification alongside co-fermentation of hexose sugars and pentose sugars in one step and within one system (Kang *et al.*, 2010). The SSCF system reveals to be a technology with great promises with respect to cost reduction in bioethanol production procedure, and likewise on reduction of inhibitory effects of xylose as well as consumption of pentoses which translates into an overall increase in efficiency (Zhang *et al.*, 2010). The advantageous position of SSCF over SHF is exhibited as the glucose that is liberated is fermented immediately yielding a low glucose concentration within the fermentation medium. This automatically reduces the inhibition of end

product during enzymatic action as well as increasing the ratio between xylose and glucose concentration redirecting fermenting microorganisms to xylose fermentation (Olofsson *et al*., 2010).

2.5 Enzyme systems involved in hydrolysis of lignocellulosic biomass

Lignocellulose degrading enzymes utilize the mechanisms of hydrolytic activities by acting mainly on hemicellulose, and acting on lignin in an oxidoreductive mechanism in their total conversion of lignocelluloses. In fact, most cellulose and hemicellulases are carbohydrate hydrolases that are dependent on keeping the mechanisms yielding products of similar anomeric configuration after the de-bonding of the glycosidic bonds employing the mechanism of 'double displacement' or 'inverting' which leads to the production of opposite anomeric configuration. The dissolution of the glycosidic bond occurs via a 'single nucleophilic-displacement' hydrolysis which involves two different amino acid residues (Glu and Asp) functioning as acid-like donor of protons or base-like nucleophiles (Vocadlo *et al.,* 2008). Some hydrolases could also function as glycosyl transferases; however, the lignin targeting enzymes are peroxidases containing a heme and some Manganese co-active cores. The lnolytic enzymes can also be phenol oxidases which are oxidoreductases that contain copper which chiefly rely on transfer of electron from lignin to high valence Fe(V/VI)-oxo, Mn(III), or Cu(II), a process that can result in radicalization, and lignin derivatization as well as bond scission (Martinez *et al.,* 2005). An overview of the constructive elements of these lignocellulolytic enzymes is given below.

2.5.1   Cellulases

Cellulases are involved in the hydrolytic cleavage of the β(1→4) glucosidic bond present in cellulose, resulting in glucose monomers and short cellodextrins. Cellulases are a family of enzymes constituting cellobiohydrolases (CBH), *endo*-1,4-β-D-glucanase (EG), and β-glucosidase (BG) types depending on the substrate affinity and source of enzyme. Although cellulose is a relatively simple substrate as it consists of only glucose subunits and carries a morphology which is primarily amorphous and crystalline, the hydrolases that cleave the bonds between the glucose units can vary based on different reactions applied in breaking the bonds (Zhang *et al*., 2010; Banerjee *et al.,* 2010). Short notes on different cellulase classes are given below.

2.5.1.1 Cellobiohydrolase

Cellobiohydrolase (CBH) is a class of cellulase widely used in the industrial lignocelluloses lysis, thus stand very indispensable. CBHs are found to be in the Glycoside hydrolase (GH) 6, 7and 48 families. Based on genomic data, GH7 CBH is regarded to be present in all cellulolytic fungi, while GH6 CBH is found not in all, but in many cellulolytic fungi. According to reports by Herpoel-Gimbert *et al.* (2008), about 70% weight of all secreted proteins and enzymic compounds of cellulolytic fungi may be CBHs. Specifically speaking, GH7 CBH targets the cellulose structure at its reducing end, while the GH6 CBH is specific for activity on the non-reducing end. This makes the two enzyme forms best candidates for co-enzymatic synergy when applied on a common substrate (Sweeny and Xu, 2012). Catalytic activity of CBHs shows that they possess a topological feature of core tunnel-like active sites giving them the property of cellulose hydrolysis in a processive manner (Vocadlo *et al*., 2008). Liu *et al*. (2011) determined that the enzyme twines into the extreme end of the cellulose chain via the active site and subsequently nicks off a cellobiosyl unit as it proceeds down the cellulose chain. Beckham *et al.* (2010) also suggested in addition that a carbohydrate binding molecule (CBM) may aid the catalytic core with the activity of processivity. This kind of processivity coupled with the insoluble property of cellulose confers on the CBH kinetics a model noncompliant with the Michaelis-Menten model, showing substantial fractal/local jamming consequence (Igarashi, *et al*., 2011). However, it has been stated that the processive movement of CBH can be hindered by the cellulose surface thus hampering CBH efficiency (Kurasin *et al.,* 2011). Inherent means of getting over this is the cellodextrin/cellulose binding mechanism of the GH7 CBH-I which functions based on the possession of approximately 10 anhydro-Glc-binding subsites present within the tunnel of activity made possible by the hydrogen bonding and π-stacking with some major residues of amino acids.

2.5.1.2 Endo-1,4-β-Glucanase

Endoglucanases (EG) act by degrading the amorphous cellulose in an organised unlike the CBH which act by 'on-off' hydrolysis of glycosidic bonds in the cellulose, thus making EGs very effective in breaking intricately shielded portions of the cellulose chain, thereby providing broken chains of cellulose for CBH to subsequently act on

them. This thus shows a form of synergistic enzymatic relationship between CBHs and EGs (Li *et al.,* 2010). This synergistic activity has been directed for application in the industrial biomass-conversion. EGs are basically secreted by cellulolytic fungi (about 20% weight of proteins secreted), and they contain a conformational orientation of a catalytic core of a little above ten glycoside hyrolase (GH) families – containing GH5, 7, 9, 12, 45, and 48 as represenatatives (Herpoel-Gimbert *et al*., 2008). EGs are common in fungi. However, some cellulolytic bacteria have been researched and found to synthesize a variety of EGs, which vary in their mechanisms of action (inverting for GH6, 9, 45, 48 and retaining mechanisms for G5,7, 12 EGs) even when fed with same substrate, probably because of the different side activities as they target hemicelluloses (Vlasenko *et al.,* 2010). The EG enzymes may also react in a synergistic manner (Wilson *et al*., 2008). Sweeney and Xu (2012) stated that the sites of activity of EGs are formed like grooves within which cellodextrin or a segment of cellulose may bind for onward enzymatic activity, in addition to this there are also CBMs on other domains within the enzyme and the CBMs have the tendency to direct EGs activity.

2.5.1.3 β-Glucosidases

Beta-glucosidases (BG) also known as cellobiose hydrolase are involved in the degradation of cellobiose and other cellodextrins. They differ from CBH and EG in their structure as they lack a modular distinct CBM, but rather possess pocket-shaped sites of activity which target the non-reducing glucose units from cellobiose and /or cellodextrin (Langston *et al.*, 2006). According to Eyzaguirre, *et al.* (2005), BGs are a member of the families of GH1, 3,and 9, and unlike other biomass degrading enzymes, BGs act upon soluble and not insoluble substrates as shown by enzyme kinetics studies (Jeoh *et al.*, 2005). Herpoel-Gimbert *et al.* (2008) submitted that BGs make up about 1% of proteins secreted by cellulolytic fungi which is quite lower than the percentages secreted for CBH and EG. Despite this, BGs play a functional role as an efficient group of enzymes in the lignocelluloses dregradation system. This is amplified by their ability to act on cellobiose reducing the generation of inhibitors for the CBH and EG enzymes. BGs are also more resistant to glucose inhibition than other enzymes (Sweeny and Xu, 2012). Their resistance to glucose, and their activity on cellobiose which increases functionality of other enzymes thus makes it imperative to supplement BGs with other lignocellulose/cellulose degrading enzymes in a bid to achieve higher biomass conversion efficiencies in the industries (Kristensen *et al.*, 2009).

2.5.2 Hemicellulases

Based on the plant cell wall structure, celluloses are enmeshed and coated by hemicelluloses which have a basic difference (from celluloses) in their polysaccharide formulation based on their different glyco-units and glycosidic bonds. Hemicellulases which function in the hydrolysis of hemicelluloses therefore frees the interlocked celluloses and also transforms the hemicelluloses into valuable sugars. The hemicellulases as an enzyme class can also be an array of enzymes with synergistic capabilities (Sweeny and Xu, 2012). According to nomenclature some of the hemicellulases and their sugars are given thus: β-glucan/β-glucanase; xylan/xylanase; xyloglucan/ xyloglucanase; arabinoxylan/arabinoxylanase; mannan/mannanase; arabinan/arabinase; polygalacturonan/polygalacturonase e.t.c. (Gao *et al.,* 2010). A cursory look at the broader classes of the hemicellulases based on their modes of action shows that hemicellulases like glycoside hydrolase can hydolyse glycosidic bonds; while carbohydrate esterases hydrolyse ester bonds. Polysaccharide lyases nick glycosidic bonds while *endo*-Hemicellulases attack internal/structural glycosidic bonds, and other glycosidases cleave the side chains. According to Gao *et al*. (2010), different plants have varied constellation of hemicelluloses like acetylated galacto-glucomannan, however the glucuronoxylans and arabinoxylans make up the major hemicelluloses like wood and grass usually applied in production of bioethanol (Gao *et al*., 2010). This means that several hemicellulase combinations are needed for industrial biomass conversion based on synergistic action (Banerjee *et al*., 2010; Gao *et al.*, 2010; Kumar and Wyman, 2009).

2.5.2.1 Endo-β-Xylanase and β-Xylosidases

Endoxylanases (EX) are involved in the breakdown of (glucurono)(arabino)xylan, which are a category of β(1→4) linked D-xylopyranosyl (Xyl) polysaccharides having varied O-substitutions by acetyl, arabinosyl (Ara), glucuronoyl (GlcU), or other substituent groups. EXs are commonly found in plants, archaea, bacteria and fungi. They have a catalytic core which belongs to the family of GH8, 10, 11, 30 and 43 (Polet *et al*., 2010). There is a difference in specificity of substrate between GH10 EX and GH11 EX as the produce shorter and longer oligosaccharides when they act on xylan (Ustinov *et al*., 2008). CBM domains are also found on EXs (Verjans *et al*., 2010). Just as BGs do for EGs, betaglucosidases function in hydrolysisng xylobiose as

they are being produced by EX activities on xylan (Jordan and Wagscha, 2010). EXs are also reported to have catalytic cores that have α-L-arabinofuranosidase activity. These enzymes also utilize the inverting or retaining mechanism depending on the nucleophile and an acid conformation for their catalytic activity. Xylanases are responsible for less than 1% weight of fungal cellulolytic enzymes produced, and have the potential for synergistic activity among themselves and when they interact with hemicellulases and cellulases. Some of the xylanases may produce large xylooligosaccharides for other xylanases to act upon. In some cases, when acting on hemicellulase, it is possible for a debranching xylanase enzyme to remove substituents to enhance enzymatic activity of other xylanases. EXs may also degrade xylan in lignocelluloses exposing the covered cellulose for subsequent cellulose activity (Pastor *et al*. 2007), thus a blend of both lignocellulolytic and hemicellulolytic enzymes are functionally attractive options in the industrial application in hydrolysis of grass or hardwood feedstocks enriched with arabinoxylan.

2.5.2.2 Acetyl Xylan Esterases, Glucuronoyl Esterase, and Feruloyl Esterase

Important ester substituents (e.g acetyl and feruloyl) are present in xylan or other hemicelluloses, and need to be eliminated for valuable endo-hemicellulase activity. This elimination action is usually mediated by acetyl xylan esterase (AXE), feruloyl esterase (FAE), and glucuronoyl esterase (GE), respectively. Acetyl xylan esterase functions in the deacetylation of substituted O_2 or O_3 sites of glycosyl backbones in xylan and some more hemicellulose substances (Biely *et al.,* 2011). According to Koseki *et al*. (2009), ferulolyl esterase is primarily focused on the hydrolysis of feruloyl esters at α-L-Arabinose, β-D-galactosyl, or α-D-Xylose side chains of arabinan, arabinoxylan, rhamnogalacturonan, or xyloglucan. For glucuronoyl esterase, Duranova *et al.* (2009) revealed that the enzyme belongs to the carboxyl esterase-15 family (CE 15), and functions in the removal of methyl groups from methyl glucuronoyl/xylose linkages of the glucuronoarabinoxylan. The Ser-His-Asp unique for catalytic acitivity in esterases, lipases and serine proteases are also unique to these set of esterases (Sweeny and Xu, 2012).

Also, feruloyl Esterases tend to have a combination of carbohydrate binding molecules with their catalytic core for its functionality as well as possessing different levels of specificity with respect to different hydroxycinnamoyl ester bonds responsible for the

link between hemicelluloses and lignin (Benoit *et al*., 2008). It is also determined that there is a synergistic cooperation between different acetyl xylan esterases, glucuronoyl esterases, and feruloyl esterases as they act on complex hemicelluloses structures. The feruloyl esterases or acetyl xylan esterases aid *endo*-hemicellulases in delignification and deacetylation as well as deferulating hemicellulases. Another form of synergy is depicted in the assistance rendered by glucuronoyl esterases to α-glucuronidase by hydrolysis of esters. Industrial application of these synergies can be observed in the breakdown of acetylated hardwood xylan or ferulated grass arabinoxylan by the bioaugmentative addition of acetyl xylan esterases as it enhances endo-hemicellulases' activity (Sweeny and Xu, 2012).

2.5.2.3 α-L-Arabinofuranosidase, α-Galactosidase and α-Glucuronidase

Alpha-L-arabinofuranosidase (AF) is responsible for the deduction of the arabinose substituent, however with varied specificity as some AFs can contain many CBMs and show different preferences for the site they attack (Saha, 2000). Alpha- galactosidase are focused on the removal of galactose substituent coupled together to galactomannan, or some other hemicelluloses through α-glycosidic bonds. There is also a reliance on NAD+ cofactors by the galactosidases (Yip and Whitters, 2006). Some alpha glucuronidases are also involved in the removal of methyl esters in xylan. The glucuronidases possess a catalytic core belonging to GH67 with over 100 sub-families (Chong *et al*., 2011). There is also a report that some alpha glucuronidases possess larger specificity to glucuronated xylooligosaccharides, whereas some are specific to polymeric glucuronoxylan (Pastor *et al*., 2007).

Alpha-L-arabinofuranosidase, α-galactosidase, and α-glucuronidases help in the overall function of xylanase, pectinase, and other hemicellulases usually by removing the branches of their polymeric substrates. In the treatment of softwood (containing arabinoglucuronoxylan) or grass (with abundant arabinoxylan) as target feedstock, supplementing AF to enzyme mixes may be beneficial to degrade lignocellusic complexes (Sweeny and Xu, 2012).

2.5.2.4 Glucanases, Mannanase, Xyloglucan Hydrolases and Pectinases

Non endo glucases/beta glucosidase β-glucanases can act as degraders of β(1→3), (1→4), or (1→6) glucan because they are a dynamic category of *endo* or *exo* enzymes possessing catalytic cores classed as GH3, 5, 12, 16, 17, 55, 64, and 81 family groups

(Martin *et al*., 2007). A good number of cellulolytic microorganisms possess secretory β-glucanases, which makes them able to function in catalysis of complex β-glucans present in the structural backbone with glycosidic bonds.

Mannanase are involved in the breakdown of (galacto)(gluco)mannans, β(1→4)-D-mannosyl or manno/glucopyranosyl polymers possessing variable α(1→6) D-Gal side chain. Mannanases are hydrolytic enzymes which have wide natural distribution in microorganisms and possess a catalytic core of GH5, 26, and 113 families (Moreira and Filho, 2008). Short chain saccharides released after mannanase degradation can be further broken down by β-mannosidases which possess catalytic regions belonging to the GH1,2 and 5 families. They also have a functional carbohydrate binding molecule which is mannan/cellulose specific. Along with the secretion of mannanase, many mannolytic microorganisms also secrete cellulases and xylanases as well as other ancillary enzymes (Herpoel-Gimbert *et al*., 2008). Enzyme cocktails containing enough quantity of mannanases can be applied in the soft wood industry, especially on galacto-glucomannan containing woods as feedstock to yield effective bioconversion of substrate.

Xyloglucan hydrolases with their catalytic portions belonging to GH5, 12, 16, 44, and 74 families degrade xyloglucan. In the degradation, β(1→4) glucan together with α(1→6) linked xylose are switched with any of α(1→2) L-arabinose or β(1→2) D-galactose units. Baumann (2007) stated that xyloglucan hydrolases are a part of a larger super family of xyloglucan transferase/hydrolase (XTH). Vlasenko *et al*. (2010) also confirmed that a lot of xyloglucan hydrolases possess a small fraction of minor endoglucanase activity as well as the endoglucanases also have minor xyloglucan-hydrolyzing property. According to Kaida *et al*. (2009) arabinofuranosidases and esterases can remove the branch in xyloglucan leading to more effective xyloglucan hydrolase functionality. The degradation of xyloglucan potentially can aid accessibility of cellulase to cellulose, thus making xyloglucan hydrolases important industrial enzymes (Kaida *et al*., 2009).

Pectinolytic enzymes bring about lysis of the pectic polymers which consist of α(1→4) poly-α-(rhamno) galacturonic acids consisting of arabinose and galactose on branching side chains as well as a dynamic scaffolding structure of methylation/acetylation (Lombard *et al*., 2010). Pectinolytic enzymes commonly include pectin methyl

esterases (having a catalytic core in the class of the CE8 family); polygalacturonase (possessing a GH28 family of binding site); pectin lyase (which have a catalytic site belonging to the pectinlyase 1,2,3,9 and 10) (Kaida *et al*., 2009). Pectin lyases, in contrast with polygalacturonases, attack an O-C4 glycosidic bond with the aid of a C6 uronic acid forming C=C bond positioned at the non-reducing portion of the galacturonoyl like most other enzymes classed together, most pectinolytic enzymes act in unison as a with the endo/exo enzymes acting in synergy (Sweeny and Xu, 2012). Pectin lyases and pectinolytic hydrolases act on both the pectate and pectin forms. The hydrolases and lyases potentially function on separate angles of the pectin/pectate while the methyl esterases remove the pectin to boost the function of enzymes that are pectate-specific. Other enzymes like galactosidases and arabinofuranosidases, may assist the functioning of pectinolytic enzymes by cutting off the side chains of polyrhamnogalacturonan which has abundant pectin polysaccharides (Lombard *et al*., 2010).

2.5.3 Lignocellulose Oxidoreductases

Researchers have stated that the secretome of a good number of cellulose degrading microbes have the secretory potentials for oxidoreductases alongside hydrolytic enzymes, which significantly underscores the value of both enzymes as co-functional in biological lignocelluloses degradation, and subsequently bioethanol industry (Martinez *et al*., 2005; Wymelenberg *et al.*, 2010). Oxidoreductases function in this case in the degradation of lignin which is quite difficult to break down due to its highly heterogenous nature. Ligin also possess aromatic polymers consisting of various units of syringyl, guaiacyl, or other hydroxyphenyl, thereby making it quite recalcitrant. These recalcitrant units are enmeshed in hemicelluloses/cellulose and also quite inhibitory to hemicellulases and/or cellulases. For industrial activity and biomass conversion, degradation of lignin is quite important as it aids in opening up access to the (hemi) celluloses (Wymelenberg *et al.*, 2010; Sweeny and Xu, 2012).

Lignocellulose peroxidases are mostly secreted by fungi. Examples and classes of these enzymes are Lignin peroxidase , Mn peroxidase , and versatile peroxidase as well as other extracellular heme peroxidases, with all having potentials of oxidatively breaking down lignin. The mechanism of the enzymes' activity is based on the fact that when they interact with H_2O_2, they form Fe(V) or Fe(IV)-oxo species that are

highly reactive and involved in the abstraction of electrons from lignin causing oxidation or radicalization. Laccases, another form of these enzymes secreted by a vast array of lignocellulosic fungi are multi-copper oxidases. They function by direct/indirect oxidation of phenolic/non-phenolic components of lignin using suitable redox-active mediators (Sweeny and Xu, 2012).

Other enzymes like aryl-alcohol oxidase, glyoxal oxidase, and carbohydrate oxidases also are linked to lignocelluloses degradation as they generate H_2O_2 from O_2 concomitantly oxidizing aromatic alcohol, glyoxal and reducing carbohydrates. The hydrogen peroxide they generate aids in the activity of lignin degrading peroxidases and thus correlatively degrading lignin.

Cellobiose dehydrogenases are also enzymes of interest in this regard and are produced by many lignocellulolytic fungi. They generate flavoheme enzyme which belongs to the lignocelluloses oxidoreductase family; the enzyme functions by dehydrogenating or oxidizing cellobiose or other cellodextrins yielding aldonolactones. They also cause the reduction of quinone and oxygen to phenol and hydrogen peroxide respectively. Previously, the catalytic function of cellobiose hydrogenase was considered from a Fenton chemistry perspective or the retardation in inhibition of products of cellulases by cellobiose alone (Bey *et al*., 2011). However, subsequent research showed that cellobiose dehydrogenases stimulate glycoside hydrolases 61 enzyme activity thus rearranging the concepts of its earlier thought function (Langston *et al*., 2011).

As the lignolytic oxidoreductases enzymes function, they generate oxidative species which in turn bind to inhibitors of industrial sugar conversion to ethanol, thus increasing the efficiency of the system (Parawira and Tekere, 2011); although their use should be limited to calculated and earlier determined activity units because of their tendency to also attack hemicelluloses/cellulose and sometimes even their corresponding hemicellulases (Bendl *et al*., 2008). Another limitation to the use of peroxidases is that they can also become autooxidises or inactivated thereby limiting their performance, thus optimizing their functionality requires further research for enhanced benefit in industrial systems.

2.6 Some microbial species of importance in bioethanol production

2.6.1 Bacteria

2.6.1.1 *Clostridium thermocellum*

Clostridium thermocellum is a much known bacterial specie used in bioethanol production through the consolidated bioprocess production system. It functions by extracellularly producing cellulases which are multi-enzyme complexes harbouring a variety of glycosyl hydrolases in the form of cellulases, hemicellulases and carbohydrates esterases (Kumagai *et al*., 2014). Increased potential of *C. thermocellum* to hydrolyze various materials which contain cellulose including wheat straw, poplar, and switch grass have been reported (Zhao *et al.*, 2012). A research work applied steam and wet disk milling on soft wood and hard wood before subsequently treating with *C. thermocellum* ATCC 27405 for bioethanol production, and achieved 79.4 mg/g cellulose from soft wood and 73.1 mg/g – cellulose from hard wood (Kumagai *et al.*, 2014).

2.6.1.2 *Clostridium phytofermentans*

In experiments conducted by Jin *et al*. (2012), *C. phytofermentans* ATCC 700394 was applied as an ethanologenic strain after using it in the bioconversion of AFEX-treated corn stover. Fermentation conditions were optimized and the strain gained the potential to hydrolyse 76% glucan and 86% xylan leading to a 2.8 g/l ethanol yield. Interestingly, the yield was about 71.8% more than that yielded by SSCF method. It was later revealed that AFEX-treated corn stover could be employed as the only source of carbon without additional source of nutrients. Identical sugar transformation were obtained when compared with nutrient supplementation experiments as it showed that xylan and glucans were converted at 77.9% and 48.9% respectively yielding 7.0 g/l of ethanol at the end of 264 h.

2.6.1.3 *Thermoanaerobacterium sp.*

Thermoanaerobacterium species has been a good candidate due to its xylanolytic properties and the innate capability to ferment glucose, xylose, galactose, and mannose. This makes the species hemicellulolytic as opposed to the cellulolytic *Clostridium* species (Shaw *et al.,* 2008). For growth, they have a temperature preference of 45 ^{o}C to 65 ^{o}C and pH tolerance of 4.0 to 6.5. Common enzymes which they synthesize include endoxylanase (for lysing xylan chains into xylobiose in conjuction with xylotriose), and β-xylosidase (for breaking oligosaccharides down to xylose). There are also some different xylanolytic enzymes which have simpler functions (Shaw *et al.,* 2008). In ethanol production using this organism, Sigurbjornsdottir and Orlygsson (2012) dicovered a strain, *T. aciditolerans* AK54, which has a potential of both biohydrogen and bioethanol production properties. From their experiments, it was confirmed that the isolate utilized xylose, fructose, glucose, galactose, sucrose, mannose, and lactose, subsequently producing lactate, ethanol, carbon dioxide, acetate, and hydrogen gas. The strain was also applied in fermenting complex matter like grass, cellulose, hemp, newspaper, and barley straw; with highest bioethanol yield of 24.2 mM obtained from cellulose fermentation. Other workers have isolated an earlier unidentified thermophilic anaerobic *T. calidifontis sp. nov.* strain (RX1) from Chinese hot springs which had a good ethanologenic ability. This strain could ferment xylose, glucose, xylan and starch for ethanol production with yield of about 81% and 58% calculated based on xylose and glucose contents respectively, achieved within 48 hours. Carbon dioxide/Hydrogen gases, lactate, and acetate, also produced (Shang *et al*., 2013).

2.6.2 Fungi

Filamentous fungi have also been severally reported to be involved in the production of lignocellulolytic enzymes that serve a great deal of functionality in bioethanol production. Examples of such fungi include: *Aspergillus* sp.*, Fusarium* sp., *Paecilomyces* sp., *Trichoderma* sp.*, Rhizopus sp., Neurospora* sp.*, Mucor sp.* as well as a good number of white-rot basidiomycetes (Lubbehusen *et al*., 2004). With regard to the hypothetical view of fungal co-evolution with plants, it is reported that the fungi have abilities to transform plant sugars into ethanol. Some of these characteristic fungi are as follows;

2.6.2.1 *Mucor circinelloides*

Mucor circinelloides was first isolated by Inokuma *et al.* (2013), and was characterised based on its ability to utilize N-Acetylglucosamine (GlcNAc) as well as chitin for nutrients (carbon source) for a straight forward manufacture of ethanol. A particular strain NBRC 6746 yielded about 18.6 g/l ethanol using 50 g/l of GlcNAc as substrate under a 72 h production period. A good ethanol yield was also observed with another strain; NBRC4572 as it had the production ability of 6 g/l ethanol using 50 g/l of chitin within 12 days incubation. From these experiments, it is opined that chitinous wastes can be used for bioethanol production as *Mucor* strains-aided bioconversion. However, much more progress needs to be made in this regard as not a good number of chitin based bioethanol research has been carried out using *Mucor* spp. This is partly due to the generally low yield obtained by some other strains of *Mucor*. Chitinase based engineering of *Mucor* strains is also proposed so as to enhance ethanol yield (Jouzani and Taherzadeh, 2015).

2.6.2.2 *Fusarium oxysporum*

As a plant pathogen, *F. oxysporum* has been evolved as an efficient converter of lignocellulosic biomass to ethanol, as few research work have targeted using it for the production of bioethanol from various pre-treated and untreated substrates (Xiros *et al*., 2011). Research has proven that *F. oxysporum* possesses an efficient cellulolytic system that produce essential enzymes necessary for breaking down lignocellulosic matter into simple saccharides and likewise possess hexose sugars and pentose sugars fermentation ability with the aid of anaerobic or microaerophilc conditions with about 0.35 g ethanol/g cellulose on the average (Ali *et al*., 2014). Due to the diverse host range of this fungus, it has great potentials in hydrolytic and fermentative activities in lignocelluloses bioconversion to ethanol. *Fusarium oxysporum* also has the ability to withstand inhibitory compounds like lignocellulosic hydrosylates and acetic acid occurring as a by-product (Xiros *et al*., 2011), thus giving it an advantage over some other organisms. Studies have also been concerned about the means of increasing ethanol production by *F. oxysporum* (Ali *et al.,* 2014).

2.6.2.3 *Fusarium verticillioides and Acremonium zeae*

A report published by de Almeida *et al*. (2013) utilized endophytic fungi, *F. verticillioides* and *Acremonium zeae* singly and as co-cultures in bioethanol production. According to the study, the use of xylose, glucose and a combination of both sugars yielded 0.46, 0.46 and 0.50 g ethanol/g sugar for *F. verticillioides* while values of 0.37, 0.39 and 0.48 g ethanol/g sugar were obtained for the respective sugar fermentation using *A. zeae.* Using pretreated sugarcane bagasse as substrate, an ethanol yield of 3.9 and 4.6 g/l was obtained when *A. zeae* and *F. verticillioides* were used respectively after a total of 40 g/l of substrate was fed in, leading to an average yield efficiency of approximately 0.31 g ethanol/g sugar consumed. Another observation obtained by the study was that the two fungi had ability to ferment glucose and xylose co-fermentatively leading to high yields, thus enhancing their suitability for bioethanol production from lignocelluloses (de Almeida *et al*., 2013).

2.6.2.4 *Aspergillus oryzae*

This fungus is a major one having good potentials for enzyme production utilizable in bioethanol fermentations. Optimization of ethanol yield has been reported using dilute alkaline peroxide on pre-treated and non-pretreated corn stover with *A. oryzae* using a continuous stirred bioreactor operated in batch mode (Machida *et al*., 2008).

2.6.2.5 *Paecilomyces variotii*

This is a soil-borne Ascomycete fungus that was evaluated by Zerva *et al*. (2014) as an important fungus for bioethanol production from lignocellulose matter. It was confirmed that the fungus had an efficient fermentation rate for both xylose and glucose with observed results not far from the theoretical yields. The fungus has also been characterised as a good degrader of wood as well as creosote - treated wood (Houbraken *et al.,* 2010) thus making it an important organism in the bioethanol production procedure. The exploitation of the inherent enzyme factory within the organism for the degradation of common ago-based lignocellulosic biomass is thus advocated (Zerva *et al*., 2014).

2.6.2.6 *White rot Basidiomycetes*

White rot Basidiomycetes or 'white-rot fungi' are competent degraders of lignin as they have been associated with the first line degradation on plant litter in municipal and terrestrial environments (Okamura *et al.,* 2001). They can synthesize extracellularly a number of enzymes namely: laccase, manganese peroxidase, lignin peroxidase, and dexterous peroxidases. They also have the ability to synthesis alcohol dehydrogenases and are well suited for application in SSF or bio-pretreatment systems for lignocelluloses (Shi*et al*., 2009). Further research work also revealed white-rot fungi like *Peniophora cinerea, Phanerochaete chrysosporium, Trametes versicolor, Tramates hirsute* and *Trametes suaveolens* to have good ethanologenic capabilities when fed with hexose sugars and low-level xylose (Okamoto *et al*. 2011).

2.6.2.6.1 *Trametes versicolor*

Characterization of *T. versicolor* as white-rot fungi with capability of efficient conversion of hexoses and xylose to bioethanol was carried out by Okamoto *et al*. (2014). They assessed the organisms's efficiency for consolidated bioprocess for bioethanol production using 20 g/l of unpretreated cellulose, corn starch, xylan, rice straw and wheat bran. Ethanol yield ranging between 4.4 – 9.8 g/l for all substrates used were obtained. Due to its suitability in fermenting a wide variety of carbon sources especially xylose-containing substrates, and its efficiency in acting without the aid of pretreatment, the organism was seen to be more superior to *S. cerevisiae* and *Pichia stipitis* for both efficiency and economic reasons (Okamoto *et al*., 2014).

2.6.2.6.2 *Flammulina velutipes*

Another Basidiomycete, a mushroom *Flammulina velutipes* has a good acceptance and application in the food industry. It possesses fermentative properties with a good level of ethanol tolerance, making it essential in bioethanol production. Extra abilities of this organism in fermentation of cellobiose, fructose, mannose, sucrose, maltose, and glucose to ethanol also give it an advantage. *Flammulina velutipes* however lacks fermentative ability for galactose and pentose sugars, and is unable to bioconvert them to ethanol (Mizuno *et al*., 2009a). Exploiting its properties in ethanol biosynthesis, Mizuno *et al*. (2009a) assessed the capability of the fungi in bioethanol production using glucose, and an 88% recovery rate was achieved. Considering sugar

fermentation profile of the mushroom, there is some similarity with the yeast *S. cerevisiae*. However, *F. velutipes* differs in its ability to efficiently bioconvert maltose, cellotriose, cellotetraose and cellobiose into bioethanol (Mizuno *et al*., 2009a). Further assessments of the fermentative abilities of *F. velutipes,* led to the study involving the use of two varieties of sorghum (wild type and brown mid-rib (*bmr*) mutated). An ethanol yield of 180 and 200 grams per liter were obtained for the wild type and the mutated substrates (Mizuno *et al*., 2009b). It was made clear that genetic / metabolic engineering of these specific fungi for enhanced cellulose activity could be very important in the ethanol production system. Another observation with these fungi was that they could tolerate up to 120 g/l much more than a stable value for *C. thermocellum* (Okamura *et al*., 2001).

2.6.2.6.3 Phlebia sp.

According to Kamei *et al*. (2012a), *Phlebia sp.* (MG60) could bioconvert lignocellulose to bioethanol when subjected to sub-aerobic conditions making it a suitable organism for consolidated bioprocess production of bioethanol. Cultivating the fungi in 20 g/l of paper waste or hardwood kraft pulp that has not undergone bleaching, a yield of 4.2 g/l and 8.4 g/l was obtained within 168 and 216 hours of fermentation resulting in an ethanol yield efficiency of 0.42 and 0.20 g/g lignocellulose. In addition, ethanol outputs ranging between 0.33 − 0.44g/g of sugar was obtained when glucose; fructose, galactose, xylose, and mannose had complete assimilation by the strain. In a follow-up experiment direct degradation of lignin was achieved and the delignified from oakwood, and subsequently the delignified oakwook was subjected to aerobic solid-state fermentations leading to bioethanol production (Kamei *et al*., 2012a). In another study, they were involved in an integrated fermentation process using fungi in a system of unified aerobic delignification coupled with anaerobic saccharification and fermentation of wood substrate by the fungi. The wood was effectively fermented as there was a shift from aerobic conditions involving bio-delignification pretreatment, to sub-aerobic conditions during saccharification and fermentation (Kamei *et al*., 2012b). Kamei *et al*. (2014a) stated that in the course of optimization of ethanol yield, higher concentrations of cellulosic matter in form of unbleached hardwood kraft pulp in concentrations of 2.0, 4.7, 9.1, and 16.5% (w/w) was used for production of bioethanol. In cultures containing 9.1% substrate, 2.5 g/l of ethanol was the highest value observed. The provision for a small aeration was done

by temporarily removing the plugs of the flasks and this yielded about 37.3 g/l ethanol. It was also reported that spent mushroom waste from *Lentinula edodes* which was generated by cultivation in consolidated bioprocess fermentation using *Phlebia* sp was used. It was subsequently opined that the combined approach of cultivation of edible mushrooms and bioethanol fermentation has potentials of becoming a new and cost-effective method (Kamei *et al*., 2014b). An integrated fermentative system involving fungi was developed by Khuong *et al*. (2014a) for biological delignification and fermentation, which had the capacity to optimize the ethanol that is being produced from sugarcane bagasse using *Phlebia sp.* MG-60. The water activity (75%) of bagasse was optimized for selective lignin removal and production of ethanol. In furtherance, some additives such as basal media, organic minerals and compounds, all affected biological delignification of bagasse using the test strain. The use of inorganic chemical agents (Fe^{2+}, Mn^{2+}, or Cu^{2+}) was also observed to improve both delignification and ethanol production. In a subsequent experiment, Khuong *et al*., 2014b gave a report that alkaline pretreatment of bagasse led to an improvement in direct bioethanol production. Cultivating the organism in 20 g/l of alkali-pretreated bagasse, a yield of 210 mg ethanol/g of the original substrate was generated after 240 hours of fermentation.

2.6.2.6.4 Peniophora cinerea and Trametes suaveolens

Two white-rot Basidiomycetes (*Peniophora cinerea* and *Trametes suaveolens*) were used by Okamoto *et al*. (2010) for ethanol production using hexose sugar as substrate. Under both aerobic and sub-aerobic conditions, *P. cinerea* produced ethanol after assimilating glucose, fructose, mannose, sucrose, fructose, cellobiose, galactose and maltose with quantity of ethanol produced within 0.19 - 0.45 g/g hexose. In a case of *T. suaveolens*, ethanol yields were low in aerobic conditions, but increased in semi-aerobic conditions yielding a range of ethanol concentration for the different sugars between 0.13 - 0.39 g ethanol/g hexose.

2.6.3   Yeasts
2.6.3.1 *Kluyveromyces marxianus*

Mesophilic conditions (28 – 37 ^{o}C) are generally preferred by most microorganisms for ethanol production. This is at variance with optimum activity for cellulases occurring at higher temperatures (50 °C). Thus, the use of mesophiles in the process leads to a

decrease in the efficiency of ethanol production. An input for correction is the introduction of thermotolerant microorganisms with good growth and fermentative potentials at high temperatures resulting in valuable rise in the quality of ethanol production process (Yanase *et al*., 2010). *Kluyveromyces marxianus* is a thermotolerant yeasts used to produce ethanol (Yuan *et al*., 2012). Strains of the organism are able to thrive very well at temperatures as high as 52°C, with short doubling times compared to molds. They have the potentials for converting quite a number of substrates, such as xylose and different other feedstocks to ethanol especially at high temperatures (Yuan *et al*., 2012). A research by Hu *et al*. (2012) characterized a *K. marxianus* PT-1 (CGMCC AS2.4515) isolated during fermentation of Jerusalem artichoke tuber flour to ethanol. A yield for extracellular inulinase activity from an inulin-type oligosaccharides leading to 73.6 g ethanol/l was obtained at 40 °C. The quality of *K. marxianus* as a viable organism for bioethanol production was found in this study. Using another inulinase producing strain *K. marxianus* Y179, Yuan *et al*. (2012), with huge success, obtained ethanol from artichoke tubers which are rich in inulin during fermentation operated in a fed-batch system. The fermentation experiment was conducted in an integrated mode as all steps of inulinase production, inulin saccharification and ethanol production were all integrated. They proved that the yeast had a preference for anaerobic conditions. By virtue of optimization of medium constituents, an optimum ethanol yield of 93.2 g/l was also achieved.

2.6.3.2 *Clavispora*

It is a fact that *S. cerevisiae* has a major difficulty breaking down cellobiose, thus requiring the external introduction of β-glucosidase for the breakdown of cellobiose to glucose for utilization by the yeast. In addition, optimum temperatures required during enzymatic hydrolysis are much higher compared to the required microbial temperature needed as safety/comfort zones for their fermentation activity. A strain of *Clavispora* was isolated and characterized by Liu *et al*. (2012); the strain has the capability of cellobiose utilization as sole carbon source and for the production of adequate β-glucosidase for synthesis of bioethanol using SSF on cellulosic matter. In the study, the *Clavispora* strain had a good tolerance to inhibitors produced as a result of the pretreatment process. From 25% solids loading of xylose-extracted corn cob residue, 23 g/l ethanol was produced using a SSF setup with the strain at temperature of 37 °C, without an externally added β-glucosidase (Liu *et al*., 2012).

2.6.3.3 Cryophilic yeast (*Mrakia blollopis*)

In cold regions like the Arctic and Antarctic, *Mrakia* spp. are cryophilic yeasts that are prominently cultured. Tsuji *et al.* (2014) studied *M. blollopis* SK-4 isolate from East Antarctica, and demonstrated the fermentation abilities of this strain on sugars like glucose, fructose, sucrose, raffinose, maltose, and fructose at cold temperatures. The fermentative ability of the strain when fed with lignocellulosic materials and glucose was assessed in the presence of Tween 80 at 10 ^{o}C. In the absence of Tween 80, the ethanol yield final concentration for glucose, Eucalyptus and Japanese Cedar were 48.2 g/l, 7.2 g/l and 12.5 g/l respectively, whereas in the presence of Tween 80 (1% v/v); ethanol concentration was increased by about 1.1–1.6 fold in comparison with that without Tween 80 (Tsuji *et al*., 2014). They confirmed that the addition of 1% (v/v) Tween 80, together with 5 U/g lipase, raised the amount of bioethanol from 1.4 to 2.4 fold in comparison with the one with no lipase and Tween 80.

2.7   Bioethanol production from sugarcane bagasse

Sugarcane is a tall perennial grass with about 6 to 37 species of the family *Saccharum*. (family Poaceae, tribe Andropogoneae). A native plant to warm temperate climates like India, Africa, Brazil, and Asia pacific. The plant is basically composed of straw and stem and the straw is include the fresh leaves, dry leaves, and tops. A general composition of sugarcane as described by Mutton (2008) is shown in figure 2.3. Milling the sugar cane stem is used to obtain the cane juice used for sugar production as well as bioalcohol. After milling, the left over fraction from the stem is called 'bagasse'. Sugarcane bagasse (SB) and Sugarcane straw (SS) are suitable for burning in supply of heat energy and for ethanol prcduction. Utilization of both substrates can enhance ethanol production from each hectare of plantation cultivated (Mutton, 2008).

Cane

Fiber
(10–16%)

Broth
(84–90%)

Cellulose   Pentose   Lignin   Soluble solids
(10–25%)

Water
(75–82%)

Sugars
(15.5–24%)

No sugars
(1–2.5%)

Sucrose   Glucose   Fructose
(14.5–24%) (0.2–1%)   (0–0.5%)

Organics
(0.8–1.8%)
Starch, wax, amino acids,
organic acids, phenolic
compounds, etc

Inorganics
(0.8–1.8%)
SiO_2, K_2O, P_2O_5,
Fe_2O_3, etc
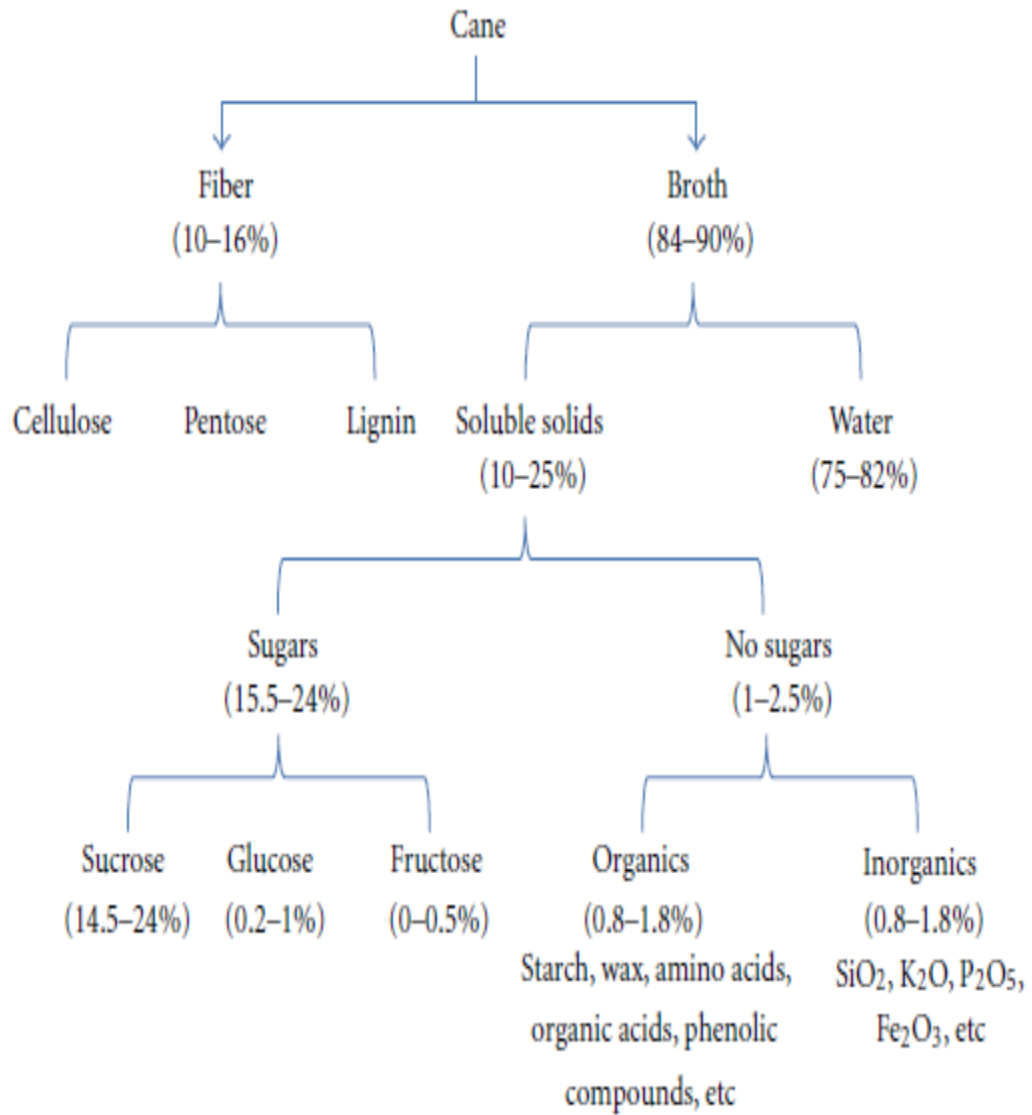
Figure 2.3: General composition of sugar cane plant (Mutton, 2008)

Sugarcane bagasse is composed of about 38.4 – 45.5% cellulose, 22.7 – 27% hemicellulose and 19.1 – 32.4% lignin. Since cellulose and hemicelluloses portions have a mixture of Carbohydrate polymers, strategies are designed for the conversion of the polysaccharides into sugars for fermentation. The hemicelluloses fraction can be hydrolysed with the addition of dilute acids subsequently followed by hydrolysis of cellulose using enzymatic actions. A chain of glucose units make up the cellulosic fraction while the hemicellulosic fraction is composed of arabinose, xylose and glucose which can be fermented into ethanol. The general biological process for converting the lignocelluloses matter into ethanol fuel is shown in figure 2.4 and involves:

(1) pretreatment of lignin/hemicellulose to liberate cellulose;

(2) depolymerization and debranching of carbohydrate polymers to generate free sugars via a cellulase mediated action;

(3) fermentation of the sugars (hexose and/or pentose) sugars to yield ethanol;

(4) Purification of the ethanol via distillation process.

Bioethanol produced using sugarcane residues is one of the most viable candidates for complementing/partial replacement of fossil fuels as it provides energy in renewable form with less carbon generating systems like that of gasoline (Canilha *et al.*, 2012).
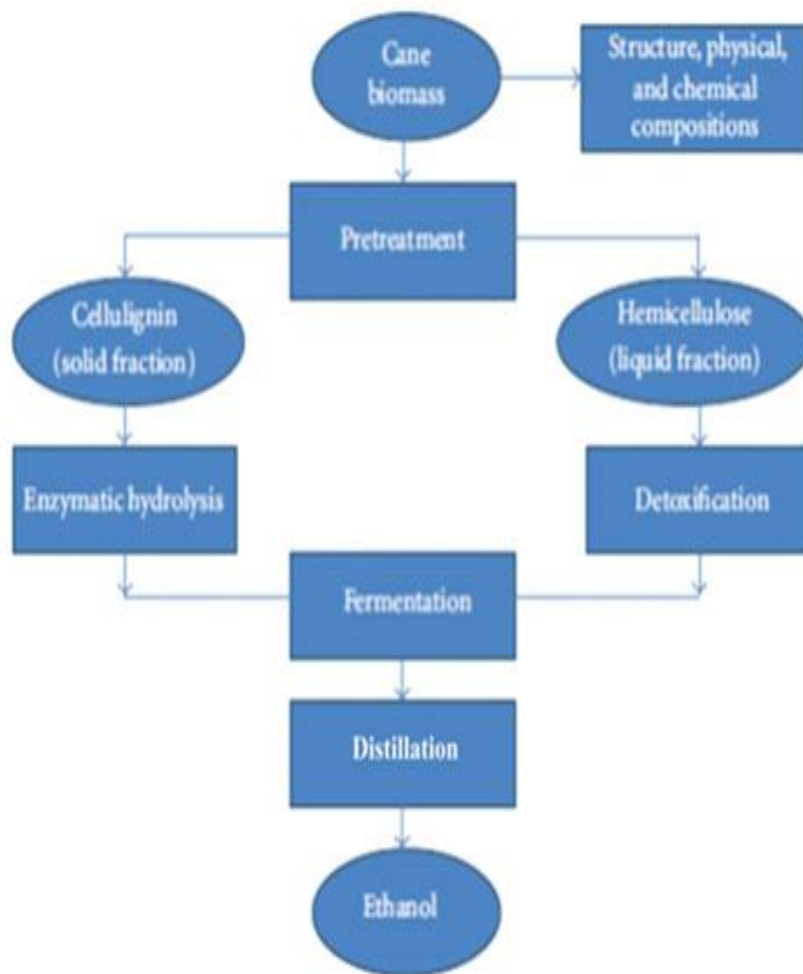
Figure 2.4: Schematic representation of ethanol production from sugar cane biomass (Canilha *et al.*, 2012)

2.8   Bioethanol production in Nigeria

The oil and gas is the major economic stay of the Nigerian economic system. However, the exploration systems are only dominant in just a portion of the country. This leads to barely few job opportunities resulting to below 1% of the over 150 million people inhabiting the country. There is a direct correlation between this and a surge in poverty levels among rural communities leading to an unbalanced rural-urban drift and migration over recent times. In a bid to balance out and mitigate these problems, an incorporation of biofuel production has been indicated by the Nigerian government, with special reference to bioethanol as a good option. Production of this energy source could enhance industrialization, increase automotive efficiency and generate domestic power in highly demanding rural areas (Azih, 2007).

The essence of this development was to make sure the common man was fully catered for as the country's economy was shaped. This led to a prompting of the Biofuels Policy (2007) which addresses the essential measures to be adopted in achieving a successful biofuels production and utilization syatem. The main target of the policy is for the reduction of the country's overdependence on imported petrol, as well as the environmental pollution emanating from the process and creating a commercially viable industry that will attract investors and consumers. All these will be geared towards more sustainable job opportunities that empower the common citizens. The aim to ensure a synergistic fusion of the downstream petroleum industry and agriculture is also targeted (Galadima *et al.*, 2010).

2.81   Nigerian Biofuels Policy and Incentives

In line with the program of 'Automotive Biomass for Nigeria', the central government of Nigeria mandated NNPC to draft the policy in August 2005, so as to target the overdependence on crude oil/oil and gas earnings and the environmental threats linked with fossil fuels exploitation and use, thus reducing to considerable practicable levels. The mandate given requires that the policy is tailor made to allow the future consumption of biofuels in a more impactful way with respect to gasoline, diesel and demanded quality improvements on petroleum products. The Nigerian National Petroleum Commission (NNPC) Nigeria's national oil company drafted the Nigerian Biofuels Policy and Incentives in 2007, which it describes as the first of its kind to be established in the country. As stated above, there was a target of integrating agriculture

with oil/gas exploration and production which has been lacking since the discovery of commercial volumes of oil in 1956. The policy focuses on key governmental agenda for bioethanol (and biodiesel as well) throughout the country from the conception, research and development stage to optimal and large scale productions subsequently tuning in investments (Galadima *et al.,* 2011).

CHAPTER THREE

3.0   MATERIALS AND METHODS

3.1.1   Collection of samples

Sugarcane bagasse was obtained from the waste disposal site of Savannah Sugar Company Limited, Numan, Adamawa State of Nigeria and transferred to the laboratory in polyethylene bags.

3.1.2   Media preparation

The media used were Yeast Extract Peptone Dextrose Agar (YEPD) for yeasts and Potato Dextrose Agar (PDA) for both yeasts and fungi. Malt extract broth and Czapek dox medium were also used for rejuvenation and selection of microbial isolates respectively. Media were prepared according to the manufacturers' specification but modifications were made in composition where necessary according to the requirement of a specific experiment. Media were sterilised at 121 ^{o}C for 15 minutes. In some cases, vitamins and other heat-sensitive additives were filter sterilised.

Medium used for reactivating yeast isolates prior to nitrogen source fortification for glucose/xylose conversion to ethanol was composed of glucose (20 g/l), xylose (10 g/l), ammonium sulphate (1g Nitrogen per litre), magnesium sulphate (0.5 g/l), Calcium chloride (0.1 g/l), potassium phosphate (0.2 g/l), 1ml/l vitamin solution (1 g/l riboflavin, 1 g/l nicotinic acid, 0.5 g/l para-aminobenzoic acid (PABA), 0.5 g/l pyridoxine, 1 g/l thiamine, 0.5 g/l pantothenic acid, 0.5 g/l biotin) and the pH was adjusted to 5.5.

The fermentation medium used for nitrogen source fortification was composed of 30 g/l of either glucose or xylose, magnesium sulphate (0.5 g/l), Calcium chloride (0.1 g/l), potassium phosphate (0.2 g/l), 1 ml/l vitamin solution (1 g/l riboflavin, 1 g/l nicotinic acid, 0.5 g/l PABA, 0.5 g/l pyridoxine, 1 g/l thiamine, 0.5 g/l pantothenic acid, 0.5 g/l biotin) and each nitrogen source under investigation at a concentration of 1g Nitrogen per litre (1 g-N/L).

3.1.2.1 Aseptic techniques

In order to prevent contamination of cultures by microorganisms from the environment or the contamination of the environment by microorganisms, the following aseptic precautions were observed:

- Laboratory surfaces were cleaned and disinfected prior to use and hands were always wiped with 70% ethanol.

- Media and cultures were handled under the laminar flow unit to prevent contamination. In few instances where experiments were carried out on the open laboratory bench, Bunsen burner was used to create a relatively sterile environment on the laboratory bench.

- Media were sterilised at 121 ^{o}C for 15 minutes before use. In some cases, heat-sensitive additives like vitamins were filter sterilised.

- In cases where sterile disposable inoculating loops were not used, the reusable inoculating loops and other equipment which came into contact with cultures and media were effectively sterilised.

- Breathing on cultures or sterile instruments was avoiding.

- Sterile glass or disposable plastic pipettes and a pipette tips were used only once to avoid cross contamination.

- Experiments with moulds were only carried out in a dedicated laboratory to prevent fungal spores from contaminating other laboratory rooms

3.1.3 Chemical composition of sugarcane bagasse sample

3.1.3.1 Raw sample

Sugarcane bagasse was crushed using a laboratory size hammer beater mill (Mini hammer mill, Rajeshwari Engineering Works, India) of mesh size 1 mm to convert it into powdery form. This was stored in plastic containers and used at different stages of the study.

The samples were extracted with organic solvents in order to remove extractives before determining their content. This was done by mixing the bagasse with organic solvent (ethanol/cyclohexane, 2:1 v/v) and washed with water at 70 ^{o}C. The dried

solid sample was used to determine the content of lignin, cellulose and hemicelluloses according to Ioelovich (2015).

3.1.3.2 Determination of lignin

The extracted biomass sample, 0.6 g, was mixed with 8 ml of 72% sulfuric acid in 150 ml Erlenmeyer flask and pre-hydrolyzed at room temperature (25 $^{\circ}$C) for 2 hours using a water bath. The concentrated acid was mixed with 45 ml distilled water and stirred to dilute it. The sample was hydrolyzed with dilute acid on a magnet hot plate at boiling temperature for 2 hours. After cooling to room temperature, the magnet was carefully taken out from the flask and the contents of the flask was poured out into dry 50 ml polypropylene tubes (PP) tubes and centrifuged at 4000 rpm for 15 min to precipitate lignin. The transparent acidic liquid phase was carefully poured out.

The sediment of lignin was washed twice with distilled water and then centrifuged. Sodium bicarbonate (50 mg) was added to the tube while stirring using a glass rod until neutralization before subsequently washing twice with distilled water. The liquid phase was separated from lignin by centrifugation. The wet lignin was dried in the PP-tube at 60 $^{\circ}$C overnight and at 100 $^{\circ}$C for 3 to 4 hours till constant weight was achieved. The percentage content of lignin (L) in the extracted biomass sample was calculated by the equation:

$$L = 100\%(P_d - P_t)/P_o$$

where P_d is weight of dry lignin together with PP-tube; P_t is weight of empty PP-tube; and P_o is weight of extracted and dried biomass sample.

3.1.3.3 Determination of holocellulose or total polysaccharides

The extracted biomass sample (0.6 g) was placed in a 150 ml Erlenmeyer flask, and 50 ml distilled water, 0.7 g sodium chlorite ($NaClO_2$) and 1 ml glacial acetic acid were added carefully. The flask covered with Petri dish was heated at boiling temperature using a hot plate with magnetic stirrer for 45 min. An additional portion of 0.7 g sodium chlorite and 1 ml acetate buffer was added, and the treatment was continued for 45 min. After cooling at room temperature, the magnetic stirrer was removed and the content was poured into 50 ml PP tubes and centrifuged at 4000 rpm for 10 min. The transparent acidic liquid phase was carefully poured out. The sediment of

holocellulose was washed with distilled water two times and centrifuged. Sodium bicarbonate (50 mg) was added while stirring with glass rod to neutralization and washed two times with distilled water. The liquid phase was separated from holocellulose sediment by centrifugation. The sediment was rinsed with 96% ethanol and centrifuged. The wet holocellulose was dried in the PP-tube at 60 ^{o}C overnight and at 100 ^{o}C for 3 to 4 hours till constant weight was achieved. The percentage content of holocellulose HC in the extracted biomass sample was calculated by the equation:

$$HC = 100\%(P_d - P_t)/P_o$$

where P_d is weight of dry holocellulose together with PP-tube; P_t is weight of empty PP-tube; and P_o is weight of extracted and dried biomass sample

3.1.3.4 Determination of cellulose and hemicellulose

The obtained holocellulose in the experiment above was weighed and hydrolyzed with 2% hydrochloric acid to remove hemicelluloses. The dried holocellulose sample was mixed with 50 ml of 2% HCl in 150 ml Erlenmeyer flask, and the sample was hydrolyzed with the dilute acid at boiling temperature for 2 hours using hot plate with magnetic stirrer.

After cooling to room temperature, the acidic dispersion of cellulose was poured out into 50 ml PP-tubes and centrifuged at 4000 rpm for 10 min. The sediment of cellulose was washed with distilled water and centrifuged two times. Sodium bicarbonate (50mg) was added to the tube while stirring with glass rod for neutralization and washed two times with distilled water. The liquid phase was separated from cellulose sediment by centrifugation. The sediment was rinsed with 96% ethanol and centrifuged. The wet cellulose was dried in the PP-tube at 60 ^{o}C overnight and at 100 ^{o}C for 3 to 4 hours to constant weight. The percentage content of cellulose C and hemicellulose H in the extracted biomass sample was calculated by the equation:

$$C = HC(P_d - P_t)/P_o$$

$$H = HC - C$$

where HC is percentage of holocellulose; P_d is weight of dry cellulose together with PP-tube; P_t is weight of empty PP-tube; and P_o is weight of extracted and dried biomass sample.

3.1.4 Method of isolation

Sugarcane bagasse samples were buried in the soil (depths of 8 – 12 cm) at three different spots on the campus of Modibbo Adama University of Technology, Yola, Adamawa State. The spots were chosen based on an observed presence of massive microbial degradation of plants materials. The spots were properly demarcated with wooden planks and tagged to prevent human trespass. The bagasse was left in the soil to undergo degradation for three months before isolation. Serial dilution method was employed for isolation. Seven test tubes labelled 1 to 7 were used. One gram (1 g) of buried sugarcane bagasse sample from each spot was weighed into 9 ml of sterile distilled water in tube 1 and shaken thoroughly to make 10^{-1}. Dilutions were subsequently made up to $10^{-6.}$ Inoculation was done under the laminar flow cabinet using the pour plate technique. One millilitre of selected dilutions of samples (10^{-4}, 10^{-5} and 10^{-6}) was placed in sterile petri dishes and 15 ml of sterilised media added and allowed to solidify. Streptomycin (100 μg/l) was added to both media, PDA and YEPD, after sterilisation before use to prevent bacterial growth. Propionic acid supplement was also added to YEPD to inhibit the growth of moulds. Potato Dextrose Agar plates were incubated for five (5) days for moulds while YEPD was incubated for forty-eight (48) hours for yeasts at 30 °C. After incubation, the plates were observed for growth and distinct colonies were selected. Selected colonies were subcultured 2 – 4 times to obtain pure cultures which were thereafter maintained on PDA slants at 4 °C. Colonial morphology of pure isolates was directly observed and recorded.

3.1.5 Selection for cellulose and hemicellulose degrading fungi

Modified Czapek-Dox Agar was used for screening the fungal isolates for ability to degrade cellulose and hemicellulose. The medium was composed of 30g xylan or carboxymethylcellulose, 2 g $NaNO_3$, 1g KH_2PO_4, 0.5g $MgSO_4.7H_2O$ and 0.01 g $FeSO_4.7H_2O$ and 20 g Agar in 1 litre of water (Oxoid, England) and 1 ml of trace solution (containing 1 g $ZnSO_4$ and 0.5 g $CuSO_4.5H_2O$ L^{-1}).

Isolates were inoculated in media containing either xylan (hemicellulose) or carboxymethylcellulose (CMC) (cellulose) as the only carbon source and incubated at 29 ±1 °C for 48 hours after which media were flooded with Congo red dye for 15 mins.

Excess dye was removed by washing with 1M NaCl and the plates were fixed with 1N HCl. Production of extracellular enzymes and hence the ability to degrade the carbon sources was indicated by cleared zones around the colonies.

3.1.6 Selection for lignin degrading fungi

Screening of fungal isolates for lignin degrading ability was performed using PDA plates (Dhouib *et al*., 2005). A polymeric dye, 0.1 g/l Poly-R478 (Sigma) was added to potato dextrose agar. The pH was adjusted to 5.5 before autoclaving at 121 ^{o}C for 15 min. Plates were inoculated with fungal isolates and incubated at 30 ^{o}C for 25 days. The change in colour of Poly R-478 dye from purple to yellow signifies the presence of lignin-degrading fungi.

3.1.7 Selection of fungal isolates for endoglucanase, xylanase and Abeta glucosidase production in liquid cultures

Isolates were grown in small-scale liquid cultures of Czapek-Dox mineral solution supplemented with either cellulose or soluble xylan as sole carbon sources. Using a flamed and cooled cork borer (5 mm), two discs of fungal hyphae from leading edge of actively growing colonies on malt extract agar were cut and introduced into 100 ml of media in 250 ml Erlenmeyer flasks. The flasks were covered with sterile cotton wool and incubated at 29 ± 1 ^{o}C for 5 days. After incubation, the cultures were harvested by filtration through Whatman NO.1 filter paper. The culture supernatants served as crude enzyme extracts and activity determined using the following methods**:**

3.1.7.1 Endoglucanase assay

Endoglucanase assay was conducted according to the procedure of Jeffries (1996). Crude enzyme was diluted in 0.05M citrate buffer, pH 4.8. A mixture of enzyme diluted in buffer (0.1 ml) and CMC (1.9 ml) was incubated for 30min at 50 ^{o}C. Two millilitre of dinitrosalicylic acid (DNSA) was added. The tubes were placed in boiling water bath for 5 min and volume made up to 16 ml with distilled water. The tubes were cooled and the reducing sugar content determined by measuring the absorbance at 540nm using a Spectrophotometer (Lamda 25 UV/Vis Spectrophotometer) (Miller, 1959). One enzyme unit (U) equals to 1µmol of glucose released per minute

3.1.7.2 Xylanase assay

In this assay, 0.1 ml of the enzyme solution was added to 1.9 ml xylan solution. Xylan solution was prepared by dissolving Birchwood xylan (Himedia) in 50 mM sodium acetate buffer (pH 5.0) and incubated at 50 ^{o}C for 30 min. The reaction was terminated by adding 2 ml of DNSA and the contents boiled for 5 min. Distilled water was added to make the content up to 16 ml. Observation of the absorbance of the final solution was made and recorded at 540nm using a spectrophotometer (Lamda 25 UV/Vis Spectrophotometer). Activity of xylanase enzyme was measured using xylose method. One unit of xylanase activity was defined as 1 μmol of xylose equivalents released per minute.

3.1.7.3 Beta-glucosidase assay

Assay for beta-glucosidase was done according to Mahapatra *et al*. (2016). To 1 ml of the crude enzyme, 1 ml of 5 mM p-Nitrophenyl- β-D-glucopyranoside (pNPG) solution was added, which was then incubated at 45 °C for 10 min using a water bath shaker. The reaction was stopped by adding 1 ml of 2 M Na_2CO_3. The activity of b-glucosidase was estimated spectrophotometrically where absorbance was measured at 410 nm. The p-nitrophenol content of the filtrate was calculated by reference to a calibration curve plotted from results obtained with standards containing known concentrations of p-nitrophenol. Unit of enzyme activity is U/ml, where U is the amount of 1mol of p-NP (para-nitrophenol) produced per minute.

3.1.8 Selection of yeast isolates for pentose and hexose fermentation

All the isolated yeast strains were streaked on a solid medium which consisted of 1.0 g/l yeast extract and 20 g/l carbon source. The carbon sources used were xylose and glucose. Xylose served as carbon source for pentose while glucose served as carbon source for hexose respectively (Abo-State *et al*., 2013). Yeasts which grew on both carbon sources were selected for further experiments.

3.1.9 Molecular identification of selected isolates

Molecular identification of isolates was carried out at the Microbial Biotechnology Laboratory, Department of Botany, Faculty of Biology, National and Kapodistrian University of Athens, Zografou, Greece.

3.1.9.1 DNA extraction

Isolation of DNA was carried out following the protocol of Hoffman and Winston (1987). Fungal hyphae were harvested from 5 days old Malt Extract broth culture and turned into powdery form by grinding in liquid nitrogen using mortar and pestle. Cells of selected yeast isolates were inoculated into yeast extract peptone medium and kept in a rotaryshaker (ORBI SAFE orbital shaker, Sanyo Gallenkamp PLC, Loughborough, UK) at 29 ^{o}C for 24 hours. Cells from 1500μL overnight cultures were pelleted in microcentrifuge tubes using Thermo Scientific Sorvall Lynx 6000 super-speed centrifuge at 10000 rpm for 5 minutes. Harvested fungal hyphae and yeast cells were resuspended in 800μL extraction buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). (For proper lysis of yeast cells, tubes were immersed in -80 °C freezer for two minutes, then immersed in a 95 °C waterbath for 1 minute to thaw quickly. This was done twice. Tubes were vortexed vigorously for 20 minutes. Phenol (800 μl) was added and vortexed. Tubes were centrifuged for 5 minutes at 12000 rpm after which the aqueous phase was collected into Eppendorf tubes. Chloroform (800 μl) was added, vortexed and centrifuged for 5 minutes at 12000 rpm. Again, the upper aqueous layer was transferred into Eppendorf tubes (and the lower phase discarded). Equal volume of isopropanol (pH 5.3) and one tenth volume of sodium acetate were added and centrifuged for 5 minutes at 12000 rpm. At this point, the supernatant was removed and discarded while 200 μl of 70% ethanol was added to the lower phase without mixing. This was centrifuged for 2 minutes at 12000 rpm and the supernatant removed. Pellets were air-dried at 50 ^{o}C to evaporate ethanol and the resultant DNA was re-suspended in 25 – 50 μlwater.

3.1.9.2 Polymerase Chain Reaction (PCR)

The primers used to amplify the rDNA internal transcribed spacer (ITS) were NS1 (5-GTAGTCATATGCTTGTCTC-3) and NS8 (5-TCCGCAGGTTCACCTACGGA-3) as described by Ueno *et al.* (2002). The amplification reaction was done in a 50 μL volume containing 0.5 μmol of each primer, 250 ng of genomic DNA template, 200 μmol each dNTPs (dATP, dCTP, dGTP and dTTP), 10μL of 5x Phusion HF buffer (this provides 1.5 mmol $MgCl_2$ in final reaction concentration), and 0.02 U/μL of Phusion High Fidelity DNApolymerase. The thermal cycler program for each PCR reaction comprises 35 cycles with denaturation at 98 °C for 10 seconds, annealing at

52 °C for 30 seconds and extension at 72 °C for 54 seconds. An initial denaturation lasted 30 seconds at 98 °C and final extension was at 72 °C for 5 minutes. The DNA template was replaced with an equal amount of nuclease free H_2O in negative controls

3.1.9.3 Agarose Gel Electrophoresis of PCR products

Agarose gel solution (0.8% concentration) containing ethidium bromide prepared in 1% TAE buffer was used to resolved the PCR poducts. Polymerase Chain Reaction products were prepared by adding 10 µL of loading buffer (6x concentration) to 50 µL PCR product giving a final volume of 60 µL. Forty microliter (40µL) of each reaction product/loading buffer mixture was loaded in wells and run at 90 volts for 15 minutes. Separation was visualized using ultraviolet light at 100 nm wavelenth.

3.1.9.4 DNA extraction from agarose gels

Products of PCR were purified following the protocols of NucleoSpin Gel and PCR Clean-up Kit (Machery-Nagel, Germany). Deoxyribonucleic acid fragment from agarose gel was carefully excised with a clean scalpel and transferred into pre-weighed clean tubes so as to determine the weight of the gel slice. For each 100 mg of gel, 200 µL buffer NT1 was added and incubated for 10 minutes at 50 °C briefly vortexing every 3 minutes until gel slice was completely dissolved. NucleoSpin Gel and PCR Clean-up Column were placed in a 2 ml collection tube and loaded up to 700µL of dissolved sample. This was centrifuged for 30seconds at 11,000x g. The flow-through was discarded and column placed back into the collection tube. Then, 700 µL Buffer NT3 was added to the column and centrifuged for 30seconds at 11,000 x g. The flow-through was again discarded and column placed back into the collection tube.To ensure total removal of residual ethanol from Buffer NT3, the column was incubated for 3 minutes at 70 °C prior to elution. To elute the DNA, columns were placed into new 1.5 ml microcentrifuge tubes and 30µL of Buffer NE (5 mM Tris/HCl, pH 8.5) was added. This was incubated 25 °C for 1 minute and centrifuged at 11,000 x g for 1 minute. Purified DNA templates collected in microcentrifuge tubes were stored at -20 °C pending sequencing.

3.1.9.5 Sequencing of purified PCR products

Sequencing of purified amplified products of PCR was performed at Vienna Biocenter (VBC), Vienna, Austria. The sequence similarity search was done for the rDNA

sequences using online search tool called Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/). The unknown organism was identified using the maximum aligned sequence through the BLAST search. Phylogenetic and molecular evolutionary analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA) version 6 (Tamura *et al.* 2013).

3.2 Conditions required for optimal efficiency for yeast isolates

3.2.1 Nitrogen source fortification for glucose/xylose conversion to ethanol

Inocula were prepared by reactivating yeast isolates in 250 ml Erlenmeyer flasks containing 100 ml culture medium which consist of glucose (20 g/l), xylose (10 g/l), ammonium sulphate (1g Nitrogen per litre), magnesium sulphate (0.5 g/l), Calcium chloride (0.1 g/l), potassium phosphate (0.2 g/l), 1 ml/l vitamin solution (1 g/l riboflavin, 1 g/l nicotinic acid, 0.5 g/l para-aminobenzoic acid (PABA), 0.5 g/l pyridoxine, 1 g/l thiamine, 0.5 g/l pantothenic acid, 0.5 g/l biotin) and the pH was adjusted to 5.5. Incubation was done aerobically at 200 rpm at 30 $^{\circ}$C for 15 hours. Cells were harvested by centrifugation at 8000 rpm for 5minutes and re-suspended in sterile phosphate buffer (pH 5.5) which was then adjusted to an optical density (OD) of 5 at 600 nm. In the main experiment, 1 ml (8.66 x 10^{-7} cells/ml) of this was used as inoculum.

The effects of five nitrogen sources were investigated. The nitrogen sources were potassium nitrate (KNO_3), ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3) as inorganic sources while urea and yeast extract served as organic sources.

The alcoholic fermentation was done anaerobically in 50 ml fermentation tube containing 50 ml total fermentation volume. The fermentation medium was composed of 30 g/l of either glucose or xylose, magnesium sulphate (0.5 g/l), Calcium chloride (0.1 g/l), potassium phosphate (0.2 g/l), 1 ml/L vitamin solution (1 g/l riboflavin, 1 g/l nicotinic acid, 0.5 g/l PABA, 0.5 g/l pyridoxine, 1 g/l thiamine, 0.5 g/l pantothenic acid, 0.5 g/l biotin) and each nitrogen source under investigation at a concentration of 1 g Nitrogen per litre (1 g-N/L). Incubation was at 150 rpm at 30 $^{\circ}$C. Fermentation set-ups with glucose were examined after 24 hours while those of xylose were examined after 48 hours. Fermentation efficiency was calculated thus:

Fermentation Efficiency $= \dfrac{ethanol\ produced\frac{g}{l}}{reducing\ sugar\ consumed\frac{g}{l}} x \left(\dfrac{1}{0.51}\right) x\ 100,$

Where 0.51 g of ethanol is the theoretical yield from 1 g of glucose

The quantity of sugar (glucose or xylose) consumed was determined by the dinitrosalicyclic acid (DNS) method by measuring the absorbance at 540 nm using a Spectrophotometer (Lamda 25 UV/Vis Spectrophotometer) (Miller, 1959).

Ethanol produced was measured by gas chromatography (Perkin Elmer XL model) equipped with flame ionisation detector. A sample of 0.8 ml was introduced into the chromatography system. The initial oven temperature was 65 °C and it was held there for 5 minutes. The temperature increased to 150 °C at a rate of 4 °C/min and held for 5 minutes before later raised to 250 °C at a rate of 4 °C/min and held for a further 5 minutes and then set on split. The injector temperature was set at 175 °C and detector temperature set at 250 °C. Area of peaks observed after the first five minutes were recorded for each sample. In order to determine the concentration of ethanol in the unknown samples, a standard was generated using absolute ethanol as a reference solution. Diluted solutions of ethanol ranging from 0.5 g/l to 36 g/l were prepared. For each ethanol dilution, the area of peak was plotted against the ethanol concentration and the slope was determined. The slope was used to calculate concentrations of ethanol in the unknown samples.

3.2.2 Effect of pH on growth of selected yeasts

The effect of pH on the growth of three selected yeasts in a culture medium containing glucose as the carbon source was investigated. The pH was varied within a range of pH 3 to 9 with appropriate buffers. Medium sterilisation was done at 121 °C for 15 minutes before inoculation. Inoculation was done with a loopful of yeast cells in log phase harvested from a 24 hour culture on YPD agar and suspended in sterile water. Each growth flask of 250 ml capacity contained 100 ml of culture medium. Incubation was at 30 °C on an orbital shaker (ORBI SAFE orbital shaker, Sanyo Gallenkamp PLC, Loughborough, UK) at 200 rpm. Growth was observed as change in optical density at 600 nm after 48 hours incubation.

3.2.3 Effect of temperature on growth of selected yeasts

Thermotolerance assessment of isolates was performed at different temperatures ranging from 30 ^{o}C to 50 ^{o}C in yeast extract peptone dextrose (YPD) broth. Each growth flask of 250 ml capacity contained 100 ml of culture medium. The set-ups were incubated on an orbital shaker (ORBI SAFE orbital shaker, Sanyo Gallenkamp PLC, Loughborough, UK) at 200 rpm for 48 hours. Optical density at 600 nm was used to measure cell growth.

3.2.4 Effect of glucose concentration on growth of selected yeasts

Effect of glucose concentration was tested following the method of Ekunsanmi and Odunfa (1990). The medium contained yeast extract (10 g/l), Peptone (10 g/l), glucose (100, 150, 200, 250 or 300 g/l). Culture medium was sterilised at 121 ^{o}C for 15 minutes. Vitamins solution was filter-sterilised and added to the culture medium after autoclaving. Inoculation was done with a loopful of 24 hour yeast culture at a log phase harvested from YPD agar and suspended in sterile water. Erlenmeyer flasks of 200 ml volume were used for the experiment. Each Erlenmeyer flask contained 70 ml of culture medium. Incubation was at 30 ^{o}C on an orbital shaker (ORBI SAFE orbital shaker, Sanyo Gallenkamp PLC, Loughborough, UK) at 200 rpm. Growth was observed as the turbidity which was measured by spectrophometer (Lamda 25 UV/Vis Spectrophotometer) at 600 nm at every 12 hours till 60 hours.

3.2.5 Effect of ethanol concentration on growth of selected yeasts

The selected yeast strains were tested for tolerance to different concentrations of ethanol in YPD broth. Medium was sterilised by autoclaving at 121 ^{o}C for 15 minutes and left to cool. The culture medium and absolute ethanol were then dispensed into Erlenmeyer flasks with appropriate adjustment to give different concentrations of ethanol and a final volume of 70 ml in each flask. Concentrations of ethanol used were 10%, 12.5%, 15%, 17.5% and 20% (v/v). A control setup which contained only YPD broth was included in the experiment for each of the selected strains. Each experiment was done in duplicate. Change in optical density from start and end of the experiment was observed after 48 hours of incubation at 30 ^{o}C.

3.2.6   Acetic acid tolerance

Yeast extract peptone dextrose broth was prepared containing varying concentrations (0 g/l to 10 g/l) of acetic acid. The blank medium without acetic acid (0 g/l) served as the control. Each Erlenmeyer flask of 200 ml capacity contained 70 ml of the culture medium. Each flask was inoculated by a loopful of yeast cells and incubated at 30 ^{o}C for 48 hours. Growth as the optical density was measured every 12 hours using a spectrophotometer (Lamda 25 UV/Vis Spectrophotometer) at 600 nm.

3.2.7   Tolerance to furfural

Yeast extract peptone dextrose broth was prepared containing varying concentrations (0 g/l to 8 g/l) of furfural (2-furaldehyde). The blank medium without furfural (0 g/l) served as the control. Each Erlenmeyer flask of 200 ml capacity contained 70 ml of the culture medium. Each flask was inoculated by a loopful of yeast cells and incubated at 30 ^{o}C for 48 hours. Growth was measured every 12 hours as the optical density by a spectrophotometer at 600 nm.

3.3 Pretreatment experiments

3.3.1 Chemical treatment: Simultaneous effect of potassium hydroxide, time and temperature on sugarcane bagasse (SB) pretreatment

The particle size of bagasse was reduced to <1 mm using a laboratory size hammer beater mill. Prior to weight measurement, SB was dried until constant weight was achieved in an infrared dryer (KELT$^®$). Three hundred mg (300mg) of dried SB were placed in 15 ml polypropylene tubes. Two ml of KOH solution was added in order to allow for four different levels of KOH/SB ratios, namely 0.00, 0.05, 0.10 and 0.15 g/g.

For every KOH/SB ratio, the effect of three processing length of time (30, 120 and 210 mins) was examined at three different temperature regimes (51, 86 and 121 ^{o}C). This corresponded to 3 x 3 x 3 = 27 different pretreatment conditions. Each condition was done in triplicate.

3.3.1.1 Hydrolysis with commercial enzyme

At the end of incubation, each tube was neutralized with the addition of an equivalent amount of HCl solution (2 ml) and the pH was adjusted to 5 by the addition of 5 ml of

200 mM citrate-phosphate buffer (total volume of 9 ml). Two hundred μl (200 μl) of commercial cellulase/hemicellulose mixture (Novozyme) was added for hydrolysis and the tubes were incubated in a rotating chamber at 45 ^{o}C for 24 ($\pm$ 2 h).

Following hydrolysis, the samples were centrifuged (10000 x g) and the supernatant was filtered (0.45 μm) and stored at -20 ^{o}C until further analysis. Total reducing sugars concentration in the supernatant was determined through the DNS method (Miller 1959). Glucose and xylose concentration was determined through High Performance Liquid Chromatography (HPLC) (LC-10AD; Shimadzu, Kyoto, Japan) with Aminex HPX-87P column (Bio-Rad Laboratories, Hercules, CA, USA) equipped with a refractive index detector. Filtered sample (50 μl) was injected into the column and separated at a flow rate of 0.6 ml/minute for a total running time of 35 minutes at column temperature of 60 ^{o}C. The mobile phase was double-distilled water. The results were fitted in a quadratic second order polynomial model of the form:

$$y = a_0 + a_1 \cdot x_1 + a_2 \cdot x_2 + a_3 \cdot x_3 + a_{11}$$
$$\cdot x_1^2 + a_{22} \cdot x_2^2 + a_{33} \cdot x_3^3 + a_{12} \cdot x_1 \cdot x_2 + a_{13} \cdot x_1 \cdot x_3 + a_{23} \quad \cdot x_2 \cdot x_3 \quad (1)$$

where,

y, is the final hydrolysis yield expressed as reducing sugars equivalent

x_1, corresponds to the normalized duration of pretreatment in min

x_2, corresponds to the normalized temperature (^{o}C), and

x_3, corresponds to the normalized KOH load; mg KOH per g SB

α_o is the intercept, α_1, α_2, and α_3 are linear coefficients of factors x_1, x_2 and x_3 respectively and they indicate the influence of individual factors, α_{11}, α_{11}, α_{22} and α_{33} are the respective quadratic coefficients, α_{12}, α_{13} and α_{23} are the second order interaction coefficients.

The independent variable normalized space is given in Table 3.1.

Table 3.1 Experimental range and levels of independent variables in terms of coded factors

Variable	Independent variable levels		
	-1	**0**	**1**
Time x_1, min	30	120	210
Temperature x_2, ^oC	51	86	121
KOH Concentration x_3, mg/g	50	100	150

3.3.2 Biological treatment: Pretreatment and hydrolysis using enzymes from fungi

Inoculation of 2 ml spore suspension (1 x 10^6 spores/ml) of selected fungal isolate was made into two grams of chipped, ground and autoclaved raw bagasse using solid state hydrolysis. Incubation of inoculated flasks was carried out at 30 °C for 15 days. After incubation, the flask contents were harvested, filtered and then total reducing sugars was determined

3.3.3 Scanning electron microscopy (SEM) of raw and pretreated samples

In order to observe the modifications caused by pretreatment on the sugarcane bagasse fibers, scanning electron microscope (JEOL 35, Tokyo, Japan) was used (Corrales *et al.*, 2012). Bagasse samples were thinly adhered to carbon tape and sputter-coated with gold. This was viewed in the scanning electron microscope at acceleration voltage of 20KV and a working distance of 38 mm. To ascertain the reproducibility of results, several SEM images were obtained on different areas of the samples.

3.3.4 Factors affecting reducing sugar production from pretreated sugarcane bagasse using *Aspergilus niger* XY

3.3.4.1 Submerged versus solid state culture:

Sugarcane bagasse was pretreated with potassium hydroxide under the optimized operating conditions as determined by the response surface methodology and was subjected to fungal hydrolysis using solid state and sub-merged condtions. Hydrolysis was carried out in an Erlenmeyer flask with 2 g of pretreated sugarcane bagasse inoculated with 2 ml of fungal spore suspended in Czapex Dox mineral solution (1x10^6 spores/ml).

In the solid state culture, the moisture content of the sugarcane bagasse was adjusted such that the ratio of mineral solution and bagasse was 4:1. Hydrolysis was conducted under stationary conditions at 30 °C. Manual shaking of flasks was done once every day in order to maintain sample uniformity. The content of the flasks was extracted at days 5, 10, 12, 15 and 20 of hydrolysis with 100 ml of a citrate buffer (50 mM, pH 4.8). The flask was agitated at 150 rpm for 1 hour, and subsequently the content was centrifuged at 4°C at 10000 rpm for 10 min. The supernatant obtained from

centrifugation was filtered, and thereafter the reducing sugar concentration in the filtrate determined by DNS method by measuring the absorbance at 540nm using a Spectrophotometer (Lamda 25 UV/Vis Spectrophotometer) (Miller, 1959). The submerged fermentation consisted of total mineral solution of 100 ml and was incubated on a rotary shaker at 30°C and 165 rpm. Sampling was done at days 2, 5, 10, 12 15 and 20.

3.3.4.2 Effect of Initial pH on reducing sugar production in solid state culture

The effect of the initial pH of the basal medium was estimated by adjusting the pH of the Czapek-Dox mineral solution used to six different values (3.5, 4.0, 5.0, 5.5, 6.0 and 7.0). Hydrolysis was carried out in an Erlenmeyer flask containing 2 g of pretreated sugarcane bagasse inoculated with 2 ml of fungal spore suspended in Czapex Dox mineral solution ($1x10^6$ spores/ml). The buffers used included 50 mM sodium citrate buffer (pH 3.5-6.0) and 50 mM Sodium phosphate buffer (pH 6.0-7.0). Moisture content of the sugarcane bagasse was adjusted such that the ratio of mineral solution and bagasse was 4:1. Incubation was conducted under stationary conditions at 30 °C. The contents of the flasks were extracted at days 5, 10, 12, 15 and 20 of hydrolysis with 50 ml of citrate buffer (50 mM, pH 4.8). The flask was then agitated at 150 rpm for 1 hour, and subsequently the content was centrifuged at 4°C at 10000 rpm for 10 min. The supernatant obtained from centrifugation was filtered, and thereafter the reducing sugar concentration in the filtrate determined using DNS method by measuring the absorbance at 540 nm using a Spectrophotometer (Lamda 25 UV/Vis Spectrophotometer) (Miller, 1959).

3.3.4.3 Effect of Incubation Temperatures on reducing sugar production in solid state culture

The effect of incubation temperatures ranging from 30 °C, 35 °C and 40 °Cwas studied. Hydrolysis was carried out in an Erlenmeyer flask containing 2 g of pretreated sugarcane bagasse inoculated with 2 ml of fungal spore suspended in Czapex Dox mineral solution ($1x10^6$ spores/ml). The pH was maintained at 5 with 50 mM sodium citrate buffer. Moisture content of the sugarcane bagasse was adjusted such that the ratio of mineral solution and bagasse was 4:1 v/v. Incubation was conducted under stationary condition. The content of the flasks was extracted at days 5, 10, 12, 15 and 20 of hydrolysis with 50 ml of a citrate buffer (50 mM, pH 5). The flask was then

agitated at 150 rpm for 1 hour, and subsequently the contents poured in polypropylene tubes and centrifuged at 4°C at 10000 rpm for 10 min. The supernatant was filtered, and thereafter the reducing sugar concentration in the filtrate determined by DNS method by measuring the absorbance at 540nm using a Spectrophotometer (Lamda 25 UV/Vis Spectrophotometer) (Miller, 1959).

3.3.4.4 Effect of fortification of nitrogen source on reducing sugar production in solid state culture

The effect of five nitrogen sources was investigated. They were ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3) as inorganic sources while urea, peptone and yeast extract served as organic sources. Each one was used as substitute for the nitrogen source ($NaNO_3$) in the Czapek-Dox mineral solution and examined for their effects on production of reducing sugars.The pH was maintained at 5 with 50 mM Sodium citrate buffer. Moisture content of the sugarcane bagasse was adjusted such that the ratio of mineral solution and bagasse was 4:1. Incubation was done under stationary condition at 35 °C. The contents of the flasks were extracted after 13 days of fermentation with 50 ml of a citrate buffer (50 mM, pH 5). The flask was agitated at 150 rpm for 1 hour, and subsequently the content poured in polypropylene tubes and centrifuged at 4 °C at 10000 rpm for 10 min. The supernatant was filtered, and thereafter the reducing sugar concentration in the filtrate determined by DNS method by measuring the absorbance at 540 nm using a Spectrophotometer (Lamda 25 UV/Vis Spectrophotometer) (Miller, 1959).

3.3.5   Factors affecting ethanol production from sugarcane bagasse

3.3.5.1 Separate hydrolysis and fermentation (SHF)

3.3.5.1.1 Effect of monoculture and coculture of yeasts on ethanol production through separate hydrolysis and fermentation

Three isolates *Pichia kluveryomyces* Y2, *Candida tropicalis* Y5 and *Saccharomyces cerevisiae* Y10 were used as fermenting organisms both in single culture and mixed culture to determine the effect of monoculture and coculture of the yeasts on the efficacy of ethanol production. Prior to fermentation, pretreatment of 3.3 g sugarcane bagasse was done using the optimum pretreatment conditions obtained from earlier

experiments. Optimum conditions for pretreatment were 0.1628 gKOH/g bagasse at 93.3 ^{o}C for 211 minutes.

Thereafter, each tube was neutralized with the addition of an equivalent amount of HCl solution and the pH was adjusted to 5 by the addition of 200 mM citrate-phosphate buffer (total volume of 50 ml). A commercial cellulase coctail was added, and the tubes were incubated in a rotating chamber at 45 ^{o}C for 24 ($\pm$ 2 h).

Fermentation of hydrolysates of total volume of 50 ml was carried out in 150 ml Erlenmeyer flasks. The flasks were incubated in a rotator shaker at 30 °C and 200 rpm for 72 h. After incubation, ethanol produced was measured by gas chromatography.

3.3.5.1.2 Effect of addition of urea on ethanol production by *C. tropicalis* Y5 through separate hydrolysis and fermentation (SHF)

The effect of addition of urea to enzymatic hydrolysate on the level of ethanol production was determined using *C. tropicalis* Y5 based on the experiment above.

3.3.5.1.3 Effect of incubation temperature on ethanol production by *C. tropicalis* Y5 through separate hydrolysis and fermentation (SHF)

Effect of temperatures on fermentation was carried out to determine the optimum temperature for ethanol production.

3.3.5.2 Simultaneous saccharification and fermentation (SSF)

3.3.5.2.1 Effect of temperature on ethanol production from pretreated bagasse by *C. tropicalis* Y5 through simultaneous saccharification and fermentation (SSF)

Simultaneous Saccharification and Fermentation of 3.3 g pretreated sugarcane bagasse was performed in 150 ml Erlenmeyer flasks containing 50 ml of fermentation medium. Urea supplement (1 g Nitrogen per litre) was added to the medium. Saccharification of pretreated bagasse was achieved with an appropriate amount of cellulase/hemicellulose mixture. During the first 6 hours, enzymatic hydrolysis was carried out at 45°C in a rotator chamber. After 6 hours of enzymatic pre-hydrolysis, 1 ml (adjusted to optical density of 5.0 at 600 nm) of selected yeast isolate was inoculated. Fermentation was allowed to go on at 200 rpm for 96 hours but at varying incubation temperatures. The incubation temperatures used were 35 °C, 45 °C, and periodical change between 35 °C

and 45 °C. Experiments were carried out in triplicates. Fermentation with raw bagasse was also carried out as a control experiment for each set of fermentation conditions. After fermentation, ethanol produced was measured by gas chromatography.

CHAPTER FOUR

4.0   RESULTS

4.1   Chemical composition of sugarcane bagasse

The chemical composition of sugarcane bagasse used in this study was determined (Table 4.1). The total polysaccharide present in the bagasse was 60.34%. This is composed of 33.46% cellulose and 26.88% hemicellulose. Lignin content of the sugarcane bagasse was observed to be 16.58% while the organic solvent soluble fraction was 23.08%.

4.2 Isolation, selection and identification of cellulose and hemicellulose degrading fungi, and hexose and pentose fermenting yeasts.

4.2.1 Isolation

Twenty one moulds and one hundred and twenty yeasts were isolated in all from decaying sugarcane bagasse.

4.2.2 Enzyme production ability of fungal isolates

All the moulds displayed ability to produce cellulase on the cellulose agar at varying levels. Isolates SB6 and XY had highest clearance zones of 26 mm. This was followed by SB7 and SB5 with clearance zones of 23 mm and 22 mm respectively. SB16 had the lowest clearance zone of 5 mm (Table 4.2). On the xylan containing agar, isolate XY had the highest clearance zone diameter of 14 mm followed by isolates SB6 and SB10 both with clearance zones of diameter 11 mm. Isolates SB1, SB4, SB7, SB8, SB12, SB14, SB15, SB16, and SB18 showed no xylanase producing ability in xylan agar (Table 4.3). Clearance zone index was calculated for both assays in cellulose and xylan containing agar by dividing the clearance zone diameter by the colony diameter. Isolate XY had the highest clearance zone indexes of 4.33 and 2.80 on both cellulose containing agar and xylan containing agar respectively followed by isolate SB6 with

Table 4.1: Chemical constituents of sugarcane bagasse

Component	Sugarcane bagasse %
Total Polysaccharides	60.34
Cellulose	33.46
Hemicellulose	26.88
Lignin	16.58
Total Extractive fraction and other materials	23.08

Table 4.2: Isolated fungi capable of producing cellulase on cellulose Agar

ISOLATE CODE	Colony diameter (mm)	Clearance Zone diameter (mm)	Clear zone index
SB1	9.00±0.05[c]	20.00±2.50[d]	2.22±0.34[a]
SB2	10.00±0.50[c]	19.00±5.01[a]	1.90±0.17[a]
SB3	6.00±0.15[a]	9.00±2.24[b]	1.50±0.55[a]
SB4	9.00±0.02[c]	19.00±2.00[a]	2.11±0.62[a]
SB5	10.00±0.85[c]	22.00±3.00[d]	2.20±0.23[a]
SB6	7.00±0.59[b]	26.00±3.00[e]	3.71±0.30[b]
SB7	8.00±0.54[b]	23.00±1.00[d]	2.88±0.12[a]
SB8	5.00±0.23[a]	11.00±2.00[b]	2.20±0.04[a]
SB9	6.00±0.65[a]	7.00±1.20[a]	1.17±0.11[a]
SB10	6.00±0.23[a]	20.00±4.50[d]	3.33±0.09[b]
SB11	7.00±0.12[b]	11.00±2.03[b]	1.57±0.63[a]
SB12	7.00±0.35[b]	15.00±2.46[a]	2.14±0.21[a]
SB13	6.00±0.68[a]	10.00±2.34[b]	1.67±0.66[a]
SB14	6.00±0.23[a]	9.00±0.22[b]	1.50±0.12[a]
SB15	8.00±0.74[b]	9.00±0.12[b]	1.13±0.33[a]
SB16	4.00±0.14[a]	5.00±0.15[a]	1.25±0.25[a]
SB17	9.00±0.35[c]	11.00±0.55[b]	1.22±0.23[a]
SB18	7.00±0.02[b]	10.00±0.59[b]	1.43±0.70[a]
SB19	10.00±0.24[c]	15.00±2.11[c]	1.50±0.40[a]
SB20	6.00±0.54[a]	9.00±1.50[b]	1.50±0.20[a]
XY	6.00±0.52[a]	26.00±1.50[e]	4.33±0.50[b]

Data are presented in means ± standard deviation of results. Values with same superscript along the same column are not significantly different.

Table 4.3: Isolated fungi capable of producing xylanase on xylan containing Agar

ISOLATE CODE	Colony diameter (mm)	Clearance Zone diameter (mm)	Clear zone index
SB1	7.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
SB2	5.00±0.00[a]	7.00±0.40[a]	1.40±0.02[a]
SB3	4.00±0.03[a]	6.00±0.00[a]	1.50±0.00[a]
SB4	7.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
SB5	6.00±0.00[a]	7.00±0.00[a]	1.17±0.00[a]
SB6	4.00±0.01[a]	11.00±0.46[a]	2.75±0.33[a]
SB7	8.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
SB8	5.00±0.27[a]	0.00±0.00[a]	0.00±0.00[a]
SB9	3.00±0.00[a]	5.00±0.00[a]	1.67±0.55[a]
SB10	6.00±0.00[a]	11.00±0.40[a]	1.83±0.02[a]
SB11	3.00±0.33[a]	4.00±0.08[a]	1.33±0.01[a]
SB12	4.00±0.00[a]	0.00±0.00[a]	0.00±0.02[a]
SB13	3.00±0.00[a]	5.50±0.34[a]	1.83±0.01[a]
SB14	2.00±0.0[a]	0.00±0.00[a]	0.00±0.00[a]
SB15	3.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
SB16	2.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
SB17	5.00±0.00[a]	8.00±0.80[a]	1.60±0.21[a]
SB18	2.00±0.01[a]	0.00±0.00[a]	0.00±0.00[a]
SB19	4.00±0.02[a]	5.00±0.03[a]	1.25±0.01[a]
SB20	3.00±0.05[a]	5.00±0.01[a]	1.67±0.12[a]
XY	5.00±0.08[a]	14.00±0.22[a]	2.80±0.20[a]

Data are presented in means ± standard deviation of results. Values with same superscript along the same column are not significantly different.

clearance zone indexes 3.71 and 2.75 on cellulose agar and xylan agar respectively. None of the fungal isolates gave a positive result for ability to degrade lignin.

4.2.3 Quantitative assessment of enzymes

Fungal isolates with both cellulolytic and xylanolytic abilities were further subjected to a more quantitative screening for endoglucanase, beta glucosidase and xylanase in liquid cultures. Isolate XY gave the highest production of the three enzymes with 60.34 ± 0.72, 82.67 ± 0.65 and 14.29 ± 0.02 U/ml of endoglucanase, xylanase and beta-glucosidase respectively. This was followed by isolate SB6 with 43.48 ± 0.84, 74.30 ± 0.04 and 10.49 U/ml of endoglucanase, xylanase and beta glucosidase respectively (Figure 4.1). Isolate SB11 however had a beta glucosidase unit of 11.25 U/ml which is slightly higher than that of isolate SB6.

4.2.4 Growth of yeast isolates on glucose and xylose

One hundred and twenty yeast strains were isolated and screened for their level of growth on glucose and xylose carbon sources. All the isolates grew on glucose agar while only eleven grew on xylose agar. Table 4.4 shows the growth of the eleven isolates in a medium composed of 20 g/l carbon source (glucose or xylose). Isolate Y5 had the best growth on xylose while isolates Y2 and Y12 grew moderately on xylose agar. The other nine isolates showed weak growth.

4.2.5 Identification

Identification of selected isolates was done using morphological and molecular methods. After a BLAST search through the GenBank of National Center for Biotechnology Information (NCBI), it was found that the genetic sequence of the 18S rDNA of isolate XY had 100% similarity with *Aspergillus niger* strain HKS11 while isolate SB6 was also highly associated with *Aspergillus awamori* strain 07-12. Isolates Y1, Y2, Y3, Y6, Y7, Y11, and Y13 are different strains of *Pichia kudriavzevii*. Y10 was identified as *Saccharomyces cerevisiae*, while Y4, Y5 and Y12 were identified as *Candida tropicalis* (Table 4.5).
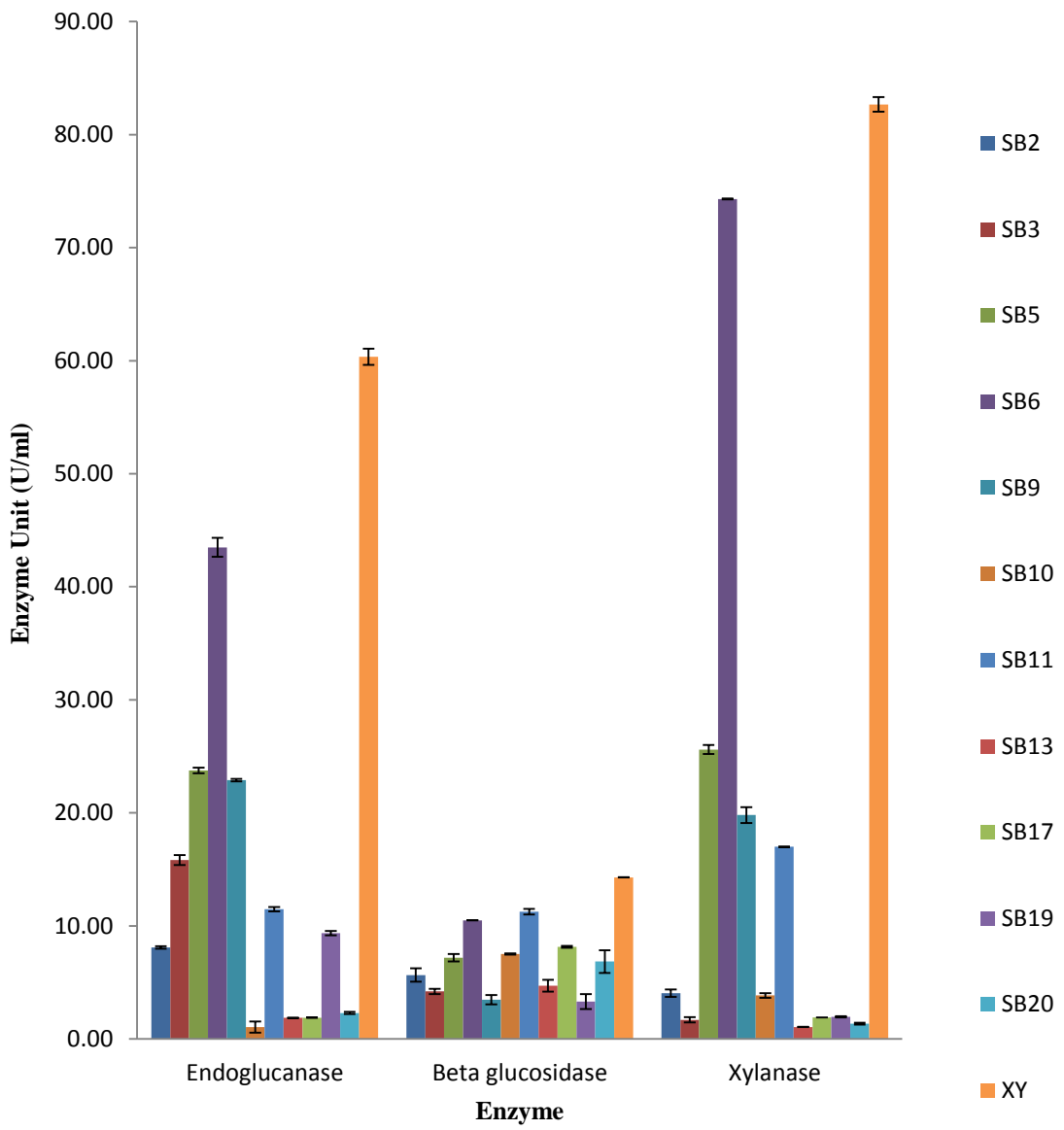
Figure 4.1: Quantitative screening of moulds isolated from decaying sugarcane bagasse for endoglucanase, beta-glucosidase and xylanase production

Table 4.4: Growth of yeast isolates on glucose and xylose Agar

Isolates	Glucose	Xylose
Y1	++++	+
Y2	++++	++
Y3	++++	+
Y4	++++	+
Y5	++++	+++
Y6	++++	+
Y7	++++	+
Y10	++++	+
Y11	++++	+
Y12	++++	++
Y13	++++	+

(-) no growth, (+) weak growth, (++) moderate growth, (+++) good growth, (++++) excellent growth

Table 4.5: Identities of selected isolates with accession numbers obtained from National Center for Biotechnology Information (NCBI)

Isolate Code	Cultural Characteristics	Identity	Accession Number
Y1	Colonies are light cream coloured and butyrous, ovoid in appearance and occur singly or in pairs.	*Pichia kudriavzevii*Y1	MG321582
Y2	White, ovoid to elongate colonies which appear singly or in pairs	*Pichia kudriavzevii*Y2	MG321583
Y3	Light cream colonies, ovoid, appear mostly in pairs though few appear singly	*Pichia kudriavzevii*Y3	MG321584
Y4	Greyish white, ovoid with shiny appearance. Colonies appear singly, in pairs and small groups	*Candida tropicalis*Y4	MG321585
Y5	oval colonies existing singly or in small groups with white colour, smooth edges and dull in appearance	*Candida tropicalis*Y5	MG321586
Y6	White, ovoid to elongate colonies which appear singly or in pairs	*Pichia kudriavzevii*Y6	MG321587
Y7	White, ovoid to elongate colonies which appear singly or in pairs. Colonies appear dry and dull	*Pichia kudriavzevii*Y7	MG321588
Y10	Colonies are flat with smooth edges, moist, creamy and glistening in appearance	*Saccharomyces cerevisiae*Y10	MG321589
Y11	White, ovoid to elongate colonies which appear singly or in pairs	*Pichia kudriavzevii*Y11	MG321590
Y12	White, ovoid with shiny appearance. Colonies appear singly, in pairs and small groups	*Candida tropicalis*Y12	MG321591
Y13	White, ovoid to elongate colonies which appear singly or in pairs	*Pichia kudriavzevii*Y13	MG321592
SB6	Black colony with cream/brown reverse	*Aspergillus awamori*SB6	MG211804
XY	Black colony with brown/cream reverse	*Aspergillus niger*XY	MG211803

4.30   Fermentative profile of yeasts
4.3.1   Nitrogen source fortification for glucose/xylose conversion to bioethanol

The effects of five nitrogen sources on conversion of glucose and xylose to bioethanol were investigated. These include potassium nitrate (KNO_3), ammonium chloride (NH_4Cl), and ammonium nitrate (NH_4NO_3) as inorganic sources while urea and yeast extract served as organic sources. None of the isolates grew nor produce any detectable amount of bioethanol with KNO_3 as the nitrogen source. Table 4.6 shows the effect of NH_4Cl on glucose and xylose conversion to bioethanol by the eleven yeast isolates. All the isolates nearly consumed all glucose present in the medium with very high sugar to ethanol conversion rates. *Pichia kudriavzevii* Y2 had the highest fermentation efficiency of 100% while *P. kudriavzevii* Y11 had the lowest fermentation efficiency of 97.72%. Consumption of xylose was highest in *C. tropicalis* Y12 (28.89 g/l), *C. tropicalis* Y4 (27.55 g/l), and *C. tropicalis* Y5 (23.06 g/l). However, xylose sugar to ethanol conversion was highest in isolate Y5 with fermentation efficiency of 34.40%. *Pichia kudriavzevii* Y1, *P. kudriavzevii* Y2, *P. kudriavzevii* Y3, *P. kudriavzevii* Y6, *P. kudriavzevii* Y11, and *P. kudriavzevii* Y13 did not produce any detectable bioethanol in the presence of NH_4Cl in this experiment.

Table 4.7 shows the effect of NH_4NO_3 on glucose and xylose conversion to bioethanol. Consumption of glucose by the eleven isolates was also high. *Candida tropicalis* Y12 had the highest production of ethanol of 14.47±0.30 g/l with fermentation efficiency of 99.48±3.00% followed by *S. cerevisiae* Y10 which produced ethanol of 14.43±0.02 g/l with fermentation efficiency of 96.16±2.00%. In the presence of NH_4NO_3 as nitrogen source, none of the isolates gave any measurable amount ethanol when xylose was used as the carbon source.

The effect of urea on glucose and xylose conversion to bioethanol is presented in Table 4.8. *Saccharomyces cerevisiae* Y10 and *P. kudriavzevii* Y2 had highest production of ethanol of 14.50±0.30 g/l (99.31±5.20% fermentation efficiency) and 14.23±1.20 g/l (98.54±2.00% fermentation efficiency) respectively. However, *C. tropicalis* Y5 with an ethanol yield of 13.61±0.00 g/l had the highest fermentation efficiency of 100.14±5.40%. *Candida tropicalis* Y5 in the presence of urea also converted xylose to produce 4.83±0.50 g/l of bioethanol as well as fermentation efficiency calculated to give 41.16±5.67% which is the highest yield from xylose sugar.

Table 4.6: Effect of NH$_4$Cl on bioconversion of glucose and xylose to bioethanol by yeasts isolated from decaying sugarcane bagasse

	GLUCOSE				XYLOSE			
Isolate code*	Total glucose Consumed (g/l)	Ethanol Yield (g/l)	Ethanol yield (g/g glucose)	Fermentation efficiency (%)	Total xylose Consumed (g/l)	EthanolYield (g/l)	Ethanol yield (g/g xylose)	Fermentation efficiency (%)
Y1	28.65±0.32^a	14.54±0.00^a	0.51±0.03^a	99.46±0.16^b	5.19±0.00^a	0.00±0.00^a	0.00±0.00^a	0.00±0.00^a
Y2	28.45±0.02^a	14.74±0.00^a	0.52±0.01^a	101.60±0.00^c	9.08±2.70^b	0.00±0.00^a	0.00±0.00^a	0.00±0.00^a
Y3	29.01±0.50^a	14.55±0.36^a	0.50±0.76^a	98.31±0.00^a	4.94±0.50^a	0.00±0.00^a	0.00±0.00^a	0.00±0.00^a
Y4	29.18±0.20^a	14.87±0.00^a	0.51±0.50^a	99.93±0.22^b	27.55±3.00^f	0.54±0.23^a	0.02±0.00^a	3.84±0.20^b
Y5	29.54±0.30^a	15.04±0.22^a	0.51±0.65^a	99.83±0.00^b	23.06±3.30^e	4.05±0.39^c	0.18±0.01^a	34.40±0.70^e
Y6	28.97±0.04^a	14.72±0.00^a	0.51±0.90^a	99.63±0.66^b	13.99±1.00^c	0.00±0.00^a	0.00±0.00^a	0.00±0.00^a
Y7	29.25±0.02^a	14.88±0.00^a	0.51±0.65^a	99.73±0.80^b	18.52±0.02^d	2.02±0.24^b	0.09±0.03^a	17.20±0.80^d
Y10	29.11±0.23^a	14.80±0.13^a	0.51±0.55^a	99.68±0.00^b	16.25±0.00^d	1.01±0.00^a	0.04±0.02^a	8.60±0.20^c
Y11	28.60±0.00^a	14.25±0.00^a	0.50±0.21^a	97.72±0.43^a	9.96±0.11^b	0.00±0.00^a	0.00±0.00^a	0.00±0.00^a
Y12	29.41±0.12^a	14.75±0.11^a	0.50±0.25^a	98.31±0.21^a	28.89±2.00^f	0.13±0.00^a	0.00±0.00^a	0.85±0.09^a
Y13	28.30±0.00^a	14.17±0.08^a	0.50±0.77^a	98.16±0.00^a	11.42±2.00^a	0.00±0.00^a	0.00±0.00^a	0.00±0.00^a

Data are presented in means ± standard deviation of results. Values with same superscript along the same column are not significantly different.

*Y1, Y2, Y3, Y6, Y7, Y11, Y13 (*Pichia kudriavzevii*), Y4, Y5, Y12 (*Candida tropicalis*), Y10 (*Saccharomyces cerevisiae*)

Table 4.7: Effect of NH_4NO_3 on bioconversion of glucose and xylose to bioethanol by yeasts isolated from decaying sugarcane bagasse

Isolate code*	GLUCOSE				XYLOSE			
	Total glucose Consumed (g/l)	Ethanol Yield (g/l)	Ethanol yield (g/g glucose)	Fermentation efficiency (%)	Total xylose Consumed (g/l)	Ethanol Yield (g/l)	Ethanol yield (g/g xylose)	Fermentation efficiency (%)
Y1	28.64±2.00^a	12.63±0.23^b	0.44±0.00^a	86.47±4.00^f	2.44±0.20^b	0.00±0.00*	0.00±0.00*	0.00±0.00*
Y2	29.81±1.00^a	12.32±0.68^b	0.41±0.00^a	81.04±2.00^d	2.14±0.04^b	0.00±0.00*	0.00±0.00*	0.00±0.00*
Y3	29.31±4.00^a	10.82±0.12^a	0.37±0.00^a	72.50±5.00^a	3.33±0.21^b	0.00±0.00*	0.00±0.00*	0.00±0.00*
Y4	29.56±1.00^a	11.57±0.24^a	0.39±0.02^a	76.77±4.00^c	2.73±0.10^b	0.00±0.00*	0.00±0.00*	0.00±0.00*
Y5	29.43±2.00^a	11.20±1.20^a	0.38±0.03^a	74.64±6.00^b	3.03±0.03^b	0.00±0.00*	0.00±0.00*	0.00±0.00*
Y6	29.49±2.00^a	11.38±0.70^a	0.39±0.00^a	75.70±7.00^b	2.88±0.30^b	0.00±0.00*	0.00±0.00*	0.00±0.00*
Y7	28.78±0.90^a	12.70±0.65^b	0.44±0.00^a	86.46±3.00^f	2.53±0.01^b	0.00±0.00*	0.00±0.00*	0.00±0.00*
Y10	29.42±1.00^a	14.43±0.02^c	0.49±0.02^a	96.16±2.00^g	0.00±0.00^a	0.00±0.00*	0.00±0.00*	0.00±0.00*
Y11	28.85±2.00^a	12.99±0.44^b	0.45±0.02^a	88.24±1.00^f	3.71±0.05^b	0.00±0.00*	0.00±0.00*	0.00±0.00*
Y12	28.52±1.00^a	14.47±0.30^c	0.51±0.20^a	99.48±3.00^g	8.90±0.70^d	0.00±0.00*	0.00±0.00*	0.00±0.00*
Y13	28.60±8.50^a	10.37±0.55^a	0.36±0.00^a	71.09±7.00^a	5.80±0.02^c	0.00±0.00*	0.00±0.00*	0.00±0.00*

Data are presented in means ± standard deviation of results. Values with same superscript along the same column are not significantly different.

*Y1, Y2, Y3, Y6, Y7, Y11, Y13 (*Pichia kudriavzevii*), Y4, Y5, Y12 (*Candida tropicalis*), Y10 (*Saccharomyces cerevisiae*)

Table 4.8: Effect of urea on bioconversion of glucose and xylose to bioethanol by yeasts isolated from decaying sugarcane bagasse

Isolate code*	GLUCOSE				XYLOSE			
	Total glucose Consumed (g/l)	Ethanol Yield (g/l)	Ethanol yield (g/g glucose)	Fermentation efficiency (%)	Total xylose Consumed (g/l)	Ethanol Yield (g/l)	Ethanol yield (g/g xylose)	Fermentation efficiency (%)
Y1	27.98 ± 0.90^b	14.20 ± 3.20^a	0.51 ± 0.02^a	99.52 ± 3.00^b	10.86 ± 0.00^e	0.04 ± 0.00^a	0.00 ± 0.00^a	0.70 ± 0.00^a
Y2	28.32 ± 2.00^c	14.23 ± 1.20^b	0.50 ± 0.10^a	98.54 ± 2.00^b	5.33 ± 0.20^d	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
Y3	25.78 ± 3.00^a	13.08 ± 2.00^a	0.51 ± 0.04^a	99.53 ± 1.30^b	2.50 ± 0.03^b	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
Y4	26.98 ± 0.50^b	13.58 ± 0.00^a	0.50 ± 0.05^a	98.71 ± 0.20^b	6.66 ± 0.04^d	0.08 ± 0.01^a	0.01 ± 0.00^a	2.19 ± 0.40^b
Y5	26.64 ± 0.50^b	13.61 ± 0.00^a	0.51 ± 0.11^a	100.14 ± 5.40^c	22.99 ± 3.00^g	4.83 ± 0.50^b	0.21 ± 0.01^a	41.16 ± 5.67^c
Y6	25.58 ± 1.00^a	12.48 ± 2.00^a	0.49 ± 0.43^a	95.63 ± 2.00^a	4.99 ± 0.50^c	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
Y7	24.95 ± 2.00^a	12.08 ± 2.04^a	0.49 ± 0.21^a	95.33 ± 3.00^a	2.13 ± 0.02^b	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
Y10	28.62 ± 0.90^c	14.50 ± 0.30^b	0.51 ± 0.13^a	99.31 ± 5.20^b	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
Y11	26.49 ± 0.30^b	12.79 ± 3.00^a	0.48 ± 0.11^a	94.58 ± 1.30^a	1.43 ± 0.30^a	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
Y12	26.81 ± 2.00^b	13.11 ± 1.00^a	0.49 ± 0.01^a	95.88 ± 1.70^a	13.01 ± 1.40^f	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
Y13	26.41 ± 1.00^b	13.48 ± 2.00^a	0.51 ± 0.03^a	100.09 ± 2.40^c	4.81 ± 0.50^c	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a

Data are presented in means ± standard deviation of results. Values with same superscript along the same column are not significantly different.

*Y1, Y2, Y3, Y6, Y7, Y11, Y13 (*Pichia kudriavzevii*), Y4, Y5, Y12 (*Candida tropicalis*), Y10 (*Saccharomyces cerevisiae*)

Other isolates showed some level of xylose consumption too but they did not synthesize any significant amount of ethanol.

Table 4.9 shows the effect of yeast extract on glucose and xylose conversion to bioethanol. All the isolates completely consumed the glucose in the medium but *P. kudriavzevii* Y2 gave the highest ethanol yield of 15.32±0.34 g/l with fermentation efficiency of 100.10±4.50%. Xylose consumption was more pronounced among many of the isolates when yeast extract was employed as the source of nitrogen but there was not a significant yield of ethanol.

4.3.2   pH effect on growth of selected yeast isolates

Three isolates (*C. tropicalis* Y5, *P. kudriavzevii* Y2 and *S. cerevisiae* Y10) were selected for further experiments. *C. tropicalis* Y5 was selected due to his confirmed ability to ferment xylose, which is a pentose sugar, to ethanol in the presence of urea. *P. kudriavzevii* Y2 was efficient in glucose conversion to ethanol. *Saccharomyces cerevisiae* Y10 was also efficient in glucose conversion to ethanol. In order to investigate the effect of pH on the growth of these three selected isolates, culture media, using appropriate buffers were set within the pH range of 3 to 9.

Growth of the three isolates was favourable under acidic (lower pH) conditions (Figure 4.2). *P. kudriavzevii* Y2 was more tolerant to the lowest pH than *C. tropicalis* Y5 and *S. cerevisiae* Y10 in this experiment. It grew fast up to optimum at pH 3 and was stable within pH 3 and 5. There was significant growth between pH 5 and 6 despite the observed decline in growth rate. A sharp and steady decline in growth was observed after pH 6. *Candida tropicalis* Y5 also was able to grow well within pH 3 and pH 6 with optimum within a narrow range of pH 5 to 6. There was a drastic decline in growth immediately after pH 6. *Saccharomyces cerevisiae* Y10 grew with constant rate from pH 3 up to pH 4 where its growth was optimum. A steady decline in growth was thereafter observed.

4.3.3   Temperature effect on growth of selected yeast isolates

Figure 4.3 shows the effect of temperature on growth of three selected yeasts. The isolates were observed to tolerate temperatures up to 45 ^{o}C with *C. tropicalis* Y5 having a better growth rate at that temperature. Differences in growth of *P. kudriavzevii* Y2 and *S. cerevisiae* Y10 was not significant at 45 ^{o}C.

Table 4.9: Effect of yeast extract on bioconversion of glucose and xylose to bioethanol by yeasts isolated from decaying sugarcane bagasse

	GLUCOSE				XYLOSE			
	Total glucose Consumed (g/l)	Ethanol Yield (g/l)	Ethanol yield (g/g glucose)	Fermentation efficiency (%)	Total xylose Consumed (g/l)	Ethanol Yield (g/l)	Ethanol yield (g/g xylose)	Fermentation efficiency (%)
Y1	$30.00\pm0.30^{*}$	13.77 ± 0.50^{b}	0.46 ± 0.33^{a}	89.97 ± 1.43^{d}	8.96 ± 2.00^{c}	0.04 ± 0.04^{a}	0.00 ± 0.00^{a}	0.88 ± 0.02^{a}
Y2	$30.00\pm0.23^{*}$	15.32 ± 0.34^{c}	0.51 ± 0.02^{a}	100.10 ± 4.50^{e}	13.00 ± 3.00^{d}	0.07 ± 0.02^{a}	0.01 ± 0.01^{a}	0.99 ± 0.11^{a}
Y3	$30.00\pm0.14^{*}$	14.20 ± 0.55^{b}	0.47 ± 0.05^{a}	92.81 ± 2.00^{a}	6.90 ± 1.00^{b}	0.03 ± 0.05^{a}	0.00 ± 0.00^{a}	0.71 ± 0.01^{a}
Y4	$30.00\pm0.44^{*}$	11.95 ± 1.22^{a}	0.40 ± 0.02^{a}	78.10 ± 1.00^{a}	16.20 ± 4.00^{e}	0.11 ± 0.02^{a}	0.01 ± 0.00^{a}	1.34 ± 0.03^{a}
Y5	$30.00\pm0.55^{*}$	12.49 ± 0.22^{a}	0.42 ± 0.10^{a}	81.60 ± 5.00^{b}	15.36 ± 0.55^{e}	0.08 ± 0.34^{a}	0.01 ± 0.00^{a}	1.02 ± 0.01^{a}
Y6	$30.00\pm0.61^{*}$	13.23 ± 2.00^{b}	0.44 ± 0.03^{a}	86.44 ± 2.00^{d}	6.23 ± 0.46^{b}	0.03 ± 0.12^{a}	0.00 ± 0.00^{a}	0.80 ± 0.02^{a}
Y7	$30.00\pm0.11^{*}$	13.30 ± 1.30^{b}	0.44 ± 0.11^{a}	86.93 ± 1.00^{d}	15.71 ± 2.00^{e}	0.05 ± 0.01^{a}	0.00 ± 0.00^{a}	0.62 ± 0.02^{a}
Y10	$30.00\pm2.00^{*}$	12.87 ± 0.20^{a}	0.43 ± 0.01^{a}	84.12 ± 4.60^{c}	0.22 ± 0.01^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Y11	$30.00\pm1.00^{*}$	13.14 ± 3.00^{b}	0.44 ± 0.03^{a}	85.88 ± 1.70^{c}	16.10 ± 0.32^{e}	0.08 ± 0.01^{a}	0.00 ± 0.00^{a}	0.92 ± 0.05^{a}
Y12	$30.00\pm0.70^{*}$	13.55 ± 1.01^{b}	0.45 ± 0.00^{a}	88.53 ± 4.23^{a}	18.96 ± 0.40^{f}	0.07 ± 0.00^{a}	0.00 ± 0.00^{a}	0.72 ± 0.06^{a}
Y13	$30.00\pm0.40^{*}$	12.10 ± 2.04^{a}	0.40 ± 0.04^{a}	79.08 ± 1.55^{a}	19.03 ± 0.12^{f}	0.07 ± 0.21^{a}	0.00 ± 0.00^{a}	0.67 ± 0.02^{a}

Data are presented in means ± standard deviation of results. Values with same superscript along the same column are not significantly different. The asterisks "*" indicates that there is no observable difference in the measured variable hence no significance was observable.
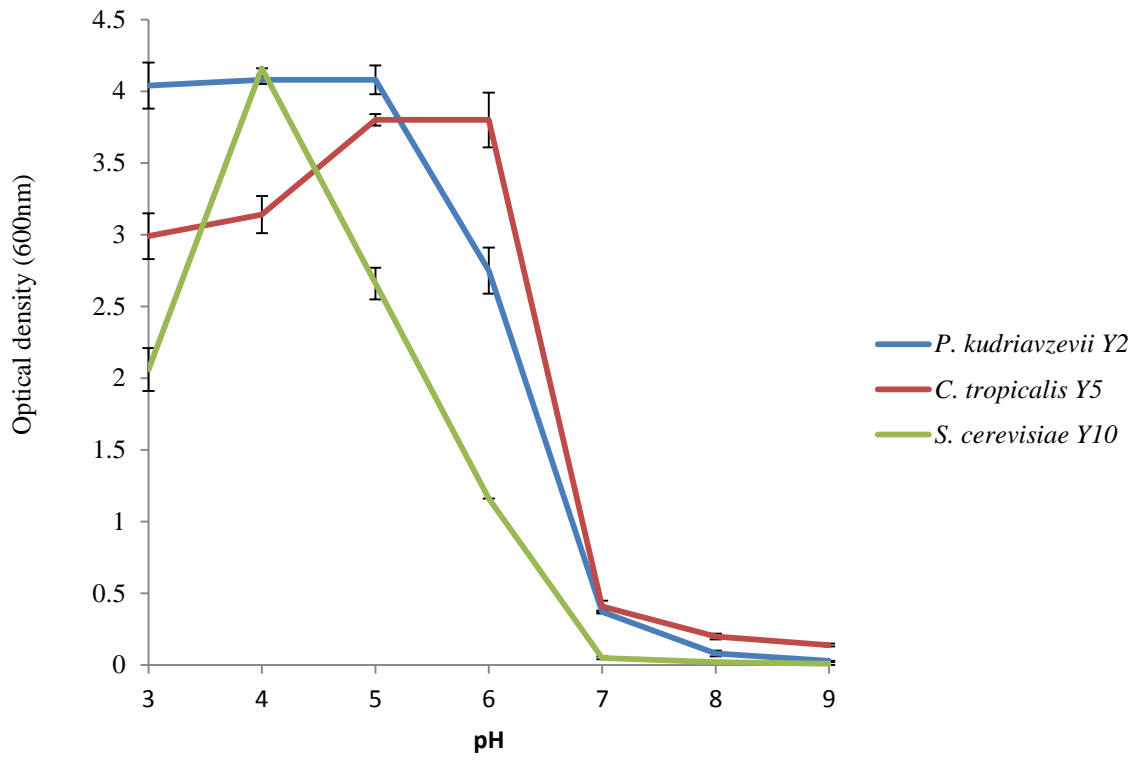
Figure 4.2: Effect of pH on the growth of selected yeasts isolated from decaying sugarcane bagasse in glucose medium
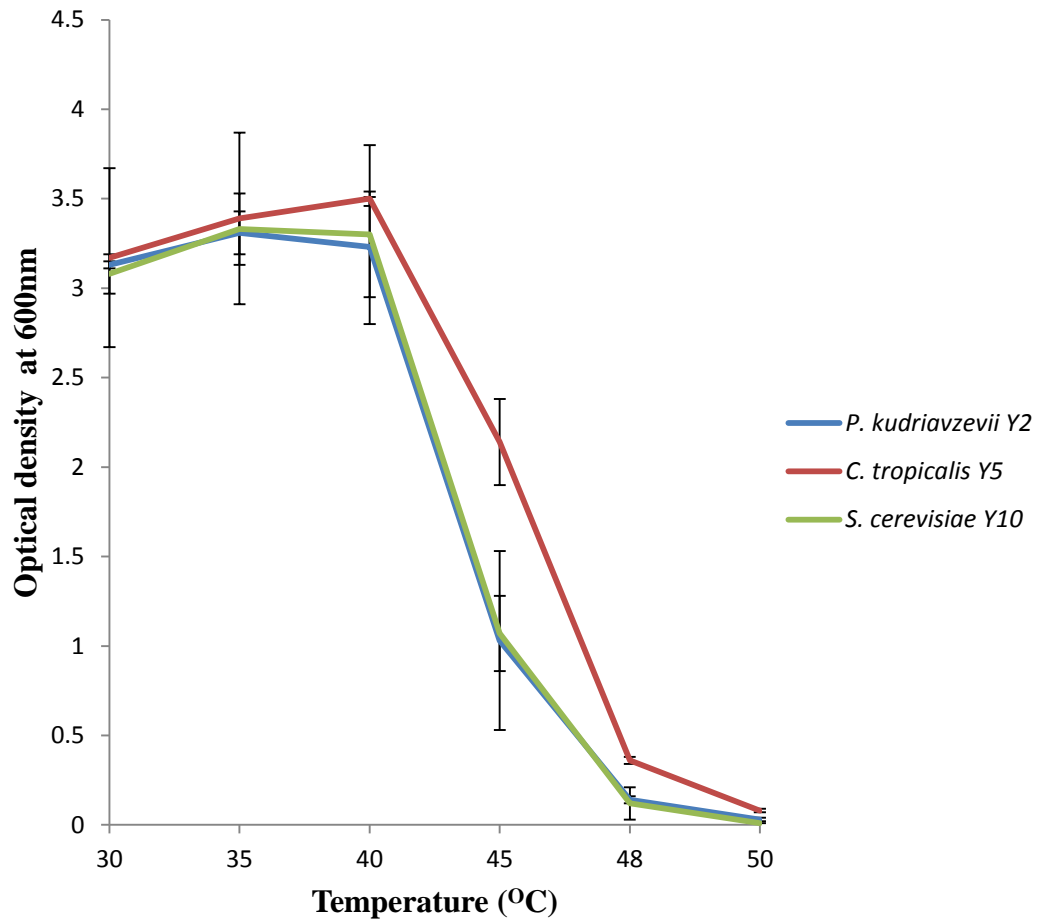
Figure 4.3: Effect of temperature on the growth of selected yeasts isolated from decaying sugarcane bagasse in glucose medium

4.3.4 Concentrations effect of glucose on growth of selected yeasts

All the three isolates were able to grow well at all the sugar concentrations without any observable delay in growth or lag phase as from the twelfth hour of incubation. *Pichia kudriavzevii* Y2 in all glucose concentrations from 100 g/l to 300 g/l had a similar growth pattern up to the 48th hour of growth (Figure 4.4). Differences in growth rate were not pronounced in 100, 150 and 200 g/l glucose concentrations but clearly obvious between the trio and the next two higher concentrations (250 and 300 g/l). At 100, 150 and 200 g/l, a peak was reached and there was a decline in growth after the 48 hours of incubation while growth did not totally cease throughout the period of the 60 hours experiment at 250 and 300 g/l glucose concentrations. There was no well-defined and direct relationship between the sugar concentration and growth rate in *C. tropicalis* Y5 (Figure 4.5). At 100 and 150 g/l glucose concentrations, there was no significant difference in growth rate. An unusual behaviour was observed when at 36 hours of incubation, growth at 250 g/l glucose was higher than growth at other concentrations thereby distorting the hitherto observed direct relationship between sugar concentration and growth rate. However, growth at 300 g/l remained lowest. Figure 4.6 shows the growth pattern of *S. cerevisiae* Y10 at different sugar concentrations. Here, growth of the yeast isolate did not follow a specific pattern among different concentrations of sugar but it was obvious that the isolate could tolerate well all the concentrations of sugar used.

4.3.5 Effect of ethanol concentration on growth of selected yeasts

The three selected yeasts were grown at 0 to 20% ethanol concentrations. A similar trend was observed among the isolates across the different concentrations as there was a decrease in growth rate as ethanol concentration increases (Figure 4.7). At 0, 10 and 12.5% ethanol concentration, very high growth was recorded for the three isolates. Between 12.5 and 15% concentrations, there was a decrease in growth by 66, 49 and 55% for *P. kudriavzevii* Y2, *C. tropicalis* Y5 and *S. cerevisiae* Y10 respectively. Thereafter, there was a rapid decline in growth of the three isolates at 17.5% ethanol till 20% where there was no significant growth of any of the isolates.
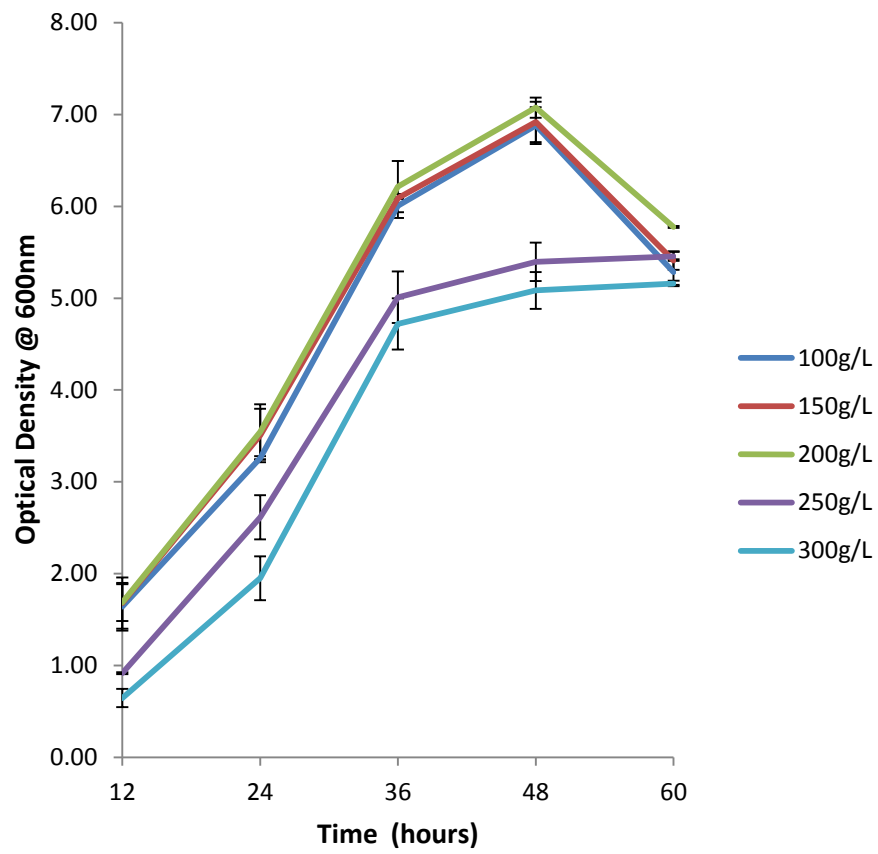
Figure 4.4: Effect of different glucose concentrations on growth of *P. kudriavzevii* Y2 at different time intervals
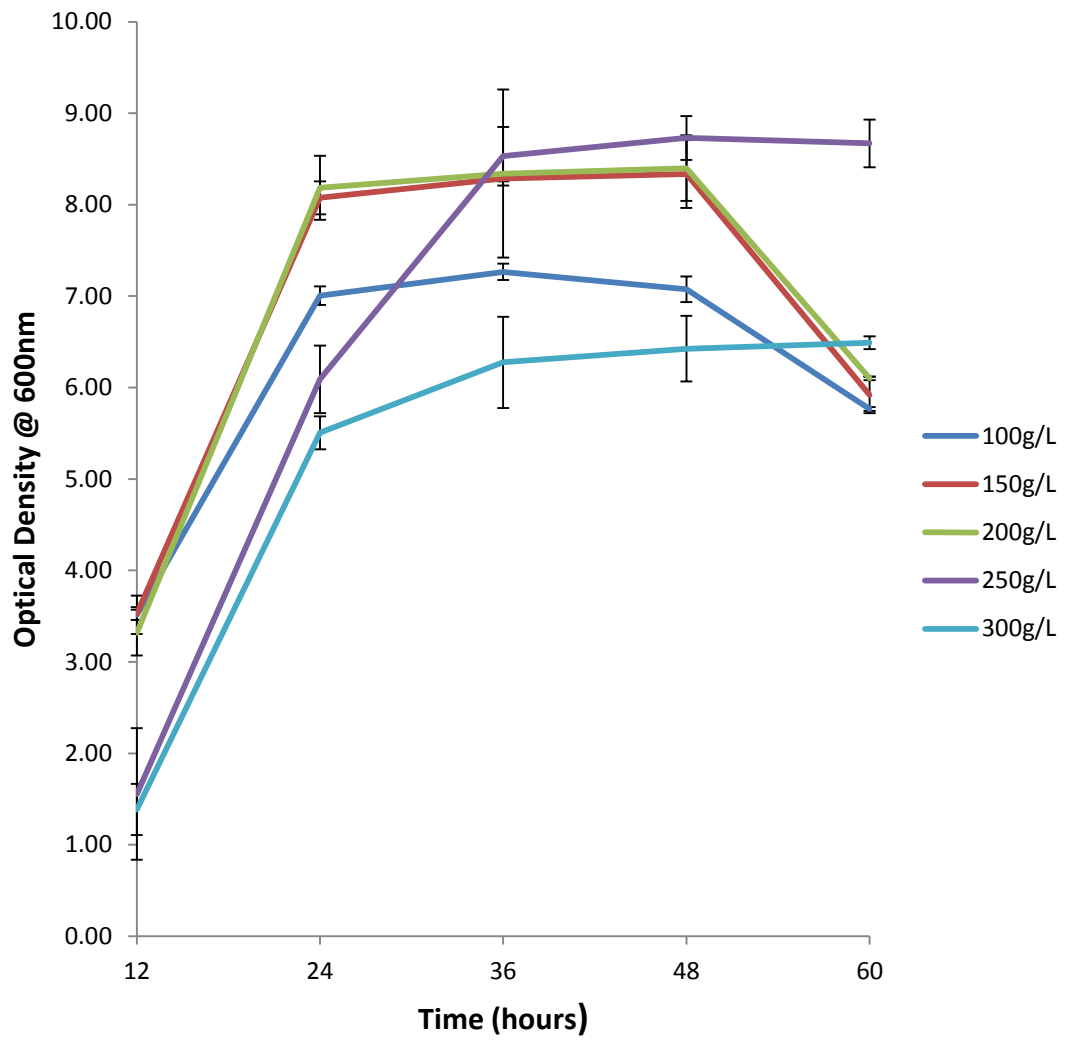
Figure 4.5: Effect of different glucose concentrations on growth of *C. tropicalis* Y5 at different time intervals
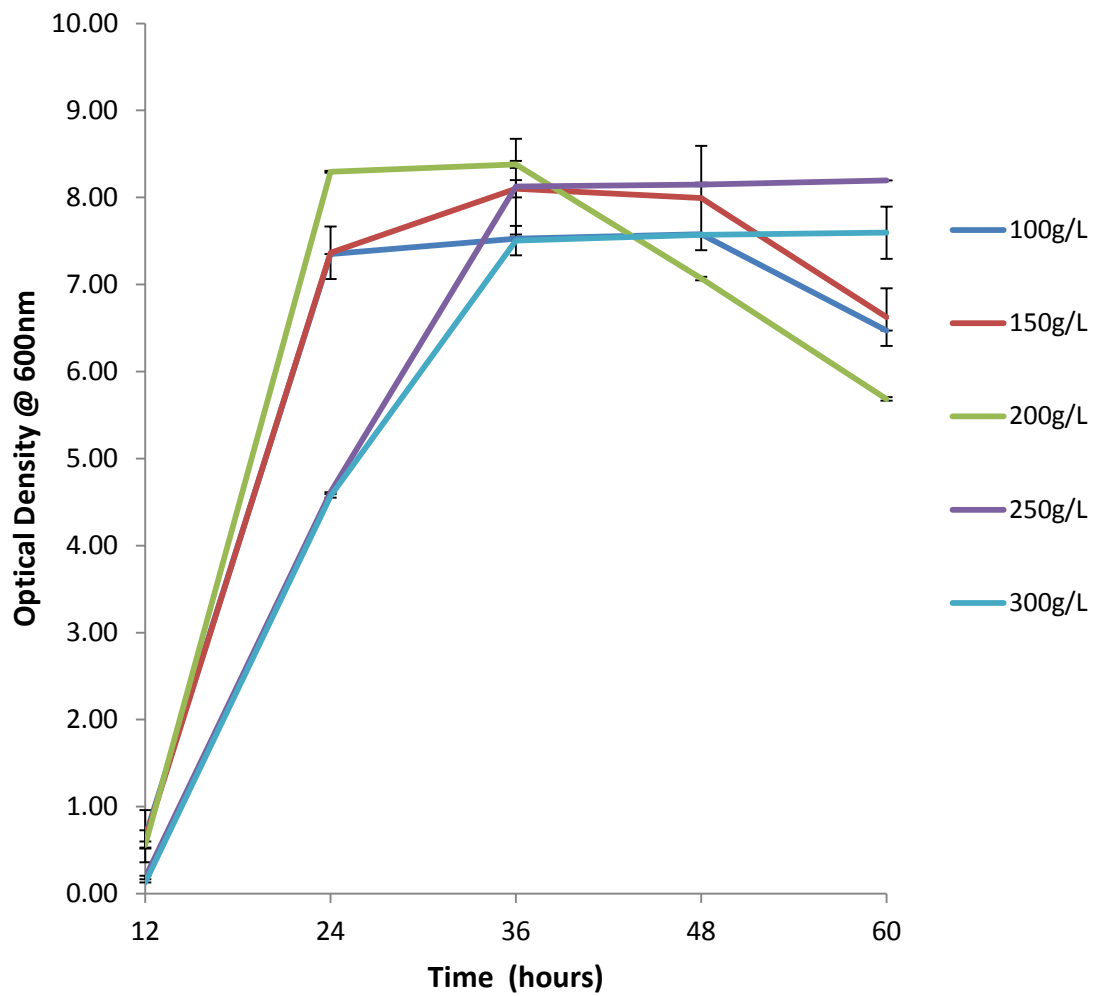
Figure 4.6: Effect of different glucose concentrations on growth of *S. cerevisiae* Y10 at different time intervals
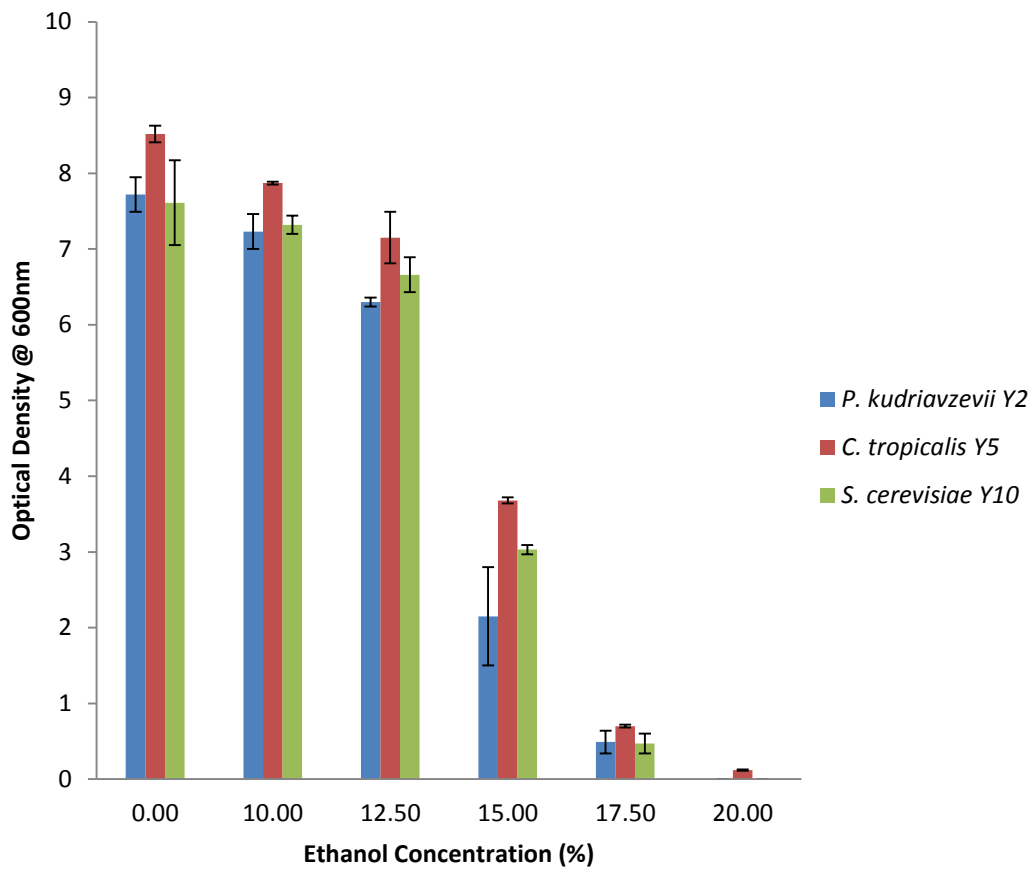
Figure 4.7: Effect of ethanol concentration on growth of selected yeasts isolated from decaying sugarcane bagasse

4.3.6 Effect of acetic acid concentrations on growth of selected yeasts

Isolates were subjected to different concentrations of acetic acid (2, 4, 6, 7, 8 and 10 g/l) in their growth medium and growth was measured as optical density at 600 nm every 12 hours up till 48 hours. Observations were compared with that of control in medium which contained no acetic acid. Percentage reduction in growth was calculated and plotted against the incubation time. Figures 4.8, 4.9 and 4.10 showed the effect of acetic acid on *P. kudriavzevii* Y2, *C. tropicalis* Y5 and *S. cerevisiae* Y10 respectively. At 2 g/l concentration of acetic acid, the isolates were inhibited at different rates in the first 12 hours of growth. *Pichia kudriavzevii* Y2 had the least inhibition of 12% while *C. tropicalis* Y5 and *S. cerevisiae* Y10 were inhibited by 35 and 30% respectively. However the isolates became more adapted to the inhibitor with time with *P. kudriavzevii* Y2 having almost a total tolerance by the 24th hour and *C. tropicalis* Y5 at the 36th hour but *S. cerevisiae* Y10 still displayed about 15% reduction in growth at the end of the experiment. All three isolates were able to get adapted to acetic acid inhibition over time up to 6 g/l concentration. Only *C. tropicalis* Y5 was able to display an ability to adapt to 7 g/l acetic acid concentration as percentage inhibition gradually reduced from 87% down to 66% at the end of the incubation time. At 8 and 10 g/l acetic acid concentrations, the three isolates suffered near 100% inhibition of growth.

4.3.7 Effect of furfural (2-furaldehyde) concentrations on growth of selected yeasts

Concentrations of furfural used in this experiments were 2, 3, 4, 6 and 8 g/l. Growth medium with 0 g/l of furfural was used as a control on which the comparison to assess the level of inhibition caused by different concentrations of furfural on the yeast isolates was based. Observations were also made every 12 hours and percentage inhibition was plotted against time. The isolates showed adaption in furfural up to 3g/l furfural concentration (Figures 4.11, 4.12 and 4.13). Inhibition was usually more pronounced at the first 12 hours; after which the isolates started displaying their ability to adapt to the presence of the inhibitor. The isolate *C. tropicalis* Y5 gave the highest adaptation at 2 g/l furfural concentration as percentage reduction falls from 61% at the twelfth hour to 9% at the twenty-fourth hour down to 2% at the forty-eighth hour (Figure 4.12). From 4g/l to 8g/l furfural concentrations, percentage reduction in growth was tending towards 100% except in *C. tropicalis* Y5 which displayed a very low
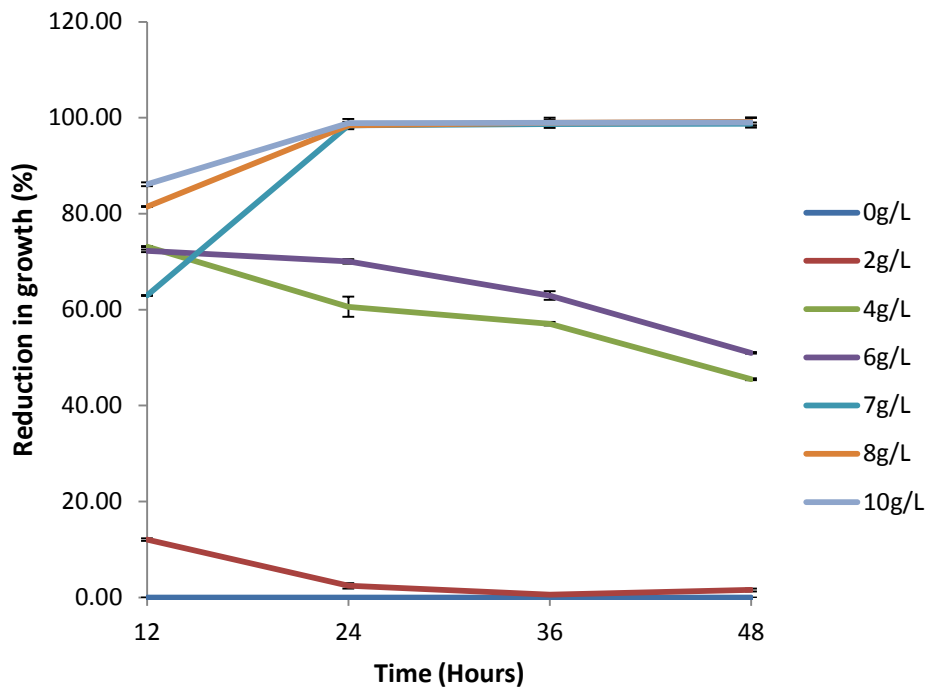
Figure 4.8: Effect of different concentrations of acetic acid on growth of *P. kudriavzevii* Y2 at different times.
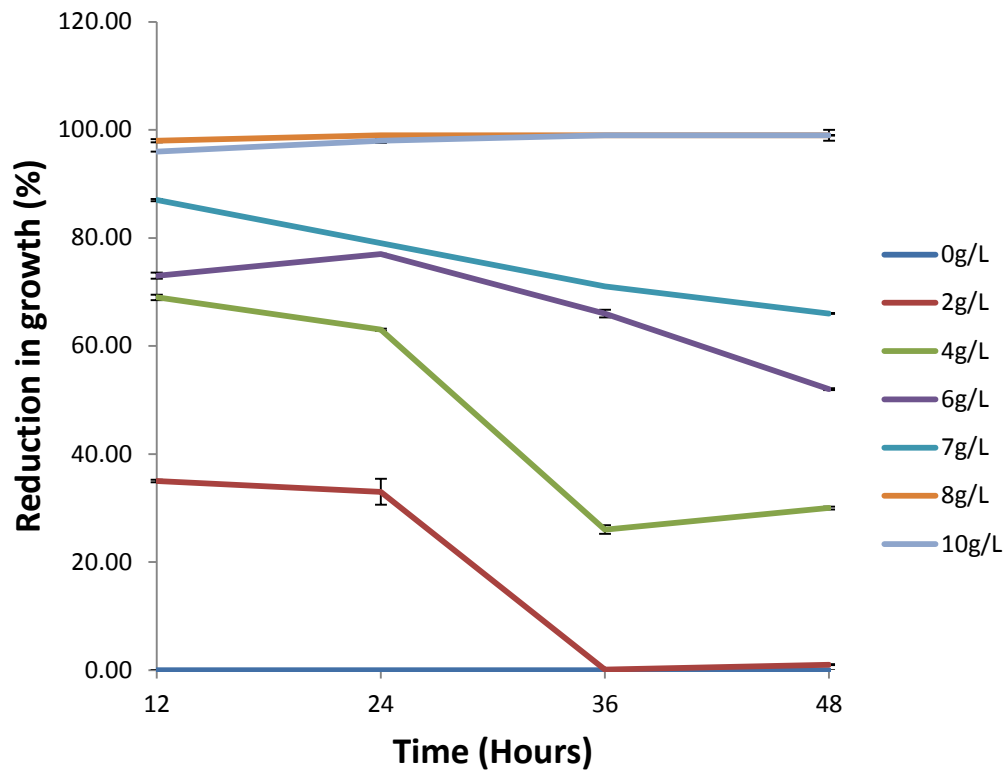
Figure 4.9: Effect of different concentrations of acetic acid on growth of *C. tropicalis* Y5 at different time intervals
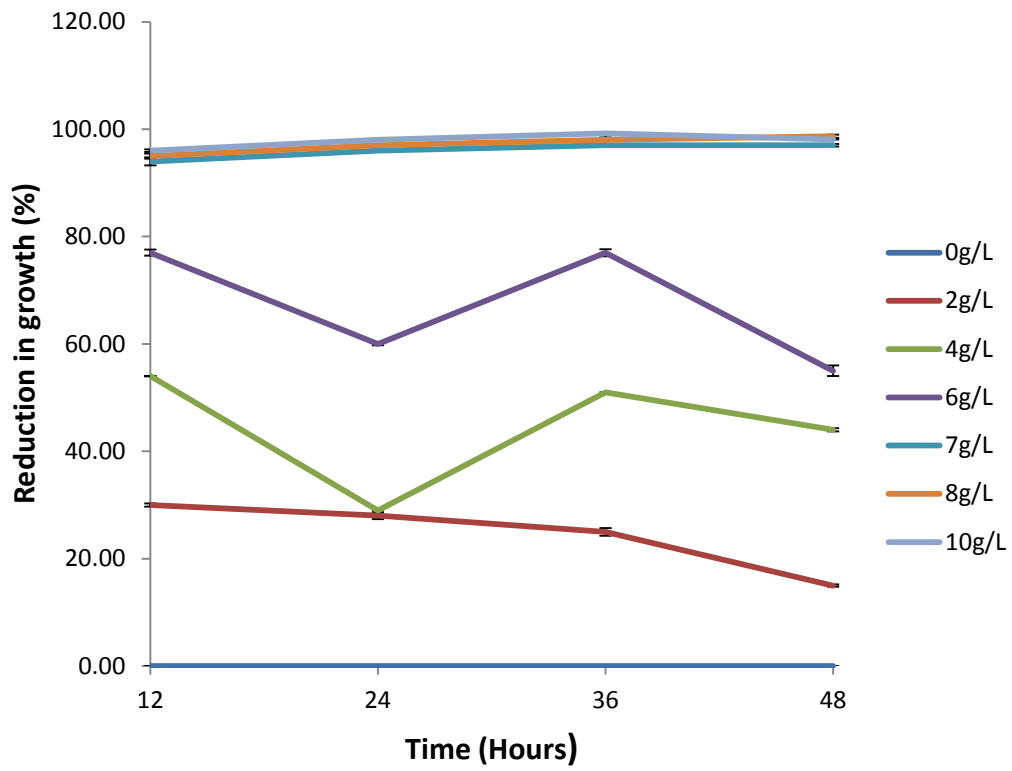
Figure 4.10: Effect of varying concentrations of acetic acid on growth of *S. cerevisiae* Y10 at different time intervals
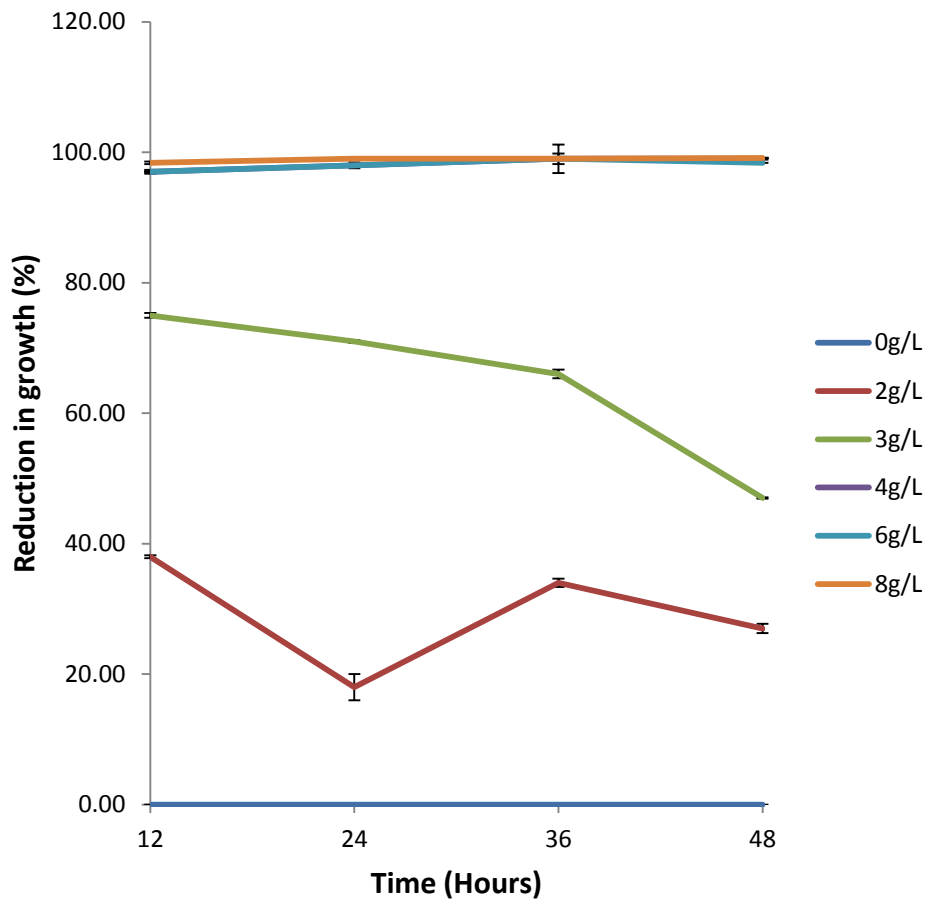
Figure 4.11: Effect of varying concentrations of furfural (2-furaldehyde) on growth of
P. kudriavzevii Y2 at different time intervals
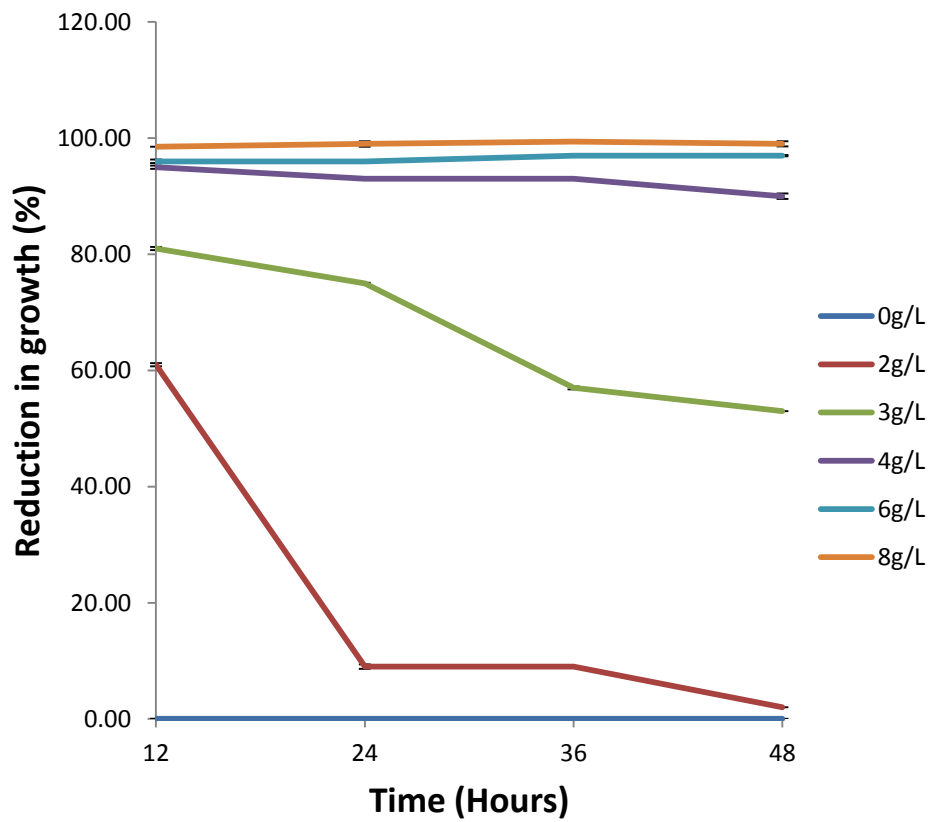
Figure 4.12: Effect of different concentrations of furfural (2-furaldehyde) on growth of
C. tropicalis Y5 at different time intervals.
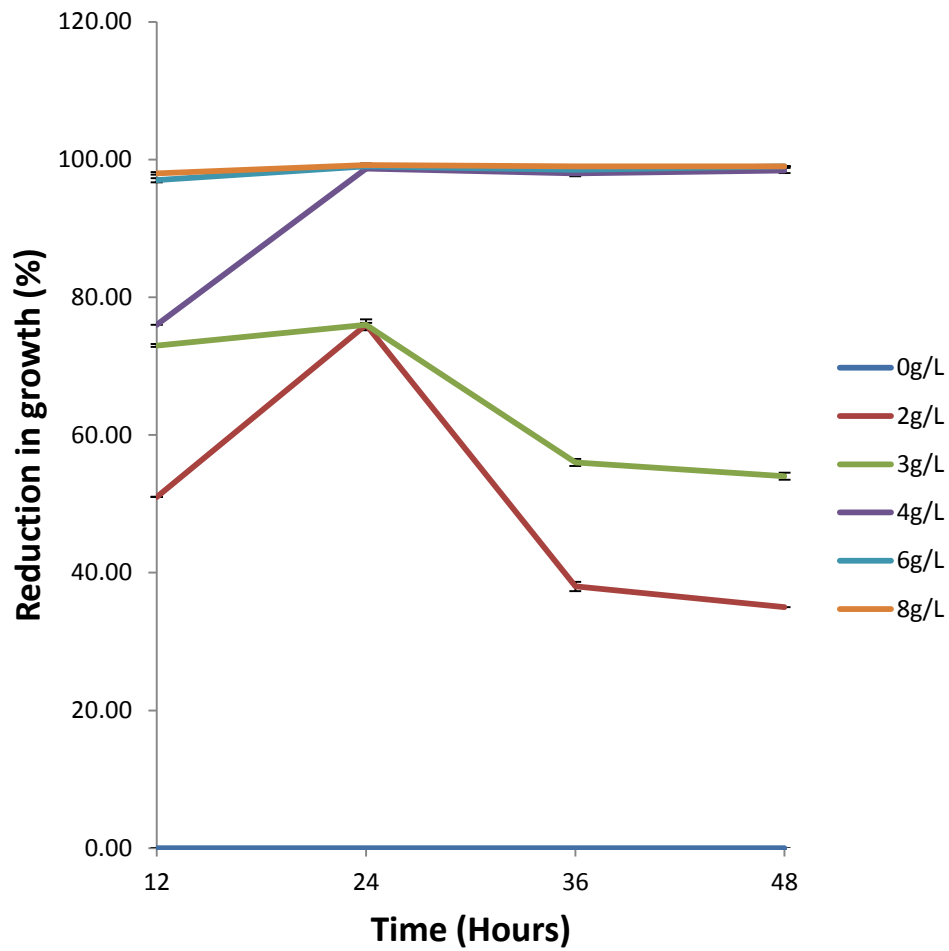
Figure 4.13: Effect of different concentrations of furfural (2-furaldehyde) on growth of *S. cerevisiae* Y10 at different time intervals

ability to adapt to 4 g/l furfural concentration as the percentage reduction in growth gradually decreases from 95% at the twelfth hour to 90% after 48 hours.

4.4.0   Sugarcane bagasse pretreatment and hydrolysis

4.4.1   Simultaneous effect of KOH, treatment time and temperature on sugarcane bagasse hydrolysis using commercial enzyme

Sugarcane bagasse pretreatment was done using potassium hydroxide at different concentrations, treatment times and temperatures. The preatreated bagasse was thereafter hydrolysed using Novozyme cellulase/hemicellulase mixture. Total reducing sugar yield was determined by DNS method. Glucose, xylose and arabinose concentrations were also determined through HPLC (Table 4.10).

Data from Table 4.10 were fitted to Equation (1) of section 3.3.1.1 of the materials and methods by using the regression tool of SigmaPlot software. Results of the three regressions performed using the same set of independent variables, (x_1, x_2 and x_3) and three dependent variables, namely the concentration of total reducing sugars, the concentration of glucose and the concentration of pentoses (xylose plus arabinose) are summarized in Table 4.11. For all responses studied, the values of α_3 were larger than the values of α_1, and α_2. This implied that KOH concentration virtually controlled the pretreatment process. The three responses had high coefficients of determination (R^2 = 0.9214, 0.8672 and 0.9261 respectively) for total reducing sugar, glucose and pentoses. The 3-D response surface plots and contour plots are shown in Figures 4.14 to 4.22. The 3-D response surface plot has three axes. The y axis is the end product (the dependent parameter) while the x and z axes are the independent parameters. When KOH concentration is one of the parameters in the graphs (Figures 4.14a, 4.15a, 4.17a, 4.18a, 4.20a and 4.21a), the end product varies significantly more than when there are both time and temperature (Figures 4.16a, 4.19a and 4.22a) at the x and z axes. The contour plots have two axes (x and y) which contain two standardized values of independent parameters at a time while the third independent parameter is kept constant. The multiple wave-like lines in each graph with numbers in between indicate the values of the end-products. Figures 4.14b, 4.15b, 4.17b, 4.18b, 4.20b and 4.21b show that increasing KOH concentration leads to increase in end product while increase in time or temperature does not necessarily lead to increase in the end product. In Figures 4.16b, 4.19b and 4.22b, KOH concentration is constant but

interaction between time and temperature do not bring about significant changes in the end products. These surface graphs therefore reveal that the dependent variables (total reducing sugar as glucose equivalent, pentoses and glucoses) are more influenced by KOH concentration than other two parameters that is, temperature (T) and time (t) which have similar effects on product output. Based on the regression results, the optimum values of studied parameters for total reducing sugar were as follows; pretreatment time 211 minutes, pretreatment temperature 93.3 ^{o}C and KOH concentration 162.8 mg/g bagasse. Theoretical prediction of total reducing sugar yield based on the revealed optimum conditions was 19.167 g/l. However, upon experimental trials using the revealed optimum pretreatment conditions, total reducing sugar of 20 g/l was observed. This is just about 4.35% higher than the statistically predicted value. As time and temperature were observed to be less important, an experiment was set up using less time from the predicted optimum (120 mins), 93.3 ^{o}C and KOH 150 mg/g bagasse. Total reducing sugars obtained under these conditions were not significantly different (Figure 4.23).

4.4.2 Growth of *Aspergillus niger* XY and reducing sugar production from raw unpretreated sugarcane bagasse

Aspergillus niger XY was selected among the other fungi for further hydrolysis experiment due to its ability to secrete highest quantities of tested hydrolytic enzymes in earlier experiments. In order to test if it was possible to hydrolyse the bagasse without any pretreatment, *A. niger* XY was inoculated on raw, unpretreated bagasse and incubated for 15 days. There was neither growth nor yield of reducing sugars throughout the incubation period.

Table 4.10: Pretreatment process variables and experimental values of responses
(dependent variables)

Process variables			Responses			
x_1	x_2	x_3	Total reducing sugars, g/l	Glucose, g/l	Xylose, g/l	Arabinose, g/l
30	51	50	9.845	5.361	1.249	0.159
120	51	50	9.450	4.895	1.374	0.212
210	51	50	10.100	4.896	1.555	0.212
30	86	50	11.008	5.149	1.770	0.222
120	86	50	9.285	4.046	1.260	0.158
210	86	50	10.600	5.040	1.598	0.615
30	121	50	10.463	4.638	1.628	0.207
120	121	50	10.956	5.307	1.982	0.364
210	121	50	10.928	5.212	2.128	0.338
30	51	100	12.515	8.781	2.682	0.365
120	51	100	15.285	6.558	3.309	0.473
210	51	100	14.558	6.769	3.601	0.437
30	86	100	14.403	8.295	4.098	0.489
120	86	100	18.113	6.766	3.509	0.449
210	86	100	18.438	7.835	4.332	0.541
30	121	100	14.008	7.455	3.944	0.404
120	121	100	16.455	7.808	4.198	0.498
210	121	100	14.175	7.347	4.648	0.583
30	51	150	15.093	6.895	3.341	0.374
120	51	150	16.048	7.415	3.937	0.455
210	51	150	17.513	7.626	4.176	0.491
30	86	150	15.253	9.067	5.017	0.590
120	86	150	19.450	9.037	5.384	0.698
210	86	150	18.655	8.589	4.670	0.597
30	121	150	16.235	9.032	5.071	0.738
120	121	150	17.990	6.919	3.951	0.424
210	121	150	18.095	9.504	6.644	0.588

Note: Data represents six replicates (three replicate pretreatments $\times$ two replicates per pretreatment replicate). x_1, x_2 and x_3 are treatment time (minutes), temperature (^{o}C) and KOH concentration (mg/g bagasse)

Table 4.11. Coefficients of regression of developed model

Coefficients	Dependent variable, y		
	Reducing sugars, g/l	Glucose, g/l	Pentoses, g/l
α_0	16.5853 ± 0.5871	7.3422 ± 0.37	4.2631 ± 0.27
α_1	0.7675 ± 0.2674	-0.0973 ± 0.17	0.3128 ± 0.12
α_2	0.5317 ± 0.2698	0.2165 ± 0.17	0.5737 ± 0.12
α_3	3.4053 ± 0.2688	1.6390 ± 0.30	1.6708 ± 0.12
α_{11}	-0.7368 ± 0.4843	0.5464 ± 0.29	0.3240 ± 0.23
α_{22}	-1.1871 ± 0.4658	-0.2834 ± 0.29	-0.3133 ± 0.22
α_{33}	-1.6073 ± 0.4651	-0.9223 ± 0.29	-0.7191 ± 0.22
α_{12}	-0.1500 ± 0.3267	0.2150 ± 0.20	0.0341 ± 0.15
α_{13}	0.6023 ± 0.3267	0.0548 ± 0.20	0.0655 ± 0.15
α_{23}	0.0932 ± 0.3294	0.2958 ± 0.21	0.2334 ± 0.15
R/R^2	0.9599/0.9214	0.9312/0.8672	0.9624/0.9261
x_1, at optimum Reduced – Real	1.013 - **211.2 min**	0.024- **122.16 min**	-0.692-**57.72 min**
x_2, optimum Reduced – Real	0.209 - **93.3ºC**	0.1-**89.5 ºC**	1.382-**134.37 ºC**
x_3, optimum Reduced – Real	1.255 - **162.8 mg/g**	0.905-**145.25 mg/g**	1.355-**167.75 mg/g**
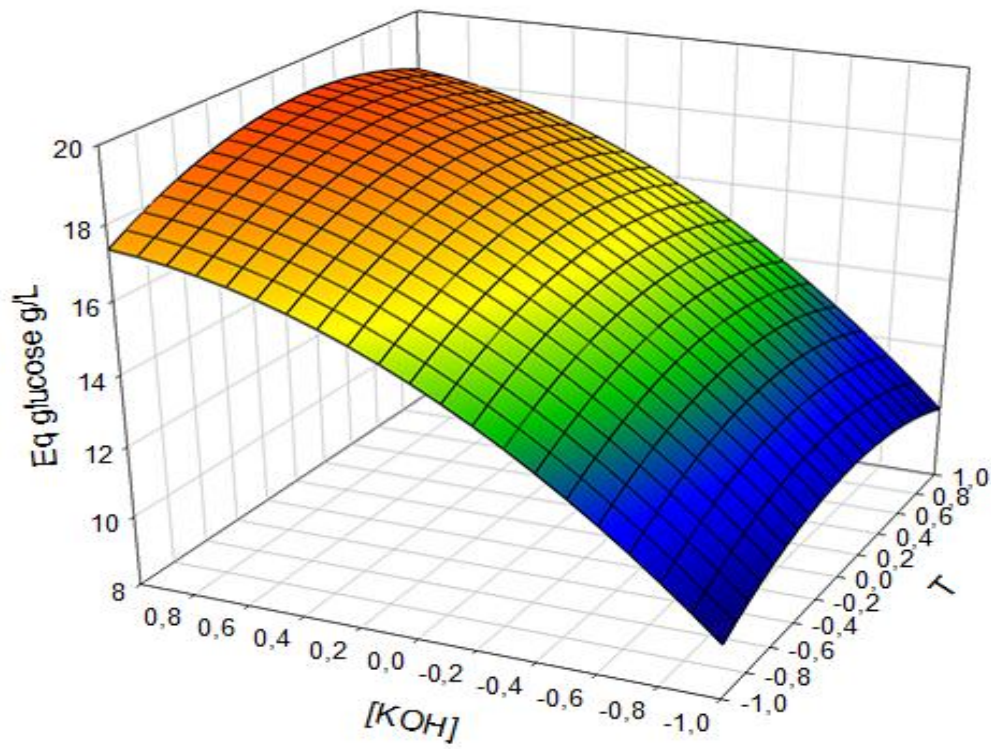
Figure 4.14a: Response surface plot showing the effect of concentration of potassium hydroxide [KOH] and pretreatment temperature (T) on total reducing sugars (Eq glucose) with pretreatment time (t) kept at optimum
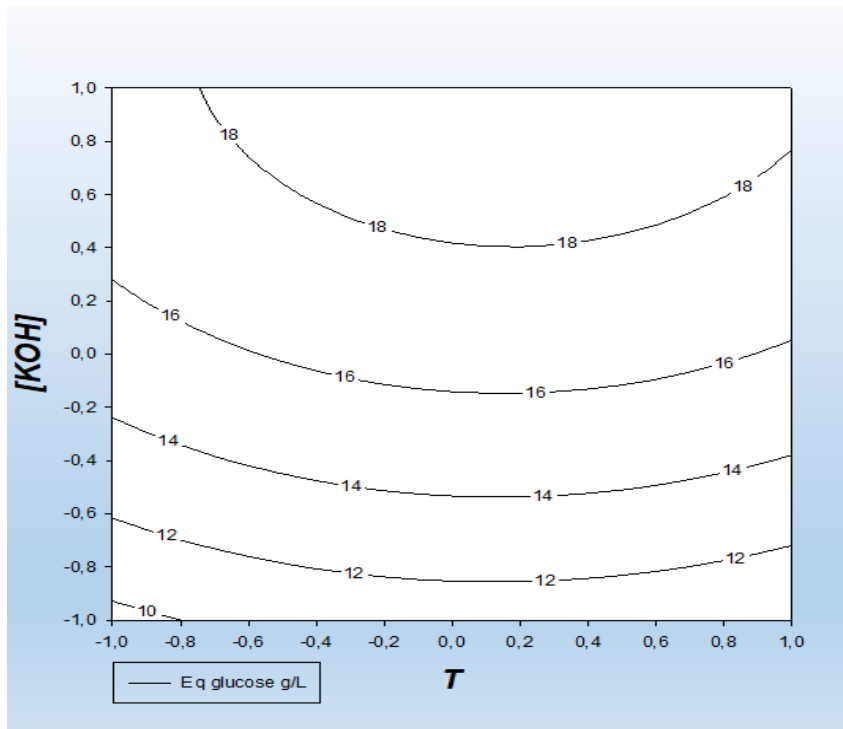
Figure 4.14b: Contour plot showing the effect of concentration of potassium hydroxide [KOH] and pretreatment temperature (T) on total reducing sugars (Eq glucose) with pretreatment time (t) kept at optimum
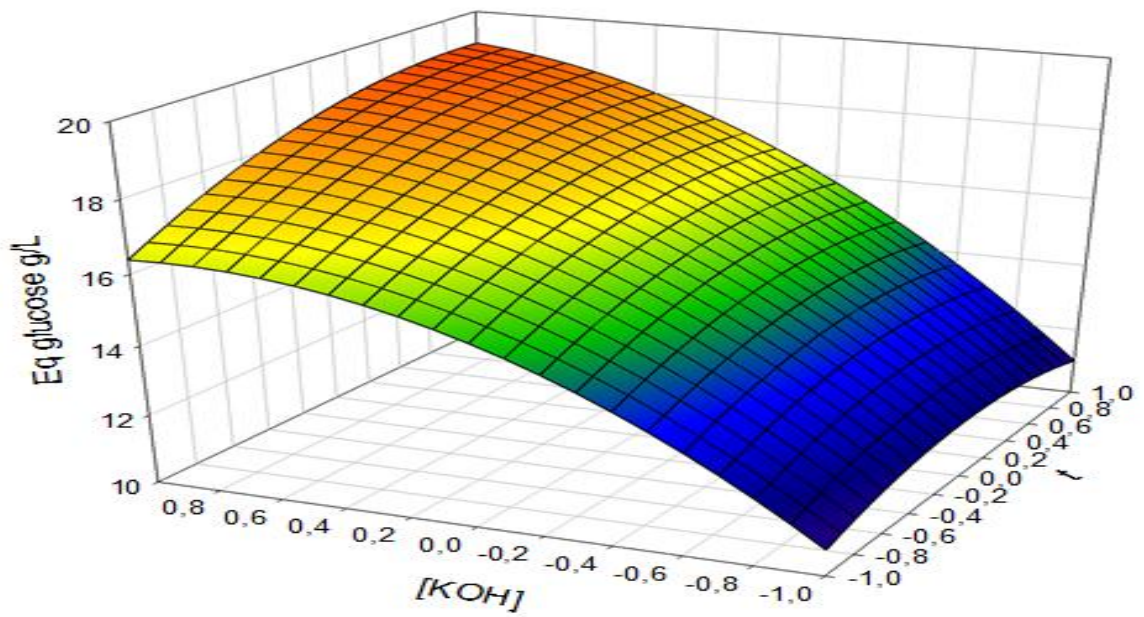
Figure 4.15a: Response surface plot showing the effect of concentration of potassium hydroxide [KOH] and pretreatment time (t) on total reducing sugar (Eq glucose) with pretreatment temperature (T) kept at optimum
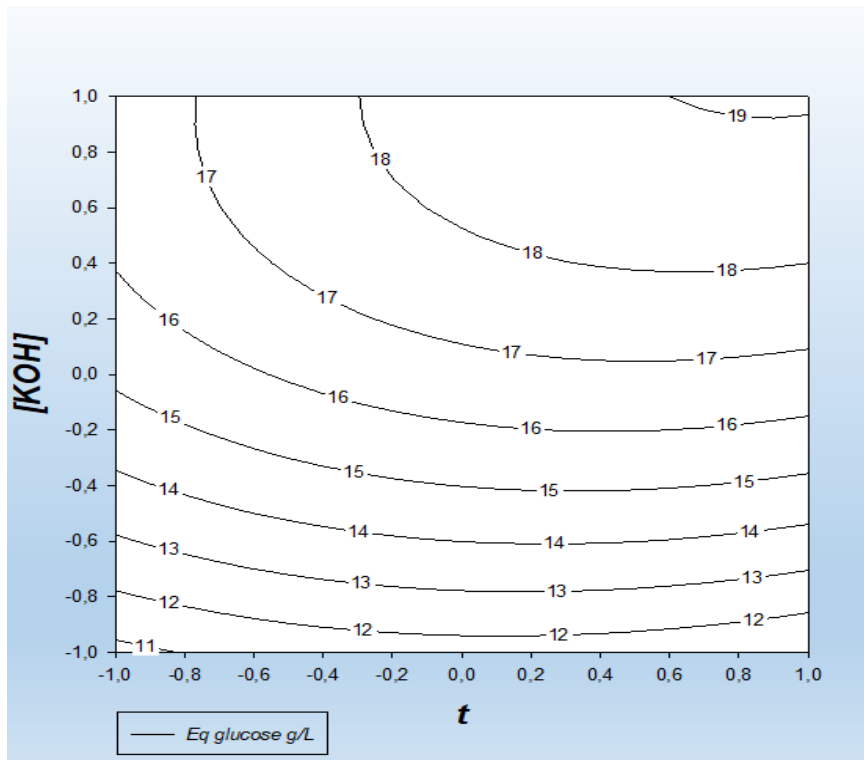
Figure 4.15b: Contour plot showing the effect of concentration of potassium hydroxide [KOH] and pretreatment time (t) on total reducing sugars (Eq glucose) with pretreatment temperature (T) kept at optimum
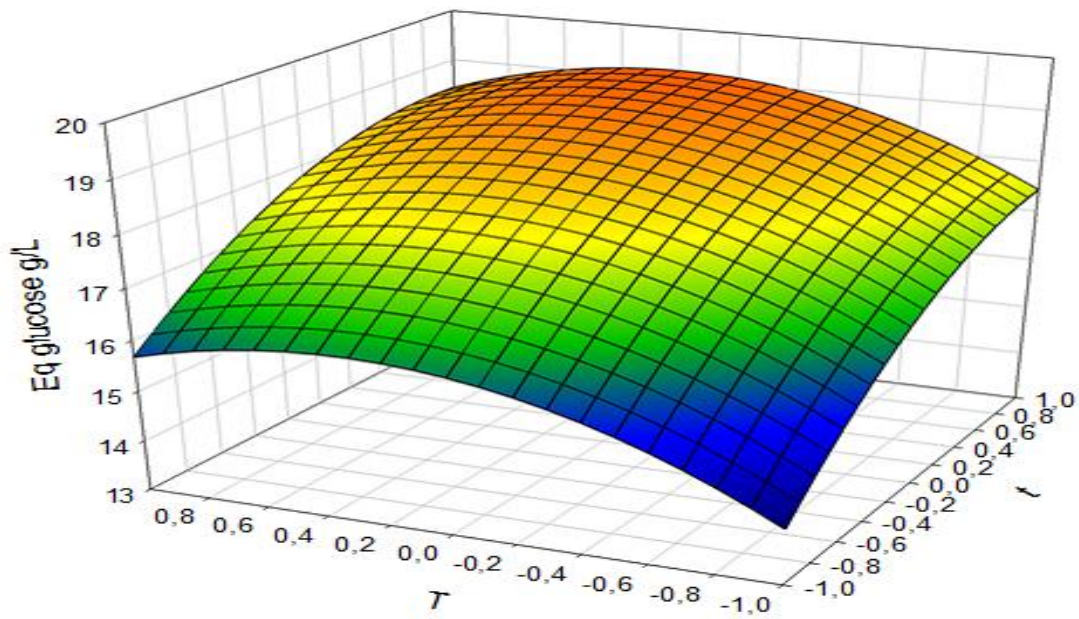
Figure 4.16a: Response surface plot showing effect of pretreatment temperature (T) and pretreatment time (t) on total reducing sugar (Eq glucose) with concentration of potassium hydroxide [KOH] kept at optimum
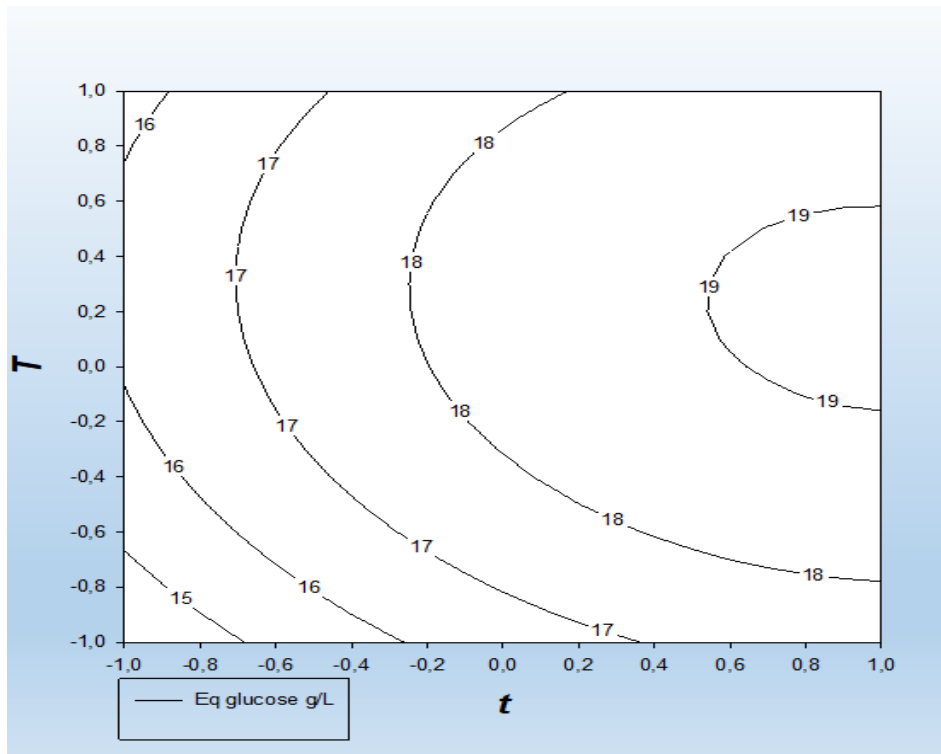
Figure 4.16b: Contour plot showing effect of pretreatment temperature (T) and pretreatment time (t) on total reducing sugar (Eq glucose) with concentration of potassium hydroxide [KOH] kept at optimum
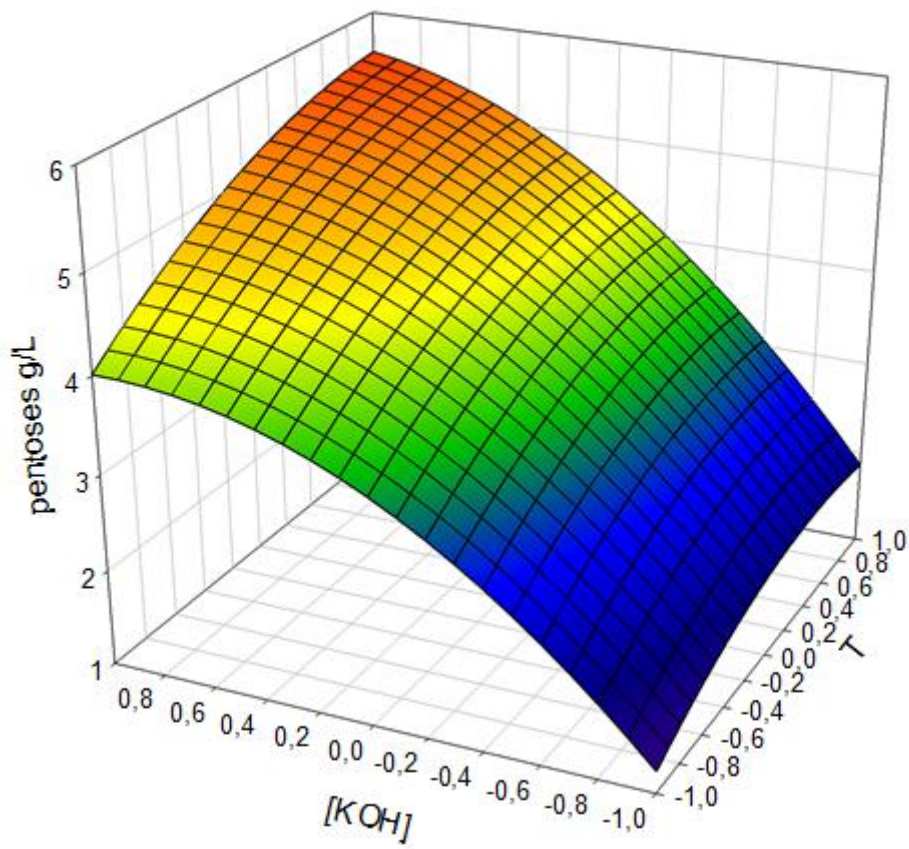
Figure 4.17a: Response surface plot showing the effect of concentration of potassium hydroxide [KOH] and pretreatment temperature (T) on pentoses (xylose + arabinose) with pretreatment time (t) kept at optimum
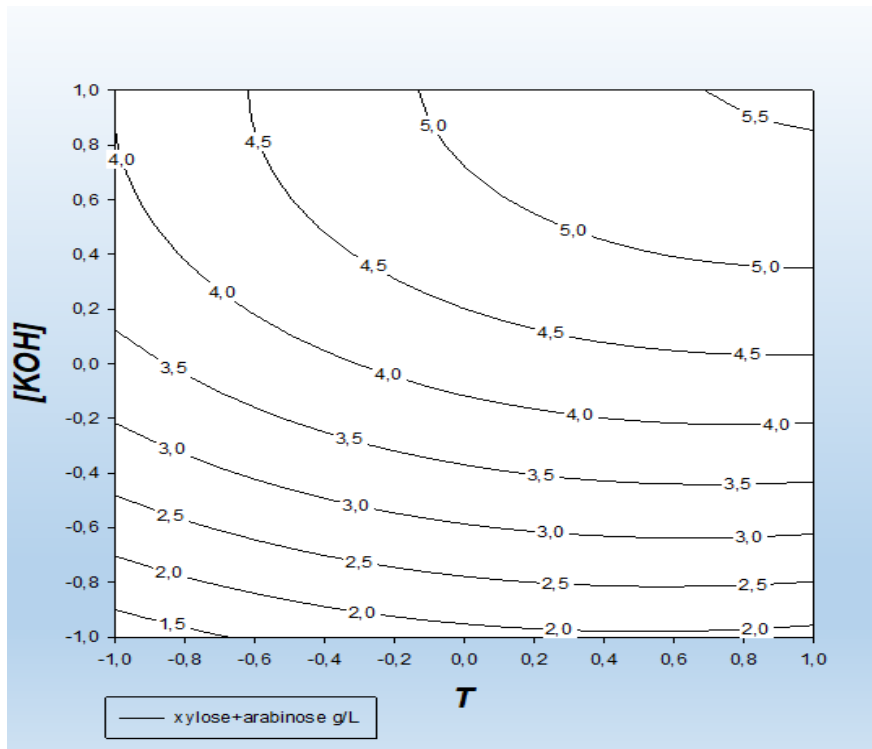
Figure 4.17b: Contour plot showing the effect of concentration of potassium hydroxide [KOH] and pretreatment temperature (T) on pentoses (xylose + arabinose) with pretreatment time (t) kept at optimum
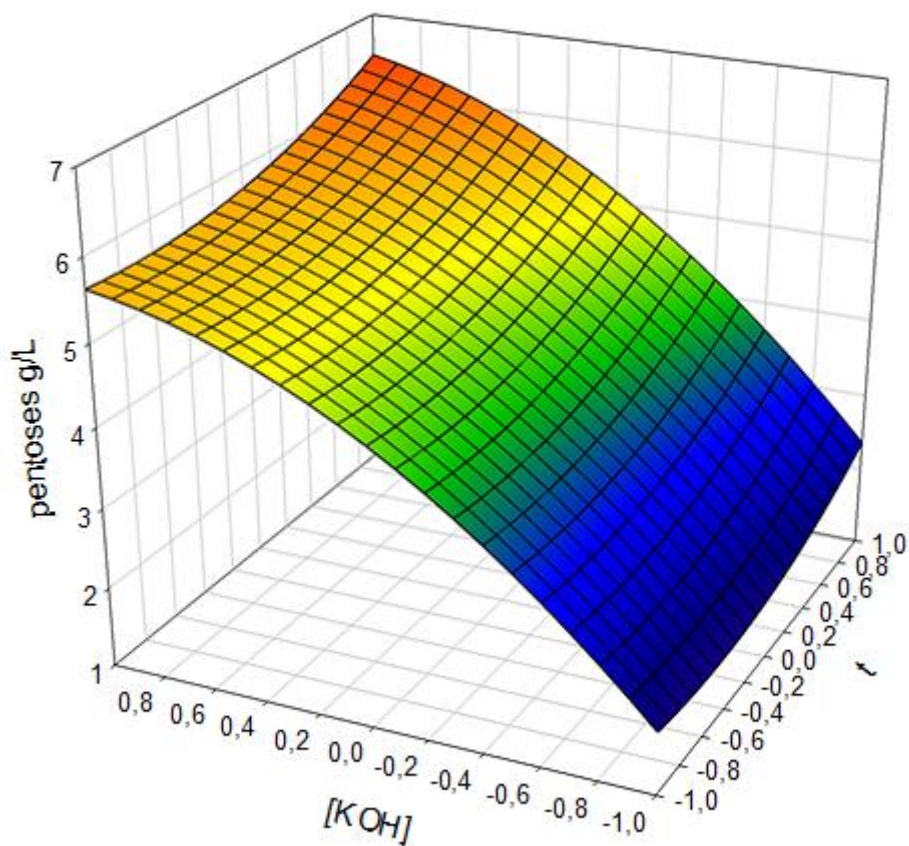
Figure 4.18a: Response surface plot showing the effect of concentration of potassium hydroxide [KOH] and pretreatment time (t) on pentoses (xylose + arabinose) with pretreatment temperature (T) kept at optimum
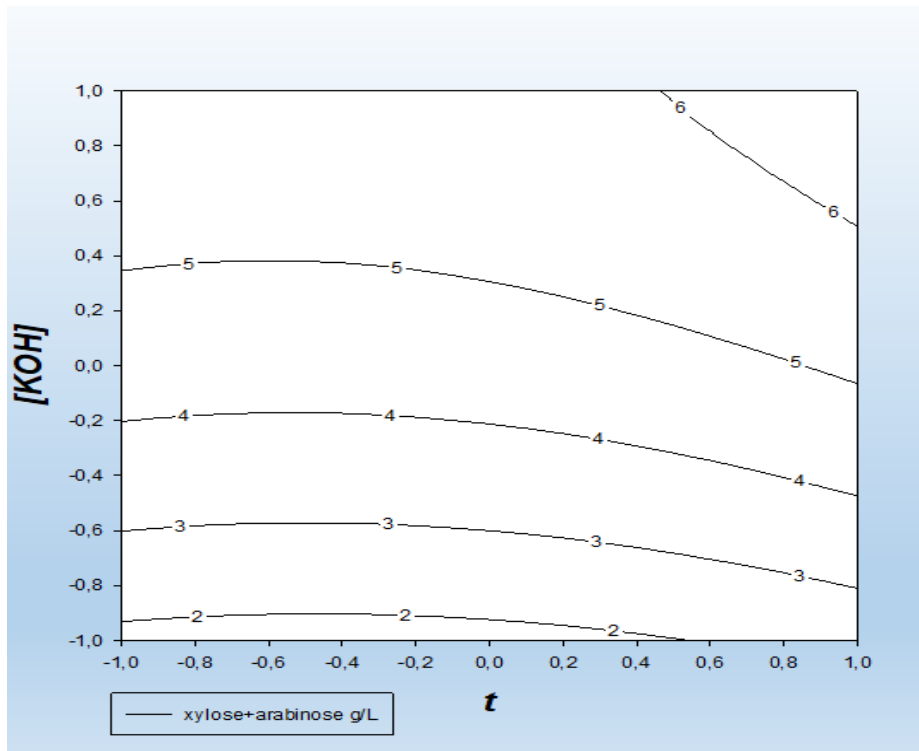
Figure 4.18b: Contour plot showing the effect of concentration of potassium hydroxide [KOH] and pretreatment time (t) on pentoses (xylose + arabinose) with pretreatment temperature (T) kept at optimum
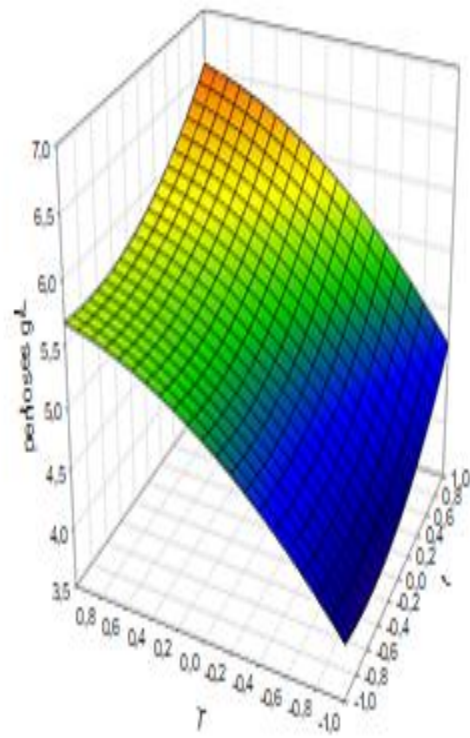
Figure 4.19a: Response surface plot showing the effect of pretreatment temperature (T) and pretreatment time (t) on pentoses (xylose + arabinose) with concentration of potassium hydroxide [KOH] kept at optimum.
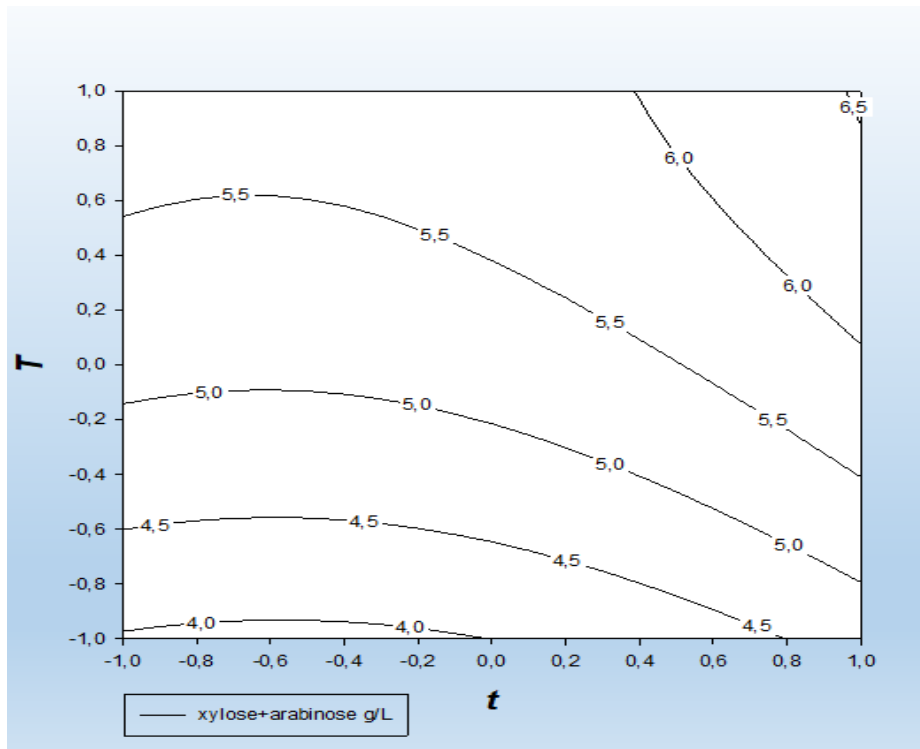
Figure 4.19b: Contour plot showing the effect of pretreatment temperature (T) and pretreatment time (t) on pentoses (xylose + arabinose) with concentration of potassium hydroxide [KOH] kept at optimum.
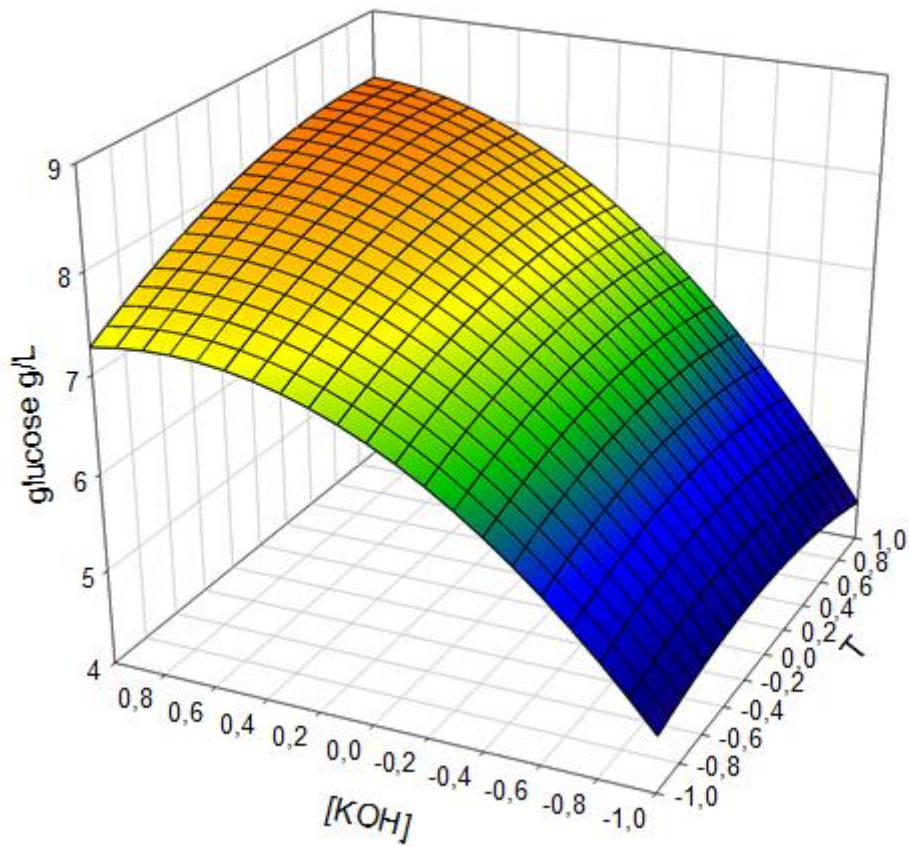
Figure 4.20a: Response surface plot showing the effect of concentration of potassium hydroxide [KOH] and pretreatment temperature (T) on glucose with pretreatment time (t) kept at optimum.
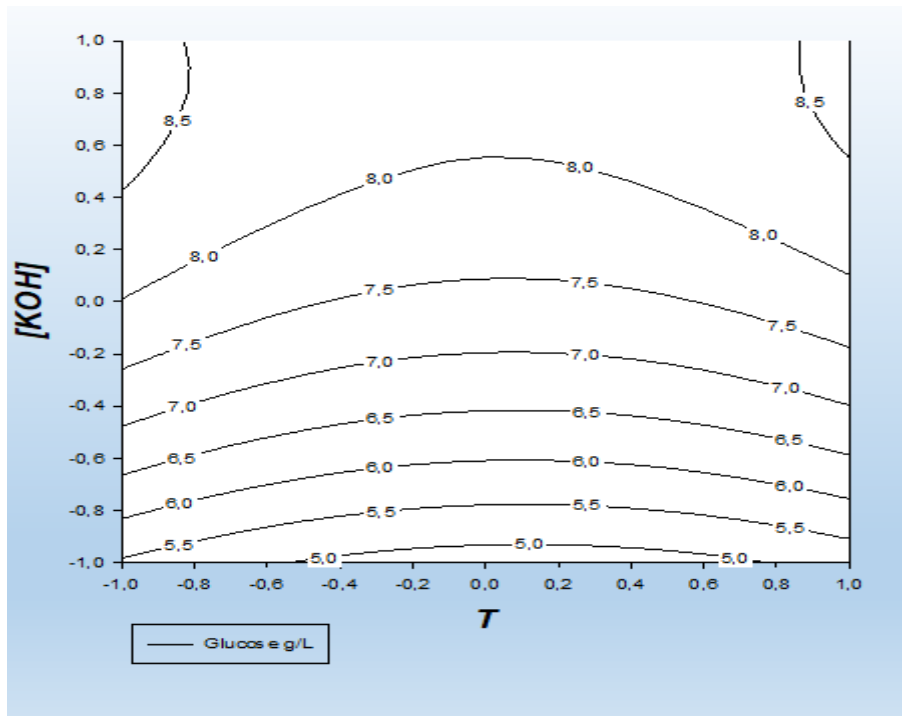
Figure 4.20b: Contour plot showing the effect of concentration of potassium hydroxide [KOH] and pretreatment temperature (T) on glucose with pretreatment time (t) kept at optimum.
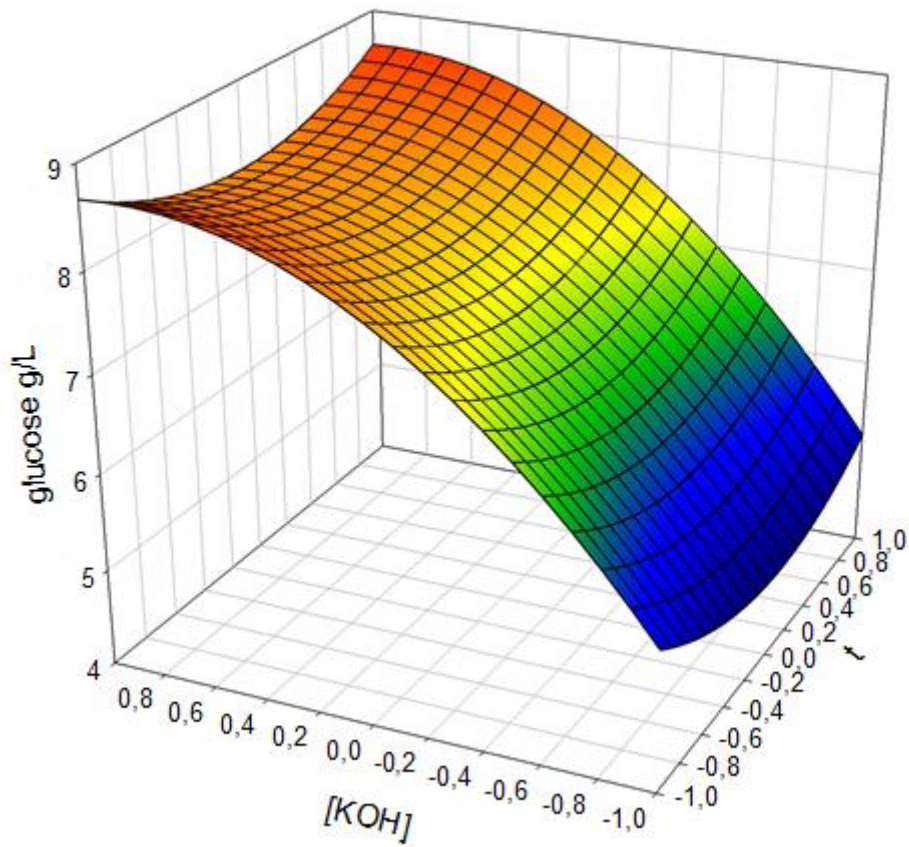
Figure 4.21a: Response surface plot showing the effect of concentration of potassium hydroxide [KOH] and pretreatment time (t) on glucose with pretreatment temperature (T) kept at optimum
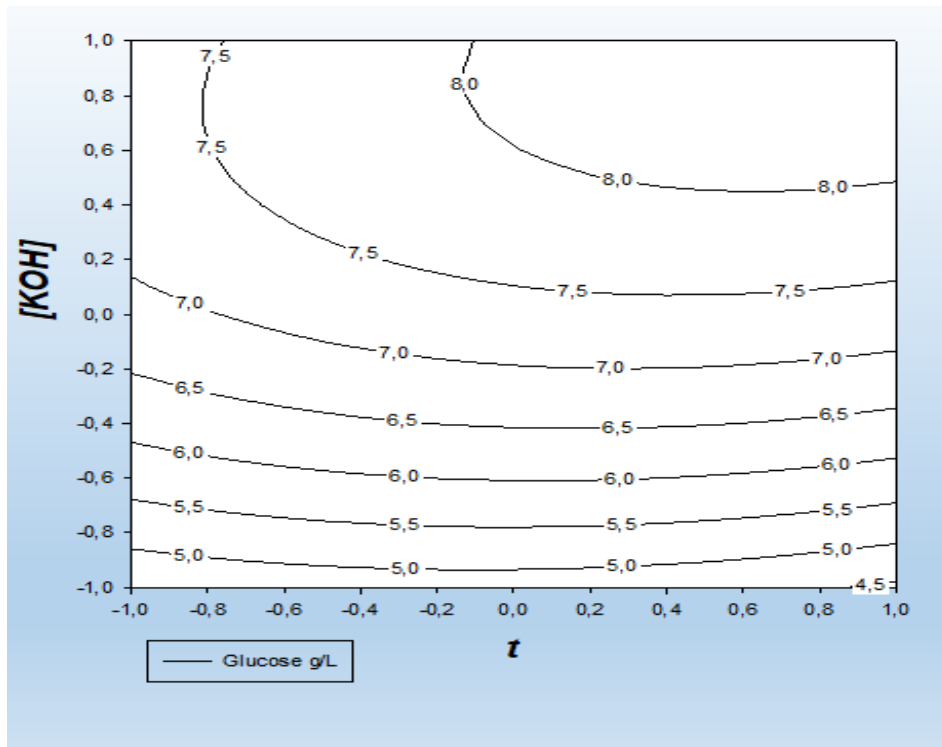
Figure 4.21b: Contour plot showing the effect of concentration of potassium hydroxide [KOH] and pretreatment time (t) on glucose with pretreatment temperature (T) kept at optimum
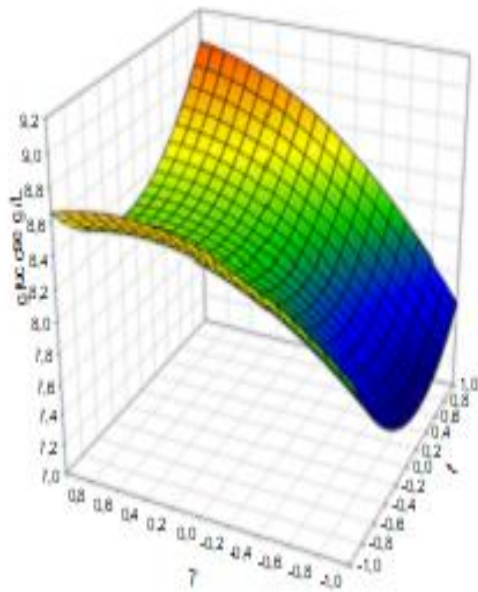
Figure 4.22a: Response surface plot showing the effect of pretreatment temperature (T) and pretreatment time (t) on glucose with concentration of potassium hydroxide [KOH] kept at optimum.
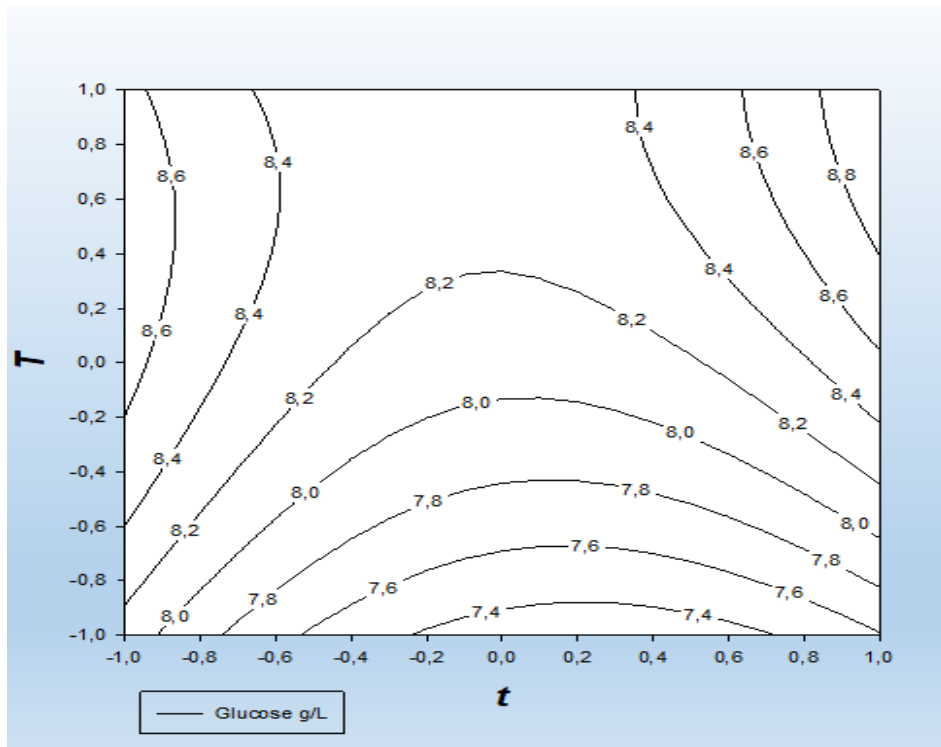
Figure 4.22b: Contour plot showing the effect of pretreatment temperature (T) and pretreatment time (t) on glucose with concentration of potassium hydroxide [KOH] kept at optimum.
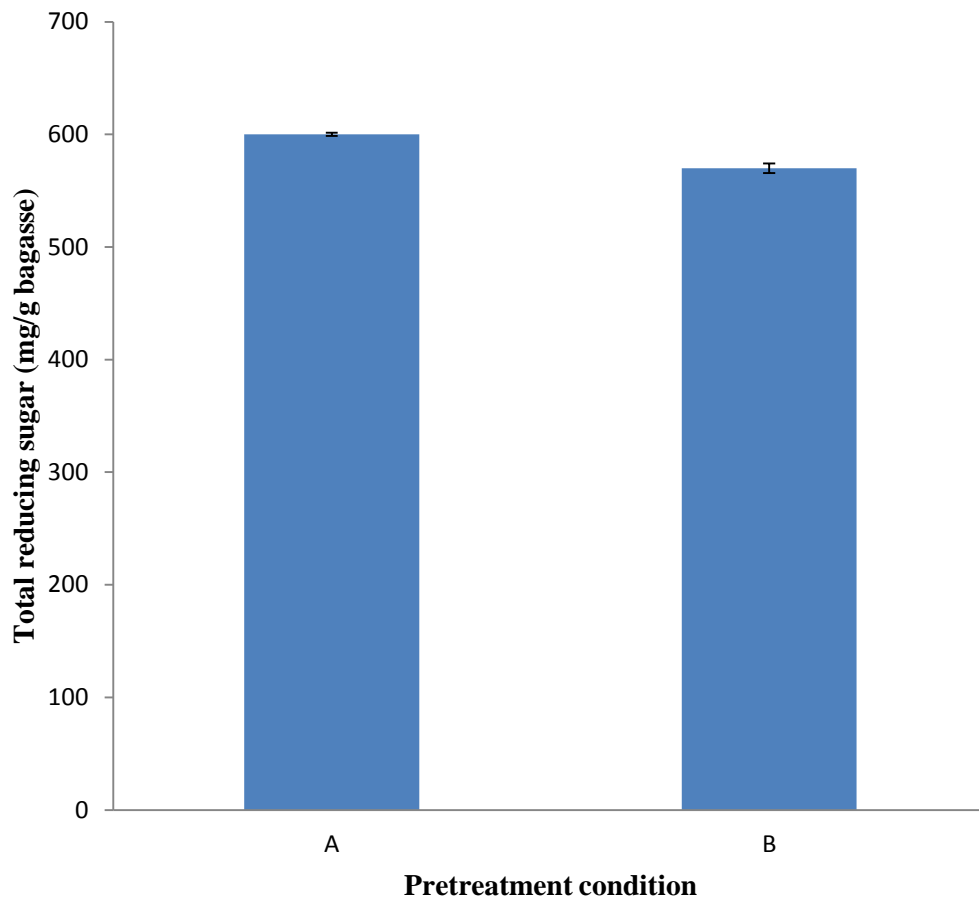
Figure 4.23: Hydrolysis of sugarcane bagasse with commercial Cellulase using the best optimised pretreatment conditions. A(211 mins, 93 ^{o}C, [KOH] 162 mg/g bagasse), B(120mins, 86 ^{o}C, [KOH] 150 mg/g bagasse)

4.5 Scanning electron microscopy of raw and pretreated samples

Scanning electron microscopy of raw bagasse and alkali pretreated bagasse at the optimum conditions was carried out to compare the changes in structure of the bagasse after alkali pretreatment. Two main morphological features were revealed; the pith and the fiber structures. Plate 4.1a shows the general view of the bagasse indicating the pith and the fiber before pretreatment while Plates 4.1b and 4.1c show the raw, untreated bagasse with focus on the surfaces of the pith and the fiber structures respectively. The fiber surface is well covered with residual materials (extractives) and arranged in parallel stripes. After pretreatment, the parallel stripes of the fiber structure became more exposed as the packing was loosened and the fiber getting detached from one another. The pith was no more conspicuous (Plate 4.2).
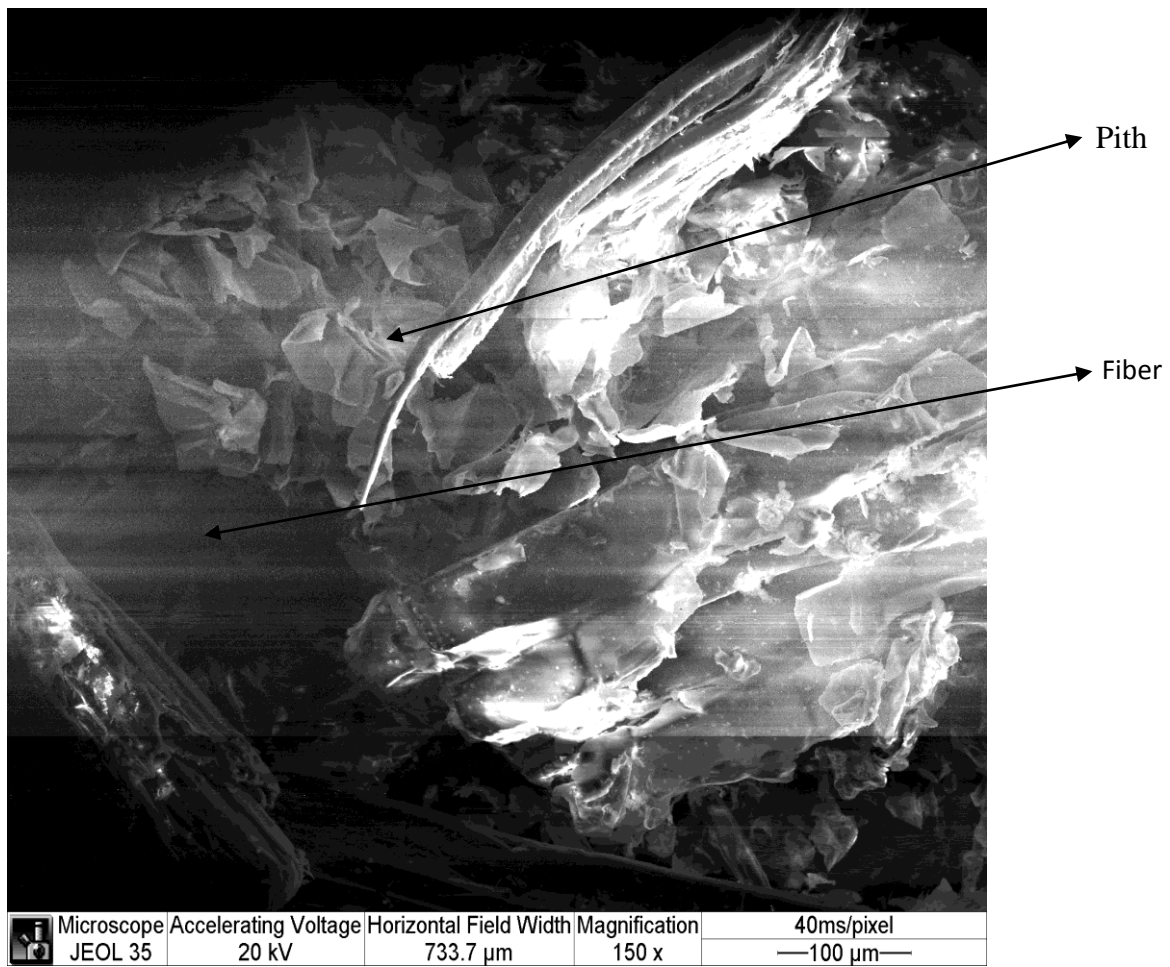
Plate 4.1a: Scanning electron micrograph of raw bagasse before pretreatment showing the pith and the fiber
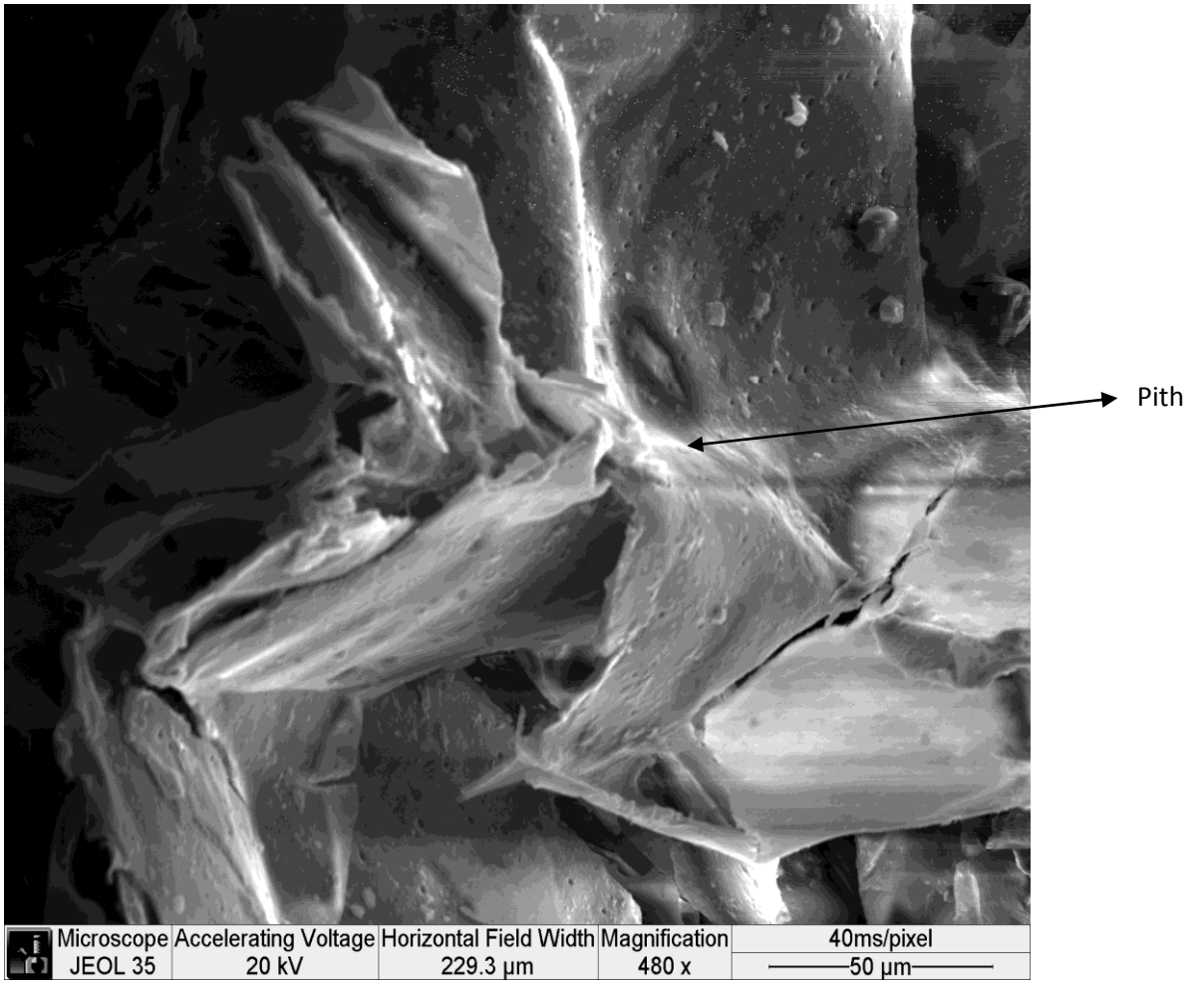
Plate 4.1b: Scanning electron micrograph of raw bagasse showing the pith
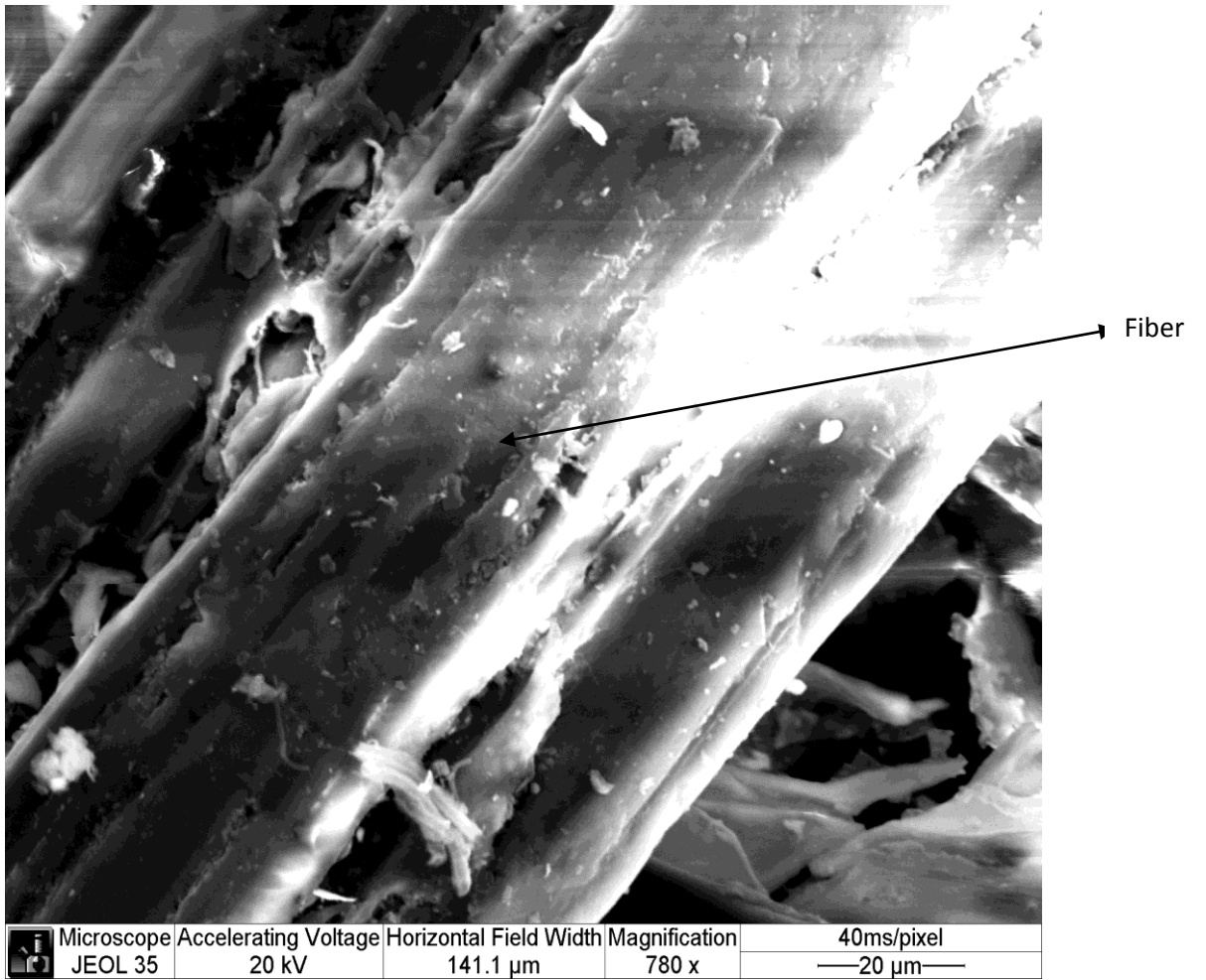
Fiber

Plate 4.1c: Scanning electron micrograph of raw bagasse showing the fiber structure

Microscope	Accelerating Voltage	Horizontal Field Width	Magnification	40ms/pixel
JEOL 35	20 kV	183.4 µm	600 x	—20 µm—

Plate 4.2: Scanning electron micrograph of pretreated bagasse showing mainly the fiber structure

4.6.0   Hydrolysis of pretreated sugarcane bagasse with *A. niger* XY

4.6.1   Submerged versus solid state hydrolysis of pretreated bagasse

Figure 4.24 shows the effect of solid state and submerged fermentation on pretreated sugarcane bagasse hydrolysis by *A. niger* XY. Sample was not taken for analysis at day 2 for solid state hydrolysis. At day 5, reducing sugar yield was not significantly different though solid state culture gave a higher yield of 6.84 mg/g as against 5.71mg/g yield from submerged culture. Subsequently at all other days of the experiment, solid state hydrolysis gave higher yields which were significantly different. In the solid state hydrolysis, there was a sharp increase in reducing sugar yield between day 5 and day 10 and thereafter, a gradual increase until day 12 when a peak of 14.95 mg/g reducing sugar was attained followed by a gradual decline in the yield.

4.6.2   Effect of initial pH on reducing sugar production by *A. niger*XY in solid state hydrolysis of pretreated bagasse

A pH range of 3.5 to 7 was tested in the experiment (Figure 4.25). The fungus yielded lowest levels of reducing sugar at pH 7. At each day, the highest range of reducing sugar yield was observed between pH 4 and pH 5.5. At pH 5 and 5.5, the fungus gave the highest reducing sugar yield of 15.09 mg/g on the 12th day.
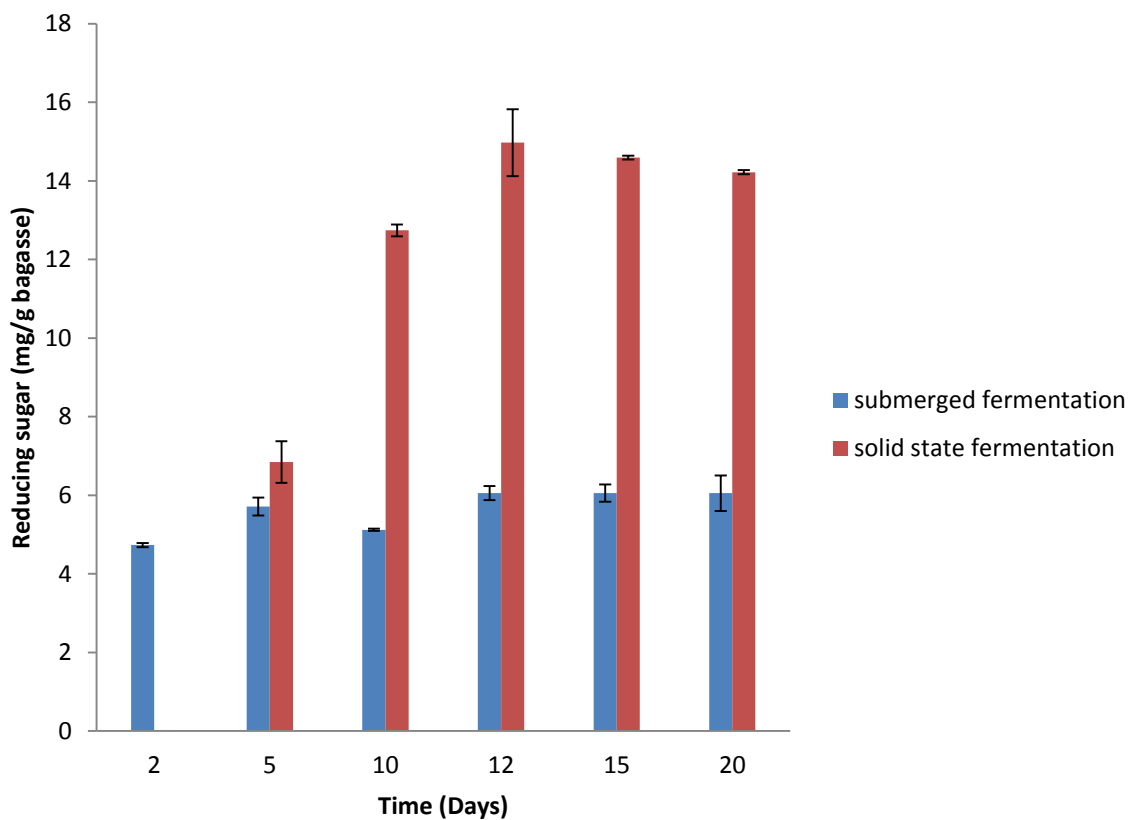
Figure 4.24: Effect of solid state and submerged culture conditions on pretreated sugarcane bagasse hydrolysis by *Aspergillus niger* XY
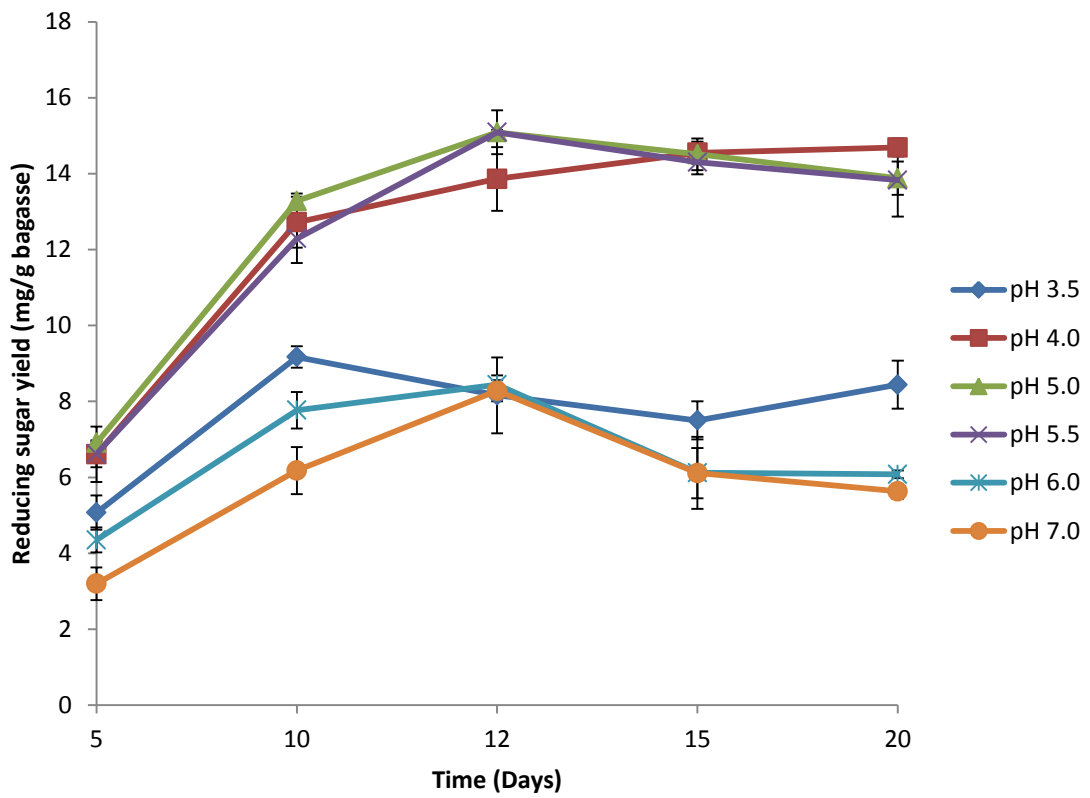
Figure 4.25: Effect of initial pH on reducing sugar production in solid state hydrolysis of pretreated sugarcane bagasse by *A. niger*XY

4.6.3 Incubation temperature effect on reducing sugar production by *A. niger*XY in solid state hydrolysis of pretreated bagasse

Figure 4.26 shows the effect of temperature on the hydrolysis of pretreated bagasse by *A. niger* XY. Optimum temperature was observed to be 35 ^{o}C at which reducing sugar level rose from 7.07 mg/g at the 5th day up to a peak of 15.80 mg/g at the 12th day.

4.6.4 Nitrogen source fortification effect on reducing sugar production by *A. niger* XY in solid state hydrolysis of pretreated bagasse

Nitrogen sources tested for their effect on the level of reducing sugar yield by *A.* niger XY in the hydrolysis of pretreated bagasse include NH_4Cl, NH_4NO_3, urea, peptone and yeast extract. Urea was observed to give the highest reducing sugars yield of 18.80 mg/g bagasse (Figure 4.27). NH_4Cl, NH_4NO_3 and yeast extract gave 15.88, 15.81 and 14.36 mg/g respectively. These values were not significantly different. Peptone was observed to give the least reducing sugar yield of 12.89 mg/g bagasse.
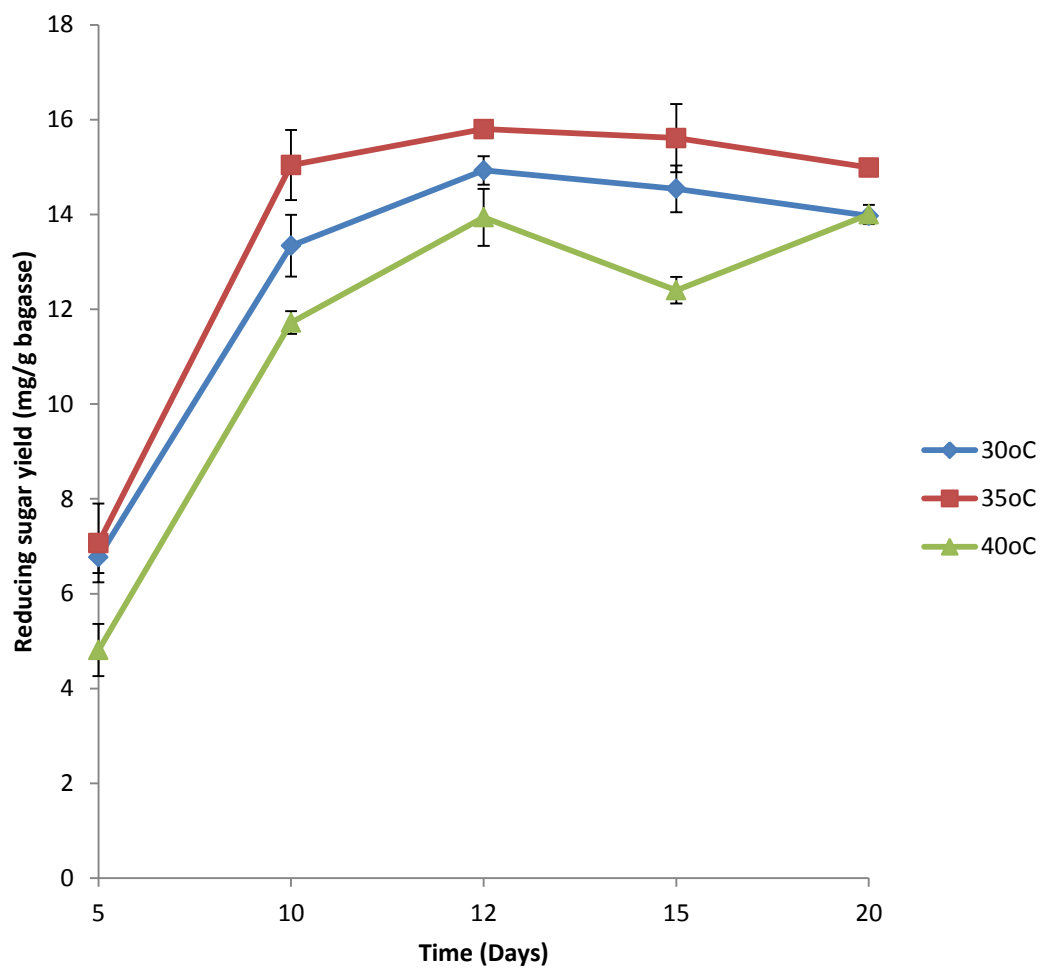
Figure 4.26: Effect of temperature on reducing sugar production under solid state hydrolysis of pretreated bagasse by *A. niger* XY
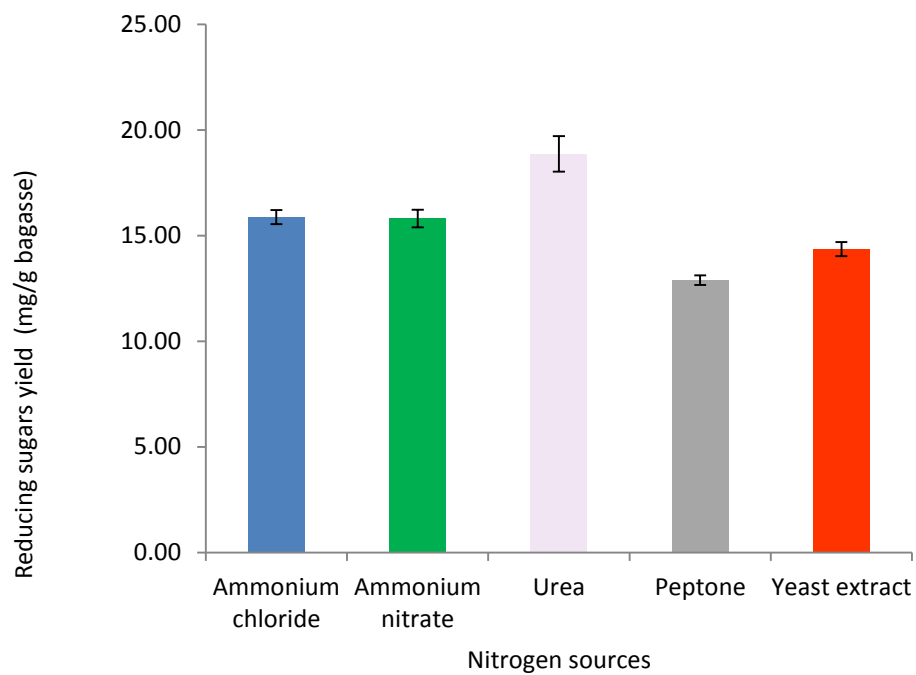
Figure 4.27: Effect of nitrogen sources on reducing sugars production in solid state hydrolysis of pretreated bagasse by *A. niger* XY after 13 days of incubation.

There was no significant difference ($p < 0.05$) in average reducing sugars yield

4.7.0   Factors affecting ethanol production

4.7.1   Monoculture and coculture effect of yeasts on ethanol production through separate hydrolysis and fermentation

Table 4.12 shows the effect of monoculture and co-cultures of selected yeasts on ethanol production. *C. tropicalis* Y5 was cultured singly and in combination with *P. kudriavzevii* Y2 and *S. cerevisiae*Y10. Total reducing sugar consumed was calculated as the difference between the reducing sugar content of the hydrolysate before fermentation and that of the sample after fermentation. Ethanol yield was measured using gas chromatography. Ethanol yield per one gram of glucose was calculated by dividing the ethanol yield in gram per litre by the total reducing sugar consumed. Calculation of fermentation efficiency was done as the percentage of ethanol yield per one gram of glucose of the sample divided by the theoretical ethanol yield per one gram of glucose. Theoretical ethanol yield per gram glucose is constant with value 0.51 (Nadeem *et al.*, 2015).

At the end of fermentation, there were just slight differences in the ethanol yield by the various combinations of the isolates (values are not significantly different). Single culture of *C. tropicalis* Y5 gave ethanol yield of 12.03 g/l and the coculture of *P. kudriavzevii* Y2 and *C. tropicalis* Y5 which gave ethanol yield of 12.46 g/l. Coculture of the three isolates gave ethanol yield of 12.15 g/l while coculture of *C. tropicalis* Y5 and *S. cerevisiae*Y10 gave ethanol yield of 11.10 g/l. Single cultures of *P. kudriavzevii* Y2 and *S. cerevisiae* Y10 gave ethanol yield of 9.16 g/l and 8.65 g/l respectively.

4.7.2 Effect of addition of urea on ethanol production by *Candida tropicalis* Y5 through separate hydrolysis and fermentation

C. tropicalis Y5 was selected for further experiment because of its ability to ferment xylose and as its coculture with other yeasts did not produce a significant increase in ethanol yield. Addition of urea as nitrogen source produced a significant difference in the yield of ethanol (Table 4.13). Fermentation setup which contained urea gave ethanol yield of 18.56 g/l with fermentation efficiency of 97.41% as against that with no urea additive which gave an ethanol yield of 12.27 g/l fermentation efficiency of 72.95%.

Table 4.12: Monoculture and co-culture effect of selected yeasts on ethanol production through separate hydrolysis and fermentation

Isolates	Total Reducing Sugar Consumed (g/l)	Ethanol Yield (g/l)	Ethanol yield (g/g sugar consumed)	Fermentation efficiency (%)
Y2	25.14 ± 1.20^b	9.16 ± 1.06^b	0.36 ± 0.01^a	71.42 ± 2.04^a
Y5	32.28 ± 2.00^a	12.03 ± 1.01^a	0.37 ± 0.02^a	73.10 ± 3.50^a
Y10	19.25 ± 1.00^c	8.65 ± 1.02^b	0.45 ± 0.02^b	88.07 ± 1.50^b
Y2,Y5	34.87 ± 1.00^a	12.46 ± 2.10^a	0.36 ± 0.06^a	70.06 ± 1.10^a
Y5,Y10	33.75 ± 1.00^a	11.10 ± 1.50^a	0.33 ± 0.22^a	64.53 ± 2.20^a
Y2,Y5,Y10	34.63 ± 1.50^a	12.15 ± 1.00^a	0.35 ± 0.21^a	68.76 ± 2.11^a

Data are presented in means ± standard deviation of results. Values with same superscript along the same column are not significantly different.

Y2 (*P. kudriavzevii*), Y5(*C. tropicalis*), Y10(*S. cerevisiae*)

Table 4.13: Effect of addition of urea on ethanol production by *C. tropicalis* Y5 through separate hydrolysis and fermentation

	Total glucose Consumed (g/l)	Ethanol Yield (g/l)	Ethanol yield (g/g sugar consumed)	Fermentation efficiency (%)
No urea	33.97 ± 1.23^a	12.27 ± 0.55^a	0.37 ± 0.04^a	72.95 ± 1.83^a
Urea	37.36 ± 1.14^b	18.56 ± 1.01^b	0.50 ± 0.07^b	97.41 ± 1.25^b

Data are presented in means ± standard deviation of results. Values with same superscript along the same column are not significantly different.

4.7.3 Incubation temperature effect on ethanol production by *Candida tropicalis* Y5 through separate hydrolysis and fermentation

Table 4.14 shows the effect of varying temperatures on ethanol production from sugarcane bagasse hydrolysate. At 28 and 35 ^{0}C, observations made were 18.53 g/l ethanol yield with 96.88% fermentation efficiency and 18.99 g/l ethanol with 98.21% respectively. Though higher yield was observed at 35 ^{0}C, ethanol production at these two temperatures was not significantly different. At 42 ^{0}C, yield was lower (11.37 g/l ethanol with 66.38% fermentation efficiency) and significantly different from other temperatures.

4.7.4 Effect of temperature on ethanol production from pretreated bagasse by *Candida tropicalis* Y5 through simultaneous saccharification and fermentation

Observations during a simultaneous saccharification and fermentation of pretreated bagasse are presented in Table 4.15. Three temperature schemes which include 35 ^{0}C, intermittent change in temperature between 35 ^{0}C and 45 ^{0}C; and 45 ^{0}C alone were examined. Optimum temperature was observed to be 35 ^{0}C with ethanol yield of 30.42 g/l and 99% fermentation efficiency. This was followed by intermittent change in temperature between 35 ^{0}C and 45 ^{0}C. Here, 23.22 g/l of ethanol and 79.62% fermentation efficiency.

Table 4.14: Temperature effect on ethanol production from urea supplemented bagasse hydrolysate by *C. tropicalis* Y5 through separate hydrolysis and fermentation

Temperature	Total reducing sugar Consumed (g/l)	Ethanol Yield (g/l)	Ethanol yield (g/g sugar consumed)	Fermentation efficiency (%)
28^0C	$37.52\pm.1.55^b$	$18.54\pm.1.00^b$	$0.49\pm.0.30^b$	$96.88\pm.1.20^b$
35^0C	$37.92\pm.2.12^b$	$18.99\pm.1.40^b$	$0.50\pm.0.01^b$	$98.21\pm.4.80^b$
42^0C	$33.54\pm.1.00^a$	$11.37\pm.2.80^a$	$0.34\pm.0.01^a$	$66.38\pm.1.00^a$

Data are presented in means ± standard deviation of results. Values with same superscript along the same column are not significantly different.

Table 4.15: Temperature effect on ethanol production from pretreated bagasse by *C. tropicalis* Y5 through simultaneous saccharification and fermentation

Temperature	Total glucose Consumed (g/l)	*Ethanol Yield (g/l)	Ethanol yield(g/g sugar)	Fermentation efficiency (%)
35 ^{0}C	59.27±.3.00^c	30.21±.1.20^c	0.50±.0.02^b	98.01±.4.00^c
35/45^{0}C	57.23±.2.50^b	23.22±.1.10^b	0.41±.0.50^b	79.62±.2.20^b
45^{0}C	47.72±.1.79^a	13.82±.1.20^a	0.29±.0.01^a	56.76±.1.50^a

Data are presented in means ± standard deviation of results. Values with same superscript along the same column are not significantly different.

*values are resultant values after deduction of ethanol yield from control experiments with unpretreated bagasse

CHAPTER FIVE

5.0   DISCUSSION

The cellulose, hemicellulose and lignin contents of the sugarcane bagasse used in this study were found to be 33.46%, 26.88% and 16.58% respectively. That makes the total carbohydrate present to be 60.34%. The cellulose and hemicellulose contents fall within the range of values in previous studies published for similar samples, that is, amounts of carbohydrates (60 – 70%), mostly in the form of two polysaccharide molecules, cellulose (33 – 45%) and hemicellulose (28 – 35%) (Paixao *et al*., 2016). Klason lignin was measured in this study and the value is close to that (17.8%) reported by del Rio *et al*. (2015) though many other researchers reported 20 – 30% lignin (Rezende *et al*., 2011; Rocha *et al*., 2015). Lower value of lignin in the sugarcane bagasse used in this study makes it a good choice for consideration for ethanol production. However, unlike many other researches, the 23.08% value for extractives present in the sample used in this study is rather high. Attempt was made to find out if the extractive was majorly composed of residual reducing sugar using the DNS method but the result was negative. No attempt was made to unravel the composition of the extractives afterwards. The use of 95% ethanol as solvent which could extract more polar compounds and later washing in hot water could have led to the high value of total extractives content; as explained by del Rio *et al*., (2015).

Aspergillus niger XY and *Aspergillus awamori* SB6 gave promising results in terms of cellulase and xylanase production. However, *A. niger* XY gave the highest clearance zone index in both refined cellulose and xylan agar. Clearance zone index which is the ratio of clearance zone diameter and colony diameter was calculated and used as a comparative measure to assess the enzymatic potential of the isolates. Behara *et al.* (2017) in their review on microbial cellulases listed moulds such as *Aspergillus*, among others, as common sources of microbial cellulases. Moretti *et al*. (2012) earlier implicated filamentous fungi to high abilities rate in the production of lignocellulose degrading enzymes, especially when grown in solid state culture using lignocellulosic residues as substrates.

None of the moulds tested for lignin degradation in this study gave a positive result. The complex and recalcitrant nature of lignin has made it a serious challenge in bioconversion researches as enzymes capable of metabolizing it are not well distributed among microorganisms in nature. Few microorganisms have been reported to have the innate ability to degrade lignin. Unlike the finding in this study, Yanq *et al*. (2011) isolated *Aspergillus sp* strain F-3 which was able to degrade alkali lignin and few other microbes including *Penicillium oxysporum*, *Fusarium solani*, *Chrysonilia sitophila* have been associated with lignin degradation though white rot fungi still remains more efficient than other reported microorganisms (Madadi and Abbas, 2017). In a review on enzyme degradation of lignin in soil, Datta *et al.* (2017) revealed that the most efficient organisms for lignin mineralisation are white-rot fungi. However, Pamidipati and Ahmed (2017) reported a locally isolated *Neurospora discrete* which produced about 1.5 times the amount of lignin degradation products than white-rot fungus in submerged culture using sugarcane bagasse as substrate; making it the only reported fungus that can perform better than white fungus at the current time.

Initially, one hundred and twenty yeast strains were isolated and screened for their level of growth using glucose and xylose as carbon sources. It was not surprising that all the yeast isolates grew very fast in glucose while eleven of the isolates were able to grow on xylose agar. Glucose, being a hexose sugar is known to be well fermented by most yeast whereas xylose belongs to the group of pentose sugars which are five carbon sugars that present difficulties for many types of yeast to ferment (Mohd-Azhar *et al*., 2017).

Candida tropicalis Y5 had the best growth in xylose agar. The fact that the other *Candida tropicalis* strains; *Candida tropicalis* Y4 and *Candida tropicalis* Y12 used in this study showed a weak ability to consume xylose indicated the importance of slight genetic differences among different strains in their metabolism. *Pichia kudriavzevii* Y2 and *Pichia kudriavzevii* Y12 showed moderate growth while *Saccharomyces cerevisiae* Y10 and other strains of *Pichia kudriavzevii* in this study showed very poor growth on xylose agar. Generally, wild type *Saccharomyces cerevisiae* are not known to be good fermenters of five carbon sugars though recombinant strains which are capable of fermenting pentose sugars have been recently generated (Ho *et al*., 1998). However, there remain several challenges on effect of inhibitors and high osmolarity

which must be overcome during fermentation (Moyses *et al.*, 2016). Some non-saccharomyces yeasts have been reported capable of overcoming these challenges. Some of such non-saccharomyces yeasts include *Candida spp*, *Pichia kudriavzevii, Zygosaccharomyces rouxii, Kluyveromyces marxianus and Ogataea (Hansenula) polymorpha, Dekkera bruxellensis,* and *Z. bailii* (Radecka *et al*., 2015). However, studies are on-going to consider these yeasts for large scale processes.

Unlike some lignocellulosic materials which can serve as both carbon and nitrogen sources, sugarcane bagasse has very low nitrogen content. Infact, Janke *et al*. (2015) noted a lack of nitrogen in sugarcane bagasse. Hence, it is important to supplement sugarcane bagasse biomass with nitrogen so as to aid the performance of the fermenting microorganisms. In order to find out the influence of different nitrogen sources in the bioconversion of glucose and xylose to bioethanol by the eleven yeast isolates, five nitrogen sources including potassium nitrate (KNO_3), ammonium chloride (NH_4Cl), and ammonium nitrate (NH_4NO_3), urea and yeast extract were used.

When potassium nitrate (KNO_3) was used, none of the isolates grew nor produced any detectable amount of ethanol with both glucose and xylose as carbon sources. Using glucose as the sole carbon source, the other four nitrogen sources gave good ethanol yields with fermentation efficiencies of 74% and above with the eleven isolates. Differences in ethanol yields and hence fermentation efficiencies among the isolates were more pronounced when xylose was used as the sole carbon source. Only *Candida tropicalis* strain Y5 in the presence of urea as nitrogen source gave a significant yield of ethanol with fermentation efficiency of 41% followed by 34% when ammonium chloride was used as nitrogen source. Chowdary *et al*. (2017) also reported urea as the best organic nitrogen source for *Candida tropicalis* CJ for ethanol production though their best inorganic nitrogen source reported which was ammonium nitrate gave a better product yield than urea. *Pichia kudriavzevii* Y7 also showed reduced tendency to convert xylose to ethanol when ammonium chloride was the nitrogen source. Depletion of xylose was observed to varying levels in all cases using the four nitrogen sources (ammonium chloride (NH_4Cl), and ammonium nitrate (NH_4NO_3), urea and yeast extract) even though xylose was not in most cases converted to ethanol. The fact that isolates in earlier studies all also showed tendencies to grow on xylose agar may be a confirmation that most of them have tendencies to utilise xylose for growth alone without necessarily producing ethanol. The inability of all the isolates to grow in the

presence of potassium nitrate could be due to lack of an enzymatic system responsible for nitrate reduction and also partly due to incubation conditions. Induction of nitrate reductases can be influenced by light whereas light was not provided in the incubator during this experiment. Many other researchers have reported different nitrogen compounds as the preferred nitrogen sources. Therefore, observations in this study can support the opinion of Gobert *et al*. (2017) that nitrogen demand is dependent on yeast strain, sugar content and fermentation conditions.

A pH range of 3 to 9 was used to investigate the effect of pH on the growth of the three selected isolates. Many available research reports on *Pichia kudriavzevii* did not focus on pH as a growth parameter but rather the organism has been used in different products fermentation experiments at different pH values (Elahi and Rehman, 2018). It is an indication that the organism can tolerate a wide range of acidic pH. In this study, *Candida tropicalis* Y5 grew optimally within a range of pH 5 and 6 after which there was a sharp decline in growth. This is in line with the observation of Chenga *et al*. (2009) who recorded a highest yield of biomass of *Candida tropicalis* at pH 6. Chowdary *et al*., (2017) also reported pH 5.5 as optimum pH for *Candida tropicalis* CJ. *Saccharomyces cerevisiae* Y10 had its optimum growth at pH 4 in this study and did not appear to tolerate a wide range of pH. A number of strains reported in some other researches had shown similar results (Arroyo-Lopez *et al*., 2009).

Growth of yeasts at elevated temperatures is important in ethanol industries so as to keep away undesired microorganisms which are contaminants from the production vessel. In this study, optimum growth of *Pichia kudriavzevii* Y2 and *Saccharomyces cerevisiae* Y10 was observed at 35 $^{\circ}$C while that of *Candida tropicalis* Y5 was observed at 40 $^{\circ}$C. They were all able to grow at temperatures as high as 45 $^{\circ}$C. Difference in growth between *Pichia kudriavzevii* Y2 and *Saccharomyces cerevisiae* Y10 at 45 $^{\circ}$C was not significant but *Candida tropicalis* Y5 grew significantly better than them at that temperature. The ability of the yeasts to grow at temperatures above 40 $^{\circ}$C classifies them as thermotolerant yeasts. The observation in this study is in line with that of Kiran Sree *et al*. (2000) who reported a capability of some strains of *Saccharomyces cerevisiae* to grow at 44 $^{\circ}$C. Talukder *et al*. (2016) also reported some strains of *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *Candida tropicalis* capable of growing at elevated temperatures for the purpose of ethanol production.

In order to obtain high ethanol yield during fermentation, use of concentrated sugar is very important. However, high substrate concentrations are inhibitory to fermentation due to osmotic stress. Mohd-Azhar *et al*. (2017) submitted that the increase in sugar concentration to a certain level would cause fermentation rate to increase but use of excessive sugar concentration will cause slow fermentation rate. This is because the concentration of sugar is beyond the uptake capacity of the microbial cells. Though substrate inhibition due to high concentration can be avoided by gradual addition of substrates, a high initial concentration can still be used if the fermenting microorganisms exhibit a good sugar tolerance (Ekunsanmi and Odunfa, 1990; Fakruddin *et al*., 2013). In this study, the three yeast isolates were able to tolerate all the sugar concentrations tested as lag phase was not observed to be prolonged beyond the first twelve hours of commencement of fermentation (Figures 4.4, 4.5 and 4.6). In the work of D'Amato *et al*. (2006), Sugar concentrations from 200 g/l to 300 g/l decreased *S. cerevisiae* growth rate. These authors found the lowest growth rate at the higher glucose concentrations. The same trend was observed with *Pichia kudriavzevii* Y2 in this study but some overlaps were observed with *Saccharomyces cerevisiae* Y10 and *Candida tropicalis* Y5. For instance, at 36 hours of fermentation, growth of *Candida tropicalis* Y5 in 250 g/l glucose rose higher than those of lower sugar concentrations (Figure 4.5). This observation however still indicates that the yeasts exhibit high sugar tolerance.

Unlike substrate inhibition which can be avoided by stepwise addition of substrate, ethanol inhibition can hardly be avoided during fermentation. Hence, it is very important for the fermenting organisms to exhibit ethanol tolerance. Ekunsanmi and Odunfa (1990) once asserted that ethanol tolerance is an advantage when yeast is being considered for industrial use especially where ethanol is the target product. The three selected yeasts were screened for ethanol tolerance at 0, 12.5, 15, 17.5 and 20% ethanol. Increase in ethanol concentration led to decrease in growth but significant growth was observed for the three isolates up to 15% ethanol. Effect of ethanol concentration was conspicuous at 17.5% ethanol as growth of the three yeasts was very slow and there was no growth observed at 20% ethanol. Therefore, the results obtained in this study showed a range of tolerance level between 12.5% and 17.5% ethanol in the three selected strains. This is in line with the work of Fakruddin *et al*. (2013) who reported two *Saccharomyces* strains which could tolerate up to 18% ethanol. Yang*et*

al. (2018) observed no tolerance of parent yeasts at 20% ethanol as it was observed in the current study but they were able to develop yeast hybrids which exhibited a 6.2% survival rate at 25% ethanol. It is therefore possible that the selected yeasts in the current study can be improved in terms of ethanol tolerance by applying some ethanol domestication strategies.

Acetic acid is a potential inhibitor of yeast growth and metabolism, therefore an utmost concern during ethanol production. Production of acetic acid is unavoidable in the ethanol fermentation process (Skinner and Leathers, 2004). Acetic acid is commonly found in lignocellulose hydrolysates after pretreatment procedures which involve dilute acid hydrolysis or steam exploision due to the release of acetyl group present in hemicellulose as acetic acid (Keating *et al*., 2006; Parawira and Tekere, 2011). Minor quantities of acetic acid are produced by yeast during alcoholic fermentation but acetic acid at toxic concentrations may also be produced by contaminating microorganisms (Schell *et al*., 2007). In this study, isolates were subjected to different concentrations of acetic acid (2, 4, 6, 7, 8 and 10 g/l). All three isolates were able to adapt to acetic acid inhibitor over time up to 6 g/l concentration. Only *Candida tropicalis* Y5 was able to adapt to 7 g/l acetic acid concentration as percentage inhibition gradually reduced from 87% down to 66% at the end of the incubation period. At 8 and 10 g/l acetic acid concentrations, the three isolates suffered near 100% inhibition of growth. Fakruddin *et al*. (2013) also reported total inhibition of yeast growth at 1% acetic acid concentration. Acetic acid and other inhibitory compounds have been shown to cause increased lag times, decreased growth and fermentation rates, reduced biomass and ethanol yields, and even cell death in *S. cerevisiae* cultures grown in various media (Gonçalves *et al*., 2015). Gonçalves *et al*. (2015) further explained the toxicity of acetic acid to be attributed to inhibition of glycolytic enzymes by intracellular acidification and accumulation of acetates which occurs when acetic acid transits through the plasma membrane and dissociate into acetate and protons. Therefore, *Candida tropicalis* Y5 which displayed a higher acetic acid tolerance in this study may further be improved through bioengineering procedures to restrict the influx of acetic acid into the intracellular region.

Certain pretreatment conditions on lignocellulosics result in formation of furan derivatives. These include hydroxymethylfurfural (HMF) and furfural (2-furaldehyde). Pentoses and uronic acid resulting from hydrolysis of hemicelluloses can undergo

dehydration resulting in furfural formation, while hexoses are dehydrated to form 5-hydroxymethyl-2-furaldehyde. HMF and furfural are known to have damaging effects on RNA, DNA, proteins and membranes even at low concentrations (Radecka *et al*., 2015; Jonsson and Martin, 2016) This has posed a major challenge in the ethanol industry as growth inhibition of fermenting yeasts brings about less ethanol yield. Improvement in ethanol production therefore calls for availability or development of furan tolerant yeast strains. In this study, furfural was used to assess the resistance ability of selected yeasts to furan compounds. Concentrations of furfural used include 2, 3, 4, 6 and 8 g/l. *Candida tropicalis* Y5 gave the highest adaptation at 2 g/l furfural concentration as percentage reduction falls from 61% at the twelfth hour to 9% at the twenty-fourth hour down to 2% at the forty-eighth hour. From 4 g/l to 8 g/l furfural concentrations, percentage reduction in growth was tending towards 100% except in *Candida tropicalis* Y5 which displayed a very low ability to adapt to 4 g/l furfural concentration as the percentage reduction in growth gradually decreased from 95% at the twelfth hour to 90% after 48 hours. Wang *et al*., (2016) reported a *Candida tropicalis* strain which could hardly grow up in the presence of 5 g/l furfural at the beginning of fermentation but was still able to survive as incubation progressed with time even in the presence of 5 and 7 g/l furfural. Certain non-saccharomyces yeasts have the potential to detoxify furan derivatives and thus can be greatly useful in conversion of hydrolysates resulting from lignocellulosics pretreatment into biofuels. In their review, Radecka *et al*. (2015) implicated *Pichia kudriavzevii*, *Candida stellate*, *C. ethanolica*, and *P. fermantans* (but with emphasis on *P. kudriavzevii*) as yeasts of choice in terms of furan tolerance. It is noteworthy though that *the S.* cerevisiae Y10 in this study gave similar tolerance with *P. kudriavzevii* Y2 and *C. tropicalis* Y5 at 3 g/l furfural whereas it was more sensitive at lower concentration. Some of the strains in earlier researches however were reportedly able to withstand higher concentrations of furfural than those in this study (Ruyters *et al*., 2015). Such differences might be due to differences in culture conditions.

During bioconversion of lignocellulosic biomass to useful substances, the primary goal of pretreatment is to improve the enzymatic hydrolysis of celluloses and hemicelluloses in order to attain an optimum amount of sugars. Various methods of pretreatment have been put forward. These included acid treatment, steam explosion, grinding, autohydrolysis or hot water treatment, alkali treatment etc. (Kim *et al*.,

2016). Due to some desirable features which include the use of chemicals that are non-polluting, non-corrosive and the ability of alkaline reagents to remove lignin more efficiently, alkaline pretreatment has become one of the leading preferred pretreatment methods (Kim *et al*., 2016). Calcium hydroxide (lime), sodium hydroxide, sodium carbonate and ammonia are examples of alkali commonly employed in pretreatment technology but most studies had focussed on sodium hydroxide as it was believed to give a better result than others (Ahmadi *et al*., 2016; Rezende *et al*., 2011). However, Paixao *et al*., (2016) have demonstrated that potassium hydroxide, even at very low concentrations, could be a better alternative to sodium hydroxide based on its different reactivity patterns.

In this study, pretreatment experiments on sugarcane bagasse were carried out by examining the effects of processing times, temperatures and KOH concentrations. Kim*et al*., (2008) earlier showed the reagent (ammonia) concentration, time and temperature to affect delignification of barley hull. After each pretreatment process, the setup was neutralised by HCl solution followed by introduction of enzyme for hydrolysis. This step was a deviation from the common cumbersome practice in many researches (Liu *et al*., 2016; Paixao *et al*., 2016) in which the pretreated bagasse is washed severally with water to neutrality before enzymatic hydrolysis. The goal to eliminate the washing steps between pretreatment and hydrolysis is believed to reduce the operating cost and time demand that may be imposed by this several washing procedure.

After the pretreatment experiments and subsequent hydrolysis by commercial enzymes, sugar values recorded were analysed using the response surface methodology to determine the optimum conditions and also to understand how each independent parameter influenced the amount of sugar released after hydrolysis. Traditionally, optimization has been done by studying one parameter at a time while keeping other parameters constant. This could be time consuming and failed to account for interactive effect among the independent variables under study. The advent of response surface method as a tool for optimization has helped to eliminate these limitations (Shankar *et al*., 2015). This method has been employed with great success to optimize processes in biorefinery including pretreatment processes (Ahmadi *et al*., 2016)

Three regressions using the same set of independent variables, time, temperature and KOH concentration (x_1, x_2 and x_3 respectively) and three dependent variables, namely the concentration of total reducing sugars, the concentration of glucose and the concentration of pentoses (xylose plus arabinose) were made. The values of multiple correlation R (0.9599, 0.9312 and 0.9620) and high coefficients of determination R^2 (0.9214, 0.8672 and 0.9261) indicate that the variables were adequately fitted to the experimental data and explain approximately 92%, 87% and 93% of the whole variation of the response (Polak-Berecka et al., 2010; Idowu and Aworh, 2014). The 3-D response surface plots and the contour plots help to illustrate the combined effects of the independent variables and the combined effect of each independent variable upon the response (dependent) variables. The dependent variables (total reducing sugars, pentoses and glucoses) are more influenced by variation in KOH concentration than other two parameters that is, temperature (T) and time (t) which have similar and less effects on product output. This implies that more attention needs be paid to KOH concentration during the pretreatment process than the other two factors. This observation agrees with that of Paixao et al., (2016) who also reported that KOH concentration was more important in sugarcane bagasse pretreatment than time. Experimenting with optimum values of studied parameters for total reducing sugar (pretreatment time 211 minutes, temperature 93 $^{\circ}$C and KOH concentration 162.8 mg/g bagasse), highest value of total reducing sugar of 20 g/l was recorded in this study. This equals 600 mg sugar/g biomass. Approximately 20 g/l was also recorded when the time was reduced from 211 minutes to 120 minutes and at KOH concentration of 150mg/g bagasse. As time was a less important variable, more than 1 hour was saved and still achieving the same result. In their study, Paixao et al. (2016) reported a maximum sugar value of 880mg sugar/g biomass. It should be noted that their raw biomass feedstock was richer; containing 71.39% total carbohydrate than the biomass feedstock used in this study which comprised only 60.34% total carbohydrate. Also, unlike in this study where pretreatment and hydrolysis were done without stopping; skipping the biomass washing step after pretreatment, Paixao et al. (2016) used a previously pretreated, washed and dried biomass sample for their hydrolysis experiment making their biomass sample more concentrated and comprised more than 95% total carbohydrate and thus a higher sugar yield after hydrolysis. However, it is opined that carrying out the pretreatment and hydrolysis without stopping; avoiding

the washing and drying steps in-between is more economically important at an industrial setting than the difference in sugar yields.

Attempt was made to hydrolyse raw, unpretreated bagasse using *A. niger* XY in a vessel where both enzyme production and hydrolysis occur concurrently. According to Hu *et al*. (2011), commercial cellulolytic enzymes are produced from filamentous fungi belonging to the genera *Trichoderma* and *Aspergillus* and that *A. niger* along with *A. oryzae* are the two most important fungi used worldwide among the members of the *Aspergillus* genera for biotechnological applications. Pirota *et al*. (2013) earlier showed that cost could be reduced by using whole fermentation medium which contained enzymes, mycelia and residual solid substrate for hydrolysis of lignocellulosic biomass. Inoculation of spores of *A. niger* XY in raw sugarcane bagasse did not yield any obvious growth after 15 days of incubation and no significant reducing sugar was detected. This was not unexpected as it was earlier observed in this study that the fungus could not produce ligninases necessary to disrupt the tight bond caused by lignin in order to have access to celluloses.

Images obtained by scanning electron microscopy on the surfaces of untreated sugarcane bagasse and the pretreated one showed the influence of the pretreatment on the morphology of the bagasse. Fibres of the untreated bagasse were intact with no disruption of the cell wall while the fibres of treated bagasse were more exposed and more detached from one another. As the pith is more fragile in structure, its presence was not conspicuous after the pretreatment. These changes indicated an increase in surface area within the bagasse and making it more suitable for enzymatic hydrolysis. In this study, KOH concentration of 150 mg/g bagasse which is equivalent to KOH 2.3% obtained as optimum KOH concentration was used for pretreatment and images of scanning electron microscopy obtained were similar to those obtained by Paixao *et al*. (2016) who used KOH 10%. The bagasse sample used by Paixao *et al*. (2016) contained 18.61% lignin. Lower lignin content (16.58%) of the sugarcane bagasse used in this study might make it more susceptible to a lower KOH concentration.

National Renewable Energy Laboratory (NREL) partnering with two leading enzyme companies, Novozymes and Genencor in 2010 announced in their bulletin a sharp fall by a factor of 10 in the cost of commercial cellulases and it was believed to be a major step toward commercializing large scale biomass-to-ethanol production. Despite that,

there has been a continuous effort geared towards prospecting for an enzyme system with a potential to reduce cost associated with biomass hydrolysis down further.

This study was able to confirm that solid state culture was more viable for reducing sugar production from treated bagasse using a filamentous fungus (Singhania *et al*., 2009). Other optimization procedures were carried out to determine best conditions including initial pH, temperature and nitrogen source required by the studied fungus for optimum total reducing sugar production. Highest quantity of total reducing sugar produced at the optimum conditions (pH 5, 35 ^{o}C and Urea as nitrogen source) was 18.80 mg/g bagasse (Figure 4.27). Endoglucanase and xylanase activities in the fermentation broth were measured at the peak of the experiment with *A. niger* XY in order to compare the total reducing sugar yield with that when commercial enzyme was used and it was found that the commercial enzyme used in this study was about twenty times more concentrated than the crude enzyme (fermentation broth). Going by proportion, experiment with *A. niger* XY must have produced total reducing sugar of about 30 mg/g bagasse for it to match the result obtained, that is 600 mg/g bagasse (Figure 4.23), when commercial enzyme was used. Hydrolysis was also more time consuming with *A. niger* XY (12 days) compared with commercial enzyme (20 hours). Johnson (2016) in his review buttressed the importance of an integrated system of ethanol production, which is still very much a focus of on-going researches, in which the same pre-treated cellulosic feedstock is used for both cellulase synthesis and ethanol production with native or recombinant microbial strains. However, attempt to promote such integrated system at the hydrolysis stage was not successful in this study and commercial enzyme was considered as the enzyme of choice for hydrolysis in further experiments.

Two fermentation techniques were employed to convert pre-treated sugarcane bagasse to bioethanol: the separate hydrolysis and fermentation (SHF) and the simultaneous saccharification and fermentation (SSF). In the SHF experiments, effects of monoculture and cocultures of selected yeast isolates, effect of addition of urea as nitrogen source and effect of fermentation temperature were examined.

Many researchers have reported the positive synergistic influence of employing a consortium of microbes during fermentation. As sugarcane bagasse comprises both hexoses and a reasonable amount of pentoses, the experimental trials were such that *C.*

tropicalis Y5, which had earlier been proven in this study to exhibit a good ability to ferment both hexoses and pentoses was used singly and in combination with other two selected yeasts which were also good hexose fermenters. Comparing sugar consumption between *P. kudriavzevii* Y2 and *S. cerevisiae* Y10, there is an indication that *P. kudriavzevii* Y2 could metabolise some quantity of pentose sugars though may not be converting it to ethanol. This is because sugar consumption by *P. kudriavzevii* Y2 (25.15 g/l) was significantly higher than that of *S. cerevisiae* Y10 (19.25 g/l) whereas ethanol yields of 9.16 g/l and 8.65 g/l respectively were not significantly different. Therefore, *S. cerevisiae* Y10 had higher fermentation efficiency based on glucose consumed and the ethanol yield. Ethanol yield and fermentation efficiency from single culture of *C. tropicalis* Y5 and co-cultures of it with *P. kudriavzevii* Y2 and *S. cerevisiae* Y10 were not significantly different. This confirms further that *C. tropicalis* Y5 can ferment both hexoses and pentoses without the synergy of other yeasts and therefore, *C. tropicalis* Y5 only was used in subsequent fermentations. For emphasis, fermentation efficiency is a percentage measure of the ethanol yield based on the sugar consumed in relation to the theoretical ethanol yield (0.51 g/l). Therefore the highest fermentation efficiency (88.07%) exhibited by *S. cerevisiae* Y10 should not be deceptive. It only implies that *S. cerevisiae* Y10 could convert a large proportion of its limited consumed sugar (mainly hexose sugars) to ethanol. Table 4.12 clearly showed that *S. cerevisiae* Y10 consumed the lowest amount of sugar. Over the years, *S. cerevisiae* have been popularly employed in industrial fermentation. However, due to its inability to ferment pentose sugars, recent researchers have focussed on some promising non-saccharomyces strains for that purpose. Abo-State *et al*. (2013) and Hermansyah *et al*. (2015) reported a *C. tropicalis* strain which could ferment both pentoses and hexoses from lignocellulosic hydrolysate. Unlike the *P. kudriavzevii* Y2 in this study which feeds on xylose but with no significant yield of ethanol, *Pichia kudriavzevii* has been implicated to ferment xylose and as such, a suitable candidate for bioethanol production (Talukde *et al.*, 2016). Further research into the metabolic pathways of pentose assimilation could lead into its proper manipulation so as to make the *P. kudriavzevii* Y2 isolated in this study more efficient for ethanol production from pentoses.

Addition of urea as a nitrogen source produced a significant difference in ethanol yield when *C. tropicalis* Y5 was used singly to ferment pretreated sugarcane bagasse

hydrolysate. A difference of approximately 51% increase in ethanol yield was observed when urea was introduced. Unlike in this study, Sopandi and Wardah (2017) observed that introduction of nitrogen source did not bring about significant increase in ethanol yield. Rice husk used in their study was reported to contain some proteins and nitrogen content though. It is important to note though that sugar cane bagasse does not have enough nitrogen content necessary for microbial growth (Janke *et al*., 2015) and so it is essential that a source of nitrogen be introduced during its hydrolysate conversion to ethanol. Similar to the finding in this study, Yue *et al*. (2010) observed that urea increased the ethanol yield and reduced the formation of side products during fermentation.

Earlier in this study, *C. tropicalis* Y5 was observed to grow optimally between 30°C and 40 ^{o}C and was still able to grow well at a temperature of 45 ^{o}C. Ethanol fermentation is documented to commonly proceed at low temperatures (25 ^{o}C to 37 ^{o}C) with most *S. cerevisiae* strains (Radecka *et al*., 2015). Fermentation of sugarcane bagasse hydrolysate with *C. tropicalis* Y5 at a set of temperatures; 28 ^{o}C, 35 ^{o}C and 42 ^{o}C, it was observed that maximum ethanol yield could be achieved within 28°C and 35 ^{o}C.

In SSF, saccharification of polymeric sugars by enzymes and fermentation of resulting reducing sugars by yeasts are made to happen concurrently. As optimum temperature conditions for hydrolytic enzymes and fermenting organisms are usually different, a challenge arises as to which temperature would be the best compromise between the two optimum temperatures. Therefore, SSF of sugarcane bagasse was performed using three temperature schemes which include 35 ^{o}C, intermittent change in temperature between 35 ^{o}C and 45 ^{o}C; and 45 ^{o}C alone. The enzyme mix used in this study as supplied freely by Novozyme, Denmark has optimum temperature at 45 ^{o}C; hence, the choice of 45 ^{o}C as among the variables. Optimum temperature was observed to be 35 ^{o}C with ethanol yield of 30.21 g/l and 98% fermentation efficiency. This was followed by intermittent change in temperature between 35 ^{o}C and 45 ^{o}C with 23.22 g/l ethanol and 79.62% fermentation efficiency.

As expected, SSF gave a higher maximum yield of ethanol than SHF in this work. During SSF, glucose is being rapidly converted to ethanol and so gives higher yield than SHF because low residual sugar relieves inhibition on the hydrolytic enzymes.

The advantages of SSF over SHF are well documented (Dahnum *et al*., 2015; Suttikul *et al*., 2016). The *C. tropicalis* Y5 reported in this study appears to be more efficient than many other yeast strains already documented for ethanol production. Nadeem *et al*. (2015) reported a *S. cerevisiae* G1 strain with highest yield of ethanol of 0.15 g/g and fermentation efficiency of 29.86%. Yuan *et al*. (2017) reported *P. kudriavzevii* SI which produced ethanol 33.4 g/l representing 75% of the theoretical yield at 42°C. The maximum ethanol value of 30.21 g/l in the current study represents about 98% of the theoretical yield. Ethanol yield in this study is therefore economically important. Moreover, Yuan *et al*., (2017) used only the cellulose-rich solid residue of dilute-acid-treated biomass for SSF in their experiment. It implies that only fermentation of hexoses was accounted for. For easier comparison of ethanol yields during SSF among different studies, it is important that consideration be put into quantities and types of reducing sugar consumed by the organism which is converted to ethanol and not just the fermentation efficiency as used in several reports. Fermentation efficiency is calculated thus:

$$\text{Fermentation Efficiency} = \frac{ethanol\ produced \frac{g}{l}}{reducing\ sugar\ consumed \frac{g}{l}} \ x \left(\frac{1}{0.51}\right) x\ 100,$$

Where 0.51 g of ethanol is the theoretical yield from 1g of glucose.

Therefore, in a fermentation system, a non-pentose fermenter can rapidly convert the hexose sugars to ethanol, resulting in high fermentation efficiency leaving the pentose sugars unused. Whereas an organism which ferments both hexose and pentose sugars might produce more ethanol with relatively less fermentation efficiency based on the equation above. However, such hexose and pentose fermenter should be preferable for ethanol fermentations which involve substrates containing both hexose and pentose sugars.

Brazil has the world's lowest cost for bioethanol production, in the range of US$0.68–US$0.95 per gallon using sugarcane as feedstock (Shapouri and Salassi, 2006). In the USA over 90% of bioethanol produced is from corn (Dias De Oliveira *et al*., 2005) and there was a significant increase in bioethanol production from 3.54 billion gallons in 2004 to 4.58 billion gallons in 2006 (Balat *et al*., 2008). However, Sugarcane bagasse is renewable and its use for bioethanol production is economically sustainable than the use of food crops which are being used by many current bioethanol companies in

Brazil and other developed nations like the United States. In this way, wastes usually available free can be turned in to wealth thereby providing a safer and cleaner way of waste disposal. Awoyale and Lokarht (2018) revealed in their review that in Nigeria, lignocellulosic bioethanol production potential from agricultural residues amounts to about 7.556×10^9 L per annum with more than 62% generated from process residues and that sub-Saharan Africa generate about 30% of the sugarcane produced in Africa. Nigerians demand for sugarcane was estimated to be 1.5 billion tonnes annually (Galadima et al., 2011). One tonne of sugarcane stems can generate close to 270 kg of bagasse, which is a reasonable quantity for processing to produce bioethanol (Awoyale and Lokarht, 2018). Techno-economic feasibility study of bioethanol production using sugarcane was recently carried out in Nigeria by Oyegoke and Dabai (2018). They reported that a 148 million litres/annum sugarcane-based bioethanol plant in Nigeria is economically feasible with estimated benefit/cost ratio, net present worth, payback period and return on investment value(s) of 1.46, $4.29 million, 10 years and 8% respectively. However, the feedstock considered in their analysis included both sugarcane juice and the bagasse. In the current study, only sugarcane bagasse was used to produce bioethanol with a promising ethanol yield of 30.42 g/l which is equivalent to 0.46 g ethanol per one gram of bagasse used during the simultaneous saccharification and fermentation. Therefore, 1.5 billion tonnes of sugarcane would generate 405 million tonnes of bagasse which in turn can produce 186.3 million tonnes (233.1 million litres) of bioethanol per annum. However, a holistic cost analysis of all factors including both fixed and circulating capital inputs which affect production is necessary to be carried out for bioethanol production from sugarcane bagasse.

CONCLUSION

Environmental concerns have made it imperative for production of renewable lignocellulose based fuels to replace the current widely used fossil fuels. However there still exist some bottlenecks which do not make the production process economically viable. Simplifying the process to be economically viable would include combination of approaches to develop biocatalyst with improved resistance to inhibitors or inhibitors free fermentation system, reduce energy demands at various levels of production and co-fermentation of hexose and pentose sugars in the same vessel using appropriate microbial strains.

Among one hunded and twenty (120) yeasts isolated in this study, the selected eleven (11) which were able to grow on both glucose and xylose agar were identified as *Saccharomyces cerevisiae* (1), *Pichia kudriavzevii* (7) and *Candida tropicalis* (3). These isolates converted glucose to ethanol with high efficiency. They tolerated high temperature of 48 ^{o}C, ethanol concentration of 17.5% and adapted to acetic acid and furfural at varying concentrations for growth. *Candida tropicalis* also converted xylose to ethanol. These isolates are suitable for industrial use in the production of ethanol from cellulosic materials.

This study demonstrated the use of low concentration of KOH 2.3% (w/v) to pretreat sugarcane bagasse to expose maximum cellulosic contents. The use of this alkaline solution prevented the production of inhibitory substances that could have been produced if acid was used for the pretreatment. It was also shown in this study that the washing steps that commonly follow pretreatment experiments before hydrolysis in many other similar research reports could be avoided by neutralizing with hydrochloric acid. This is believed to reduce the cost of energy required for mixing during washing. The price of cellulase for enzymatic hydrolysis is also an important factor that determines the overall cost of bioethanol production. Despite the sharp drop in the cost of enzyme as announced by the Novozyme enzyme company in 2010, it is still recommended that further studies be carried out to establish the economic benefits of an on-site production of cellulase such that the enzyme is produced in the same vessel where bioethanol production takes place thereby removing the cost required for enzyme processing and transportation.

It is generally believed that coculture of selected yeasts could improve the outcome of fermentation due to co-fermentation of hexose and pentose sugars. However, it was observed in this study that *Candida tropicalis* Y5 could compete well with many other reported ethanol producing yeasts in terms of tolerance to stress factors and could, in a monoculture, ferment both hexose and pentose sugars to ethanol considerably well. Metabolic engineering of *C. tropicalis* Y5 to enhance conversion of xylose to bioethanol and subsequent evaluation of synergy between metabolically engineered *C. tropicalis* Y5 and efficient hexose fermenters would hopefully bring about a drastic increase in bioethanol yield.

Sugarcane bagasse being a waste and a renewable material makes its use for bioethanol production economically viable than the use of food crops as we have in the first generation biofuels. Transportation of the bagasse to bioethanol plants however may add to the cost of production. Therefore, siting of bioethanol plants at the premises of sugar factories is also preferable.

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APPENDIX
Yeast Extract Peptone Dextrose Agar (YEPD) (Sigma)

Ingredients	(g/l)
Peptone	20
Yeast extract	10
Glucose	20
Agar	15

Potato Dextrose Agar (PDA) (Himedia)

Ingredients	(g/l)
Potatoes infusion from	200
Dextrose	20
Agar	15

Figure 4.1: Quantitative screening of moulds isolated from decaying sugarcane bagasse for endoglucanase, beta-glucosidase and xylanase production using liquid medium

Isolates	Endoglucanase (U/ml)	Beta-glucosidase (U/ml)	Xylanase (U/ml)
SB2	8.09 ± 0.12^c	5.65 ± 0.60^b	4.05 ± 0.33^b
SB3	15.82 ± 0.43^e	4.20 ± 0.23^a	1.69 ± 0.23^a
SB5	23.75 ± 0.24^f	7.17 ± 0.33^c	25.60 ± 0.40^d
SB6	43.48 ± 0.84^g	10.49 ± 0.02^d	74.30 ± 0.04^e
SB9	22.88 ± 0.11^f	3.46 ± 0.41^a	19.80 ± 0.70^c
SB10	1.04 ± 0.50^b	7.51 ± 0.05^c	3.84 ± 0.20^b
SB11	11.48 ± 0.20^d	11.25 ± 0.24^d	17.00 ± 0.01^c
SB13	1.86 ± 0.01^a	4.71 ± 0.52^a	1.05 ± 0.01^a
SB17	1.88 ± 0.01^a	8.15 ± 0.09^c	1.90 ± 0.01^a
SB19	9.36 ± 0.20^c	3.31 ± 0.66^a	1.95 ± 0.05^a
SB20	2.29 ± 0.10^b	6.84 ± 1.00^{ab}	1.35 ± 0.08^a
XY	$\mathbf{60.34\pm0.72^h}$	$\mathbf{14.29\pm0.02^e}$	$\mathbf{82.67\pm0.65^f}$

Data are presented in means ± standard deviation of results. Values with same superscript along the same column are not significantly different.

Figure 4.4: Effect of different glucose concentrations on growth of *P. kudriavzevii* Y2 at different time intervals

Glucose concentration (g/l)	12hr	24hr	36hr	48hr	60hr
100	1.64±0.26[b]	3.26±0.02[c]	6.01±0.13[c]	6.88±0.20[a]	5.29±0.14[a]
150	1.68±0.28[b]	3.51±0.29[c]	6.09±0.01[c]	6.92±0.22[ab]	5.41±0.10[a]
200	1.69±0.20[b]	3.55±0.30[c]	6.22±0.28[c]	7.08±0.11[b]	5.78±0.01[a]
250	0.92±0.01[a]	2.62±0.24[b]	5.01±0.28[ab]	5.4±0.21[a]	5.46±0.05[a]
300	0.65±0.10[a]	1.95±0.24[a]	4.72±0.28[a]	5.09±0.20[a]	5.16±0.03[a]

Figure 4.5: Effect of different glucose concentrations on growth of *C. tropicalis* Y5 at different time intervals

Glucose concentration (g/l)	12hr	24hr	36hr	48hr	60hr
100	3.52 ± 0.21^b	7.01 ± 0.10^{bc}	7.27 ± 0.09^{ab}	7.08 ± 0.14^{ab}	5.77 ± 0.02^a
150	3.53 ± 0.07^b	8.08 ± 0.18^c	8.29 ± 0.03^b	8.34 ± 0.37^b	5.92 ± 0.20^a
200	3.32 ± 0.25^b	8.19 ± 0.35^c	8.34 ± 0.92^b	8.40 ± 0.36^b	6.1 ± 0.02^b
250	1.56 ± 0.72^a	6.09 ± 0.37^b	8.53 ± 0.32^b	8.73 ± 0.24^b	8.67 ± 0.26^c
300	1.39 ± 0.28^a	5.51 ± 0.18^a	6.28 ± 0.50^a	6.43 ± 0.36^a	6.49 ± 0.07^b

Figure 4.6: Effect of different glucose concentrations on growth of *S. cerevisiae* Y10 at different time intervals

Glucose concentration (g/l)	12hr	24hr	36hr	48hr	60hr
100	0.66 ± 0.30^b	7.35 ± 0.00^b	7.53 ± 0.02^a	7.58 ± 0.01^a	6.47 ± 0.00^b
150	0.63 ± 0.10^b	7.37 ± 0.30^b	8.10 ± 0.10^b	8.00 ± 0.60^b	6.63 ± 0.33^b
200	0.56 ± 0.04^b	8.30 ± 0.01^b	8.38 ± 0.04^b	7.07 ± 0.02^a	5.69 ± 0.02^a
250	0.19 ± 0.02^a	4.62 ± 0.00^a	8.13 ± 0.55^b	8.15 ± 0.02^b	8.20 ± 0.00^c
300	0.13 ± 0.00^a	4.57 ± 0.02^a	7.51 ± 0.17^a	7.57 ± 0.00^a	7.60 ± 0.30^b

Data are presented in means ± standard deviation of results. Values with same superscript along the same column are not significantly different.

Figure 4.7: Effect of ethanol concentration on growth of selected yeasts isolated from decaying sugarcane bagasse

Isolate	0.0%	10.0%	12.5%	15.0%	17.5%	20.0%
Y2	7.72 ± 0.23^a	7.23 ± 0.23^a	6.30 ± 006^a	2.15 ± 0.65^a	0.49 ± 0.15^a	0.00 ± 0.00^a
Y5	8.52 ± 0.11^a	7.87 ± 0.02^a	7.15 ± 0.34^a	3.68 ± 0.04^a	0.70 ± 0.02^a	0.12 ± 0.01^a
Y10	7.61 ± 0.56^a	7.32 ± 0.12^a	6.66 ± 0.23^a	3.03 ± 0.06^a	0.47 ± 0.13^a	0.00 ± 0.01^a

Figure 4.8: Effect of different concentrations of acetic acid on growth of *P. kudriavzevii* Y2 at different times.

Acetic acid concentration (g/l)	12 hours	24 hours	36 hours	48 hours
0	$0.00\pm0.00^*$	$0.00\pm0.00^*$	$0.00\pm0.00^*$	$0.00\pm0.00^*$
2	12.04 ± 0.25^a	2.42 ± 0.57^a	0.59 ± 0.24^a	1.55 ± 0.28^a
4	73.14 ± 0.10^b	60.60 ± 2.10^b	57.06 ± 0.38^b	45.49 ± 0.22^b
6	72.23 ± 0.27^b	70.05 ± 0.44^c	62.94 ± 0.93^b	50.99 ± 0.18^c
7	62.96 ± 0.11^{ab}	98.39 ± 0.77^d	98.68 ± 0.05^b	98.73 ± 0.30^d
8	81.48 ± 0.03^d	98.39 ± 0.45^d	98.97 ± 0.64^b	99.15 ± 0.83^d
10	86.11 ± 0.40^e	98.85 ± 0.90^d	$98.97\pm1.0b^b$	99.01 ± 1.05^d

Data are presented in means ± standard deviation of results. Values with same superscript along the same column are not significantly different.

The asterisks "*" indicates that there is no observable difference in the measured variable hence no significance was possible.

Figure 4.9: Effect of different concentrations of acetic acid on growth of *C. tropicalis* Y5 at different time intervals

Acetic acid concentration (g/l)	12hours	24hours	36hours	48hours
0	$0.00\pm0.00^*$	$0.00\pm0.00^*$	$0.00\pm0.00^*$	$0.00\pm0.00^*$
2	35.00 ± 0.23^a	33.00 ± 2.40^a	0.10 ± 0.04^a	1.00 ± 0.10^a
4	69.00 ± 0.50^b	63.00 ± 0.22^b	26.00 ± 0.80^b	30.00 ± 0.26^b
6	73.00 ± 0.55^c	77.00 ± 0.05^c	66.00 ± 0.70^c	52.00 ± 0.18^c
7	87.00 ± 0.21^d	79.00 ± 0.04^c	71.00 ± 0.09^d	66.00 ± 0.10^d
8	98.00 ± 0.30^e	99.00 ± 0.10^d	99.00 ± 0.20^e	99.00 ± 0.05^e
10	96.00 ± 0.00^f	98.00 ± 0.40^d	99.00 ± 0.06^e	99.00 ± 1.00^e

Figure 4.10: Effect of different concentrations of acetic acid on growth of *S. cerevisiae* Y10 at different time intervals

Acetic acid concentration (g/l)	12hours	24hours	36hours	48hours
0	$0.00\pm0.00^*$	$0.00\pm0.00^*$	$0.00\pm0.00^*$	$0.00\pm0.00^*$
2	30.00 ± 0.28^a	28.00 ± 0.62^a	25.00 ± 0.70^a	15.00 ± 0.23^a
4	54.00 ± 0.03^b	29.00 ± 0.00^a	51.00 ± 0.00^b	44.00 ± 0.29^b
6	77.00 ± 0.57^c	60.00 ± 0.23^b	77.00 ± 0.65^c	55.00 ± 1.00^c
7	94.00 ± 0.75^d	96.00 ± 0.24^c	97.00 ± 0.18^d	97.00 ± 0.24^d
8	95.00 ± 0.48^d	97.00 ± 0.25^c	98.00 ± 0.81^d	98.70 ± 0.30^d
10	96.00 ± 0.26^d	98.00 ± 0.05^c	99.20 ± 0.14^d	98.10 ± 0.00^d

Data are presented in means ± standard deviation of results. Values with same superscript along the same column are not significantly different.

Figure 4.11: Effect of different concentrations of furfural on growth of *P. kudriavzevii* Y2 at different time intervals

Furfural concentration (g/l)	12hours	24hours	36hours	48hours
0	$0.00\pm0.00^{*}$	$0.00\pm0.00^{*}$	$0.00\pm0.00^{*}$	$0.00\pm0.00^{*}$
2	38.00 ± 0.20^{a}	18.00 ± 0.90^{a}	34.00 ± 0.62^{a}	27.00 ± 0.72^{a}
3	75.00 ± 0.36^{b}	71.00 ± 0.22^{b}	66.00 ± 0.66^{b}	47.00 ± 0.10^{b}
4	97.00 ± 0.10^{c}	98.00 ± 0.00^{c}	99.00 ± 2.20^{c}	99.00 ± 0.10^{c}
6	97.00 ± 0.30^{c}	98.00 ± 0.45^{c}	99.00 ± 0.82^{c}	98.40 ± 0.00^{c}
8	98.40 ± 0.20^{c}	99.00 ± 0.23^{c}	99.00 ± 0.00^{c}	99.10 ± 0.05^{c}

Figure 4.12: Effect of different concentrations of furfural on growth of *C. tropicalis* Y5 at different time intervals.

Furfural concentration (g/l)	12hours	24hours	36hours	48hours
0	$0.00\pm0.00^*$	$0.00\pm0.00^*$	$0.00\pm0.00^*$	$0.00\pm0.00^*$
2	$6\ 1.00\pm0.27^a$	9.00 ± 0.40^a	9.00 ± 0.12^a	2.00 ± 0.00^a
3	81.00 ± 0.28^b	75.00 ± 0.10^b	57.00 ± 0.26^a	53.00 ± 0.02^b
4	95.00 ± 0.28^c	93.00 ± 0.12^c	93.00 ± 0.00^c	90.00 ± 0.045^c
6	96.00 ± 0.30^c	96.00 ± 0.21^c	97.00 ± 0.26^c	97.00 ± 0.11^c
8	98.50 ± 0.00^c	99.00 ± 0.46^c	99.40 ± 0.21^c	99.00 ± 0.04^c

Data are presented in means ± standard deviation of results. Values with same superscript along the same column are not significantly different.

Figure 4.13: Effect of different concentrations of furfural on growth of *S. cerevisiae* Y10 at different time intervals.

	12hours	24hours	36hours	48hours
0g/l	$0.00\pm0.00^*$	$0.00\pm0.00^*$	$0.00\pm0.00^*$	$0.00\pm0.00^*$
2g/l	51.00 ± 0.02^a	76.00 ± 0.27^a	38.00 ± 0.66^a	35.00 ± 0.00^a
3g/l	73.00 ± 0.20^b	76.00 ± 0.80^a	56.00 ± 0.50^b	54.00 ± 0.50^b
4g/l	76.00 ± 0.00^c	98.70 ± 0.08^d	98.00 ± 0.44^c	98.40 ± 0.36^c
6g/l	97.00 ± 0.30^d	99.00 ± 0.10^d	98.50 ± 0.10^c	99.00 ± 0.00^c
8g/l	98.00 ± 0.19^d	99.20 ± 0.24^d	99.00 ± 0.00^c	99.00 ± 0.04^c

Figure 4.24: Effect of solid state and submerged fermentation conditions on sugarcane bagasse hydrolysis by *Aspergillus niger*XY

	SmF	SSF
Day 2	4.73±0.05[a]	0.00±0.00[a]
Day 5	5.71±0.23[b]	6.84±0.53[b]
Day 10	5.12±0.03[b]	12.74±0.15[c]
Day 12	6.05±0.18[c]	14.97±0.85[d]
Day 15	6.05±0.22[c]	14.59±0.05[d]
Day 20	6.05±0.45[c]	14.22±0.35[d]

Figure 4.25: Effect of initial pH on reducing sugar production in solid state hydrolysis by *Aspergillus niger* XY

	pH3.5	pH4	pH5	pH5.5	pH6	pH7
Day 5	5.07 ± 0.45^a	6.61 ± 0.73^a	6.91 ± 0.03^a	6.62 ± 0.35^a	4.35 ± 0.33^a	3.20 ± 0.43^a
Day 10	9.17 ± 0.28^c	12.72 ± 0.67^b	13.28 ± 0.20^b	12.29 ± 0.64^{ab}	7.77 ± 0.48^{bc}	6.18 ± 0.62^c
Day 12	8.16 ± 1.00^{bc}	13.86 ± 0.84^{bc}	15.09 ± 0.06^{bc}	15.09 ± 0.58^b	8.44 ± 0.25^c	8.28 ± 0.28^d
Day 15	7.50 ± 0.50^b	14.55 ± 0.29^c	14.51 ± 0.42^c	14.30 ± 0.32^b	6.12 ± 0.95^{bc}	6.11 ± 0.66^c
Day 20	8.44 ± 0.63^{bc}	14.69 ± 0.24^c	13.88 ± 0.44^b	13.83 ± 0.96^b	6.08 ± 0.10^{bc}	5.63 ± 0.20^b

Figure 4.27: Effect of nitrogen sources on reducing sugar production in solid state hydrolysis of bagasse by *Aspergillus niger* XY after 13 days of incubation.

	Average reducing sugar (mg/g bagasse)
NH_4Cl	15.88 ± 0.33^b
NH_4NO_3	15.81 ± 0.42^b
Urea	18.8 ± 0.84^c
Peptone	12.89 ± 0.23^a
yeast extract	14.36 ± 0.33^b

Sequence of *Pichia kudriavzevii*Y1

CATTATACGGTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTTATTT
GATAGTTCCGTTCTACATGGATAACCGTGGAAAATCTAGAGCTAATACATG
CGTAAAGCCCCGACTTCGGGAGGGGTGTATTTATTAGATAAAAAATCAAT
GCCCTCGGGCCTTTTGATGATTCATAATAACTTTTCGAAGCTCATGGCCTTG
CGCCGGAGCTGGTTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGG
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CGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTGACAATATATAAC
GATACAGGGCCTTTGGTCTTGTAATTGGAATGAGTACAATGTAAATACCTT
AAC GAGGAACAATTGGAGGGCAA GTCTGGTGCCA

Sequence of *Pichia kudriavzevii*Y3

CATTATACGGTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTTATT
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TTT GC CCA CTC GGCAC CTTAC

Sequence of *Candida tropicalis* Y5

TGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTTATTTGATAGTACCTTACT
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TCATAATAACTTTTCGAATCGCATGGCCTTGTGCTGGCGATGGTTCATTCAAATT
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AGGAAAG GGGGCAACTCC ATTCTGGAACCGAGAA

Sequence of *Pichia kudriavzevii* Y2

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TTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTTTT
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Sequence of *Pichia kudriavzevii*Y6

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ACTTTCCCATGGGGCCGAG

Sequence of *Saccharomyces cerevisiae* Y10

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Sequence of *Pichia kudriavzevii*Y11

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AGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTGACAATAT
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Sequence of *Candida tropicalis*Y12

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Sequence of *Pichia kudriavzevii*Y13

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Sequence of *Aspergillus niger*XY

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