

Adaptation of xylose fermenting yeasts, isolated from various sources in the Limpopo province, to improve ethanol production in the biofuel industry

by

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DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo for the degree of Master of Science in Microbiology has not been previously submitted by me for a degree at this or any other University, that this is my work in design and execution, and that all material contained herein has been acknowledged.

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Signature: _____

DEDICATION

This work is dedicated to my son, who continues being my inspiration and encourages me to work hard and my wonderful parents: the late Mashudu Michael Tshivhase and loving mother Shonisani Elizabeth Tshivhase who introduced me to the joys of reading and importance of education, enabling such a study to take place.

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ABSTRACT

The recent oil crisis and environmental concerns over fossil fuels has led to the development of biofuels from lignocellulosic materials. Two main sugars from lignocellulose that can be used for bioethanol production are glucose and xylose. Xylose is problematic, because there are few yeasts that can utilise and ferment it. Xylose fermentation is not as efficient compared to glucose fermentation. Some of the factors that affect xylose fermentation include rate of xylose consumption, aeration, temperature and inhibitors. To improve ethanol production and fermentations and to make the process economically viable at industrial scale, there is a need to find a robust microorganism that can ferment efficiently in harsh industrial conditions. Therefore, the aim of this study was to investigate by means of evolutionary engineering (adaptation), the adaptability of seven locally isolated yeasts in terms of growth on high xylose concentration, in the presence of acetic acid as well as at elevated temperatures. Seven yeast strains (*Candida guilliermondii* MBI2, *Candida* sp. Kp6.2ey, *Candida tropicalis* Kp21ey, *Candida tropicalis* Kp42ey, *Candida tropicalis* Kp43ey, *Ogatea methanolica* Kp2ey and *Pichia kudriavzevii* Kp34ey) were adapted to ferment 60 g/L xylose as sole carbon source in the presence of 3 g/L acetic acid at 37°C. *P. kudriavzevii* Kp34ey was the only yeast to adapt to these conditions. The adapted *P. kudriavzevii* Kp34ey was compared with the parental strain (unadapted) and a reference strain, *Scheffersomyces stipitis* NRRLY-7124, using different volumetric oxygen transfer coefficient (K_La) rates. *P. kudriavzevii* Kp34ey (adapted and parental strain) and *S. stipitis* NRRLY-7124 produced the highest ethanol concentrations at a K_La value of 3.3. Overall, for all K_La values tested, the adapted strain performed better than the parental strain and *S. stipitis* NRRLY-7124. The adapted *P. kudriavzevii* Kp34ey yielded 4.03 g/L ethanol on 60 g/L xylose with 3 g/l acetic acid at 37°C at a K_La value of 3.3 and was the only yeast tested to grow under these conditions.

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CHAPTER 1

INTRODUCTION

The decrease in fossil fuel supply and the increase in greenhouse gas emissions have led to the search for non-conventional fuel produced from bio-renewable energy sources, such as plant material (Jeffries, 2016). Bioethanol can be produced from different first, second and third generation sources of biomass (Ohgren *et al.*, 2016). The production of biofuel from plant biomass has also received worldwide interest from the transport sector (Girio *et al.*, 2010). Lignocellulosic biomass, derived from plant material, if used for the production of bioethanol is a promising alternative source for fuel, which could minimise emission of greenhouse gases resulting in great socio-economic benefits (Vleet and Jeffries, 2015). Concerns of fossil fuel use have led to enormous research activity to obtain alternatives that would save a country's dependence on imported fuel, not damage the environment and provide much needed transportation energy (Sims *et al.*, 2016).

First generation biofuel is produced from crops that are also used as food sources (mostly sugar cane and corn). Lignocellulose consists of the carbohydrate polymers: lignin, hemicelluloses and cellulose and is an abundant source of fermentable sugars for the production of second generation biofuel (Balat, 2011). Lignocellulosic biomass does not compete with food or animal feed production. Hence, lignocellulose contributes to environmental sustainability (Jeffries, 2016). Lignocellulose is abundantly available, but it is resistant to bioconversion due to its complex structural properties (Kuhad *et al.*, 2011).

The steps involved in producing bioethanol from lignocellulosic biomass include pretreatment, hydrolysis of complex carbohydrates, fermentation, and distillation for product recovery (Zhang and Geng, 2016). Fermentable sugars (mainly hexose and pentose sugars) are released from lignocellulose through pretreatment and hydrolysis, but inhibitory compounds that fermenting microbes need to resist are also produced (Tomas-Pejo *et al.*, 2010). The lack of microorganisms that are able to efficiently ferment both hexose (mostly glucose) and pentose (mainly xylose) sugars released during pretreatment and hydrolysis of lignocellulose material, is a substantial factor which limits the industrial use of lignocelluloses for biofuel production (Balat, 2011). Commercial ethanol production ideally needs a robust organism that would be able to ferment both hexose and pentose sugars, produce high ethanol yields, tolerate inhibitors present in the lignocellulose hydrolysates after pre-

treatment, resist high concentrations of ethanol and also ferment at elevated temperatures (Balat, 2011; Salinas and Grauslund, 2013).

Saccharomyces cerevisiae is the best fermenting microbe concerning the bioethanol industry. However, it cannot ferment pentose sugars (Hahn-Hagerdal *et al.*, 2014). There are a number of organisms that are able to ferment pentoses (xylose), but the problem is that some, like bacteria, produce various by-products and are unable to tolerate high ethanol concentrations. Some yeasts and moulds ferment xylose slowly and the latter also produce by-products. Most yeasts are unable to ferment at elevated temperatures and also in the presence of inhibitors (Fan *et al.*, 2013).

Genetic engineering is one approach to produce the ideal fermenting microbe, whereby the pentose phosphate pathway in yeast cells is modified, so that the yeasts are able to convert xylose to ethanol (Koppram *et al.*, 2012). However, during longer application in the laboratory, the stability of recombinant yeast strains is not guaranteed. Moreover, global acceptance of genetically modified organisms is also problematic (Ling *et al.*, 2014). The use of non-genetically engineered yeast strains that are able to utilize and convert xylose efficiently for ethanol production by use of natural selection and breeding would be beneficial (Mussatto *et al.*, 2010). Adaptation or evolutionary engineering is an effective approach to adapt fermenting yeasts to improve the efficiency of ethanol production (Tomas-Pejo *et al.*, 2010). It is therefore the emphasis of this study to evaluate the adaptability of locally isolated yeasts for effective xylose fermentation.

The aim of this study was to adapt locally isolated pentose fermenting yeasts to ferment at high sugar concentrations, elevated temperatures and in the presence of the inhibitory compound acetic acid. This was followed by fermentation studies adopting the best adapted yeast strain.

The main objectives of this study were to:

- (i) Adapt locally isolated yeast strains to high xylose concentrations.
- (ii) Adapt locally isolated yeast strains to high temperatures.
- (iii) Adapt locally isolated yeast strains to high concentrations of acetic acid.
- (iv) Conduct fermentation studies in a bioreactor using the best adapted yeast strain.

CHAPTER 2

LITERATURE REVIEW

2.1 Biofuels

Biofuels have attracted much attention in recent years, because it is renewable and environmentally friendly compared to fossil fuel, which is harmful to the environment and leads to inflation, as a result of its high price and scarcity (Balat *et al.*, 2016). The biofuel in this study will refer to bioethanol, a renewable liquid that is produced from biomass material by microorganisms. However, biofuels also include any hydrocarbon fuel that is produced from recently living organic matter. Biofuels can either be in a liquid or gas state (Eva-Mari, 2016).

Biogas is produced when organic matter is converted to a gas by microorganisms in the absence of oxygen (Willis *et al.*, 2006). Its production is through anaerobic digestion of biodegradable materials, such as municipal waste, manure, plant material and sewage by anaerobic bacteria. This gas consists of carbon dioxide and methane, mostly with small amounts of hydrogen sulphide and moisture present. Hydrogen, methane and carbon monoxide can be oxidized or combusted with oxygen and the energy released allows biogas to be used as a fuel. The biogas energy can be used in a gas engine to convert the energy in the gas into electricity and heat (Angelidaki and Sanders, 2016).

Liquid biofuels are mostly used in the transport sector and include bioethanol and biodiesel. Bioethanol is ethanol produced from plant biomass such as agricultural waste, wood, wheat, sugar cane, etc. Biofuel is an alternative fuel that is presently produced mainly from food crops. Biofuel can be used in motor vehicles in its pure form and it signifies a vital renewable fuel (Brad *et al.*, 2016). Bioethanol has been used mostly in the United States and Europe, up until the 1900s, but became costly to produce compared to petroleum fuel. Since then, bioethanol was ignored until the oil crisis began in the 1970s and a growing interest in bioethanol as an alternative transportation fuel grew since the 1980s (Vleet and Jeffries, 2015).

There are certain standards that must be met with reference to greenhouse gas (GHG) benefits to ensure bioethanol is produced responsibly. Those standards are: (1) Annual farming of feedstock crops must not be done on land that is rich in carbon, both below and

above ground, such as peat soils used as permanent grassland; (2) bioethanol plants must use biomass and not fossil fuels; (3) nitrous oxide emissions should be minimal and this can be achieved by using efficient strategies of fertilization and (4) by-products should be used resourcefully so as to maximize their energy and GHG benefits (Sun and Cheng, 2012;Choi and Sang, 2016).

The term “fuel” is referred to as any material that stores energy in a form that can be released and used as heat energy. Fuels are mostly used for transportation and power generation (Chandel *et al.*, 2014). Fossil fuels are slowly becoming depleted, because of the high demand; due to population growth in the world, increasing use of transport and electricity usage (Alvira *et al.*, 2015). There are fuel alternatives that are considered, such as: wind power, solar power, electric cars and biofuels (ethanol, biodiesel, methanol, butanol). Biofuels are divided into four categories in terms of the feedstock used in their production. The generations include first, second, third and fourth generation, with first and second generation being investigated the most (Akita *et al.*, 2016).

2.1.1. First generation biofuels

First generation biofuels are also called conventional biofuels, and are produced from different plant materials that are also used as food sources, such as starch, sugar and vegetable oil (Naik *et al.*, 2010). Any biofuel made from a feedstock that can be consumed by humans is referred to as first generation biofuel. First generation biofuels cause food insecurities and also require a large portion of land to grow food crops for bioethanol production. This has led to more crops being used for ethanol production instead of being used in the food industry and has led to a global increase in food prices over the last few years. Also, using food crops for bioethanol production raises nutritional and ethical concerns, because about 60% of humans worldwide are reported to be malnourished. First generation biofuel crops for ethanol fuel production also use up valuable land and waste water and energy resources that can be used to produce food for human consumption (Hahn-Hagerdal *et al.*, 2014).

First generation bioethanol is the most used biofuel in the world, its use dates back to 1894 in France and Germany where it was used by the emerging industry of internal combustion engines (ICEs). Brazil has been using bioethanol since 1925. The use of this fuel was prevalent in the United States and Europe until the 1900s when it became costly to produce. However, there has been a growing interest because of the oil crisis that began in the 1970s.

Currently, first generation biofuel is being applied as a gasoline additive or gasoline improver, and is usually blended with gasoline (1:9 bioethanol to gasoline). This blend is known as E10 or gasohol. The European Union (EU) quality standard suggests a blend of 5% bioethanol with petrol because the blend does not require any engine modification and is also covered by vehicle warranties (Naik *et al.*, 2010).

Eva-Mari (2016) reported that the EU is looking into banning the use of first generation biofuels, because it appears that their use does not efficiently reduce greenhouse gas emissions and they put agriculture, food and the natural ecosystem at risk. Hence there is a dire need to successfully implement the use of either second, third or fourth generation biofuels.

2.1.2. Second generation biofuels

Second generation biofuels are produced from non-food plant materials (lignocellulose), such as municipal solid wastes, agricultural residues (rice straw) forestry waste and energy crops like grass. The production of second generation biofuels was encouraged as first generation biofuels are highly controversial, because they result in the rise of food prices, deforestation and food availability. The advantage of lignocellulosic biofuels is the use of agricultural waste and energy crops on marginal land that does not compete with food production (Sarkar *et al.*, 2012).

Presently, production of second generation biofuels is not cost effective due to technical barriers faced, such as overcoming the recalcitrance of the lignocellulose material, costs in the production of lignocellulolytic enzymes and finding robust microorganisms able to handle fermentative stress conditions, such as high temperatures and inhibitors produced during the pretreatment step. Numerous countries, including South Africa are involved in overcoming these technical barriers (Dias *et al.*, 2015).

2.1.3. Advantages of using bioethanol

Bioethanol from lignocellulose is a good transport fuel as ethanol has distinctive and desirable features. Bioethanol has a high octane number and is not toxic, which is a measure of the gasoline quality that can be used to prevent early ignition which leads to engine knock, and additionally its flammability limits are broad. The high oxygen content in

ethanol permits better oxidation and helps in reducing carbon monoxide and aromatic compound emissions, which are harmful to the environment (D'Amore *et al.*, 2014). Biomass has the potential to offer a secure source of raw material for the production of bioethanol as it has minimal fossil fuel inputs and it has very little conflict with the use of land for production of food.

2.1.3.1. Environmental and economic importance

Presently, the world is greatly reliant on various fossil energy sources such as oil, coal, natural gas, etc. (Martin *et al.*, 2007). Biofuels are vital because they decrease the dependence on fossil fuels, lower greenhouse gas emissions and bring business opportunities to rural communities (Jin and Jeffries, 2014). Biofuels could be an economical substitute in the coming years in many countries with oil prices increasing and improvement of cheaper and efficient technologies in lignocellulose conversion to ethanol (Balat, 2011).

Production of biofuels for transport can assist in reducing the accumulation of CO₂. The key advantage of bioethanol is its greenhouse gas (GHG) benefits. Ethanol is low in volatility, toxicity, and photochemical reactivity, which results in the reduction of ozone formation (Ohgren *et al.*, 2016).

2.1.3.2. Ethanol vs gasoline

Ethanol, when compared with gasoline has a higher octane rating, which means that it has the ability to resist compression, which enables combustion engines to run at a high compression ratio and this gives it a high performance rate. Moreover, the heat vaporization and the vapour pressure of ethanol are greater than that of gasoline (Chu and Lee, 2014). Conversely, due to the oxygen content of ethanol, it has 33% less energy than gasoline. Ethanol also emits acetaldehyde, which causes respiratory tract infections. Petrol causes air pollution because of the incomplete combustion that occurs. Ethanol produces 60-90% less carbon dioxide compared to petrol (Anderlei *et al.*, 2015).

2.2. Lignocellulose

Lignocellulose refers to plant material, also called plant biomass. These materials are readily available and are a favourable feedstock for industrial production of low-cost fuel ethanol (Martin *et al.*, 2007). The three main components of lignocellulose material are cellulose (35-50% of dry weight), hemicellulose (15-35% of plant dry weight) and lignin (15-25% of dry weight). The lignocellulosic complex is the most plentiful biopolymer on earth. Lignocellulose comprises of about 50% of the biomass in the world and it is estimated that 20-60 billion litres of bioethanol can be produced sustainably annually (Mussatto and Teixeira, 2010; Girio *et al.*, 2010).

The lignocellulose material that can be used for ethanol production is divided into four different groups (figure 2.1) namely: hardwood (poplar, etc.), crop residues (wheat straw, corn stover or rice straw, municipal solid waste, etc.), softwood (pine), and herbaceous biomass, such as switchgrass (Sims *et al.*, 2016).

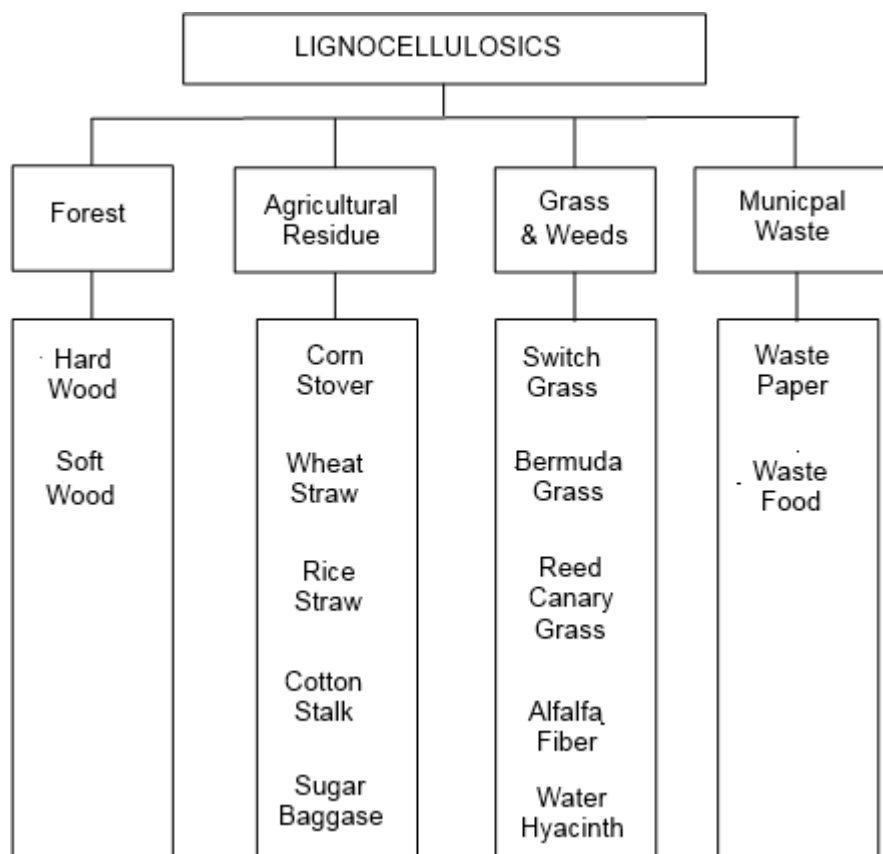


Figure 2.1: The main lignocellulosic materials that are/ have been explored for production of biofuels (Blanca-Ocreto, 2013)

2.2.1. Cellulose

Cellulose is an abundant component of natural biomass and has attracted interest due to its characteristic of being a good source for biofuel production, such as bioethanol. It is a water insoluble plant biomass found in terrestrial plants, agricultural wastes and algae (Pulidindi *et al.*, 2014). Cellulose is comprised of anhydrous glucose that is linked by β (1,4)-glycosidic bonds, with the disaccharide cellobiose being the basic repeating unit (Alvira *et al.*, 2015).

Cellulose can be hydrolyzed by cellulases to yield glucose monomers, which can in turn be fermented to bioethanol. Cellulases used are mainly fungal based and are a multi-enzyme complex composed of three enzymes, which act in synergy for complete cellulose hydrolysis. These enzymes include endoglucanases, β -glucosidases and exoglucanases (Sukumaran *et al.*, 2012). Hydrolysis of plant biomass breaks down celluloses to simple fermentable sugars by cleaving β -1, 4-glycosidic bonds within cellulose through catalysis of hydrogen ions (Yanuar *et al.*, 2014).

In order to produce glucose for subsequent fermentation to ethanol, hydrolysis of cellulose can either be achieved enzymatically or by using concentrated or dilute acid. For ethanol produced from cellulose to be economical certain requirements should be considered: (i) the efficiency and cost of conversion of lignocellulose to ethanol and cost; (ii) the product revenue, and (iii) the feedstock cost (Sun and Cheng, 2012).

2.2.2 Hemicellulose

Hemicelluloses are a heterogeneous class of polymers which makes up 15-35% of the total dry weight of wood. Hemicelluloses include xylan, arabinan, mannan and galactan, the classification is dependent on sugar moieties that are present. The main monomers are D-xylose and D-arabinose which are pentoses, however other monomers such as D-glucose, D-mannose and D-galactose, the hexoses, are also present. Hemicellulose is hydrolyzed easily by xylanases to monomeric sugars under mild conditions (Sarkar *et al.*, 2012).

Hemicellulose is hydrolyzed by dilute acid, alkali and by hemicellulases. Hydrolysis of hemicelluloses release pentose and hexose monomers that can be converted to ethanol or valuable acids, such as levulinic acid and formic acid (Girio *et al.*, 2010). The two main

pentoses found in hemicelluloses are D-xylose and L-arabinose with D-xylose being the most abundant pentose sugar in hemicelluloses followed by L-arabinose. The D-xylose content in hemicellulose constitutes about 24% of dry weight (du Preez *et al.*, 2008).

2.2.3. Lignin

Lignin is a heterogeneous aromatic polymer and also a macromolecule of phenolic character that is plentiful in nature, around 40 to 50 billion tons is produced annually. It is a dehydration product of monomeric alcohols; coniferyl alcohol, coumaryl alcohol and sinapyl alcohol. It binds the fibres, cells and vessels which make up the plant material (Abdel-Hamid *et al.*, 2013).

The spaces between hemicellulose, cellulose and pectin components are sealed by lignin. Lignin is covalently linked to hemicellulose thereby giving the whole plant mechanical strength. Hence, it is difficult to degrade lignin effectively. White rot fungi are able to degrade lignin by producing laccase enzymes and extracellular peroxidases (Buqq *et al.*, 2011).

2.3. Ethanol production from lignocellulosic biomass

Ethanol is known to be a suitable alternative to the common transport fuel, petrol, because it is renewable. Extensive research is being carried out in order to achieve an efficient conversion of lignocellulose to ethanol. The conversion is achieved through a series of steps, namely:

1. Pretreatment - this includes breaking down the lignocellulose material to improve hydrolysis
2. Hydrolysis - the breaking down of complex polymers into simpler sugars
3. Fermentation - yeast metabolic process of converting sugars into alcohol
4. Distillation - whereby ethanol is purified in order to meet fuel specifications and be used in automobiles

The main challenges being tackled are the pretreatment, hydrolysis, and fermentation steps (McMillan 2015).

2.3.1. Pretreatment

The first step in the conversion of lignocellulose to bioethanol is pretreatment. The main aim of pretreatment is to destroy the lignin shell protecting cellulose and hemicellulose (figure 2.2), to increase porosity, to decrease crystallinity of cellulose and also to allow enzymes access to the substrate. The following requirements are needed for a successful pretreatment (1) minimal loss of carbohydrates (2) increased sugar formation or the capability to form sugars by enzymatic hydrolysis (3) minimal formation of inhibitors and (4) cost efficiency (Koppram *et al.*, 2012).

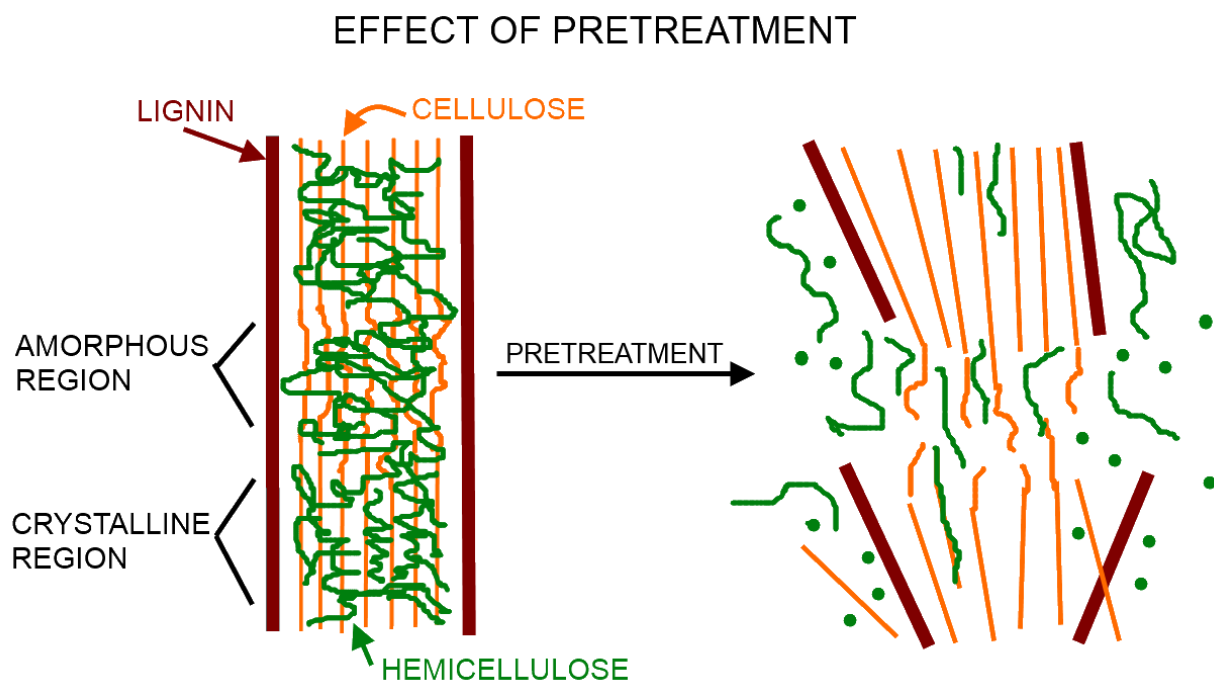


Figure 2.2: Breaking down of the lignocellulosic component during pretreatment (Blanca-Ocreto, 2013).

There are various technologies that have been proposed on how to pretreat lignocellulosic material. These technologies can be classified into biological pretreatment, physical pretreatment, physico-chemical pretreatment and chemical pretreatment. Biological pretreatment employs the use of microorganisms to degrade the lignocellulose material with

white-rot fungi, such as *Phanerochaete chrysosporium*. The main objective of physical pretreatment is to reduce the particle size of lignocellulose in order to increase the surface area and reduce the degree of polymerization. This can be achieved by grinding, milling or chipping. The particle size ranges from 0.2-30 mm depending on the physical method used. Examples of chemical pretreatment are alkali pretreatment and acid pretreatment, whereby the aim is to solubilize the hemicellulose fraction and make cellulose more accessible to enzymes (Alvira *et al.*, 2015). During the pretreatment step, the process of secondary decomposition leads to the formation of by-products that are inhibitory or even toxic to microorganisms responsible for fermenting the sugars released, this inhibitory compounds include phenolics, organic acids and furans (Novy *et al.*, 2014).

2.3.2. Hydrolysis

Hydrolysis is a vital process used in the conversion of pre-treated biomass to fermentable sugars. The two methods used in this process are the use/application of enzymes and acids, commonly referred to as enzyme hydrolysis and acid hydrolysis (Zheng, 2014).

Enzymatic hydrolysis is a more effective and favourable method, because it is less toxic, less corrosive and has a high conversion rate (Blanch *et al.*, 2014). Enzymes also use less energy and require environmental conditions that are mild. Most importantly, enzymatic hydrolysis does not form inhibitory compounds. However, cellulolytic enzymes are substrate specific and are unstable when exposed to high temperatures. The high cost of enzyme production and the recovery process from the reaction mixture could also be problematic (Kumar *et al.*, 2013).

Acid hydrolysis of lignocellulose results in compounds that inhibit fermenting microorganisms. Some of the inhibitors that are present in hydrolysates of lignocellulosic biomass are furaldehydes, such as furfural and hydroxymethyl furaldehydes (HMF), aromatic acids such as phenols, and aliphatic acids (figure 2.3) such as levulinic acid, formic acid and acetic acid (Mosier *et al.*, 2005). Elimination of inhibitory compounds increases the fermentability of lignocellulosic hydrolysates. Detoxification is one method that is used in reducing inhibitors in order to improve fermentation of hydrolysates, but the detoxification process must be limited due to economic reasons. Adapting microorganisms that can ferment lignocellulosic hydrolysates in the presence of inhibitors is a potential strategy that can be used to deal with inhibitory compound problems (Talebnia *et al.*, 2011).

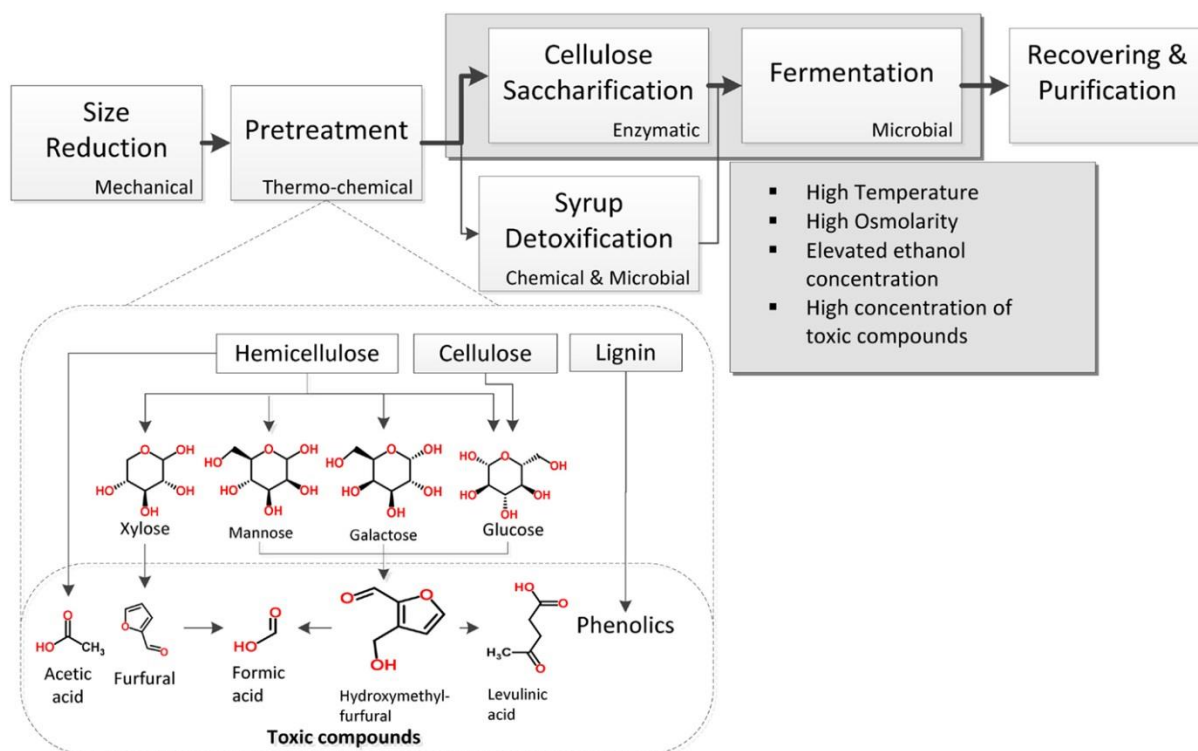


Figure 2.3.: Formation of inhibitors during pretreatment of lignocellulosic material (Caspeta *et al.*, 2015).

2.3.3. Fermentation

Fermentation is a process that converts the hexoses and pentoses released during hydrolysis to ethanol. This process can either be carried out by fungi (mostly yeasts) or bacteria. Ethanol fermentation of lignocellulosic materials entails that the microorganism should preferably be able to ferment both hexose and pentose sugars. However, most microorganisms are unable to efficiently ferment pentose sugars. For ethanol production to be considered economically viable, the fermenting microorganisms should be able to utilise all the carbon sources that are present in the hydrolysates, yield a high amount of ethanol, tolerate inhibitors in the hydrolysates and be able to withstand high temperatures, high sugar concentrations and high concentrations of ethanol (Sun and Cheng, 2012).

Fermentation of monosaccharides from the hydrolysis of plant biomass is mostly done by either (1) separate hydrolysis and fermentation (SHF) or (2) simultaneous saccharification and fermentation (SSF). The former occurs when enzyme hydrolysis and fermentation are

performed consecutively, whereas with the latter the hydrolysis and fermentation step take place at the same time (Dias *et al.*, 2015).

The SHF process has been employed previously in the fermentation process, but SHF yields low ethanol probably because the end products (glucose and cellobiose) released in cellulose hydrolysis strongly inhibit the efficiency of cellulase. Glucose inhibits β -glucosidase which results in an increase in cellobiose since β -glucosidase catalyses the hydrolysis of cellobiose to glucose. Cellobiose itself has an inhibiting effect on cellulases and thereby reduces cellulase activity. To achieve a reasonable ethanol yield, lower solid loadings and higher enzyme additions could be needed (Balat, 2011). Another disadvantage with SHF is the risk of contamination. Due to the relatively long residence time (one to four days) for the hydrolysis process, there is a risk of microbial contamination of the sugar solution. However, SSF has been shown to be more effective than SHF because it yields higher ethanol compared to SHF as there are very little monomeric sugars lost during the whole process and it reduces the risk of contamination. SSF exhibits low inhibition of the enzymes due to the simultaneous fermentation and does not require separate reactors, which makes SSF more economical. The disadvantage of SSF lies in the different temperatures that have to be used for hydrolysis and for fermentation (Olofsson *et al.*, 2008).

2.4. Pentose fermentation

Microorganisms converting pentose sugars (D-xylose and L-arabinose) to ethanol follow the isomerase pathway (bacteria) or the dehydrogenase/reductase pathway (Fungi) (Figure 2.4) (Aristidou and Pentilla, 2013).

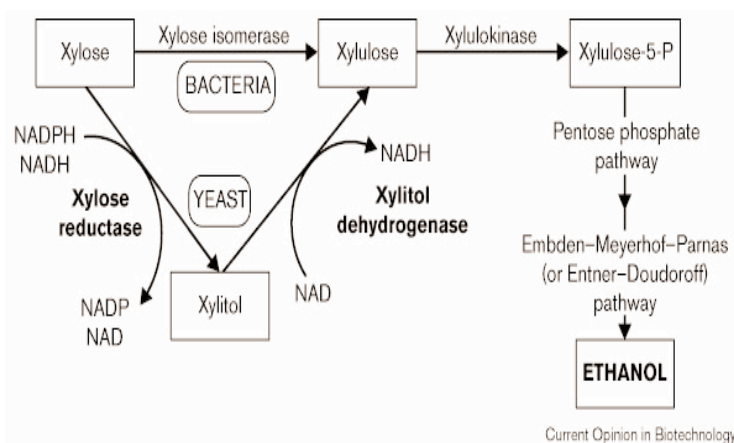


Figure 2.4: D-Xylose assimilation by bacteria and yeast (Aristidou and Pentilla, 2013).

Fungi (moulds and yeasts) reduce D-xylose to xylitol by an NADPH linked xylose reductase or by an NADH linked xylose reductase. Xylitol is oxidized to xylulose with NAD⁺ by xylose dehydrogenase. Xylulokinase (XK) phosphorylates xylulose at the C5–OH position to yield xylulose-5-phosphate (X5P), which is then channelled into glycolytic intermediates such as fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (GA3P) through the pentose phosphate pathway (PPP). Once these intermediates are in the Embden–Meyerhof– Parnas pathway, they are converted to pyruvate (Hahn-Hagerdal *et al.*, 2014). Under anaerobic conditions, pyruvate is decarboxylated to acetaldehyde by pyruvate decarboxylase which is then reduced to ethanol by alcohol dehydrogenase (Margeot *et al.*, 2009).

The enzyme xylose reductase which is responsible for reducing xylose to xylitol uses either the NADH or NADPH as reducing cofactors in yeast, the latter is preferred. When the dissolved oxygen concentration in the medium increases, the xylose reductase becomes more NADPH-dependent and this could imply that phosphate is a rate limiting factor under strictly aerobic conditions (Yong *et al.*, 2002).

There are three different metabolic routes that the yeasts take according to the oxygen availability. In aerobic conditions, cell biomass is favoured and ethanol production decreases because it requires a reducing environment. But under strictly anaerobic conditions, which are achieved by replacing oxygen with nitrogen, xylitol formation is highly favoured because the NAD⁺ or NADP⁺ that is formed by xylose reductase is then reduced to NADH or NADPH and will not be available for the sequential reaction that needs to be catalysed by xylitol dehydrogenase which is needed for growth sustained by the pentose phosphate pathway. In this condition, the enhancement of ethanol is not sufficient (Converti *et al.*, 2000). However, microaerophilic conditions have proved to be the best condition for ethanol formation, because the scarce oxygen that is available is completely consumed for yeast growth thus increasing ethanol productivity (Cong *et al.*, 2012).

2.5. Xylose fermenting microorganisms

Microorganisms that are capable of efficiently fermenting xylose to ethanol are essential in the production of biofuel, since they would contribute in increasing the final yield of ethanol from lignocellulosic materials (Tanimura *et al.*, 2012). Much research has been done on microorganisms that can ferment xylose and many have been reported to be able to directly ferment xylose to ethanol, but with lower yields compared to glucose fermentation. Factors

influencing the performance of the xylose fermenting microbes are pH, temperature, inhibitor tolerance, ethanol tolerance and growth rate (Olofsson *et al.*, 2013).

Microorganisms used for bioethanol production include, bacteria, yeast and moulds. These microorganisms should possess certain traits that will make them desirable for use in the large scale production of ethanol. These traits include the GRAS status (generally regarded as safe) of the organism, ability to be recycled, growth at low pH to reduce the risk of contamination (especially at pH less than 5.0), be thermotolerant and ferment in the presence of inhibitors (Sukumaran *et al.*, 2012; Zaldivar *et al.*, 2011).

2.5.1. Bacteria

Bacteria, such as *Zymomonas mobilis* as well as genetically engineered *Escherichia coli* and *Klebsiella oxytoca* have gained much attention, because they can ferment sugars faster than yeasts. *Z. mobilis*, which is a Gram-negative bacterium, is well known for its efficient production of bioethanol from glucose, fructose and sucrose at high rates (Hahn-Hagerdal *et al.*, 2014). When compared to *Saccharomyces cerevisiae*, the efficiency of *Z. mobilis* to produce bioethanol from glucose was very high, showing that it can attain about five percent higher bioethanol yields and up to 5-fold higher bioethanol volumetric productivity compared to *S. cerevisiae* (Sanchez *et al.*, 2010).

However, *Z. mobilis* is not suitable for bioethanol production, as it only ferments glucose, sucrose and fructose (Chan *et al.*, 2012; du Preez *et al.*, 2008). Most bacteria efficiently produce bioethanol from the hexose sugars glucose and fructose, but not from pentose sugars. The other disadvantages of using bacterial cultures are the limited and neutral pH growth range (6.0–8.0), undesirable by-products, and public perceptions regarding bacterial species (Bachmann *et al.*, 2015).

2.5.2 Moulds

Moulds like *Fusarium*, *Trichoderma* and *Rhizopus* are able to ferment pentoses and hexoses to ethanol. The advantage in using moulds is that they are able to convert cellulose or hemicellulose to ethanol in a single step reducing the cost of fermentation significantly.

However, the fermentation process is slow and often produces by-products (Kuhad *et al.*, 2011).

2.5.3 Yeasts

A number of yeast that metabolise xylose are unable to produce ethanol from it. Under aerobic conditions, almost half of all yeasts will grow on xylose but only a few will be able to ferment xylose, and within that, a very small number will produce ethanol at a level that is economically significant (Jeffries and Alexander, 2008).

A small group of known yeasts that are able to ferment xylose directly to ethanol are *Scheffersomyces stipitis*, *Pichia kudriavzevii*, *Candida shehatae*, and *Pachysolen tannophilus*. These yeasts can assimilate and ferment xylose, but the ethanol levels produced are lower when compared to glucose fermentation by *S. cerevisiae*. Additionally, these yeasts need a cautious control of low oxygen maintenance in the culture medium that is required for their oxidative metabolism. Moreover, these yeasts have been reported to successfully ferment pure xylose and not the D-xylose from aqueous hemicellulose streams after lignocellulose pretreatment, probably because of the presence of inhibitors (Gray *et al.*, 2013).

2.5.3.1. *Scheffersomyces stipitis*

S. stipitis is a haploid, homothallic yeast producing hat-shaped ascospores and pseudomycelia able to ferment xylose and glucose, including various other constituents such as L-arabinose, that are present in industrial cellulosic sugars (Willis *et al.*, 2006). *S. stipitis* is commonly found in the gut of passalid beetles (du Preez *et al.*, 1985) and ferments sugars under oxygen limited conditions (Kuhad *et al.*, 2011).

S. stipitis has the capabilities of catabolising glucose, xylose, arabinose, rhamnose, cellobiose, mannose, galactose and also some lignin related compounds. *S. stipitis* is the best yeast in producing ethanol from xylose. When compared to other yeast species, some added advantages of *S. stipitis* are that it has simple growth requirements and a strong resistance to contamination as well as detoxification of inhibitors derived from biomass. *S. stipitis* can produce high amounts of ethanol with a maximum yield of 0.48 g/g xylose (Liu *et*

al., 2012). Despite these properties *S. stipitis* also has a few disadvantages which include, slow consumption of xylose, difficult control of precise oxygenation and it cannot handle concentrations of ethanol above 0.6 g/g sugar as the ethanol becomes toxic to it (Slininger *et al.*, 2015).

2.5.3.2. *Pichia kudriavzevii*

Pichia kudriavzevii was previously known as *Issatchenkia orientalis*, (anamorph *Candida krusei*). This yeast has been isolated from various fruit and food sources such as sourdoughs and orange juice, fermented pineapple juice, etc. (Kurtzman *et al.*, 1980). In 2012, Chan and colleagues drafted the genome of *P. kudriavzevii* to investigate its commercial viability. They revealed the presence of genes coding for xylitol dehydrogenase, xylulokinase and xylose reductase. These enzymes are responsible for converting xylose to xylulose, which is further taken into the pentose phosphate pathway for production of ethanol. This strain is principally convenient because it has a strong NADH-linked aldose reductase activity which yields a more favourable cofactor balance in the xylose conversion to xylulose thereby resulting in high yields of ethanol as opposed to having an NADPH-linked aldose reductase. (Bruinenberg *et al.*, 2004).

P. kudriavzevii has been reported to be able to utilise a wide range of sugars such as xylose, arabinose, galactose, mannose and many more. This yeast is thermotolerant meaning it can tolerate temperatures ranging up to 42 °C; it additionally exhibits multi-stress tolerance characteristics such as tolerance to acid concentrations, high ethanol concentrations, high salt concentrations and high temperatures. It is able to produce high ethanol concentrations when subjected to high temperatures (Yuangsaard *et al.*, 2013).

2.6. Inhibitors

Inhibitors are produced during the lignocellulose pretreatment step. The presence of inhibitors negatively affects the fermentation process by microbes resulting in lower levels of ethanol being produced (Lee, 2007). The concentration and nature of these inhibitors vary greatly according to the conditions of pretreatment applied (such as the concentration of chemicals used), and also according to the raw material used. The inhibitory compounds are divided into three main groups (figure 2.5), namely furaldehydes (furfural and

hydroxymethylfurfural (HMF)), weak acids (acetic acid and levulinic acid) and phenolic compounds, such as vanillin and coniferyl aldehyde (Oberoi *et al.*, 2014).

The presence of acetic acid has been reported to significantly decrease the productivity and ethanol yield in fermentations (Sun and Cheng, 2012). Acetic acid, HMF and furfural are the three main inhibitors affecting fermentation. However, most yeasts have been reported to grow in the presence of HMF and furfural, with acetic acid being the most inhibitory compound (Fan *et al.*, 2013).

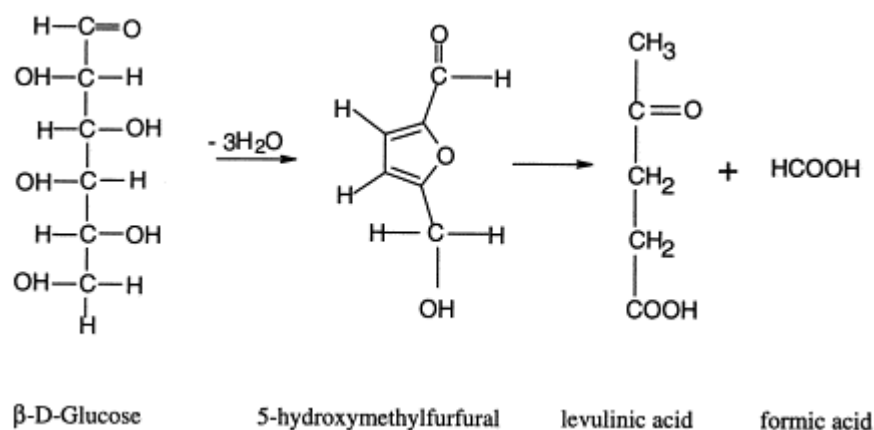


Figure 2.5: Formation of levulinic acid and formic acid during acid hydrolysis (Larsson *et al.*, 2000).

2.6.1. Acetic acid

Acetic acid is formed during the de-acetylation of hemicellulose and also during the breakdown of HMF. The acetyl groups are released as acetic acid. Acetic acid reduces the biological activities of the yeast by interfering with the enzymes (nucleases), which in turn break down the DNA and inhibit RNA and protein synthesis (Oberoi *et al.*, 2014). According to Maiorella *et al.* (2014), acetic acid inhibits the metabolism of yeast by chemically interfering with the transportation of phosphate through the cell membrane, which requires the use of ATP. Acetic acid interference leads to an increase in the ATP required for this maintenance function, and it also interferes with the morphology of the cell. Fermentation inhibition by acetic acid is caused by the un-dissociated, uncharged form of acetic acid (Olofsson *et al.*, 2008). The un-dissociated acid moves through the cell membrane by passive diffusion, then dissociates when it enters the cell, decreasing the internal pH of the yeast (Parawira and Tekere, 2011).

Tolerance of acetic acid is achieved by pumping protons out of the cytoplasm to maintain the intracellular pH catalysed by increasing ATPase activity. Acetic acid inhibits fermentation at concentrations above 100 mM, while fermentations with concentrations lower than 100 mM improved ethanol production (Margeot *et al.*, 2009).

Fermentation inhibition can be overcome by biological detoxification whereby microorganisms or their enzymes act on toxic compounds that are found in biomass hydrolysates and change the structure or composition and make it less toxic. There is a great necessity to explore other strategies such as evolutionary engineering or genetic engineering to make microorganisms more tolerant towards acetic acid (Caspeta *et al.*, 2015).

2.7. Strain improvement

One of the most important requirements for industry is the enhancement of eukaryotic or prokaryotic cells for bioethanol production. Some of the industrial needs include increasing the product yield, eliminating by products or/and inhibitors and efficient production of ethanol under harsh conditions for the producing microorganism (Nyanga *et al.*, 2013).

Ethanol production on an industrial scale needs microorganisms that can cope with conditions such as an increase in osmotic pressure, quick temperature fluctuations, low nutrient supply and high presence of ethanol, which affect yeast cell dynamics in an unfavourable manner. Therefore, for efficient fermentation of ethanol to occur, there is need for a robust strain, which can handle the above mentioned process conditions (Sims *et al.*, 2016).

Strain improvement is defined as the technology used to manipulate and improve microbial strains in order to improve their metabolic capacities for biotechnological applications. Yeast strain improvement is targeted to enhance: growth rate, carbon source utilization, genetic stability and productivity, amongst others (Nigam, 2012).

There are a number of methods used in strain improvement. (1) Mutagenesis is a process whereby gene information of a microorganism causes an enhancement in phenotype and genotype performance (Steensels *et al.*, 2014). (2) Transduction is the transfer of DNA from one cell to another using a bacteriophage (Shi *et al.*, 2014). (3) Transformation is where genetic information of the recipient cell being altered due to absorbing and integrating with

the genome of the donor cell (Bengtsson *et al.*, 2009). (4) Protoplast fusion occurs when two different microorganisms are fused together, when dividing cells have lost their cell wall, to exchange genetic materials and now each contains the characteristics of both (Sanchez *et al.*, 2010). (5) Recombinant DNA technology occurs when genes are isolated, amplified, altered and then put in the genome of another organism (Slininger *et al.*, 2015). (6) Evolutionary engineering is adapting cells under selective pressure over a long period of time, resulting in modification of a certain population of cells that will have an advantage over the original dominating cells (Steensels *et al.*, 2014).

While most of these approaches have been technically successful, the yield and rates of fermentation using mixed sugars with presence of inhibitors (acetic acid) so far has not reached feasible commercial targets (Tofighi *et al.*, 2014). In this study evolutionary engineering (adaptation) will be used to enhance isolated yeasts.

2.7.1 Evolutionary engineering

Evolutionary engineering, otherwise known as adaptation is a strain improvement approach which aims at increasing genetic diversity and also screens large populations so as to acquire desired phenotypes. The advantages of using evolutionary engineering as opposed to other strain improvement methods is that there is very little to no necessity of detailed genetic background information for the trait of interest and there is no need for the detailed knowledge of the complex nature and action of inhibitors that the microbes will be exposed to (Koppram *et al.*, 2012). Additionally, a strain adapted to tolerate a particular condition (lignocellulose inhibitors) may tolerate more different conditions other than the one it has been previously subjected to (Harner *et al.*, 2015).

Adaptation is done through experimental evolution of microorganisms over time (Kahr *et al.*, 2011). The adaptation process of the microbial population to distinct environments is currently experimented at high resolutions in various laboratories (Ooi and Lankford, 2012). In these experiments, populations are sampled and archived over time as they evolve. Representative genomes from the samples are re-sequenced and compared to the ancestors' genome so as to classify the mutant alleles that have accumulated. In large microbial populations, it is known that vast numbers of mutant alleles exist at very low frequencies. The mutational actions that have happened in the beginning in some populations are possibly more than the number of nucleotide sites in the genome. The rates

of mutation are not constant throughout the genome. However, the diversity in mutant alleles is a rich source of distinction on which natural selection may occur (Satomura *et al.*, 2016).

Traditionally, adaptation has been used to screen for growth of an organism, however, another improvement factor required for is fermentation, which is why adapted strains that exhibit enhanced growth are also assessed for their ability to ferment different sugars and in the presence of lignocellulosic inhibitors. Adaptation can either be done sequentially (whereby a strain is subjected to one environmental stress at a time, before moving on to the next) or simultaneously (whereby a strain is subjected to multiple stress at the same time). (Harner *et al.*, 2015). Nigam (2012) did a study wherein the fermentation performance of *P. stipitis* NRRLY-7124 was investigated in a synthetic pre-hydrolysate medium. The strain was sequentially adapted in different concentrations of acetic acid as well as xylose; concentrations were gradually increased over time. The adaptation approach (sequential) showed an improved adapted strain which could ferment xylose in a shorter period of time and yielded fifteen-fold better ethanol production than its parental strain. Moore *et al.* (2014) did a study using *S. cerevisiae* which was simultaneously adapted to concentrations of glucose, xylose, furfural and HMF. The strain yielded very low amounts of ethanol, and was unable to grow and ferment when a combination of the sugars and inhibitors was used.

Adaptation experiments can be carried out in shake flasks or on agar plates, with each method having its advantages and disadvantages. Adaptation in liquid culture can be done to propagate microbial cells at regular intervals (daily) whereby an aliquot of the culture is transferred to a new flask with fresh new medium for another round of growth. This setup has the advantage of using affordable equipment and also has advantage of controlling environmental factors such as spatial culture homogeneity and temperature. However, this set up also has a few shortcomings which include fluctuating nutrient supply and growth rate, unstable environmental conditions such as pH and aeration as well as population density that varies. Adaptation on solid agar plates is done by streaking the microbial cells on an agar plate at regular intervals and picking the biggest colony and transferring it to a new plate, which is repeated for 50 cycles. The whole procedure of agar plating is cheap and is not tedious, it allows for selection of improved phenotypes (by simply picking up the biggest colony) and it also shows any contaminating organisms. The disadvantage of using agar plates is that they tend to dry after a few days and therefore it is difficult to detect any growth delays (Dragosits and Mattanovich, 2013; Kurtzman *et al.*, 2010).

Evolutionary engineering is an important and efficient strategy for obtaining a desired strain, since there is no naturally occurring organism that can meet all the required specifications for bioethanol production, such as high productivity, high yield, effective utilisation of

substrates, ethanol tolerance and tolerance to inhibitors present in hydrolysates (Demeke *et al.*, 2013).

CHAPTER 3

MATERIAL AND METHODS

3.1 Yeast strains

Six xylose fermenting yeast strains, previously isolated from the dung of wild herbivores in the Kruger National Park, Limpopo Province, South Africa (Makhuvele *et al.*, 2017) and one strain isolated from sugar cane were used in this study (Table 3.1). *Pichia stipitis* NRRLY-7124 was included as a reference strain.

Table 3.1: Yeasts used in this study

Yeast	Strain number	Origin
<i>Candida guilliermondii</i>	MB12	Outer part of sugar cane, UL, SA
<i>Candida</i> sp.	Kp6.2ey	Buffalo dung, KNP, SA
<i>Candida tropicalis</i>	Kp21ey	Elephant dung, KNP, SA
<i>Candida tropicalis</i>	Kp42ey	Rhino dung, KNP, SA
<i>Candida tropicalis</i>	Kp43ey	Rhino dung, KNP, SA
<i>Ogataea methanolica</i>	Kp2ey	Elephant dung, KNP, SA
<i>Pichia kudriavzevii</i>	Kp34ey	Dassie dung, KNP, SA

KNP: Kruger National Park; SA: South Africa; UL: University of Limpopo

All yeast strains were stored in cryogenic vials at -80°C in 15% glycerol and were revived in 250 ml Erlenmeyer flasks containing YPX broth (3 g/L yeast extract, 20 g/L xylose 3 g/L malt extract, 5 g/L peptone and 0.1 g/L chloramphenicol). The inoculated flasks were incubated at 200 rpm and 30°C for 48 hours.

All the yeast strains were then streaked on xylose agar plates (3 g/L yeast extract, 3 g/L malt extract, 20 g/L xylose, 20 g/L agar and 0.2 g/L chloramphenicol). The plates were incubated for 48 hours at 30°C. Colonies were examined under a light microscope for purity and were re-streaked on xylose agar plates followed by incubation as before until pure colonies were obtained. Pure yeast cultures were stored on YM slants at 4 °C.

3.2. Preliminary screening of yeast strains

3.2.1 Screening of yeast strains on xylose

Confirmation of growth of the seven yeast strains with xylose as main carbon source was done using test tubes (150 x 12 mm), with 5 ml media, containing YM broth (20 g/L xylose, 3 g/L yeast extract and 3 g/L malt extract). The test tubes were incubated at 30°C for 24 hours. Yeast strains indicating growth (turbidity) were selected for further studies.

3.2.2. Determination of maximum temperature for growth

The strains were inoculated and grown in test tubes with YM broth (as indicated in 3.2.1) and incubated at 30°C, 35°C, 37°C and 40°C for 24 - 48 hours (Kurtzman *et al.*, 2010). Detection of growth at the highest temperature was noted as the maximum temperature for growth.

3.2.3. Growth of yeast strains in the presence of acetic acid

Yeast strains able to grow at 40°C were inoculated in test tubes containing YM broth (as indicated in 3.2.1) with acetic acid added at concentrations of 1 to 4 g/L. The test tubes were incubated at 37°C and 40°C for 24 - 48 hours. An increase in turbidity was noted as a positive result.

3.3. Adaptation tests on selected yeast strains

Yeast strains able to grow on xylose as sole carbon source, at 37°C or 40°C and in the presence of acetic acid were selected for adaptation experiments. The adaptation experiments were done by streaking the yeasts on agar plates, incubated for 24 hours and selected the fastest growing colony (visual inspection) on each plate to be re-streaked on the same conditions. The process continued for 50 repetitions (Kahr *et al.*, 2011). The adapted

yeast was then subjected to the next round of conditions. Adaptations were performed on increased xylose concentrations, followed by higher temperatures and lastly by increasing the acetic acid concentration.

The first round of adaptation was performed using agar plates containing 50 g/L xylose and 6.7 g/L YNB without amino acids. The plates were incubated at 30°C for 24 hours. A colony was selected and transferred to the next plate. This was repeated until 50 repetitions were completed. The xylose concentration was then increased to 60 g/L and 50 repetitions followed.

The best performing yeasts were then streaked on plates containing 60 g/L xylose and 6.7 g/L YNB without amino acids at 35°C. The temperature was increased to 37°C after 50 repetitions followed by 40°C for 50 repetitions.

Yeasts adapted to 40°C were then incubated on plates containing 6.7 g/L YNB without amino acids, 60 g/L xylose and 2 g/L acetic acid at 37°C (no growth was detected at 40°C). The acetic acid concentration was increased to 3 g/L after 50 repetitions and followed the same adaptation process. The yeast strain able to adapt the best (fastest growing in 24 hours) on these conditions (60 g/L xylose, 37°C and 3 g/L acetic acid) was further investigated.

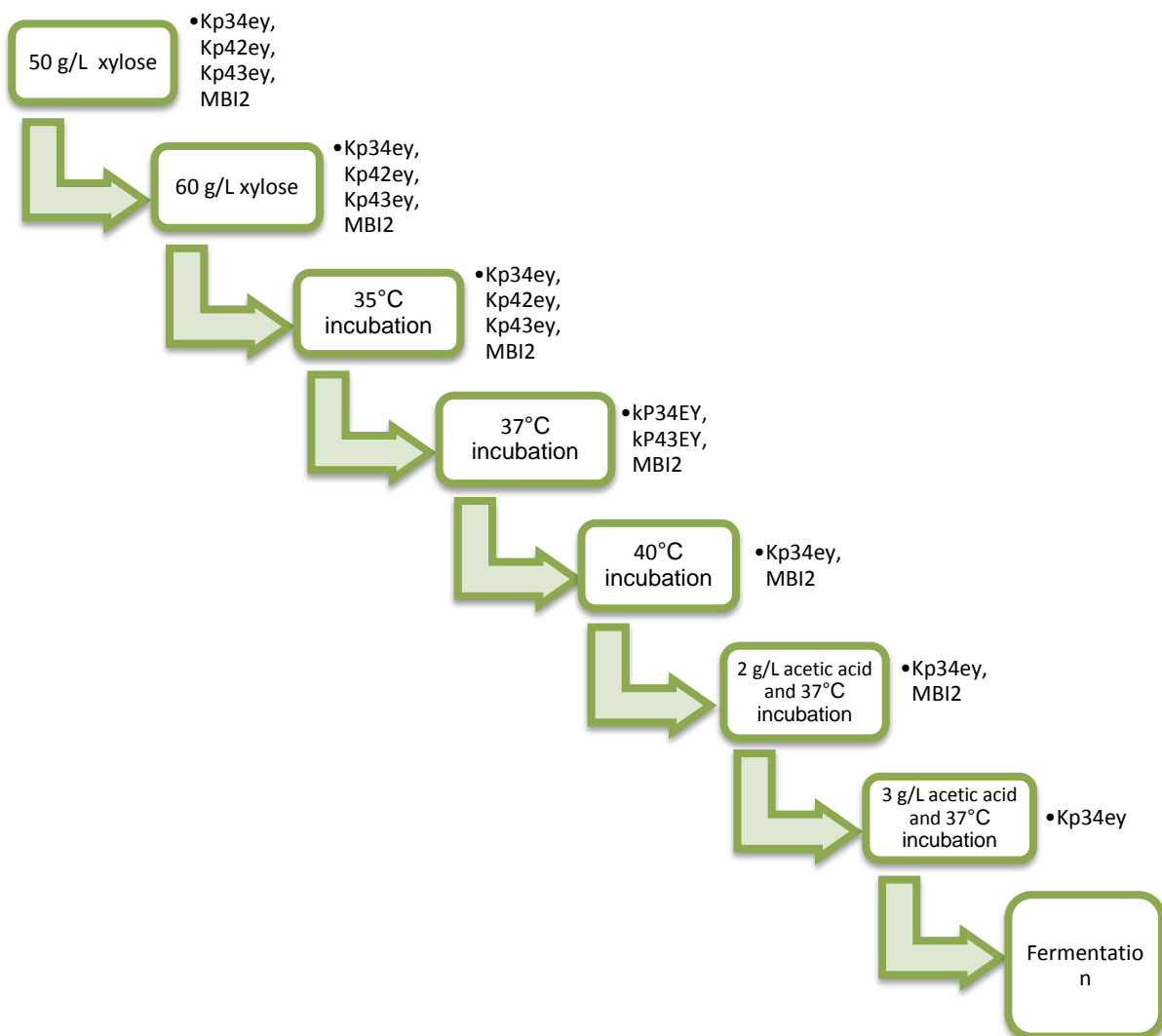


Figure 3.1: Schematic illustration of stepwise adaptation process carried out. Yeast cultures were continually cultivated 50 times in agar media containing 6.7 g/L YNB at different temperatures (35 and 37°C), xylose concentrations (50 and 60 g/L) and acetic acid concentration (2 and 3 g/L).

3.4. Fermentation studies

3.4.1 Confirmation of ethanol production

The yeast strains ability to ferment xylose after every adaptation step was tested using xylose fermentation media (20 g/L, 50 g/L or 60 g/L xylose, 10 g/L yeast extract, 2 g/L KH_2PO_4 , 2 g/L $(\text{NH}_4) \text{SO}_4$, 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g/L of ZnSO_4 and 0.2 g/L of chloramphenicol) adopted from Okamoto *et al.* (2012).

Two colonies of each yeast strain were inoculated into 250 ml Erlenmeyer flasks, as pre-inoculum, containing 50 ml of xylose fermentation media. The flasks were incubated at 30°C for 24 hours in a rotary shaker at 150 rpm. These flasks were used to inoculate 50 ml of fresh xylose fermentation media in 250 ml flasks to a starting $\text{OD}_{600\text{nm}}$ of 0.1, and were incubated at 30°C and 150 rpm on a rotary shaker, with sampling done after 24 hours.

The best performing yeast strain after adaptation was selected for further studies. *S. stipitis* NRRLY-7124 was included as a reference to compare with the best adapted yeast strain and its parental strain.

The yeast strains were also tested for xylose fermentation after the preliminary screening.

3.4.2. Bioreactor studies

Fermentation studies were conducted using a 2.5L BioFlo®/CelliGen® 115 Benchtop Fermentor & Bioreactor, using control modules to monitor pH, temperature and dissolved oxygen (DO). The pH of the fermentation medium was kept at 5.5 using 1M of potassium hydroxide (KOH). The measurement of DO was done using a polarographic (pO_2) probe, with the measurements corresponding to the relationship between saturated oxygen and dissolved oxygen concentration in the liquid media.

The volumetric oxygen transfer coefficient (K_La) was determined as proposed by Wise (1951). Using nitrogen and air sparging, respectively, the polarographic oxygen probe was calibrated at the atmospheric pressure by setting saturation at 0% and 100%. Nitrogen was sparged in the media in order to completely remove oxygen from the fermentor. The time course for oxygen saturation was monitored and recorded under the stirring conditions and

air flow conditions that were to be used for the fermentation. The formula that was proposed by Stanbury *et al* in 1995 was used to calculate the K_{La} , value whereby the K_{La} value was equal to the slope of the resulting straight line representation of $\ln(C^* - CL)$ vs time. In order to determine the values of K_{La} , the fermentor settings were configured to be the same one that will be used in the fermentation run of the micro-organisms.

$$\ln(C^* - CL)$$

Where $\ln = \ln$

C^* = is the saturated dissolved oxygen concentration

CL = concentration of dissolved oxygen in the fermentation broth

The K_{La} values investigated were 2.5, 3.3 and 4.0 (aeration rate of 0.1, 0.5 and 1.0 vvm and agitation speed of 100 rpm, 150 rpm and 450 rpm respectively). The temperature was set at 30°C or 37°C. The fermentation vessel contained 1000 ml media consisting of 60 g/L xylose, 10 g/L, 2 g/L KH_2PO_4 , 2 g/L $(NH_4) SO_4$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L of $ZnSO_4$ and 0.2 g/L of chloramphenicol. Experiments were conducted with or without the addition of 3 g/L acetic acid.

The fermentation vessel with media was inoculated with the yeast strain to an OD_{600nm} of 0.1. Sampling was done every 6 hours for the first 24 hours followed by 12 hour intervals until stationary phase was reached. The samples were analysed to determine growth (OD_{600nm}), ethanol, xylitol and xylose.

3.5. Analytical methods

Two millilitre samples from the Erlenmeyer flasks and fermentation vessel were collected as indicated above. Cell biomass was measured spectrophotometrically at an OD of 600 nm. The samples were then filtered using a 0.22 μm filter membrane and stored at -20°C until analysis was done for ethanol production, xylose consumption and xylitol production.

3.5.1 High Performance Liquid Chromatography (HPLC) analysis

High performance liquid chromatography (HPLC) (Shimadzu) was used to determine xylose consumption and xylitol production. The HPLC was equipped with a Rezex RCM monosaccharide H+ (300 mm × 7mm) column and deionized water was used as the mobile phase. The temperature was set at 85°C and the flow rate was 0.6 ml/min with a sample volume of 20 µL. A Shimadzu RID10A refractive index detector was used to detect separated components. External standards of xylose and xylitol were used to determine the concentration of the compounds.

3.5.2 Gas Chromatography (GC) analysis

Ethanol content was determined by capillary gas chromatography on a Shimadzu GC-2010 Plus gas chromatograph (Kyoto, Japan) equipped with AOC 20Si Auto ampler and AOC 20i auto injector and a flame ionisation detector. A ZB-WAX plus column (30 m × 0.25 mm ID x 0.25 µm) at a column flow rate of 1.29 ml/min. Nitrogen was used as the carrier gas. A volume of 1 µl of the sample was injected in the GC using a splitless injection. The concentration of ethanol in the samples was determined using known ethanol standards.

CHAPTER 4

RESULTS

4.1. Preliminary screening of yeast strains

The preliminary screening of all yeast strains was done to confirm that the selected yeast strains (Table 3.1) were able to utilise xylose as a carbon source and to determine the maximum growth temperature as well as the concentration of acetic acid these yeast strains were able to tolerate (Table 4.1). Ethanol produced by the yeast strains was determined where growth occurred at the highest temperature and tolerance of acetic acid concentration.

Table 4.1: Preliminary screening of the seven selected yeast strains used, subjected to different test conditions (high xylose concentration (40 g/L), temperatures of 30, 35, 37 and 40 °C as well as acetic acid concentration of 1, 2 and 3 g/L) in test tubes. Only three yeast strains produced ethanol after being subjected to high xylose concentration with a mixture of acetic acid, *P. kudriavzevii* produced the highest ethanol in medium consisting of 3 g/L acetic acid at 37 °C with 40 g/L of xylose as the sole carbon source

Yeast strain	Xylose (g/L)	Temperature (°C)				Acetic acid (g/L)				Ethanol (g/L)	
		40	30	35	37	40	1	2	3		4
<i>Candida guilliermondii</i> Mbi2	++	++	++	++	++	-	++	+	+	-	0.6
<i>Candida sp.</i> Kp6.2ey	+	++	++	-	-	-	+	-	-	-	0
<i>Candida tropicalis</i> Kp21ey	+	++	++	+	-	-	+	+	-	-	0
<i>Candida tropicalis</i> Kp42ey	++	++	++	+	-	-	++	+	-	-	0.1
<i>Candida tropicalis</i> Kp43ey	++	++	++	+	-	-	++	+	+	-	0
<i>Pichia kudriavzevii</i> Kp34ey	++	++	++	++	++	-	++	+	+	-	1.2
<i>Pichia sp.</i> Kp2ey	+	++	++	+	-	-	+	-	-	-	0

++: growth after 24 hours

+: growth after 72 hours

-: no growth after 72 hours

4.1.1. Adaptation studies

The following results shown in table 4.2 are of the four organisms (MBI2, kp42ey, kp43ey and kp34ey) that were able to grow at high xylose concentrations, high temperature and in the presence of acetic acid during the preliminary screening. The adaptation was done in a sequential manner, whereby the organisms were grown in 50 g/L xylose with no acetic acid and were incubated at 35 °C, the adaptation was done/repeated for 50 cycles (50 times in the same condition). Once the adaptation was done, it was followed by 60 g/L of xylose without acetic acid at 35 °C (50 cycles), then the temperature was increased to 37 and 40 °C still with no addition of acetic acid, lastly acetic acid was added gradually and the organisms were adapted at 37 and 40 °C. The organisms grew poorly at 40 °C with 60 g/L xylose and 3 g/L acetic acid and therefore the adaptation was stopped at 37 °C with 60 g/L xylose and 3 g/L acetic acid.

Table 4.2: Growth and ethanol produced after adaptation at different temperatures (35, 37 and 40 °C), xylose concentrations (50 and 60 g/L) and in the presence of different acetic acid concentrations (2, 3 and 4 g/L). *P. kudriavzevii* was the best strain to adapt to all these conditions as it showed the best growth rate at 37 °C in 60 g/L of xylose and 3 g/L of acetic acid producing 2.3 g/L of ethanol under these test conditions.

Yeast strain	Xylose (g/L)		Temperature (°C)			Acetic acid (g/L)			Ethanol (g/L)
	50	60	35	37	40	2	3	4	
<i>C. guilliermondii</i> MBI2	++	++	++	++	+	++	+	-	1.1
<i>C. tropicalis</i> kp42ey	++	++	+	-	-	-	-	-	0
<i>C. tropicalis</i> kp43ey	++	++	++	+	+	+	-	-	0.4
<i>P. kudriavzevii</i> kp34ey	++	++	++	++	+	++	++	-	2.3

++: growth detected within 24 hours

+ : growth detected after 24 hours

- : no growth

4.2. Fermentation studies

Fermentation was conducted in a benchtop bioreactor as indicated earlier using the adapted and parental *P. kudriavzevii* Kp34ey strains and *S. stipitis* NRRLY-7124, a known pentose fermenter. Fermentation studies were performed using different $K_L a$ values, temperatures and acetic acid concentrations.

4.2.1. Fermentation at a K_La of 2.5

Fermentation of the three yeasts (parental strain of *P. kudriavzevii* Kp34ey, the adapted strain of *P. kudriavzevii* Kp34ey and the reference strain *S. stipitis* NRRLY-7124) was conducted at a K_La of 2.5 and 60 g/L xylose at two temperature values (30°C and 37°C) and acetic acid concentrations (0 g/L and 3 g/L) (Fig 4.1 - 4.3). The adapted strain was able to grow at both temperatures and both acetic acid concentrations, while the parental strain and reference strain did not grow when incubated at 30°C and 37°C in the presence of acetic acid. Ethanol and OD values were recorded until the stationary phase was reached, xylose values were recorded over 72 hours, and xylitol recorded for as long as ethanol was.

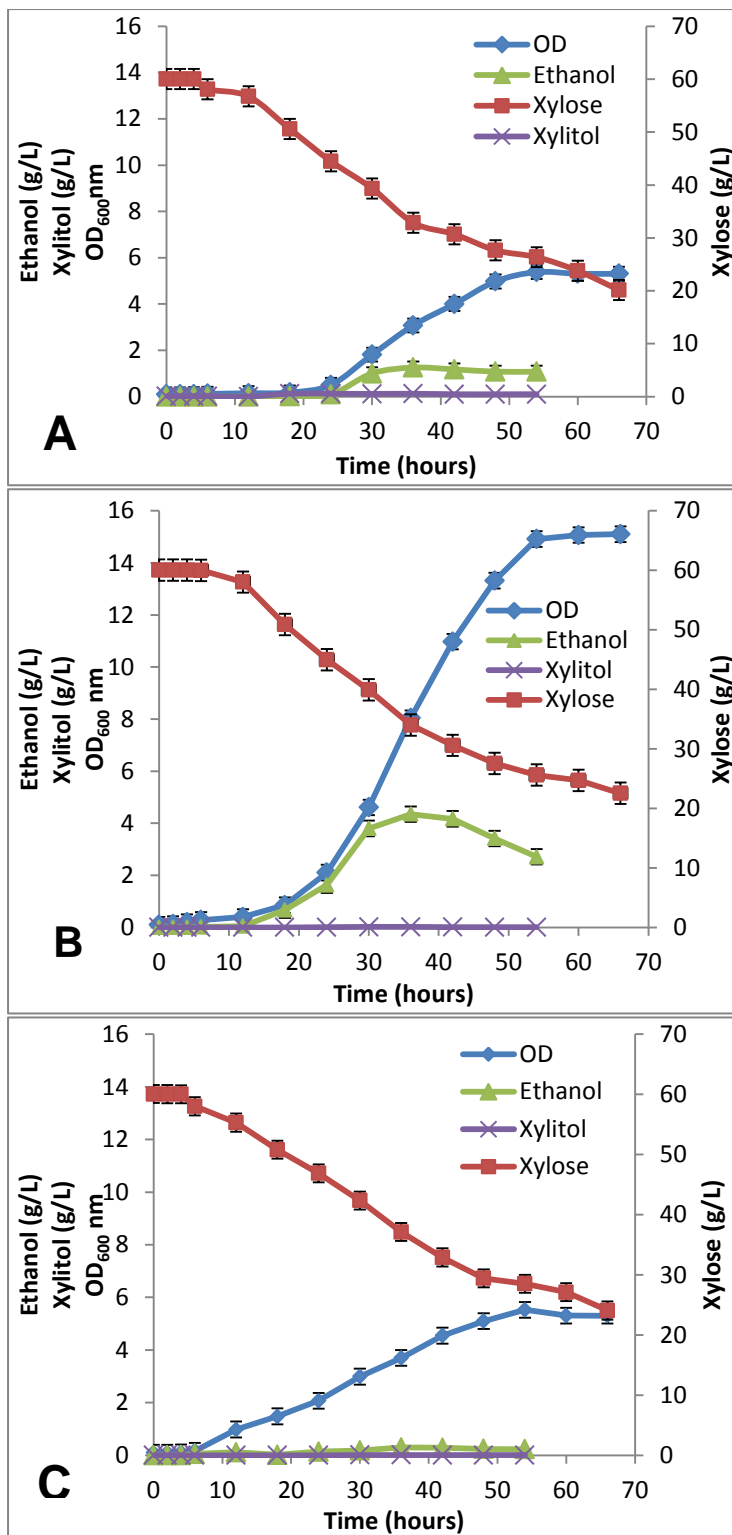


Figure 4.1: Fermentation of xylose at $K_L a$ 2.5 in the presence of 60 g/L xylose at 30°C, in the absence of acetic acid. *P. kudriavzevii* Kp34ey (parental) (A), *P. kudriavzevii* Kp34ey (adapted) (B) and *S. stipitis* NRRLY-7124 (reference) (C).

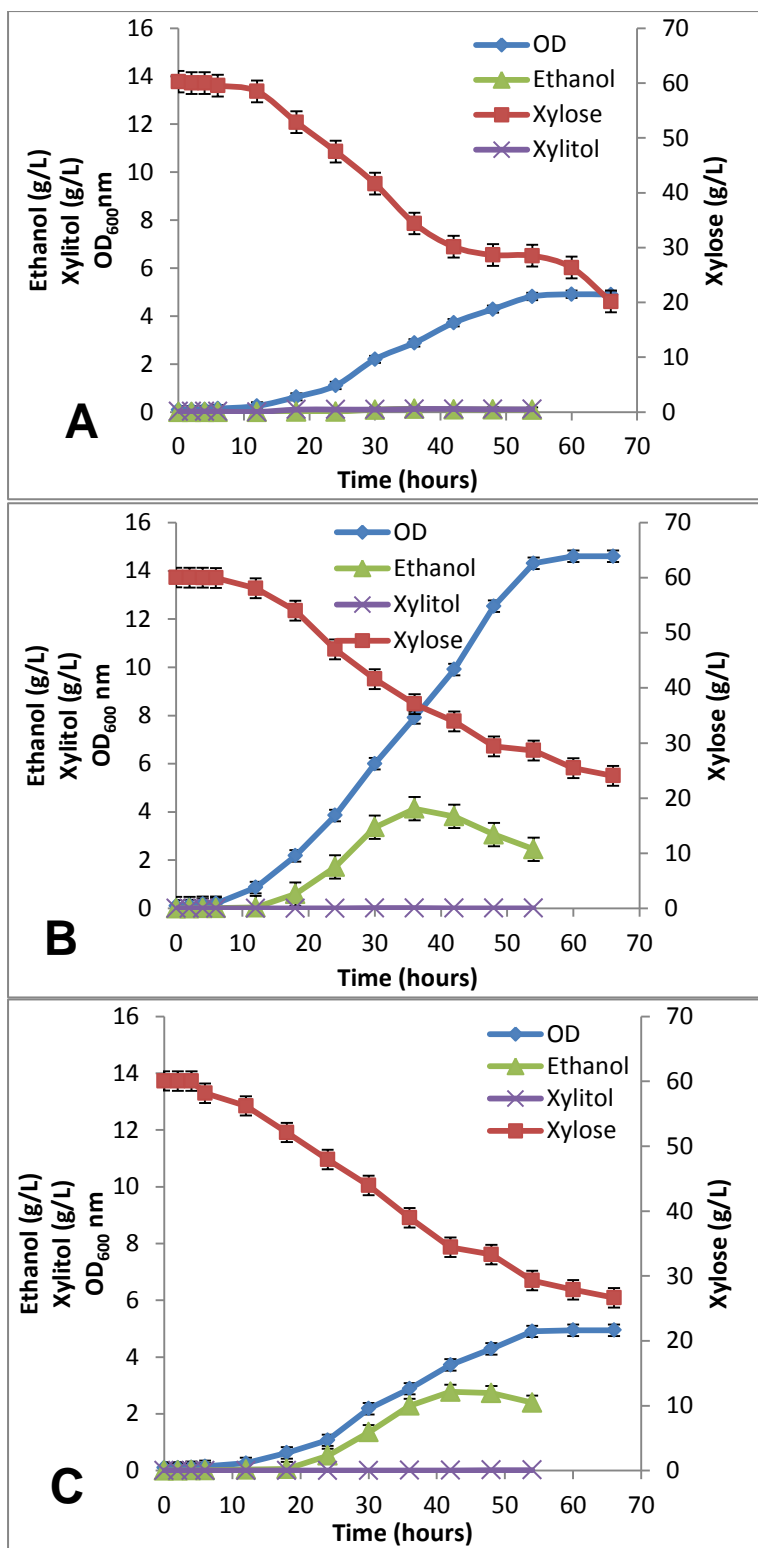


Figure 4.2: Fermentation of xylose at $K_L a$ 2.5 in the presence of 60 g/L xylose at 37°C, in the absence of acetic acid by the parental strain *P. kudriavzevii* Kp34ey (A), adapted strain *P. kudriavzevii* Kp34ey (B) and reference strain *S. stipitis* NRRLY-7124 (C).

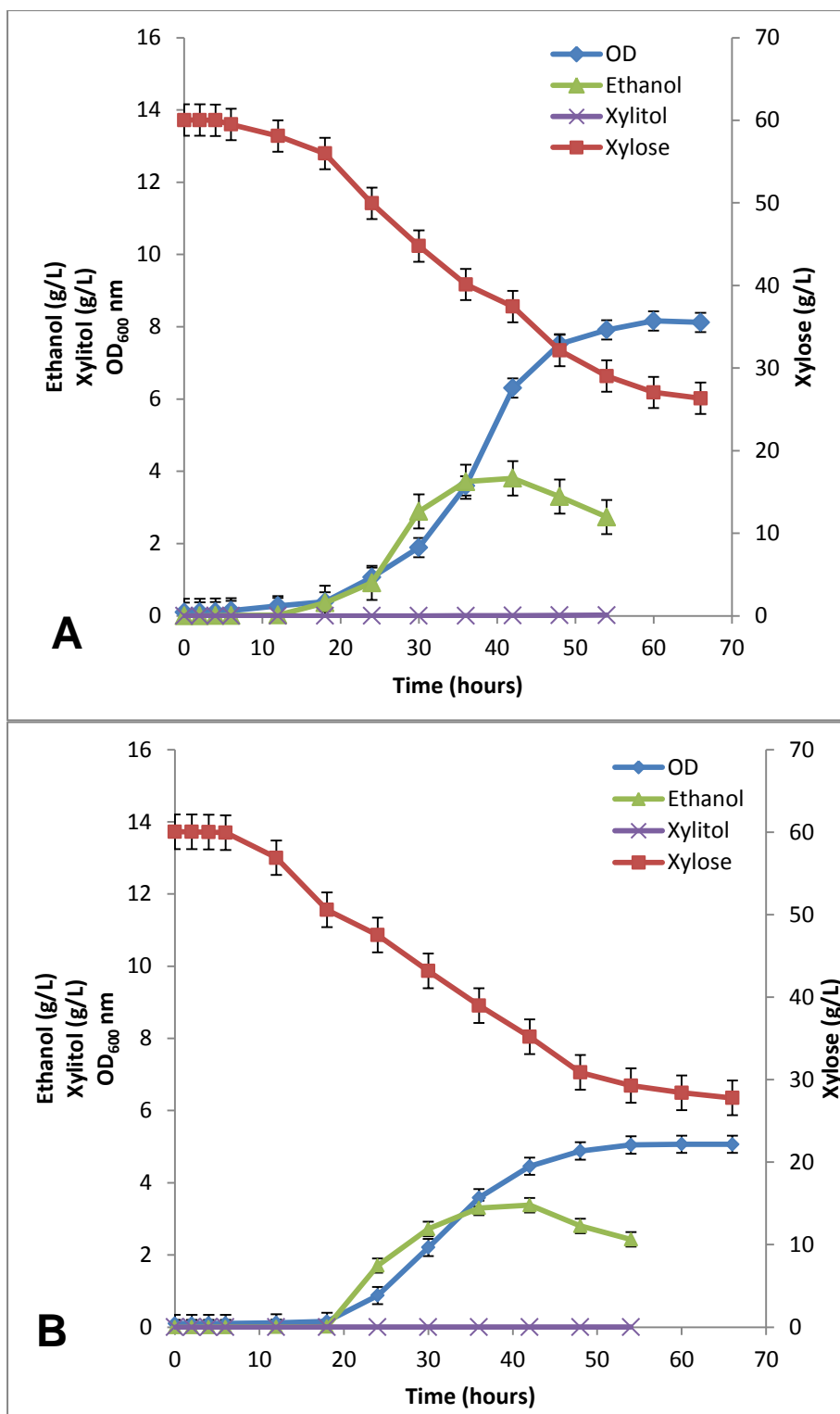


Figure 4.3: Fermentation of xylose at K_{La} 2.5 in the presence of 60 g/L xylose with the addition of 3 g/L acetic acid by parental strain *P. kudriavzevii* Kp34ey at 30°C (A) , adapted strain *P. kudriavzevii* Kp34ey at 37°C (B).

4.2.2. Fermentation at a K_La of 3.3

Fermentation of the three yeasts (parental strain of *P. kudriavzevii* Kp34ey, the adapted strain of *P. kudriavzevii* Kp34ey and the reference strain *S. stipitis* NRRLY-7124) was conducted at a K_La of 3.3 and 60 g/l xylose. Temperature values varied between 30°C and 37°C and the absence or presence of acetic acid at 3 g/L were tested (Fig 4.4 - 4.7). The yeasts produced higher amounts of ethanol at this K_La value compared to K_La of 2.5 and K_La of 4.0. All three yeasts produced higher ethanol values, when fermented in media without acetic acid and at 30°C, with the highest ethanol produced being 6.07 g/L by the adapted *P. kudriavzevii* Kp34ey strain after 30 hours. *S. stipitis* NRRLY-7124 produced 4.9 g/L of ethanol after 30 hours, while the parental strain of *P. kudriavzevii* Kp34ey producing 3.3 g/L ethanol after 36 hours. It was only the adapted strain of *P. kudriavzevii* Kp34ey that was able to grow and ferment at 37°C with 3 g/L of acetic acid and produced 4.0 g/L of ethanol after 36 hours.

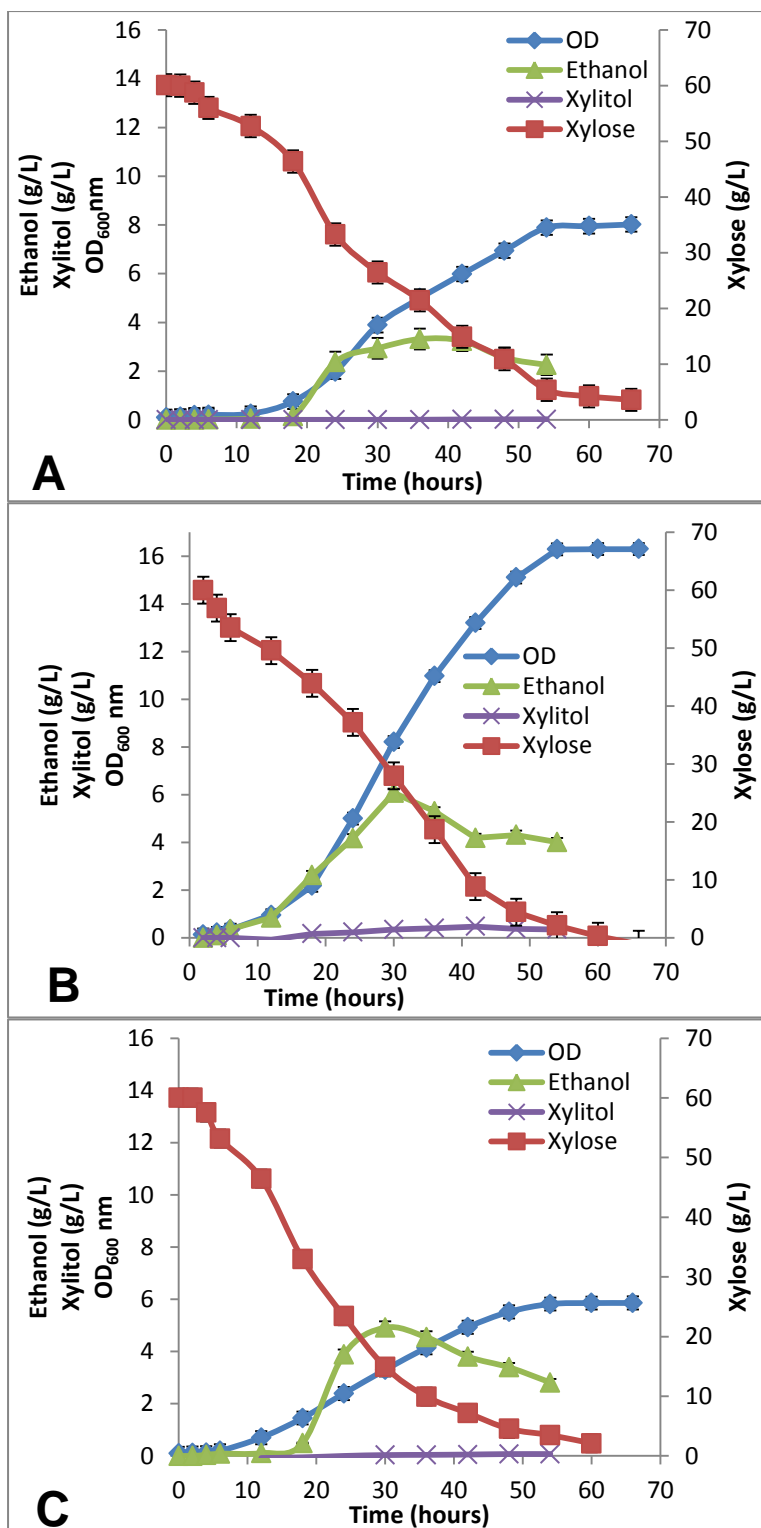


Figure 4.4: Fermentation of xylose at K_{La} 3.3 in the presence of 60 g/L xylose at 30°C without the addition of acetic acid by the parental strain of *P. kudriavzevii* Kp34ey (A), the adapted strain of *P. kudriavzevii* Kp34ey (B) and *S. stipitis* NRRLY-7124 (C).

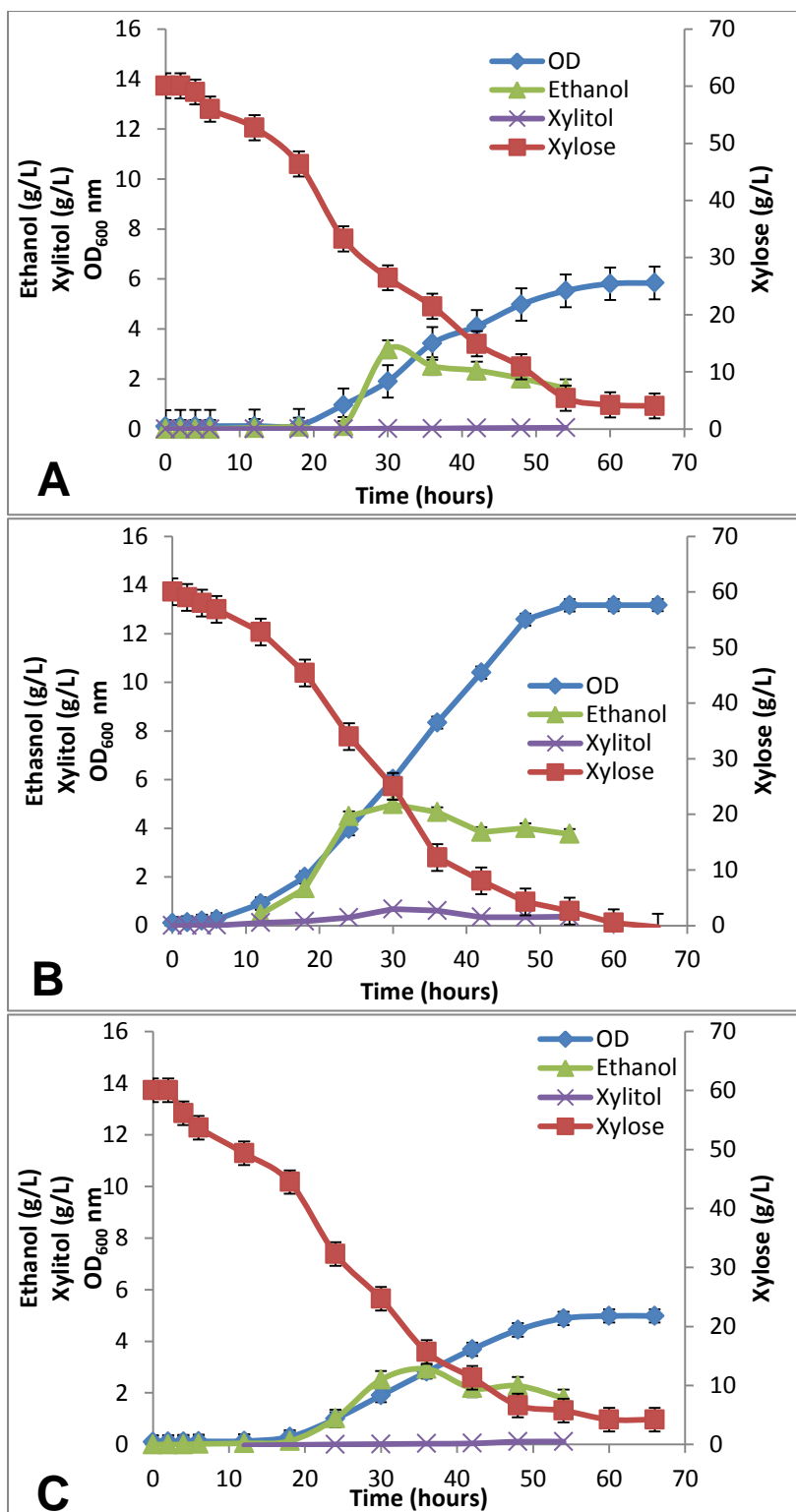


Figure 4.5: Fermentation of xylose at $K_L a$ 3.3 in the presence of 60 g/L xylose at 37°C without the addition of acetic acid by the parental strain of *P. kudriavzevii* Kp34ey (A), adapted strain of *P. kudriavzevii* Kp34ey (B) and the reference strain *S. stipitis* NRRLY-7124 (C).

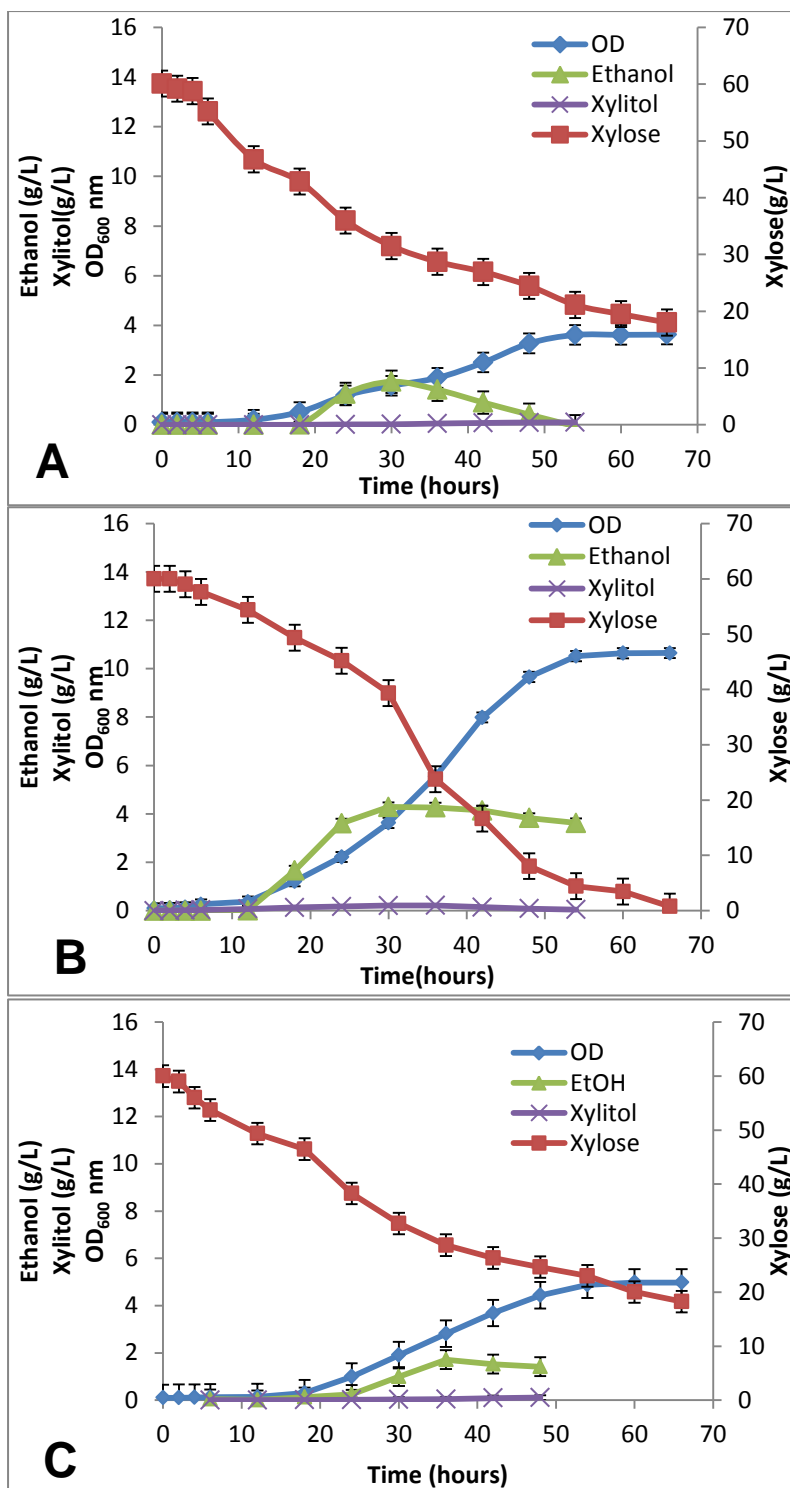


Figure 4.6: Fermentation of xylose at $K_L a$ 3.3 in the presence of 60 g/L xylose at 30°C with the addition of 3 g/L acetic acid by the parental strain of *P. kudriavzevii* Kp34ey (A), adapted strain of *P. kudriavzevii* Kp34ey (B) and the reference strain *S. stipitis* NRRLY-7124 (C).

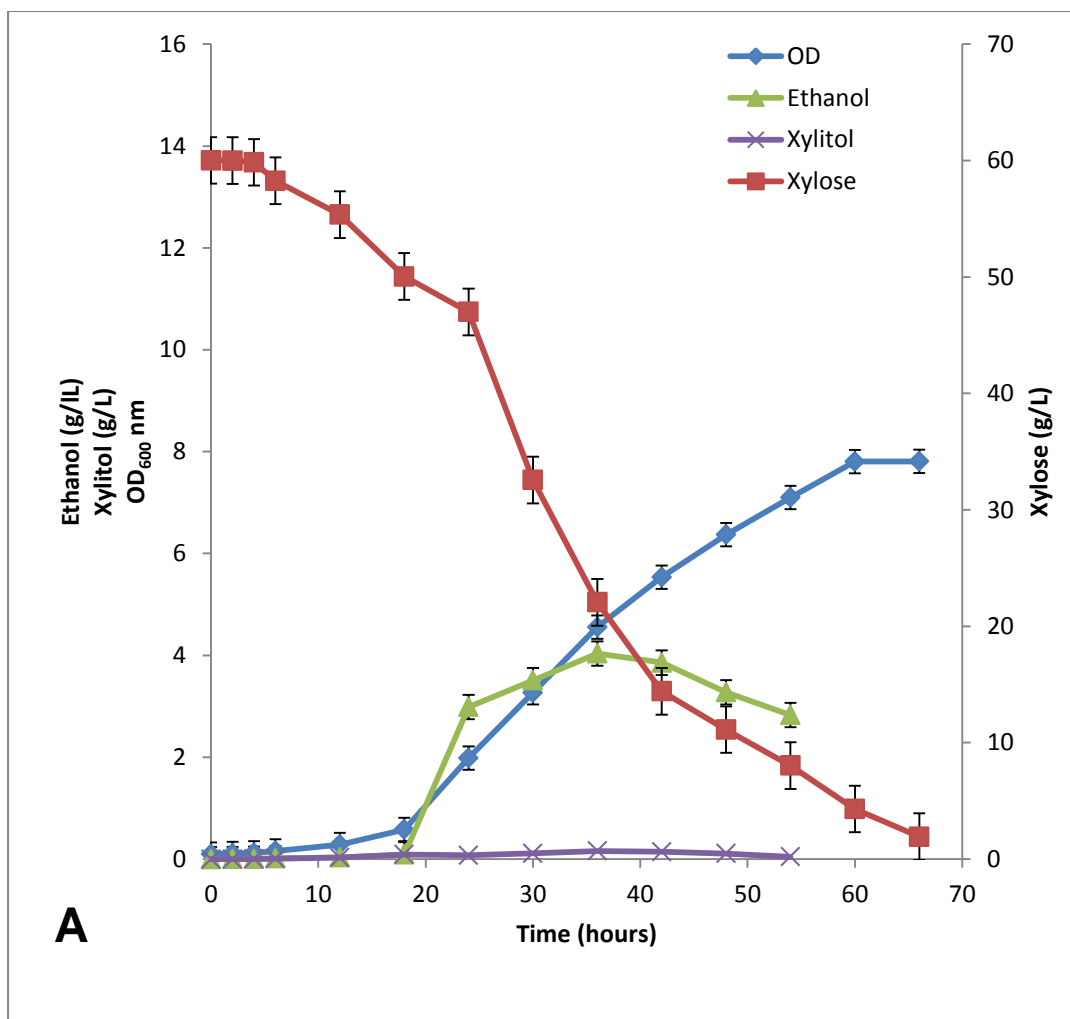


Figure 4.7: Fermentation of xylose by the adapted strain of *P. kudriavzevii* Kp34ey at $K_L a$ 3.3 in the presence of 60 g/L xylose at 37°C with the addition of 3 g/L acetic acid.

4.2.3: Fermentation at a K_La of 4.0

Fermentation by the three yeasts (parental strain of *P. kudriavzevii* Kp34ey, the adapted strain of *P. kudriavzevii* Kp34ey and the reference strain *S. stipitis* NRRLY-7124) was conducted at a K_La of 4.0 and 60 g/l xylose. Fermentation was done at either 30°C or 37°C with the absence or presence of 3 g/L acetic acid (Fig 4.8 - 4.11). All the yeasts were able to grow and ferment in the tested conditions i.e. low and high temperature and in the presence and absence of acetic acid. The ethanol production (1.7 g/L) by *S. stipitis* NRRLY-7124 was lower at K_La 4.0 than K_La 2.5 and K_La 3.3, at 30°C, however biomass was higher compared to K_La 2.5 and 3.3 and no xylitol was produced. The parental and adapted strains for *P. kudriavzevii* Kp34ey produced very low to no xylitol and the biomass was the highest for K_La 4.0. The maximum ethanol produced at 30°C without acetic acid was 3.6 g/L and 2.4 g/L by the adapted strain and parental strain of *P. kudriavzevii* Kp34ey, respectively. The amount of ethanol produced at 37°C with 3 g/L acetic acid was very low for all the yeasts, with the least being 0.2 g/L produced by the parental strain of *P. kudriavzevii* Kp34ey followed by 0.7 g/L by *S. stipitis* NRRLY-7124 and lastly 2.4 g/L by the adapted strain of *P. kudriavzevii* Kp34ey.

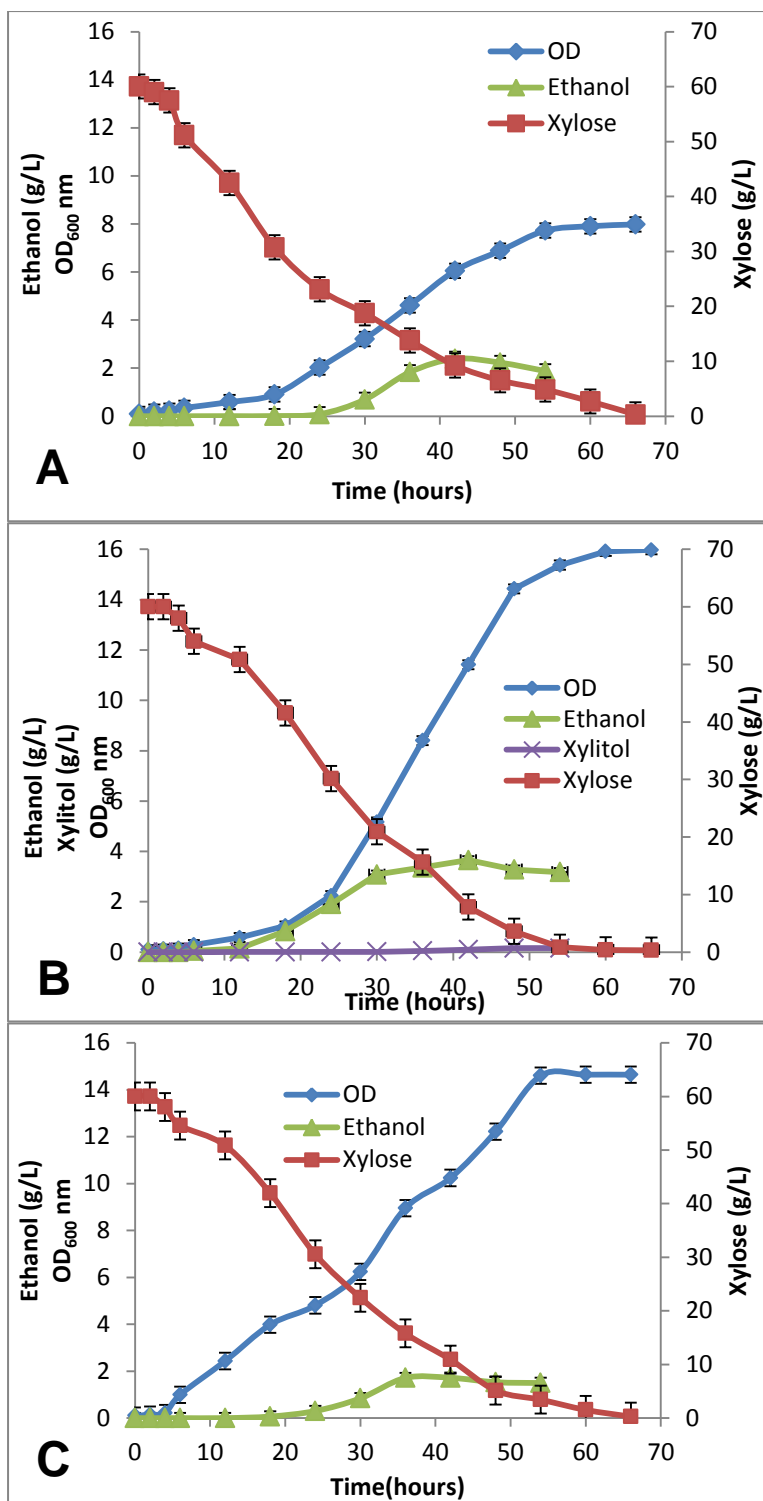


Figure 4.8: Fermentation of xylose at $K_L a$ 4.0 in the presence of 60 g/L xylose at 30°C without the addition of 3 g/L acetic acid by the parental strain of *P. kudriavzevii* Kp34ey (A), adapted strain of *P. kudriavzevii* Kp34ey (B) and the reference strain *S. stipitis* NRRLY-7124 (C).

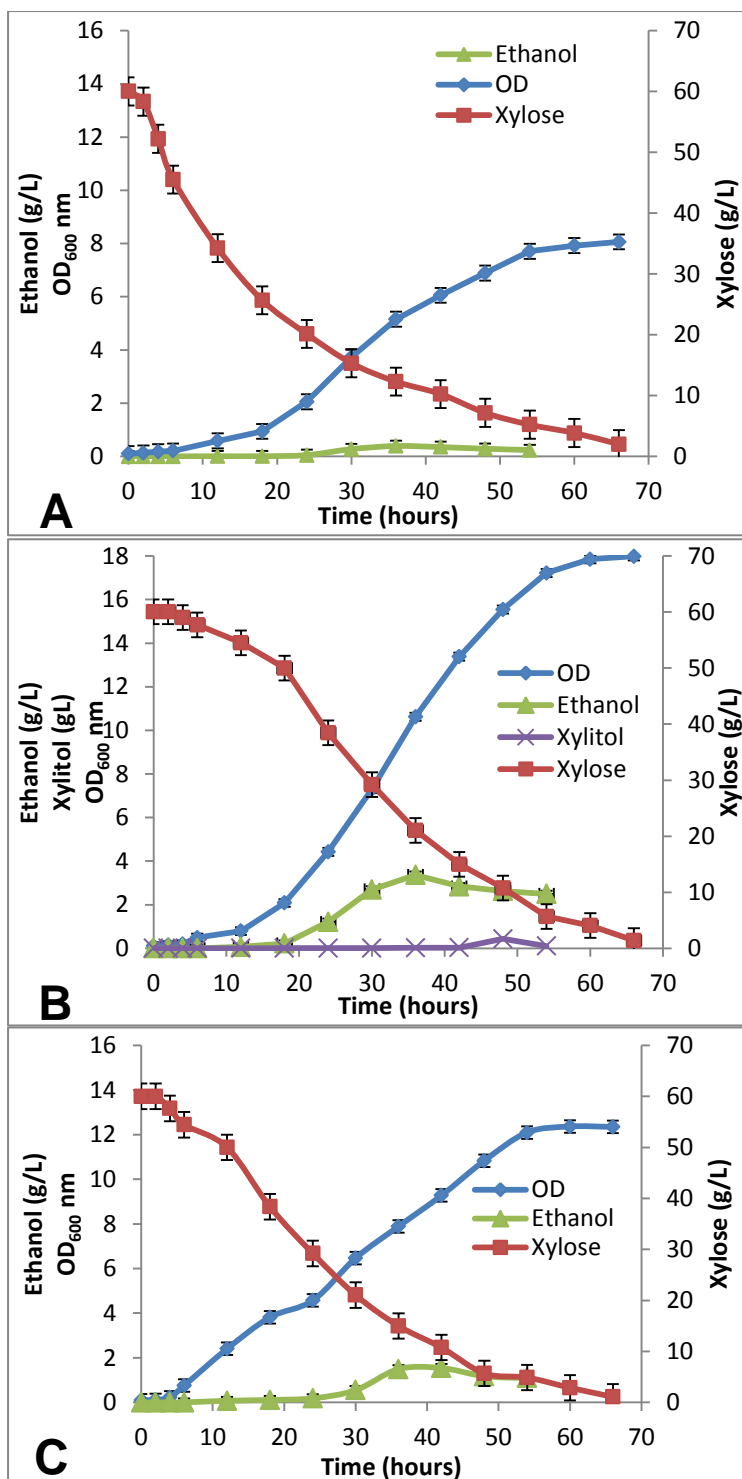


Figure 4.9: Fermentation of xylose at $K_L a$ 4.0 in the presence of 60 g/L xylose at 37°C without the addition of acetic acid by the parental strain of *P. kudriavzevii* Kp34ey (A), adapted strain of *P. kudriavzevii* Kp34ey (B) and the reference strain *S. stipitis* NRRLY-7124 (C).

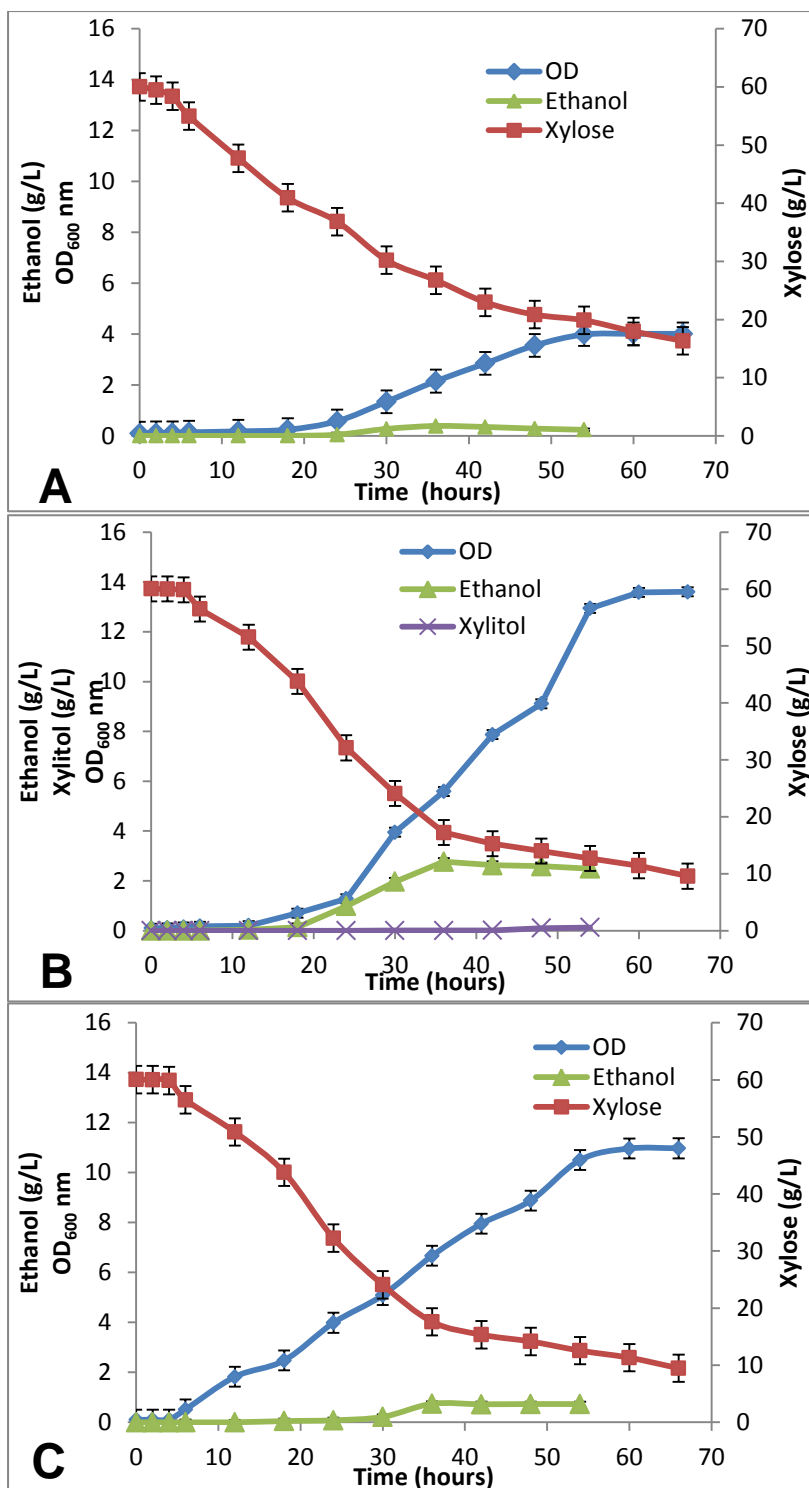


Figure 4.10: Fermentation of xylose at $K_L a$ 4.0 in the presence of 60 g/L xylose at 30°C with the addition of 3 g/L acetic acid by the parental strain of *P. kudriavzevii* Kp34ey (A), adapted strain of *P. kudriavzevii* Kp34ey (B) and the reference strain *S. stipitis* NRRLY-7124 (C).

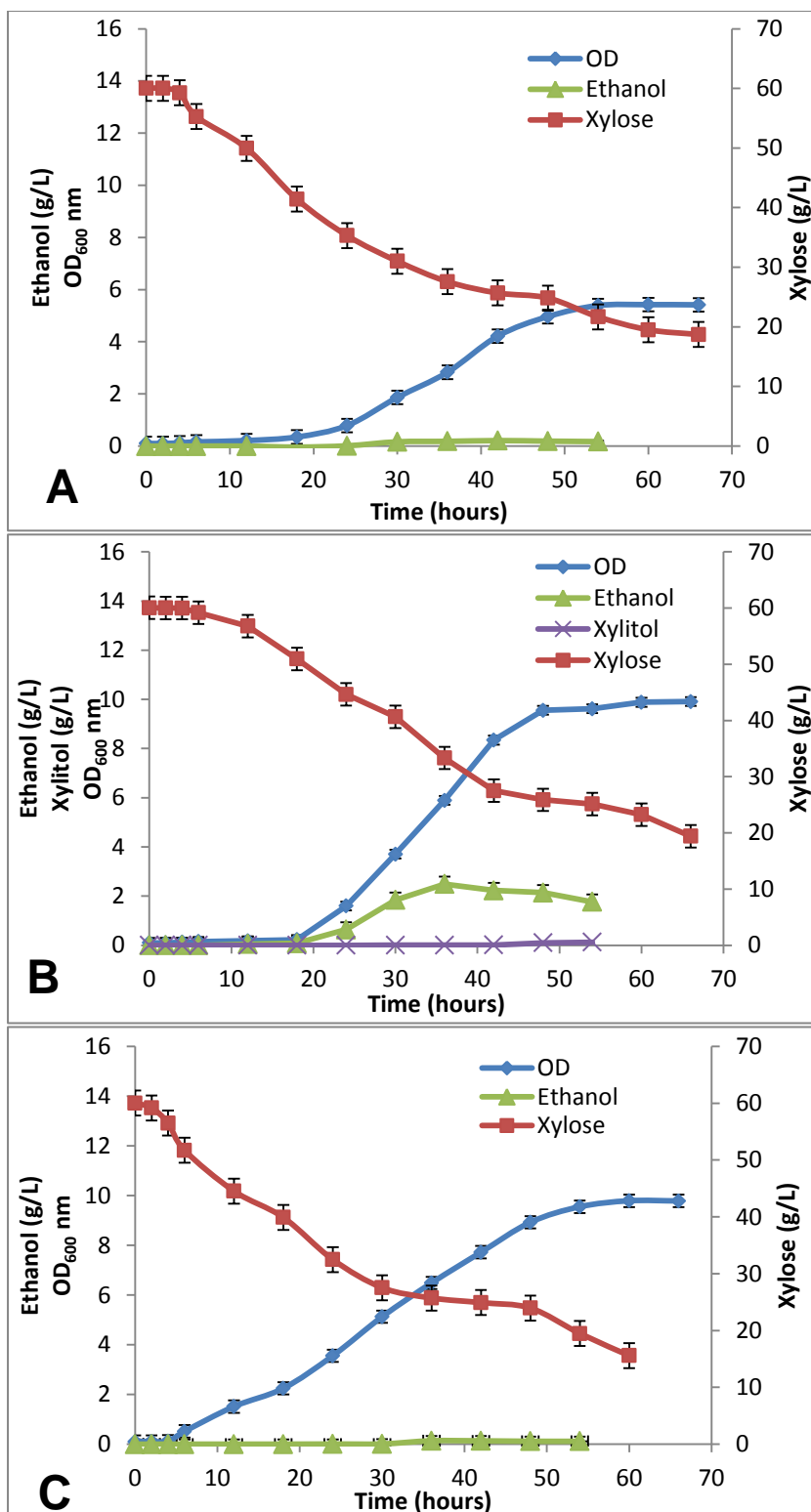


Figure 4.11: Fermentation of xylose at $K_L\alpha$ 4.0 in the presence of 60 g/L xylose at 37°C with the addition of 3 g/L acetic acid by the parental strain of *P. kudriavzevii* Kp34ey (A), adapted strain of *P. kudriavzevii* Kp34ey (B) and the reference strain *S. stipitis* NRRLY-7124 (C).

4.3. Summary of the fermentation studies

Table 4.3 is a summary of the fermentation studies conducted and it shows that the adapted strain of *P. kudriavzevii* KP34ey performed better than the parental strain as well as the reference strain in all the conditions tested. Temperature was measured as degrees Celsius (°C); acetic acid, ethanol produced and xylose consumed were measured in grams per litre (g/L); volumetric oxygen transfer coefficient was measured as K_La (h^{-1}) and maximum specific growth rate was measured as μ_{max} (h^{-1}).

Table 4.3: Fermentation results for *P. kudriavzevii* (parental and adapted) and *S. stipitis*

NRRLY-7124

Yeast	Temperature (°C)	Acetic acid (g/L)	K_{La} (h^{-1})	μ_{max} (h^{-1})	Maximum ethanol produced (g/L)	Xylose consumed (60-n g/L)	Standard deviation of EtOH
<i>P. kudriavzevii</i> kp34ey parental strain	30	0	2.5	0.03±0.00	1.26±0.02	39.8±0.01	2.51±0.02
	37	0		0.10±0.00	0.14±0.01	37.7±0.00	2.56±0.02
	30	3		-	-	-	-
	37	3		-	-	-	-
<i>P. kudriavzevii</i> kp34ey adapted strain	30	0	2.5	0.12±0.02	4.34±0.03	37.47±0.02	2.65±0.02
	37	0		0.17±0.01	4.14±0.04	35.95±0.01	4.24±0.04
	30	3		0.13±0.01	3.81±0.03	33.65±0.04	2.62±0.02
	37	3		1.17±0.02	3.38±0.02	32.2±0.03	2.54±0.03
<i>S. stipitis</i> NRRLY-7124 reference strain	30	0	2.5	0.04±0.00	2.77±0.00	35.93±0.00	0.57±0.00
	37	0		0.10±0.00	0.30±0.00	33.36±0.01	0.57±0.00
	30	3		-	-	-	-
	37	3		-	-	-	-
<i>P. kudriavzevii</i> kp34ey parental strain	30	0	3.3	0.16±0.01	3.32±0.03	56.0±0.03	3.21±0.00
	37	0		0.09±0.00	3.19±0.04	55.95±0.02	3.64±0.01
	30	3		0.04±0.00	1.73±0.04	42.0±0.04	4.35±0.02
	37	3		-	-	-	-
<i>P. kudriavzevii</i> kp34ey adapted strain	30	0	3.3	0.16±0.01	6.08±0.00	59.5±0.01	1±0.00
	37	0		0.16±0.01	4.99±0.01	59±0.01	2.30±0.01
	30	3		0.13±0.01	4.29±0.01	57.32±0.00	2.01±0.00
	37	3		0.10±0.01	4.03±0.01	56.74±0.00	2±0.00
<i>S. stipitis</i> NRRLY-7124 reference strain	30	0	3.3	0.12±0.00	4.91±0.02	47.8±0.02	2.49±0.01
	37	0		0.08±0.01	2.93±0.02	45.8±0.04	2.51±0.01
	30	3		0.09±0.00	1.75±0.02	42.4±0.04	2.51±0.01
	37	3		-	-	-	-
<i>P. kudriavzevii</i> kp34ey parental strain	30	0	4.0	0.09±0.01	2.39±0.01	58.5±0.00	1.52±0.01
	37	0		0.12±0.01	0.39±0.01	54.24±0.00	1.54±0.01
	30	3		0.08±0.00	0.39±0.01	50.09±0.00	1.53±0.01
	37	3		0.09±0.00	0.21±0.01	48.69±0.00	2±0.00
<i>P. kudriavzevii</i> kp34ey adapted strain	30	0	4.0	0.13±0.01	3.65±0.00	59.1±0.01	0.57±0.00
	37	0		0.15±0.00	3.36±0.00	57.8±0.01	0.61±0.00
	30	3		0.12±0.00	2.77±0.00	54.67±0.00	0.59±0.00
	37	3		0.05±0.00	2.49±0.00	53.29±0.00	0.57±0.00
<i>S. stipitis</i> NRRLY-7124 reference strain	30	0	4.0	0.11±0.00	1.71±0.00	58.69±0.01	0.59±0.00
	37	0		0.13±0.00	1.54±0.00	57.12±0.01	0.57±0.00
	30	3		0.12±0.00	1.53±0.00	56.02±0.00	0.55±0.00
	37	3		0.09±0.00	0.74±0.00	50.05±0.00	0.59±0.00

EtOH- Ethanol**n-** Concentration of xylose remaining after fermentation **K_{La}** - Volumetric oxygen transfer coefficient **μ_{max}** - Maximum specific growth rate

CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1 Discussion

The depletion of fossil fuels, currently the main source of transportation fuel, along with the negative ecological impact it has due to greenhouse gas emissions, has led to the development of a sustainable, cleaner and in future perhaps cheaper fuel, namely bioethanol (Liu *et al.*, 2012). Recently, biofuels from lignocellulosic biomass has been extensively acknowledged as one of the most attractive alternatives (Lee, 2007; Ling *et al.*, 2014; Naik *et al.*, 2010; Sims *et al.*, 2016). However, to ensure efficient production of biofuels from lignocellulose, there must be a robust microorganism, that is able to efficiently consume and ferment all the sugars present in the lignocellulosic plant material, particularly xylose and glucose. This microorganism should also be able to withstand high fermenting temperatures, the presence of inhibitors, particularly acetic acid, and be able to ferment in the presence of high sugar concentrations (Koppram *et al.*, 2012). Hence, the aim of this study was to adapt a yeast that will withstand such conditions, for industrial use.

The microorganisms listed in table 4.1 have all been reported to grow in the presence of xylose (da Silva *et al.*, 2008; Cadete *et al.*, 2012; Eken *et al.*, 2000; Arroyo-Lopez *et al.*, 2010, respectively) and in temperatures above 30°C, with *Candida guilliermondii* and *Pichia kudriavzevii* being further studied using adaptation approaches (da Silva *et al.*, 2008; Silva and Roberto, 2001). None of the yeasts used in the above studies were grown in xylose with acetic acid and at elevated temperatures. *Candida tropicalis* was grown in xylose without inhibitors and yielded an ethanol concentration of 8.35 g/L (Eken *et al.*, 2000) compared to the 0.1 g/L in table 4.1 obtained after it was grown on xylose, 2g/L acetic acid and at 37°C. *Candida guilliermondii* produced 1.9 g/L ethanol in 10 g/L of xylose and 2 g/L acetic acid (Sene *et al.*, 2001) compared to the 0.6 g/L in this study (Table 4.1). The ethanol yields were very low compared to published literature (Silva *et al.*, 2011; Cadete *et al.*, 2012; Demeke *et al.*, 2013). This could be attributed to the incubation period (24 hours) and the inclusion of acetic acid and elevated temperatures. Also, this part of the study was conducted to compare the yeast strains under similar conditions and not necessarily optimal conditions.

Adaptation or evolutionary engineering is used successfully to improve the effectiveness of yeasts to produce ethanol (Demeke *et al.*, 2013; Kahr *et al.*, 2011; Nigam, 2012). Initially, seven xylose fermenting yeasts were used and went through a series of different adaptations (high sugar concentration, high temperatures and presence of the inhibitor acetic acid). The adaptation approach was done using one extreme condition at a time to allow the microorganisms to gradually adapt to the environment they were subjected to. For each round of adaptation, the cycle was repeated 50 times to ensure that the microorganisms were fully adapted and would not revert back to their wild type state (Kahr *et al.*, 2011). Media consisting of Yeast Nitrogen Base (without amino acids) and xylose was used to make sure that the only carbon source used would be xylose. This will ensure that no other carbon source was contributing to the growth and adaptation of the microorganisms. At the end of the adaptations, only one yeast strain, *P. kudriavzevii* Kp34ey was able to grow and ferment in 60 g/L xylose, 3 g/L acetic acid and at 37°C. This yeast was further used in fermentation studies using a benchtop stirred tank bioreactor, whereby it was compared to its parental strain (non-adapted) as well as the well-known pentose fermentor, *S. stipitis*. *P. kudriavzevii* has been adapted on high concentrations of galactose and xylose (Yuangsaard *et al.*, 2013), adapted on lignocellulosic hydrolysates (Oberoi *et al.*, 2014), amongst many, which shows its potential as an efficient xylose fermenter. Arroyo Lopez *et al.* (2010) studied the effects of pH, temperature and high sugars (glucose, xylose, glucose with xylose) on fermentation by *P. kudriavzevii* and compared it with the parental strain and *S. cerevisiae* as a control strain. The adapted strain of *P. kudriavzevii* performed four times better than its parental and two times better than the control strain. This is indicated by the increased ethanol production when the adapted strain is fermented in high sugar concentrations, low pH and high temperatures.

Studies in bioreactors have an advantage, because it allows for the accurate monitoring/evaluation of fermentation parameters as compared to shake flasks in a rotary incubator. The parameters used for this study were: 60 g/L xylose, pH 5.5, K_La of 2.5, 3.3, and 4.0, temperatures 30 and 37°C and 0 and 3 g/L acetic acid. Media adopted from Okamoto *et al.* (2012) was used in this study for the fermentation process because Nabais *et al.* (2008) indicated that a combination of several media compositions such as carbon sources, nitrogen sources, trace elements, vitamins, mineral salts, peptides, amino acids and/or other growth factors led to the improvement of alcoholic fermentations.

There are many factors that affect xylose fermentation; the most notable factor is aeration (Dussan *et al.*, 2016). In this study, different aeration rates calculated as K_La were used

similarly to Branco *et al.*, (2008) to determine which oxygen concentration yields high ethanol yields. The yeasts (parental and adapted *P. kudriavzevii* Kp34ey and *S. stipitis* NRRLY-7124) performed better at a K_La of 3.3 in terms of increased ethanol yields (Figures 4.4 - 4.7). The best ethanol concentration of 6.08 g/L was obtained by the adapted *P. kudriavzevii* strain at 30°C. Yuangsaard *et al.* (2013) also adapted a *P. kudriavzevii* strain at high sugar (xylose, xylose + galactose) and high temperatures (37°C and 40°C) at a K_La value of 3.3. They reported a drastic reduction in ethanol production at elevated temperatures and attributed it to a decrease in cell viability, hence the low ethanol production. Gallardo *et al.* (2016) reported *P. kudriavzevii* as a potential yeast for increasing ethanol production under high temperatures, after having adapted it to high temperatures (37°C) and it yielded more ethanol compared to its parental strain at a K_La of 2.5, with an increase in xylitol production. In the oxido-reductase pathway, xylose reductase reduces xylose to xylitol using NADPH or NADH cofactors, xylitol is then oxidised to xylulose by xylitol dehydrogenase using cofactor NAD^+ . Xylulose is then phosphorylated by xylulose kinase to form xylulose-5-phosphate, which will go into the pentose phosphate pathway, eventually yielding ethanol. Oxygen limiting conditions cause cofactor imbalance, which results in the accumulation of xylitol when there is insufficient regeneration of NAD^+ (Yong *et al.*, 2002).

S. stipitis is a respiration-fermentative yeast and consequently surplus oxygen can lead to low production of ethanol (Kuhad *et al.*, 2011). *S. stipitis* can yield ethanol anaerobically, however micro-aerobic conditions for ethanol fermentation seemed to be optimal (Silva *et al.*, 2011). This tendency was also observed in this study for the yeast strains investigated in the bioreactor.

Acetic acid is probably the most toxic agent found in lignocellulosic hydrolysate and has negative effects on the fermentative yeast (Banat *et al.*, 2008; Zhao *et al.*, 2009). Yeast cells are unable to grow and ferment in media containing acetic acid, because of the undissociated form of acetic acid that interferes with the cell membrane of the yeast, thus killing it (Gasch *et al.*, 2000). Not much information is available on the in depth mechanism of yeast tolerance to acetic acid and much focus has been put on the ability of yeast to divide and grow in the presence of acetic acid (Arneborg *et al.*, 2005; Arneborg *et al.*, 2007; Bauer *et al.*, 2013). From the results, *P. kudriavzevii* has shown an improved tolerance to this toxic agent, (this is seen by an increase in biomass and ethanol). This could be because of the progressive biomass adaptation that has led to an induction of enzymes responsible for breakdown of acetic acid which in turn improves production of ethanol and biomass (Casal *et al.*, 2006). During natural adaptation process, several yeast cellular changes occur, some of which include an increase in activities related to cell structure repair and repair of

damaged molecules (DNA, lipids, protein); increase in anabolic pathways, protein synthesis and cell proliferation linked activities. All of these could be possible reasons to improved yeast growth in acetic acid (Almeida *et al.*, 2009).

The adapted strain of *P. kudriavzevii* Kp34ey consumed more sugar in all fermentation conditions compared to the parental strain (Fig 4.1-4.11). Dhaliwal *et al.* (2012) also reported improved sugar consumption in the adapted strain of *P. kudriavzevii* and increased ethanol production compared to the parental strain. The adapted strain of *P. kudriavzevii* Kp34ey was the only yeast in this study able to ferment xylose in the presence of acetic acid at 37°C and is the first report on the adaptation of *P. kudriavzevii* in the presence of acetic acid.

5.2 Conclusions

Effective viable production of second generation biofuels and rapid consumption of xylose is vital. It is of great significance to consider all the potential factors that affect the production of ethanol during xylose fermentation (Balat, 2011). Finding a robust microorganism that can efficiently ferment xylose is one good approach and in this study seven microorganisms were evaluated for xylose fermentation.

The application of evolutionary engineering or adaptation led to the improvement of the yeast strain *P. kudriavzevii* Kp34ey. This adaptation enabled the yeast to grow and ferment at 37°C and in the presence of 60 g/L xylose and the inhibitor acetic acid. The adapted strain of *P. kudriavzevii* Kp34ey compared well with *S. stipitis* in terms of ethanol production and both yeasts performed the best at a K_{La} of 3.3. The adapted strain of *P. kudriavzevii* Kp34ey was also able to ferment xylose in the presence of acetic acid at 37°C. This is the first report on the adaptation of this yeast on acetic acid and indicates its ability to adapt fast in a changing environment. This could be investigated further to improve ethanol production at elevated temperatures to possibly be applied in the conversion of lignocellulose to ethanol.

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