THE IMPROVEMENT OF BIOETHANOL PRODUCTION BY PENTOSE FERMENTING YEASTS PREVIOUSLY ISOLATED FROM HERBAL PREPARATIONS, DUNG BEETLES AND MARULA WINE

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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Surname, Initials (Mr)	**Date**

PUBLICATION AND PRESENTATIONS

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DEDICATION

I dedicate this work to my wonderful and supportive mother, Motolla Salphinah Mabjalwa and my brothers Moremi Clement Sello and Moremi Thabo. Their gratitude and encouragement, motivated me to accomplish this study. This study is mostly dedicated to the late Prof I. Ncube (Co-supervisor), who trained me on the use of HPLC and GC analysis and his scientific contribution will forever be remembered.

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ABSTRACT

Production of bioethanol from lignocellulosic biomass has gained significant attention worldwide as an alternative fuel source for the transportation sector without affecting food supply. Efficient conversion of pentose sugars (L-arabinose and D-xylose) produced during hydrolysis of hemicellulose to ethanol can enhance the economic viability. In this study, a total of 390 yeasts isolated from Marula wine, the gut of dung beetles, herbal concoctions and banana residues were screened for the ability to ferment L-arabinose and D-xylose. Fourteen yeasts were able to ferment both pentose sugars and ten strains were subjected to an adaptation process in the presence of acetic acid using L-arabinose as carbon source. Four adapted strains of *Meyerozyma caribbica* were able to ferment L-arabinose to ethanol and arabitol in the presence of 3 g/L acetic acid at 35 °C. *Meyerozyma caribbica* Mu 2.2f fermented D-xylose, L-arabinose and a mixture of D-xylose and L-arabinose to produce 1.7, 3.0 and 1.9 g/L ethanol, respectively, compared to the parental strain with 1.5, 1.0 and 1.8 g/L ethanol, respectively, in the absence of acetic acid. The adapted strain of *M. caribbica* Mu 2.2f produced 3.6 and 0.8 g/L ethanol from L-arabinose and D-xylose, respectively in the presence of acetic acid while the parental strain failed to grow. In the bioreactor, the adapted strain of *M. caribbica* Mu 2.2f produced 5.7 g/L ethanol in the presence of 3 g/L acetic acid with an ethanol yield and productivity of 0.338 g/g and 0.158 g/L/h, respectively at a K_La value of 3.3 h^{-1}. The adapted strain produced 26.7 g/L arabitol with a yield of 0.900 g/g at a K_La value of 4.9 h^{-1}. *Meyerozyma caribbica* Mu 2.2f could potentially be used to produce ethanol and arabitol under stressed conditions.

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Abbreviations

KH_2PO_4	Potassium dihydrogen phosphate
$MgSO_4$	Magnesium sulfate
HCL	Hydrochloric acid
GC	Gas Chromatography
HPLC	High Pressure Liquid Chromatography
OD	Optical Density

CHAPTER 1

INTRODUCTION

Biofuels are increasingly becoming a renewable alternative to fossil fuels and it is estimated that by 2035 approximately one-quarter of the world's energy will be generated from biomass (Kalyani *et al*., 2017). Bioethanol is a liquid fuel produced from biomass using different conversion technologies (Zhao *et al*., 2018). First generation bioethanol is produced from food crops, such as wheat, barley, maize, potato and sugarcane (Alfenore and Molina-jouve, 2018). However, first generation bioethanol raises a concern about environmental impacts and carbon balances, which sets limits in the increasing production of first generation biofuels. The main disadvantage of first generation bioethanol is the food versus fuel debate, which could lead to food shortages and rising food prices. Furthermore, this result in insufficient land required for food and feed production (Kumari and Singh, 2018). Second generation bioethanol is produced from lignocellulosic biomass, such as forest resources, agricultural residues, municipal and industrial wastes. These biomass sources are abundant, cheaper and do not compete with food resources (Nguyen *et al*., 2016; Kalyani *et al*., 2017; Guerrero *et al*., 2018). Therefore, the conversion of abundant lignocellulosic biomass to transportation fuel will help improve energy security globally (Ramanjaneyulu and Reddy, 2019).

Lignocellulose is composed of three polymeric components namely, cellulose, hemicellulose and lignin. Hemicellulose and cellulose contribute up to 70% of plant biomass and are covalently linked to lignin (Kanta *et al*., 2017). Production of lignocellulosic-based ethanol involves pre-treatment, hydrolysis and fermentation. Pre-treatment of lignocellulosic biomass is necessary for enzymes to access the polysaccharides (hemicellulose and cellulose) in plant biomass in order to ensure efficient saccharification (Kalyani *et al*., 2017). Unfortunately, pre-treatment sometimes result in the release of toxic and acidic compounds, which has a negative effect on the yeasts in the fermentation step (Kumari and Singh, 2018). The hydrolysis or saccharification step involves the release of simple sugars (pentoses, mainly L-arabinose and D-xylose, and

hexose, mainly glucose, mannose and galactose) from the cellulose and hemicellulose components, which can then be converted to ethanol during the fermentation step (Aditiya *et al*., 2016).

Hemicellulose is a heterogeneous polymer composed mainly of pentose sugars, which are released after hydrolysis of hemicellulose by either hemicellulases or acids (Ahmed and Sarkar, 2018). D-xylose and L-arabinose released from hemicellulose generally constitute a significant fraction (nearly 16-19% and 3-15%, respectively) of lignocellulosic biomass. Therefore, their conversion to bioethanol is essential for an economically feasible production process. The concentrations of D-xylose and L-arabinose depend on the nature of lignocellulosic biomass and the type of pre-treatment employed (Alfenore and Molina-jouve, 2018; Koti *et al*., 2016). Fermentation of glucose to bioethanol by yeasts is well known, while the ability of microorganisms to convert D-xylose and L-arabinose to ethanol is often problematic. This is because of the lack of robust microorganisms that can ferment pentose sugars effectively in the presence of inhibitors and high temperatures (Chandel *et al*., 2011; Modi *et al*., 2018). One approach to improve pentose fermenting yeasts in the presence of inhibitors and high temperatures is adaptation or evolutionary engineering (Saini *et al*., 2018). Therefore, there is a need to develop yeast strains able to ferment a wide variety of sugars (pentoses and hexoses) in a highly inhibitory environment and high temperatures, while maintaining a high ethanol yield and production rate (Saini *et al*., 2017).

Aim

The aim of the study is to evaluate and improve yeasts isolated from the gut of dung beetles, herbal concoctions, Marula wine and banana wastes for their ability to ferment D-xylose and L-arabinose in the presence of inhibitors and high temperatures.

Objectives

- To evaluate the ability of locally isolated yeasts to ferment pentose sugars (D-xylose or L-arabinose), previously isolated from the gut of dung beetles, herbal concoctions, Marula wine and banana wastes.
- To adapt selected yeasts to improve ethanol fermentation at higher temperatures.

- To optimise aeration for ethanol production in a bioreactor by the best-adapted yeast.

CHAPTER 2

LITERATURE REVIEW

2.1 Biofuel as an alternative to fossil fuels

Biofuels are defined as a liquid, solid or gas that is generated from biomass. Among the three renewable energies, liquid biofuels receive the most attention as it contributes about 40% of the total energy consumption in the world. Bioethanol (biofuel) is widely used in the world as an alternative fuel to fossil fuel. Bioethanol significantly contributes to the reduction of crude oil consumption, environmental pollution, global warming and the creation of job opportunities (Hajar *et al*., 2017). In an attempt to promote sustainability and independence from fossil fuel, lignocellulosic-based ethanol is now favoured as a blend or fossil fuel substitute (Aditiya *et al*., 2016). Lignocellulosic based ethanol can be generated from different sources of biomass feedstocks like molasses, wheat straw, grass and agricultural wastes (Jahnavi *et al*., 2017).

2.2 Liquid biofuels

Renewable sources used for the production of liquid biofuels (bioethanol) are classified largely into the first generation (sugar and starch), second generation (lignocellulosic biomass) and third generation (algae) bioethanol (Zabed *et al*., 2017). Bioethanol obtained from the edible feedstock, such as sugar cane, maize, wheat, rice, cassava and sweet potato is classified as first generation bioethanol. Second generation bioethanol is produced from non-edible feedstocks that includes wood, straw and grasses (Hajar *et al*., 2017). First and second generation bioethanol have potential on a commercial scale (Zabed *et al*., 2017). Production of third-generation bioethanol from algae is still in a developmental stage and limited to laboratory research.

2.2.1 First generation liquid biofuels

Ethanol (as liquid biofuel) produced from sucrose or starch is classified as first generation bioethanol. Sucrose from sugar cane and starch from maize have been used for bioethanol production in countries such as Brazil and the USA (Zabed *et al*., 2017). The

production of bioethanol from starch-containing materials consists of four steps; pre-treatment, fermentation, distillation and dehydration. The conversion of starch into fermentable sugars is a simple process compared to lignocellulosic material. Pre-treatment and the dehydration steps are the key steps that affect bioethanol production efficiency and the total cost of the process (Bayrakci *et al*., 2017). However, the use of edible biomass has limited the production of first generation bioethanol because the use of edible biomass results in a price increase in food suitable for human consumption (Ahmed and Sarkar, 2018; Kumari and Singh, 2018).

2.2.3 Second generation liquid biofuels

Second generation bioethanol is derived from lignocellulosic biomass, such as wood, straw and grasses. These feedstocks are abundant, cheap and do not compete directly with food production (Hajar *et al*., 2017; Kalyani *et al*., 2017). Bioethanol generated from these feedstocks is regarded as enviromentally friendly, safe and clean. Production of second generation bioethanol also involves four major steps; pre-treatment, hydrolysis, fermentation and distillation (Guerrero *et al*., 2018). Different pre-treatment methods have been applied to enhance enzyme accessibility to lignocellulose substrates. During the hydrolysis process, complex carbohydrates such as celluloses and hemicelluloses are converted into free monomer molecules (hexose and pentose sugars), which are further converted into ethanol by microorganisms during the fermentation process (Alfenore and Molina-jouve, 2018; Carrillo-Nieves *et al*., 2019).

2.3 Lignocellulosic biomass

Lignocellulosic biomass is the primary building block of plant cell walls, which mainly consists of hemicellulose, cellulose and lignin (Fig 2.1) and the content of these molecules in the cell wall vary with different plant sources (Table 2.1). Lignin is covalently linked to hemicellulose and cellulose to form a complex network, which is highly robust and recalcitrant to depolymerisation (Zabed *et al*., 2016). This makes the production of ethanol from lignocellulosic biomass more complex than starchy raw material (Ishizaki and Hasumi, 2014).

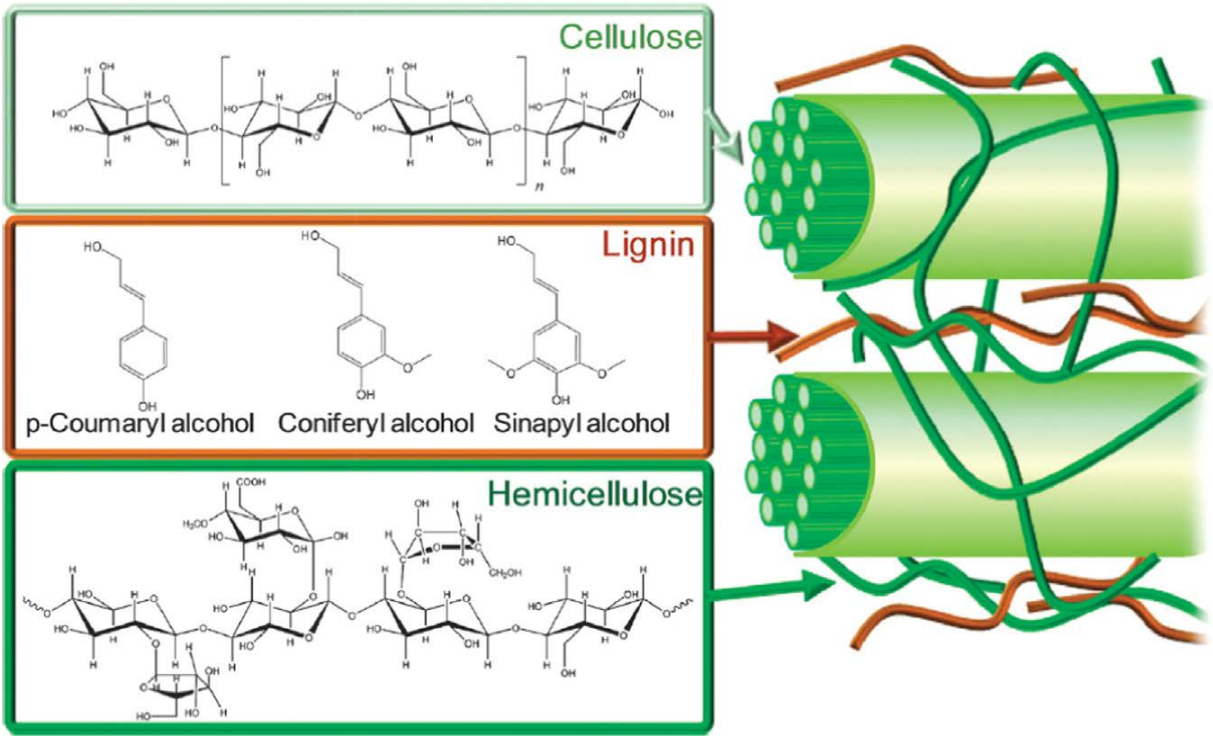

Figure 2.1. Lignocellulose structure (Bach and Skreiberg, 2016).

Table 2.1. The composition of lignocellulosic biomass from different plant sources (Khattak *et al*., 2012; Ramanjaneyulu and Reddy, 2019).

Lignocellulose source	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**
Coniferous wood	40-50	20-30	25-35
Wheat straw	35-38	26-32	23-32
Bagasse	52	19-26	23-32
Corn Stalks	35-47	26-31	3-5
Rice straw	28-36	22-28	12-14
Corn Stover	40-43	21-28	7-21

2.3.1 Cellulose

Cellulose is the most abundant component of lignocellulose in the environment and is a linear homopolymer consisting of D-glucose units that are joined together in a chain by β-1,4–glycosidic linkages. It constitutes 15 – 30% of the dry mass of the primary cell wall and up to 40% of the secondary cell wall in the plant cells (Zabed *et al*., 2016). Cellulose serves as the main carbon source for bioethanol production from lignocellulosic biomass followed by hemicellulose (Tian *et al*., 2018). Cellulose is present in both crystalline and amorphous forms, which makes it water-insoluble and resistant to depolymerisation (Zabed *et al*., 2016).

2.3.2 Hemicellulose

Hemicelluloses are a heterogeneous class of polymers representing 15 – 30% of plant biomass and contain a mixture of pentoses (mainly D-xylose and L-arabinose) and hexoses (D-mannose, D-glucose and D-galactose). Woody biomass is composed of a higher content of hemicellulose than those of herbaceous and agricultural waste (Luo *et al*., 2019). D-xylose and L-arabinose generally constitute a significant fraction (nearly 16-19% and 3-15%, respectively) of lignocellulosic biomass and their utilization is essential for a feasible bioethanol production process. Hence, the importance to ferment pentose sugars along with glucose to make the process economically viable (Koti *et al*., 2016).

2.3.2 Lignin

Lignin is a phenolic polymer covalently linked with cellulose and hemicelluloses in plant biomass. Lignin contributes between 10 and 20% of the lignocellulosic biomass depending on the plant species. Lignin is composed of three phenylpropanoid monomers; p-coumaryl, coniferyl and sinapyl. Alcohols contribute 15 - 35% (w/w) of the lignocellulose complex. Lignin plays a unique role in strengthening the plant structure and also protects both cellulose and hemicellulose from pathogens and insect attack (Kumari and Singh, 2018; Naidu *et al*., 2018; Carrillo-Nieves *et al*., 2019). The presence of lignin limits the rate of enzymatic hydrolysis by acting as a physical barrier and preventing the digestible parts of the substrate to be hydrolysed (Tian *et al*., 2018). Chen *et al*. (2006) have indicated that genetically engineered lignin in plants reduces the lignin formation and

enhances ethanol production, but reduces the plant's resistance to pathogens and insect infestation.

2.4 Lignocellulosic bioethanol

Bioethanol production from lignocellulosic biomass involves four steps, namely pre-treatment, hydrolysis of complex carbohydrates, fermentation, and distillation for product recovery (Kuhad *et al*., 2011) as indicated in Fig 2.2.

2.4.1 Pre-treatment

Lignocellulosic plant biomass has a complex and recalcitrant structure, which requires a pre-treatment step prior to enzymatic hydrolysis. The aim of the pre-treatment step is to degrade the lignin structure and disrupt the crystalline structure of cellulose to enhance the accessibility of enzymes to cellulose during the hydrolysis step (Bhatia *et al*., 2017; Mohapatra *et al*., 2017). This increases substrate porosity with lignin redistribution in the cell wall and enables maximal exposure of cellulose surface area for the enzymes to attain effective hydrolysis with minimal energy consumption and maximal sugar recovery (Mohapatra *et al*., 2017).

Hemicelluloses are sensitive to some pre-treatment processes, such as steam explosion which degrade hemicellulose and generate toxic compounds that could affect the subsequent hydrolysis and fermentation steps (Alvira *et al*., 2010; Ge *et al*., 2018). Temperature and retention times are the important parameters required to be controlled during the pre-treatment process to avoid the formation of unwanted inhibitors, such as furfurals and hydroxymethyl furfurals, which has a negative effect on the fermenting organism (Haghighi Mood *et al*., 2013).

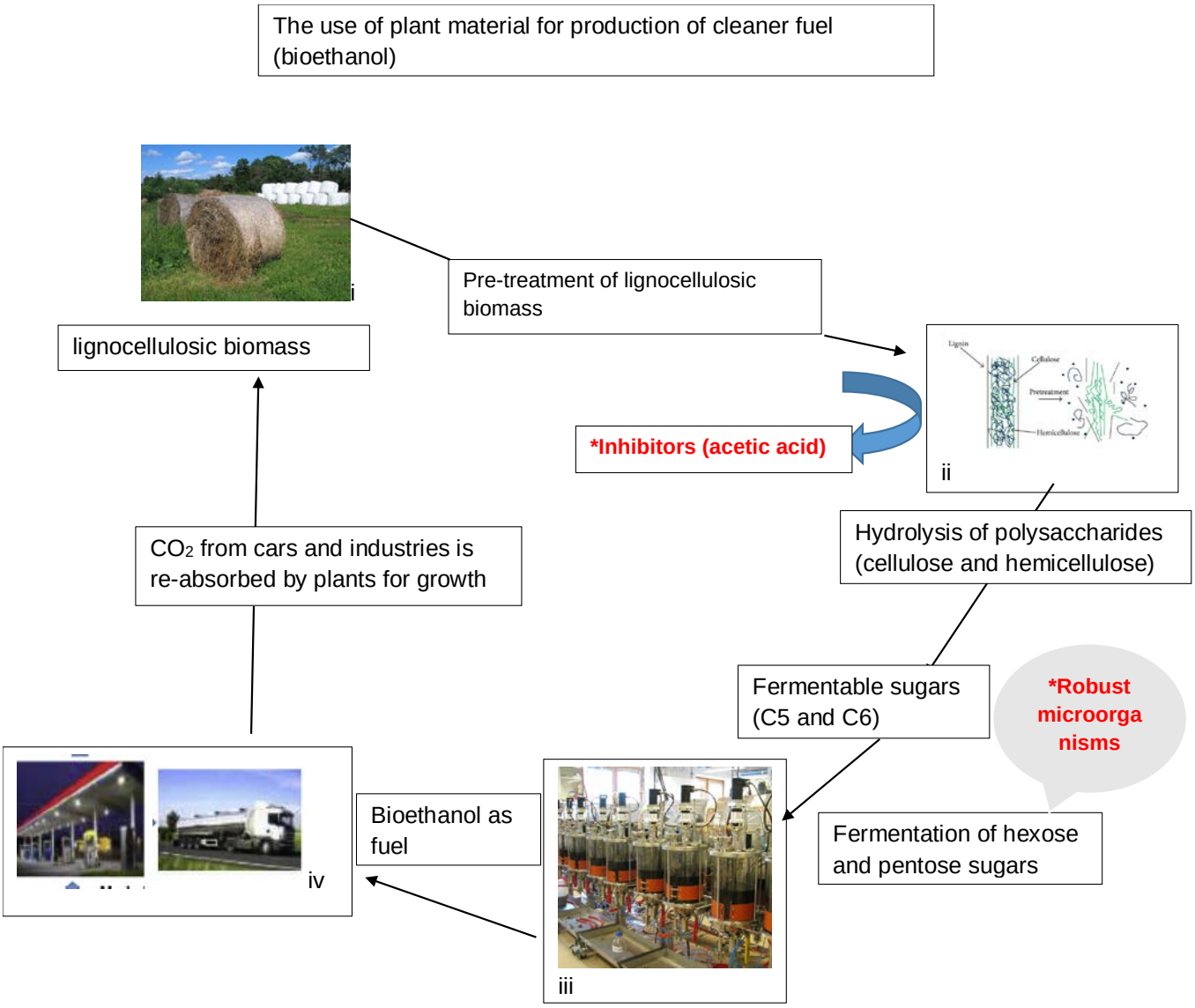

Figure 2.2 Represent overall ethanol production from plant material and factors (marked in red) that hinders ethanol yields. (i), (iii) (Sun, 2015), (ii) (Sànchez Nogué and Karhumaa, 2015), (iv) (Ahmed and Sarkar, 2018)

2.5. Inhibitors released from lignocellulose during pre-treatment

Dehydration of hexose and pentose sugars during pre-treatment results in the formation of the inhibitory compounds (Fig 2.3). These chemicals can affect the performance of microorganisms during fermentation. Formation of inhibitory substances depends on the type of pre-treatment used on lignocellulose. These inhibitors are classified into three groups: furans, carboxylic acids and phenolic compounds (Koppram *et al*., 2012).

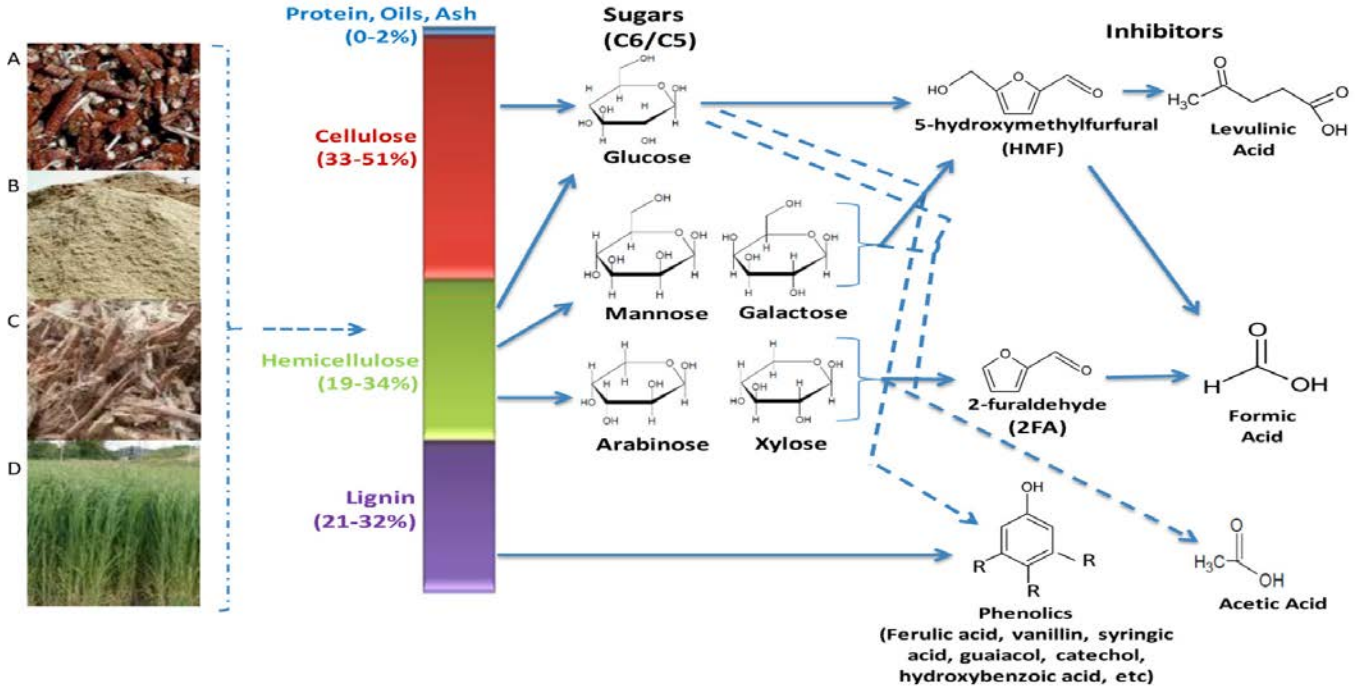

Figure 2.3. Different sugars and inhibitory compounds produced during pre-treatment. (A) maize cobs (B) sawdust (C) sugarcane bagasse (D) fast-growing grasses (Ibraheem and Ndimba, 2013).

2.5.1. Carboxylic acids

The major carboxylic acids (also known as aliphatic acids) released during pre-treatment of lignocellulose are acetic acid, formic acid, and levulinic acid. Acetic acid is the most studied inhibitor due to its negative effect on fermentative yeasts and its inability to convert acetic acid to less harmful compounds as compared to other inhibitors (Wei *et al*., 2015; Yuan *et al*., 2017). Acetic acid is produced during the hydrolysis of acetyl groups from hemicellulose (Fig 2.3). Formic acid is formed during the degradation of furfural and 5-

hydroxymethylfurfural (HMF), while levulinic acid is formed through the degradation of HMF (Fig 2.3) (Jönsson *et.al*, 2013).

Feedstocks, such as agricultural residues and hardwood have a high content of acetylated xylan, which releases high concentrations of aliphatic acids (more than 100 mM) and results in the inhibition of growth and fermentation. Softwood hydrolysate is composed of low concentrations of carboxylic acid (below 100 mM), which could improve ethanol production (Jeon *et al*., 2016). Palmqvist *et al*. (1999) showed that acetic acid significantly inhibited the growth of the yeast *Candida shehatae* NJ 23 when growing on a medium containing glucose as a major carbon source. A study by Hasunuma *et al*. (2011) indicated that the addition of acetic acid results in a reduction of D-xylose consumption and in the production of ethanol, xylitol and glycerol. Undissociated weak acids such as acetic acid diffuse across the plasma membrane into the cytosol, where the dissociation of the acids takes place due to the neutral intracellular pH. The dissociation of the acid results in the decrease in the cytosolic pH, which may lead to cell death (Fletcher *et al*., 2017, Palmqvist and Hahn-Hägerdal, 2000; Jönsson *et al*., 2013). In order to retain the intracellular pH, more ATP is rapidly generated and hydrolysed for pumping out protons, which results in the loss of energy that affects cell growth of microorganisms negatively (Wei *et al*., 2013; Wang, *et al*., 2018).

2.5.2. Furans

Furfural and 5-hydroxymethylfurfural (HMF) are the main furan compounds derived from the dehydration of pentose and hexose sugars, respectively, during pre-treatment of lignocellulose Furan compounds inhibit the glycolysis pathways of many organisms by interacting with protein and RNA synthesis (Barakat *et al*., 2012; Luo *et al*., 2019; Taherzadeh *et al*., 2000). Furfural negatively affected the specific growth rate of cells by inhibiting the glycolytic enzymes and causing damage to mitochondria, vacuoles, actin, and nuclear chromatin. Some microorganisms (such as *S. cerevisiae*) can convert furfural into less toxic compounds, such as furfuryl alcohol and furoic acid, which reduce the inhibition caused by furfural (Wang *et al*., 2016). Wang *et al*. (2016) indicated that *Candida tropicalis* continued to grow and consume D-xylose in the presence of furfural (3 and 5

g/L) implying that D-xylose transport or metabolism was not affected by furfural at low concentrations, while 9 g/L furfural was lethal for *C. tropicalis*.

2.5.3 Phenolic compounds

Phenolic compounds are formed from lignin (vanillyl alcohol, hydroxybenzoic acid and syringic acid) or sugars (catechol) during acid-catalysed hydrolysis or pre-treatment of lignocellulose (Larsson *et al*., 2000; Jönsson *et al*., 2013; Wang *et al*., 2018). Lignin content varies with the type of plant material used, therefore the concentration of inhibitors formed will also vary (Amen-chen *et al*., 2001). Phenolic compounds have an inhibitory effect on both microbial growth and ethanol yield (Palmqvist and Hahn-Hägerdal, 2000). Phenolic compounds with low molecular weight are more toxic than those with higher molecular weight because they diffuse rapidly and easily through the cell and inhibit cell growth and ethanol production (Lin *et al*., 2015). The mechanism of toxicity has not been elucidated yet, but Keweloh *et al*. (1990) suggested one possible mechanism could be the interference with the cell membrane by influencing its function and changing its protein-to-lipid ratio. It seems yeast are able to convert the inhibitory state of the phenolic compounds into a non-inhibitory state. Larsson *et al*. (2000) studied the influence of aromatic compounds on oxygen-limited and ethanolic fermentation by *S. cerevisiae*. They indicated that *S. cerevisiae* was able to convert coniferyl aldehyde to coniferyl alcohol and dihydroconiferyl alcohol, which are less toxic compounds.

2.6 Hydrolysis

The hydrolysis process occurs after pre-treatment to convert polysaccharides (cellulose or hemicellulose) into fermentable sugars for ethanol production. The hydrolysis process is required since microorganisms used for fermentation can only ferment simpler sugars (Aditiya *et al*., 2016; Hajar *et al*., 2017). Two common hydrolysis methods used are acid or enzymes. Enzymatic hydrolysis is known for its economical challenge due to the high cost of enzymes and considered impractical for commercial purposes. However, in comparison with acid hydrolysis, enzymes work in a mild environment, hence lower equipment maintenance cost. Moreover, the disposal system for acid hydrolysis is essential to consider and it requires additional cost. Another major problem is the ability

of the acid to gradually degrade the sugar monomers once they are formed in a hostile acidic environment at high temperatures (Aditiya *et al*., 2016).

2.6.1 Enzymatic hydrolysis of cellulose

Cellulase is a complex of enzymes that act together to completely convert cellulose into monomeric glucose. The cellulase system is composed of three major groups of enzymes: endoglucanase, exoglucanase and β–glucosidase (Kumar *et al*., 2015; Zabed *et al*., 2017). Endoglucanase, randomly cleaves β–1,4 glycosidic linkages of cellulose, resulting in the decrease in chain length and release of free chain ends, while exoglucanase splits cellobiose units from the ends of cellulose molecules. The end result of both endoglucanase and exoglucanase are short cello-oligosaccharides and cellobiose, which are catalytically cleaved to produce glucose through the action of β–glucosidase (Qing *et al*., 2010). Cellulase yields 53 - 94% of glucose depending on the type of feedstock and pre-treatment technique utilised (Khattak *et al*., 2012). Cellulases are thermostable with an optimal temperature ranging from 50 - 60 ºC and are stable at a pH range of between 4.5 - 5 (Srivastava *et al*., 2018). Ko *et al*. (2015) obtained a 70% glucose yield from cellulose using Cellic Ctec2 at 50 ºC in 72 hours.

2.6.2 Enzymatic hydrolysis of hemicelluloses

Hemicellulose, unlike cellulose, is easier to hydrolyse due to its amorphous structure. Xylan, as one of the most abundant components in hemicellulose, is a polysaccharide that is composed of pentose sugars (mainly D-xylose and L-arabinose). Degradation of xylan requires multiple enzymes broadly called xylanases (Aditiya *et al*., 2016; Zabed *et al*., 2017). Softwood hemicelluloses are composed of xyloglucans, arabinogalactans, arabinoglucuronoxylans and glucomannans while hardwood is composed of xylans and glucomannans (Sindhu *et al*., 2016). The xylanase system is composed of endo-xylanase, exo-xylanase, β–xylosidase, α–arabinofuranosidase, α–glucuronidase, acetyl xylan esterase, and ferulic acid esterase. Endo and exo–xylanase act on the main chains of xylans and hydrolyse them into smaller chains. The enzyme β–xylosidase attacks xylo-oligosaccharides and produces 69-77% of xylose. The α–arabinofuranosidase and α–glucuronidase act on the xylan backbone and removes L-arabinose (35%) and 4–o–methyl glucuronic acid, respectively (Khattak *et al*., 2012; Zabed *et al*., 2016).

2.7 Fermentation

Fermentation is a metabolic process that converts simple sugars (pentoses and hexoses) into ethanol (Zabed *et al*., 2017). Pentose sugars (D-xylose and L-arabinose) generated from lignocellulosic biomass cannot be fermented by *S. cerevisiae* commonly used in the ethanol industry. Hence, other yeasts and bacteria are used to increase fermentation yields of ethanol derived from biomass sugars (both pentose and hexose). Hydrolysates derived from lignocellulosic biomass tend to contain fermentation inhibitors, such as acetic acid and furfural that lower ethanol yield. Therefore, robust strains that are resistant to inhibitors are required (Gray *et al*., 2006).

2.7.1 Ethanol production from L-arabinose and D-xylose

Bioethanol production from lignocellulosic biomass depends on the availability of robust microorganisms that can ferment all sugars available in the feedstock, including D-xylose and L-arabinose. Currently, *S. cerevisiae* is a robust ethanol producer of D-glucose, but cannot ferment pentose sugars (Bettiga *et al*., 2009).

The pentose sugars, L-arabinose and D-xylose enter the pentose phosphate pathway (PPP) after they are converted to D-xylulose 5-phosphate (Fig 2.4). In bacteria, L-arabinose is converted to L-ribulose, and then to L-ribulose-5-phosphate and finally to D-xylulose-5-phosphate (substrate of the pentose phosphate pathway), catalysed by L-arabinose isomerase, L-ribulokinase and L-ribulose-5-P 4-epimerase, respectively (Fig. 2.4).

D-xylose is directly isomerized to D-xylulose by D-xylose isomerase, followed by phosphorylation to form D-xylulose-5-phosphate that enter the pentose phosphate pathway (Bettiga *et al*., 2009; Chandel *et al*., 2011). Bacteria can convert D-xylose to ethanol under anaerobic conditions. The major drawback of using ethanologenic bacteria is the inhibition of the fermentation process due to high concentrations of ethanol and sugars and the production of unwanted by-products (Chandel *et al*., 2011).
In fungi, L-arabinose is converted to L-arabitol and D-xylose is converted to xylitol by the same enzyme aldose reductase. L-arabitol is converted to L-xylulose by L-arabitol

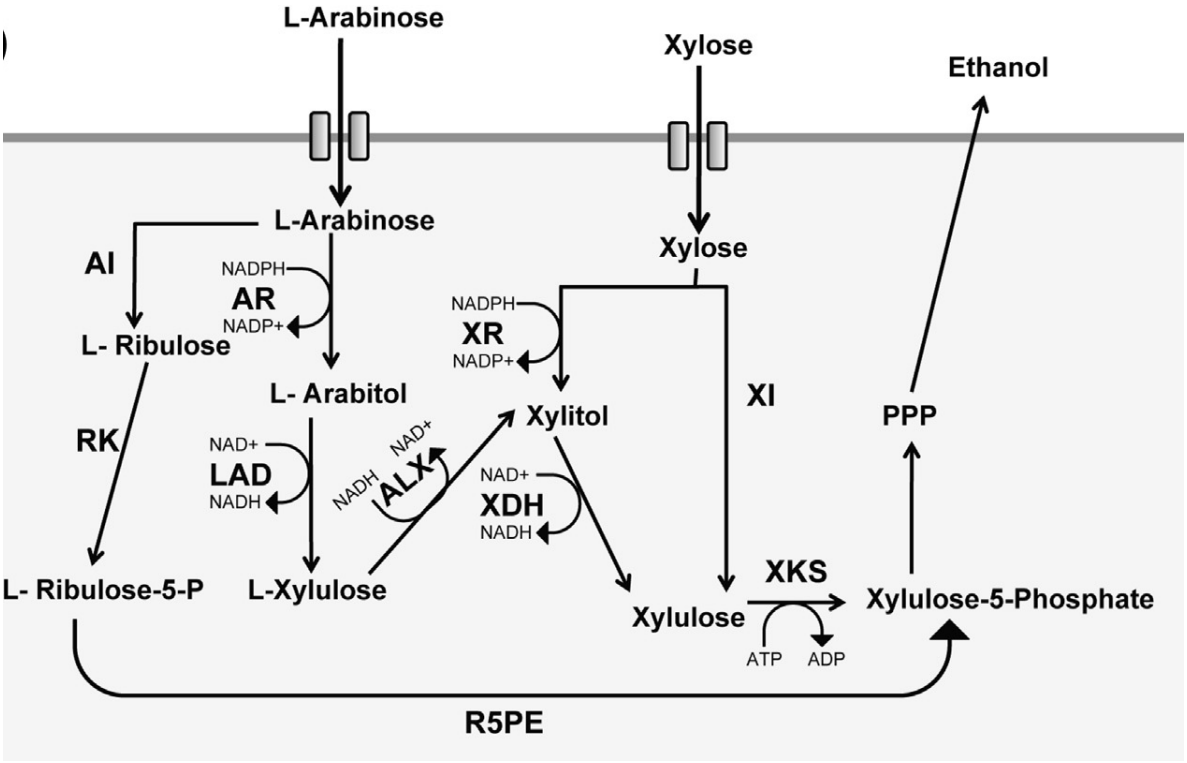

Figure 2.4. Bacterial and fungal pentose (L-arabinose and D-xylose) catabolism pathway. AI: L-arabinose isomerase; ALX: L-xylulose reductase; AR: aldose reductase; LAD: L-arabitol dehydrogenase; R5PE: ribulose 5-phosphate epimerase; RK: ribulokinase; XDH: xylitol dehydrogenase; XI: D-xylose isomerase; XKS: xylulokinase, XR: xylose reductase (Kricka *et al*., 2015).

dehydrogenase, which is further reduced to xylitol by L-xylulose reductase. Thus, the L-arabinose pathway converges with the D-xylose pathway at the level of the achiral compound xylitol (Bettiga *et al*., 2009). Xylitol is converted to D-xylulose by xylitol dehydrogenase, which is further oxidized to form D-xylulose-5-phosphate (Fig. 2.4). D-Xylulose is further metabolized via the pentose phosphate pathway (PPP), in which the non-oxidative rearrangements of α−xylose-5-phosphate by ribulose phosphate-3-epimerase, transaldolase (TAL) and transketolase (TK) results in the generation of glyceraldehyde-3-phosphate and fructose-6-phosphate, which can be converted to ethanol by fermentative reactions of the Embden–Meyerhoff–Parnas (EMP) pathway. Most fungi need microaerophilic conditions to ferment pentose sugars to prevent co-factor imbalances (Kuhad *et al*., 2011).

2.7.2 Pentose fermenting yeasts

The fermentation of pentose sugars from lignocellulosic biomass is critical for the economical production of lignocellulosic ethanol (Guarnieri *et al*., 2017). Traditional yeast (*S. cerevisiae*) can efficiently ferment glucose to ethanol, but cannot ferment D-xylose, which is a dominant sugar in hemicellulose (Kim *et al*., 2013; Sharma *et al*., 2017).

Yeasts isolated from different locations, such as tree exudates, wood-boring insects, decaying wood, rotten fruit and tree bark have been reported to produce ethanol from D-xylose. Known natural D-xylose fermenting yeasts include *Scheffersomyces* (*Pichia*) *stipitis*, *Candida shehatae*, *Candida lignosa*, *Candida insectosa*, *Candida tenuis*, *Kluyveromyces marxianus*, *Pachysolen tannophilus*, *Spathaspora passalidarum* and *Spathaspora arborariae*. Among these yeasts, *S*. *stipitis* and *S. passalidarum* are considered the best ethanol producers from D-xylose (Cadete *et al*., 2012; Hou, 2012). However, these yeasts cannot produce ethanol from D-xylose effectively in the presence of inhibitors at elevated temperatures (Cadete *et al*., 2012; Romero-García *et al*., 2016).

Natural L-arabinose fermenting yeasts are poorly characterized by the limited information available (Fonseca *et al*., 2007; Silva *et al*., 2014). The study of McMillan and Boynton. (1994) showed that *S*. *stipitis*, *Schizosaccharomyces pombe*, *Candida tropicalis*, and *P. tannophilus* were able to utilize L-arabinose and produce L-arabitol as a major end-product, rather than ethanol. This study was supported by Nigam (2001a), who indicated that *S. stipitis* assimilated low amounts of L-arabinose, but could not ferment it. According to a study conducted by Fonseca *et al*. (2007), *Candida arabinofermentans* PYCC 5603T grew on fermentation medium containing 80 g/L L-arabinose and produce 2.4 g/L of ethanol after 48 hours

2.8 Conversion of pentose sugars to ethanol

The major factors that influence the conversion of pentose sugars to ethanol include aeration, temperature, pH, incubation time, initial substrate concentration and inhibitors present in the hydrolysate.

2.8.1 Temperature

Temperature is an important parameter and is regulated as it affects the performance of cells during fermentation. Most yeast fermentation processes operate in the temperature range between 20 and 36 ˚C (Imtiaz *et al*., 2013). High temperatures (above 45 ˚C) negatively affect the fermentation process. Yeast cells produce heat-shock proteins and inactivate its cellular ribosomes in response to this stress condition (Cazetta *et al*., 2007; Zabed *et al*., 2017). Cazetta *et al*. (2007) reported that 40 ˚C was negative for the fermentative process, resulting in lower ethanol production. On the other hand, much lower temperatures (15 - 20 ˚C) during fermentation caused lower specific growth rates of the cells and resulted in lower tolerance to ethanol. The ideal temperature for bioethanol production differs depending on the yeast. The ideal temperature for *S. stipitis*, *S. cerevisiae*, and *K. marxianus* is 25 - 26, 30 and 45 ˚C respectively (Hajar *et al*., 2017; Santos *et al*., 2016). However, finding a thermotolerant yeast able to ferment at high temperatures would potentially reduce cooling costs, increase saccharification and fermentation rates and minimise contamination (Signori *et al*., 2014).

2.8.2 pH

The pH is another key factor with a significant influence on ethanol fermentation, as it directly affects yeast cells and cellular processes (Masiero *et al*., 2014; Zabed *et al*., 2017). In the fermentation media, a pH below or above a certain level can alter the overall charge on the plasma membrane, which affects the permeability of some essential nutrients into the cells. A pH of 4.0 - 5.0 is normally the range for ethanol production by yeasts. The optimum pH range during the fermentation process may differ depending on the type of substrate utilised and fermenting yeast used (Lin *et al*., 2012a). Recent studies indicated that *S. cerevisiae* can grow sufficiently and produce ethanol from date juice at a pH of 3.8 (Louhichi *et al*., 2013). Dussán *et a*l. (2016) reported that both *S. stipitis* and *S. shehatae* yeasts were able to grow on fermentation medium consisting of D-xylose with an initial pH of 6.50 and produced maximum ethanol concentrations of 7.34 and 18 g /L with a final pH of 6.98 and 6.91, respectively.

2.8.3 Incubation time

A short or long incubation time affect ethanol yields. Cadete *et al*. (2012) indicated that *S. passalidarum* strains and *S. stipitis* UFMG-XMD-15.2 produced ethanol optimally from D-xylose (18 and 12.3 g/L, respectively) in 24 hours. A study by du Preez *et al*. (1986) indicated that *C. shehatae* CBS 2779 consumed 100% D-xylose and 99% L-arabinose in 28 and 211 hours, respectively. Less ethanol was produced from D-xylose, while no ethanol was detected from L-arabinose. *Scheffersomyces shehatae* UFMG-HM 52.2 consumed 100% of pentose sugars present in the sugarcane bagasse and produced 10 g/L ethanol after 48 hours (Antunes *et al*., 2014). However, recently it has been reported that *Meyerozyma caribbica* continued to produce ethanol from L-arabinose when the fermentation time was increased from 72 to 96 hours (Sukpipat *et al*., 2017).

2.8.4 Initial substrate concentration

Initial substrate concentration has a direct effect on the rate of fermentation and the growth of yeast cells (Singh *et al*., 2014). The yeast starves and ethanol productivity decreases when the D-xylose concentration is below 1 g/L. A high sugar concentration (normally 150 g/L) causes the fermentation rate to increase. However, the use of excessive sugar (above 150 g/L) will cause a steady fermentation rate, since the uptake capacity of the microbial cells is at a maximum rate (Hajar *et al*., 2017). It should be noted that different fermenting microbes respond differently to the initial substrate concentration (Lin *et al*., 2012a).

Scheffersomyces guilliermondii PYCC 3012 did not produce any ethanol in complex medium containing 80 g/L L-arabinose under oxygen-limited conditions (Fonseca *et al*., 2007). Dien *et al*. (1996) showed that *Ambrosiozyma monospora* grew on media containing 80 g/L L-arabinose and produced 4.1 g/L ethanol after 12 days. It has been reported that *S. stipitis* NRRL Y-7124 consumed 90% of D-xylose when grown on fermentation media with 50 g/L D-xylose and produced a maximum ethanol yield of 0.35 g/g after 24 hours (Cadete *et al*., 2012). Modi *et al*. (2018) reported that *C. tropicalis* BE grew on fermentation medium containing 2% D-xylose and achieved a 74% ethanol yield. However, ethanol yield of less than 10% was obtained when 9% of D-xylose was utilised. The decrease in ethanol yield was due to osmotic stress implemented by a high sugar

concentration that acted as a fermentation inhibitor. Currently, no studies have been conducted on the effect of different L-arabinose concentrations on ethanol production.

2.8.5 Aeration

Aeration is important for pentose fermentation by yeasts because the aeration level sets the carbon flow division between cell growth and product formation (Dussán *et al*., 2016). At low aeration or micro-aeration condition, yeasts produce either arabitol or xylitol instead of ethanol from D-xylose or L-arabinose. This scenario is due to the imbalance of the co-factors NAD^+ and NADPH. NADPH is a co-factor for xylose reductase that reduces L-arabinose or D-xylose to arabitol or xylitol, respectively. Arabitol or xylitol is further oxidized to form xylulose via arabitol or xylitol dehydrogenase that uses NAD^+ as co-factor. Therefore, the cell accumulates more arabitol or xylitol due to the insufficient conversion of NADH to NAD^+ (Makhuvele *et al*., 2017; Martins *et al*., 2018; Seiboth and Metz, 2011).

Hou (2012) demonstrated that *Spathaspora passalidarum* produced a high ethanol yield, fast cell growth, and rapid sugar consumption with D-xylose being consumed after glucose depletion, while *S. stipitis* was unable to utilize D-xylose under anaerobic conditions. The reason for *S*. *passalidarum* to consume D-xylose under the anaerobic condition is that the strain preferred NADH (instead of NAPDH) as a cofactor for dual D-xylose reductase and NAD^+ for xylitol dehydrogenase. Therefore, sufficient NAD^+ required by xylitol dehydrogenase were generated through re-oxidation of NADH by xylose reductase. Unrean and Ngyen (2012) reported that D-xylose fermentation by *S. stipitis* under micro-aeration increased the ethanol yield to 0.40 g/g from D-xylose. High oxygen levels resulted in an increase in specific growth rate and lower ethanol yields. Lin *et al*. (2012b) noted that *S. stipitis* produced a maximum ethanol yield of 0.44 g/g at an aeration rate of 0.05 vvm with an increase in aeration resulting in lower ethanol yields, with an increase in biomass.

Fonseca *et al*. (2007) indicated that *C. arabinofermentans* PYCC 5603(T) and *P. guilliermondii* PYCC 3012 produced biomass when grown on media containing L-arabinose under aerobic conditions. When microaerobic conditions were applied, both yeasts produced L-arabitol of 0.09 g/g and 0.38 g/g, respectively, instead of ethanol. This

is normally due to insufficient regeneration of the cofactor NAD^+ that is required by either xylitol or arabitol dehydrogenase.

2.9 Adaptation or evolutionary engineering

Evolutionary engineering or adaptation is a tool to enhance a trait of a microbial population under selective pressure (Parachin, 2011). One of the most interesting features of microorganisms is their ability to adapt rapidly to different environmental conditions. Adaptive laboratory evolution has been used with great success to gain insights into the genetic basis and dynamics of adaptation (Portnoy *et al*., 2011). In addition, adaptation has also become widely used for biotechnological applications, improving yields, and reducing costs in industrial settings. The normal range of subculturing is between 50 and 100 repetitions (Harner *et al*., 2015; Huang *et al*., 2009).

A classical method for evolutionary engineering is the development of a robust strain from the parental strain by direct sequential transfer of a strain on a solid culture medium (Fig 2.5) that provides the selective pressure, such as high inhibitor concentrations and temperatures (Çakar *et al*., 2012). Serial transfer in shake flasks or tubes (Fig 2.5) is another approach for yeast evolutionary engineering. Serial transfer selects for mutants with a higher maximum specific growth rate, and with improved rates of substrate consumption and product formation (Mans *et al*., 2018).

Adaptation of yeast strains to inhibitor compounds is an alternative approach to the detoxification of hydrolysates and will prevent the loss of fermentable sugars during detoxification (Alvira *et al*., 2010). Moreover, adaptation approaches have also been used to improve fermentation capabilities and ethanol yield in recombinant strains such as *S. stipitis* and *S. cerevisiae* (Kuhad *et al*., 2011). Some of the recognized evolutionary approaches include an adaptation of microorganisms to whole fermentable sugars with high concentrations of inhibitors or to defined media composed of one or more synthetic inhibitors (Koppram *et al*., 2012).

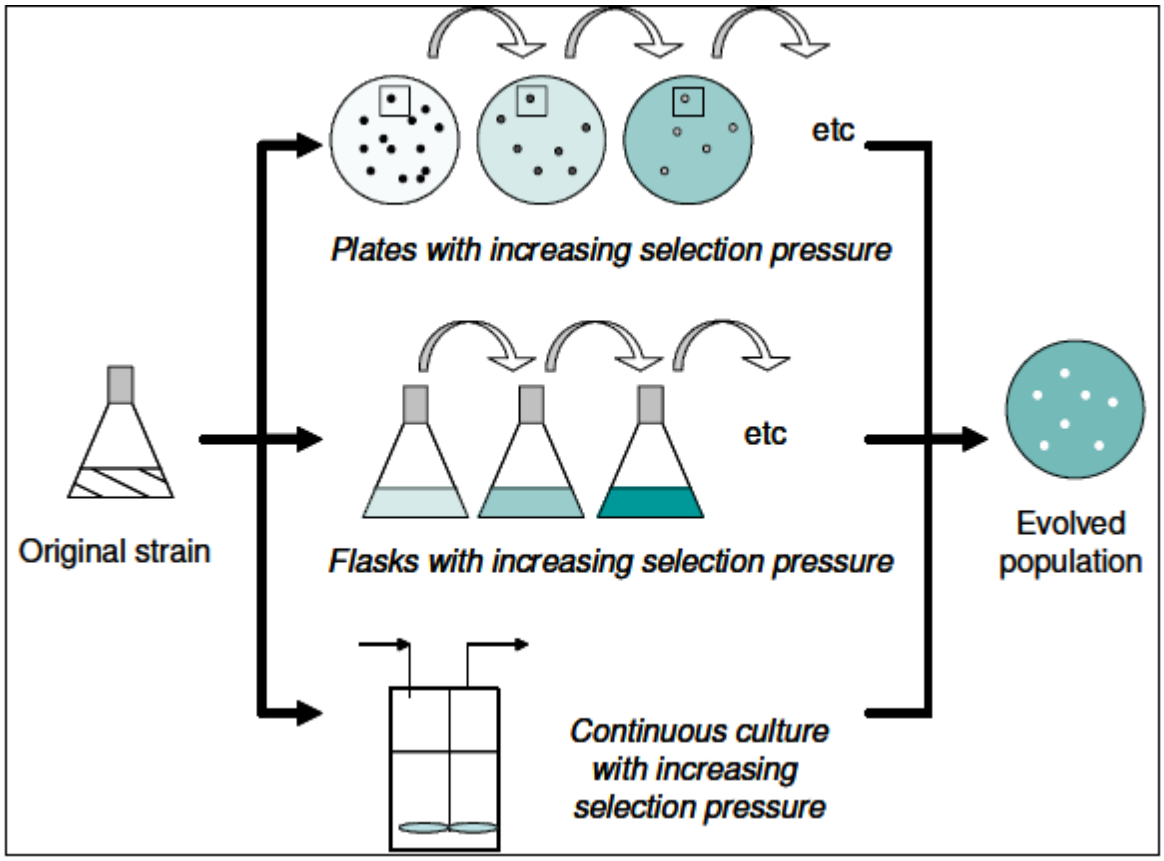

Figure 2.5 Different approaches to evolutionary engineering to obtain the desired phenotype (Hahn-Hägerdal *et al*., 2005)

Silva *et al*. (2014) reported that a non-adapted *S. stipitis* consumed only 51% of D-xylose while an adapted strain consumed D-xylose 96% better than the non-adapted strain. Martı *et al*. (2007) showed that the adapted recombinant xylose-utilizing *S. cerevisiae* strain was able to consume D-xylose and produce ethanol in a shorter period as compared to the parental strain. The adapted strain was also able to convert fermentation inhibitors (furfural and HMF) faster to non-toxic compounds, as compared to the non-adapted strain (Martı *et al*., 2007; Silva *et al*., 2014). Morales *et al*. (2017) found that after 380 generations of adaptation for *Spathaspora passalidarum* it produced 5.8 g/L ethanol (0.31 g/g ethanol yield) in the presence of 3.5 g/L acetic acid in a synthetic medium containing a mixture of glucose and D-xylose. The native strain in comparison obtained an ethanol yield of 0.21 g/g. No studies for adapting fermenting yeast for L-arabinose fermentation have been found in the literature.

Chapter 3

Materials and Methods

3.1 Yeasts

Three hundred and ninety yeasts, previously isolated from banana waste, the gut of dung beetles, herbal concoctions and Marula wine were streaked on D-xylose or L-arabinose agar plates (10 g/L D-xylose or L-arabinose, 15 g/L bacteriological agar, 6.7 g/L yeast nitrogen base (YNB) and 0.2 g/L chloramphenicol) and incubated at 30 ˚C for 120 hours. Freshly inoculated yeast malt agar (YM) slants (10 g/L glucose, 0.2 g/L chloramphenicol, 3 g/L malt extract, 3 g/L yeasts extract, 5 g/L peptone and 15 g/L bacteriological agar) with yeasts that grew on both D-xylose and L-arabinose were incubated at 30 ˚C for 24 hours and stored at 4 ˚C (Barathikannan *et al*., 2016). All the pentose sugars in this study were autoclaved separately and mixed afterwards with the rest of the medium to prevent caramelization. The purity of the yeast cultures was regularly checked by microscopic examination and colony morphology throughout the study.

3.2 Selection of D-xylose and L-arabinose fermenting yeasts

All yeasts able to grow on both D-xylose and L-arabinose agar plates were inoculated into test tubes with a Durham tube. The test tubes containing modified fermentation media described by Silva *et al*. (2012) consisted of 30 g/L sugar (D-xylose or L-arabinose), 5 g/L peptone, 3 g/L yeast extract, 2.3 g/L urea, 3 g/L KH_2PO_4, 1 g/L $MgSO_4$ and 0.2 g/L chloramphenicol. The fermentation test tubes were incubated at 30 °C for 120 hours. Positive results were noted by the presence of a bubble in the Durham tube. *Scheffersomyces stipitis* NRRL Y7124 was used as a positive control for both D-xylose and L-arabinose fermentation. Experiments were performed in duplicates.

3.3 Yeast identification using ITS and D1/D2 sequencing

The isolated yeasts fermenting D-xylose and L-arabinose were identified by means of DNA sequencing. The yeast isolates were streaked on YM agar plates and incubated at 30 ˚C. The cultures were sequenced by Inqaba Biotechnical Industries (Pty) Ltd (South

Africa). The ZR Fungal/Bacterial DNA MiniPrepTM Kit (Zymo Research) was utilised for DNA extraction, according to the instructions of the manufacturer. The ITS1 region of all selected yeasts was amplified using the PCR primers ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Barathikannan *et al*., 2016). Amplification was done in 25 µl reactions using the EconoTaq Plus Green Master Mix (Lucingen). The following PCR conditions were performed: 35 cycles including an initial denaturation at 95 ºC for 2 min. Thereafter denaturation was done at 95 ºC for 30 s, annealing at 50 ºC for 30 s and extension at 72 ºC for 1 min. A final extension at 72 ºC for 10 min was followed by holding it at 4 ºC. The D1/D2 domain of the 26S rDNA region was also amplified for all yeast isolates using primers NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') as described above. The DNA sequencing was done with ABI V3.1 Big dye according to the manufacturer's instructions on the ABI 3500 XL Instrument.

The sequences were cleaned using Chromas, followed by Bioedit to produce consensus sequences. The yeast isolates were identified by comparing the obtained sequences with that of the NCBI National Center for Biotechnology Information's searching databases (http://www.ncbi.nlm.nih.gov/BLAST/) using the BLAST sequence analysis tool. The ITS1 and D1/D2 sequences were compared using nucleotide-nucleotide BLAST (blastn) with default settings.

Biochemical properties of selected yeast isolates were identified using the VITEK 2 automated microbiology system as described by Pincus (2010). The VITEK 2 system was applied to compare the biochemical tests of the yeast isolates in these study with the reported biochemical tests of similar yeasts. The study was accomplished by suspension of a pure culture on to the test kit (VITEK 2 YST) and the kit was placed in the cassette which was incubated in the VITEK incubator. The test kit was specifically used for yeast biochemical tests and the biochemical reaction was analysed as the culture grow occurs in the VITEK incubator.

3.4 Determination of ethanol production from pentose-fermenting yeasts

A pre-inoculum was prepared in 250 ml Erlenmeyer flasks containing 25 ml of the modified fermentation media as described in 3.2 with D-xylose or L-arabinose used as carbon source. The flasks, inoculated with yeasts able to ferment both D-xylose and L-arabinose, were incubated at 30 °C and 200 rpm for 48 hours. The cultures were used to inoculate Erlenmeyer flasks (250 ml) containing 100 ml of the same media with a starting optical density (OD) of 0.1 at 600 nm. The flasks were incubated in the same manner as the pre-inoculum for a period of 96 hours. Two-millilitre samples were withdrawn at 24, 48, 72 and 96 hours to determine the ethanol concentration using gas chromatography (GC). Experiments were performed in duplicates.

3.5 Acetic acid and thermotolerance of pentose-fermenting yeasts

Pentose fermenting yeasts able to ferment both the pentose sugars were grown on slants containing 6.7 g/L YNB and 20 g/L of L-arabinose and incubated at different temperatures (35 °C, 37 °C and 40 °C, respectively) to determine the maximum growth temperature. Different concentrations of acetic acid (1, 2 and 3 g/L) were added in the same media (used for temperature evaluation) to determine the ability of the yeasts to grow in the presence of acetic acid during incubation at 30 °C. Yeasts with acetic acid tolerance and thermotolerance were further used in the adaptation experiments. The experiments were done in duplicate.

3.6 Adaptation (evolutionary engineering) of yeasts on L-arabinose.

Yeast strains able to ferment both pentose sugars and grew in the presence of 3 g/L acetic acid were selected for adaptation. The combination of pentose sugars, higher temperatures and the presence of acetic acid were used to improve the selected yeasts. The yeasts were inoculated onto agar plates containing 6.7 g/L YNB supplemented with 30 g/L L-arabinose and 3 g/L acetic acid and incubated at 35 °C for 24 hours at pH 5.0. Colonies were re-streaked onto the same media and incubated at 35 °C for 24 hours and the process was repeated 50 times. The process was repeated 50 times at 37 °C followed by 40 °C under the same conditions as described for 35 °C. The process of re-streaking

for each of the changed parameters was done according to the method of Silva *et al*. (2014).

3.7 Screening of adapted yeasts for ethanol production

The best adapted yeast strains (ability to ferment in the presence of acetic acid at elevated temperatures) were screened for ethanol production at different temperatures (35, 37 and 40 °C) in 250 ml Erlenmeyer flasks containing the same modified fermentation medium (3 g/L yeast extract, 2.3 g/L urea, 1 g/L $MgSO_4$, 30 g/L L-arabinose, 5 g/L peptone, 3g/L KH_4PO_2 and 0.2 g/L chloramphenicol) with 3 g/L acetic acid. Each flask contained L-arabinose as carbon source and sampling was done every 24 hours for 120 hours.

The best-adapted yeast strain was selected based on high ethanol production along with the acetic acid tolerance and thermotolerance. The best-adapted yeast strain was then compared with its parental strain in 250 ml Erlenmeyer flasks using different pentose sugars in the presence and absence of acetic acid. The different pentose concentrations used in the flasks contained either D-xylose (50 g/L), L-arabinose (40 g/L), or a mixture of D-xylose (50 g/L) and L-arabinose (40 g/L) with or without the addition of 3 g/L acetic acid. The sampling was done regularly for 5 days and the experiments were conducted in triplicates. HPLC was used to determine D-xylose, L-arabinose, arabitol and xylitol concentrations, with GC used for ethanol determination.

3.8 Fermentation studies

The best-adapted yeast strain was further evaluated for ethanol production in a Bioflow New Brunswick Bioreactor using a three-litre fermenting vessel containing one litre of media. Control modules used included aeration, pH, agitation and temperature. The same fermenting media as indicated earlier (section 3.7) was used with L-arabinose as a carbon source. Fermentation in the bioreactor was conducted at a fixed pH of 5.0 by adding 3M HCl to prevent an increase in media pH at 35 °C. Aeration and agitation were tested using different fixed K_La values (2.3, 3.3 and 4.9 h^{-1}). The experiments were conducted in triplicate for a period of 120 hours. Sampling was done regularly to determine the biomass, ethanol, L-arabinose and arabitol concentrations in the bioreactor. Biomass determinations were done using dry weight in grams, whereas sugar and ethanol

concentrations were determined using high-performance liquid chromatography (HPLC) and gas chromatography (GC), respectively.

3.9 Determination of volumetric oxygen transfer coefficient (K_La)

Various aeration rates and agitation speeds were used to determine the effect of oxygen on ethanol production by the selected adapted yeast strain. The dynamic gassing-out method was applied to determine the different K_La (2.3, 3.3 and 4.9 h^{-1}) values. In this method, the oxygen concentration in the non-fermented medium was reduced to zero by gassing in nitrogen gas through the bioreactor. The deoxygenated medium was re-aerated and agitated at a fixed agitation speed and aeration rate using a calibrated polarographic oxygen sensor to measure dissolved oxygen in the medium. The polarographic oxygen sensor was previously calibrated at atmospheric pressure according to the instructions of the manufacturer. The concentration of dissolved oxygen in the medium was monitored using the equation:

$$\frac{dC\mathrm{L}}{dt} = K_La\ (C^*_L - C_L) \quad (1)$$

The K_La values were calculated using $\ln(C^*_L - C_L)$ versus time, where C_L is the concentration of dissolved oxygen in the fermentation broth and C^*_L is the saturated dissolved oxygen concentration in the fermentation medium (Bellido et al., 2013). The K_La values tested during fermentation of L-arabinose were 2.3 h^{-1}, 3.3 h^{-1} and 4.9 h^{-1} with air that was introduced into the bioreactor at 0.1 vvm for all K_La values with the agitation speed at 100 rpm for 2.3 h^{-1}, 150 rpm for 3.3 h^{-1} and 200 rpm for 4.9 h^{-1}. The range of different K_La values used was the same as described by Silva *et al*., (2012) where D-xylose was used as carbon source.

3.10 Analytical methods

3.10.1 GC analysis

The ethanol content was determined by a GC-2010 Plus Shimadzu Gas Chromatograph. A ZB-WAX plus column was used at a starting temperature of 40 °C and raised to 140 °C after sample injection at a rate of 20 °C/min. It was then raised to 200 °C at a rate of 50 °C/min and kept at this temperature for 2 min. Nitrogen was used as carrier gas at a flow

rate of 17.6 mL/min and at a pressure of 100 kPa. The temperature of the detector was set at 255 °C. For each sample, a volume of 1 µL was automatically injected onto the GC column using a split syringe AOC-20i + s. GC solution operation analysis database was used to process the data samples. The ethanol in the samples was measured by comparing it with the known ethanol standards (Nguyen *et al*., 2016).

3.10.2 HPLC analysis

A Shimadzu prominence 20 (Tokyo, Japan) high-performance liquid chromatography (HPLC) instrument equipped with a RID 10A Refractive Index detector was used to determine the D-xylose, L-arabinose, arabitol and xylitol concentrations. A Razex RHM monosaccharide H+ (300 mm x 7mm) column was utilised 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. A sample volume of 20 µl was injected into the HPLC using a SIL-20A autosampler. LC Solution Operation analysis was used to process the sample data. Known standards of D-xylose, L-arabinose, arabitol and xylitol were used to calculate the concentration of the samples (Makhuvele *et al*., 2017).

3.10.3 Calculations of fermentation parameters

The fermentation parameters, arabitol, cell biomass, ethanol and xylitol yield, ethanol productivity D-xylose and L-arabinose consumption were determined as described below. Arabitol (g/g), ethanol (g/g) and xylitol (g/g) yields were calculated as described by Cadete *et al.* (2012), which correlated to the products generated ($\Delta P_{\text{arabitol}}$, $\Delta P_{\text{ethanol}}$, $\Delta P_{\text{xylitol}}$) with the substrates ($\Delta S_{\text{L-arabinose}}$, $\Delta S_{\text{D-xylose}}$) consumed. The ethanol productivity was calculated by the ratio between maximum ethanol concentration and fermentation time (h) at which high ethanol was generated. Cell concentrations were determined by correlating the optical density (OD) measurements spectrophotometrically at 600 nm with a standard curve of dry weight against optical density previously constructed. The biomass yield was determined by the ratio between cell concentration (g/L) and substrate utilised (g/L).

CHAPTER 4
RESULTS

In this study, a total of 390 yeasts, previously isolated from banana waste, the gut of dung beetles, Marula wine and herbal concoctions were evaluated for their ability to grow and ferment both D-xylose and L-arabinose. Twenty-seven yeasts were able to grow on plates containing either D-xylose or L-arabinose and 13 yeasts were able to ferment both sugars. Yeasts with preferred characteristics (growth at elevated temperatures and in the presence of acetic acid) were selected for adaptation experiments. The adapted yeasts were further evaluated to select the best ethanol producing yeast strain. The best adapted yeast strain was evaluated in a bioreactor to determine different rates of aeration for ethanol production.

4.1 Identification and biochemical characterization of selected yeast

Thirteen yeast isolates capable of D-xylose and L-arabinose fermentation were identified using ITS-5.8S and D1/D2 domain sequencing (Table 4.1). The identified yeasts indicated 98 - 100 % similarities with that found on the GenBank database and the sequences (ITS1 and D1/D2) of all identified yeasts in this study were deposited in the GenBank database with accession numbers as indicated in Table 4.1. Most of the yeast isolates identified belong to *Meyerozyma caribbica* (D28L3, D14W2, D28L4, D14YE6, D14YE1, D14YE2, D4WPO1 and Mu2.2f) followed by *Cryptococcus terrestris* (C11Y, C12Y, CW1 and CW2) and *Candida tropicalis* (Kp34ey).

The identified yeasts were compared and confirmed using biochemical characteristics according to the VITEK 2 system (Pincus, 2010) and the results are depicted in Table 4.2. The biochemical profile for the *Meyerozyma caribbica* strains (D28L3, D14W2, D28L4, D14YE6, D14YE1, D14YE2, D4WPO1 and Mu2.2f) were comparable to that found by Kurtzman *et al*. (2010) and confirmed the utilisation of both D-xylose and L-arabinose. All the identified yeast strains were able to assimilate cellobiose, D-xylose, D-glucose and L-arabinose, found in lignocellulosic biomass. *Cryptococcus terrestris* C12y assimilated D-xylose, D-glucose and L-arabinose but failed to assimilate cellobiose.

Table 4. 1 Identification of selected pentose fermenting yeast isolates.

Source	Species name	Isolate	Accession number	Similarity (%) ITS	Similarity (%) D1/D2
1	*Candida tropicalis*	Kp34ey	MH626009	99	99
2	*Cryptococcus terrestris*	C11Y	MH606241	99	100
		C12y	MH605570	99	100
		CW1	MH606235	99	100
		CW2	MH606220	100	100
3	*Meyerozyma caribbica*	D4WPO1	MH607123	100	98
4		D14W2	MH606144	100	100
		D14YE1	MH607117	100	98
		D14YE2	MH607121	100	99
		D14YE6	MH608311	100	99
		D28L3	MH605998	100	98
		D28L4	MH606146	100	98
		Mu 2,2f	MH625960	99	100

1 – Dung beetle, 2 - Herbal concoctions, 3 - Banana wastes, 4 - Marula wine

Table 4.2. Biochemical characterisation of selected pentose fermenting yeast strains.

.Biochemical tests	1	2	3	4	5	6	7	8	9	10	11	12	13	14
D-gluconate assimilation	w	-	+	+	-	+	+	+	+	+	+	+	+	+
N-acetyl-glucosamine assimilation	-	-	-	-	+	+	+	-	+	+	+	+	-	-
Esculin hydrolyse	+	+	w	+	-	+	+	+	+	+	+	+	+	+
L- glutamate assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Xylose assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D- Lactate assimilation	-	-	-	-	+	w	-	-	-	-	-	-	+	-
Acetate assimilation	+	-	+	-	w	+	+	+	+	+	+	+	+	+
Citrate(Sodium) assimilation	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Glucoronate assimilation	w	w	+	+	+	+	+	+	w	w	+	+	+	+
L-Proline assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2-Keto-D-Gluconate assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Raffinose assimilation	+	+	-	+	+	+	+	+	+	+	+	+	+	+
PNP-N-acetyl-BD-galactosaminidase	-	+	w	+	-	-	-	-	-	-	w	+	-	-
D-Mannose assimilation	-	-	+	-	+	+	+	+	+	+	+	+	+	+
D- Melizitose assimilation	-	-	w	-	-	+	+	+	+	+	+	+	+	-

.Biochemical tests	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L- Sorbose assimilation	-	-	-	-	+	-	-	-	-	-	-	-	-	-
L- Rhamnoze assimilation	+	+	-	+	-	+	+	+	+	+	+	+	+	+
Xylitol assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Sorbitol assimilation	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Sucrose assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Alphaglucosidase	-	-	-	-	+	+	+	+	+	+	+	+	+	+
D-Turanase assimilation	-	nd	-	-	+	+	+	+	+	+	+	+	+	+
D-Trehalose assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate assimilation	-	-	+	-	-	-	-	-	-	-	-	-	-	-
L- Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D- Galacturanate assimilation	w	+	+	+	+	+	+	+	+	+	+	+	+	+
Lysine-Arylamidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Malate assimilation	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Leucine-Arylamidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arginine	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Erythritol assimilation	+	w	-	-	-	-	-	-	-	-	+	-	-	+

.Biochemical tests	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Glycerol assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tyrosine- Arylamidase	-	-	+	-	w	-	-	-	-	-	-	-	-	-
Beta- Acetyl- Glucosaminidase	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Arbutine assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amygdaline assimilation	w	-	-	-	-	+	+	+	w	+	+	+	+	+
D-Galactose assimilation	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Gentiobiose assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucose assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose assimilation	+	w	-	+	+	+	+	+	+	+	+	+	+	+
Methyl-A-D- Glucopyranosidase assimilation	-	-	-	-	-	+	+	+	+	+	+	+	+	-
Cellobiose assimilation	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Gamma-glutamyl-transferase	+	+	+	+	-	+	+	+	+	+	+	+	+	+
D-maltose assimilation	+	+	+	+	nd	+	+	+	+	+	+	+	+	+

Yeasts; 1, *Candida. tropicalis* Kp42ey; 2, *Cryptococcus. terrestris* C11y 3, *C. terrestris* c12y; 4, *C. terrestris* CW1; 5, *C. terrestris* CW2; 6, *Meyerozyma. caribbica* D4WPO1; 7, *M. caribbica* D14W2; 8, *M. caribbica* D14YE1; 9, *M. caribbica* D14YE2; 10, *M. caribbica* D14YE6, 11, *M. caribbica* D28L3; 12, *M. caribbica* D28L4; 13, *M. caribbica* MU 2.2f; 14, *Scheffersomyces stipitis* NRRL Y7124.

+ Positive, - negative, nd - not determined, w –weak growth

4.2 Ethanol production, acetic acid tolerance and maximum growth temperatures

Thirteen yeasts able to ferment both D-xylose and L-arabinose were evaluated for ethanol production. The ethanol concentration indicated in Table 4.3 is the maximum ethanol produced after a certain fermentation time. *Meyerozyma caribbica* D14YE6 produced 3.9 g/L ethanol from L-arabinose, followed by *M. caribbica* D14W2 (1.9 g/L) and *M. caribbica* Mu2.2f (0.7 g/L) after 72 hours. As expected, *S. stipitis* NRRL Y7124 produced the most ethanol (4.5 g/L) from D-xylose after 24 hours, followed by *M. caribbica* D14W2 and *C. tropicalis* Kp42ey with 1.2 g/L and 1.0 g/L of ethanol after 48 hours, respectively. The other yeasts produced less than 1 g/L of ethanol from D-xylose.

The thirteen yeast isolates were tested for the ability to grow at elevated temperatures and in the presence of acetic acid with L-arabinose as carbon source (Table 4.4). D-xylose was not used further as carbon source because of the low levels of ethanol produced by the yeast isolates compared to *S. stipitis* NRRL-Y7124. All yeasts belonging to *Meyerozyma caribbica* (D28L3, D14W2, D28L4, D14YE6, Mu 2.2f, D14YE1, D14YE2 and D4WPO1) were able to grow in the presence of 3 g/L acetic acid on agar slants with growth only observed after two to four days of incubation at 30 °C. The yeast strains *C. tropicalis* KP42ey and *S. stipitis* NRRL Y7124 were able to grow in the presence of 1 g/l acetic acid, while all the strains of *C. terrestris* (C12y, CW1, CW2 and C11Y) failed to grow in the presence of acetic acid. *Meyerozyma caribbica* D28L3, *M. caribbica* D14W2, *M. caribbica* D28L4, *M. caribbica* D14YE6 and *M. caribbica* Mu 2.2f were able to grow at a maximum temperature of 40 ˚C, with the yeasts *M. caribbica* D14YE1 and *M. caribbica* D14YE2 growing at 37 °C. *Candida tropicalis* KP42ey and *S. stipitis* NRRL Y7124 were able to grow at 35 °C, while all four strains belonging to *C. terrestris* (C12y, CW1, CW2 and C11Y) grew at 30 °C along with *M. caribbica* D4WPO1.

Table 4.3 Ethanol production from D-xylose and L-arabinose by fourteen yeasts able to ferment both sugars.

Yeast	Maximum ethanol from L-arabinose (g/l)	Maximum ethanol from D-xylose (g/l)	Time (hours) for L-arabinose	Time (hours) for D-xylose
C. tropicalis Kp42ey	0.6 ± 0.03	1.0 ± 0.38	96	48
C. terrestris C11y	0.6± 0.01	0.8 ± 0.03	72	48
C. terrestris C12y	0.6 ± 0.05	0.8 ± 0.01	72	24
C. terrestris CW1	0.5 ± 0.00	0.9 ± 0.05	72	48
C. terrestris CW2	0.5 ± 0.00	0.7 ± 0.01	48	48
M. caribbica D4WPO1	0.5 ± 0.01	0.9 ± 0.10	96	48
M. caribbica D14W2	1.9 ± 0.12	1.2 ± 0.20	72	48
M. caribbica D14YE1	0.5 ± 0.00	0.8 ± 0.02	72	48
M. caribbica D14YE2	0.6 ± 0.03	0.8 ± 0.03	72	48
M. caribbica D14YE6	3.9 ± 0.83	0.9 ± 0.12	72	48
M. caribbica D28L3	0.5 ± 0.01	0.9 ± 0.13	72	24
M. caribbica D28L4	0.5 ± 0.01	0.9 ± 0.02	48	48
M. caribbica Mu 2.2f	0.7 ± 0.21	0.8 ± 0.52	72	48
S. stipitis NRRL Y7124	0.5 ± 0.01	4.5 ± 0.02	72	24

Table 4.4. Effect of acetic acid and temperature on growth of selected yeasts with L-arabinose as carbon source.

Yeasts	Acetic acid (g/l)[1]	Temperature (ºC)[2]
C. tropicalis Kp42ey	1	35
C. terrestris C11y	-	30
C. terrestris C12y	-	30
C. terrestris CW1	-	30
C. terrestris CW2	-	37
M. caribbica D4WPO1	3	30
M. caribbica D14W2	3	40
M. caribbica D14YE1	3	37
M. caribbica D14YE2	3	37
M. caribbica D14YE6	3	40
M. caribbica D28L3	3	40
M. caribbica D28L4	3	40
M. caribbica Mu 2.2f	3	40
S. stipitis NRRL Y7124	1	35

1 – Maximum acetic acid concentration at which the yeast was able to grow.

2 – Maximum temperature where growth still occurred.

4.3 Adaptation of selected yeast strains

Yeast strains with the ability to grow in the presence of acetic acid and at temperatures above 30 °C were adapted on YNB agar plates containing L-arabinose. Ten yeast strains (*C. tropicalis* Kp42ey, *M. caribbica* D28L3, D14W2, D28L4, D14YE6, Mu 2.2f, D14YE1, D14YE2, D4WPO1 and *S. stipitis* NRRL Y7124) were selected for adaptation. For the initial step of adaptation, the selected yeasts were grown on agar plates containing 3 g/L acetic acid at 35 °C. Yeast strains that grew during the first stage of adaptation, were further adapted further with 3 g/L acetic acid at 37 °C and subsequently on agar plates with 3 g/L acetic acid at 40 °C. The yeast strains *C. tropicalis* Kp42ey and *S. stipitis* NRRL Y7124 failed to grow on agar plates containing 3 g/L acetic acid when incubated at 35 °C, while strains of *M. caribbica* D14YE2, D4WPO1, D14W2 and D28L4 failed to grow on agar plates with 3 g/L acetic acid at 40 °C. Only four strains belonging to *M. caribbica* (D28L3, D14YE1, D14YE6 and Mu 2.2f) were able to adapt to the most stringent conditions.

4.4 Shake flask fermentation studies on adapted yeast strains

The four adapted *M. caribbica* strains (D28L3, D14YE1, D14YE6 and Mu 2.2f) were screened for L-arabinose (30 g/L) fermentation at 35 °C and 37 °C. Strangely, no growth was observed for any of the adapted yeasts when fermenting at 40 °C in shake flasks compared to growth at 40 °C on agar plates during adaptation. The fermentation of L-arabinose at 35 °C in the presence of 3 g/L acetic acid is indicated in Table 4.5. *Meyerozyma caribbica* Mu 2.2f produced 4.3 g/L ethanol followed by *M. caribbica* D14YE1 with 1.2 g/L ethanol after 24 hours (with acetic acid) compared to 0.7 g/L and 0.5 g/L ethanol respectively, for the parental strains without the addition of acetic acid (Table 4.3). The ethanol yield for the adapted *M. caribbica* strains on L-arabinose varied between 0.030 and 0.160 g/g with *M. caribbica* Mu 2.2f having the highest ethanol yield. It was also noted that *M. caribbica* Mu 2.2f had the highest ethanol productivity of 0.180 g/L/h followed by *M. caribbica* D14YE1 with an ethanol productivity of 0.050 g/L/h. Both these ethanol productivity values were calculated after 24 hours. The arabitol yield was between 0.783 and 0.764 g/g with *M. caribbica* D14YE6 producing 22 g/L arabitol

followed by *M. caribbica* D14YE1 with 21.6 g/L arabitol after 72 and 96 hours, respectively.

Ethanol production, yield and productivity of the adapted yeasts grown on fermentation medium containing acetic acid at 37 °C is presented in Table 4.6. All four yeast strains were able to grow with *M. caribbica* Mu 2.2f producing 1.7 g/L ethanol after 48 hours, compared to *M. caribbica* D14YE6 producing 0.8 g/L ethanol after 24 hours. Ethanol production in the adapted strain of *M. caribbica* Mu 2.2f increased from 0.7 g/L in the parental strain (without acetic acid), while the parental strain of *M. caribbica* D14YE6 produced more ethanol (3.7 g/L), without acetic acid, than the adapted strain. The adapted strain of *M. caribbica* Mu 2.2f produced ethanol at a yield and productivity of 0.221 g/g and 0.047 g/L/h, respectively. The yeast *M. caribbica* D28L3 consumed most of the L-arabinose (24.7 g/L, data not shown) and converted it mostly to arabitol (19.2 g/l) rather than ethanol (0.6 g/L). This strain had an arabitol yield of 0.792 g/g as compared to *M. caribbica* D14YE6 (second best arabitol producer) producing 6.6 g/L arabitol with a yield of 0.299 g/g. It was decided to investigate *M. caribbica* Mu 2.2f further, since it produced the most ethanol in the presence of acetic acid at 35 and 37 °C.

Table 4.5 Fermentation of L-arabinose by the adapted strains of *M. caribbica* in the presence of 3 g/L acetic acid at 35 °C.

Strain number	Maximum ethanol (g/L)	Ethanol yield (g/g)	Maximum arabitol (g/L)	Arabitol yield (g/g)	Ethanol productivity (g/L/h)
Mu 2.2f	4.3 ± 0.60[a]	0.160	20.4 ± 3.1[d]	0.680	0.180
D28L3	0.8 ± 0.01[b]	0.030	22.2 ± 3.07[c]	0.783	0.022
D14YE1	1.2 ± 0.86[a]	0.050	21.6 ± 0.44[c]	0.764	0.050
D14YE6	0.8 ± 0.18[a]	0.030	22 ± 1.37[d]	0.733	0.033

Ethanol and arabitol yield was determined at maximum ethanol or arabitol concentrations.

a = after 24 hours of fermentation.

b = after 48 hours of fermentation

c = after 72 hours of fermentation

d = after 96 hours of fermentation

Table 4.6 Fermentation of L-arabinose by the adapted strains of *M. caribbica* in the presence of 3 g/L acetic acid at 37 °C.

Stain number	Maximum ethanol (g/L)	Ethanol yield (g/g)	Maximum arabitol (g/L)	Arabitol yield (g/g)	Ethanol productivity (g/L/h)
Mu 2.2f	1.7 ± 0.12[b]	0.221	2.2 ± 0.4[b]	0.117	0.047
D28L3	0.6 ± 0.01[b]	0.040	19.2 ± 0.11[d]	0.792	0.017
D14YE1	0.7 ± 0.01[a]	0.060	6.6 ± 0.71[c]	0.299	0.029
D14YE6	0.8 ± 0.18[a]	0.046	2.2 ± 0.008[d]	0.088	0.033

Ethanol and arabitol yield were determined at maximum ethanol or arabitol concentrations.

a = after 24 hours of fermentation.

b = after 48 hours of fermentation

c = after 72 hours of fermentation

d = after 96 hours of fermentation

4.4 Comparison of the adapted and parental strains of *M. caribbica* Mu 2.2f

The adapted and parental strains of *M. caribbica* Mu 2.2f were subjected to the fermentation of pentose sugars containing either 3 g/L acetic acid or without acetic acid as shown in table 4.7. Both strains were able to grow and ferment in a medium containing 50 g/L D-xylose or 40 g/L L-arabinose or a 90 g/L pentose mixture (50 g/L D-xylose and 40 g/L L-arabinose), without the addition of acetic acid. The adapted strain produced 1.9 g/L ethanol compared to 1.8 g/L ethanol for the parental strain from the mixed pentose sugars after 36 hours of fermentation with ethanol yields of 0.059 and 0.052 g/g, respectively. The ethanol productivity of the adapted strain was slightly higher (0.053 g/L/h) compared to 0.04 g/L/h for the parental strain. The parental strain produced more xylitol (6.5 g/L) from the pentose mixture than the adapted strain (4.6 g/L), while the adapted strain produced more arabitol (4.7 g/L) than the parental strain (2.2 g/L). The arabitol and xylitol yields of the parental strain was 0.381 g/g and 0.150 g/g, respectively. Whereas, the adapted strain had yields of 0.347 g/g and 0.084 g/g for arabitol and xylitol. It consumed less of the pentose sugars compared to the parental strain (data not shown).

The adapted strain produced a maximum of 3.0 g/L ethanol from L-arabinose after 48 hours of incubation compared to the maximum of 1.0 g/L ethanol for the parental strain after 24 hours (Table 4.7). The adapted strain also produced a higher ethanol yield and productivity of 0.148 g/g and 0.062 g/L/h respectively, compared to the parental strain (0.076 g/g and 0.043 g/L/h, respectively) with L-arabinose as carbon source. The adapted strain produced more arabitol (16.8 g/L) and a higher yield (0.494 g/g) compared to the parental strain that produced 7.4 g/L ethanol at a yield of 0.325 g/g.

The adapted strain produced 1.7 g/L ethanol with a yield of 0.042 g/g from D-xylose compared to 1.5 g/L ethanol and a yield of 0.044 g/g for the parental strain. The maximum ethanol productivity for the adapted strain was 0.071 g/L/h compared to 0.063 g/L/h for the parental strain. However, the parental strain produced a higher xylitol concentration of 8.5 g/L and a xylitol yield of 0.2 g/g compared to 2.8 g/L ethanol produced with a yield of 0.06 g/g for the adapted strain.

The parental strain could not grow on any of the pentose sugars in the presence of acetic acid. The adapted strain could also not grow on a combination of D-xylose and L-arabinose in the presence of acetic acid. However, the adapted strain fermented 50 g/L D-xylose or 40 g/L L-arabinose in the presence of 3 g/L acetic acid. The adapted strain produced more ethanol from L-arabinose (3.6 g/L) than from D-xylose (0.8 g/L) after 36 hours of fermentation (Figure 4.7). The ethanol yield and productivity of the adapted strain was also higher on L-arabinose with 0.181 g/g and 0.100 g/L/h, respectively, than on D-xylose with an ethanol yield of 0.04 g/g and a productivity of 0.02 g/L/h. It was also noted that the adapted strain produced 3.8 g/L xylitol with a yield of 0.172 g/g from D-xylose and 20.8 g/L arabitol with a yield of 0.657 g/g from L-arabinose. It was decided to evaluate different aeration conditions using the adapted strain of *M. caribbica* Mu 2.2f with 30 g/L L-arabinose as carbon source in the presence of acetic acid at 35 C°, since these conditions resulted in a higher ethanol production, when acetic acid was present.

Table 4.7 Fermentation parameters of the adapted and parental yeast strains from *M. caribbica* Mu 2.2f with D-xylose, L-arabinose or a mixture of the pentose sugars at 35 °C with or without acetic acid.

Fermentation parameters	Absence of acetic acid						3 g/L Acetic acid	
	Adapted			Parental			Adapted	
	D-xylose	L-arabinose	Mixture	D-xylose	L-arabinose	Mixture	D-xylose	L-arabinose
Maximum Ethanol (g/L)	1.7±0.5[a]	3.0±0.4[c]	1.9±0.2[b]	1.5±0.7[a]	1.0±0.11[a]	1.8±0.5[b]	0.8±0.1[b]	3.6±0.1[b]
Ethanol yield (g/g)	0.042	0.148	0.059	0.044	0.076	0.052	0.040	0.181
Maximum arabitol (g/L)	-	16.8±1.4[d]	4.7±3.1[d]	-	7.4±0.14[c]	2.2±0.1[b]	-	20.2±1.5[e]
Xylitol (g/L)	2.8±0.4[b]	-	4.6±1.6[e]	8.5±0.4[b]	-	6.5±1.3[d]	3.8±0.7[b]	-
Arabitol yield (g/g)	-	0.494	0.347	-	0.325	0.381	-	0.657
Xylitol yield (g/g)	0.06	-	0.084	0.20	-	0.150	0.172	-
Ethanol productivity (g/L/h)	0.071	0.062	0.053	0.063	0.043	0.040	0.020	0.100

Ethanol and arabitol yield were determined at maximum ethanol or arabitol concentrations.

a = after 24 hours of fermentation

b = after 36 hours of fermentation

c = after 48 hours of fermentation

d = after 72 hours of fermentation

e = after 96 hours of fermentation

4.5 Effect of aeration on L-arabinose fermentation

The adapted strain of *M. caribbica* Mu 2.2f was evaluated at different aeration rates in a bioreactor in order to determine the ideal aeration rate for high ethanol production and L-arabinose consumption. The temperature of the bioreactor was controlled at 35 °C and the pH kept at 5.0 with 30 g/L of L-arabinose as carbon source. The adapted strain produced the highest ethanol concentration of 5.7 g/L at a K_La value of 3.3 h^{-1} after 36 hours compared to 4.2 g/L for K_La 4.9 h^{-1} and 3.3 g/L for K_La 2.3 h^{-1} as indicated in Table 4.8 and Figure 4.1. The ethanol yield was the highest at K_La 3.3 h^{-1} (0.338 g/g). The ethanol productivity at K_La 4.9 h^{-1} was 0.175 g/L/h, compared to 0.158 g/L/h for K_La 3.3 h^{-1} and 0.106 g/L for 2.3 h^{-1}. The maximum ethanol produced at K_La 4.9 h^{-1} was obtained after 24 hours, which contributed to the higher ethanol productivity, while at K_La 2.3 h^{-1} and 3.3 h^{-1} the maximum ethanol production was observed after 36 hours.

However, the adapted strain produced 26.7 g/L arabitol at a K_La value of 4.9 h^{-1} and 18.2 g/L at K_La 3.3 and 9.9 g/L at K_La 2.3 h^{-1}. Similarly, the arabitol yield was found to be 0.9 g/g at K_La 4.9 h^{-1}, compared to 0.66 g/g for 3.3 h^{-1} and 0.37 g/g for K_La 2.3 h^{-1}. Furthermore, the L-arabinose present in the fermentation medium was fully consumed at a K_La value of 4.9 h^{-1} (Figure 1C) after 120 hours of fermentation.

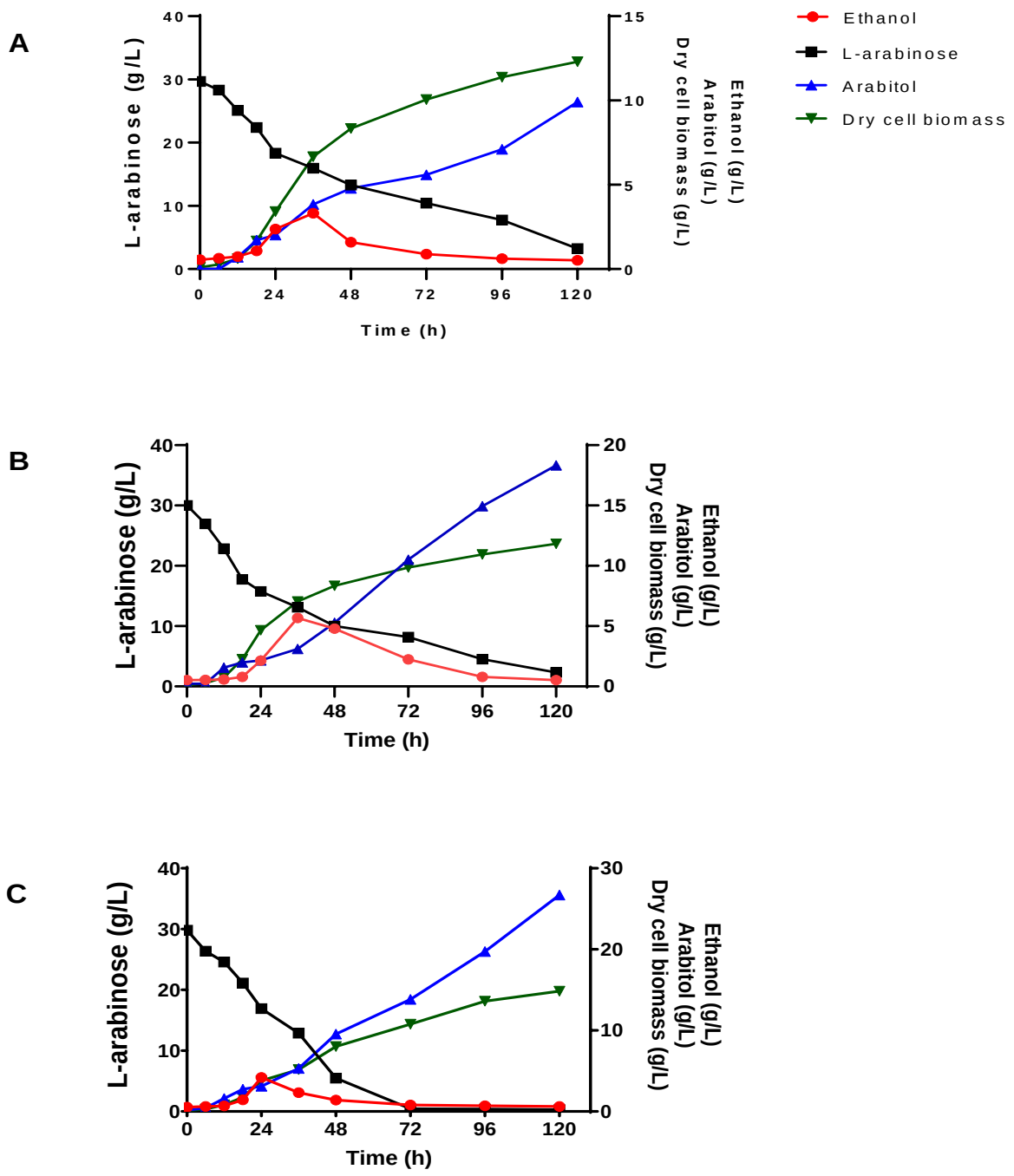

Figure 4.1. Fermentation of L-arabinose by the adapted strain of *M. caribbica* Mu 2.2f at K_La values of 2.3 h-1 (A), 3.3 h-1 (B) and 4.9 h-1 (C) in the presence of 30 g/L L-arabinose at 35 °C with the addition of 3 g/L acetic acid.

Table 4.8 Fermentation of L-arabinose by the adapted strain of *M. caribbica* Mu 2.2f at different K_La values with the addition of 3 g/L acetic acid at 35 °C.

K_La (h^{-1})	**Maximum ethanol (g/L)**	**Ethanol yield (g/g)**	**Highest arabitol (g/L)**	**Arabitol yield (g/g)**	**Ethanol productivity (g/L/h)**
2.3	3.8 ± 0.1[b]	0.270	9.9 ± 0.8[c]	0.370	0.106
3.3	5.7 ± 0.5[b]	0.338	18.3 ± 1.1[c]	0.660	0.158
4.9	4.2 ± 0.1[a]	0.321	26.7 ± 2.3[c]	0.900	0.175

The ethanol and arabitol yield were determined at maximum ethanol or arabitol concentrations.

a = after 24 hours of fermentation

b = after 36 hours of fermentation

c =after 120 hours of fermentation

CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1 DISCUSSION

The excessive usage of non-renewable fossil fuel as an energy source around the world has resulted in the increased release of greenhouse gases into the atmosphere that is leading to global warming (Günan Yücel and Aksu, 2015). Biofuel (mainly ethanol) has been regarded as an alternative clean and renewable energy source to fossil fuels. Efficient bioethanol production from plant materials in second generation biofuels requires efficient conversion of the sugars (pentose and hexose) present in hemicellulose. The hexose sugars (glucose, galactose and mannose) in hemicellulose are efficiently converted to ethanol by traditional *Saccharomyces cerevisiae* strains, while pentose sugars (D-xylose and L-arabinose) are not naturally fermented by *S. cerevisiae*. It is crucial to convert both hexose and pentose sugars to bioethanol for the process to be economically feasible (Modi *et al*., 2018).

Yeasts previously isolated from the gut of dung beetles, herbal concoctions, banana waste and Marula wine were screened to ferment both D-xylose and L-arabinose. Seven of the yeast isolates used in this study were obtained from Marula wine. Yeasts associated with Marula wine should be good fermenters of sugars. Molelekoa *et al*. (2018) isolated non-*Saccharomyces* yeast from Marula fruit and found *Pichia kudriavzevii* to be present, a known pentose fermenting yeast. This yeast was investigated by several authors for its ability to produce ethanol from D-xylose (Charoensopharat *et al*., 2010; Elahi and Rehman, 2018; Nweze *et al*., 2019; Yuan *et al*., 2017). Four yeast isolates (CW1, CW2, C12y and C11y) were isolated from herbal concoctions. There is no information available on the screening of yeasts associated with herbal concoctions in terms of pentose fermentation and these could be a good source for screening since it is a plant related source. Strangely, only one yeast isolate from the gut of dung beetles and banana waste respectively were able to ferment both pentose sugars. These sources are known to be associated with pentose fermenting organisms. Suh *et al*. (2003) isolated

xylose assimilating and fermenting yeasts (*C. shehatae*, *C. ergatensis, S stipitis and* S. *segobiensis*) from passalid beetles. Makhuvele *et al*. (2017) isolated 6 xylose assimilating yeasts belonging to *Candida tropicalis* from the dung of dassie, kudu, rhino and wildebeest. Santa-Maria *et al*. (2013) determined the pentose concentrations in different parts of banana waste, pseudostem (5-11% D-xylose and 2-3% L-arabinose), leaves (7-11% D-xylose and 3-4% L-arabinose) and rachis (8-11% D-xylose and 3-4% L-arabinose). However, Brooks (2008) isolated 8 yeasts from banana peels for the production of ethanol and all isolates failed to ferment D-xylose and L-arabinose.

Of the 390 yeast isolates screened in this study, only 13 were able to ferment both D-xylose and L-arabinose in the test tubes with Durham tubes (data not shown). It is known that only a few yeasts will ferment pentose sugars when screening D-xylose or L-arabinose assimilating yeasts. Alves Araújo *et al*. (2019) screened xylose-fermenting ability among 205 yeast isolates obtained from fruit pulp and plants of Cerrado. They found that only 3 isolates were able to ferment D-xylose in the test tubes and one of the 3 isolates were identified as *Meyerozyma guilliermondii,* a close relative of *M. caribbica*. Martini *et al*. (2016) isolated 350 yeasts from sugarcane, only one isolate fermented both D-xylose and L-arabinose in test tubes and yeast isolate was identified as *Meyerozyma guilliermondii*. Species of *Meyerozyma* isolated from Marula wine dominated, as 8 isolates were identified to be *Meyerozyma caribbica* and four strains were identified as *Cryptococcus terrestris* with only one strain of *Candida tropicalis* isolated (Table 4.1). Martini *et al*. (2016) isolated yeasts from sugarcane juice and the best pentose-fermenting yeast fermented both D-xylose and L-arabinose and was identified as *M. guilliermondii*. There is not much information available on the fermentation of pentoses by *M. caribbica* with investigators indicating that low or no ethanol was detected (Saha and Bothast, 1996; Hande *et al*., 2013; Sukpipat *et al*., 2017). *Meyerozyma caribbica* is regarded as a safe and harmless yeast as it is used in Mexico for the production of tequila (Saucedo-Luna *et al*., 2011).

Four basidiomycetous yeasts isolated from herbal concoctions were identified as *Cryptococcus terrestris* (Table 4.1). Yeasts belonging to *Cryptococcus* were previously identified as ethanol producers from D-xylose. Rao *et al*. (2008) isolated xylose-

fermenting yeasts from the bark of trees and found *Candida saitoi*, *Candida albidosimilis* and *Candida albidus* to ferment D-xylose. All yeast isolates in this study assimilated a broad range of substrates (L-arabinose, D-xylose and glucose) found in the lignocellulosic biomass as shown in Table 4.2. Barathikannan *et al*. (2016) isolated 33 yeasts from different fermented fruits, 22 were able to assimilate L-arabinose, D-xylose and glucose based on the biochemical characterization.

In this study the strains of *M. caribbica* were able to ferment both D-xylose and L-arabinose (Table 4.3). The most ethanol produced from D-xylose was 1.2 g/L (after 48 hours) by *M. caribbica* D14W2 and 3.9 g/L (after 72 hours) from L-arabinose by *M. caribbica* D14YE6 (Table 4.3). This is the first report of this yeast associated with Marula wine with the ability to ferment both D-xylose and L-arabinose. It is important to note that previous authors found L-arabinose fermentation to be slow for wild yeast, whereas in this study the maximum ethanol concentration was obtained after 72 hours. *Candida arabinofermentans* YB-1984 produced 1.9 g/L ethanol from L-arabinose after 14 days (Kurtzman and Dien, 1998) . The study conducted by Dien *et al*. (1996) on L-arabinose fermentation, showed that *Ambrosiozyma monospora* NRRL Y-148 produced a maximum ethanol concentration of 4.1 g/L from L-arabinose after 12 days. Sukpipat *et al*. (2017) used *M. caribbica* 5XY2, that was previously isolated from an alcohol starter fermentation, to ferment D-xylose and L-arabinose and the yeast produced less than 0.6 g/L of ethanol from both pentose-sugars after 120 hours.

Inhibitory compounds such as furans, weak acids and phenolic compounds are normally produced during pretreatment of lignocellulosic biomass and have a negative effect on microorganisms involved during the fermentation of lignocellulose. Acetic acid is the inhibitor mostly studied due to its occurrence and severity of inhibition on the fermentation process (Pan *et al*., 2018). All eight strains of *M. caribbica* were able to grow on plates with the addition of 3 g/L acetic acid (Table 4.4). The study conducted by Perna *et al*. (2018) showed that *M. guilliermondii* CCT7783 grew on media containing L-arabinose and D-xylose in the presence of 10 g/L acetic acid and suggested that the species used acetic acid as a carbon source. Charoensopharat *et al*. (2010) investigated the effect of acetic acid (4, 6, 8, and 10 g/L) on yeasts isolated from Jerusalem artichoke when

inoculated on YM agar plates. In their study, species of *M. caribbica* was among the yeast isolates able to grow on plates containing up to 4 g/L acetic acid.

The application of thermotolerant pentose-fermenting yeasts for bioethanol production has more advantages over low-temperature ethanol fermentation. These include, a higher hydrolysis rate for enzymes, ethanol yield, low contamination risk, and lower cooling costs (Nweze *et al*., 2019). Five strains of *M. caribbica* (D14W2, D14YE6, D28L3, D28L3 and Mu 2.2f) grew at 40 °C on agar plates using L-arabinose as carbon source and these strains were included in the adaptation process (Table 4.4). Sukpipat *et al*. (2017) reported similar results where the strain of *M. caribbica* 5XY2 grew at 40 °C. Charoensopharat *et al*. (2010) also investigated the effect of temperature on yeast isolates from Jerusalem artichoke and *M. caribbica* could grow at 40 °C on YM agar plates. The results in this study are supported by Kurtzman *et al*. (2010) as they reported that species of *M. caribbica* can grow up to a maximum temperature of 42 °C on agar slants.

Evolutionary engineering or adaptation is used to improve the traits of microorganisms, such as the production of bioethanol from lignocellulosic substrates (Parachin, 2011; Saini *et al*., 2017). In this study, ten yeast strains (8 strains of *M. caribbica*, *C. tropicalis* KP24ey and *Scheffersomyces stipitis* NRRL Y7124) were subjected to the adaptation process with four yeasts, belonging to *M. caribbica* (D28L3, D14YE1, D14YE6 and Mu 2.2f), adapting up to 40 °C in the presence of 3 g/l acetic acid. Strangely, no growth was observed when the yeasts were incubated in shake flasks at 40 °C. It is known that oxygen solubility decreases with an increase in temperature in liquids (Finn,1954). Therefore, it is possible that at 40 °C in shake flasks, sufficient oxygen was not supplied to these yeasts to produce biomass. Similar results were reported by Abdel-Banat *et al*. (2010), where *Kluyveromyces marxianus* DMKU3-1042 grew on YPD plates at 48 °C, but failed to grow at the same temperature when inoculated in flasks. This study is the first report on the adaptation of *M. caribbica* on acetic acid, high temperature with L-arabinose as a carbon source.

When the adapted yeasts were evaluated for ethanol production at 35 and 37 °C, it was found that 35 °C gave better results (Table 4.5). The adapted strain of *M. caribbica* Mu

2.2f produced 4.3 g/L ethanol (after 24 hours) and 20.4 g/L arabitol (after 96 hours) from L-arabinose in the presence of acetic acid at 35 °C with an ethanol yield and productivity of 0.160 g/g and 0.180 g/L/h, respectively. In comparison, the adapted strain of *M. caribbica* Mu 2.2f produced 1.7 g/L ethanol with an ethanol yield and productivity of 0.221 g/g and 0.047 g/L/h, respectively when incubated at 37 °C with the other adapted yeasts producing less than 1 g/L ethanol (Table 4.6). Similar results were obtained for *Pichia kudriavzevii* CM4.2 with more ethanol concentration being produced at 37 °C than at 40 °C (Charoensopharat *et al*., 2010). Watanabe *et al*. (2012) evaluated the effect of temperature on yeasts isolated from soil with *Candida* sp. NY7122 producing 1.92 and 0.75 g/L ethanol with an ethanol yield of 0.11 and 0.04 g/g at 30 and 37 °C, respectively. It was decided to further investigate the adapted strain of *M. caribbica* Mu 2.2f due to its higher ethanol concentration, when compared to the other adapted strains.

All the adapted *M. caribbica* strains produced a significant amount of arabitol (20 – 22.2 g/L) during growth on L-arabinose with *M. caribbica* D28L3 producing the most arabitol (22.2 g/L) with a yield of 0.783 g/g (Table 4.5). Kordowska-Wiater *et al*. (2017a) isolated arabitol producing yeasts from raspberry with *C. parapsilosis* 27RL-4 producing 10.72 g/L arabitol with a yield of 0.53 g/g. Kordowska-Wiater *et al*. (2017b) reported that *S. shehatae* 20BM-3 from rotten wood produced 7.97 g/L arabitol with a yield of 0.36 g/g from 30 g/L L-arabinose. Dien *et al*. (1996) reported that *C. succiphila* Y-1998 and *C. auringiensis* Y-11848 produced 81 and 73 g/L arabitol from L-arabinose, respectively.

The adapted strain of *M. caribbica* Mu 2.2f produced 3.6 g/L and 0.8 g/L ethanol from L-arabinose and D-xylose respectively, while the parental strain was unable to ferment L-arabinose in the presence of acetic acid (Table 4.7). Similar results were obtained by Nigam (2001b), who adapted *S. stipitis* NRRL Y-7124 on hardwood hemicellulose acid hydrolysate with the adapted strain producing 8.3 g/L in the presence of 5 g/L acetic acid with the parental strain failing to produce ethanol. In the absence of acetic acid, the adapted strain *M. caribbica* Mu 2.2f produced 3-fold more ethanol and 2.2-fold more arabitol from L-arabinose when compared to the parental strain. The ethanol produced from D-xylose was similar for the adapted and the parental strain. This was expected since *M. caribbica* Mu 2.2f was only adapted on L-arabinose and not D-xylose. Similar

results were obtained by various authors when *S. stipitis* was adapted on D-xylose, with the adapted yeast producing more ethanol than the parental strain (Nigam, 2001b; Silva *et al*., 2014).

Bioreactor studies are used to control parameters such as agitation speed, aeration rate, pH and temperature, which has an impact on the production of fermentation products (ethanol, arabitol, cell biomass, xylitol, etc.) (Bellido *et al*., 2013; Silva *et al*., 2016). In order to determine the ideal aeration for optimal ethanol production during batch fermentation by the adapted strain of *M. caribbica* Mu 2.2f, different K_La values (2.3 h^{-1}, 3.3 h^{-1}, 4.9 h^{-1}) were applied in a bioreactor (Fig 4.1 and Table 4.8). Ethanol production by the adapted *M. caribbica* Mu 2.2f was improved in the bioreactor as compared to the experiments done in the flasks with a K_La value of 3.3 h^{-1} providing the best ethanol results (5.7 g/L ethanol at a yield of 0.338 g/g). Most studies have been conducted to investigate the effect of K_La values on ethanol production from D-xylose using yeasts such as *S. stipitis*, *S. hagerdaliae* and *S. shehatae* (Bellido *et al*., 2013; Dussán *et al*., 2016; Silva *et al*., 2016, 2012). There are no previous studies done on the optimisation of aeration on L-arabinose fermentation. Bellido *et al*. (2013) obtained the highest ethanol concentration, yield and productivity of 22.3 g/L, 0.40 g/g and 0.30 g/L/h respectively, from D-xylose at a K_La value of 3.3 h^{-1} for *S. stipitis* DSM 3651 after 72 hours in the absence of acetic acid. Silva *et al*. (2012) investigated the ideal K_La value(s) for high ethanol production by *S. stipitis* NRRL Y-7124 and found that the strain produced 26.5 g/L ethanol from a sugar mixture (D-xylose and glucose) at K_La values between 2.3 and 4.9 h^{-1} after 96 hours. The adapted *M. caribbica* Mu 2.2f consumed all the L-arabinose at a K_La value of 4.9 h^{-1} and cell biomass was also the highest at K_La 4.9 h^{-1} (Fig. 4.1a-c). Application of a high K_La value results in high sugar consumption without improving ethanol production (Lin *et al*., 2012a).

Arabitol is a sugar alcohol molecule with five carbons that are used as a natural sweetener in food and the pharmaceutical industry and it also prevents dental caries (Himabindu *et al*., 2014). In this study, a higher yield was obtained for arabitol compared to ethanol from L-arabinose (0.900 g/g and 0.338 g/g, respectively). Sukpipat *et al*. (2017) investigated arabitol producing yeasts isolated from Thai starter and noted *Meyerozyma caribbica*

5XY2 produced a high arabitol concentration of 30.3 g/L with a yield of 0.61 g/g from 50 g/L L-arabinose. Kumdam *et al*. (2013) examined the production of L-arabitol from several lignocellulosic biomass (sucrose, glucose, L-arabinose, fructose and glycerol) using *Debaryomyces nepalensis* NCYC 3413 and also noted that less ethanol (2.43 g/L) and more arabitol (22.7 g/L) was produced from L-arabinose with ethanol and arabitol yields of 0.03 and 0.26 g/g, respectively. Saha and Bothast (1996) reported that strains of *Candida entomaea* NRRL Y-7785 and *S. guilliermondii* NRRL Y-2075 produced 33.0 and 31.5 g/L L-arabitol, respectively, from 50 g/L L-arabinose at 34 °C with an L-arabitol yield of 0.66 and 0.63 g/g, respectively. Sundaramoorthy and Gummadi (2019) isolated arabitol producing yeasts from seawater and soil samples, with the yeast *P. manchurica* from seawater producing 24.6 g/L arabitol while the two soil yeast isolates produced 22.5 g/L with yields of 0.615 and 0.563 g/g respectively. Watanabe *et al*. (2012) reported that the strain of *Candida* sp. NY7122 produced 10.69 g/L arabitol from 20 g/L L-arabinose when incubated at 37 °C after 72 hours with a yield and productivity of 0.537 g/g and 0.148 g/L/h, respectively.

The results obtained for ethanol and arabitol production for the adapted *M. caribbica* Mu 2.2f was compared to similar yeasts in Table 5.1 and Table 5.2. In this study *M. caribbica* Mu 2.2f produced an ethanol concentration of 5.7 g/L with a yield and productivity of 0.338 g/g and 0.158 g/L/h, respectively, in the presence of acetic acid after 36 hours. *Ambrosiozyma monospora* produced 4.1 g/L ethanol with a yield and productivity of 0.150 g/g and 0.014 g/L/h respectively, in the absence of acetic acid after 14 days (Table 5.1). McMillan and Boynton (1994) reported that *C. tropicalis* NRRL Y-11860 produced 8.4 g/L arabitol from L-arabinose with a high yield of 1.02 g/g and they reported that the arabitol yield was equal to the maximum theoretical arabitol yield from L-arabinose. In this study, the adapted strain of *M. caribbica* Mu 2.2f produced a maximum arabitol yield of 0.900 g/g after 96 hours which is close to the maximum theoretical yield (90%) as shown in Table 5.2. The adapted strain of *M. caribbica* Mu 2.2f has the ability to ferment L-arabinose in a shorter period to produce ethanol and arabitol when compared to other L-arabinose fermenting yeasts. The ability of yeasts to ferment the pentose sugars (D-xylose or L-arabinose) found in the lignocellulosic biomass in the presence of inhibitors produced during pretreatment could be applicable for second generation bioethanol

(Makhuvele *et al*., 2017). Arabitol is considered to be of industrial importance, as it is used as natural sweetener and clinical applications (Kordowska-Wiater, 2015; Martini *et al*., 2016).

Meyerozyma caribbica Mu 2.2f could be considered for ethanol and arabitol production in the presence of acetic acid. Further investigation needs to be conducted on this yeast to improve ethanol and arabitol production. Adaptation could be considered in shake flasks at higher temperatures to overcome the problems observed in this study.

5.2 CONCLUSIONS

L-arabinose fermentation by yeasts is poorly documented as compared to D-xylose. Four adapted strains of *M. caribbica* were able to ferment L-arabinose to ethanol and arabitol in the presence of 3 g/L acetic acid at 35 °C. Adaptation improved the production of ethanol from L-arabinose by *M. caribbica* strains. The adapted *M. caribbica* Mu 2.2f strain produced 5.7 g/L ethanol with a yield of 0.338 g/g from L-arabinose with a K_La value of 3.3 h^{-1}. More arabitol than ethanol was produced at a K_La 4.9 h^{-1} with a high yield of 0.900 g/g. The adapted *M. caribbica* Mu 2.2f strain could be a potential candidate in the fermentation of pentose rich lignocellulosic biomass, such as sugarcane bagasse, wheat straw, wheat bran, corn fibre or brewery's spent grain. Therefore, an adapted *M. caribbica* Mu 2.2f strain could prove to be a promising yeast candidate for both bioethanol and arabitol production under stressed conditions as compared to the documented pentose fermenting yeasts under normal conditions.

Table 5. 1 Comparison of ethanol production by the adapted *M. caribbica* Mu 2.2f with other reported L-arabinose fermenting yeasts.

Species	Yeast strain	$Y^{et}_{p/s}$ (g/g)[1]	Qp^{et} (g/L/h)[2]	Maximum ethanol (g/L)	Time (hours or days)[3]	References
M. caribbica	Mu 2.2f	0.338	0.660	5.7	36 hrs	This study
M. caribbica	D14YE6	0.120	0.051	3.7	72 hrs	This study
Debaryomyces nepalensis	*NCYC 3413*	0.03	0.020	2.43	120 hrs	(Kumdam *et al*., 2013)
Candida sp.	NY7122	0.040	0.031	0.75	72 hrs	(Watanabe *et al*., 2012)
Ambrosiozyma monospora	NRRL Y-148	0.150	0.014	4.1	12 days	(Dien *et al*., 1996)
M. caribbica	5XY2	0.011	0.005	0.6	120 hrs	(Sukpipat *et al*., 2017)

[1] Ethanol yield, $Y^{et}_{p/s}$ (g/g) - the relationship between ethanol ($\Delta P_{ethanol}$) formed from consumed L-arabinose ($\Delta S_{arabinose}$)

[2] Ethanol productivity, Qp^{et} (g/L/h)- correlation between ethanol titre (g/L) and fermentation time (h)

[3] Fermentation time at which maximum ethanol (g/L) was produced towards the end or at the end of the fermentation process

Table 5. 2 Comparison of arabitol production by adapted *M. caribbica* Mu 2.2f with previously reported arabitol producing yeasts.

Yeast	Yeast strain	$Y^{ara}_{p/s}$ (g/g)[1]	Qp^{ara} (g/L/h)[2]	Maximum arabitol (g/L)	Time (hours)[3]	References
M. caribbica	Mu 2.2f	0.900	0.175	26.7	96 hrs	This study
M. caribbica	D14YE6	0.230	0.096	6.9	72 hrs	This study
Candida tropicalis	NRRL Y-11860	1.02	0.091	8.4	92 hrs	(McMillan and Boynton, 1994)
M. caribbica	5XY2	0.010	0.002	0.37	120 hrs	(Sukpipat *et al*., 2017)
Candida sp.	NY7122	0.537	0.148	10.69	72 hrs	(Watanabe *et al*., 2012)
C. arabinofermentans	PYCC 5603^T	0.600	0.200	58	270 hrs	(Fonseca, *et al*., 2007)

[1] Arabitol yield, $Y^{ara}_{p/s}$ (g/g) - the relationship between ethanol ($\Delta P_{arabitol}$) formed from consumed L-arabinose ($\Delta S_{arabinose}$)

[2] Arabitol productivity, Qp^{ara} (g/L/h) - correlation between arabitol titre (g/L) and fermentation time (h)

[3] Fermentation time at which maximum arabitol (g/L) was produced towards the end or at the end of the fermentation process

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