ETHANOL PRODUCTION BY YEAST FERMENTATION OF AN *OPUNTIA FICUS-INDICA* BIOMASS HYDROLYSATE

by

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This dissertation is dedicated to my mother

She sacrificed everything so her children could have opportunities she never had.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

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1. INTRODUCTION

The industrial and societal developments recorded during the 20th century have been greatly influenced by the discovery of petroleum and its derivates. Petroleum has for a long time been an abundant and cheap raw material for the production of fine chemicals and, more important, transport fuels (van Maris *et al.*, 2006). However, petroleum is a non-renewable resource and with the depletion of this crucial energy reserve it is clear that current supply can no longer meet the ever increasing global energy demands (Sánchez & Cardona, 2008). Recurring crises in major crude-oil producing areas such as the Middle East and the Niger delta, and spectacular growth experienced within the major Asian economies, especially China in recent years, among other factors have helped push crude-oil prices constantly above 60 dollars per barrel (Sánchez & Cardona, 2008; Skeer & Wang, 2007). This has raised concerns about the security of oil supplies, requiring national governments to reconsider their dependence on foreign oil reserves (Fofana *et al.*, 2009; van Maris *et al.*, 2006). For instance, in countries that are heavily dependent on oil imports, e.g. South Africa, where approximately 70% of its required liquid petroleum fuel is imported (the remaining 30% is produced locally by Sasol Limited and PetroSA) and 98% of energy in the transport sector is based on petroleum products, the country's economy is exposed to oil supply risks and its unsustainability in the long term (Vanderschuren *et al.*, 2010).

Furthermore, the combustion of fossil fuels such as coal and oil has led to a steady increase in the levels of greenhouse gas emissions which are a major cause of climate change, particularly global warming (Ragauskas *et al.*, 2006). Motor vehicles already account for 70% of global carbon monoxide (CO) emissions and 19% of carbon dioxide (CO_2) emissions worldwide. There are an estimated 700 million automobiles, minivans and light trucks on roadways globally and these numbers are projected to increase to 1.3 billion by 2030 and to more than 2 billion vehicles by 2050. Such growth will in no doubt affect the availability of global oil reserves as well as the stability of ecosystems and global climate (Balat & Balat, 2009). These concerns require intensified efforts to diversify our energy sources and focus more on alternative clean and carbon-neutral fuels that can be sustainable in the long term.

Interest in alternative energy sources has increased since 1997 when the Kyoto protocol, of which South Africa is a signatory, was proposed to limit the global net emission of CO_2. The European Union adopted a white paper to substitute progressively 20% of fossil fuels

with alternative fuels in the transport sector by 2020, with an intermittent goal set at 5.75% by the end of 2010 (Hahn-Hägerdal *et al.*, 2006). Among other alternatives, one solution is to harness solar energy in the form of plant biomass to produce biofuels (Sánchez & Cardona, 2008). The term biofuel generally refers to any solid, liquid or gaseous fuel that is predominantly produced from biomass through biochemical or thermochemical processes (Balat, 2007). Biomass sources include plant matter and lignocellulosic residues, such as forestry and agricultural by-products as well as municipal wastes (Balat & Balat, 2009).

Biofuels appear to be an attractive option for several reasons, one of which is that biomass is a renewable resource (Kumar *et al.*, 2009). Biomass is geographically more evenly distributed than fossil fuels; hence biofuel production will to a large extent be domestic and will ensure security of supply (Hahn-Hägerdal *et al.*, 2006). Moreover, the use of biofuels can contribute to the mitigation of greenhouse gas (GHG) emissions. They are cleaner-burning than fossil fuels and the CO_2 released will be that which has already been fixed from the atmosphere by photosynthesis, thereby making their use carbon neutral (Figure 1.1) (Lin & Tanaka, 2006). Beyond the energy and environmental benefits, biofuels promote rural economies by providing employment in those areas that produce the biomass raw material (Demirbas & Balat, 2006).

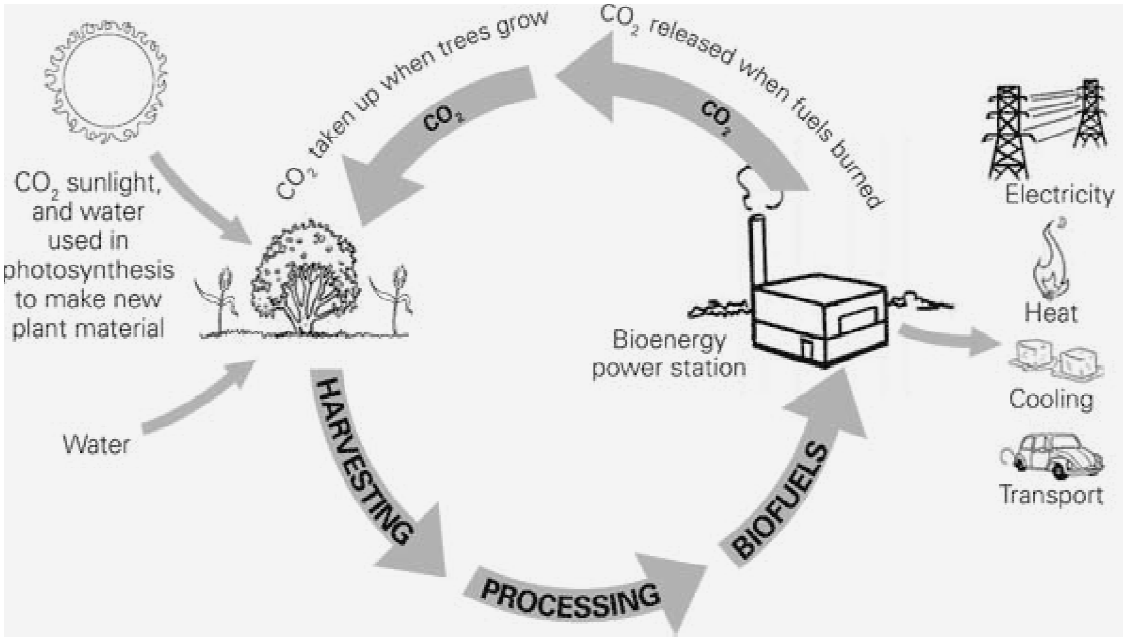


Figure 1.1 The biofuels closed loop carbon cycle
(http://www.nicholas.duke.edu/thegreengrok/graphics/biofuelscarboncycle/image_view_fullscreen)

Liquid biofuels can be grouped into (a) bioalcohols, (b) vegetable oils and biodiesels, and (c) biocrude (Demirbas, 2009). Ethanol produced through the fermentation of sugars is currently the most predominant liquid biofuel and is already a well established biofuel in the transport and industry sectors of some countries, notably Brazil, the USA and the European Union (Galbe & Zacchi, 2007). Bioethanol can be used as a neat alcohol fuel or blended with petrol. Several positive effects are achieved when ethanol is blended with petrol: as an oxygenated petrol additive, bioethanol has a higher octane number (93-113) than petrol, thus reducing the need for toxic octane-enhancing additives (Bothast & Schlicher, 2005). Its oxygen content ensures that petrol burns better with the consequent reduction in the emissions of carbon monoxide and non-combusted hydrocarbons (Sánchez & Cardona, 2008). Furthermore, ethanol is an excellent fuel for advanced hybrid or flexi-fuel vehicles (FFV) (Hahn-Hägerdal *et al.*, 2006).

The world's ethyl alcohol production has reached about 70 billion litres per annum, with the USA and Brazil accounting for close to 90% of the global output (Renewable Fuels Association, http://www.ethanolrfa.org). The European Union, China, India, Canada and Thailand are also major bioethanol producing countries. Bioethanol is presently produced from sugar sources such as sugar cane juice (Brazil), molasses (India) and sugar beet (France), and also starch sources such as maize (USA, Canada), wheat (Germany, Spain, Sweden) and cassava (Thailand) (Antoni *et al.*, 2007; Purwadi *et al.*, 2007). However, these raw materials, which require prime agricultural land for cultivation and which are also used for human food and animal feed, will not be sufficient to meet the rising demand for fuel ethanol (Chang, 2007; Hahn-Hägerdal *et al.*, 2006). Moreover, their utilisation as ethanol feedstock has also led to an increase in global food prices (Frow *et al.*, 2009).

On the other hand, largely abundant lignocellulosic materials such as agricultural wastes and municipal paper waste or dedicated energy crops such as switchgrass constitute sustainable and cheap feedstocks for bioethanol production (Purwadi *et al.*, 2007). A significant portion of marginal lands that are not suitable for intense agriculture for food production can become useful for producing lignocellulosic biomass (Doran-Peterson *et al.*, 2008). In spite of its availability and obvious advantages, the main limitation of lignocellulosic biomass as feedstock is its complex structure and composition. Its polymers have to be broken down into fermentable sugars by complex and energy consuming methods before conversion to ethanol can take place. However, there is ongoing intensive research into cheaper and more effective methods of lignocellulose degradation (Galbe & Zacchi, 2007; Sánchez & Cardona, 2008).

Most energy crops do not require land meant for cultivation or intensive agricultural practices. This implies that non-fertile land or land in arid regions can become productive when converted into land for growing energy crops. In South Africa, one plant with such potential is the spineless prickly pear cactus (*Opuntia ficus-indica*). It possesses the remarkable quality of being able to take up and store water within a short time, enabling it to thrive in the harsh conditions present in arid and semi-arid regions (Middleton & Beinart, 2005; Oelofse *et al.*, 2006).

1.1 Objectives of this study

The main objective of this study was to investigate the feasibility of utilising *Opuntia ficus-indica* cladodes as a lignocellulosic biomass feedstock for bioethanol production. However, since an understanding of the structure and composition of the feedstock is essential for any biomass conversion process, the first step was to determine the chemical composition of the *O. ficus-indica* cladode. The second objective was to establish a procedure for the conversion of the *Opuntia* cladode polysaccharides into fermentable monomers through pretreatment and enzymatic hydrolysis. The third and final objective was to assess ethanol production from the resulting sugars using *Saccharomyces cerevisiae* and compare its performance to that of *Kluyveromyces marxianus,* a thermotolerant yeast able to use a wider range of substrates.

This dissertation is the first to my knowledge where the simultaneous saccharification and fermentation (SSF) of the *O. ficus-indica* cladode hydrolysate is described, and only the second report on ethanol production from the *Opuntia* cladodes. Furthermore, this dissertation provides data on the performance of *Kluyveromyces marxianus* during fermentation under both non-aerated and oxygen-limited conditions.

Where this section provides the background to the study, including the importance of alternative fuel sources, the first part of Chapter 1 provides a survey of literature regarding the current status of biomass conversion to ethanol, with the focus on lignocellulosic materials. In the second part of Chapter 1, attention is given to the feedstock used in this study, *Opuntia ficus-indica* cladodes, where its origin, composition, uses, merits and challenges are discussed. Chapter 2 focuses on the chemical composition, dilute acid pretreatment and enzymatic hydrolysis of the *O. ficus-indica* cladode to produce a fermentable hydrolysate. In Chapter 3, the batch fermentation profiles of *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* are investigated under non-aerated and oxygen-limited conditions in a chemically-defined medium containing a sugar mixture similar in

composition to the *O. ficus-indica* cladode enzymatic hydrolysate. In Chapter 4, separate hydrolysis and fermentation of the cladode is performed using both yeast strains under the different aeration conditions and the results compared to those of the simultaneous saccharification and fermentation of the pretreated cladode, which is regarded as a procedure for improving sugar conversion and ethanol production. In the final summary, general conclusions are drawn with further proposals for research.

2. LITERATURE REVIEW

2.1 Ethanol from biomass

Bioethanol is produced from biomass feedstocks which include plant materials that are rich in sucrose or storage polysaccharides (e.g. starch), as well as from lignocellulossic biomass which contains structural polysaccharides that need to be broken down into fermentable sugars (Cardona & Sánchez, 2007).

2.1.1 First generation feedstocks

Sugar-based and starch-based crops, generally referred to as first generation feedstocks, are the primary raw materials for bioethanol production on a commercial scale. These include sugar cane, sugar beet and sweet sorghum, whereas the commonly used starch-based crops include maize, wheat, rice and cassava (Prasad *et al.*, 2007; Sánchez & Cardona, 2008). Sugar cane juice is directly fermentable due to its high content of reducing sugars (mainly sucrose) and it requires no pretreatment except for size reduction and pressing. Cane molasses (the left over syrup after sugar has been crystallized from the juice), is also rich in sucrose but contains high concentrations of salts and other compounds that increase the osmolarity of the fermentation media, thereby inhibiting fermentation (Wackett, 2008; Wilkie *et al.*, 2000).

Starch-based crops, especially maize, are high yield feedstocks for ethanol production, but they require enzymatic hydrolysis to convert the starch into readily fermentable sugars. Commercial thermostable α-amylase, added at 90-110^{o}C, is employed to liquefy the starch kernels into dextrins and small amounts of glucose, followed by saccharification through addition of glucoamylase at lower temperatures (60-70^{o}C). The resulting glucose-containing hydrolysate may then be supplemented with ammonium sulphate or urea to provide nitrogen and is readily fermented at 30-32^{o}C using *Saccharomyces cerevisiae* (Hahn-Hägerdal *et al.*, 2006).

Commercial bioethanol production from first generation feedstocks is now successfully established in some countries, notably Brazil, the USA and certain EU countries. However, the dependence on such crops as the primary raw materials for ethanol production has several drawbacks: feedstocks such as maize, wheat, barley, cassava, rice and sugar cane currently also serve as staple food in many parts of the world. The increased demand for these crops for both human and animal consumption in addition to feedstock for bioethanol production, could lead to sharp increases in global food prices (Reijnders,

2009). Moreover, the demand for these feedstocks impacts negatively on the production of other agricultural crops where, for example, prime agricultural land formerly used to cultivate wheat is converted to maize cultivation for bioethanol production, thereby directly decreasing the availability of wheat and indirectly increasing its price (Frow *et al.*, 2009). Thus, for bioethanol to be economically sustainable on a global scale, biomass sources that are abundant in nature and do not compete as food or for agricultural land, must be explored (Sun & Cheng, 2002).

2.1.2 Lignocellulosic biomass

Lignocellulosic materials are known as second generation feedstocks for producing bioethanol. The various forms of lignocellulosic feedstocks can be grouped into six main categories (Table 1.1). They account for nearly 50% of world biomass with an estimated annual production of 10 to 50 billion tonnes, making lignocellulose arguably the most abundant and renewable organic component of the biosphere (Claassen *et al.*, 1999). Considering this abundance, there is huge potential for the use of lignocellulosic biomass as energy sources, especially bioethanol production. Utilising lignocellulosic raw materials would also minimize the potential competition between land use for food and for energy feedstock production (Champagne, 2007; Hahn-Hägerdal *et al.*, 2006). For countries where cultivation of energy crops for bioethanol is difficult, lignocellulosic biomass offers an attractive option (Cardona & Sánchez, 2007). This kind of raw material is also less expensive when compared to conventional agricultural feedstocks such as maize, and it can be produced with a lower input of fertilizers, pesticides and energy (Hahn-Hägerdal *et al.*, 2006; Öhgren *et al.*, 2006). To harness these advantages, however, the technological and economical challenges facing the lignocellulose-to-ethanol processes must be addressed.

Table 1.1 Lignocellulosic biomass categories. (Adapted from Lin & Tanaka, 2006; Sánchez & Cardona, 2008; Sun & Cheng, 2002)

Biomass Category	Common Examples
Industrial cellulosic waste	Saw mail and paper mill waste, furniture industry discards
Municipal solid waste	Newsprint and office waste paper
Agricultural residues	Wheat straw, corn stover, rice hulls, sugar cane bagasse
Dedicated herbaceous biomass	Alfalfa hays, switchgrass, Bermuda grass, reed canary grass, Timothy grass
Hardwoods	Aspen, poplar
Softwoods	Pine, spruce

2.2 Polymer composition of lignocellulose

Lignocellulose occurs within plant cell walls which consists primarily of an intermeshed and intricate matrix of three main polymers, namely cellulose, hemicelluloses, lignin and, depending on the feedstock, pectins (van Maris *et al.*, 2006) (Table 1.2).

Cellulose, the major constituent of lignocellulosic biomass (33-51%), is a straight chain homopolysaccharide composed of approximately 2 000 to 15 000 D-glucose units linked by β-(1→4)-glycosidic bonds (Figure 1.2) (Kumar *et al.*, 2008; van Maris *et al.*, 2006). The multiple hydroxyl groups on the glucose residues from one chain bind together with the oxygen molecules of another chain, to form tightly packed and highly crystalline structures called microfibrils that are linked together by hydrogen bonds and van der Waals forces. This structural integrity, as well as its close association with other plant substances, renders cellulose highly water insoluble and resistant to depolymerisation (Pauly & Keegstra, 2008; van Maris *et al.*, 2006).

Table 1.2 Polymer composition of lignocellulosic biomass (van Maris *et al.*, 2006)

Polymers	Content in Lignocellulose (%)	Major monomers
Cellulose	33-51	Glucose
Hemicelluloses	19-34	Xylose, glucose, mannose, arabinose, rhamnose, galactose
Lignin	20-30	Aromatic alcohols
Pectins (when present)	2-20	Galacturonic acid and rhamnose

The hemicellulose is a complex of heterologous polymers consisting of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and uronic acids (4-O-methyl-glucuronic, galacturonic and glucuronic acids). The sugars are linked together by β-1,4- and sometimes by β-1,3-glycosidic bonds (Figure 1.2). It has a lower molecular weight than cellulose and is more easily hydrolyzed to its constituent monosaccharides (Pérez *et al.*, 2002; van Maris *et al.*, 2006). Hemicellulose hydrogen bonds together with cellulose microfibrils form a network that provides the structural backbone of plant cell wall (Kumar *et al.*, 2008; Mosier *et al.*, 2005; Pérez *et al.*, 2002). The composition of hemicellulose varies with plant source (van Maris *et al.*, 2006). Xylans are the principal hemicelluloses found in hardwoods while glucomannans are more common in softwoods (Kumar *et al.*, 2008; Pérez *et al.*, 2002).

Lignin, which constitutes about 20-30% (dry wt) of lignocellulose, consists of phenolic residues such as *trans-ρ*-coniferyl alcohol and *trans-ρ*-sinapyl alcohol (Figure 1.2) (Kumar *et al.*, 2008; Pérez *et al.*, 2002; van Maris *et al.*, 2006). Lignin confers structural support, impermeability, resistance to microbial attack and oxidative stress to the plant cell wall (Mosier *et al.*, 2005; Pérez *et al.*, 2002). However, these attributes result in the presence of lignin to obstruct enzymatic hydrolysis of polysaccharides present in the biomass (Kumar *et al.*, 2008; Mosier *et al.*, 2005).

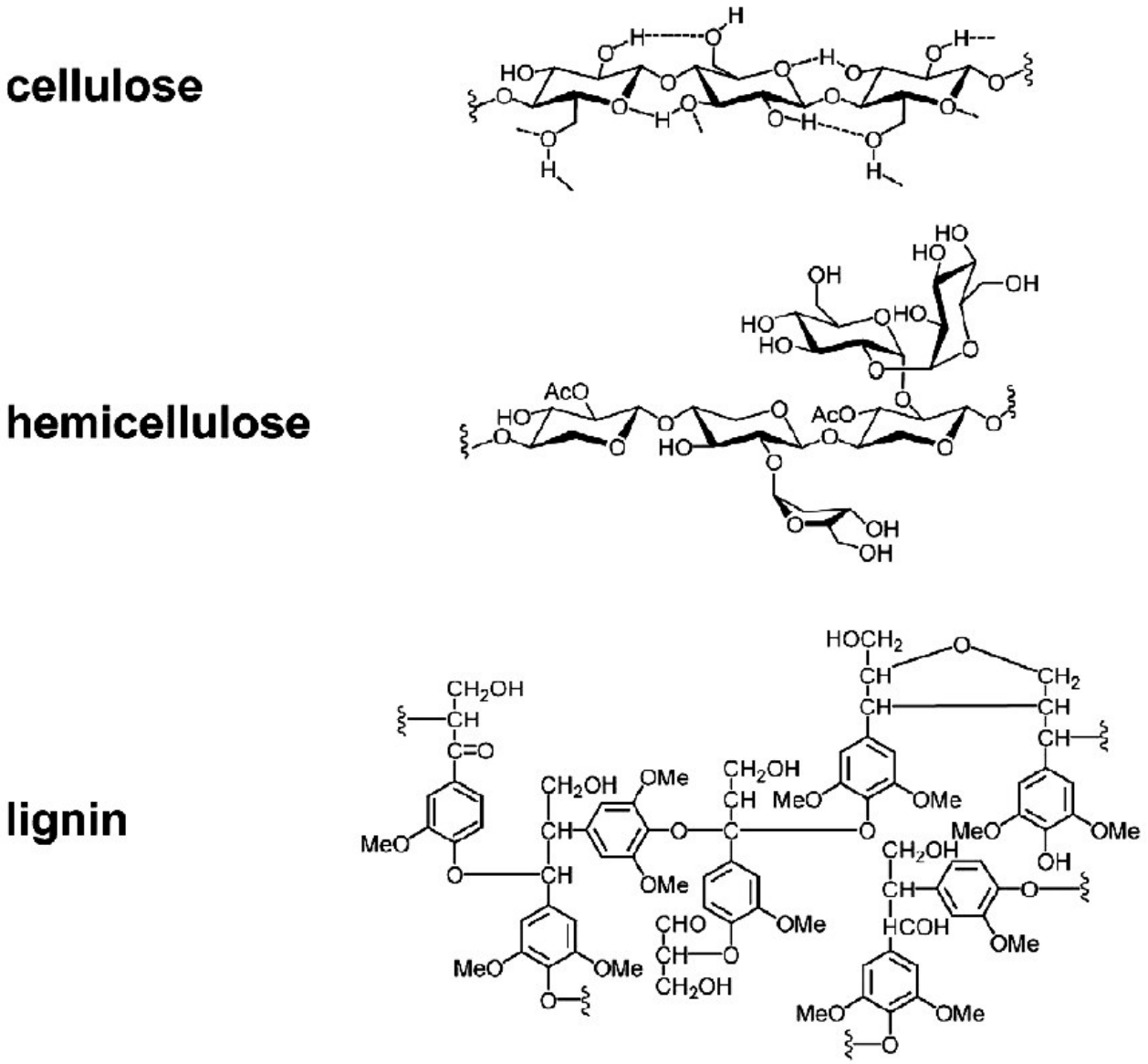


Figure 1.2 General structure of cellulose, hemicellulose and lignin biopolymers found in lignocellulosic biomass (Chang, 2007)

Pectins are complex structural polysaccharides that serve as hydrating and cementing agents for the cellulose matrix in plant cell wall (Blanco *et al.*, 1999; van Maris *et al.*, 2006). They consist of a backbone of partially esterified α-D-galacturonic acid subunits linked by (1→4) glycosidic bonds (Figure 1.3) (Cárdenas *et al.*, 2008; Ridley *et al.*, 2001). Other constituent sugars, most commonly D-galactose, L-arabinose and D-xylose, are attached in side chains to the pectin backbone, while D-glucose, D-mannose, L-fucose and and D-glucuronic acid occur less frequently (Goycooleal & Cárdenas, 2003). Pectins are more prominent in agricultural residues such as citrus peels and sugar beet pulp (Grohmann *et al.*, 1998; van Maris *et al.*, 2006).

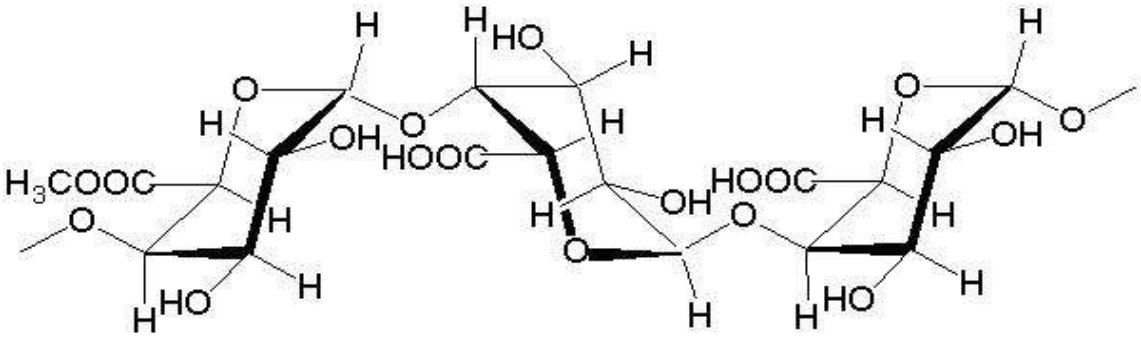


Figure 1.3 Schematic illustration of the structure of pectin showing uronic acid residues and methylated groups (Benhura & Chidewe, 2011)

2.3 Ethanol production from lignocellulosic biomass

For a lignocellulosic ethanol process to be economically competitive with starch or sugar-based processes, all of the sugars present in the cellulose and hemicellulose have to be available to the fermenting organism (Hahn-Hägerdal *et al.*, 2007a). However, due to the structure and composition of the plant cell wall as described above, this process entails a much higher degree of complexity, leading to high ethanol production costs (Cardona & Sánchez, 2007). There are three major stages involved in the conversion of lignocellulose to ethanol (Figure 1.4): (1) pretreatment, (2) enzymatic hydrolysis, (3) fermentation of the resulting hydrolysate by bacteria, yeasts or filamentous fungi (Balat & Balat, 2009). Before any of the above steps can be embarked upon, analysis of the structure and composition of the potential biomass is important. Information gained from such analysis aids the design of biomass conversion/ethanol production processes that are specific for the feedstock.

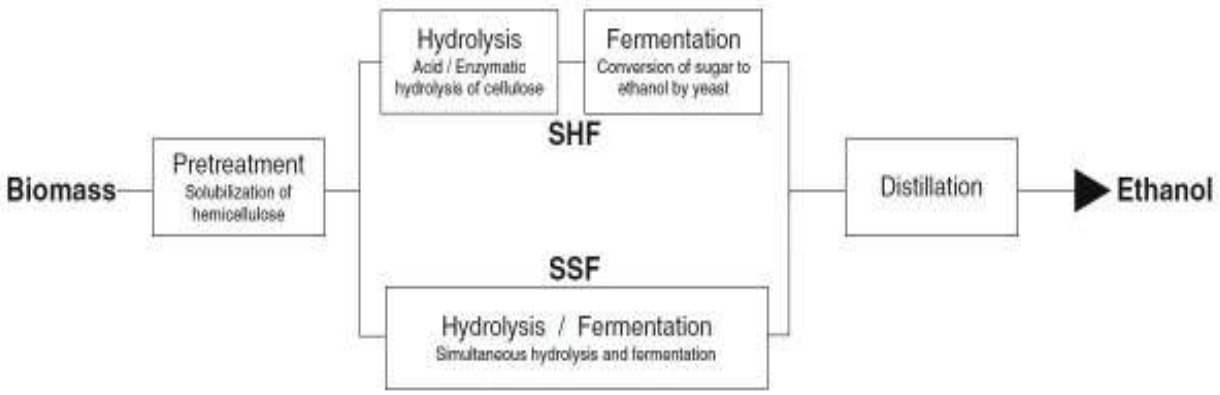


Figure 1.4 A schematic illustration of the process design for ethanol production from lignocellulosic biomass. *SHF:* separate hydrolysis and fermentation; *SSF:* simultaneous saccharification and fermentation (Hahn-Hägerdal *et al.*, 2007a)

2.4 Pretreatment

Pretreatment of lignocellulose is required to alter its complex structure, thereby increasing its surface area which facilitates rapid and efficient hydrolysis of the polymer to fermentable sugars (Figure 1.5) (Chen *et al.*, 2007; van Maris *et al.*, 2006). An effective pretreatment method is one that aims to improve the formation and availability of sugars through hydrolysis, avoid the degradation or loss of carbohydrates, avoid the formation of by-products that could be inhibitory to the fermentation process and also minimize energy consumption and operational costs (Kumar *et al.*, 2009; Taherzadeh & Karimi, 2008). Since lignocellulosic materials have complex structures, their pretreatment is not simple (Pauly & Keegstra, 2008). Pretreatment can be the most expensive stage in the biomass-to-ethanol process. However, there is potential for improvements in the areas of efficiency and cost reduction through further research and development (Kumar *et al.*, 2009). Pretreatment methods can be classified as physical, chemical, physico-chemical and biological (Galbe & Zacchi, 2007).

2.4.1 Physical pretreatment

Several mechanical and non-mechanical methods can be used for the physical pretreatment of biomass. Mechanical methods involve biomass comminution by a combination of chipping, grinding and milling to reduce biomass size and cellulose crystallinity (Kumar *et al.*, 2009). The energy required for mechanical pretreatment depends on the final particle size and biomass characteristics. However, in most cases this energy consumed is higher than the theoretical energy present in the biomass (Kumar *et al.*, 2009; Sun & Cheng, 2002). Non-mechanical methods such as irradiation have also been tested. Irradiation of the cellulose by gamma-rays results in the cleavage of β-1,4-glycosidic bonds, giving a larger surface area and lower crystallinity. This method is, however, far too expensive to be used in a full-scale process and doubts remain about its feasibility (Galbe & Zacchi, 2007; Kumar *et al.*, 2009). Pyrolysis has also been evaluated as a physical pretreatment method. When biomass is treated at temperatures above 300^{o}C, cellulose rapidly decomposes to gaseous products and residual char. At lower temperatures, the decomposition is much slower and less volatile products are formed. The high temperatures used and the cooling costs of the system, however, render pyrolysis an extremely expensive method (Bridgwater *et al.*, 1999).

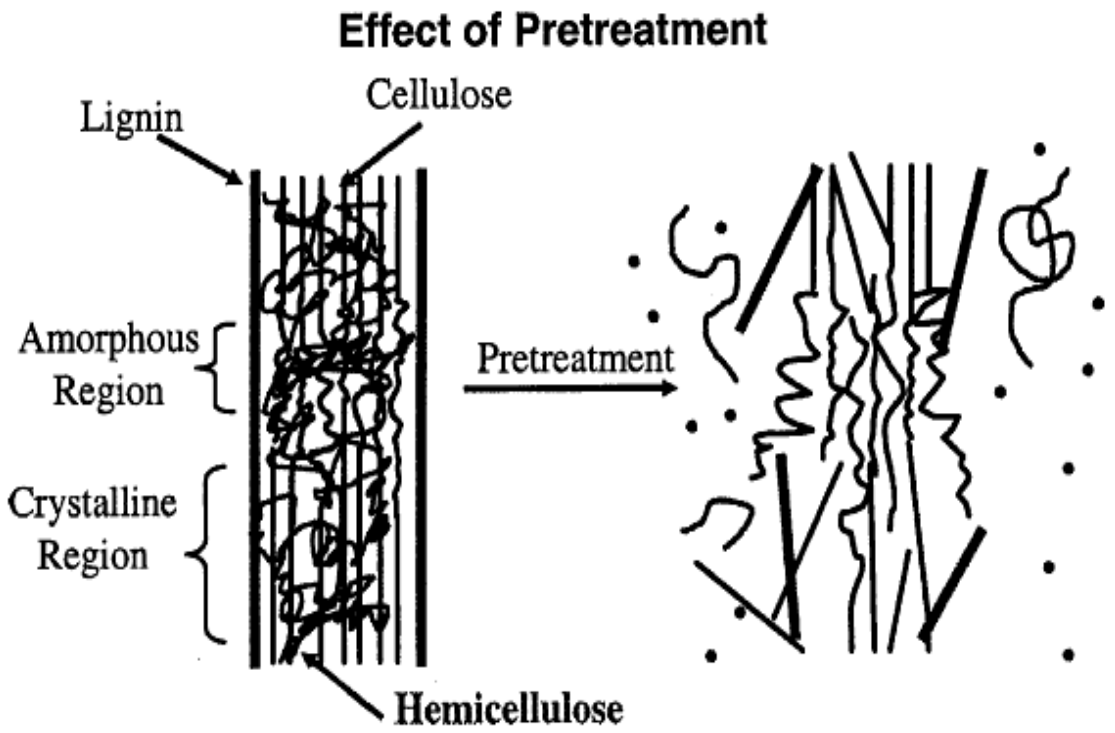


Figure 1.5 Schematic of the effect of pretreatment on lignocellulosic biomass (Mosier *et al.*, 2005)

2.4.2 Chemical Pretreatment

Chemical pretreatment involves the use of different chemical agents such as ozone, acids, alkalis, hydrogen peroxide and organic solvents to release lignin and degrade the hemicellulose (Sánchez & Cardona, 2008).

Ozonolysis

The most significant effect of treating lignocellulosic biomass with ozone is on the degradation of lignin. Ozone pretreatment effectively decreases the amount of lignin and thus increases the *in vitro* digestibility of the biomass. Hemicellulose is partially degraded while the cellulose is hardly affected (Silverstein *et al.*, 2007; Sun & Cheng, 2002). In contrast to other chemical pretreatment methods, it does not produce toxic residues and the reactions can be carried out at room temperature and pressure (Sun & Cheng, 2002). Ozonolysis is, however, a very expensive procedure due to the large amount of ozone required (Kumar *et al.*, 2009). The efficiency of ozone treatment can also be affected by insufficient reaction time, low ozone concentration and uneven ozone distribution throughout the lignocellulosic material (Silverstein *et al.*, 2007).

Dilute-acid pretreatment

The use of dilute acid has been successfully developed for the pretreatment of lignocellulose (Sun & Cheng, 2002). Dilute H_2SO_4 pretreatment, especially at concentrations below 4% w/w, has been used in most studies because it is inexpensive and can achieve high reaction rates (Galbe & Zacchi, 2007). Nitric acid, hydrochloric acid and phosphoric acid have also been tested (Azzam, 1987; Xiao & Clarkson, 1997). Dilute acid effectively hydrolyses the hemicellulose component of the lignocellulosic biomass and most of it is recovered as monomeric sugars. Removal of hemicellulose enhances cellulose digestibility in the residual solids and glucose yields of up to 100% can be obtained when the hemicellulose is completely hydrolysed (Kumar *et al.*, 2009). There are primarily two types of dilute acid pretreatment processes: (i) a high temperature (> 160^{o}C) continuous-flow process used for low solids loadings (i.e. weight of solids/weight of reaction mixture equals 5-10%) and (ii) a low temperature (< 160^{o}C) batch process used for high solids loadings of about 10-40% (Esteghlalian *et al.*, 1997; Taherzadeh & Karimi, 2008). Recently developed dilute acid processes make use of less severe conditions and achieve a high conversion of xylan to xylose. This is important for favourable overall process economics as xylan accounts for nearly 30% of the total carbohydrate in many lignocellulosic materials (Hinman *et al.*, 1992; Sun & Cheng, 2002). Although dilute acid pretreatment can significantly enhance cellulose hydrolysis, it has been shown that the hydrolysate may be difficult to ferment because of the presence of toxic substances (Galbe & Zacchi, 2007). Furthermore, the combined costs of building non-corrosive reactors, using high pressures, neutralizing and conditioning the hydrolysate prior to hydrolysis and fermentation all contribute to make dilute acid pretreatment a more expensive process than, for example, steam explosion or the AFEX method (Kumar *et al.*, 2009).

Alkaline pretreatment

This form of pretreatment utilises alkaline solutions such as NaOH, KOH, NH_4OH, or $Ca(OH)_2$ (Taherzadeh & Karimi, 2008). Sodium hydroxide is the most commonly studied pretreatment alkali and is seen as an alternative to sulphuric acid (Kumar *et al.*, 2009; Silverstein *et al.*, 2007). Compared to acid processes, alkaline pretreatment causes less sugar degradation and much of the caustic salts can be recovered or regenerated. Alkaline pretreatment also requires lower temperatures and pressures than other pretreatment technologies. However, it is much slower and the pretreatment times are in the order of hours and sometimes days rather than minutes and seconds (Kumar *et al.*, 2009). The mechanism of alkali pretreatment is thought to be saponification of intermolecular ester

bonds crosslinking xylan, lignin and other hemicelluloses (Silverstein *et al.*, 2007). Dilute NaOH treatment causes the biomass to swell, leading to an increase in internal surface area, a decrease in cellulose crystallinity and degree of polymerization, as well as a separation of structural linkages between lignin and carbohydrates (Sun & Cheng, 2002). Alkaline pretreatment is, however, less effective for softwoods when the lignin content is above 26% (Yamashita *et al.*, 2010).

Oxidative delignification

The oxidative delignification process involves the addition of an oxidizing compound such as H_2O_2 (hydrogen peroxide) or peracetic acid to the biomass in a water suspension (Hendriks & Zeeman, 2009). Lignin degradation is catalyzed by the peroxidase in the presence of H_2O_2. Azzam (1989) reported a significant increase in the susceptibility of sugar cane bagasse to enzyme hydrolysis after pretreatment with hydrogen peroxide. About 50% of the lignin and most of the hemicellulose was solubilized when treated with 2% H_2O_2 at 30^{o}C for 8 h (Azzam, 1989). This helped to achieve a 95% glucose recovery from cellulose in the subsequent hydrolysis with cellulase. A total sugars yield of 604 milligrams per gram, corresponding to 94% of theoretical, was obtained after alkaline peroxide pretreatment and enzymatic saccharification of barley straw (Saha & Cotta, 2010). Inhibitors such as furfural and hydroxy-methylfurfural were not observed following oxidative delignification treatment (Kumar *et al.*, 2009). However, hydrogen peroxide decomposes in the presence of water at high temperatures and this may lead to a decreased solubilization of lignin and xylan (Silverstein *et al.*, 2007).

Organosolv process

The organosolv (organosolvation) process is a promising pretreatment strategy that employs an organic or aqueous organic solvent mixture with inorganic solvent catalysts such as HCl or H_2SO_4 to break the internal lignin and hemicellulose bonds (Sun & Cheng, 2002; Zhao *et al.*, 2009). Methanol, ethanol, acetone, ethylene glycol and tetrahydrofurfuryl alcohol (THFA) are common organic solvents that can be used in the process. Cellulose is partially hydrolysed into smaller fragments that remain insoluble in the liquor, hemicellulose is hydrolysed mostly into soluble components such as oligosaccharides, monosaccharides and acetic acid, while lignin is hydrolysed primarily into lower molecular weight fragments that dissolve in the aqueous ethanol liquor (Kumar *et al.*, 2009). After pretreatment, the solvents used need to be drained from the reactor, evaporated, condensed and recycled to reduce operating costs. Moreover, removal of

solvents from the system is necessary to prevent them inhibiting enzyme hydrolysis, growth of microorganisms as well as fermentation (Sun & Cheng, 2002).

2.4.3 Physico-chemical pretreatment

This category includes methods that are a mixture of purely physical and chemical methods, the most common of which are steam pretreatment and ammonia fibre explosion (AFEX) (Galbe & Zacchi, 2007).

Steam pretreatment

Steam provides an effective means to rapidly heat up materials to the target temperature without excessively diluting the resulting sugars (Mosier *et al.*, 2005). Steam pretreatment is one of the most widely used methods for pretreating lignocellulose. This method was formerly known as 'steam explosion' because it was believed that an explosive action on the fibres was necessary to render the material amenable to hydrolysis. However, it is more likely that the hemicellulose is hydrolyzed by the acetic acid and other acids released during the steam pretreatment (Galbe & Zacchi, 2007; Mosier *et al.*, 2005). The biomass is subjected to high pressure saturated steam (0.69-4.83 MPa) at a temperature of 160-260^{o}C which is maintained for several seconds to a few minutes, after which the pressure is released (Sun & Cheng, 2002). The process causes solubilisation of the hemicellulose and lignin transformation, thus improving the accessibility of the cellulose fibrils to the enzymes during hydrolysis (Mosier *et al.*, 2005; Sun & Cheng, 2002). During steam pretreatment, parts of the hemicellulose hydrolyze and form acids, which could serve as a catalyst for further hydrolysis of the hemicellulose. This situation in which the acids formed *in situ* catalyze the process itself, is known as 'auto-cleave' steam pretreatment (Hendriks & Zeeman, 2009). Sometimes an acid catalyst such as H_2SO_4 or SO_2 can also be directly added to produce an effect similar to dilute acid hydrolysis, thereby increasing the hemicellulose sugar recovery and digestibility of the solid residue (Galbe & Zacchi, 2007).

The use of an acid catalyst is especially important when pretreating softwood, since it is in general more difficult to degrade (Galbe & Zacchi, 2007). Steam pretreatment has a low energy requirement when compared to mechanical methods such as biomass comminution. The conventional mechanical methods require 70% more energy than steam pretreatment to achieve the same size reduction. Furthermore, steam pretreatment neither incurs recycling costs nor does it have a negative impact on the environment (Sun & Cheng, 2002). Limitations of steam pretreatment include destruction of a portion of the xylan fraction which decreases sugar recovery, incomplete disruption of the lignin-

carbohydrate matrix and formation of inhibitory compounds. After pretreatment, the biomass needs to be washed to remove the inhibitory materials along with water soluble hemicellulose. About 20-25% of the initial dry matter is removed by the water wash, resulting in a decrease in the overall sugar yield after saccharification (McMillan, 1994)

Ammonia fibre explosion (AFEX)

Ammonia fibre explosion is a pretreatment method which, similar to the steam pretreatment process, involves operating at high pressures (Balan *et al.*, 2009). AFEX involves exposing the material to liquid ammonia (1-2 kg ammonia per kg of dry biomass) at temperatures below 100^{o}C, and pressure above 3 MPa for 10-60 min after which the pressure is suddenly reduced (Galbe & Zacchi, 2007; Sun & Cheng, 2002). During pretreatment, only a small amount of the hemicellulose is solubilized and the lignin is not removed. The hemicellulose is degraded into oligomeric sugars and deacetylated, which could be why no solubilization occurs with the hemicellulose. However, the pretreatment alters the material's structure, resulting in an increased water retention capacity and improved digestibility (Galbe & Zacchi, 2007). Liquid ammonia causes cellulose swelling and a phase change in the crystal structure from cellulose I to cellulose III (Mosier *et al.*, 2005). The AFEX pretreatment method has been used for herbaceous and agricultural residues including alfalfa, wheat straw, corn stover, municipal solid waste, switchgrass and sugar cane bagasse. However, this method only works moderately well on hardwoods and is not effective on materials with a higher lignin content such as newspaper and aspen chips which contain up to 25% lignin (Mosier *et al.*, 2005; Sun & Cheng, 2002). AFEX does not produce inhibitors that may affect the downstream processes, thus a water wash is not necessary after pretreatment. Moreover, the pretreatment does not require the biomass to be in small particle sizes for efficacy (Sun & Cheng, 2002).

2.4.4 Biological pretreatment

Biological pretreatment processes exploit the ability of microorganisms such as brown, white and soft-rot fungi to degrade lignin and hemicellulose (Sun & Cheng, 2002). Brown rots attack cellulose, whereas white and soft rots attack both cellulose and lignin. White-rot fungi are the most effective for biological pretreatment of lignocellulosic materials. Lignin degradation by white-rot fungi occurs through the action of lignin-degrading enzymes such as peroxidases and laccase (Lee *et al.*, 2007). These enzymes are regulated by carbon and nitrogen sources; for instance, the white rot fungus *Phanerochaete chrysosporium* produces lignin peroxidases and manganese-dependent peroxidases as secondary metabolites in response to carbon or nitrogen limitation (Sun & Cheng, 2002). Biological

pretreatment is considered to be environmentally friendly and energy saving since it requires no chemicals and is performed at low temperatures. However, the rate of hydrolysis is very slow and some material is lost as these organisms to an extent can consume lignocellulose (Hsu, 1996). Nonetheless, biological pretreatment can be incorporated as a step preceding some of the other types of pretreatment methods (Galbe & Zacchi, 2007).

2.4.5 Overview of pretreatment methods

Table 1.3 summarizes the advantages and limitations of the different pretreatment methods discussed in this section. The challenge is in choosing a method that can combine effective solubilization of hemicellulose, lignin alteration, production of fewer inhibitors and at a low cost. It must be emphasized that it is not always possible to transfer the results of pretreatment from one type of material to another. A method that is efficient for a particular type of biomass may not work on another. While methods such as ozonolysis, dilute acid and AFEX are capital intensive, some other methods such as fungal delignification are very slow. Furthermore, factors such as energy balance, solvent recycling and environmental effects have to be carefully considered for any selected method. Nevertheless, despite their disadvantages, chemical methods such as dilute acids and alkali, and physico-chemical pretreatment methods such as steam pretreatment and AFEX are among the most effective and promising processes for industrial applications.

Table 1.3 Summary of the advantages and limitations of various pretreatment methods

Pretreatment Method	Pretreatment Process	Advantages	Limitations and disadvantages
Physical pretreatment	Mechanical comminution	Reduces cellulose crystallinity and increases biomass surface area	Energy required usually higher than inherent biomass energy
	Pyrolysis	Produces gas and liquid products	High temperature; ash production
	Ozonolysis	Reduces lignin content; toxic substances are not produced	Expensive; ozone required in large amounts
Chemical pretreatment	Dilute acid	Hydrolyses hemicellulose to xylose and other sugars; alters lignin structure	High cost; corrosion of equipment; forms inhibitors
	Alkali	Removes hemicellulose and lignin; increases biomass surface area	Long residence times; irrecoverable salts formed and incorporated into biomass; not effective on softwoods
	Hydrogen peroxide	Solubilises lignin; does not produce inhibitors	Hydrogen peroxide decomposes at high temperature, causing a decrease in lignin and hemicellulose solubilisation
	Organosolv	Hydrolyses lignin and hemicellulose	High cost; solvents need to be recovered and recycled
Physico-chemical pretreatment	Steam pretreatment	Causes hemicellulose degradation and lignin transformation; short residence time; cost effective	Destroys a portion of the xylan fraction; incomplete destruction of the lignin-carbohydrate matrix; formation of toxic compounds
	AFEX	Increases biomass surface area; removes hemicellulose and lignin to an extent; inhibitory compounds are not formed	Not effective for biomass with a high lignin content; ammonia is expensive and hazardous
Biological pretreatment	Fungal delignification	Degrades lignin and hemicellulose; low energy required	Slow reaction rate; loss of cellulose

2.5 Enzymatic hydrolysis of cellulose

Enzymatic cellulose hydrolysis is currently carried out using highly specific cellulases under mild conditions (pH 4-5, 45-50^{o}C) (Sun & Cheng, 2002). The end products are glucose and other reducing sugars that can be fermented by yeasts or bacteria to ethanol (Sun & Cheng, 2002).

The term cellulases refers to enzymes from three major groups: (i) endoglucanase (EG, endo-1,4-D-glucanohydrolase), which randomly attacks the regions of low crystallinity on the cellulose chain, creating free chain ends; (ii) exoglucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiodehydrolase), which degrades the cellulose further by removing cellobiose units from the free chain ends; (iii) β-glucosidase, which hydrolyses cellobiose to produce two glucose molecules (Duff & Murray, 1996; Lynd *et al.*, 2002; Prasad *et al.*, 2007).

Commercial cellulases are mainly obtained from aerobic cultivations of *Trichorderma reesei* and to a lesser extent *Aspergillus niger* (Prasad *et al.*, 2007; Sánchez & Cardona, 2008). Other fungi that have been reported to produce cellulases include species of *Sclerotium, Schizophyllum* and *Penicillium*. Some bacteria, e.g. species of *Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus, Bacteroides, Erwinia, Acetovibrio, Microbispora* and *Streptomyces*, can produce cellulases with high a specific activity but low enzyme titres (Sun & Cheng, 2002).

Several factors can influence the enzymatic hydrolysis of cellulose. A low substrate concentration would result in a low overall glucose yield (Hamelinck *et al.*, 2005). An increase in the substrate concentration would lead to an increased glucose yield as well as an increased rate of reaction. However, a high substrate concentration can cause substrate inhibition, which would substantially decrease the rate of the hydrolysis, and the extent of substrate inhibition depends on the ratio of total substrate to total enzyme. A high cellulase dosage would also significantly raise process costs (Prasad *et al.*, 2007).

The susceptibility of cellulosic substrates to enzymatic hydrolysis depends on the structural feature of the substrate, including cellulose crystallinity, degree of polymerization, surface area and lignin content (Sun & Cheng, 2002; Taherzadeh & Karimi, 2008). Lignin interferes with hydrolysis by acting as a shield, preventing access of cellulases to cellulose and hemicellulose, thereby resulting in extended reaction times to achieve high conversions. On top of that, lignin irreversibly adsorbs a large portion of the

cellulase, rendering it unavailable for further hydrolysis of cellulose (Qing *et al.*, 2010). Therefore, removal of lignin during pretreatment is essential to dramatically increase the hydrolysis rate (McMillan, 1994; Prasad *et al.*, 2007). Also, removal of hemicellulose increases the mean pore size of the substrate, thereby increasing cellulase accessibility to cellulose (Hendriks & Zeeman, 2009).

Cellulase activity decreases during hydrolysis, partially due to the irreversible adsorption of cellulases on cellulose (Sun & Cheng, 2002). An enzyme dosage of about 10 FPU/g (filter paper units per gram cellulose) is often used for laboratory studies because it provides high yields within a reasonable time (48-72 h) at a reasonable cost (Sun & Cheng, 2002).

It has been reported that additives such as non-ionic surfactants (e.g. Tween 20 and Tween 80), non-catalytic protein (e.g. bovine serum albumin) and polymers (e.g. polyethylene glycol) can drastically enhance the enzymatic conversion of cellulose into fermentable sugars and decrease the amount of enzymes required for hydrolysis (Kristensen *et al.*, 2007; Kumar & Wyman, 2009; Qi *et al.*, 2010; Qing *et al.*, 2010). The positive effect of surfactant addition on lignocellulose is generally believed to be through the following: (i) altering the substrate structure and making it more accessible to enzymes, (ii) stabilizing the enzymes and preventing their denaturing during hydrolysis, (iii) increasing surface interaction between substrates and enzymes, and (iv) reducing non-productive absorption of enzymes (Eriksson *et al.*, 2002; Kim *et al.*, 2007a). However, a mechanism that can consistently explain how surfactants improve enzymatic hydrolysis has yet to be developed (Qing *et al.*, 2010).

Cellulase mixtures from different organisms or a mixture of cellulase and other enzymes has also been used to improve hydrolysis yields and increase the rate of reaction (Sun & Cheng, 2002). A mixture of hemicellulases or pectinases together with cellulases also exhibits a significant increase in the extent of cellulose and hemicellulose conversion (Grohmann *et al.*, 1995; Sun & Cheng, 2002; Wilkins *et al.*, 2007). Even though cellulases produced by *T. reesei* contain some β-glucosidase, which is responsible for hydrolysing the formed cellobiose into glucose, this enzyme has a low activity. Unfortunately, during enzymatic hydrolysis end-product inhibition of cellobiohydrolases occurs when cellobiose is formed. Therefore, β-glucosidase from other sources should be added to complement the activity of the cellulases from this fungus (Murray, 1987; Sánchez & Cardona, 2008). Intermediate and end-product inhibition can also be reduced by using a higher concentration of enzymes, removal of sugars during hydrolysis by ultrafiltration or by

simultaneous saccharification and fermentation (SSF, see section 2.1.5). Enzymes can, moreover, be recovered and recycled to reduce enzyme cost, although enzyme quality will decrease gradually with each recycling step (Hamelinck *et al.*, 2005).

2.6 Inhibitory compounds in lignocellulosic hydrolysates

During the pretreatment of lignocellulose, especially with dilute acid, numerous degradation products are generated, many of which inhibit microbial growth and metabolism. The inhibitors formed during pretreatment can be assigned into three main groups based on origin: furan derivatives, weak acids and phenolic compounds (Liu, 2006; Palmqvist & Hahn-Hägerdal, 2000a).

Aromatic compounds that occur from sugar degradation are predominantly furan derivatives, the most prominent of which are furfural from pentoses and hydroxymethyl furfural (HMF) from hexoses. Furans are formed in high concentrations during severe acid pretreatment conditions (Klinke *et al.*, 2004; Taherzadeh *et al.*, 1997) and are considered to be the most potent inhibitors of yeast growth and fermentation (Olsson & Hahn-Hägerdal, 1996; Taherzadeh *et al.*, 2000). Acetic acid is ubiquitous in hemicellulose hydrolysates where the hemicellulose and to some extent the lignin is acetylated. Hydrocarboxylic acids such as glycolic acid and lactic acid are common degradation products of alkaline pretreatment. Formic acid is produced from sugar degradation, whereas levulinic acid is formed by 5-HMF degradation (Klinke *et al.*, 2004; Palmqvist & Hahn-Hägerdal, 2000b). Phenolic compounds are formed by the solubilization and hydrolytic or oxidative cleavage of lignin. The most common phenolic compounds found in lignocellulosic hydrolysates include 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, vanillin, dihydroconiferyl alcohol, coniferyl aldehyde, syringaldehyde and syringic acid (Klinke *et al.*, 2004).

Furfural and HMF can be converted into furfuryl alcohol and 5-hydroxymethyl furfuryl alcohol, respectively, by yeasts such as *S. cerevisiae.* The presence of furfural and HMF has been shown to increase the lag phase, reduce the specific growth rate and the cell mass yield on ATP as well as the volumetric and specific ethanol productivities. Cell growth is more sensitive to furfural than ethanol concentration (Palmqvist & Hahn-Hägerdal, 2000b). It has been suggested that the presence of furfural and HMF inhibits NADH-dependent yeast alcohol dehydrogenase (ADH), leading to intracellular acetaldehyde accumulation, which is believed responsible for the long lag phase in

microbial growth (Modig *et al.*, 2002; Palmqvist & Hahn-Hägerdal, 2000b). Undissociated weak organic acids act on the cell by readily diffusing across biological membranes, thereby disturbing the membrane potential and acidifying the cytoplasm which has to be compensated for by ATP-dependent proton pumping (Heer & Sauer, 2008). In addition, intracellular accumulation of the anion, either by itself or together with intracellular acidification, can lead to toxic effects. For instance, acetic acid has been demonstrated to induce apoptosis. Phenolic compounds are also known to compromise the integrity of the cell membrane (van Maris *et al.*, 2006). In general, degradation products reduce enzymatic and biological activities, break down DNA, inhibit protein and RNA synthesis and reduce ethanol yield (Modig *et al.*, 2002). Therefore, to facilitate fermentation processes, detoxification procedures are often required to remove inhibitory compounds from the hydrolysate. However, these additional steps increase the costs and complexity of the process and generate extra waste products (Liu, 2006).

To solve the problem of inhibitors present in the hydrolysate, several physical, chemical or biological methods of hydrolysate detoxification prior to fermentation have been investigated (Palmqvist & Hahn-Hägerdal, 2000a). These include the addition of activated charcoal, extraction with organic solvents, ion exchange, ion exclusion, molecular sieves, overliming and steam stripping (Olsson & Hahn-Hägerdal, 1996). Treatment of the hydrolysate with alkali such as calcium hydroxide (overliming) is the most common detoxification method used for removing furaldehydes and phenolic compounds (Sánchez & Cardona, 2008). In this process the pH of the hydrolysate is increased up to pH 10 by adding $Ca(OH)_2$ or some other hydroxide. After mixing, the resulting precipitate is removed and the hydrolysate pH is readjusted to pH 5.5 with H_2SO_4 (Olsson & Hahn-Hägerdal, 1996; Palmqvist & Hahn-Hägerdal, 2000a). The detoxifying effect of overliming is due both to the precipitation of toxic components and to the instability of some inhibitors at high pH values (Palmqvist & Hahn-Hägerdal, 2000a). The effectiveness of any detoxification method depends on the type of lignocellulosic hydrolysate, as each has a different level of toxicity, depending on the raw material and pretreatment conditions (Carvalho *et al.*, 2006). Other alternative approaches to detoxification include adapting the fermenting organism to the hydrolysate or isolating strains from natural and industrial habitats or harsh environments. A newer approach is the development of inhibitor-tolerant strains through genetic modification and metabolic engineering. However, due to the synergistic interactions among inhibitors and poor knowledge of the mechanisms of these interactions, it is not clear against which inhibitor resistance is desired (Olsson & Hahn-

Hägerdal, 1996; Sánchez & Cardona, 2008; van Maris *et al.*, 2006). To facilitate the development of specific, efficient and cheap detoxification methods, intense research is required to identify the key inhibitory substances as well as their inhibitory mechanisms. This information will also enable the modification of pretreatment and hydrolysis processes to minimize the formation of the most potent inhibitors (Olsson & Hahn-Hägerdal, 1996).

2.7 Fermentation processes of biomass hydrolysates

After pretreatment and enzymatic hydrolysis of lignocellulose, the next step involves ethanol production by fermentation of the sugars in the hydrolysate, usually using yeasts. In the classic set-up, the hydrolysis of cellulose and the fermentation are performed sequentially in separate units. This configuration is referred to as separate hydrolysis and fermentation (SHF). However, the two process steps can be performed simultaneously in a single unit, i.e. simultaneous saccharification and fermentation (SSF) (Öhgren *et al.*, 2007; Sánchez & Cardona, 2008). The ethanol produced is then recovered from the culture broth by distillation.

2.7.1 Separate hydrolysis and fermentation (SHF)

When a sequential process of hydrolysis and fermentation is utilized, the pretreated biomass first undergoes enzymatic hydrolysis (saccharification) followed by ethanolic fermentation (Sánchez & Cardona, 2008). A major advantage of SHF is that hydrolysis and fermentation can be performed at optimum operating conditions. The enzymes are, however, end-product inhibited when cellobiose and glucose accumulate (see section 2.5) (Hahn-Hägerdal *et al.*, 2006; Sun & Cheng, 2002).

2.7.2 Simultaneous saccharification and fermentation (SSF)

Concern about product inhibition occurring during SHF was the rationale behind the first report on simultaneous saccharification and fermentation (Takagi *et al.,* 1977, cited by Öhgren *et al.,* 2007). In SSF, hydrolysis and fermentation are performed in a single process unit allowing reducing sugars produced to be immediately consumed by the fermenting organism. Thus, the effect of end-product inhibition by sugars is neutralized (Hahn-Hägerdal *et al.*, 2006; Sánchez & Cardona, 2008). SSF also seems to decrease the inhibition of enzymes by toxic by-products present in pre-hydrolysate after pretreatment (Tengborg *et al.*, 2001). This improves the overall ethanol yield and productivity. Furthermore, SSF compared to the two-stage SHF process has several other advantages that include (i) a lower enzyme requirement (ii) a reduced risk of contamination, since glucose is removed immediately and ethanol is produced (iii) a shorter process time (iv)

less reactor volume because a single reactor is used and (v) lower capital costs (Sun & Cheng, 2002).

However, there are some drawbacks of the SSF process, one of which is the difficulty encountered with yeast recirculation due to the presence of lignin residues in the hydrolysate (Öhgren *et al.*, 2007). A major disadvantage of SSF is that the optimum temperature condition for enzyme hydrolysis (45-50^{o}C) is much higher than what is required for fermentation (e.g., 30^{o}C for *S. cerevisiae*). Therefore, a compromise temperature of around 38^{o}C is employed, meaning hydrolysis is usually the rate-limiting process in SSF (Philippidis & Smith, 1995; Sun & Cheng, 2002). This problem can be alleviated by the use of a thermotolerant yeast such as *Kluyveromyces marxianus*, which is able to efficiently produce ethanol at temperatures higher than 40^{o}C (Ballesteros *et al.*, 2004; Kádár *et al.*, 2004)

An improvement of the SSF technology called SSCF (simultaneous saccharification and co-fermentation) is targeted at ethanol production from both hexose and pentose sugars in one step (Hahn-Hägerdal *et al.*, 2006; Zhang & Lynd, 2010). SSCF offers increased potential for a more streamlined processing and lower capital costs. The success of SSCF and co-fermentation of hexose and pentoses in general requires the construction of genetically engineered microorganisms able to co-ferment glucose and xylose concurrently with enzymatic hydrolysis of cellulose and hemicellulose. However, this is no easy task and in the past decades there has been extensive research to develop microorganisms with such capabilities. The recent advances are discussed in the following section.

2.8 Microorganisms suitable for bioethanol production

Unlike sucrose and starch-based bioethanol, which is produced from one or two sugar monomers, lignocellulose-based ethanol is obtained through fermentation of a mixed sugar hydrolysate, i.e. hexoses and pentoses (Hahn-Hägerdal *et al.*, 2006; Zaldivar *et al.*, 2001). As a result, for lignocellulose to be economically competitive with sugar cane or grains, all types of sugars in cellulose and hemicellulose must be efficiently converted to ethanol (Hahn-Hägerdal *et al.*, 2007a; Jeffries, 2006; Öhgren *et al.*, 2006). This implies that the potential fermenting organism must, besides the traits listed in Table 1.4, be able to meet such demands (Senthilkumar & Gunasekaran, 2005; Zaldivar *et al.*, 2001). Historically, the best known microbes used for ethanolic fermentation of hexoses such as

glucose and galactose have been yeasts. Of these, *Saccharomyces cerevisiae* (baker's yeast) is the preferred choice due to its ability to produce ethanol up to concentrations reaching 18% (w/v) and its high tolerance of up to 150 g ethanol l^{-1} (Claassen *et al.*, 1999). However, *S. cerevisiae* is unable to ferment the hemicellulose-derived pentoses such as xylose and arabinose and is, therefore, of limited use for lignocellulose substrates with a high content of such sugars. The same restriction applies to the ethanologenic bacterium *Zymomonas mobilis* which is comparable to *S. cerevisiae* in terms of ethanol yield on glucose as well as ethanol tolerance (Sommer *et al.*, 2004; Zaldivar *et al.*, 2001). Xylose is the most prominent pentose sugar in the hemicellulose of hardwoods and crop residues (25% of dry weight) and is second only to glucose in natural abundance, whereas arabinose constitutes only 2-4% of dry weight, although it can reach up to 20% in many herbaceous crops (McMillan & Boynton, 1994). Because of their high content in lignocellulose, the efficient utilization of pentoses is important to significantly reduce production costs (Prasad *et al.*, 2007). There are naturally occurring xylose-fermenting yeasts such as *Candida shehatae, Pichia stipitis* and *Pachysolen tannophilus*. However, their use is limited due to slow rates of ethanol production, strict oxygen requirement, poor inhibitor tolerance and by-product formation (du Preez *et al.*, 1984; du Preez, 1994; Hahn-Hägerdal *et al.*, 2006; Sommer *et al.*, 2004)

While no microbial strain meets all the requirements listed in Table 1.4, there have been efforts to develop the 'ideal' organism through metabolic engineering (Dien *et al.*, 2003; Hahn-Hägerdal *et al.*, 2006).

Table 1.4 Important traits for efficient fermentation of lignocellulose. Modified from Dien *et al.*, (2003), Senthilkumar & Gunasekaran, (2005), Zaldivar *et al*., (2001)

Essential Parameters	**Other desirable traits**
Broad substrate utilization range	Simultaneous sugar utilization
High ethanol yield (greater than 90% of theoretical)	Hemicellulose and cellulose hydrolysis
High ethanol tolerance (at more than 40 g l^{-1})	GRAS status
High ethanol productivity (more than 1 g l^{-1} h^{-1})	Recyclable
Minimal by-product formation	Minimal nutrient supplementation required
Increased tolerance to inhibitors	Tolerance to acidic pH and high temperature
Tolerance to process hardiness	Tolerance to high osmotic pressure

The main goals of metabolic engineering can be summarized as follows: (a) improvement of yield, productivity and overall cellular physiology, (b) extension of the substrate range, (c) deletion or reduction of by-product formation and (d) introduction of pathways leading to new products (Kern *et al.*, 2007). Thus, through metabolic engineering, several of the traits listed in Table 1.4 have been transferred to adequate hosts. As a result, a variety of organisms displaying attractive features for fermentation of lignocellulosics have been engineered in the last three decades, with most effort concentrated on the three most promising microbial platforms, namely *Z. mobilis, Escherichia coli* and *S. cerevisiae* (Dien *et al.*, 2003; Hahn-Hägerdal *et al.*, 2006; Zaldivar *et al.*, 2001). In the following section the ethanolic properties and genetic improvements of these three strains are discussed with the most emphasis on *S cerevisiae*. *Kluyveromyces marxianus*, one of the two yeasts investigated in this study, is also discussed.

2.8.1 *Zymomonas mobilis*

Zymomonas mobilis is an important ethanol producing bacterium isolated from 'pulque', the fermented juice of agave (Claassen *et al.*, 1999). This gram-negative bacterium is notable for its ability to produce ethanol rapidly and efficiently from glucose-based feedstocks (Dien *et al.*, 2003). *Z. mobilis* has a homoethanol fermentation pathway and can tolerate an ethanol concentration of up to 120 g l^{-1}. It also exhibits a high sugar tolerance of up to 400 g l^{-1}, can ferment sugars at a low pH value (pH 3.5-7.5) and is generally regarded as safe (GRAS) (Dien *et al.*, 2003; Mohagheghi *et al.*, 2002). Compared to *Saccharomyces cerevisiae, Z. mobilis* gives 5-10% more ethanol per gram of fermented glucose and a 2.5-fold higher specific ethanol productivity (Lee *et al.*, 1979; Rogers *et al.*, 1979). An ethanol yield of up to 97% of the theoretical maximum and ethanol concentrations of up to 12% (w/v) have been reported in glucose fermentations using *Z. mobilis* (Lynd et al., 1996). This high yield and productivity have been attributed to its unique physiology: *Zymomonas* is one of the few facultative anaerobic bacteria that metabolize glucose using the Entner-Doudoroff (ED) pathway (Figure 1.6) as opposed to the Embden-Meyerhoff-Parnas (EMP) or glycolytic pathway (Panesar *et al.*, 2006). In terms of ethanol production the ED pathway is so efficient that up to 1 µmol of glucose is converted to ethanol and carbon dioxide per min per mg of cell protein (even in cell free extracts), while producing only 1 ATP per mole of glucose, which is half of the ATP produced in the EMP pathway (Sprenger, 1996). Consequently, *Zymomonas* produces less biomass than yeasts and more carbon is channelled to fermentation products (Dien *et al.*, 2003).

All the enzymes involved in fermentation are expressed constitutively and they comprise as much as 50% of the cell's total protein (Sprenger, 1996). Thus the low ATP yield, its facilitated diffusion system (instead of active transport system) and its elevated levels of glycolytic and ethanologenic enzyme pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH), enable *Z. mobilis* to maintain a high glucose flux and efficient conversion of glucose to ethanol (Dien *et al.*, 2003; Mohagheghi *et al.*, 2002; Panesar *et al.*, 2006; Zhang *et al.*, 1995). *Zymomonas* also has simple nutritional needs, with some strains requiring only panthothenate and biotin for growth (Dien *et al.*, 2003). In spite of all its advantages as an ethanologen, *Z. mobilis* is not well suited for biomass conversion because it has a limited substrate range, fermenting only glucose, fructose and sucrose. As such, wild type strains are not naturally suited for xylose and arabinose fermentation because they lack the necessary pentose assimilation and metabolism pathways (Mohagheghi *et al.*, 2002). *Z. mobilis* would also require pathways for the metabolism of mannose and galactose, which constitute a considerable portion of some lignocellulosic raw materials. However, being a prokaryote, *Z. mobilis* is more amenable to genetic manipulation than yeast species (Panesar *et al.*, 2006).

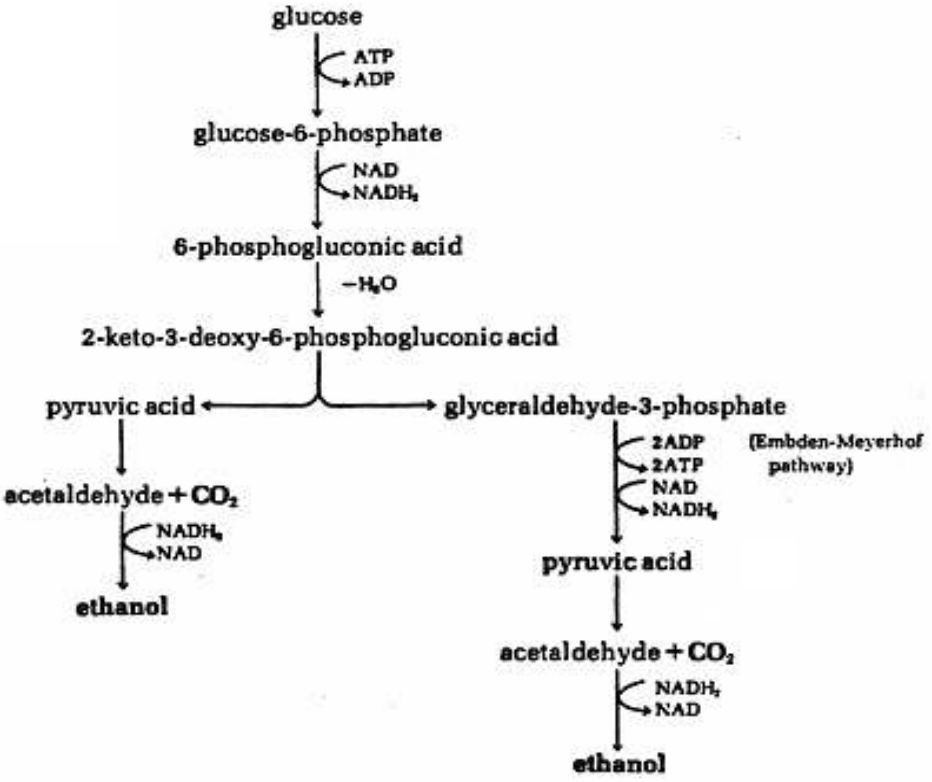


Figure 1.6 Entner-Doudoroff pathway in *Zymomonas*

(http://www.textbookofbacteriology.net/metabolism_3.html)

Recombinant strains of *Z. mobilis* capable of fermenting xylose (Zhang *et al.*, 1995), arabinose (Deanda *et al.*, 1996) and both sugars, together with glucose (Mohagheghi *et al.*, 2002) in detoxified lignocellulosic hydrolysates, have been successfully constructed. Xylose fermentation by *Z. mobilis* required the introduction and expression of four *E. coli* genes: xylose isomerase (*xylA)*, xylulose kinase (*xylB*), transketolase (*tktA*) and transaldolase (*talB*) (Zhang *et al.*, 1995). Xylose isomerase and xylulose kinase convert xylose to xylulose-5-phosphate, which is then converted into intermediates of the ED pathway by transketolase and transaldolase (Zhang *et al.*, 1995). The same strategy was used to engineer *Z. mobilis* to ferment arabinose. In this case, a plasmid was constructed with five genes also isolated from *E. coli*: L-arabinose isomerase (*araA*), L-ribulose kinase (*araB*), L-ribulose-5-phosphate-4-epimerase (*araD*), transketolase (*tktA*) and transaldolase (*talB*) (Deanda *et al.*, 1996). The first three enzymes are required to convert arabinose to xylulose-5-phosphate and, as before, xylulose-5-phosphate was converted to ED pathway intermediates via transketolase and transaldolase. The strains constructed grew on the individual sugars with ethanol yields of 86% (xylose) and 98% (arabinose) of the theoretical. Since this breakthrough, more recombinant strains have been constructed with improved yields and productivities (Joachimsthal & Rogers, 2000). The recently developed *Z. mobilis* strain AX101 can ferment both xylose and arabinose and carries the seven necessary recombinant genes (*xylA, xylB, araA, araB, araD, tktA, talB*) as part of its chromosomal DNA (Mohagheghi *et al.*, 2002). In mixed sugar fermentations, this strain utilised glucose, then xylose and finally arabinose although it was hampered by acetic acid toxicity and only 75% of the arabinose was utilised (Lawford & Rousseau, 2002; Mohagheghi *et al.*, 2002). However, in more recent studies, acetate tolerant *Z. mobilis* mutant strains (AcR) were developed (Yang *et al.*, 2010), paving the way for a more robust biocatalyst for the fermentation of lignocellulosic hydrolysates.

2.8.2 *Escherichia coli*

Escherichia coli is one of several bacteria (including *Klebsiella oxytoca, Erwinia sp.* and *Clostridium saccharolyticum*) that can naturally utilise hexoses, pentoses and uronic acids (galacturonic acid and glucuronic acid). Its broad substrate utilisation range as well as other advantages, such as its easily manipulated genetic system, non-requirement for complex growth factors and history of industrial use (e.g., for production of recombinant protein) make *E. coli* a potential biocatalyst for ethanol production (Dien *et al.*, 2003; Lin & Tanaka, 2006). *E. coli* however, grows poorly on xylose and the end product of

fermentation is not only ethanol, but also a range of by-products including lactate, acetate, succinate and formate (Figure 1.7) (Clark, 1989).

E. coli produces ethanol from the central metabolite pyruvate, using pyruvate formate lyase (PFL). However, this fermentation pathway is unbalanced because one mole of NADH is generated from each mole of pyruvate made from sugars and two moles of NADH are required to convert pyruvate into ethanol. Therefore, *E. coli* has to balance its fermentation by also producing acetate and succinate. By contrast, *Z. mobilis* and yeasts convert pyruvate to ethanol utilising pyruvate decarboxylase (PDC), which only requires one mole of NADH for each ethanol produced, enabling them to be homoethanol fermentative (Dien *et al.*, 2003). To increase ethanol production by *E. coli*, several metabolic engineering strategies were devised to redirect the carbon flux towards ethanol production (Zaldivar *et al.*, 2001).

An *E. coli* strain transformed with a plasmid expressing *pdc* and the alcohol dehydrogenase II (ADH II) gene (*adhB),* isolated from a genomic library of *Z. mobils,* was able to produce ethanol almost exclusively (Ingram *et al.*, 1987). The *Z. mobilis* PDC has a lower K_m for pyruvate than lactate dehydrogenase and PFL, effectively shifting the carbon flux to ethanol production (Figure 1.7). The genes were co-expressed under the control of a single promoter, creating the PET (production of ethanol) operon (Ingram *et al.*, 1987) which was subsequently integrated into the chromosomes of several bacterial hosts leading to the development of *E. coli* strain KO11 (Bothast *et al.*, 1999; Ohta *et al.*, 1991). Strain KO11 and its derivatives such as LY01 have been used in laboratory investigations to ferment glucose, xylose and arabinose in many types of lignocellulosic substrates including rice hulls (Moniruzzaman & Ingram, 1998), sugar cane bagasse, agricultural residues (Asghari *et al.*, 1996), orange peel, pectin rich pulp (Doran *et al.*, 2000) and brewery waste (Rao *et al.*, 2007), in addition to several other sources, with the authors reporting ethanol yields of 0.4 g per g sugars and above. However, the use of a recombinant organism for large-scale ethanol production is viewed by some as an obstacle to commercialization. Recently, ethanologenic *E. coli* strains lacking foreign genes have been described (Kim *et al.*, 2007b). The strains produced ethanol yields of 82% and 90% of theoretical respectively, from glucose and xylose.

In spite of these improvements, more work is required to address the drawbacks of using *E. coli* in large scale fermentations due to its narrow and neutral pH requirement of between pH 6.0-8.0, sensitivity to inhibitors, intolerance to high ethanol concentrations

compared to *S. cerevisiae* and public perceptions regarding the danger of *E. coli* strains (Dien *et al.*, 2003).

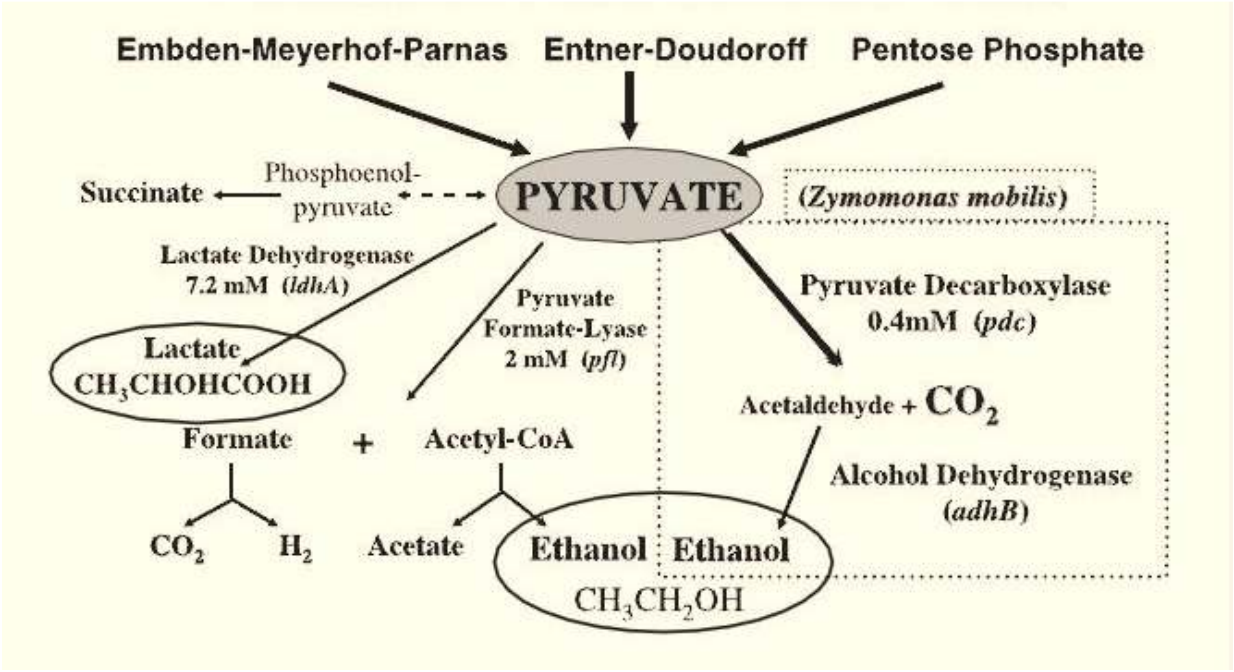


Figure 1.7 Hexose and pentose sugar conversion to ethanol by recombinant *E. coli* in conjunction with the *Z. mobilis* ethanol pathway. A solid arrow indicates the natural *E. coli* reactions; a dashed arrow indicates those of *Z. mobilis* (Doran-Peterson *et al.*, 2008)

2.8.3 *Saccharomyces cerevisiae*

Baker's yeast, *Saccharomyces cerevisiae,* has traditionally been the preferred organism for the large-scale fermentation of sugar and starch-based raw materials. *S. cerevisiae* produces ethanol at stoichiometric yields in addition to a remarkable tolerance to ethanol, a wide spectrum of inhibitors and an elevated osmotic pressure (Hahn-Hägerdal *et al.*, 2007a). These unusual properties are a result of its adaptation to efficient ethanol production from hexoses during thousands of years (Olsson & Hahn-Hägerdal, 1996; Piskur *et al.*, 2006; Wackett, 2008). Presently, sugar and starch-based fermentations operate almost exclusively with *S. cerevisiae* as the production organism and it is possible to integrate these with large-scale lignocellulosic ethanol processes, thereby substantially reducing the production cost. Despite these advantages, the most important challenge of using *S. cerevisiae* for lignocellulosic fermentation is its inability to metabolize and ferment xylose and arabinose (Barnett, 1976). Consequently, converting *S. cerevisiae* into an efficient xylose and arabinose-fermenting yeast has long been one of the major challenges in yeast metabolic engineering (Kuyper *et al.*, 2005b).

Xylose utilization

Although *S. cerevisiae* naturally possesses genes for xylose utilization, they are expressed at such low levels that they do not support growth on xylose (Richard *et al.*, 1999; Toivari *et al.*, 2004). However, the xylose keto isomer, xylulose, can be utilised and fermented by *S. cerevisiae*, albeit ten times slower than glucose (Chiang *et al.*, 1981; Senac & Hahn-Hägerdal, 1990). Yeasts such as *Pichia stipitis, Candida shehatae* and *Pachysolen tannophilus* can assimilate pentoses, but their rate of ethanol production from glucose is at least five-fold less than that of *S. cerevisiae*. Moreover, their use for ethanolic fermentation requires strict control of aeration and their ethanol tolerance is much lower (Claassen *et al.*, 1999; du Preez, 1994). In such yeasts, xylose metabolism is initiated by two redox reactions that convert xylose to xylulose (Figure 1.8b). The first reaction is catalysed by a generally NADPH-dependent aldose reductase or xylose reductase (XR), that reduces xylose to xylitol, while in the second step xylitol is oxidised to xylulose via a generally NAD^+-dependent xylitol dehydrogenase (XDH). Xylulose is then phosphorelated to yield xylulose-5-phosphate, an intermediate of the pentose phosphate pathway (PPP) (Figure 1.8b).

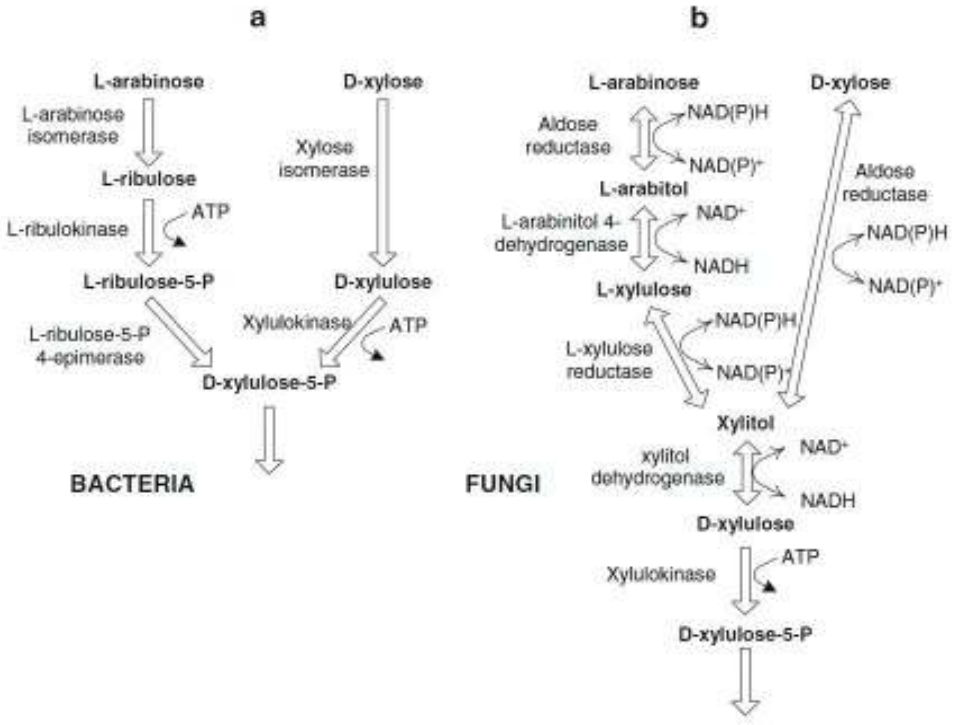


Figure 1.8 D-xylose and L-arabinose metabolism in (a) bacteria and (b) fungi (Hahn-Hägerdal *et al.*, 2007b)

Based on this knowledge, numerous metabolic-engineering strategies have been explored in laboratory strains of *S. cerevisiae*. The *P. stipitis* genes *XYL1* and *XYL2,* which code for XR and XDH, respectively, have been introduced into *S. cerevisiae*, resulting in biomass formation from xylose (Kötter *et al.*, 1990). Through additional genetic modifications, including the overexpression of an endogenous *XKS1* gene encoding xylulokinase, (XK) and pentose-phosphate pathway enzymes, xylose fermentation was achieved, albeit with the inevitable production of substantial amounts of xylitol and/or glycerol (Eliasson *et al.*, 2000; Ho *et al.*, 1998; Kötter & Ciriacy, 1993; Tantirungkij *et al.*, 1993; Toivari *et al.*, 2001). The xylitol formed was as a consequence of an imperfect match of the cofactor specificities of XR and XDH. For every mole of NADPH used by XR, one mole of NADH needs to be reoxidized, and the only way for the yeast to do so is to produce xylitol (Bruinenberg *et al.*, 1984; Kuyper *et al.*, 2004). Decreasing xylitol excretion by XR and XDH-expressing *S. cerevisiae* strains has been approached by several metabolic engineering strategies, including optimising the expression levels of XR and XDH (Eliasson *et al.*, 2001; Karhumaa *et al.*, 2007) changing the cofactor affinity of XR (Jeppsson *et al.*, 2006; Watanabe *et al.*, 2007), and modifying the redox metabolism of the host cell (Verho *et al.*, 2003).

An interesting alternative was to circumvent this intrinsic redox problem through the expression of a heterologous gene encoding xylose isomerase (XI) (Figure 1.8a). In most bacteria, xylose is converted to xylulose in one step by this enzyme. Cofactors are not required in the isomerisation, thus the redox balance is not an issue. Bacterial xylose isomerase encoded by the XlyA gene has been expressed in *S. cerevisiae*, but with little success, mainly attributed to improper protein folding, posttranslational modifications, disulphide bridge formation and the internal pH of the yeast (Amore *et al.*, 1989; Gárdonyi & Hahn-Hägerdal, 2003; Ho *et al.*, 1983; Moes *et al.*, 1996; Walfridsson *et al.*, 1996). A notable exception was the xylose isomerase from *Thermus thermophilus* which was functionally expressed in *S. cerevisiae,* generating small amounts of ethanol from xylose (Walfridsson *et al.*, 1996). However, the optimum temperature of this enzyme was 85^{o}C and it had a low activity at 30^{o}C, which is the optimum temperature for *S. cerevisiae.* Even when all enzymes involved in the conversion of xylose to glycolysis intermediates were over-expressed, the XI activity was too low for efficient xylose metabolism (Karhumaa *et al.*, 2005; van Maris *et al.*, 2006).

Because of the disappointing results of the XI research, more attention was given to the XR/XDH approach, mainly because contrary to XI obtained from archaea and bacteria,

these eukaryotic genes could be functionally expressed in *Saccharomyces*, enabling the yeast to metabolise xylose. However, an important breakthrough was achieved with the more recent discovery of a fungal xylose isomerase (Harhangi *et al.*, 2003). This xylose isomerase obtained from the obligate anaerobic fungus, *Piromyces* sp. E2 was the first of its kind to be successfully expressed in *S. cerevisiae,* followed by the recently discovered XI from the fungus *Orpinomyces* (Madhavan *et al.*, 2009). The resulting *S. cerevisiae* strain transformed with the *Piromyces* XI was adapted through prolonged cultivation techniques resulting in anaerobic growth on xylose at a specific growth rate of 0.03 h^{-1} accompanied by an ethanol yield of 0.42, although it had a very low specific ethanol productivity of 0.14 g g^{-1} h^{-1} (Harhangi *et al.*, 2003; Kuyper *et al.*, 2003; Kuyper *et al.*, 2004). Further improvements led to the development of another strain that, in addition to *XYLA,* over-expressed all the genes involved in the conversion of xylose into the intermediates of glycolysis. In addition, the *GRE3* gene encoding native aldolase reductase in *S. cerevisiae* was deleted to further minimize xylitol production, as xylitol inhibits the activity of XR (Kuyper *et al.*, 2005a). This strain, RWB 217, had a maximum specific growth rate of 0.09 h^{-1} and a specific ethanol productivity of 0.46 g g^{-1} h^{-1} (Kuyper *et al.*, 2005a). Co-utilisation of mixed substrates was improved through further evolutionary engineering by prolonged anaerobic cultivation on mixtures of glucose and xylose for 35 cycles. The new strain was able to completely ferment a sugar mixture containing 20 g l^{-1} each of glucose and xylose within 24 h. (Kuyper *et al.*, 2005b).

The success achieved through the XR/XDH as well as the XI approach show that the kinetics of xylose fermentation are no longer a bottleneck for the production of bioethanol. Current research is now focussed on successfully transferring strains and concepts from laboratory to industrial conditions, as industrial strains of *S. cerevisiae* are usually diploid or polyploid and lack auxotrophic markers (Kern *et al.*, 2007; van Maris *et al.*, 2006; Wang *et al.*, 2004). An example is the engineered xylose-fermenting strain TMB 3400, which in addition to introduction of the *P. stipitis* xylose utilisation pathway, was subjected to chemical mutagenesis with ethyl methanesulfonate. Strain TMB 3400 was found to be inhibitor tolerant and had a maximum specific growth rate of 0.14 h^{-1} with an ethanol yield and productivity of 0.25 and 0.1 g g^{-1} h^{-1}, although with a high glycerol yield (Wahlbom *et al.*, 2003). Nevertheless, these outcomes demonstrated that results obtained from laboratory strains can be reproduced in industrial strains, paving the way for metabolic engineering of superior xylose-fermenting *S. cerevisiae* strains.

Arabinose utilisation

Naturally occurring *S. cerevisiae* strains cannot utilise arabinose (Barnett, 1976). Although many yeasts are capable of aerobic growth on arabinose, only four species, including *Candida arabinofermentans* and *Ambrosiozyma monospora*, have been reported to produce trace amounts of ethanol in yeast extract medium with a high L-arabinose content (Dien *et al.*, 1996; Kurtzman & Dien, 1998). Similar to xylose, arabinose is converted to the PPP intermediate, D-xylulose 5-phosphate. However, the metabolic pathways to convert L-arabinose to D-xylulose 5-phosphate are distinctively different in bacteria and fungi (Figure 1.8). In the fungal pathway, L-arabinose first has to be converted into the corresponding polyol, and L-arabitol is subsequently oxidised to L-xylulose, which requires an L-xylulose reductase for conversion into xylitol, the intermediate compound common to the catabolic pathways of both xylose and arabinose (Fonseca *et al.*, 2007a; Richard *et al.*, 2003). The two oxidations and two reductions are coupled to NAD^+ and NADPH consumption, respectively, (Figure 1.8b), and there is limited cellular capacity to regenerate NAD^+ under oxygen limited conditions, which may result in the accumulation of arabitol (Bruinenberg *et al.*, 1983; Dien *et al.*, 1996; Fonseca *et al.*, 2007a). This redox imbalance is proposed as the reason why L-arabinose fermentation is rare among yeasts (Dien *et al.*, 1996; van Maris *et al.*, 2006).

Several studies have been aimed at the metabolic engineering of *S. cerevisiae* for L-arabinose fermentation, including combining all the structural genes in the fungal L-arabinose pathway and overexpressing them in *S. cerevisiae* (Richard *et al.*, 2003). Ethanol production from arabinose was achieved, albeit at a very low ethanol productivity of 0.00035 g g^{-1} h^{-1}. This was possibly due to an imbalance of the redox cofactors or arabinose transport into the cell (Kern *et al.*, 2007; Richard *et al.*, 2003; Verho *et al.*, 2004). Alternatively, the bacterial L-arabinose pathway, in which no redox reactions are involved in the initial steps of L-arabinose metabolism, was expressed in *S. cerevisiae* (Figure 1.8a). Integration of the *E. coli* genes involved in L-arabinose conversion to D-xylulose-5-P into *S. cerevisiae* was only partly successful, as the engineered strain accumulated L-arabitol and did not produce ethanol from L-arabinose, although there was enzyme activity (Sedlak & Ho, 2001). Expression of these genes plus overexpression of the *S. cerevisiae* galactose permease gene (*GAL2*) yielded better results, although ethanol production was only possible under oxygen-limited conditions (Becker & Boles, 2003; Jeffries & Jin, 2004). Extensive evolutionary engineering further improved the performance of *S. cerevisiae*, leading to the development of strains such as IMS0002 (Wisselink *et al.*,

2007) which exhibited high rates of arabinose consumption and ethanol production, together with a high ethanol yield of 0.43 g g^{-1} during anaerobic growth on arabinose. In addition, it was able to efficiently ferment a sugar mixture of both glucose and arabinose, although the ability to ferment xylose was lost during long term selection for improved arabinose fermentation. Nevertheless, this represented a significant breakthrough in the metabolic engineering of arabinose metabolism in yeast.

Co-utilisation of pentose sugars by *S. cerevisiae* has been the focus of several research groups in recent years. In industrial strains where plasmid usage is not applicable, a method for chromosomal integration of *AraA*, *AraB* and *AraD* in the rDNA region was applied. The resultant strains simultaneously consumed xylose and arabinose during aerobic and anaerobic batch cultivations (Karhumaa *et al.*, 2006). Recently, xylose and arabinose-fermenting recombinant *S. cerevisiae* strains expressing an improved fungal pentose utilisation pathway have also been constructed (Bera *et al.*, 2010; Bettiga *et al.*, 2009). Bettiga and co-workers overexpressed the L-arabinose pathway in *S. cerevisiae* strain TMB3043 (Karhumaa *et al.*, 2005) which harboured an overexpression of PPP and XK in addition to a deletion of *GRE3,* whereas in the engineered yeast of Bera *et al*. (2010) only the genes that encoded L-arabitol 4-dehydrogenase and L-xylulose reductase were overexpressed. In both cases, the yeast strains were able to co-ferment pentoses with an increased ethanol yield. L-arabitol production was still an issue, though it was considerably lower than what had been reported for previous D-xylose reductase expressing strains.

Evolutionary engineering strategies have proven to be successful tools for selecting recombinant *S. cerevisiae* strains capable of anaerobicaly utilizing xylose and arabinose (Becker & Boles, 2003; Kuyper *et al.*, 2004; Sonderegger & Sauer, 2003; Wisselink *et al.*, 2007). In most cases, the techniques have been primarily focused on the utilisation of single sugars. However, when selecting for mutants capable of utilising mixed pentoses, complications may arise due to the different metabolic pathways involved that require unequal selective pressures (Sauer, 2001). For instance, previous evolutionary engineering on an engineered *S. cerevisiae* strain for anaerobic arabinose utilization as the sole carbon source resulted in the strain losing its xylose-utilizing capacities (Wisselink *et al.*, 2007; Wisselink *et al.*, 2009). To minimize the effect of unequal selection pressure on the utilization of glucose, xylose and arabinose, two new selection strategies have been described: (a) repeated anaerobic batch cultivations in media with alternating combinations of glucose, xylose and arabinose as sole carbon sources (rather than a fixed mixture) that would allow for a more even distribution of the cultivation times on each

carbon source even when the cells may prefer one substrate over another (Garcia Sanchez *et al.*, 2010; Wisselink *et al.*, 2009) and (b) increasing the dilution rate which could subsequently help to increase the specific growth rate, productivity and ethanol yield of the yeast in a continuous cultivation containing xylose and arabinose as carbon sources (Garcia Sanchez *et al.*, 2010). It is hoped that through creative integration of metabolic engineering, evolutionary engineering and process design there would be more breakthroughs in the search for faster and more efficient anaerobic alcoholic fermentation of lignocellulosic hydrolysates by engineered *S. cerevisiae* strains.

2.8.4 *Kluyveromyces marxianus*

Kluyveromyces marxianus is a homothallic, hemiascomycetous yeast that is a sister species of *Kluyveromyces lactis* and is phylogenetically related to *Saccharomyces cerevisiae* (Lachance, 1998; Llorente *et al.*, 2000). *K. marxianus* occurs naturally in tequila fermentation and is frequently isolated from fresh agave plant (*Agave* spp.), agave molasses and fermenting agave must (Lachance, 1995). The population of *K. marxianus* in agave must is dominant and peaks during the early stages of fermentation: afterwards it is gradually replaced by *S. cerevisiae* as the ethanol concentration reaches 5.5% (w/v) (Lachance, 1995). *K. marxianus*, unlike *S. cerevisiae*, is capable of assimilating and utilising lactose as carbon source, leading to its frequent isolation from dairy products such as fermented milks, yoghurt and cheeses (Jakobsen & Narvhus, 1996; Rohm *et al.*, 1992). This long history of safe association with food products resulted in *K. marxianus* (as well as *K. lactis* and *S. cerevisiae*) being granted GRAS (Generally Regarded As Safe) and QPS (Qualified Presumption of Safety) status, meaning that there are few restrictions to its application and thereby greatly enhancing its potential in the biotechnology sector (Hensing *et al.*, 1994; Lane & Morrissey, 2010). However, unlike the other two well-established species, *K. marxianus* has received much less attention from the scientific community; instead there is more interest in its biotechnological applications. Consequently there is no public genome sequence, commercial cloning system or an adopted strain of *K. marxianus* for basic research purposes (Fonseca *et al.*, 2008; Rocha *et al.*, 2010). The ploidy status of *K. marxianus* is also not fully resolved. Traditionally it was considered to be haploid. However, there have been suggestions that the commonly used strain CBS 6556 may be diploid (Ribeiro *et al.*, 2007). Recent molecular approaches indicate that *K. marxianus* exists in both haploid and diploid forms in research and industrial settings (Hong *et al.*, 2007; Nonklang *et al.*, 2009). Therefore, the ploidy of the

most common strains needs to be determined as this has direct implication for the application of molecular methodologies (Lane & Morrissey, 2010).

Sugar metabolism and physiology

K. marxianus is classified as a facultatively fermentative and Crabtree negative yeast (Castrillo & Ugalde, 1993; Dijken *et al.*, 1993). Several reports focussing on the regulation of respiration and fermentation and on the Crabtree effect in yeasts have shown that *K. marxianus* exhibits a strong Crabtree negative character in contrast to what is commonly observed with *S. cerevisiae* (Fonseca *et al.*, 2008). This conclusion arose from the fact that there was no ethanol production detected in experiments involving the application of a glucose pulse to respiring cells in carbon-limited chemostat cultures (Bellaver *et al.*, 2004; Van Urk *et al.*, 1990; Verduyn *et al.*, 1992). It has been shown that when respiro-fermentative metabolism occurs in *K. marxianus* as a function of increasing glycolytic flux, the maximum respiratory capacity of the cells has not yet been achieved. This is in contrast to the situation in *S. cerevisiae*, in which respiro-fermentative metabolism starts once it reaches its maximum respiratory capacity. However, it is important to note that *K. marxianus* cannot grow under strictly anaerobic conditions and ethanol production is almost exclusively linked to oxygen-limited growth conditions (Bellaver *et al.*, 2004; Dijken *et al.*, 1993; Fonseca *et al.*, 2008). It must also be considered that several related factors can influence the Crabtree effect and these may not exert themselves equally in all species (Lane & Morrissey, 2010). Thus, there is a variation between extreme Crabtree positive and Crabtree negative yeasts, which might explain why some but not all of *K. marxianus* strains are very effective ethanol producers (Hong *et al.*, 2007; Lane & Morrissey, 2010; Nonklang *et al.*, 2008). *K. marxianus* has also been shown to possess the highest tricarboxylic acid cycle (TCA) flux among hemiascomycetous yeasts (Blank *et al.*, 2005).

Another important aspect of the physiology of *K. marxianus* is that it has one of the highest growth rates among yeasts. For example, during a chemostat study the specific growth rate of *K. marxianus* CBS 6556 was reported to be 0.6 h^{-1}, equivalent to a doubling time of 70 min, and by using a pH-auxostat, an even faster growing variant with a μ_{max} value of 0.8 h^{-1}, equivalent to a doubling time 52 min, could be selected (Groeneveld *et al.*, 2009). *K. marxianus*, however, requires the availability of growth factors when cultivated in a mineral salts medium. Supplementation of the medium with a vitamins cocktail containing essential growth factors such as nicotinic acid, calcium panthothenate and biotin is necessary for the cultivation of this yeast (Fonseca *et al.*, 2007b; Verduyn *et al.*, 1992).

Industrial exploitation

K. marxianus is a very versatile yeast that could be economically exploited for a wide range of applications, including the production of enzymes such as inulinase, pectinase and lactase (Cruz-Guerrero *et al.*, 1995; Hensing *et al.*, 1994; Kushi *et al.*, 2000), single-cell protein production (Schultz *et al.*, 2006), aroma compounds (Fabre *et al.*, 1995; Medeiros *et al.*, 2000), baking yeast (Caballero *et al.*, 1995; Dimitrellou *et al.*, 2009) and, of importance to this dissertation, bioethanol production (Ballesteros *et al.*, 2004; Kádár *et al.*, 2004; Limtong *et al.*, 2007). The underlying interest in the use of this yeast for biotechnological applications is due to some of its important physiological properties, namely its thermotolerance, its capacity to catabolize a wider range of non-glucose carbon sources such as lactose and inulin than *S. cerevisiae*, its rapid growth rate and its high secretory capacity (Fonseca *et al.*, 2008; Lane & Morrissey, 2010; Nonklang *et al.*, 2008; Rocha *et al.*, 2010).

For fuel ethanol production, *S. cerevisiae* is cultivated at optimal temperatures of 30-35^{o}C (Lin & Tanaka, 2006). However, among other ethanologenic yeast species used in fermentation processes, *K. marxianus* has been reported to have the best performance in terms of growth and ethanol production at elevated temperatures (Abdel-Banat *et al.*, 2010). Many thermotolerant strains of *K. marxianus* are capable of achieving maximum growth temperatures as high as 47^{o}C to 52^{o}C and can efficiently produce ethanol at temperatures varying between 38^{o}C and 45^{o}C (Ballesteros *et al.*, 2004; Banat & Marchant, 1995; Gough *et al.*, 1996; Limtong *et al.*, 2007; Nigam *et al.*, 1997; Nonklang *et al.*, 2008; Singh *et al.*, 1998). The benefits to this characteristic include decreased cooling costs, a more efficient saccharification and fermentation, the possibility of continuous ethanol removal during the fermentation and a decreased risk of contamination (Abdel-Banat *et al.*, 2010). *K. marxianus*, however, exhibits a lower ethanol tolerance than *S. cerevisiae* (Gough *et al.*, 1996; Hack & Marchant, 1998).

Recombinant DNA technology

Conventional biotechnology has always applied physiological and biochemical methods to improve processes and to identify and select more efficient strains. However, it is apparent that recombinant DNA technology is required for successful industrial processes, either to make the process more economically feasible or to transform the organism for the expression of heterologous proteins (Fonseca *et al.*, 2008; Lane & Morrissey, 2010). Transformation of *K. marxianus* by inserting foreign DNA is by no means new. One major limitation is the lack of comprehensive genome sequence information and there is still a

strong reliance on a partial genome sequence of strain CBS 712 and comparison to the completed *K. lactis* genome sequence (Lane & Morrissey, 2010; Llorente *et al.*, 2000). Thus, to date there are only a few reports on engineered *K. marxianus* strains with new industrially relevant functions, as most molecular studies focused on proof of concept and development of molecular tools.

Nevertheless, a number of foreign genes have been successfully cloned in different strains of *K. marxianus* using *S. cerevisiae* promoters, leading to the expression of heterologous proteins, including α-galactosidase (Bergkamp *et al.*, 1993), endopolygalcturonase (Siekstel *et al.*, 1999), lactate dehydrogenase (Pecota *et al.*, 2007), endo-beta-1,4-glucanase, cellobiohydrolase and β-glucosidase (Hong *et al.*, 2007), α-amylase (Nonklang *et al.*, 2008) and glucose oxidase (Rocha *et al.*, 2010). The study by Hong and co-workers (2007) was particularly significant in that it led to the construction of a strain that could simultaneously express the genes involved in cellulose degradation, enabling it to grow on cellobiose or carboxymethyl-cellulose (CMC) as sole carbon sources. The strain also exhibited a high ethanol yield on cellobiose. Moreover, the genes involved encoded thermostable enzymes, allowing *K. marxianus* to grow and produce ethanol at temperatures of up to 45^{o}C (Fonseca *et al.*, 2008; Hong *et al.*, 2007). Recently, a *K. marxianus* strain expressing *S. cerevisiae FLO1, 5, 9* and *10* flocculation genes was constructed (Nonklang *et al.*, 2009). The *K. marxianus FLO9* strain showed stable flocculation properties and an efficient fermentation ability at 40^{o}C. These examples illustrate that with molecular tools it is possible to improve on several aspects of this yeast that are important for industrial processes and that *K. marxianus* is a promising alternative host for the expression of heterologous compounds.

The number of *K. marxianus* strains that have been investigated is quite large and many of them are not available in the most common international culture collections worldwide. Thus, significantly different values of growth parameters such as μ_{max} and $Y_{x/s}$ have been reported not only for different strains within the species, but also for the same strain when investigated in different laboratories (Fonseca *et al.*, 2007b; Rocha *et al.*, 2010) The lack of an organized research community behind this species makes it difficult to acquire fundamental information about its metabolism and physiology. In this sense it would be necessary for researchers to use less strains and place more attention on the ones with characteristics that have given *K. marxianus* a clear advantage over other yeasts: thermotolerance, a high growth rate, being Crabtree negative, and a broad substrate spectrum (Fonseca *et al.*, 2008).

3. The prickly pear cactus [*Opuntia ficus-indica* (L.) Mill.]

3.1 Introduction

The prickly pear cactus, *Opuntia ficus-indica* (Figure 1.9) is the most commonly known and agriculturally important of approximately 1600 species of cactus plants which make up the Cactaceae family (Inglese *et al.*, 2002). The genus *Opuntia* contains about 300 species and is the largest in the subfamily Opuntioideae (Nobel, 2002). *O. ficus-indica* exists as both spiny and spineless forms (Brutsch & Zimmermann, 1993). *O. ficus-indica*, together with other members of the Opuntioideaes, is a xerophyte commonly found in arid and semi-arid regions. It is adaptable to a wide range of soil conditions and can tolerate high and low temperatures. The presence of *O. ficus-indica* in places such as Canada, the USA, Mexico, Chile, Brazil, the Caribbean, Italy, Spain, the Middle East, Tunisia, Morocco, Ethiopia, South Africa, India and Australia suggests that it can survive in many climatic conditions (Oelofse *et al.*, 2006; Stintzing & Carle, 2005).

Such adaptation to extreme habitats has been aided by several evolutionary modifications to its form and function, notably nocturnal stomatal opening, meaning that net CO_2 uptake and transpiration occur during the cooler part of the 24 hour cycle. This gas exchange pattern is referred to as crassulacean acid metabolism (CAM) (Nobel, 2002). The CO_2 taken up is incorporated into a three-carbon compound to form a four-carbon organic acid such as malate, using phosphoenol pyruvate carboxylase. The accumulated acids are stored overnight in large vacuoles of the chlorenchyma cells; thus the tissue of CAM plants becomes more acidic at night and their osmotic pressure increases (Nobel, 2002). *O ficus-indica* has a greater water retention ability than most plants and it has up to a five-fold greater efficiency of water conversion to biomass than other plants species such as maize, giving it the possibility to reach extremely high productivity of 50 t dry mass ha^{-1} yr^{-1} and 5 to 6 t dry mass ha^{-1} yr^{-1} under water-limited conditions (Nobel, 2002; Russell & Felker, 1987).

3.2 Origin and distribution

Prickly pear cactus is generally believed to have originated from central Mexico where it was an important agro-economic and cultural crop alongside maize (*Zea mays)* and agave (Casas & Barbera, 2002). Spanish conquistadores first encountered the plant and its fruits on arrival in the Mexican plateau in 1519. The collection of curious plants found in the New World by the European travellers, notably Christopher Columbus, led to the introduction of

O. ficus-indica to Spain and within a short time it had naturalized throughout the country. By the end of the eighteenth century it had spread to the rest of the Mediterranean region, including Italy and Greece. Later it was introduced to other parts of the world, including the rest of the Americas, India, Australia, Northern Africa and South Africa (Casas & Barbera, 2002; Russell & Felker, 1987)

In South Africa, *O. ficus-indica* was first introduced to the Cape region around the seventeenth century. This plant is very invasive and 60 years ago about 900 000 hectares of veldt, mostly in the Eastern Cape and Karoo, had been infested by the spiny prickly pear cactus. There is evidence to suggest that it was the spineless forms that were originally introduced to South Africa but that these reverted back to the spiny forms over a period of nearly 200 years (Brutsch & Zimmermann, 1993).

The Conservation of Agricultural Resources Act No. 43 of 1983 declared the naturalized spiny forms as weeds and instituted measures of controlling and eradicating them. Major success was achieved with biological control by introducing three natural insect enemies: the prickly pear moth (*Cactoblastis cactorum*), the prickly pear cochineal insect (*Dactylopius opuntiae*) and the prickly pear weevil (*Metamasius spinolae*) with *D. opuntiae* contributing to clearing nearly 70% of the infested areas. Chemical measures were employed in areas where biological control was not so effective; for instance, in the vicinity of Uitenhage along the coastal regions of the Eastern Cape where cooler and wetter conditions prevail, which are less favourable for cochineal development. However, this act did not apply to the cultivated spineless forms which had been introduced into the Karoo region in 1914 (Barbera, 1995). Since 1980, intensive and specialized plantations of spineless prickly pear cacti have been set up, mostly in the Northern Province and Ciskei regions, stretching from Gauteng province to Mpumalanga (Brutsch, 1997; Brutsch & Zimmerman, 1993). Popular prickly pear cactus growing areas include Paarl, Uitenhage, Lovedale, Kroonstad, Middelburg and Nylstroom (Brutsch, 1997). The total area cultivated in South Africa is about 1 000 ha, with an annual production of about 8 000 tons (wet weight) (Inglese *et al.*, 2002).

No doubt the uncontrolled invasion of *O. ficus-indica* in the early century was a national disaster and the loss of agricultural land far outweighed the benefits that the plant had to offer. The current emphasis in research on *O. ficus-indica* has shifted towards utilizing the remaining populations of the spiny forms and it is now quite feasible to reverse its status from that of a weed to an economic plant (Brutsch & Zimmermann, 1993)

Figure 1.9 Prickly pear cactus, *Opuntia ficus-indica* **A.** Spiny variety **B**. Spineless cladode with fruits (http://www.cepolina.com/photo/opuntia_ficus_indica_leaf_fruit.htm)

3.3 Morphology

Opuntia ficus-indica can be identified by its morphology (Figure 1.9), namely: (a) Arborescent succulent stems known as cladodes that can grow up to 5 m high and 60 - 150 cm wide. Each cladode has a dark green colour, is covered by a layer of wax and is oblong with obovate joints. The typical dimensions of the cladodes are 30 - 60 cm long, 20- 40 cm wide and 19-28 mm thick. (b) Areoles bearing glochids that are short, smooth, barbed deciduous leaf spines that are easily dislodged. Its areoles are also the origin of

the flowers which vary in colour from orange to yellow; (c) Edible fruits (tunas) are produced which could be yellow, orange, red or purple, with a fleshy pulp and thin skin (Sudzuki-Hills, 1995).

3.4 *O. ficus-indica* fruit

3.4.1 Fruit composition

The *O. ficus-indica* fruit (prickly pear) weighs between 140 and 240 g (wet wt) and consists of approximately 48% peel, 45% pulp and 7% seeds (Hamdi, 1997). The pulp has a pH of 5.8 and a total solids content of 16%, which competes favourably with the pulp of other common fruits such as prunes, apples, peaches and cherries (Hamdi, 1997; Sáenz, 2000). Glucose and fructose are the predominant sugars in the pulp at a 60:40 ratio. They constitute 11 to 13% fresh weight and more than 50% of the pulp dry weight (El Kossori *et al.*, 1998; Gurrieri *et al.*, 2000; Hamdi, 1997; Russell & Felker, 1987). Sucrose is not detected in the prickly pear fruit, even though its presence at some point during fruit development and/or ripening cannot be completely ruled out. It is suggested that the absence of sucrose could be as a result of activity by the enzyme β-fructofuranosidase-fructohydrolase or invertase which hydrolyses sucrose into equal amounts of glucose and fructose (Gurrieri *et al.*, 2000). In the prickly pear skin, glucose is the main sugar present, but small amounts of fructose can also be found (El Kossori *et al.*, 1998). The fibre content of the prickly pear fruit differs in the pulp and skin (Table 1.5). The pulp fibre is rich in pectin and only contains trace amounts of lignin (El Kossori *et al.*, 1998). Analysis of the pectin revealed that it is a complex mixture of polysaccharides that are structurally related to the mucilage of the prickly pear cladode (Habibi *et al.*, 2004; Matsuhiro *et al.*, 2006). Prickly pear fruit has a caloric value of 198 kJ 100 g^{-1} (wet wt) of pulp, which is comparable to that of pure sucrose or other fruits such as pears, apricots and oranges (Hamdi, 1997). The fruit's ascorbic acid content can reach levels of 400 mg l^{-1}, which is about half the ascorbic acid concentration of some vitamin C-rich fruits such as oranges and lemons. Its mineral composition is also comparable to other fruits in terms of nutritive value (Sáenz, 2000).

Table 1.5 Crude composition of the prickly pear fruit (% w/w, dry matter)
(El Kossori *et al.,* 1998)

Constituent	Pulp	Skin
Protein	5.13	8.3
Non-protein nitrogen	0.025	0.024
Lipids	0.97	2.43
Total fibre	20.5	40.8
Ash	8.5	12.1
Ethanol soluble carbohydrates	58.3	27.6
Starch	4.55	7.12

3.4.2 Uses of the prickly pear fruit

Also known as cactus pears, the prickly pear fruit has become an important fruit crop in semi-arid regions where it plays a strategic role in both commercial and subsistence agriculture (Hamdi, 1997). As human food, the fresh pulpy pear can be consumed as is or can be dried and eaten as relish or pickles. It as an excellent dietetic fruit because it aids digestion, provides dietary fibres, organic acids and certain vitamins that aid metabolism. It can also help to cure diarrhoea and constipation. Prickly pear fruit is used for the manufacture of marmalade, jam, gels, sweeteners, fruit juice and nectars (Sáenz, 2000). It is also a source of natural food pigments such as betanin and isobetanin which are responsible for the red beet colour of the fruit (Hamdi, 1997; Turker *et al.*, 2001). An alternative use of the cactus pear is for the production of alcoholic beverages; for example, “Colonche”, mainly produced in Mexico by fermenting the juice and pulp in wooden barrels. “Colonche” is a low-alcohol drink and is best when freshly fermented, as it quickly turns acidic (Sáenz, 2000). Because prickly pear juice contains fermentable sugars (glucose and fructose), it has also been considered as a raw material for single cell protein (SCP) production with *Candida utilis* (Paredes-López *et al.*, 1976). Furthermore, it could serve as an alternative substrate to molasses for baker’s yeast production, helping to decrease the environmental problems associated with molasses due to the high biological oxygen demand (BOD) of the bioprocess effluent. Cactus pear peel, which makes up roughly 48% (wet weight) of the fruit is a by-product used as animal feed while the seed can be processed into seed oil. This oil is similar to other edible vegetable oils such as grape-seed oil and it has a high level of unsaturated acids such as lineoleic acid (73.4%), palmitic acid (12%), oleic acid (8.8%) and stearic acid (5.8%) (Hamdi, 1997; Sáenz, 2000).

3.5 *O. ficus-indica* cladodes

3.5.1. General composition of the cladodes

There is a considerable variation in the literature regarding the chemical composition of *O. ficus-indica* cladodes. These variations could be because cladode composition is influenced by agronomic conditions (e.g., soil type, climate and growing conditions), type of cultivar, the season and the age of the plant. Therefore, data on the respective constituents should not be taken as absolute values (Nefzaoui & Ben Salem, 2002; Stintzing & Carle, 2005). Malainine *et al*. (2003) reported a composition of 19.6 g ash, 7.2 g fat and wax, 3.6 g lignin and 69.6 g polysaccharides in 100 g (dry wt.) of despined dry cladode biomass, but crude protein was not determined. Table 1.6 shows the typical range of the constituents of *O. ficus-indica* cladodes.

Table 1.6 Mean chemical composition of despined *Opuntia ficus-indica* cladodes (adapted from Stintzing & Carle (2005)

Constituent	Fresh weight (g/ 100 g)	Dry weight (g/100 g)
Water	88-95	0
Carbohydrate (total polysaccharides)	3-7	64-71
Ash	1-2	19-23
Crude fibre	1-2	18
Protein	0.5-1	4-10
Lipid	0.2	1-4

On a dry biomass basis, *O. ficus-indica* cladodes contain approximately 22% cellulose, 13% hemicellulose and 34% acidic polysaccharides (mucilage and pectins), giving roughly 69% total carbohydrates (Malainine *et al*., 2005). Since cellulose and hemicelluloses are been discussed in section 2.2 of this review, this section will focus on the acidic polysaccharides generally referred to as mucilage.

3.5.2 Mucilage component

The cactaceae family is characterized by the production of a hydrocolloid commonly referred to as mucilage (Matsuhiro *et al*., 2006), which forms molecular networks that have the ability of retaining large amounts of water (Sepulveda *et al*., 2007). Mucilage is present

in both the cladode and fruit of the plant. In the cladode it is stored in mucilaginous cells that are located within the chlorenchyma and parenchyma (Terrazas & Mauseth, 2002; Saenz, 2004). The chemical composition of mucilage in *O. ficus-indica* cladodes has been the subject of various studies with major contradictions. Amin *et al.* (1970) described it as a neutral polysaccharide composed of arabinose, rhamnose, galactose and xylose, but no galacturonic acid. On the other hand, the water soluble polysaccharide extract of *O. ficus-indica* 'Burbank's cv spineless' contain neutral fractions of glucans and glycoproteins and an acidic polysaccharide fraction composed of arabinose, galactose, rhamnose, xylose and galacturonic acid (Paulsen & Lund, 1979). These apparent contradictions may have been due to contamination of the mucilage with other compounds found in the cell wall and/or the purification methods used not being completely effective (Saenz, 2004). In general, the mucilage is a high molecular weight polysaccharide comprising variable amounts of L-arabinose, D-galactose, L-rhamnose and D-xylose as the major neutral sugars, as well as D-galacturonic acid (Medina-Torres *et al.*, 2000; Sáenz *et al.*, 2004; Sepúlveda *et al.*, 2007). According to Nobel *et al*. (1992), the sugar composition in *O. ficus-indica* mucilage was approximately (dry weight) 42% arabinose, 22% xylose, 21% galactose, 8% galacturonic acid and 7% rhamnose. Quite similar results were also reported by Medina-Torres *et al*. (2000).

The suggested primary structure of the mucilage is a linear repeating core chain of (1→4) linked α-D-galacturonic acid and (1→2) linked β-L-rhamnose with lateral chains of (1→6)-β-D-galactose attached to O-4 of rhamnose residues. The galactose side residues present further branching in the O-3 or both the O-3 and O-4 positions (McGarvie & Parolis, 1979a), (Figure 2.0). The composition of the acid-labile peripheral chains is complex with at least 20 different types of oligosaccharides, mostly disaccharides and trisaccharides, being identified. These invariably contain (1→5) linked L-arabinose residues and D-xylose as terminal groups, giving a xylose:arabinose ratio of approximately 1:2. Rhamnose and galacturonic acid are presumed to be confined to the acid resistant backbone of the mucilage as they do not appear after hydrolysis of the mucilage (McGarvie & Parolis, 1979b).

The physico-chemical and rheological properties of the mucilage extracted from *O. ficus-indica* cladodes have been subjected to research by several groups (Forni *et al.,* 1994; Madjoub *et al.,* 2001; McGarvie & Parolis, 1979, Medina-Torres *et al.,* 2000). Due to the presence of D-galacturonic acid, several authors have indistinctly referred to the mucilage

in *Opuntia* cladodes as pectin or a pectinoid. Contradicting this is the discovery of two types of water soluble materials which were extracted from the mucilage of *Opuntia* cladodes and identified by size-exclusion chromatography (SEC) analysis: a high molecular weight fraction (HWS) (10% by weight) with an average molecular weight of 14.2 x 10^6, and another low molecular weight fraction (LWS) (90% by weight) with a molecular weight of 4 x 10^3 (Cárdenas *et al.*, 2008; Goycooleal & Cárdenas, 2003; Majdoub *et al.*, 2001). LWS was poorly water soluble and contained 80% protein while no protein was detected in the HWS fraction (Majdoub *et al.,* 2001).

LWS is now known to be mucilage material easily identified as the slimy fluid that appears as soon as the pad is cut, whereas HWS is a pectin polysaccharide. Mucilage, in contrast to the pectin, has a low degree of elasticity and does not show gelling activity in the presence of calcium ions. Moreover, up till now, the mucilage polysaccharides do not seem to be chemically associated, either covalently or otherwise, with the structural cell wall pectins (Saenz, 2004; Goycoolea & Cardenas, 2003). Since an unpurified mucilage extract displayed visco-elastic properties, the conclusion was that the protein fraction and the polysaccharides interacted by intermolecular bonding (Stintzing & Carle, 2005).

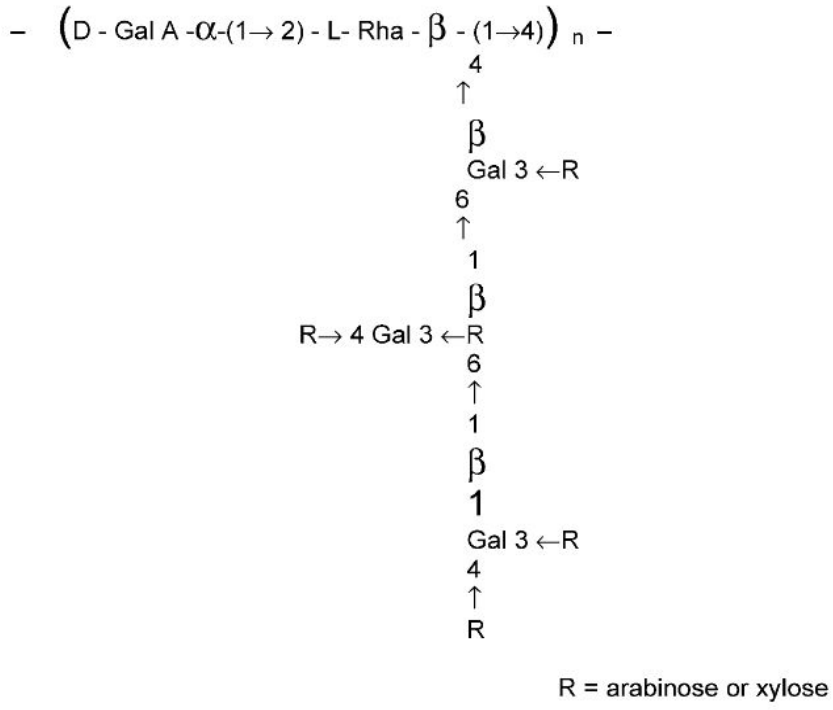


Figure 2.0 Proposed partial structure for *Opuntia ficus-indica* mucilage (Mcgarvie & Parolis, 1981, cited by Saenz *et al*. (2004)

3.5.3 Uses of O. ficus-indica cladodes

Human consumption

In Mexico and some parts of the United States, young fresh cladodes (traditionally called "nopalitos") are widely consumed as vegetables and for many years have been used to prepare a diversity of dishes including sauces, salads, soups, stews, beverages and snacks (Hamdi, 1997). After trimming and chopping, the cladodes may be eaten fresh or cooked. Boiled cladodes have a taste similar to green beans. Nopalitos are very perishable with a storage life of only 1 day at room temperature, but can be preserved for up to 6 days when packed in polythene bags and stored at 5^{o}C (Guevara *et al.*, 2001; Sáenz, 2002). Other products derived from cladodes include jams, chutney, candied nopales, nopalitos in brine and pickled nopalitos (Sáenz, 2000). The importance of "healthy foods" which are low in calories, cholesterol and fat, and high in dietary fibre has not been lost on consumers (Ayadi *et al.*, 2009). Regular consumption of adequate amounts of dietary fibre contributes to the control of blood cholesterol, thereby reducing the risks of degenerative diseases like diabetes and coronary heart disease as well as prevention and control of gastrointestinal disorders associated with low fibre intake (Van Horn, 1997). Cactus cladodes when dried, milled and sifted can be added to wheat flour to make bakery products with a higher fibre content than those made with pure wheat flour (Ayadi *et al.*, 2009; Shedbalkar *et al.*, 2010)

Use as forage

During severe drought periods when grasses are depleted or have become overgrazed, *O. ficus-indica* cladodes are used as emergency feed for livestock in Mexico, Brazil, Chile, USA, Morrocco, Tunisia and South Africa (Russell & Felker, 1987; Stintzing & Carle, 2005). The cladodes provide a considerable amount of the animals' energy and water requirements, although they are low in protein and minerals, requiring supplementation (de Kock, 2002). Addition of straw or alfalfa hay to the cladodes before feeding livestock is recommended to reduce the laxative effect attributed to a high oxalic acid content in cladodes (de Kock, 2002).

Other uses

Prickly pear cactus pads are used as hosts for growing *Dactylopius coccus* Costa, the insect that produces cochineal pigment which is used as a natural colourant in foods, cosmetics and textiles. *O. ficus-indica* cladode powder is sold in capsules to regulate blood sugar or increase the general fibre intake (Stintzing & Carle, 2005). The prickly pear

cladode also has several potential pharmaceutical benefits and is used in traditional medicines for its antihypoglycemic and hypolipidemic, antioxidant, antiulcerogenic and anti-inflammatory properties. For instance, similar to *Aloe vera*, it is used to soothe inflamed insect bites and as a topical application to superficial wounds such as cuts and bruises. Juice from cladodes may also be found in cosmetics. In North Africa the 'spineless cactus–alley cropping system', which involves planting of cactus in the inter row spaces between food crops, is used to limit land degradation and produce inexpensive and drought resistant sources of feed (Alary *et al.*, 2007). Prickly pear cactus plants are also explored for use in the phytoremediation of toxic heavy metals (Figueroa *et al.*, 2007). It is believed that they would be effective pollutant scavengers because of their very shallow and massive roots that spread out widely near to the soil surface. The chances of such "toxic" cacti entering into the food-chain are limited, as grazing animals will generally avoid eating non-processed cacti (Shedbalkar *et al.*, 2010).

3.6 Prospects and challenges facing ethanol production from *O. ficus-indica* cladodes

Like any other potential lignocellulosic biomass feedstock for industrial use, efficient conversion of the polysaccharides in the cladode into monomeric sugars with low by-product formation is a challenge. This procedure would involve pretreatment and enzymatic hydrolysis. One advantage of the cladode is its low lignin content, reported to be about 4% (dry wt) (Stintzing & Carle, 2005) and this could be expected to result in easier hydrolysis of the hemicellulose and cellulose constituents than in biomass with a higher lignin content. The high moisture content in the cladode could result in dilution of its constituent sugars, however. In addition, the mucilage which makes up about 34% (dry wt) of the cladode may reduce the accessibility of the polysaccharides to hydrolysis and reduce overall sugar yields. A likely cost effective way of achieving moisture removal would be sun drying, while depolymerisation of the pectin could be done through the addition of pectinases.

In spite of these challenges, the prickly pear cactus has several other advantages that could benefit its use as a prospective lignocellulosic biomass feedstock for bioethanol production. These include its non-competition for prime agricultural land due to its adaptation to arid and semi-arid environments. A further implication of this is that deforestation would not be required for planting this energy crop and otherwise marginal

land could become useful through the cultivation of *Opuntia* cladodes for ethanol production.

3.7 Conclusions and motivation for research

There are very few reports on the production of ethanol from prickly pear cladodes. In fact, to the best of my knowledge, only one documented report exists in literature (Retamal *et al.*, 1987). The authors attempted fermentation of the fresh cladode, dried material from the cladode as well as the prickly pear fruits. However, they obtained only 14 g ethanol l^{-1} from the dried cladodes using *S. cerevisiae.* Information was not provided to enable the evaluation of aspects such as the ratio of biomass to added water. The low ethanol concentration recorded could be as a result of poor sugar recovery, which may have been a result of using a low solids loading, the authors' approach of carrying out enzymatic hydrolysis before acid pretreatment or the presence of inhibitors in the hydrolysate. A starting point for further research would be to accurately identify and quantify the constituent sugars in the *Opuntia ficus-indica* cladode. Although several authors have published data on its overall composition, few details are given of the individual sugars. Thus, a comprehensive study using standard methods to further elucidate the sugar composition of the prickly pear cladode is required. With these questions raised, it is evident that more research is required to properly establish the potential of *O. ficus-indica* cladodes as a raw material for second generation bioethanol production.

3.8 References

Abdel-Banat, B., Hoshida, H., Ano, A., Nonklang, S. & Akada, R. (2010). High-temperature fermentation: how can processes for ethanol production at high temperatures become superior to the traditional process using mesophilic yeast? *Appl Environ Microbiol* **85**, 861-867.

Alary, V., Nefzaoui, A. & Jemaa, M. B. (2007). Promoting the adoption of natural resource management technology in arid and semi-arid areas: Modelling the impact of spineless cactus in alley cropping in central Tunisia. *Agr Syst* **94**, 573-585.

Amore, R., Wilhelm, M. & Hollenberg, C. P. (1989). The fermentation of xylose - an analysis of the expression of *Bacillus* and *Actinoplanes* xylose isomerase genes in yeast. *Appl Microbiol Biotechnol* **30**, 351-357.

Antoni, D., Zverlov, V. I. & Schwarz, W. (2007). Biofuels from microbes. *Appl Microbiol Biotechnol* **77**, 23-35.

Asghari, A., Bothast, R. J., Doran, J. B. & Ingram, L. O. (1996). Ethanol production from hemicellulose hydrolysates of agricultural residues using genetically engineered *Escherichia coli* strain KO11. *J Ind Microbiol Biotechnol* **16**, 42-47.

Ayadi, M. A., Abdelmaksoud, W., Ennouri, M. & Attia, H. (2009). Cladodes from *Opuntia ficus-indica* as a source of dietary fiber: Effect on dough characteristics and cake making. *Ind Crop and Prod* **30**, 40-47.

Azzam, A. M. (1987). Saccharification of bagasse cellulose pretreated with $ZnCl_2$ and HCl. *Biomass* **12**, 71-77.

Azzam, A. M. (1989). Pretreatment of cane bagasse with alkaline hydrogen peroxide for enzymatic hydrolysis of cellulose and ethanol fermentation. *J Environ Sci Health B* **24**, 421-433.

Balan, V., Bals, B., Chundawat, S. P., Marshall, D. & Dale, B. E. (2009). Lignocellulosic biomass pretreatment using AFEX. *Method Mol Biol* **581**, 61-77.

Balat, M. (2007). An overview of biofuels and policies in the European Union. *Energ Sourc B Energ Econ Plann* **2**, 167-181.

Balat, M. & Balat, H. (2009). Recent trends in global production and utilization of bio-ethanol fuel. *Appl Energ* **86**, 2273-2282.

Ballesteros, M., Oliva, J. M., Negro, M. J., Manzanares, P. & Ballesteros, I. (2004). Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SSF) with *Kluyveromyces marxianus* CECT 10875. *Process Biochem* **39**, 1843-1848.

Banat, I. M. & Marchant, R. (1995). Characterization and potential industrial applications of five novel, thermotolerant, fermentative, yeast strains. *World J Microbiol Biotechnol* **11**, 304-306.

Barnett, J. A. (1976). The utilization of sugars by yeasts. *Adv Carbohydr Chem Biochem* **32**, 125-234.

Becker, J. & Boles, E. (2003). A modified *Saccharomyces cerevisiae* strain that consumes L-arabinose and produces ethanol. *Appl Environ Microbiol* **69**, 4144-4150.

Bellaver, L. H., de Carvalho, N. M. B., Abrahão-Neto, J. & Gombert, A. K. (2004). Ethanol formation and enzyme activities around glucose-6-phosphate in *Kluyveromyces marxianus* CBS 6556 exposed to glucose or lactose excess. *FEMS Yeast Res* **4**, 691-698.

Benhura, M. A. N. & Chidewe, C. (2011). Characterization of the polysaccharide material that is isolated from the fruit of *Cordia abyssinica*. *Afr J Biochem Res* **5**, 95-101.

Bera, A., Sedlak, M., Khan, A. & Ho, N. (2010). Establishment of L-arabinose fermentation in glucose/xylose co-fermenting recombinant *Saccharomyces cerevisiae* 424A(LNH-ST) by genetic engineering. *Appl Microbiol Biotechnol* **87**, 1803-1811.

Bergkamp, R. J. M., Bootsman, T. C., Toschka, H. Y., Mooren, A. T. A., Kox, L., Verbakel, J. M. A., Geerse, R. H. & Planta, R. J. (1993). Expression of an α-galactosidase gene under control of the homologous inulinase promoter in *Kluyveromyces marxianus*. *Appl Microbiol Biotechnol* **40**, 309-317.

Bettiga, M., Bengtsson, O., Hahn-Hagerdal, B. & Gorwa-Grauslund, M. F. (2009). Arabinose and xylose fermentation by recombinant *Saccharomyces cerevisiae* expressing a fungal pentose utilization pathway. *Microb Cell Fact* **8**, 40.

Blanco, P., Sieiro, C. & Villa, T. G. (1999). Production of pectic enzymes in yeasts. *FEMS Microbiol Lett* **175**, 1-9.

Blank, L. M., Lehmbeck, F. & Sauer, U. (2005). Metabolic-flux and network analysis in fourteen hemiascomycetous yeasts. *FEMS Yeast Res* **5**, 545-558.

Bothast, R. J. & Schlicher, M. A. (2005). Biotechnological processes for conversion of corn into ethanol. *Appl Microbiol Biotechnol* **67**, 19-25.

Bothast, R. J., Nichols, N. N. & Dien, B. S. (1999). Fermentations with new recombinant organisms. *Biotechnol Prog* **15**, 867-875.

Bridgwater, A. V., Meier, D. & Radlein, D. (1999). An overview of fast pyrolysis of biomass. *Org Geochem* **30**, 1479-1493.

Bruinenberg, P. M., de Bot, P. H. M., Van Dijken, J. P. & Scheffers, W. A. (1983). The role of redox balances in the anaerobic fermentation of xylose by yeasts. *Appl Microbiol Biotechnol* **18**, 287-292.

Bruinenberg, P. M., Bot, P. H. M., Dijken, J. P. & Scheffers, W. A. (1984). NADH-linked aldose reductase: the key to anaerobic alcoholic fermentation of xylose by yeasts. *Appl Microbiol Biotechnol* **19**, 256-260.

Brutsch, M. O. (1997). Climatic data of selected cactus pear (*Opuntia ficus-indica*) growing areas in South Africa. *Acta Hortic* **438**, 13-20.

Brutsch, M. & Zimmermann, H. (1993). The prickly pear (*Opuntia ficus-indica* [Cactaceae]) in South Africa: Utilization of the naturalized weed, and of the cultivated plants. *Econ Bot* **47**, 154-162.

Caballero, R., Olguín, P., Cruz-Guerrero, A., Gallardo, F., García-Garibay, M. & Gómez-Ruiz, L. (1995). Evaluation of *Kluyveromyces marxianus* as baker's yeast. *Food Res Int* **28**, 37-41.

Cárdenas, A., Goycoolea, F. M. & Rinaudo, M. (2008). On the gelling behaviour of 'nopal' (*Opuntia ficus-indica*) low methoxyl pectin. *Carbohydr Polym* **73**, 212-222.

Cardona, C. A. & Sánchez, O. J. (2007). Fuel ethanol production: Process design trends and integration opportunities. *Bioresour Technol* **98**, 2415-2457.

Carvalho, G. B., Mussatto, S. I., Cândido, E. J. & Almeida e Silva, J. (2006). Comparison of different procedures for the detoxification of eucalyptus hemicellulosic hydrolysate for use in fermentative processes. *J Chem Technol Biotechnol* **81**, 152-157.

Casas, A. & Barbera, G. (2002). Mesoamerican domestication and diffusion. In *Cacti: Biology and Uses*, pp. 143-162. Edited by P. S. Nobel. Berkely: University of California Press.

Castrillo, J. I. & Ugalde, U. O. (1993). Patterns of energy metabolism and growth kinetics of *Kluyveromyces marxianus* in whey chemostat culture. *Appl Microbiol Biotechnol* **40**, 386-393.

Champagne, P. (2007). Feasibility of producing bio-ethanol from waste residues: A Canadian perspective: Feasibility of producing bio-ethanol from waste residues in Canada. *Resour Conserv Recy* **50**, 211-230.

Chang, M. C. (2007). Harnessing energy from plant biomass. *Curr Opin Chem Biol* **11**, 677-684.

Chen, Y., Sharma-Shivappa, R. & Chen, C. (2007). Ensiling agricultural residues for bioethanol production. *Appl Biochem Biotechnol* **143**, 80-92.

Chiang, L. C., Gong, C. S., Chen, L. F. & Tsao, G. T. (1981). D-xylulose fermentation to ethanol by *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **42**, 284-289.

Claassen, P. A. M., van Lier, J. B., Lopez Contreras, A. M., van Niel, E. W. J., Sijtsma, L., Stams, A. J. M., de Vries, S. S. & Weusthuis, R. A. (1999). Utilisation of biomass for the supply of energy carriers. *Appl Microbiol Biotechnol* **52**, 741-755.

Clark, D. P. (1989). The fermentation pathways of *Escherichia coli*. *FEMS Microbiol Lett* **63**, 223-234.

Cruz-Guerrero, A., Garcia-Peña, I., Barzana, E., Garcia-Garibay, M. & Gomez-Ruiz, L. (1995). *Kluyveromyces marxianus* CDBB-L-278: A wild inulinase hyperproducing strain. *J Ferment Bioeng* **80**, 159-163.

de Kock, G. C. (2002). The use of *Opuntia* as a fodder source in arid areas of Southern Africa. In *Cactus (Opuntia spp.) as forage*, pp. 101-106. Edited by C. Mondragón-Jacobo & S. Pérez-González. Rome: FAO.

Deanda, K., Zhang, M., Eddy, C. & Picataggio, S. (1996). Development of an arabinose-fermenting *Zymomonas mobilis* strain by metabolic pathway engineering. *Appl Environ Microbiol* **62**, 4465-4470.

Demirbas, A. (2009). Biofuels securing the planet's future energy needs. *Energ Convers Manage* **50**, 2239-2249.

Demirbas, M. F. & Balat, M. (2006). Recent advances on the production and utilization trends of bio-fuels: A global perspective. *Energ Convers Manage* **47**, 2371-2381.

Dien, B. S., Cotta, M. A. & Jeffries, T. W. (2003). Bacteria engineered for fuel ethanol production: current status. *Appl Microbiol Biotechnol* **63**, 258-266.

Dien, B. S., Kurtzman, C. P., Saha, B. C. & Bothast, R. J. (1996). Screening for L-arabinose fermenting yeasts. *Appl Biochem Biotechnol* **57-58**, 233-242.

Dijken, J. P., Weusthuis, R. A. & Pronk, J. T. (1993). Kinetics of growth and sugar consumption in yeasts. *Antonie van Leeuwenhoek* **63**, 343-352.

Dimitrellou, D., Kandylis, P., Kourkoutas, Y., Koutinas, A. A. & Kanellaki, M. (2009). Evaluation of thermally-dried *Kluyveromyces marxianus* as baker's yeast. *Food Chem* **115**, 691-696.

Doran, J., Cripe, J., Sutton, M. & Foster, B. (2000). Fermentations of pectin-rich biomass with recombinant bacteria to produce fuel ethanol. *Appl Biochem Biotechnol* **84-86**, 141-152.

Doran-Peterson, J., Cook, D. M. & Brandon, S. K. (2008). Microbial conversion of sugars from plant biomass to lactic acid or ethanol. *Plant J* **54**, 582-592.

du Preez, J. C. (1994). Process parameters and environmental factors affecting D-xylose fermentation by yeasts. *Enzyme Microb Technol* **16**, 944-956.

du Preez, J. C., Prior, B. A. & Monteiro, A. M. T. (1984). The effect of aeration on xylose fermentation by *Candida shehatae* and *Pachysolen tannophilus*. *Appl Microbiol Biotechnol* **19**, 261-266.

Duff, S. J. B. & Murray, W. D. (1996). Bioconversion of forest products industry waste cellulosics to fuel ethanol: A review. *Bioresour Technol* **55**, 1-33.

El Kossori, R. L., Villaume, C., El Boustani, E., Sauvaire, Y. & Méjean, L. (1998). Composition of pulp, skin and seeds of prickly pears fruit (*Opuntia-ficus indica* sp.). *Plant Food Hum Nutr* **52**, 263-270.

Eliasson, A., Christensson, C., Wahlbom, C. F. & Hahn-Hagerdal, B. (2000). Anaerobic xylose fermentation by recombinant *Saccharomyces cerevisiae* carrying XYL1, XYL2, and XKS1 in mineral medium chemostat cultures. *Appl Environ Microb* **66**, 3381-3386.

Eliasson, A., Hofmeyr, J. H., Pedler, S. & Hahn-Hägerdal, B. (2001). The xylose reductase/xylitol dehydrogenase/xylulokinase ratio affects product formation in recombinant xylose-utilising *Saccharomyces cerevisiae*. *Enzyme Microb Technol* **29**, 288-297.

Eriksson, T., Börjesson, J. & Tjerneld, F. (2002). Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. *Enzyme Microb Technol* **31**, 353-364.

Esteghlalian, A., Hashimoto, A. G., Fenske, J. J. & Penner, M. H. (1997). Modeling and optimization of the dilute-sulfuric-acid pretreatment of corn stover, poplar and switchgrass. *Bioresour Technol* **59**, 129-136.

Fabre, C. E., Duviau, V. J., Blanc, P. J. & Goma, G. (1995). Identification of volatile flavour compounds obtained in culture of *Kluyveromyces marxianus*. *Biotechnol Lett* **17**, 1207-1212.

Figueroa, J. A. L., Afton, S., Wrobel, K., Wrobel, K. & Caruso, J. A. (2007). Analysis of phytochelatins in nopal (*Opuntia ficus*): a metallomics approach in the soil-plant system. *J Anal At Spectrom* **22**, 897-904.

Fofana, I., Chitiga, M. & Mabugu, R. (2009). Oil prices and the South African economy: A macro-meso-micro analysis. *Energ Policy* **37**, 5509-5518.

Fonseca, C., Romao, R., Rodrigues de Sousa, H., Hahn-Hagerdal, B. & Spencer-Martins, I. (2007a). L-arabinose transport and catabolism in yeast. *FEBS J* **274**, 3589-3600.

Fonseca, G., Heinzle, E., Wittmann, C. & Gombert, A. (2008). The yeast *Kluyveromyces marxianus* and its biotechnological potential. *Appl Microbiol Biotechnol* **79**, 339-354.

Fonseca, G. G., Gombert, A. K., Heinzle, E. & Wittmann, C. (2007b). Physiology of the yeast *Kluyveromyces marxianus* during batch and chemostat cultures with glucose as the sole carbon source. *FEMS Yeast Res* **7**, 422-435.

Frow, E., Ingram, D., Powell, W., Steer, D., Vogel, J. & Yearley, S. (2009). The politics of plants. *Food Sec* **1**, 17-23.

Galbe, M. & Zacchi, G. (2007). Pretreatment of lignocellulosic materials for efficient bioethanol production. *Adv Biochem Eng Biotechnol* **108**, 41-65.

Garcia Sanchez, R., Karhumaa, K., Fonseca, C., Sanchez Nogue, V., Almeida, J., Larsson, C., Bengtsson, O., Bettiga, M., Hahn-Hägerdal, B. & other authors (2010). Improved xylose and arabinose utilization by an industrial recombinant *Saccharomyces cerevisiae* strain using evolutionary engineering. *Biotechnol Biofuels* **3**, 13-24.

Gárdonyi, M. & Hahn-Hägerdal, B. (2003). The *Streptomyces rubiginosus* xylose isomerase is misfolded when expressed in *Saccharomyces cerevisiae*. *Enzyme Microb Technol* **32**, 252-259.

Gough, S., Flynn, O., Hack, C. J. & Marchant, R. (1996). Fermentation of molasses using a thermotolerant yeast, *Kluyveromyces marxianus* IMB3: simplex optimisation of media supplements. *Appl Microbiol Biotechnol* **46**, 187-190.

Goycooleal, F. M. & Cárdenas, A. (2003). Pectins from *Opuntia* spp.: A short review. *J Prof Assoc Cactus* **5**, 17-29.

Groeneveld, P., Stouthamer, A. H. & Westerhoff, H. V. (2009). Super life - how and why 'cell selection' leads to the fastest-growing eukaryote. *FEBS J* **276**, 254-270.

Grohmann, K., Cameron, R. G. & Buslig, B. S. (1995). Fractionation and pretreatment of orange peel by dilute acid hydrolysis. *Bioresour Technol* **54**, 129-141.

Grohmann, K., Manthey, J. A., Cameron, R. G. & Buslig, B. S. (1998). Fermentation of galacturonic acid and pectin-rich materials to ethanol by genetically modified strains of *Erwinia*. *Biotechnol Lett* **20**, 195-200.

Guevara, J. C., Yahia, E. M. & Brito de la Fuente, E. (2001). Modified atmosphere packaging of prickly pear cactus stems (*Opuntia* spp.). *Lebensm Wiss Technol* **34**, 445-451.

Gurrieri, S., Miceli, L., Lanza, C. M., Tomaselli, F., Bonomo, R. P. & Rizzarelli, E. (2000). Chemical characterization of Sicilian prickly pear (*Opuntia ficus-indica*) and perspectives for the storage of Its juice. *J Agric Food Chem* **48**, 5424-5431.

Habibi, Y., Mahrouz, M., Marais, M. F. & Vignon, M. R. (2004). An arabinogalactan from the skin of *Opuntia ficus-indica* prickly pear fruits. *Carbohydr Res* **339**, 1201-1205.

Hack, C. J. & Marchant, R. (1998). Characterisation of a novel thermotolerant yeast, *Kluyveromyces marxianus* var *marxianus*: development of an ethanol fermentation process. *J Ind Microbiol Biotechnol* **20**, 323-327.

Hahn-Hägerdal, B., Galbe, M., Gorwa-Grauslund, M. F., Lidén, G. & Zacchi, G. (2006). Bio-ethanol - the fuel of tomorrow from the residues of today. *Trends Biotechnol* **24**, 549-556.

Hahn-Hägerdal, B., Karhumaa, K., Fonseca, C., Spencer-Martins, I. & Gorwa-Grauslund, M. (2007a). Towards industrial pentose-fermenting yeast strains. *Appl Microbiol Biotechnol* **74**, 937-953.

Hahn-Hägerdal, B., Karhumaa, K., Jeppsson, M. & Gorwa-Grauslund, M. F. (2007b). Metabolic engineering for pentose utilization in *Saccharomyces cerevisiae*. *Adv Biochem Eng Biotechnol* **108**, 147-177.

Hamdi, M. (1997). Prickly pear cladodes and fruits as a potential raw material for the bioindustries. *Bioprocess Biosyst Eng* **17**, 387-391.

Hamelinck, C. N., van Hooijdonk, G. & Faaij, A. P. C. (2005). Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long term. *Biomass Bioenerg* **28**, 384-410.

Harhangi, H. R., Akhmanova, A. S., Emmens, R., van der Drift, C., de Laat, W. T. A. M., van Dijken, J. P., Jetten, M. S. M., Pronk, J. T. & Op den Camp, H. J. M. (2003). Xylose metabolism in the anaerobic fungus *Piromyces* sp. strain E2 follows the bacterial pathway. *Arch Microbiol* **180**, 134-141.

Heer, D. & Sauer, U. (2008). Identification of furfural as a key toxin in lignocellulosic hydrolysates and evolution of a tolerant yeast strain. *Microb Biotechnol* **1**, 497-506.

Hendriks, A. T. W. M. & Zeeman, G. (2009). Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresour Technol* **100**, 10-18.

Hensing, M., Vrouwenvelder, H., Hellinga, C., Baartmans, R. & Dijken, H. (1994). Production of extracellular inulinase in high-cell-density fed-batch cultures of *Kluyveromyces marxianus*. *Appl Microbiol Biotechnol* **42**, 516-521.

Hinman, N., Schell, D., Riley, J., Bergeron, P. & Walter, P. (1992). Preliminary estimate of the cost of ethanol production for SSF technology. *Appl Biochem Biotechnol* **34-35**, 639-649.

Ho, N. W. Y., Rosenfeld, S., Stevis, P. & Tsao, G. T. (1983). Purification and characterization of the D-xylose isomerase gene from *Escherichia coli*. *Enzyme Microb Technol* **5**, 417-420.

Ho, N. W., Chen, Z. & Brainard, A. P. (1998). Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. *Appl Environ Microbiol* **64**, 1852-1859.

Hong, J., Wang, Y., Kumagai, H. & Tamaki, H. (2007). Construction of thermotolerant yeast expressing thermostable cellulase genes. *J Biotechnol* **130**, 114-123.

Hsu, T. A. (1996). Pretreatment of Biomass. In *Handbook on bioethanol: production and utilization*, p. 179. Edited by C. E. Wyman. Washington, DC: Taylor & Francis.

Inglese, P., Basile, F. & Schirra, M. (2002). Cactus pear fruit production. In *Cacti: Biology and Uses*, pp. 163-184. Edited by P. S. Nobel. Berkely: University of California Press.

Ingram, L. O., Conway, T., Clark, D. P., Sewell, G. W. & Preston, J. F. (1987). Genetic engineering of ethanol production in *Escherichia coli*. *Appl Environ Microbiol* **53**, 2420-2425.

Jakobsen, M. & Narvhus, J. (1996). Yeasts and their possible beneficial and negative effects on the quality of dairy products. *Int Dairy J* **6**, 755-768.

Jeffries, T. W. & Jin, Y. S. (2004). Metabolic engineering for improved fermentation of pentoses by yeasts. *Appl Microbiol Biotechnol* **63**, 495-509.

Jeffries, T. W. (2006). Engineering yeasts for xylose metabolism. *Curr Opin Biotechnol* **17**, 320-326.

Jeppsson, M., Bengtsson, O., Franke, K., Lee, H., Hahn-Hägerdal, B. & Gorwa-Grauslund, M. F. (2006). The expression of a *Pichia stipitis* xylose reductase mutant with higher KM for NADPH increases ethanol production from xylose in recombinant *Saccharomyces cerevisiae*. *Biotechnol Bioeng* **93**, 665-673.

Joachimsthal, E. & Rogers, P. (2000). Characterization of a high-productivity recombinant strain of *Zymomonas mobilis* for ethanol production from glucose/xylose mixtures. *Appl Biochem Biotechnol* **84-86**, 343-356.

Kádár, Z., Szengyel, Z. & Réczey, K. (2004). Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. *Ind Crops Prod* **20**, 103-110.

Karhumaa, K., Wiedemann, B., Hahn-Hagerdal, B., Boles, E. & Gorwa-Grauslund, M. F. (2006). Co-utilization of L-arabinose and D-xylose by laboratory and industrial *Saccharomyces cerevisiae* strains. *Microb Cell Fact* **5**, 18.

Karhumaa, K., Fromanger, R., Hahn-Hägerdal, B. & Gorwa-Grauslund, M. F. (2007). High activity of xylose reductase and xylitol dehydrogenase improves xylose fermentation by recombinant *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* **73**, 1039-1046.

Karhumaa, K., Hahn-Hägerdal, B. & Gorwa-Grauslund, M. F. (2005). Investigation of limiting metabolic steps in the utilization of xylose by recombinant *Saccharomyces cerevisiae* using metabolic engineering. *Yeast* **22**, 359-368.

Kern, A., Tilley, E., Hunter, I. S., Legisa, M. & Glieder, A. (2007). Engineering primary metabolic pathways of industrial micro-organisms. *J Biotechnol* **129**, 6-29.

Kim, H., Kim, S. & Kim, C. (2007a). The effects of nonionic surfactants on the pretreatment and enzymatic hydrolysis of recycled newspaper. *Biotechnol Bioprocess Eng* **12**, 147-151.

Kim, Y., Ingram, L. O. & Shanmugam, K. T. (2007b). Construction of an *Escherichia coli* K-12 mutant for homoethanologenic fermentation of glucose or xylose without foreign genes. *Appl Environ Microbiol*.

Klinke, H. B., Thomsen, A. B. & Ahring, B. K. (2004). Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. *Appl Microbiol Biotechnol* **66**, 10-26.

Kötter, P., Amore, R., Hollenberg, C. P. & Ciriacy, M. (1990). Isolation and characterization of the *Pichia stipitis* xylitol dehydrogenase gene *XYL2*, and construction of a xylose-utilizing *Saccharomyces cerevisiae* transformant. *Curr Genet* **18**, 493-500.

Kötter, P. & Ciriacy, M. (1993). Xylose fermentation by *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* **38**, 776-783.

Kristensen, J. B., Börjesson, J., Bruun, M. H., Tjerneld, F. & Jørgensen, H. (2007). Use of surface active additives in enzymatic hydrolysis of wheat straw lignocellulose. *Enzyme Microb Technol* **40**, 888-895.

Kumar, P., Barrett, D. M., Delwiche, M. J. & Stroeve, P. (2009). Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Ind Eng Chem Res* **48**, 3713-3729.

Kumar, R., Singh, S. & Singh, O. (2008). Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. *J Ind Microbiol Biotechnol* **35**, 377-391.

Kumar, R. & Wyman, C. E. (2009). Effect of additives on the digestibility of corn stover solids following pretreatment by leading technologies. *Biotechnol Bioeng* **102**, 1544-1557.

Kurtzman, C. P. & Dien, B. S. (1998). *Candida arabinofermentans*, a new L-arabinose fermenting yeast. *Antonie van Leeuwenhoek* **74**, 237-243.

Kushi, R. T., Monti, R. & Contiero, J. (2000). Production, purification and characterization of an extracellular inulinase from *Kluyveromyces marxianus* var. *bulgaricus*. *J Ind Microbiol Biotechnol* **25**, 63-69.

Kuyper, M., Harhangi, H. R., Stave, A. K., Winkler, A. A., Jetten, M. S. M., Laat, W. T. A. M., Ridder, J. J. J., Op den Camp, H. J. M., Van Dijken, J. P. & other authors (2003). High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? *FEMS Yeast Res* **4**, 69-78.

Kuyper, M., Hartog, M. M. P., Toirkens, M. J., Almering, M. J. H., Winkler, A. A., Van Dijken, J. P. & Pronk, J. T. (2005a). Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. *FEMS Yeast Res* **5**, 399-409.

Kuyper, M., Toirkens, M. J., Diderich, J. A., Winkler, A. A., Van Dijken, J. P. & Pronk, J. T. (2005b). Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. *FEMS Yeast Res* **5**, 925-934.

Kuyper, M., Winkler, A. A., Dijken, J. P. & Pronk, J. T. (2004). Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle. *FEMS Yeast Res* **4**, 655-664.

Lachance, M. A. (1998). *Kluyveromyces* van der Walt emend. van der Walt. In *The Yeasts, A Taxonomic Study*, 4 edn, pp. 227-247. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.

Lachance, M.-A. (1995). Yeast communities in a natural tequila fermentation. *Antonie van Leeuwenhoek* **68**, 151-160.

Lane, M. M. & Morrissey, J. P. (2010). *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. *Fungal Biol Rev* **24**, 17-26.

Lawford, H. & Rousseau, J. (2002). Performance testing of *Zymomonas mobilis* metabolically engineered for cofermentation of glucose, xylose, and arabinose. *Appl Biochem Biotechnol* **98-100**, 429-448.

Lee, J. W., Gwak, K. S., Park, J. Y., Choi, D. H., Kwon, M. & Choi., I.-G. (2007). Biological pretreatment of softwood *Pinus densiflora* by three white-rot fungi. *J Microbiol* **45**, 485-491.

Lee, K. J., Tribe, D. E. & Rogers, P. L. (1979). Ethanol production by *Zymomonas mobilis* in continuous culture at high glucose concentrations. *Biotechnol Lett* **1**, 421-426.

Limtong, S., Sringiew, C. & Yongmanitchai, W. (2007). Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. *Bioresour Technol* **98**, 3367-3374.

Lin, Y. & Tanaka, S. (2006). Ethanol fermentation from biomass resources: current state and prospects. *Appl Microbiol Biotechnol* **69**, 627-642.

Liu, Z. (2006). Genomic adaptation of ethanologenic yeast to biomass conversion inhibitors. *Appl Microbiol Biotechnol* **73**, 27-36.

Llorente, B., Malpertuy, A., Blandin, G., Artiguenave, F., Wincker, P. & Dujon, B. (2000). Genomic exploration of the hemiascomycetous Yeasts: 12. *Kluyveromyces marxianus* var. *marxianus*. *FEBS Lett* **487**, 71-75.

Lynd, L., Elamder, R. & Wyman, C. (1996). Likely features and costs of mature biomass ethanol technology. *Appl Biochem Biotechnol* **57-58**, 741-761.

Lynd, L., Weimer, P., van Zyl, W. & Pretorius, I. (2002). Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiol Mol Biol Rev* **66**, 739.

Madhavan, A., Tamalampudi, S., Ushida, K., Kanai, D., Katahira, S., Srivastava, A., Fukuda, H., Bisaria, V. & Kondo, A. (2009). Xylose isomerase from polycentric fungus *Orpinomyces*: gene sequencing, cloning, and expression in *Saccharomyces cerevisiae* for bioconversion of xylose to ethanol. *Appl Microbiol Biotechnol* **82**, 1067-1078.

Majdoub, H., Roudesli, S. & Derantani, A. (2001). Polysaccharides from prickly pear peel and nopals of *Opuntia ficus-indica*: extraction, characterization and polyelectrolyte behaviour. *Polym Int* **50**, 552-560.

Matsuhiro, B., Lillo, L. E., Sáenz, C., Urzúa, C. C. & Zárate, O. (2006). Chemical characterization of the mucilage from fruits of *Opuntia ficus indica*. *Carbohydr Polym* **63**, 263-267.

McGarvie, D. & Parolis, H. (1979a). The mucilage of *Opuntia ficus-indica*. *Carbohydr Res* **69**, 171-179.

McGarvie, D. & Parolis, H. (1979b). The mucilage of *Opuntia ficus-indica*. Part 2.I The degraded polysaccharide. *J Chem Soc , Perkin Trans 1*, 1464-1466.

McMillan, J. D. (1994). Pretreatment of Lignocellulosic Biomass. In *Enzymatic Conversion of Biomass for Fuels Production*, pp. 292-324. Edited by M. E. Himmel, J. O. Baker & R. P. Overend. Washington, DC: American Chemical Society.

McMillan, J. & Boynton, B. (1994). Arabinose utilization by xylose-fermenting yeasts and fungi. *Appl Biochem Biotechnol* **45-46**, 569-584.

Medeiros, A. B. P., Pandey, A., Freitas, R. J. S., Christen, P. & Soccol, C. R. (2000). Optimization of the production of aroma compounds by *Kluyveromyces marxianus* in solid-state fermentation using factorial design and response surface methodology. *Biochem Eng J* **6**, 33-39.

Medina-Torres, L., Brito-De La Fuente, E., Torrestiana-Sanchez, B. & Katthain, R. (2000). Rheological properties of the mucilage gum (*Opuntia ficus-indica*). *Food Hydrocolloid* **14**, 417-424.

Middleton, K. & Beinart, W. (2005). Cactus pear as dryland fodder: Ambovombe farm, Madagascar and Wellwood farm, South Africa compared, c.1920–1950. *J Agrar Change* **5**, 251-280.

Modig, T., Lidén, G. & Taherzadeh, M. J. (2002). Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. *Biochem J* **363**, 769-776.

Moes, C. J., Pretorius, I. S. & Zyl, W. H. (1996). Cloning and expression of the *Clostridium thermosulfurogenes* D-xylose isomerase gene (*xyLA*;) in *Saccharomyces cerevisiae*. *Biotechnol Lett* **18**, 269-274.

Mohagheghi, A., Evans, K., Chou, Y.-C. & Zhang, M. (2002). Cofermentation of glucose, xylose,and arabinose by genomic DNA-integrated xylose/arabinose fermenting strain of *Zymomonas mobilis* AX101. *Appl Biochem Biotechnol* **98**, 885-898.

Moniruzzaman, M. & Ingram, L. O. (1998). Ethanol production from dilute acid hydrolysate of rice hulls using genetically engineered *Escherichia coli*. *Biotechnol Lett* **20**, 943-947.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M. & Ladisch, M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour Technol* **96**, 673-686.

Murray, W. D. (1987). Effects of cellobiose and glucose on cellulose hydrolysis by both growing and resting cells of *Bacteroides cellulosolvens*. *Biotechnol Bioeng* **29**, 1151-1154.

Nigam, P., Banat, I. M., Singh, D., McHale, A. P. & Marchant, R. (1997). Continuous ethanol production by thermotolerant *Kluyveromyces marxianus* IMB3 immobilized on mineral Kissiris at 45^{o}C. *World J Microbiol Biotechnol* **13**, 283-288.

Nobel, P. S. (2002). Ecophysiology of *Opuntia ficus-indica*. In *Cactus (Opuntia spp.) as forage*, pp. 13-20. Edited by C. Mondragón-Jacobo & S. Pérez-González. Rome: FAO.

Nonklang, S., Abdel-Banat, B. M. A., Cha-aim, K., Moonjai, N., Hoshida, H., Limtong, S., Yamada, M. & Akada, R. (2008). High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042. *Appl Environ Microbiol* **74**, 7514-7521.

Nonklang, S., Ano, A., Abdel-Banat, B. M. A., SAITO, Y., Hoshida, H. & Akada, R. (2009). Construction of flocculent *Kluyveromyces marxianus* strains suitable for high-temperature ethanol fermentation. *Biosci Biotechnol Biochem* **73**, 1090-1095.

Oelofse, R. M., Labuschagne, M. T. & Potgieter, J. P. (2006). Plant and fruit characteristics of cactus pear (*Opuntia* spp.) cultivars in South Africa. *J Sci Food Agric* **86**, 1921-1925.

Öhgren, K., Bengtsson, O., Gorwa-Grauslund, M. F., Galbe, M., Hahn-Hägerdal, B. & Zacchi, G. (2006). Simultaneous saccharification and co-fermentation of glucose and xylose in steam-pretreated corn stover at high fiber content with *Saccharomyces cerevisiae* TMB3400. *J Biotechnol* **126**, 488-498.

Öhgren, K., Bura, R., Lesnicki, G., Saddler, J. & Zacchi, G. (2007). A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. *Process Biochem* **42**, 834-839.

Ohta, K., Beall, D. S., Mejia, J. P., Shanmugam, K. T. & Ingram, L. O. (1991). Genetic improvement of *Escherichia coli* for ethanol production: chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. *Appl Environ Microbiol* **57**, 893-900.

Olsson, L. & Hahn-Hägerdal, B. (1996). Fermentation of lignocellulosic hydrolysates for ethanol production. *Enzyme Microb Technol* **18**, 312-331.

Palmqvist, E. & Hahn-Hägerdal, B. (2000a). Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. *Bioresour Technol* **74**, 17-24.

Palmqvist, E. & Hahn-Hägerdal, B. (2000b). Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresour Technol* **74**, 25-33.

Panesar, P. S., Marwaha, S. S. & Kennedy, J. F. (2006). *Zymomonas mobilis*: an alternative ethanol producer. *J Chem Technol Biotechnol* **81**, 623-635.

Paredes-López, O., Camargo-Rubio, E. & Ornelas Vale, A. (1976). Influence of specific growth rate on biomass yield, productivity and composition of *Candida utilis* in batch and continuous culture. *Appl Environ Microbiol* **31**, 487-491.

Pauly, M. & Keegstra, K. (2008). Cell-wall carbohydrates and their modification as a resource for biofuels. *Plant J* **54**, 559-568.

Pecota, D. C., Rajgarhia, V. & Da Silva, N. A. (2007). Sequential gene integration for the engineering of *Kluyveromyces marxianus*. *J Biotechnol* **127**, 408-416.

Pérez, J., Muñoz-Dorado, J., de la Rubia, T. & Martínez, T. (2002). Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *Int Microbiol* **5**, 53-63.

Philippidis, G. & Smith, T. (1995). Limiting factors in the simultaneous saccharification and fermentation process for conversion of cellulosic biomass to fuel ethanol. *Appl Biochem Biotechnol* **51-52**, 117-124.

Piskur, J., Rozpedowska, E., Polakova, S., Merico, A. & Compagno, C. (2006). How did *Saccharomyces* evolve to become a good brewer? *Trends Genet* **22**, 183-186.

Prasad, S., Singh, A. & Joshi, H. C. (2007). Ethanol as an alternative fuel from agricultural, industrial and urban residues. *Resour Conserv Recy* **50**, 1-39.

Purwadi, R., Brandberg, T. & Taherzadeh, M. J. (2007). A possible industrial solution to ferment lignocellulosic hydrolyzate to ethanol: continuous cultivation with flocculating yeast. *Int J Mol Sci* **8**, 920-932.

Qi, B., Chen, X. & Wan, Y. (2010). Pretreatment of wheat straw by nonionic surfactant-assisted dilute acid for enhancing enzymatic hydrolysis and ethanol production. *Bioresour Technol* **101**, 4875-4883.

Qing, Q., Yang, B. & Wyman, C. E. (2010). Impact of surfactants on pretreatment of corn stover. *Bioresour Technol* **101**, 5941-5951.

Ragauskas, A. J., Williams, C. K., Davison, B. H., Britovsek, G., Cairney, J., Eckert, C. A., Frederick, W. J., Jr., Hallett, J. P., Leak, D. J. & other authors (2006). The path forward for biofuels and biomaterials. *Science* **311**, 484-489.

Rao, K., Chaudhari, V., Varanasi, S. & Kim, D. S. (2007). Enhanced ethanol fermentation of brewery wastewater using the genetically modified strain *E. coli* KO11. *Appl Microbiol Biotechnol* **74**, 50-60.

Reijnders, L. (2009). Acute View Transport biofuels: Can they help limiting climate change without an upward impact on food prices? *J Vebr Lebensm* **4**, 75-78.

Retamal, N., Duran, J. M. & Fernandez, J. (1987). Ethanol production by fermentation of fruits and cladodes of prickly pear cactus (*Opuntia ficus-indica* (L.) Miller). *J Sci Food Agric* **40**, 213-218.

Ribeiro, O., Gombert, A. K., Teixeira, J. A. & Domingues, L. (2007). Application of the Cre-loxP system for multiple gene disruption in the yeast *Kluyveromyces marxianus*. *J Biotechnol* **131**, 20-26.

Richard, P., Toivari, M. H. & Penttilä, M. (1999). Evidence that the gene YLR070c of *Saccharomyces cerevisiae* encodes a xylitol dehydrogenase. *FEBS Lett* **457**, 135-138.

Richard, P., Verho, R., Putkonen, M., Londesborough, J. & Penttila, M. (2003). Production of ethanol from L-arabinose by *Saccharomyces cerevisiae* containing a fungal L-arabinose pathway. *FEMS Yeast Res* **3**, 185-189.

Ridley, B. L., O'Neill, M. A. & Mohnen, D. (2001). Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* **57**, 929-967.

Rocha, S., Abrahão-Neto, J., Cerdán, M., González-Siso, M. & Gombert, A. (2010). Heterologous expression of glucose oxidase in the yeast *Kluyveromyces marxianus*. *Microb Cell Fact* **9**, 4.

Rogers, P. L., Lee, K. J. & Tribe, D. E. (1979). Kinetics of alcohol production by *Zymomonas mobilis* at high sugar concentrations. *Biotechnol Lett* **1**, 165-170.

Rohm, H., Eliskases-Lechner, F. & Bräuer, M. (1992). Diversity of yeasts in selected dairy products. *J Appl Microbiol* **72**, 370-376.

Russell, C. & Felker, P. (1987). The prickly-pears (*Opuntia* spp., Cactaceae): A source of human and animal food in semiarid regions. *Econ Bot* **41**, 433-445.

Sáenz, C. (2000). Processing technologies: an alternative for cactus pear (*Opuntia* spp.) fruits and cladodes. *J Arid Environ* **46**, 209-225.

Sáenz, C. (2002). Cactus pear fruits and cladodes: a source of functional components for foods. *Acta Hortic* **581**, 253-263.

Sáenz, C., Sepúlveda, E. & Matsuhiro, B. (2004). *Opuntia* spp mucilage's: a functional component with industrial perspectives. *J Arid Environ* **57**, 275-290.

Saha, B. C. & Cotta, M. A. (2010). Comparison of pretreatment strategies for enzymatic saccharification and fermentation of barley straw to ethanol. *N Biotechnol* **27**, 10-16.

Sánchez, O. J. & Cardona, C. A. (2008). Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresour Technol* **99**, 5270-5295.

Sauer, U. (2001). Evolutionary engineering for industrially important microbial phenotypes. *Adv Biochem Eng Biotechnol* **73**, 129-169.

Schultz, N., Chang, L., Hauck, A., Reuss, M. & Syldatk, C. (2006). Microbial production of single-cell protein from deproteinized whey concentrates. *Appl Microbiol Biotechnol* **69**, 515-520.

Sedlak, M. & Ho, N. W. Y. (2001). Expression of *E. coli* araBAD operon encoding enzymes for metabolizing L-arabinose in *Saccharomyces cerevisiae*. *Enzyme Microb Technol* **28**, 16-24.

Senac, T. & Hahn-Hägerdal, B. (1990). Intermediary metabolite concentrations in xylulose- and glucose-fermenting *Saccharomyces cerevisiae* cells. *Appl Environ Microbiol* **56**, 120-126.

Senthilkumar, V. & Gunasekaran, P. (2005). Bioethanol production from cellulosic substrates: Engineered bacteria and process integration challenges. *J Sci Ind Res* **64**, 845-853.

Sepúlveda, E., Sáenz, C., Aliaga, E. & Aceituno, C. (2007). Extraction and characterization of mucilage in *Opuntia* spp. *J Arid Environ* **68**, 534-545.

Shedbalkar, U., Adki, V., Jadhav, J. & Bapat, V. (2010). *Opuntia* and other cacti: applications and biotechnological insights. *Trop Plant Biol* **3**, 136-150.

Siekstel, R., Bartkevicitute, D. & Sasnauskas, K. (1999). Cloning, targeted disruption and heterologous expression of the *Kluyveromyces marxianus* endopolygalacturonase gene (EPG1). *Yeast* **15**, 311-322.

Silverstein, R. A., Chen, Y., Sharma-Shivappa, R. R., Boyette, M. D. & Osborne, J. (2007). A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. *Bioresour Technol* **98**, 3000-3011.

Singh, D., Banat, I. M., Nigam, P. & Marchant, R. (1998). Industrial scale ethanol production using the thermotolerant yeast *Kluyveromyces marxianus* IMB3 in an Indian distillery. *Biotechnol Lett* **20**, 753-755.

Skeer, J. & Wang, Y. (2007). China on the move: Oil price explosion? *Energ Policy* **35**, 678-691.

Sommer, P., Georgieva, T. & Ahring, B. K. (2004). Potential for using thermophilic anaerobic bacteria for bioethanol production from hemicellulose. *Biochem Soc Trans* **32**, 283-289.

Sonderegger, M. & Sauer, U. (2003). Evolutionary engineering of *Saccharomyces cerevisiae* for anaerobic growth on xylose. *Appl Environ Microbiol* **69**, 1990-1998.

Sprenger, G. A. (1996). Carbohydrate metabolism in *Zymomonas mobilis*: a catabolic highway with some scenic routes. *FEMS Microbiol Lett* **145**, 301-307.

Stintzing, F. C. & Carle, R. (2005). Cactus stems (*Opuntia* spp.): A review on their chemistry, technology, and uses. *Mol Nutr Food Res* **49**, 175-194.

Sudzuki-Hills, F. (1995). Anatomy and morphology. In *Agro-ecology, cultivation and uses of cactus pear*, 1 edn, pp. 28-35. Edited by G. Barbera, P. Inglese & E. Pimienta. Rome: FAO.

Sun, Y. & Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresour Technol* **83**, 1-11.

Taherzadeh, M. J., Gustafsson, L., Niklasson, C. & Lidén, G. (2000). Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* **53**, 701-708.

Taherzadeh, M. J., Eklund, R., Gustafsson, L., Niklasson, C. & Liden, G. (1997). Characterization and fermentation of dilute-acid hydrolyzates from wood. *Ind Eng Chem Res* **36**, 4659-4665.

Taherzadeh, M. J. & Karimi, K. (2008). Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. *Int J Mol Sci* **9**, 1621-1651.

Tantirungkij, M., Nakashima, N., Seki, T. & Yoshida, T. (1993). Construction of xylose-assimilating *Saccharomyces cerevisiae*. *J Ferment Bioeng* **75**, 83-88.

Tengborg, C., Galbe, M. & Zacchi, G. (2001). Reduced inhibition of enzymatic hydrolysis of steam-pretreated softwood. *Enzyme Microb Technol* **28**, 835-844.

Toivari, M. H., Aristidou, A., Ruohonen, L. & Penttilä, M. (2001). Conversion of xylose to ethanol by recombinant *Saccharomyces cerevisiae*: importance of xylulokinase (XKS1) and oxygen availability. *Metab Eng* **3**, 236-249.

Toivari, M. H., Salusjarvi, L., Ruohonen, L. & Penttila, M. (2004). Endogenous xylose pathway in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **70**, 3681-3686.

Turker, N., Coskuner, Y., Ekiz, H. I., Aksay, S. & Karababa, E. (2001). The effects of fermentation on the thermostability of the yellow-orange pigments extracted from cactus pear (*Opuntia ficus-indica*). *Eur Food Res Technol* **212**, 213-216.

Van Horn, L. (1997). Fiber, lipids, and coronary heart disease : a statement for healthcare professionals from the nutrition committee, American heart association. *Circulation* **95**, 2701-2704.

van Maris, A., Abbott, D., Bellissimi, E., van den Brink, J., Kuyper, M., Luttik, M., Wisselink, H., Scheffers, W., van Dijken, J. & other authors (2006). Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. *Antonie van Leeuwenhoek* **90**, 391-418.

Van Urk, H., Voll, W. S. L., Scheffers, W. A. & van Dijken, J. P. (1990). Transient-state analysis of metabolic fluxes in Crabtree-positive and Crabtree-negative yeasts. *Appl Environ Microbiol* **56**, 281-287.

Vanderschuren, M. J. W. A., Lane, T. E. & Wakeford, J. J. (2010). Can the South African transport system surmount reduced crude oil availability? *Energ Policy* **38**, 6092-6100.

Verduyn, C., Postma, E., Scheffers, W. A. & van Dijken, J. P. (1992). Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* **8**, 501-517.

Verho, R., Londesborough, J., Penttila, M. & Richard, P. (2003). Engineering redox cofactor regeneration for improved pentose fermentation in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **69**, 5892-5897.

Verho, R., Putkonen, M., Londesborough, J., Penttilä, M. & Richard, P. (2004). A novel NADH-linked L-xylulose reductase in the L-arabinose catabolic pathway of yeast. *J Biol Chem* **279**, 14746-14751.

Wackett, L. P. (2008). Microbial-based motor fuels: science and technology. *Microb Biotechnol* **1**, 211-225.

Wahlbom, C. F., van Zyl, W. H., Jönsson, L. J., Hahn-Hägerdal, B. & Otero, R. R. (2003). Generation of the improved recombinant xylose-utilizing *Saccharomyces cerevisiae* TMB 3400 by random mutagenesis and physiological comparison with *Pichia stipitis* CBS 6054. *FEMS Yeast Res* **3**, 319-326.

Walfridsson, M., Bao, X., Anderlund, M., Lilius, G., Bulow, L. & Hahn-Hägerdal, B. (1996). Ethanolic fermentation of xylose with *Saccharomyces cerevisiae* harboring the *Thermus thermophilus* xylA gene, which expresses an active xylose (glucose) isomerase. *Appl Environ Microbiol* **62**, 4648-4651.

Wang, Y., Shi, W. L., Liu, X. Y., Shen, Y., Bao, X. M., Bai, F. W. & Qu, Y. B. (2004). Establishment of a xylose metabolic pathway in an industrial strain of *Saccharomyces cerevisiae*. *Biotechnol Lett* **26**, 885-890.

Watanabe, S., Abu Saleh, A., Pack, S. P., Annaluru, N., Kodaki, T. & Makino, K. (2007). Ethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing protein-engineered NADH-preferring xylose reductase from *Pichia stipitis*. *Microbiology* **153**, 3044-3054.

Wilkie, A. C., Riedesel, K. J. & Owens, J. M. (2000). Stillage characterization and anaerobic treatment of ethanol stillage from conventional and cellulosic feedstocks. *Biomass Bioenerg* **19**, 63-102.

Wilkins, M. R., Widmer, W. W., Grohmann, K. & Cameron, R. G. (2007). Hydrolysis of grapefruit peel waste with cellulase and pectinase enzymes. *Bioresour Technol* **98**, 1596-1601.

Wisselink, H. W., Toirkens, M. J., Rosario Franco Berriel, M., Winkler, A. A., Van Dijken, J. P., Pronk, J. T. & van Maris, A. J. A. (2007). Engineering of *Saccharomyces cerevisiae* for efficient anaerobic alcoholic fermentation of L-arabinose. *Appl Environ Microbiol* **73**, 4881-4891.

Wisselink, H. W., Toirkens, M. J., Wu, Q., Pronk, J. T. & van Maris, A. J. A. (2009). Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains. *Appl Environ Microbiol* **75**, 907-914.

Xiao, W. & Clarkson, W. W. (1997). Acid solubilization of lignin and bioconversion of treated newsprint to methane. *Biodegradation* **8**, 61-66.

Yamashita, Y., Sasaki, C. & Nakamura, Y. (2010). Effective enzyme saccharification and ethanol production from Japanese cedar using various pretreatment methods. *J Biosci Bioeng* **110**, 79-86.

Yang, S., Land, M. L., Klingeman, D. M., Pelletier, D. A., Lu, T. Y., Martin, S. L., Guo, H. B., Smith, J. C. & Brown, S. D. (2010). Paradigm for industrial strain improvement identifies sodium acetate tolerance loci in *Zymomonas mobilis* and *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* **107**, 10395-10400.

Zaldivar, J., Nielsen, J. & Olsson, L. (2001). Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. *Appl Microbiol Biotechnol* **56**, 17-34.

Zhang, J. & Lynd, L. R. (2010). Ethanol production from paper sludge by simultaneous saccharification and co-fermentation using recombinant xylose-fermenting microorganisms. *Biotechnol Bioeng* **107**, 235-244.

Zhang, M., Eddy, C., Deanda, K., Finkelstein, M. & Picataggio, S. (1995). Metabolic engineering of a pentose metabolism pathway in ethanologenic *Zymomonas mobilis*. *Science* **267**, 240-243.

Zhao, X., Cheng, K. & Liu, D. (2009). Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. *Appl Microbiol Biotechnol* **82**, 815-827.

CHAPTER 2

CHEMICAL COMPOSITION, PRETREATMENT AND ENZYMATIC HYDROLYSIS OF *OPUNTIA FICUS-INDICA* CLADODE BIOMASS

CONTENTS

1. Abstract

The cladodes of *Opuntia ficus-indica* (prickly pear cactus) are a potential biomass feedstock for bioethanol production, especially in arid and semi arid regions. Compositional analysis indicated that it had low lignin content of 8%. The content of readily fermentable carbohydrates in the cladode (34.3%) was also comparable to other conventional lignocellulosic biomass sources such as sugar cane bagasse and corn stover. This study focussed on the production of a fermentable hydrolysate from the cladode using dilute acid pretreatment and enzymatic hydrolysis. A statistical design experimental approach was applied to investigate the influence of acid concentration and contact time on the enzymatic digestibility of dried and milled *O. ficus-indica* cladode. Enzymatic hydrolysis experiments were also performed with varied enzyme loadings of cellulase and β-glucosidase with or without the addition of pectinase. The optimum conditions were pretreatment with 1.5% (w/w) sulphuric acid at 120^{o}C for 50 min at a substrate loading of 30% (w/v) followed by hydrolysis with 15 FPU cellulase, 15 IU β-glucosidase and 100 IU pectinase per gram of dry biomass. These parameters yielded an *O. ficus-indica* hydrolysate containing (per litre) 45.5 g glucose, 6.3 g xylose, 9.1 g galactose, 10.8 g arabinose and 9.6 g fructose. The galacturonic acid content in the hydrolysate was not quantified.

2. Introduction

Biofuels such as bioethanol produced from sugar-based and starch-based crops, as well as from lignocellulosic biomass, are regarded as a potential replacement to some of the liquid fossil fuels used in transportation (Öhgren *et al.*, 2007). Bioethanol also provides an opportunity for non-oil producing countries to be less dependent on foreign imports. The use of lignocellulosic ethanol (i.e. ethanol from lignocellulosic biomass) is, however, desirable over the use of traditional sugar or starch derived bioethanol due to the abundance of lignocellulose and non-competition for food and feed production. Moreover, the use of lignocellulose-derived ethanol diminishes CO_2 emissions compared to fossil fuels. There is much interest in the utilization of easily available, inexpensive and renewable lignocellulosic biomass for the production of bioethanol that does not compete for agricultural land (Ragauskas *et al.*, 2006). South Africa, for example, is a relatively dry country with a mean annual rainfall of less than 500 mm and with the vast areas of

marginal land available in semi-arid areas, it would be advantageous to make the land more productive through the cultivation of energy crops.

One such plant is *Opuntia ficus-indica*, also known as the prickly pear cactus. *O. ficus-indica* produces a high yield of biomass, has a high efficiency of water use and is highly drought resistant; thus it usually requires little or no irrigation (Inglese *et al.*, 2002).

The main steps involved in the bioconversion of lignocellulosic biomass are pretreatment, enzymatic hydrolysis, fermentation and distillation. Pretreatment accounts for the major part of the overall production costs and effective pretreatment could significantly reduce operational costs (Ragauskas *et al.*, 2006; Yang & Wyman, 2008). Among different pretreatment methods investigated, chemical pretreatment through the use of dilute acid is one of the most extensively studied and commonly used method (Mosier *et al.*, 2005b; Sun & Cheng, 2002). Dilute acid solubilises the hemicellulose, alters the lignocellulose structure and improves its accessibility to enzymes, which in turn increases the yield of fermentable sugars. Cellulolytic enzymes such as cellulase and β-glucosidase hydrolyse cellulose and cellobiose into glucose. In pectin rich biomass, pectinase solubilises the pectin, which in turn improves cellulose accessibility and overall sugar yield, when used in conjunction with cellulase and β-glucosidase (Grohmann *et al.*, 1995).

However, even before any form of bioconversion of lignocellulose can occur, it is important to understand the nature and composition of the potential biomass feedstock with emphasis on its constituent carbohydrate polymers and lignin. This information would provide insight on how to adapt pretreatment and enzymatic hydrolysis technologies to suit the biomass. In the case of the *O. ficus-indica* cladode, there is ample information in literature on its general composition but very little regarding its constituent sugars. Several authors reported its chemical composition in the form of crude fibre, total fibre and total polysaccharides (Ginestra *et al.*, 2009; Majdoub *et al.*, 2001; Malainine *et al.*, 2003; Stintzing & Carle, 2005).

Consequently, the first aim of this study was to determine the composition of the *O. ficus-indica* cladode by identifying its carbohydrates and their respective amounts in the cladode. The influence of pretreatment parameters (dilute acid concentration and contact time) on the enzymatic digestibility of the biomass was investigated to obtain optimum pretreatment conditions. Steam pretreatment or AFEX were the preferred options for pretreatment, but dilute acid was used instead due to technical constraints. Enzymatic hydrolysis with different enzyme combinations was also investigated to further improve

overall sugar yield. The optimum conditions were finally applied to produce a fermentable hydrolysate for the purpose of investigating bioethanol production.

3. Materials and Methods

Raw material

Fresh cladodes of *Opuntia ficus-indica* (cultivar “Algerian”) were harvested from a prickly pear cactus farm outside Bloemfontein, South Africa. The cladodes were cut into strips using a mechanical shredder and sun-dried, after which they were further processed by hammer milling to a particle size of 1 mm (Figure 2.1). The dried and milled cladode with a dry matter content of 96.3 ± 0.1% was thoroughly mixed to ensure representative samples and stored in a sealed container at room temperature until further use.

Figure 2.1 Mechanical pretreatment of *O. ficus-indica* cladodes

Chemical composition of O. ficus-indica *cladode*

Compositional analysis of the cladode flour, which included total solids, ash, cellulose, structural carbohydrates, lignin and extractives, was determined using standard NREL laboratory analytical procedures for biomass analysis provided by the National Renewable Energy Laboratory (NREL) (Sluiter *et al.*, 2004a; Sluiter *et al.*, 2004b; Sluiter *et al.*, 2006). The starch content was determined through starch hydrolysis as per the protocol of the manufacturer of the assay kit (Megazyme K-TSTA kit, Megazyme, Bray, Ireland). The nitrogen content was determined by a micro-Kjeldahl procedure as described by the Association of Official Analytical Chemists (AOAC) and the factor 6.25 was used to convert nitrogen content to crude protein content (AOAC, 2005). These analyses were performed at the Department of Forestry and Wood Science, Stellenbosch University, South Africa. Analytical determinations of the chemical composition were carried out in triplicate and mean values calculated.

Dilute acid pretreatment experimental design

A central composite design was used to evaluate the pretreatment of *O. ficus-indica* cladode powder. The parameters investigated were dilute sulphuric acid at concentrations of 0.9 to 5.1% (w/w) and a contact time of 20 to 80 min. Although thermogravimetric analysis indicated that the raw material was stable up to 160^{o}C, the temperature was fixed at 120^{o}C due to technical constraints and the substrate loading was fixed at 20% (w/v). The central composite design was used as a preliminary study to evaluate responses and optimize the parameters that had significant effects on the glucose yield, yield of overall sugars and by-product formation at that temperature. The statistical analysis was performed using the commercial software STATISTICA 7.1 (Statsoft Inc., Tulsa, USA). Results were obtained through analysis of variance (ANOVA), standardized Pareto chart and response surface methodology.

Pretreatment apparatus

Dilute acid pretreatment experiments were carried out in batch tube reactors described elsewhere (Jacobsen & Wyman, 2001; Lloyd & Wyman, 2003; Lloyd & Wyman, 2005). The dimensions of the reactor tubes were 152.0 mm x 11.8 mm x 12.7 mm (length, inner diameter, outer diameter) and were made of Hastelloy C-276, (Hayes International Inc. Kokomo, Indiana, USA), a nickel-molybdenom-chromium wrought alloy that is generally considered versatile and corrosion resistant. Heat was provided by two 28.8 cm wide x 35

cm deep 4-kW model SBL-2D fluidized sand baths (Techne, Minneapolis, USA), fitted with a TC-8D temperature controller (Techne) which maintained the temperature within a 0.3^{o}C variance of the set point. The temperature within the reactor tubes was monitored by a temperature probe fitted inside a reactor tube loaded with biomass sample. The pretreatment experiments were conducted at the Department of Process Engineering, Stellenbosch University, South Africa, while subsequent pretreatment of larger biomass samples were carried out in the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Bloemfontein, using a Hiclave HV-110 autoclave (Hirayama, Saitama, Japan) with adjustable temperature settings.

Pretreatment trials

For pretreatment using reactor tubes, the required dilute H_2SO_4 solution was added to 1.2 g *Opuntia* cladode flour at a solids concentration of 20% (w/v) and loaded into each reactor tube. The tubes were sealed with Swagelok (Salon, Ohio, USA) stainless steel end caps, protected from the acid by specially designed Teflon plugs inserted into the tube ends (Lloyd & Wyman, 2003), and were left overnight at room temperature to allow the acid to penetrate the biomass for improved pretreatment. The tubes were subsequently loaded into a wire basket suspended by a chain hoist and lowered first into a sand bath set at 320^{o}C (Figure 2.2). This procedure allowed rapid heating up of the reactor contents to the target temperature of 120 (± 1)^{o}C in less than 2 min. As the target temperature was approached, the basket was raised out of the sand bath and transferred to a second sand bath which was maintained at the required temperature. Here it was held for the specified amount of time, removed from the sand bath and immediately immersed into a water bath filled with water at room temperature to halt the reaction. Each pretreatment was run in duplicate to produce enough pretreated biomass for the analysis of water insoluble solids (WIS) as well as for enzymatic hydrolysis. After cooling, the contents of the reactors were washed with 100 ml of deionized water and vacuum filtered through a wire mesh for recovery of the solid fraction (WIS) and liquid fraction (prehydrolysate). The WIS fraction was oven-dried at 40^{o}C for 24 h and used for subsequent enzymatic hydrolysis experiments. A portion of the WIS was also kept for analysis of its structural components while the liquid fraction was analyzed for sugars and by-product concentration. Prior to analysis of its carbohydrate content, the liquid fraction was further subjected to a mild acid hydrolysis using 4% (v/v) H_2SO_4 for 30 min at 120^{o}C (García-Aparicio *et al.*, 2006; Shevchenko *et al.*, 2000). This facilitated the conversion of soluble oligomers into their respective monomers.

Figure 2.2 Dilute acid pretreatment of *O. ficus-indica* cladode biomass using fluidized sand baths. The reactor tubes loaded in the metal basket can be seen.

Enzymes

Commercial enzyme preparations Celluclast 1.5L, Novozym 188 and Pectinex Ultra SP-L were kindly provided on request by Novozymes A/S (Bagsvaerd, Denmark) while Spezyme CP was provided by Genencor (Leiden, The Netherlands).

Determination of cellulase and β-glucosidase activities were performed as recommended by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987). Pectinase activity was quantified as described by Phutela *et al*. (2005). The reaction mixture containing 1 ml of 1% citrus pectin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.05 M citrate buffer (pH 4.4) and suitably diluted enzyme was incubated at 50^{o}C for 30 min in a reciprocal shaking water bath. A 3 ml volume of DNS reagent (Miller, 1959) was added to stop the reaction and the tubes were kept in a boiling water bath for 10 minutes. Afterwards, the tubes were immediately cooled in ice and the developed colour was read at 575 nm using a Helios Gamma UV-Visible spectrophotometer (Thermo Electron

Corporation, Altrincham, UK). The amount of released reducing sugar was quantified using galacturonic acid (Sigma) as standard (Phutela *et al.*, 2005). One unit of pectinase activity was defined as the amount of enzyme required to release 1 µmol of reducing sugars (galacturonic acid equivalents) per minute (U ml^{-1}) under the assay conditions.

Enzymatic digestibility of water insoluble solids (WIS)

The washed WIS fractions from different pretreatment conditions generated by the central composite design were enzymatically hydrolyzed at an enzyme loading of 15 FPU cellulase and 15 IU β-glucosidase per gram (dry wt) of pretreated *Opuntia* biomass. Enzymatic digestibility experiments were performed in 24 ml glass tubes containing 10 ml 0.05 M sodium citrate buffer (pH 4.8) at a substrate concentration of 2% (w/v). Sodium azide was added at a concentration of 0.02% (w/v) to prevent microbial contamination. The tubes were incubated at 50^{o}C in a reciprocal shaking water bath at 150 rpm. Samples were withdrawn at 24, 48 and 72 hours, centrifuged at 10 600 x *g* for 10 min and the supernatants analysed for sugar content by high-performance liquid chromatography (HPLC) as described later.

Production of an* O. ficus-indica *cladode enzymatic hydrolysate

To produce a stock hydrolysate for future fermentation experiments, a trial run was first performed in a 1 litre bioreactor (Sartorius Stedim Biotech, Goettingen, Germany). Subsequently, a 15 l stainless steel bioreactor with a 10 l working volume was used. A mass of 2.5 kg was mixed into 8.4 l of dilute H_2SO_4 solution to obtain a solids loading of 30% (w/v). The bioreactor was sealed and left overnight. Afterwards, the contents were autoclaved *in situ* at 120^{o}C for the required period. The bioreactor was cooled to 50^{o}C and the pH was adjusted and maintained at pH 4.8 by automatic addition of 3M KOH or 3N H_2SO_4. The stirrer speed was maintained at 300 rpm. The enzymes were added directly to the slurry and hydrolysis was performed *in situ* for up to 48 h. Samples were withdrawn at intervals for sugar analysis. At the end of the experiment, the enzymatic hydrolysate was collected in sterile 1 l bottles while the reactor was continuously stirred to ensure even distribution of the hydrolysate in all the bottles. The hydrolysate was stored at -20^{o}C until further use.

Analytical procedures

Due to the complexity of the lignocellulosic biomass, several analytical procedures were initially used for sugar identification and quantification. This included thin layer

chromatography (TLC), HPLC as well as a high performance anion exchange (HPAE) system with an electrochemical detector (ECD).

TLC: Samples taken from the *O. ficus-indica* cladode hydrolysate were diluted five-fold to reduce viscosity and spotted onto aluminium 20 x 20 cm silica gel 60 F_{254} plates (Merck Chemicals, Modderfontein, RSA) using chloroform-butanol-methanol-water-acetic acid (9:15:10:3:3) as the mobile phase. The plates were sprayed with a visualization reagent and afterwards baked at 100^{o}C for sugar detection. The visualization reagent consisted of 4 ml concentrated H_2SO_4, 0.2 g naphthoresorcinol and 0.4 g diphenylaline dissolved in 100 ml of 96% ethanol.

HPLC: Sugars and by-products (acetic acid, HMF and furfural) in the liquid fraction, as well as the sugars released in the enzyme-hydrolysed WIS, were analyzed first with an Aminex HPX-87H ion exclusion column equipped with a cation-H cartridge (Bio-Rad, Hercules, CA, USA). Subsequent analyses of the hydrolysate obtained after combined pretreatment and saccharification of the *O. ficus-indica* cladode were, however, performed with an Aminex HPX-87P or a Rezex RPM-Monosaccharide Pb+2 cation exchange column (Phenomenex, Torrance, CA, USA) for better peak resolution. The sugars cellobiose, glucose, xylose, galactose, arabinose and fructose were quantified with a Waters refractive index detector (Microsep, Johannesburg, RSA), whereas by-products were analyzed with a Waters UV detector at 280 nm (Microsep). The HPX-87H column was operated at an oven temperature of 65^{o}C, with a mobile phase of 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1} and an injection volume of 10 µl. The Aminex HPX-87P and the Rezex Pb columns, which were similar anion exchange columns, were equipped with guard columns and operated at an oven temperature of 85^{o}C using deionized water as the mobile phase at a flow rate of 0.4 ml min^{-1}. Pretreated samples were neutralized with $Ca(OH)_2$ to precipate the sulphates, whereas enzymatic hydrolysate slurries were diluted to reduce the viscosity of the sample and prolong the life of the column. All samples were centrifuged at 10 600 x *g* for 10 min and the supernatants filtered using 0.45 µm acetate filters. Samples of 10 µl each were automatically injected into the HPLC system. Individual and mixed sugar standards were also prepared and analysed according to the conditions above.

HPAE-ECD: A high performance anion exchange system with an electrochemical detector (HPAE-ECD) was used to confirm the sugars identified by TLC and HPLC. A CarboPac PA-1 column (Dionex Corporation, Sunnyvale, California, USA), protected by a guard

column, was used with two eluents, 250 mM NaOH and deionized water, at a flow rate of 1 ml min^{-1}, while the injection volume was 10 µl and the run time was 41 min.

4. Results

4.1 Chemical composition of *O. ficus-indica* cladode

The composition and structural carbohydrates of the dried and milled cladode are highlighted in Table 2.1 below. Compositional analysis data from literature, as well as the composition of some conventional lignocellulosic biomass feedstocks, are also listed in the table for comparison. The carbohydrate content of the cladode amounted to 42% (dry wt), comprising mainly glucan, xylan, galactan, arabinan and fructan oligomers. The glucan fraction in the cladode comprised 9.6 ± 0.2% starch and 13.5 ± 1.1% cellulose. The total sugar content of the cladode observed in this study was higher than reported by Ginestra *et al*. (2009) (Table 2.1). The authors also reported on fucan and rhamnan fractions which were, however, not detected in this study. Instead, the presence of a fructan fraction was observed during analysis of the cladode (Figure 2.3, Table 2.3). The low xylan content observed in the cladode was similar to the results of Ginestra *et al*. (2009), who reported a content of 1.9% xylose.

The total sugar content of the *O. ficus-indica* cladode was low compared to those of other lignocellulosic biomass feedstocks such as sugar cane bagasse (Neureiter *et al*., 2002), corn (maize) stover (Ruth & Thomas, 2003) and barley straw (García-Aparicio *et al*., 2011) (Table 2.1), mainly due to the lower glucan and xylan content of the cladode. The lignin content of 7.95% (dry wt) was significantly lower than that of the other feedstocks. The biomass had a high content of extractives (24.3% dry wt) consisting of mainly galacturonic acid subunits. The composition of other constituents such as ash, lignin, and protein were similar to previous analytical data on the *Opuntia* cladode (Stintzing & Carle, 2005).

Table 2.1 Mean chemical composition of *O. ficus-indica* cladode and comparison with some conventional lignocellulosic biomass feedstocks

Constituent (% dry wt.)	*O. ficus-indica* cladode (This study)	*O. ficus-indica* cladode[a]	Sugar cane bagasse[b]	Corn stover[c]	Barley straw[d]
*Glucan	23.1	15.3	40.2	37.4	37.1
Xylan	3.9	1.9	22.5	21.1	21.3
Arabinan	3.8	4.0	2.0	2.9	3.8
*Galactan	6.4	3.4	1.4	2.0	1.2
*Fructan	4.8	-	-	-	-
Fucan	-	0.07			
*Mannan	Trace	1.4	0.5	1.6	-
Rhamnan	-	0.7	-	-	-
Total sugars	42.0	26	66.6	65.0	63.4
*Fermentable sugars	34.3	19.7	42.1	41.0	38.3
Lignin	7.9	16	25.2	18.0	19.2
Ash	16.8	n/a	10-15	5.2	8.2
Protein	7.5	6.42	n/a	3.1	n/a
Extractives	24.3	17.7	n/a	4.7	15.4
Total	98.5				

*Total readily fermentable constituent sugars
[a]Ginestra *et al*. (2009). Note: The author used a mixture of three cultivars, Surfarina, Muscaredda and Sanguigna which were cultivated in Italy.
[b]Neurieter *et al*. (2002)
[c]Ruth & Thomas (2003)
[d]García-Aparicio *et al*. (2011)

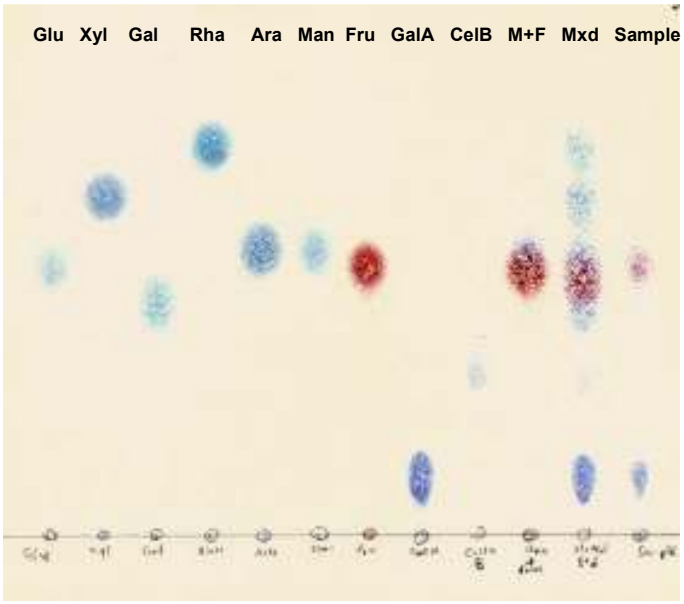


Figure 2.3 TLC plate showing the presence of fructose (pink spot) in a diluted *O. ficus-indica* cladode hydrolysate sample. Glucose (Glu), Xylose (Xyl), Galactose (Gal), Rhamnose (Rha), Arabinose (Ara), Mannose (Man), Fructose (Fru), Galacturonic acid (GalA), Cellobiose (CelB), Mannose and Fructose (M+F), Mixed sugar standards (Mxd), *Opuntia* hydrolysate (Sample).

4.2 Pretreatment and enzymatic digestibility based on central composite design

The *Opuntia* cladode flour was subjected to dilute acid pretreatment using process parameters generated in the central composite design (Table 2.2). The aim was to evaluate the effect of pretreatment conditions on the overall sugar yield obtained after enzymatic hydrolysis and on cellulose susceptibility to enzymatic hydrolysis of the solid fraction.

The solids recovery (remaining solids after pretreatment divided by original oven-dried weight) and the sugars obtained after different pretreatment conditions are given in Table 2.2. As expected, the total solids recovery, which ranged from 31.7 to 52.3%, was negatively influenced by an increase in the acid concentration. This effect was mainly attributed to the solubilization of the hemicellulose fraction and water soluble extractives during pretreatment. Arabinan recovery in the liquid fraction ranged from 26.3 to 76.3% of the theoretical amount present in the original biomass and both acid concentration and pretreatment time contributed to its release. The cladode flour had a pH of 5.2 and did not drop below pH 3.5 after acid pretreatment. Total by-products did not exceed 0.92 g per

100 g dry powder in the liquid fraction. These included acetic acid (0.68 g), HMF (0.19 g), and furfural (0.05 g). The low value represents an added advantage of using *Opuntia* cladode biomass as feedstock for bioethanol production, considering the toxic effects of by-products on the fermenting organism during ethanol production.

Table 2.2 Conditions used and mean values of sugars released during dilute acid pretreatment of *O. ficus-indica* cladode flour

Pretreatment				Sugar recovery (g 100 g biomass^{-1})		Sugar released (% of theoretical)	
Sulphuric acid (% w/w)	**Time (min)**	**Solids recovery (%)**	**pH**	**Arabinan**	**Glucan***	**Arabinan**	**Glucan**
0.9	20.0	52.3	4.9	1.0	2.2 (5.2)	26.3	32.0
0.9	80.0	47.5	4.9	2.3	3.8 (5.2)	60.5	39.0
5.1	20.0	37.5	3.7	2.4	4.0 (4.0)	63.2	34.6
5.1	80.0	33.9	3.7	2.5	4.8 (5.2)	65.8	43.3
0.0	50.0	49.4	5.2	2.1	3.4 (4.7)	55.3	39.4
6.0	50.0	31.7	3.5	2.9	5.4 (5.3)	76.3	46.3
3.0	7.6	49.8	4.2	1.4	3.4 (4.9)	36.8	35.9
3.0	92.4	40.3	4.2	1.9	4.7 (5.9)	50.0	45.9
3.0	50.0	39.2	4.2	2.2	4.3 (6.0)	57.9	44.6
3.0	50.0	41.5	4.2	2.0	4.4 (6.1)	52.6	45.5
3.0	50.0	38.9	4.2	2.1	4.5 (6.0)	55.3	45.5
3.0	50.0	39.0	4.2	2.1	4.5 (5.9)	55.3	45.0

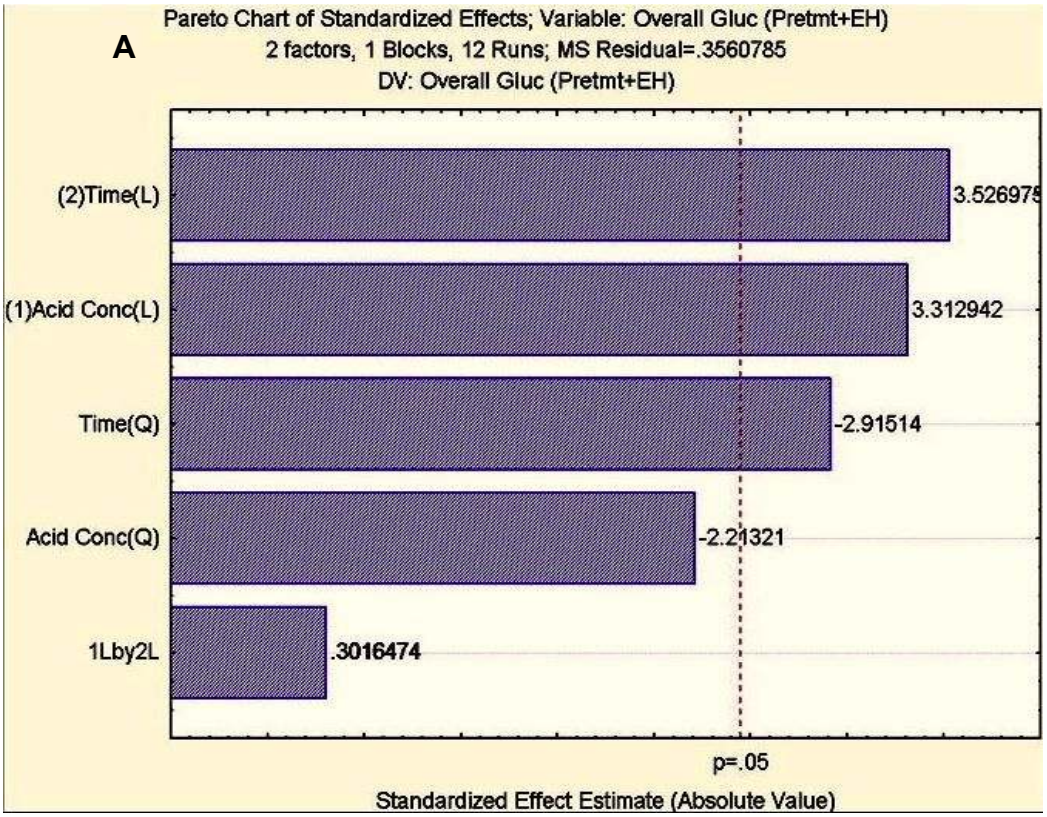
The data in parentheses represent glucose values after enzymatic hydrolysis

To evaluate the effect of different pretreatment conditions on the digestibility of pretreated *Opuntia* cladode solid residue (WIS fraction), enzymatic hydrolysis tests were performed using cellulase and β-glucosidase. Celluclast 1.5L FG and Spezyme CP, both cellulose-hydrolysing enzymes, had measured cellulase activities of 70 FPU ml^{-1} and 80 FPU ml^{-1}, respectively, whereas Novozym 188 had a β-glucosidase activity of 750 IU ml^{-1}. The yields take into consideration the amount of sugar released during both pretreatment and enzymatic hydrolysis of the WIS fraction. For all the pretreatment conditions tested, the

sugar yields obtained with enzymatic hydrolysis were slightly higher compared to non-pretreated *Opuntia* cladode by about 14.2% (data not shown). The conditions used, however, only yielded a maximum glucose value of 10.7 g per 100 g dry biomass, corresponding to approximately 46% of the theoretical glucose content (Table 2.2). The optimum pretreatment time during which the highest sugar yields were achieved, appeared to be 50 min. Enzymatic hydrolysis after pretreatment with 6% acid produced the highest overall glucose value, but 3% acid also yielded comparable results. Moreover, with prolonged pretreatment time 0.9% acid produced almost 40% of the theoretical glucose, suggesting that a lower acid concentration would still be able to release a comparable amount of glucose as well as reduce operational costs. The glucose released during pretreatment likely originated from non-cellulosic glucose in the form of starch (Ginestra *et al.*, 2009) that amounted to 9.6 g starch per 100 g biomass (dry wt), corresponding to 42% of the theoretical glucose in the cladode biomass.

The statistical significance of the effect of acid concentration and residence time on overall glucose yield was determined by ANOVA. The results are presented in a standardized Pareto chart (Figure 2.4a) where both residence time and acid concentration showed a positive effect ($P < 0.05$) with contact time being the more significant of the two. Figure 2.4b shows a response surface plot which was calculated using a second-order polynomial model. An increase in the enzymatic hydrolysis yield was obtained by increasing pretreatment time and, to a lesser extent, acid concentration.

Temperature, an important parameter during pretreatment, was not included in the statistical design due to technical constraints and, as stated earlier, 120^{o}C was the fixed temperature used. The total amount of glucose released by pretreatment was too low to proceed with fermentation trials. Therefore, it was necessary to optimize the glucose yield based on the results of initial pretreatment and enzymatic hydrolysis.



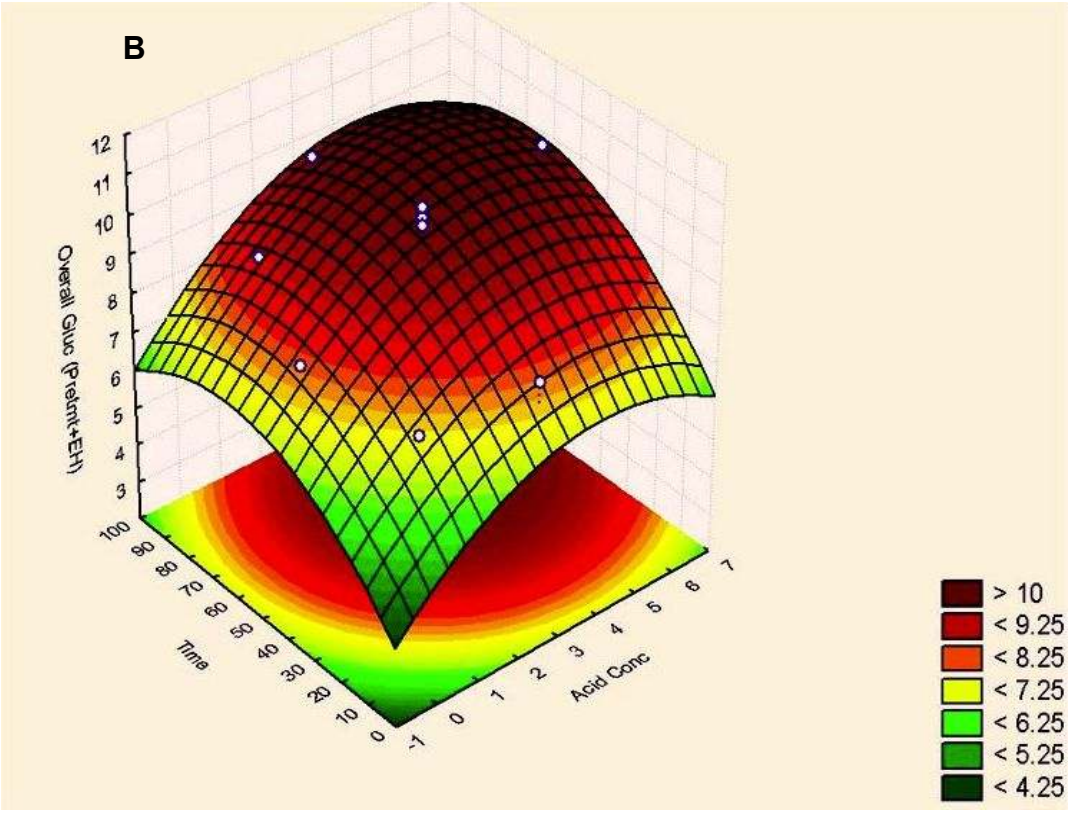


Figure 2.4 A. Standardized Pareto chart for overall glucose yield (% dry biomass). Standardized effects were calculated by dividing the effect by its standard error. **B.** Estimated response surface for overall glucose yield showing the influence of time and sulphuric acid concentration at a fixed temperature of 120^{o}C

4.3 Optimization of pretreatment

From this stage onwards, pretreatment was performed at the University of the Free State. It also became possible to identify and quantify other sugars such as xylose, galactose and fructose because of the availability of an Aminex HPX-87P HPLC column.

Pretreatment of the *Opuntia* cladode flour was performed in an autoclave using 3% (w/w) sulphuric acid for 50 min and a solids loading of 20% and 30% (w/v), respectively, to determine if these results would be comparable with those of conditions previously used in the tube reactors. A 30% solids loading was used in an attempt to increase the final concentration of sugars in the hydrolysate. Under these conditions, 3.1 g arabinose (79% of theoretical) and 6.2 g glucose per 100 g biomass (dry wt) were liberated, compared to mean values of 2.1 g arabinose and 4.4 g glucose when tube reactors were used (Table 2.3). This result indicated that the autoclave could be employed for further pretreatment processes. However, the higher arabinose and glucose values observed after autoclaving could have been due to the prolonged pretreatment of the material because of the time it took to heat up to the appropriate temperature as well as to cool down afterwards. Nevertheless, the autoclave allowed the simultaneous pretreatment of more than one sample and larger volumes.

By increasing the solids loading from 20% to 30%, an acid hydrolysate with a higher sugar content was obtained (Table 2.3). Further attempts to increase the solids loading beyond 30% were unsuccessful because the biomass was immiscible and probably was poorly penetrated by the acid solution.

The next step was to determine whether conditions milder than using 3% sulphuric acid would prove as effective. The biomass was subjected to pretreatment with 1, 1.5, 2 and 2.5% (w/w) sulphuric acid at 120^{o}C for 50 min with a 30% solids loading. Afterwards, enzymatic hydrolysis was performed on the dry WIS as described in the methods section, using cellulase and β-glucosidase with a 2% WIS loading. Comparable yields of the hemicellulose sugars xylose and arabinose were obtained. However, there was a slight increase in the final values of glucose and galactose with increased acid concentration (Table 2.4). A sulphuric acid concentration of 1.5% (w/w) was chosen for pretreatment because, among other factors (see discussion), the sugar yields obtained were comparable to those obtained using 2 and 3% acid. Although the acid concentration was less significant than reaction time and sugars were released even when the material was pretreated without acid, it was observed that besides slightly higher values, the acid

hydrolysate was less viscous and more of a slurry than the non-acid pretreated hydrolysate.

Table 2.3 Mean values of sugars released by pretreatment of *O. ficus-indica* cladode flour in an autoclave compared to values obtained using a tubular reactor

	Sugars (g 100 g dry biomass^{-1})				
Pretreatment	**Glucose**	**Xylose**	**Galactose**	**Arabinose**	**Fructose**
Tube reactor (0% acid)	**3.4**	n/a	n/a	**2.1**	n/a
Autoclave (0% acid)	**3.9**	0.9	1.7	**2.7**	1.1
	3.6*****	0.8*	1.7*	2.6*****	1.1*
Tube reactor (3% acid)	**4.4**	n/a	n/a	**2.1**	n/a
Autoclave (3% acid)	**6.2**	1.3	2.3	**3.0**	1.0
	7.3*****	1.4*	3.0*	3.6*****	1.4*

Note: The values in bold (glucose and arabinose) are directly comparable between both pretreatment apparatus at 20% solids loading. The values indicated by an asterisk were obtained using a 30% solids loading

Table 2.4 Final sugar yield as a function of dilute acid concentration after pretreatment in an autoclave and subsequent enzymatic hydrolysis of WIS at a 2% (w/v) loading

	Sugars (g 100 g dry biomass^{-1})				
Acid concn (% w/w)	**Glucose**	**Xylose**	**Galactose**	**Arabinose**	**Fructose**
0	8.3	2.0	3.8	3.0	2.9
1.0	9.0	2.2	4.7	3.2	2.9
1.5	9.8	2.4	5.8	3.5	3.2
2.0	10.5	2.4	6.0	3.6	3.1
2.5	10.5	2.4	6.2	3.6	3.2
3	10.4	2.2	6.0	3.6	3.2

4.4 Optimization of enzymatic hydrolysis to improve overall sugar yield

Cellulase and β-glucosidase at 15 FPU and 15 IU, respectively, per g dry biomass were the only enzymes used up to this point for hydrolysis of the pretreated biomass. To improve the sugar yield through enzymatic hydrolysis, 100 IU per g biomass of Pectinex

Ultra-SPL (pectinase) was included in the enzyme mix. The pectinase had an activity of 1064 IU ml^{-1}. Hydrolysis of dried WIS pretreated with 1.5% (w/w) sulphuric acid for 50 min was performed as described earlier. Moreover, cellulase and β-glucosidase loadings of 20 and 30 FPU per g biomass were included in the trials to determine if the sugar yield could be improved by increasing the enzyme concentration. The effect of these enzymes was also determined in *Opuntia* flour pretreated without acid, for comparison.

The glucose yield was not significantly affected by the inclusion of pectinase when compared with hydrolysis using only two enzymes. In fact, the difference between the maximum amount of glucose obtained from the two sets of enzyme combinations was only 0.3 g. However, when pectinase was added, about 4 g more of sugars other than glucose were released, compared to hydrolysis with only cellulase plus β-glucosidase (Figure 2.5). A similar trend was observed with the enzyme combinations used for non-acid-pretreated biomass compared to those pretreated with acid, except for a slight improvement in glucose values when the enzyme loading of all three enzymes was increased. An increase in enzyme loading from 15 up to 30 FPU also did not improve the glucose and overall sugar yield of the acid-treated biomass either in the cellulase plus β-glucosidase hydrolysis or when pectinase was added to the enzyme mix (Figure 2.5). Addition of excess enzymes to the non-acid-pretreated biomass did not produce as much sugars as with acid pretreatment (Figure 2.5), further justifying the importance of acid for the release of sugars in pectin rich biomass.

Based on the results obtained from pretreatment and enzymatic hydrolysis trials thus far and considering the economic implications of using excess enzymes, 15 FPU cellulase, 15 IU β-glucosidase and 100 IU pectinase per g of pretreated substrate seemed optimal for enzymatic hydrolysis after dilute acid pretreatment with 1.5% H_2SO_4 at 120^oC for 50 min. These conditions resulted in a recovery of theoretical sugar contents of 63.2% glucose, 66.7% xylose, 79.7% galactose, 78.5% arabinose and 85.4% fructose, respectively. However, the glucose recovery in particular, was still low.

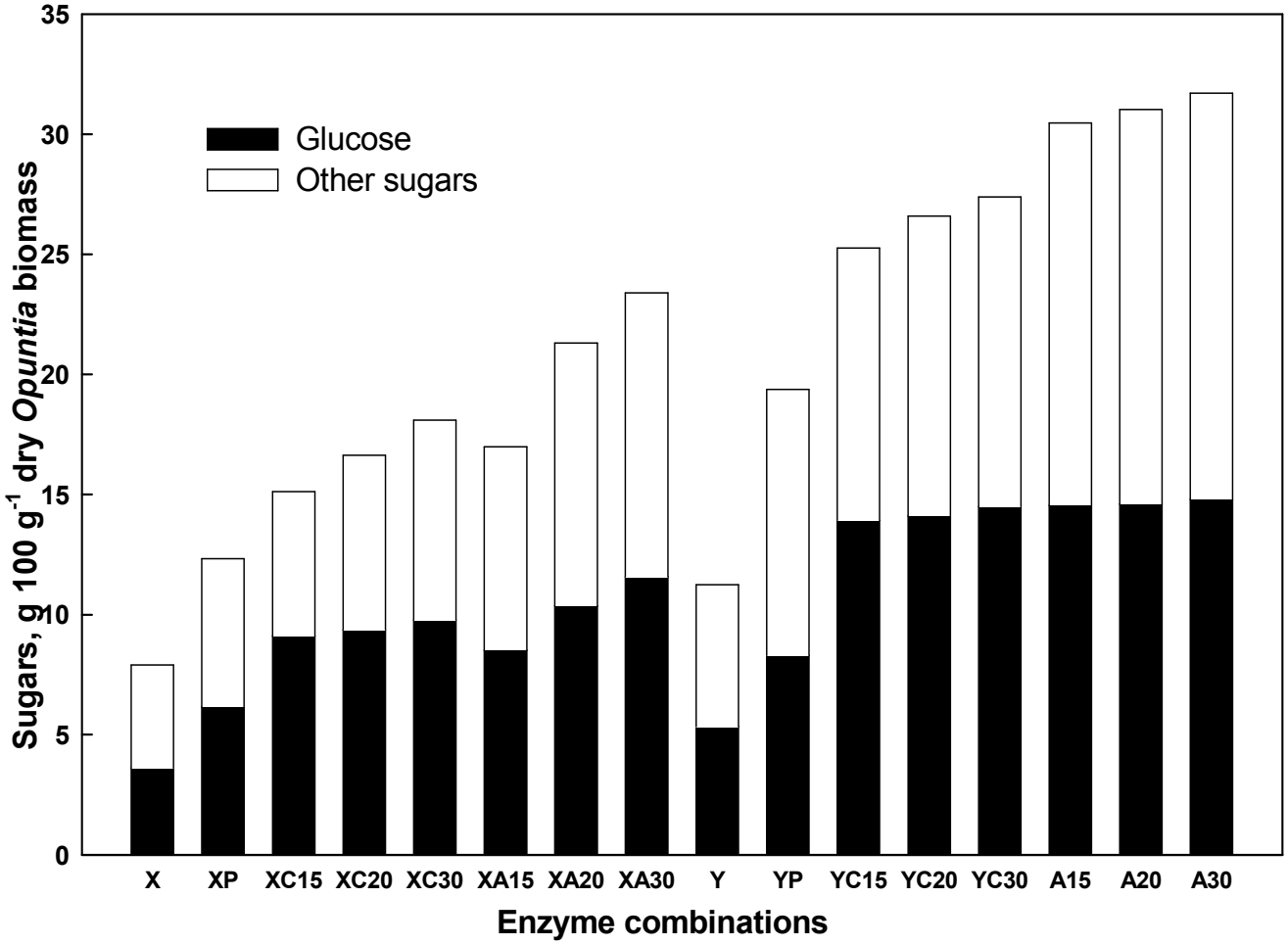


Figure 2.5 Overall yields of glucose and other sugars (xylose, galactose, arabinose and fructose) after pretreatment and enzymatic hydrolysis as a function of different enzyme combinations: pretreatment without acid (X), pretreatment with acid (Y), and subsequent saccharification with cellulase and β-glucosidase (C), pectinase (P), all three enzymes (A). The numbers 15, 20 and 30 represent the cellulase (FPU g^{-1}) and β-glucosidase (IU g^{-1}) enzyme loadings.

4.5 Production of an *O. ficus-indica* cladode hydrolysate

A cocktail of enzymes consisting of cellulase, β-glucosidase and pectinase at 15 FPU, 15 IU and 100 IU, respectively, per g dry biomass were used to hydrolyse the pretreated slurry. Samples were withdrawn at intervals for sugar analysis during the 48 h enzymatic hydrolysis.

The sugar concentrations after pretreatment were as follows (per litre of hydrolysate): 7.4 g glucose, 3.9 g xylose, 2.3 g galactose, 4.0 g arabinose and 5.0 g fructose. There was a time interval of approximately 10 min between the enzyme addition and collection of the first sample. This was to allow for proper mixing and to obtain a homogenous sample. This time frame was, surprisingly, long enough to allow the glucose concentration to increase up to 24.5 g l^{-1}. This rapid rate of glucose release continued for the first two hours of hydrolysis, producing 35 g glucose l^{-1} (Figure 2.6 A). A further 10 g l^{-1} was subsequently produced, giving a total glucose concentration of 45 g l^{-1} after 48 h. The other sugar oligomers were also converted into their monomeric forms and their hydrolysis proceeded similar to the release of glucose. (Figure 2.6 A, B).

The combined pretreatment and enzymatic hydrolysis produced an *Opuntia* cladode hydrolysate containing (per litre) 45.5 g glucose, 6.3 g xylose, 9.1 g galactose, 10.8 g arabinose and 9.6 g fructose (Table 2.5). The total sugar concentration was 81.3 g l^{-1}, of which 64.2 g were readily fermentable (glucose, galactose and fructose). Hydrolysis with cellulase, β-glucosidase and pectinase enzymes released 78% of theoretical sugars and close to 80% of the theoretical glucose in the biomass.

Table 2.5 Final sugar concentration in *O. ficus-indica* hydrolysate in relation to the theoretical concentrations in the original biomass

	Sugars				
Parameters	**Glucose**	**Xylose**	**Galactose**	**Arabinose**	**Fructose**
Concn. (g l^{-1})	45.5	6.3	9.1	10.8	9.6
Concn. (g per 100 g dry biomass)	18.2	2.5	5.8	2.6	3.8
% of theoretical	78.8	64.4	90.6	67.5	79.7

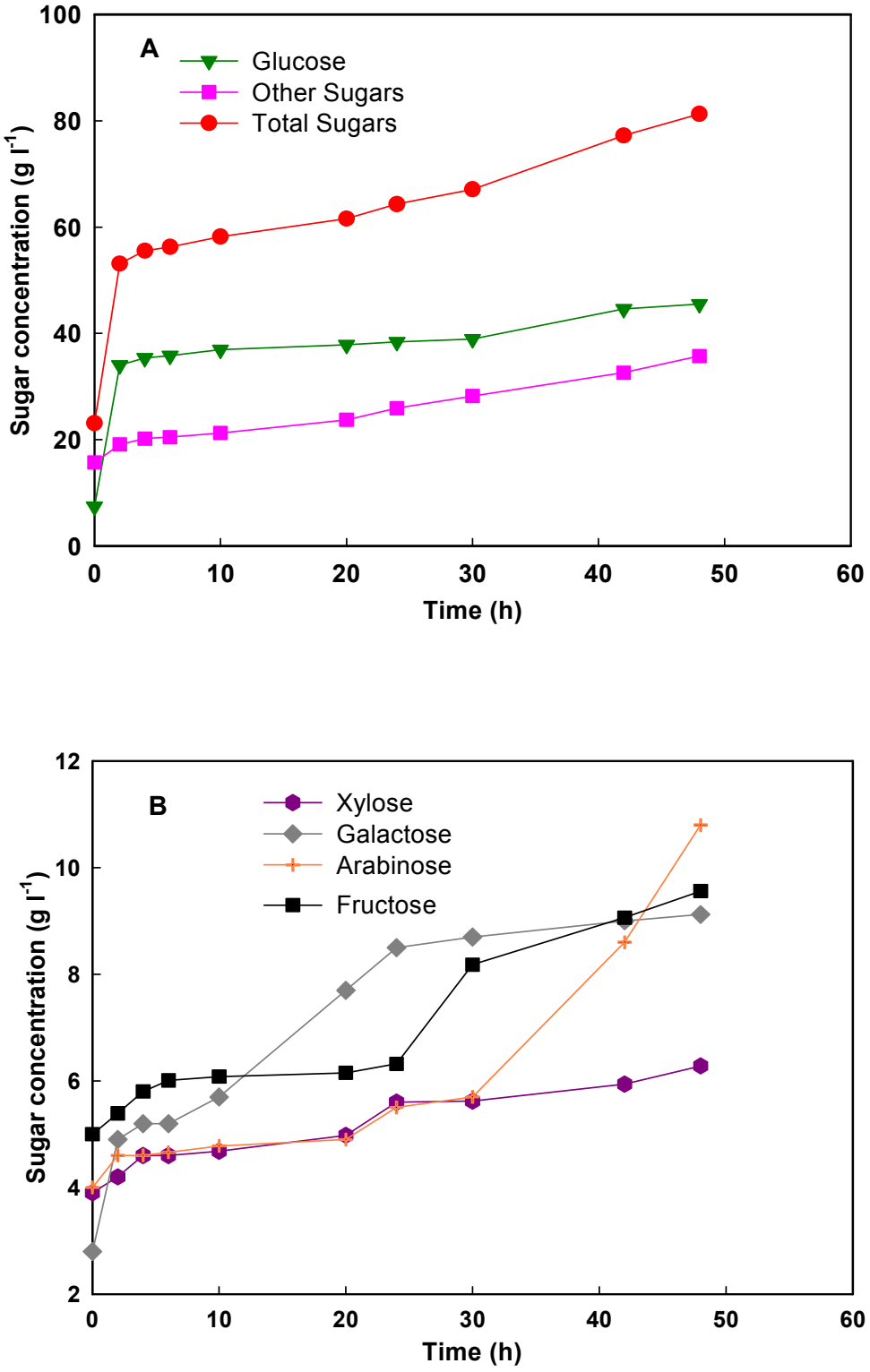


Figure 2.6 Glucose and other sugars released during 48 h enzymatic hydrolysis of dilute acid pretreated *O. ficus-indica* cladode flour. **A.** Glucose, other sugars and total sugars. **B.** Other sugars (xylose, galactose, arabinose and fructose). Time 0 h is actually 10 min after the addition of enzymes.

5. Discussion

O. ficus-indica produces abundant biomass and is a highly drought resistant plant, making its cladodes a potential feedstock for bioethanol production in semi-arid regions. The main disadvantage is, however, the high water content of the fresh cladode (about 88 to 95%). This problem was addressed by first sun-drying and milling the cladode, giving a more concentrated raw material as well as facilitating easier storage and handling.

The total carbohydrate content of 42 g per 100 g biomass was comparable to the 36 g per 100 g biomass reported by Ginestra *et* al. (2009). However, according to other reports, the carbohydrate content of dry cladode biomass ranged between 64 to 71 g per 100 g (Stintzing & Carle, 2005). This variation could be attributed to either of, or a combination of, two reasons. Firstly, agricultural and environmental factors as well as the age of the cladodes influenced the composition. For instance, younger cladodes tend to contain a higher carbohydrate content (Ginestra *et al.*, 2009; Rodriguez-Felix & Cantwell, 1988; Rodríguez-Garcia *et al.*, 2007; Stintzing & Carle, 2005). Secondly, as pointed out previously in this dissertation, the analytical methods used and the manner in which values were reported, varied with different authors. An example can be found in a previous article where the authors included acidic polysaccharides, mucilage and pectin (which consists of mainly D-galacturonic acid subunits) in the polysaccharide composition of the cladode to obtain a value of 69% (dry wt) total polysaccharides (Malainine *et al.*, 2005). In this study, the water soluble extractives, which made up 24.3% of the dry weight, consisted mainly of galacturonic acid units. The extractives and carbohydrates would total to 66.3% polysaccharides, which was within the range reported in literature (Table 2.1).

In a previous report by Ginestra *et al.* (2009) describing the cladode composition, mannan was present (1.3%, dry wt) and no fructan fraction was detected, whereas in this study only trace amounts of mannan were discovered while a fructan fraction was identified in the cladode (Table 2.1). The fructan fraction was confirmed by TLC (Figure 2.3), HPAE-ECD and HPLC analysis using an Aminex HPX-87P column. Its monomer, fructose, happened to be the third most abundant sugar (Table 2.1) in the cladode biomass after glucose and galactose. Due to the numerous polysaccharides present in the *O. ficus-indica* cladode, identification and quantification of the individual fractions that make up its carbohydrate profile proved quite a challenge and not one particular analytical method was sufficient to provide all the necessary information.

The total sugar content, especially the glucan content in the cladode, was lower than those of other lignocellulosic biomass feedstocks (Table 2.1). However, this study showed that the *Opuntia* cladode biomass had a high content of galactan and fructan that compensated for the low glucan content. Their monomeric units (galactose and fructose) are readily fermentable and, together with glucose, they made up 34.3% (dry wt) of the cladode biomass, which was close to those of the other feedstocks (Table 2.1). The low xylan content found in the *Opuntia* cladode biomass during this study (Table 2.1) corresponded with findings reported in literature (Ginestra *et al.*, 2009). This indicated that the hemicellulose backbone of *O. ficus-indica* cladode is unlike the other feedstocks which usually have a xylo-arabinan or xylo-mannan structure. Thus, for ethanol production from the *Opuntia* cladode biomass, a xylose fermenting yeast may not necessarily be a requisite. Although Ginestra *et al*. (2009) reported a lignin content of 16% (dry wt) in the *O. ficus-indica* cladode (Table 2.1), the authors mentioned that it included a high amount of calcium oxalate which could not be separated, and that the actual lignin content could be lower. In this study, the lignin content of the cladode was 7.95% (dry wt). This value was significantly lower than in other feedstocks and would result in the formation of less toxic inhibitors such as phenolic compounds following dilute acid pretreatment.

Initial constraints in this investigation regarding HPLC analysis of hemicellulose sugars due to poor peak separation, however, meant that only arabinose could be accurately quantified. Thus, arabinose was the main sugar taken into consideration during pretreatment, whereas the subsequent enzymatic hydrolysis focused on glucose. The release of arabinose and xylose from hemicellulose is an important indicator for the efficacy of acid pretreatment. The arabinan fraction is usually the least stable sugar during pretreatment (Shevchenko *et al.*, 2000) and it was solubilised even in the absence of acid treatment (0% acid, 50 min). The presence of glucose in the liquid fraction could be as a result of starch saccharification during pretreatment. This observation is also consistent with that of another report by a previous author (Ginestra *et al.*, 2009). Non-cellulosic glucose from starch constituted 9.6% of the raw material and about 42% of the total glucan (Table 2.1). The pH value of the pretreated cladode did not decrease below pH 3.5 during the pretreatment trails, suggesting that the material had a strong buffering capacity even at a high acid concentration of up to 6% (w/w) and would require only a small amount of alkali to adjust its pH for the purpose of enzymatic hydrolysis. There is a possibility that its high ash content, which comprises potassium and calcium, played a role in stabilizing the pH.

The use of acid for pretreating the cladode was justified by the production of a less slimy hydrolysate than when no acid was added. It has been reported that addition of acid helped to solubilise some of the pectin by disrupting the glycosidic bonds between galacturonic acid units (Grohmann *et al.*, 1995). A less viscous hydrolysate would be more accessible to enzymes, which would increase the efficiency of subsequent enzymatic hydrolysis (Grohmann *et al.*, 1995). However, for economic reasons, a concentration of 3% sulphuric acid is too high for pretreatment and there remains the risk of producing toxic by-products as a result of sugar degradation. Therefore, a compromise had to be reached. A decrease of the acid concentration by half (i.e. 1.5% acid) gave results closer to the yields obtained with 2% and 3% acid and produced slightly higher sugar yields than lower acid concentrations. Under these conditions, 1.5% sulphuric acid at 120^{o}C for 50 min was chosen as the pretreatment condition and more emphasis was placed on improving sugar yield through enzymatic hydrolysis. Pretreatment at a higher temperature decreased the concentration of acid required and also shortened retention time. However, technical constraints restricted the pretreatment temperature used in this study to 120^{o}C.

For complete conversion of all carbohydrates to monomeric sugars in pectin-rich plant biomass such as *O. ficus-indica*, which contains galacturonic acid units in its cell wall (Cárdenas *et al.*, 2008; Goycooleal & Cárdenas, 2003), it is necessary to include pectinases in the enzyme mixture (Grohmann *et al.*, 1995). Pectinase is, however, not effective on its own for the liberation of sugars, as only 3 g glucose per 100 g biomass (Figure 2.5) was released during enzymatic hydrolysis, the rest coming from pretreatment. The high cost of enzymes, and the minimal difference in yields obtained with 15 FPU and 30 FPU cellulase per g dry biomass, meant that the obvious enzyme loading was 15 FPU cellulase together with 15 IU β-glucosidase and 100 IU pectinase per g dry biomass for hydrolysis of the pretreated slurry. A cellulase loading of 15 FPU per gram of WIS has been used in several studies, and is close to the enzyme dosage of 10 FPU per g often used in laboratory-scale hydrolysis experiments (Ballesteros *et al.*, 2004; García-Aparicio *et al.*, 2006; Hu & Wen, 2008; Mosier *et al.*, 2005a; Sun & Cheng, 2002). These conditions were, therefore, used to produce the final *Opuntia* hydrolysate for use in future experiments. Due to technical constraints during HPLC and HPAE-ECD analysis, however, the galacturonic acid concentration in the hydrolysate could not be accurately quantified.

The lower glucan content of the *Opuntia* cladode biomass compared to other feedstocks (Table 2.1) meant that a high biomass loading would be required to produce a hydrolysate

with appreciable glucose content. For instance, in this study 250 g l^{-1} of dried and milled cladode was used to produce a hydrolysate that contained 45.5 g glucose l^{-1}. By contrast, 130 g l^{-1} of sugar cane bagasse was required to produce 65 g glucose l^{-1} in a pilot bioethanol plant in Brazil (Bon & Ferrara, 2007). Nevertheless, O. *ficus-indica* has the advantage of being able to produce abundant biomass as well as thrive in places where sugar cane, for instance, would not grow, which is the main reason behind the investigation of its use as a potential bioethanol feedstock.

With the pretreatment and enzymatic hydrolysis conditions used in this study, the theoretical ethanol yield obtainable from 1 kg dry *Opuntia* flour is 0.16 l ethanol, assuming that the hexose sugars were completely utilised, and 0.21 l ethanol if pentose sugars were fermented. A theoretical ethanol concentration of 30 g l^{-1} may be expected from the readily fermentable sugars and 40.7 g l^{-1} from total sugars. These yields are low for an industrial ethanol plant. Therefore, to increase sugar concentrations in the hydrolysate, which would inadvertently improve ethanol yields during fermentation, as well as to achieve an economic process, further investigations into more efficient pretreatment and enzymatic hydrolysis technologies are required. Such investigations should be targeted at process engineering to optimize pretreatment through the use of more efficient methods such as steam pretreatment. Nevertheless, the final sugar concentrations of the hydrolysate obtained during this study are a good starting point and the *O. ficus-indica* cladode biomass remains a promising potential feedstock for bioethanol production.

6. References

AOAC (2005). Chapter 4: Animal Feed. In *Official Methods of Analysis of AOAC International*, 18 edn, pp. 12-21. Edited by W. Harwitz & G. W. Latimer. Arlington, VA: AOAC International.

Ballesteros, M., Oliva, J. M., Negro, M. J., Manzanares, P. & Ballesteros, I. (2004). Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SSF) with *Kluyveromyces marxianus* CECT 10875. *Process Biochem* **39**, 1843-1848.

Bon, E. P. S. & Ferrara, M. A. (2007). Ethanol production via enzymatic hydrolysis of sugar-cane bagasse and straw. In *The role of agricultural biotechnologies for production of bioenergy in developing countries*, 1 edn. Rome: FAO.

Cárdenas, A., Goycoolea, F. M. & Rinaudo, M. (2008). On the gelling behaviour of 'nopal' (*Opuntia ficus-indica*) low methoxyl pectin. *Carbohydr Polym* **73**, 212-222.

García-Aparicio, M. P., Oliva, J. M., Manzanares, P., Ballesteros, M., Ballesteros, I., González, A. & Negro, M. J. (2011). Second-generation ethanol production from steam exploded barley straw by *Kluyveromyces marxianus* CECT 10875. *Fuel* **90**, 1624-1630.

García-Aparicio, M., Ballesteros, I., González, A., Oliva, J., Ballesteros, M. & Negro, M. (2006). Effect of inhibitors released during steam-explosion pretreatment of barley straw on enzymatic hydrolysis. *Appl Biochem Biotechnol* **129**, 278-288.

Ghose, T. K. (1987). Measurement of cellulase activities. *Pure Appl Chem* **59**, 257-268.

Ginestra, G., Parker, M. L., Bennett, R. N., Robertson, J., Mandalari, G., Narbad, A., Lo Curto, R. B., Bisignano, G., Faulds, C. B. & other authors (2009). Anatomical, chemical, and biochemical characterization of cladodes from prickly pear [*Opuntia ficus-indica* (L.) Mill.]. *J Agric Food Chem* **57**, 10323-10330.

Goycooleal, F. M. & Cárdenas, A. (2003). Pectins from *Opuntia* spp.: A short review. *J Prof Assoc Cactus* **5**, 17-29.

Grohmann, K., Cameron, R. G. & Buslig, B. S. (1995). Fractionation and pretreatment of orange peel by dilute acid hydrolysis. *Bioresour Technol* **54**, 129-141.

Hu, Z. & Wen, Z. (2008). Enhancing enzymatic digestibility of switchgrass by microwave-assisted alkali pretreatment. *Biochem Eng J* **38**, 369-378.

Inglese, P., Basile, F. & Schirra, M. (2002). Cactus pear fruit production. In *Cacti: Biology and Uses*, pp. 163-184. Edited by P. S. Nobel. Berkely: University of California Press.

Jacobsen, S. & Wyman, C. (2001). Heat transfer considerations in design of a batch tube reactor for biomass hydrolysis. *Appl Biochem Biotechnol* **91-93**, 377-386.

Lloyd, T. & Wyman, C. (2003). Application of a depolymerization model for predicting thermochemical hydrolysis of hemicellulose. *Appl Biochem Biotechnol* **105**, 53-67.

Lloyd, T. A. & Wyman, C. E. (2005). Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids. *Bioresour Technol* **96**, 1967-1977.

Majdoub, H., Roudesli, S. & Derantani, A. (2001). Polysaccharides from prickly pear peel and nopals of *Opuntia ficus-indica*: extraction, characterization and polyelectrolyte behaviour. *Polym Int* **50**, 552-560.

Malainine, M. E., Dufresne, A., Dupeyre, D., Mahrouz, M., Vuong, R. & Vignon, M. R. (2003). Structure and morphology of cladodes and spines of *Opuntia ficus-indica*. Cellulose extraction and characterisation. *Carbohydr Polym* **51**, 77-83.

Malainine, M. E., Mahrouz, M. & Dufresne, A. (2005). Thermoplastic nanocomposites based on cellulose microfibrils from *Opuntia ficus-indica* parenchyma cell. *Compos Sci Technol* **65**, 1520-1526.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* **31**, 426-428.

Mosier, N., Hendrickson, R., Ho, N., Sedlak, M. & Ladisch, M. R. (2005a). Optimization of pH controlled liquid hot water pretreatment of corn stover. *Bioresour Technol* **96**, 1986-1993.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M. & Ladisch, M. (2005b). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour Technol* **96**, 673-686.

Neureiter, M., Danner, H., Thomasser, C., Saidi, B. & Braun, R. (2002). Dilute-acid hydrolysis of sugarcane bagasse at varying conditions. *Appl Biochem Biotechnol* **98-100**, 49-58.

Öhgren, K., Bura, R., Lesnicki, G., Saddler, J. & Zacchi, G. (2007). A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. *Process Biochem* **42**, 834-839.

Phutela, U., Dhuna, V., Sandhu, S. & Chadha, B. S. (2005). Pectinase and polygalacturonase production by a thermophilic *Aspergillus fumigatus* isolated from decomposting orange peels. *Braz J Microbiol* **36**, 63-69.

Ragauskas, A. J., Williams, C. K., Davison, B. H., Britovsek, G., Cairney, J., Eckert, C. A., Frederick, W. J., Jr., Hallett, J. P., Leak, D. J. & other authors (2006). The path forward for biofuels and biomaterials. *Science* **311**, 484-489.

Rodriguez-Felix, A. & Cantwell, M. (1988). Developmental changes in composition and quality of prickly pear cactus cladodes (nopalitos). *Plant Food Hum Nutr* **38**, 88-93.

Rodríguez-Garcia, M., de Lira, C., Hernández-Becerra, E., Cornejo-Villegas, M., Palacios-Fonseca, A., Rojas-Molina, I., Reynoso, R., Quintero, L., Del Real, A. & other authors (2007). Physicochemical characterization of nopal pads (*Opuntia ficus-indica*) and dry vacuum nopal powders as a function of the maturation. *Plant Food Hum Nutr* **62**, 107-112.

Ruth, M. F. & Thomas, S. R. (2003). The effect of corn stover composition on ethanol process economics. http://www.eere.energy.gov/biomass/pdfs/34040.pdf.

Shevchenko, S. M., Chang, K., Robinson, J. & Saddler, J. N. (2000). Optimization of monosaccharide recovery by post-hydrolysis of the water-soluble hemicellulose component after steam explosion of softwood chips. *Bioresour Technol* **72**, 207-211.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J. & Templeton, D. (2004a). *Determination of ash biomass*. Golden, CO.: National Renewable Energy Laboratory.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. & Crocker, D. (2004b). *Determination of extractives in biomass*. Golden, CO: National Renewable Energy Laboratory.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. & Crocker, D. (2006). *Determination of structural carbohydrates and lignin in biomass*. Golden, CO: National Renewable Energy Laboratory.

Stintzing, F. C. & Carle, R. (2005). Cactus stems (*Opuntia* spp.): a review on their chemistry, technology and uses. *Mol Nutr Food Res* **49**, 175-194.

Sun, Y. & Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresour Technol* **83**, 1-11.

Yang, B. & Wyman, C. E. (2008). Pretreatment: the key to unlocking low-cost cellulosic ethanol. *Biofuels Bioprod Bioref* **2**, 26-40.

CHAPTER 3

FERMENTATION PROFILES OF *KLUYVEROMYCES MARXIANUS* AND *SACCHAROMYCES CEREVISIAE* IN A SIMULATED *OPUNTIA FICUS-INDICA* CLADODE BIOMASS HYDROLYSATE

CONTENTS

1. Abstract

The fermentation profiles of *Kluyveromyces marxianus* Y-2791 and *Saccharomyces cerevisiae* Y-0528 were evaluated for 48 h at 40^{o}C and 35^{o}C, respectively, in a chemically-defined medium containing (per litre) 46 g glucose, 6 g xylose, 9 g galactose, 11 g arabinose and 10 g fructose. The sugars were similar in concentration to an enzymatic hydrolysate of *Opuntia ficus-indica* cladode biomass. Both yeasts were cultivated under non-aerated conditions, whereas *K. marxianus* was also cultivated using oxygen-limited conditions at a dissolved oxygen tension controlled between 0.5 to 1% of saturation. Under non-aerated conditions, *K. marxianus* produced a maximum ethanol concentration of 25 g l^{-1}, which was comparable to the 25.8 g l^{-1} obtained with *S. cerevisiae*, albeit at a volumetric ethanol productivity that was almost four-fold lower. In contrast to *S. cerevisiae*, *K. marxianus* assimilated xylose and arabinose, but failed to completely utilise galactose. However, during oxygen-limited cultivation, *K. marxianus* depleted all of the hexose and pentose sugars completely. *K. marxianus* produced 22.1 g ethanol l^{-1} at a volumetric productivity similar to that of *S. cerevisiae*, but consumed the ethanol once the sugars were depleted. This study demonstrated the importance of oxygen control during fermentation using *K. marxianus*, as well as the potential of this yeast as a viable alternative to *S. cerevisiae* for bioethanol production from mixed sugar substrates at elevated temperatures.

2. Introduction

The production of bioethanol from lignocellulosic biomass is an alternative to the use of conventional feedstocks such as sugar cane and maize, also referred to as first generation feedstocks (Champagne, 2007; Farrell *et al.*, 2006; Gray *et al.*, 2006). Unlike these, lignocellulosic biomass has the advantage of being abundant and its use as feedstock for fuel production does not compete with human food or animal feed. First generation feedstocks normally contain sucrose or starch as their main carbohydrates, and the respective sugar monomers are easily fermented by yeasts. However, lignocellulosic materials yield a mix of different hexoses (e.g. glucose, fructose, galactose, mannose), and pentoses (e.g. xylose, arabinose) after pretreatment and enzymatic hydrolysis (Kumar *et al.*, 2009; Mosier *et al.*, 2005; Sánchez & Cardona, 2008). Thus, the fermentation of lignocellulosic biomass hydrolysates requires a micro-organism that can efficiently convert

all the available sugars to ethanol to be economically competitive (Hahn-Hägerdal *et al.*, 2007; Jeffries, 2006; Sánchez & Cardona, 2008; van Maris *et al.*, 2006).

Saccharomyces cerevisiae is the most established micro-organism used in existing commercial-scale ethanol industries, mainly due to its ability to produce ethanol at stoichiometric yields from hexose sugars, in addition to its remarkable tolerance to high ethanol concentrations, toxic inhibitors and high osmotic pressures (Claassen *et al.*, 1999; Hahn-Hägerdal *et al.*, 2007; Jeffries & Jin, 2004). The inability of *S. cerevisiae* to utilise or ferment pentose sugars, except after extensive metabolic or evolutionary engineering, is a major drawback facing the use of this yeast for lignocellulosic-ethanol production. In addition, the lower optimum temperature range of 25 to 35^{o}C required for fermentations with *S. cerevisiae* creates an obstacle for processes such as SSF (simultaneous saccharification and fermentation) which are best operated at 45 to 50^{o}C (Sun & Cheng, 2002). Fermentation at elevated temperatures has several advantages, including lower cooling costs, enabling continuous ethanol recovery during fermentation, having a minimal risk of microbial contamination, and also resulting in an improved efficiency of the SSF process (Abdel-Banat *et al.*, 2010; Anderson *et al.*, 1986; Fonseca *et al.*, 2008; Limtong *et al.*, 2007).

One yeast capable of high temperature fermentation is the thermotolerant *Kluyveromyces marxianus*, which has been reported to produce ethanol at temperatures above 40^{o}C and to have a maximum growth temperature of up to 52^{o}C (Anderson *et al.*, 1986; Banat & Marchant, 1995; Nonklang *et al.*, 2008; Rodrussamee *et al.*, 2011). *K. marxianus* can also assimilate a greater range of lignocellulosic sugars such as cellobiose, xylose and arabinose, in addition to having a higher growth rate than *S. cerevisiae* (Lane & Morrissey, 2010; Nonklang *et al.*, 2008). *K. marxianus* is, however, less ethanol tolerant than *S. cerevisiae* and it requires oxygen to utilise some sugars (Fonseca *et al.*, 2008). Unlike *S. cerevisiae, K. marxianus* cannot grow under strictly anaerobic conditions (Bellaver *et al.*, 2004). It is also Crabtree negative and ethanol production is almost exclusively linked to an oxygen limitation (Bellaver *et al.*, 2004; Fonseca *et al.*, 2008). Nevertheless, its advantages render *K. marxianus* a viable alternative to *S. cerevisiae* as an ethanol producer from lignocellulosic hydrolysates.

The purpose of this study was to evaluate the performance of *K marxianus* as an ethanol producer at elevated temperatures in a sugar mixture containing hexoses and pentoses. *S. cerevisiae* was used as the benchmark. Since the *Opuntia ficus-indica* cladode was the

lignocellulosic biomass feedstock focused on in this dissertation, the sugar concentrations in the medium were constituted similar to the cladode enzymatic hydrolysate for comparison. This study also provided information on the fermentation profiles of *K. marxianus* under both oxygen-limited and non-aerated conditions.

3. Materials and Methods

Yeast strains

Kluyveromyces marxianus UOFS Y-2791, isolated from the agave plant (*Agave americana)* and *Saccharomyces cerevisiae* UOFS Y-0528, a commercial wine yeast strain obtained from the University of the Free State MIRCEN yeast culture collection, were used in the fermentation experiments. Pure colonies of the yeast strains were maintained on GPY agar slants containing (per litre): 40 g glucose, 5 g peptone, 5 g yeast extract and 20 g agar.

Inoculum preparation

Pre-cultures of *K. marxianus* and *S. cerevisiae* were cultivated in a sterile semi-defined chemical medium containing (per litre): 30 g glucose, 0.25 g citric acid, 3 g yeast extract, 5 g $(NH_4)_2SO_4$, 9.6 g KH_2PO_4, 0.76 g K_2HPO_4, 0.75 g $MgSO_4{\cdot}7H_2O$, 0.05 g $CaCl_2{\cdot}2H_2O$, 0.1 g NaCl and 1 ml of a trace elements stock solution. The trace elements stock contained (per litre) 0.035 g $FeSO_4{\cdot}7H_2O$, 0.007 g $MnSO_4{\cdot}7H_2O$, 0.011 g $ZnSO_4{\cdot}7H_2O$, 0.001 g $CuSO_4{\cdot}5H_2O$, 0.002 g $CoSO_4{\cdot}6H_2O$, 0.0013 g $Na_2MoO_4{\cdot}2H_2O$, 0.002 g H_3BO_3, 0.0004 g KI and 0.0016 g $Al_2(SO_4)_3{\cdot}18H_2O$. The pH of the medium was adjusted to pH 5.5 by addition of 3 M KOH. A loopful of cells from 24 h agar slants was inoculated into 500 ml side-arm flasks with cotton wool plugs containing 50 ml of the growth medium, and incubated at 34^{o}C on an orbital shaker at 200 rpm. These pre-cultures were grown until the cells reached late exponential phase, which had previously been determined to be after 9 and 11 h for *K. marxianus* and *S. cerevisiae*, respectively. Active cultures for inoculating the fermentation medium were subsequently prepared by inoculating 1 ml from each pre-culture into another set of shake flasks containing the above medium. They were also incubated until late exponential phase and immediately used to inoculate the fermentation medium.

Fermentation medium

A chemically-defined medium containing a sugar mixture similar in composition to the *O. ficus-indica* cladode enzymatic hydrolysate was used for fermentation purposes. The medium contained (per litre) 46 g glucose, 6 g xylose, 9 g galactose, 11 g arabinose, 10 g fructose, 0.5 g citric acid, 5 g $(NH_4)_2HPO_4$, 0.75 g KH_2PO_4, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.1 g NaCl, 1 ml of a trace elements stock solution as described above and 4 ml of a filter-sterilized vitamin stock solution. The vitamin stock solution contained (per litre) 0.025 g biotin, 0.5 g calcium pantothenate, 0.5 g nicotinic acid, 0.1 g *p*-aminobenzoic acid, 0.5 g pyridoxine hydrochloride, 0.5 g thiamine hydrochloride, and 12.5 g *m*-inositol. The sugar mixture was transferred into a 500 ml Erlenmeyer flask, whereas the mineral salts solution was transferred into the bioreactor to be used for the experiment and the pH adjusted to pH 5.0 by addition of 3 M KOH. Both were sterilized by autoclaving at 121^{o}C for 20 min. After cooling, the vitamin stock solution was added to the sugar mixture in a laminar air flow cabinet and aseptically transferred into the bioreactor.

Fermentation conditions

Fermentations were carried out in a 1.6 l Biostat® B-plus stirred tank reactor (Sartorius Stedim Biotech, Göttingen, Germany) (Figure 3.1) using a 1 l culture volume. An inoculum size of 5% (v/v) was used by aseptically adding 50 ml of inoculum to 950 ml chemically-defined medium. The starting optical density at 690 nm (OD_{690}) was 0.26, equivalent to a biomass concentration of 0.12 g l^{-1} of *K. marxianus* cells and 0.1 g l^{-1} of *S. cerevisiae* cells. The pH was maintained at pH 5.0 by automatic titration with either 3 M KOH or 3 N H_2SO_4. *S. cerevisiae* was grown at 35^{o}C, whereas 40^{o}C was used for *K. marxianus*. These temperatures were based on the upper limit of optimum values obtained from temperature profile experiments previously performed on both yeasts (see Discussion). Both yeasts were cultivated under non-aerated conditions and an additional cultivation under oxygen-limited conditions was carried out with *K. marxianus*. During the non-aerated cultivation the culture was kept homogenous by maintaining a constant stirrer speed of 100 rpm, whereas during cultivation under oxygen-limited conditions, the culture was sparged with sterile air at an aeration rate of 0.3 l min^{-1} and the dissolved oxygen tension (DOT) was maintained at 0.5 to 1% of saturation by automatic adjustment of stirrer speed. The exhaust gas condenser was cooled to 4^{o}C. Cultivation was done for up to 48 h and samples were

collected at regular intervals for analyses. All fermentations were carried out in duplicate and mean values are reported.

Figure 3.1 The Biostat B-plus reactor and control unit used for the fermentation experiments

Analytical procedures

The composition of the exhaust gas during fermentation was continuously monitored using an Uras 10E infrared and a Magnos 6G paramagnetic gas analyser (Hartman and Braun, Frankfurt, Germany) for determination of CO_2 and O_2, respectively. Cell concentrations were monitored by measuring culture turbidity against a medium blank with a Photolab S6 spectrophotometer (WTW, Weilheim, Germany) at a wavelength of 690 nm (OD_{690}). The dry cell weight was determined using duplicate 10 ml samples that were centrifuged, washed with distilled water and dried overnight at 105^{o}C. Samples collected for determining sugar utilisation and product formation were immediately cooled on ice before centrifugation at 10 600 *x g* using an Eppendorf 5430 R centrifuge (Eppendorf AG, Hamburg, Germany) at 4^{o}C. The supernatants were filtered through a 0.45 µm filter prior to chromatographic analysis. Supernatants not immediately analyzed were stored at -20^{o}C. The concentrations of glucose, xylose galactose, arabinose and fructose were

determined by HPLC using an Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA) at 85^{o}C at a flow rate of 0.4 ml min^{-1} with milliQ water as eluent. The ethanol concentration was determined with a Shimadzu GC 2010 gas chromatograph (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a Phenomenex ZB wax column (Phenomenex, Torrance, CA, USA) and a flame ionization detector (FID), with hydrogen as carrier gas at 35 cm^{3}. The oven temperature was 80^{o}C for 2.5 min, ramped at 25^{o}C min^{-1} to 180^{o}C with a 2 min isothermal period. The injection volume was 0.6 µl at a 50:1 split ratio. The injector and detector temperatures were 150^{o}C and 300^{o}C, respectively.

4. Results

4.1 Non-aerated cultivation of *K. marxianus* and *S. cerevisiae*

Under non-aerated conditions, a maximum ethanol concentration of 25.8 g l^{-1} (mean value) was reached after 36 h of cultivation with *S. cerevisiae*, whereas 25.0 g l^{-1} was obtained with *K. marxianus* at 40^{o}C after 48 h due to its maximum volumetric ethanol productivity (Q_p) being almost four-fold lower than that of *S. cerevisiae* (Figure 3.2; Table 3.1). *S. cerevisiae* gave a specific ethanol productivity (q_p) that ranged between 3.27 and 4.14 g g^{-1} h^{-1} calculated during the first 7 h of ethanol production, whereas the specific productivity of *K. marxianus* was much lower and in the range of 0.33 and 0.93 g g^{-1} h^{-1} between 5 and 21 h of fermentation (Figure 3.3). Ethanol yield coefficients on utilised sugars corresponding 0.38 and 0.39 were obtained with *K. marxianus* and *S. cerevisiae*, respectively. Moreover, the ethanol yield coefficients on total sugars were also comparable: 0.30 with *K. marxianus* and 0.31 with *S. cerevisiae*. *K. marxianus* produced 1.9 g biomass l^{-1}, which was lower than the 2.6 g l^{-1} produced by S*. cerevisiae* (Figure 3.2a,b; Table 3.1). *K. marxianus* had a maximum specific growth rate (μ_{max}) of 0.47 h^{-1} which was higher than that of *S. cerevisiae* (0.38 h^{-1}), but only over a brief initial time period. The maximum volumetric biomass productivity ($Q_{p\ biomass}$) of *K. marxianus* (0.39 g l^{-1} h^{-1}) was also higher than that of *S. cerevisiae* (0.27 g l^{-1} h^{-1}).

As expected, both yeasts utilised glucose and fructose first, whereas assimilation of galactose only increased after their depletion (Figure 3.2a,b). *S. cerevisiae* completely consumed both glucose and fructose within 12 h, whereas *K. marxianus* required 20 h for fructose and 30 h for glucose depletion. Galactose was also completely utilised by *S. cerevisiae*. However, only 3.4 g galactose l^{-1}, which was about 30% of the initial

galactose concentration, was utilised by *K. marxianus* (Figure 3.2e). The pentoses xylose and arabinose were also poorly utilised by *K. marxianus*. Some decrease in the xylose and, to a lesser extent, arabinose concentration was detected during cultivation with this *S. cerevisiae* strain (Figure 3.2d).

4.2 Oxygen-limited cultivation of *K. marxianus*

Under this aeration condition an ethanol concentration of 22.1 g l^{-1} (mean value) was achieved after 12 h of cultivation. This was the lowest concentration obtained during the fermentation experiments, although it was produced at a maximum ethanol productivity of 2.98 g l^{-1} h^{-1}, which was close to the 3.28 g l^{-1} h^{-1} achieved by *S. cerevisiae* under non-aerated conditions (Table 3.1). However, the ethanol produced by *K. marxianus* was assimilated once the hexoses sugars were depleted (Figure 3.2c). The specific ethanol productivity of *K. marxianus* decreased over time and was within the range of 1.27 to 3.20 g g^{-1} h^{-1} (Figure 3.3). The ethanol yield coefficients on utilised as well as on total sugars (0.31 and 0.26, respectively) were lower than what was obtained when the same yeast was cultivated without aeration. This was probably as a result of the yeast channelling the ethanol and the residual sugars towards biomass production. *K. marxianus* produced the highest biomass concentration of 8.6 g l^{-1} with a corresponding biomass yield coefficient of 0.1, as well as the highest maximum specific growth rate of 0.52 h^{-1}, under oxygen limitation (Figure 3.2c, Table 3.1). The maximum biomass productivity of *K. marxianus* under these conditions was 0.55 g biomass l^{-1} h^{-1}, which higher than that of the same yeast when cultivated without aeration (0.39 g l^{-1} h^{-1}), as well as that of *S. cerevisiae* (0.27 g l^{-1} h^{-1}).

In contrast with the non-aerated cultivation profile, galactose was completely utilised, as were xylose and arabinose. The uptake rate of the pentoses increased following depletion of the hexoses (Figure 3.2c).

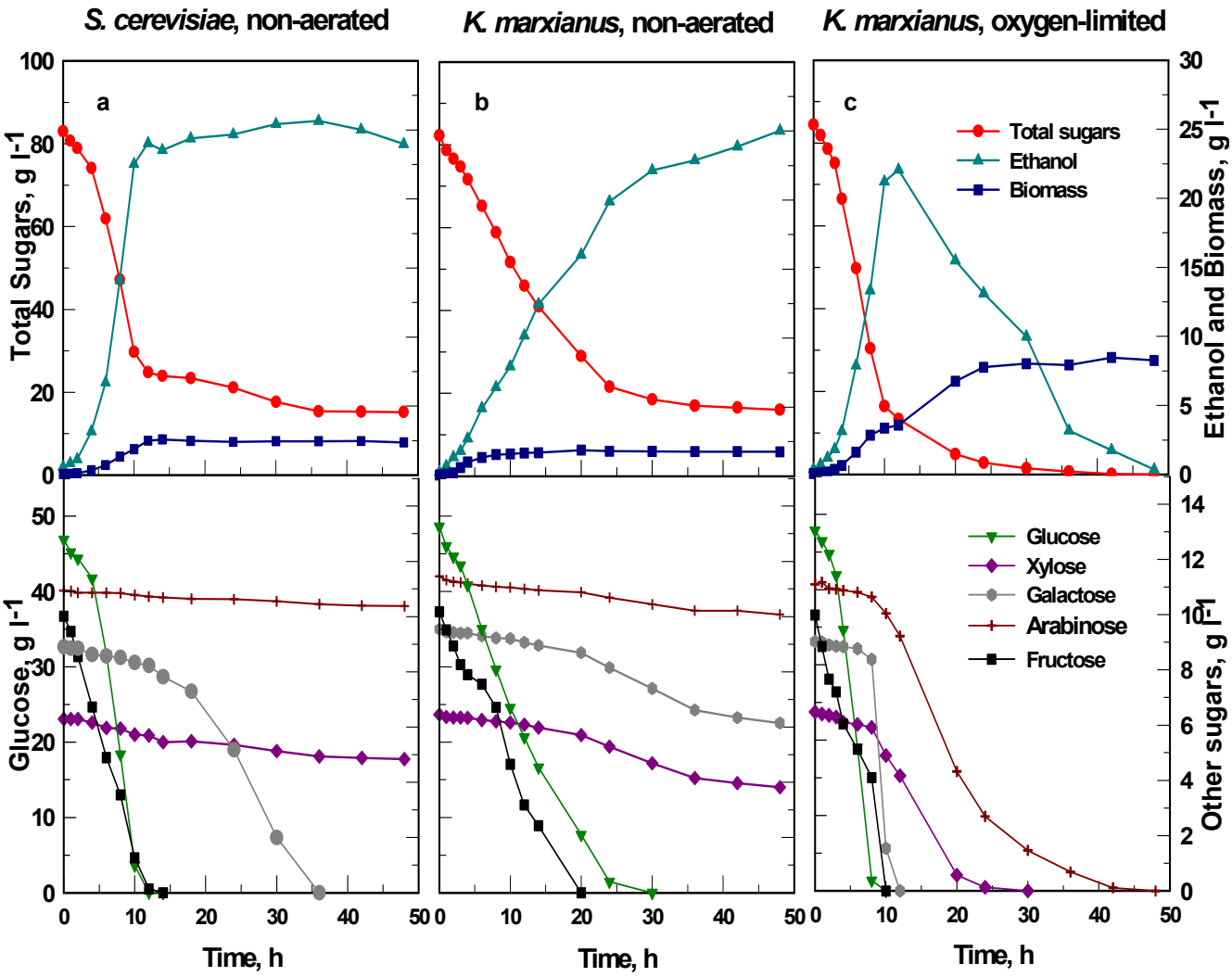


Figure 3.2 Fermentation profiles of *S. cerevisiae* Y-0528 and *K. marxianus* Y-2791 in a chemically defined medium containing a sugar mixture similar to an enzymatic hydrolysate of *O. ficus-indica* cladode biomass: **(a)** *S. cerevisiae,* non-aerated. **(b)** *K. marxianus,* non-aerated. **(c)** *K. marxianus,* oxygen-limited.

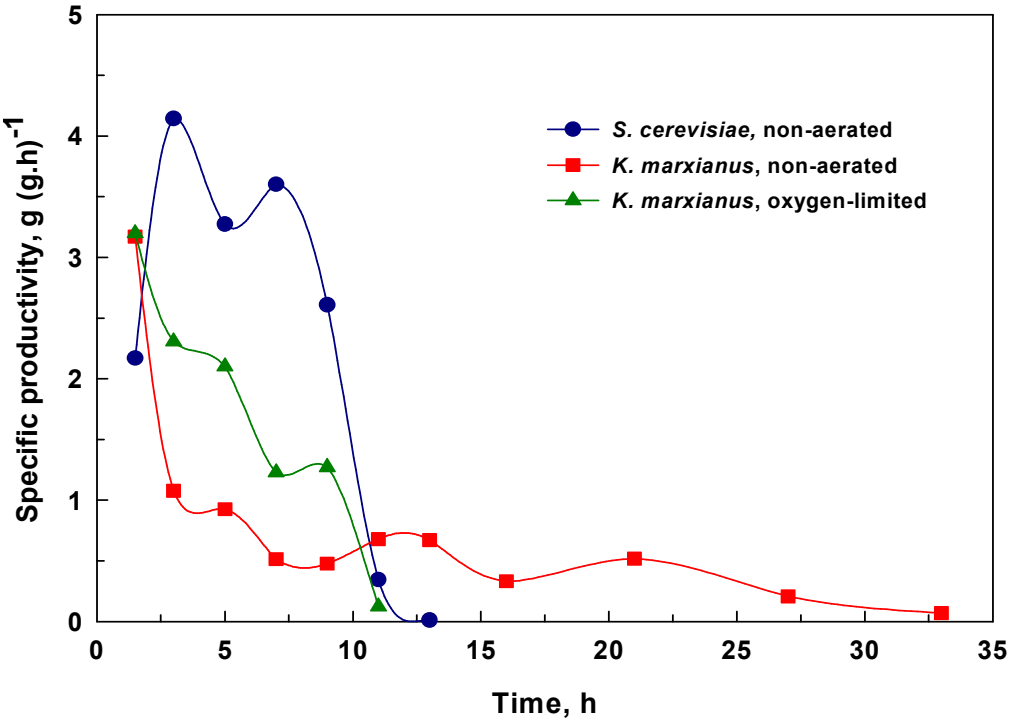


Figure 3.3 Profiles of specific ethanol productivity versus time during cultivation of *S. cerevisiae* and *K. marxianus* under non-aerated and oxygen-limited conditions.

Table 3.1 Fermentation parameters of *S. cerevisiae* Y-0528 and *K.* marxianus Y-2791 in a chemically defined medium containing a sugar mixture resembling an enzymatic hydrolysate of *O. ficus-indica* cladode biomass.

	S. cerevisiae	***K. marxianus***	
Parameter	**Non-aerated**	**Non-aerated**	**Oxygen-limited**
Residual sugars, g l^{-1}	15.3	18.2	0.0
μ_{max}, h^{-1}	0.38	0.47	0.52
Q_p, g ethanol l^{-1} h^{-1}	3.28	0.85	2.98
Q_p, g biomass l^{-1} h^{-1}	0.27	0.39	0.55
Maximum ethanol, g l^{-1}	25.8	25.0	22.1
Maximum biomass, g l^{-1}	2.6	1.90	8.5
Ethanol yield on utilised sugars	0.39	0.38	0.31
Ethanol yield on total sugars	0.31	0.30	0.26
Biomass yield on utilised sugars	0.04	0.04	0.1
Biomass yield on total sugars	0.03	0.03	0.1

5. Discussion

The temperature and ethanol tolerance of both yeasts used in this study were previously investigated by another member of the research group (unpublished). According to those results, *K. marxianus* Y-2791 had a higher optimum temperature range of 37 to 40^{o}C and a maximum growth temperature of 47^{o}C compared to the 32 to 35^{o}C optimum temperature range and 39^{o}C maximum growth temperature of *S. cerevisiae* Y-0528. The temperature values recorded for *K. marxianus* are in agreement with previous reports on its superior thermotolerance over *S. cerevisiae* (Anderson *et al.*, 1986; Banat *et al.*, 1996; Nonklang *et al.*, 2008). According to reports in literature, *K. marxianus*, however, has a lower tolerance to ethanol than *S. cerevisiae* (Banat *et al.*, 1996; Rosa & Sá-Correia, 1992). Similarly, from previous unpublished experiments in our laboratory, *S. cerevisiae* Y-0528 exhibited an ethanol tolerance of up to 112 g l^{-1}, this value representing the calculated upper limit for growth, whereas the ethanol tolerance of *K. marxianus* Y-2791 was only up to 84 g l^{-1}. Nevertheless, this value exceeded the ethanol concentrations likely to be reached during the fermentation of lignocellulosic hydrolysates. Therefore, the ethanol tolerance of *K. marxianus* Y-2791 was deemed sufficient for use of this yeast in the fermentation of lignocellulosic biomass feedstocks.

In this study, the non-aerated cultivation of *K. marxianus* was not completely anaerobic because of oxygen diffusion into the medium through several channels such as the silicone rubber tubing and air filter, all aided by continuous mixing of the medium at 100 rpm. No precautions to ensure strict anaerobic conditions, such as sparging with nitrogen gas, were taken. The dissolved oxygen tension in the medium dropped from 100% to 0.2% of saturation during the first 30 minutes after inoculation due to cell respiration. The above factors presumably created a severely oxygen-limited situation and the rate of oxygen leakage into the *K. marxianus* culture was sufficient for ethanol production, albeit at very slow rates (Figures 3.2b, 3.3). Under these conditions *K. marxianus* probably used respiro-fermentative metabolism. This behaviour of *K. marxianus* appeared to be similar to what was observed with its sister species, *Kluyveromyces lactis*, when cultivated under similar conditions (Merico *et al.*, 2009). The maximum ethanol concentration of 25 g l^{-1} as well as the ethanol yield achieved by *K. marxianus* without aeration was comparable to those of *S. cerevisiae,* although *K. marxianus* required a fermentation time of up to 48 h to reach that concentration, whereas *S. cerevisiae* required only 36 h to reach its maximum ethanol concentration. This was as a result of the greater specific and volumetric ethanol productivity of *S. cerevisiae* compared to *K. marxianus* (Figure 3.3, Table 3.1). This high

fermentation rate is one of the reasons why *S. cerevisiae* is often preferred over other yeasts such as *K. marxianus* for fermentation under semi-anaerobic/anaerobic conditions.

K. marxianus exhibited a higher μ_{max} value than *S. cerevisiae*, similar to previous reports (Fonseca *et al.*, 2007; Lane & Morrissey, 2010) and had a shorter lag phase than *S. cerevisiae* during the fermentation (results not shown). However, this higher growth rate did not necessarily translate into more biomass production, unless oxygen was available. The dissolved oxygen present in the medium at the start of the experiment was sufficient for *K. marxianus* to produce up to 1 g biomass l^{-1} during the first 6 h of cultivation. As the biomass increased, the oxygen limitation also became much more stringent in the bioreactor because the volumetric oxygen uptake rate increased whereas the rate of oxygen diffusion into the culture broth remained essentially constant. At this point the DOT decreased to below the detection limit. On the other hand, when the DOT was controlled between 0.5 to 1% of saturation, *K. marxianus* produced up to 3.6 g of biomass l^{-1} during the first 12 h of cultivation, at which time the hexoses became depleted. It then exhibited diauxic growth, similar to what occurs with *S. cerevisiae* in aerobic glucose batch cultures (Käppeli, 1986), producing a further 4.9 g of biomass l^{-1} from ethanol, xylose and arabinose. This behaviour further highlighted the importance of DOT control during cultivations with *K. marxianus*.

The control of the DOT at between 0.5 to 1% of saturation enhanced the specific as well as the volumetric ethanol productivity of *K. marxianus* compared to cultivation without aeration. Thus the difference in the Q_p values recorded during non-aerated and oxygen-limited cultivation of *K. marxianus* was not only a consequence of the higher biomass concentration obtained under oxygen-limited conditions; the availability of oxygen enhanced ethanol production. The drawback of growing *K. marxianus* with aeration was, however, that as soon as the hexoses were depleted, the produced ethanol was subsequently utilised for biomass production (Figure 3.2c). Even at such very low DOT levels, *K. marxianus* was capable of aerobic utilisation of ethanol. To prevent ethanol assimilation, aeration would have to be cut off at some point during the fermentation, or a stricter DOT control measure would have to be put in place. However, the latter would require the use of a more sensitive dissolved oxygen probe and controller. The maximum DOT that would not permit ethanol assimilation is still unclear and further investigation is required.

Considering the utilisation of sugars under the two different aeration conditions investigated, glucose and fructose, the substrates capable of sustaining the highest growth rate, were the first to be assimilated as expected, due to catabolyte repression in addition to the higher affinity of both yeasts for these sugars in mixed sugar cultivations (Bisson & Fraenkel, 1983; Postma & Van den Broek, 1990; Reifenberger *et al.*, 1997). Galactose utilisation appeared to be repressed by glucose and only increased when glucose was almost completely exhausted.

K. marxianus consumed galactose poorly under non-aerated conditions, but completely utilised it when aeration was provided (Figure 3.2b,c). A similar observation was reported by Rodrussamee *et al*. (2011), who performed fermentations in stationary flasks using *K. marxianus* in YP medium supplemented with a sugar mixture containing equal concentrations of glucose and galactose. The authors reported that glucose was completely consumed, whereas less than half the available galactose was utilised. A likely explanation for this behaviour could be that *K. marxianus* exhibited the phenomenon referred to as the Kluyver effect.

The initial definition of the Kluyver effect was that certain yeasts could utilize particular disaccharides aerobically but not anaerobically, although these yeasts could use one or more of the component hexoses anaerobically (Sims & Barnett, 1978). More recently the Kluyver effect was redefined as 'the inability to ferment certain disaccharides to ethanol and CO_2, even though respiratory metabolism of the disaccharides and alcoholic fermentation of the component hexose(s) can occur' (Weusthuis *et al.*, 1994). In recent times, however, several other authors have described the Kluyver effect to include sugars other than disaccharides, notably galactose (Barnett & Entian, 2005; Fukuhara, 2003; Goffrini *et al.*, 2002). The rationale behind this respiration-dependent assimilation of certain sugars is still not clear but it is believed to be due to the interplay of several factors involving a decreased rate of transport and metabolism of certain sugars under anaerobic or anoxic conditions (Fukuhara, 2003; Goffrini *et al.*, 2002; Rodrussamee *et al.*, 2011). *S. cerevisiae* does not, however, exhibit the Kluyver effect on galactose.

It also has been suggested that the poor utilisation of the pentoses xylose and arabinose by *K. marxianus* in the absence of aeration was due to the incapability of NADH oxidation by the mitochondrial electron transport chain (Rodrussamee *et al.*, 2011).

S. cerevisiae does not utilise xylose or arabinose. However, there have been some earlier reports indicating the detection of low activities of xylose reductase (XR) and xylitol

dehydrogenase (XR) in *S. cerevisiae*, enabling it to utilise some xylose when another substrate such as glucose or galactose is co-metabolised (Batt *et al.*, 1986; van Zyl *et al.*, 1989). The disappearance of about 1.5 g xylose l^{-1} from the medium during the 48 h cultivation of *S. cerevisiae* Y-0528 might suggest that this commercial strain had some capacity for xylose utilisation, or it could have been as a result of slight discrepancies in the HPLC analysis. However, no firm conclusion can be drawn and further investigation is required. The apparent decrease of 0.6 g l^{-1} in the arabinose concentration was also probably due to HPLC analytical error, rather than uptake by *S. cerevisiae*.

These results have shown that with the appropriate control of the DOT, *K. marxianus* had the ability to produce ethanol at rates close to what was obtainable with *S. cerevisiae*, in addition to the complete utilisation of hexose and pentose sugars. Even without DOT control, *K. marxianus* still produced an ethanol concentration comparable to that obtained with *S. cerevisiae*, at a higher temperature of 40^{o}C. Fermentations will, however, have to be carried out on the actual *O. ficus-indica* hydrolysate and compared with these results to further assess the potential of *K. marxianus* as an alternative to *S. cerevisiae* for bioethanol production at elevated temperatures.

6. References

Abdel-Banat, B., Hoshida, H., Ano, A., Nonklang, S. & Akada, R. (2010). High-temperature fermentation: how can processes for ethanol production at high temperatures become superior to the traditional process using mesophilic yeast? *Appl Environ Microbiol* **85**, 861-867.

Anderson, P. J., McNeil, K. & Watson, K. (1986). High-efficiency carbohydrate fermentation to ethanol at temperatures above 40^{o}C by *Kluyveromyces marxianus* var. *marxianus* isolated from sugar mills. *Appl Environ Microbiol* **51**, 1314-1320.

Banat, I. M. & Marchant, R. (1995). Characterization and potential industrial applications of five novel, thermotolerant, fermentative, yeast strains. *World J Microbiol Biotechnol* **11**, 304-306.

Banat, I. M., Singh, D. & Marchant, R. (1996). The use of a thermotolerant fermentative *Kluyveromyces marxianus* IMB3 yeast strain for ethanol production. *Acta Biotechnol* **16**, 215-223.

Barnett, J. A. & Entian, K. D. (2005). A history of research on yeasts 9: regulation of sugar metabolism. *Yeast* **22**, 835-894.

Batt, C. A., Caryallo, S., Easson, D. D., Akedo, M. & Sinskey, A. J. (1986). Direct evidence for a xylose metabolic pathway in *Saccharomyces cerevisiae*. *Biotechnol Bioeng* **28**, 549-553.

Bellaver, L. H., de Carvalho, N. M. B., Abrahão-Neto, J. & Gombert, A. K. (2004). Ethanol formation and enzyme activities around glucose-6-phosphate in *Kluyveromyces marxianus* CBS 6556 exposed to glucose or lactose excess. *FEMS Yeast Res* **4**, 691-698.

Bisson, L. F. & Fraenkel, D. G. (1983). Involvement of kinases in glucose and fructose uptake by *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* **80**, 1730-1734.

Champagne, P. (2007). Feasibility of producing bio-ethanol from waste residues: A Canadian perspective: Feasibility of producing bio-ethanol from waste residues in Canada. *Resour Conserv Recy* **50**, 211-230.

Claassen, P. A. M., van Lier, J. B., Lopez Contreras, A. M., van Niel, E. W. J., Sijtsma, L., Stams, A. J. M., de Vries, S. S. & Weusthuis, R. A. (1999). Utilisation of biomass for the supply of energy carriers. *Appl Microbiol Biotechnol* **52**, 741-755.

Farrell, A. E., Plevin, R. J., Turner, B. T., Jones, A. D., O'Hare, M. & Kammen, D. M. (2006). Ethanol can contribute to energy and environmental goals. *Science* **311**, 506-508.

Fonseca, G., Heinzle, E., Wittmann, C. & Gombert, A. (2008). The yeast *Kluyveromyces marxianus* and its biotechnological potential. *Appl Microbiol Biotechnol* **79**, 339-354.

Fonseca, G. G., Gombert, A. K., Heinzle, E. & Wittmann, C. (2007). Physiology of the yeast *Kluyveromyces marxianus* during batch and chemostat cultures with glucose as the sole carbon source. *FEMS Yeast Res* **7**, 422-435.

Fukuhara, H. (2003). The Kluyver effect revisited. *FEMS Yeast Res* **3**, 327-331.

Goffrini, P., Ferrero, I. & Donnini, C. (2002). Respiration-dependent utilization of sugars in yeasts: a determinant role for sugar transporters. *J Bacteriol* **184**, 427-432.

Gray, K. A., Zhao, L. & Emptage, M. (2006). Bioethanol. *Curr Opin Chem Biol* **10**, 141-146.

Hahn-Hägerdal, B., Karhumaa, K., Fonseca, C., Spencer-Martins, I. & Gorwa-Grauslund, M. (2007). Towards industrial pentose-fermenting yeast strains. *Appl Microbiol Biotechnol* **74**, 937-953.

Jeffries, T. W. & Jin, Y. S. (2004). Metabolic engineering for improved fermentation of pentoses by yeasts. *Appl Microbiol Biotechnol* **63**, 495-509.

Jeffries, T. W. (2006). Engineering yeasts for xylose metabolism. *Curr Opin Biotechnol* **17**, 320-326.

Käppeli, O. (1986). Regulation of carbon metabolism in *Saccharomyces cerevisiae* and related yeasts. *Adv Microb Physiol* **28**, 181-209.

Kumar, P., Barrett, D. M., Delwiche, M. J. & Stroeve, P. (2009). Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Ind Eng Chem Res* **48**, 3713-3729.

Lane, M. M. & Morrissey, J. P. (2010). *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. *Fungal Biol Rev* **24**, 17-26.

Limtong, S., Sringiew, C. & Yongmanitchai, W. (2007). Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. *Bioresour Technol* **98**, 3367-3374.

Merico, A., Galafassi, S., Piskur, J. & Compagno, C. (2009). The oxygen level determines the fermentation pattern in *Kluyveromyces lactis*. *FEMS Yeast Res* **9**, 749-756.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M. & Ladisch, M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour Technol* **96**, 673-686.

Nonklang, S., Abdel-Banat, B. M. A., Cha-aim, K., Moonjai, N., Hoshida, H., Limtong, S., Yamada, M. & Akada, R. (2008). High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042. *Appl Environ Microbiol* **74**, 7514-7521.

Postma, E. & Van den Broek, P. J. (1990). Continuous-culture study of the regulation of glucose and fructose transport in *Kluyveromyces marxianus* CBS 6556. *J Bacteriol* **172**, 2871-2876.

Reifenberger, E., Boles, E. & Ciriacy, M. (1997). Kinetic characterization of individual hexose transporters of *Saccharomyces cerevisiae* and their relation to the triggering mechanisms of glucose repression. *Eur J Biochem* **245**, 324-333.

Rodrussamee, N., Lertwattanasakul, N., Hirata, K., Suprayogi, S., Limtong, S., Kosaka, T. & Yamada, M. (2011). Growth and ethanol fermentation ability on hexose and pentose sugars and glucose effect under various conditions in thermotolerant *Kluyveromyces marxianus*. *Appl Microbiol Biotechnol* **90**, 1573-1586.

Rosa, M. F. & Sá-Correia, I. (1992). Ethanol tolerance and activity of plasma membrane ATPase in *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*. *Enzyme Microb Technol* **14**, 23-27.

Sánchez, O. J. & Cardona, C. A. (2008). Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresour Technol* **99**, 5270-5295.

Sims, A. P. & Barnett, J. A. (1978). The requirement of oxygen for the utilization of maltose, cellobiose and D-galactose by certain anaerobically fermenting yeasts (Kluyver effect). *J Gen Microbiol* **106**, 277-288.

Sun, Y. & Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresour Technol* **83**, 1-11.

van Maris, A., Abbott, D., Bellissimi, E., van den Brink, J., Kuyper, M., Luttik, M., Wisselink, H., Scheffers, W., van Dijken, J. & other authors (2006). Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. *Antonie van Leeuwenhoek* **90**, 391-418.

van Zyl, C., Prior, B. A., Kilian, S. G. & Kock, J. L. F. (1989). D-Xylose utilization by *Saccharomyces cerevisiae*. *J Gen Microbiol* **135**, 2791-2798.

Weusthuis, R. A., Visser, W., Pronk, J. T., Scheffers, W. A. & van Dijken, J. P. (1994). Effects of oxygen limitation on sugar metabolism in yeasts: a continuous-culture study of the Kluyver effect. *Microbiology* **140**, 703-715.

CHAPTER 4

ETHANOL PRODUCTION FROM *O. FICUS-INDICA* CLADODE HYDROLYSATE USING *KLUYVEROMYCES MARXIANUS* AND *SACCHAROMYCES CEREVISIAE*

CONTENTS

1. Abstract

Kluyveromyces marxianus UOFS Y-2791 and *Saccharomyces cerevisiae* UOFS Y-0528, cultivated at 40^{o}C and 35^{o}C respectively, were investigated for bioethanol production using an *Opuntia ficus-indica* cladode hydrolysate slurry with a water-insoluble solids content or 14% as biomass feedstock. Both yeasts were cultivated without aeration and the performance of *K. marxianus* was also investigated under oxygen-limited conditions where the dissolved oxygen tension (DOT) was controlled at less than 1% of saturation. Their fermentation profiles were compared using separate hydrolysis and fermentation (SHF) and simultaneous hydrolysis and fermentation (SSF) process configurations. *K. marxianus* utilised galactose poorly in the absence of aeration, whereas it completely consumed all the hexoses under oxygen-limited conditions. Both *K. marxianus* and *S. cerevisiae* gave similar ethanol yields in SHF and SSF under non-aerated conditions, although *K. marxianus* had a lower volumetric ethanol productivity than *S. cerevisiae*. Under oxygen-limited conditions *K. marxianus* gave a lower ethanol yield than obtained with both yeasts cultivated without aeration. However, *K. marxianus* achieved maximum volumetric ethanol productivities of 2.3 g l^{-1} h^{-1} and 1.57 g l^{-1} h^{-1} in SHF and SSF, respectively, which were higher than when it was cultivated without aeration, although the ethanol produced was consumed upon sugar depletion. The overall ethanol productivity in SSF was double that of SHF. The effect of inhibitors in the biomass hydrolysate appeared negligible. The highest ethanol concentration of 20.6 g l^{-1}, corresponding to approximately 70% of the theoretical maximum yield on total sugars in the hydrolysate, was an improvement on the 14 g l^{-1} previously reported elsewhere. This study demonstrated the feasibility of producing ethanol from *O. ficus-indica* cladodes, albeit at low concentrations, and that *K. marxianus* had potential as an alternative to *S. cerevisiae* for bioethanol production, especially at elevated temperatures, which is beneficial for SSF bioprocesses.

2. Introduction

The conversion of lignocellulosic biomass to ethanol involves pretreatment, enzymatic hydrolysis, fermentation of the released monomeric sugars by microorganisms, and lastly ethanol distillation (Hahn-Hägerdal *et al.*, 2006). The overall success of this process depends on the volume of ethanol produced per dry weight of the raw material, and obtaining an ethanol concentration of at least 4% (w/v) in the fermentation medium (García-Aparicio *et al.*, 2011). The sugar concentration in the enzymatic hydrolysate plays

a major role in the amount of ethanol produced during fermentation. A higher substrate content usually leads to higher concentration of hydrolysed sugars which should result in a high final ethanol concentration (García-Aparicio *et al.*, 2011; Sun & Cheng, 2002). In addition, starting with a high solids loading would enhance the economy of the process, because less energy would be required in the subsequent distillation and evaporation steps (Linde *et al.*, 2007; Olsson & Hahn-Hägerdal, 1996). Nevertheless, increasing the solids loading presents some difficulties such as end-product inhibition of the enzymes by glucose and cellobiose, higher amounts of inhibitors originating from pretreatment, and mass transport problems caused by the viscosity of the pretreated material (Linde *et al.*, 2007).

When enzymatic hydrolysis and fermentation are carried out in sequence, the process is referred to as separate hydrolysis and fermentation (SHF). However, both processes can be performed in one step known as simultaneous saccharification and fermentation (SSF) (Öhgren *et al.*, 2007; Takagi *et al.*, 1977). The SSF process is a promising option because it reduces operating costs, since both hydrolysis and fermentation are performed in the same vessel. Moreover, end-product inhibition of the enzymes is minimized due to fermentation occurring concurrently with enzymatic hydrolysis, thereby allowing a higher solids loading for hydrolysis (García-Aparicio *et al.*, 2011; Olofsson *et al.*, 2008). SSF processes, however, usually operate at sub-optimal conditions because the optimum temperature required for enzymatic hydrolysis (45 to 50^{o}C) is higher than for fermentation (25 to 35^{o}C) (García-Aparicio *et al.*, 2011; Öhgren *et al.*, 2007).

In this chapter, both SHF and SSF process configurations were applied to the cladode biomass of *Opuntia ficus-indica*, a plant capable of drought resistance and a high biomass productivity in arid and semi-arid regions. To the best of my knowledge, only one paper on bioethanol production from *O. ficus-indica* cladodes has been published (Retamal *et al.*, 1987). These authors achieved a final ethanol concentration of 1.4% (w/v) using *Saccharomyces cerevisiae* as the fermenting organism. Interestingly, enzymatic hydrolysis preceded the acid treatment step.

The focus of this study was to investigate the feasibility of utilising the *O. ficus-indica* cladodes as potential bioethanol feedstock and evaluating both the SHF and SSF processes. The thermotolerant yeast *Kluyveromyces marxianus* was used to ferment the hydrolysate at a temperature of 40^{o}C, which was closer to optimum for saccharification. The cultivation of *K. marxianus* was carried out under non-aerated and oxygen-limited

conditions, and its performance was compared to *Saccharomyces cerevisiae* which served as benchmark.

3. Materials and methods

Feedstock

All the experiments were performed using *Opuntia ficus-indica* cladode flour as biomass feedstock. The cladode flour was obtained through shredding, sun-drying and hammer-milling of fresh *O. ficus-indica* cladodes (cultivar "Algerian"), harvested from a farm outside Bloemfontein, South Africa. The composition of the cladode biomass, on a dry mass basis, was 23.1% glucan, 3.9% xylan, 3.8% arabinan, 6.4% galactan, 4.8% fructan, 7.9% lignin, 24.3% extractives and 16.8% ash. The composition was determined as described in Chapter 2.

Dilute acid pretreatment

The conditions and procedures used to obtain an acid pretreated slurry for subsequent enzymatic hydrolysis to produce a hydrolysate for SHF experiments are given in detail in Chapter 2. Briefly, the pretreatment of the biomass feedstock consisted of mixing 2.5 kg of *O. ficus-indica* cladode flour with 8.4 l of 1.5% (w/w) H_2SO_4 to achieve a solids loading of 30% (w/v), and soaking overnight in a 15 l (10 l working volume) stainless steel Biostat-C bioreactor (Sartorius Stedim Biotech, Göttingen, Germany). Afterwards, the slurry was autoclaved *in-situ* at 120^{o}C for 50 min. The solid fractions generated by pretreatment were analysed in the same way as the raw material, whereas the liquid fraction was analysed for monomeric and oligomeric sugars. A similar but slightly scaled down approach was used to obtain the SSF pretreated slurry. A 200 g aliquot of flour was soaked in 672 ml dilute acid in a 1.6 l (1 l working volume) Biostat B-plus bioreactor vessel, conforming to the same solids loading of 30% (w/v). Subsequently the slurry was autoclaved in a Hiclave HV-110 autoclave (Hirayama, Saitama, Japan) at 120^{o}C for 50 min. Samples were taken after pretreatment and the solid and liquid fractions were separately analysed for sugars.

The pretreated slurry was subsequently used for either separate hydrolysis and fermentation (SHF), or simultaneous saccharification and fermentation (SSF). An overview of the experimental set-up is schematically shown in Figure 4.1.

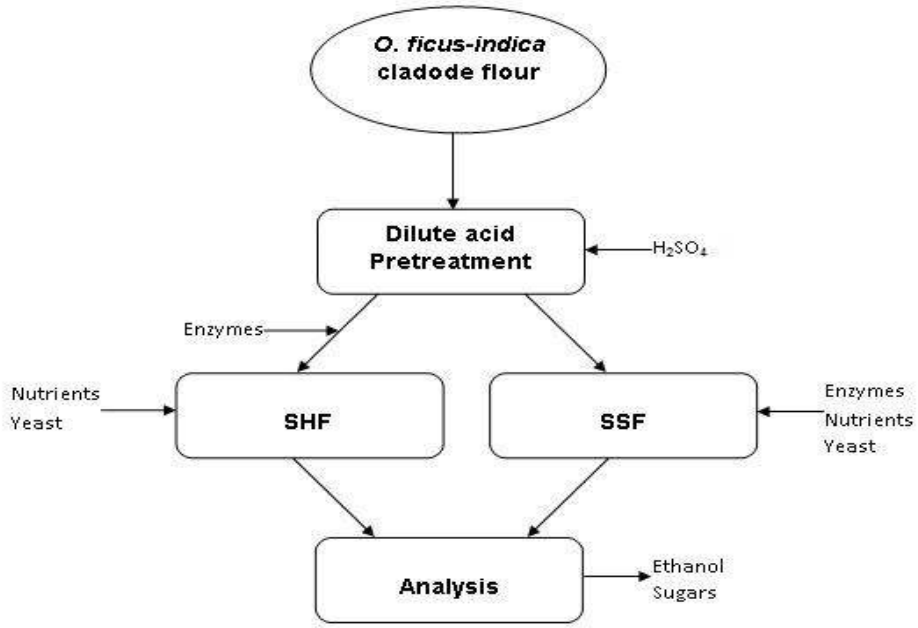


Figure 4.1 The experimental procedure used to convert cladodes of *O. ficus-indica* into ethanol

Yeast inoculum preparation

Kluyveromyces marxianus UOFS Y-2791, a thermotolerant yeast isolated from the agave plant (*Agave americana)* and *Saccharomyces cerevisiae* UOFS Y-0528, a commercial wine yeast strain obtained from the University of the Free State MIRCEN yeast culture collection, were used in the SHF and SSF experiments. Pure cultures of the yeast strains were maintained on GPY agar slants containing (per litre): 40 g glucose, 5 g peptone, 5 g yeast extract and 20 g agar. Pre-cultures and inocula used for fermentation were prepared as described in Chapter 3.

General fermentation conditions

Both the enzyme hydrolysate used for SHF and the acid-pretreated slurry used for SSF were supplemented with mineral salts, trace elements and vitamins. The nutrient concentrations in the cultivation medium were (per litre) 0.5 g citric acid, 5 g $(NH_4)_2HPO_4$, 0.75 g KH_2PO_4, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.1 g NaCl, 1 ml of a trace elements stock solution and 4 ml of a filter-sterilized vitamin stock solution (see Chapter 3). A Biostat B-plus stirred tank bioreactor was used for the fermentations. The pH in the reactor was maintained at pH 5.0 by automatic titration with either 3 M KOH or 3 N H_2SO_4. *K. marxianus* was grown at 40^{o}C, which is closer to the temperature optima of

45^{o}C to 50^{o}C for enzymatic hydrolysis, whereas *S. cerevisiae* was grown at 35^{o}C. In the SHF and SSF experiments both yeasts were subjected to non-aerated cultivation conditions with the stirrer speed maintained at 100 rpm to ensure a homogenous mixture. *K. marxianus* was also grown under oxygen-limited conditions, where the culture was sparged with air at an aeration rate of 0.3 l min^{-1} and the dissolved oxygen tension (DOT) maintained at 0.5 to 1% of saturation by automatic adjustment of stirrer speed.

Enzymes

Celluclast 1.5L, Novozym 188 and Pectinex Ultra SP-L enzyme preparations were kindly provided on request by Novozymes A/S (Bagsvaerd, Denmark). The determinations of enzyme activities of cellulase, β-glucosidase and pectinase have been described in Chapter 2.

Separate hydrolysis and fermentation (SHF)

Following pretreatment, the temperature of the acid pretreated slurry was decreased to 50^{o}C and the pH adjusted and maintained at pH 5 by automatic titration with either 3 M KOH or 3 N H_2SO_4. Enzymatic hydrolysis was carried out on the whole slurry without separation of the liquid fraction from the water insoluble solids (WIS). An enzyme cocktail containing (per g dry biomass), 15 FPU cellulase, 15 IU β-glucosidase and 100 IU pectinase was added to the pretreated slurry and the stirrer speed was maintained at 300 rpm. The hydrolysis was performed for up to 48 h to allow maximum release of monomeric sugars. Afterwards, the resulting *O. ficus-indica* cladode hydrolysate was collected in sterile 1 l bottles while the reactor was continuously stirred to ensure even distribution of the hydrolysate in all the bottles. Each 1 litre hydrolysate stock contained 46 g glucose, 6 g xylose, 9 g galactose, 11 g arabinose and 10 g fructose. The bottles were stored at -20^{o}C until further use.

The *O. ficus-indica* cladode hydrolysate was used as substrate for the fermentation experiments. An 800 ml volume of the hydrolysate was sterilized by mild autoclaving at 110^{o}C for 10 min, after which it was cooled to the desired cultivation temperature and supplemented with 150 ml of a sterile nutrients solution. The nutrients solution also served as diluent to obtain a more miscible slurry. The reactor was inoculated with an inoculum size of 5% (v/v), corresponding to a biomass concentration of 0.16 or 0.15 g l^{-1} in the case of *K. marxianus* and *S. cerevisiae,* respectively. All SHF experiments were conducted for 48 h and samples were withdrawn at regular intervals for ethanol and sugar analysis.

Simultaneous saccharification and fermentation (SSF)

SSF experiments were performed in the Biostat B-plus reactor using the whole slurry from the pretreatment stage (Figure 4.1). The slurry used for each SSF experiment was pretreated separately. Temperature and pH were adjusted and the hydrolysate was supplemented with nutrients and sterile deionized water to ensure a final WIS concentration of 140 g l^{-1}, thereby conforming to the same solids loading used during the hydrolysis stage of SHF. An enzyme mixture similar to what was used for enzymatic hydrolysis during SHF was added to the pretreated slurry.

There was a time interval of about 10 min between the addition of enzymes and inoculation to allow some decrease in viscosity of the slurry and to improve mixing. During that period, the stirrer speed was set at 300 rpm and decreased to 150 rpm just before inoculation. After addition of enzymes, mineral salts and inoculum, the final working volume was 1 l. The SSF experiments were performed for 80 h and samples were collected at intervals for analysis. To enable comparison of the ethanol yields obtained in SSF and SHF, the calculated yields on total sugars were based on the final sugar concentration in the cladode enzymatic hydrolysate (see Chapter 2).

Analytical procedures

Hydrolysate samples used for measuring sugar and ethanol concentrations were collected and analysed as described in Chapters 2 and 3. All experiments were performed in duplicate and mean values are reported.

4. Results

4.1 Enzymes

Celluclast 1.5 FG had a measured cellulase activity of 70 FPU ml^{-1}, Novozym 188 a β-glucosidase activity of 750 IU ml^{-1} and Pectinex Ultra SP-L a pectinase activity of 1064 IU ml^{-1}.

4.2 Fermentation of *O. ficus-indica* cladode enzymatic hydrolysate

The SHF fermentation profiles of *K. marxianus* and *S. cerevisiae* under non-aerated conditions, as well as that of *K. marxianus* under oxygen limited conditions, are shown in Figure 4.2a-c. As previously mentioned in this dissertation, dilute acid pretreatment of the

O. ficus-indica cladode biomass was performed at a 30% solids loading to produce a hydrolysate with sugar content closer to what is obtained from other conventional lignocellulosic biomass feedstocks. However, after pretreatment, separation of the solid fraction and liquid fraction was rather complicated because of the small particle size of the biomass and the large volumes involved. Moreover, the pretreated biomass was not exactly a slurry, but rather resembled a mass of wet, swollen, gummy material, difficult to remove from the pretreatment vessel or to filter unless it was further diluted. Because of these challenges, enzymatic hydrolysis was performed directly on the pretreated cladode hydrolysate and the whole slurry was used without separation. Dilution of the hydrolysate to improve mixing resulted in a decrease in the total sugar concentration in the hydrolysate. The hydrolysate medium used in each SHF experiment had a total sugar content of 59.5 g l^{-1}, consisting of (per litre), 30.7 g glucose, 5.6 g xylose, 8.7 g galactose, 5.9 g arabinose and 8.6 g fructose.

S. cerevisiae and *K. marxianus*, grown in the absence of aeration, produced comparable maximum ethanol concentrations of 19.6 and 19.5 g l^{-1} (mean values), respectively, corresponding to ethanol yields of 80% and 66% of the theoretical yield based on utilised sugars and total sugars, respectively (Figure 4.2 Table 4.1). However, under these conditions *K. marxianus* gave a volumetric productivity of 0.93 g ethanol l^{-1} h^{-1} which was lower than that of *S. cerevisiae* (1.41 g l^{-1} h^{-1}). The sugar utilisation pattern observed in these experiments was similar to that of previous cultivations using a chemically defined medium with a similar sugar composition as in the undiluted hydrolysate (See Chapter 3, Fig. 3.1). Both yeasts completely consumed glucose and fructose and in the *S. cerevisiae* cultures the residual galactose concentration was less than 0.5 g l^{-1}. By contrast, the residual galactose concentration in the *K. marxianus* cultures was 4.8 g l^{-1} (Figure 4.2b). *K. marxianus* also utilised xylose and arabinose poorly under non-aerated conditions. Not surprisingly, *S. cerevisiae* did not utilise arabinose, although it seemed that the particular strain used in this study exhibited a very slight ability to utilise xylose, resulting in the disappearance of about 2 g xylose l^{-1} from the hydrolysate during the 48 h fermentation period.

When *K. marxianus* was grown in the hydrolysate under oxygen-limited conditions, it completely consumed galactose (Figure 4.2c). Even under these conditions xylose and arabinose were poorly utilised, although the uptake of xylose and, to a lesser extent, arabinose was faster towards the end of the fermentation. This was in contrast to what had been observed previously during cultivation in a chemically-defined medium where both

sugars were completely consumed (Chapter 3, Fig. 3.1c). *K. marxianus* produced an ethanol concentration of 14.7 g l^{-1} which was lower than the 19.5 g l^{-1} produced by the same yeast under non-aerated conditions (Figure 4.2c). The ethanol was assimilated after depletion of glucose and fructose. Nevertheless, *K. marxianus* achieved a maximum ethanol volumetric productivity of 2.23 g ethanol l^{-1} h^{-1}, which was more than double the value obtained during non-aerated cultivation and also exceeded that of *S. cerevisiae* (Table 4.1).

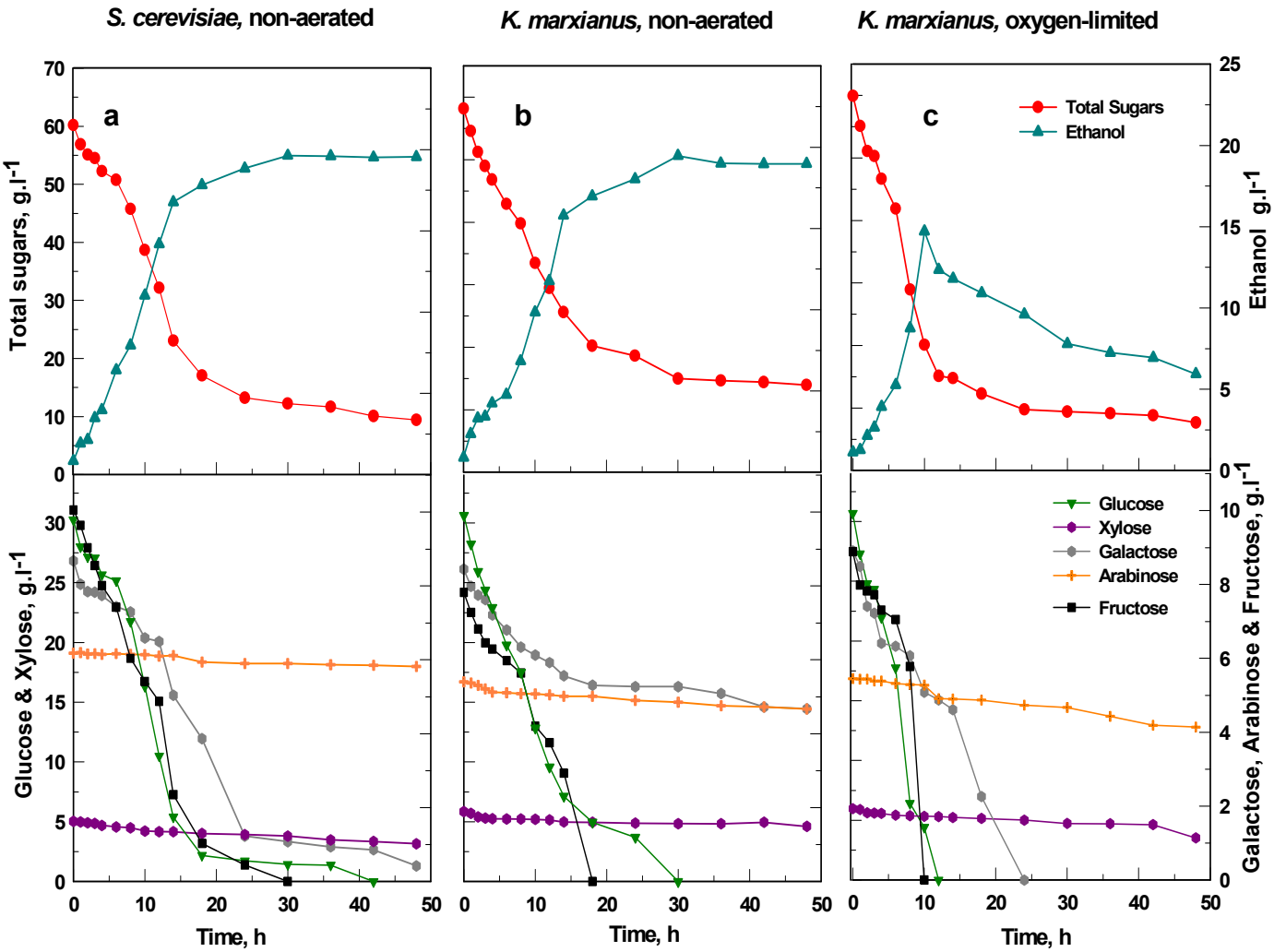


Figure 4.2 SHF fermentation profiles of *S. cerevisiae* Y-0528 and *K. marxianus* Y-2791 in *O. ficus-indica* cladode hydrolysate using different conditions of aeration: **(a)** *S. cerevisiae,* non-aerated, **(b)** *K. marxianus,* non-aerated, **(c)** *K. marxianus,* oxygen-limited. Time zero indicates time of inoculation following 48 h of enzymatic hydrolysis at 50°C.

4.3 Simultaneous saccharification and fermentation of pretreated *O. ficus-indica* cladode biomass

SSF fermentation profiles of *K. marxianus* and *S. cerevisiae* in acid pretreated *O. ficus-indica* cladode biomass at a WIS content of 140 g l^{-1} are shown in Figure 4.3a-c. The performance of *K. marxianus* was investigated under both under non-aerated and oxygen-limited conditions.

At the start of fermentation, the total sugar content of the SHF and SSF hydrolysates were similar. This may have been because only 800 ml of the original SHF hydrolysate stock was used and made up to 1 l with the concentrated nutrient solution, which diluted the sugars slightly, whereas the SSF hydrolysate benefited from the ten minute time interval between enzyme addition and yeast inoculation, during which saccharification already occurred. In this short space of time a large portion of the oligomeric sugars, corresponding to a total sugar concentration of about 55 g l^{-1}, of which glucose amounted to 25 g l^{-1}, were released in their monomeric form (Figure 4.3). This release of sugars within a short period was also observed during enzymatic hydrolysis to produce a fermentable *O. ficus-indica* cladode hydrolysate (see Chapter 2). The total sugar concentration in the hydrolysate continued to increase during the first few hours of fermentation, mainly due to the accumulation of glucose, demonstrating that hydrolysis of glucan was occurring faster than glucose uptake for growth and ethanol production (Figure 4.3). After 4 h, ethanol production proceeded faster than hydrolysis, resulting in a decrease in the total sugar content. The viscosity of the hydrolysate gradually decreased over time, presumably because of the loss of cellulosic structure and water binding capacity due to cellulose degradation (Rosgaard *et al.*, 2007) as well as pectin solubilization (see Discussion).

Even at a high solids loading of 140 g l^{-1} the lag phase was very short, indicating that the yeasts adapted well to the conditions in the hydrolysate. After 36 h of non-aerated cultivation, *S. cerevisiae* produced an ethanol concentration of 20.6 g l^{-1}, whereas *K. marxianus* achieved a slightly lower but comparable concentration of 19.3 g l^{-1}. The values corresponded to 70% and 64% respectively, of the theoretical yield on total sugars in the hydrolysate. *K. marxianus*, however, exhibited a maximum volumetric productivity of 1.08 g ethanol l^{-1} h^{-1}, whereas *S. cerevisiae* performed considerably better, achieving 1.41 g ethanol l^{-1} h^{-1} (Figure 4.3a, b, Table 4.1). During the utilisation of glucose and fructose, the enzymatic conversion of other oligomers into their respective monomeric forms

occurred concurrently (Figure 4.3 a-c). Galactose, for instance, gradually increased in concentration; however, once glucose and fructose were almost completely depleted, its concentration decreased, indicating a shift towards galactose utilisation by the yeasts (Figure 4.3 a-c). Similar to what was observed during SHF and a previous cultivation in a chemically defined medium (Chapter 3), *K. marxianus* poorly utilised galactose, xylose and arabinose under non-aerated conditions.

By contrast, these sugars were better utilised by *K. marxianus* during growth under oxygen-limited conditions (Figure 4.3c). Although galactose was incompletely utilised, the residual concentration after 40 h was only about 1 g l^{-1}, whereas xylose was completely depleted after 70 h. Ethanol production with *K. marxianus* under oxygen-limited conditions peaked at 20 h, reaching 14.2 g l^{-1}. Again, the ethanol produced was assimilated upon glucose and fructose depletion (Fig. 4.3c). Of the two aeration conditions, the highest maximum volumetric productivity of 1.57 g ethanol l^{-1} h^{-1} was achieved with *K. marxianus* during growth under oxygen-limited conditions.

4.4 Comparison of the SHF and SSF processes

Ethanol concentrations, volumetric productivities and yield coefficients of *K. marxianus* and *S. cerevisiae* under non-aerated and oxygen-limited conditions, using the SHF and SSF process configurations, are compared in Table 4.1. During SHF and SSF under non-aerated conditions, the ethanol concentration produced by *K. marxianus* was comparable to that of *S. cerevisiae* (Figs. 4.2 and 4.3, Table 4.1). The ethanol concentration produced by *K. marxianus* grown under oxygen-limited conditions was also similar in both SHF and SSF. Whereas the highest mean maximum ethanol concentration of 20.6 g l^{-1} was achieved with *S. cerevisiae* during SSF, *K. marxianus* produced a comparable concentration of 19.3 g l^{-1} using the same process, and 19.5 g l^{-1} using SHF. The ethanol yields achieved by *K. marxianus* grown in the absence of aeration were also quite similar to the yields obtained with *S. cerevisiae* in SHF and SSF (Table 4.1). *K. marxianus* completely consumed galactose during SHF under oxygen-limited conditions, whereas there was residual galactose of about 1 g l^{-1} in the hydrolysate after 80 h of SHF (Figs 4.2c and 4.3c). The maximum ethanol volumetric productivity of *S. cerevisiae* during SHF was comparable to what was achieved during SSF. When *K. marxianus* was grown without aeration, it also achieved comparable maximum ethanol volumetric productivities with both SHF and SSF, whereas under oxygen-limited conditions it exhibited a volumetric

productivity that was almost 1.5 times higher during SHF than for SSF. Under both non-aerated and oxygen-limited conditions, the maximum overall productivity achieved by either yeast using SSF was more than double the values recorded for SHF (Table 4.1).

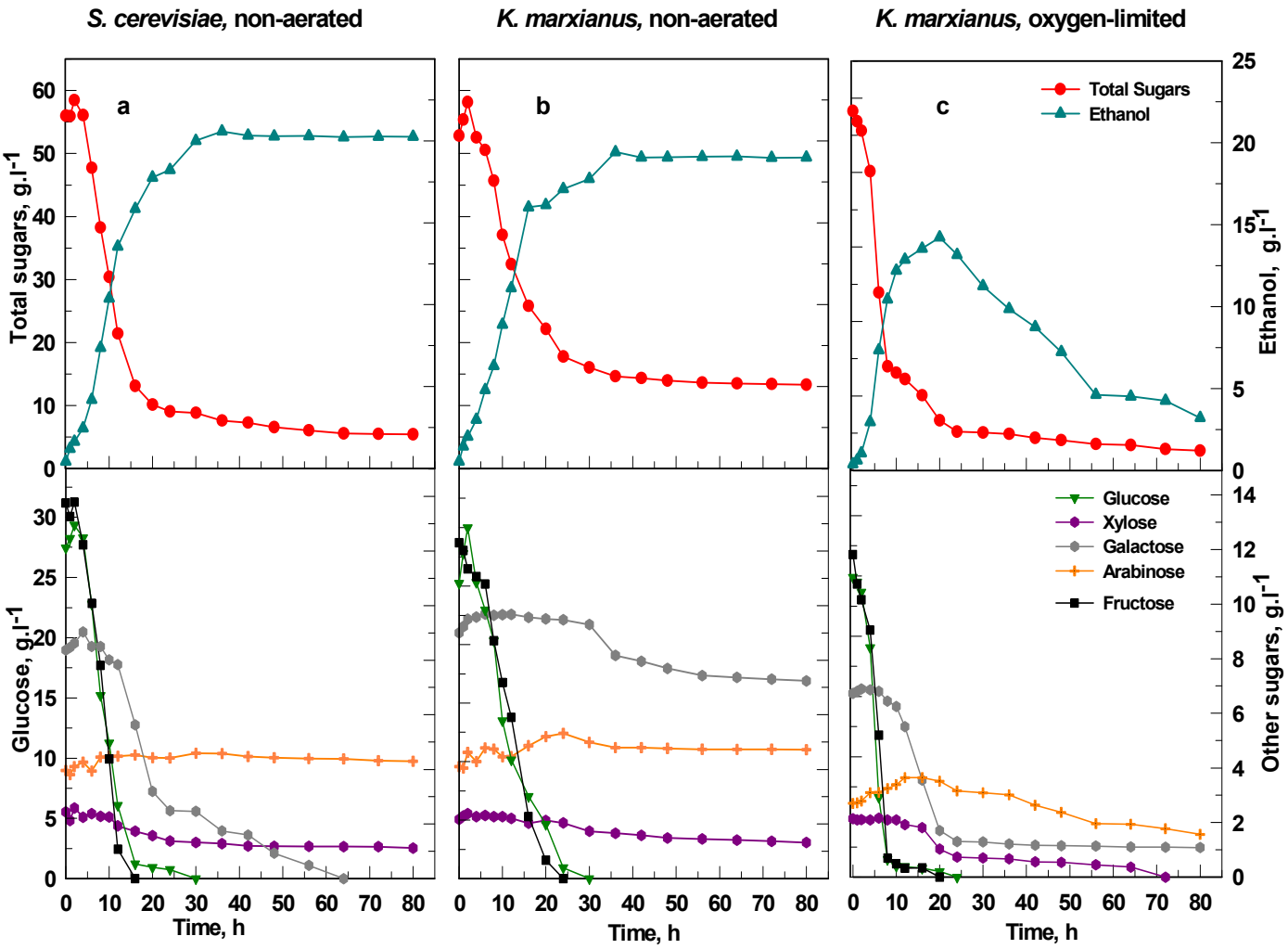


Figure 4.3 SSF fermentation profiles of *S. cerevisiae* Y-0528 and *K. marxianus* Y-2791 in *O. ficus-indica* cladode hydrolysate using different conditions of aeration: **(a)** *S. cerevisiae,* non-aerated, **(b)** *K. marxianus,* non-aerated, **(c)** *K. marxianus,* oxygen-limited. Time zero indicates time of inoculation, 10 minutes after addition of enzymes.

Table 4.1 Fermentation parameters of *S. cerevisiae* and *K. marxianus* during SHF and SSF of *O. ficus-indica* cladode hydrolysate

Parameter	SHF			SSF		
	S. cerevisiae	*K. marxianus*		*S. cerevisiae*	*K. marxianus*	
	Non-aerated	Non-aerated	Oxygen-limited	Non-aerated	Non-aerated	Oxygen-limited
Residual sugars, g l^{-1}	9.4	13.5	7.7	5.4	13.3	2.7
$Q_{p\ (EtOH)}$, g l^{-1} h^{-1}	1.48	0.93	2.23	1.41	1.08	1.57
$Q_{p\ (overall)}$, g l^{-1} h^{-1}	0.25	0.25	0.24	0.57	0.54	0.71
Max. ethanol, g l^{-1}	19.6	19.5	14.7	20.6	19.3	14.2
Y_{EtOH}, utilised sugars	0.40 (80%)*	0.42 (84%)*	0.28 (56%)*	nd	nd	nd
Y_{EtOH}, total sugars	0.33 (66%)*	0.33 (66%)*	0.25 (50%)*	0.35 (70%)*	0.3 (64%)*	0.24 (48%)*
Y_{EtOH}, theoretical fermentable sugars	0.25 (50%)*	0.24 (48%)*	0.18 (36%)*	0.26 (52%)*	0.24 (48%)*	0.18 (36%)*
Y_{EtOH}, theoretical total sugars	0.19 (38%)*	0.18 (36%)*	0.14 (28%)*	0.19 (38%)	0.18 (36%)*	0.13 (26%)*

Note: The values in parentheses are the percentage of maximum theoretical ethanol yields obtainable (0.51 g ethanol per g potential sugar in substrate). nd: not determined.

5. Discussion

In this study, the capability of *K. marxianus* Y-2791 and *S. cerevisiae* Y-0528 to ferment the constituent sugars in an *O. ficus-indica* cladode hydrolysate was evaluated using SHF and SSF procedures. Additionally, the performance of *K. marxianus* was investigated under non-aerated and oxygen limited conditions. It was observed that, unlike *S. cerevisiae, K. marxianus* exhibited a poor uptake of galactose in the absence of aeration. This behaviour may have been due to the yeast exhibiting the Kluyver effect which, simply put, is the inability of yeasts to effectively ferment certain sugars in the absence of oxygen or respiration (Fukuhara, 2003). Interestingly, *K. lactis*, which is the most closely related yeast species to *K. marxianus*, is known to exhibit the Kluyver effect on galactose.

One of the advantages that *K. marxianus* has over *S. cerevisiae* is its ability to assimilate substrates such as xylose and arabinose. However, it can utilise these sugars only under

aerobic or oxygen-limited conditions (Lane & Morrissey, 2010; Nonklang *et al.*, 2008). The control of the DOT at low levels, for example below 1% of saturation, is quite a challenge when working with lignocellulose hydrolysates. Less consistent oxygen diffusion into the medium could be the likely reason why *K. marxianus* incompletely utilised xylose and arabinose under oxygen-limited conditions in the *Opuntia* hydrolysate, but totally consumed these sugars in a chemically-defined medium (Chapter 3). It has also been suggested that the utilisation of pentose sugars by *K. marxianus* required respiratory activity to sustain cofactor balance and ATP level (Rodrussamee *et al.*, 2011). Proper control of DOT and oxygen transfer is, therefore important during cultivations with *K. marxianus.* The minimum DOT at which complete utilisation of these sugars would occur while also preventing ethanol assimilation, however, remains unclear and further investigation is required. Strategies for preventing ethanol assimilation based on these experiments include shutting off aeration just before 10 h in SHF and before 20 h in SSF, or using more strict aeration control. However, carrying out the latter might be impractical in such a hydrolysate and at such a high solids loading.

The higher volumetric ethanol productivity observed with both yeasts during SHF compared to SSF could be attributed to the greater extent of enzymatic hydrolysis and solubilization of the SHF slurry before it was inoculated, which rendered the hydrolysate less viscous and more miscible, facilitating oxygen and mass transfer. However, the overall productivity in SSF was more than double the values recorded for SHF (Table 4.1) Thus, using the SSF process could potentially save energy costs. Further advantages of SSF over SHF include prevention of end-product inhibition of the enzymes (Linde *et al.*, 2007).

The release of most of the sugars within the first few hours of SSF (also observed during the enzymatic hydrolysis stage of SHF) opened an avenue for future experiments to determine the possibility of using lower enzyme loadings for hydrolysis of the *O. ficus-indica* cladode. If comparable sugar yields were obtained with lower enzyme loadings, operating costs be significantly reduced (Wingren *et al.*, 2003). Although no enzymatic hydrolysis of the pretreated slurry was performed using pectinase alone, it is believed that the inclusion of pectinase in the enzyme cocktail ensured a decreased viscosity of the hydrolysate. A mixture of pectinase and cellulase enzymes is necessary for pectin solubilization and the complete release of sugars (Grohmann *et al.*, 1995). The *O. ficus-indica* cladode biomass is very rich in pectin, which is hydrolysed into galacturonic acid units by pectinase. However, neither of the yeasts used in this study grew on galacturonic

acid as the sole carbon source (results not shown). The use of genetically modified yeasts capable of galacturonic acid fermentation could improve the ethanol final concentration obtained from the *O. ficus-indica* cladode hydrolysate and also add more value to the biomass feedstock.

The performance of *S. cerevisiae* and particularly *K. marxianus* based on the maximum ethanol concentrations and ethanol yields on both utilised and total sugars in the hydrolysate compared favourably with results of cultivations in a chemically defined medium using similar sugar concentrations. This suggested that inhibitory compounds such as furfural and hydroxymethylfurfural (HMF), produced from sugar degradation during pretreatment, did not pose a significant problem to the yeasts, even though the whole slurry was used for hydrolysis and fermentation. The 14% WIS content used in this study rendered stirring and mixing difficult, mainly at the early stages of enzymatic hydrolysis and SSF, due to the high viscosity of the hydrolysate. This is one major problem encountered when using a high solids loading during fermentation (Ballesteros *et al.*, 2004; Rosgaard *et al.*, 2007). Nevertheless, the ethanol yields of *K. marxianus* on total sugars in the hydrolysate, even at such a high solids loading, corresponded well with those obtained when other strains of *K. marxianus* were used for SSF at a lower WIS content of feedstocks such as barley straw (García-Aparicio *et al.*, 2011), switchgrass (Faga *et al.*, 2010) and spruce (Bollók *et al.*, 2000).

Although *K. marxianus* exhibited a lower volumetric productivity than *S. cerevisiae* under non-aerated conditions, the results obtained from these experiments agreed with several other reports showing that *K. marxianus* could achieve ethanol yields and concentrations similar to those of *S. cerevisiae* during fermentation of lignocellulosic biomass hydrolysates, with the added advantage of fermentation at elevated temperatures of 40^{o}C and above, which is advantageous for SSF bioprocesses (Ballesteros *et al.*, 2004; Faga *et al.*, 2010; Tomás-Pejó *et al.*, 2009). In addition, the capacity of *K. marxianus* to ferment at higher temperatures than *S. cerevisiae* has the advantage of lower cooling costs, less possibility of microbial contamination, as well as enabling simultaneous fermentation and ethanol recovery which cannot be done below 40^{o}C (Abdel-Banat *et al.*, 2010; Cardona & Sánchez, 2007; Rodrussamee *et al.*, 2011; Sánchez & Cardona, 2008).

In this study, the highest ethanol concentration of 20.6 g l^{-1}, corresponding to approximately 70% of the theoretical yield on total sugars in the *O. ficus-indica* cladode hydrolysate, was an improvement on the 14 g l^{-1} previously reported by Retamal *et al*.

(1987). This showed that with process configurations such as SSF, more ethanol could be produced from the *O. ficus-indica* cladode biomass. However, the ethanol concentration achieved, which was equivalent to 2.6% (w/v), was still short of the acceptable minimum limit for an economic process (Wingren *et al.*, 2003). Nevertheless, the value could be improved through more efficient technologies such as steam pretreatment which would optimize the sugar content of the hydrolysate, and possibly lead to increased final ethanol concentration.

6. References

Abdel-Banat, B., Hoshida, H., Ano, A., Nonklang, S. & Akada, R. (2010). High-temperature fermentation: how can processes for ethanol production at high temperatures become superior to the traditional process using mesophilic yeast? *Appl Environ Microbiol* **85**, 861-867.

Ballesteros, M., Oliva, J. M., Negro, M. J., Manzanares, P. & Ballesteros, I. (2004). Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SSF) with *Kluyveromyces marxianus* CECT 10875. *Process Biochem* **39**, 1843-1848.

Bollók, M., Réczey, K. & Zacchi, G. (2000). Simultaneous saccharification and fermentation of steam-pretreated spruce to ethanol. *Appl Biochem Biotechnol* **84-86**, 69-80.

Cardona, C. A. & Sánchez, O. J. (2007). Fuel ethanol production: Process design trends and integration opportunities. *Bioresour Technol* **98**, 2415-2457.

Faga, B. A., Wilkins, M. R. & Banat, I. M. (2010). Ethanol production through simultaneous saccharification and fermentation of switchgrass using *Saccharomyces cerevisiae* D5A and thermotolerant *Kluyveromyces marxianus* IMB strains. *Bioresour Technol* **101**, 2273-2279.

Fukuhara, H. (2003). The Kluyver effect revisited. *FEMS Yeast Res* **3**, 327-331.

García-Aparicio, M. P., Oliva, J. M., Manzanares, P., Ballesteros, M., Ballesteros, I., González, A. & Negro, M. J. (2011). Second-generation ethanol production from steam exploded barley straw by *Kluyveromyces marxianus* CECT 10875. *Fuel* **90**, 1624-1630.

Grohmann, K., Cameron, R. G. & Buslig, B. S. (1995). Fractionation and pretreatment of orange peel by dilute acid hydrolysis. *Bioresour Technol* **54**, 129-141.

Hahn-Hägerdal, B., Galbe, M., Gorwa-Grauslund, M. F., Lidén, G. & Zacchi, G. (2006). Bio-ethanol - the fuel of tomorrow from the residues of today. *Trends Biotechnol* **24**, 549-556.

Lane, M. M. & Morrissey, J. P. (2010). *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. *Fungal Biol Rev* **24**, 17-26.

Linde, M., Galbe, M. & Zacchi, G. (2007). Simultaneous saccharification and fermentation of steam-pretreated barley straw at low enzyme loadings and low yeast concentration. *Enzyme Microb Technol* **40**, 1100-1107.

Nonklang, S., Abdel-Banat, B. M. A., Cha-aim, K., Moonjai, N., Hoshida, H., Limtong, S., Yamada, M. & Akada, R. (2008). High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042. *Appl Environ Microbiol* **74**, 7514-7521.

Öhgren, K., Bura, R., Lesnicki, G., Saddler, J. & Zacchi, G. (2007). A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. *Process Biochem* **42**, 834-839.

Olofsson, K., Bertilsson, M. & Liden, G. (2008). A short review on SSF - an interesting process option for ethanol production from lignocellulosic feedstocks. *Biotechnol Biofuels* **1**, 1-14.

Olsson, L. & Hahn-Hägerdal, B. (1996). Fermentation of lignocellulosic hydrolysates for ethanol production. *Enzyme Microb Technol* **18**, 312-331.

Retamal, N., Duran, J. M. & Fernandez, J. (1987). Ethanol production by fermentation of fruits and cladodes of prickly pear cactus (*Opuntia ficus-indica* (L.) Miller). *J Sci Food Agric* **40**, 213-218.

Rodrussamee, N., Lertwattanasakul, N., Hirata, K., Suprayogi, S., Limtong, S., Kosaka, T. & Yamada, M. (2011). Growth and ethanol fermentation ability on hexose and pentose sugars and glucose effect under various conditions in thermotolerant *Kluyveromyces marxianus*. *Appl Microbiol Biotechnol* **90**, 1573-1586.

Rosgaard, L., Andric, P., Dam-Johansen, K., Pedersen, S. & Meyer, A. (2007). Effects of substrate loading on enzymatic hydrolysis and viscosity of pretreated barley straw. *Appl Biochem Biotechnol* **143**, 27-40.

Sánchez, O. J. & Cardona, C. A. (2008). Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresour Technol* **99**, 5270-5295.

Sun, Y. & Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresour Technol* **83**, 1-11.

Takagi, M., Abe, S., Suzuki, S., Emert, G. H. & Yata, N. (1977). A method for production of alcohol direct from cellulose using cellulase and yeast. In *Proceedings of the Bioconversion Symposium IIT, New Delhi*, pp. 551-571.

Tomás-Pejó, E., Oliva, J. M., González, A., Ballesteros, I. & Ballesteros, M. (2009). Bioethanol production from wheat straw by the thermotolerant yeast *Kluyveromyces marxianus* CECT 10875 in a simultaneous saccharification and fermentation fed-batch process. *Fuel* **88**, 2142-2147.

Wingren, A., Galbe, M. & Zacchi, G. (2003). Techno-economic evaluation of producing ethanol from softwood: comparison of SSF and SHF and identification of bottlenecks. *Biotechnol Progr* **19**, 1109-1117.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

General Discussion and Conclusions

The ability of *Opuntia ficus-indica* to survive in arid and semi-arid regions is due to several unique characteristics of the plant, including its high water retention capacity. As a result, the cladodes are made up of 88 to 95% water and only contain 3 to 7% polysaccharides on a wet weight basis. Such a high water content meant that moisture removal to concentrate the carbohydrates, as well as to obtain a dried biomass feedstock, was the first challenge faced in this study. The problem was solved by processing the fresh cladodes into dry flour by cutting them into strips followed sun-drying and hammer milling. The dried and milled cladode flour had a moisture content of 3.7%.

Several authors have published data on the composition of the *O. ficus-indica* cladode, but information on the carbohydrate profile, and in particular the individual sugars, was not always clearly stated. This was largely due to the different analytical procedures used (Malainine *et al.*, 2003; Malainine *et al.*, 2005; Retamal *et al.*, 1987; Rodriguez-Felix & Cantwell, 1988). Consequently, the first objective of this dissertation was to carry out a standardized and comprehensive investigation to elucidate the general composition of the *O. ficus-indica* cladode. The combined glucan, fructan and galactan content of the cladode, which amounted to 34.3% (dry wt), was within the range of values reported for the total hexose sugar content of other conventional biomass feedstocks such as in sugar cane bagasse (42.1%) (Neureiter *et al.*, 2002), corn stover (41%) (Ruth & Thomas, 2003), barley straw (38.3%) (García-Aparicio *et al.*, 2011) and switch grass (33.23%) (Hamelinck *et al.*, 2005). The low xylose content of cladode biomass used in this study, compared to that of other feedstocks, suggested that the hemicellulose structure of the *O. ficus-indica* cladode was not a xylan, but more of an arabinan. Therefore, considering the composition of the *O. ficus-indica* cladode biomass, ethanol production would occur mainly from the hexoses, thus a xylose fermenting yeast strain may not necessarily be a requisite for an economically viable bioprocess.

Dilute acid pretreatment of the *O. ficus-indica* cladode flour was effected with 1.5% (w/w) H_2SO_4 at 120^{o}C for 50 min at a solids loading of 30% (w/v). The fluidized sand bath initially used for the pretreatment trials was capable of heating the *O. ficus-indica* cladode biomass up to 160^{o}C, which was the maximum temperature at which the biomass remained stable. The above equipment could, however, only accommodate a limited sample size of about 2 g. Since it was necessary to produce sufficient hydrolysate for fermentations in bioreactors, and because the only available equipment during these

experiments that could heat up such large volumes was an autoclave, the pretreatment temperature was restricted to 120±1^{o}C. Hence, during the pretreatment trials based on the central composite design, temperature was not included as a variable, while acid concentration and reaction time were the only factors investigated. In this study, the whole pretreatment slurry was used during enzymatic hydrolysis without separation of the liquid and solid fractions. That was largely because of the difficulties encountered with filtration due to the paste-like nature of the pretreated slurry, which was attributed to its 1 mm particle size and the presence of mucilage. The pretreated slurry required further dilution for the liquid fraction to be separated from the water insoluble solids (WIS), which would decrease the sugar content. Enzymatic hydrolysis of the pretreated slurry at a solids loading of 14% (w/v) with an enzyme loading of 15 FPU cellulase, 15 IU β-glucosidase and 100 IU pectinase per g dry biomass yielded a hydrolysate that could be used for fermentation experiments, thereby fulfilling the second objective of this dissertation. The inclusion of pectinase in the enzyme mix helped to decrease the viscosity of the slurry during hydrolysis. Although the glucose content in the hydrolysate was lower than that of other conventional biomass feedstocks such as sugar cane bagasse, it is possible that with optimization of the pretreatment and hydrolysis stages through improved technologies such as steam pretreatment, the sugar content of the *O. ficus-indica* cladode hydrolysate could be improved. It would also be of interest to investigate the possibility of obtaining a less viscous pretreated biomass slurry following steam pretreatment. If that could be achieved, it would decrease the required enzyme dosage particularly that of pectinase, used during hydrolysis, resulting in a significant decrease in the operational costs.

The fermentation of a simulated *O. ficus-indica* cladode hydrolysate provided a bench mark for the performance of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* in the actual hydrolysate. The ability of *K. marxianus* to produce ethanol at higher temperatures than *S. cerevisiae* has several benefits for process configurations such as simultaneous saccharification and fermentation (SSF), particularly a decrease in operating costs. The higher overall productivity achieved during fermentation using SSF compared to the separate hydrolysis and fermentation (SHF) process also gave credence to why SSF is the preferred process configuration.

The fermentation profile of *K. marxianus* during cultivation in the absence of aeration in a chemically defined medium, as well as in the *Opuntia* hydrolysate using both SHF and SSF, showed that the yeast could produce ethanol at concentrations comparable to those obtained with *S. cerevisiae*. However, the poor utilisation of galactose by *K. marxianus*

under non-aerated conditions was a major concern. For a biomass feedstock such as the *O. ficus-indica* cladode flour that contained galactose as its third most abundant sugar, it is crucial that *K. marxianus* should be able to completely ferment galactose to further justify its potential as an alternative to *S. cerevisiae*. Incomplete utilisation of galactose would result in lower ethanol yields than with *S. cerevisiae.* It appeared that galactose utilisation by *K. marxianus* was respiration-dependent, because during oxygen-limited cultivations in a chemically-defined medium the yeast completely consumed galactose as well as xylose and arabinose. Unlike *S. cerevisiae*, *K. marxianus* is a Crabtree-negative yeast and ethanol production is linked to an oxygen-limitation (Bellaver *et al.*, 2004; Fonseca *et al.*, 2008). This implies that the control of DOT would be a very important parameter to optimize for optimal ethanol production using *K. marxianus*. Thus, if too little oxygen is available, the yeast would utilise the sugars poorly, as was the case with galactose, whereas if too much oxygen is provided, not only would less ethanol would be produced, but in addition to the yeast utilising the available sugars for biomass production, it would also assimilate the produced ethanol once the sugars were depleted. In this study it was not completely understood what the critical dissolved oxygen level was where ethanol assimilation would occur, because even when the dissolved oxygen tension (DOT) was controlled at between 0.5 and 1% of saturation, it seemed that even such a low DOT value exceeded the critical DOT for ethanol production, and was sufficient for the yeast to utilise ethanol as a carbon source. Further investigations would be required to determine the DOT at which ethanol assimilation would not occur. Nevertheless, these experiments have shown that under oxygen-limited conditions, *K. marxianus* was capable of achieving a volumetric ethanol productivity and yield comparable to that of *S. cerevisiae*.

Based on the experimental results of this study, the *O. ficus-indica* cladode seemed promising as a lignocellulosic biomass feedstock for bioethanol production, provided a process could be devised that would result in a higher fermentable carbohydrate concentration in the fermentation broth. Process configurations such as SSF also increased the maximum ethanol concentration achievable from the cladode hydrolysate up to 2% (w/v) ethanol. Nevertheless, further improvements are needed to obtain an ethanol concentration of at least 4% (w/v), which is considered as the benchmark for decreasing the energy demand in the distillation step to achieve an economic process (Wingren *et al.*, 2003). It could also be concluded from this study that thermotolerant *K. marxianus* Y-2791 had potential as an alternative to *S. cerevisiae* for ethanol production from lignocellulosic biomass. Moreover, with appropriate control of DOT, *K. marxianus* can completely utilise

the hexoses and pentoses available in the biomass feedstock, in addition to ethanol production at rates comparable to *S. cerevisiae.*

References

Bellaver, L. H., de Carvalho, N. M. B., Abrahão-Neto, J. & Gombert, A. K. (2004). Ethanol formation and enzyme activities around glucose-6-phosphate in *Kluyveromyces marxianus* CBS 6556 exposed to glucose or lactose excess. *FEMS Yeast Res* **4**, 691-698.

Fonseca, G., Heinzle, E., Wittmann, C. & Gombert, A. (2008). The yeast *Kluyveromyces marxianus* and its biotechnological potential. *Appl Microbiol Biotechnol* **79**, 339-354.

García-Aparicio, M. P., Oliva, J. M., Manzanares, P., Ballesteros, M., Ballesteros, I., González, A. & Negro, M. J. (2011). Second-generation ethanol production from steam exploded barley straw by *Kluyveromyces marxianus* CECT 10875. *Fuel* **90**, 1624-1630.

Hamelinck, C. N., van Hooijdonk, G. & Faaij, A. P. C. (2005). Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long term. *Biomass Bioenerg* **28**, 384-410.

Malainine, M. E., Dufresne, A., Dupeyre, D., Mahrouz, M., Vuong, R. & Vignon, M. R. (2003). Structure and morphology of cladodes and spines of *Opuntia ficus-indica*. Cellulose extraction and characterisation. *Carbohydr Polym* **51**, 77-83.

Malainine, M. E., Mahrouz, M. & Dufresne, A. (2005). Thermoplastic nanocomposites based on cellulose microfibrils from *Opuntia ficus-indica* parenchyma cell. *Compos Sci Technol* **65**, 1520-1526.

Neureiter, M., Danner, H., Thomasser, C., Saidi, B. & Braun, R. (2002). Dilute-acid hydrolysis of sugarcane bagasse at varying conditions. *Appl Biochem Biotechnol* **98-100**, 49-58.

Retamal, N., Duran, J. M. & Fernandez, J. (1987). Ethanol production by fermentation of fruits and cladodes of prickly pear cactus (*Opuntia ficus-indica* (L.) Miller). *J Sci Food Agric* **40**, 213-218.

Rodriguez-Felix, A. & Cantwell, M. (1988). Developmental changes in composition and quality of prickly pear cactus cladodes (nopalitos). *Plant Food Hum Nutr* **38**, 88-93.

Ruth, M. F. & Thomas, S. R. (2003). The effect of corn stover composition on ethanol process economics. http://www.eere.energy.gov/biomass/pdfs/34040.pdf.

Wingren, A., Galbe, M. & Zacchi, G. (2003). Techno-economic evaluation of producing ethanol from softwood: comparison of SSF and SHF and identification of bottlenecks. *Biotechnol Progr* **19**, 1109-1117.

Summary

Opuntia ficus-indica, the prickly pear cactus, is well adapted for cultivation in arid and semi-arid regions, with a yield of 10 to 40 tonnes (dry wt) cladode biomass per ha. The cladodes (the “leaves”, which in fact are the stems) might serve as lignocellulosic biomass feedstock for second generation bioethanol production, without competing for agricultural land or replacing significant natural vegetation. The main objective of this study was to investigate the feasibility of bioethanol production from an enzymatic hydrolysate of *O. ficus-indica* cladodes. The potential of a *Kluyveromyces marxianus* isolate UOFS Y-2791, a yeast capable of utilising a wider range of carbon substrates and of ethanol production at higher temperatures than *Saccharomyces cerevisiae*, was investigated for bioethanol production using an *O. ficus-indica* cladode enzymatic hydrolysate as feedstock. *S. cerevisiae* UOFS Y-0528, a wine yeast strain, was used as benchmark.

Compositional analysis of the cladode biomass indicated that it had a low lignin content of 8% (dry wt). The content of readily fermentable carbohydrates in the cladode, which was 34.3 g per 100 g dry biomass of which 23 g was glucose, was comparable to other conventional biomass feedstocks such as sugar cane bagasse and corn stover, whereas it had a low xylose content. By applying a statistical design experimental approach where acid concentration and contact time were varied, optimum conditions for dilute acid pretreatment of the dried and milled cladode were determined to be 1.5% (w/w) sulphuric acid for 50 min at a temperature of 120^{o}C and a dry biomass loading of 30% (w/v). Enzymatic hydrolysis experiments were performed with varied enzyme loadings of cellulase and β-glucosidase with or without the addition of pectinase, and the enzyme loadings chosen were 15 FPU cellulase, 15 IU β-glucosidase and 100 IU pectinase per gram of dry biomass. These parameters yielded an *O. ficus-indica* hydrolysate containing (per litre) 45.5 g glucose, 6.3 g xylose, 9.1 g galactose, 10.8 g arabinose and 9.6 g fructose.

Using a chemically-defined medium with a sugar composition similar to the hydrolysate as benchmark, *K. marxianus* and *S. cerevisiae* were grown in the *O. ficus-indica* hydrolysate at 40^{o}C and 35^{o}C, respectively, under non-aerated conditions, whereas the performance of *K. marxianus* was also investigated under oxygen-limited conditions where the DOT was controlled at less than 1% saturation. The fermentation profiles of both yeasts were compared using separate hydrolysis and fermentation (SHF) and simultaneous hydrolysis and fermentation (SSF) process configurations, at a water-insoluble solids (WIS) content

of 14%. Both yeasts achieved comparable ethanol yields in SHF and SSF under non-aerated conditions, although *K. marxianus* exhibited a lower volumetric ethanol productivity than *S. cerevisiae*. *K. marxianus*, cultivated under oxygen-limited conditions, achieved a lower ethanol yield than both yeasts cultivated without aeration. However, *K. marxianus* exhibited the highest volumetric ethanol productivity of 2.3 g l^{-1} h^{-1} and 1.57 g l^{-1} h^{-1} in SHF and SSF, respectively, although the ethanol produced was assimilated upon hexose depletion. *K. marxianus* utilised galactose poorly in the absence of aeration, but completely consumed the sugar under oxygen-limited conditions. The overall ethanol productivity of SSF was double that of SHF. An ethanol concentration of 20.6 g l^{-1}; the highest concentration achieved in this study, was an improvement on the 14 g l^{-1} previously reported elsewhere.

This study provided more information on the chemical composition of the *O. ficus-indica* cladode, particularly regarding its constituent carbohydrates, and also highlighted the feasibility of ethanol production from the cladodes, albeit at low concentrations from an industrial point of view. *K. marxianus* demonstrated its potential as an alternative to *S. cerevisiae* for bioethanol production from lignocellulosic biomass.

Keywords: *Opuntia ficus-indica,* bioethanol, lignocellulose, pretreatment, enzyme, *Kluyveromyces marxianus, Saccharomyces cerevisiae,* DOT, SHF, SSF

Opsomming

Opuntia ficus-indica, die turksvy kaktus, is goed aangepas vir verbouïng in droë en semi-ariede streke, met 'n opbrengs van 10 tot 40 ton (droë massa) kladode biomassa per hektaar. Die kladodes (die "blare", wat in werklikheid die stamme is) kan dien as ‘n lignosellulose-biomassa grondstof vir tweede-generasie bioetanol-produksie, sonder om vir landbougrond mee te ding of om beduidende natuurlike plantegroei te vervang. Die hoofdoel van hierdie studie was om die haalbaarheid van bioetanol-produksie vanaf 'n ensiem-hidrolisaat van *O. ficus-indica* kladodes te ondersoek. Die potensiaal van *Kluyveromyces marxianus* isolaat UOVS Y-2791, 'n gis wat in staat is om ‘n wyer reeks koolstofsubstrate te benut en tot etanolproduksie by hoër temperature as *Saccharomyces cerevisiae*, is vir bioetanol-produksie vanaf 'n *O. ficus-indica* kladode ensiem-hidrolisaat ondersoek. *S. cerevisiae* UOVS Y-0528, 'n wyngis ras, is as vergelykende maatstaf gebruik.

Die analise van die samestelling van die kladode biomassa het aangedui dat dit 'n lae lignien inhoud van 8% (droë massa) gehad het. Die inhoud van maklik-fermenteerbare koolhidrate in die kladode, wat 34,3 g per 100 g droë biomassa, waarvan 23 g glukose was, was vergelykbaar met ander konvensionele biomassa-voerstowwe soos suikerrietbagasse en mieliereste, terwyl dit 'n lae xilose-inhoud gehad het. Deur die toepassing van 'n statistiese ontwerp eksperimentele benadering waar suurkonsentrasie en kontaktyd gevarieer is, is die optimum toestande vir die verdunde suurbehandeling van die gedroogde en gemaalde kladode vasgestel as 1,5% (w/w) swaelsuur vir 50 minute by 'n temperatuur van 120^{o}C en ‘n droë biomassa-lading van 30% (m/v). Ensimatiese hidrolise-eksperimente is met verskillende ensiemladings van sellulase en β-glukosidase met of sonder die toevoeging van pektinase uitgevoer, en die ensiemladings wat gekies is, was 15 FPU sellulase, 15 IU β-glukosidase en 100 IU pektinase per gram droë biomassa. Hierdie parameters het 'n *O. ficus-indica* hidrolisaat wat (per liter) 45,5 g glukose, 6,3 g xilose, 9,1 g galaktose, 10,8 g arabinose en 9,6 g fruktose bevat het, gelewer.

Met 'n chemies-gedefinieerde medium met ‘n suikersamestelling soortgelyk aan die hidrolisaat as maatstaf, is *K. marxianus* en *S. cerevisiae* in die *O. ficus-indica* hidrolisaat by 40^{o}C en 35^{o}C, onderskeidelik, onder nie-deurlugte toestande gekweek, terwyl die prestasie van *K. marxianus* ook onder suurstofbeperkte toestande waar die vlak van opgeloste suurstof by minder as 1% versadiging beheer is, ondersoek is. Die fermentasie-profiele van beide giste in aparte hidrolise en fermentasie (SHF) en gelyktydige hidrolise

en fermentasie (SSF) konfigurasies, met 'n water onoplosbare vastestof-inhoud van 14%, is vergelyk. Beide giste het vergelykbare etanol-opbrengste in SHF en SSF onder nie-deurlugte toestande gelewer, alhoewel *K. marxianus* 'n laer volumetriese etanolproduktiwiteit as *S. cerevisiae* getoon het. Onder suurstof-beperkte toestande het *K. marxianus* 'n laer etanol-opbrengs as beide giste wat sonder deurlugting gekweek is, bereik. *K. marxianus* het egter die hoogste volumetriese etanolproduktiwiteit van 2,3 g l^{-1} h^{-1} en 1,57 g l^{-1} h^{-1} in die SHF en SSF prosesse, onderskeidelik, getoon, alhoewel die geproduseerde etanol na uitputting van die heksoses opgeneem is. In die afwesigheid van deurlugting het *K. marxianus* galaktose swak benut, maar onder suurstof-beperkte toestande is dit volledig opgeneem. Die algehele etanolproduktiwiteit van SSF was dubbel dié van SHF. 'n Etanolkonsentrasie van 20.6 g l^{-1}, die hoogste konsentrasie wat in hierdie studie bereik is, was 'n verbetering op die 14 g l^{-1} wat vroeër elders gerapporteer is.

Hierdie studie het meer inligting oor die chemiese samestelling van die *O. ficus-indica* kladode verskaf, veral met betrekking tot die samestellende koolhidrate, en het ook die haalbaarheid van etanolproduksie uit die kladodes, alhoewel teen lae konsentrasies vanuit 'n nywerheids-oogpunt, uitgelig. *K. marxianus* het sy potensiaal as 'n alternatief vir *S. cerevisiae* vir bioetanol produksie vanuit lignosellulose-biomassa gedemonstreer.

Sleutelwoorde: *Opuntia ficus-indica,* bioetanol, lignosellulose, voorbehandeling, ensiem, *Kluyveromyces marxianus, Saccharomyces cerevisiae,* suurstofvlak, SHF, SSF