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Biobutanol production from cellulosic and sugar-based feedstock from the corn plant

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Abstract

In this thesis, biobutanol production by biological fermentation was investigated from the corn plant, integrating two approaches. The first one was to utilize corn cobs, a cellulosic-based material. The second, using a new sugar-based material, sugarcorn juice. Utilizing suitable Clostridia strains for each substrate, these approaches converged into a biorefinery concept to produce renewable biofuels in Ontario, Canada.

The corn cob pretreatment was carried out by a dilute acid method resulting in temperature as the variable with most significant effect towards glucose liberation. The enzymatic hydrolysis was performed utilizing a very low concentration of an enzymatic stock with approximately 44% of hydrolysis conversion. Biobutanol fermentation was pursued utilizing a *Clostridium beijerinckii* strain and cellulosic biobutanol was produced in a concentration of 4.42 g L^{-1} at 48 h with 97% of reducing sugars utilization.

Different ABE fermentations by *Clostridium saccharobutylicum* ATCC BAA-117 using glucose, fructose, sucrose, and a mix of them, resulted in butanol production as high as 12-14 g L^{-1}.

For the first time, sugarcorn juices from Canadian corn hybrids, were characterized and proven as a suitable medium for biobutanol production. Variation in sugar composition of sugarcorn juices across different hybrids and growth seasons were observed during this study, from 102 to 145 g L^{-1}, with fructose, glucose and sucrose accounting for about 80%.

Clostridium beijerinckii 6422 produced 8.49 g L^{-1} of butanol over 257 h of fermentation utilizing sugarcorn juice as substrate. It had a biphasic fermentation where acids accumulation happened at the beginning of fermentation. Interestingly, at the end of the fermentation butyric acid was reactivated and the butanol production shifted towards butyric acid production.

Clostridium saccharobutylicum produced 11.05 g L^{-1} of butanol over 227h of fermentation utilizing sugarcorn juice as substrate. Both strains could utilize sucrose, fructose and glucose concomitantly. There is enough evidence to agree that *Clostridium saccharobutylicum* has a PTS-sucrose system which allows the cell to transport sucrose inside the cell.

The proposed Canadian sugarcorn (CANSUG) biorefinery can commercially generate biofuels and biochemicals while limiting wastes, offer environmental benefits to the energy sector, and strengthen the Canadian bio-economy.

Keywords

Biobutanol production, sugarcorn juice, *Clostridium saccharobutylicum, Clostridium beijerinckii*, corn cobs, ABE fermentation.

Co-Authorship Statement

Chapter 3: Fermentation of simple sugars by Clostridia species

A version of the above chapter is to be submitted to Biochemical Engineering journal.

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[2, 3] supervision, coordination of the study, scientific expertise, critical review of the manuscript

Chapter 4: Pretreatment, hydrolysis of corn cobs, cellulases experiments and biobutanol fermentation

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[2,3] supervision, critical review

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[1a 1b] equal contribution to the submitted manuscript

[1a 1b] equal contribution to characterization experiments, data analysis, draft preparation

[1a] biobutanol production- design, execution of tests, data analysis, draft preparation

[1b] bioethanol production- design, execution of experiments, data analysis, draft preparation

[2a 2b] sugarcorn growth, juice supply, agronomic inputs, critical review of the manuscript

[3a 3b] sugarcorn hybrid development, agronomic inputs, critical revision of the manuscript

[*] supervision, coordination of the study, scientific expertise, critical review of the manuscript

Chapter 7: Evaluation of sugarcorn as a potential Canadian energy crop

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[3a 3b] agronomic inputs, critical revision of manuscript

[*] supervision, scientific expertise, critical review of the manuscript

Dedication

To my beloved parents, Reyna & Raymundo, for their unconditional love and encouragement through all my life.

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Abbreviations

AAFC	Agriculture and Agri-Food Canada
ABE	Acetone butanol ethanol
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
CANSUG	Canadian Sugarcorn
CBP	Consolidated Bioprocessing
CCR	Carbon catabolite repression
CFU	Colony forming units
C. acetobutylicum	*Clostridium acetobutylicum*
C. beijerinckii	*Clostridium beijerinckii*
C. saccharobutylicum	*Clostridium saccharobutylicum*
C. saccharoperbutylacetonicum	*Clostridium saccharoperbutylacetonicum*
C. perfringes	*Clostridium perfringes*
Clostridium sp	Clostridium species
CNM	Clostridial Nutrient Medium
DNS	Dinitrosalicylic acid
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures)
EM	Erlenmeyer
GC	Gas chromatography
GHG	Greenhouse gases
HPLC	High performance liquid chromatography
IEA	International Energy Agency
PDB	Potato dextrose broth
PEP	Phosphoenolpyruvate
PS	Phenol-sulfuric acid
PTS	Phosphoenolpyruvate dependent phosphotransferase system

RID	Refractive Index Detector
S. cerevisiae	*Saccharomyces cerevisiae*
SCJ	Sugarcorn juice
SCJdil	Diluted sugarcorn juice
SCJ-P2	Sugarcorn juice-P2 medium
SCJM	Sugarcorn juice mixed
TEA	Trimethylamine
FFV	Flex-fuel vehicles

Chapter 1

1 Introduction

1.1 Background

Growing population and growth in per capita energy usage (industrialization and transportation of our society) have contributed to the rise in energy consumption and will dictate the future energy demand [Hallenbeck, 2014]. Evidence of increasing in anthropogenic emissions of greenhouse gases (GHG) have been ever strengthening, as well as their impacts on climate change and serious environmental problems. Currently, most of the global energy is supplied by fossil fuels (petroleum, natural gas, and coal) which accounts for over 80% of the world's energy supply, and is one of the major sources of GHGs emissions [Stern, 2008].

It has been shown that energy use and GHGs emissions are closely related. The majority of GHGs emitted in Canada are a result of the burning of fossil fuels. In this regard, the energy provided by the fossil fuels is used to heat homes and businesses, transport goods and people, and to power industrial equipment. In 2015, the emissions from fossil fuels accounted for 81% of Canadian GHG emissions. The remaining emissions are from non-energy sources such as agricultural and industrial processes, and waste handling [National-Energy-Board, 2017].

In an effort to reduce emissions, sustainable, low carbon and renewable energy alternatives to fossil fuels have been explored, of which plant-derived biomass represents an abundant and inexpensive source [Stern, 2008]. 'Bioenergy' has been defined as the energy derived from the conversion of renewable organic substrates from animal or plant sources ('biomass'). Furthermore, bioenergy is expected to play a crucial role, meeting 30% of global energy demand by 2050 [Guo et al., 2015].

For instance, biofuels are solid, liquid or gaseous fuels derived from biomass. Liquid biofuels include bioethanol, biobutanol, and biodiesel, whereas biomethane and biohydrogen are common gaseous biofuels. Plant biomass contains cellulosic sugars which can serve as a substrate for microbial conversion to biofuels [Demain et al., 2005; Speight and Singh, 2014; EIA, 2016].

Butanol (C_4H_9OH) is a colourless and flammable alcohol, also known as 1-butanol, butyl alcohol or n-butanol. It is a chiral molecule with four isomers, n-butanol, 2-butanol, iso-butanol and tert-butanol [National-Center-for-Biotechnology-Information, 2015]. Butanol can be used as a fuel in internal combustion engines or as an industrial chemical commodity, a diluent or extractant [Durre, 2007;Cascone, 2008]. 'Biobutanol' refers to n-butanol produced from renewable biomass. The biological production of butanol can be achieved by microbial Clostridia via the ABE –Acetone, Butanol, Ethanol– fermentation process [Durre, 2008]. These species can consume a variety of carbon substrates including pentoses, hexoses, starches and even complex substrates like cellulosic residues [Jones and Woods, 1986; Mitchell, 1998].

Figure 1.1 shows an overview of the ABE fermentation process from its early development. It was established in Manchester, England in 1912 and its industrial development was boosted during World War I (1914-1918) and World War II (1939-1945), to provide solvents to chemical industries [Awang et al., 1988]. During World War II, the industrial ABE production using solventogenic Clostridia was a very successful industrial fermentation [Jones and Woods, 1986].

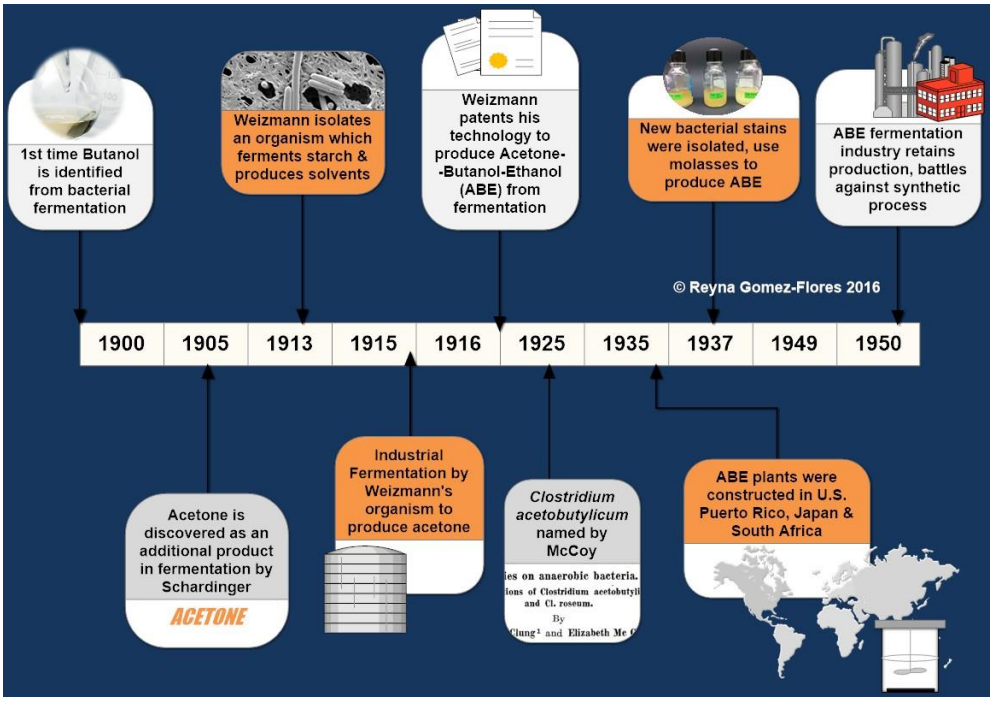

Figure.1.1 Timeline of microbial butanol production: an overview from 1900-1953. Data collected from [Jones and Woods, 1986;Awang et al., 1988].

In the 1950s the biggest plant was located in Illinois, USA which consisted of 96 fermenters (189,000 L per fermenter) [Durre, 2007]. There were also plants in South Africa and Russia, which operated until 1980s [Zverlov et al., 2006]. In the United States and Europe, all production ceased in the middle of 1950s.

Meanwhile, China launched its ABE fermentation process in the 1950s, peaked in the 1980s and stopped by the late 1990s. China restarted and increased its butanol fermentative production in 2005, after a hold in production in 2008; the Country resumed its industry in 2010. Consequently, Cathay Industrial Biotech (a Chinese corporation) was the top worldwide biobutanol manufacturer in 2012 [SBI, 2012; Chiao and Sun, 2007].

In 2000-2010, several companies worldwide revived their interest in the research of biobutanol production to be used as chemical precursor and biofuel, as an alternative to an oil-based economy. Some examples are Gevo™, Cobalt™, Butamax™ (BP™-DuPont™), Green Biologics LTD™, Syntec Biofuel™, Butalco™, Russian Technologies (Russian State Owned Company) and new research platforms like Plantaonix W2™, Energy, ZeaChem™, Energy Quest™, Metabolic Explorer ™, Optinol™, Abengoa™, Celtic Renewables™.

For instance, Cobalt and Dupont are using new bacterial strains in the ABE process with lignocellulosic biomass feedstock [SBI, 2012]. However, in 2012, Gevo opened its production facilities in Minneapolis, based on its proprietary technology to produce isobutanol as an alternative to the ABE process [Gevo, 2012].

Another critical technology has been established by TetraVitae Bioscience, a spin-off company from the University of Illinois, US. The enterprise developed its fermentation process to produce n-butanol and acetone by an enhanced microorganism platform. As of 2011, the Company operates as a subsidiary of Eastman Renewable Materials [McClenathan, 2010].

In late 2016, Green Biologics™ (Abingdon, UK) started its renewable n-butanol US production at Little Falls, Minnesota, utilizing corn as a feedstock. The plant was a retrofit from a former ethanol plant. The company has targeted the n-butanol as a building block for chemicals, rather than as a biofuel. This effort is the result of the initial work from Manchester in the 1912 and was successful due to the genetic optimization of its patented *Clostridium*

strain and advancement in its fermentation process. It also produces acetone [Green-Biologics, 2016].

Accordingly, policies in Canada directly promote the use of liquid biofuels by supporting research and development, commercialization assistance, tax incentives and mandatory use regulation (5% ethanol in gasoline since 2010) [Goverment-of-Canada, 2010]. Although, there is no existing mandate for butanol in gasoline. Currently, in Canada, efforts have been made to set up a biobutanol pilot plant facility in Sarnia, Ontario by KmX Corporation [Morden, 2012].

One of the main driving forces in biobutanol production, when designed to be used as biofuel, around the world is the petrochemical industry. Hence, ABE fermentation plant profitability is closely related to the butanol price and further intertwined with the cost of oil [Green, 2011]. Alternative chemical production method involves catalytic condensation of ethanol to produce butanol through the Guerbet reaction or via the Oxo reaction (petrochemical process) where propylene reacts with synthesis gas to form butyl aldehyde and then hydrogenated to produce butanol [Matar and Hatch, 2001].

Technological factors that hinder fermentative biobutanol production are low butanol yield, costly recovery stage and the cost of the substrate. As an alternative to pure substrates such as glucose, biobutanol can be produced from starchy corn grain, agricultural lignocellulosic wastes, energy crops or forest residues [Durre, 2007]. Additionally, ‘sugarcorn,' which are corn hybrids with high stalk sugar concentration developed by researchers from Agriculture and Agri-Food Canada (Reid et al. 2015), has been intended as a potential Canadian energy crop. As such, this research studies for the first time the production of biobutanol from sugarcorn juice.

Different strategies are proposed to increase biobutanol yields and optimize its bioprocesses, such as the discovery of new Clostridia strains, metabolic engineering, and novel bioreactors configurations [Zhu and Yang, 2010]. Ultimately, biobutanol production would be economically viable if considered within the integrated process technology or a biorefinery one.

1.2 Problem Statement

Biobutanol production via microbial fermentation has been studied and intermittently produced in industrial scale over than 100 years [Jones and Woods, 1986; Durre, 2007]. Nevertheless, lignocellulosic biomass has not been fully utilized for the biobutanol industrial production due to the additional cost of pretreatment and enzymatic or chemical hydrolysis steps before fermentation. Additionally, the cluster of different sugars derived from the lignocellulosic biomass is a heterogeneous mix. The study of biobutanol fermentation from lignocellulosic hydrolysate (pentoses and hexoses) is necessary to understand possible inhibition due to by-products of the pretreatment and to optimized biobutanol yields [Mitchell and Tangney, 2005].

Remarkable efforts have been made by Agricultural and Agri-food Canada (AAFC, Ottawa, Ontario) researchers over several years to develop a Canadian energy crop, as an alternative to starchy corn grain and cellulosic feedstocks. The strategy focused on utilizing the inherent characteristics of corn inbreeds to generate new corn hybrids with high stalk sugar concentration, named ‘sugarcorn’. There is reasonable postulation to explore the potential of sugarcorn juice as substrate to produce biofuels, such as bioethanol and biobutanol; as well, as other bio-based materials [Reid et al., 2015]. A sugar characterization of sugarcorn juice should be made as the first step to study further microbial fermentations, and the effect of its sugars in the Clostridial metabolism.

1.3 Research Objectives

The main goal of this research was to investigate the biobutanol fermentation using two different feedstocks from the corn plant, lignocellulosic corn cob and sugarcorn juice, a novel corn hybrid designed to be a potential Canadian energy crop. More specifically, this work includes:

- Study of butanol production in serum bottle experiments to compare selected strains of Clostridium in the chemical-defined medium.
- Develop a pretreatment and enzymatic hydrolysis process for lignocellulosic corn cob and study the biobutanol production using corn cob hydrolysate as the carbon source.
- Study of corn cob pretreatment and production of a corn cob hydrolysate.
- Biobutanol production using Clostridia species cultivated in corn cob hydrolysate medium.
- Characterization of juice extracted from sugarcorn plants.
- Evaluation of biobutanol production using Clostridia species cultivated in sugarcorn juice medium.
- Assessment of sugarcorn as a sugar-based Canadian feedstock.

1.4 Thesis Organization

The present work contains seven chapters and follows the "integrated article" format as outlines in the Thesis Regulation Guide by the School of Graduate and Postdoctoral Studies (SGPS) of the University of Western Ontario. The chapters are explained below:

Chapter 1 presents the general introduction and background, research objectives and contribution.

Chapter 2 encloses the literature review on biobutanol production from lignocellulosic biomass, chemical and biological hydrolysis of the biomass. The Canadian sugar-based sugarcorn development was discussed, and the metabolism of the butanol producing Clostridia was explained.

Chapter 3 describes the study of the fermentation of simple sugars by Clostridia species.

Chapter 4 presents the pretreatment of corn cobs (lignocellulosic biomass), cellulase activity experiments and biobutanol fermentations.

Chapter 5 describes the characterization of sugarcorn juice based on total solids, total dissolved solids, moisture content, density, and sugar concentration. The effect of autoclaving and filtration on sugars juice was examined. An analysis of variation across different sugarcorn hybrids was carried out.

Chapter 6 presents a study of biobutanol fermentation using sugarcorn juice by three different Clostridia strains.

Chapter 7 encloses an evaluation of sugarcorn as a potential Canadian energy crop, typical growth, and juice characteristics are compared with sugarcane, energy cane and sweet sorghum. A Canadian sugarcorn (CANSUG) biorefinery was proposed.

Chapter 8 summarizes the general conclusions of the research and recommendations for future work based on the results of this study are given.

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Chapter 2

2 Literature Review

2.1 Introduction

In an effort to reduce GHGs emissions, sustainable, low carbon and renewable energy alternatives to fossil fuels have been explored, of which plant-derived biomass represents an abundant and inexpensive source [Stern, 2008]. The sustainable processing of renewable biomass to produce bio-based chemicals, biofuels and energy is referred to as Biorefining [Cherubini et al., 2009;Saddler et al., 2012]. It can support rural communities by strengthening agriculture and forest-based economy, and helping to diversify energy sources [Dale et al., 2014].

Canada is currently consolidating a series of strategic actions to pursue the reduction of GHGs emissions through the increased use of lower carbon fuels and alternative technologies, such as electricity, renewable natural gas, hydrogen, and renewable fuels. Among them, the creation of a Clean Fuel Standard is under development by Environment and Climate Change Canada (ECCC) [National-Energy-Board, 2017]. Biobutanol, as a renewable fuel, has numerous advantages over bioethanol and has been undertaking increased activity in R&D. Among the technologies to produce biobutanol, microbial fermentation can utilize carbohydrates from lignocellulosic agricultural wastes and converted to n-butanol, acetone, ethanol, and organic acids. Alternatively, energy crops can be used as a substrate for biological fermentations [Durre, 2008].

2.2 Canadian climate policy framework for reducing GHGs emissions

Although Canada has one of the biggest oil sands reserve (Alberta, Canada) in the world, there have been projects, such as ecoENERGY Innovation Initiative (Canada's Economic Action Plan, October 2012), to invest in the development of renewable energy and cleaner energy technologies in order to diversify energy sources, ending in 2017 [Goverment-of-Canada, 2012].

Recently, both federal and provincial governments made major policy announcements throughout 2016 to advance Climate policies in Canada. Amid these policies are pricing carbon pollution, amendments to federal regulations to phase out traditional coal-fired generation by 2030, and a plan to develop a Clean Fuel Standard.

Currently, new regulatory requirements for a Clean Fuel Standard are being developed under the umbrella of the Canadian Environmental Protection Act (CEPA, 1999), and it is being led by the Environment and Climate Change Canada (ECCC), a Governmental department responsible for coordinating environmental policies and programs. The primary objective of the Clean Fuel Standard is to achieve 30 megatonnes of annual reductions in GHG emissions, targeting a 30% emissions reductions below 2005 levels by 2030 [National-Energy-Board, 2017]. The standard will include requirements for the regulatees to reduce the GHG emissions from the fuel they supply and will be set to minimize the lifecycle carbon intensity of fuels provided on a given year, based on lifecycle analysis.

Canadian provinces follow the Federal Renewable Fuel Mandate, as shown in Figure 2.1, under which a minimum amount of renewable fuel is required to be blended into traditional petroleum fuel. The regulations require petroleum fuel producers and importers to have an average renewable content of at least 5% based on their volume of gasoline (E5) and an average renewable content of at least 2% based on their amount of diesel (B2) fuel and heating distillate oil [Goverment-of-Canada, 2010;National-Energy-Board, 2017].

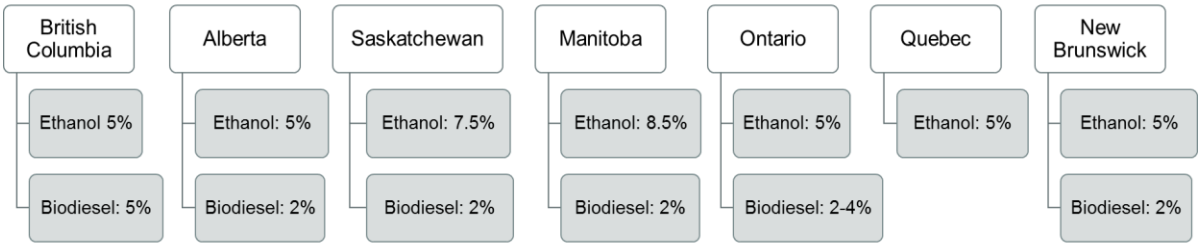

Figure 2.1. Provincial mandates of biofuels in conventional fuels blend in Canada

The Gasoline regulation in Ontario, implemented in 2007, requires at least 5% ethanol in gasoline and provides a regulatory incentive for cellulosic ethanol (1-liter cellulosic ethanol is equivalent to 2.5 liters of ethanol). Based on 2016 Climate Change Action Plan, Ontario wants to increase the availability and use of lower-carbon fuels including the renewable fuel content of gasoline [National-Energy-Board, 2017].

The Canadian government is setting up a Clean Fuel Standard for 2018 that considers ethanol-butanol-gas blends, aiming to achieve 30 Mt of annual reductions in GHG emissions. In April 2018, the O. Reg. 535/05 (Ethanol in Gasoline) included changes that requires gasoline suppliers to maintain an average of 10% starting in 2020 [Ontario, 2018]

The importance of the creation of new Canadians policies is giant; it brings certainty to the Canadian biofuels companies and its investors; the polices are a substantial commitment for a healthy and robust atmosphere to support Research & development & innovation in renewable biofuels throughout the country.

2.3 n-Butanol: A Chemical and biofuel

Butanol can be used as a fuel in automobiles or as an industrial chemical commodity, as a diluent or extractant [Durre, 2007;Cascone, 2008]. Butanol (C_4H_9OH) is a colorless and flammable alcohol, also known as 1-butanol, butyl alcohol or n-butanol. It is a chiral molecule with four isomers, n-butanol, 2-butanol, iso-butanol, and tert-butanol, each of which has different properties such as boiling points, densities, melting points, and octane numbers [National-Center-for-Biotechnology-Information, 2015].

Present industrial butanol production is achieved through two petrochemical processes. One involves catalytic condensation of ethanol to produce butanol via the Guerbet reaction; the second one consists of the oxo reaction [Matar and Hatch, 2001]. In industries, butanol is predominantly used as a chemical precursor to produce polymers and plastics, like butyl acetate and butyl glycol ether. It is used as a solvent in the production of cosmetics, detergent formulations, hormones, antibiotics, vitamins, and drugs. Also, it is used as a direct solvent in paints and diluent for hydraulic fluids [Durre, 2007;Green, 2011].

Butanol has several intrinsic properties which make it an exciting biofuel over ethanol, including that n-butanol is less hygroscopic and corrosive. Besides, butanol air-to-fuel ratio (A/F) and energy content is closer to that of gasoline, as shown in Table 2.1 [Liu et al., 2013]. Butanol could be used in pure form or blended with gasoline in automotive spark-ignition engines without significant modifications [Ramey and Yang, 2004], in comparison to ethanol, in which modified vehicles have to be available for the user. Commercial E85 flex-fuel vehicles (FFVs) have been designed to utilize ethanol-gasoline volume blends of 85% ethanol-15% gasoline. FFVs are vastly available in North America, Europe, and Brazil. However, in non-FFVs, only 10% ethanol-90% gasoline volume blends are the highest concentration legally permitted for use in the USA and Europe, and 5% ethanol- 95% gasoline blends currently in Canada [Yanowitz and McCormick, 2009;Environment-Canada, 2010].

Table 2.1. Comparison of fuel properties [Dernotte et al., 2010;Liu et al., 2013]

Fuel	Chemical formula	Octane number	Low heating value (MJ/kg)	RVP (kPa)	Evaporation heat (MJ/kg)	A/F stoichio-metric	Energy density (MJ/L)	Flammability Limits (% vol)
Gasoline	~$C_8H_{15.6}$	80-99	43.5	60-90	0.36	14.7	32	0.6-0.8
Ethanol	C_2H_6O	108	26.8	19.3	0.92	9.0	19.6	4.3-19
n-Butanol	$C_4H_{10}O$	96	32	18.6	0.43	11.1	29.2	1.4-11.2

For instance, butanol's energy density is closer to that of gasoline and contains 33% more energy than ethanol, which is convenient for running the automotive for longer distance. Butanol is less volatile and flammable than ethanol, which makes butanol a safer and an easier liquid to be transported through existing pipelines and infrastructure with little risk of corrosiveness [Liu et al., 2013]. However, ethanol has a higher-octane rating, and a higher heat of evaporation when compared to butanol, as shown in Table 2.1.

The study of butanol/gasoline blends and its effects on the greenhouse gas emissions (CO, SOx, and NOx) in internal combustion engine have been studied. Results exposed that when butanol-gas blends remained under B60 (60% butanol- 40% gasoline), CO emissions were

lower than using gas alone. However, NOx emission continued very close when butanol-gas blend or gasoline alone was used in an ICE [Dernotte et al., 2010].

In 2015, n-butanol was cleared to be blended with gasoline up to 12.5% in the USA [ASTM, 2015]. Current strategies to introduce biobutanol into E15 and E85 gasoline-ethanol blends have been found to satisfy European Standards (EN 228 and CEN/TS 15293/2011) [Lapuerta et al., 2017]. Thereby, successful approval of n-butanol-gasoline and n-butanol-ethanol-gasoline blends will encourage the commercial production of biobutanol as a bio-based fuel.

2.4 Feedstocks available for biofuels

The North American model of ethanol production from corn grain has been successful, especially in the United States, which is next only to Brazil in global ethanol production. Brazil has successfully utilized sugarcane for about 30 years to produce ethanol from the sugars extracted from the stalk, and burning the resultant bagasse to generate electricity [White et al., 2012]

However, the starch-based ethanol process can achieve only one-ninth of the energy conversion efficiency of ethanol from sugarcane [Reid et al., 2015]. In recent years, 40% of United States corn production has been redirected for use as a cellulosic raw material in the biotechnological production of ethanol. As a result, an imminent worldwide debate has been set regarding the use of food crops to produce biofuels [Wallace, 2005].

Cellulose-based residues from forests and agriculture are used in the developing second-generation biofuels sector [Mohr and Raman, 2013]. The primary challenge in the process has been the cost of feedstock [Mabee, 2014] and the development of sustainable pretreatment processes [Mosier et al., 2005]. It is worth to note that for a cellulosic biofuel industry to be successful, well-planned supply chain and logistics of feedstocks (harvest and transport) are two crucial aspects to plan [Dale, 2015]. The cellulosic feedstock can be converted into biofuel through either biochemical or thermochemical route. The former generally requires the use of expensive enzymes, whereas the latter mandates high capital costs, which currently limits the use of cellulosic biofuels in large scale [Wright, 2014].

2.4.1 Lignocellulosic biomass composition

Lignocellulosic biomass encompasses three major biopolymers: cellulose (14-47%), hemicellulose (19-50%) and lignin (5-30%). A representation of a typical lignocellulosic structure is shown in Figure 2.2. Cellulose is a linear homo-polysaccharide of glucose units connected by strong β-1,4 glycosidic bonds. Adjacent cellulose chains are held together firmly via hydrogen bonding, resulting in a high degree of crystallinity. Several cellulose fibrils cluster together, yielding larger microfibrils, which in turn assemble to form even larger macrofibrils, the integral constituents of a plant cell wall. The macrofibrils encloses several microfibrils of several glycosidic units.

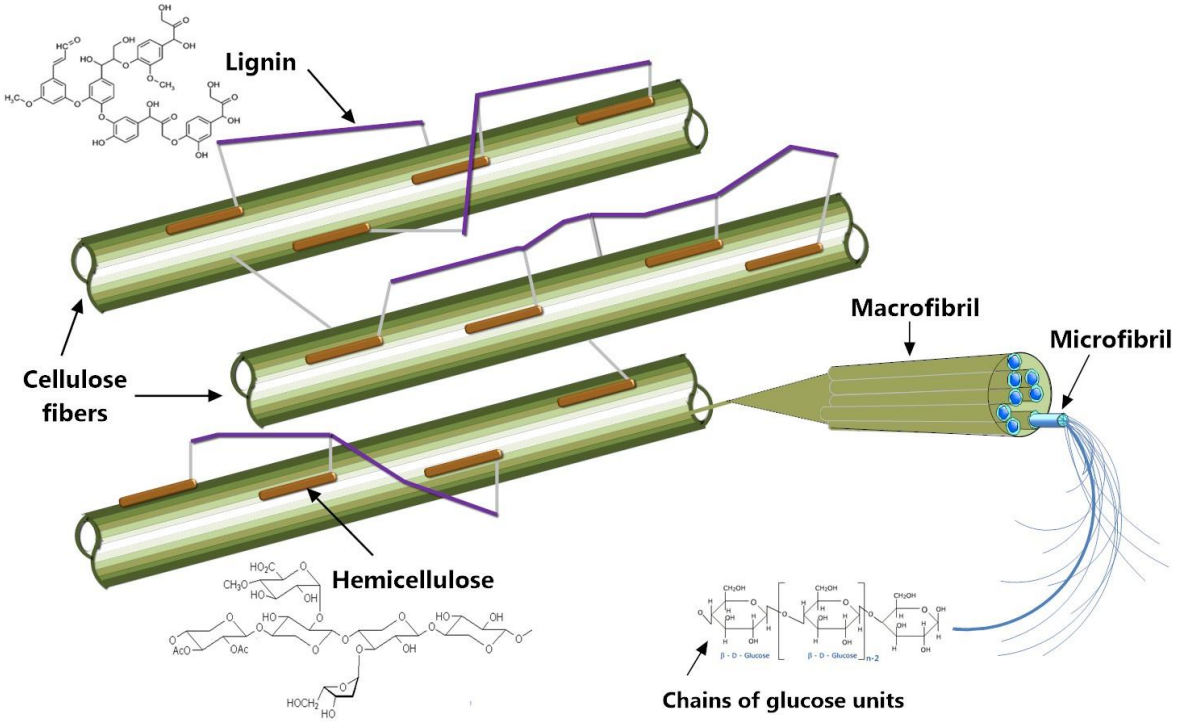

Figure 2.2 Representation of a typical lignocellulosic structure and its biopolymers.
(figure created using Smartdraw® software)

Hemicellulose, on the other hand, is an amorphous and branched heteropolymer, characterized by a low degree of polymerization and low thermal stability. It includes monomer units such as pentoses (arabinose and xylose), hexoses (glucose, galactose, mannose, rhamnose, and fucose) and uronic acids (galacturonic, glucuronic and methyl glucuronic). Lignin is a highly cross-linked aromatic polymer of phenylpropane units, whose size may vary depending on the

feedstock. Lignin building blocks include monolignols, phenylpropane, ρ-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol [Demain et al., 2005;Keshwani, 2010].

Lignocellulosic biomass possesses a complex structure of cellulose interconnected with hemicellulose and surrounded by a lignin sea. The lignin has a limited covalent association with amorphous hemicellulose and the inherent crystalline-cellulose structure, resulting in a rigidly packed arrangement [Demain et al., 2005;Mosier et al., 2005].

2.4.2 Lignocellulosic biomass

Lignocellulosic biomass comprises forestry wastes (e.g. wood chips, and sawdust), agricultural residues (e.g. corn stover, corn cobs, wheat straw, barley straw, rice straw, sorghum straw, and sugarcane bagasse), energy crops (sweet sorghum, energy cane, miscanthus, switchgrass, and recently, sugarcorn hybrids), industrial wastes and municipal solid wastes [Mosier et al., 2005;Jansen and Lübberstedt, 2012b;Reid et al., 2015]. Among suitable Canadian energy crops, the native Switchgrass (*Panicum virgatum L*) of Western Canada has shown promise as a viable feedstock, along with other warm-season grasses including big bluestem (*Andropogon gerardii*) and little bluestem (*Schizachyrium scoparium*) [Mabee, 2013].

Microalgae-biodiesel-residues dried distillers' grains, and agave bagasse are among other polysaccharide-rich residues for biofuel production [Ezeji and Blaschek, 2008;Hernandez-Salas et al., 2009;Cheng et al., 2015].

Common lignocellulosic agricultural residues and their typical compositions are shown in Table 2.2. These residues present an attractive, low-cost and non-food option to produce biofuels and bio-based chemicals, offering environmental, social and economic benefits. Selection of biomass for a commercial bioprocess requires the evaluation of its qualities, such as cost, availability, uniformity, purity and cellulosic yield [Tolan, 2002].

Table 2.2. Typical composition of different agricultural lignocellulosic materials (% of total dry material)

Material	Physical structure	Cellulose	Hemicellulose	Lignin	Ash	Information Ref.	Picture Ref.
Barley straw		33	24	16	na	[Tomas-Pejo et al., 2008]	[Roberts, 2012]
Dried distillers' grains		17	28	28	5	[Mussatto and Roberto, 2005]	[Pinkerton, 2015]
Corn cob		42	33	18	1.5	[Schwietzke et al., 2009]	[Gomez-Flores, 2015]
Corn fiber		14-35	30-35	na	na	[Saha and Cotta, 2006]	[Hochman, 2014]
Corn stover		37	28	23	na	[Tomas-Pejo et al., 2008]	[Austin, 2009]
Rice hulls		21.5	23	14.6	na	[Megawati et al., 2011]	[Young, 2016]
Rice straw		32-47	19-27	5-24	10-17	[Binod et al., 2010]	[Yonezawa, 2011]
Sorghum straw		35	24	25	na	[Téllez-Luis et al., 2002]	[Texturez, 2008]
Sugarcane bagasse		43	26	22	1.4	[da Silva et al., 2010]	Maari, 2013]
Sugarcane straw		33	29	32	5.7	[da Silva et al., 2010]	[Golovaty, 2011]
Wheat straw		36	28	29	na	[Qureshi et al., 2008]	Zcool, 2010]

na = not available

2.5 Corn

Maize (*Zea mays* L.), most commonly known as ‘corn’ in the United States and Canada, is a Mexican-native giant grass that employs the C4 pathway for photosynthesis and widely grown grain in the Americas [Matsuoka et al., 2002], it is also broadly cultivated all around the world because of its ability to grow in varied environments. In some countries, for example, Mexico, corn is the main component of their daily diet [Wellhausen, 1952].

It exhibits a high efficiency of carbon fixation, water use, and nitrogen (N) economy. It can utilize carbon dioxide, water and nutrients to produce sugars to serve as energy reserves for growth and metabolism. As the corn plant grows, the sugars that are initially stored in the stalk and leaves ultimately get accumulated in the grain as starch [Abendroth et al., 2011].

2.5.1 Corn growth and development

The plant growth and development are terms often used interchangeably, yet each has a particular meaning. Growth refers to the increase in size of an individual plant or plant component. Development refers to the plant’s progression from earlier to later stages of maturity based on specific criteria that must happen to validate that the plant has reached a particular stage [Abendroth et al., 2011].

Temperature, moisture stress, weed pressure, and adequate fertility will affect the corn growth. Moreover, the progression of corn developmental stages can be predicted and it is related to temperature [Abendroth et al., 2011].

Corn expresses a determinate growth habit, which is defined by the single stalk terminating in the tassel, at top. Vegetative structures (leaves and stalk) are initiated and then continue to grow while the reproductive structures (male tassel and female ears) are initiated and rising, as seen in Figure 2.3. Regularly, different portions of the plan are growing, but the plant is staged only by what is identifiable at a specific point in time without dissection.

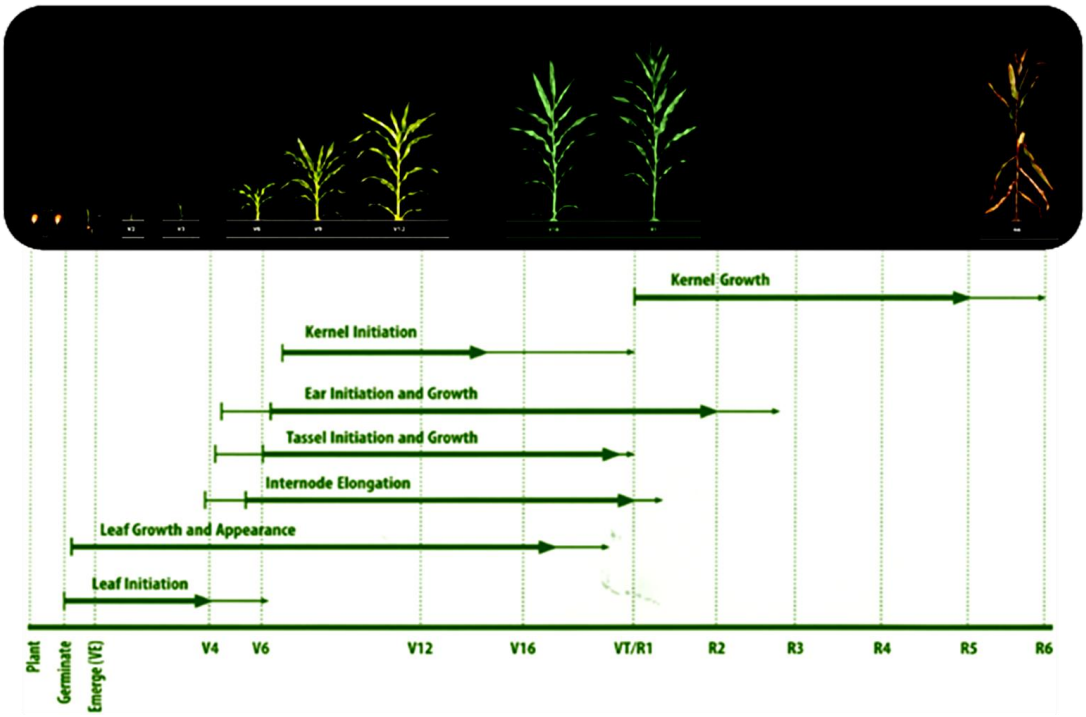

Figure 2.3. Diagram of initiation and growth of corn plant (planting to physiological maturity, R6). Bold arrows denote the primary period when corn initiates a new stage; thin arrows are possible variations. Adapted from [Abendroth et al., 2011]

Corn is first staged based on its vegetative development (V). Once that is complete, its stage is based on the development of the reproductive (R) structure (ears), and on established in visual indicators of kernel development. Corn has male and female flowers separated by a distance on the plant as the tassel and ears respectively. The highest and final leaf on a plant varies with hybrid, planting date, and location (most hybrids produce 19 to 20 leaves). VE means emergence and VT, tasseling. Reproductive stages are named with "R" followed by the numbers 1 to 6, as displayed in Figure 2.3. Kernel initiation referred to the initiation of florets, which may eventually become kernels if pollinated and fertilized [Abendroth et al., 2011].

To fully understand the mechanisms, the development of the corn plant is correlated with the air temperature. Therefore, development varies from year to year if the calendar is only accounted. Prediction of corn development becomes obtainable within and across growing seasons when evaluated using thermal time. This concept represents the length of time the corn spends within a defined temperature range considered optimum for that crop. The time required for corn to progress from one developmental stage to another is based on the amount of heat accumulated. From the several models for measuring corn thermal time, the most used technique is called growing degree days (GDD) [Abendroth et al., 2011].

2.5.2 Corn cob

Leftover biomass from corn harvestings, such as corn cobs and corn stover, are currently waste materials but could offer an affordable lignocellulosic biomass source. In the past, corn cob and corn stover residues were left behind on cornfields to maintain soil quality [Jansen and Lübberstedt, 2012a]. However, a U.S. Department of Agriculture report from January 2013, indicates that soil quality would not decrease if the cobs/stover are removed. Furthermore, this agricultural waste makes up 20 % of the corn residue by weight [UPI, 2013].

Corn cob is the central core of a maize ear and is considered an agricultural waste like corn stover. The physiology of the cob can be described, as shown in Figure 2.4 [Sehgal and Brown, 1965;Nickerson, 1954], as concentrically tubes and four zones can be seen in the cross-section of a corn cob:

1. White-inner pith containing thin-walled parenchyma cells.
2. Woody ring or mid-cob, shaped of rachis nodes, inner and outer vascular system.
3. Coarse chaff, basically basal portions of the first and second glumes or rudimentary leaves.
4. Fine chaff, consisting of flimsy lemmas and paleas.

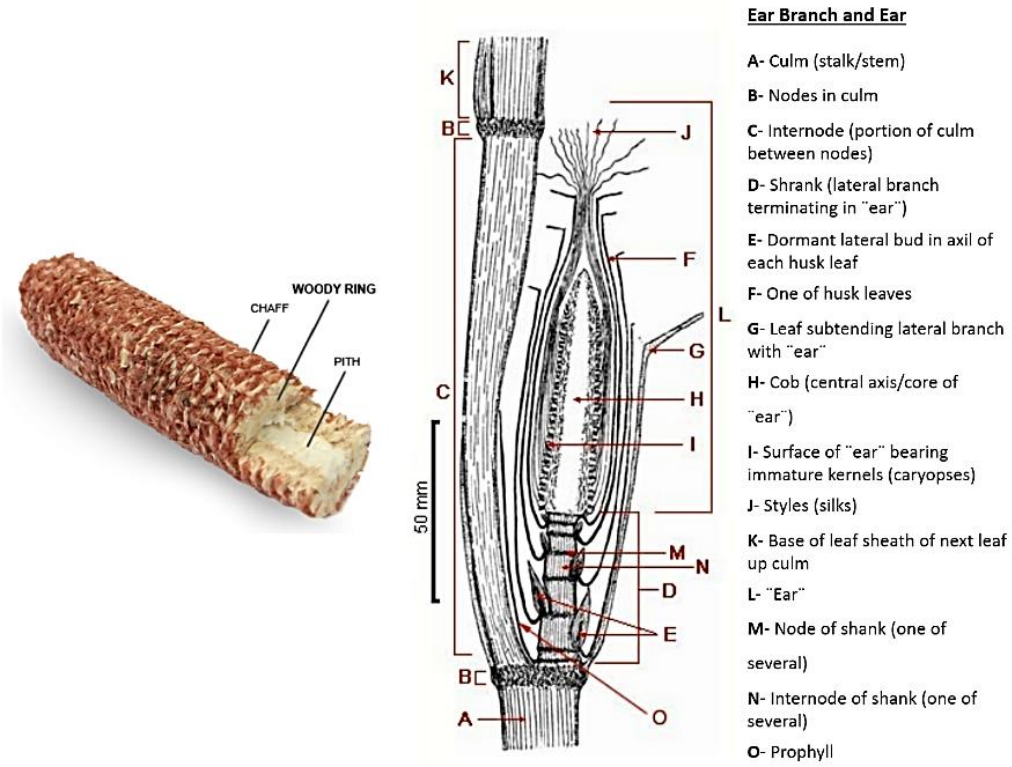

Figure 2.4. Structure of corn and corn cob [Weatherwax, 1955]

The cob mass percentage has been reported as follows 1.9% pith, 60.3% woody ring, 33.7% coarse chaff, and 4.1% fine chaff [Weatherwax, 1955], yet these percentages may change based on the corn hybrid and inbred. Furthermore, the range of cob measurements differs widely by genotypes, geographical location, variety, climate conditions and harvest methods [Lens, 1948]. The mean cob length is 15.42 cm, mean diameter is 2.5 cm, mean density is 0.28 g/mL, mean mass is 61.87 g and mean volume is 78.30 mL [Floey and Vander Hooven, 1981].

The elemental analysis of corn cobs resulted in the following composition: carbon 48.1%, hydrogen 6.0%, nitrogen 0.4%, sulphur 0.1%, oxygen 44% and ash 1.5% [Preto, 2010]. Interestingly, corn cobs can be used to produce furfural, an important chemical in the manufacture of resins for automotive brakes, or fiberglass. Furthermore, dry corn cobs have a high absorption capacity, higher than clay, and as such are used for spill cleanups (Tin Win 2005). Corn cobs, despite variation of its chemical contents, are abundantly available agricultural waste and are perfect candidates as a substrate for biobutanol fermentation.

2.5.3 Corn in Canada and Ontario

In Canada, the provinces of Ontario and Quebec are the two main corn producers. In 2017, Canada's corn for grain production was estimated to be 14.3 million metric ton, with a harvested area of 1,417,200 ha (planted area of 1,447,200 ha) and a corn production yield of 10.1 ton/ha. The overall movements from 2010 to 2017 are shown in Figure 2.5 [CANSIM, 2017].

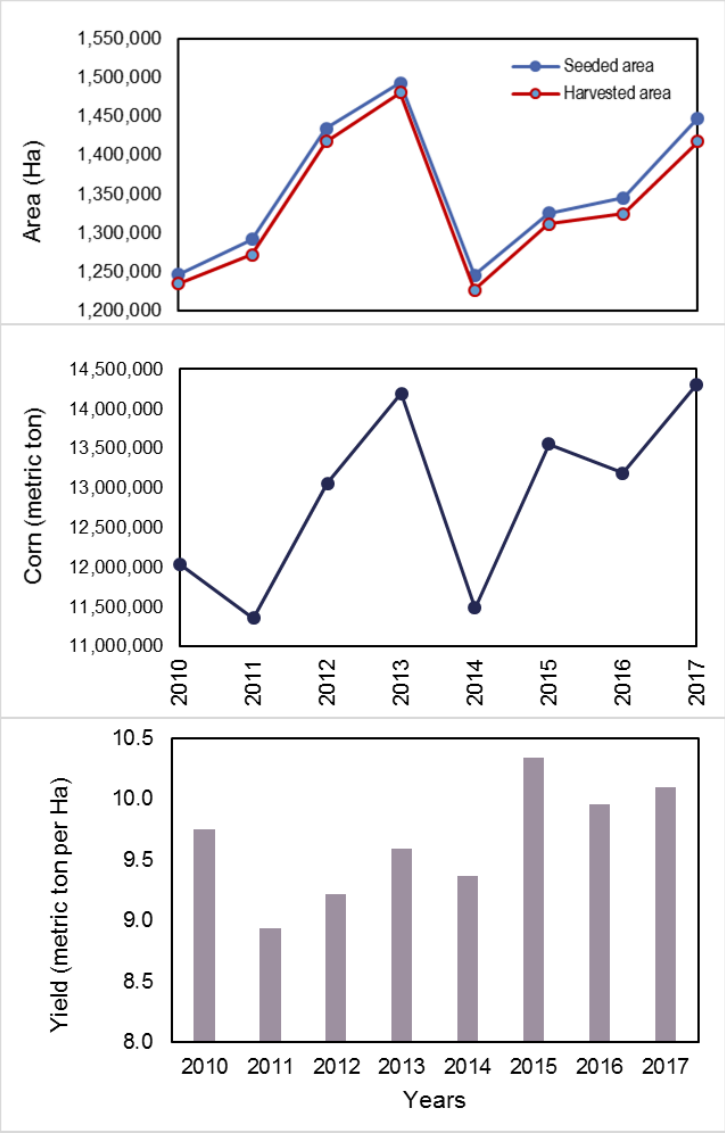

Figure 2.5. Estimated areas, yields, and production of corn in Canada from 2010-2017. Created using information from [CANSIM, 2017;Statistics-Canada, 2017b]

A Canadian map showing the geographical location of corn for grain is displayed in Figure 2.6. The map was produced using remote sensing and geospatial analysis from Statistics Canada with information from the 2011 Census of Agriculture. As shown, almost all the corn-for-grain production is carried out in the A (southwestern Ontario) and B (southern Quebec) areas.

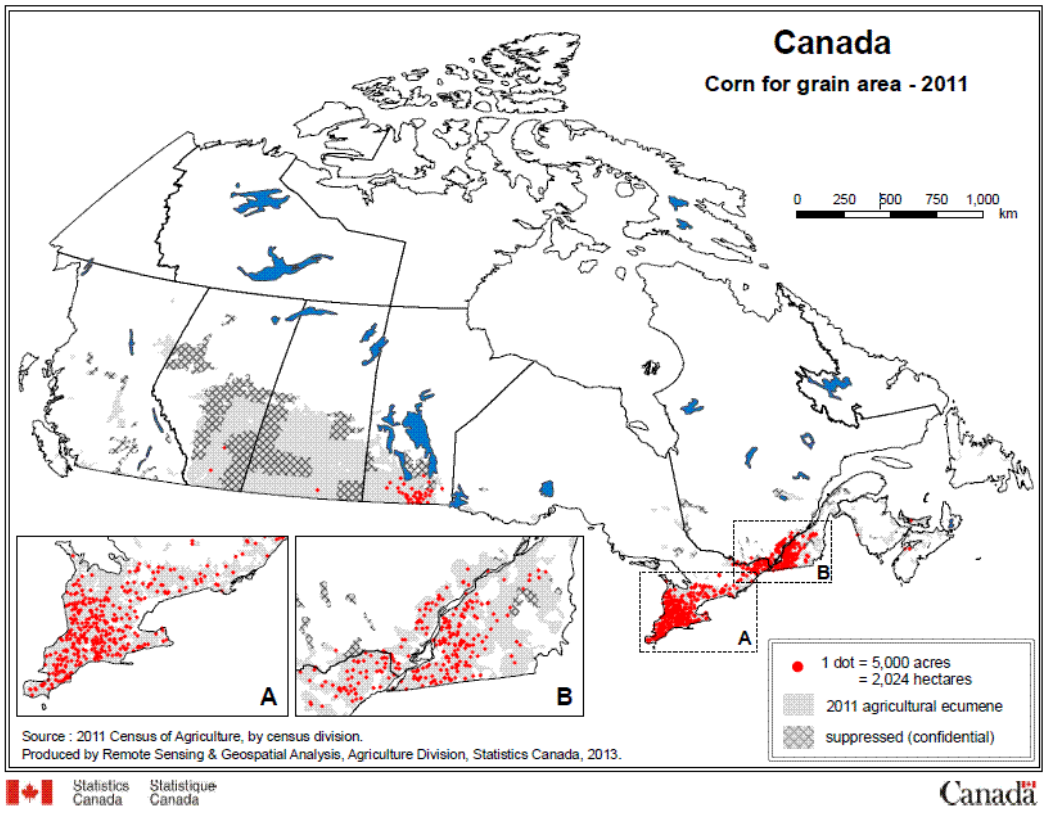

Figure 2.6. Canadian map showing the geographical location of corn for grain.

(From Census of Agriculture 2011, prepared by Statistics Canada, 2013)

Statistic Canada released recently information that Ontario planted 58% (849,841 ha), Quebec 26% (380,000 ha), Manitoba 11% (165,921 ha) and other provinces 4% (61,108 ha) of corn for grain. An overall increase of 7.5% in cultivation area occurred from 2016 to 2017 [Statistics-Canada, 2017a;Statistics-Canada, 2017b].

2.6 Sugarcorn

Corn hybrids can be tailored to enhance specific characteristics to suit different applications. For example, hybrids particularly rich in proteins, thiamin, niacin, pantothenic acid or folate have been developed for food industry [Xu and Crouch, 2008]. For Canada, in particular, the climatic conditions in most regions demand short growth periods, which is a major challenge in finding a viable fuel crop to help meet the country's energy demand [Mabee, 2013;Reid et al., 2015].

As an alternative to corn grain and lignocellulosic corn stover, corn hybrids with high stalk sugar content referred to as 'sugarcorn' have been developed by Agriculture and Agri-Food Canada (AAFC, Ottawa, Ontario), as pictured in Figure 2.7. Sugarcorn was envisioned as potential Canadian energy crop suited for short growing seasons in the country. AAFC agronomists carefully chose these corn hybrids from various inbred corn lines along several years [Reid et al., 2015;Reid et al., 2016].

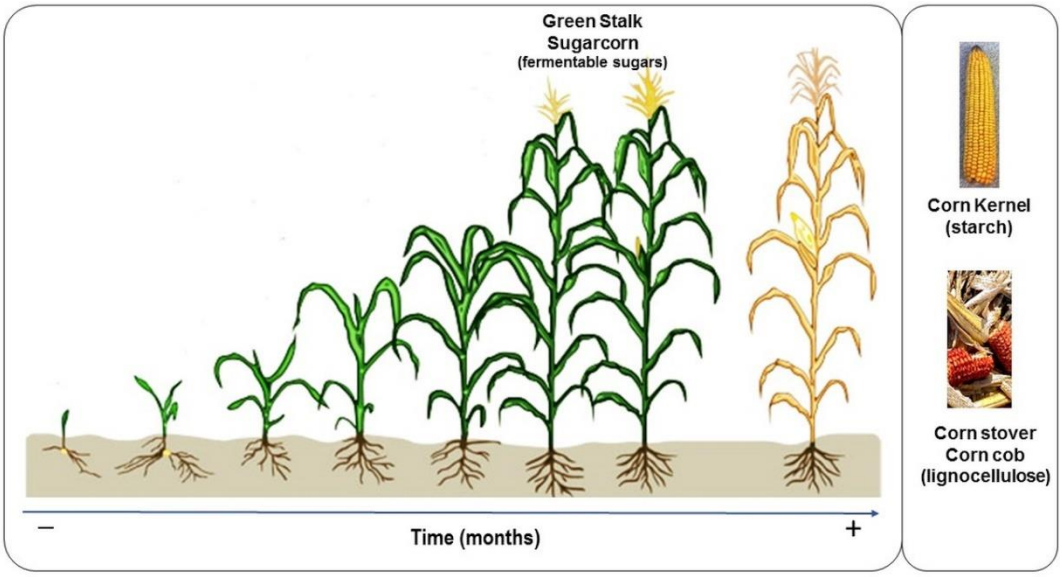

Figure 2.7. Representation of Canadian Sugarcorn (sugar-based feedstock) an alternative to corn grain (starch-based feedstock) and corn stover/cob (cellulosic-based feedstock).

Corn stalks accumulate sucrose, glucose, and fructose as well as other soluble solids [Loomis, 1945] until 2-3 weeks after silking. Over time, the sugar concentration declines due to translocation of metabolites from stalk to grain and is converted to starch, unless there is severe drought stress, pollination is prevented, or by ear removal [Hume and Campbell, 1972;Abendroth et al., 2011]. Stalk sugar content is a genetically influenced trait and corn hybrids resistant to cold injury and stalk rot have been known to reach high sugar concentrations [Van Reen and Singleton, 1952;Reid et al., 2015].

Sugarcorn plants reach high concentrations of stalk sugars in the days following silking, facilitating an earlier harvest before corn maturity, thereby saving agronomic resources. The germplasm is adapted to Canadian short growth seasons from May to September, particularly suitable for the primary corn growing regions of Southwestern Ontario and Southern Quebec [Reid et al., 2015;Reid et al., 2016].

2.6.1 Sugarcorn juice

Sugarcorn stalks contain readily fermentable sugars that can facilitate a direct bioconversion process which can circumvent the need for enzymes, unlike processes based on starch and cellulosic feedstocks. The juice extracted from the green stalks can supply sugars for the production of liquid biofuels, such as bioethanol and biobutanol [Reid et al., 2015]. A similar route, fermentation of rich sucrose juice, has been used by the Brazilian bioeconomy to produce biofuels [Pereira et al., 2015].

2.7 Hydrolysis, pretreatment of lignocellulosic biomass

The entire lignocellulose deconstruction process is a combination of physical, chemical, and biological treatments, which may vary based on residue characteristics and the desired soluble sugars. The first stage of biomass deconstruction is the pretreatment, which aims to alter the highly-organized lignocellulosic matrix into an accessible substrate for enzymatic hydrolysis or microorganisms [Mosier et al., 2005].

Pretreatment methods involve reduction of the biomass particle size by mechanical milling, followed by hydrothermal, chemical or physicochemical processes. Popular pretreatments that have been investigated through the years include steam explosion, carbon dioxide explosion, liquid hot water, ionic liquids, diluted and concentrated acids, alkalis, organosolvation, ammonia fiber explosion -AFEX^{TM}-, ammonia recycle percolation and ozonolysis [Mosier et al., 2005; Yang and Wyman, 2008]. Each process serves to disrupt the lignin seal, break the hemicellulose structure, and alter the cellulose structure in different arrangements. Pretreatment techniques for lignocellulosic biomass have been extensively reviewed in the literature [Mosier et al., 2005; Chundawat et al., 2010; Jönsson and Martín, 2016].

Figure 2.8 shows compounds commonly generated from pretreatment of lignocellulosic agricultural residues. Some of these compounds are known inhibitors, which may interfere with Clostridial cell growth and butanol production, requiring a subsequent detoxification step. There are three major groups of inhibitory compounds: (1) furan derivatives, with 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (HMF) being the most abundant compounds, (2) weak carboxylic acids, mainly acetic, formic and levulinic acid, and (3) phenolic compounds, such as, vanillin and syringaldehyde from the degradation of lignin.

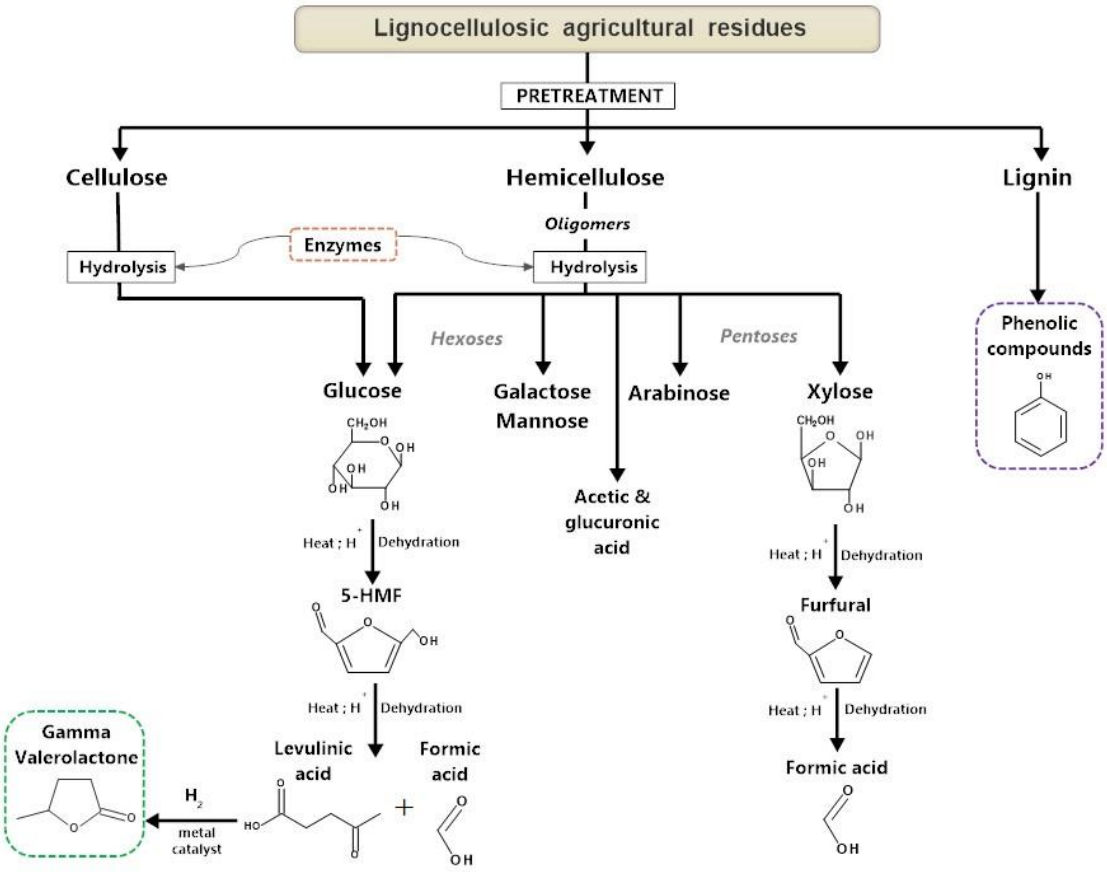

Figure 2.8. Compounds commonly generated from pretreatment of lignocellulosic agricultural residues.

The furan derivatives, HMF, and furfural, are dehydration products of hexoses and pentoses, formed during the treatment of lignocellulosic materials at high temperatures and pressures [Chundawat et al., 2010;Jönsson and Martín, 2016]. Weak acids are formed through deacetylation of hemicellulose, or through further degradation of HMF and furfural, whose decomposition products lead to the formation of levulinic acid and formic acid [Jönsson and Martín, 2016], as shown in Figure 2.8.

Several detoxification processes have been employed, including biological (fungi and fungal enzymes), physical (evaporation and adsorption), and chemical processes (alkali-NaOH, KOH, $Ca(OH)_2$ and, overliming) [Jönsson and Martín, 2016]. The use of an extra detoxification step adds cost to the overall process and will depend on the concentration of the inhibitors and their effects on biobutanol fermentation process.

2.7.1 Dilute acid pretreatment

Dilute acid hydrolysis is the most widely used pretreatment for conversion of lignocellulose [Tolan, 2002]. Among the mineral acids used are sulfuric, hydrochloric, and phosphoric acids. The pretreatment is a dry-to-slurry process which results in high recovery of the hemicellulosic sugars in the pretreated liquor, and a solid cellulose fraction with modified surface [Jönsson and Martín, 2016]. Dilute acid (DA) process is typically carried out using 0.5-2.5 wt% sulfuric acid, with temperatures around 120 to 250 ˚C, under pressures of 15 to 75 psi, and with reaction time varying from 1-120 min [Mosier et al., 2005;Tolan, 2002]. The rate of hydrolysis is affected by the amount of lignin in the biomass, with higher lignin content leading to a slower rate [Megawati et al., 2015].

After pretreatment, the acidic solution must be neutralized before further process [Mosier et al., 2005]. DA hydrolysates contain mainly xylose, arabinose, glucose, galactose, and mannose, in conjunction with furans, phenolics, weak acids, and other compounds, [Chandel et al., 2012].

DA hydrolysis does not result in significant corrosion of the equipment, and as only a small amount of acid is used, there is no economic or regulatory need to recover it. The pretreatment step produces material with a high surface area suitable for further enzymatic hydrolysis [Tolan, 2002]. DA of lignocellulosic materials has been used for the commercial production of furfural from hemicellulose-derived xylose [Mosier et al., 2005;Peterson et al., 2008].

Several pilot-scale continuous and large-scale batch reactors have been developed [Chundawat et al., 2010;Tolan, 2002;Chandel et al., 2012]. Pilot-scale DA studies (190°C, ~2 wt.% H_2S0_4) of corn stover have shown the formation of degradation products, such as 5-HMF (15.7 mg/g), furfural (7.94 mg/g), levulinic acid (3.65 mg/g), formic acid (3.17 mg/g), p-coumaric acid (1.83 mg/g) and ferulic acid (1.31 mg/g) [Chundawat et al., 2010]. As some of the compounds may inhibit or interfere with microbial fermentation, several detoxification techniques have been employed. These may be biological (fungi and fungal enzymes), physical (evaporation and extraction), or chemical (alkali-NaOH, KOH, $Ca(OH)_2$ and overliming) [Peterson et al., 2008] treatments. To overcome the high rate of sugar degradation reactions, short residence times (10 s to 1 min) at high temperatures (240-400 °C) have been proposed to obtain high yields of

glucose [Luterbacher et al., 2014]. Detailed reviews of DA hydrolysis and its chemistry are available in the literature [Qian et al., 2005;Peterson et al., 2008;Chandel et al., 2012].

2.7.2 Enzymatic Hydrolysis or Saccharification

Cellulases, commonly used for the depolymerization of cellulose to glucose, consists of three major classes: endoglucanases (EC 3.2.1.4), exoglycanases, including cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21). Xylanases are enzymes that can hydrolyze hemicellulose to xylose, arabinose, mannose, glucose, galactose, and acetate. Bacteria, fungi, and actinomycetes are major producers of xylanases. Among fungi, *Aspergillus* and *Trichoderma spp.* have been utilized for commercial production of the enzymes [Demain et al., 2005;Banerjee et al., 2010;Zhang and Zhang, 2013]. Most companies have developed their proprietary enzyme cocktails, with enzymatic activities as high as 180 FPU/mL [Eckard, 2015].

The actual cost of commercial enzymes ($/unit activity, or $/kg enzyme preparation) is not visibly marketed and fluctuates. In 2012, there was a baseline estimated production cost of cellulase to be $10.14/kg [Klein-Marcuschamer et al., 2012]. Factors such as substrate loading, enzyme loading, enzyme thermostability, and hydrolysis time are crucial and impact the amount of enzyme needed for each lignocellulosic biomass [Singhania et al., 2007;Zhang and Zhang, 2013;Eckard, 2015]. Examples of pretreatments, enzymatic conditions, and sugar yields are presented in Table 2.3.

Table 2.3. Acetone-Butanol-Ethanol (ABE) production, yield, and productivity from pretreated lignocellulosic agricultural residues by different *Clostridium* species

Agricultural residue	Pretreatment & Hydrolysis	Inhibitor Removal	Microorganism	Bioreactor type	Working volume (mL)	C_{ABE} (g L^{-1})	$Y_{ABE/S}$ (g g^{-1})	P_{ABE} (g L^{-1} h^{-1})	C_{BTOH} (g L^{-1})	Time (h)	Ref.
Cassava bagasse	Water + Enzymes	None	*C. acetobutylicum* JB200	250 FBB and 1 L flask	1000	15.41	0.34	0.39	9.71	40	Lu et.al. 2012
Corn cob + glucose	$Ca(OH)_2$ + Enzymes	None	*Clostridium beijerinckii* NCIMB 8052	250 ml bottle	50	16	0.32	0.33	8.2	48	Zhang et. al. 2012
Corn cob	WDM + Enzyme	None	*C. acetobutylicum* SE-1	100 ml bottle	50	14.12	0.36	0.20	9	72	Zhang et. al. 2013
Corn fiber§ +xylose	Enzymatic	None	*C. acetobutylicum* P260	50 ml test tube	30	24.6	0.44	0.47	NS	NS	Qureshi et. al. 2006
Corn fiber	Dilute H_2SO_4 + Enzymes	XAD -4	*C. beijerinckii* BA101			9.3	0.39	0.10			Qureshi et. al. 2008a
Corn fiber	Enzymatic	None	*C. beijerinckii* BA101			8.6	0.35	0.10			Qureshi et.al. 2008a
De-oiled rice bran	Dilute H_2SO_4 + Enzymes	XAD-4	*C. saccharo perbutylacetonicum*N1-4			12.13	0.44	0.1			Al-Shorgani et al. 2012
Mix of agricultural waste	-	None	*C. beijerinckii* BA101			14.8	0.37	0.20			Jesse et. al. 2002
Wheat straw	1 % dilute $H2SO_4$ Enzymes wo sediments	None	*C. beijerinckii* P260	125 ml bottle	100	25	0.42	0.60	12.0	42	Qureshi et.al. 2007
Wheat straw	Alkaline peroxide Enzyme	Electro dialysis	*C. beijerinckii* P260	50 ml bottle	30	22.17	0.42	0.55	12.33	40	Qureshi et. al. 2008b
Wheat straw	Dilute H_2SO_4,, Enzymes w/sediments		*C. beijerinckii* P260	250 ml bottle	100	13.12	0.32	0.14	8.09	72	Qureshi et al. 2008c
Wheat straw	Dilute H_2SO_4 pH = 6.5 w/sediments		*C. beijerinckii* P260 SSF	2500 ml bioreactor.GS	1000	21.42	0.41	0.31		71	Qureshi et. al. 2008c

GS= Gas Stripping, WDM = wet disk milling, C_{ABE}= Total ABE production, $Y_{ABE/S}$= ABE yield, P_{ABE}=ABE productivity, C_{BTOH}= Butanol production, §= integrated hydrolysis, fermentation and recovery process, NS=Not specified.

Different methods have been proposed for the reduction of the amount of enzyme needed, such as enzyme immobilization, enzyme recycling, and the addition of surfactants, lipids, or metal ions [Eckard, 2015]. Non-ionic surfactants such as Tween 20 and Tween 80 have been demonstrated to enhance enzymatic hydrolysis from pretreated lignocellulosic residues [Tabka et al., 2006; Partida-Sedas et al., 2016]. The influence may be due to the surfactant's action to decrease the adverse adsorption of cellulase to crystalline cellulose and lignin. Surfactants are further known to prevent cellulase denaturation due to thermal deactivation during incubation by reducing surface tension and viscosity of the liquid, thereby enhancing the contact of the enzyme with the air-liquid interface [Eckard, 2015]. Furthermore, the application of nanomaterial principles opens new possibilities to increase both productivity and yields of enzymatic hydrolysis of cellulosic materials [Verma et al., 2014]. The use of nanomaterials (such as silica, gold-doped silica, magnetic) to immobilize cellulase enzymes is a promising new method, because they provide the enzyme with a stable environment for its enzymatic activity, making the enzyme-substrate bonding more specific [Verma et al., 2014].

2.8 Biobutanol production: Fermentative butanol production by *Clostridium spp.*

Biobutanol refers to n-butanol produced from renewable biomass, via microbial fermentation. The biological production of butanol can be achieved naturally by Clostridia species via the ABE fermentation process [Durre, 2008].

Clostridia are gram-positive, strictly anaerobic bacteria with a particular rod shape bacillus, varying in size from 0.5-1.5 and 1.5-6 μm, spore-forming and motile by peritrichous flagella. During sporulation, cells swell markedly and store granulose, a polysaccharide that serves as carbon and energy source during solventogenesis [Dürre, 2005]. Solventogenesis serves as a kind of emergency reaction to let the cells acquire time to complete endospore formation and, therefore, to guarantee long-time survival [Dürre, 2005].

Only few *Clostridium* species can produce butanol and are referred to as solventogenic clostridia [Durre, 2008;Lee et al., 2008b;Mitchell, 2015]. *Clostridium acetobutylicum* ATCC 824 has historically represented the ABE fermentation since its industrial production in 1916

[Moon et al., 2016]. Molecular taxonomic studies have classified solventogenic clostridia into four species: *C. acetobutylicum, C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* [Keis et al., 2001]. All of these were originally classified as *Clostridium acetobutylicum,* and were isolated in different part of the world from soil and vegetable material for the purpose of converting carbohydrate material into the industrially important acetone and butanol [Jones and Woods, 1986]. These species can consume a variety of carbon substrates including pentoses, hexoses, starches and even, complex substrates like cellulosic residues [Jones and Woods, 1986; Mitchell, 1998], which gives them an excellent advantage for industrial fermentations.

C. beijerinckii NCIMB 8052, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* were mistakenly designated as *C. acetobutylicum* in early work [Dürre, 2005]. Interestingly, strains of *C. acetobutylicum* and *C. beijerinckii* are more suitable to metabolize sugars from corn wastes, while *C. saccharobutylicum* and *C. saccharoperbutylacetonicum* can better utilize molasses-derived sugars, as substrates for ABE production [Durre, 2008; Mitchell, 2016].

The optimal temperature for Clostridia growth and fermentation is between 35 and 37 $^{\circ}$C, and the optimal pH is 4.5–7.0, at an atmosphere of pure CO_2, N_2 or a 1:9 mixture of N_2 and CO_2 [van Andel et al., 1985; Zigova and Sturdík, 2000]. However, many factors including the substrate concentration, pH, H_2 partial pressure, acetate, and butyrate, impact cell growth rate, final product concentration and the relative proportions of the products [Kong et al., 2006; Rodriguez et al., 2006;Jo et al., 2008]. Excess carbon source often affects osmotic dehydration of microorganisms in a fermentation process.

Nutrients in the fermentation media play an essential role in the growth and biobutanol production in Clostridia. While complex nitrogen sources such as yeast extract facilitate rapid growth and solvent production, the nutrient requirements for the growth are rather simple [Monot et al., 1982]. Clostridia require high redox potential to produce butanol (and ethanol) and the supply of additional reducing power results in increased butanol and ethanol formation with reduced acetone formation [Mitchell, 1998]. The oxidation-reduction (redox) potential (Eh) provides the most useful scale for measuring the degree of anaerobiosis. Simple stated, the Eh is a measure of the tendency of a solution to give or take up electrons (i.e. to be oxidized or reduced). Studies on *C. acetobutylicum* ATCC 824 using a synthetic medium provided with

sugars, salts, and vitamins show that some of these components could enhance growth and butanol and/or solvent production in Clostridia [Monot et al., 1982].

2.8.1 Anaerobic Fermentation by Clostridia

2.8.1.1 Sugars uptake by the solventogenic clostridia

The ability of solventogenic clostridia to metabolize different carbon sources is one of the most industrial attractiveness of the ABE fermentation. The understanding of the mechanisms of sugar uptake and its regulation in the cell is critical for further developments, and it is yet under research.

The main mechanism of sugar uptake is the PEP-dependent phosphotransferase system (PTS), which transports and phosphorylates its sugar substrates and it's the cornerstone in metabolic regulation [Mitchell, 2016].

PTS is a multicomponent phosphoryl transfer chain containing two general proteins, enzyme I (EI) and HPr, and two system-specific proteins (or domains) denoted to as IIA and IIB. A final system-specific protein/domain IIC (in some cases together with an additional protein/domain IID) provides the channel by which the sugar crosses the membrane to be phosphorylated as it enters the cytoplasm [Mitchell, 2016].

The recent clostridial genome sequences have shown that the presence of several phosphotransferases systems for the uptake of hexoses, hexoses derivatives and disaccharides. The genomes of *C. acetobutylicum* and *C. beijerinckii* have 13 PTS and 43 PTS, respectively. A lot of the PTS function have been only inferred on the basis of genome context and sequence analysis [Mitchell, 2015]. On the contrary, uptake of pentoses happens via non-PTS mechanisms. Numerous non-PTS transporters that could potentially be involved in sugar uptake are encoded in the genomes of all solventogenic clostridial strains.

Finally, the phenomenon of carbon catabolite repression (CCR) is present in solventogenic bacteria. Therefore, readily metabolized sugar applies an extensive inhibitory effect on uptake and metabolism of alternative substrates. CCR depends on the catabolite control protein (CcpA), which is driven to bind to regulatory target sites called catabolite responsible elements, as a result of interaction with a phosphorylated form of the PTS phosphocarrier protein HPr [Mitchell, 2016].

2.8.1.2 Clostridial fermentative pathways

The hexose sugars are metabolized via the Embden-Meyerhof pathway (EMP) with the conversion of 1 mole of hexose to 2 moles of pyruvate, with the net production of 2 moles of adenosine triphosphate (ATP) and 2 moles of reduced nicotinamide adenine dinucleotide (NADH). Conversely, pentoses are catabolized by the pentose phosphate pathway (PPP) [Fortman et al., 2008; Keasling and Chou, 2008; Fischer et al., 2008]. In this regard, the pentoses, within the cells, are converted to pentose 5-phosphate and dissimilated, resulting in the production of fructose-6-phosphate and glyceraldehyde 3-phosphate, which enter the glycolytic pathway. The utilization of 3 moles of pentose yields 5 moles of ATP and 5 moles of NADH [Rogers et al., 2006; Lee et al., 2008a].

The pyruvate resulting from glycolysis is cleaved in the presence of coenzyme A (CoA) to yield carbon dioxide, acetyl-CoA, and reduced ferredoxin, by pyruvate ferredoxin oxidoreductase, as shown in Figure 2.9A. Subsequently, this reduced ferredoxin works as an electron donor to reduce NAD^+ to NADH by NADH-ferredoxin oxidoreductase or to produce H_2 by transferring electrons to the hydrogenase complex, which is a characteristic byproduct of these metabolisms [Lopez-Contreras et al., 2012].

Acetyl-CoA is the primary intermediate of the fermentation, leading to both acid and solvent production. Acetic acid is produced via a branched pathway during butyric acid production. Acetyl-CoA is converted into butyryl-CoA by following a condensation of two acetyl-CoA molecules to produce acetoacetyl-CoA and CoA. Subsequently, acetoacetyl-CoA is reduced to form 3-hydroxybutyryl-CoA, which is then dehydrated to produce crotonyl-CoA and finally reduced to produce butyryl-CoA [Jang et al., 2012].

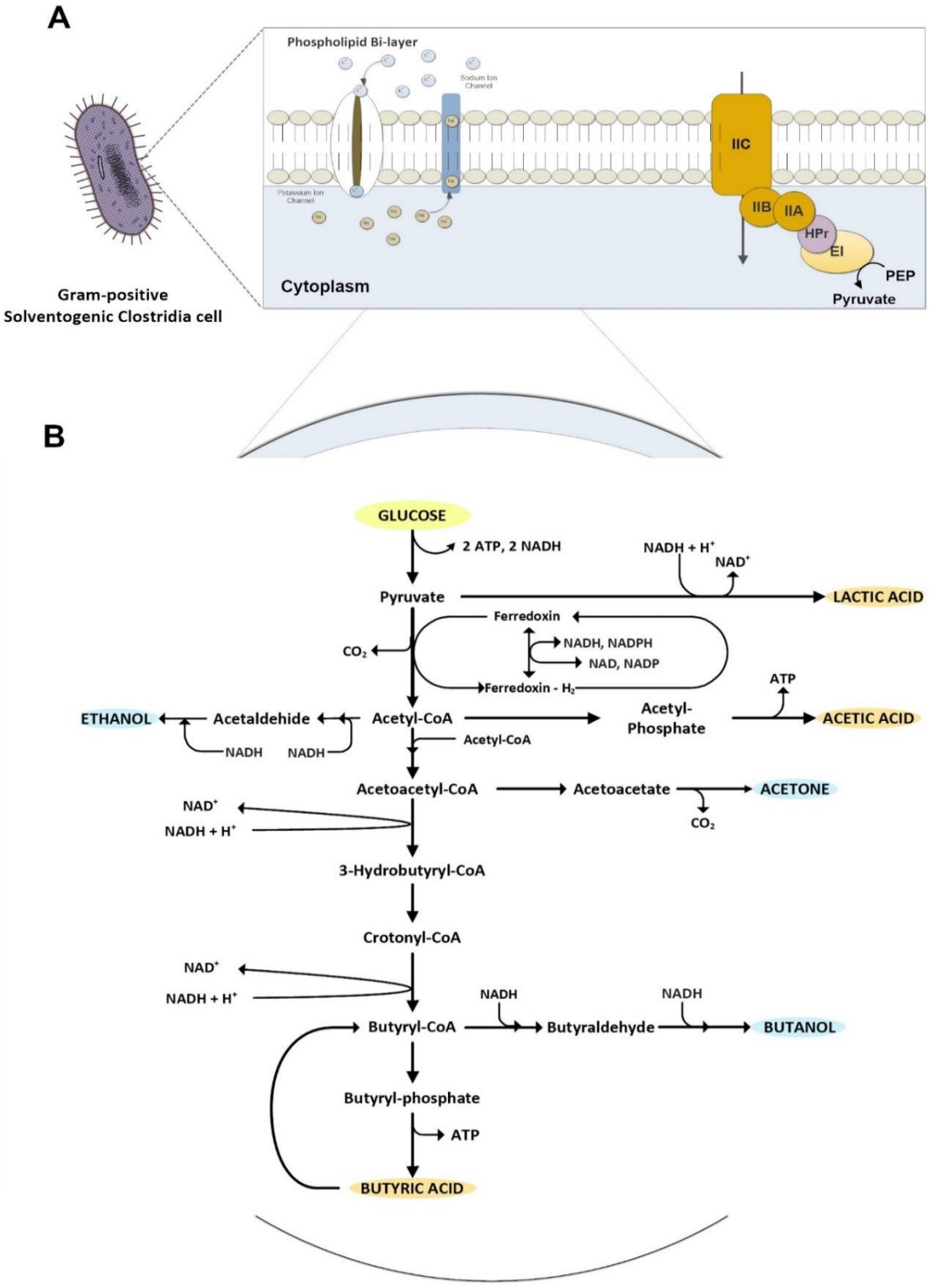

Figure 2.9. (A) PEP-dependent phosphotransferase system (PTS) on Clostridia (B) Simplified catabolic pathways of acid and solvent formation in *Clostridium acetobutylicum.* Adapted from [Mitchell, 2015;Lopez-Contreras et al., 2012]

For butyrate pathway, phosphotrans butyrylase and butyrate kinase catalyze the reactions from butyryl-CoA to butyrate. Including an additional ATP is formed from butyryl-phosphate, a total of 3 ATPs and 1 butyrate are produced from glucose. Along the acetate branch, 4 ATPs are formed during the conversion of glucose to two acetic acids. Acetate is the main product, especially during the cell growth phase. Towards the end of exponential growth, a major metabolic pathway switch is known to take place, resulting in slow down of acetate production, and utilization of excreted acetate to produce butyrate [Canganella et al., 2002]. The purpose of this type of recycling in the organism may be related to detoxification of the medium by reducing total hydrogen ion concentration, which occurred when one butyrate was substituted for two acetates. Hence, the metabolism shifts from more energy producing formation acetate to less H_2-producing butyrate formation. Finally, butyryl-CoA is converted to butyraldehyde and subsequently to butanol by two dehydrogenases [Jones and Woods, 1986].

2.8.1.3 Clostridial biphasic fermentation

In a batch culture, a typical feature of the solvent producing *Clostridium* species is the biphasic fermentation. The first phase is the acidogenic phase, during which the acids forming pathways are activated, and acetate, butyrate, hydrogen, and carbon dioxide are produced as major products, decreasing the pH of the culture medium. This acidogenic phase usually occurs during the exponential growth phase [Andersch et al., 1983; Hartmanis et al., 1984]. As the culture enters the stationary growth phase, the metabolism of the cells undergoes a 'metabolic switch' to solvent production (solventogenic phase). During the solventogenic phase, acids are re-assimilated concomitantly with the continued consumption of carbohydrate to produce acetone, butanol and ethanol (isopropanol instead of acetone in some *C. beijerinckii* strains), which normally results in an increase in the pH of the culture medium [Soni et al., 1982; Dabrock et al., 1992; Grupe and Gottschalk, 1992].

As optimum pH conditions for acidogenesis and solventogenesis differ, they are adjusted depending on the objective of the Clostridial fermentation [Grupe and Gottschalk, 1992]. Different pH values can affect the distribution of produced acids, cell membrane transport behavior and cell lysis [Zigova and Sturdík, 2000]. Solvent formation is affected by several factors, such as the requirement of a low pH in the medium, threshold concentrations of acetate

and butyrate, and a suitable growth-limiting factor such as phosphate or sulfate [Lopez-Contreras et al., 2012]. Interestingly, solvent formation appears to be associated with the availability of ATP and NAD(P)H, and can be controlled in continuous culture by varying the pH in the bioreactor [Andersch et al., 1983; Millat et al., 2013].

In a typical batch fermentation process, acetic acid is metabolized by Clostridia into butyrate. The addition of acetate in the medium enhances the consumption of glucose, leads to a faster cell growth, and increases the final biomass and butyrate concentration [Canganella et al., 2002; Zhang et al., 2009].

Studies of *Clostridium acetobutylicum* have shown that both cellular growth and solvent production are strongly pH dependent. For instance, at pH 4.5 solvents were dominant metabolites, and both acetic and butyric acid, produced during the first growth phase, are partly re-assimilated for solvent production. At pH 6.0, a single growth-related acetic and butyric production phase with negligible solvent production was observed. The concentration of butyric acid at the end of the first exponential growth phase (when the initial specific growth rate starts to decrease) was evaluated at different pH, between pH 4.0 and 5.5 solvent formation always started at an undissociated butyric acid concentration between 1.6 and 1.9 g L^{-1}. [Monot et al., 1984].

The butyrate-producing metabolic pathway is inhibited by the end product-butyrate. Undissociated butyric acid passes through the bacterial cytoplasmic membrane via diffusion and dissociates inside the cell. This affects the transmembrane pH gradient and decreases the amount of energy available for biomass growth but has a favorable influence on the production of acetone and butanol. Therefore, underlining the essential role of undissociated butyric acid on the induction of solvent production [Zigova and Sturdík, 2000].

Solventogenic *Clostridium* species, have a spore-forming life cycle which limits the efficiency of industrial fermentations [Hu et al., 2011]. During solventogenesis the active cells become endospores as accumulation of solvents reach toxic levels for the cell. Solvent formation appears to be associated with the *spoOA* gene and its DNA-binding protein, SpoOA, are jointly involved in solvent production and sporulation in *C. beijerinckii* [Ravagnani et al., 2000].

In *C. acetobutylicum* ATCC 824, the genome consists of a 3.94-Mb chromosome and most of the genes involved in solvent production are located on a megaplasmid of 192-kb (pSOL1) [Nolling et al., 2001]. The loss of this megaplasmid results in asporogenic strains unable to make solvents [Cornillot et al., 1997]. Finally, previous studies have shown that *C. acetobutylicum spoOA* inactivation mutant stops producing spores and solvent, while over-expression of *spoOA* gene can enhance solvent production since OA binding boxes have been identified in the promoter regions of solvent formation genes [Harris et al., 2002].

2.8.1.4 Metabolic engineering of *Clostridium* spp.

Interestingly, many Clostridia contain a complete or partial cellulosome plus xylan degradation enzymes. A cellulosome is a complex multi-enzymatic system, consisting of cellulase catalytic modules, carbohydrate binding domains, dockerins, and cohesins. These serve to connect the catalytic and carbohydrate binding domains to the surface of the bacterial cell expressing the cellulosome [Wackett, 2008]. As a result, some *Clostridium* species have the biochemical tools to utilize cellulose directly.

Among various advances for enhancing biobutanol fermentation is the Consolidated Bioprocess (CBP), which involves enzyme production, cellulose hydrolysis and microbial fermentation within the Clostridia cells in one step. This can be achieved by genetic engineering of Clostridia species. CBP has the potential to lower significantly the overall production cost of biobutanol [Lynd et al., 2005]. Cellulolytic Clostridia such as, *Clostridium cellulolyticum, Clostridium thermocellum,* and recently *Clostridium termitidis* have been studied to screen CBP, with promising results for biofuel production [Demain et al., 2005; Lu et al., 2006; Gomez-Flores et al., 2015].

Efforts have been made to develop a *C. acetobutylicum* strain that can utilize cellulose directly. There is evidence that *C. acetobutylicum* ATCC 824 might have a cellulosome [Moraïs et al., 2012]. *C. acetobutylicum* ATCC 824, however, has no cellulolytic activity, suggesting that elements of the cellulosome are either missing or not expressed. To make *C. acetobutylicum* utilize cellulose directly, the cellulase gene from *C. cellulovorans,* or the gene encoding the scaffold protein from *C. cellulolyticum* and *C. thermocellum,* was introduced into *C. acetobutylicum.* However, the level of expressed heterologous cellulase was rather low [Perret et al., 2004].

2.9 Research approach

In this thesis, biobutanol production by biological fermentation was investigated from the corn plant, integrating two different approaches. The first one was to utilize corn cobs, a cellulosic-based material to produce biobutanol. The second approach was to investigate biobutanol production using a sugar-based material, sugarcorn juice, as depicted in figure 2.10.

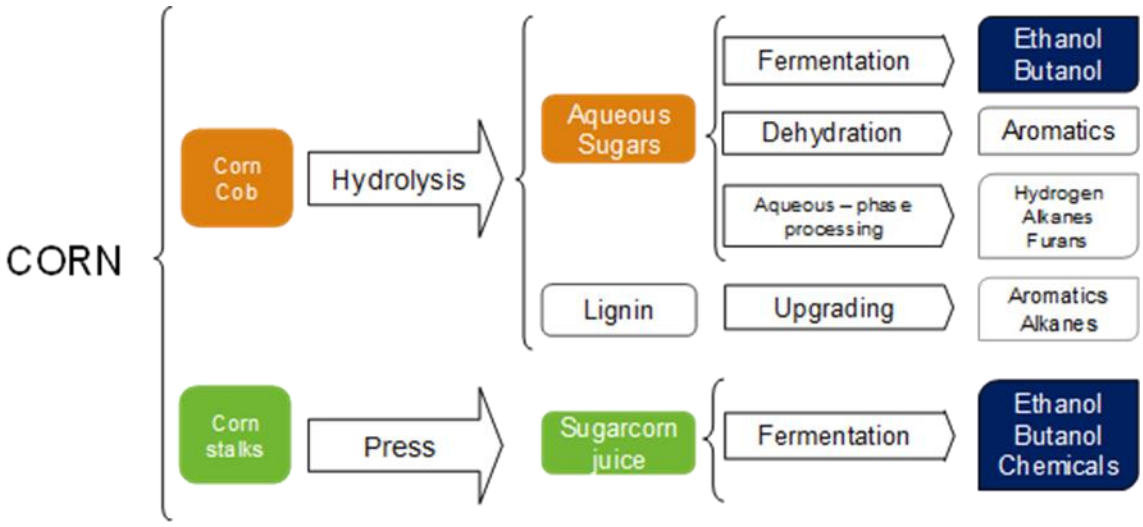

Figure 2.10. Research approaches to produce biobutanol.

Both approaches were designed to investigate productivities, yields and titers of biobutanol production, utilizing suitable Clostridia strains for each substrate. The two approaches converged into a biorefinery concept to produce renewable biofuels in Ontario, Canada, and offers new scenarios for crucial bioeconomy development.

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Chapter 3

3 Pretreatment, hydrolysis of corn cobs, cellulases experiments and biobutanol fermentation

3.1 Background

The conversion of lignocellulosic agro-industrial residues, such as corn cobs, into value-added products by microbial fermentation can contribute to the development of sustainable bio-based products, and consequently, support bioeconomy. A corn cob is the core of a maize ear. It is a non-edible lignocellulosic material and its structure is represented by a) white-inner pith containing thin-walled parenchyma cells, b) woody ring or mid-con, shaped of rachis nodes, inner and outer vascular system, c) coarse chaff, mainly basal portions of the first and second glumes or rudimentary leaves and d) fine chaff, consisting of flimsy lemmas and paleas [Nickerson, 1954; Sehgal and Brown, 1965].

It is primarily constituted of cellulose, hemicellulose, and lignin, as shown in table 3.1. In general, cellulose and hemicellulose are integrated by different sugars, such as glucose, xylose, manse, arabinose, and rhamnose.

Table 3.1 Cell wall composition of corn cobs (% of the dry material)

Components (%)				References
Cellulose	**Hemicellulose**	**Lignin**	**Ash**	
42.5	34.3	18.4	-	[Cheng et al., 2009]
32	35	20	4	[Garrote et al., 2001]
47.5	37.3	6.8	1.2	[Zhu et al., 2006]
42	33	18	1.5	[Schwietzke, 2009]

Sugars entraped within the corn cob matrix can be released by a chemical pretreatment, followed by enzymatic hydrolysis, also known as saccharification, to release soluble fermentable sugars. Then, a microbial fermentation is carried out to produce biobutanol.

Previously, lignocellulosic biomass has been utilized to produce ethanol. In this work, an alternative biofuel was studied., butanol. Biobutanol fermentation has the advantage of using bacteria such as *Clostridium beijirinckii* and *Clostridium acetobutylicum*, which can metabolize pentoses and hexoses. Thus, they can tolerate byproducts of pretreated lignocellulosic biomass, such as furfural and hydroxymethyl furfural.

Sulfuric acid has been the most widely tested acid, although few tests have been conducted with nitric acid, hydrochloric acid, phosphoric acid as well [Grohmann and Bothast, 1997;Brink, 1993]. Sulfuric acid has been utilized to remove hemicellulose from lignocellulosic materials by enhancing the digestibility of cellulose [Sherrard and Kressman, 1945;Brownell et al., 1986].

Dilute sulfuric acid pretreatment has different effects on the chemical composition and physical structure of lignocellulosic biomass. This pretreatment has a minor impact on lignin, but major effect on altering the hemicellulose structure, and has little effect on cellulase crystallinity [Huang et al., 2011].

Table 3.2 shows dilute sulfuric acid experiments from literature. Considering these results, pretreatment conditions, i.e. temperature, time, and loading ratio were selected as parameters for hydrolysis studies. Sulfuric acid concentration was set at 1% (v/v) and the particle diameter was 2 mm.

Table 3.2 Corn residues pretreatment comparison

Biomass	Temperature (°C)	Time (min)	Acid concentration	Loading ratio (%)	Comments	Reference
Corn stover, poplar, switchgrass	140-160	1-60	0.6-1.2	10	Parr Reactor	[Esteghlalian et al., 1997]
Agricultural residues	120-150	2-90	0.44-1.9	6	Parr Reactor	[Chen et al., 1996]
Corn stover	165-183	3-12	0.5-1.41	20	Pilot Plant	[Schell et al., 2003]
Corn cob	121	120	1.25	10	Autoclave	[Guo et al., 2010]
Corn cob	121	40	1	6	Autoclave	[de Carvalho Lima et al., 2002]
Corn fiber	100,120,140,160	10-60	2-10	-	Parr reactor	[Grohmann and Bothast, 1997]
Corn stover	140-190	1-5	1	21.3-22%	Dionex Solvent Extractor	[Moxley et al., 2012]

Working in laboratory settings allows ideal conditions for the development of a process. Nevertheless, on large scale the cost of specialty materials, such as enzymes, surfactants, are sensitive for the economic viability of the process [Kazi et al., 2010].

The high price of cellulolytic enzymes could increase the cost of the production of the lignocellulosic biofuels, as it makes the enzymatic hydrolysis an expensive step in the overall process. Hydrolysis is affected by multiple factors, such as enzyme loading, reaction time, temperature, substrate composition, and inhibitor concentration [Rosales Calderon et al., 2014]. Therefore, it is a key factor to study the enzyme load per lignocellulosic material. In this study, the enzyme loading to the corn cobs was low for potential industrial scenarios.

In an effort to evaluate the biobutanol production from corn cobs cellulosic sugars, Clostridial fermentation was carried out with soluble sugars derived from corn cobs, as the carbon source.

3.2 Materials and methods

3.2.1 Collection of corn cobs

Corncobs from a local farm near London, Ontario (42°50'54.1"N 81°04'05.4"W) were generously provided by Lunn Family Farm (Malahide, Ontario, Canada). The cobs were collected from the soil, brought to the laboratory and air-dried overnight. Next day, corncobs were finely grounded using a Thomas Wiley Laboratory Mill Model 4, equipped with a rotary grinder and stationary blades with a 2-mm screen sieve. Finally, the milled corncobs were stored in clean containers at room temperature until use. An elementary particle distribution quantification was done to the collected 2-mm milled corn cobs.

A set of 4 sieves with sieve numbers #8, #16, #25, #80 (Canadian Standard Sieve Series W.S. Tyler Company of Canada Limited, St. Catharines, Ontario), having opening sizes of 2360, 1000, 710, 180 μm, respectively, were used on a rotating platform at a rotating speed of 350 rpm for 30 min.

3.2.2 Corn cob pretreatments

A set of screening experiments were performed to study the variable with main effect on liberated glucose from corn cob. The pretreatment was carried out in a 75 mL Parr reactor (Parr Instrument, USA). A full factorial design of the experiments was completed, utilizing three factors, time (X_1), temperature (X_2) and loading ratio (X_3), and three levels. The design of the experiment was based on a 3^3 three-level, full factorial design and the analysis was done by Minitab 18.1 (©2017 Minitab, Inc.). The response variable, Y, was set as the glucose yield (glucose concentration in grams per liter over grams of corn cob). These pretreatments aimed to study the effect of the temperature, time and loading ratio (mass of corn cob loaded into the reactor to the total mass of the slurry).

Corn cobs were soaked in a 1% w/w sulfuric acid solution in three different loading ratios (5, 10, 20) and manually stirred until homogenization, then loaded to the reactor with reaction times of 10, 20 and 30 minutes at 120, 180 and 240 °C (Figure 3.3). The hydrolysates were stored in a refrigerator until further use.

Table 3.3 Loading ratio in corn cob pretreatment experiments

Code	Total mass (g)	Corn cob mass (g)	Sulfuric acid solution mass (g)
LR5	56	3	53
LR10	56	6	50
LR20	56	12	44

About 40 grams of water was added to each hydrolysate, and the resultant pH was measured. Then 2%(w/v) of sodium hydroxide was used to neutralize the samples until a final pH between 4-5 was reached. Finally, each hydrolysate was brought up to 100 g with distilled water. The mixture was then filtered through a Whatman No. 1 filter in a Buchner funnel to separate the hydrolysate from the wet biomass. The glucose concentration was measured using a UV-test kit (Sekisui Diagnostics). The samples were kept in -20 °C freezer.

Table 3.4 3^3 full factorial design

Variable	Low (-1)	Standard (0)	High (+1)	Units
Time (X1)	10	20	30	Minutes
Temperature (X2)	120	180	240	°C
% Loading ratio (X3)	5.36	10.71	21.43	% Loading ratio

After pretreatment studies, vacuum filtration separated the liquid solution and solid. Glucose in the liquor was analyzed by enzymatic kit to quantify the released glucose (figure 3.1).

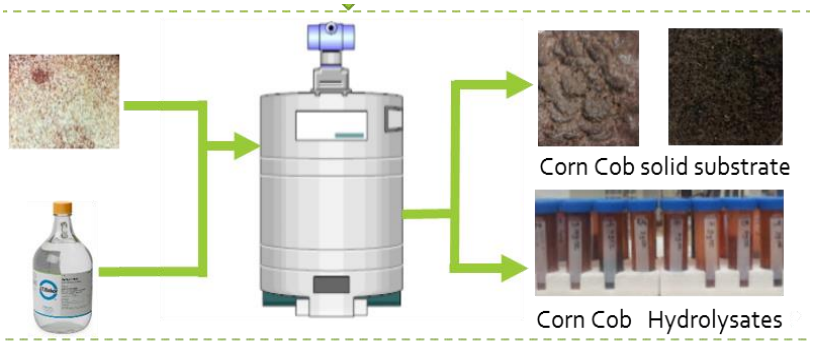

Figure 3.1 Overall corn cob pretreatment experiments

3.2.3 Corn cob pretreatment for biobutanol production

Corn cobs were treated with a two-step pretreatment method in 100 ml serum bottles.

Two grams of dry corn cobs (2 mm diameter) were weighted into a 100-ml serum bottle, then -mL of 1% (v/v) sulfuric acid solution were added and manually stirred., the bottles were closed with butyl rubber stoppers and the clamped with aluminum seals. The first cycle was run in an autoclave, at 121 °C for 30 min with a loading ratio of 16. Once it was finished, the mix was left to cool down, and a cannula-syringe set was used to withdraw the liquid. The wet corn cobs were washed with sterile distilled water three times.

Next, the wet corn cobs were soaked with a 0.125 M NaOH solution, and stirred until homogenization. The glass bottles were clamped with aluminum caps and set into the autoclave for a second cycle at 121 °C for 60 min, with a loading ratio of 16. The mix was allowed to cool down, and with a cannula, the liquid was washed with sterile distilled water (pH 5.0), thrice, to get a pH close to 5. After each cycle, the glucose amount liberated per cycle in the solution was measured. This experiment was run in duplicate, and the pretreated corn cobs were immediately subjected to enzymatic hydrolysis step (Figure 3.2).

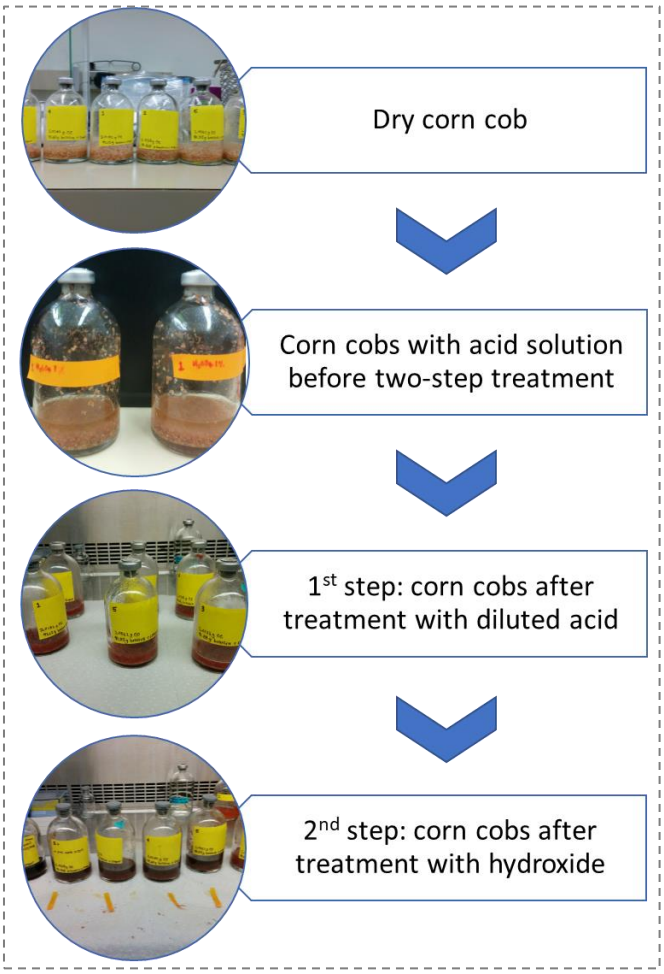

Figure 3.2 Two-step corn cob pretreatment for biobutanol fermentation

3.2.4 Cellulase activity determination

To quantify the cellulase activity, the procedure was designed to measure cellulase activity in "filter paper units" (FPU) per milliliter of original (undiluted) enzyme solution. For quantitative results, the enzyme preparations were compared by significant and equal conversion. The value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper (4% conversion) in 60 minutes has been designated as the intercept for calculating filter paper cellulose units (FPU) by IUPAC. This procedure follows IUPAC guidelines and determines enzyme activity as filter paper units in a cellulase preparation. For the measurement of cellulase activities, the standard laboratory analytical procedures were used [Adney, 2008].

Commercial enzyme cocktails utilized are listed below, all of them were stored at 4 °C until use:

- ENMEX (Mexico City, Mexico. http://www.enmex.com.mx/)
    - Celuzyme – from *Trichoderma longibrachiatum*, a solid compound with cellulase, hemicellulase and beta-glucanase activity.
    - Celuzyme XB – from *Trichoderma longibrachiatum*, a liquid compound with xylanase, beta-glucanase and cellulase activity.
- VISCOZYME L (Sigma-Aldrich from Novozymes) – A liquid cocktail from *Aspergillus spp*. Multienzyme complex with arabinase, cellulase, beta-glucanase, hemicellulase, and xylanase.
- Cellic® CTec2 (Novozymes - Basgværd, Denmark) – A liquid cocktail from *Trichoderma*, with cellobiohydrolases I (Cel7A), endoglucanase I (Cel7B) and beta-glucosidase.

3.2.5 Enzymatic hydrolysis or saccharification

An enzyme-buffer stock solution (1:20) was prepared with 19 mL of sterilized 0.09 M citrate buffer (pH 4.8) and 1 mL of concentrated Cellic CTec2 liquid enzyme plus 40 µL of Triton X-100 (0.2%). Triton is a nonionic surfactant, and it could enhance the cellulase activity and enzymatic hydrolysis of pure cellulose or lignocellulosic biomass [Eckard, 2015]. The pretreated wet corn cobs (H_2SO_4/NaOH) mentioned above, were immediately utilized for this enzymatic hydrolysis step to avoid supplementation of the citrate buffer solution with antibiotics or other chemicals over the enzymatic reaction [Selig et al., 2008].

The wet corn cobs inside the serum bottles were added with ten mL of distilled water (pH 5.0) and set up into a water bath at 50 °C. A magnetic stirrer was put to keep an agitation for a homogeneous reaction. From this enzyme-buffer stock solution, 0.135 mL (5.4 FPU) were added to the pretreated corn cobs hydrolysate (2g) bottles. The preparation was dosed based on [Cannella et al., 2012]. The enzymatic kinetics were carried out for 72h and sampled at 12, 24, 48 and 72h. The procedure is shown in Figure 3.3.

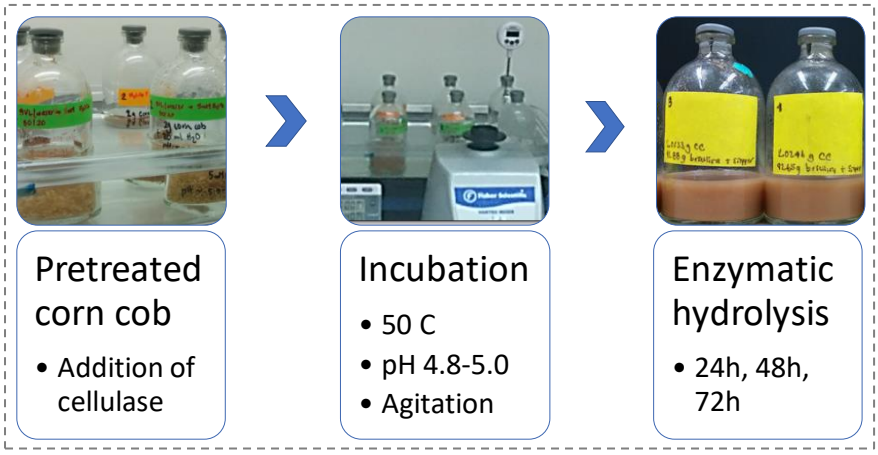

Figure 3.3 Overall corn cob enzymatic hydrolysis or saccharification procedure

3.2.6 Biobutanol fermentation

From the enzymatic hydrolysate, the liquor was used as the carbon source for the biobutanol fermentations. The strain used in this experiment was the asporogenic strain *Clostridium beijerinckii* ATCC 6422, purchased from America Type Culture Collection. All chemicals for preparation of media were obtained from Sigma-Aldrich Co. The Clostridial Nutrient Medium (CNM, Fluka Analytical) was used as growing media. This medium contained per liter of solution in distilled water: Meat extract, 10 g; peptone, 5 g; yeast extract, 3 g; D (+) glucose, 5 g; starch, 1 g; sodium chloride, 5 g; sodium acetate, 3 g; L-cysteine hydrochloride, 0.5 g; agar, 0.5 g and, resazurin solution (1 g L^{-1}), 0.25 mL; final pH of 6.8.

Modified P2 culture medium [Qureshi and Blaschek, 1999] was composed of the following separately prepared solutions (in grams per 100 ml of distilled water, unless otherwise indicated). Carbon source solution: corn cob hydrolysate (120 mL); yeast extract, 0.1198 g. 1.24 mL of buffer solution: KH_2PO_4, 5 g; K_2HPO_4, 5 g; ammonium acetate, 22 g. 1.24 mL of vitamins solution: p-aminobenzoic acid, 0.01 g; thiamine, 0.01 g; biotin, 0.0001 g. 1.24 mL of minerals solution: $MgSO_4$-$7H_2O$, 2 g; $MnSO_4$-$7H_2O$, 0.1 g; $FeSO_4$-$7H_2O$, 0.1 g; NaCl, 0.1 g.

The culture media was sterilized by filtration (Nalgene filtration system). The pH was adjusted to 6.8. Following this, 0.059 g L-cysteine hydrochloride (Sigma) and 100 uL of resazurin solution (1 g L^{-1}) were added to the final media to reduce the culture medium for optimal growth. The bottles were sparged with ultrapure nitrogen to make the environment anaerobic.

For fermentation, a 10% inoculum was utilized, following 14-16 hours of incubation. The fermentation was carried out for 120 h, in an orbital shaker at 90 rpm at 37 °C in 60 mL serum bottles, with a working volume of 30 mL. The procedure is shown in Figure 3.4.

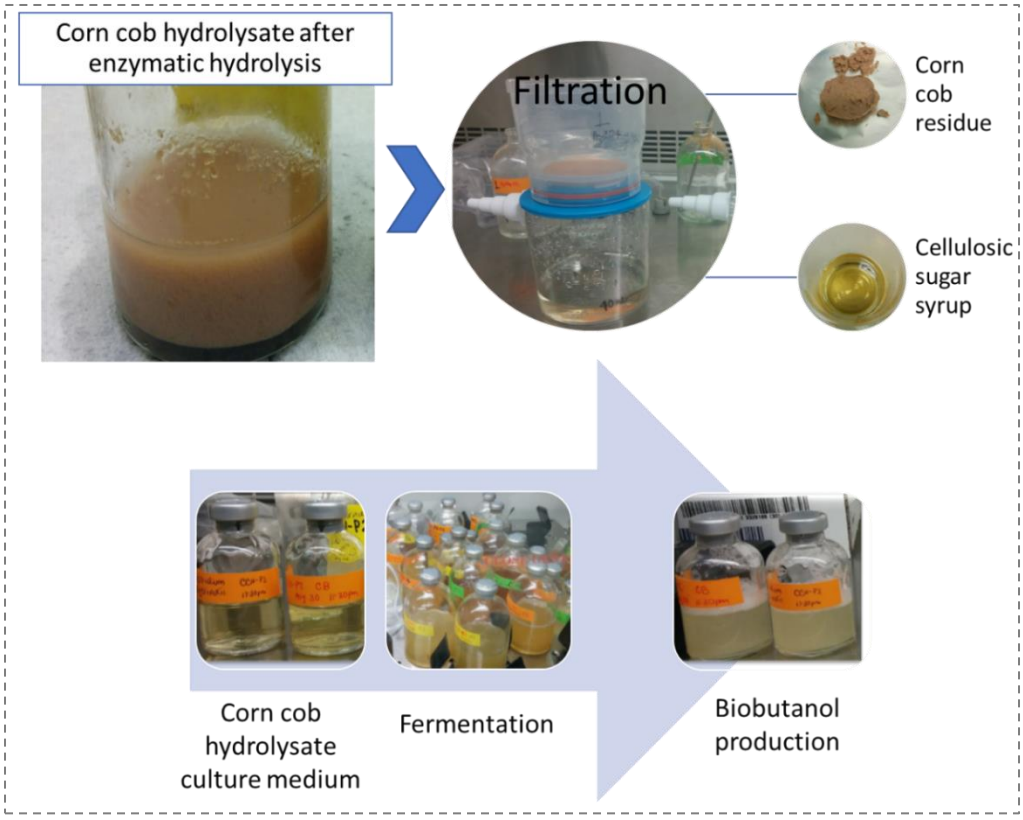

Figure 3.4 Diagram of corn cob hydrolysate fermentation process

3.2.7 Analytical methods

pH was measured by a pH-meter (VWR). Reducing sugars were measured by the DNS method [Miller, 1959]. Glucose concentration was measured using Genzyme Diagnostics Reagent kit. Metabolite concentrations were quantified using a Gas Chromatograph System (Hewlett Packard 6890 Series) coupled with a Flame Ionization Detector (FID). Analytical methods are fully described in Appendix A.

3.3 Results and discussion

3.3.1 Corn cob particle size distribution

The results of the particle size distribution of corn cobs by weight are shown in Figure 3.5. Most of the corn cob particles were in the range between 180 to 1000 µm, while no particle size was above 2,360 µm.

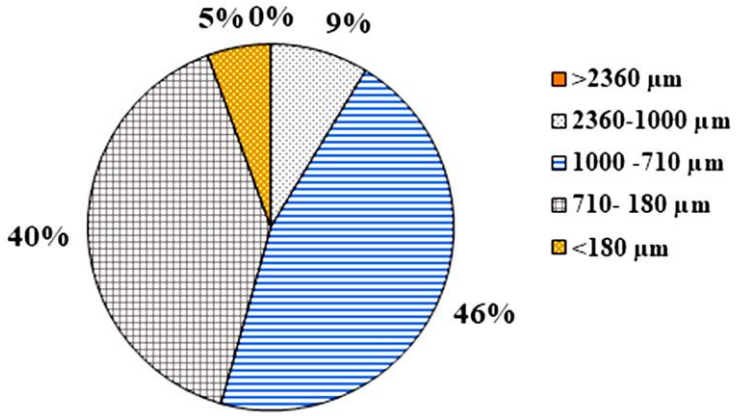

Figure 3.5 Corn cob Average Particle Size distribution

3.3.2 Corn cob pretreatments

In Table 3.5, the released glucose for each corn cob pretreatment in the liquid part of the hydrolysate is shown.

From table 3.5, it can be seen that pretreatments numbers (Exp #) 4, 5, 9, 16, 20 and 27 had greater liberated glucose concentration per g of corn cob. PT4 (10min, 180 °C, LR10) = 2.85 (g L^{-1})/g, PT5 (10min, 180 C, LR5) = 2.951 (g L^{-1})/g, PT9 (30min, 180 C, LR5) = 3.192 (g L^{-1})/g, PT16 (20min, 180 C, LR5) = 2.734 (g L^{-1})/g, PT20 (30min, 180 C, LR20) = 1.698 (g L^{-1})/g, PT27 (20min, 180C, LR10) = 1.673 (g L^{-1})/g.

Table 3.5 Experimental results for dilute acid pretreatment of corn cobs

Experiment #	Time (min)	Temperature (°C)	Loading ratio (LR)	Yield (g glucose L^{-1}) / g corn cob
1	20	120	20	0.294
2	10	120	20	0.203
3	30	120	20	0.331
4	10	180	10	2.852
5	10	180	5	2.951
6	30	240	10	0.117
7	20	120	5	0.379
8	20	120	10	0.290
9	30	180	5	3.192
10	30	240	20	0.061
11	20	180	10	1.475
12	10	240	5	0.218
13	30	120	10	0.402
14	30	240	5	0.222
15	10	240	20	0.067
16	20	180	5	2.734
17	20	180	20	1.665
18	10	240	10	0.122
19	30	120	5	0.418
20	30	180	20	1.698
21	20	240	10	0.034
22	10	120	5	1.361
23	10	180	20	0.060
24	20	240	20	0.084
25	10	120	10	0.226
26	20	240	5	0.234
27	20	180	10	1.673

As it can be seen, the majority to the temperature of 180 °C. An ANOVA analysis was carried in Minitab, and when using a P-value = 0.05, all the P-values less than 0.05 were significant. The results for each variable are shown in table 3.6.

Table 3.6 P-values for time, temperature and loading ration in the corn cob hydrolysis

Variable	P-value
Time	0.801
Temperature	0.000
Loading ratio	0.083

The temperature was the factor that influenced most the hemicellulose solubilization, thereby liberating glucose, as well as other monosaccharides, such as xylose and arabinose. As shown in Figure 3.6, the interaction plot gives a clear picture of that temperature (180 °C) and loading ratio 5 were important variables. Moreover, the interaction plot of time and temperature confirms that temperature of 180 °C as a constant operating temperature will result in a good glucose yield.

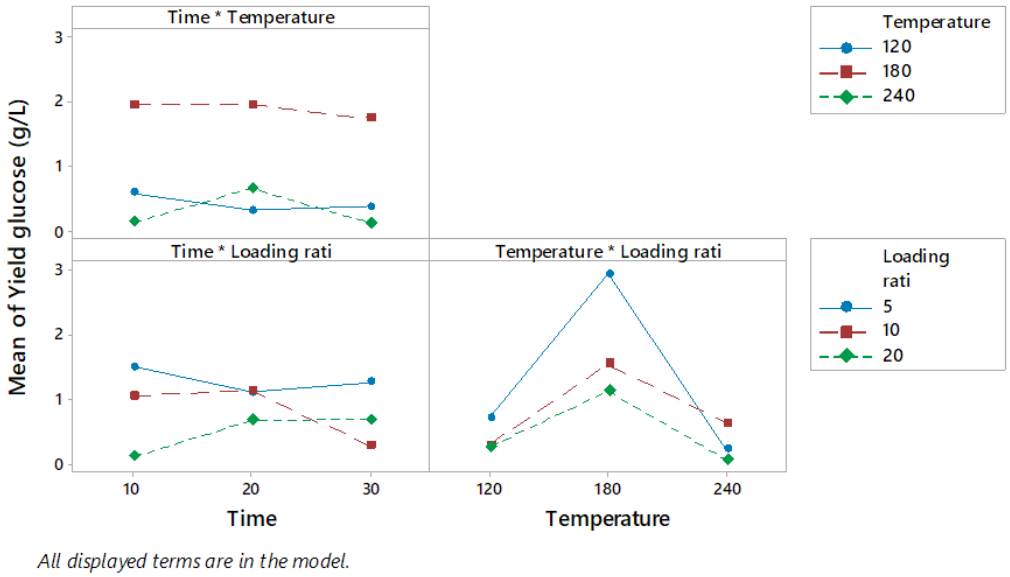

Figure 3.6 Interaction plot for liberated glucose yield (g/L) – fitted means

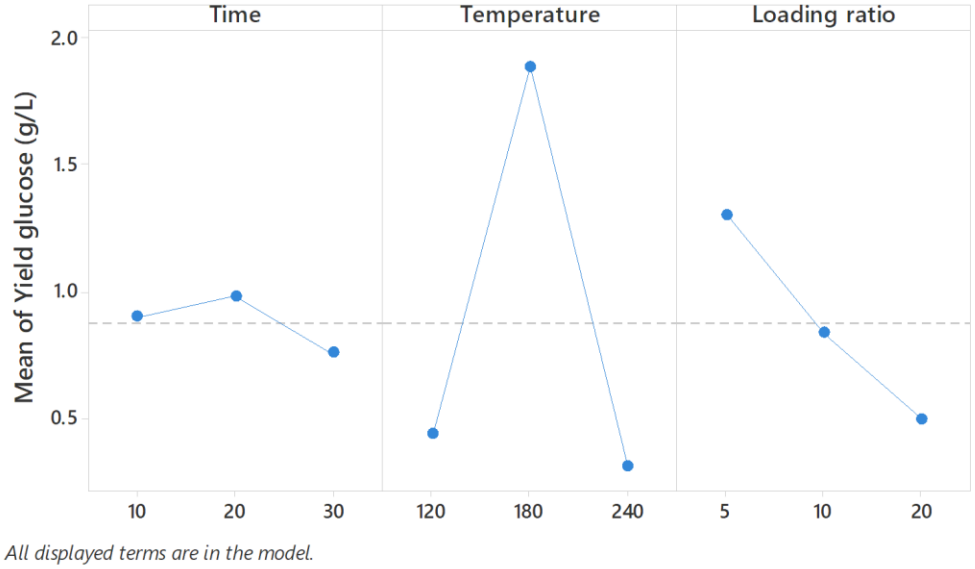

Figure 3.7 Main effects plot for glucose yield (g/L)

Ideally selecting the diluted sulfuric acid pretreatment will be based on the maximum glucose liberated from the corn cob lignocellulosic matrix. Diluted sulfuric acid pretreatment focuses on weakening the hemicellulose structure, and in these experiments, the glucose yield in the liquid part of the corn cob hydrolysate was a direct observation of the partly dissolved hemicellulose layer.

The best result was Pretreatment 9 (30 min, 180 °C, LR5), with a glucose yield of 3.192 (g/L)/g. In a laboratory scale, this idea may be experimented, but in an industrial level, it might not be economically feasible, due to the several batches needed to be carried out per corn cob mass pretreated. A more logical and cost-efficient industrial approach would have been to deal with the highest amount of corn cobs per cycle (either loading ratio of 10 or 20) or in these experiments a loading ratio of 20, 21.43 % to completely soak the corn cobs in the acidic solution.

From these set of experiments, released glucose was measured in the corn cobs hydrolysates. Due to technical constrains, the Parr reactor for furthers studies was not available and needed to shift the next step to utilize an autoclave which can only give us a maximum process temperature of 121-122 °C. The aim was to develop a parallel process, which could result in a

yield of 1.55 grams of liberated glucose per liter of corn cob hydrolysate, per gram of corn cob used.

3.3.3 Corn cob dilute acid pretreatment

A preferred loading ratio between 10-20 was set as LR=16, and a two-step process, all in duplicates, was designed as: Step 1: 2 grams of dry corn cobs, in a 10-mL reaction volume, with 1% (v/v) sulfuric acid solution, process time of 30 min in an autoclave at 120 °C. Three washes with distilled water were carried out. Step 2: 1.95 grams of wet corn cobs (from the previous step) in a 10-mL reaction volume, with 0.125 M of NaOH solution, process time of 60 min in an autoclave for 120 °C. The experiments were carried out in duplicate.

A combined effect of both pretreatments resulted in a yield of 1.33 (g glucose L^{-1}) per gram of corn cob. This value is relatively in the medium yields compared with the 2-3 (g glucose L^{-1}) / g of corn cob of the most significant result from the screening experiments.

3.3.4 Cellulase activity measurement

In Table 3.7 the filter paper unit per milliliter (FPU/mL) of the studied enzymatic solutions is shown. These measurements were done to compare the hydrolytic potential of each enzymatic cocktail available for the study. The liquid samples were diluted in citrate buffer in a ratio of 1:20, whereas the solid samples were diluted 1:40 (w/w) in citrate buffer.

Table 3.7 Experimental results of cellulolytic enzymes

Enzyme	FPU/mL	Description
Sigma liquid enzyme	17.72	Arabanase, cellulase, β-glucanase, hemicellulase, and xylanase
Enmex liquid enzyme	11.14	Xylanasa, beta-glucanasa, celulasa
Enmex powder enzyme	64.65	Celulasa, Hemicelulasa, beta-glucanasa
Cellic CTec2 liquid enzyme	40.5	Cellobiohydrolase I, endoglucanase I, beta-glucosidase

Previous reports show that Cellic CTec2 can achieve a high enzymatic activity range from 120-223 FPU/mL [Rosales Calderon et al., 2014]. Since our sample of Cellic CTec2 enzyme only achieved a 40.5 FPU/mL, a degradation of the enzymatic cocktail could have happened and cellulase hydrolytic power measured as filter paper unit was less than the expected (table 3.8).

Table 3.8 Properties of commercial Cellic CTec2 (Novozymes)

Protein content (mg/mL)	Cellulose	FPU/mL	Enzyme/g solid	Ref.
	Cellic CTec2	223	10 FPU /g cellulose	[Rodrigues et al., 2015]
Not available	Cellobiohydrolase I (Cel7A)			
	Endoglucanase I (Cel7B)			
	B-glucosidase			
161	Cellic CTec2	120.5	7.5 FPU/g dry matter	[Cannella et al., 2012]
	Cellobiohydrolase I (Cel7A)			
	Endoglucanase I (Cel7B)			
	B-glucosidase	2731 (U/mL)		

3.3.5 Enzymatic hydrolysis or Saccharification

In this regard, Cellic CTec2 from Novozymes was selected to carry on the process as it has been proved by several studies that could have a good hydrolysis yield. The results are given in Figure 3.8. There was a concentration of 15.40 g L^{-1} of glucose liberated after 72 h of enzymatic hydrolysis, nevertheless, since 48h, the reaction does not release more glucose. The expected released glucose amount would have been 38-40 g L^{-1}, as a concentrated cellulosic syrup. In this case, a 40% cellulose conversion was achieved at 48 h. The glucose in this step was measured using an enzymatic kit and glucose was selectively measured.

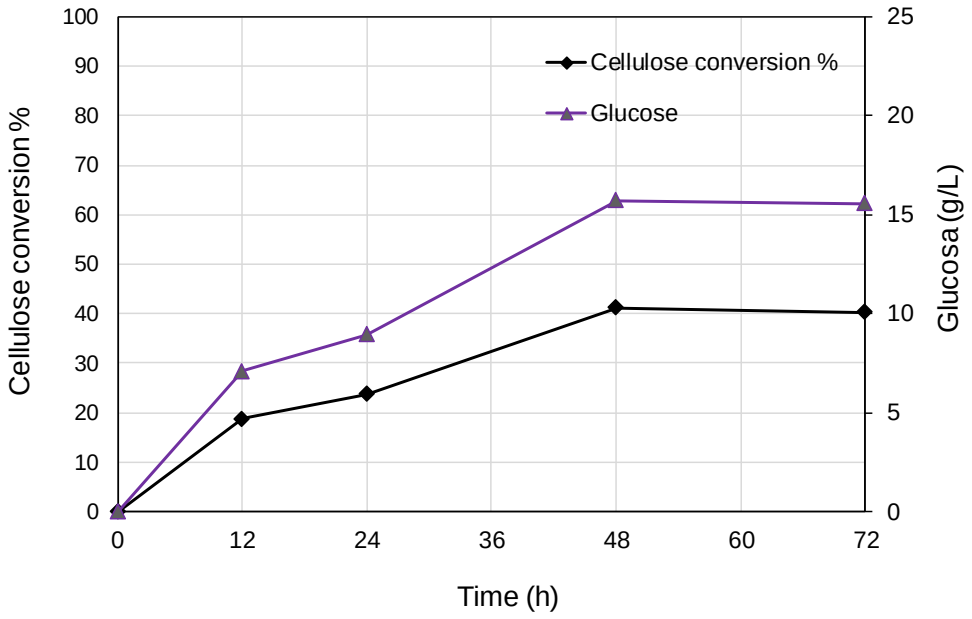

Figure 3.8 Corn cob cellulase conversion and glucose release by enzymatic hydrolysis (Cellic CTec2)

3.3.6 Butanol fermentation

For butanol fermentation from corn cob hydrolysates, reducing sugars were measured using DNS method. This method measures glucose, xylose and arabinose. By an arithmetic difference, the first reducing sugars from the corn cob hydrolysate was subtracted by the amount of glucose liberated; we have an estimated amount of 5.5 g L^{-1} in the corn cob hydrolysate that could be xylose and arabinose in some extent. About 97% of the reducing sugars were entirely depleted by 120 h of fermentation.

As seen in Figure 3.9, biobutanol was produced utilizing cellulosic sugars from corn cob hydrolysate. By 48 h, the maximum titer was reached at 4.42 g L^{-1}, a yield of 0.21 g butanol / g reducing sugars and productivity of 0.036 (g L^{-1})-h. It is worth noting that there was not an evident acetic and butyric acid accumulation at the beginning of the fermentation, and it was not clear, reaching maximum concentrations of 1.39 g L^{-1} and 1.24 g L^{-1}, respectively. Acetone had a titer of 3.54 g L^{-1} at 96 h. The maximum concentration of ABE was 8 g L^{-1}at 48 h.

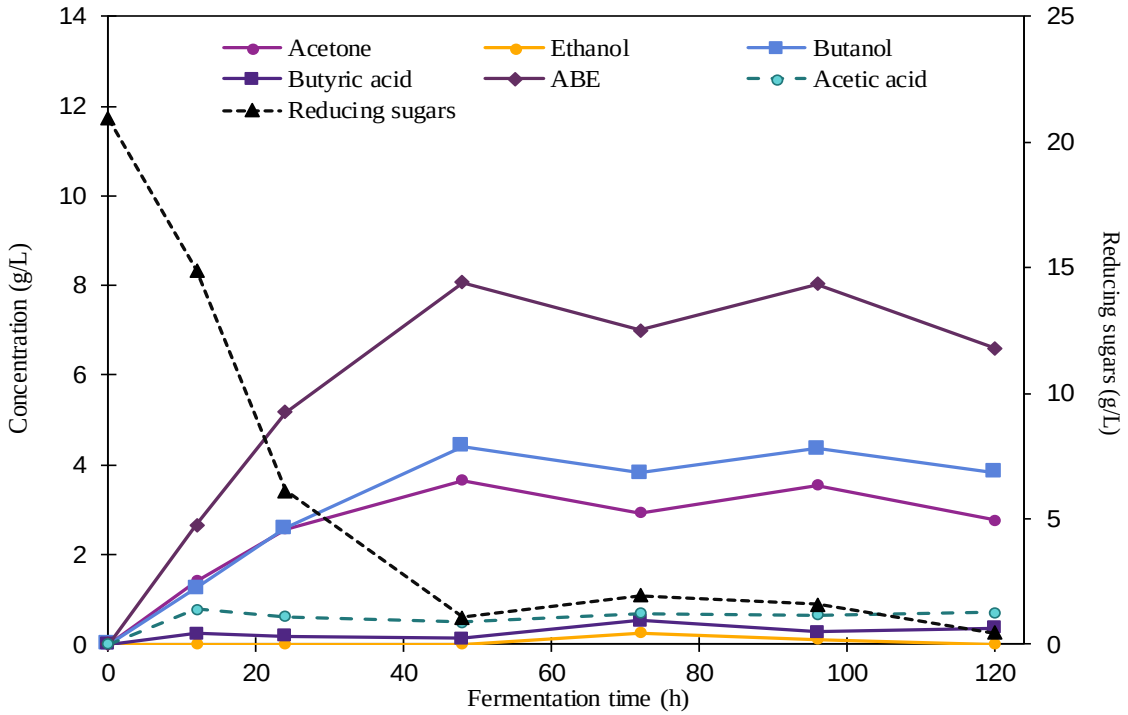

Figure 3.9 Biobutanol fermentation of corn cob hydrolysate sugars by *Clostridium beijerinckii*

3.4 Conclusion

The dilute acid pretreatment with sulfuric acid of corn cobs showed that temperature is the variable with most significant effect towards hemicellulose solubilization and glucose liberation. Even though, the need to use an alternative temperature as of technical difficulties arise, a two-step pretreatment designed to pretreat the corn cobs.

The enzymatic hydrolysis was carried out utilizing a very low concentration of an enzymatic stock solution of Cellulic C2Tec from Novozymes to hydrolyse the cellulose from the corn cobs. The hydrolysis conversion % was around 44%, indicating the possibility of improvement in the second round of enzymatic hydrolysis and increase the enzyme dose. Pursuing the enzymatic hydrolysis in a sterile environment and right after of the pretreatment was done allowing to skip the use of any antibiotic. After the enzymatic hydrolysis, there was no removal of inhibitors, only a physical separation of the liquids and solids.

Biobutanol fermentation was carried out utilizing a *Clostridium beijerinckii* strain and cellulosic biobutanol was produced with titer of 4.42 g L^{-1} at 48 h of fermentation with 97% of reducing sugars used by this time. It is worth noting that there was not a visible acidogenesis phase or acid accumulations at the beginning of the fermentation.

3.5 References

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Chapter 4

4 Fermentation of simple sugars by Clostridia species

4.1 Introduction

Acetone, butanol, and ethanol (ABE) fermentation presents the following challenges: i) low butanol titer (below 12 g L^{-1}); ii) low butanol productivity; iii) high cost of the fermentation feedstock; and iv) the high cost of butanol recovery from broth [Lee et al., 2008]. Metabolic engineering and *in situ* product recovery could be two options to overcome these specific fermentation challenges. Availability of cost-efficient substrate is a crucial factor for any industrial fermentation and will influence the overall cost of butanol [Kumar et al., 2012]. The understanding of sugars uptake helps explaining metabolism of each solventogenic Clostridia and provides the first step to develop insight of the ABE fermentation kinetics and the effect of complex substrate in butanol production.

This chapter shows the results of ABE fermentation by *Clostridium saccharobutylicum* (ATCC® BAA-117) or NCP262 (DSM 13864), using individual sugars (glucose, fructose, and sucrose) and a mix of the three of them. *C. saccharobutylicum* was selected as it can produce ABE solvents from various sugars, such as fructose, cellobiose, sucrose, and mannose. Thus, it has been used for industrial butanol production by a South African company [Poehlein et al., 2013].

Clostridia assimilates mono and disaccharides using different transport mechanisms. Most of studies have found a phosphoenolpyruvate-dependent phosphotransferase system (PTS) to be responsible to transfer carbohydrates from outside to inside the cell. In fact, the system performs a simultaneous effort to transport and to chemically modify the sugar via phosphorylation. Although there is evidence that there are both PTS and non-PTS transport systems (ATP-driven transporters and other non-PTS permease) in *Clostridium acetobutylicum*, there are no such studies on *Clostridium saccharobutylicum* [Mitchell, 1998;Reid, 2005].

The selection of these sugars was based on the composition of sugarcorn juice (Chapter 5). The vast majority of plant juices contain sucrose, and understanding of its uptake within

Clostridia could help find avenues to improve butanol production. The primary goals of this chapter were to study the kinetic parameters of each fermentation, butanol titer, butanol productivity and the effect of a sugar mixture in the assimilation of the other sugars, as well to study possible synergistic effects during the fermentation of the mix of the sugars.

4.2 Materials and methods

4.2.1 Microorganism and media

The strain used in this experiment was the sporogenic strain *Clostridium saccharobutylicum*, ATCC BAA-117 purchased from American Type Culture Collection. All chemicals for preparation of media were obtained from Sigma-Aldrich Co. The Clostridial Nutrient Medium (CNM, Fluka Analytical) was used as growing media. This medium contained per liter of solution in distilled water: Meat extract, 10 g; peptone, 5 g; yeast extract, 3 g; D (+) glucose, 5 g; starch, 1 g; sodium chloride, 5 g; sodium acetate, 3 g; L-cysteine hydrochloride, 0.5 g; agar, 0.5 g and, resazurin solution (1 g L^{-1}), 0.25 mL; final pH of 6.8.

Stock cultures were cultivated in CNM and adequately kept following the ATCC procedures and stored at -80 °C. For these fermentations, *C. saccharobutylicum* spores were stored at 4 °C and reactivated heating them at 80 °C for 10 min. After this, they were aseptically transferred to 1L glass bottle containing 500 mL of glucose-P2 medium and cultivated at 90 rpm and 37 °C in an orbital shaker (Thermo scientific MaxQ^{TM} 4338, Marietta, USA).

P2 culture medium [Qureshi and Blaschek, 1999] was composed of the following separately prepared solutions (in grams per 100 ml of distilled water, unless otherwise indicated). 1. Sugar solution: glucose, or fructose, or sucrose or mixed sugars, 62 g; yeast extract, 1.031 g in 970 ml of distilled water. 2. Buffer solution: KH_2PO_4, 5 g; K_2HPO_4, 5 g; ammonium acetate, 22 g. 3. Vitamins solution: p-aminobenzoic acid, 0.01 g; thiamine, 0.01 g; biotin, 0.0001 g. 4. Minerals solution: $MgSO_4$-$7H_2O$, 2 g; $MnSO_4$-$7H_2O$, 0.1 g; $FeSO_4$-$7H_2O$, 0.1 g; NaCl, 0.1 g. For these experiments, all the culture media were prepared with sterilized distilled water (121 °C for 15 min) and filter-sterilized using filter units (Thermo Scientific Nalgene, model 1208M78, with a membrane of 0.2 μm). Ten milliliters each of filter-sterilized P2 medium

nutrient solutions (buffer, vitamins and minerals solutions) were added to 970 ml of sugar solution. The pH was adjusted to 6.8 with a 5N NaOH solution. Subsequently, 0.5 g L^{-1} L-cysteine hydrochloride (Sigma) and 0.25 ml L^{-1} of resazurin solution (1 g L^{-1}) were added to the final media to reduce the environment for optimal growth. All autoclaving cycles were done in an AMSCO 2014 autoclave.

4.2.2 Fermentation conditions

The fermentations were carried out in 1L glass-bottle (Wheaton™) with screw butyl rubber cap, containing 500 ml of culture medium. All the bottles were purged with nitrogen to eliminate the oxygen out and make the environment anaerobic. The inoculum was 10% (v/v) of an active cell culture of 16 h of *C. saccharobutylicum*, which were cultivated at 90 rpm and 37 °C in an orbital shaker (Thermo scientific MaxQ™ 4338, Marietta, USA), and uncontrolled pH. All experiments were carried out in duplicates and the reported results are the mean values between them. Three milliliters of culture medium were periodically sampled under a microbial cabinet.

4.2.3 Analytical methods

From collected fermentation samples, cell growth was measured as optical absorbance at 600 nanometers (OD600) using a spectrophotometer (Genesys™ 10S UV-Vis, Thermofisher Scientific). pH was measured by a pH-meter (VWR Symphony SB70P, Beverly, USA). Gas volume was recorded by releasing the gas pressure in the bottles using appropriately sized glass syringes to equilibrate with the ambient pressure [Owen et al., 1979].

The broth was further centrifuged for 10 min at 10,000 rpm to separate the biomass and obtain a clear supernatant, which was later diluted, mixed and filtered through a 0.45 μm syringe filter (Acrodisc 13 mm, Pall) for further quantifications. Reducing sugars were measured by DNS method [Miller, 1959]. Metabolite concentrations were measured using a gas chromatograph (Hewlett Packard 6890 Series) with a Flame Ionization Detector (FID). Sucrose was measured by HPLC (Waters Alliance System) with a refractive index detector (RID). The specific analytical conditions are presented in Appendix A.

4.3 Results and discussion

4.3.1 Fermentation using glucose as the carbon source

The fermentation tests were carried out in a standard P2 medium with different carbon sources – glucose, fructose, sucrose and a mixture of them-.

Figure 4.1 shows the profiles of the pH and of the glucose and metabolites from the glucose-based fermentation of *Clostridium saccharobutylicum* measured in a batch culture. The analysis of the data confirms that there are an acidogenic phase and solventogenic phase, as in *Clostridium acetobutylicum* [Lopez-Contreras et al., 2012] and it produced butanol up to a titer of 13.05 g L^{-1} after 227 h of fermentation. The initial pH at the beginning of the fermentation was 6.44 and dropped down to 4.86 after 32 h. Following that point, pH was controlled within the range of 4.90-5.2. Solventogenic *Clostridium* species have shown that cellular growth and solvent production are strongly pH dependent. After about 24 h of fermentation, it has been shown that pH reaches 4.86 and by that time the butyric acid accumulated, 1.46 g L^{-1} This resulted in triggering the biochemical production of butanol [Gottwald and Gottschalk, 1985]. On the other hand, the acetic acid titer reaches a maximum concentration of 5.66 g L^{-1}, at 24h.

After this point, *Clostridium saccharobutylicum* shifted its metabolism to acetone and ethanol production (solventogenesis phase), reaching at 227 h a maximum concentration of 8.84 g L^{-1} butanol and 0.66 g L^{-1} ethanol. The maximum concentration of ABE (acetone, butanol, and ethanol) at 227 h was 22.56 g L^{-1}.

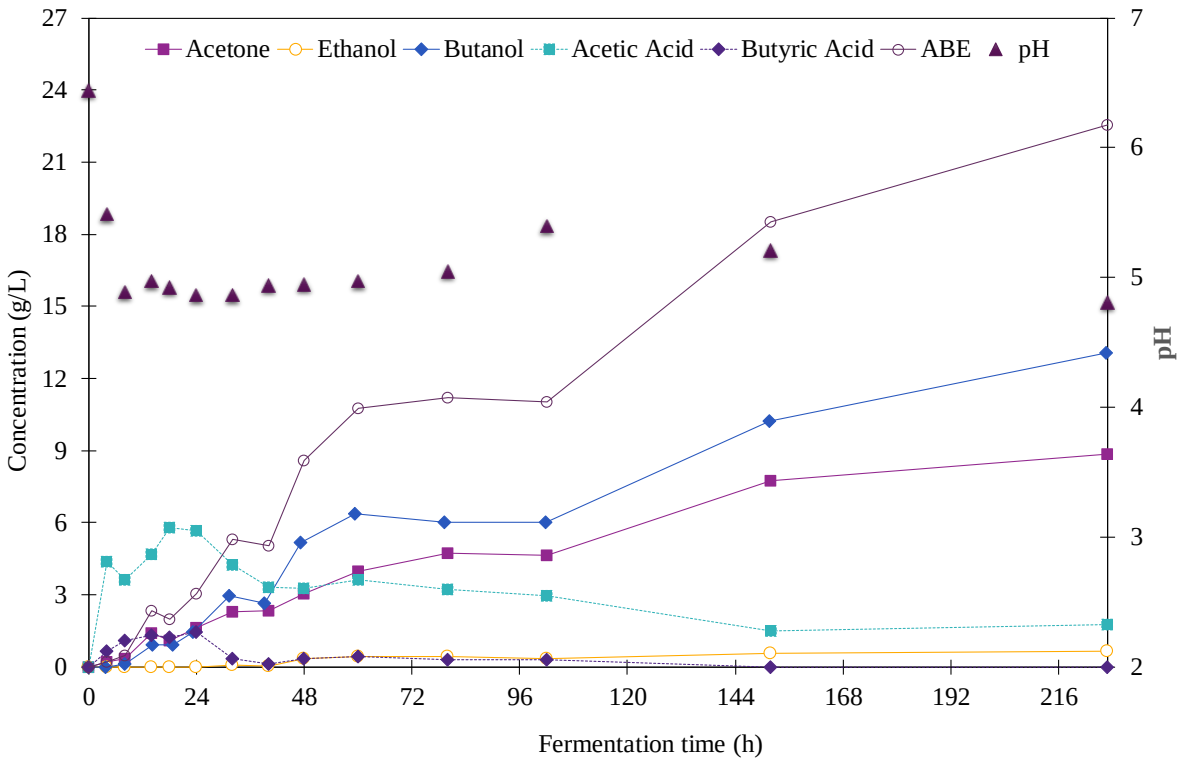

Figure 4.1 Experimental kinetics measured during *C. saccharobutylicum* fermentation in the glucose-P2 medium.

Figure 4.2 reports the cell growth as dry cell weight or also called cellular biomass (g L^{-1}) of *C. saccharobutylicum*, butanol production, and glucose consumption. There was a lag phase of 8 hours followed by an exponential growth phase lasting 14 h. A maximum of 2.63 g L^{-1} biomass was detected after 48 h. As a side note, solvents produced within the cell become toxic up to a certain level in each strain and slows their own production. Solventogenesis serves as an emergency response and its entangled with the sporulation of the cell, which will allow the cell to survive [Dürre, 2005]. Butanol production is associated to the stationary growth phase, and even when the cells concentration reached a plateau, internally the cells keep producing butanol. Meantime its physiological state turns it into a spore. The maximum cell concentration was recorded as 2.90 g L^{-1} at 102 h (Fig. 4.2).

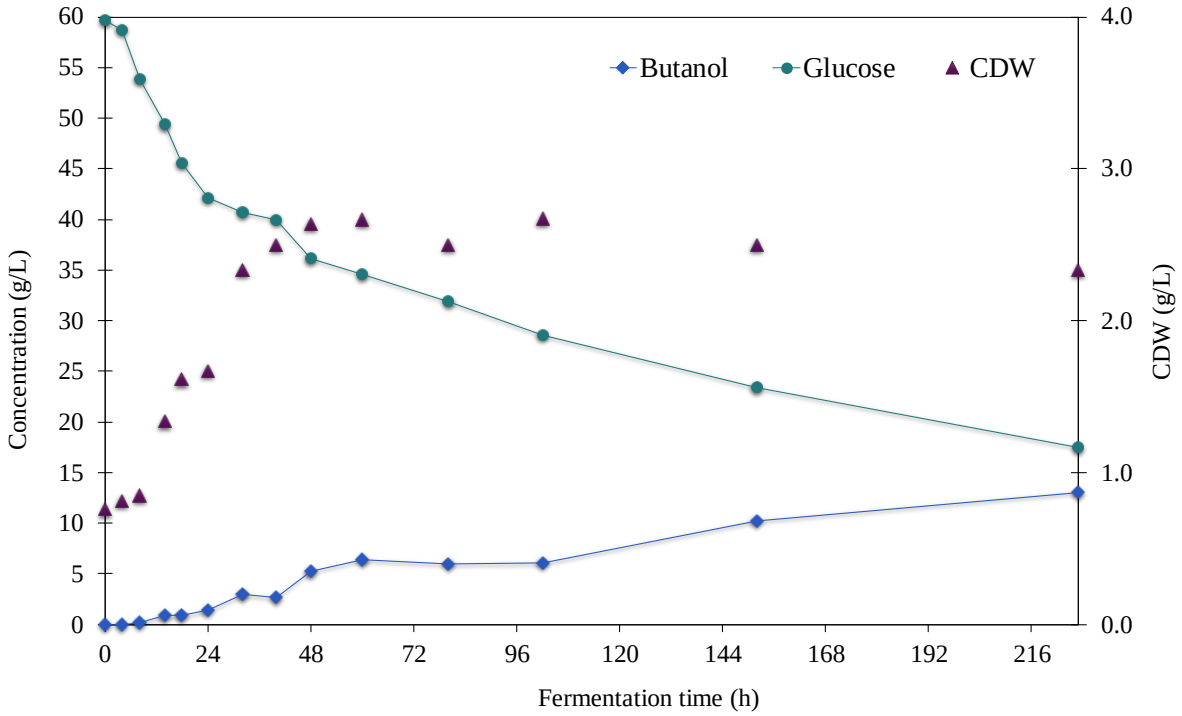

Figure 4.2 Butanol production, glucose consumption and cellular growth by *C. saccharobutylicum*

Glucose is a hexose and its considered the most efficient substrate for cellular growth. Clostridia spp. uses the central carbohydrate-degrading pathway, well known as glycolysis or Embden-Meyerhof-Parnas pathway, when dealing with 6C substrates. Glucose concentration a inoculation was 59.7 g L^{-1} and its final concentration was 17.5 g L^{-1}, giving an overall glucose utilization of 70% and a glucose utilization rate of 0.185 g $L^{-1}h^{-1}$.

In figure 4.3 the cumulative gas production of *C. saccharobutylicum* is shown. Clostridial is a biphasic fermentation, as in the case of *C. acetobutylicum*, and these bacteria produce acetic acid, butyric acid, hydrogen and carbon dioxide as the major products during the exponential growth phase. Following the exponential phase, there is a transition to the solventogenic phase (Figure 4.2); in this experiment it lasted about 48h, where the formation of acids decreases, and acetone, butanol and ethanol are produced in greater amounts. The cumulative gas production at 48 h reached 2,700 mL and from that point in only increased to 4,600 mL at the

end of the fermentation (227 h). That trend was also observed during the fermentation of *C. saccharobutylicum.*

The measured optical density at 600 nm during the exponential phase (until 48 h) was 2.0. After that, the optical density remained nearly constant, increasing to only 2.2 at the end of the fermentation.

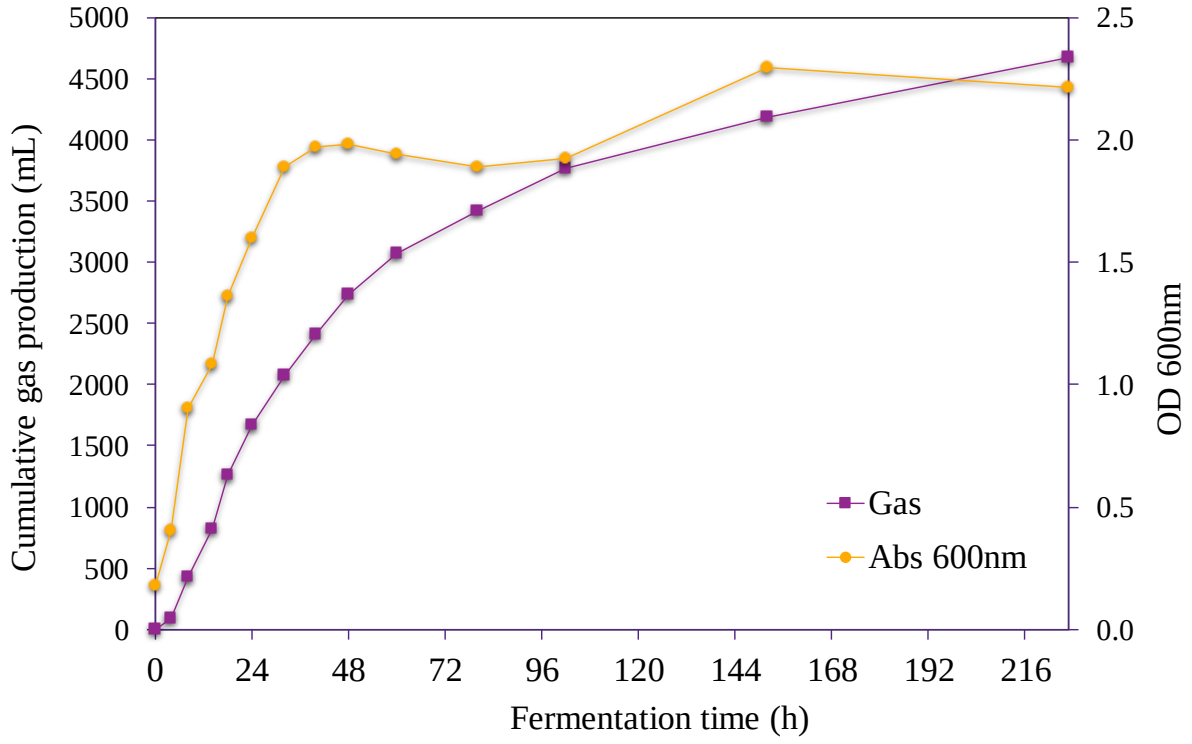

Figure 4.3 Cumulative gas production and cellular growth (OD 600nm) during *C. saccharobutylicum* fermentation on glucose-P2 medium.

4.3.2 Fermentation using fructose as the carbon source

Fructose its another interesting carbon source to produce biobutanol. It is another hexose that once inside the cell, is phosphorylated and follows the glycolysis pathway. As shown in Figure 4.4, butanol was produced from fructose by *Clostridium saccharobutylicum* during a batch fermentation, reaching 9.81 g L^{-1} at 102 h and 14.27 g L^{-1} at 227 h. A good ABE titer was found, achieving an overall 24 g L^{-1} at 227 h. Acetic and butyric acids were produced during the acidogenic phase, reaching a 3.20 g L^{-1} and 2.82 g L^{-1} at 14 h, after that time butyric acid

was reassimilated and the cell metabolism started the production of butanol. The pH started at 6.6 and as expected, it decreased to 4.8-4.9 during the acidogenic phase. At 48 h the pH increased to 5.0 and remained at that range for the rest of the fermentation without any control. Acetone and ethanol had maximum concentrations of 9.15 g L^{-1} and 0.63 g L^{-1}, respectively at the end of the fermentation.

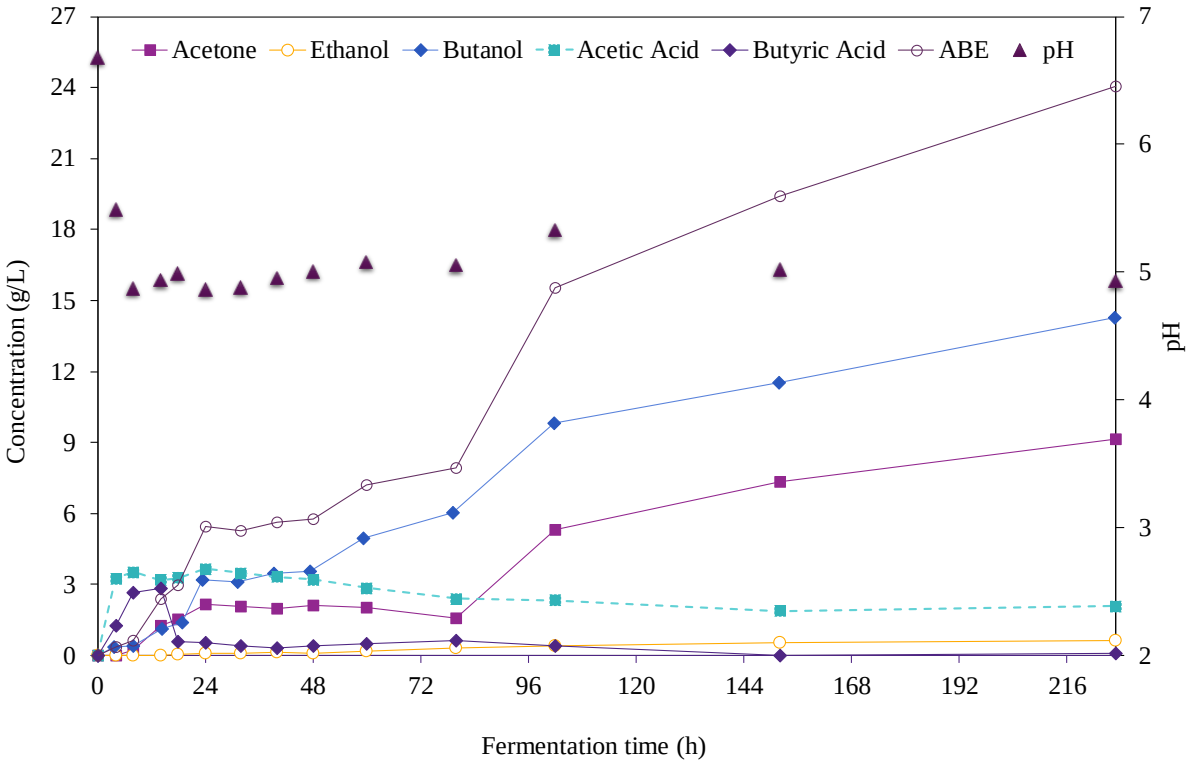

Figure 4.4 Batch fermentation of *C. saccharobutylicum* on fructose-P2 medium.

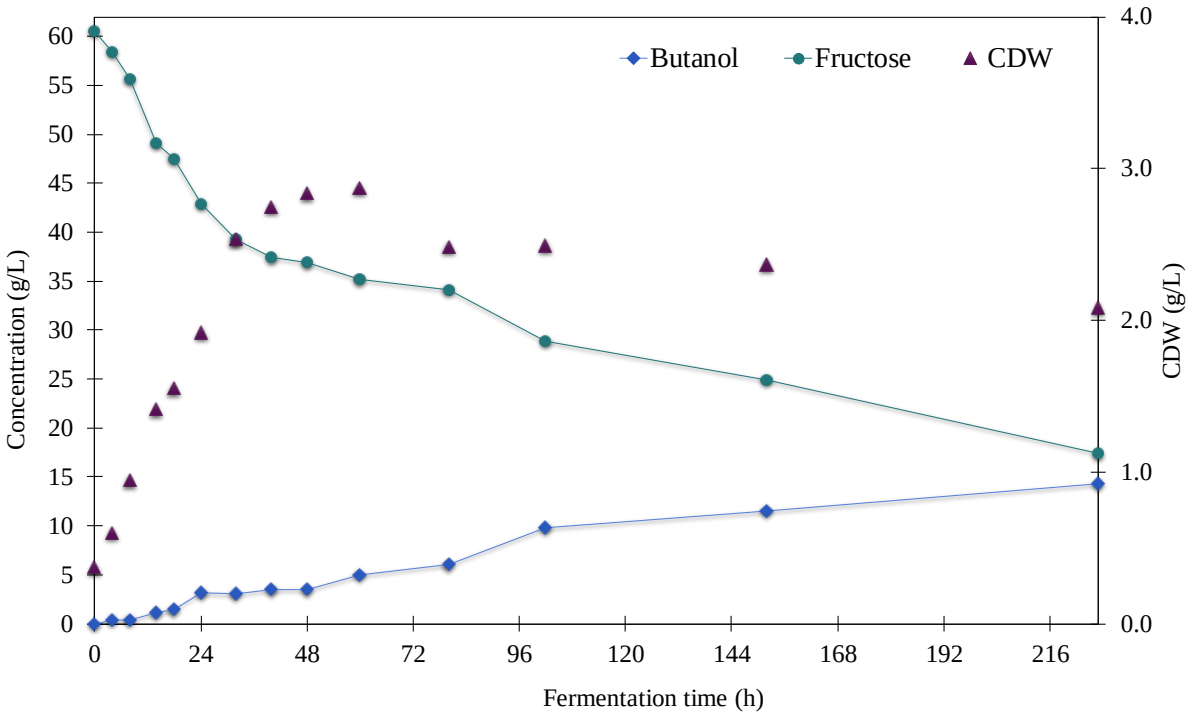

Figure 4.5 Butanol production, fructose consumption and cellular growth by *C. saccharobutylicum*

Figure 4.5 displays the fructose consumption during the fermentation time. Its concentration started at 60.56 g L^{-1}. After 32 h, the fructose concentration was 39 g L^{-1}, with a fructose utilization of 35%. By the 227 h, the final fructose consumption reached 72%. The cellular growth was exponential up to 40 h, reaching 2.75 g L^{-1} cells. It remained high and started to diminish after 80 h of fermentation. Significant butanol production started at 24 h and showed steady progress.

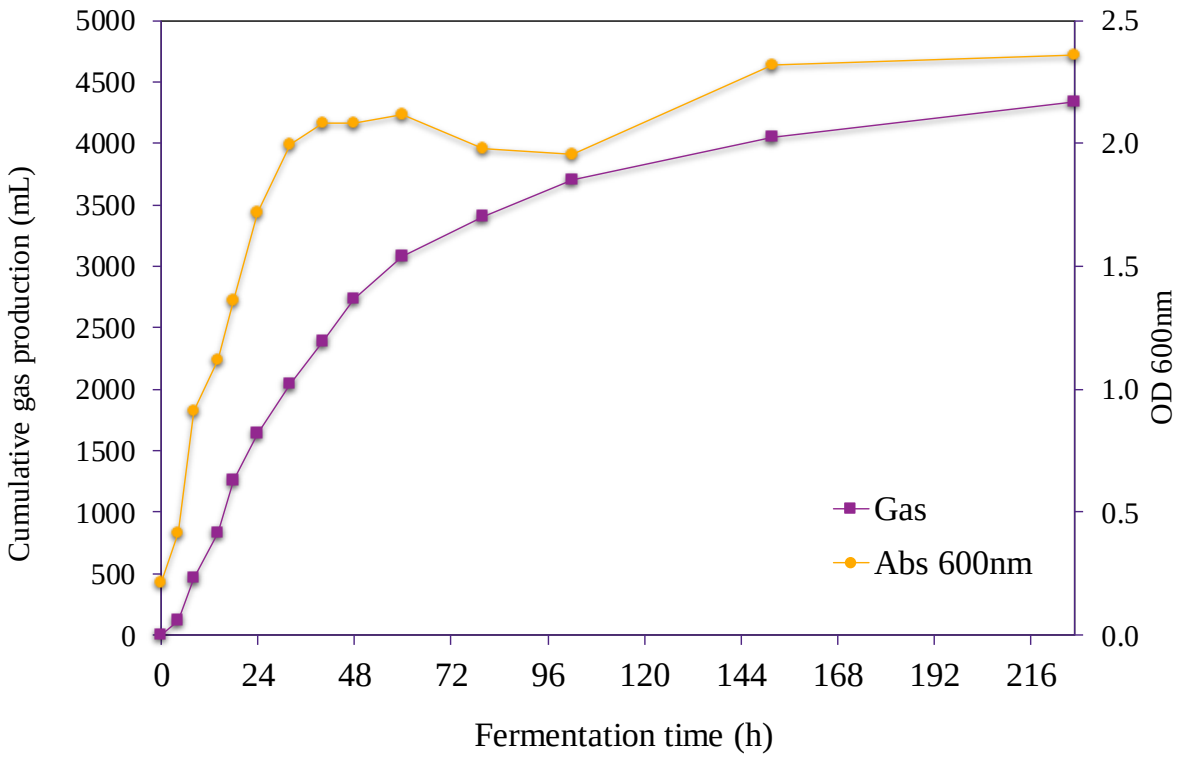

Figure 4.6 Cumulative gas production and cellular growth (OD 600nm) by *C. saccharobutylicum* on fructose-P2 medium.

Clostridium saccharobutylicum grown on fructose had a maximum optical density of 2.0 at 32h. The cumulative gas (H_2 and CO_2) volume was 2,000 mL of as shown in Figure 4.6. At 48 h the accumulated gas was 2,700 mL, similar to the case with glucose. At the end of the fermentation, the gas volume reached 4,300 mL and the optical density was 2.3. Fructose and glucose used as carbon source for butanol production showed very similar values, and both followed biphasic fermentation.

4.3.3 Fermentation using sucrose as the carbon source

Sucrose is a disaccharide most commonly found in higher plant tissues and includes fructose and glucose within its molecule. Biobutanol production was achieved using sucrose as a carbon source, as shown in Figure 4.7.

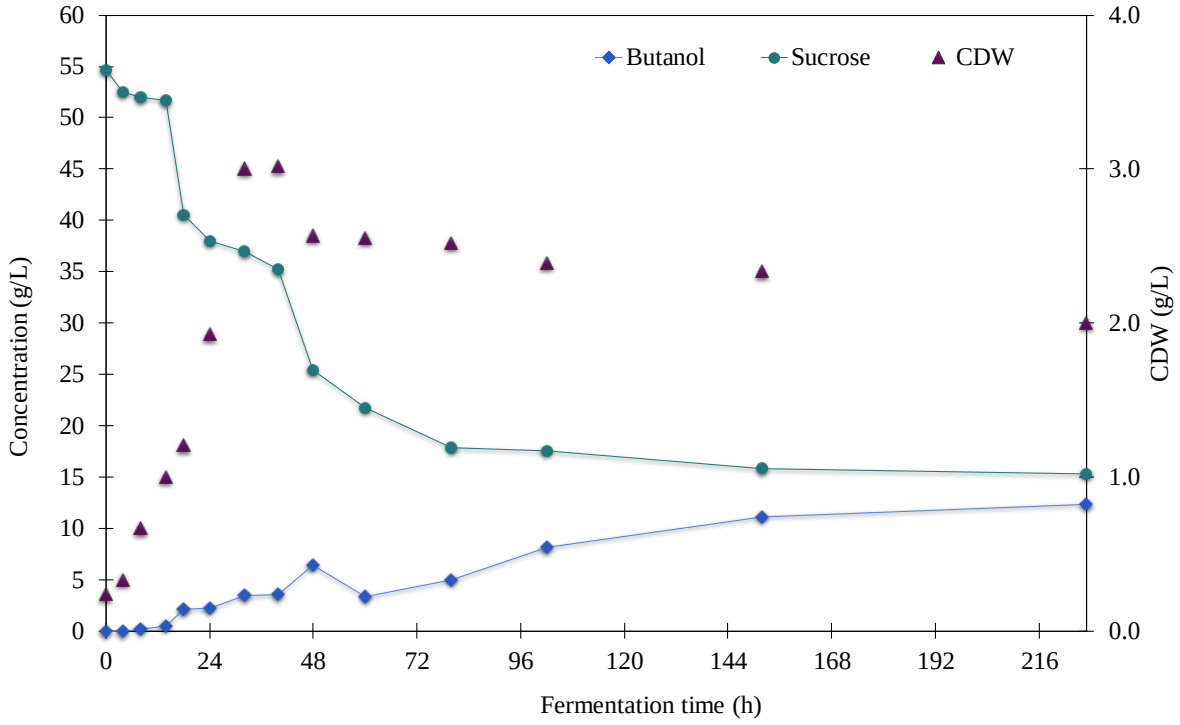

Figure 4.7 Butanol production, glucose consumption and cellular growth by *C. saccharobutylicum*

The starting pH of the sucrose-based experiment was 6.6 and decreased quickly reaching 4.87 by the 8th hour of fermentation. Following that, pH remained nearly constant in the range of 4.8-5.0 until 60 h, when it started increasing up to 5.3 at 102 h. Butanol concentration reached a level of 8.18 g L^{-1} by 102 h and the final butanol concentration was 12.38 g L^{-1} at 227 h, just below fructose-based fermentation (14.27 g L^{-1}). The production of acetic acid was greater that butanol during the acid phase, having a concentration of 4.2 g L^{-1} at 4 h, and then was varying until 60 h, when it started to decrease until reaching 1.90 g L^{-1} at 227 h. Acetone and ethanol concentrations at the end of the fermentation were 10.56 g L^{-1} and 0.74 g L^{-1}, respectively.

Butyric acid was not produced significantly during the solventogenic phase and reached a maximum titer of 1.69 g L^{-1} at 14 h, as shown in Figure 4.7.

Figure 4.8 shows the sucrose consumption by *C. saccharobutylicum* starting from 54.66 g L^{-1} at the beginning of fermentation. Initially, until 14h, the sucrose was consumed slowly. The potential explanation of for this situation can be based on the sucrose uptake from the culture medium to the cell. There are different transport routes for sucrose in Clostridia. For instance, the phosphoenolpyruvate (PEP)-dependent phosphotransferase system, also known as PEP-PTS, which has been identified in *C. acetobutylicum* and *C. beijerinckii* for sucrose uptake [Tangney et al., 1998;Mitchell, 2015]. Once its senses the sucrose in the culture medium, it triggers the formation of the enzymatic cluster to phosphorylate the sucrose molecule outside the cell in order to be able to use the sucrose molecule. After 14 h, sucrose consumption sees a sharp decrease until 48 h, reaching a concentration of 25.37 g L^{-1}. From that point on, the sucrose concentration decreases slowly until the end of the fermentation at 227 h, reaching a final concentration of 15.29 g L^{-1}. The average sucrose utilization rate was 0.17 g L^{-1}-h while the sucrose utilization was 72%.

C. saccharobutylicum grown on sucrose reached a maximum cell concentration of 3.01 g L^{-1} at 40 h and decreased over time until the fermentation finalized with a concentration of 2.0 g L^{-1} at 227 h.

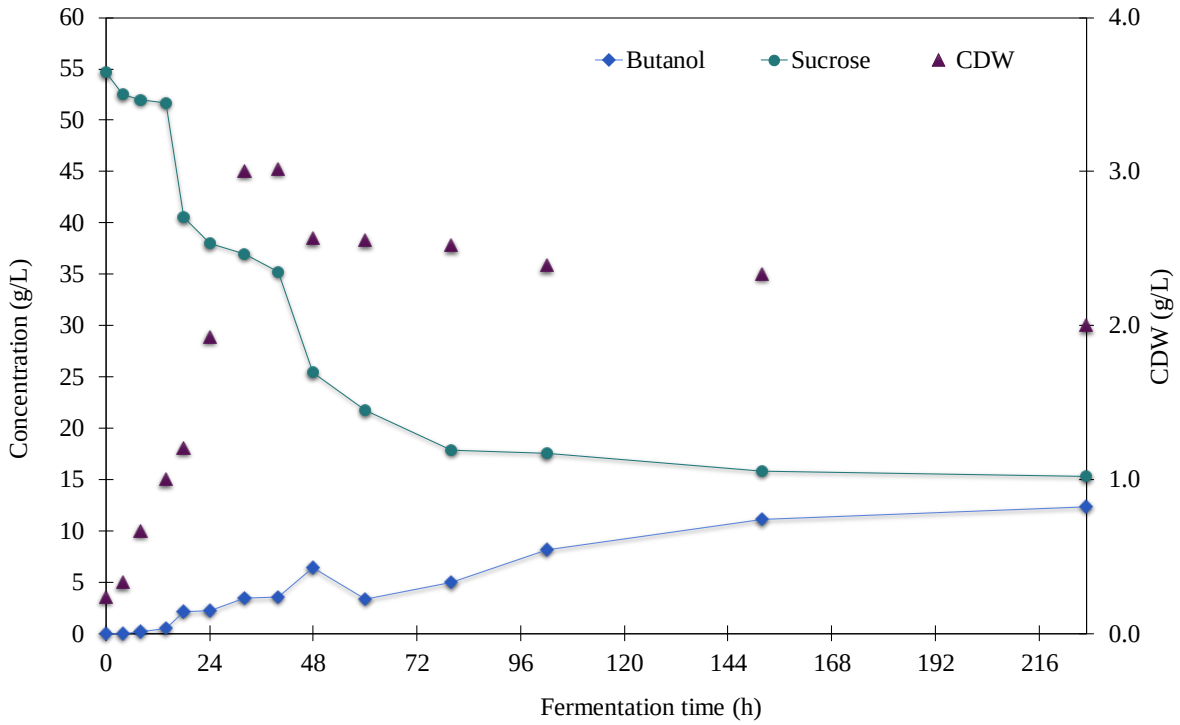

Figure 4.8 Comparison of butanol production, carbon source consumption and cellular growth during *C. saccharobutylicum* fermentation on sucrose-P2 medium.

Figure 4.9 shows the cumulative gas production, which reached a maximum of 2,700 mL at 48 h. The optical density was 2.1, which is consistent with the exponential growth phase displayed in Figure 4.8.

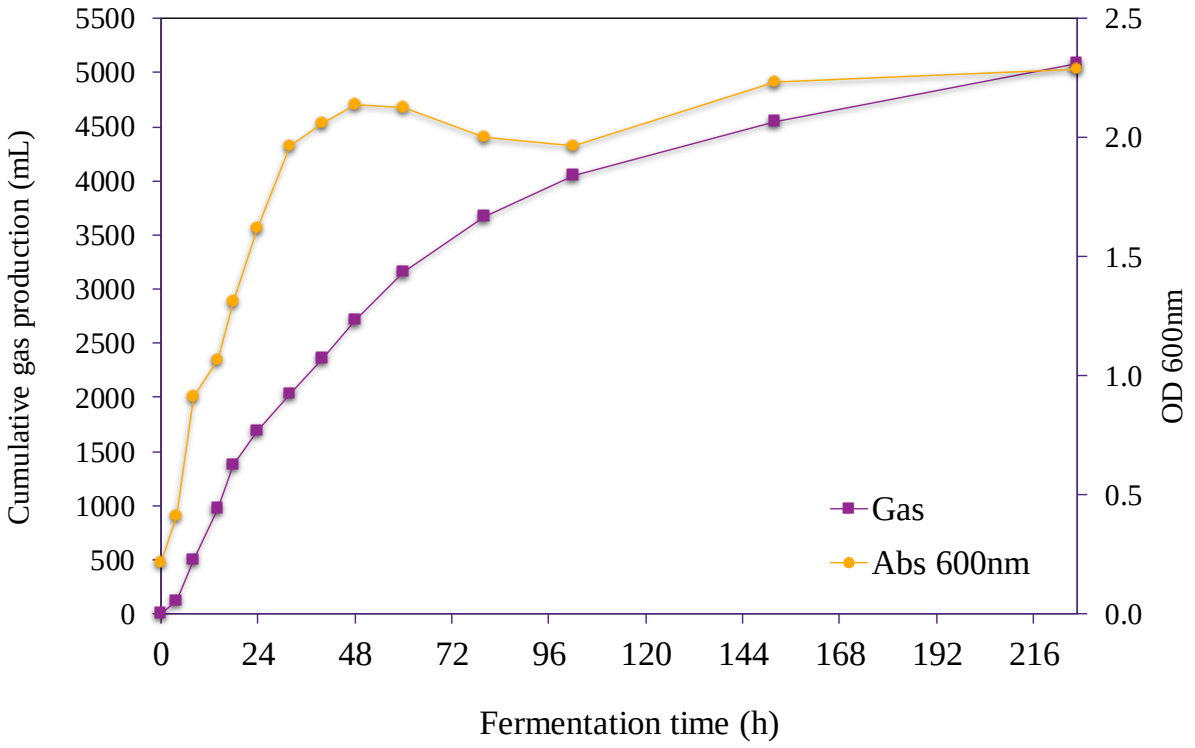

Figure 4.9 Cumulative gas production and cellular growth (OD 600nm) by *C. saccharobutylicum* on sucrose-P2 medium.

4.3.4 Fermentation using mixed sugars as a carbon source

Sugarcorn juice contains a mix of sugars, mainly, glucose, fructose and sucrose [Gomez-Flores et al., 2018]. For Clostridia species, glucose is the monosaccharide preferred for healthy fermentation and biobutanol production. Nevertheless, sugarcorn juice, from different sugarcorn hybrids, could contain different amounts of glucose, fructose and sucrose, mainly depending on the age of the plant and the kind of hybrid [Reid et al., 2015]. When working with anaerobic solventogenic bacteria, the challenge is greater than when dealing with ethanol-producing and novel yeasts. As seen in the above experiments, sucrose has a slower consumption rate than glucose or fructose that might be due to the different sugar uptake mechanisms and internal metabolism. In this experiment, we explored the butanol production and growth of *C. saccharobutylicum* when cultivated on a mix of sugars.

In Figure 4.10, butanol production from a mixed sugar source is shown. Butanol reached a titer of 9.81 g L^{-1} at 102 h and a final titer of 14.27 g L^{-1}. Acetic acid had a maximum concentration of 3.91 g L^{-1} by the 32nd hour, whereas the butyric acid was always maintained a very low concentration of 2.82 g L^{-1} at 14 h and after that time it was always below 1 g L^{-1}. This is an interesting observation since common solventogenic fermentation is carried out during the acidogenesis phase. However, it seems that the mix of sugars enhances some specific mechanisms within the cell that hinder butyric acid production, while the butanol production remains active and normal. The ABE final concentration reached 23.43 g L^{-1} and was similar to that for single sugars fermentations. Acetone concentration was 8.58 g L^{-1} at 152h, whereas ethanol concentration was not more than 0.6 g L^{-1} over the entire fermentation. The pH of this experiment started at 6.4 and decreased sharply at the beginning until reaching 4.9 at 8 h; then it remained almost constant until 102 h when it increased to 5.3 while the butanol concentration kept rising.

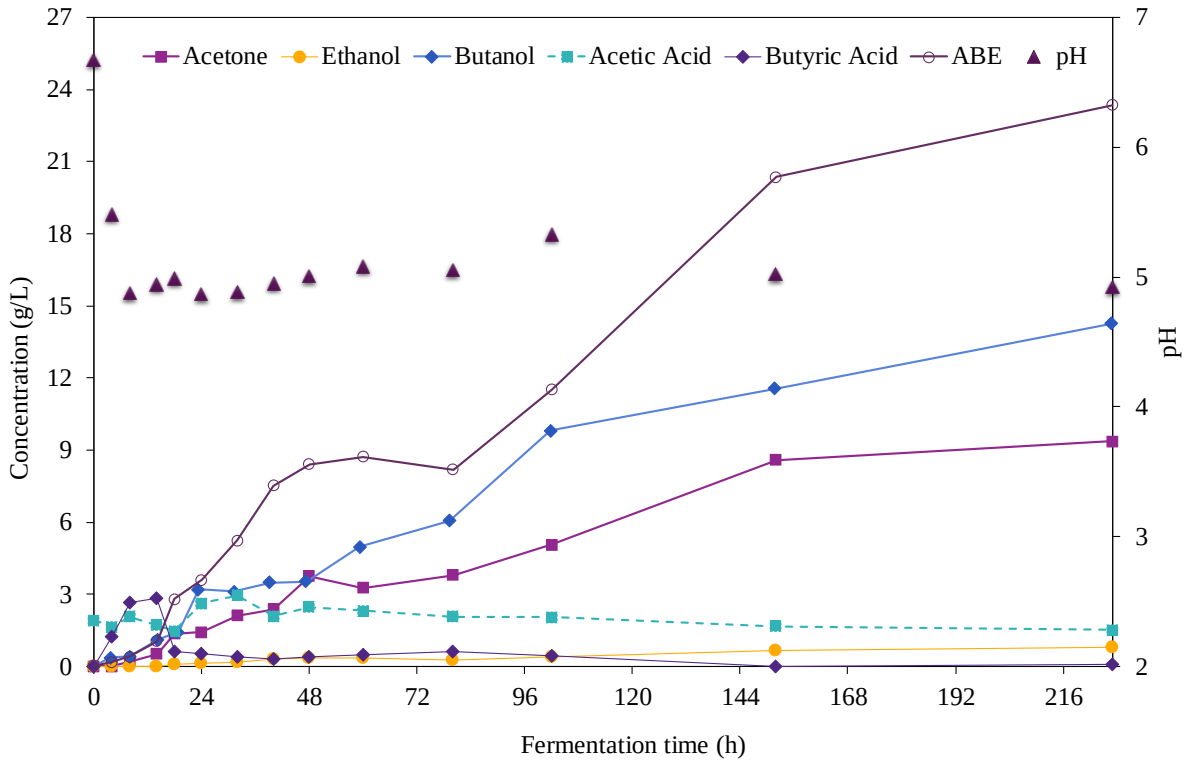

Figure 4.10 Experimental kinetics measured during *C. saccharobutylicum* fermentation on mix sugars-P2 medium.

Figure 4.11 shows the sugar consumption by *C. saccharobutylicum* over time. The three sugars were consumed and glucose seemed to be the first sugar consumed, as expected in the case of *C. acetobutylicum* [Durre, 2008]. In our case, with an initial concentration of 22.78 g L^{-1} at inoculation time, glucose was the first sugar consumed in the first 8 hours. Following that, a low fructose consumption started after 8 hours. By 14 h of fermentation glucose and fructose are concurrently being absorbed by the cell. Sucrose concentration started to be consumed after 32 h. After 40 h, sucrose its clearly being depleted over time. This behavior can be explained by the difference of sucrose-PTS system when compared to the sucrose-based fermentation results. Another important observation is that *Clostridium saccharobutylicum* did not wait until both monosaccharides had been depleted to trigger the construction of the PTS-system. Once the cell senses the sucrose in the culture medium, its mechanisms begin to uptake the disaccharide, even if there are glucose and fructose available in the medium. This observation

did not happen in *C. beijerinckii*, where sucrose was not utilized in the presence of glucose until the glucose was depleted [Reid et al., 1999].

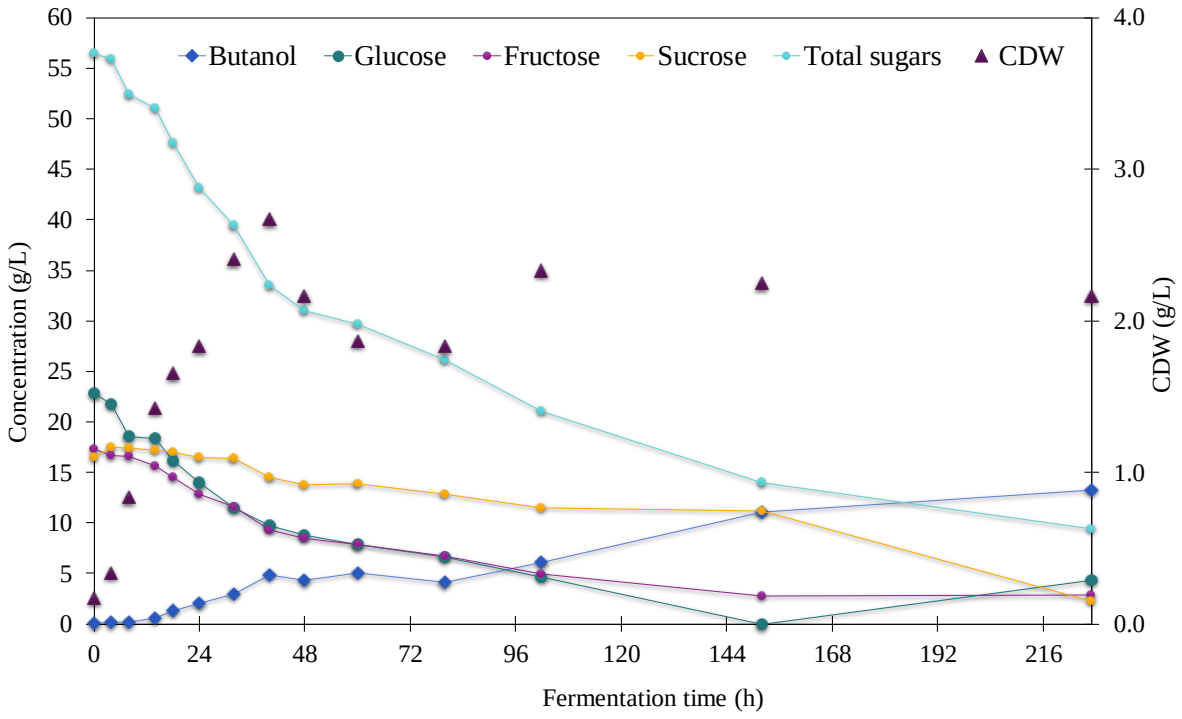

Figure 4.11 Comparison of butanol production, carbon source consumption and cellular growth during *C. saccharobutylicum* fermentation on mixed sugars-P2 medium.

By 152 h, glucose has been consumed entirely, and a 2.5 g L^{-1} of fructose remains in the media. Sucrose was still present at a concentration of 11.17 g L^{-1}, and the consumption of the disaccharide will provide the remaining energy for the metabolic activities and for butanol production.

In Figure 4.12, the cumulative gas production and the optical density are shown. The maximum optical density was 2.0 at 48 h and the cumulative gas production was 2,500 mL. The optical density had a small increase by the end of the fermentation, reaching 2.4 at 227 h.

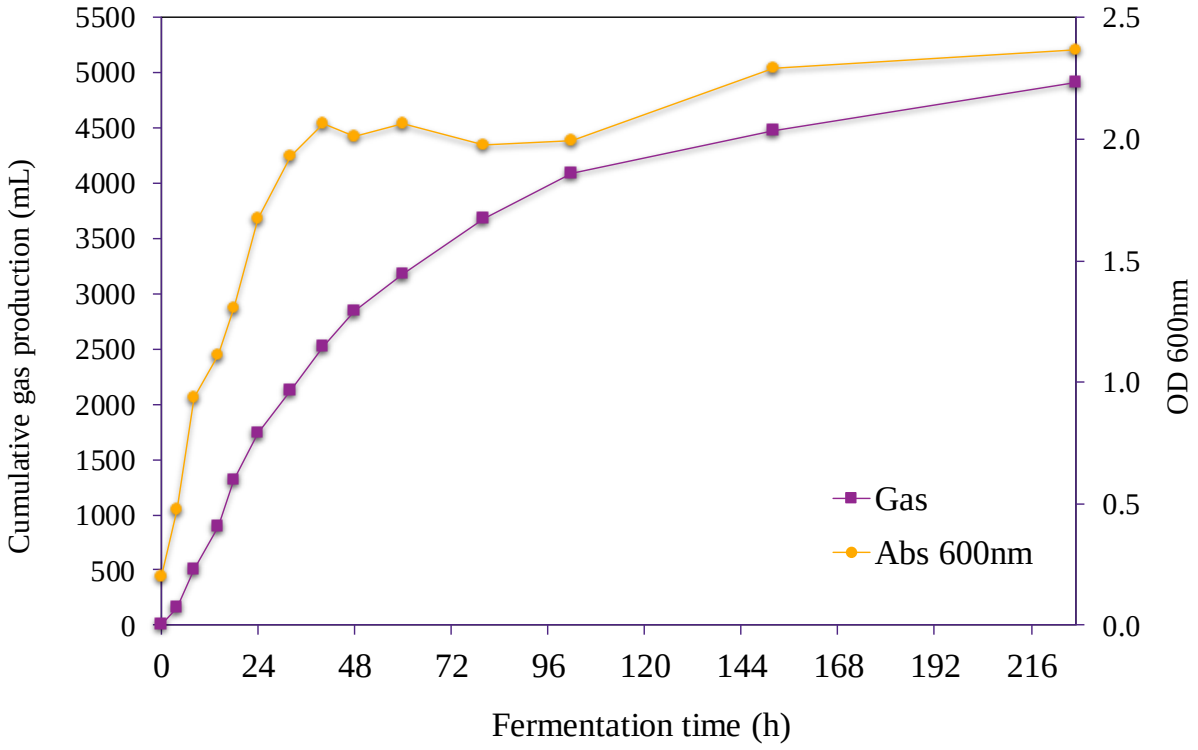

Figure 4.12 Cumulative gas production and cellular growth (OD 600nm) by *C. saccharobutylicum* on mixed sugars-P2 medium.

4.3.5 Cross-Species Comparison: sucrose degradation I (sucrose-PTS)

Utilizing the Pathway Tools, version 21.5 software from SRI international (BIOCYC14, https://biocyc.org), a simple comparative analysis across *C. acetobutylicum, C. beijerinckii and C. saccharobutylicum* was performed. This software compared a single metabolic pathway across different organism's databases. For each reaction step, identified enzymes and genes are listed. A pathway may not be present in an organism database even if enzymes have been identified for one or more of its reactions, and its indicated for each case. The objective was to compare between the three Clostridia species, if genes for PTS systems were present.

Table 4.1 shows the pathways that are shared between three different solventogenic Clostridia species. The analysis was narrowed to only sucrose phosphotransferase.

The bacterial sucrose transport inside the cell by the phosphoenolpyruvate-dependent phosphotransferase system (PTS) allows the translocation of the sucrose from the exterior to the interior of the cell, and chemically modifies the sucrose molecule by phosphorylation. Each PTS is composed of two energy-coupling proteins, Enzyme I and HPr, and several sugar-specific Enzyme II proteins (EIIA, EIIB, and EIIC), and transport and phosphorylation of the sugar happens when a phosphoryl group donated by PEP is passed via EI, HPr, EIIA, EIIB, and EIIC to the sucrose (or any sugar) [Reid, 2005].

For *C. saccharobutylicum,* a sucrose-PTS system has been identified, as shown in table 4.1. This information supports the hypothesis of the way the sucrose is consumed. The data show that the PTS system has an Enzyme II protein, B, and C, which its indeed comprised in the *scrA* gene. Overall, from the results of our experiments, we can conclude that the sucrose uptake in *C. saccharobutylicum* is not being hindered by glucose and fructose presence, nor that glucose needed to be completely depleted to start the sucrose uptake.

Table 4.1 Comparative analysis of enzymes and genes among solventogenic Clostridia

Organism	Enzymes for sucrose degradation	Genes for sucrose degradation by PTS-sucrose
***C. acetobutylicum* ATCC 824**	EC 3.2.1 levanase/invertase EC 2.7.1.4 fructokinase	-
***C. beijerinckii* NCIMB 8052**	EC 2.7.1.4 fructokinase	EC 2.7.1.211 PTS sucrose transporter subunit IIBC
***C. saccharobutylicum* DSM 13864**	-	EC 2.7.1.211 PTS sucrose EIIBC component ScrA

4.4 Conclusion

Different ABE fermentations by *Clostridium saccharobutylicum* ATTC BAA-117 (*Clostridium saccharobutylicum* DSM 13864), using three individual sugars (glucose, fructose, and sucrose) and a mix of these were carried out. The selection of these sugars was based on the study of the composition of sugarcorn juice, a new and potential Canadian energy crop. The study found that under an initial concentration of sugars at 60 g L^{-1}, glucose, fructose, and sucrose are good substrates for biobutanol production utilizing this Clostridia strain. The results based on butanol production are summarized in table 4.2.

Table 4.2 Summary of the results of biobutanol fermentations by *C. saccharobutylicum*

Substrate	**Yield (g butanol / g sugar)**	**Q_p (g/L)-h**	**Titer (g/L)**	**% of the theoretical yield**
Glucose	0.306	0.057	13.05	72.85
Fructose	0.330	0.062	14.27	78.57
Sucrose	0.314	0.054	12.38	74.76
Mix	0.28	0.058	13.20	66.66

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Chapter 5

5 Characterization of sugarcorn juice

5.1 Introduction

Butanol (1-butanol, n-butanol) has been considered as a bulk chemical with a wide range of industrial applications. Currently, there is an increased interest for the implementation of butanol as a biofuel and as an oxygenated additive to be blended with gasoline, in a similar mode as ethanol [Lopez-Contreras et al., 2012].

To allow the fermentative biobutanol production, three different feedstocks can be utilized, starch, cellulosic and sugar-based feedstocks. In this scenario, sweet sorghum, sugarcane, and sugar beet are common sugar-based materials, which have been deployed to produce biofuels [Barcelos et al., 2016; Lopes et al., 2016; Haankuku et al., 2015].

Agriculture and Agri-Food Canada developed through selective breeding a sugar-based feedstock for Canada, named as ‘Sugarcorn’, which are corn hybrids with high sugar concentration in the stalks. Following the development of the corn plant, the sugar concentration in the stalks peaks in the weeks following silking. At this stage, sugarcorn juice can be extracted by pressing the stalks, thus providing a mix of readily fermentable sugars [Reid et al., 2015]. A crucial trait of sugarcorn is that its germplasm is adapted to Canadian weather conditions and short growth seasons of May to September, particularly suitable for the major corn growing regions of southwestern Ontario and southern Quebec [Reid et al., 2015;Reid et al., 2016].

Furthermore, farmers in these regions are familiar and knowledgeable with the seed and harvest of corn, which highlights a significant advantage for the potential implementation of this energy crop, either for biofuels or bio-materials production [Reid et al., 2015].

One of the aims of this thesis was to characterize the juice extracted from the sugarcorn plant, based on their nutrient composition and sugar concentration; similarly, to the characterization of well-known sweet sorghum, sugarcane, and sugar beet sugars. Further use of sugarcorn juice will focus on the design of a culture media suitable for biobutanol microbial fermentations.

The study was carried out in two sugarcorn batches, and different efforts were made to study the variation of juice sugars across different plant ages and hybrid types of the sugarcorn plant.

One of the main industrial challenges when working with plant juices is its susceptibility to spoilage due to inherent microbes or contamination from the environment. Among spoilage microorganisms, such as bacteria, molds and yeasts are found within sugarcane juices and are responsible for the alteration of the juice. To prevent spoilage or contamination, a sterilization step using acidification, thermal treatment (pasteurization) and filtration is necessary to inhibit the growth of any microorganism that might damage the quality of the juice [Silva et al., 2016].

In this chapter, carbon filtration and autoclaving methods were selected to asses them as sugarcorn juice primary pretreatment.

5.2 Materials and methods

5.2.1 Materials

Four different sugarcorn hybrids, AAFC-SC1, AAFC-SC2, AAFC-SC3, AND AAFC-SC4 were the results of several corn inbreeds efforts from Dr. Lana Reid's team at Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada in Ottawa (Ontario, Canada).

These four hybrids were seeded, grown and harvested in Ridgetown, Ontario at the Centre for Agricultural Renewable Energy and Sustainability of the University of Guelph (42°26'N, 81°53'W) in the years 2014 and 2015.

The sugarcorn plants were harvested 5 to 10 days after silking, and the stalk of the plant was cut about 12-13 cm above the soil level, the ears were manually removed and the sugarcorn stalks -including leaves- were feed through a three-roller press to extract the sugarcorn juice. Once the juice was collected, it was stored at -20 °C. Dr. Robert Nicol and Dr. Brandon Gilroyed were responsible for the after-mentioned process.

The sugarcorn juice was then transported to University of Western Ontario in London (Ontario, Canada). For the characterization and fermentation studies, the sugarcorn juice was thawed at room temperature and filtered through cheesecloth to remove plant residues. Once filtered, the

juices were transferred to 1-liter pre-washed plastic containers, sealed, weighed and stored at -20 °C until further use.

For the 2014 year, two sugarcorn juices batches were brought to the University of Western Ontario laboratory. They were named sugarcorn juice A (SCJ A) and sugarcorn juice B (SCJ B), harvested two weeks apart in September 2014 (Table 5.1).

Table 5.1 Sugarcorn juice harvested years and properties

Year	Planting	Harvesting	Age of the plants (days)	Crop heat Unit (CHU)	Difference in days
2014	26-Jun	Silking Approx.	68		
		12-Sep	78	1834	0
		25-Sep	91	1999	13
2015	13-May	Silking Approx.	88		
		10-Aug	98	1907	0
		19-Aug	107	2140	9
		1-Sep	112	2401	22

For the year 2015, two sugarcorn juice batches were taken to the University of Western Ontario laboratory we called them sugarcorn juice C (SCJ C) and sugarcorn D (SCJ D), harvested on August 2015. It is important to note that in 2014 each batch contained a mixture of juice from the four sugarcorn hybrids grown at Ridgetown campus. In 2015, individual samples of each sugarcorn hybrid were brought to the University.

All characterization procedures were carried out in triplicates, using Sugarcorn juices from 2014, unless otherwise specified. Some biobutanol fermentations were performed utilizing sugarcorn juice form 2014, as well 2015 samples.

5.2.2 Analytical methods

Total solids, total dissolved solids, moisture and ash content (on a weight basis) were calculated using National Renewable Energy Laboratory protocols (NREL) [Sluiter et al., 2008;Hames et al., 2008]. To determine the percentage of total solids, total dissolves solids and moisture, two sets of sugarcorn juice samples, one set filtered through 0.1 μm Whatman®

membrane, and the second set unfiltered, were dried at 105 °C (Thermofisher scientific® oven) until constant weight was achieved. Once the samples were dried, then ash content was measured by setting the samples in a muffle furnace at 575 °C for four hours, weighed until constant weight was performed to determine ash content in sugarcorn juice. The density of the juice was estimated gravimetrically with an uncertainty of 1 mg, using an analytical balance and a 50-millimeter pycnometer. Calibration of the glassware was done with distilled water at 20°C.

The pH of the sugarcorn juice was measured with a pH meter (VWR Symphony SB70P, Beverley, USA), previously calibrated with standard buffer solutions (pH 4, pH 7, pH 10). Elemental analysis (carbon, nitrogen, hydrogen and oxygen content) for sugarcorn juice was determined utilizing a Flash EA 1112 Series- Elemental Analyzer (Thermoscientific®, Waltham, USA) at the Institute for Chemical and Fuels from Alternative Resources (ICFAR). The protein part of the sugarcorn juice was quantifies following the Bradford method [Kruger, 1996].

The Brix measurements were made using a Brix refractometer (Leica Auto ABBE, Buffalo, USA) with temperature compensation. Sucrose standards were used, as well as distilled water serving as blank. Total carbohydrates were determined using the phenol-sulfuric acid method [Dubois et al., 1956] and reducing sugars using dinitrosalicylic acid or DNS [Miller, 1959] (Appendix A). To determine sucrose, fructose and glucose concentrations in sugarcorn juices, liquid chromatography (HPLC, Waters Alliance System, New Castle, USA) was used. These methods are fully described in Appendix A.

5.2.2.1 Microbiology of sugarcorn juice

About 1 ml of sugarcorn juice was serially diluted up to 1 x $10^{-8,}$ and 100 µL were plated into a nutrient broth agar plate and dispersed using a triangular loop. The Petri dishes were incubated at 30 °C for 48 h. These broad microbiology tests aimed to investigate the possibility of not having to autoclave the stalk juice and use it as is for further butanol fermentation.

5.2.2.2 Sugarcorn juice treatments

The effect of activated carbon filtration was investigated by filtering solutions of SCJ A and SCJ B through a bed of granular activated carbon (Calgon Carbon Corporation, Pittsburgh, USA) with a 3:1 ratio by weight. Granular activated carbon utilized in these experiments had an Iodine number of at least 1000 mg/g, and a sufficient pore size of 0.55 to 0.75 mm.

Effect of autoclaving on sugars in sugarcorn juice was studied, for which SCJ A and SCJ B were taken separately in tightly aluminum crimped 30 ml serum bottles and autoclaved at 121°C and 15 psi for 15 minutes (Autoclave AMSCO 2041). Total carbohydrates, reducing sugars and concentration of sucrose, glucose, and fructose were determined before and after autoclaving, The variation of carbohydrates in sugarcorn juices samples from 2015 were studied across each hybrid and plant maturity. The juice samples were selected such that, the plants were grown for 98 and 112 days. A simple study was carried out to understand the effect of age on sugarcorn stalk carbohydrates with limited juice samples from 98, 107 and 112 days of growth.

5.3 Results and discussion

5.3.1 Sugarcorn juice characterization

Sugarcorn juice is a light yellowish to brownish-green colored liquid (Figure 5.1) with fresh cut grass odor and sweet aroma, closely resembling sugarcane juice.

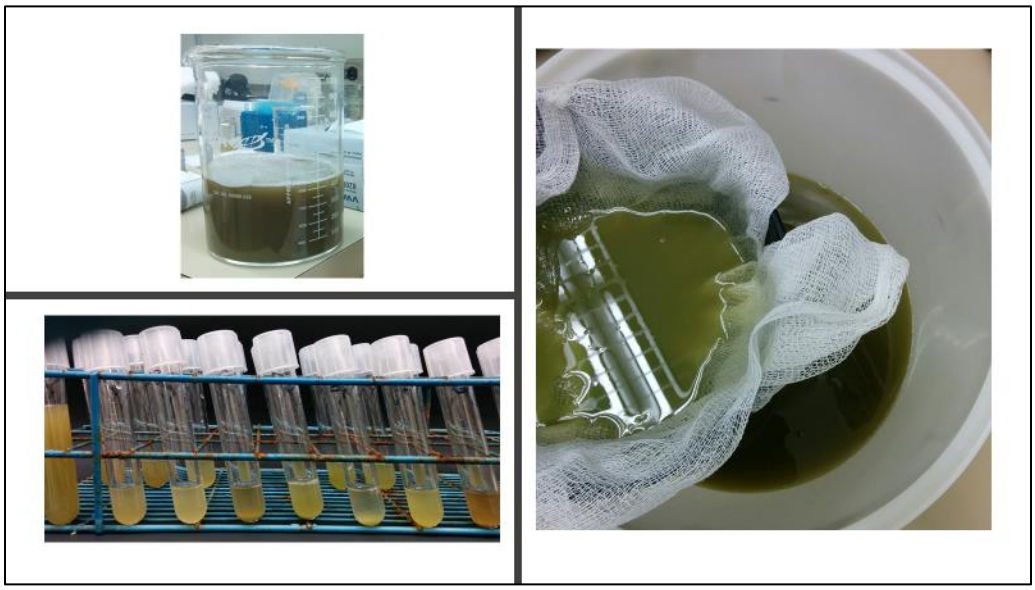

Figure 5.1 Picture of sugarcorn juice

It has a mild acidity (pH>4.89) and is composed of approximately 91% water and 9% total dissolved solids. The physical and chemical properties of the juice, in general, varied distinctly between SCJA (78 days) and SCJB (91 days) as shown in Table 5.1. Sugarcorn juice samples had an ash content of 5.9 and 6.4 wt.%, though ash content is known to vary based on factors such as soil type, hybrid, growth conditions, fertilizers used and maturity [Samson et al., 2008].

Carbon content was about 3 to 4.5%, whereas hydrogen content in sugarcorn juice varied between 4.8-7.5%. Oxygen content was between 38.45 to 41.92% in sugarcorn samples with vastly settling solids. Sugarcorn juice samples evaluated had nitrogen and protein concentration between 0.04 and 0.78%. The low carbon content highlights the aqueous nature of the sugarcorn juice and provides evidence of a diluted liquid.

Table 5.2 Composition of sugarcorn juice from 2014 samples

Physical property	SCJ A (78 days) 2014	SCJ B (91 days) 2014
Moisture content (wt%)	90.57 ± 0.04	91.9 ±0.01
Ph	5.08 ±0.02	4.89 ± 0.01
Specific gravity (dimensioless)	1.04 ± 0.00	1.04 ± 0.00
Ash (wt%)	5.94 ± 0.12	6.44 ± 0.04
Total solids (wt%)	9.44 ± 0.04	8.73 ± 0.18
Total dissolved solids (wt%)	9.39 ± 0.06	8.10 ± 0.10
Composition (wt%)		
Carbon	4.44 - 4.52	3.05 – 3.52
Hydrogen	4.79 – 6.35	6.09 – 7.51
Oxygen	Not detected	38.45 – 41.92
Nitrogen	0-0.04	0.20 – 0.78
Protein	0.08 ± 0.0	0.09 ± 0.0

Among the dissolved solids, it should be noted that for the year 2014 at 78 days, the mix of the four sugarcorn hybrids had a composition of sucrose (4.6%), glucose (3.0%) and fructose (2.4%). Interestingly, when the sugarcorn plant was harvested at 91 days, the juice composition changed, for example, sucrose concentration reached up to 7.0%, whereas glucose and fructose decreased.

As discussed later in Figure 5.4, A is the concentration of total carbohydrates (TC) in the mix of 4 sugarcorn hybrids varied from 86 to 145 g L^{-1} comparing sugarcorn juice from 78 days to 91 days. Previous studies with sugarcorn hybrids grown in 2008 in Ottawa (ON, Canada) achieved concentrations as high as 125 to 180 g L^{-1} [Reid et al., 2015]. Our sugarcorn juices samples had a concentration of reducing sugars of 66 g L^{-1} and 28.5 g L^{-1} at 78 days and 91 days, respectively.

Sucrose, glucose, and fructose were the prominent sugars. The tetra saccharide stachyose and the trisaccharide maltotriose were among the other sugars identified. Organic derivatives such as succinic acid, methylmalonic acid, lactic acid, and glycolaldehyde were also present in small amounts.

The sugarcorn sugar's variability it is not within the scope of this research. The variations in sugars can be explained to the difference in the extraction process, hybrid types, plant maturity, soil and temperature conditions within seasons [Van Reen and Singleton, 1952;Reid et al., 2016].

Agronomic science has developed different systems to calculate corn development. One of them is the crop heat unit (CHU), which is an energy term determined for each day and accumulated from planting to the harvest date. The plant growth is dependent on the total amount of heat to which the crop is subjected during its lifetime [Brown and Bootsma, 1993]. One crucial trait about sugarcorn juice is that it was developed to prosper in southwestern Ontario and parts of Quebec. Nevertheless, the importance of the temperature variations over 2014 and 2015 and the heat the sugarcorn received in different seasons it is illustrated in Figure 5.2, and it is correlated with the sugar concentration of the corn hybrids. The crop heat unit quantification was made by Dr. Rob Gilroyed research team, and it was a personal communication. As shown in Figure 5.3, sugarcorn hybrids grown in 2014 had a CHU of 1,999, whereas the 2015 hybrids had 2,401 and consequently total carbohydrates and reducing sugars decreased. Temperature records for 2015 displayed an arid season, compared with 2014 and the sugarcorn hybrids showed drought stress indicators since early morning (Rob Gilroyed 2017 - Personal Communication).

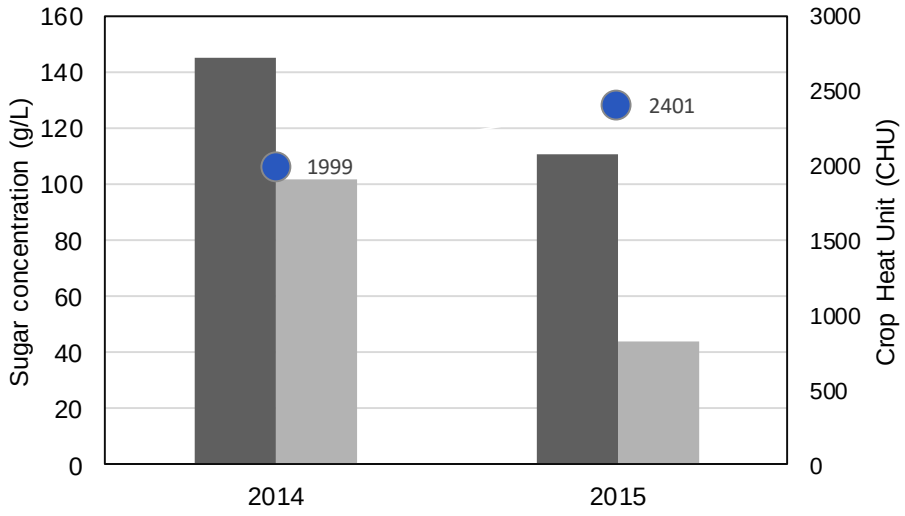

Figure 5.2 Comparison between sugarcorn juice samples, harvested in 2014 and 2015 and its crop heat unit

The amount of stalk sugars will accumulate during growth development, and over time, the sugar concentration will decline due to translocation of metabolites from stalk to grain. During 2015, we had specific samples of each sugarcorn hybrids at two different harvest times, 98 days (August 10, 2015) and 112 days (September 1st, 2015).

Figure 5.3 shows that the highest translocation happened for hybrid AAFC-SC1 from 12.4 to 10.8 Bx. AAFC-SC2 also presented a decrease in stalk sugars andf or AAFC-SC3 the sugars were practically the same concentration, 11.7 Bx. Lastly, for AAFC-SC4 it appears that the sugars were more concentrated at 112 days, 11.4-12 Bx. This phenomenon could have happened due to the drought stress that the sugarcorn plant experienced in 2015.

Amongst the four hybrids grown at Ridgetown, ON, an average of stalk sugar concentration was found to be 11.7±0.5 Bx. This example shows that the planting of sugarcorn was made May 13, harvested on September 1st, 2015 (2,401 CHU), in almost four months. This hybrid provided an 11.7 Bx or about 118 g L^{-1} of fermentable sugars for further biobutanol fermentations. Comparing both seasons, 2014 and 2015, sugarcorn juice hybrids could yield up to 145 g L^{-1} of readily fermentable sugars in less than four months.

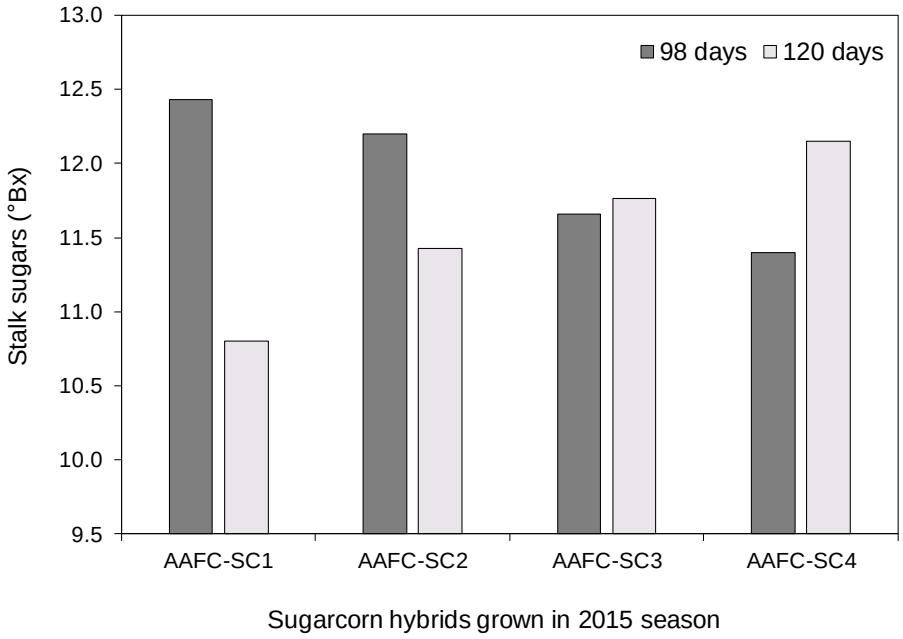

Figure 5.3 Variation of stalk sugars in Brix across the sugarcorn hybrids AAFC-SC1, AAFC-SC2, AAFC-SC3 and AAFC-SC4 in 2015 harvested at 98 and 112 days

5.3.2 Sugarcorn juice pretreatment

Juices with high sugar content such as sweet sorghum juice and sugarcane juice are susceptible to spoilage. Hence the juices are treated to limit microbial contamination before processing (Kumar et al. 2015; Quintero et al. 2008). These treatments also serve to clarify the juice by reducing turbidity. Sugarcorn juice samples were plated in nutrient agar to evaluate microbial contamition inherent to the juice; the results are presented in table 5.3.

Table 5.3. Colony forming units present in sugarcorn juices

Code	Medium	Number of colonies	Dilution factor	CFU/mL
SCJ 2015 mix from 4 sugarcorn hybrids	Nutrient broth	267	1.00E-03	2.67E+06
AAFC SCJ 3 2015	Nutrient broth	238	1.00E-03	2.38E+06
AAFC SCJ 4 2015	Nutrient broth	128	1.00E-03	1.28E+06

Biological juices, like sweet sorghum stalk juice, have a short shelf life (4-5 h) post crushing due to their high fermentable sugar content and the rapid sugar degradation during storage is due to the metabolic activities of contaminating spoilage bacteria [Ganesh Kumar et al., 2015]. Sugarcorn juices were autoclaved subsequently and results are shown in Figure 5.4.

Sterilization of sugarcorn juice via autoclaving was performed and resulted in a reduction of total carbohydrates by 20% and 15% for SCJ A and SCJ B. Also, reducing sugars in the juice increased by 24% for SCJ B and 3% for SCJ A. The results are shown in Figures 5.4-A and 5.4-B.

Autoclaving causes hydrolysis of glycosidic bonds in sucrose, forming equimolar amounts of the constituent monosaccharides, fructose and glucose [Chauhan, 2008]. Oligosaccharides and polysaccharides in the medium are also hydrolyzed, which explains the observed increase in reducing sugars. Over autoclaving process, some monosaccharides present in the medium could degrade [Wang and Hsiao, 1995], to furfural or hydroxymethyl furfural. Individual sucrose, glucose and fructose concentrations measured by HPLC are shown in Figures 5.4-D and 5.4-E. The graphs show an increase in glucose and fructose concentration in the juice, and a decreased sucrose concentration. agree with the above discussion, showing an increase in amounts of glucose and fructose and

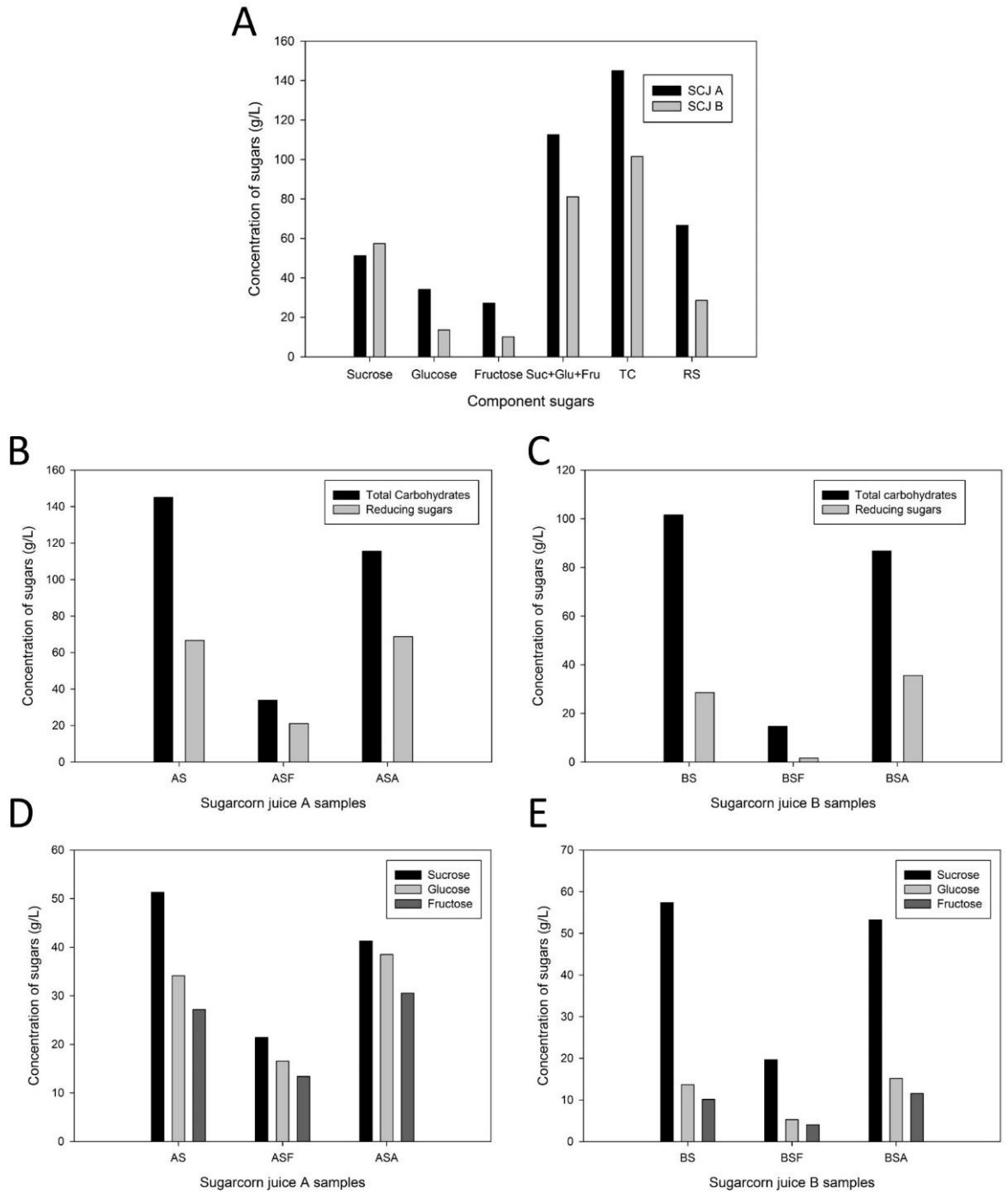

Figure 5.4. (A) The concentration of sugars in sugarcorn juice from 2014 harvest, SCJ A= 78 days and SCJ B=91 days. (B) and (C) Effect of filtration (ASF and BSF) and autoclaving (ASA and BSA) on concentration of reducing sugars and carbohydrates in SCJ A (AS)and SCJ B (BS), respectively. (D) and (E) Effect of filtration and autoclaving on the concentration of glucose, fructose and sucrose in SCJ A and B. Analyses were carried out in triplicates.

Filtration through granular activated carbon (GAC) has been used for clarification and purifying natural sugary juices prior to syrup formation or alcoholic fermentations [Urbanic, 1985].

Visible de-colorization due to the removal of pigments occurred to the sugarcorn juice when filtrated through a GAC bed and the effects in sugars concentration are presented in figure 5.5D and 5.5E and noticeable in figure 5.7. Most of the sugars were adsorbed by the filtration bed, resulting in a 77% and 83% reduction in carbohydrates for SCJ A and SCJ B respectively.

Sucrose, glucose and fructose concentrations decreased after GAC filtration (Figures 5.4D and E). Activated carbon filtration was found to be disadvantageous as a pretreatment method for sugarcorn juice, and no further experiments followed.

Figure 5.5 Comparison of sugarcorn juice A as is (left), and sugarcorn juice A filtered through GAC (right)

5.4 Conclusion

Relevant physical and chemical characterization of sugarcorn juice was done, as a potential substrate for biobutanol fermentations. High sucrose concentration was expected in the juice, but essential amounts of glucose and fructose were also accounted in the juice. Variation in sugar composition of sugarcorn juices across different hybrids and growth seasons were observed during this study. Studies on the effect of autoclaving may help to account for differences in sugar compositions between fresh juice and juice sterilized via autoclaving. For biobutanol production, concentrations of different sugars as a mix are a crucial factor for Clostridial fermentations, because Clostridia sugar uptake metabolism could affect butanol productivity. Sucrose, glucose and fructose accounted for 80% of the total carbohydrates in the sugarcorn juice.

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Chapter 6

6 Biobutanol fermentation using sugarcorn juice

6.1 Introduction

The juice extracted from sugarcorn can have sugar concentrations as high as 145 g L^{-1}, with potential to be a useful commodity in the biorefining sector. Figure 6.1 illustrates the process steps for production of bio-based products from corn. In this part of the study, biochemical production of biobutanol by *Clostridium spp.* was performed utilizing the sugarcorn juice as a carbon source for the solventogenic bacteria. This work is the first to use sugarcorn juice to produce biobutanol. In this chapter, 3 different Clostridial strains were investigated.

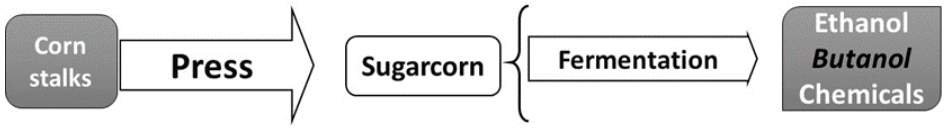

Figure 6.1. Flow diagram of sugar extraction from corn stalks juice for biobutanol production

6.2 Fermentation in Bioreactor utilizing *Clostridium beijerinckii* 55025 asporogenic strain

6.2.1 Materials and methods

6.2.1.1 Feedstocks

Sugarcorn hybrids were grown and harvested in Ridgetown, Ontario at the Centre for Agricultural Renewable Energy and Sustainability of the University of Guelph (42°26'N, 81°53'W) in the years 2014 and 2015. The sugarcorn plants were harvested 5 to 10 days after silking, and the stalk of the plant was cut about 12-13 cm above the soil level, the ears were manually removed and the sugarcorn stalks -including leaves- were feed through a three-roller

press to extract the sugarcorn juice. Once the juice was collected, it was stored at -20 °C. Dr. Robert Nicol and Dr. Brandon Gilroyed were responsible for the after-mentioned process.

The juice, after few days of frozen storage, was transported to the University of Western Ontario in London (Ontario, Canada). For the characterization and fermentation studies, the sugarcorn juice was thawed at room temperature and filtered through cheesecloth to remove plant residues. Once filtered, the juices were transferred to 1-liter clean plastic containers, sealed, weighed and stored at -20 °C until further use.

6.2.1.2 Microbial strain and media

The strain used in this experiment was the asporogenic strain *Clostridium beijerinckii* ATCC 55025, purchased from America Type Culture Collection. All chemicals for preparation of media were obtained from Sigma-Aldrich Co. The Clostridial Nutrient Medium (CNM, Fluka Analytical) was used as growing medium. This medium contained per liter of solution in distilled water: Meat extract, 10 g; peptone, 5 g; yeast extract, 3 g; D (+) glucose, 5 g; starch, 1 g; sodium chloride, 5 g; sodium acetate, 3 g; L-cysteine hydrochloride, 0.5 g; agar, 0.5 g and, resazurin solution (1 g L^{-1}), 0.25 mL; final pH of 6.8.

P2 culture medium [Qureshi and Blaschek, 1999] was composed of the following separately prepared solutions (in grams per 100 ml of distilled water, unless otherwise indicated). Sugar solution: glucose, 62 g; yeast extract, 1.031 g in 970 ml of distilled water. Buffer solution: KH_2PO_4, 5 g; K_2HPO_4, 5 g; ammonium acetate, 22 g. Vitamins solution: p-aminobenzoic acid, 0.01 g; thiamine, 0.01 g; biotin, 0.0001 g. Minerals solution: $MgSO_4$-$7H_2O$, 2 g; $MnSO_4$-$7H_2O$, 0.1 g; $FeSO_4$-$7H_2O$, 0.1 g; NaCl, 0.1 g. The sugar solution was sterilized at 121 °C for 15 min. After cooling to room temperature, ten milliliters each of filter-sterilized P2 medium nutrient solutions (buffer, and minerals solutions) were added to 970 ml of sugar solution. The pH was adjusted to 6.8. Following this, 0.5 g L^{-1} L-cysteine hydrochloride (Sigma) and 0.25 ml L-1 of resazurin solution (1 g L^{-1}) were added to the final media to reduce the culture medium for optimal growth.

Sugar corn juice medium, (SCJ-P2 medium) was prepared with sugarcorn juice A (Code: Jan 2015 Batch 1/11, 2/11 and 3/11). The juice was diluted at a ratio of 1:1 with distilled water and

it became the sugar solution in SCJ-P2 culture medium and proceeded with the addition of the P2 stock solutions.

6.2.1.3 Strain revival

About 200 mL of CNM was prepared out of which 50 ml was transferred to a 100-ml serum bottle, and the remaining 150 mL to a 250-mL glass bottle to be used for the subculture medium. The serum bottle was closed with a butyl rubber stopper and crimped with an aluminum seal, while the glass bottle was tightly capped with a rubber stopper. Both bottles were degassed by applying vacuum, then highly purity nitrogen gas was sparged into the bottles, and finally they were autoclaved at 121 °C for 15 min.

Two milliliters of frozen culture (kept at -80 °C) was aseptically transferred to the serum bottle with 50 mL CNM medium. Inoculation was carried out under a cannula system with a gentle stream of nitrogen gas flowing through the tubes and bottles. The culture was incubated (Thermo Scientific MaxQ4000 Incubator) at 37 °C for 24 h, with a shaker speed of 90 rpm.

6.2.1.4 Subculture

From the strain revival culture, the fresh cells were transferred 10%(v/v) to 150 mL Subculture medium (CNM) in the glass bottle and incubated for 14-16 h at 37 °C and 90 RPM.

6.2.1.5 Inoculum

Duplicate bottles, each containing 225 ml of P2 medium were tightly capped with rubber butyl caps, degassed by applying vacuum and sparged with high purity nitrogen gas to provide an anaerobic atmosphere. The medium in the bottles was aseptically inoculated with 25 mL of fresh bacterial subculture and incubated for 14-16 h at 37 °C and 100 RPM.

6.2.1.6 Fermentation conditions

Fermentation experiments were carried out in two 3L bioreactors (New Brunswick BioFlo 110) equipped with online dissolved oxygen and pH monitoring. About 1,350 ml each of P2 Culture medium (glucose concentration 60 g L^{-1}, buffer solution 10% v/v) and diluted sugarcorn juice medium (sugar concentration 75-76 g L^{-1}, buffer solution 10% v/v) were prepared and

transferred to the two fermenters. The contents of the two bioreactors were autoclaved and cooled to room temperature. The other nutrient solutions were sterilized (0.2 µm filter) by filtration and aseptically added to the sterile media. L-cysteine hydrochloride, 0.5 g L^{-1} and, 0.5 mL resazurin solution (1 g L^{-1}), were added to the final media to reduce the redox potential in both cases. Nitrogen was sparged into the bioreactor vessel for about 30 min and the media were inoculated with 10% v/v inoculum and incubated at 37 °C, 150 rpm and an initial pH of 6.8.

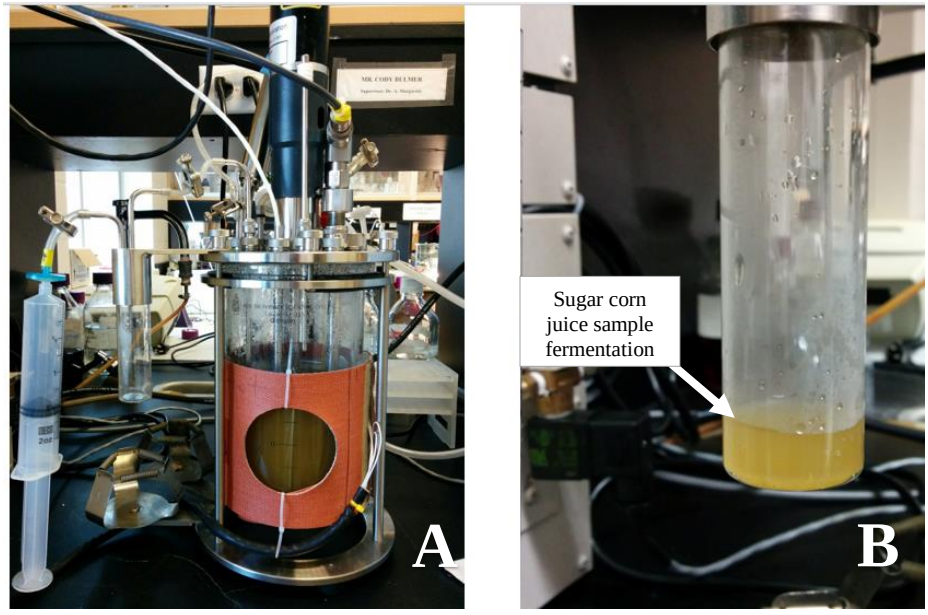

Figure 6.2. A. Photograph of 3 L bioreactor with growing *Clostridium beijerinckii* 55025, B. Close-up of a withdrawn sample of sugar corn juice fermentation

6.2.1.7 Analytical Methods

Cell growth was tracked by measuring optical density at 600 nm in a spectrophotometer and dry weight measurement by filtration using 0.45 µm membrane. Reducing sugars and total carbohydrates were measured by DNS and phenol-sulfuric method, respectively. HPLC was used to measure the concentration of sucrose, glucose, and fructose. The products were measured using a gas chromatograph Hewlett Packard 6890 Series with a flame ionization detector (FID). Fully described method are found in Appendix A.

6.2.2 Results and discussion

In a typical batch culture, a characteristic feature of the solvent producing *Clostridium* species is biphasic fermentation; the metabolism is composed of two phases: an acid phase and a solvent one, as shown in Figure 6.3.

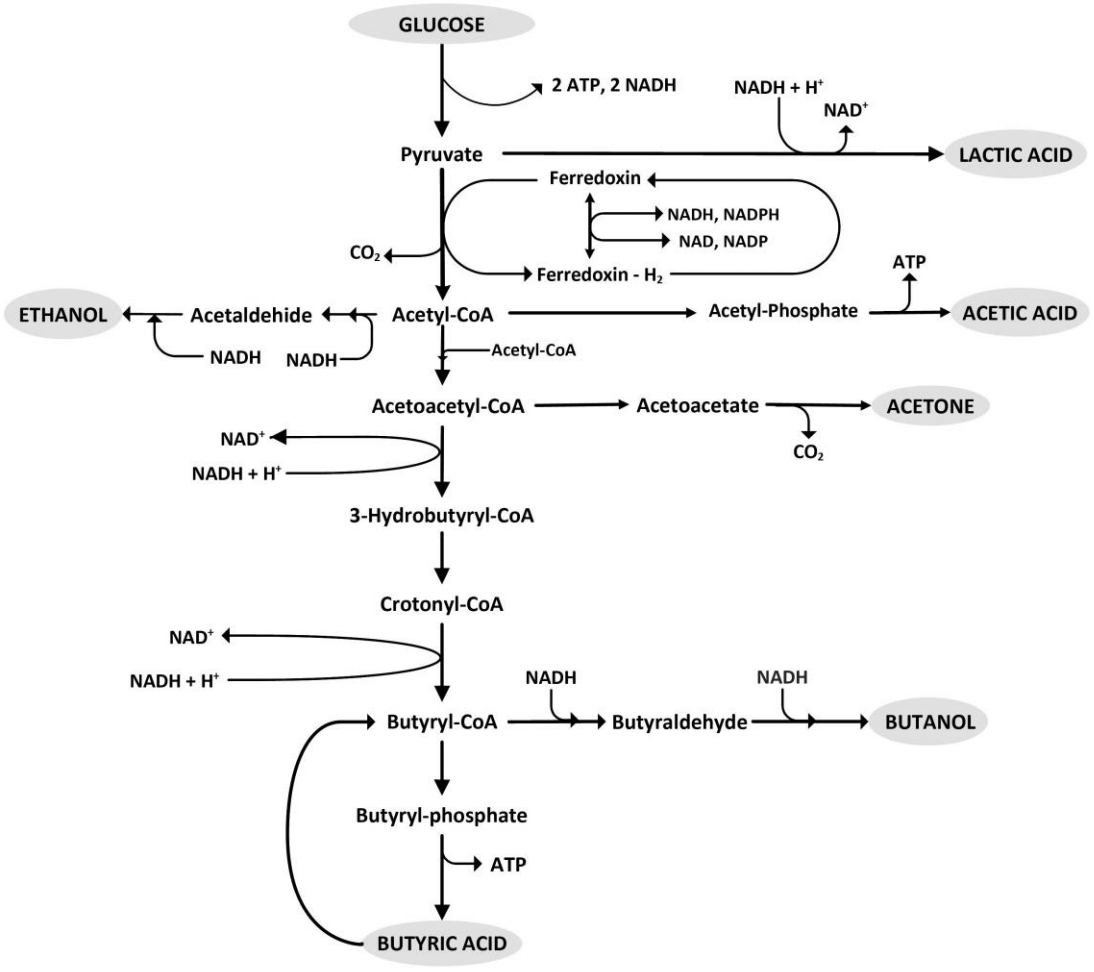

Figure 6.3. Simplified biochemical pathways in *Clostridium acetobutylicum*
Adapted from [Lopez-Contreras et al., 2012]

The first phase is the acidogenic phase, during which the acids forming pathways are activated, and acetate, butyrate, hydrogen, and carbon dioxide are produced as primary products, which results in a decrease in the pH of the culture medium. This acidogenic phase usually occurs during the exponential growth phase [Andersch et al., 1983; Hartmanis et al., 1984]. As the culture enters the stationary growth phase, the cellular metabolism undergoes a shift to solvent

production (solventogenic phase). During the solventogenic phase, acids are re-assimilated concomitantly with the regular consumption of sugars for the production of acetone, butanol, and ethanol (or isopropanol instead of acetone in some *C. beijerinckii* strains), which usually increases the pH of the culture medium. The relationship between the breakpoint in the pH of the fermentation and the onset of solvent production, which occurs at the beginning of the second phase of the fermentation, was identified early on in the development of the industrial fermentation processes [Soni et al., 1982; Dabrock et al., 1992; Grupe and Gottschalk, 1992].

Clostridium beijerinckii 55025, is an asporogenic mutated strain which produces acetone, butanol, and ethanol. The sporulation gene Spo0A is closely related to the environmental stress that a Clostridial cell can suffer, either from starvation or solvent toxicity. This gene plays a principal role in controlling several aspects of the transition from exponential growth to stationary phase in *C. beijerinckii.* These include initiation of sporulation, accumulation of the storage polysaccharide, granulose, and production of acetone and butanol. [Wilkinson et al., 1995].

a) Fermentation using glucose as substrate

As shown in Figure 6.4, the starting pH was 5.7 which decreased to 4.25 in 32 hours of fermentation. After that, unregulated pH was maintained at 4.35 until the end of the experiment. The dissolved oxygen within the bioreactor was planned to be kept at zero levels, by sparging pure nitrogen to the vessel before the inoculation. After inoculation, nitrogen was sparged sporadically to clean the pipes after sampling. Although, at 41 h, the dissolved oxygen readings increased until reaching a value of 2.

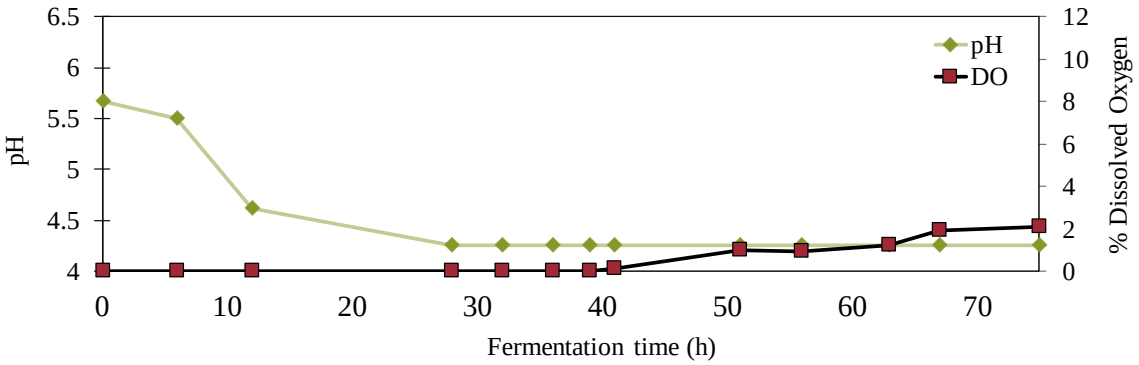

Figure 6.4 Dissolved oxygen and pH profile using glucose-P2 medium, by *Clostridium beijerinckii* 55025.

The pH values of a Clostridia fermentation depends on the process objective, as the optimum pH for acidogenesis and solventogenesis differs [Grupe and Gottschalk, 1992]. The results shown in Figure 6.5, exhibit the starting of butanol and acetone production at 39 hours, acetic acid starts at time cero mainly can comes from the inoculum. The starting pH of 5.67 was not expected after inoculation. The suggested pH initial value should have been around 6.8.

The graphic shows how the organic acids, acetic and butyric acid, start being produced since initial stages of fermentation (pH 5.67), until approximately pH of 4.25, and eventually their concentration diminished and butanol and acetone are being produced and the solventogenesis stage starts. Acetone, butanol, and ethanol reached their maximum concentration at 56 hours of fermentation, where 0.97, 0.36 and 2.6 g L^{-1} respectively were produced.

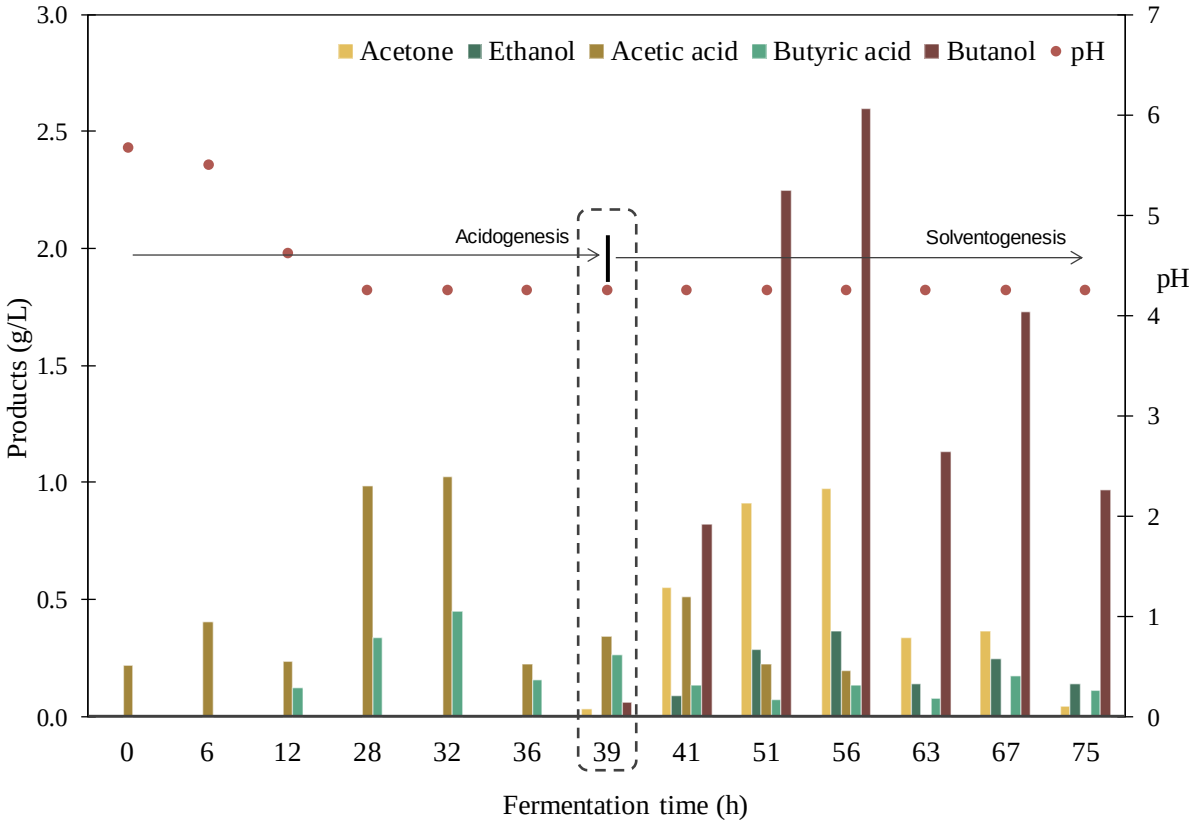

Figure 6.5. Acetone, butanol, ethanol, acetate and butyrate production by *Clostridium beijerinckii* 55025, in Glucose-P2 culture medium

A pH of about 6.0 is beneficial for the cell growth and butyric acid biosynthesis, especially in *Clostridium butyricum* [He et al., 2005]. For *Clostridium tyrobutyricum*, variation in pH can change the distribution of the metabolic flux. At pH 6.3, the highest butyrate concentration is produced, compared to that at pH 6.0 and 6.7 [Zhu and Yang, 2004; Jo et al., 2008]. In our

results, it took around 12 hours for the cell to start producing butyric acid, precisely when the pH dropped from over 6 to 4.82.

Figure 6.6 shows the bacterial growth of *Clostridium beijerinckii* 55025. Dry cell weight (g L^{-1}) and absorbance at 600 nm were measured, and both follows the same growth trend. The maximum dry cell weight was 1.6 g L^{-1} at 39 hours of fermentation.

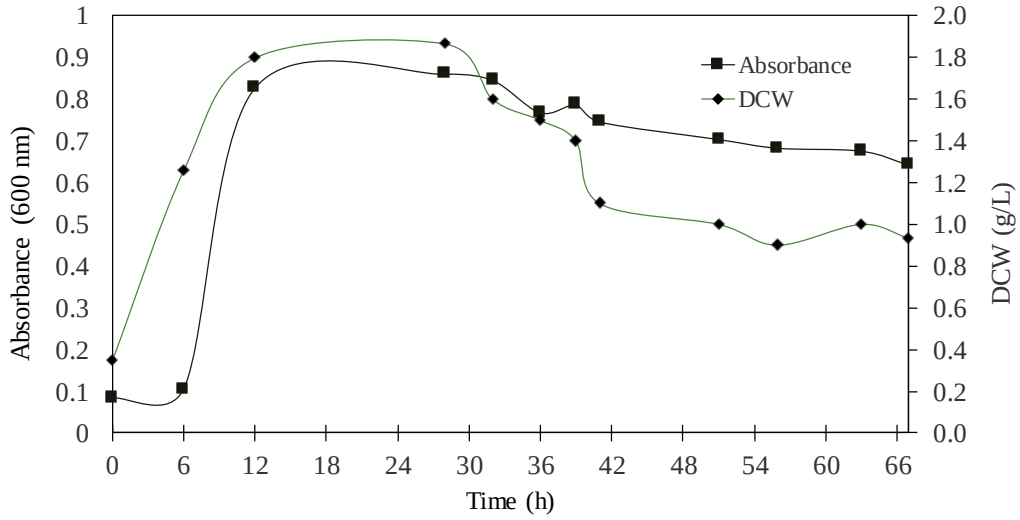

Figure 6.6. *Clostridium beijerinckii* 55025 growth profile during ABE fermentation, using Glucose-P2 medium

The phosphorylated form of the Spo0A protein has been shown to regulate sporulation in *C. acetobutylicum, C. perfringens and C. beijerinckii*, and apparently does so in all Clostridia. Inactivation of spo0A in *C. beijerinckii* and *C. acetobutylicum* blocks solvent, spore and granulose formation, and prevents sporulation and enterotoxin production in *C. perfringens*.[Paredes et al., 2005]. Nevertheless, the asporogenic Clostridia 55025, with parent strain *Clostridium acetobutylicum* ATCC 4259, has good growth, the onset of solventogenic phase, the bacteria only consume the already produced acids, yet further consumption of glucose is only about 3 g L^{-1}.

In Table 6.1 the fermentation parameters for each product are shown. Also given are the yield of product per cell biomass produced $Y_{(P/X)}$ and per substrate consumed $Y_{(P/S)}$. Butanol titer was 1.73 g L^{-1} and its productivity 0.026 g L^{-1}- h, at 67 hours of fermentation time.

On the filed patent document, *Clostridium beijerinckii* 55025 reached a butanol concentration of 13 g L^{-1} and 0.33 g L^{-1}-h, at 35 h. It's worth noting that the fermentation was done without controlling the pH, whereas in the patent, the pH was maintained between 5.0 and 5.2 through all the fermentation time and could be a parameter to consider in further experiments.

Table 6.1 Fermentation Parameters from *Clostridium beijerinckii* 55025

Fermentation Parameters			
At 67 h	**Acetone**	**Ethanol**	**Butanol**
Titer (g/L)	0.37	0.25	1.73
$Y_{(P/X)}$ (g g^{-1})	0.17	0.12	0.79
$Y_{(P/S)}$ (g g^{-1})	0.019	0.029	0.135
Productivity (g L^{-1}·h)	0.005	0.004	0.026

b) Fermentation using sugarcorn juice as substrate

The second bioreactor had sugarcorn medium (SCJ-P2) and was inoculated with the strain *C. beijerinckii* 55025 as well. Figure 6.8, presents the % dissolved oxygen and pH profile during 62 h fermentation. %DO over the fermentation varied and the system monitored at about 10% DO at 20 h and then the reading decreased. This behavior could be due to air coming into the bioreactor or, a fail in the DO probe. It could also be because the minerals dissolved in the sugarcorn juice plant interaction with the DO probe.

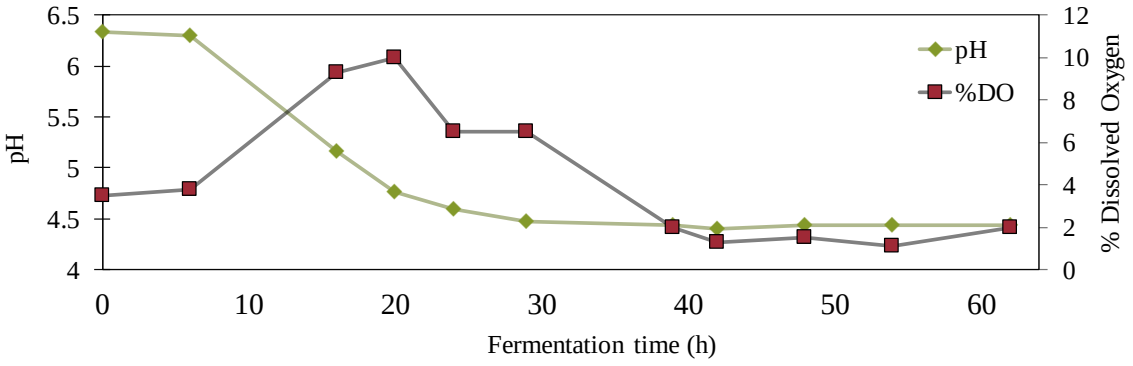

Figure 6.7. Dissolved oxygen and pH profile during ABE fermentation in a bioreactor, using Sugar corn juice medium as a substrate, by *Clostridium beijerinckii* 55025

Figure 6.8 shows the characteristic biphasic fermentation, the pH dropped from 6.33 to 4.44. Butanol was initially detected at 24 h and pH of 4.59, earlier when comparing with the results in glucose-P2 culture medium. Butanol was produced in minimal quantities (0.11 g L^{-1}) in the SCJ-medium. The metabolism of the strain *Clostridium beijerinckii* 55025 grown in sugar corn was affected. Acetone is produced only in minuscule amounts and ethanol was not detectable.

On the patent information, *Clostridium beijerinckii* 55025 reached a butanol concentration of 13 g L^{-1} and 0.33 g L^{-1}-h, at 35 h. It's worth noting that the fermentation was done without controlling the pH, whereas in the patent, the pH was maintained between 5.0 and 5.2.

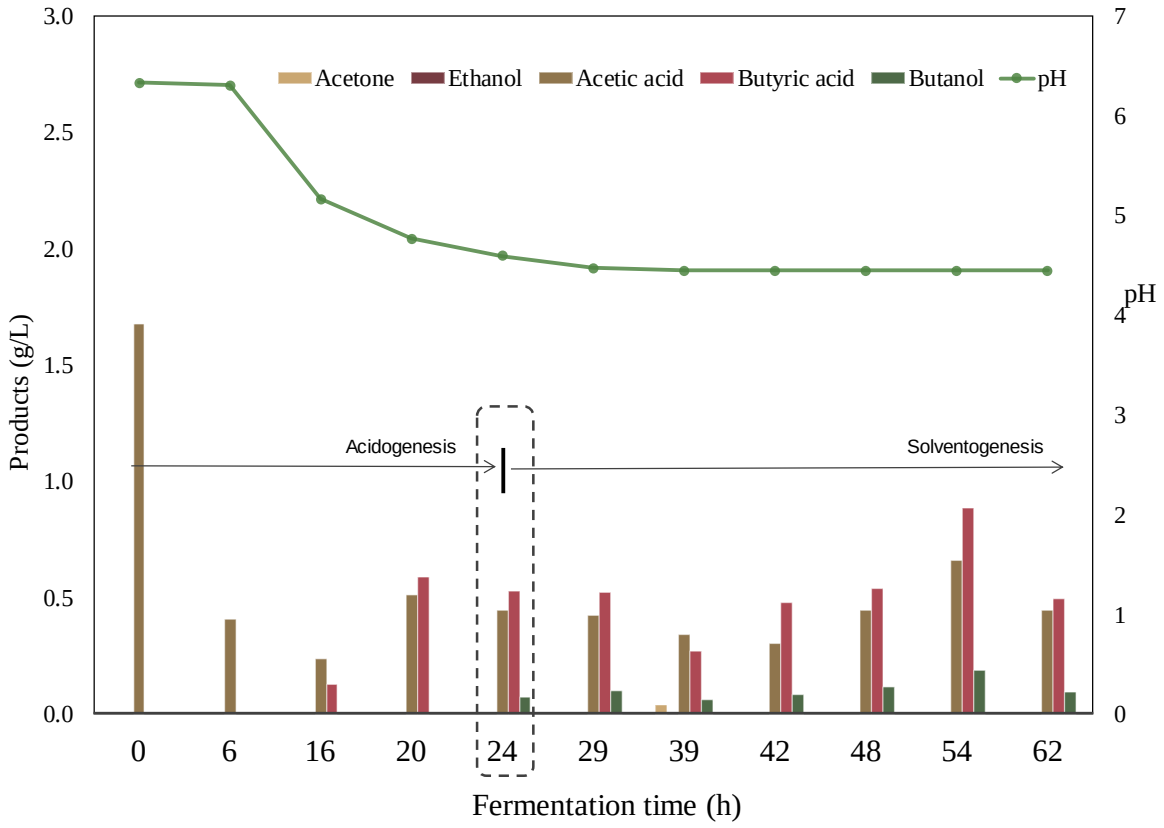

Figure 6.8 Acetone, butanol, ethanol, acetate and butyrate production by *Clostridium beijerinckii* 55025, on SCJ – P2 medium

The SCJ-P2 consumption of substrates was monitored measuring reducing sugars, total carbohydrates, sucrose, glucose and fructose, all of them in concentration units of grams per liter. This culture medium had to be diluted to be in the range of 50-60 g $L^{-1}$of initial reducing sugars concentration. Nevertheless, the number of total carbohydrates was higher than in

glucose-P2 medium, as high as 82-84 g L^{-1}. This situation could have been contributed to the lower butanol titer and productivity, shown in Table 6.2. One theory is that the strain *Clostridium beijerinckii* 55025, was unable to produce the enzymatic complex to degrade the polysaccharides within the sugarcorn juice quickly.

For the case of sugarcorn juice medium, there are three primary saccharides sucrose, fructose, and glucose. Sucrose is the major carbon source present in sugar cane and sugar beet and has traditionally been used as a substrate for industrial-scale ABE fermentation using solventogenic clostridia. Studies on sucrose utilization by *C. beijerinckii,* as well as *C. acetobutylicum* ATCC 824, have revealed that sucrose uptake in these organisms takes place by sucrose phosphoenolpyruvate dependent phosphotransferase system (PTS) (Figure 6.10).

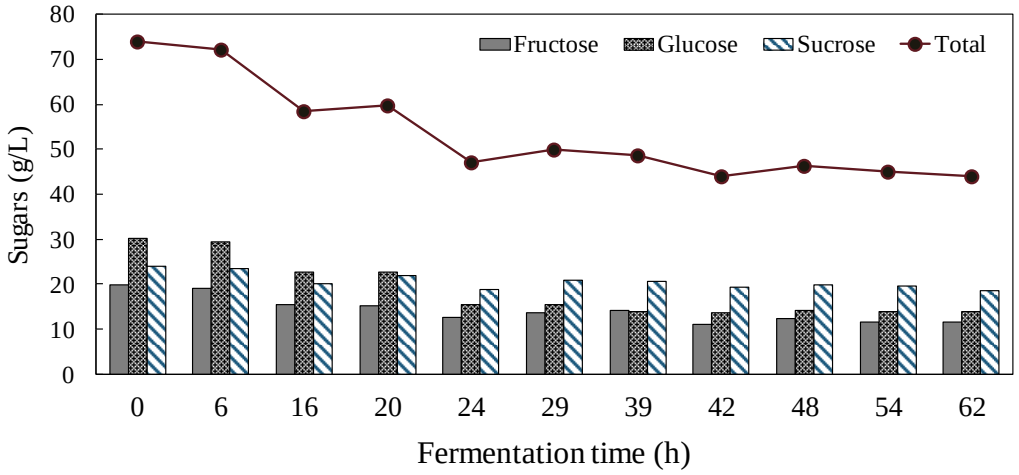

Figure 6.9. Fructose, glucose and sucrose consumption by *Clostridium beijerinckii* 55025 during ABE fermentation, using Sugar corn juice medium

The entire sucrose enzyme II complex was present within the membrane of this organism. Additionally, sucrose-6-phosphate hydrolase and fructokinase activities were detected in sucrose-grown cultures of *C. beijerinckii*. The genes encoding the proteins of the sucrose utilization pathway were identified from the *C. acetobutylicum* genome sequence: the scrAKB genes encoding EII of the sucrose PTS, fructokinase, and sucrose 6-phosphate hydrolase. Although the sucrose metabolism is conserved between *C. acetobutylicum* and *C. beijerinckii*, the operons show considerable differences in organization and regulatory elements [Tangney et al., 1998]. There are no studies on sucrose consumption by *Clostridium beijerinckii* 55025 in literature. Unfortunately, sucrose conversion percentage was only 22%, in 62 hours a

remaining concentration of 18 g L^{-1} was detected in the culture medium. Similarly, none of the monosaccharides, fructose, and glucose, were completely depleted; with 41.81% and 54.01% of conversion, respectively.

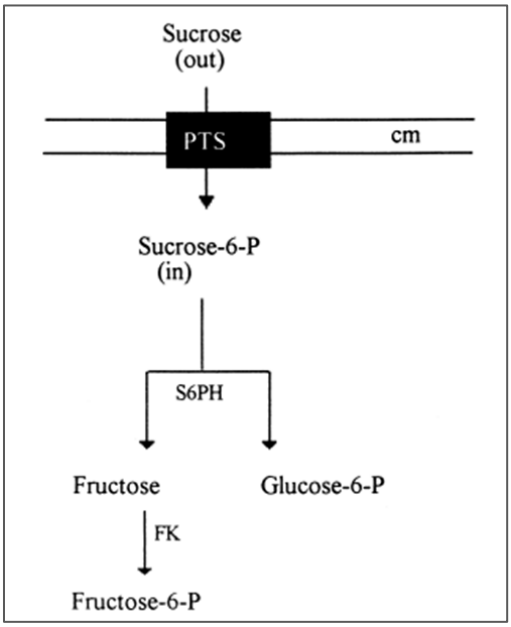

Figure 6.10. Schematic representation of the pathway for sucrose transport and metabolism in *Clostridium beijerinckii* NCIMB 8052. Abbreviation: cm = cell membrane; PTS=phosphotransferase system; S6PH = sucrose-6-phosphate hydrolase; FK= fructokinase [Tangney et al., 1998]

Sucrose is a disaccharide, formed from glucose and fructose. An important study can be applied to the understanding of the regulatory mechanism of sugars of *Clostridium beijerinckii*, a solventogenic Gram-positive bacterium. The phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) was described an enzymatic cluster that leads the translocation and phosphorylation of sucrose and prompts its assimilation into the cell, through the cytoplasmic membrane. The PTS, an enzymatic cluster is being composed of two general cytosolic proteins, called Enzyme I and HPr, and Enzyme II. Reported observations indicated that glucose might regulate sucrose utilization. Glucose also regulates cellobiose and lactose metabolism [Mitchell, 1998]. The sucrose transport via the PTS, starts with sucrose phosphorylation, yielding sucrose-6-phosphate by the presence of the sucrose-6-phosphate hydrolase and further fructokinase enzymes. Phosphoenolpyruvate (PEP) regulates the PTS

system, whereas fructokinase enzyme is ATP dependent and phosphorylates the fructose conversion into fructose-6-phosphate [Tangney et al., 1998].

Table 6.2 Results of the bioreactor fermentation of sugarcorn juice medium by *Clostridium beijerinckii 55025*

Products	***Fermentation parameters***	**Fructose *62 h***	***Glucose* *62 h***	***Sucrose* *62 h***
Acetone	Titer (g/L)	0	0	0
	Y (P/X) (g/g)	0	0	0
	Y (P/S) (g/g)	0	0	0
	Productivity (g/L ·h)	0	0	0
Ethanol	Titer (g/L)	0	0	0
	Y (P/X) (g/g)	0	0	0
	Y (P/S) (g/g)	0	0	0
	Productivity (g/L ·h)	0	0	0
Butanol	Titer (g/L)	0.09	0.09	0.09
	Y (P/X) (g/g)	0.02	0.02	0.02
	Y (P/S) (g/g)	0.011	0.005	0.017
	Productivity (g/L ·h)	0.001	0.001	0.001
Acetic Acid	Titer (g/L)	0.44	0.44	0.44
	Y (P/X) (g/g)	0.06	0.06	0.06
	Y (P/S) (g/g)	0.028	0.014	0.043
	Productivity (g/L ·h)	0.007	0.007	0.007
Butyric Acid	Titer (g/L)	0.49	0.49	0.49
	Y (P/X) (g/g)	0.13	0.13	0.13
	Y (P/S) (g/g)	0.059	0.030	0.093
	Productivity (g/L ·h)	0.008	0.008	0.008

6.2.3 Conclusions

Results show that *Clostridium beijerinckii* 55025 is not a good strain for sucrose uptake, maybe because of the mutation suffered from its own DNA. The mutation on this strain was aimed to increase butanol tolerance, which is closely regulated by the sporulation control system. It is suggested that a new trial with different Clostridia strain should be pursued. Hence, for further fermentation sugarcorn juice will be diluted, as there could be another variable that is altering cell growth and butanol production.

6.3 Sugarcorn juice fermentation in 250 mL bottles by *C. beijerinckii* 6422 and *C. saccharobutylicum*

6.3.1 Materials and Methods

6.3.1.1 Feedstocks

Sugarcorn hybrids were grown and harvested in Ridgetown, Ontario at the Centre for Agricultural Renewable Energy and Sustainability from University of Guelph (42°26'N, 81°53'W) in the years 2014 and 2015. The sugarcorn plants were harvested 5 to 10 days after silking, and the stalk of the plant was cut about 12-13 cm above the soil level, the ears were manually removed and the sugarcorn stalks -including leaves- were feed through a three-roller press to extract the sugarcorn juice. Once the juice was collected, it was stored at -20 °C. Dr. Robert Nicol and Dr. Brandon Gilroyed were responsible for the after-mentioned process.

Days later was transported to the University of Western Ontario in London (Ontario, Canada). For the characterization and fermentation studies, the sugarcorn juice was thawed at room temperature and filtered through cheesecloth to remove plant residues. Once filtered, the juices were transferred to 1-liter pre-washed plastic containers, sealed, weighed and stored at -20 °C until further use.

6.3.1.2 Microbial strain and Media

The strain used in this experiment was the sporogenic strain *Clostridium beijerinckii* ATCC 6422, and *Clostridium saccharobutylicum* ATCC BAA-117. The strains were purchased from America Type Culture Collection. All chemicals for media and substrates were obtained from Sigma-Aldrich Co. The Clostridial Nutrient Medium (CNM, Fluka Analytical) was used as growing medium. This medium contained (per liter of distilled water): Meat extract, 10 g; peptone, 5 g; yeast extract, 3 g; D (+) glucose, 5 g; starch, 1 g; sodium chloride, 5 g; sodium acetate, 3 g; L-cysteine hydrochloride, 0.5 g; agar, 0.5 g and, resazurin solution (1 g L^{-1}), 0.25 mL; final pH of 6.8.

P2 culture medium [Qureshi and Blaschek, 1999] was composed of the following separately prepared solutions (in grams per 100 ml of distilled water, unless otherwise indicated). Sugar solution: glucose, 62 g; yeast extract, 1.031 g in 970 ml of distilled water. Buffer solution: KH_2PO_4, 5 g; K_2HPO_4, 5 g; ammonium acetate, 22 g. Vitamins solution: p-aminobenzoic acid,

0.01 g; thiamine, 0.01 g; biotin, 0.0001 g. Minerals solution: $MgSO_4$-$7H_2O$, 2 g; $MnSO_4$-$7H_2O$, 0.1 g; $FeSO_4$-$7H_2O$, 0.1 g; NaCl, 0.1 g. The sugar solution was sterilized at 121 °C for 15 min. On cooling to room temperature, ten milliliters of each filter-sterilized P2 medium nutrient solutions (buffer, and minerals' solutions) were added to 970 ml of sugar solution. The pH was set up at 6.8. Then, 0.5 g L^{-1} L-cysteine hydrochloride (Sigma) and 0.25 ml L^{-1} of resazurin solution (1 g L-1) were subsequently added to the final media to reduce the culture medium for optimum growth.

Sugar corn juice Medium, (SCJ-P2) was composed by sugarcorn juice A (Code: Jan 2015 Batch 7/11 & Jan 2015 Batch 9/11) and sugarcorn juice B (Code: July 2015 Batch 2-3). For aiming to get a sugar corn juice medium of approximating 60 g L^{-1} sugar concentration was done:

$$C1V1 + C2V2 = C3V3$$

$$C3 = \frac{C1V1 + C2V2}{V3} = \frac{(66.68\ g\ L^{-1})(1.25\ L) + (28.58\ g\ L^{-1})(0.25\ L)}{(1.5\ L)} = 60.33\ g\ L^{-1}$$

A 1L medium with SCJ A and SCJ B in 5:1 proportion was prepared, and diluted with 0.3 L water to prepare 1.3 L of. diluted sugar corn juice. This was further diluted 1:1 with water to prepare 500 mL medium. This sugar corn juice was taken as the sugar solution in P2 culture medium. The final culture medium (SCJ-P2 medium) had a density of 1.017±0.0075 g/mL.

6.3.1.3 Strain revival

Two-hundred milliliters of CNM were prepared and 50 ml were placed in a 100 mL serum bottle to be used for the inoculum. The serum bottle was closed with a butyl rubber stopper and crimped with aluminum seals, the glass bottle was tightly capped with rubber stoppers. Both were degassed by applying vacuum and sparged with highly purity N2 gas (Praxair), and autoclaved at 121 °C for 15 min and then cooled to room temperature. Two milliliters of frozen culture (kept at -80 °C), were aseptically transferred to the 50 ml-serum bottles with CNM medium. All the inoculations were done under a cannula system with a gentle stream of nitrogen gas flowing through the tubes and bottles. Then, the culture was incubated (Thermo Scientific MaxQ4000 Incubator) at 37 °C for 24 h, with a shaking speed of 90 rpm.

6.3.1.4 Subculture

From the strain revival culture, the fresh cells were transferred 10%(v/v) to the Subculture medium (CNM) and incubated for 14-16 h at 37 °C and 90 rpm.

6.3.1.5 Inoculum

Batch anaerobic inoculum was performed in duplicate bottles with a working volume of 50 ml of liquid medium. Bottles containing 50 ml of P2 medium were tightly capped with rubber butyl caps, degassed by applying vacuum and sparged with high purity nitrogen gas to provide an anaerobic atmosphere. Finally, inoculation of the culture was done with 10% (v/v) of fresh bacteria from the subculture step. The inoculum was incubated for 14-16 h at 37 °C and 75 rpm.

6.3.1.6 Fermentation conditions

Fermentation experiments were carried in duplicate, on a 250 mL glass bottles (Wheaton) with 135 ml of P2 medium. These bottles were inoculated with 10% (v/v) of fresh inoculum, previously described. The fermentation was monitored for 18 days (446h) with *Clostridium beijerinckii*, whereas for *Clostridium saccharobutylicum* it was 8 days (188h).

6.3.1.7 Analytical Methods

Cell growth was monitored by measuring the OD600 value and dry weight. Duplicates using the same media with the same concentration of glucose or sugar corn juice, without the culture, served as controls. The products, such as acetic acid, butyric acid, acetone, butanol, and ethanol were measured utilizing a Gas Chromatograph System Hewlett Packard 6890 Series coupled with a Flame Ionization Detector (FID). 0.5 microliters of sample, all injections were done manually in duplicate. (See Appendix A)

Quantification of sugar content in °Brix

The sugar content in sugarcorn juice was quantified using a Leica Auto Abbe refractometer. A blank was prepared using distilled water. The amount of dissolved solids (in this case, the amount of dissolved sugars) in the sugarcorn juice was determined by correlating to a standard curve prepared with sucrose solutions of up to 52.63 % (w/w).

6.3.2 Results and discussion

6.3.2.1 Sugarcorn juice fermentation by *C. beijerinckii* 6422

Sugars uptake and its regulation are critical aspects of control of bacterial fermentation, and a thorough characterization can make a significant contribution towards the future development of an effective ABE process [Mitchell, 2016].

ABE products and pH variation during 446 h of fermentation are illustrated in figure 6.11. The initial media pH was 6.4, which diminished to 5.7 after 35.5 hours. Following this, pH remained at 5.5 for several days, until after 10 days of fermentation, when it dropped to 5.3 and later to 5.26 after 18 days. When the pH dropped below 5.5, a marked increase in acetic and butyric acid concentrations was observed.

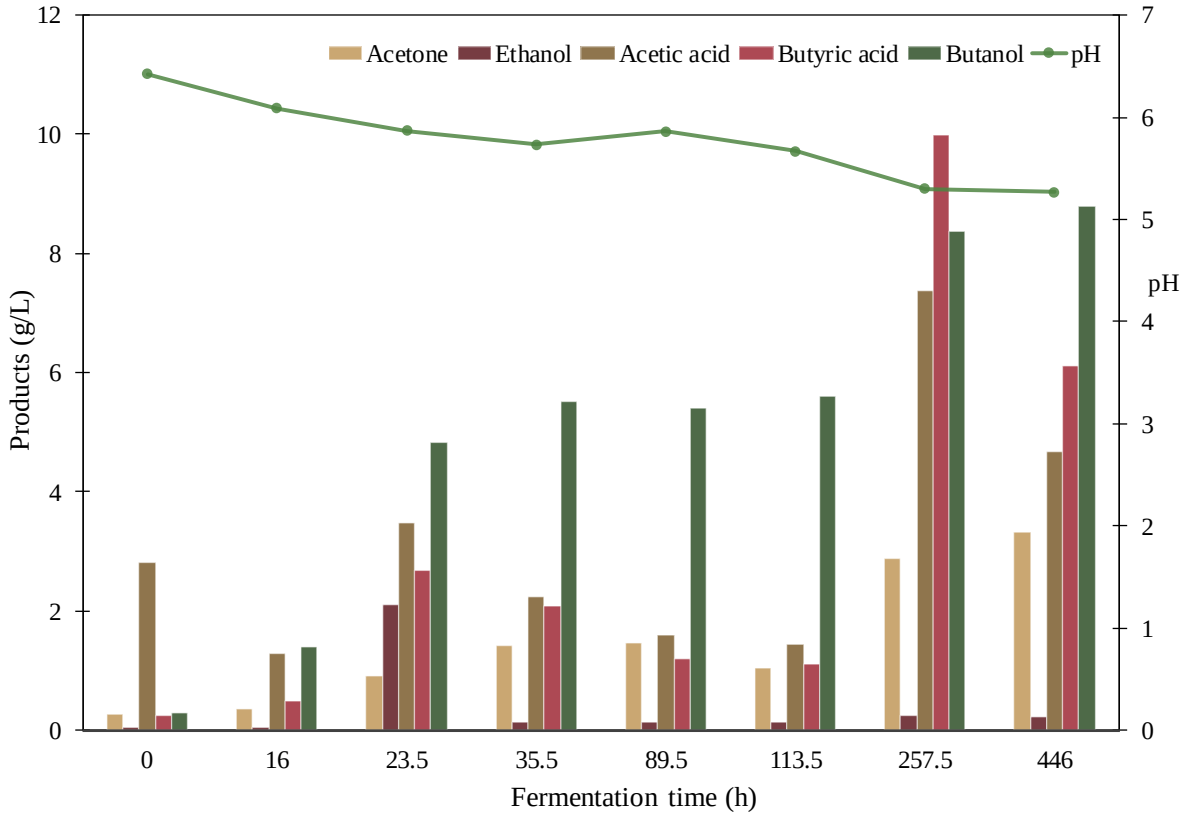

Figure 6.11. ABE fermentation profile, using Sugar corn juice medium as substrate, by *Clostridium beijerinckii* 6422

As shown figure 6.12, fermentation media initially contained 17.4 g L^{-1} glucose, 11.4 g L^{-1} fructose, and 8.1 g L^{-1} sucrose. Glucose and fructose were utilized before sucrose, with glucose

clearly being the most preferred carbon source. Studies have shown the presence of a glucose-dependent PTS system in *C. acetobutylicum, C. beijerinckii and C. perfringes*, however, glucose utilization was enhanced despite reduced PTS activity. Hence, it has been suggested that an alternative non-PTS transport system may contribute to preferential glucose utilization in Clostridium species, such as ATP-driven transporters or gluconate:H+ transporters.

During fermentation, it was evident that CCR in *C. beijerinckii* prevented uptake of sucrose until glucose in the medium was almost depleted. Sucrose hydrolase and fructokinase activities have been detected in sucrose-grown cultures of C. beijerinckii, as shown in chapter 4, table 4.1. It is proposed that once inside the cell, sucrose-6-phosphate is hydrolyzed to yield glucose-6-phosphate and fructose, following which the latter gets phosphorylated before it enters glycolysis along with the former.

A passive sucrose uptake was observed from 16 h to 89.5 h (less than 1 g L^{-1}), which may be explained by the presence of other mechanisms for sucrose transport without chemical modification, such as non-PTS permease activity in the cell [Reid, 2005].

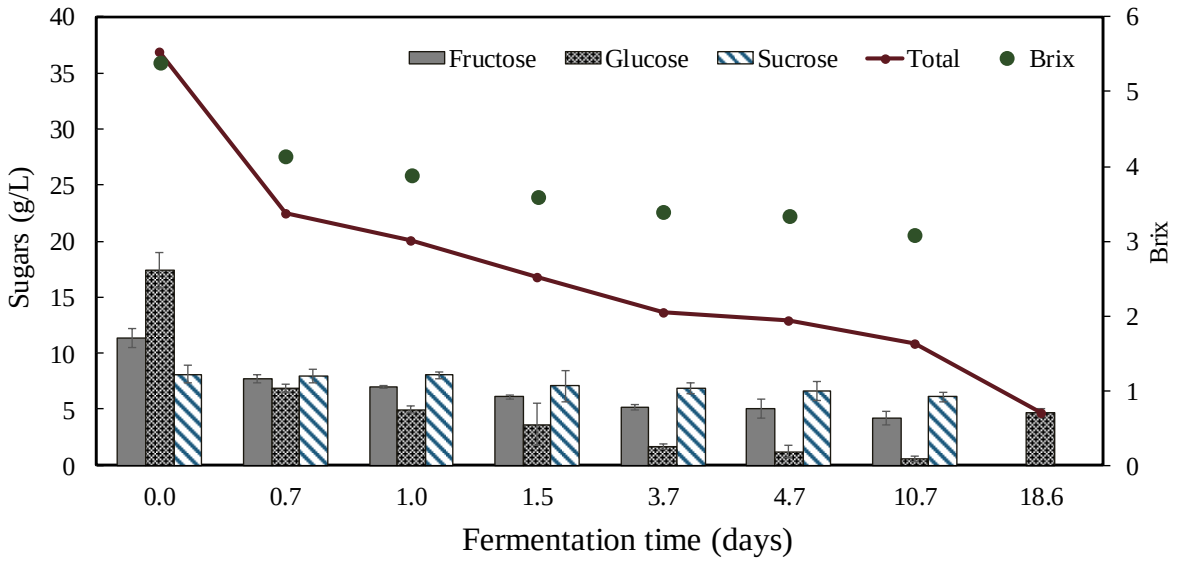

Figure 6.12. Fructose, glucose and sucrose consumption by *Clostridium beijerinckii* 6422 during ABE fermentation, using Sugar corn juice medium

After 257.5 h (10.7 days), the amount of glucose, fructose and sucrose dropped to 0.56 g L^{-1}, 4.18 g L^{-1} and 6.11 g L^{-1}, respectively. Though fructose and sucrose had been completely consumed in 446 h (18 days), interestingly, 4.67 g L^{-1} of residual glucose was detected. The inability of *Clostridium beijerinckii* to assimilate the remaining glucose, may be due to the decreased influence of the glucose-PTS system in the Clostridial metabolism towards later stages of the fermentation

Figure 6.13 shows the growth profile for *Clostridium beijerinckii* 6422, when grown in sugarcorn juice culture media, the cell concentration was 3.2 g L^{-1} at the end of fermentation.

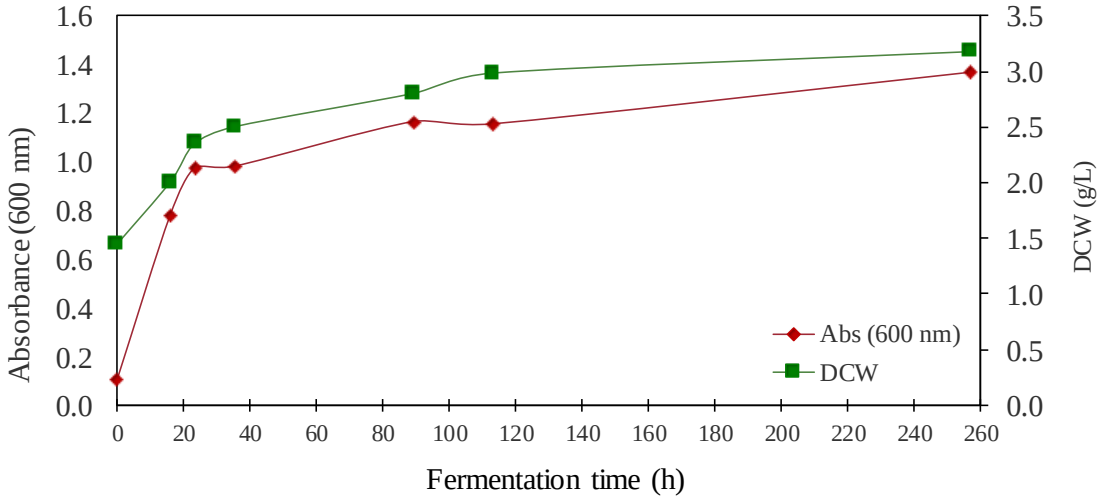

Figure 6.13. *Clostridium beijerinckii* 6422 growth profile during ABE fermentation, using SCJ-P2 medium

Tables 6.3 and 6.4 summarize the parameters and metabolites of interest for the fermentation

Table 6.3. Fermentation parameters from *Clostridium beijerinckii 6422*

Products	*Fermentation parameters*	*257.5 h (10.7 d)*
Acetone	Titer (g/L)	2.88
	Y (P/X) (g/g)	1.52
	Y (P/S) (g/g)	0.008
	Productivity (g/L ·h)	0.011
Ethanol	Titer (g/L)	0.25
	Y (P/X) (g/g)	0.126
	Y (P/S) (g/g)	0.101
	Productivity (g/L ·h)	0.001
Butanol	**Titer (g/L)**	**8.37**
	Y (P/X) (g/g)	4.66
	Y (P/S) (g/g)	0.310
	Productivity (g/L ·h)	0.032
Acetic Acid	Titer (g/L)	7.38
	Y (P/X) (g/g)	2.64
	Y (P/S) (g/g)	0.176
	Productivity (g/L ·h)	0.029
Butyric Acid	Titer (g/L)	9.99
	Y (P/X) (g/g)	5.62
	Y (P/S) (g/g)	0.374
	Productivity (g/L ·h)	0.039
ABE	**Titer (g/L)**	**11.50**
	Y (P/X) (g/g)	6.299
	Y (P/S) (g/g)	0.420
	Productivity (g/L ·h)	0.045

Table 6.4 Summary of fermentation parameters of butanol production

Time (h)	**$Y_{P/S}$ ($g\ g^{-1}$)**	**Q_P ($g\ L^{-1}\ .h^{-1}$)**	**Titer ($g\ L^{-1}$)**	**Total ABE ($g\ L^{-1}$)**	**Substrate utilization**			
					Glucose	Fructose	Sucrose	Total sugars
257	0.310	0.032	8.37	11.50	97%	63%	25%	71%
446	0.264	0.019	8.80	12.33	73%	100%	100%	87%

6.3.2.2 Sugarcorn juice fermentation by *Clostridium saccharobutylicum* ATCC BAA-117

The biobutanol fermentation was carried out in duplicate in a sugarcorn juice-based P2 medium. The objective of this experiment was to evaluate a different Clostridia strain and investigate if more butanol can be produced with sugarcorn juice. From previous fermentations with pure sugars, this strain had a good butanol production, but sugars were not completed consumed when a starting with a concentration of about 60 g L^{-1} was utilized. The experiment aimed to determine if *Clostridia* could completely utilize the glucose, fructose and sucrose from the sugarcorn juice. If so, there could be a window of opportunity to design a semi-batch mode coupled to an *in-situ* recovery strategy, to avoid the solvents toxicity within the cell during butanol production.

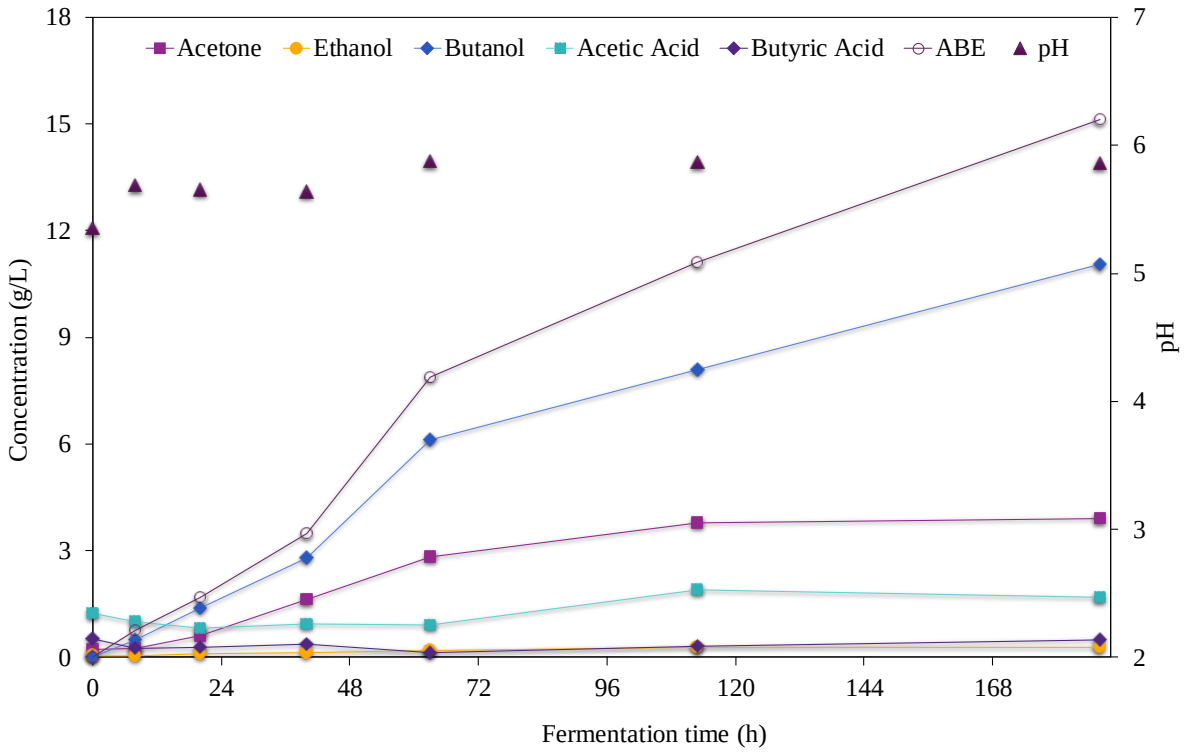

Figure 6.14 Experimental fermentation of *C. saccharobutylicum* fermentation on SCJ-P2 medium.

Figure 6.14 shows the profile of the pH during fermentation and the produced metabolites. Interestingly, when dealing with sugarcorn juice as the carbon source, the pH was set at 6.8

before autoclaving, but once the reading was done the initial fermentation pH was below. In this case, the first reading was 5.35. Interestingly, the pH increased, but it was established between 5.6-5.8 over the fermentation course. This behavior underpins the importance of working with plant juices that will contain certain chemicals that can have a positive effect on the biochemical production of biobutanol, and in this example by *Clostridium saccharobutylicum.*

The production of butyric acid over time in this fermentation was almost null; its concentration was no more than 1.88 g L^{-1} at 113 h and no apparent sign of production at the acidogenesis (Figure 6.14). Ethanol concentration was very low, never exceeding 0.3 g L^{-1}. Finally, the butanol titer was 8.1 g L^{-1} at 113 h and reached a maximum concentration of 11.05 g L^{-1}. ABE concentration was 15.12 g L^{-1} at the 188 h.

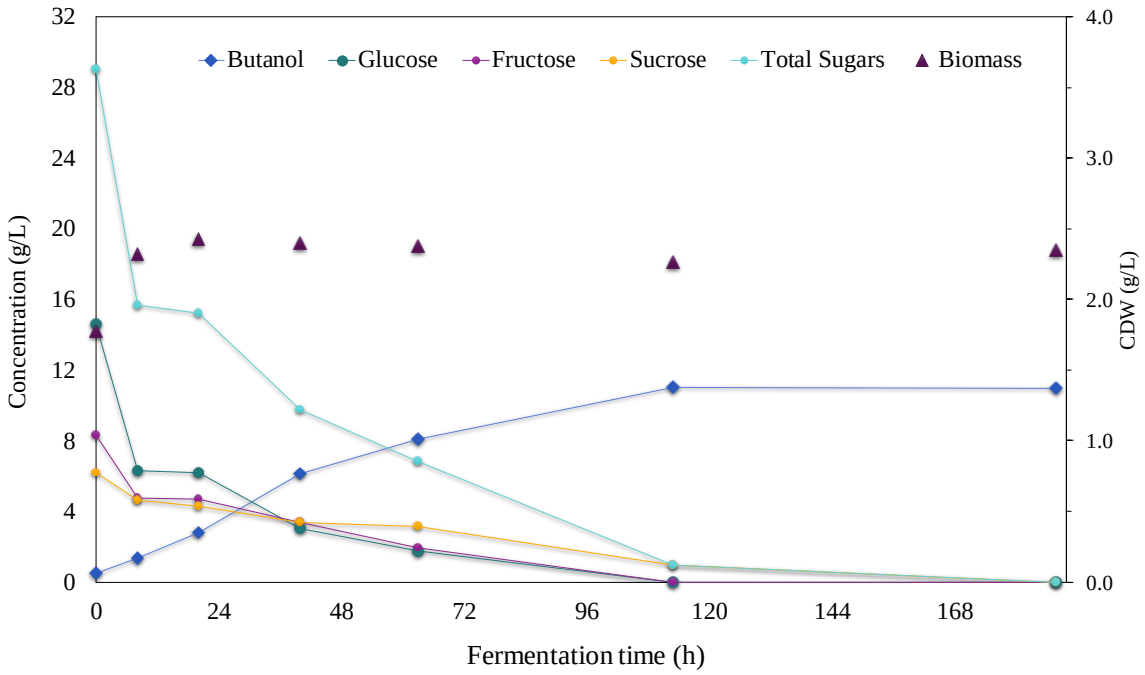

Figure 6.15 Butanol production, sugar consumption and cellular growth by *C. saccharobutylicum* fermentation on SCJ-P2 medium.

Figure 6.15 shows the consumption of sugarcorn sugars during the fermentation. Glucose and fructose were completed depleted by 113 h. The remaining sucrose was depleted for 188 h and even by 168 h in looked like there was no left sucrose in the culture medium. The initial concentration of total sugars was 29.05 g L^{-1}, half of that of the experiments with pure sugars discussed in chapter 4.

6.3.3 Conclusions

In this chapter, we studied the production of biobutanol using sugarcorn juice as the carbon source. Sugarcorn juice is the juice extracted from a potential Canadian energy crop developed by Agriculture and Agri-Food Canada (AAFC) researchers as a strategy for Ontario farmers.

ABE fermentation by different Clostridia was studied and the key findings are listed below.

- *Clostridia beijerinckii* 55025 was not able to utilize the sugarcorn juice to produce butanol. Its metabolism was shifted to produce a considerable amount of biomass rather than any solvent. This is an asporogenic strain.
- *Clostridium beijerinckii* 6422 produced 8.49 g L^{-1} of butanol over 257 h of fermentation utilizing sugarcorn juice as substrate. It had a biphasic fermentation where acids accumulation happened at the beginning of fermentation. Interestingly, at the end of the fermentation butyric acid was reactivated and the butanol production shifted towards butyric acid production.
- *Clostridium saccharobutylicum* produced 11.05 g L^{-1} of butanol over 227 h of fermentation utilizing sugarcorn juice as substrate.
- Both strains, *C. beijerinckii 6422* and *C. saccharobutylicum* could utilize sucrose, fructose and glucose concomitantly. There is enough evidence to agree that *Clostridium saccharobutylicum* has a PTS-sucrose system which allows the cell to transport sucrose inside the cell.

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Chapter 7

7 Evaluation of sugarcorn as a viable Canadian energy crop

7.1 Background

In order to assess the potential of sugarcorn plant for biobutanol production, the following information was discussed in relation to energy crops with sugar-rich stalks, such as sweet sorghum and energy cane: (1) typical crop features, (2) juice characteristics, and (3) processes for butanol production.

Table 7.1 Comparison of typical crop features in sugary feedstocks

Properties	Sugarcorn	Sugar cane	Energy cane	Sweet sorghum
Crop cycle (months)	3-4	10-12	10-15	3.5
Number of cycles/year	One	One	One	Two
Yield (t/ha/year)	80a	70	100	60
Brix (% juice)	11-16 a	13-15	10-12	11-13

[Reid et al., 2015;Kim and Day, 2011;McKaig, 1936;Aragon, 2013]

Table 7.2 Comparison of juice characteristics in sugary feedstocks

	Sugarcorn Juice A	Sugarcorn juice B	Energy cane	Sweet sorghum
Juice (% total)	49 [a]	49 [a]	53.6	71.9
Sucrose (% juice)	4.8	5.4	8.1[d]	7.5[d]
Glucose (% juice)	3.2	1.3	0.7[d]	1.2[d]
Fructose (% juice)	2.6	1.0	0.7[d]	0.7[d]
Total (% juice)	10.6	7.7	9.5[d]	9.4[d]
Ash (wt.%)	5.9	6.4	2.9[d]	2.7[d]

[a] From Reid 2015 assuming values from genotype C103(1)
[b] Adapted from Kim, 2011 (2)
[c] McKaig, 1936 (3)
[d] Aragon 2013 (4)

Sugarcorn can accumulate a stalk sugar content (11-16 °Bx) comparable with sugarcane and energy cane, achieved in one-third the crop cycle. Furthermore, the yield per hectare of sugarcorn plant is 80 metric tons, next only to energy cane, among the energy crops compared (Table 7.1).

The juice extractability of sugarcorn was 49%, slightly lower than energy cane, which has 53.6%. The ash content of SCJ was more than double that of energy cane and sweet sorghum juices. The content of sucrose, glucose, and fructose in the juice amounted to about four-fifth of the total carbohydrates for SCJ and was comparable with energy cane juice. It was interesting that, like sweet sorghum, SCJ had a good proportion of glucose and fructose too along with sucrose, the primary sugar in the juice (Table 7.2).

Comparison of a sugarcorn-based bioprocess for production of butanol with that of corn kernel and corn stover is shown in Figure 7.1. The major difference between the three lies in the upstream processing steps. Most of ethanol plants in USA use dry milling to convert corn kernel to ethanol and was used as an example for what could be butanol plants. This kind of technology involves the addition of different amylases during the process, to hydrolyze the starch to oligosaccharides and subsequently to monosaccharides, as substrates for fermentation. On the other hand, the corn stover is milled, pretreated to hydrolyze the lignocellulose, conditioned and saccharified by cellulase, prior to fermentation.

A bioprocess for sugarcorn may require juice extraction from the sugarcorn plants, separation of coarse residues from juice, followed by juice treatment to minimize contamination. For instance, for butanol production from *Clostridium saccharobutylicum*, finding the right initial sugarcorn juice dilution may serve to have a good fermentation and butanol production, while also favoring cell growth. The treated sugarcorn juice will not require the use of expensive enzymes, as it contains sugars that are readily-assimilable by *Clostridium spp.* Further, it will reduce costs associated with chemical or biochemical catalysts and equipment.

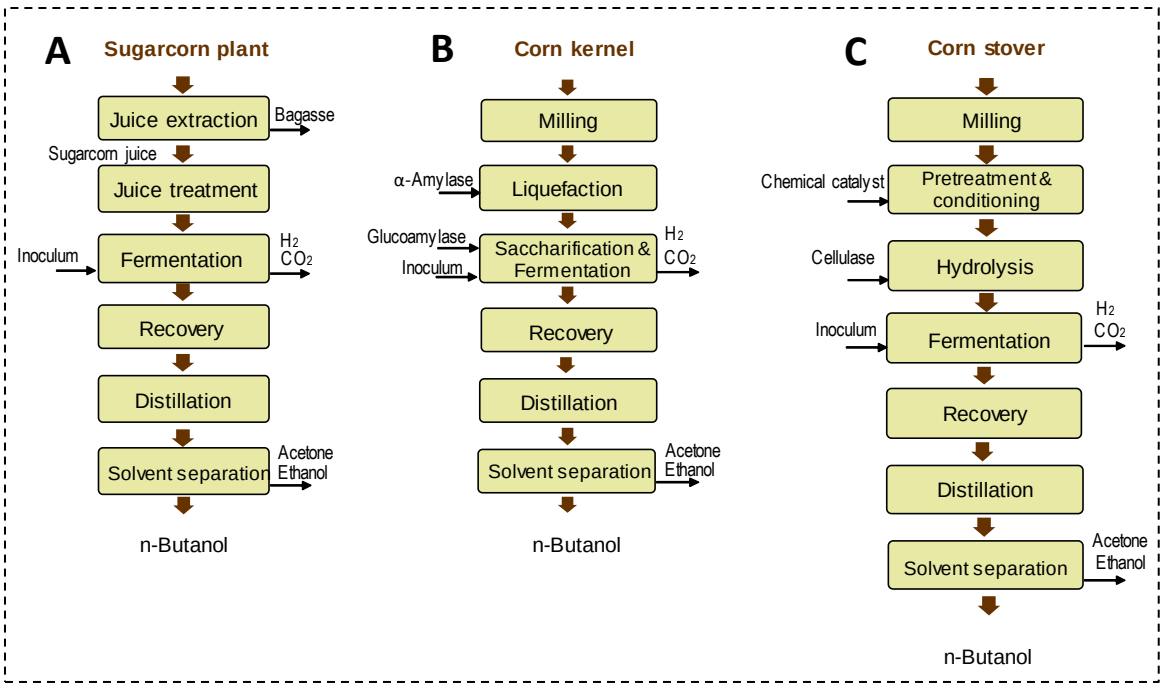

Figure 7.1 Process flow diagram for butanol production from (A) sugarcorn plant, (b) starchy corn kernel, and (c) lignocellulosic corn stover. B and C adapted from [Pfromm et al., 2010;Kumar et al., 2012]

A biorefinery system is described as a conversion pathway from feedstock to products, via platforms and processes. The platforms are intermediates from which final products are derived. This study was focused on the concept of a biorefinery system motivated on the large-volume production of transportation biofuel, which can be blended with gasoline or diesel [Cherubini et al., 2009].

IEA Bioenergy Task-42 researchers [Cherubini et al., 2009] developed a classification approach for energy driven biorefineries and based on our results where the characteristics and potential of sugarcorn juice have been highlighted, a Canadian Sugarcorn Biorefinery (CANSUG Biorefinery) is proposed, as shown in Figure 7.2.

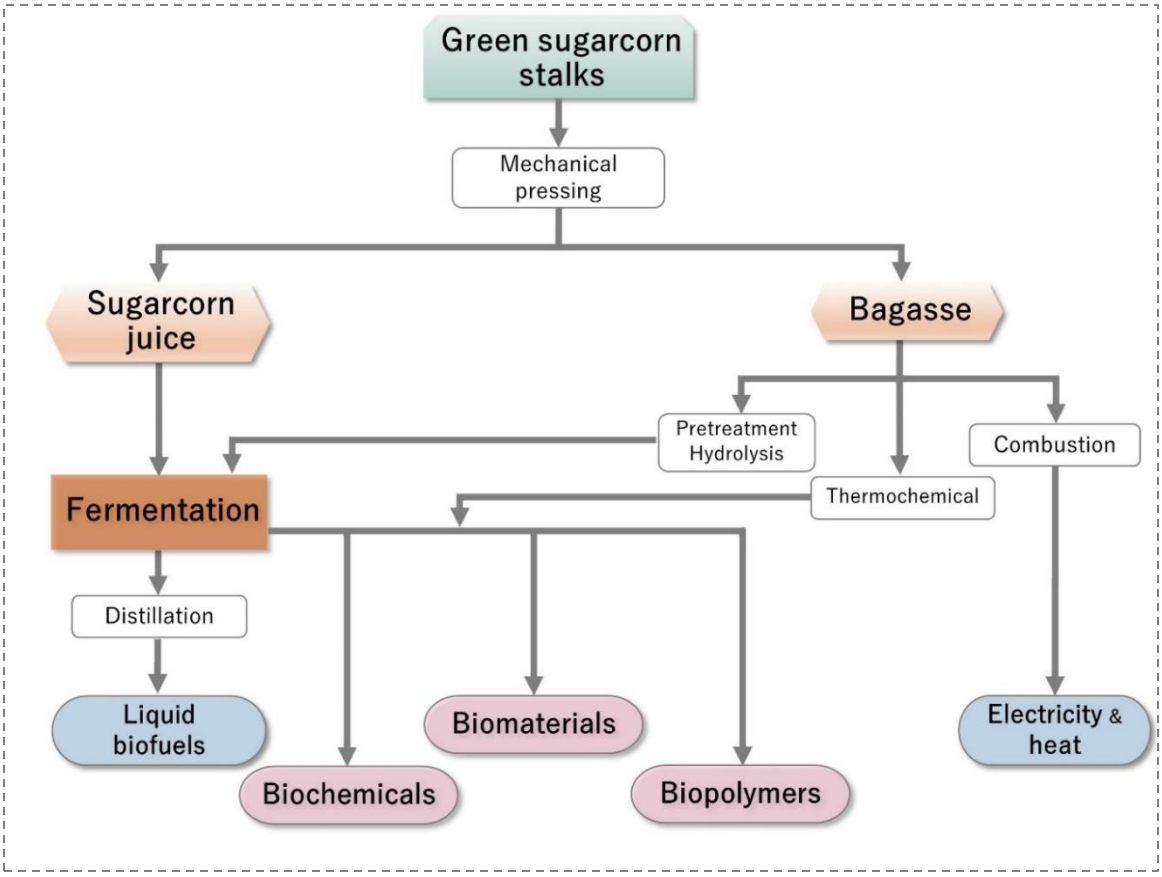

Figure 7.2 Flow diagram of proposed CANSUG Biorefinery (the original figure was designed by RGF, TNT, AM).

(This figure was first published in Thiruvengadathan 2017 thesis, and it has its permission to use it, as both of us developed it)

Comparing Figure 2.7 and Figure 7.2, production of sugarcorn as valuable biomass can open new revenue opportunities for farmers and industries. The sugarcorn plants, while still green, will be milled to extract juice, which can then be biochemically converted to renewable transportation biofuels. The process can also produce useful co-products such as biomaterials and biochemicals that can offer additional economic and environmental benefits. The cellulose in the bagasse can be treated and enzymatically hydrolyzed to generate a new sugary stream for the fermentation or, it can be combusted to provide the heat and electricity required by the biorefinery plant.

7.2 Conclusions

The sugar characterization results highlighted that sugarcorn juice has abundant fermentable sugars, characteristic of established substrates like sugarcane juice and sweet sorghum juice. Sugarcorn juice showed promise regarding yield for both bioethanol and biobutanol fermentations [Gomez-Flores et al., 2018]. With further optimization of the medium and process, higher fermentation efficiency and productivity can be achieved. Sugarcorn is a new feedstock that can potentially reduce the cost of energy and enzyme inputs currently used in the conventional biomass-to-biofuel processes. Given the familiarity of corn in the agricultural sector, sugarcorn may be deployed faster as a Canadian energy crop to support the Canadian bioeconomy.

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Chapter 8

8 Conclusions and Recommendations

8.1 Conclusions

The following findings summarize the major outcomes of this research:

- ABE fermentations by *Clostridium saccharobutylicum* ATTC BAA-117 (also known as *Clostridium saccharobutylicum* DSM 13864), using three individual sugars (glucose, fructose, and sucrose) and a mix of the three were carried out. All of them resulted in the production of butanol as high as 12-14 g L^{-1}. This strain can metabolize the three sugars concomitantly.

- The dilute acid pretreatment with sulfuric acid to corn cobs showed that temperature is the variable with most significant effect towards glucose formation. A two-step pretreatment was designed for corn cobs.

- The enzymatic hydrolysis was carried out utilizing a very low concentration of an enzymatic stock solution of Cellulic C2Tec from Novozymes to hydrolysis the cellulose from the corn cobs. The hydrolysis conversion was around 44%, indicating the possibility of improvement in the second round of enzymatic hydrolysis and with the increase of the enzyme dose.

- Biobutanol fermentation was pursued utilizing g a *Clostridium beijerinckii* strain and cellulosic biobutanol was produced in a titer of 4.42 g L^{-1} at 48h of fermentation with 97% of reducing sugars used by this time. It is worth noting that there was not a visible acidogenesis phase or acid accumulations at the beginning of the fermentation.

- For the first time, sugarcorn juices from Canadian corn hybrids, were characterized and proven as a suitable medium for biobutanol production. Variation in sugar composition of sugarcorn juices across different hybrids and growth seasons were observed during

this study, from 102 g L^{-1} and 145 g L^{-1}, with fructose, glucose, and sucrose together accounting for about 80% of reducing sugars.

- ABE fermentation by different Clostridia strains was studied, and critical remarks were found. *Clostridia beijerinckii* 55025 was not able to utilize the sugarcorn juice to produce butanol. Its metabolism was shifted to generate a considerable amount of biomass than any solvent. This is an asporogenic strain. *Clostridium beijerinckii* 6422 produced 8.49 g L^{-1} of butanol over 257 h of fermentation utilizing sugarcorn juice as substrate. It had a biphasic fermentation where acids accumulation happened at the beginning of fermentation. Interestingly, at the end of the fermentation butyric acid was reactivated and the butanol production shifted towards butyric acid production. *Clostridium saccharobutylicum* produced 11.05 g L^{-1} of butanol over 227h of fermentation utilizing sugarcorn juice as substrate. Both strains, *C. beijerinckii 6422* and *C. saccharobutylicum,* could utilize sucrose, fructose, and glucose concomitantly. There is enough evidence to agree that *Clostridium saccharobutylicum* has a PTS-sucrose system which allows the cell to transport sucrose inside the cell.

- Sugarcorn can be used for fermentative production of butanol and other useful fuels and chemicals. The proposed Canadian sugarcorn (CANSUG) biorefinery can generate commercially valuable products while limiting wastes and can offer social, economic and environmental benefits to the energy sector, while also strengthening the growing Canadian bio-economy.

8.2 Recommendations and future work

Based on the finding of this study, further work should address the following:

- Test other cellulases following the developed two-step pretreatment design to compare if better enzymatic hydrolysis can be achieved.
- Optimize the dilution of the sugarcorn juice to obtain the highest possible sugars concentration without inhibiting the biobutanol production.
- Develop a kinetic model for *Clostridium saccharobutylicum* using simple sugars to describe the cellular growth.

- Immobilization strategy for *Clostridium saccharobutylicum* fermentation can improve the biobutanol final titer, and a continuous fermentation strategy can be achieved.
- An *in-situ* recovery strategy can be studied to avoid sporulation and increase butanol production.

Appendix A

Gas chromatography was utilized to determine the concentrations of acetone, butanol, ethanol, acetic acid and butyric acid. Samples from fermentation were centrifuged and the supernatant was used. Samples were diluted 1 to 10 with distilled water, mixed and filtered through a 0.45 µm syringe filter (Acrodisc 13 mm, Pall). The samples were analyzed with a GC System Hewlett Packard 6890 Series.

The gas chromatograph (GC System Hewlett Packard 6890 Series) was coupled to a flame ionization detector (FID), GC Chemstation (Agilent Technologies) and a HP-Innowax column (length 30 m, diam. 0.25mm ID, and 0.25 µm film thickness) using helium as the carrier gas at a flow rate of 1.5 mL min^{-1}. The GC operation proceed with an injector temperature of 220°C and, the detector temperature was set up at 250 °C. The column temperature ramp started at 40 °C for 2 min, raised to 45°C at increasing rate of 5°C/min, finally raised to 225°C at increasing rate of 20° C/min, and held at 225°C for 3 min. A volume of 1 uL sample was manually injected, the split ratio was 1:25. All the injections were done in duplicate.

Methods: Two methods were developed for the products quantification:

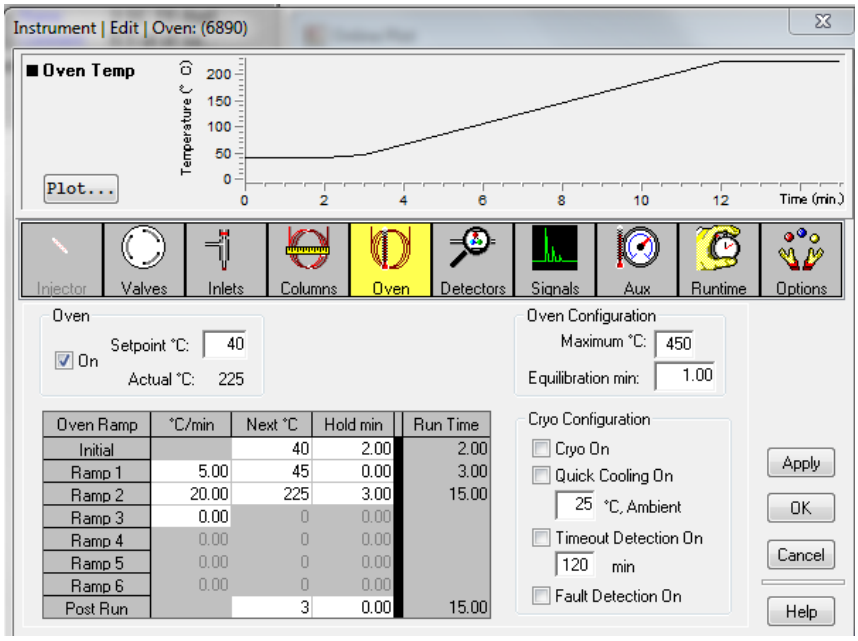
1) GC Method ABE
2) GC Method ABE Short

1-GC Method: ABE

Oven Ramp (°C/min)	Temperature (°C)	Hold (min)	Run Time (min)	Injector Temperature (°C)	Detector Temperature (°C)
-	35	5			
5	150	2		220	250
20	250	1	37		

2-GC Method: ABE SHORT

Oven Ramp (°C/min)	Temperature (°C)	Hold (min)	Run Time (min)	Injector Temperature (°C)	Detector Temperature (°C)
-	40	2			
5	45	0		220	250
20	225	3	15		



Example of GC software utilized, oven temperature utilized for ABE short method

GC Method: ABE Short. Volume injection: 0.5 uL. Running time: 15 min

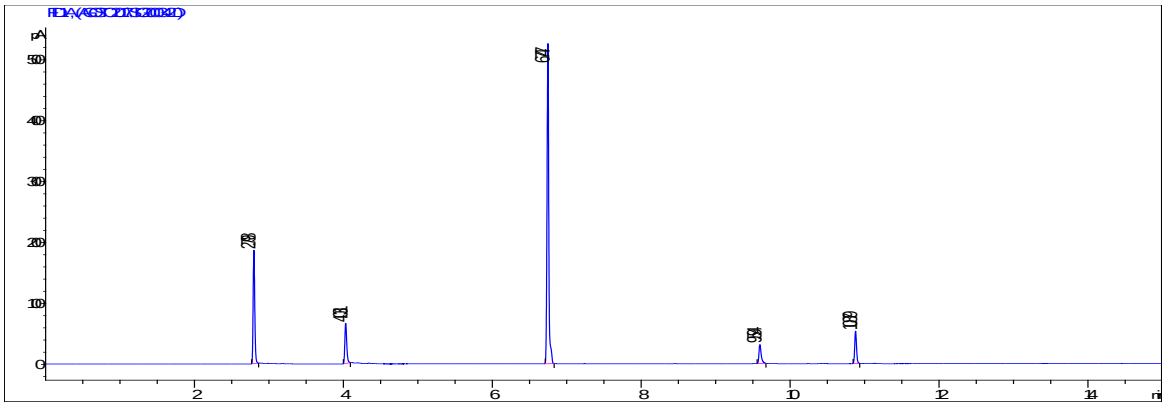
Analyte	Vol inj (uL)	Time1	Time2	Concentration (g/L)	Area 1	Area 2	Area avg	Std error
Acetone	0.5	2.799	2.798	1.25	152.2	122.1	137.2	21.3
Acetone	0.5	2.801	2.798	0.83	98.2	109.6	103.9	8.1
Acetone	0.5	2.8	2.802	0.58	73.8	71.4	72.6	1.7
Acetone	0.5	2.804	2.758	0.25	31.9	45.6	38.8	9.7
Acetone	0.5	2.804	2.758	0	0	0	0	0.0
Ethanol	0.5	4.031	4.032	0.75	73.9	63.4	68.7	7.4
Ethanol	0.5	4.033	4.033	0.5	45.4	74.4	59.9	20.1
Ethanol	0.5	4.034	4.034	0.35	36.2	34.1	35.2	1.5
Ethanol	0.5	4.037	3.982	0.15	16.3	20.3	18.3	2.8
Ethanol	0.5	4.037	3.982	0	0	0	0	0.00
Butanol	0.5	6.717	6.705	2.5	514	452.3	483.2	43.6
Butanol	0.5	6.71	6.755	1.67	345.4	371.6	358.5	18.5
Butanol	0.5	6.722	6.712	1.17	273.4	252.4	262.9	14.9
Butanol	0.5	6.713	6.659	0.5	120.1	143.5	131.8	16.5
Butanol	0.5	6.713	6.659	0	0	0	0	0.00
Acetic Acid	0.5	9.589	9.588	1.5	74.1	66	70.1	5.7
Acetic Acid	0.5	9.59	9.595	1	57.4	52.2	54.8	3.7
Acetic Acid	0.5	9.59	9.592	0.7	41	42.6	41.8	1.1
Acetic Acid	0.5	9.594	9.597	0.3	28.6	19.4	24	6.5
Acetic Acid	0.5	9.594	9.597	0	0	0	0	0.0
Butyric Acid	0.5	10.878	10.877	1	101.7	92.2	97.0	6.7
Butyric Acid	0.5	10.878	10.879	0.67	74.8	83.7	79.3	6.3
Butyric Acid	0.5	10.878	10.878	0.47	66.1	55.3	60.7	7.6
Butyric Acid	0.5	10.879	10.889	0.2	33.1	19.8	26.5	9.4
Butyric Acid	0.5	10.879	10.889	0	0	0	0	0.0

GC Method: ABE-Short Volume injection: 1uL. Running time: 15 min

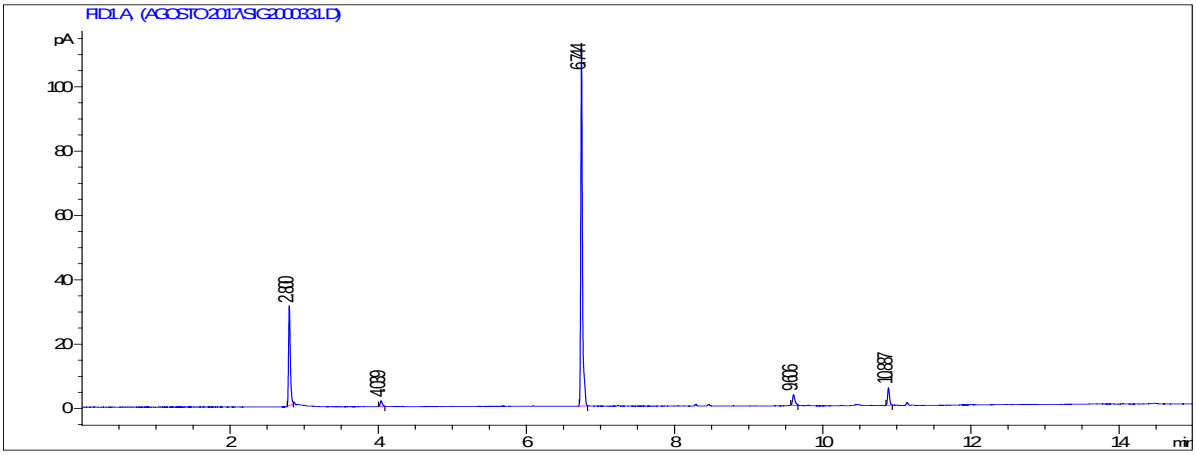
Analyte	Vol inj (uL)	Time1	Time2	Concentration (g/L)	Area 1	Area 2	Area avg	Std error
Acetone	1	2.798	2.805	1.25	282.7	248	265.35	24.54
Acetone	1	2.801	2.794	0.83	88.5	121.6	105.05	23.41
Acetone	1	2.801	2.798	0.58	64.6	75.8	70.2	7.92
Acetone	1	2.801	2.802	0.25	48.6	52.4	50.5	2.69
Acetone	1	2.801	2.802	0	0	0	0	0.00
Ethanol	1	4.031	4.039	0.75	116.8	117.7	117.25	0.64
Ethanol	1	4.034	4.03	0.5	49	68.3	58.65	13.65
Ethanol	1	4.036	4.033	0.35	35.8	38.4	37.1	1.84
Ethanol	1	4.035	4.036	0.15	22.6	23.8	23.2	0.85
Ethanol	1	4.035	4.036	0	0	0	0	0.00
Butanol	1	6.747	6.762	2.5	853.2	842.1	847.65	7.85
Butanol	1	6.75	6.761	1.67	388.5	463.2	463.2	52.82
Butanol	1	6.749	6.735	1.17	258.1	276.2	267.15	12.80
Butanol	1	6.732	6.736	0.5	200.9	174.6	187.75	18.60
Butanol	1	6.732	6.736	0	0	0	0	0.00
Acetic Acid	1	9.594	9.6	1.5	66.6	69.7	68.15	2.19
Acetic Acid	1	9.596	9.595	1	58.5	58.9	58.7	0.28
Acetic Acid	1	9.599	9.6	0.7	40.3	33.1	36.7	5.09
Acetic Acid	1	9.601	9.601	0.3	26.8	21	23.9	4.10
Acetic Acid	1	9.601	9.601	0	0	0	0	0.00
Butyric Acid	1	10.879	10.885	1	90.4	103	96.7	8.91
Butyric Acid	1	10.88	10.879	0.67	75.7	82.6	79.15	4.88
Butyric Acid	1	10.881	10.881	0.47	50.4	45.4	47.9	3.54
Butyric Acid	1	10.883	10.883	0.2	34	25.5	29.75	6.01
Butyric Acid	1	10.883	10.883	0	0	0	0	0.00

GC Method: ABE. Injection volume: 0.5 uL. Running time: 37 min

Analyte	Vol inj (uL)	Time1	Time2	Concentration (g/L)	Area 1	Area 2	Area avg	Std error
Acetone	0.5	2.985	2.994	1.25	147.6	140.5	144.05	5.02
Acetone	0.5	2.984	2.989	0.83	103.2	94	98.6	6.51
Acetone	0.5	2.988	2.997	0.58	68.1	65	66.55	2.19
Acetone	0.5	2.994	2.991	0.25	24.7	30.3	27.5	3.96
Acetone	0.5	2.994	2.991	0	0	0	0	0.00
Ethanol	0.5	5.074	5.086	0.75	79	67.9	73.45	7.85
Ethanol	0.5	5.068	5.076	0.5	50.6	41.9	46.25	6.15
Ethanol	0.5	5.078	5.089	0.35	31.6	29.5	30.55	1.48
Ethanol	0.5	5.085	5.082	0.15	11.3	14.6	12.95	2.33
Ethanol	0.5	5.085	5.082	0	0	0	0	0.00
Butanol	0.5	12.215	12.209	2.5	539.1	503.9	521.5	24.89
Butanol	0.5	12.199	12.202	1.67	360.2	334	347.1	18.53
Butanol	0.5	12.196	12.206	1.17	244.7	231.8	238.25	9.12
Butanol	0.5	12.183	12.191	0.5	108.8	112.9	110.85	2.90
Butanol	0.5	12.183	12.191	0	0	0	0	0.00
Acetic Acid	0.5	21.946	21.957	1.5	58.3	48.4	53.35	7.00
Acetic Acid	0.5	21.954	21.952	1	40	46.3	43.15	4.45
Acetic Acid	0.5	21.963	21.97	0.7	29	34.2	31.6	3.68
Acetic Acid	0.5	21.969	21.975	0.3	20.5	21.7	21.1	0.85
Acetic Acid	0.5	21.969	21.975	0	0	0	0	0.00
Butyric Acid	0.5	26.58	26.589	1	92.2	77.9	85.05	10.11
Butyric Acid	0.5	26.583	26.585	0.67	59.9	62.9	61.4	2.12
Butyric Acid	0.5	26.588	26.596	0.47	43	45.7	44.35	1.91
Butyric Acid	0.5	26.589	26.597	0.2	28.7	26	27.35	1.91
Butyric Acid	0.5	26.589	26.597	0	0	0	0	0.00



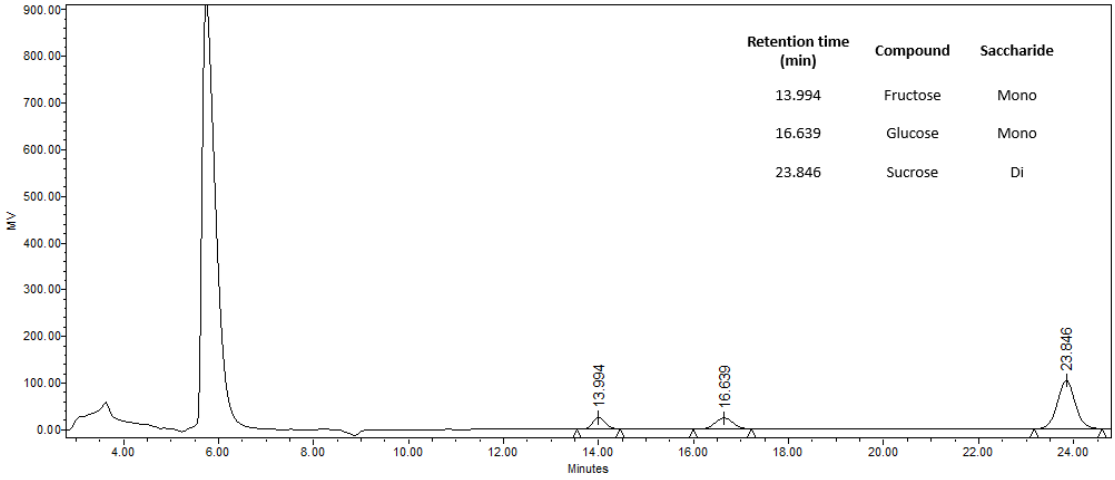
GC Chromatogram of calibration curve at point 1 from standards solution containing acetone, ethanol, butanol, acetic acid and butyric acid, using GC Method: ABE-Short. Volume injection: 1uL



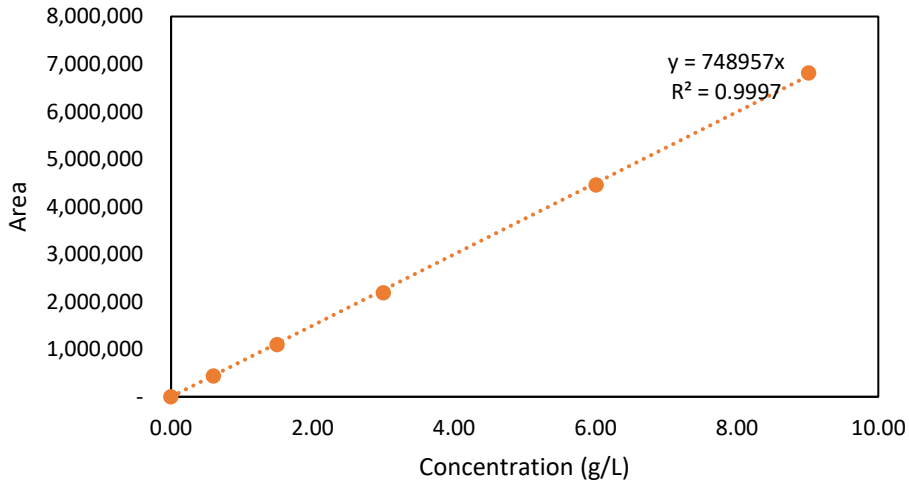
Example of GC Chromatogram from Sample: Bottle 1, Mix sugars, *Clostridium beijerinckii* 6422 at 120h of fermentation time. Using GC Method: ABE-Short. Volume injection: 1uL. Sample was diluted 10 times in distilled water.

HPLC

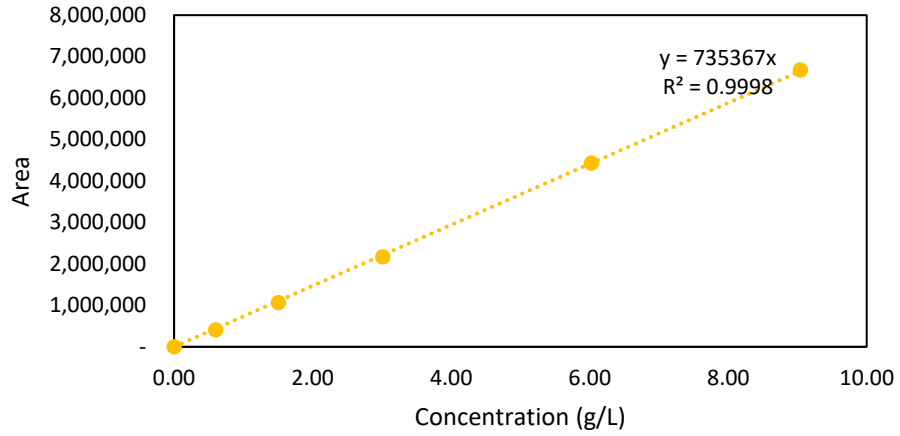
Liquid chromatography (HPLC, Waters Alliance System, New Castle, USA) was used to measure glucose, fructose and sucrose. The system was coupled with a refractive index detector (RID). An XBridge Amide column (3.5µm, 4.6 x 250 mm) with a mobile phase of 75/25 (v/v) acetonitrile/water, 0.2% triethylamine, working at a rate of 0.6 mL per minute was utilized for the quantification. Samples were diluted with equal volume of 50/50 (v/v) acetonitrile/water and filtered through a 0.45µm filter (Acrodisc 13mm, Pall) and finally loaded into an HPLC vial.



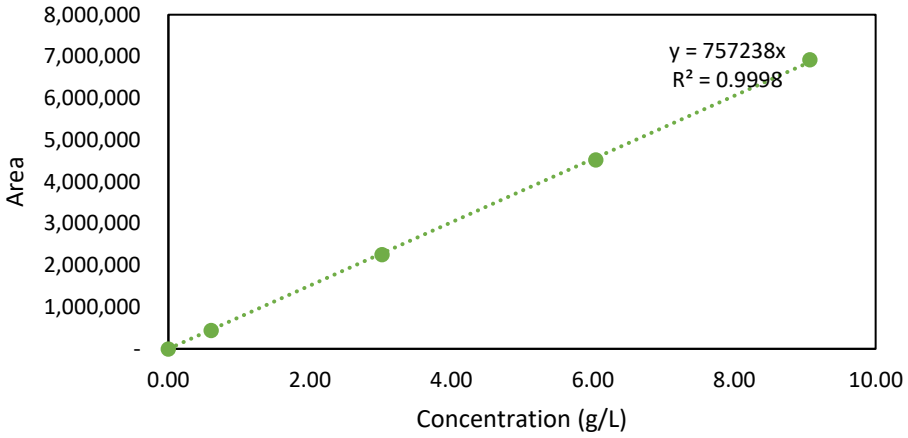
Example of HPLC Chromatogram from Sample of sugarcorn juice A



Glucose calibration curve HPLC Column XBridge Amide



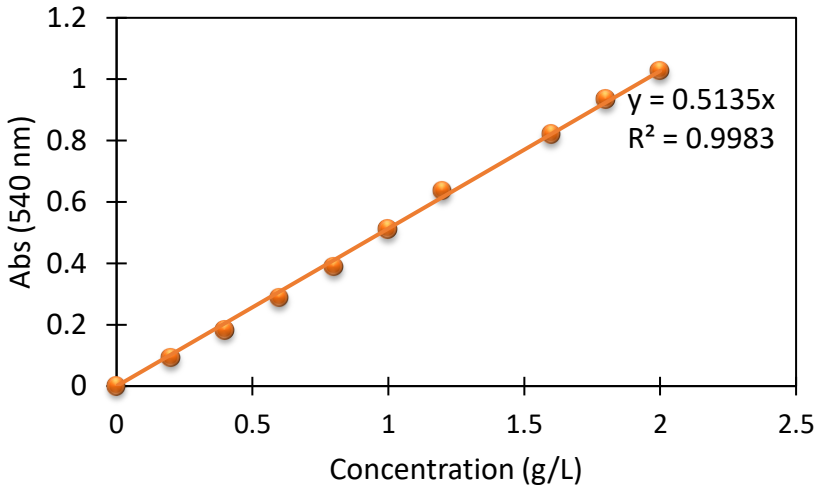
Fructose calibration curve HPLC Column XBridge Amide



Sucrose calibration curve HPLC Column XBridge Amide

Dinitro salicylic acid (DNS) method for reducing sugars measurement

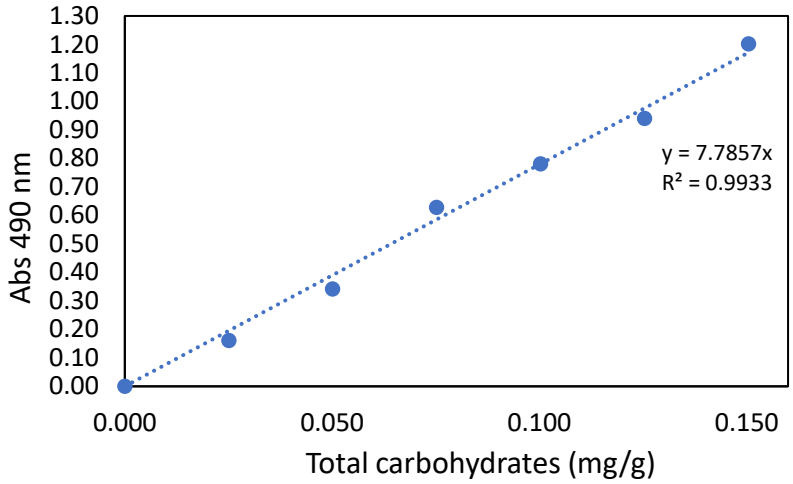
Reducing sugar was quantified by DNS method. 500 µL of appropriately diluted sample was added into an assay tube and mixed with 500 µL of DNS reagent, vortexed for 5 seconds. The assay tubes were set into boiling water for 5 min. Following this, the tubes were kept in cold water for 5 min to stop the reaction. Five mL of distilled water was added to each tube, and the solution was vortexed. Finally, the optical density at 540 nm was measured in a spectrophotometer (Genesys 10S UV-Vis Thermo scientific) and the absorbance values were recorded. Water was used in place of the sample to prepare the blank. The amount of reducing sugars was determined using a standard curve made with D-glucose up to 2 mg/mL [Miller, 1959].



DNS Calibration curve using glucose solution as standard

Phenol-Sulfuric method

An aliquot (0.5 mL) of the broth sample was added to a wide glass test tube, then 0.5 mL of 5% aqueous solution of phenol was added, finally 2.5 mL of concentrated sulfuric acid was carefully added to the surface of the previous solution mix using a bench dispenser (Dispensette™ Organic, Brand). Vigorous vortex of 1 min was applied to the solution mix. It was left to rest at room temperature for 10 min. Immediately, the mix was put in ice water for 10 more min. After 20 min, the absorbance was determined at 490 nm in spectrophotometer (Genesys 10S UV-Vis Thermoscientific). A blank was prepared using distilled water. The amount of sugars was determined by reference to a standard curve prepared with solutions containing up to 0.2 mg/L of D-glucose.



Phenol sulfuric acid Calibration curve using glucose solution as standard

Genzyme Diagnostics Reagent kit for glucose quantification

The fermentation broth or corn cob hydrolysate was diluted if needed previous to be used. The sample was filtered through 0.45 μm (Acrodisc 13mm, Pall). The filtered sample was mixed with the Genzyme reagent to spectrophotometer vials in the ratio 1:100 (e.g. 25 μL:2.5 mL) and then mixed. Finally, the mix was let to resto for 10-15 min and the absorbance at 505 nm was measured in a spectrophotometer (Genesys 10S UV-Vis Thermoscientific).

Curriculum Vitae

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Publications:

R. Nicol, D. Young, L. McNea, K. Van Overloop, B. Gilroyed, T. Nambi, **R. Gomez-Flores,** A. Margaritis, M. Morrison and L. Reid, Fermentation of juice from sugarcorn, a new feedstock for the ethanol industry", Recent Advancements in Fermentation technology, SIMB. (November 2015, USA)

Gomez-Flores, R., Thiruvengadathan. T.N, Nicol, R., Gilroyed, B., Margaritis, A. "Bioethanol and biobutanol production from sugarcorn juice" 70th Northeast Corn Improvement Conference. (February 2017, Ottawa, ON).

Gomez-Flores, R., Thiruvengadathan. T.N, Nicol, R., Gilroyed, B., Morrison, M., Reid, L.M., Margaritis, A. "Bioethanol and biobutanol production from sugarcorn: a new Canadian energy crop for biofuels" Published on Biomass and Bioenergy 2018.