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Bioethanol Production Using Saccharomyces cerevisae Cultivated In Sugarcorn Juice

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Abstract

For the first time, juice extracted from sugarcorn, a new Canadian energy crop, was used for bioethanol production. Physical and chemical characteristics of sugarcorn juice (SCJ) were determined. SCJ contained a maximum of 145 g/L of carbohydrates, with sucrose, glucose and fructose together contributing 80%. Effect of autoclaving and carbon filtration on juice sugars were investigated.

Shake flask fermentations using *Saccharomyces cerevisiae* grown in yeast extract supplemented SCJ produced a maximum of 45.6 g/L ethanol in 72 h. Bioreactor studies using un-supplemented SCJ achieved 40 g/L ethanol in 26 h, yielding a maximum of 0.46 g ethanol/g fermentable sugars, representing 90.4% of theoretical yield.

Sugarcorn's crop features and juice characteristics were compared with those of sugarcane, sweet sorghum and energy cane. A proposed sugarcorn based bioethanol process was compared with corn and corn stover based processes. A Canadian sugarcorn (CANSUG) biorefinery was proposed for production of renewable fuels and chemicals.

Keywords

Co-Authorship Statement

Chapter 3: Characterization of sugarcorn juice

Chapter 4: Bioethanol production using *Saccharomyces cerevisiae* cultivated in sugarcorn juice medium: shake flask experiments

A version of the above chapters has been **submitted** as part of a manuscript to Biomass and Bioenergy.

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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 experiments, 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 manuscript

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

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

Chapter 5: Bioethanol production and yield improvement using *Saccharomyces cerevisiae* ATCC 26603 cultivated in sugarcorn juice: bioreactor studies

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[1] design, execution of experiments, data analysis, manuscript preparation

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

Chapter 6: Evaluation of sugarcorn as a potential Canadian biofuel feedstock.

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To my family- Ambuja, Thiruvengadathan, Raman and Shruti

for their love and support

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Abbreviations

GHG   Greenhouse gases

AAFC   Agriculture and Agri-Food Canada

SCJ   Sugarcorn juice

ATP   Adenosine tri-phosphate

SCJ A   Sugarcorn juice A

SCJ B   Sugarcorn juice B

SCJ C   Sugarcorn juice C

PS   Phenol-sulfuric acid

DNS   Dinitro-salicylic acid

PDB   Potato dextrose broth

YE   Yeast extract

EM   Erlenmeyer

CFU   Colony forming units

YM   Yeast malt

ATCC   American type culture collection

TC   Total carbohydrates

FS   Fermentable sugars

CANSUG   Canadian sugarcorn

Chapter 1

1   Introduction

1.1   Sustainability and global energy scenario

Scientific developments have presented mankind with different ways to utilize resources to improve the quality of life. A development is 'sustainable' if it "meets the needs of the present without compromising the ability of the future generations to satisfy their own needs"(Brundtland 1987). Preference of unsustainable alternatives, along with the ever-rising world population has resulted in depletion of resources. The world population reached 7.3 billion in 2015, and projected to increase by 33% to reach 9.7 billion in 2050, and by 53% to cross 11.2 billion in 2100 (Melorose, Perroy, and Careas 2015). To meet the energy demand of such a growing population has been earmarked as one of the major challenges facing humanity (Richard Smalley 2003).

Combustion of fossil fuels, namely, oil, coal and natural gas is the source of most of the global energy. In 2015, these fuels accounted for 86 % of the energy consumed (World Energy Council 2016). Fossil fuels are non-renewable energy sources and their global supplies are unlikely to last more than 120 years (International Energy Agency 2013) if consumed at current rate of consumption. Further, burning of fossil fuels is one of the major sources of greenhouse gases (GHG), whose adverse impacts on climate change and global warming are well documented and forecasted.

In an attempt to reduce GHG emissions, and to supplement fossil fuels, low-carbon and clean energy alternatives are being developed and deployed. The growing energy demand, insecurity of fossil fuel supply, together with favorable policy preferences, federal mandates and associated subsidies (Guo, Song, and Buhain 2015; International Energy Agency 2015) remain key drivers in the diversification of energy sources and gradual shift in balance towards renewable energy. These include energy from solar, nuclear, hydrothermal, geothermal, wind and biomass, which have grown unexpectedly over the last 15 years (World Energy Council 2016) and continue to appeal to investors, facilitating rapid advancements in terms of technology and infrastructure.

1.2   Bioenergy, biorefining and biofuels

The energy derived by the conversion of renewable organic substances from animal or plant sources ('biomass') to energy or energy-containing compounds is referred to as 'bioenergy'. Modern bioenergy utilizes highly efficient and sustainable conversion processes compared to the low efficient traditional bioenergy that involves combustion of unprocessed wood, straws or manures (Gurung and Oh 2013). In 2014, the global total primary energy supply (TPES) was 5.7 x 10^{20} J, out of which bioenergy accounted for 10 %, making it the largest renewable energy source (International Energy Agency 2016; World Energy Council 2016).The particular interest drawn towards bioenergy can be attributed to: 1. The abundance of biomass (global bioenergy potential of land excluding agriculture lands, infrastructure, wilderness and forests is sufficient to meet one-third of the current global energy demand). 2. Availability of established infrastructure and processes and 3. Biomass remaining the sole feedstock for large scale liquid biofuels production (Guo et al. 2015). Global bioenergy consumption will continue increase in the following decades, and is estimated to supply as much as 30% of global energy in 2050 (Guo et al. 2015)

Sustainable processing of biomass to produce biofuels, biochemicals and bioenergy is referred to as biorefining (Saddler, J.N., Mabee, W.E., Simms, R. and Taylor 2011). Biorefineries can be considered as environment-friendly analogues of oil refineries. They are designed to minimize waste by efficient utilization of every fraction of the biomass, with separate conversion steps to process each fraction (Menon and Rao 2012). The value added bioproducts serve to offset biofuel production costs and improve profitability of the industry. By 2020, biorefineries are estimated to generate a global revenue of US$295 billion across the biomass value chain (King, Inderwildi, and Williams 2010).

Biofuels are liquid or gaseous fuels derived from biomass. Liquid biofuels include bioethanol, biobutanol, biodiesel and biopropanol, while biomethane and biohydrogen are common gaseous biofuels. Production of biofuels is expected to amass US$80 billion in revenue by 2020(King et al. 2010) and its sustainable production can reduce CO_2 emissions by 2.1 Gt annually (Eisentraut, Brown, and Fulton 2011). The contribution is expected to

be critical especially in transportation, fueling 27% of the sector's demand by 2050 (Eisentraut et al. 2011).

1.3   Bioethanol

Bioethanol is the largest produced liquid biofuel in the world. As a transportation fuel, it can be either used in blended form along with gasoline (gasohol) or as pure ethanol. Ethanol fermentation is one of the most mature and well established bioprocesses (Swana et al. 2011). With global production crossing 100 billion liters in 2016 (Renewable Fuels Association 2016), ethanol is expected to remain the most prominent and cost-effective biofuel for the foreseeable decades, with prices approaching that of gasoline(Eisentraut et al. 2011).

Bioethanol is produced via microbial degradation of carbohydrate rich substrates by yeast, bacteria and fungi. *Saccharomyces cerevisiae* is the most commonly used yeast in small scale as well as industrial bioethanol production. *S. cerevisiae* produces ethanol as its major fermentation product. The robust yeast can operate in a wide pH range, and can tolerate high levels of ethanol and other inhibitory compounds when compared to other fermentative microbes (Almeida et al. 2007; Lin et al. 2012; Prasertwasu et al. 2014; Tesfaw and Assefa 2014).

First generation bioethanol production utilizes edible crops such as, sugarcane, corn, wheat, rice and sorghum as feedstock. Majority of the ethanol plants across the world are first generation. Lignocellulosic materials and residues from agriculture and forests are used to produce second generation bioethanol, while third generation biofuels use algal feedstocks (Jambo et al. 2016).

US and Brazil together account for more than 85% of the world's ethanol, however, they employ largely different processes. While Brazil uses a year-round supply of sugarcane feedstock, North American ethanol industries produce ethanol from corn grain. In comparison, the process of corn grain to ethanol in US achieves only one-sixth of the energy efficiency (energy invested to energy returned) of sugarcane to ethanol (Goldemberg and Goldemberg 2007; Reid et al. 2015). The potential of increase in prices

of corn (Niewöhner et al. 2016; Rathmann 2010), a food crop, which along with wheat and rice contributes to two-third of the world's calorific intake, underlines the need for developing alternate energy crops for ethanol production.

1.4 Bioethanol feedstocks in Canada

If we consider Canada's scenario, most of ethanol in Canada is produced from corn. Sugarcane does not grow in Canada and sweet sorghum is not familiar to Canadian farmers. The climatic conditions in most arable regions of Canada demand short growth periods, which is the major challenge in developing a viable energy crop (Reid et al. 2015). Native feedstocks such as switchgrass, big and little blue stem, have shown promise (Mabee 2013). As large scale cellulosic ethanol facilities are not yet a reality in Canada (Reid et al. 2015) due to high production costs, the ethanol industry is reliant on corn.

1.5 Sugarcorn, a new Canadian energy crop

As an alternative to starchy corn grain and cellulosic feedstocks, 'sugarcorn', which are corn hybrids with high stalk sugar concentration were developed by researchers from Agriculture and Agri-Food Canada (AAFC, Ottawa, Ontario) (Reid et al. 2015).

Corn stalks are known to accumulate sugars up to 2-3 weeks following silking. The stalk sugars translocate to corn grain as the corn plant matures (Abendroth et al. 2011; Hume and Campbell 1972; Loomis 1945). Corn stalk sugar content is a genetically influenced trait, which can be tailored to be enhanced, for instance, hybrids resistant to stalk rot and cold injury are known to reach high stalk sugar concentrations(Reid et al. 2015).

In an attempt to enhance the potential of corn stalks as a biofuel feedstock, sugarcorn hybrids were developed using high stalk sugar corn varieties and select inbred lines by AAFC. Sugarcorn germplasm is adapted to short growth seasons in Canada (usually from May to September), particularly suited for the primary corn producing regions in southwestern Ontario and southern Quebec. Sugarcorn plants accumulate high concentration of sugars in the stalks, which peak in the weeks following silking. The plant can be harvested at this stage instead of waiting till the end for grain corn to mature, thereby saving time and agronomic resources. Following harvest, the juice extracted from the

sugarcorn stalks can be used as a medium rich in sugars, physiologically similar to sugarcane juice (Reid et al. 2015). The sugarcorn, due to its characteristics, has been identified as a viable biofuel crop for Canada, a theory which this research attempts to experimentally verify.

1.6   Thesis objectives

- Characterization of juice extracted from sugarcorn plants

- Bioethanol production using *Saccharomyces cerevisiae* cultivated in sugarcorn juice medium and improvement of ethanol yield (Shake flask experiments)

- Bioethanol production using the flocculating yeast strain *Saccharomyces cerevisiae* ATCC 26603 cultivated in sugarcorn juice medium and improvement of ethanol yield (Bioreactor studies)

- Evaluation of sugarcorn as a potential Canadian biofuel feedstock

1.7   Thesis overview

An overview of the thesis is presented below:

Chapter 1 Introduction

The background of the area of research, the need and motivation for the research, as well as its relevance to Canada's energy scenario, are elaborated. The objectives of the research are enlisted.

Chapter 2 Literature review

The rationale behind the use of bioethanol as a fuel, and an account of prominent feedstocks used for bioethanol production is provided. Different feedstock options available to Canada and the potential of sugarcorn is discussed. Ethanol production pathway in *Saccharomyces cerevisiae* and selection of strain of interest are outlined in brief

Chapter 3 Characterization of sugarcorn juice

Characterization of sugarcorn juice was performed to determine total solids, total dissolved solids, moisture content, ash content, density, viscosity, pH, N, C, H, O content, concentration of total carbohydrates, reducing sugars and fermentable sugars. Effect of autoclaving and activated carbon filtration on sugars in the juice was studied. Variation of stalk carbohydrates across different hybrids and age was analyzed.

Chapter 4 Bioethanol production using *Saccharomyces cerevisiae* cultivated in sugarcorn juice medium: shake flask experiments

Two sets of fermentation experiments were carried out in shake flasks. Commercial dry yeast was revived and cultivated using pure sugarcorn juice, diluted sugarcorn juice and sugarcorn juice supplemented with yeast extract. Consumption of carbohydrates and production of ethanol were studied, and yield values were compared. Inoculum enrichment via yeast extract supplement and improvement of yield constituted the second set of experiments. The variation of pH, as well as the concentration of sugars, viable cells and ethanol were followed as a function of time.

Chapter 5 Bioethanol production and yield improvement using Saccharomyces cerevisiae ATCC 26603 cultivated in sugarcorn juice: bioreactor studies

Preserved *Saccharomyces cerevisiae* ATCC 26603 cells were revived and cultivated in yeast malt medium. Concentration of cells was increased via growth in diluted sugarcorn juice. The inoculum was transferred to pure sugarcorn juice in a stirred tank bioreactor and fermentation was carried out for 72 hours. pH, viable cell count, dry cell weight, concentrations of carbohydrates, reducing sugars, fermentable sugars and ethanol were measured as a function of time. Ethanol yield was determined.

For yield enhancement, *Saccharomyces cerevisiae* ATCC 26603 cells revived in yeast malt medium were propagated for a longer incubation time (18 hours) using a diluted sugarcorn juice supplemented with yeast extract. The inoculum was transferred to pure sugarcorn juice in a stirred tank bioreactor and fermentation was performed for 72 hours. The experiment was repeated using yeast extract supplemented sugarcorn juice as fermentation medium. In addition to the above mentioned analyses, sugar estimation by brix

refractometer was studied and tested for use as a rapid and resource-efficient analytical method.

Chapter 6 Evaluation of sugarcorn as a potential Canadian biofuel feedstock

Typical growth and juice characteristics of sugarcorn were compared with that of the established feedstocks, sugarcane, energy cane and sweet sorghum. A process for bioethanol production from sugarcorn juice was suggested and compared to bioethanol production processes for corn and corn stover. A Canadian sugarcorn (CANSUG) biorefinery was proposed and the potential social, economic and environmental benefits are outlined.

Chapter 7 Conclusions and Recommendations

A summary of the key findings of the research is provided. Recommendations are made for future fermentation research using sugarcorn juice.

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Chapter 2

2   Literature review

2.1   Introduction

Depletion of fossil fuel reserves has triggered the worldwide surge to diversify our energy sources, with particular interest towards renewable resources, as they represent abundant, cleaner and seemingly inexhaustible energy available to be tapped, provided economically viable technologies are in place. Energy derived from biomass is expected to be a key contributor to the future energy sector, which, together with other renewables, can help supply energy to billions who lack it (Lin and Tanaka 2006). Biomass is also the only known source for liquid biofuels (Guo, Song, and Buhain 2015). As 40% of world's energy consumption is in the form of the liquid fuels, diesel and gasoline (Tan, Lee, and Mohamed 2008), liquid biofuels are considered a natural alternative to supplement conventional oil derived fuels.

2.2   Bioethanol as a renewable fuel

Bioethanol is a liquid biofuel produced from biomass via fermentation process. Unlike gasoline, ethanol can be completely burned, and is a cleaner fuel. It can be used either as an independent fuel or fuel enhancer (Sánchez and Cardona 2008).

The engine performance, emissions and material compatibility for the use of multiple ethanol-gasoline and ethanol-diesel blends have been studied (Agarwal 2007; Masum et al. 2013; Stein, Anderson, and Wallington 2013; Surisetty, Dalai, and Kozinski 2011; Thangavelu, Ahmed, and Ani 2016). Bioethanol is an oxygenated fuel, meaning it facilitates better oxidation of hydrocarbons, leading to lesser emissions of carbon monoxide and aromatics (Sánchez and Cardona 2008). It has been proven to limit particulate emissions in compression ignition and spark-ignition engines (Agarwal 2007). Though bioethanol has a relatively lower energy density, it has a higher octane number (113) than gasoline (87-93) (Renewable Fuels Association 2017). This, along with other desirable properties, such as, higher flame speeds, broader flammability range and higher heat of vaporization (Balat, Balat, and Öz 2008) when compared to gasoline, can improve

the engine efficiency of the blend (Balat et al. 2008; Renewable Fuels Association 2017), and enhance its suitability for use in modern engines that operate on higher compression ratios (Balat et al. 2008; Masum et al. 2013). In low blend form, ethanol from corn can help reduce GHG emissions by 3-4% when compared to pure gasoline. Ethanol can be blended with gasoline for up to 10-15% (E10 or E15) (Moriarty and Yanowitz 2015) without modifications to conventional automobile engines. On the other hand, flex-fuel motors in Brazil have engines with self-calibrating electronic control units which can adjust to fuel blends consisting of anywhere between 0% to 100% ethanol (Goldemberg 2008). Apart from its environmental benefits, use of bioethanol as fuel or as a low-cost octane-boosting additive for gasoline also has economic benefits, as it can create new jobs, support agriculture based economy and help meet the energy needs of developing countries that lack sufficient fossil fuel reserves.

2.3 Feedstocks for bioethanol production

Commonly employed feedstocks for bioethanol production across the world can be produced from sugar-based feedstocks (such as sugarcane, sweet sorghum and sugarbeet) that are rich in fermentable sugars, or feedstocks which are rich in polysaccharides sugars that are subsequently hydrolyzed to supply the fermentable sugars for ethanol production. The latter can be starch based feedstocks (such as corn, wheat and rice), or feedstocks containing a complex of cellulose, hemicellulose and/or other polysaccharides (lignocellulosic biomass). The biochemical reactions which may be involved in conversion of vegetative biomass to bioethanol are shown below (Cardona and Sánchez 2007; Guo et al. 2015; Sánchez and Cardona 2008).

Hydrolysis of starch, cellulose or similar polysaccharides to hexose sugars (glucose and fructose)

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6 \qquad \qquad \ldots (2.1)$$

Hemicellulose hydrolysis to pentose sugars (xylose, mannose, arabinose, etc.)

$$(C_5H_8O_4)_n + nH_2O \rightarrow nC_5H_{10}O_5 \qquad \qquad \ldots (2.2)$$
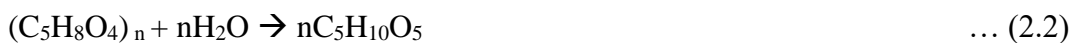
Hydrolysis of sucrose to glucose and fructose in *Saccharomyces cerevisiae*, catalyzed by the enzyme invertase.

$$C_{12}H_{22}O_{11} + H_2O \rightarrow C_6H_{12}O_6 + C_6H_{12}O_6 \qquad \qquad \dots (2.3)$$
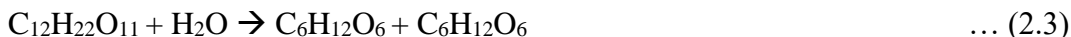
Conversion of hexoses and pentoses to ethanol via the following exothermic reactions (enthalpy of formation of ethanol, $\Delta_f H^{\circ} = -278$ kJ/mol)

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \qquad \qquad \dots (2.4)$$

$$C_5H_{10}O_5 \rightarrow 5C_2H_5OH + 5CO_2 \qquad \qquad \dots (2.5)$$

The reaction of ethanol production from hexose sugar has a maximum theoretical yield of 0.511 g ethanol per g of glucose utilized. Industrial first generation ethanol processes operate at > 90% (Gombert and van Maris 2015) of theoretical yield.

2.3.1 Sugar or Sucrose-based feedstocks

Most of the world's ethanol is produced from sugar crops. Conversion of sucrose to ethanol is more direct than starch to ethanol process. The disaccharide sucrose can be hydrolyzed using invertase enzymes secreted by yeasts to produce the readily fermentable sugars, glucose and fructose. The hydrolysis can also be partially achieved during the juice conditioning step. Commercial sucrose-based energy crops are sugarcane, sugarbeet and sweet sorghum (Cardona and Sánchez 2007; Zabed et al. 2014).

Sugarcane (*Saccharum officinarum*) is a C4 crop (capable of high efficient carbon fixation that avoids photorespiration, with a four carbon acids formed as its first product) and is the most important feedstock employed for ethanol production in tropical and subtropical countries. It is used for ethanol production either as sugarcane juice (as in Brazil) or as molasses (as in India), a non-crystalline by-product of sucrose purification. The sugarcane juice has a fermentable sugar content between 12-17%, more than 90% of which is composed of sucrose. The juice also contains organic nutrients and minerals in minute quantities which are conducive for microbial growth. The sugarcane based Brazilian

ethanol industry represents a robust renewable energy model, consistently achieving at least twice the renewable energy produced per fossil fuel consumed (RER) ratio than the maximum RER achieved by any US ethanol plant based on corn (Astolfi-Filho et al. 2011; Cardona and Sánchez 2007; Chum et al. 2014; Ergun and Ferda Mutlu 2000; Ghosh and Ghose 2003; Laluce et al. 2016; Zabed et al. 2014).

Sugarbeet (*Beta vulgaris*) is a crop which grows in temperate climatic conditions, particularly common in European countries. It requires 35-40% lesser water and fertilizer than sugarcane. The raw juice extracted from sugarbeet, as well as its by-product from sugar industry, beet molasses, are sources of fermentable sugars for ethanol production. Sugar beet juice contains 16.5% sucrose (Ogbonna, Mashima, and Tanaka 2001), and as around 85-90% of its sugars are fermentable, the juice can be used directly after pH adjustment, making it a convenient substrate (Balat et al. 2008; Dodić et al. 2012; Ergun and Ferda Mutlu 2000; Zabed et al. 2014).

Sweet sorghum (*Sorghum bicolor* L.) is a C4 crop, capable of growing in both temperate and tropical climatic conditions. The crop's grain as well as stalk juice can be used for ethanol production. Sweet sorghum juice has a fermentable sugar content of around 13 to 17% sugars, 10 to 14% of which consists of sucrose (Akbulut and Özcan 2008), with reducing sugars predominantly contributing the rest. The juice also contains micronutrients that can enhance yeast growth and metabolism (Cao, Gao, and Gu 2006). Sweet sorghum possesses several advantages when compared to other biofuel feedstocks, such as, high photosynthetic efficiency, high tolerance to drought and cold temperatures, lower nitrogen and fertilizer requirements, high carbon assimilation and short growth cycles (3.5 months) (Kim and Day 2011). Further, it has the highest stalk juice extractability (71.9%) (Kim and Day 2011) among all sugar crops, and the crop as a whole can potentially achieve an ethanol yield of up to 8000 L ha^{-1}, which is twice that of corn and 30% higher than sugarcane (Deesuth et al. 2012), making it a promising feedstock for bioethanol production (Andrzejewski et al. 2013; Barcelos et al. 2016; Kumar et al. 2013; Laopaiboon et al. 2009; Yu, Zhang, and Tan 2009; Zabed et al. 2014).

2.3.2   Starch-based feedstocks

Starch is a long chain homopolymer of D-glucose, which is hydrolyzed to obtain glucose syrup suitable for ethanol production, a process common in North America and Europe (Balat et al. 2008). Corn and wheat are the principal starch-based feedstocks. Other starchy feedstocks used for ethanol production include sweet potato, potato, cassava, rice and barely.

Corn (*Zea mays L.*) is a Mexican-native giant C4 grass, and the most grown grain in the Americas (Matsuoka et al. 2002). The stalk of the plant contains sugars, which on maturity accumulate in the corn kernel as starch (Abendroth et al. 2011; Taylor et al. 2010). The starchy kernel is either processed by dry milling, which aims to achieve maximum capital return per liter of ethanol, or wet milling, which uses higher capital investments to produce useful products from corn grain prior to ethanol fermentation step (Bothast and Schlicher 2005). Both processes use amylase enzyme to breakdown the complex starch network to glucose, which in turn is used to produce bioethanol. US is the largest producer of ethanol in the world, 95% of which is produced from corn starch (Renewable Fuels Association 2017).

Wheat (*Triticum aestivum*) is a grass grown for its grain, and is the most produced food crop in the world in terms of area harvested. The wheat grain used for ethanol processing is dried to about 14% moisture content, milled into the starchy flour, which is enzymatically hydrolyzed to glucose, which in turn is fermented to ethanol, similar to corn milling process (Mortimer and Elsayed 2004; Murphy and Power 2008).

2.3.3   Lignocellulosic feedstocks

As first generation ethanol uses food crops, the feedstock functionality is restricted. Agriculture and forest residues, fast growing trees and energy crops, which together represent world's most abundant and renewable resource, constitute the lignocellulosic biomass available for bioethanol production (Balat et al. 2008; Guo et al. 2015; Mohr and Raman 2013; Refaat 2012).

Currently the most common lignocellulosic feedstocks used for commercial ethanol production are by-products or wastes of sugar-based or starch-based ethanol processes and serve to improve the overall ethanol yield of the plant (Mortimer and Elsayed 2004). In some plants, they also are used to partially replace fossil fuels used to supply energy for these processes, thereby reducing emissions and facilitating a higher RER (Chum et al. 2014; Gallagher, Yee, and Baumes 2016; Mortimer and Elsayed 2004). Such feedstocks include, bagasses from sugarcane (Cardona, Quintero, and Paz 2010; Dasgupta et al. 2013; Pandey et al. 2000) and sweet sorghum (Barcelos et al. 2016; Goshadrou, Karimi, and Taherzadeh 2011), straws from wheat (Kaparaju et al. 2009; Karagöz and Özkan 2014; Mortimer and Elsayed 2004; Murphy and Power 2008) and sugar beet (Mortimer and Elsayed 2004), sugar beet pulp (Foster, Dale, and Doran-Peterson 2001; Zheng et al. 2013) and corn stover (Gallagher et al. 2016; Humbird et al. 2011; Luo, Van Der Voet, and Huppes 2009).

One advantageous strategy under research to generate lignocellulosic biomass has been to use low quality marginal land unsuitable for cultivation of food crops, for growing cellulose-rich grasses, crops and trees. Perennial energy crops and grasses such as switchgrass (Schmer et al. 2008; Tao et al. 2011), miscanthus (Heaton, Dohleman, and Long 2008), energy cane (a fiber rich variety of sugarcane) (Kim and Day 2011; Matsuoka et al. 2002; Qiu, Aita, and Walker 2012), giant reed (Lemons e Silva et al. 2015), napier grass (Liu et al. 2017) and shrub willow (Zamora, Apostol, and Wyatt 2014) can achieve high ethanol yields at low costs. Biomass from fast growing trees such as eucalyptus, black locust, pine and hybrid popular can generate wood chips rich in cellulose and hemicellulose (Balat et al. 2008; Bomgardner 2013; Guo et al. 2015).

Lignocellulosic biomass require pretreatment prior to fermentation, which help reduce material size and crystallinity, providing easier access for hydrolysis. Different pretreatment strategies are being extensively studied (Aditiya et al. 2016; Alvira et al. 2010; Refaat 2012).

The potential ethanol yields from sugar, starch and lignocellulosic feedstocks is show in Table 2.1.

Table 2.1 Potential ethanol yields from different feedstocks

Feedstock	Bioethanol yield potential L/ton
Sugarcane	70
Sugar beet	110
Sweet sorghum	60
Sweet potato	125
Potato	110
Cassava	180
Corn	360
Rice	430
Barley	250
Wheat	340
Cellulosic biomass	280

Source: (Balat et al. 2008)

Among the types of feedstocks discussed, sugar-based feedstocks contain readily fermentable sugars, which are preferable from the processing standpoint. The Table 2.2 and Table 2.3 summarize some key batch studies on bioethanol fermentations using juice extracted from the prominent commercial sugar-based feedstocks, namely sugarcane, sugarbeet and sugarcane.

Table 2.2 Literature on batch bioethanol productions from prominent fermentable juices

Feedstock (reactor volume)	Microorganisms	Sugars (g/L)	Supplements used (g/L)	Conditions	Time (h)	Yield (g/g)	Productivity (g/L/h)	Reference
Sorghum juice–sucrose (500 mL)	*Saccharomyces cerevisiae* NP01	280	Yeast extract, 3; peptone, 5	4.9, 30˚C, 200 rpm	60	0.51	2.01	(Laopaiboon et al. 2009)
Sorghum juice - molasses (500 mL)	*S. cerevisiae* NP01	280	Yeast extract, 3; peptone, 5	4.9, 30˚C, 200 rpm	40	0.45	1.52	(Laopaiboon et al. 2009)
Sweet sorghum juice (2L)	*S. cerevisiae* NP01	290	Yeast extract, 9:	4.8, 30˚C, 200 rpm, 2.5 vvm air for 4h	52	0.50	2.55	(Khongsay et al. 2012)
Sorghum juice (2L)	*S. cerevisiae* NP01	270	Yeast extract, 9; Zn^{2+}, 0.01; Mg^{2+}, 0.05; Mn^{2+}, 0.04	4.8, 30˚C, 100 rpm	48	0.49	2.51	(Deesuth et al. 2012)
Sugarcane juice (150 mL)	*S. cerevisiae AS2.1190* immobilized on sugarcane pieces	174	None	3.9, 30˚C	32	0.51	2.48	(Liang et al. 2008)
Sugarcane juice (250 mL)	*Kluyveromyces marxianus DMKU 3-1042*	220	0.5 g/L $(NH_4)_2$	5.0, 40˚C, 150 rpm	72	0.42	0.92	(Eiadpum, Limtong, and Phisalaphong 2012)
Sugarcane juice (250 mL)	*K. marxianus DMKU 3-1042* immobilized on silk cocoon	220	0.5 g/L $(NH_4)_2$	5.0, 40˚C, 150 rpm	72	0.44	0.89	(Eiadpum et al. 2012)

Table 2.3 Literature on batch bioethanol productions from prominent fermentable juices- continued

Feedstock (reactor volume)	Microorganisms	Sugars (g/L)	Supplements used (g/L)	Conditions	Time (h)	Yield (g/g)	Productivity (g/L/h)	Reference
Sorghum juice (250 mL)	*S. cerevisiae* CICC 1308 immobilized on calcium alginate beads	85-156 initial reducing sugars	None	4.0, 31˚C, 150 rpm	9-13	0.38-0.42	1.3-1.5	(Jin, Liu, and He 2012)
Sorghum juice (5 L)	*S. cerevisiae* CICC 1308 immobilized on calcium alginate beads	156 initial reducing sugars	None	4.0, 31˚C, 150 rpm	13	0.42	1.5	(Jin et al. 2012)
Sugar beet juice (2L)	*S. cerevisiae* IR-2 immobilized on loofa sponge	200	None	6.5, 30˚C, 200 rpm	-	0.37-0.41	6.5-11.1	(Ogbonna et al. 2001)
Sugar beet raw juice (300 mL)	*S. cerevisiae* KF-7	152	$CaCl_2 \cdot 2H_2O$, 1.0; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $(NH4)_2SO_4$, 0.5.	6.3, 30˚C	24	~0.32	~2.2	(Tan et al. 2015)
Diluted sugar beet thick juice (300 mL)	*S. cerevisiae* KF-7	200	$CaCl_2 \cdot 2H_2O$, 1.0; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $(NH4)_2SO_4$, 0.5.	9.1. 30˚C	48	~0.45	~1.9	(Tan et al. 2015)
Sugar beet thick juice (2L)	Commercial *S. cerevisiae*	25% w/w	None	5.0, 30˚C, 200 rpm	72	~0.38	-	(Dodić et al. 2009)

2.4   Canadian bioethanol feedstocks

Canada's energy user demand is expected to grow at a pace of 0.7% yearly till 2040 (National Energy Board of Canada 2016). A federal mandate implemented (December 15, 2010) under the *Canadian Environmental Protection Act*, in the form of Renewable Fuels Regulations, requires a renewable content (ethanol) of at least 5% in gasoline (volume basis). Provincial mandates, implemented as Renewable Fuel Standards (RFS), require either an equivalent (Alberta, British Columbia, Ontario and Quebec) or higher (Saskatchewan– 7.5% and Manitoba- 8.5%) renewable content in gasoline. In addition, British Columbia also has a Low Carbon Fuel Standard (LCFS) in place, which requires emission reductions within a defined period of time. The present estimated domestic production capacity of 1.775 billion liters of ethanol is not sufficient to meet the blending mandates. Consequently, Canada will continue to import ethanol, with total imports expected to be over 2 billion liters in 2017, almost entirely from US. The current incentive for domestic production of renewable alternatives to gasoline is 0.03 CAD$ per liter of ethanol, and is expected to sunset in the following years (Dessureault 2016; Moorhouse and Wolinetz 2016; Natural Resources Canada 2017a, 2017b).

In Canada, bioethanol is produced almost entirely from the grain crops, corn and wheat (Dessureault 2016; Sorda, Banse, and Kemfert 2010). In 2016, out of an estimated 13.2 million metric tons of corn grain (Statistics Canada 2017) produced in Canada, approximately 24.5% (Dessureault 2016) was used for fuel ethanol production. Corn processing for ethanol production has two major cost bottlenecks that lead to high production costs, one being the large amount of amylolytic enzymes, namely, α-amylase and gluco-amylase required to break down starch to glucose, and the other being high energy costs, mainly to achieve and maintain the high temperatures (140-180˚C) required for cooking (Balat et al. 2008). The potential of increase in prices of corn, due to its continued reliance by the ethanol industry is a growing concern (Rathmann 2010). This, along with the low energy efficiency of corn renders it unsustainable in the absence of federal incentives (Reid et al. 2015). As wheat is a starch-based feedstock and an important food grain, with a milling process closely resembling corn, it shares similar shortcomings.

Considering Canada's sustainable lignocellulosic biomass sources, native energy crops such as, switchgrass (*Panicum virgatum* L), Triticale (*Triticosecale Wittmack*) and Camelina (*Camelina saliva L. Crantz*), and the fast growing trees, Willow (*Salix spp*.) and poplar (*Populus spp*.) have shown promise. Agricultural residues available include wheat, barley and flax straw, while pine (*Pinus spp.*) is the most dominant tree in Canada's forests and a suitable source of forest residue. Large scale cellulosic ethanol, however, suffers from major challenges that result in high production cost, which include, 1. high feedstock prices (Mabee 2014; Mathew et al. 2014; Mussatto et al. 2010), 2. energy intensive distillation processes to separate low-titer ethanol from fermentation broth (Nikolić, Mojović, and Djukić-Vukovic 2013; Tesfaw and Assefa 2014) and 3. lack of sustainable pretreatment strategies (Mosier et al. 2005; Tao et al. 2011), as hydrolysis via thermochemical route has high capital costs and biochemical route involves use of expensive cellulases.

Tropical feedstocks such as sugarcane are not suited to Canadian conditions. While sweet sorghum can be grown in Canada, the crop is not familiar to Canadian farmers and early maturing cultivars are not sufficiently available (Reid et al. 2015). Further, Canada's climate necessitates short crop growth seasons, which is a major challenge to develop alternate feedstock to supplement grain ethanol.

2.5 Sugarcorn

In an attempt to reduce Canadian ethanol industry's reliance on corn grain, Reid et al., 2015 (Reid et al. 2015), researchers from Agriculture and Agri-Food Canada (AAFC), tested the adaptability of select varieties of corn genotypes, with potential for achieving high stalk sugars, to short growth seasons in Canada. The study also included commercial inbred lines developed by AAFC. Genotypic variation, along with the optimal selection and harvest times were investigated, for use to develop hybrids with high concentration of stalk sugars. The high stalk sugar corn hybrids or 'sugarcorn' were pressed to extract juice whose sucrose concentration was found to vary from 5.1 and 16.4 ˚Bx across the 39 genotypes evaluated in the study. Sugarcorn hybrids from locally bred inbred lines showed variability in sucrose as well as biomass, meaning, their traits can be tailored through appropriate breeding strategies to develop hybrids for ethanol as well as silage production. The

sugarcorn hybrids tested in the study were estimated to have the potential to achieve an ethanol yield of 3600 L ha^{-1}. In addition, sugarcorn hybrids were estimated to have a silage yield potential of 40 Mg ha^{-1}(Reid et al. 2015).

Sugarcorn is adapted to Canadian climatic conditions (Reid et al. 2015), suited for growth in the spring-summer temperatures of Ontario and Quebec (23.1 to 11.5˚C), the principal corn producing provinces. As shown in Figure 2.1, unlike growth of corn grain for ethanol production, the harvest need not be delayed until ear maturity. Sugarcorn can facilitate an earlier harvest, and a shorter growth cycle, as the plants can accumulate high concentrations of sugars in their stalk, which peak in the weeks following silking. The juice from the stalk, or sugarcorn juice (SCJ), can be used as sugary substrate similar to sugarcane or sweet sorghum juice, for ethanol production. The familiarity of the corn crop to Canadian farmers (Reid et al. 2015) is an added advantage.
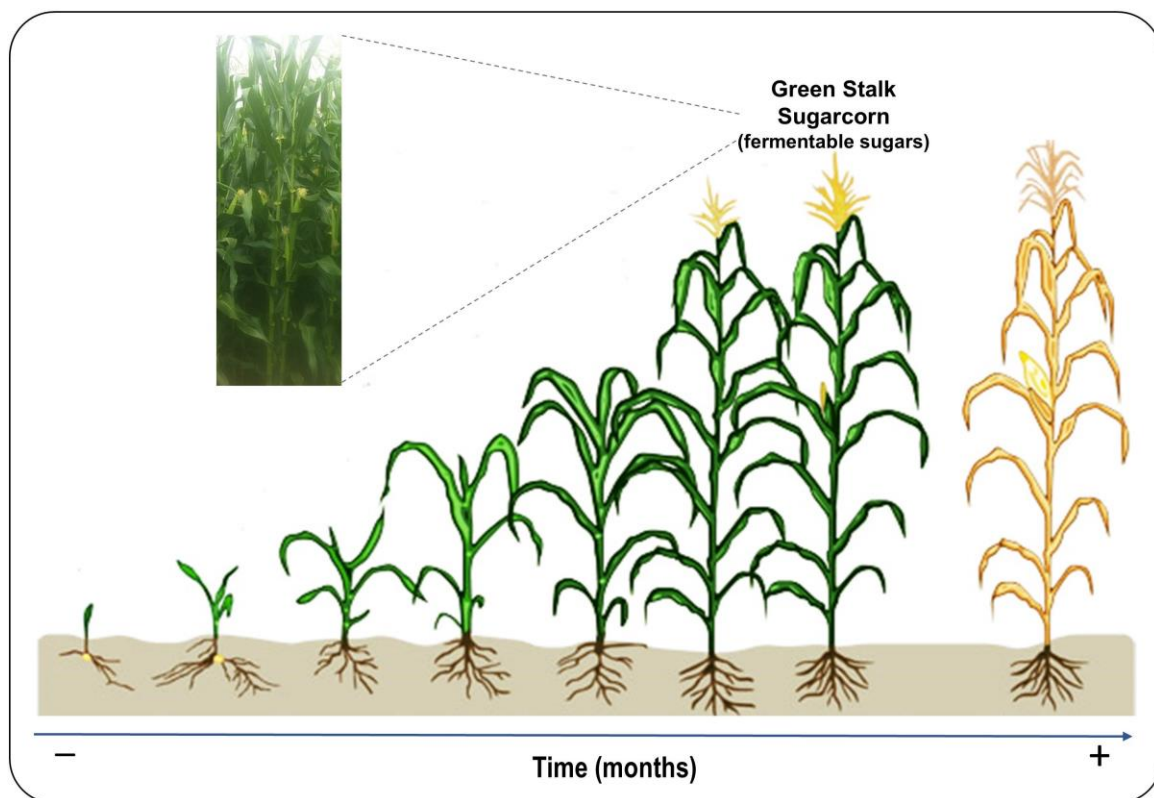
Figure 2.1 Harvest stages of grain corn and sugarcorn

2.6 *Saccharomyces cerevisiae*

Though there have been many microorganisms that have been studied for bioethanol production, including bacteria, fungi and algae, the yeast *Saccharomyces cerevisiae* still remains the most preferred species for bioethanol production.

Saccharomyces cerevisiae produces ethanol via the glycolysis pathway, which metabolizes one molecule of glucose to produce 2 pyruvate molecules. Under anaerobic conditions, the pyruvate is reduced to 0.511 ethanol by a reaction catalyzed by alcohol dehydrogenase, one which also produces 0.489 carbon di oxide. Two adenosine-triphosphate (ATP)

molecules generated during glycolysis are utilized by the cells for growth, which is crucial to drive the fermentation, without which ATPs may accumulate and inhibit phosphofructokinase, an important enzyme for glycolysis. Hence, cell growth is important to drive ethanol fermentation. Apart from CO_2, glycerol, organic acids and higher alcohols may be produced during the fermentation, which may inevitably result in production of intermediate compounds, thereby resulting in a lower ethanol yield than the theoretical (90-93%) (Bai, Anderson, and Moo-Young 2008).

Industrial corn and sugarcane based ethanol processes typically use very high gravity fermentations and cell recycle. Such fermentations, subject the yeasts to a variety of stresses, including low pH, osmotic stress, high ethanol concentrations, high temperatures, sulfites, contamination by bacteria, inhibitory compounds such as organic acids(Bai et al. 2008; Passoth 2014). Several strains of *Saccharomyces cerevisiae* have been metabolically engineered to further improve its tolerance to stresses and inhibitors, to reduce glycerol production, and to utilize a broader range of substrates including pentoses (Passoth 2014).

The flocculating yeast strain *Saccharomyces cerevisiae* ATCC 26603 was used for parts of this research to produce ethanol from sugarcorn juice. The strain has high productivity, and has been used to utilize a variety of different substrates, such as, sugar beet juice (Ogbonna et al. 2001), molasses(Haroldson and Bjrling 1981; Rose 1975), starch (Abouzied and Reddy 1986, 1987) and lignocellulosic (Kalyani et al. 2013; Lee et al. 2000; Sharma, Kalra, and Grewal 2002; Sharma, Kalra, and Kocher 2004) feedstock.

Flocculating yeasts have economic advantages in large scale, as they aid easier separation of biomass from broth, which lowers the energy expended in centrifugation, one of the most energy intensive processes (Nahvi, Emtiazi, and Alkabi 2002). Flocculation can also facilitate recycle and reuse of cells. *Saccharomyces cerevisiae* ATCC 26603   is osmotolerant and has been used to produce ethanol in concentrated substrates (Haroldson and Bjrling 1981; Rose 1975), which may favor potential process improvements and cost cuttings on scale up (Deesuth et al. 2012).

2.7 Conclusion

Sugar-based feedstocks offer significant advantages over starch-based feedstocks in terms of ease of processing. Sugarcorn was selected for this research due to its reported characteristics, suitability to Canadian conditions and estimated potential to reduce the ethanol industry's reliance on grain crops. Sugarcorn juice was characterized and evaluated for bioethanol production using the robust yeast, *Saccharomyces cerevisiae*.

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Chapter 3

3   Characterization of sugarcorn juice

3.1   Introduction

Bioethanol is widely considered a reliable renewable liquid fuel alternative to gasoline. Presently, commercial bioethanol plants are predominantly driven by sugar-based or starch-based feedstocks. The sugar-based feedstocks contain simple sugars which are readily utilized by fermentative microbes and converted to bioethanol in laboratory and industrial scale bioethanol processes. Several studies have utilized crops such as sweet sorghum (Barcelos et al. 2016; Jin, Liu, and He 2012; Khongsay et al. 2012; Kumar et al. 2013; Laopaiboon et al. 2009), sugarcane (Astolfi-Filho et al. 2011; Eiadpum, Limtong, and Phisalaphong 2012; Laluce et al. 2016; Liang et al. 2008) and sugarbeet (Dodić et al. 2009, 2012; Ergun and Ferda Mutlu 2000; Ogbonna, Mashima, and Tanaka 2001).

Sugarcorn is an emerging sugar-based feedstock for Canada, developed through extensive selective breeding by Agriculture and Agri-Food Canada. Sugarcorn is capable of accumulating high sugar concentrations in the stalks, whose levels peak in the weeks following silking. At this stage, juice can be extracted to supply readily available sugars. The sugarcorn plant has an efficient C4 photosynthetic pathway (Reid et al. 2015) similar to sugarcane and sweet sorghum, while it is also suited for Canadian climatic conditions and characteristically short growth seasons. These advantages, along with the familiarity of the corn plant to farmers, have led to it being suggested as a potential energy crop for biofuel production (Reid et al. 2015).

Juices extracted from established sugar-based feedstocks have been characterized and their physical, rheological properties as well as nutrient composition, well understood. The studies have helped optimize the composition of these substrates for microbial fermentations. The characterization depend on a multitude of factors, such as growth conditions, plant age, hybrid type and extraction strategies, meaning they are specific for each study. In order to assess the suitability of sugarcorn juice as a medium for biofuels production, the fundamental physical properties and nutrients in two different batches of

juice were determined, to get pointers towards required nutrient supplements, pre-processing steps, fermentation and analysis. An attempt was made to understand the variation of juice sugars across different plant ages and hybrid types of the sugarcorn plant.

Sugar-based feedstocks are susceptible to spoilage by microbes, as they are well adapted to the pH, nutrients and water activity of the juices extracted (Sobrinho, Cristina, and Pascoli 2011). A sterilization step involving heat treatment or filtration (Astolfi-Filho et al. 2011) are commonly employed to pretreat sugary juices, as is the clarification step that serves to reduce juice turbidity (Andrzejewski et al. 2013) of the sugary substrate. In this chapter, effect of autoclaving and carbon filtration are assessed as potential sterilization-clarification pretreatment strategies for sugarcorn juice.

3.2 Materials and methods

All materials and methods are same as described in their first mention in the thesis, unless otherwise specified.

Four different sugarcorn hybrids, namely, AAFC-SC-1, AAFC-SC-2, AAFC-SC-3, and AAFC-SC-4 were developed by Dr. Lana Reid, Dr. Malcolm Morrison and their research team at Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada. The hybrids were grown at Ridgetown, Ontario (42°26'N, 81°53'W) in 2014 and 2015. The sugarcorn plants were harvested 5 to 10 days after silking. The stalk of the plant was cut about 12-13 cm above the soil, the ears were removed and the whole plant was fed through a three-roller press to extract the juice. The sugarcorn juice extracted was initially stored at -20°C for few weeks, and later was transported to the University of Western Ontario in ice boxes. The sugarcorn growth, harvest, juice extraction and juice supply were done by Dr. Robert Nicol and Dr. Brandon Gilroyed, Center for Agricultural Renewable Energy and Sustainability. University of Guelph, Ridgetown campus, Ontario, Canada.

The juice was thawed at room temperature and filtered through cheesecloth to remove coarse residues. The filtered sugarcorn juice was then transferred to 1L plastic containers, sealed, weighed and stored at -20°C until use.

There were three sugarcorn juice batches, sugarcorn juice A (SCJ A) and sugarcorn juice B (SCJ B), harvested two weeks apart in September 2014, as well as, sugarcorn juice C (SCJ C), harvested on August 2015. Note that each batch contained a mixture of juice from all 4 sugarcorn hybrids. All characterization procedures were performed in triplicates, using SCJ A and SCJ B, unless otherwise specified. The errors are reported as standard error of the mean throughout the thesis, unless otherwise specified.

The total solids, total dissolved solids, moisture and ash content (all on weight basis) were determined using National Renewable Energy Laboratory (NREL) protocols (Sluiter, Hames, Hyman, et al. 2008; Sluiter, Hames, Ruiz, et al. 2008). Two sets of sugarcorn juice samples, one set filtered through 0.1 µm Whatman membrane, and another unfiltered, were dried to constant weight at 105°C to determine the percentage of total solids, total dissolved solids and moisture. Dried samples were burned in a muffle furnace set at 575°C and weighed to constant weight to determine the ash content in sugarcorn juice.

The density of the sugarcorn juice was estimated gravimetrically with an uncertainty of 1 mg using analytical balance and a 50 mL pycnometer. The calibration was performed with distilled water (20°C) standard. The dynamic viscosity of the sugarcorn juice at 25°C was measured using a Brookfield rotational viscometer (Model S LVDV-II+, Middleboro, USA), with a ULA spindle.

The pH of the sugarcorn juice was measured using a benchtop pH meter (VWR symphony SB70P, Beverley, USA). N, C, H and O content in the sugarcorn juice was determined using Flash EA 1112 Series elemental Analyzer (Thermoscientific, Waltham, USA) at Institute for Chemical and Fuels from Alternative Resources (ICFAR). The protein content of the juice was determined by Bradford method (Kruger 2009).

A Brix refractometer (Leica Auto ABBE, Buffalo, USA) with temperature compensation was used for rapid estimation of sugar content in the juice. Sucrose standard was used with distilled water serving as blank. Concentration of total carbohydrates was determined by phenol-sulfuric acid (PS) method (DuBois et al. 1956) and reducing sugars by dinitrosalicylic acid (DNS) method (Miller 1959). A UV-vis spectrophotometer (Thermo Fisher Scientific G10S, Madison, USA) was used for absorbance measurements involved

in both analytical methods. Concentration of sucrose, fructose and glucose in sugarcorn juice was determined by high performance liquid chromatography (Waters Alliance HPLC System, New Castle, USA) coupled with a refractive index detector. An XBridge Amide column (3.5 um, 4.6 x 250 mm) was used with 75/25 (v/v) Acetonitrile/Water + 0.2% v Triethylamine (TEA) mobile phase flowing at a rate of 0.6 mL/min. The samples were prepared by dilution with equal volume of 50/50 (v/v) Acetonitrile/Water and filtered through a 0.45µ filter.

Effect of autoclaving on sugars in sugarcorn juice was studied, for which SCJ A and SCJ B were taken separately in tightly sealed 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,

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. GAC used had an Iodine number of at least 1000 mg/g, and an effective pore size of 0.55 to 0.75 mm.

Variation of carbohydrates in sugarcorn juice C (SCJ C) across different hybrid types and plant maturity was investigated. The juice samples for this particular study were selected such that, the plants were 98 days old during harvest. An attempt was made to study the effect of age on stalk carbohydrates with limited sugarcorn juice samples from 98, 107 and 120 days old plants of AAFC-SC-3, and AAFC-SC-4. In terms of crop heat units (CHU), an agronomic energy term calculated from daily temperatures to represent crop development, 98,107 and 120 days of growth corresponded to 1907, 2140 and 2401 CHU (Nicol and Gilroyed 2016), respectively.

3.3   Results and discussion

Sugarcorn juice is a mild yellowish to brownish-green colored liquid (Figure 3.1) with fresh cut grass odour, closely resembling sugarcane juice, unsurprising , given the physiological (Reid et al. 2015) similarity between the two plants.

Figure 3.1 Sugarcorn juice filtered through cheese cloth

The physical and chemical properties of the juice, in general, varied distinctly between SCJA and SCJB as shown in Table 3.1 and Table 3.2. Moisture content in juice was over 90 wt.%, higher than that of sugarcane juice which is known to range between 78 and 86% (Sobrinho et al. 2011). Sugarcorn juice is slightly more acidic than sugarcane juice and sweet sorghum juice (Andrzejewski et al. 2013; Sobrinho et al. 2011). Sugarcorn juice samples had an ash content of 5.9 and 6.4 wt.%, comparable to that of corn stover (Demirbas 2010; Lizotte, Savoie, and De Champlain 2015), though ash content is known to vary based on factors such as soil type, hybrid, growth conditions, fertilizers used and maturity (Samson and Mehdi 1998; Soleymani and Shahrajabian 2012).

The carbon and hydrogen content in sugarcorn juice was about 3-4.5 %. There were large variations in hydrogen and oxygen content, as the solution was dilute and contained quickly settling solids. Sugarcorn juice samples analyzed had nitrogen and protein concentrations comparable with that of sugarcane juice and sweet sorghum juice (Andrzejewski et al. 2013; Sobrinho et al. 2011).

Table 3.1 Physical characterization of sugarcorn juice

Property	Sugarcorn juice A	Sugarcorn juice B
Total solids (wt.%)	9.44 ± 0.04	8.73 ± 0.01
Total dissolved solids (wt.%)	9.39 ± 0.03	8.10 ± 0.10
Moisture content (wt.%)	90.57 ± 0.04	91.90 ± 0.01
Ash (wt.%)	5.94 ± 0.12	6.44 ± 0.04
Specific gravity	1.04 ± 0.00	1.04 ± 0.00
pH	5.08 ± 0.02	4.89 ± 0.00

Table 3.2 Chemical composition of sugarcorn juice

Composition	Sugarcorn juice A	Sugarcorn juice B
Carbon (wt.%)	4.44 - 4.52	3.05 - 3.52
Hydrogen (wt.%)	4.44 - 4.52	6.09 -7.51
Oxygen (wt.%)	Not detected	7.27 - 41.92
Nitrogen (wt.%)	0 - 0.04	0.20 - 0.78
Protein (wt.%)	0.08 ± 0.0	0.09 ± 0.0

The variation of shear stress as a function of shear rate at 25˚C is provided in Figure 3.2.

For Newtonian fluids,

Shear stress, $\tau = \mu . \frac{du}{dy}$ … (3.1)

where μ is the dynamic viscosity and the differential term represents rate of the velocity gradient.

Non-Newtonian pseudoplastic and dilatant fluids follow power law,

Shear stress, $\tau = K . \left(\frac{du}{dy}\right)^{n}$ … (3.2)

where K and n are flow consistency and flow behavior indices, respectively. For dilatant fluids, the value of n is greater than 1 (McCabe et al. 2005). Taking logarithm and substituting the shear rate and shear stress values from the experiment, and performing linear regression analysis, we get K= 0.00071 Pa s^{-1} and n = 1.196 (R^2= 0.9808) for sugarcorn juice, at 25˚C. This non-Newtonian, dilatant (or shear thickening) behavior is unlike sweet sorghum juice, which is pseudoplastic (or shear thinning) (Akbulut and Özcan 2008) or sugarcane juice (Astolfi-Filho et al. 2011) ,which is Newtonian (shear stress proportional to rate of shear). However, measurements across different temperatures are required to understand better the viscosity and flow behavior of sugarcorn juice.

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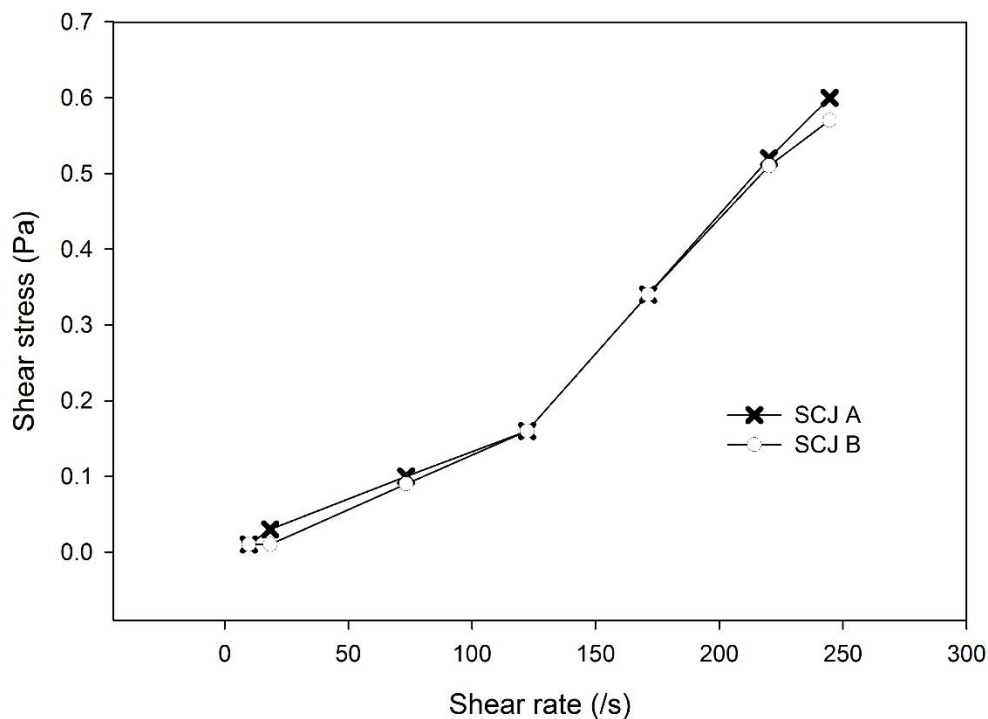
Figure 3.2 Plot of shear stress vs shear rate for sugarcorn juice at 25˚C

Brix is a measure of the grams of total solids in 100 grams of a given solution (Margalit 2012; Son et al. 2009). As more than 95% of solids in juices used for fermentation are sugars (mostly in dissolved form), brix refractometry is used as a rapid method for estimating sugar content in sugary substrates. The Brix values were 11.82 ± 0.09 °Bx for SCJ A and 10.21 ± 0.03 °Bx for SCJ B.

Concentration of total carbohydrates in sugarcorn juice samples ranged between 125 to 180 g/L. Variations in sugars may be attributed to difference in extraction conditions, hybrid types and plant maturity at the time of harvest (Reen and Singleton 1952). In SCJ A, the total carbohydrates was 145.1 g/L, reducing sugars accounting for 46% of the amount. SCJ B had a carbohydrate concentration of 101.5 g/L, 28% of which were reducing sugars (Figure 3.3).
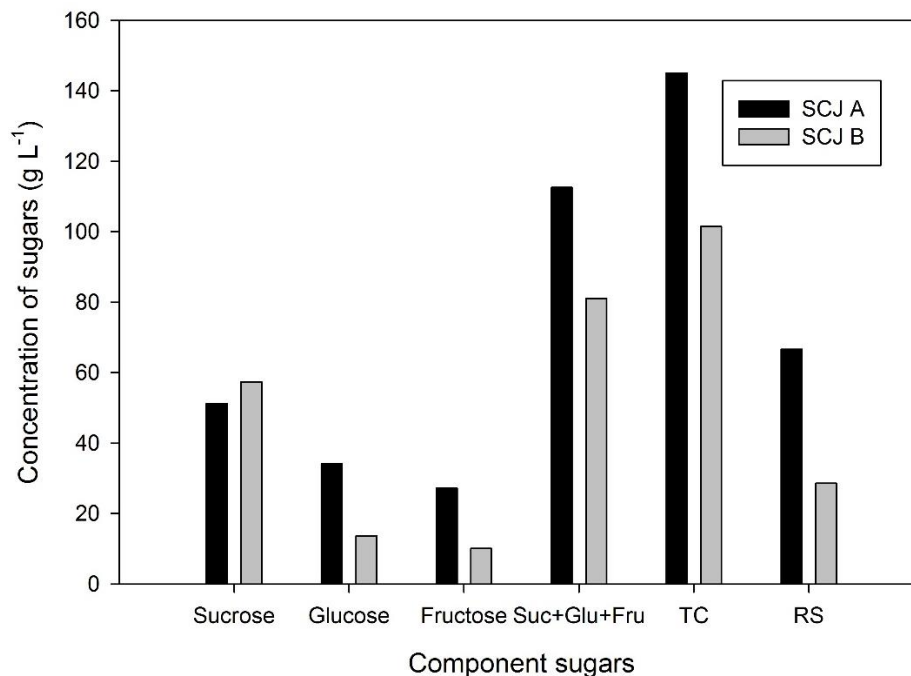
Figure 3.3 Sugar composition of sugarcorn juice

Peaks of fructose, glucose and sucrose measured for SCJ A and SCJ B are illustrated in Figure 3.4. Sucrose, glucose and fructose were the prominent sugars in both SCJ A and SCJ B, accounting for nearly four-fifth of the total sugars. These three sugars are fermented by yeast and their relative proportions are shown in Figure 3.5. The tetrasaccharide stachyose, the trisaccharide maltotriose were among the other sugars identified. Organic derivatives as succinic acid, methylmalonic acid, lactic acid and glycolaldehyde were also present in small amounts.
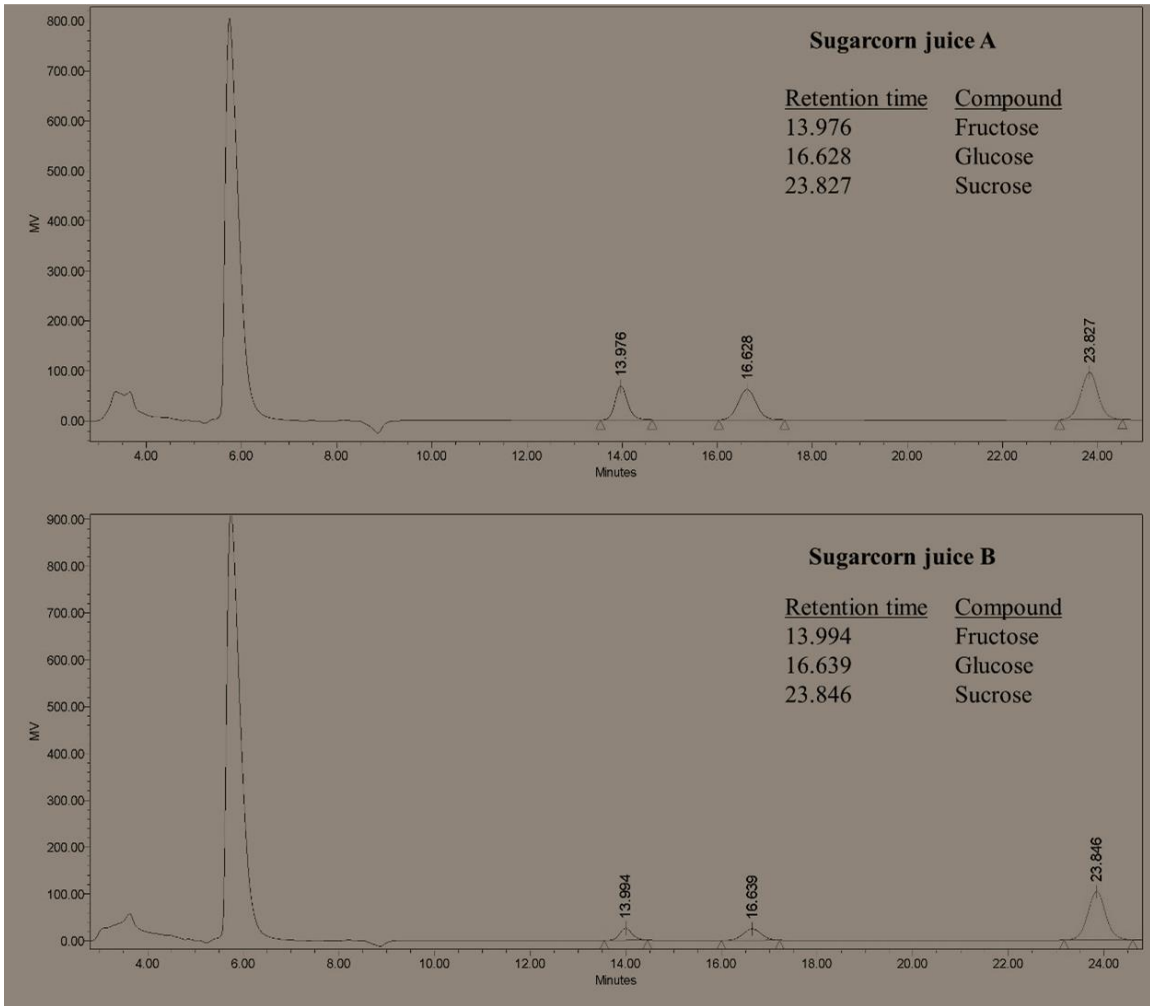
Figure 3.4 HPLC chromatogram for Sugarcorn juice
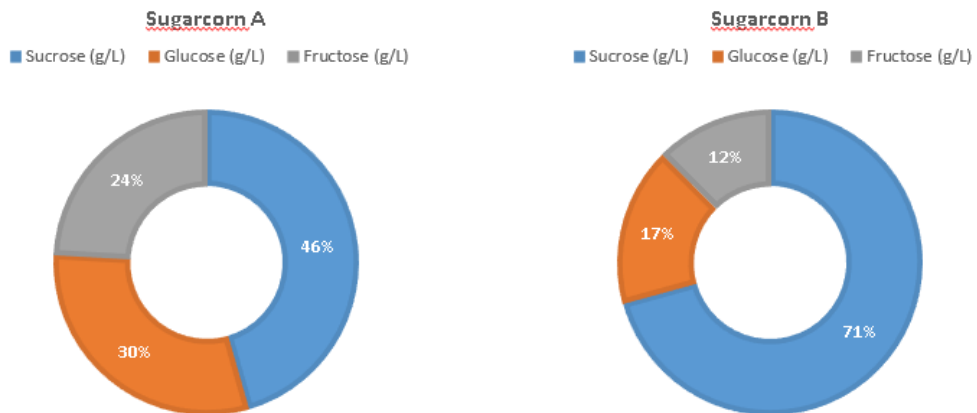
Figure 3.5 Relative proportions of fermentable sugars in sugarcorn juice

Juices with high sugar content such as sweet sorghum juice and sugarcane juice are susceptible to spoilage, hence treatments are done to limit microbial contamination prior to processing (Kumar et al. 2015; Quintero et al. 2008). These treatments also serve to clarify the juice by reducing turbidity. 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 3.6 and 3.7. Autoclaving causes hydrolysis of glycosidic bonds in sucrose, forming equimolar amounts of the constituent monosaccharides, fructose and glucose (Chauhan 2008; Martínez et al. 2014).Oligosaccharides and polysaccharides in the medium are also hydrolyzed, which explains the increase in reducing sugars observed. Further, as SCJ B contained more sucrose, it gained more reducing sugars when compared to SCJ A. Also, some monosaccharides already present in the medium can degrade (Wang and Hsiao 1995), which explains the reduction in carbohydrates on autoclaving. Individual sucrose, glucose and fructose concentrations measured by HPLC shown in Figures 3.8 and 3.9 agree with the above discussion, showing increase in amounts of glucose and fructose and decreased sucrose concentration on autoclaving.

Filtration through granular activated carbon has been used for clarification and purifying natural sugary juices prior to syrup formation or alcoholic fermentations (Urbanic 1982). The filtration of sugarcorn juice through a GAC bed caused visible de-colorization due to removal of pigments. 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 also showed large decrease in sugar concentrations as shown in the Figures 3.6 and 3.7. Activated carbon filtration was found to be disadvantageous at the dosage level used and optimization may be required if this has to be used as a pretreatment.
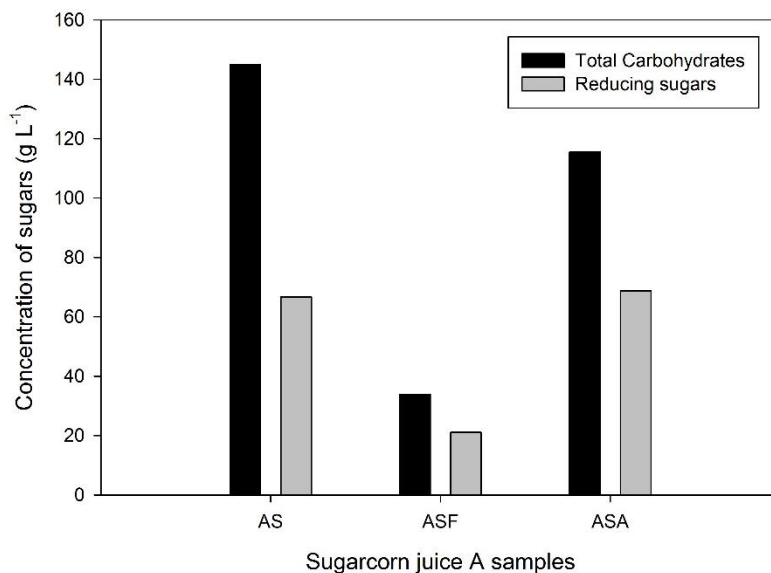
Figure 3.6 Effect of filtration and autoclaving on carbohydrates and reducing sugars in sugarcorn juice A (SCJ A) showing 1. AS- SCJ A, 2. ASF- activated carbon filtered SCJ A & 3. ASA- autoclaved SCJ A
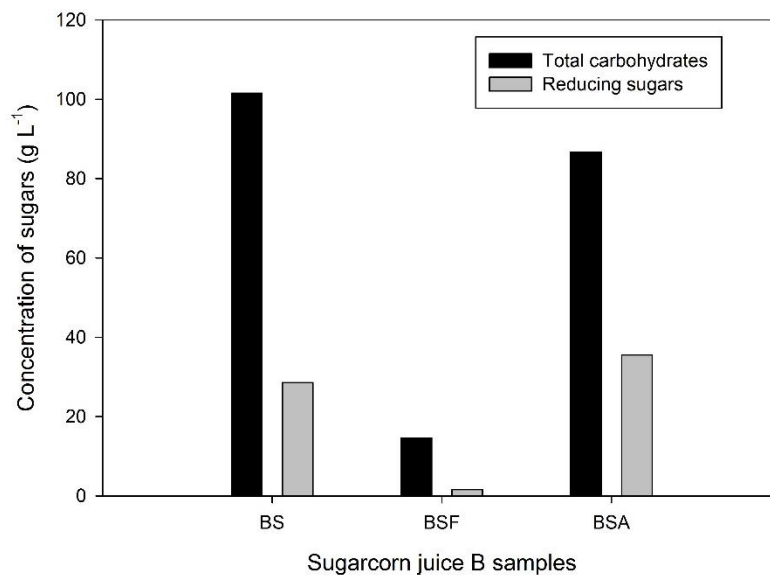
Figure 3.7 Effect of filtration and autoclaving on carbohydrates and reducing sugars in sugarcorn juice B (SCJ B) showing 1. BS- SCJ B, 2. BSF- activated carbon filtered SCJ B & 3. BSA- autoclaved SCJ B
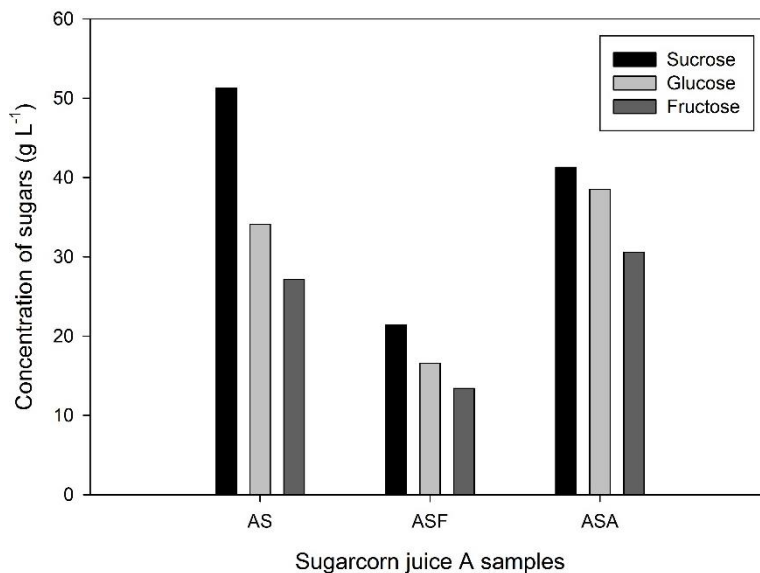
Figure 3.8 Effect of filtration and autoclaving on fermentable sugars in sugarcorn juice A (SCJ A) showing 1. AS- SCJ A, 2. ASF- activated carbon filtered SCJ A & 3. ASA- autoclaved SCJ A
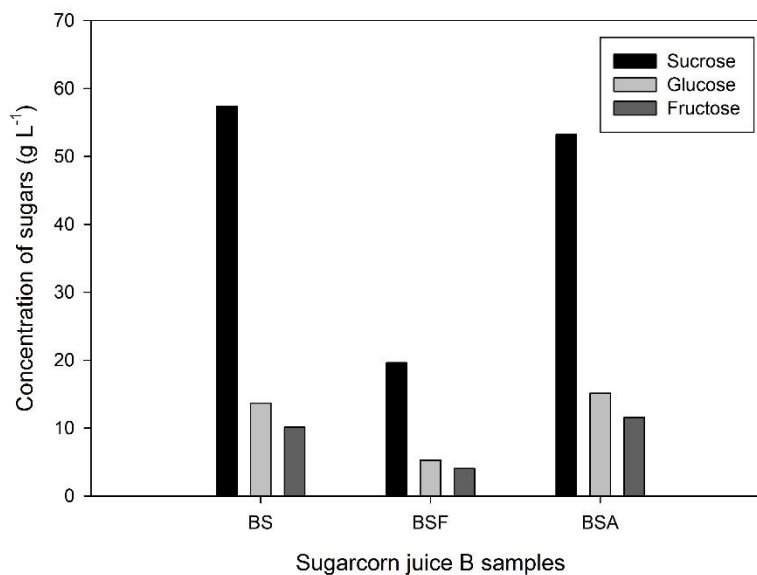
Figure 3.9 Effect of filtration and autoclaving on fermentable sugars in sugarcorn juice B (SCJ B) showing 1. BS- SCJ B, 2. BSF- activated carbon filtered SCJ B & 3. BSA- autoclaved SCJ B

The amount of carbohydrates in the sugarcorn juice varied across different sugarcorn hybrids and among plants of different ages during harvest (Figures 3.10 and 3.11). AAFC-SC-1 had the highest stalk carbohydrates concentration of 110.6 g/L with 46% reducing sugars. AAFC-SC-2 showed the highest proportion of reducing sugars which accounted for 54% of 90.2 g/L total carbohydrates. . A higher amount of reducing sugars as in the case of AAFC-SC-1 and AAFC-SC-2 might be favorable for microbial fermentations as they are readily metabolizable, energy efficient carbon sources for the cells. The total carbohydrates for all four hybrids were above 90 g/L. Earlier harvested plants had higher stalk carbohydrates, as translocation was minimized. For AAFC-SC-3 the effect of translocation on age was more prominent, with the carbohydrates dropping from 100.6 g/L in 98 days to 81.4 g/L (19% reduction) in 107 days and then to 64.9 g/L in 120 days (35% reduction). For AAFC-SC-4, the reduction in stalk sugars was 6% and 8% for the delayed harvests of 107 and 120 days, respectively.
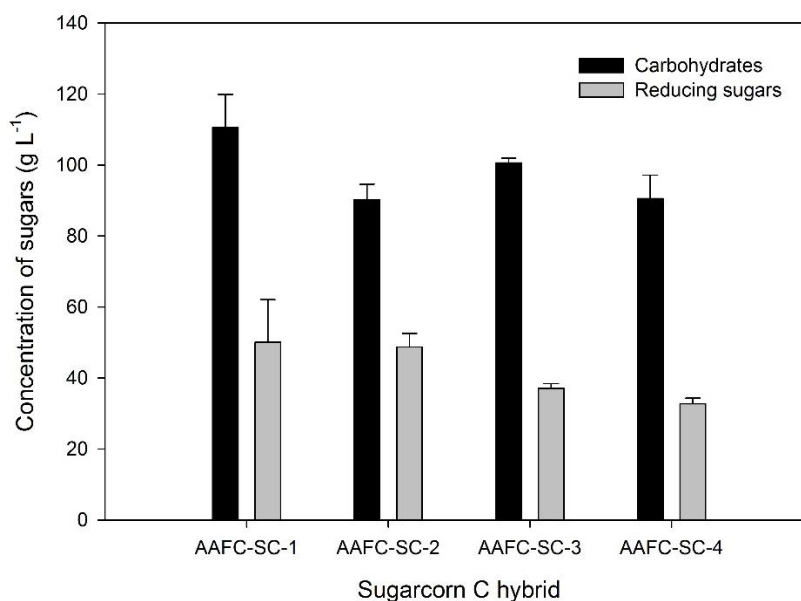
Figure 3.10 Variation carbohydrates and reducing sugars across the sugarcorn hybrids AAFC-SC-1, AAFC-SC-2, AAFC-SC-3 & AAFC-SC-4 (sugarcorn juice C). Error bars indicate standard deviation between the samples
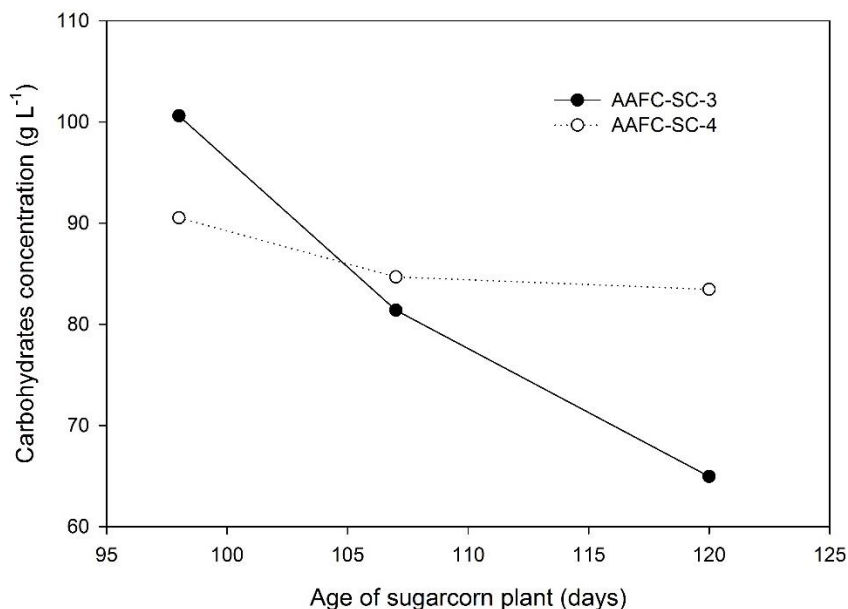
Figure 3.11 Variation of stalk carbohydrates with age of sugarcorn plant during harvest for the hybrids (sugarcorn juice C)

3.4 Conclusion

Fundamental physical and chemical characterization of sugarcorn juice was performed for its use as bioethanol production medium. While a high sucrose content for sugarcorn juice was expected, the medium also contained appreciable amounts of glucose and fructose, which are more energetically favorable substrates than sucrose for the cells. Together, the 3 fermentable sugars accounted for 80% of the total carbohydrates in the sugarcorn juice. Studies on effect of autoclaving may serve to account for differences in sugar compositions between natural juice and juice sterilized via autoclaving. Considering the wide variation in sugar characteristics of sugarcorn juice across different hybrids and growth seasons observed during characterization, fermentation experiments carried out in replicates in the chapters 4 and 5 were decided to be conducted using juice from same batch to limit the variables.

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Chapter 4

4 Bioethanol production using *Saccharomyces cerevisiae* cultivated in sugarcorn juice medium: shake flask experiments

4.1 Introduction

Bioethanol is an attractive renewable fuel and a cleaner, low-carbon alternative to gasoline. It has higher octane number, flame speeds, heat of vaporization compared to gasoline, with broader flammability limits (Balat, Balat, and Öz 2008; Renewable Fuels Association 2017). Due to these advantages, it has been widely used as an additive along with gasoline. Ethanol is the safest octane boosting additive for gasoline, which has displaced Methyl tert-butyl ether (MTBE), a toxic aromatic additive that contaminates air and water (Renewable Fuels Association 2017).

Canada's federal mandates require a minimum blend of 5% ethanol with gasoline(Natural Resources Canada 2017). Despite producing 1.7% of the world's bioethanol (Renewable Fuels Association 2016), the country's production capacity is insufficient to meet the domestic demand of 3.775 billion liters of ethanol (Dessureault 2016), more than half of which is presently supplied by imports. Out of the 17 ethanol plants currently operational, 8 use corn feedstock, 5 use other grains, with the other 4 being demonstration scale cellulosic ethanol facilities (Ethanol Producer Magazine 2017). Canada is reliant on grain based feedstock, whose energy efficiency is low when compared to sugar-based feedstock such as sugarcane (Goldemberg and Goldemberg 2007), which underlines the need for alternative energy crops.

Sugarcorn was developed as a new energy crop for Canada capable of reducing reliance on corn grain feedstock. As sugarcorn is a sugar-based feedstock, it has the potential to achieve energy efficiencies closer to that of sugarcane (Reid et al. 2015). The sugarcorn juice was characterized for the first time in our lab and found to contain a maximum of 145 g/L total sugars, most of which were readily fermentable sugars, predominantly sucrose, glucose and fructose. The physical properties and elemental composition of sugarcorn juice were also determined. As there were variations in characteristics across different batches

of sugarcorn juice, fermentations were carried out using sugarcorn juice from the same batch, discussed in this chapter.

Two shake flask fermentation experiments were conducted for bioethanol production using sugarcorn juice- one for initial evaluation of ethanol production using *Saccharomyces cerevisiae* grown in different sugarcorn juice medium compositions, followed by a fermentation with enriched inoculum to further improve the yield.

4.2   Materials and methods

Sugarcorn juice A (SCJ A) was used for ethanol fermentation experiments discussed in this chapter. Potato dextrose broth (PDB) and yeast extract (YE) were purchased from Sigma Aldrich Co. Fleischmann's active dry yeast was used for all fermentation experiments, and ethanol fermentations in this chapter were carried out in duplicate. A benchtop orbital shaker (Thermo scientific MaxQTM 4338, Marietta, USA) was used for incubation.

4.2.1   Bioethanol production using *Saccharomyces cerevisiae* cultivated in different sugarcorn juice media compositions

A 250 mL Erlenmeyer (EM) flask containing 100 mL PDB (24 g/L) was autoclaved, and aseptically inoculated with 0.5 g of dry Baker's yeast, followed by incubation at 30°C for 15 h and 200 rpm. To increase cell concentration, 10% (v/v) sample was used to inoculate 90 mL of autoclaved sugarcorn juice and incubated for 12 h with same conditions as before.

Twelve 250 mL EM flasks, each containing 90 mL medium were prepared with four different compositions, namely (1) pure sugarcorn juice, (2) sugarcorn juice with 3 g/L yeast extract, (3) sugarcorn juice with 9 g/L yeast extract and (4) sugarcorn juice medium diluted 1:1 with distilled water. The media were autoclaved, following which eight 10 mL samples from the inoculum culture were centrifuged at 5000 rpm for 15 minutes to prepare the inoculum pellets. Each pellet was transferred to each of the flasks containing sterile SCJ-based medium. The flasks were incubated for 72 h at 30°C and 200 rpm.

4.2.2 Yield improvement for bioethanol production using *Saccharomyces cerevisiae* cultivated in sugarcorn juice

Sugarcorn juice with 3 g/L yeast extract was used as the medium for both inoculum (working volume = 90 mL) and fermentation (working volume = 130 mL), with all other parameters such as strain revival medium, seed culture size and incubation conditions remaining the same.

4.2.3 Analysis methods

Broth samples from both bioethanol fermentations were centrifuged and amount of total carbohydrates and reducing sugars in the cell-free supernatants were estimated by PS method and DNS method respectively.

Supernatant samples from both fermentations were diluted, mixed and filtered through a 0.45 µm syringe filter (Acrodisc 13 mm, Pall), to prepare samples for determination of ethanol concentration. Each sample was then analyzed with a gas chromatograph (GC System Hewlett Packard 6890 Series) coupled to a flame ionization detector (FID), GC Chemstation (Agilent Technologies, Palo Alto, USA) and a HP-Innowax column (length 30 m, 0.25mm ID, and 0.25 µm film thickness) using helium as the carrier gas, at a flow rate of 1.5 mL/min. The GC operation was always started with an injector temperature and detector temperature set up at 220°C and 250°C respectively and with a split ratio of 1:25. 0.5 µL of each sample was injected in duplicate. The method for measuring ethanol concentration in GC involved setting up and maintaining the GC oven at 35°C for 7 minutes.

For the yield improvement fermentation, determination of viable *Saccharomyces cerevisiae* cells was carried out using well mixed broth sample serially diluted and plated on PDB-agar plates.

4.3   Results and discussion

4.3.1   Bioethanol production using *Saccharomyces cerevisiae* cultivated in different sugarcorn juice media compositions

The purpose of this experiment was preliminary evaluation of sugarcorn juice as an ethanol production medium for *Saccharomyces cerevisiae*. The effect of yeast extract as a nitrogen source for ethanol production was also studied.

4.3.1.1   Concentration of sugars

The key component sugars in sugarcorn juice are sucrose, glucose and fructose. Due to catabolite repression, *Saccharomyces cerevisiae* when grown in complex media such as SCJ, consume other carbon sources only after the glucose in the medium is depleted. Catabolite repression can give rise to 'Crabtree effect', a phenomenon due to which yeasts ferment sugars to ethanol even in aerobic conditions. Crabtree effect can result in high levels of ethanol, which apart from killing competing microbes, can also serve as carbon source for the yeasts in the presence of oxygen (Marques et al. 2016), during sugar stress.

All undiluted media compositions prepared had concentration of carbohydrates prior to fermentation ranging between 103 to 119 g/L, and the corresponding reducing sugars in the range of 84 to 89 g/L as shown in Figure 4.1. Yeast extract is a complex nitrogen supplement and an excellent source of amino acids. It has components like adenine, lactose and trehalose which play key roles in protein synthesis and cell growth (Zhang et al. 2003). Consequently, *Saccharomyces cerevisiae* grown in SCJ media supplemented with YE exhibited rapid consumption of reducing sugars, all of which were utilized in 18 h. For pure and diluted SCJ media, complete reducing sugar depletion was achieved in 24 h.

A similar trend was observed while studying carbohydrate concentration, with a more prominent difference in consumption rates between the broths with and without yeast extract. *Saccharomyces cerevisiae* cells grown on SCJ supplemented with 3 g/L and 9 g/L yeast extract showed 96% and 90% consumption of sugars, respectively in 18 h.
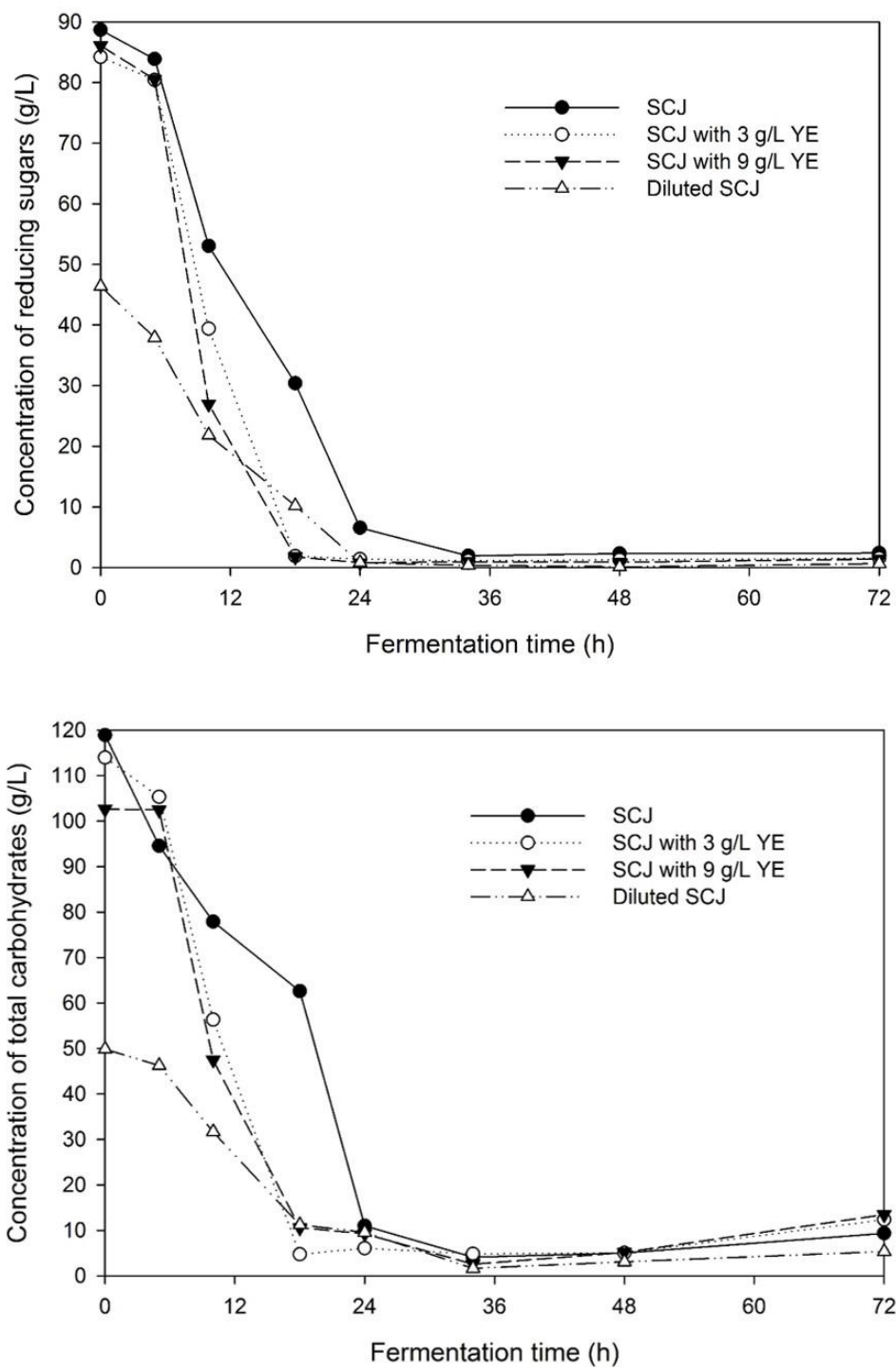
Figure 4.1 Variation of Concentration of reducing sugars (top figure) and carbohydrates (bottom figure) as a function of time for Fermentation of *Saccharomyces cerevisiae* **grown in pure SCJ, SCJ with 3 g/L YE, SCJ with 9 g/L YE and 0.5X diluted SCJ**

On the other hand, it took 24 h for pure SCJ medium to achieve 90% depletion of sugars. In 24 h, the total carbohydrate concentration dropped below 10 g/L in all media.

Given that sugars were nearly depleted by 24 h, the rise in carbohydrates between 34 h to 72 h for all SCJ media compositions may indicate a switch in the metabolism from fermentative to aerobic, characterized by consumption of ethanol (Marques et al. 2016) by the *Saccharomyces cerevisiae* cells. This may have also been due to lower accuracy of PS method at low sugar concentrations.

4.3.1.2 Ethanol concentration

Sugarcorn juice proved to be a suitable medium for ethanol production, even without additional supplementation, producing a maximum of 29.8 g/L ethanol in 34 h as shown in Figure 4.2, achieving a productivity of 0.63 g/L/h and a yield of 0.26 g/g. The diluted SCJ medium was able to produce 8.7 g/L ethanol in the same time, with a yield of 0.18 g/g, suggesting the sugar concentration might have been too low for the yeast cells, resulting in lower ethanol yield. The flasks with SCJ with YE, showed steeper increases in ethanol concentration in the first 18 h. The ethanol titer reached nearly 15 g/L and 20 g/L for medium with 3 g/L and 9 g/L YE, respectively. For SCJ medium with 3 g/L YE, ethanol concentration reached a peak value of 30.3 g/L in 72 h, with a yield of 0.27 g/g, the highest among the SCJ media compositions evaluated.
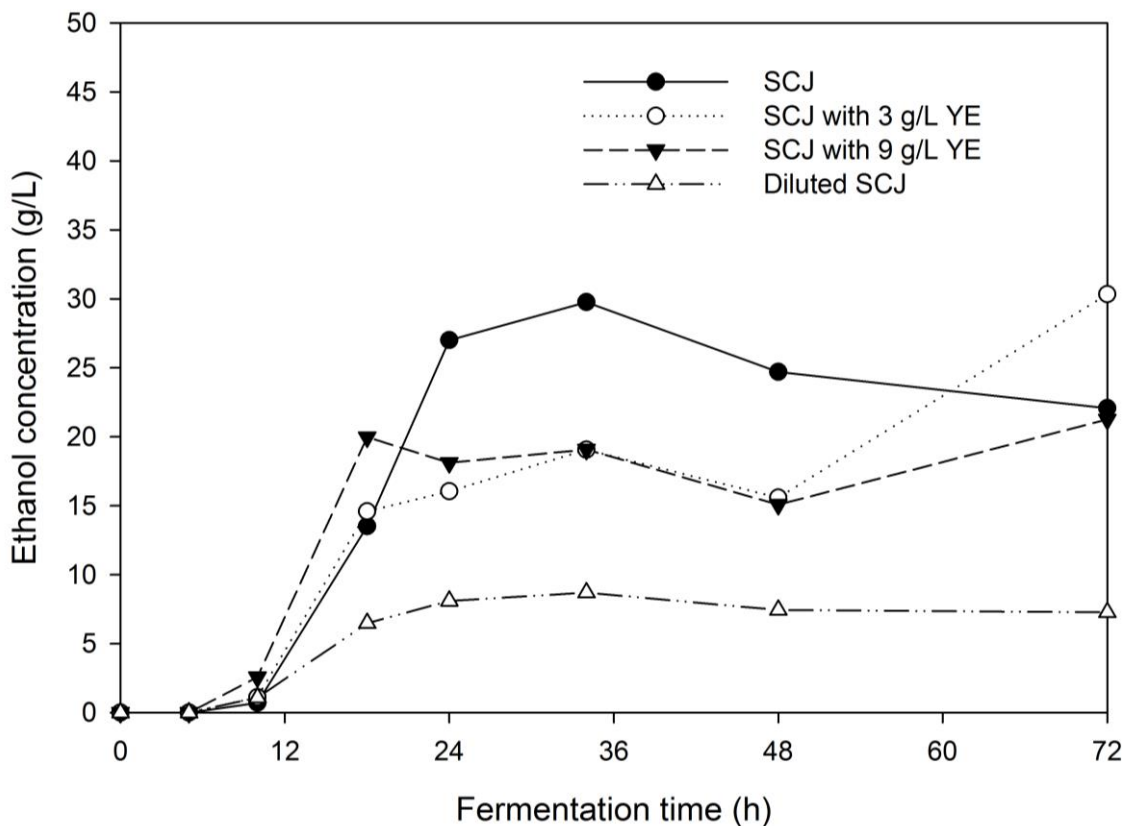
Figure 4.2 Variation of ethanol concentration as a function of time for Fermentation using *Saccharomyces cerevisiae* grown in pure SCJ, SCJ with 3 g/L YE, SCJ with 9 g/L YE and 0.5X diluted SCJ

4.3.2   Yield improvement for Bioethanol production using *Saccharomyces cerevisiae* cultivated in sugarcorn juice

4.3.2.1   pH variation

Yeasts have the tendency to maintain their intracellular pH within optimal levels irrespective of changes in extracellular pH (Narendranath and Power 2005). As most of the enzymes in *Saccharomyces cerevisiae* are active in the slightly acidic range, the initial broth pH of 5.3 was conducive for the yeast cell metabolism, ensuring active sugar utilization and ethanol production. There was steep decrease in pH until 24 hours, due to weak acids produced as by-products of sugar metabolism. The fall in pH was more gradual

from then on, until 48 h mark, when the ethanol production had slowed down and stabilized around 3.9. The variation of pH during fermentation is presented in Figure 4.3.

4.3.2.2   Viable cell concentration

The viable yeast cell count plotted as a function of time is illustrated in Figure 4.3. The maximum cell concentration of 1.42×10^8 CFU/mL was reached at 34 h. As almost all utilizable sugars in the medium had been consumed, the cell count declined gradually to 8.65×10^7 CFU/mL towards the 72 h. The specific growth rate of *S. cerevisiae* grown in sugarcorn juice medium supplemented with YE, was calculated based on its direct correlation with the linear part of the logarithmic growth curve of the viable cell concentrations as $\mu = 0.43$ /h

$$\ln \frac{N}{N_0} = \mu(T - T_0) \quad \text{(Neidhardt, Ingraham, and Schaechter 1990)} \quad \dots (4.1)$$

where N and N_0 are final and initial viable cell counts and T and T_0 are the corresponding time values.
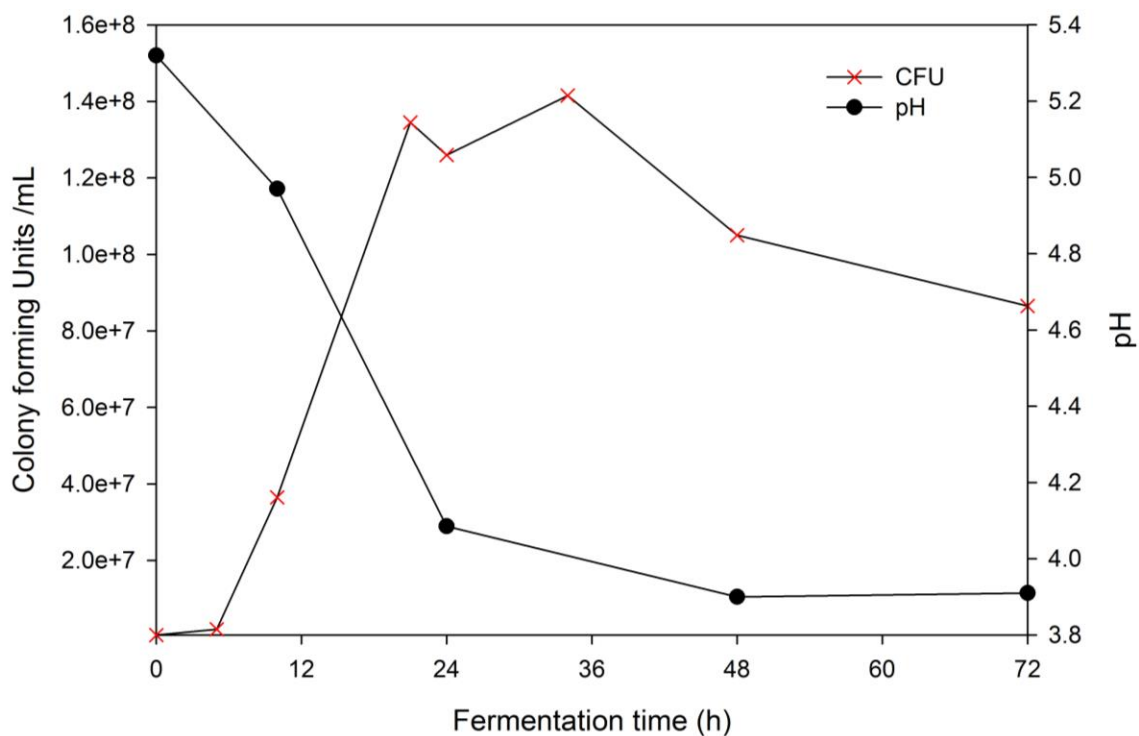
Figure 4.3 Variation of pH and CFU with time for *Saccharomyces cerevisae* cultivated in SCJ-YE medium

4.3.2.3 Concentration of sugars

The variation of sugar concentration during the ethanol fermentation is illustrated in Figure 4.4. The total carbohydrate content in broth samples during the start of fermentation was determined as 110.6 g/L, of which the concentration of reducing sugars was estimated to be more than 90 g/L. Most of the reducing sugars were consumed by yeast in the first 24 h, reaching a value of 2.3 g/L, followed by a plateau in reducing sugar consumption around 1.7 g /L, which may suggest presence of few simple sugars, which the strain of *S. cerevisiae* was not able to metabolize. The carbohydrate concentration reduced sharply to a value of 8.63 g/L in 34 h. There was a slight increase in rate of consumption of carbohydrates between 24 h and 34 h which may indicate lack of accuracy of PS method at low sugar concentrations.

4.3.2.4   Ethanol concentration

As suggested by Figure 4.4, ethanol was produced at a steady rate from the start of the fermentation and crossed 20 g/L in 24 h. Between 24 h and 34 h, rate of ethanol production was further enhanced, reaching 36.5 g/L in 34 h. This trend corresponds to a sudden increase in concentration of viable cells, as well as consumption of carbohydrates. Following this, the increase in ethanol concentration was more gradual, reaching a maximum value of 45.6 g/L following 72 h of fermentation. YE addition in inoculum to supplement SCJ, resulted in high cell concentration in inoculum, increasing the ethanol yield by 55% to 0.41 g/g and productivity by 51% to 0.63 g/L/h as shown in Figure 4.5.
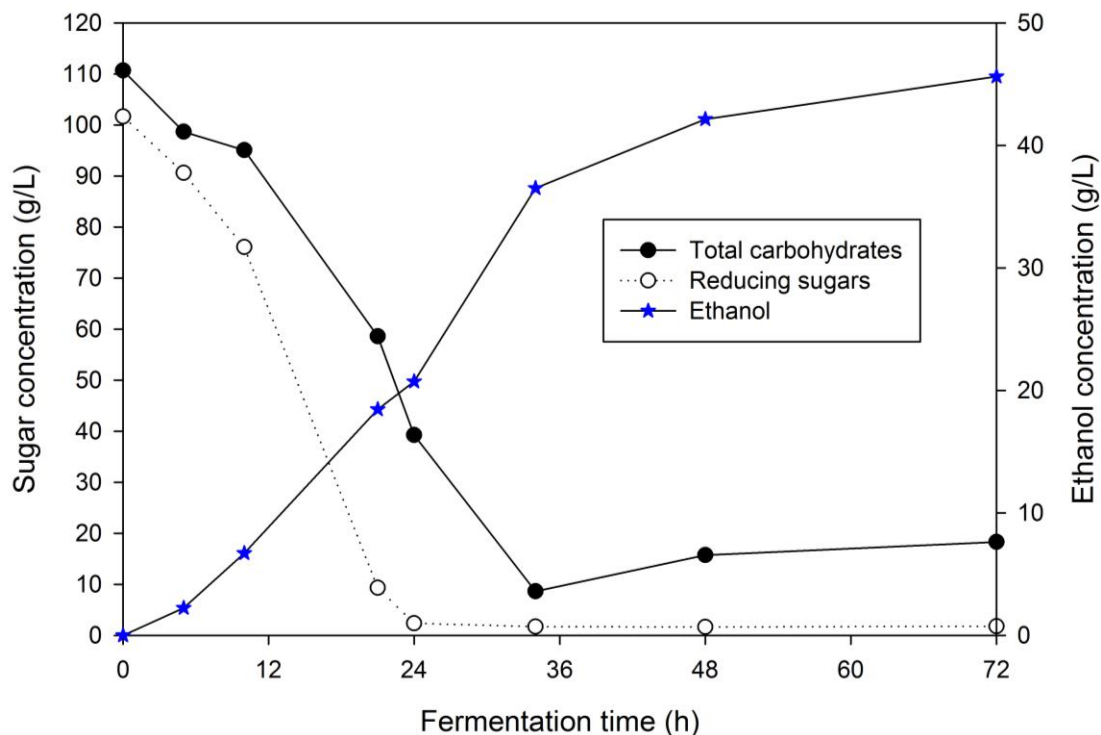
Figure 4.4 Concentration of carbohydrates, reducing sugars and ethanol as a function of time for *Saccharomyces cerevisiae* cultivated in SCJ-YE medium
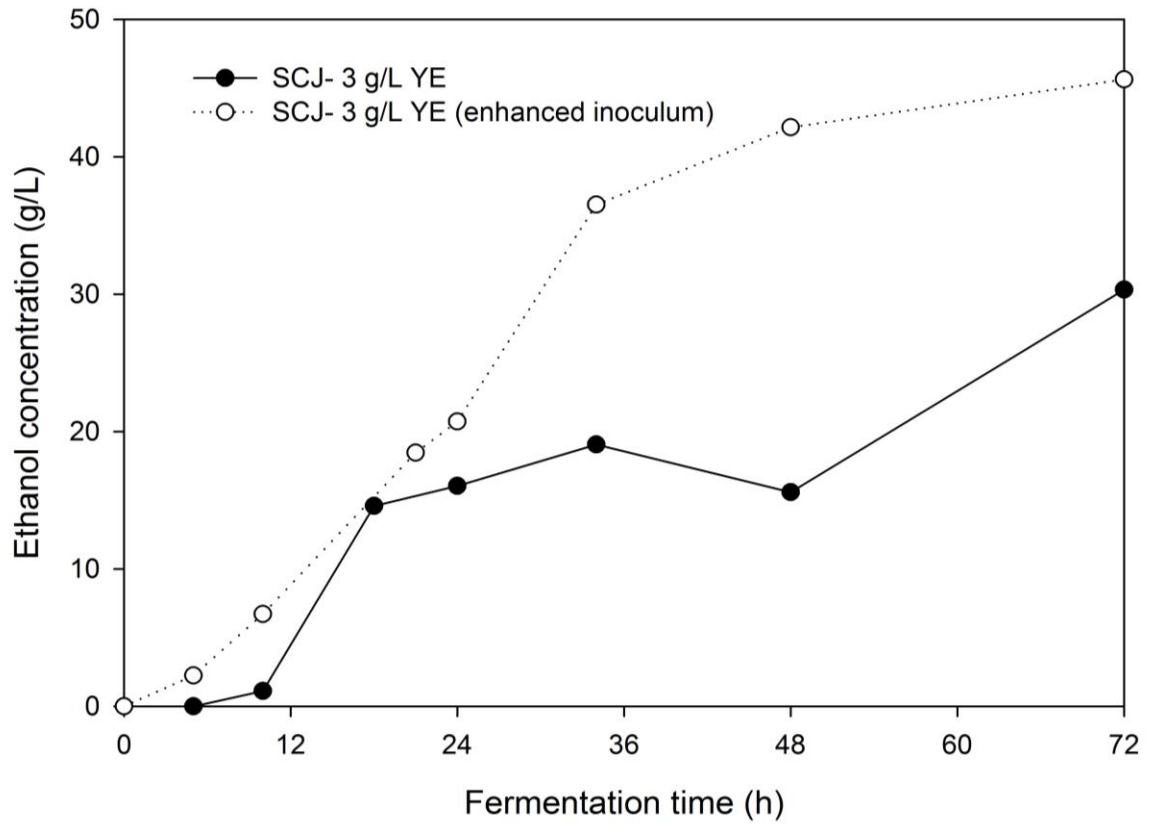
Figure 4.5 Comparison of ethanol production with and without yeast extract supplementation in the inoculum

4.4   Conclusion

For the first time, sugarcorn juice was successfully tested as an ethanol production medium for growth of *Saccharomyces cerevisiae*. Among the media compositions studied, pure sugarcorn juice achieved an ethanol concentration of 29.8 g/L ethanol in 34. The results of the yield improvement experiment underline the impact of yeast extract as a nitrogen supplement for the juice, in strengthening the inoculum and to achieve higher ethanol concentrations during fermentation. A maximum of 45.6 g/L ethanol, corresponding to a yield of 0.41 (g ethanol/g carbohydrates) was achieved in 72 h for the yield improvement experiments using sugarcorn juice medium in shake flasks.

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Chapter 5

5   Bioethanol production and yield improvement using *Saccharomyces cerevisiae* ATCC 26603 cultivated in sugarcorn juice: bioreactor studies

5.1   Introduction

Bioethanol is the largest produced liquid biofuel in the world with global production exceeding 100 billion liters in 2016 (Renewable Fuels Association 2016). Bioethanol is expected to remain the most prominent and cost-effective biofuel for the foreseeable decades, with prices approaching that of gasoline (Eisentraut, Brown, and Fulton 2011).

For Canada, a federal mandate implemented in 2010 requires a blend of a minimum of 5% ethanol in gasoline across the country, with provincial ethanol mandates implemented subsequently requiring between 5-8.5% ethanol blend (Natural Resources Canada 2017). The Canadian bioethanol industry is heavily reliant on corn grain feedstock (Reid et al. 2015). In 2016, out of an estimated 13.2 million metric tons of corn grain (Statistics Canada 2017) produced in Canada, nearly one-fourth was used for fuel ethanol production (Dessureault 2016). However, the corn grain to ethanol process has a lower conversion efficiency (Goldemberg and Goldemberg 2007; Reid et al. 2015), due to the enzyme and energy intensive processing required for the breakdown of the starchy grain. Also, as Canada's domestic production capacity is short of the current demand for bioethanol (Dessureault 2016), diversification of feedstocks may be a beneficial strategy.

Sugarcorn was developed by Reid et al, from Agriculture and Agri-Food Canada (AAFC) and suggested as a potential Canadian biofuel crop in their paper published in 2015 (Reid et al. 2015). Sugarcorn are corn hybrids which are tailored to suit Canadian climatic conditions, achieving high concentration of stalk sugars, whose amounts peak in the weeks following silking of the plant (Reid et al. 2015). Similar to sugarcane and sweet sorghum, a sugar rich juice can be extracted from the stalk that can facilitate a more direct fermentation process (Reid et al. 2015), when compared to grain ethanol. Instead of waiting for kernel maturity, it can facilitate an earlier harvest. Also, as Canadian farmers are

familiar to growing corn, sugarcorn was proposed as a viable feedstock capable of reducing dependence on corn grain feedstock (Reid et al. 2015).

Following characterization of sugarcorn juice and production of bioethanol using sugarcorn juice in shake flasks, the fermentation was tested in a stirred tank bioreactor. It was observed during the previous fermentations, that some of the reducing sugars were not consumed by the commercial baker's yeast used. This chapter of the research discusses the fermentation studies in bioreactor using a flocculating *Saccharomyces cerevisiae* strain which is known to produce ethanol with an ability to utilize a variety of carbon sources. As the initial objective was to produce ethanol and study the kinetics, sugarcorn juice was used for fermentation as such without any additional supplementation.

Certain process improvements were applied to the previous kinetic study, in order to enhance the ethanol yield. Aeration had to be reduced to favor ethanol production, which however may potentially impact cell concentrations. In order to compensate this, inoculum preparation was tinkered to increase cell concentration. A nitrogen source was required for improvement of ethanol yield, for which yeast extract was used. Based on promising results from shake flask experiments, yeast extract was used for inoculum enhancement of both SCJ compositions tested for the yield improvement study carried out in the bioreactor.

5.2   Materials and methods

5.2.1   Bioethanol production using *Saccharomyces cerevisiae* ATCC 26603 cultivated in sugarcorn juice

Sugarcorn juice C (SCJ C) was used for the fermentation experiment. A freeze dried vial of *Saccharomyces cerevisiae* ATCC 26603, the strain of interest, was purchased from American type culture collection (ATCC). Yeast malt (YM) broth (composed of peptic digest of animal tissue- 5 g/L, yeast extract- 3 g/L, malt extract- 3 g/L and dextrose- 10 g/L) was purchased from Sigma Aldrich.

5.2.1.1   Strain preservation

Two loops of freeze-dried yeast were aseptically withdrawn, each of which was rehydrated using 5 ml of sterile water at 30°C in a test tube. This was followed by successive sub-

culturing at 30°C using 100 ml YM medium (pH = 6.2), first for 12 h without agitation and later for another 24 h at 100 rpm in an incubator. The cells (mean viable cell count = 1.37 x 10^6 CFU /ml) were mixed with equal volume of 30% glycerol in cryovials and frozen at -84°C until use.

5.2.1.2 Strain revival

YM medium (200 ml each, pH adjusted to 6.2 using 2M sulphuric acid) was prepared in two 500 ml Erlenmeyer flasks and autoclaved at 121°C for 16 minutes. A cryovial of cells was transferred aseptically to inoculate each EM flask containing sterilized YM medium. Incubation was carried out at 30°C for 12 hours and 200 rpm.

5.2.1.3 Inoculum preparation

Sugarcorn juice, earlier frozen at -20°C was thawed at room temperature. The total sugar concentration was determined as 109 g/l. The medium for inoculum was prepared by diluting sugarcorn juice with an equal volume of distilled water, reducing the initial sugar concentration to 54.5 g/l. Two 500 ml Erlenmeyer flasks containing 180 ml diluted juice (pH = 5.29) were pH adjusted to 6.0 using 2M NaOH and autoclaved at 121°C for 16 minutes. The sterilized sugarcorn juice was inoculated with 10% (v/v) seed culture followed by incubation at 30°C for 13 hours and 200 rpm.

5.2.1.4 Ethanol fermentation and analysis

The sugarcorn juice medium was pH adjusted from 5.09 to 6.2, autoclaved and inoculated with 10% (v/v) incubated culture. Fermentation was carried out in a 2L bioreactor for 72 h with an agitation of 200 rpm. Temperature was maintained at 30±2°C by circulating water to the jacketed bioreactor vessel. Air at 1 Lpm was supplied continuously for the first 12h after which it was turned off to facilitate ethanol production. About 5-6 ml samples were withdrawn at regular intervals for analysis.

The sample pH was determined using a pH meter (VWR symphony SB70P, Beverley, USA). Dry weight was determined by centrifuging 1 ml samples in microfuge tubes of known weight to get the pellet, which was later oven dried and weighed for constant weight. Number of viable cells were determined by plating serially diluted culture samples

on YM agar medium. Ethanol concentration as a function of time was measured using GC (Agilent Technologies, Palo Alto, USA), while concentration of carbohydrates and reducing sugars were determined by PS and DNS methods. The consumption of the predominant constituent sugars- sucrose, fructose and glucose was followed by High performance liquid chromatography (Waters Alliance HPLC System, New Castle, USA) coupled with a refractive index detector.

5.2.2   Improvement of yield for Bioethanol production using Saccharomyces cerevisiae ATCC 26603 cultivated in sugarcorn juice

Sugarcorn juice A (SCJ A) was used for the yield improvement fermentations. SCJ A, *Saccharomyces cerevisiae* ATCC 26603, YM broth and yeast extract (YE) were procured from sources mentioned in 4.2 and 5.2.1. The fermentation steps are shown in Figure 5.1

5.2.2.1   Strain preservation

One cryovial of cells preserved as previously elaborated in section 5.2.1.1, was used to aseptically inoculate sterilized 100 ml YM medium and incubated at 30˚C with an agitation of 100 rpm for 28 h. The mean viable cell count was 2.3 x 10^7 CFU/mL. Following this, the cells were mixed with 30% glycerol in cryovials and frozen at -84°C as before, for subsequent use in yield improvement experiments.

5.2.2.2   Strain revival

YM medium (200 ml each, pH adjusted to 6.0 using 2M sulphuric acid) was prepared in two 500 ml Erlenmeyer flasks and autoclaved at 121°C for 16 minutes. A cryovial of cells was transferred aseptically to inoculate each EM flask containing sterilized YM medium. Incubation was carried out at 30°C for 12 hours and 200 rpm.

5.2.2.3   Inoculum preparation

Sugarcorn juice was thawed at room temperature, required amount was measured out and diluted with equal volume of distilled water. Two 500 ml Erlenmeyer flasks containing 180 ml diluted juice supplemented with 3 g/L YE was pH adjusted to 6.0 using 2M NaOH and

autoclaved at 121°C for 16 minutes. The sterilized sugarcorn juice was inoculated with 10% (v/v) revived culture followed by incubation at 30°C for 18 hours and 200 rpm.

5.2.2.4   Ethanol fermentation and analysis

Bioethanol fermentation experiments were carried out using (1) Sugarcorn juice and (2) Sugarcorn juice supplemented with 3 g/L YE. In each case, 900 mL medium was prepared and pH adjusted to 6.5 using 5N NaOH and autoclaved. With aeration and agitation switched on, 100 ml inoculum was used to inoculate the sugarcorn medium in a 2L stirred tank bioreactor. Fermentation was carried out for 72 h with an agitation of 200 rpm. Temperature was maintained at 30±1°C by circulating water to the jacketed bioreactor vessel. Aeration at 1 Lpm was supplied continuously for the first 5h, after which it was stopped to facilitate ethanol production. About 7 ml samples were withdrawn at regular intervals for analysis.

Measurement of pH, viable cell count, and concentration of total carbohydrates, reducing sugars, ethanol, as well as individual concentrations of sucrose, glucose and fructose were determined by methods mentioned in section 5.2.1.4. Dry cell weight was measured by filtration through a 0.45µm ReliaDisc[TM] CN-membrane and drying for constant weight in an oven at 105˚C. As an additional analysis, a digital brix refractometer was used to monitor the refractive index of cell free broth during the fermentation. A pure sucrose standard was used to calibrate the brix refractometer values to estimate sugar concentrations. Empirical equations by Son et al., 2009 (Son et al. 2009) were used for calculating true brix by accounting for alcohol interference.
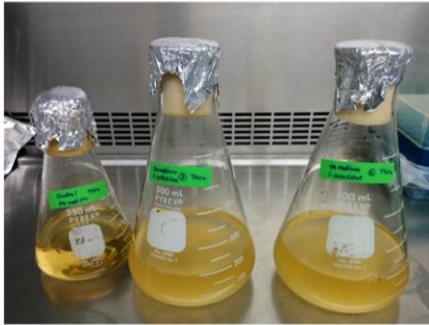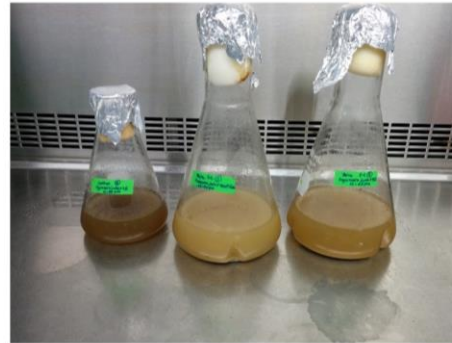
Figure 5.1 Steps in bioethanol yield improvement fermentation using *Saccharomyces cerevisiae* ATCC 26603 in sugarcorn juice medium: 1. Frozen cells 2. Strain revival or pre-inoculum preparation 3. Inoculum preparation 4. Fermentation

5.3   Results and discussion

5.3.1   Bioethanol production using *Saccharomyces cerevisiae* ATCC 26603 cultivated in sugarcorn juice

5.3.1.1   pH variation

Though the pH of the sugarcorn juice was adjusted to 6.2 prior to sterilization, it dropped drastically to 5.47 following inoculation. The broth pH decreased throughout the fermentation, ultimately reaching a value of 4.3 at the end of 72h. The pH drop was due to the accumulation of week acids which are by products formed when the yeast cells metabolize the sugars in the broth and increase in concentration (Figure 5.2).

5.3.1.2   Number of viable cells and specific growth rate

Following inoculation, the viable cell count was $3.4x10^{-5}$ CFU/mL. As the sugars were consumed the cells multiplied rapidly, and a peak in the exponential phase was reached at the 24h mark, with a concentration of $2.5x10^{-8}$ CFU/mL. The specific growth rate, μ was determined as 0.54 h^{-1}. After 24 h, the count slightly decreased due to exhaustion of sugars, characteristic of stationary phase (Figure 5.2).
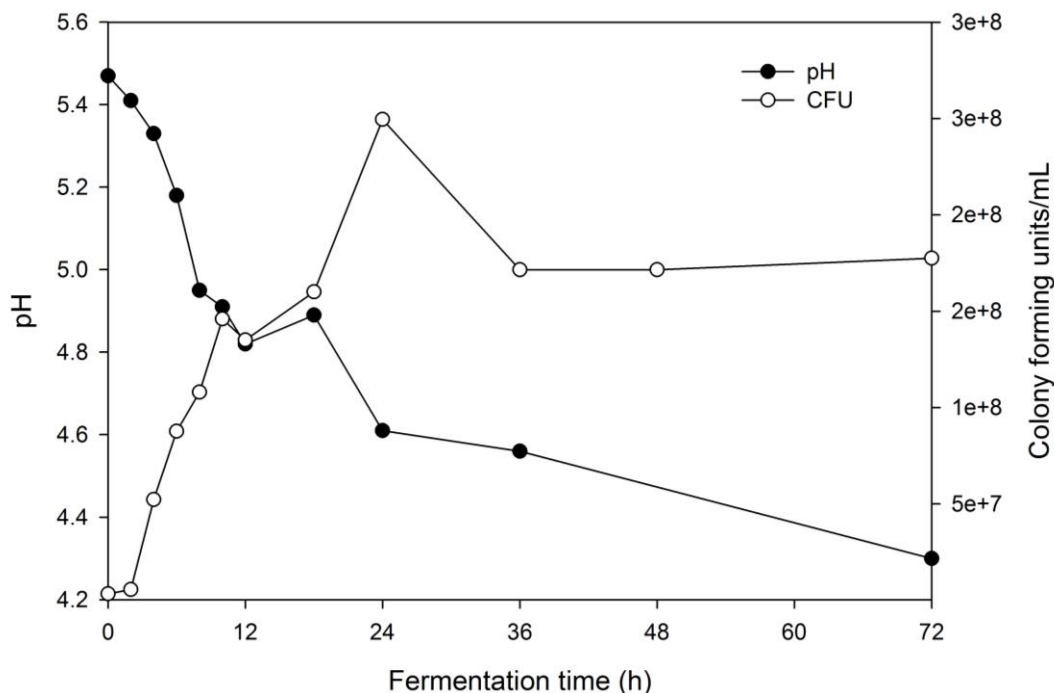
Figure 5.2 pH and viable cell concentration as a function of time for *Saccharomyces cerevisiae* ATCC 26603 cultivated in sugarcorn juice

5.3.1.3   Dry cell weight

The dry weight of the *Saccharomyces cerevisiae* cells increased steeply from an initial concentration of 5.5 g/l to a maximum value of 14.9 g/l in 6 hours. Following this, the dry cell weight dropped slightly and stabilized around 10 g/l up to 48 hours of fermentation. The dry cell concentration decreased further due to complete depletion of nutrients, reaching a value of 4.8 g/l in 72 hours (Figure 5.3).

5.3.1.4   Concentration of total sugars

The initial concentration of sugars in the sugarcorn juice prior to sterilization was 109 g/l and it reduced to 91.9 g/l following inoculation. The total sugar concentration reduced rapidly for the first 24 hours and more gradually later on, till 48 hours. Almost half the sugars were consumed within 12 hours of fermentation time with the maximum rate of

consumption occurring between the 8 and 10 hours, corresponding to the late exponential phase. All sugars in the medium were completed depleted in 48 h (Figure 5.3).

5.3.1.5   Concentration of reducing sugars and fermentable sugars

Glucose and fructose are the primary reducing sugars in the sugarcorn juice and they were utilized fast in the first 12 h due to aeration and later at a slightly slower rate till 24 h mark. The rate of consumption further slowed down towards complete consumption in 48 h.

Concentration of the primary fermentable sugars, sucrose, fructose and glucose determined by HPLC showed that the yeast preferred glucose to fructose. This was illustrated by a steady decrease in glucose levels and nearly stable fructose levels till 24 hours. Also, a very small amount of fructose still remained after 48 hours, while there were no detectable glucose or sucrose. The breakdown of sucrose to fructose and glucose was prominent after 4 hours of fermentation (Figure 5.3).

5.3.1.6   Ethanol concentration

Ethanol production in comparison with sugar consumption is shown in Figures 5.3 and 5.4.There was no ethanol production in the initial 4 hours of fermentation. Following this, the ethanol levels increased gradually till 10 hour mark, and increased sharply in the next two hours, corresponding to the late exponential phase. This agrees well with the sudden increase in sugar consumption that was observed between 8 to 10 hours. Ethanol production slowed down after 24 hours, as sugar utilization also had become slower by this time. Ethanol production stagnated after 48 hours when all sugars had depleted, reaching a maximum value of 17.9 g/L in 72h. The ethanol productivity and yield of the fermentation was determined as 0.25 g/L/h and 0.20 g ethanol/g carbohydrates. This corresponded to an ethanol yield of 0.180 g ethanol/g fermentable sugars.
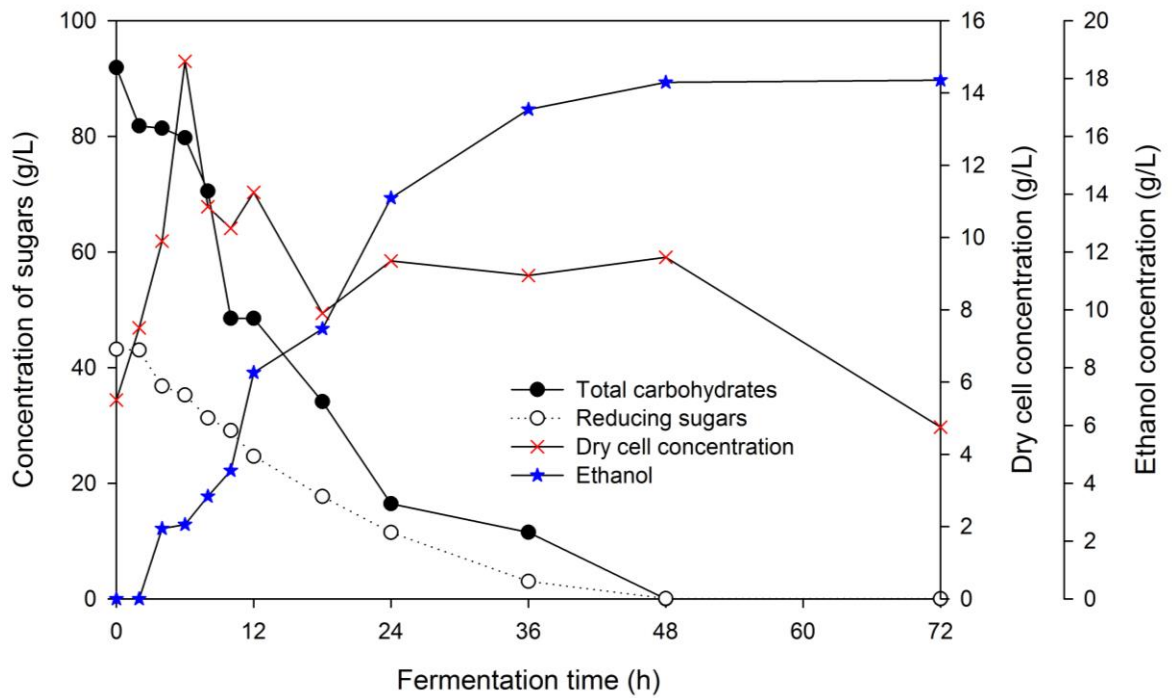
Figure 5.3 Concentration of carbohydrates, reducing sugars, biomass and ethanol as a function of time for *Saccharomyces cerevisiae* ATCC 26603 cultivated in SCJ
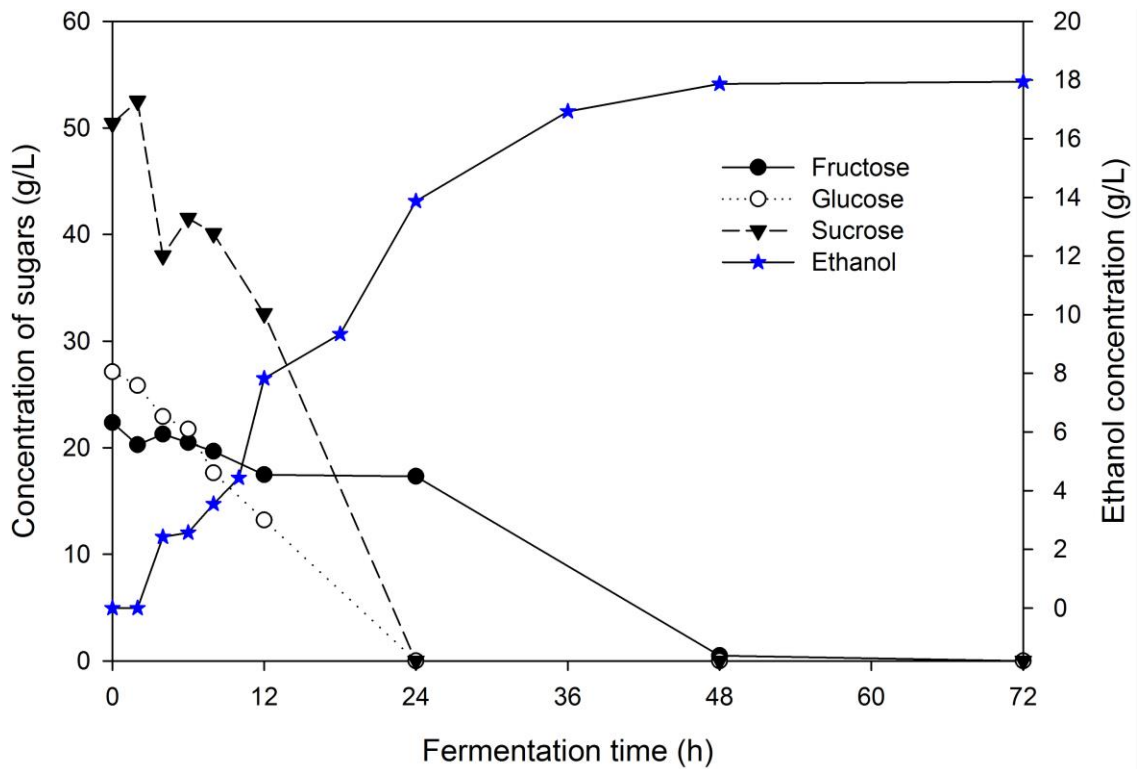
Figure 5.4 Fermentable sugars consumption and ethanol production as a function of time for *Saccharomyces cerevisiae* ATCC 26603 cultivated in SCJ

5.3.2 Improvement of yield for Bioethanol production using Saccharomyces cerevisiae ATCC 26603 cultivated in sugarcorn juice

5.3.2.1 pH variation

In both fermentation runs, the initial pH of the medium prior to autoclaving was adjusted to 6.5. Immediately after inoculation, the pH of the broth dropped to 6.0 when sugarcorn was used as such, whereas when used supplemented with YE, it dropped to 6.14. Yeasts are capable of maintaining the intracellular pH within physiologically optimal levels suited for metabolism irrespective of extracellular pH (Thomas, Hynes, and Ingledew 2002). During lag phase the intracellular pH is much less than that during exponential phase (Takeo Imai and Ohno 1995) as the protons are pumped out of the cells in the latter. In alcoholic fermentations, drop in medium pH is a result of organic acids which are released into the medium as by-products of sugar consumption by the cells (Viegas et al. 1989). Consequently, the broth pH had reduced to 4.92 after 12 hours of fermentation with or without YE supplementing the sugarcorn juice, corresponding to exponential growth phase. The concentration of protons in the broth had peaked by 18h. After 36 h, the pH of the fermentation broth started to increase slightly probably due to decrease in intracellular pH (T. Imai and Ohno 1995), characteristic of stationary phase showing decelerated growth. Sugarcorn juice, if initial pH was adjusted to pH=6.5, was able to maintain its pH in an optimal range of 5 to 6 suitable for ethanol production using *Saccharomyces cerevisiae* ATCC 26603 (Abouzied and Reddy 1986, 1987), without the need for online pH control (Figure 5.5).
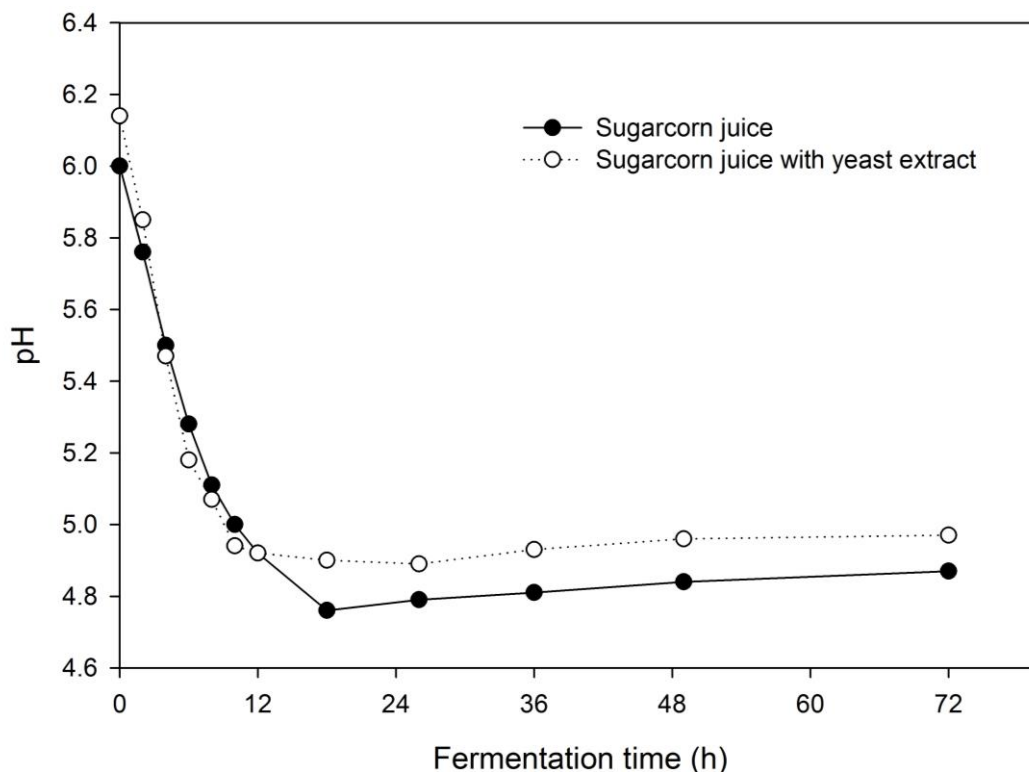
Figure 5.5 Comparison of pH variation for *Saccharomyces cerevisiae* ATCC 26603 cultivated in SCJ medium and SCJ-YE medium

5.3.2.2 Number of viable cells and specific growth rate

The flocculent *Sacchharomyces cervisiae* ATCC 26603 cells grew visibly faster when sugarcorn juice was supplemented with YE than when sugarcorn juice was used as such. The specific growth rate, μ, was 0.33 h^{-1} for the yeast cells grown in sugarcorn juice but when YE was added as a nitrogen supplement to the juice, the μ value increased to 0.92 h^{-1}. The viable cell count measured on YM agar medium showed diauxic growth for both fermentations. Diauxic growth in *Saccharomyces cerevisiae* has been reported earlier, primarily caused by catabolite repression of enzymes catalyzing alternative pathways, due to preferential utilization of glucose by the yeast (Albers, Bakker, and Gustafsson 2002; Kamatam 2007; Lavová et al. 2014). For fermentations using sugarcorn juice, rapid consumption of glucose and fructose and simultaneous breakdown of sucrose, resulted in cell growth, depleting all of the medium's reducing sugars in 18 h for fermentation using

sugar corn juice and 12 h for fermentation using sugarcorn juice and YE. There is a phase were cells suffer from sugar stress, during which the cell viability drops (between 18 to 24 hours of fermentation for sugarcorn juice and between 12 to 18 hours for sugarcorn juice supplemented with YE). Following this, the yeast may have started to consume ethanol as an alternative carbon source (Lei, Rotboll, and Jorgensen 2001; Ramon-Portugal, Pingaud, and Strehaiano 2004), resulting in an increase in cell viability which reached a peak of 1×10^9 cells/mL (26 h) for sugarcorn juice medium with YE and 6.7×10^8 cells/mL (36 h) for un-supplemented sugarcorn juice medium (Figure 5.6).
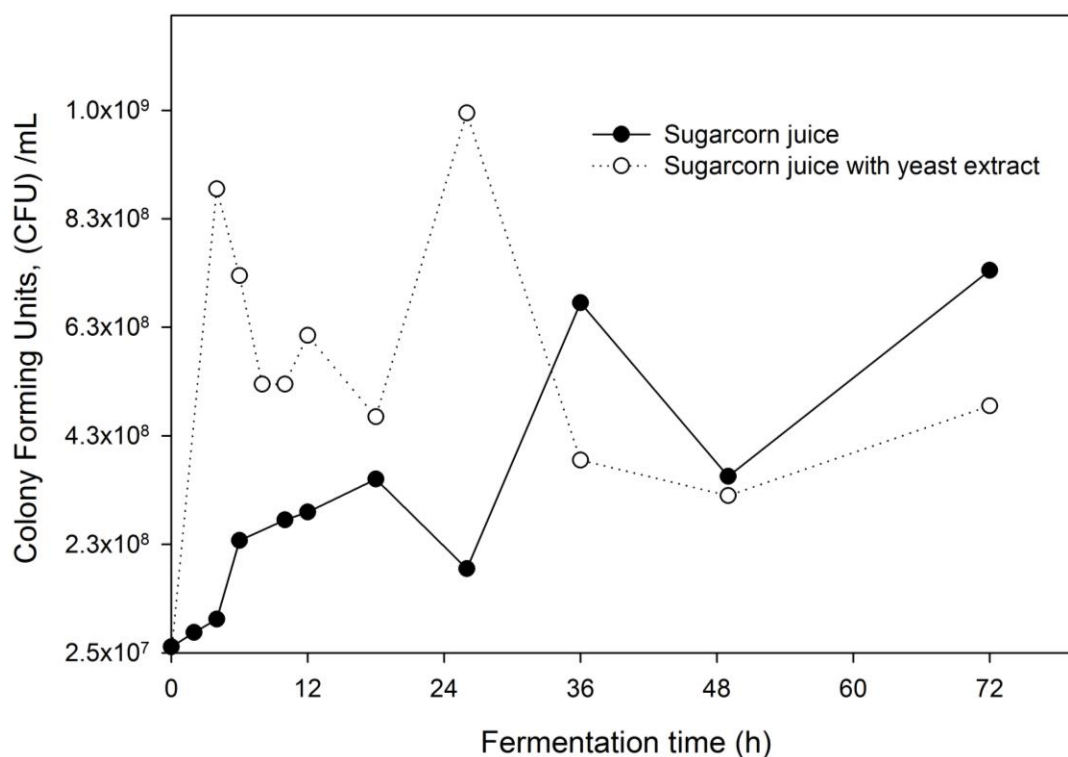
Figure 5.6 Viable cell concentration as a function of time for *Saccharomyces cerevisiae* ATCC 26603 cultivated in SCJ medium and SCJ-YE medium

5.3.2.3 Dry cell weight

Initial concentration of dry biomass was 3 g/L and 3.25 g/L respectively for fermentation using sugarcorn juice with and without YE supplement. The cells grown in sugarcorn juice showed a short lag phase of 2 h and reached maximum cell concentration of 10.8 g/L in 18 h. For the fermentation of sugarcorn juice with YE, the lag phase was not discernable in the time intervals measured and log phase showed a much steeper growth, with a peak at 13.8 g/L in 10 h. Both these peak values occur when more than 85% of total carbohydrates in the medium where consumed and almost all of the reducing sugars were depleted. YE , which is an effective organic nitrogen source with growth stimulating compounds (Hakobyan, Gabrielyan, and Trchounian 2012).When used in combination with sugarcorn juice, YE promoted faster cell growth and achieved higher concentrations of yeast biomass (Figure 5.7).
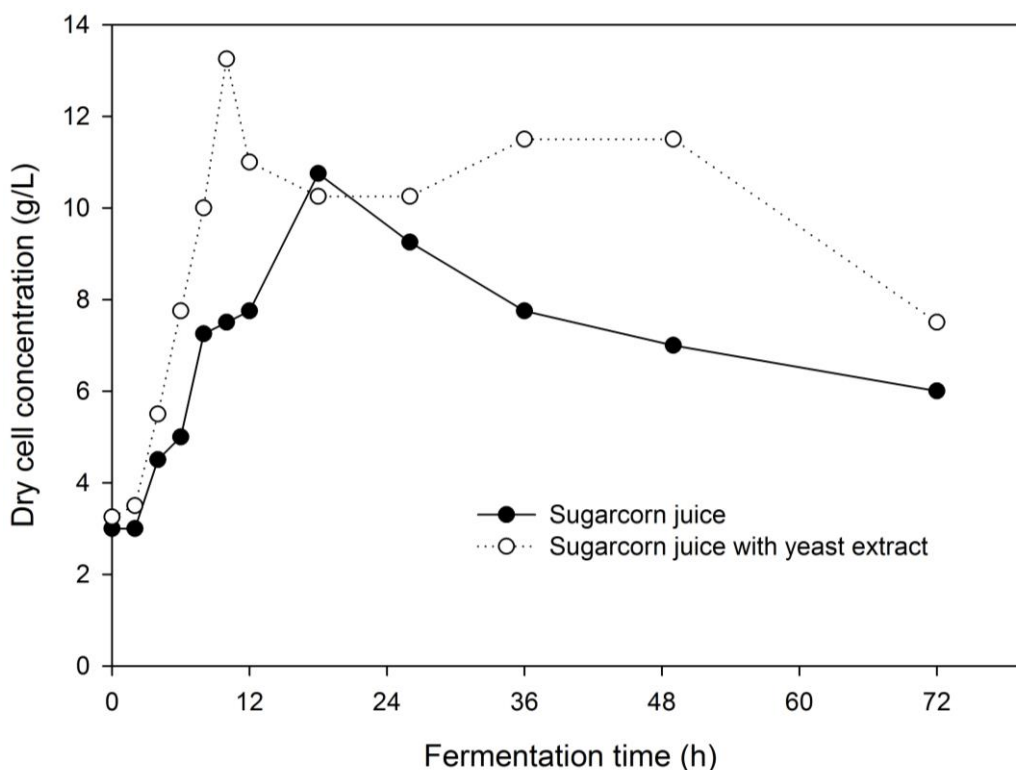
Figure 5.7 Dry cell concentration as a function of time for *Saccharomyces cerevisiae* ATCC 26603 cultivated in SCJ medium and SCJ-YE medium

5.3.2.4   Concentration of total sugars

The initial concentration of total carbohydrates in the sugarcorn juice medium was 103 g/L and 110.7 g/L respectively for fermentations with and without YE. Reducing sugars constituted 65.4 % and 62.7 % by weight of the initial total carbohydrates in the corresponding fermentations. The carbohydrates were consumed at a rapid rate until most the reducing sugars in the broth depleted. The utilization of sugars by yeast was faster in the presence of YE to supplement sugarcorn juice (Figure 5.8).
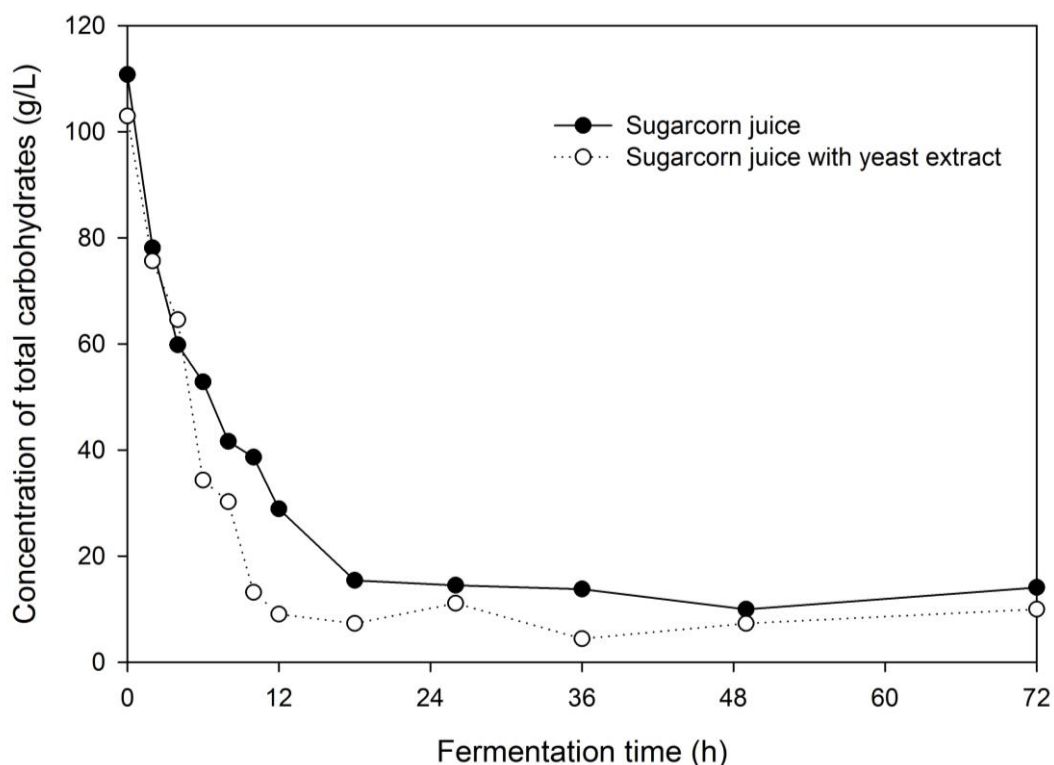
Figure 5.8 Variation of carbohydrates concentration as a function of time for
***Saccharomyces cerevisiae* ATCC 26603 cultivated in SCJ medium and SCJ-YE**
medium

5.3.2.5   Concentration of reducing sugars and fermentable sugars

Sugarcorn juice is a natural complex medium rich in reducing sugars. For the batch used for fermentation, initial concentration of total reducing sugars (glucose and fructose) in the juice was 69.5 g/L for sugarcorn juice and 67.3 g/L for sugarcorn juice with YE. The yeast cells can directly consume these sugars to produce biomass, carbon-di-oxide and ethanol. The medium when supplemented with YE showed much faster consumption of reducing sugars with over 99% utilization in 12 h. When sugarcorn juice was used, cells took 18 h of fermentation to achieve 99% consumption of reducing sugars (Figure 5.9).

Figure 5.9 Reducing sugars consumption as a function of time for *Saccharomyces cerevisiae* ATCC 26603 cultivated in SCJ medium and SCJ-YE medium

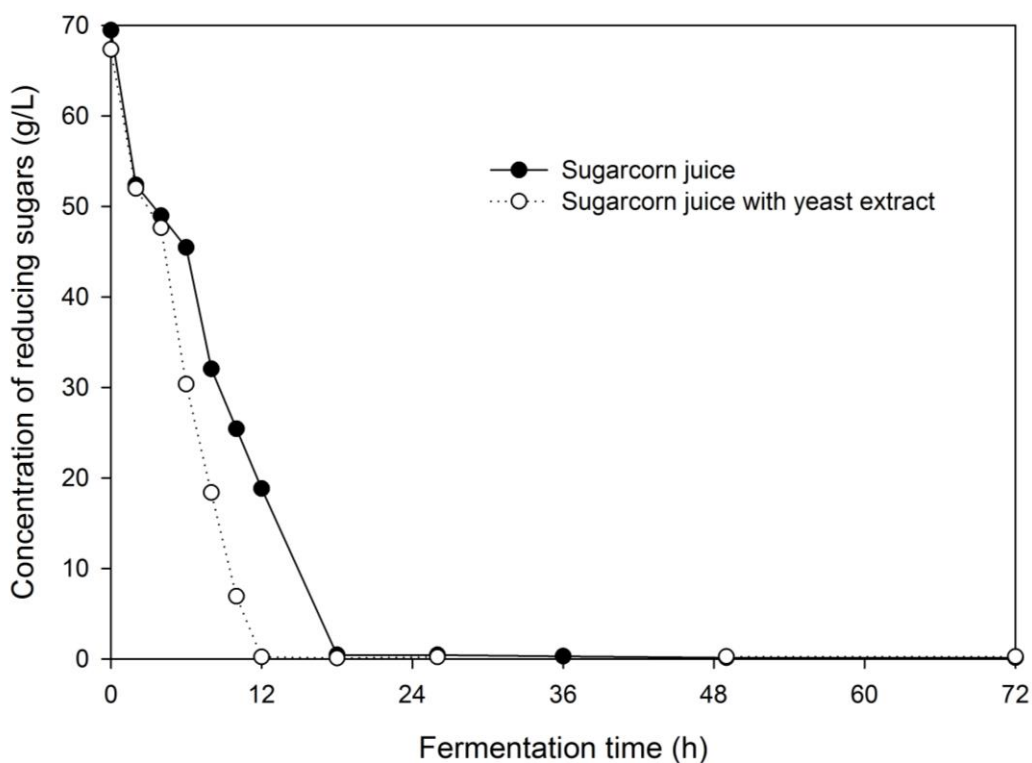The fructose, glucose and sucrose together amounted to a concentration of 88.3 g/L (80% of total sugars) and 85.5 g/L (83% of total sugars) in the broth with and without YE respectively, at the start of the fermentation. Aided by initial aeration, glucose and fructose in the medium were consumed rapidly by the yeast cells leading to complete depletion of these monosaccharides, subsequently triggering the hydrolysis of all of sucrose in the medium within 4 hours of fermentation. The effect of carbon catabolite repression (CCR) leading to preference of glucose over fructose was observed in 8 h and 12 h for fermentation with and without YE. The time for complete utilization of glucose and fructose agreed well with the reducing sugars consumption discussed earlier (Figures 5.11 and 5.12).

5.3.2.6   Ethanol concentration

Comparison of ethanol production in SCJ and SCJ-YE fermentations is shown in Figure 5.10. Ethanol production in relation to biomass consumption and sugars consumption are illustrated in Figures 5.11, 5.12, 5.13 and 5.14. The corresponding yields and productivity values are listed in Table 5.1 and 5.2. Both fermentations produced more than 20 g/L ethanol within 12 h. The ethanol production was rapid for both fermentations until the medium contained reducing sugars, after which the rate dropped. Sugarcorn juice medium produced 40 g/L in 26 h, with an ethanol yield of 0.41 g ethanol/g total carbohydrates (TC) and a productivity of 1.5 g/L/h. The ethanol concentration reached a maximum of 41.7 g/L in 49 h. Sugarcorn juice supplemented with YE produced 34.9 g/L ethanol in 36 h with a yield of 0.33 g/g. In the medium with yeast extract, *Saccharomyces cerevisiae* cells were able to degrade sugars much faster, with most used for biomass production rather than ethanol production, as illustrated by the high viable cell concentrations. This may have resulted in a lower ethanol yield with yeast extract supplementation. The maximum yields in terms of fermentable sugars (FS) consumed were 0.46 g/g for SCJ fermentation (90.4% theoretical yield) and 0.38 g/g for SCJ-YE (75.1% theoretical yield) media.

The maximum yield achieved in the study was compared with existing batch studies on bioethanol production using prominent fermentable sugar juices in Table 5.4. Clearly bioethanol fermentation using sugarcorn juice in this study achieved a competitive yield without nutrient additional nutrient supplementation in the fermentation media.

The maximum ethanol yield (0.46 g/g) and fermentable sugars (88.3 g/L) for this fermentation, along with fermentable sugar values for autoclaved SCJ A (as it was fresher) in Figure 9 (110.3 g/L) were used to extrapolate yield per hectare for sugarcorn juice. The yield is shown in comparison with that of corn and sweet sorghum in Table 5.3. The actual ethanol yield may be lower if a sugarcorn hybrid of lower juice yield is employed, the value used for calculation being 56.1 Mg/ha (Reid et al. 2015). The yield may also be reduced due to inevitable losses and process challenges introduced on scale up. The ethanol yield may be higher if sugar depletion due to storage is avoided, and if the sugarcorn is juiced and fermented within short period following harvest, similar to the sugarcane-fed ethanol industry.
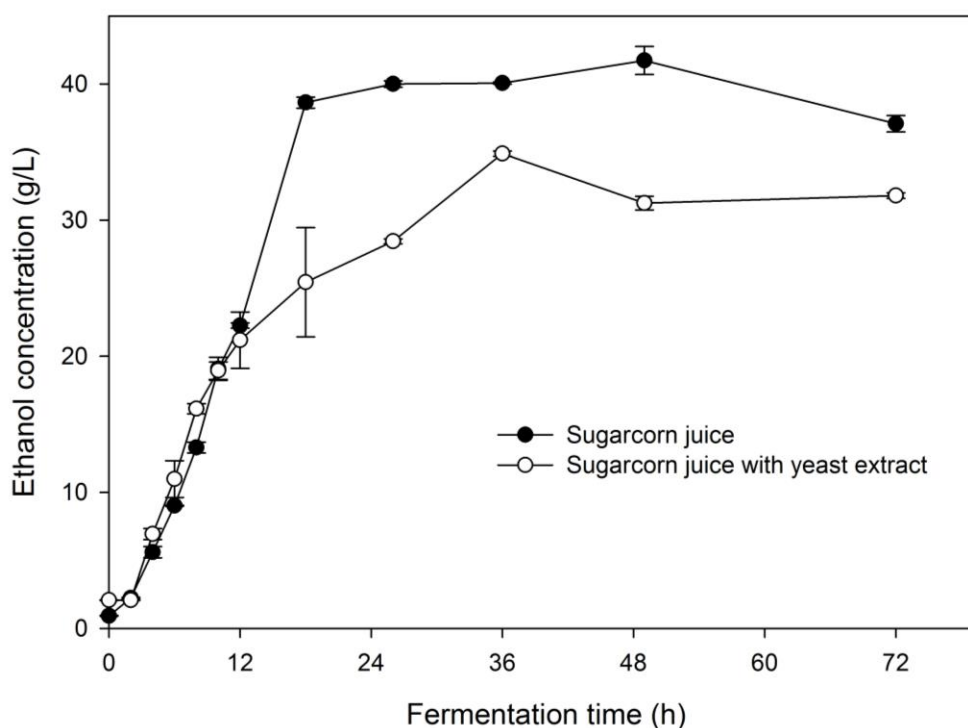
Figure 5.10 Ethanol production as a function of time for Saccharomyces cerevisiae ATCC 26603 cultivated in SCJ medium and SCJ-YE medium. Error bars indicate standard deviation.
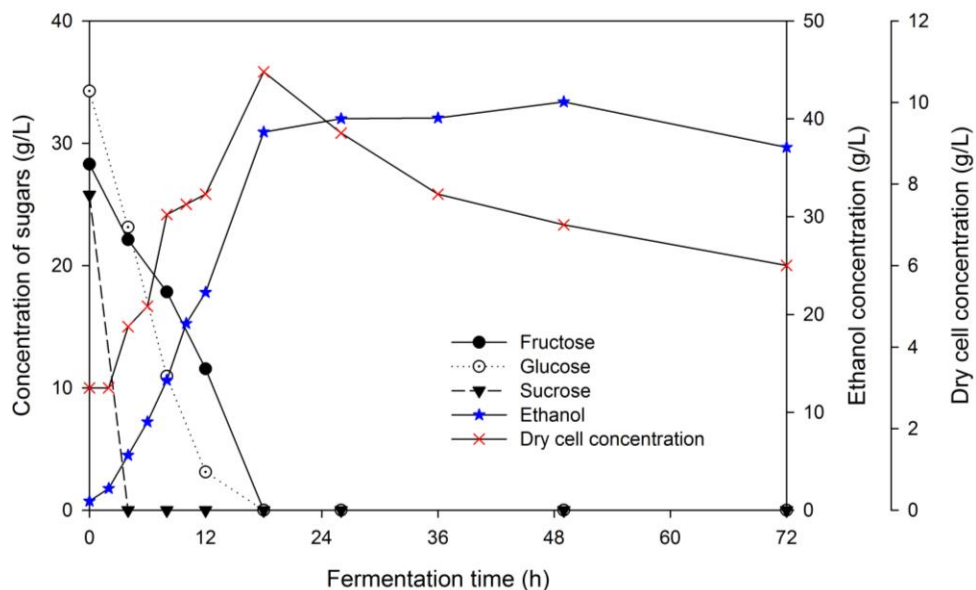
Figure 5.11 Consumption of fermentable sugars, production of biomass and ethanol as a function of time for *Saccharomyces cerevisiae* ATCC 26603 cultivated in SCJ medium
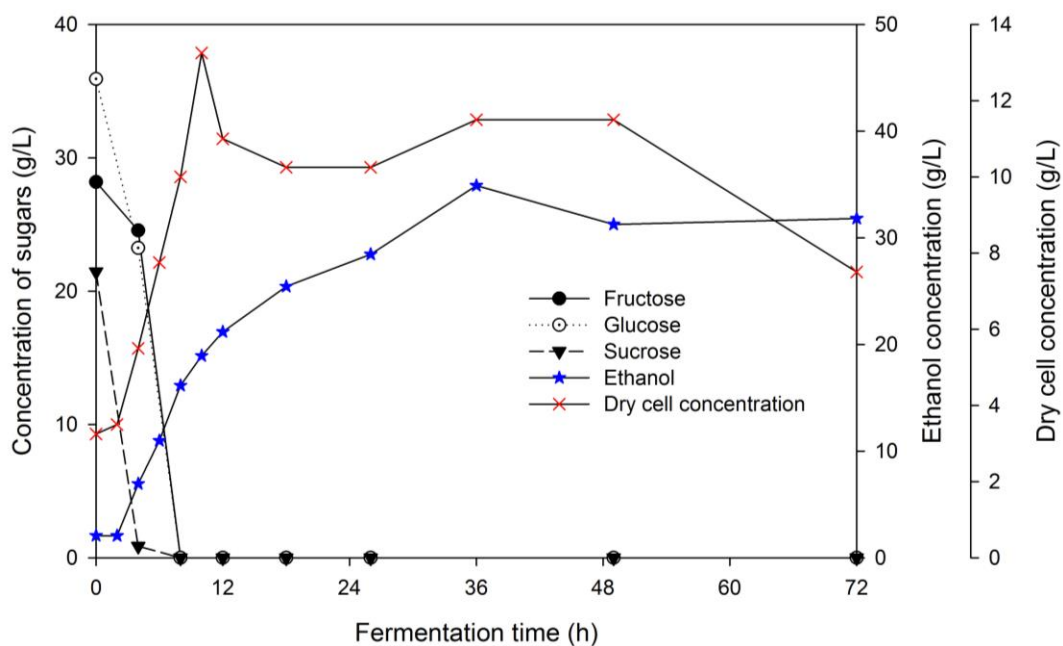
Figure 5.12 Consumption of fermentable sugars, production of biomass and ethanol as a function of time for *Saccharomyces cerevisiae* ATCC 26603 cultivated in SCJ-YE medium
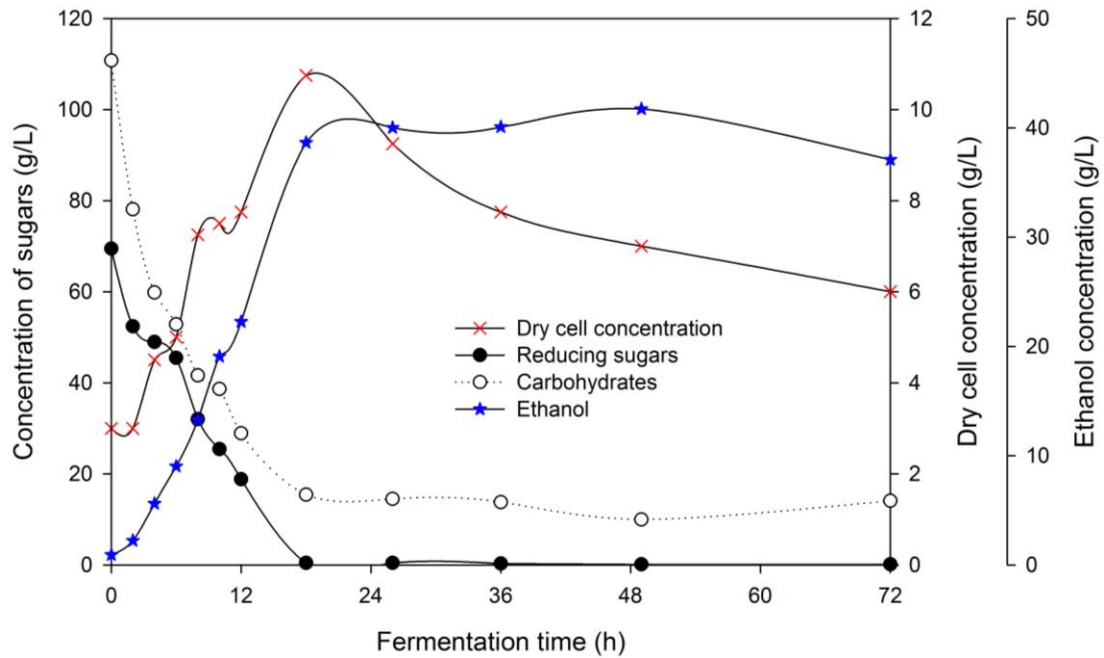
Figure 5.13 Concentration of biomass, sugars, ethanol as a function of time for
Saccharomyces cerevisiae **ATCC 26603 cultivated in SCJ medium**
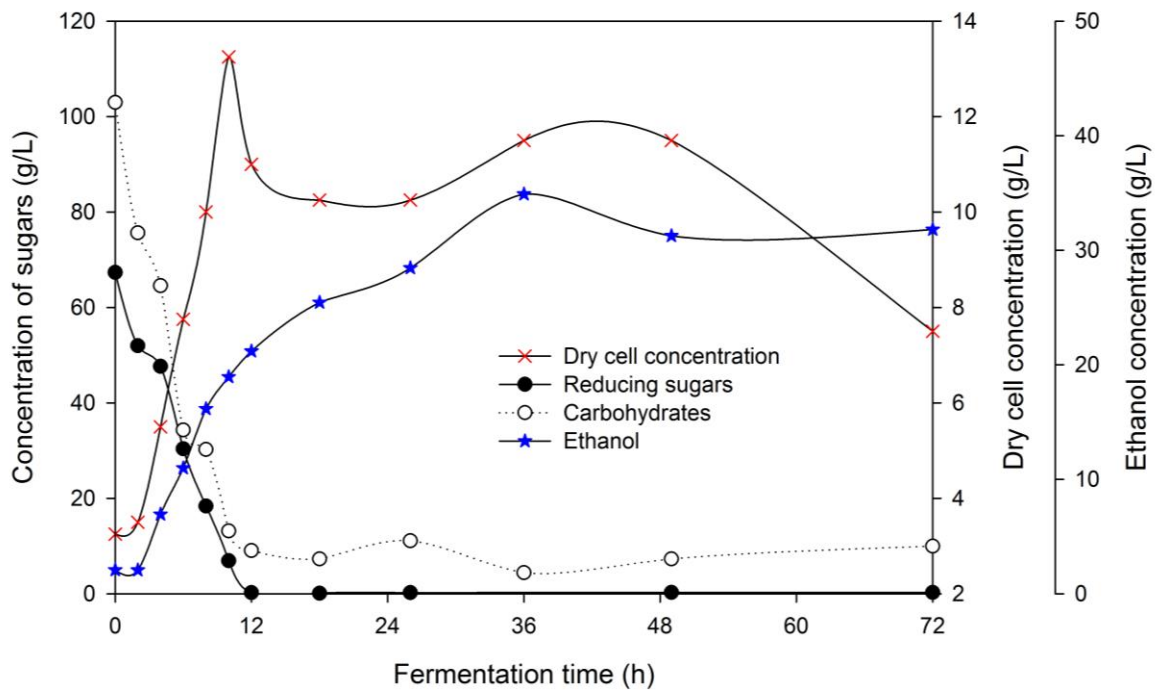
Figure 5.14 Concentration of biomass, sugars, ethanol as a function of time for
Saccharomyces cerevisiae **ATCC 26603 cultivated in SCJ-YE medium**

Table 5.1 Yield and productivity of ethanol production for *Saccharomyces cerevisiae* ATCC 26603 grown in SCJ

Time (h)	Ethanol yield (TC) (g/g)	Ethanol yield (FS) (g/g)	%theoretical yield (TC)	Productivity (g/L/h)	v/v ethanol (%)
26	0.41	0.44	86.6	1.50	4.95
36	0.40	0.44	86.7	1.09	4.96
49	0.41	0.46	90.4	0.83	5.17
72	0.37	0.41	80.1	0.50	4.58

Table 5.2 Yield and productivity of ethanol production using *Saccharomyces cerevisiae* ATCC 26603 grown in SCJ-YE

Time (h)	Ethanol yield (TC) (g/g)	Ethanol yield (FS) (g/g)	%theoretical yield (FS)	Productivity (g/L/h)	v/v ethanol (%)
26	0.29	0.31	60.4	1.01	3.34
36	0.33	0.38	75.1	0.91	4.16
49	0.31	0.34	66.8	0.60	3.70
72	0.32	0.35	68.0	0.41	3.77

Table 5.3 Ethanol yield comparison with corn and sweet sorghum

Feedstock	Sugarcorn	Corn	Sweet sorghum
Ethanol yield (L/ha)	2780-3470	2664[a]	2800[a]

[a](Reid et al. 2015)

Table 5.4 Comparison of select batch studies for bioethanol production using fermentable sugar juices

Feedstock	Microorganisms	Brix (˚Bx)	Supplements used	Initial pH	Temperature (˚C)	Agitation (rpm)	Aeration (vvm)	Time (h)	Yield (%)	Productivity (g/L/h)	Reference
Sugarcane juice	*Kluyveromyces marxianus*	22	Sucrose 0.05% (NH4)$_2$SO$_4$ 0.05% KH$_2$PO$_4$ 0.15% MgSO$_4$ · 7H$_2$O	5.0	37	300	0.2	48	57.1*	1.3	(Limtong, Sringiew, and Yongmanitchai 2007)
Sweet sorghum juice	*Saccharomyces cerevisiae* TISTR 5048	24	Sucrose 0.3% Yeast extract 0.5% peptone	4.9	30	0	-	60	82.4*	1.68	(Laopaiboon et al. 2007)
Sugar beet juice	*Saccharomyces cerevisiae* ATCC 26603	16.5	Sucrose 0.4% Yeast extract 0.064% NaCl 0.341% K$_2$HPO$_4$	6.5	30	220	-	24	76.3**	0.53	(Ogbonna, Mashima, and Tanaka 2001)
Sugarcorn juice	*Saccharomyces cerevisiae* ATCC 26603	11.3	None	6.0	30	200	1 [+]	26	80.4**	1.5	This research

+aeration for first 5 hours

* experimental ethanol yield determined as %ethanol per %sugars utilized   **experimental ethanol yield determined as g ethanol per g carbohydrates utilized

5.3.2.7   Sugar content by brix refractometer

Brix measurements are convenient to approximate the sugar content in a given sugary solution. As an alcoholic fermentation proceeds the sugar concentration in the medium drops which tends to decrease the refractive index. The ethanol produced by the cells as a product of sugar utilization has a refractive index similar to sugar and is less dense compared to water (Son et al. 2009).The refractive index increases linearly with increase in ethanol concentration, leading to an overestimation of the measured apparent brix (A.R) value (Rogerson and Symington 2006; Son et al. 2009).Son *et al* in 2009 formulated empirical equations to overcome this error. Two of the equations (mentioned below) were used to calculate true brix (T.B) for the fermentation experiments discussed in this chapter, assuming a similar alcohol interference pattern for sugarcorn juice as that of high sugar substrates used in wine production.

T.B. = −0.352 I.B. + 1.264 A.R. + 2.006

Where I.B as a function of time was calculated from the below equation, using corresponding ethanol concentrations (A) determined by GC

A = 0.967 I.B. − 0.766 A.R. − 5.793

The true brix values dropped from an initial 11.3˚Bx to 1.8˚Bx in 18 hours of fermentation for sugarcorn juice and from 10.7˚Bx to 1.6˚Bx in 12 hours when sugarcorn juice was used with yeast extract. Following this, there were more fluctuations in the brix values similar to those observed in carbohydrates measured by phenol-sulfuric (PS) method (Figure 5.15).
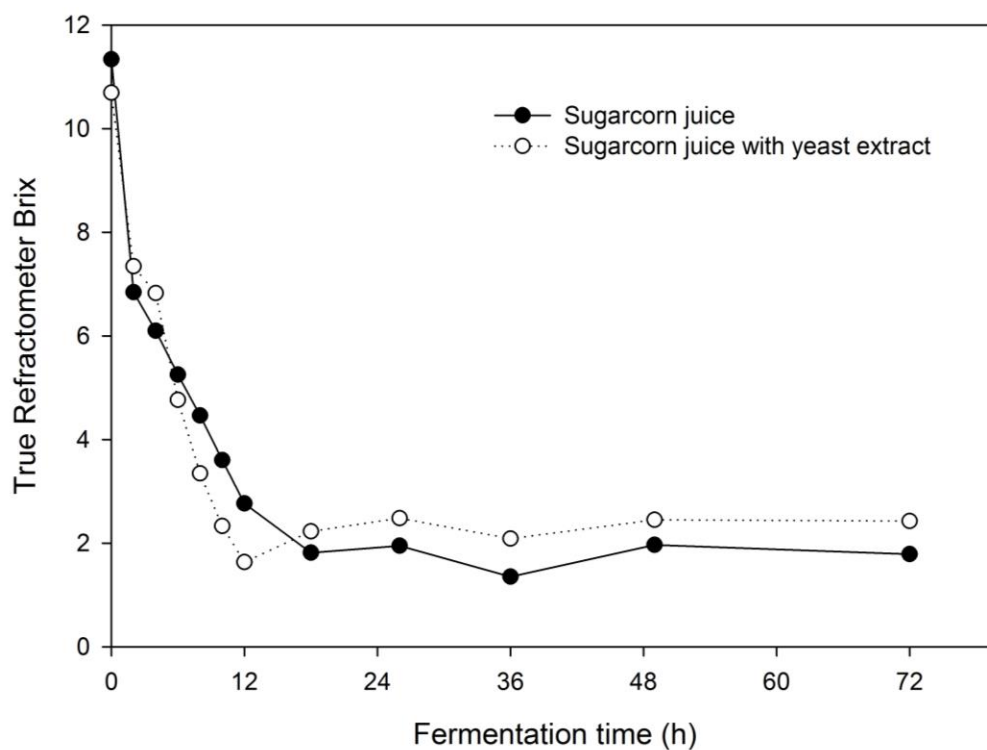
Figure 5.15 True refractometer brix as a function of time for bioethanol production using *Saccharomyces cerevisiae* ATCC 26603 grown in SCJ and SCJ-YE media

The true brix values calculated generally agreed well with the sugar concentration determined by PS method, especially for the fermentation using pure sugarcorn juice medium. For the fermentation using sugarcorn juice with yeast extract, the curves where understandably a little further apart, as the medium was much different from the sucrose standard used to calibrate the values measured by brix refractometer. The trend was however still closer to the variation in carbohydrates with respect to fermentation time, than when apparent refractometer brix values were used for estimation of sugar concentration of the cell free broth (Figure 5.16 and 5.17).
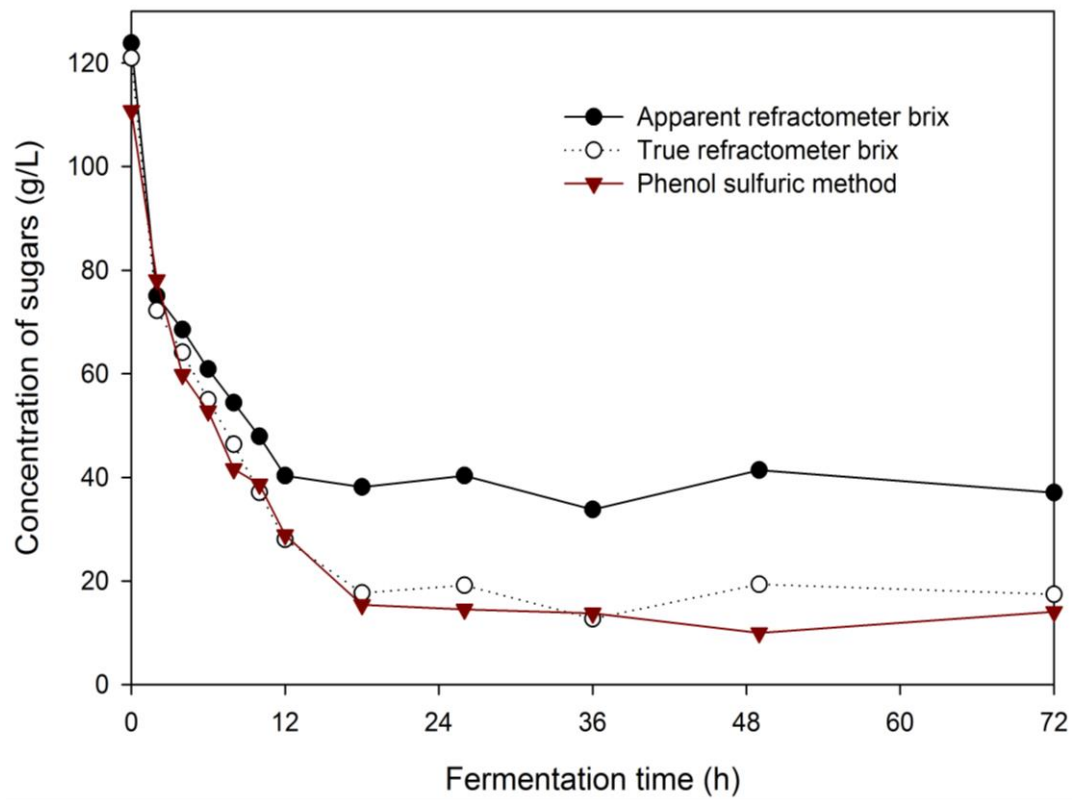
Figure 5.16 Concentration of sugars by Phenol sulfuric method and sucrose-based brix (apparent and true) concentrations vs Fermentation time for *Saccharomyces cerevisiae* ATCC 26603 in SCJ medium
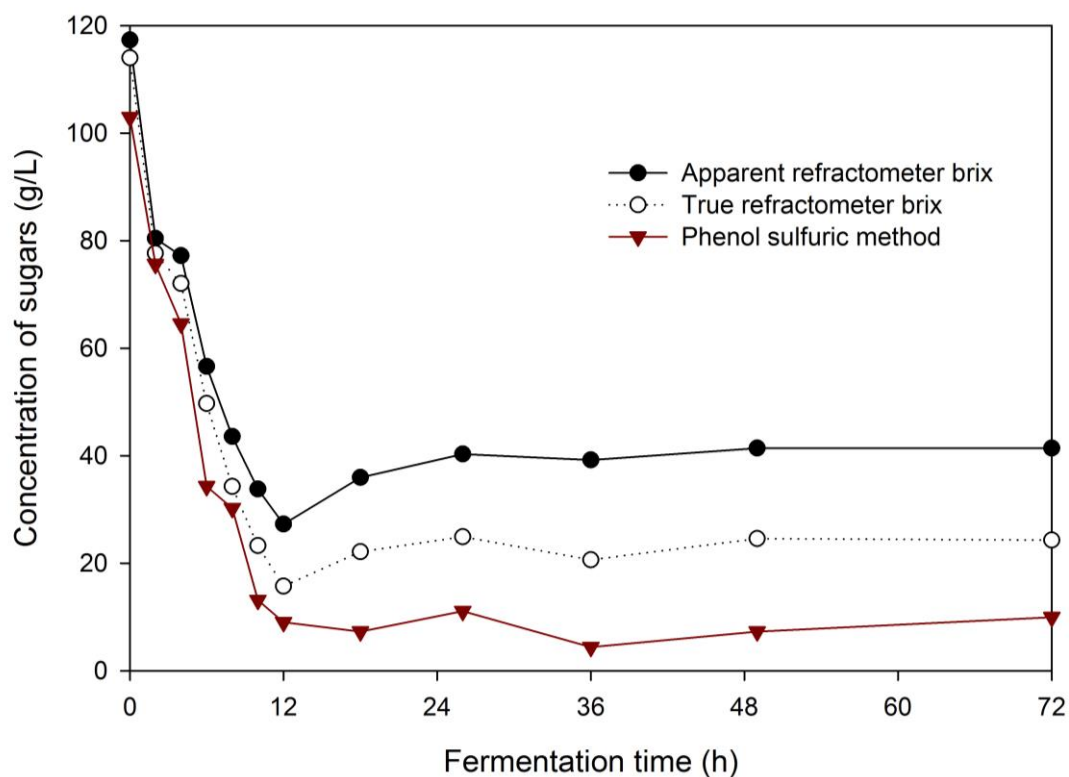
Figure 5.17 Concentration of sugars by Phenol sulfuric method and sucrose-based brix (apparent and true) concentrations vs Fermentation time for *Saccharomyces cerevisiae* ATCC 26603 in SCJ-YE medium

5.4 Conclusion

During the initial bioreactor experiment, unlike the commercial dry yeast used for the shake flask experiments, the strain *Saccharomyces cerevisiae* ATCC 26603 was able to consume all sugars in the sugarcorn juice in 48 hours, producing ethanol. This may be attributed to the ability of the selected strain to consume hexoses as well as pentoses from a variety of different substrates. Kinetics of the fermentation were studied for the first time in

bioreactor achieving an ethanol yield of 0.20 g ethanol/ g carbohydrates and 0.18 g ethanol/ g fermentable sugars.

The yield improvement experiment that followed focused on reducing the duration of aeration, while strengthening the cell concentration in the inoculum. Fermentable sugars in the sugarcorn juice were consumed by *Saccharomyces cerevisiae* ATCC 26603 within 18 hours of fermentation, while it took only 12 hours when the medium was supplemented with yeast extract. Yeast extract supplementation to sugarcorn juice helped achieve high biomass productivity, while higher ethanol production was achieved with un-supplemented sugarcorn juice. The fermentation produced 40 g/L ethanol in 26 h (Productivity= 1.5 g/L/h), and a maximum of 41.7 g/L ethanol 49 h representing a yield of 0.46 g ethanol/g fermentable sugars (90.4% theoretical) and a productivity of 0.83 g/L/h. The fermentative performance was comparable with similar batch fermentations using other fermentable juices, despite using no additional supplementation for the fermentation media.

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Chapter 6

6   Evaluation of sugarcorn as a potential Canadian biofuel feedstock

6.1   Introduction

Prominent commercial biofuel feedstocks include sugarcane, corn, sweet sorghum, energy cane, sugar beet and wheat. Sugarcorn was suggested in the paper published by Reid et al, in 2015 as a potential biofuel crop for Canada (Reid et al. 2015). The characterization studies of sugarcorn juice carried out in Chapter 3, illustrated the nutrient composition, with carbohydrates in the juice investigated extensively. Fermentation studies discussed in Chapters 4 and 5 proved the potential of sugarcorn for bioethanol production, achieving high yields with minimal or no additional nutrient supplementations.

In order to assess the suitability of sugarcorn as a feedstock for bioethanol production, it is important to evaluate its feasibility from growing the crop to the end products and by-products. For this purpose, sugarcorn was compared with select existing commercial feedstocks on the following criteria- (1) typical crop features (2) juice characteristics and (3) ethanol production process. Sugarcane and corn grain have built robust bio-economies not just due to their potential in biofuel production, but also due to other useful by-products whose commercial value has been instrumental to make the associated biorefineries profitable. A sugarcorn-based biorefinery is proposed for production of renewable chemicals and fuels from the new feedstock, and the potential benefits to Canadian bio-economy are outlined.

6.2 Comparison of typical crop growth traits and juice characteristics

Table 6.1 shows the typical crop growth features in comparison to other biofuel feedstock with sugar-rich stalks sugarcane, sweet sorghum and energy cane. Sugarcorn juice A, B or C discussed in the prior chapters are a mixture of juices extracted from 4 sugarcorn hybrids, AAFC-SC-1, AAFC-SC-2, AAFC-SC-3 and AAFC-SC-4 and typically had a brix content varying from 11-13 Brix. However, juice extracted from individual sugarcorn hybrids can have a sugar content of as much as 16 Brix (Reid et al. 2015; Reid and Morrison 2017). Sugarcorn was able to accumulate a comparable sugar content, in a growth period close to that of sweet sorghum (3.5 months), and one-third that of sugarcane and energy cane. Sugarcorn hybrids discussed earlier (SCJ C) had achieved the necessary carbohydrate content in 98 days.

Due to sugarcorn's short crop cycle, the rate of nitrogen applied during agriculture is much less than that reported for the compared energy crops. The biomass content of sugarcorn is greater than that of sweet sorghum and sugarcane, with a reported range between 85 to 115 t/ha/year (Reid and Morrison 2017). As shown in Table 6.2, sugar corn's average juice extractability of 49% (Reid et al. 2015) is however lower than that of energy cane (53.6%) or sweet sorghum (71.9%). Similar to sweet sorghum, sugarcorn juice had appreciable amounts of glucose and fructose, apart from sucrose. Glucose and fructose, readily assimilable carbon sources for fermentative microbes, accounted for 54% and 29% of the total fermentable sugars for SCJ A and SCJ B. The ash content of sugarcorn juice was interestingly more than double that of the compared energy crops.

Table 6.1 Typical features: sugarcorn, sugarcane, energy cane & sweet sorghum

Typical features	Sugarcorn	Sugarcane[b]	Energy cane[b]	Sweet sorghum[b]
Crop cycle (months)	3-4	10-12	10-15	3.5
Number of cycles/year	One	One	One	Two
N rate (kg/ha)	50	300	300	100
Yield (t/ha/year)	85-115[a]	70	100	60
Brix (% juice)	11-16[a]	13-15	10-12	11-13

Table 6.2 Juice composition: sugarcorn, energy cane & sweet sorghum

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

[a](Reid et al. 2015) [b](Kim and Day 2011) [c](Aragon, Lu, and Kochergin 2015)

6.3   Comparison of bioprocess for ethanol production

Figure 6.1 shows the comparison of a sugarcorn based process for ethanol production to that from corn and corn stover feedstock. While the downstream processing steps can be expected to remain the same for all three, the major difference lies in the required upstream processing steps.

Most of the ethanol plants in North America use dry milling (Liska et al. 2009) to convert corn to ethanol. The corn grain is washed, followed by crushing and grinding. The ground corn is then mixed with water to form a mash. During the liquefaction step, the amylose and amylopectin in the dissolved mash are subjected to partial hydrolysis by thermostable alpha-amylases to produce starch oligomers or dextrins. This is followed by gluco-amylase catalyzed hydrolysis of dextrins to glucose, and assimilation of glucose by microbial fermentation in the same reactor to produce ethanol, referred to as simultaneous saccharification and fermentation (SSF) (Bothast and Schlicher 2005; Quintero et al. 2008). The amylases cost amounts to 0.013 US$ per liter of ethanol produced (Eidman 2007). The energy costs may contribute to 20% of the total production costs, a major part of which is utilized to break down the starchy grain to glucose (Eidman 2007). This also makes the process less eco- friendly due to the fossil fuels used in at least half of the cases (Gallagher, Yee, and Baumes 2016) to supply energy required to fuel the process (Quintero et al. 2008)
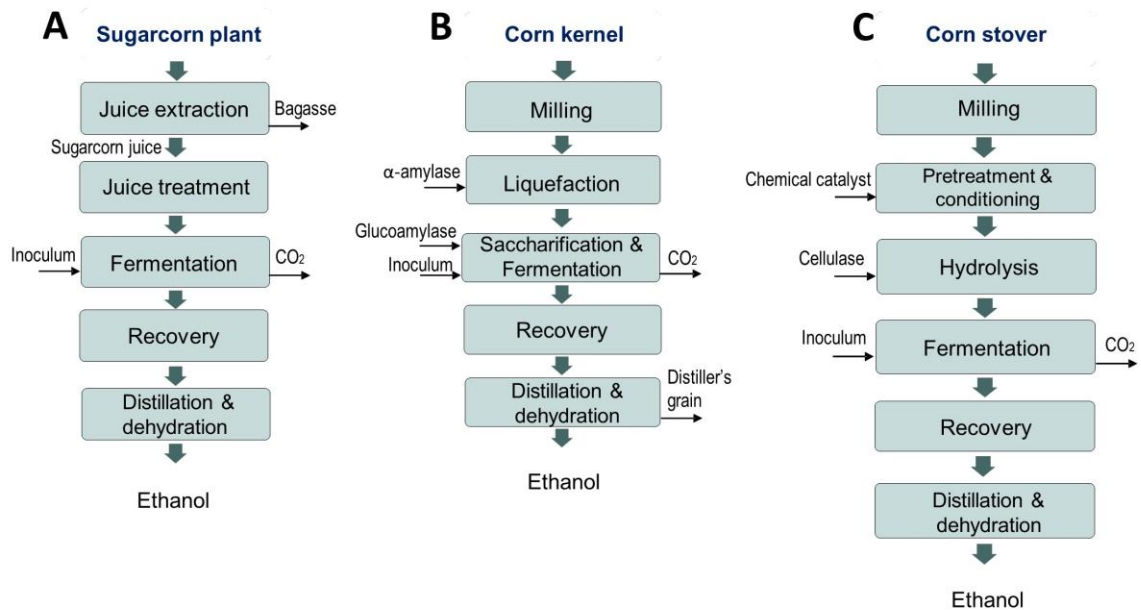
Figure 6.1 Comparison of the main bioprocesses steps for bioethanol production using A. sugarcorn B. corn kernel and C. corn stover

The corn stover pretreatment steps serve to remove lignin from the biomass, to make the cellulosic sugars susceptible to enzymatic cleavage. Chemical pretreatments such as dilute acid hydrolysis are currently more economically viable but may require detoxification. The pre-hydrolyzed corn stover is conditioned and enters a simultaneous saccharification and fermentation process. This involves saccharification of oligomers into glucose and xylose by cellulases (Aden et al. 2002), and conversion of the hexose sugars to ethanol via microbial fermentation. . The pretreatment step is highly energy intensive (Luo, Van Der Voet, and Huppes 2009), contributes to as much as 19-22% of the biofuel production costs and may result in large amounts of oligomers which may not be directly utilized by the fermentative microbes (Aden et al. 2002; Aden and Foust 2009; Yang and Wyman

2008).Cellulases cost about 0.388 US$ per liter of ethanol produced from corn stover (Klein-Marcuschamer et al. 2012; Morrison et al. 2016).

Bioethanol production process from sugarcorn will require washing and milling to extract juice from the sugarcorn to separate bagasse which can be processed separately. A juice clarification step can be carried out to remove coarse residues from sugarcorn juice, followed by a sterilization step such as heat treatment. The sterile sugarcorn juice contains readily fermentable sugars and can directly be used for fermentation by *Saccharomyces cerevisiae*. The above process can be considered similar to sugarcane processing, but unlike sugarcane in the US which has a high feedstock cost contributing to as much as 62% of the ethanol production costs (Shapouri and Salassi 2006), the sugarcorn feedstock in Canada can be expected to cost much lesser. Further, sugarcorn to ethanol process may not require the use of enzymes, unlike corn or corn stover fed processes, and can also save on energy otherwise expended to deconstruct complex starch or cellulosic chains.

6.4   Canadian sugarcorn (CANSUG) biorefinery

A biorefinery is defined as the conversion pathway from renewable feedstock (or biomass) to marketable products, via platforms and processes. A biorefinery system is generally driven by the production of large volume of biofuel, (such as bioethanol, biodiesel or biobutanol) capable of blending with gasoline or diesel. Other products from side-streams serve to generate additional revenue to offset production costs and to make the industrial venture profitable.

Based on the common classification approach for Biorefineries developed by International Energy Agency (IEA) Bioenergy Task-42 researchers in 2009 (Francesco Cherubini, Maria Wellisch, Thomas Willke, Ioannis Skiadas, René Van Ree 2009) and the inferred sugarcorn

juice characteristics and potential, a proposed two-platform Canadian sugarcorn (CANSUG) biorefinery is outlined in the Figure 6.2.
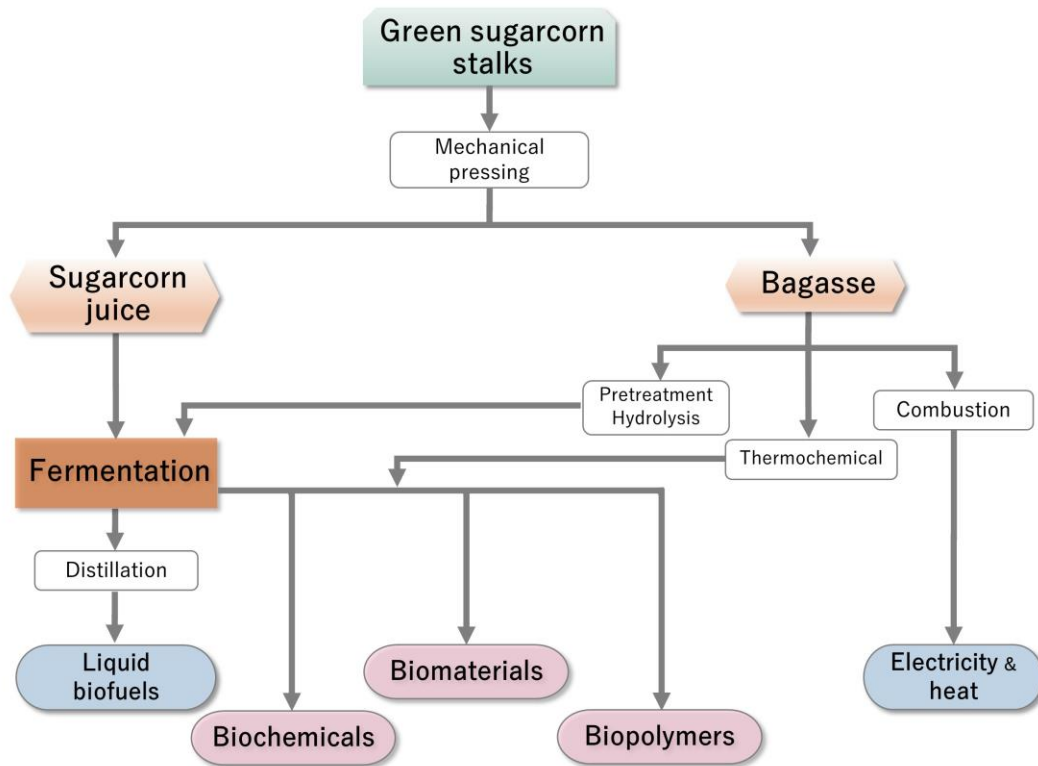
Figure 6.2 Canadian sugarcorn (CANSUG) biorefinery

The sugarcorn plants (the *biomass*), while still green, can be harvested in the weeks following silking and milled to extract sugarcorn juice. The juice serves as the main *platform* of the biorefinery and is converted via microbial fermentation *process* to the primary *product*, say, the liquid biofuel. Other compounds which may be formed during fermentations, such as organic acids and solvents, as well as spent yeasts, can serve as

useful byproducts of the main platform. Environmentally friendly biopolymers, biomaterials and biochemicals of commercial value can also be produced from fermentation stream, as primary products or as useful co-products.

The lignocellulosic sugarcorn bagasse can be pretreated and saccharified to generate a new stream for fermentation or can be combusted to produce heat and electricity, similar to the several wood chips boiler plants operating across the country. Usage of the bagasse for biogas production is also a feasible product stream. The residues of the main fermentation stream as well as the biogas fermentations can be used as a nutritious cattle fodder.

The two-platform CANSUG biorefinery can generate new revenue opportunities throughout the biomass value chain, for farmers and industries.

6.5 Conclusions

Sugarcorn hybrids can achieve plant biomass and sugar yields comparable with established sugar-based feedstocks within short growth cycle and consequently low nitrogen rate requirements. Sugarcorn juice samples used in this research on an average showed slightly higher fructose and glucose levels than sugarcane or sweet sorghum. Process for bioethanol production from sugarcorn juice does not require energy and enzyme intensive preparation steps, unlike corn ethanol or cellulosic ethanol processes, therefore may facilitate lower production costs. Sugarcorn feedstock can be used to establish sustainable biorefineries which can help strengthen the Canadian bio-economy.

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Chapter 7

7   Conclusions and Recommendations

➢ For the first time, sugarcorn juice was characterized and proven as a suitable medium for bioethanol production.

➢ Sugarcorn has abundant fermentable sugars, characteristic of established feedstocks such as sugarcane, sweet sorghum and sugarbeet. Concentration of sugars in the tested sugarcorn juice batches were 145 g/L and 102 g/L, with fructose, glucose and sucrose together accounting for about 80%.

➢ Shake flask ethanol fermentation studies using dry baker's yeast grown in sugarcorn juice medium supplemented with yeast extract achieved a maximum concentration of 45.6 g/L in 72 h, with a yield of 0.41 g ethanol/ g carbohydrates utilized and a productivity of 0.63 g/L/h.

➢ Bioreactor studies involving fermentation of sugarcorn juice by *Saccharomyces cerevisiae* ATCC 26603 produced 40 g/L ethanol in 26 h (Productivity= 1.5 g/L/h), reaching a maximum of 41.7 g/L ethanol in 49 h. The maximum ethanol yields were 0.41 g ethanol/ g carbohydrates and 0.46 g ethanol/ g fermentable sugars (90.4% of theoretical yield from glucose). Optimization of medium and the process may help replicating or improve the achieved yields on scale up. Assuming replication of maximum ethanol yields and sugar concentrations from this research, it was estimated that sugarcorn juice can potentially produce 2780-3470 L/ha ethanol, a value higher than those estimated for corn grain and sweet sorghum.

➢ The strain *Saccharomyces cerevisiae* ATCC 26603 showed good fermentative performance in sugarcorn juice medium. As the strain is known to consume a wide

range of substrates, and is adaptable to very high gravity fermentations as well as cell recycle, the strain may suit further research and scale up of production of bioethanol from sugarcorn juice.

➢ As illustrated by the proposed sugarcorn to bioethanol production process, sugarcorn juice can facilitate a direct fermentation process that circumvents the need for enzymes, unlike enzyme-driven corn grain or cellulosic bioethanol processes. Sugarcorn can also reduce energy consumption, and can help improve the energy efficiency of Canadian bioethanol plants to achieve ratios comparable to that of the Brazilian sugarcane-fed ethanol plants.

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

➢ Canadian farmers are used to growing corn (Reid et al. 2015) and possess the machinery required, hence sugarcorn can be deployed relatively faster in the agriculture sector, if a commercially viable process for bioethanol production can be developed.

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Appendix

Bioethanol fermentation for media selection: shake flask experiment (Chapter 4)

Concentration of carbohydrates, reducing sugars & ethanol for fermentations using SCJ & diluted SCJ medium

Time (h)	Sugarcorn juice			Diluted sugarcorn juice		
	Carbohydrates (g/L)	Reducing sugars (g/L)	Ethanol (g/L)	Carbohydrates (g/L)	Reducing sugars (g/L)	Ethanol (g/L)
0	119±12.6	88.7±11.7	0.00±0.00	49.8±1.3	46.4±2.1	0.00±0.00
5	94.5±0.6	83.9±1.8	0.00±0.00	46.2±7.8	37.9±0.9	0.00±0.00
10	77.9±9.5	53.1±3.3	0.71±0.09	31.7±1.2	21.8±0.2	1.08±0.35
18	62.6±7.9	30.4±1.6	13.5±0.1	11.2±1.5	10.2±0.6	6.48±0.17
24	11.0±0.5	6.54±1.01	27.0±8.6	9.56±0.31	0.78±0.19	8.09±0.64
34	4.11±0.8	1.91±0.04	29.8±11.4	1.64±0.21	0.35±0.12	8.69±1.18
48	5.04±0.7	2.30±0.04	24.7±2.3	3.08±1.64	0.08±0.00	7.43±0.28
72	9.35±5.5	2.38±0.12	22.1±3.7	5.34±0.21	0.62±0.08	7.26±0.24

Concentration of carbohydrates, reducing sugars & ethanol for fermentations using SCJ media with YE

Time (h)	Sugarcorn juice with 3 g/L yeast extract			Sugarcorn juice with 9 g/L yeast extract		
	Carbohydrates (g/L)	Reducing sugars (g/L)	Ethanol (g/L)	Carbohydrates (g/L)	Reducing sugars (g/L)	Ethanol (g/L)
0	114±13.1	84.2±0.2	0.00±0.00	103±1.4	86.1±0.1	0.00±0.00
5	105±3.4	80.4±0.5	0.00±0.00	102±2.2	80.5±1.7	0.00±0.00
10	56.3±13.4	39.4±0.1	1.11±0.16	47.5±11.7	27.0±6.3	2.57±0.38
18	4.73±0.21	1.87±0.00	14.6±0.9	10.6±0.1	1.75±0.12	20.0±0.9
24	6.06±0.31	1.36±0.12	16.0±0.3	9.25±0.21	0.78±0.70	18.1±0.3
34	4.83±0.92	1.09±0.16	19.1±0.8	2.57±2.16	0.94±0.16	19.1±1.2
48	5.04±1.95	1.25±0.23	15.6±0.7	5.14±0.21	0.86±0.08	15.1±2.1
72	12.3±2.3	1.52±0.04	30.3±1.3	13.5±3.4	1.36±0.12	21.3±6.4

Bioethanol fermentation for yield improvement: shake flask experiment (Chapter 4)

Concentration of sugars and ethanol as a function of time

Time (h)	Carbohydrates (g/L)	Reducing sugars (g/L)	Ethanol (g/L)
0	111±2.0	102±0.2	0.00±0.00
5	98.6±11.9	90.6±4.2	2.2±1.0
10	95.1±7.7	76.1±4.3	6.7±0.9
18	58.6±2.1	9.31±1.05	18.5±2.0
24	39.3±16.9	2.34±0.31	20.7±4.4
34	8.6±1.2	1.71±0.16	36.5±2.9
48	15.7±7.3	1.64±0.23	42.1±5.4
72	18.1±1.0	1.75±0.04	45.6±0.2

Bioethanol fermentation: bioreactor studies (Chapter 5)

Concentration of sugars, biomass and ethanol for *Saccharomyces cerevisiae* ATCC 26603 cultivated in sugarcorn juice

Time (h)	Fructose concentration (g/L)	Glucose concentration (g/L)	Sucrose concentration (g/L)	Reducing sugars (g/L)	Total carbohydrates (g/L)	Biomass concentration (g/L)	Ethanol concentration (g/L)
0	22.4 ± 0.9	27.1± 0.8	50.4±1.6	43.2±0.2	91.9±0.4	5.5±0.0	0.00±0.00
2	20.3± 0.9	25.8± 0.3	52.5±0.2	43.0±0.0	81.8±2.8	7.5±0.0	0.00±0.00
4	21.3± 1.8	22.9± 0.7	38.1±1.7	36.8±0.3	81.4±1.9	9.9±1.2	2.43±0.02
6	20.5± 0.3	21.7± 0.6	41.5±0.4	35.2±0.5	79.7±0.0	14.9±3.3	2.57±0.35
8	19.7± 0.5	17.6± 0.1	40.1±0.5	31.3±0.0	70.5±9.5	10.9±0.5	3.55±0.30
12	17.5± 0.1	13.2± 1.1	32.6±0.4	24.7±0.2	48.5±4.3	11.3±1.0	7.83±0.01
24	17.3± 0.1	0.00±0.00	0.00±0.00	11.5±0.3	16.4±1.2	9.35±0.85	13.9±0.6
48	0.49± 0.04	0.00±0.00	0.00±0.00	0.04±0.04	0.00±0.00	9.45±0.15	17.9±0.1
72	0.00±0.00	0.00±0.00	0.00±0.00	0.15±0.15	0.00±0.00	4.75±0.35	17.9±0.4

Bioethanol fermentation- yield improvement: bioreactor studies (Chapter 5)

Concentration of sugars, biomass and ethanol for S. ATCC 26603 fermentation in SCJ-YE medium

Time (h)	Fructose concentration (g/L)	Glucose concentration (g/L)	Sucrose concentration (g/L)	Reducing sugars (g/L)	Total carbohydrates (g/L)	Biomass concentration (g/L)	Ethanol concentration (g/L)
0	28.2±2.0	35.9±1.8	21.4±0.9	67.3±3.1	103±0.6	3.25	2.07±0.02
4	24.5±0.1	23.2±0.1	0.86±0.61	47.6±1.8	64.5±2.1	5.5	6.94±0.29
8	13.2±0.05	3.55±0.00	0.00±0.00	18.40±0.04	30.2±3.5	10	16.2±0.26
10	-	-	-	6.91±0.43	13.2±3.5	13.25	18.9±0.5
12	0.00±0.00	0.00±0.00	0.00±0.00	0.21±0.04	9.04±5.34	11	21.2±1.5
18	0.00±0.00	0.00±0.00	0.00±0.00	0.09±0.09	7.30±4.62	10.25	25.4±2.8
26	0.00±0.00	0.00±0.00	0.00±0.00	0.21±0.04	11.1±2.7	10.25	28.5±0.1
36	-	-	-	-	4.42±0.92	11.5	34.9±0.1
49	0.00±0.00	0.00±0.00	0.00±0.00	0.26±0.00	7.30±2.36	11.5	31.3±0.4
72	0.00±0.00	0.00±0.00	0.00±0.00	0.26±0.09	9.97±3.80	7.5	31.8±0.2

Concentration of sugars, biomass and ethanol for *Saccharomyces cerevisiae* ATCC 26603 grown in SCJ medium

Time (h)	Fructose concentration (g/L)	Glucose concentration (g/L)	Sucrose concentration (g/L)	Reducing sugars (g/L)	Total carbohydrates (g/L)	Biomass concentration (g/L)	Ethanol concentration (g/L)
0	28.3±0.2	34.3±0.3	25.8±0.1	69.5±2.4	111±1.9	3	0.93±0.02
4	22.1±0.2	23.1±0.1	0.00±0.00	49.0±3.1	59.8±4.9	4.5	5.60±0.29
8	17.8±0.1	11.0±0.1	0.00±0.00	32.0±1.5	41.6±1.1	7.25	13.3±0.3
10	-	-	-	25.4±1.5	38.6±4.1	7.5	19.1±0.6
12	11.6±0.2	3.1±0.19	0.00±0.00	18.8±0.8	28.9±4.4	7.75	22.3±0.1
18	0.00±0.00	0.00±0.00	0.00±0.00	0.43±0.00	15.4±3.3	10.75	38.6±0.3
26	0.00±0.00	0.00±0.00	0.00±0.00	0.43±0.09	14.5±10.2	9.25	40.0±0.2
36	-	-	-	0.30±0.04	13.8±2.9	7.75	40.1±0.1
49	0.00±0.00	0.00±0.00	0.00±0.00	0.13±0.13	9.97±1.1	7	41.7±0.7
72	0.00±0.00	0.00±0.00	0.00±0.00	0.13±0.13	14.1±2.0	6	37.1±0.4

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