Fermentation of Air Currents Assisted Particle Separation (ACAPS) by-product streams from barley for bioethanol production

by

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ABSTRACT

To offset biofuel production costs, a biorefining approach was employed that enables the co-production of high-value products. Barley starch concentrate is a by-product generated during isolation of β-Glucan from barley grains via the Air Currents Assisted Particle Separation (ACAPS) technology. This study was aimed at establishing a fermentation approach for bioethanol production from a low-value by-product stream. It is anticipated that this study will (1) minimize low-cost by-products from the β-Glucan isolation process; (2) alleviate economic stress on bioethanol production from conventional grains; and (3) increase the economic return from the ACAPS process.

The isolation of β-Glucan from Fibar barley using ACAPS technology generates a starch concentrate that is efficiently converted to ethanol (86.7 ± 3.5%) following the established fermentation protocol in our lab. Furthermore, enzymatic hydrolysis of barley starch concentrate mash is significantly more efficient compared to conventional wheat mash, producing more than 2-fold greater amount of glucose after only 2 h. Then, the timing of addition as well as the requirement of FERMGEN™ 2.5X for hydrolysis of barley starch concentrate mash were studied. It was found that barley starch concentrate does not require FERMGEN™ 2.5X for hydrolysis. Thus, the effect of omitting other enzymes during hydrolysis of barley starch concentrate mash was studied. It was observed that complete hydrolysis of barley starch concentrate mash can be achieved with STARGEN™ 002 only, and there was no specific advantage of adding FERMGEN™ 2.5X, OPTIMASH™ TBG and GC 626. Moreover, particle size distribution of wheat flour, barley flour and barley starch concentrate indicated that barley starch concentrate has a larger surface area per unit volume, which likely contributes to the higher accessibility to STARGEN™ 002.

The next step was to obtain hydrolysis kinetics for barley starch concentrate mash that were similar to the wheat benchmark by decreasing the STARGEN™ 002 concentration. In this starch hydrolysis study, it was observed that when FERMGEN™ 2.5X, OPTIMASH™ TBG and GC 626 were omitted, hydrolysis of barley starch concentrate mash with 0.5X dosage of STARGEN™ 002 showed similar enzymatic kinetics to the wheat benchmark using all four enzymes. Therefore, the impact of lower STARGEN™ 002 dosage and adding supplements on ethanol production was examined. The lower STARGEN™ 002 dosage may help generate lower sugar levels for reduced osmotic stress, thereby potentially improving ethanol yields with less enzyme cost. Here, results show that fermentations incorporating barley starch concentrate and 0.25X dosage of STARGEN™ 002 (with no FERMGEN™, OPTIMASH™ TBG and GC 626) displayed similar ethanol yield efficiency as the wheat benchmark, however, phosphorus supplementation was required.

To summarize, the barley starch concentrate examined in this study is a promising feedstock for bioethanol production compared to the wheat benchmarks commonly used in industry. This study successfully optimized the simultaneous saccharification and fermentation (SSF) while controlling rates of hydrolysis and avoiding osmotic stress on yeasts. Considerable cost saving is possible during production of bioethanol from barley starch concentrate by using decreasing dosages of STARGEN™ 002, and omitting the other enzymes typically used in wheat fermentations. This fermentation approach not only ferments barley starch concentrate efficiently but also likely creates a protein enriched distiller grains that could be valuable as animal feed. It is possible that protein would be concentrated dramatically in barley starch concentrate and higher than the protein in barley DDGS, due to the removal of fiber from ACAPS process. The development of ACAPS by-product-based biorefinery would involve effective bioconversion of

barley starch concentrate for the production of two high-value compounds, bioethanol and the co-product (DDGS), which will make the entire process more sustainable and economically feasible.

PREFACE

This thesis is an original work by Yeye Lu. No part of this thesis has been previously published. All experiments in this thesis were discussed and approved by Dr. David C. Bressler and Dr. Michael Chae. All experiments were conducted in the Biorefining Conversions and Fermentation lab at the University of Alberta. Dr. Michael Chae contributed to the thesis revisions and edits.

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ACAPS = air current assisted particle separation

acetyl-CoA = acetyl coenzyme A

ANOVA = analysis of variance

ATP = adenosine triphosphate

BSC = barley starch concentrate

BG = β-glucan

cc/min = cubic centimeters per minute

CDC = Crop Development Centre

cfu = colony-forming unit

CO_2 = carbon dioxide

CPS wheat = Canadian Prairie Spring Wheat

DDGS = dried distillers' grains with solubles

DEPC = diethyl pyrocarbonate

DM = dry mass

DP3 = cellotriosyl

DP4 = cellotetraosyl

EIA = U.S. Energy Information Administration

FID = flame ionization detector

g = grams

GC = gas chromatography

GC-FID = gas chromatography coupled with Flame ionization detector

GOPOD = glucose oxidase-peroxidase

GSHE = granular starch hydrolyzing enzymes

h = hour

HCl = hydrochloric acid

HPLC = high performance liquid chromatography

kg = kilograms

L = liters

M = mol/L

MGY = million gallons per year

min = minutes

mL = milliliters

mm = millimeter

Mt = megatonne

NADH = nicotinamide adenine dinucleotide

NaCl = sodium chloride

NaOH = sodium hydroxide

nm = nanometer

O_2 = oxygen

PGRE = Prairie Green Renewable Energy

pH = potentiometric hydrogen ion concentration

rpm = revolutions per minute

SO_2 = sulfuric acid

CAC = citric acid cycle

TDF = total dietary fiber

VHG = very high gravity

w/v = weight per unit volume percent

wt/wt = weight per unit weight percentage

µL = micro liter

1 Introduction

1.1 Project background

Bioethanol is considered as one of the most promising renewable biofuel alternatives to fossil fuels. The major bioethanol feedstocks in North America are starchy and sugary materials such as corn, wheat, sugarcane and sugar beet. However, the challenge of these feedstock is the high cost and the potential competition with food security (Kwiatkowski *et al*., 2006; Pietrzak & Kawa-rygielska, 2014). Lignocellulosic biomass is considered as a promising raw material for bioethanol production due to its abundance and also the minimized potential conflict between land use for food production and energy feedstock production (Srivastava *et al*., 2015; Zabed *et al.*, 2017). However, the access of cellulase enzymes to cellulose is difficult due to the presence of lignin and hemicellulose. Therefore, efforts should focus on low-cost substrates enriched in starch or sugar.

Barley is a crop grown for food, malting and animal feed. In 2018/19, barley was the fourth most produced cereal grain in the world after corn, wheat, rice (FAO, 2019.) Barley production is agronomically suited in much of Western Canada (Ingledew *et al*., 1995). Alberta accounts for the largest production of barley in Canada, with approximately 3.91 million metric tons in 2017/18 (Canfax, 2018). Barley is an excellent source of carbohydrates (mainly starch) and (1→3)(1→4)-β-d-Glucan (β-Glucan) (Asare *et al*., 2011a). Of the barley produced in Alberta in 2014, 80% is used for livestock feed, 19% is used for malting, while 1% of barley is for human consumption (Oliveira, 2015). However, the demonstrated health benefits of β-Glucan in lowering cholesterol levels and postprandial serum glucose levels in humans and animals has led to tremendous interest in developing efficient methods to isolate β-Glucan from barley grains.

Recently, a novel fractionation technology named air currents particle separation

technology (ACAPS) has been developed at the University of Alberta (Vasanthan, 2017). This technology enables the isolation of desirable β-Glucan at a high concentration and high yield, but at low cost. The ACAPS process generates a starch-rich by-product stream after isolation of β-Glucan concentrate from pearled barley flour. To meet the increasing demand for global bioethanol production, not only it is necessary to select a suitable raw material, but also to process it more cost-effectively (García-Aparicio *et al*., 2011). This above-mentioned barley ACAPS by-product, namely barley starch concentrate, is one of these promising substrates that could be converted into bioethanol both efficiently and economically. The attributes of elevated starch and high protein content make it a desirable substrate, as starch can be hydrolyzed to sugars and protein is likely to be concentrated in the bioethanol coproducts (distillers dried grains with solubles), which could be a valuable animal feed. In addition, barley starch concentrate mash has reduced viscosity due to the removal of β-Glucan from barley grains, which indicates that viscosity-reducing enzymes, such as β-glucanase, may not be required. Thus, bioethanol fermentation of the ACAPS by-products could be considered as a waste minimization and value-added product generation approach.

In industry, barley by-products are usually used as livestock feed or a food ingredient. By-products derived from abrasive milling of barley could be used as ruminant feed due to the high fiber, and low starch and protein (Mustafa *et al*., 1998). The pearling by-product during pearled barley production, which contains bioactive compounds (β-glucans, tocopherols, and tocotrienols), has been proposed by Marconi *et al.* (2000) to be a potential ingredient for functional foods (e.g. pastas) as a good source of dietary fiber. The yield of barley starch concentrate from barley flour is around 75%. The estimated barley starch concentrate production can reach around 900 Mt per year, taking into account that current annual production of Cerabeta^{TM} dietary fiber concentrate is

300 Mt, which is the product obtained from ACAPS process. Therefore, converting barley starch concentrate to bioethanol will add additional revenue to GrainFrac Inc, which would increase the economic return from ACAPS process.

The hypothesis of this study is that barley starch concentrate can be fermented to bioethanol at similar ethanol yields to conventional wheat flour under *S. cerevisiae* fermentation conditions. The overall objective of this study is to develop fermentation approaches to capture value from the by-product streams from the ACAPS process. The approach taken is to ferment the starch concentrate, which is also high in protein, to create a value chain including a premium enriched protein distillers grain. The short term objectives are listed below.

1.2 Objectives

1.2.1 Short-term objectives

1. To compare fermentability of wheat flour, barley flour and barley starch concentrate following the established STARGEN-based wheat-to-ethanol protocol in our lab;

2. To compare starch hydrolysis efficiency of wheat flour, barley flour and barley starch concentrate;

3. To study the effect of omitting enzymes on starch hydrolysis of barley starch concentrate;

4. To mimic hydrolysis kinetics of barley starch concentrate to that of wheat flour by optimizing levels of STARGRN™ 002;

5. To incorporate a decreasing dosage of STARGRN™ 002 into fermentation of barley starch concentrate to obtain similar/higher ethanol yield to wheat flour.

1.3 Benefits to the industry

The incorporation of barley starch concentrate into bioethanol fermentation will alleviate economic stress on ethanol production using conventional starchy or sugary materials, such as

wheat and corn. The study will provide information and scientific evidence on utilization of starch enriched by-product streams. The distiller grain remaining post fermentation is likely to consist of concentrated protein, lipid, minerals, which may help develop a new feed supplement opportunity to Alberta's agricultural crop value chains.

2 Literature Review

2.1 Ethanol's role in the world fuel economy

2.1.1 Key drivers for the development of bioethanol

An increasing energy consumption has been observed due to the growing population and continuous industrialization. Therefore, it was predicted that fossil fuels will be depleted over the next 40-50 years (Vohra *et al*., 2014). The consequence of fossil fuel deficiency will be severe for countries whose economy has dependency on crude oil. Hence, there is a great interest in exploring renewable energy resources. Bioethanol is one of the most promising alternatives to fossil fuel, and can be produced from renewable agricultural products like starchy materials (wheat, corn, barley, oats, etc.), sugary materials (glucose, sucrose and molasses, etc.) and cellulosic and lignocellulosic materials (Hossain *et al*., 2017; Puligundla *et al*., 2011). Bioethanol can be used directly as pure ethanol or blended with gasoline to produce "gasohol" (Hajar *et al*., 2017) Bioethanol offers several advantages over fossil fuel. First of all, oxygen accounts for 34.7% of bioethanol but 0% for gasoline, leading to a 15% higher combustion efficiency than gasoline (Mustafa Balata *et al*., 2008). Secondly, an environmental benefit of bioethanol is decreased carbon dioxide emissions by up to 30-50%, which can help reduce the greenhouse effect. Furthermore, the insignificant amount of sulfur content results in fewer emissions of sulfur oxide, which can help prevent acid rain (Bajpai, 2013). In contrast, the burning of fossil fuels leads to greenhouse gases that cause climate change. Thirdly, the by-products due to incomplete oxidation of ethanol, such as acetic acid and acetaldehyde, are less toxic than the by-products produced from fuel (Minteer, 2016).

The Ford Motor Company had interest in designing automobiles that run on alcohol fuels in the 1920s. The CEO of this company, Henry Ford, even quoted ethanol as "the fuel of future"

in 1925 (Bajpai, 2013). Rapid growth of the ethanol industry was observed starting from the late 1970s. For example, after the oil crisis in 1973, Brazil started a "ProAcool" program of government-mandated ethanol production in order to reduce fuel prices and the dependency on oil imports (Velasco *et al*., 2011). From the 1980s, the U.S. ethanol industry has grown tremendously in response to surging domestic use and worldwide demand. Ethanol production in the United States caught up to Brazil's for the first time in 2005, growing by 15%. As shown in Figure 2-1, there was slight growth of ethanol production from the 1980s to 2006, followed by a dramatic increase from 2007 to 2017. Between 2004 and 2017, U.S. ethanol production, virtually all from corn starch, increased from 3.4 to 15.84 billion gallons per year (Lewandrowski *et al.*, 2017)

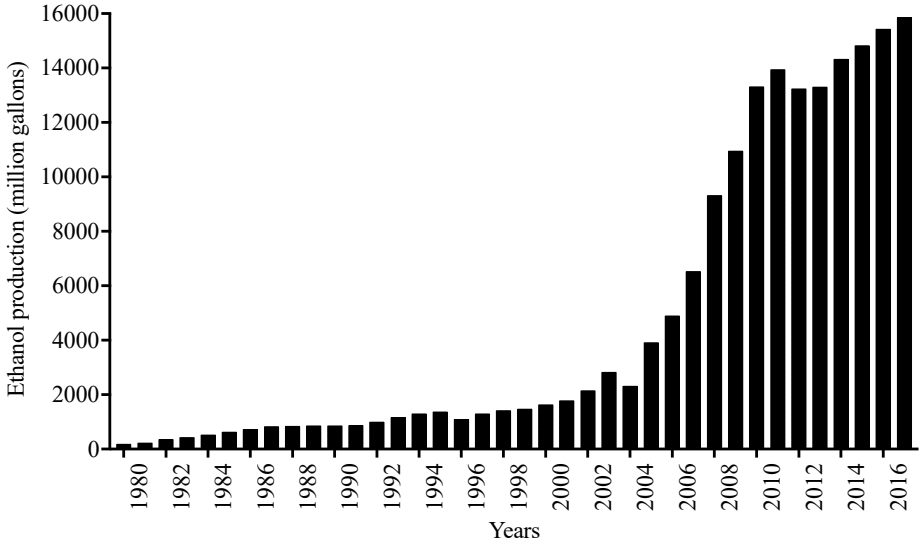


Figure 2-1 Ethanol production in the USA during 1980-2017. Reproduced with permission from Renewable Fuels Association.

In addition to the benefits of reducing carbon dioxide emissions, the key drivers for ethanol demand include energy security and social and economic pressures. There is a huge dependency on imported energy supply in the U.S. For example, Canada remained the largest exporter of total petroleum to the U.S. in March, 2019, exporting 4.4 million barrels per day (U.S. Energy Information Administration, n.d.). Secondly, bioethanol production can help the generation of

employment. A typical 100 MGY (million gallons per year) ethanol plant provides 657 direct and 650 indirect jobs (Renewable Fuels Association, 2013).

2.1.2 Global bioethanol marketplace

In order to respond to the current demand for bioethanol production, global ethanol production has increased over time as shown in Figure 2-2. Global production increased from 13.12 billions of gallons in 2007 to 27.05 billions of gallons in 2017 with a decrease in 2011 and 2012 (Renewable Fuels Association, 2018b). As shown in Figure 2-2 below, the largest ethanol producing industries, representing over 85% of the global 27.05 billon gallon produced in 2017, are located in the U.S. and Brazil. The United States is the largest bioethanol producer, with a total ethanol production of 15.8 billion gallons in 2017, which increased from 6.52 billion gallons in 2007. Brazil, the second largest producer, produces 7.8 billions of gallons of fuel ethanol annually. As shown in Figure 2-3, there was a sharp increase in ethanol production in Canada from 2007 to 2013, and reached the highest in year 2013.

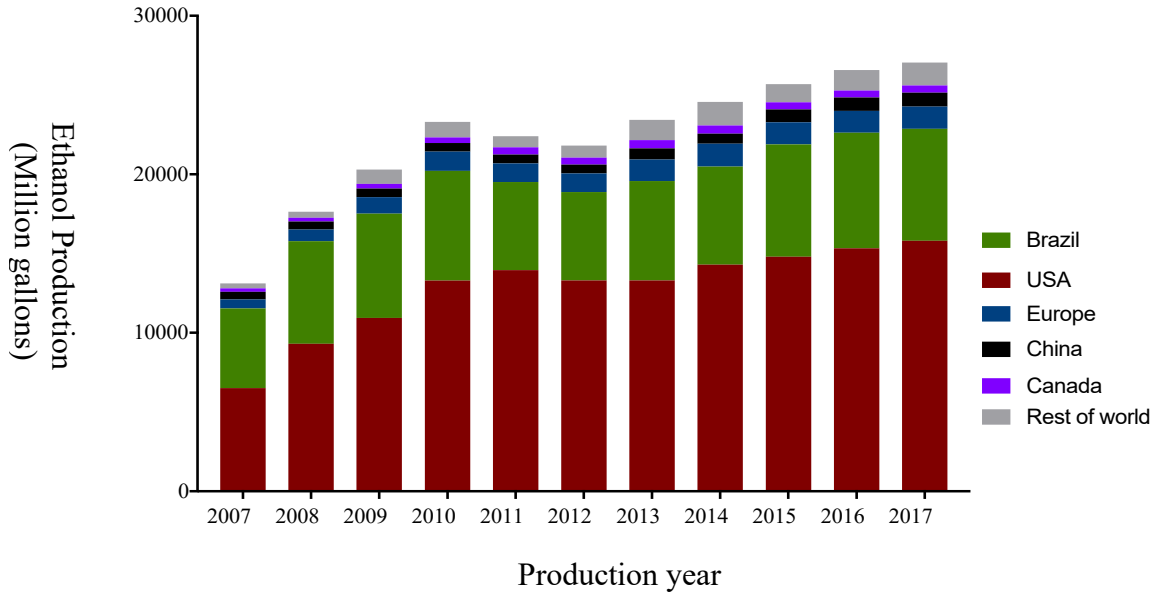


Figure 2-2 Global ethanol production in country or region during 2007-2017. Reproduced with permission from Alternative Fuels Data Center.

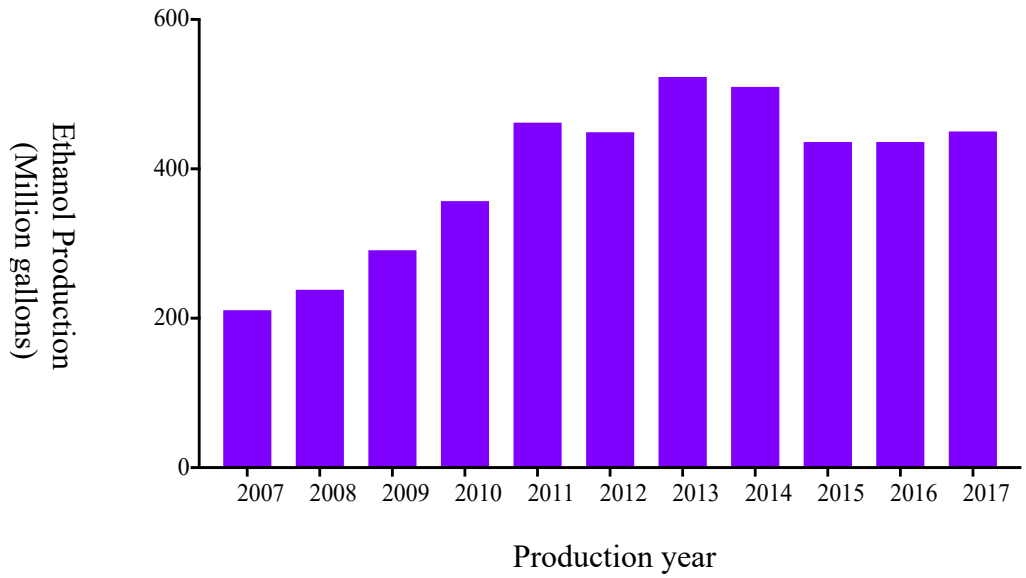


Figure 2-3 Ethanol production by Canada during 2007-2017. Reproduced with permission from Alternative Fuels Data Center.

2.2 Feedstock for bioethanol production

2.2.1 First-generation feedstocks

In North America, the first-generation bioethanol feedstock is sugary and starch-based grains. In the U.S. and Brazil, the primary fuel ethanol are produced from starchy and sugary feedstocks (Mohanty & Swain, 2019). According to the International Energy Agency (2010), over two-thirds of biofuel were produced from first-generation and land-based feedstocks. This implies the important role of first-generation feedstocks in fuel ethanol production in the global scenario.

The chief advantage of sugary crops are high sugar yields and low conversion costs, while the main disadvantage is the natural seasonal availability (Vohra *et al*., 2014). Sugary materials only require extraction processes for fermentable sugar production, such as milling and pressing for sucrose production from sugar cane. However, starchy crops require hydrolysis of starch to glucose using amylolytic enzymes, since starch cannot be utilized directly by yeast. As a result,

the energy input for bioethanol production from sugar crops is remarkably lower than that of starchy crops (Ho *et al*., 2014). However, one disadvantage of sugar crops is the disability of growing globally due to selective climate conditions and soil types.

Starchy crops are widely used for bioethanol production due to the low cost, availability across the world, storage capability for long periods and high ethanol yield. Corn is a dominant cereal crop used for bioethanol production on commercial scale worldwide (Ho *et al*., 2014). The U.S. occupies first place in terms of corn bioethanol production with a major share of around 37%, followed by China and Brazil with 21.2% and 8.3%, respectively. The ethanol plants in the United States produce bioethanol almost exclusively from corn feedstocks (Renewable Fuels Association, 2017). In 2016, 95% of total fuel ethanol produced in the U.S. was from corn starch, while 3% was from wheat.

Wheat is an ideal feedstock since it contains all the essential nutrients for yeast growth and product formation (Koutinas *et al*., 2004). It is estimated that the global production of wheat will be 734.74 million metric tons in 2018, which is less than that of corn at 1099.61 million metric tons (US Department of Agticulture, 2019). Wheat is always available and abundant in Canada (Saunders *et al*., 2012). During the 1970s, sugarcane became an industrial scale bioethanol feedstock when Brazil launched a policy aimed at developing large-scale bioethanol production. During the production, sucrose was concentrated and extracted from the juice expressed from the culms, and then the residual molasses was fermented to bioethanol. In 2018, 347,500 million liters of ethanol was produced from sugarcane in Brazil (Sergio Barros, 2017).

Wheat is unique among the grains because only wheat flour has the protein complex called ‘‘gluten’’ that can be formed into a dough (Bekes *et al.*, 2004). When wheat flour is mixed with water, gluten protein forms the skeleton of dough matrix, where starch granules are considered to

act as filler particles (Wang *et al*., 2017). As can be seen in Figure 2-4, the surfaces of two starch granules show some lipid coating. Gliadin molecules appear as single-chain molecules, contrasting with the polymers of glutenin, made up of subunits joined by disulfide bonds, either long or short coils. Gas bubbles are also present between the starch granules (Bekes *et al*., 2004).

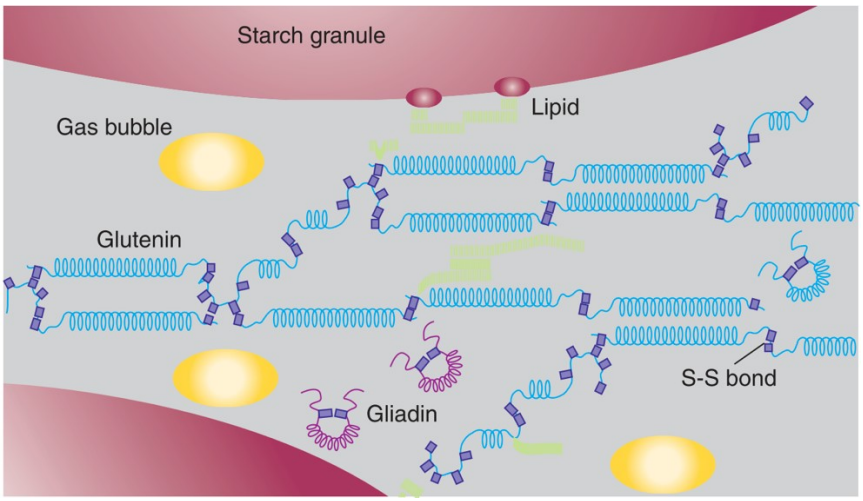


Figure 2-4 Diagrammatic representation of the molecules involved in dough formation. Republished with permission of ELSEVIER, from [WHEAT | Grain Proteins and Flour Quality, Encyclopedia of Grain Science, F.Bekes, M.C. Gianibelli, C.Wrigley and 2004]; permission conveyed through copyright Clearance Center, Inc.

There are several studies regarding the comparison of bioethanol production for small grains, such as wheat, barley, triticale and corn. Lacerenza *et al*. (2008) reported that in terms of ethanol yield per hectare, barley produced the highest level of ethanol, with soft white spring wheat ranking second. The ethanol yield per hectare of hard white spring wheat, hard red spring wheat and Durum wheat were similar and occupied last place. McLeod *et al*. (2010) conducted similar studies. In this study, samples of thirty-one cultivars of Canadian small grains from seven locations in western Canada were fermented, and their ethanol yields were compared to evaluate their potential to serve as feedstock in the bioethanol industry. As a result, the suitability of small spring grains for ethanol production ranked as: (1) hull-less barley, (2) Canada Western Soft White Spring Wheat, Canada Prairie Spring Red Wheat and Canada Prairie Spring White Wheat, (3)

triticale, (4) Canada Western Amber Durum Wheat, (5) Canada Western Red Spring Wheat, (6) hulled barley, and (7) oat.

2.2.2 Second-generation feedstocks

Although sugarcane and corn are promising sources for bioethanol production, they are not sufficient to satisfy the demand of worldwide bioethanol production. Therefore, researchers are searching for a cheaper alternative to fulfill the deficiency of bioethanol production. Second-generation feedstocks include agricultural residues, grasses, and forestry and wood residues (Vohra *et al*., 2014). Lignocellulosic biomass has attracted considerable attention in both the ethanol industry and academic research due to its availability, low cost, and lack of competition with food production (Viikari *et al.*, 2012). About 50% of the world biomass is considered as lignocellulosic biomass and its total annual production is estimated to be approximately 10–50 billion tonnes (Srivastava *et al*., 2015). Therefore, there have been tremendous efforts in the development of advanced technologies in biofuel production from second-generation feedstocks.

Lignocellulosic materials mainly contain lignin, cellulose and hemicellulose. The conversion of cellulose and hemicellulose to ethanol involves the hydrolysis of lignocellulosic biomass to produce reducing sugars through thermochemical and biochemical processes, followed by fermentation (Vohra *et al*., 2014). Although lignocellulosic biomass is considered as the most promising alternative bioenergy resource, there are technical difficulties associated with lignocellulose conversion. The intra- and intermolecular hydrogen bonds are the first reason, as these strong bonds lead to the resistance of crystalline cellulose to enzymatic hydrolysis. Secondly, the structure of hemicellulose covers crystalline cellulose, which restricts the access of cellulases to break down cellulose. Thirdly, the secondary cell wall is made up of polysaccharides bonded with lignin, which also inhibits enzymatic hydrolysis. Furthermore, lignin and its phenolic

degradation products hinder enzymatic hydrolysis (Viikari *et al*., 2012).

2.2.3 Alternative cereal grains as potential bioethanol feedstocks

The need to find a cost-effective and efficient grain source for ethanol production has amplified in significance as well. There are several potential alternative cereal grains (e.g. barley, oat, rice, sorghum) that can serve as ethanol feedstocks and provide good economic opportunities for the fuel ethanol industry.

Barley has been used as a feedstock for ethanol production in Canada and the United States (Bornet *et al*., 1987; Jones & Ingledew, 1994).The first barley-based ethanol plant was built by Osage BioEnergy LLC in Hopewell in the United States (Roy Roberson, 2009). In Europe, a fermentation procedure for barley bioethanol production has been developed by Danisco (Copenhagen, Denmark) (Roy Roberson, 2009). In Canada, Prairie Green Renewable Energy (PGRE) was built in Saskatoon that uses barley as a primary feedstock; it will produce 196 million litres of fuel grade ethanol domestically and about 228,000 tonnes of high protein/amino acid animal feed per year (Prairie Green Renewable Energy, n.d.).

Barley has several advantages in terms of bioethanol production compared to conventional feedstocks. Barley has a great adaptability to harsh environmental conditions and are able to thrive on marginal lands. In regions with mild winter, winter barley is grown as cover crop to maintain the nutrients in soil (Nghiem *et al*., 2010). Winter barley is double-cropped with corn and soybean to allow a three crop production in a two-year cycle. Barley planting will not be competing with the land used for corn production as barley grows well in some areas where corn does not grow well or at all or is too costly (Hu *et al*., 2015). Thus, barley production will benefit farmers and the rural economy outside the “corn belt” (Nghiem *et al*., 2011). The price of feed barley is relatively lower than wheat and corn and it has the potential of producing distillers dried grains with solubles

(DDGS) as animal feed with higher protein content and lysine content than that of corn (Griffey *et al*., 2010).

Increased interest in hull-less barley for food or feed utilization has resulted in the development and research of new varieties. Hull-less barley or naked grains, are developed through breeding for food and feed applications (Aldughpassi & Wolever, 2012). Hull-less barley varieties often have high digestible energy for feeding (due to increased starch content and reduced fiber levels) and high level of malt extract for brewing (Ingledew *et al*., 1995, Oliveira, 2015). Furthermore, hull-less barley is projected to be cheaper than wheat, even though it is higher than hulled barley (Ingledew *et al.*, 1995). However, the economic challenge of hull-less barley application is its relatively lower yield compared to hulled barley (Oliveira, 2015).

One barley variety, CDC Fibar, was developed at the University of Saskatchewan, Crop Development Centre's non-malting barley breeding program. This variety is a two-rowed, hull-less, waxy food barley (Canadian Grain Commission, 2019). According to Asare *et al*. (2011), CDC Fibar contains 58.1% starch, 16.6% protein, 3.6% crude lipid and 17.5% total dietary fiber. It was observed that CDC Fibar was lowest in starch, while highest in protein and dietary fiber among ten hull-less barley varieties (Asare *et al*., 2011a). Recently, chemical compositions for four hull-less barley cultivars (zero-amylose waxy, CDC Fibar; 5%-amylose waxy, CDC Rattan; normal-amylose, CDC McGwire and high-amylose, HB08302) were compared with that of one hulled barley variety (CDC Copeland). It was observed that hulled barley showed similar starch content as CDC McGwire, while both were higher than the other hull-less varieties. Furthermore, among all the varieties, CDC Fibar was highest in amylopectin (50.6% DM) and in β-Glucan (10.0% DM) (Yang *et al.,* 2013). The starch in Fibar barley contains 100% amylopectin, while normal barley contains starch with 15-25% amylose and 75-85% amylopectin (Aldughpassi & Wolever

2012, Ullrich *et al*. 1986).

The Bressler lab has examined Alberta-grown barley grains as potential feedstocks for bioethanol production (Gibreel *et al*., 2009). Ethanol yield of barley grain types including Dehulled Xena barley, Dehulled Bold barley, and Fibar barley were compared to that of benchmarks (e.g. Pioneer Hi-Bred Corn, CPS wheat). Two alternative systems, namely jet-cooking and cold starch hydrolysis, were performed. For Very High Gravity (30% solids) STARGEN^{TM}-based ethanol fermentations, dehulled Bold barley showed significantly ($P<0.05$) higher efficiency compared to dehulled Xena barley, but the difference with Fibar barley was not significant ($P>0.05$). There was a significant decrease in ethanol yield efficiency for Fibar barley when jet-cooking was applied compared to Very High Gravity (VHG, 30% solids) STARGEN^{TM}-based ethanol fermentations, possibly due to Millard reactions between amino acids and reducing sugars during jet-cooking. In addition, ethanol efficiency of barley varieties were highly comparable to benchmarks. Therefore, it was concluded that the three barley types evaluated were promising bioethanol feedstocks.

2.2.4 ACAPS by-product

2.2.4.1 The characteristics of β-glucan

β-Glucan is one of the non-starch polysaccharides that is present in the wall of endosperm cells and enclose the starch, protein and lipid of the grain. It is present in many natural sources like bacteria, yeast, fungi, algae, edible mushrooms, and cereal grains such as oats, barley, wheat, and rye (Maheshwari *et al*., 2017). The structure and scanning electron micrograph of barley grain endosperm are presented in Figure 2-5 (Vasanthan & Temelli, 2008). Among these sources, barley and oats have the highest percentage of β-glucan, varying from 3% to 11% and 3% to 7% on a dry basis, respectively. However, wheat is not considered as a source of β-Glucan due to the low content, usually below 1% (Cui & Wood, 2007).

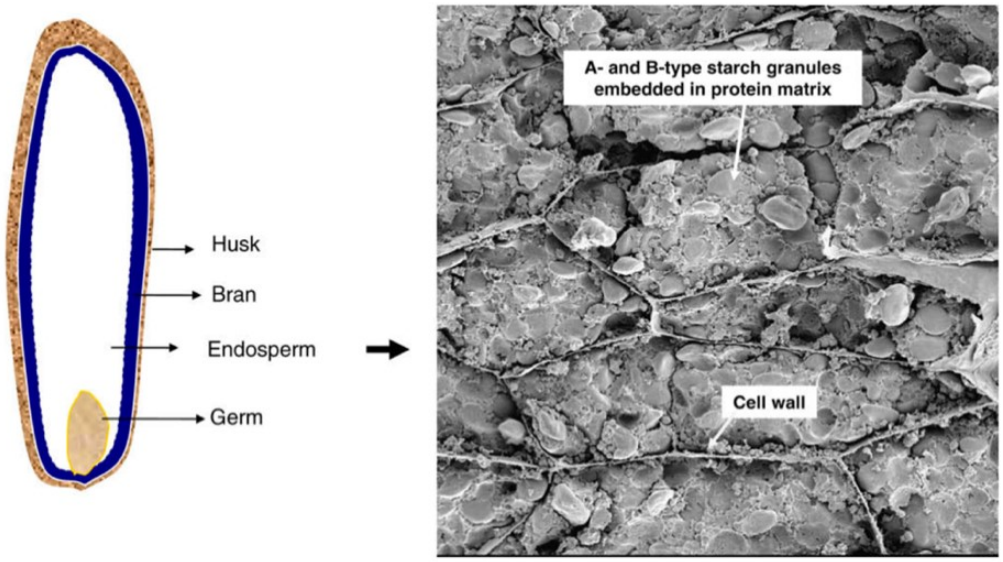


Figure 2-5 Scanning election micrograph of barley grain endosperm, showing cell walls where most β-Glucan is present. Republished with permission of Elsevier, from [Grain fractionation technologies for cereal β-Glucan concentration, Vasanthan & Temelli, volume 41 and 2008]; permission conveyed through copyright Clearance Center, Inc.

β-Glucan is composed of (1→4)-β-linked segments of mostly three (cellotriosyl, DP3) or four (cellotetraosyl, DP4) glucose units. These linear segments are linked via β-1-3 linkages, which leads to kinks in the straight chain polymer, allowing water to get in between the chains and making β-Glucan soluble in water. The solubility of β-glucans, which is inversely related to the ratio of cellotriosyl/cellotetraosyl units (DP3:DP4), is important for the viscosity of a β-Glucan solution (Skendi *et al*., 2003). A high concentration of β-Glucan produces viscous solutions, leading to problems in brewing and in the performance of animal feed (Cui & Wood, 2007).

Various studies have demonstrated the health benefits of (1→3),(1→4)-β-D-glucan, including lowering blood cholesterol levels to reduce heart disease risk, regulation of blood glucose levels for diabetes management and improvement of gut health (Forrest & Wainwright, 1977; Bornet *et al*., 1987; Gallaher *et al.*, 1993; German *et al*., 1996). The main mechanism of lowering cholesterol level is because of the soluble fibre gel that traps the bile acid. In the liver,

bile acid is produced from cholesterol, and is then released into the small intestine to digest fat. When β-Glucan is consumed, it forms a soluble fibre gel that can surround the bile acid, preventing them from being recycled back into the liver. Instead, bile acid eventually leaves the body through the digestive tract. As a result, the liver takes cholesterol out of the blood to replace lost bile acid, and thus the total blood cholesterol level decreases. Additionally, the fermentation of dietary fiber to short chain fatty acids by the bacteria in the colon may be another mechanism of providing health benefits, as it produces a large amount of propionate that significantly inhibits cholesterol synthesis (Haack *et al*., 2007, Lattimer & Haub, 2010).

Due to the documented health benefits of β-Glucan in recent years, the use of β-Glucan as a functional food ingredient from barley grains has gained attention (Mälkki, 2004; Trepel, 2004; Yokoyama *et al*., 1997; Cavallero *et al*., 2002). The demand for barley products rich in β-Glucan is increasing dramatically in the food market. Hence, development of a cost-effective process for cereal β-Glucan extraction with high yield is the current requirement for wide application in the food industry and medical uses.

2.2.4.2 Traditional technologies of β-Glucan extraction from barley grains

Various wet and dry technologies have been developed to extract cereal β-Glucan concentrate out of barley or oat grains. The wet technologies include water extraction, alkaline extraction, acidic extraction, and enzymatic extraction. Maheshwari *et al*. (2017) reviewed the above-mentioned extraction methods of β-Glucan from grain sources, and reported that water extraction and alkali extraction show better yield and recovery of extracted β-Glucan than other wet technologies. Water extraction is recommended the most by the author due to the minimization of chemical use.

Dry technologies, as well as water extraction, are considered as green technologies as no

chemicals are involved in extraction, and thus the barley fractions are considered as safe ingredient for consumers (Gómez-Caravaca *et al*., 2015a). The dry technologies include pearling, dry milling/flaking, sieving and air classification. Pearling refers to gradually removing the outer grain tissue layers, including seed coat (testa and pericarp), aleurone, subaleurone layers, and the germ to obtain a polished grain by abrasion (Marconi *et al*., 2000). Then de-hulled grains are milled to reduce particle size by dry milling. Sieving technology basically employs sieves with small openings to separate and classify milled particles by size. Air classification that is applied to the meal (from dry sieving) or the flour (from dry milling and sieving) can separate fractions enriched or depleted in β-glucan. The air-classification is based on density differences of the particles. Among the above-mentioned standard dry technologies for the processing of grains into component fractions (fiber, starch and protein), milling and sieving are the most common and economic. Nevertheless, during sieving, clogging is common with fine sieves, which slows down the feeding rate, leading to less throughput. Therefore, it has been a challenge for researchers to obtain fractionation methods with high yield and purity.

2.2.4.3 Air Currents Assisted Particle Separation Technology

Recently, an improved grain processing technology, named as "Air Currents Assisted Particle Separation" (ACAPS), was developed at the University of Alberta by Dr. Thava Vasanthan. This ACAPS technology has both design and process patents and has been internalized by a new company named GrainFrac Inc. (Edmonton, AB). Briefly, the ACAPS technology is an improved high-throughput sieving apparatus with additional air currents to facilitate the sieving process that can address the clogging problems (Vasanthan, 2017).

The apparatus used in the ACAPS process contains a top chamber separated from a bottom chamber by a sieve. By vacuum suction, milled grain particles are drawn in through inlet ports to

the top chamber. Within the top chamber, the ACAPS technology employs dynamic air currents, created under vacuum and by high pressure air pulsing, to fluidize the particles of finely ground grains allowing them to be filtered through a micron-sized sieve. As a result, fine particles pass through the openings of the sieve and enter the bottom chamber, leaving the coarse grain fractions in the top chamber. Afterwards, coarse and fine grain fractions are drawn out of the top and bottom chambers, respectively, under vacuum suction via the corresponding exit ports, thereby allowing them to be collected in the corresponding vessels. In this regard, this invention produces coarse fractions that are enriched in β-Glucan and a fine fraction. Two possible different streams are produced as fine fractions, either starch and protein concentrate (separated from milled barley and oat flours) or protein concentrate (separated from canola meal and soy meal), depending on the feedstock going into the process (Vasanthan, 2017).

The ACAPS technology is different from the normal air-classification as it is an enhanced sieving process that is based on the different size of particles, whereas normal air-classification is based on the different density of particles (Gómez-Caravaca *et al*., 2015b). For example, in the pin-milling and air-classification (PMAC) process, pin-milling is first used to disintegrate seeds to fine particles. Then air-classification is used to separate particles by differences in density, mass and projected area in the direction of air flow The ACAPS technology has substantial advantages over pin-milling and air-classification technology in the production of barley and oat β-Glucan concentrates. The ACAPS technology showed high efficiency, with respect to higher yield and higher concentration compared with pin-milling and air-classification technology, in separating β-Glucan concentrates from barley flours (Vasanthan, 2017; Figure 2-6). Secondly, the β-Glucan concentrate separated by the ACAPS technology is of high-quality. The taste and color profiles of the ACAPS β-Glucan product were neutral, which are desirable attributes for food formulations.

The β-Glucan molecules retained high molecular weight and viscosity in solution, and demonstrated excellent solubility and water binding capacity. Thirdly, the ACAPS technology is 40-50% cheaper than the existing dry processing technologies (Vasanthan, 2017). The ACAPS technology shows promise with regards to out-competing current alternatives in the food industry; ultimately this may translate to supplying new markets due to an improved affordability of this valuable concentrate.

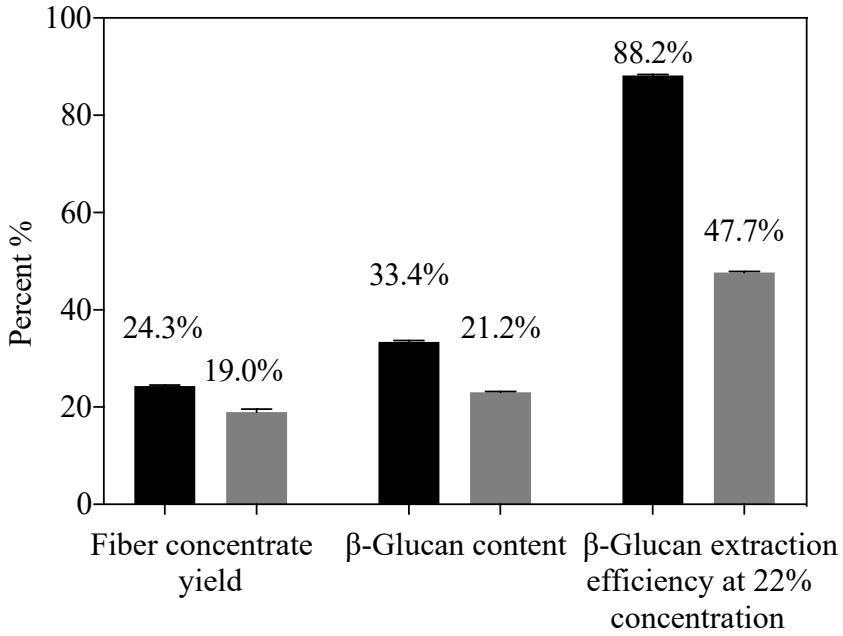


Figure 2-6 Fiber concentrate yield, β-Glucan content and β-Glucan extraction efficiency of ACAPS (black bar) and PMAC technology (grey bar). The drawing is based on the information provided by Vasanthan (2017). Tests are done on commercial scale.

2.2.4.4 Barley starch concentrate and its potential value

The composition of the ACAPS by-product may differ based on the feedstock going into the ACAPS process. When barley flour is subjected to the ACAPS process, this technology produces dietary fiber, leaving an underutilized barley by-product rich in starch, namely barley starch concentrate (Figure 2-7). It contains a significant amount of starch, which can be easily hydrolyzed into glucose using amylase. Moreover, the high content of protein can be hydrolyzed

into peptides and some beneficial amino acids, which serve as yeast nutrients and can accelerate fermentation (Thomas & Ingledew, 1990). Additionally, it contains a very low concentration of β-Glucan (1.9%) compared to normal barley grains at 5% (Vasanthan & Bhatty, 1995), suggesting that viscosity issues during ethanol fermentation will be less likely. High viscosity of the mash could impede mixing which negatively impact the distribution of yeast and nutrients (Nghiem *et al*., 2010).

Barley grains β-Glucan concentrate Barley starch concentrate

Figure 2-7 β-Glucan and barley starch concentrate and generated via the ACAPS process.

The by-product of the ACAPS process, barley starch concentrate, accounts for around 75% of the original grain. Taking into consideration that the current annual production of Cerabeta™ (trade name for β-Glucan concentrate) is 300 Mt, the estimated barley starch concentrate can reach 900 Mt per year. One of the more promising solutions for barley starch concentrate utilization is to ferment it to obtain bioethanol. At the end of fermentation, the dried distillers grains may be rich in protein, which could be an excellent animal feed. In conclusion, although the value of this barley by-product is lower than the main β-Glucan concentrate, value addition to the by-product will make the ACAPS technology more viable.

2.3 *Saccharomyces cerevisiae* and ethanol fermentation

2.3.1 Sugar utilization pathways in *Saccharomyces cerevisiae*

Yeast is a eukaryotic, single-celled organism. Yeast has a broad set of carbon sources that it can use to support growth (e.g. sugars, polyols, alcohols, organic acids and amino acids) but its

preferred carbon source is sugar (Rodrigues *et al.*, 2006). In *S. cerevisiae*, the main metabolic pathway for conversion of glucose to pyruvate is glycolysis. The pathway is operational under both fermentative and respiratory modes of metabolism. During glycolysis, one mole of glucose is catalyzed into two moles of pyruvate via several enzymes. Additionally, a net of two molecules of ATP (adenosine triphosphate) are produced in glycolysis, which can be utilized by yeast cells to drive biosynthesis and/or yeast cell growth (Bai *et al.*, 2008). Afterwards, two pathways of using pyruvate for energy production are respiration and fermentation, depending on the presence of oxygen. The pathways of carbon metabolism in yeast are shown in Figure 2-8.

2.3.1.1 Alcoholic fermentation under anerobic conditions

Under anerobic conditions, pyruvate is first decarboxylated to acetaldehyde in a reaction catalyzed by pyruvate decarboxylase. In the second step, acetaldehyde is reduced to ethanol by the action of alcohol dehydrogenase using electrons from NADH (nicotinamide adenine dinucleotide). These two key enzymes in the yeast fermentative pathway, namely pyruvate decarboxylase and alcohol dehydrogenase, have metal ion cofactor requirements of magnesium and zinc, respectively (Walker & Walker, 2018). Each mole of pyruvate will be reduced to one mole of ethanol and one mole of carbon dioxide. During the conversion of pyruvate to ethanol, there is no ATP produced (Nelson & Cox, 2008; Walker & Walker, 2018). However, the NAD+ regenerated by alcohol dehydrogenase helps maintain the redox balance and enables glycolysis to continue (Walker & Walker, 2018) .

2.3.1.2 Respiration under aerobic conditions

Respiration in cells has 3 stages, which are acetyl-CoA production, acetyl CoA oxidation, and finally electron transfer and oxidative phosphorylation (Nelson & Cox, 2008). Under aerobic conditions, in stage 1, pyruvate is firstly decarboxylated to acetyl-CoA by a pyruvate

dehydrogenase complex, releasing one molecule of CO_2. Then, in stage 2, acetyl-CoA undergoes oxidation which is carried out in the citric acid cycle (CAC). Acetyl-CoA condenses with the four-carbon acid, oxaloacetate, to produce citrate, a six-carbon acid, and subsequently, several other organic acid intermediates are formed. One turn of the cycle generates two molecules of CO_2 and regenerates oxaloacetate (Boulton *et al.*, 1999b). Furthermore, three molecules of NADH and one molecule of $FADH_2$ are generated, which are the inputs of the electron transport chain. In stage 3, electrons carried by NADH and $FADH_2$ are funneled into the electron-transport chain, ultimately reducing O_2 to H_2O (Nelson & Cox, 2008). ATP is released when electrons get transported from higher energy state to lower energy state.

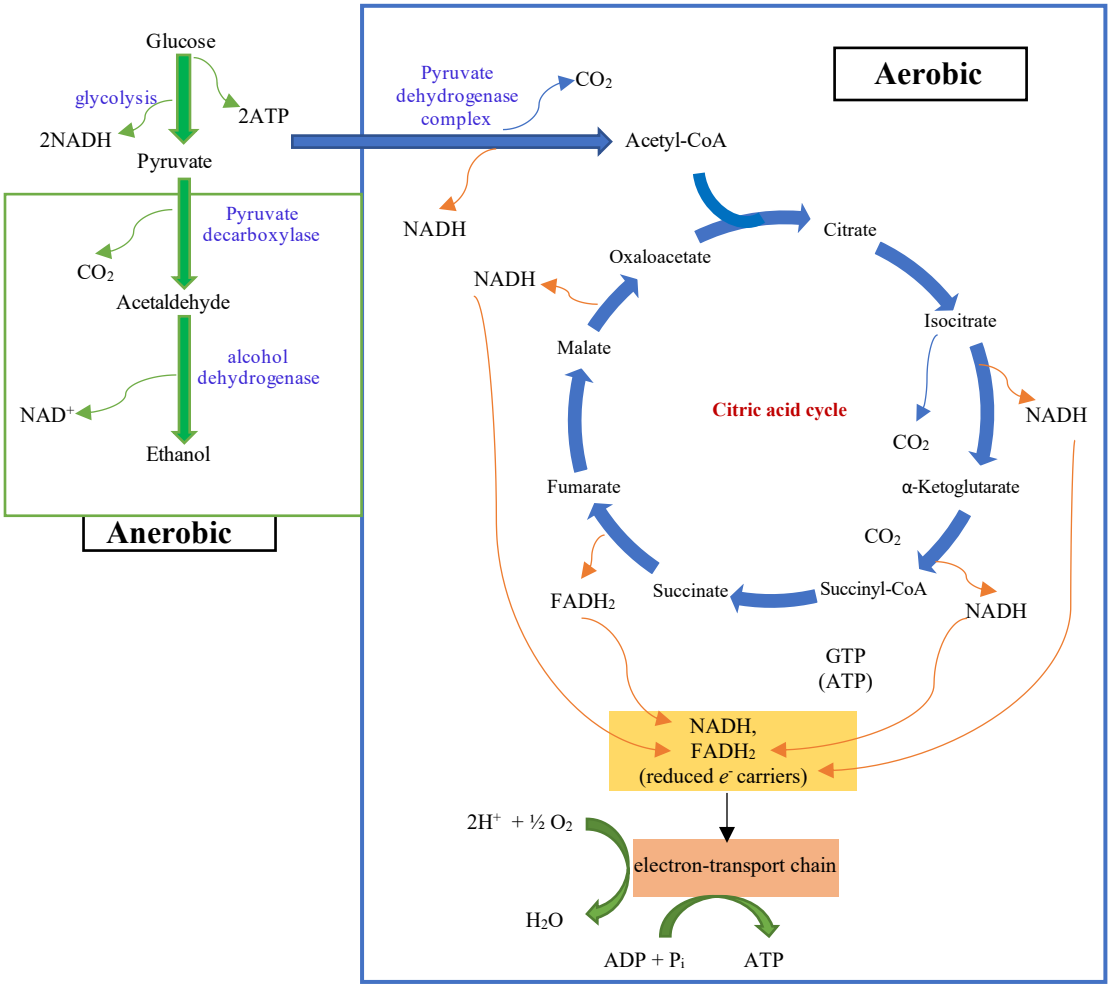


Figure 2-8 Aerobic and anaerobic respiration in yeasts (Adapted from Nelson, Lehninger & Cox, 2008)

2.3.1.3 Crabtree effect

Yeasts use fermentation as the predominant sugar catabolism pathway if sugar is present in high concentrations (e.g. above 1% sugar in the culture medium depending on the strains) despite the presence of oxygen (Jacques *et al*., 2003; Dickinson & Schweizer, 2004). Even under fully aerobic conditions, ethanol is produced by this yeast when sugars are present in excess (van Dijken *et al*., 1993). This phenomenon is called the Crabtree effect, and the yeasts expressing this trait are called Crabtree-positive yeasts (Dashko *et al.*, 2014). However, many yeasts other than *S. cerevisiae* do not exhibit the Crabtree effect (Dickinson & Schweizer, 2004). In the alcohol industry, O_2 is supplied at the beginning of ethanol production because oxygen is needed for membrane components such as unsaturated fatty acids and sterols. An alternative strategy to O_2 addition is to supplement unsaturated fatty acids and sterols into the medium (Walker & Walker, 2018).

2.3.2 Pros and cons of utilizing *S. cerevisiae* in bioethanol production

Yeast has been intensively applied in brewery and bioethanol industries for alcohol production for over a thousand years. A large variety of yeasts are utilized for bioethanol production, such as *S. cerevisiae*, *Endomicopsis burtonii*, *Scwanniomyces castelli,* etc. (Hossain *et al*., 2017). Among those yeast strains, *S. cerevisiae* is widely used for generating fuel alcohol from a variety of biomasses. *S. cerevisiae* has several desired attributes for alcoholic fermentations. For example, *S. cerevisiae* is well developed for large-scale fermentations, produces high yields of ethanol and high production rates, has robust capabilities for stress tolerance and is easy to manipulate genetically (Walker & Walker, 2018). However, there are some challenges with utilizing *S. cerevisiae* in the bioethanol industry. For instance, unmodified *S. cerevisiae* is only

able to ferment hexose, but not pentose sugars such as xylose, which is a major sugar obtained from lignocellulosic biomass (Zacchi *et al*., 2006). To solve the problem of pentose fermentation, a hybrid yeast strain with *S. cerevisiae* and xylose-fermenting yeasts like *P. tannophilus*, *C. shehatae* and *P. stipites* are being developed to yield ethanol from xylose (Hajar *et al*., 2017).

2.3.3 Main by-product of yeast fermentation

In practice, the relationship of one mole of glucose to yield two moles of ethanol is not often achieved. The reason for this may include the incomplete hydrolysis of starch, sugar consumption for cellular material, and formation of other by-products (e.g., glycerol, lactic acid, and acetic acid) (Wu *et al*., 2006). Glycerol is a major by-product during ethanol fermentation, third only to ethanol and CO_2 (Walker & Stewart, 2016). Glycerol is produced at around 1.0% (w/v) for most ethanol fermentations (Bai *et al*., 2008). High glucose concentrations increase extracellular osmotic pressure, and thus osmotolerant yeasts excrete glycerol into the medium (Petrovska *et al*., 1999). The yeast produce glycerol in order to maintain intracellular redox balance (Wang *et al*., 2007a). Briefly, glycerol derived from sugar is formed from dihydroxyacetone phohsphate (a metobolite during glycolysis). Dihydroxyacetone phohsphate is reduced to glycerol phosphate and then dephospholylated into glycerol (Boulton *et al.*, 1999b). Glycerol contributes a mouth-feel in fermented beverages, such as beer and wine, however glycerol production during bioethanol fermentations can decrease the ethanol yields (Walker & Walker, 2018).

Lactic and acetic acids are major inhibitory end-products produced by lactic acid bacteria (Beckner *et al.*, 2011). *Lactobacilli* and other lactic acid bacteria are the most common contaminants in ethanol production. It is generally believed that *Lactobacilli* causes inhibition of yeast through two mechanisms. Firstly, the contaminant in fermentation medium competes with

yeast for trace nutrients, which are required for optimal growth. Secondly, organic acids, metabolic end-products of *Lactobacilli*, inhibit the yeast growth. The undissociated form of the acid diffuses across yeast cells and then dissociates H^+ ions, resulting in an acidification of the cytoplasm (Bayrock & Ingledew, 2004). In a chemically defined mineral salts medium, lactic acid at concentrations of 0.2-0.8% (w/v) and acetic acid at concentrations of 0.05-0.1% (w/v) stressed yeast, as demonstrated by the inhibition of yeast growth, and reduction in the rate of glucose consumption and ethanol production (Narendranath *et al.,* 2001). However, industrial bioethanol is produced mainly from corn mash, which is more complex than standard growth media. Graves *et al.* (2006) studied the effect of pH and lactic acid or acetic acid on ethanol production by *S. cerevisiae* in industrially relevant corn mash media with varying solids contents. It was observed that lactic acid at 4% (w/v) decreased final ethanol concentrations in all mashes at all pH levels. The lowest concentration of acetic acid that stressed the yeast decreased from 1.6 to 0.4% (w/v) as the initial pH of the mashes declined from 5.5 to 4.0, respectively. Other metabolites produced from lactic acid bacteria are diacetyl, hydroxylated fatty acids and reuterin (an antibiotic affecting *Saccharomyces* and other fungi) (Beckner *et al.*, 2011).

2.3.4 Stress factors effecting yeast metabolism

During ethanol fermentation, yeast cells encounter various conditions, such as nutrient limitation, temperature variation, ethanol inhibition, osmotic stress, bacterial contamination, etc. These stresses can dramatically affect population dynamics and industrial fermentation, including ethanol production. Among these conditions, osmotic stress and alcohol repression are the most detrimental conditions (Zhao & Bai, 2009). Potential stress on *S. cerevisiae* during fermentation is shown in Figure 2-9.

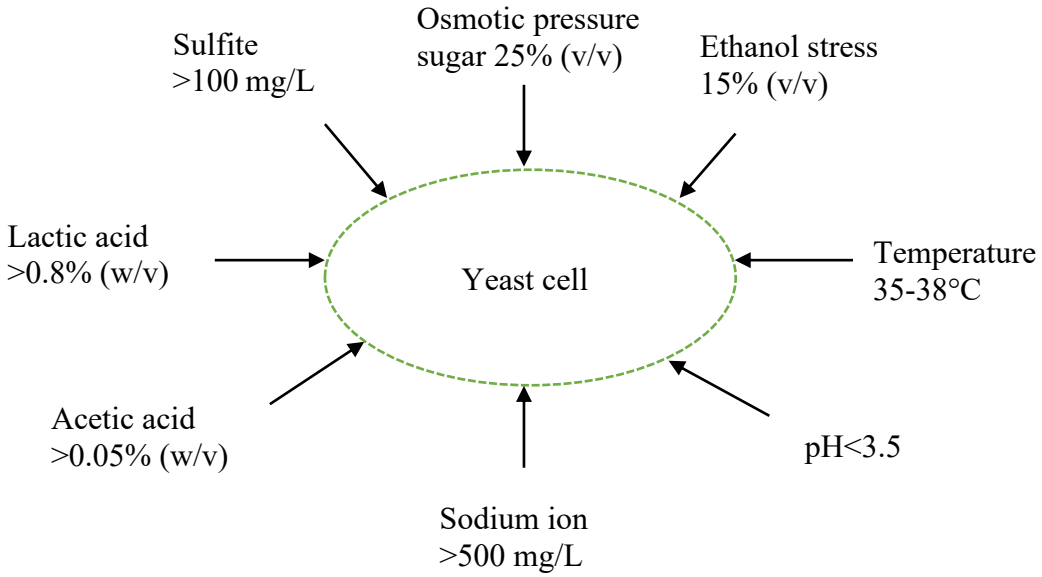


Figure 2-9 Potential stress on *S. cerevisiae* during fermentation. Republished with permission of ELSEVIER, from [Ethanol fermentation technologies from sugar and starch feedstocks, F.W. Bai a,b *et al*., volume 26 and 2008]; permission conveyed through copyright Clearance Center, Inc.

2.3.4.1 Osmotic stress

Osmotic stress is a situation where an imbalance of intracellular and extracellular osmolarities of yeast cells is sufficient to cause a detrimental change in physiology. The extent of osmotic pressure is dependent on the concentration of solutes surrounding the cell. As a response to this challenge, yeast cells will increase their cell volume to hypoosmotic stress and decrease their volume to hyperosmotic stress (Pratt *et al*., 2003). Hypoosmotic stress occurs when there is low external osmotic potential, such as in deionized water, leading to an influx of water into cell. When yeast cells are shifted to medium of higher osmotic pressure, cells will lose water from the cytoplasm, leading to a reduction of cell volume (Dickinson & Schweizer, 2004).

The major osmotic stress for yeast during alcoholic production is when they are inoculated into medium containing high amounts of sugar, such as wort during brewing (Gibson *et al.*, 2007). Yeast cells are subjected to high osmotic stress at the initial stage of fermentation when the sugar

level of the medium is over the tolerance limit (>30% w/v) (Puligundla *et al*., 2011). D'Amore *et al.* (1988) observed that the increase in osmotic pressure with glucose resulted in an increase in intracellular ethanol accumulation, associated with the decrease in yeast growth, fermentation rate and ethanol yield. The nutrient supplementation with excess peptone, yeast extract, magnesium sulfate and potassium phosphate was found to relieve the detrimental effects of high osmotic pressure. However, under these conditions, no significant effect on the intracellular and extracellular ethanol distribution was observed. This indicates the key role of nutrient limitation during fermentations with media in high osmolarity.

2.3.4.2 Alcohol repression

Ethanol is toxic to yeast cells. It was observed that the viability of yeast cells decreases with an increasing ethanol concentration from 0 to 20% (v/v) and that damage to the cell surface was observed for yeast with 10% (v/v) ethanol stress (Birch & Walker, 2000). A great deal of mechanisms have been proposed related to ethanol inhibition. It was suggested that the action of ethanol is primarily located at a hydrophobic site, possibly at a membrane (Carlsen *et al*., 1991). Ethanol can potentially interfere with hydrogen bonding within and between hydrated cell components, ultimately leading to a disruption in enzymes and membrane structure and function (Hallsworth, 1998). On the other hand, ethanol also inhibits yeast metabolism by decreasing mRNA and protein levels, reducing glycolytic enzyme activities, induction of stress response proteins, etc. (Stanley *et al*., 2010). However, yeast cells have evolved mechanisms to deal with the damage resulting from inhibition by ethanol (Jacques *et al.*, 2003).

2.3.4.3 Compound inhibition

There are some compounds in the cereal grains that may have potential inhibitory effect to yeast growth. For example, β-Glucan which is commonly present in barley mashes, may generate

the foaming problems due to its viscous elastic nature (Jacques *et al*., 2003). Some amino acids, such as glycine and lysine are inhibitory to yeast under the nitrogen-limiting growth conditions. The observation of those inhibitory effects are not observed when assimilable nitrogen is in excess. It is reported that barley mashes contains higher content of lysine compared to wheat mashes (Thomas *et al.*, 1995). Furthermore, phytic acid is known to inhibit proteolytic enzymes that may impact the hydrolysis of protein to beneficial amino acids that required by yeasts for growth. An imbalance in inorganic nutrition may cause subtle alternations of yeast metabolism and growth. For instance, a proper ratio between calcium and magnesium positively influences fermentation rates (Jacques *et al*., 2003).

2.4 Starch

2.4.1 Starch granule architecture

Four levels of organization make up the architecture of the starch granule: the amylopectin cluster, the crystalline and amorphous lamella, the blocklet and the whole starch granule (Figure 2-10) (Vamadevan & Liu, 2016). Starch granules are semi-crystalline. Crystalline lamella is formed by amylopectin double helices interspersed with amorphous lamella containing amylose and α-(1-6) branch regions of amylopectin and amylose (Tester *et al.,* 2004). The blocklet is the ordered aggregation of several crystalline-amorphous lamellae into an asymmetric structure. The size of blocklet ranges from 20 to 100 nm depending on the botanic origin of starch and location of granules. Larger blocklets occur in potato starch and are generally related to enzymatic resistance of the starch granule (Vamadevan & Liu, 2016). Aggregated large blockets and small blockets form crystalline hard shell and semi-crystalline soft shell, respectively. The arranged crystalline hard shell and semi-crystalline soft shell make up the growth ring, with an amorphous central region (hilum) consisting of amylopectin and periphery region (Naguleswaran *et al.*,

2013a).

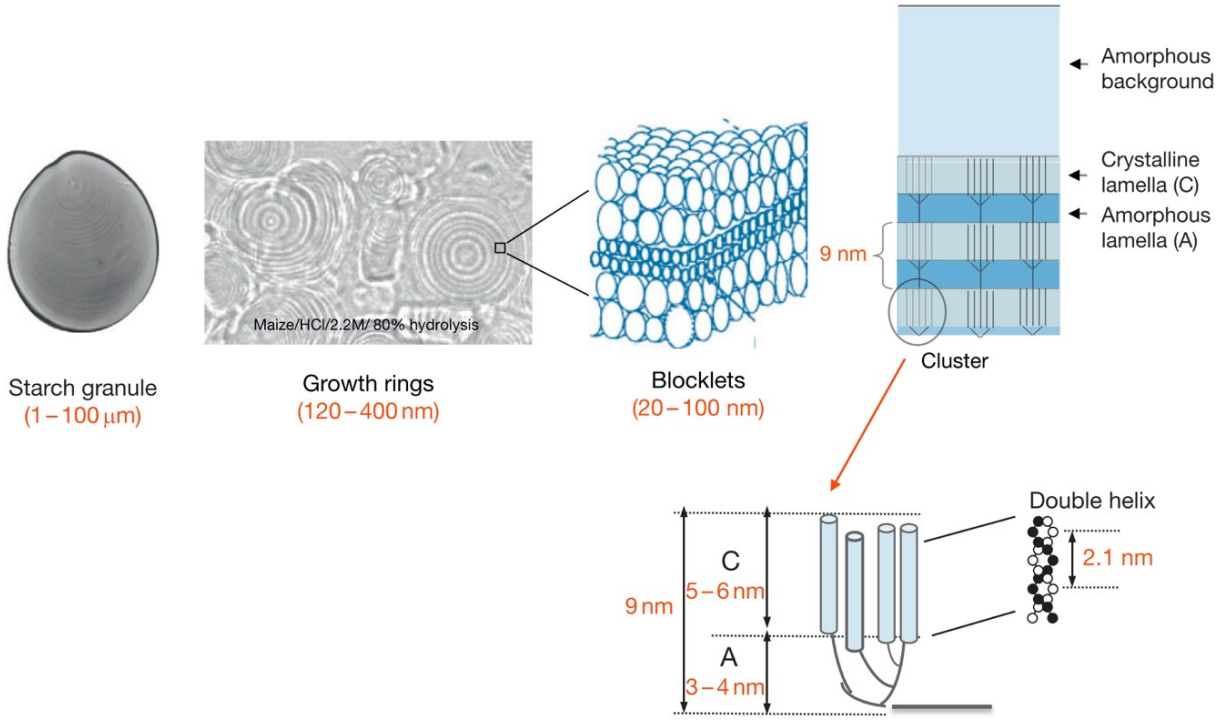


Figure 2-10 Schematic representation of the structural levels of starch. Republished with permission of ELSEVIER, from [Starch, Starch Architecture and Structure, V. Vamadevan, Q. Liu and 2016]; permission conveyed through copyright Clearance Center, Inc.

2.4.2 Starch composition

Starch is commercially isolated from many sources. The major starch source in the world is maize, followed by potato, cassava and wheat (Vasanthan *et al.*, 2016). Starch is a polysaccharide consisting of branched chains of glucose molecules. Starch granules are composed of two types of polymers: amylose and amylopectin (Figure 2-11) (Pérez *et al.*, 2009). The ratio of these two types of polysaccharides varies based on the botanical origin of starch. Amylose is a relatively long, linear chain containing 500-20,000 units of glucose joined by around ~99% (1→4)-α- and ~1% (1→6)-α-linkages. Amylopectin, a larger molecule than amylose, is heavily branched and consists of about 200,000 units of glucose joined by 95% (1→4)-α- and 5% (1→6)-α- linkages.

However, since amylopectin is extensively branched, it has unit chains that are relatively shorter than the major chain found in amylose molecules (Bertoft, 2017; Tester *et al.,* 2004). Based on the amylose concentration, starch is classified into 3 types: waxy (0-15% amylose), normal (20-35% amylose) and high-amylose (>40% amylose) (Foster Tester *et al*., 2004). Naturally occurring starch such as starch found in normal maize, wheat, and potato, contains about 70-80% amylopectin and 20-30% amylose (Pérez & Bertoft, 2010).

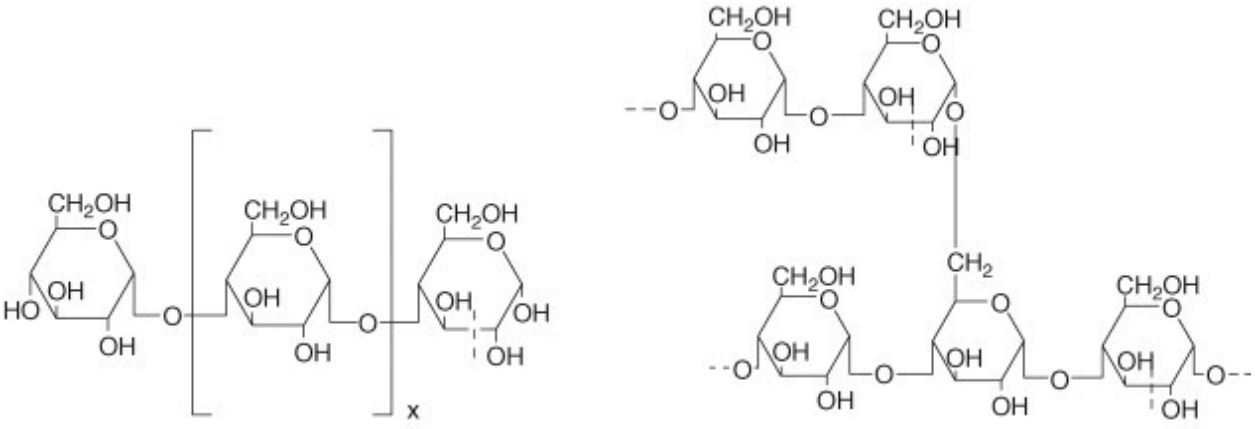


Figure 2-11 Structure of amylose and amylopectin. Republished with permission of ELSEVIER, from [Starch, Starch features of starch granules, Pérez, Baldwin and Gallant, third edition, and 2009]; permission conveyed through copyright Clearance Center, Inc.

In addition to amylose and amylopectin, there are minor components associated with starches, such as protein, lipid, moisture as well as mineral and salts. Minor components can be classified into (1) particulate material (mainly cell-wall fragments); (2) surface components (proteins, enzymes, amino acid and nucleic acids); and (3) internal components (mainly lipid). Cereal starches from wheat and maize contain 0.8-1.2% and 0.6-0.8% lipid, respectively (Buléon *et al.*, 1998). Lipids are present in starch as free fatty acids or lysophospholipids, which make amylose-lipid complexes (Pérez & Bertoft, 2010). Purified starch contains <0.6% protein, which are present on the surface and embedded within the matrix of granules. Commercially available

starch contains moisture varying from 10 to 18% depending on the source. Starch contains <0.4% minerals, including calcium, magnesium, phosphorus, potassium and sodium (Tester *et al*., 2004). The ash content is typically less than 0.5% (dry basis) (Thomas & William, 2008).

2.4.3 Barley grains structure and composition

The structure of barley grain is illustrated in Figure 2-5. The barley grain structure is similar to that of other cereals, which contain hull, endosperm and germ. The hull or husk contains cellulose, insoluble arabinoxylans, lignin, polyphenols and other minerals, and the hull contributes to 10-13% of the dry weight of barley grains making it the second largest component after endosperm. The endosperm is the major component of barley grain, taking up to 75%-80% of the total kernel weight (Hoover & Vasanthan, 2009). The endosperm consists of the starchy endosperm and a surrounding aleurone layer. Starchy endosperm is often considered as a tissue comprising cells that are rich in starch. It stores nutrients for the embryos growth during germination (Holopainen-Mantila, 2015). Starch granules are embedded in the protein matrix of endosperm wherein each cell compartment is clearly distinguished by cell walls (Naguleswaran *et al*., 2013b). Those cell walls consist of 75% β-Glucan and 20% arabinoxylan. The aleurone layer mainly consists of arabinoxylan (71%) and a minor part of β-Glucan (20%) (Ullrich, 2011). In general, the barley grain contains 60-80% carbohydrates, 9-13% nitrogenous compounds, 1-2% fat, and 10-15% water (Chibbar *et al*., 2004).

As can been seen in Figure 2-4, barley endosperm contains the starch-filled cells, which is densely packed with starch granules and storage protein bodies. The endosperm proteins can be classified as two main groups depending on their degree of association with the starch granule. The first group contains all water-soluble proteins that are not associated with the starch granule. The second group consists of the proteins that are associated with the starch granule, either on the

surface and/or internally in granule (Borén *et al.*, 2004), which are considered as granule-bond starch proteins (GBSPS).

2.4.4 Barley starches composition and applications

As the major component of carbohydrate in barley grains, starch accounts for up to 65% of kernel dry weight (Czuchajowska *et al.*, 1998). Barley starch has a diverse chemical makeup due to different genotypes, and thus can vary in the amount of amylose, lipid, ash, and phosphorus-containing compounds (Song & Jane, 2000). The compositions of barley starch can also differ based on differences in isolation and quantification methods. For example, barley starch isolates from 6 hull-less barley genotypes contain protein and ash contents varying from 0.04% to 0.30% and from 0.16 to 0.40%, respectively (Gao *et al.*, 2009). It is noteworthy that part of the protein cannot be removed with purification, and thus is retained in the starch granule interior (Hoover & Vasanthan, 2009). Barley starches contain lipids that are present on the surface and interior of starch granules, accounting for 0.1–0.2% and 0.3–1.7%, respectively (Hoover & Vasanthan, 2009). Barley starch contains ash varying from 0.10% to 0.20% (Naguleswaran *et al*., 2013).

The starch concentrate produced from barley grains is often used in feed formulations, feed pelleting and as an adhesive in paper board. There are only a few facilities producing barley starch around the world, in which milling and air-classifications are utilized to fractionate starch concentrate (up to 78% on dry weight basis) from barley grains. For example, Alko Limited, a company in Finland processes barley grains into prime starch, a lower grade starch, and by-product. Those end products are used in paper industries, potable alcohol, and animal feed, respectively.

2.4.5 Starch hydrolysis

In industry, starch is hydrolyzed to yield oligosaccharides and monomeric sugars, such as glucose and maltose, which are applied widely in food industries. For example, sweeteners and

beverage ethanol are produced from sugars hydrolyzed from starch. Acid hydrolysis and enzymatic hydrolysis are two major ways of starch depolymerization. Acid hydrolysis is simple but may form some undesirable by-products, most importantly 5-hydroxymethylfurfural (5-HMF) (Das & Kayastha, 2019). This by-product is toxic to yeast cells and inhibits yeast growth. On the other hand, enzymatic hydrolysis is eco-friendly and effective (Zabed *et al*., 2017). Figure 2-12 shows the action of amylolytic and pullulytic enzymes (Bertoldo & Antranikian, 2002).

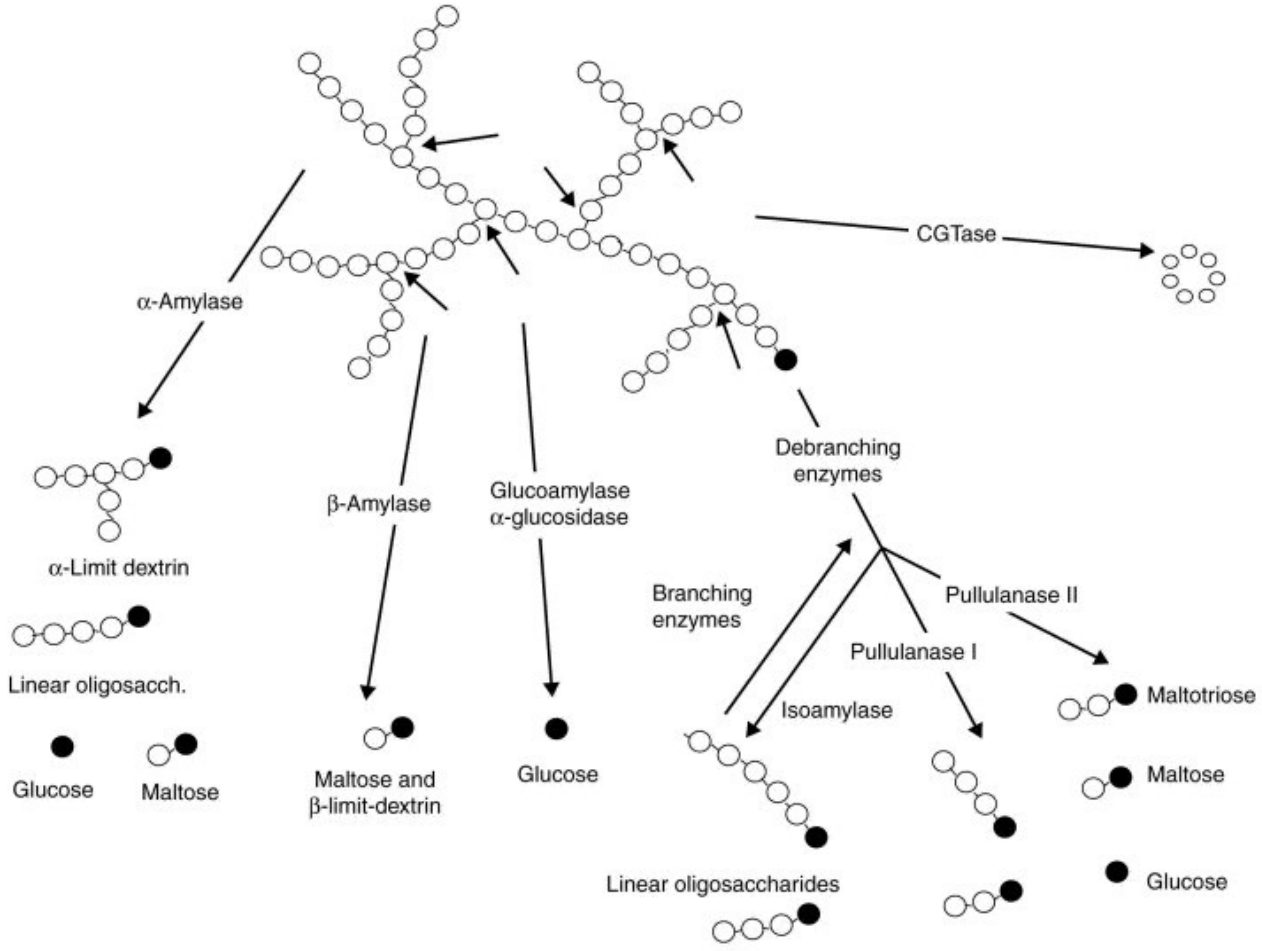


Figure 2-12 Schematic presentation of the action of amylolytic and pullulytic enzymes. Black circles indicate reducing sugars. Republished with permission of ELSEVIER, from [Starch-hydrolyzing enzymes from thermophilic archaea and bacteria, Costanzo Bertoldo & Garabed Antranikian, volume 6 and 2002]; permission conveyed through copyright Clearance Center, Inc.

2.4.5.1 Endo-acting α-Amylase hydrolysis of starch

The major category of enzyme that hydrolyzes the α-(1→4)-linkages of starch is α-Amylase. α-Amylase is found in bacteria, fungi, plants and animals (Stikvoort *et al*., 2018). α-Amylase hydrolyzes the α-D-(1,4)-glycosidic linkages in the interior of the starch polymer, which leads to the formation of linear oligosaccharides and branched and low molecular weight molecules known as α-limit dextrins, (Naguleswaran *et al.*, 2013). The action of α-Amylase on starch leads to a rapid decrease of starch solution viscosity, thus α-Amylases are called liquefying enzymes. Glucose is a minor product formed from a slow second hydrolysis of the primary maltodextrin products (Fraser-Reid *et al.*, 2014). The process of starch digestion by α-Amylase is complex and influenced by many factors. Granule size and shape are two main controlling factors as they affect the surface to area ratio. Other factors also regulate hydrolysis due to the effect on accessibility of enzymes, such as amylose content, lipid content, phosphate content, crystallinity and double helices (Tester *et al.*, 2006).

2.4.5.2 Exo-acting β-Amylase, glucoamylase and α-Glucosidase hydrolysis of starch

Exo-acting enzymes act exclusively on α-(1→4)-linkages or on both α-(1→4)-linkages and α-(1→6)-linkages at the non-reducing ends of starch chains, producing maltodextrin or glucose products. Exo-acting starch hydrolases include β-Amylase, gluco-amylase, and α-Glucosidase. β-amylase is the primary exo-acting amylase, which exclusively hydrolyzes the penultimate α-(1→4)-glycosidic bonds at the non-reducing ends of starch chains to form maltose. This enzyme is specific for amylose chains with six glucose units. Unlike α-amylase, β-amylase cannot hydrolyze maltotriose into maltose and glucose; however, a slight thermal shock can help form glucose and dextrin (Tomasik & Horton, 2012). β-Amylase is primarily found in higher plants and also some microorganisms such as *Bacillus* strains and *Clostridium thermosulfurogenes* (Sarikaya

et al., 2000). Generally, β-amylase hydrolyzes α-(1→4)-glycosidic bonds less efficiently than α-amylase.

Glucoamylase hydrolyzes the first glycosidic linkage at the non-reducing-ends of starch chains to form β-D-glucose. Glucoamylase is able to hydrolyze both α-(1→4)-linkages and α-(1→6)- glycosidic linkages, with the α-(1→6)-linkages being hydrolyzed at a faster rate. Thus, the ability to hydrolyze α-(1→6)-glycosidic linkages makes glucoamylase able to completely hydrolyze starch to glucose. However, glucoamylase has a substrate preference, as it hydrolyzes polysaccharides best (Stikvoort *et al*., 2002). Industrially, β-Amylase is combined with glucoamylase to produce high-maltose syrup (Tomasik & Horton, 2012).

α-Glucosidase is the enzyme that acts in the last step of starch degradation. It hydrolyzes the terminal non-reducing α-(1→4)-linkages in disaccharides and oligosaccharides yielding glucose. α-Glucosidase has no effect on hydrolyzing high-molecular-weight substrates such as starch or pullulan, but acts best on short maltooligosaccharides (Bertoldo & Antranikian, 2002). α-Glucosidase is found in animals, plants, bacteria or fungal species (Tomasik & Horton, 2012).

2.4.5.3 Debranching enzymes

Debranching enzymes are those that exclusively hydrolyze α-(1→6)-glycosidic bonds: isoamylase and pullulanase. Pullulanases are cable of hydrolyzing α-(1→6)-glycosidic bonds in pullulan and amylopectin, while isoamylases can only hydrolyze α-(1→6)-glycosidic bonds in amylopectin (Stikvoort *et al*., 2018). Pullulanases are classified into type 1 and 2 depending on their abilities to hydrolyze α-(1→4)-glycosidic bonds in other polysaccharides. Pullulanase type 1 specifically hydrolyzes the α-1,6-linkages in pullulan and in branched oligosaccharides, producing maltotriose and linear oligosaccharides, respectively. On the other hand. pullulanase type 2, or amylopullulanase, cleaves both α-1,4- and α-(1→6)-glycosidic bonds of polysaccharides,

producing glucose, maltose and maltotriose (Tomasik & Horton, 2012). In industry, pullulanases are usually combined with α-Glucosidases in starch saccharification due to the ability of attacking α-Glucosidase-resistant α-(1→6)-glycosidic bonds (Bertoldo & Antranikian, 2002).

2.4.5.4 Enzyme action on starch granules

Amylases react very slowly with starch granules (Nordin & Kim 1960). This is due to the highly ordered and semi-crystalline structure of starch granules (Naguleswaran *et al.*, 2013b). When starch is gelatinized (cooked) or modified, the semi-crystalline structure will become amorphous, allowing enzymes to attack starch granules and hydrolyze at a faster rate (Tester *et al.,* 2006, Wu *et al*., 2006). Generally, amylases interact with starch granules in two ways: 1) exocorrosion, which is the erosion of the surface of starch granules leading to fissures and pits; 2) endocorrosion, which is the hydrolysis of channels within granules resulting in granule disintegration (Sujka & Jamroz, 2009). The native starch hydrolysis process is generally described as follows: Firstly, enzymes randomly diffuse onto certain points of the granule surface and then hydrolysis starts. Secondly, hydrolysis continues radially, which leads to pore formation. Thirdly, the pores provide channels towards the granule core. Finally, enzymes that are trapped within the granule cause a gradual hydrolysis spreading to the surface (Tester *et al*., 2006).

2.4.5.5 Factors effecting starch hydrolysis

Several structural and physicochemical properties of starch granules, such as the granule size, granule shape, amylose content, protein content and lipid content, regulate enzymatic digestion of starch. Small native starch granules are usually digested faster than larger native granules. This is because small granules have larger surface area to volume ratio, providing a higher accessibility of enzymes to starch granules (Naguleswaran *et al*., 2012, Qi & Tester 2016). Similar to granule size, the granule shape is associated with surface area to volume ratio, thus

potentially affecting starch hydrolysis (Tester *et al.,* 2006). The ratio of amylose to amylopectin has an important impact on both starch hydrolysis and ethanol conversion efficiency. Amylose content is reported to be inversely correlated to the starch enzymatic digestibility (Asare *et al*., 2011b). Also, it was observed that waxy substrates showed higher conversion efficiency than non-waxy ones (Wu *et al*., 2006; Zhao *et al.*, 2009). This is because waxy starches are more crystalline and are more readily damaged by milling, making them more easily hydrolyzed by α-Amylase (Tester *et al*., 2004). On the other hand, for starch granules with high amylose content, the amylose chains may have been packed, which leads to hydrolysis difficulties (Naguleswaran *et al*., 2013).

Minor components associated with starch granules, such as lipid, protein and phosphate impact accessibility of enzymes to starch granules. A study in rice starch revealed that both lipid and protein may form a coating around the starch granules, which inhibits the hydrolysis by lowering the accessibility of digestive enzymes to starch granules (Ye *et al*., 2018). Naguleswaran *et al.* (2011) obtained similar results when they studied the effect of removing minor components (i.e. protein and phospholipids) on starch amylolysis of triticale and corn. In that study, protein and phospholipid were found to be mainly present on the starch granule surface, and also rich in the internal channels. Phosphate present in starches are in the form of phospholipids, phosphate monoesters, and inorganic phosphate (Jane, 1994; Tester *et al*., 2006). Theoretically, starches with high amount of phosphate usually have lower enzymatic digestibility (Naguleswaran *et al*., 2013b). Absar *et al.* (2009) observed that a higher phosphorus content was associated with a lower enzymatic digestibility of gelatinized starch ($P<0.01$). In another study, starch hydrolysis rate was reported to be significantly negatively correlated with phosphorus content in raw starch, whereas this negative correlation was weak in gelatinized starch ($P<0.05$) (Zaidul *et al*., 2008). Thus, both studies reported a negative correlation between starch hydrolysis and phosphate content however

at different significance levels. Phosphate is believed to limit the enzymatic hydrolysis by forming bonds with starch, or by blocking the active sites of starch granules from amylase attack.

2.4.6 Resistant starch

In the bioethanol industry, resistant starch (RS) is a portion of raw starch that is unavailable to enzymatic hydrolysis and does not ferment to ethanol (Sharma *et al*., 2010). In the food industry, resistant starch is defined as starch that escapes digestion in the small intestine, though it may be digested in the large intestine (Haralampu, 2000). Resistant starch that escapes from hydrolysis and fermentation processes will lower the overall ethanol yield. In support of this, Sharma *et al.* (2010) reported that a higher initial resistant starch content leads to a lower efficiency of starch conversion and thus a lower ethanol yield.

Resistant starch can be classified into 5 groups: 1) RS1, physically inaccessible to enzymes due to incomplete grain milling or chewing, mainly from whole or partly milled grains and seeds, legumes; 2) RS2, a certain type of granule that resists enzyme action due to the compact structure in raw starch (i.e., raw potato, banana, and high-amylose starch), such as ungelatinized starch; 3) RS3, retrograded starch formed during cooling of gelatinized starch, which is the most resistant starch; 4) RS4, chemically modified starch, such as cross-linked starch (Sajilata & Singhal, 2006); and 5) RS5, amylose-lipid complex which makes starch more thermally stable (Ordonio & Matsuoka, 2016). In terms of the formation of resistant starch, in particular RS2, the starch granule is tightly packed radially and is dehydrated, leading to difficulties during amylolytic attack of starch granules. This explains the resistant nature of raw starch granules (Haralampu, 2000).

There are intrinsic and extrinsic factors that influence the formation of resistant starch. In most cases, a higher amylose content results in higher resistant starch (Liljeberg & Bjo, 1998; Wu

et al., 2006). The amount of amylose also contributes to the extent and rate of retrogradation of starch, which influences the resistance to amylase digestion. In addition, the association of minor components (i.e. protein, fiber, enzyme inhibitors, sugars, ions, lipids) affects the resistant starch content. For example, interaction between starch and protein decreases resistant starch content (Sajilata & Singhal, 2006). High temperature processing, such as steam cooking, will increase the production of resistant starch content (Kulakow *et al.*, 2017). However, even though higher temperature increases the resistant starch content, the high temperature during liquefication leads to an opposite result in ethanol fermentation. Sharma *et al.* (2010) observed that a higher temperature liquefication leads to a better starch conversion, thus a higher ethanol yield and less residual starch, than lower temperature liquefication.

2.4.7 The importance of starch hydrolysis in bioethanol production

Starch hydrolysis and its enhancement are important matters in the bioethanol industry. Starch cannot be fermented directly by *S. cerevisiae* (Jacques *et al*., 2003; Walker & Walker, 2018). Thus, starch requires prehydrolysis to fermentable sugars (e.g. glucose, maltose and maltotriose) prior to fermentation (Walker & Stewart, 2016). As mentioned in section 2.3.3.5, the variation in starch structure and physicochemical properties affects the enzymatic conversion of starch to sugars, and ethanol yield. Two basic conventional steps are employed to hydrolyze starch in the ethanol industry, namely liquefication and saccharification. During liquefication, starch is firstly gelatinized under high temperature, followed by conversion of liquefied starch into short chain products using thermostable α-Amylase. Afterwards, glucoamylase works on the liquefied starch granules during saccharification, resulting in the formation of glucose from dextrins (Zabed *et al*., 2017). As an alternative, cold starch hydrolysis is an energy-saving and efficient strategy which will be described in section 2.5.2. Based on the hydrolysis of starch, the industrial integrated

technologies employed include separate hydrolysis and fermentation (SHF), and simultaneous saccharification and fermentation (SSF). SHF conducts liquefication, saccharification and fermentation steps separately, thus hydrolysis is done before fermentation (Zabed *et al*., 2017). Whereas in SSF, sugar is fermented to bioethanol as soon as it appears in the medium. SSF combines the enzymatic hydrolysis and ethanol fermentation in order to keep the glucose concentration at a low level, which avoids osmotic stress and reduces the risk of contamination (K. Liu, 2011a; Vohra *et al*., 2014).

In order to enhance hydrolysis of starch, research efforts are focused on choosing suitable starch materials and optimizing hydrolysis conditions. Tester *et al.* (2006) concluded that an enhanced enzymatic hydrolysis can be achieved by utilizing waxy starches, milling starch to fine particles, and utilizing starch materials without coating of lipid and proteins. In addition, enzyme concentrations, incubation temperature, liquefication time, pH and pretreatment are important factors in terms of optimizing hydrolysis conditions (Masiero *et al*., 2014, Shanavas *et al.*, 2010).

2.5 Technologies for starch-based bioethanol production

Overall, the ethanol production process involves 1) obtaining sugar solutions from feedstocks, 2) converting sugar to bioethanol by fermentation, and 3) separation of ethanol by distillation and purification. However, the detailed process depends on the raw materials that are used. Efficient and high yield fermentations are critical for the ethanol industry. The ethanol industry has engaged in implementing energy-saving and productivity-maximizing technologies to their existing production methods, including cold starch hydrolysis and very high gravity fermentation.

2.5.1 Wet milling and dry milling

Dry milling and wet milling are two distinctive methods commercially adopted for

bioethanol production. A corn wet mill uses corn as feedstock, and involves fractionation of corn to separate corn oil for food, and corn gluten meal and corn gluten feed for animal feed, while the starch is hydrolyzed to sugars for ethanol production (Vohra *et al*., 2014). This process includes several basic steps, which are steeping, separation and recovery of germ, fiber, protein and starch in succession (Wronkowska, 2016). Steeping involves the soaking of clean corn grains in water solutions of SO_2 (sulfur dioxide) under controlled conditions to soften the kernel texture. The next step is the separation of kernel components and washing. In this process, the steeped corn is coarsely ground and germ (the embryo of the seed) is separated using hydrocyclones, followed by the washing of germ-rich materials to extract corn oil. After the separation of germ, the degermed corn slurry is screened to separate fiber from the starch and gluten based on their different particle sizes. The last step is primary starch separation, during which gluten and starch streams are produced. Afterwards, the separated gluten is sold as animal feed while the recovered starch is hydrolyzed to sugars for bioethanol production (Rausch *et al*., 2018). Therefore, wet milling requires extensive equipment and capital investment.

Dry milling is more suitable for small-scale ethanol plants that mainly produce ethanol and distiller grains (Zabed *et al*., 2017). A classical dry milling of corn grains includes grain receiving, liquefaction, saccharification, fermentation, distillation and ethanol recovery, as well as stillage processing. In this process, the corn first is cleaned and ground in a hammer mill. Then, the ground corn is added with process water to obtain a slurry. The mixture is subjected to liquefaction, where starch is gelatinized in a jet-cooker, and converted into dextrins with thermostable α-amylase, followed by further conversion by glucoamylase to glucose. Glucose is fermented to ethanol and carbon dioxide using yeast. The solids (e.g. fiber and gluten) remaining after fermentation are dried to produce distiller grains, which is sold as animal feed.

In summary, the difference between dry milling and wet-milling is the aims. Dry-milling is focused on the capital return of ethanol production while wet-milling is concerned with the multiproduction of valuable products, before ethanol is fermented from starch (Bothast, 2005). In the U.S., around 90% of ethanol plants are dry mills (U.S. Department of Energy, n.d.).

2.5.2 Jet cooking and cold starch hydrolysis

Jet cooking is a process in which a jet cooker (steam injection heater) employs high-pressure steam, which contacts starchy materials to form a slurry of granular starch (Jeffrey, 2003). This helps fully gelatinize starch granules by destroying the semi-crystalline structure of granules, which facilitates hydrolysis of starch using enzymes (Li *et al*., 2014). Jet cooking is widely applied in the gelatinization and liquefaction step during bioethanol production from starchy materials (Septiano *et al.*, 2010). Ground starchy materials mixed with processing water, thermostable α-amylase, ammonia, and lime (to help adjust pH) are sent to a slurry tank, and then the starch is gelatinized in a jet-cooker at high temperatures (85–105°C in laboratory or up to 165°C in commercial plant), and hydrolyzed into short chains (dextrins, maltose and maltotriose) with thermostable α-amylases. During the two steps, all of the amylose is solubilized and leached out from the starch granules. As a result, the viscosity of slurry increases by 20-fold due to the swelling of starch granules, making pumping and transferring difficult. Afterwards, the gelatinized starch is liquefied with α-Amylase and thus viscosity decreases. It is estimated that the energy input of these two steps are 30% ~ 40% of the total energy for starch-based bioethanol production (Lee *et al.*, 2012). Therefore, this process is energy-intensive and costly.

The concept of an alternative hydrolysis strategy, known as non-cook starch hydrolysis, raw starch hydrolysis or cold starch hydrolysis, was introduced in the 1940s (Cinelli *et al.*, 2015). There has been substantial interest and research in cold starch hydrolysis for bioethanol production

and food research in the last decades (Wang *et al*., 2007a; Gibreel *et al*., 2009; Uthumporn *et al*., 2009; Li *et al*., 2012). The enzymes utilized for this process are known as Granular Starch Hydrolyzing enzymes (GSHE), and were developed by GENENCOR (now a Danisco Division). These enzymes hydrolyze the native starch granules into fermentable sugars at sub-gelatinization temperatures, eliminating the need for excessive heating energy required for the process of jet-cooking. Cold starch hydrolysis includes the disintegration of starch granules, the decrease of average molecular weight, the dissolving of starch polymer fragments, and the formation of fermentable sugars. This technology reduces the energy consumption since the liquefication and cooking steps are not required (Cinelli *et al*., 2015). Furthermore, the gradual release of sugar will reduce osmotic stress to microbes and the use of low heat will prevent by-product formation from the Maillard reaction, which maintains the high yield (Sun *et al*., 2010).

2.5.3 Very high gravity fermentation

In very high gravity fermentation, very high concentrations of sugars (>250 g/L) are loaded into the fermentation systems whereas normal fermentation uses mash containing less than 180 g/L of sugar (Baeyens *et al*., 2015). In the 1990s, very high gravity mash was proposed with the medium containing sugar over the level at 250 g/L to achieve over 15% (v/v) ethanol concentration (Thomas *et al.*, 1995; Thomas *et al*., 1996; Wang *et al*., 1999). Due to the reduced consumption of process water, distillation costs and stillage evaporation costs can be significantly reduced (Wang *et al.*, 1999a; Puligundla *et al.,* 2011). Besides, the high ethanol productivity can help offset the capital cost and also energy cost per liter of ethanol (Deesuth *et al.*, 2015). Another potential advantage of the very high gravity fermentation is reduced survival and proliferation of contaminating bacteria which reduce ethanol yield (Thomas *et al.*, 1996b). In very high gravity mash, yeast's stress tolerance becomes an important factor as the osmotic stress and ethanol level

can affect the yeast (Jacques *et al*., 2003). It has been proven that the detrimental impacts due to the osmotic stress on yeast cells can be effectively alleviated by nutrient supplementation (D'Amore *et al.*, 1988). For example, the addition of yeast extract to very high gravity wheat mashes leads to stimulation in fermentation rate. This seems to be mediated through to the increased growth of yeast in the mash with less than 35 g/100 mL dissolved solids, or the improved fermentation capacity of yeast cells in the mash with above 35 g/100 mL dissolved solids (Thomas *et al.*, 1993).

2.6 Distillers dried grains with solubles (DDGS)

Distillers dried grains with solubles (DDGS) is the coproduct of conventional dry-grind bioethanol production (Singh *et al*., 2002). Various feedstocks, such as sugarcane, barley, corn, oats and wheat are used for DDGS production. The detailed production procedure of DDGS is described in the literature quite intensively. Briefly, at the end of fermentation, distillation is employed to remove ethanol, whereas the remaining non-fermentables are dried to DDGS (Singh *et al.*, 2002). During distillation, a heating column is used to recover alcohol due to different boiling points (78°C for alcohol and 100°C for water). After distillation, the remaining solid and liquid is known as whole stillage. Then, the whole stillage is centrifuged to produce thin stillage (a liquid fraction) and distillers wet grains (a solid fraction). Around 15%-30% of the thin stillage is recycled as backset to be used as process water to slurry the ground grain, while the remaining portion is evaporated, and concentrated into condensed distiller solubles. Ultimately, the condensed distiller solubles is mixed with distillers wet grains to form distillers wet grains with solubles, and then dried into DDGS (Liu, 2011b; Bothast, 2005).

The ethanol fermentation co-product, DDGS contains concentrated nutrients, including proteins, fat, resistant starch, fibers, minerals and yeast cells (Li *et al*., 2014). In DDGS, starch is

decreased to about 6.0%, whereas the protein, oil, and ash contents increase about 3-fold over the original ground corn. DDGS protein (20%) is from yeast and the rest of the protein is from ground corn (Han & Keshun, 2010). Liu (2011) compared the mineral composition of DDGS with that of ground corn samples, and showed that in DDGS, concentrations of minerals, including phosphorus, potassium, magnesium, copper, and zinc demonstrated a ~3-fold increase. In addition, sodium, sulfur, calcium and iron increased 260-fold, 7.77-fold, over 5-fold, and 2.38-fold, respectively. However, the much higher sodium in DDGS may exceed the requirement of most ruminants and lead to nutritional disorders. Belyea *et al*. (2004) measured the composition of DDGS samples from a corn ethanol plant located in Minnesota, U.S. The mean concentration (g/100 g dry matter) of crude fat, protein, crude fiber, acid detergent fiber, ash and residual starch were 11.9%, 31.3%, 10.2%, 17.2%, 4.6% and 5.1%, respectively. Liu (2009) showed that the average values of six DDGS samples from fuel ethanol plants for protein, oil, ash, and residual starch were 27.4%, 11.7%, 4.4%, and 4.9% dry matter, respectively.

During starch hydrolysis, due to the incomplete conversion of starch to sugars, residual starch remained in the coproducts (Liu, 2011b). The DDGS contains over 5% of the residual starch (Plumier *et al.*, 2015). The amount of residual starch in DDGS is dependent on the physicochemical properties of starch (i.e. starch content, amylose/amylopectin ratio, crystalline structure, and association between starch and other minor components), grain species, hydrolysis conditions and quantification methods (Li *et al*., 2014). Li *et al*. (2014) studied the microstructure and explored the resistant starch origin of residual starches in DDGS. During cold starch hydrolysis, some starch granules are embedded in the protein matrix or entrapped in cells. They could be grouped as RS1 and RS2 as they were physically unavailable for complete enzymatic hydrolysis. On the other hand, during the jet-cooking process, all the types of resistant starch were formed.

Over the last decades, the production of DDGS in the U.S. has been rapidly increasing, as the number of dry-grind ethanol plants increases. As shown in Figure 2-13 (Renewable Fuels Association, 2017), the output of animal feed has increased dramatically from 2000 to 2011; however, there was a slight decrease within the next two years. In 2018, 41.30 million metric tons of co-product animal feed were produced. In the U.S., most (98%) of the DDGS is produced from dry-grind ethanol plants, while the rest is from the beverage industry.

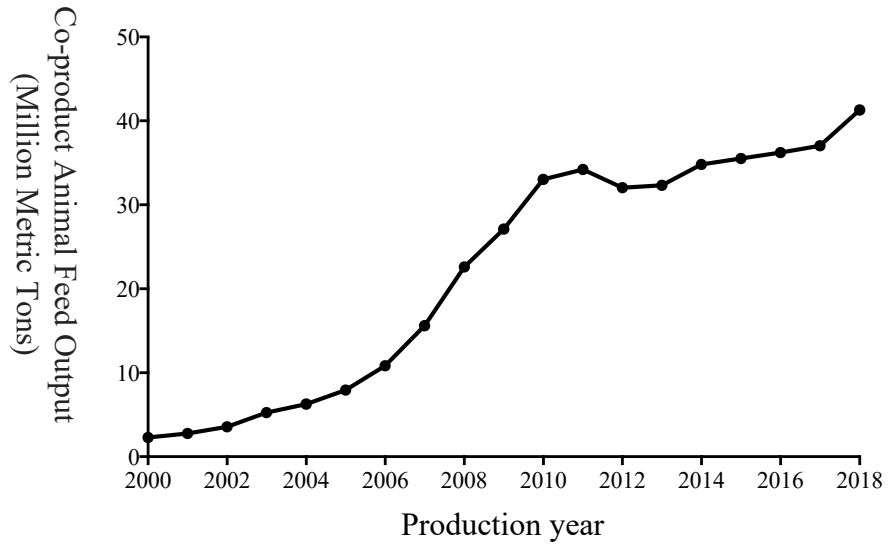


Figure 2-13 U.S. ethanol industry co-product animal feed output. Reproduced with permission from Renewable Fuels Association.

DDGS is a high-value animal feed. The attributes of high energy, protein and mineral content make it comparable to those expensive conventional animal foods, such as corn and soybean meal. It is frequently used in beef, dairy, poultry, swine, and aquaculture feeds. Economic returns from selling DDGS as animal feed can help offset much costs of ethanol production (Jacques *et al*., 2003).

Based on ethanol's value-added proposition (Renewable Fuels Association, 2018a), distiller grains added 49% of value to every bushel of corn processed. Thus, DDGS is the key to sustainability of bioethanol and livestock industries (Rosentrater, 2012). However, the challenge of utilizing DDGS as animal feed is the high variation in nutrient compositions among differenent sources (Liu, 2011b).

3 Materials and Methods

3.1 Materials

Samples of the barley by-product streams, as well as barley flour (Fibar variety) were obtained from GrainFrac Inc (Edmonton, AB, Canada) in 2017. Spring wheat (AC Andrew) was provided by Seed Solutions (Viking, AB, Canada) in 2011. As the first batch of wheat ran out, another batch of spring wheat (AC Andrew) harvested in 2017 was purchased from Galloway Seeds Ltd (Fort Saskatchewan, AB, Canada). Wheat grain, barley flour, and barley starch concentrate were kept in airtight plastic bags in the fridge at 4°C until used for experiments. Prior to the mashing process, wheat grain was ground using a laboratory hammer mill (Model 3100, Perten, Sweden) equipped with a mill feeder (Model 3170, Perten, Sweden) and a 0.5 mm sieve. The starch content (wet basis) of the wheat flour, barley starch concentrate and barley flour were determined using a Total Starch Assay Kit (Megazyme, Country Wicklow, Ireland).

3.2 Yeast, enzymes and chemicals

Active dry yeast *S. cerevisiae* supplied by Lallemand Biofuels & Distilled Spirits (Duluth, GA, USA) was used throughout this study. According to manufacturers' data, the average number of live cells per gram is 1×10^{10} (dry matter basis). Prior to inoculation, 7.5 g of yeast was rehydrated in 37.5 g of sterile water for 30 min at 30°C, 200 rpm in an incubator shaker. Commercial enzymes were provided by Genencor International (Hanko, Finland). FERMGEN™ 2.5X is an acid proteolytic enzyme with declared activity of 2500 spectrophotometric acid protease unit/g. OPTIMASH™ TBG mainly contains thermostable endo-1,3(4)- ß-glucanase that catalyzes the endohydrolysis of 1,3- or 1,4 linkages in β-D-glucan. According to the manfactors, the activity of OPTIMASH™ TBG is 5625 U/g. GC 626 is an acid α-Amylase with declared activity of 10,000 (soluble starch unit/g). STARGEN™ 002 is an enzyme blend of α-Amylase and glucoamylase

with declared activity of 570 glucoamylase unit/g. A D-Glucose Assay Kit was obtained from Megazyme (Country Wicklow, Ireland). DEPC (≥97%), urea (>98%), D-(+)-glucose (≥95%), lactic acid (88.0-92.0%) and water for HPLC were purchased from Sigma-Aldrich (St. Louis, MO). Acetic acid (≥99.7%), 1-butanol (≥99.4%), sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$; 98.0 to 100.5%), and sodium phosphate monobasic dihydrate ($NaH_2PO_4 \cdot 2H_2O$; 99.5%) were purchased from Fisher Scientific (Fair Lawn, NJ). Ethanol (anhydrous; 100%) was provided from Greenfield Global (Mississauga, Ontario, CA). To prepare mashes, Milli-Q water was used (Milli-Q, Millipore SAS, Molsheim, France). The recipe of the trace metal supplement used in fermentation experiments is shown Table 3-1.

Table 3-1 Essential minerals used in fermentation of barley starch concentrate (section 3.10)

Name Formula	Supplier Purity	Essential minerals
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	ACROS Organics, New Jersey, USA 96%	Ca^{2+}
Boric acid (H_3BO_3)	Fisher Scientific, Fair Lawn, NJ, USA ≥99.5%	B^{3+}
Manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$)	Sigma-Aldrich, St. Louis, MO, USA ≥98%	Mn^{2+}
Iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$)	Sigma-Aldrich, St. Louis, MO, USA ≥98%	Fe^{3+}
Zinc chloride ($ZnCl_2$)	MP Biomedicals, LLC, Solon, Ohio, USA ≥97%	Zn^{2+}
Sodium molybdate dihydrate ($Na_2MoO4 \cdot 2H_2O$)	Fisher Scientific, Fair Lawn, NJ, USA 99.5 to 103.0%	Mo^{6+}
Cobalt chloride Hexahydrate ($CoCl_2 \cdot 6H_2O$)	J.T.Baker, Phillipsburg, NJ, USA 99.3%	Co^{2+}
Copper chloride ($CuCl_2$)	Sigma-Aldrich, St. Louis, MO, USA 97%	Cu^{2+}

3.3 Batch fermentation of wheat, barley flour and barley starch concentrate

The general fermentation strategy is modified from Jin *et al.* (2016). The procedure of fermentation experiments is shown in Figure 3-1.

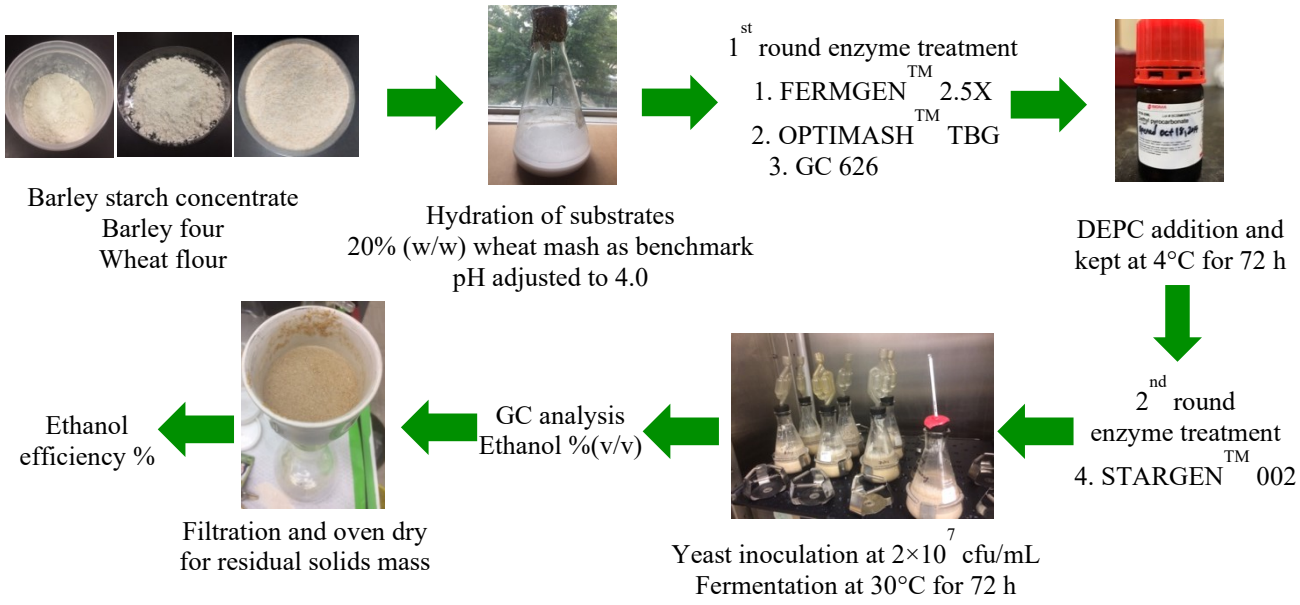


Figure 3-1 General fermentation strategy

3.3.1 Preparation of mashes

Fermentations were carried out in 500 mL shake-flasks. All prepared mashes contained the same mass of starch and same volume of water in order to maintain the same starch concentration. For the fermentation of 20% wheat (w/w, wet basis) as benchmark, 50.0 g of wheat flour, 45.3 g of barley flour and 37.9 g of barley starch concentrate were weighed into pre-weighed 500 mL Erlenmeyer flasks and mixed thoroughly with 200 mL of water. The pH of the mashes was adjusted to 4.0 using 4 N HCl. The flask was covered with foil and heated in an incubator shaker (Innova 44/44R, New Brunswick Scientific, Edison, NJ, USA) at 55°C and 200 rpm. When the temperature of mash reached 55°C, 47.0 µL of FERMGEN™ 2.5X (940 µL/kg of grain), 4 µL of OPTIMASH™ TBG (80 µL/kg of grain), and 22 µL of GC 626 (440 µL/kg of grain) were added. The flask was kept at this temperature for 1 h at 200 rpm. After that, diethyl pyrocarbonate (DEPC) (Sigma-Aldrich, ≥97%, St. Louis, MO) was added to the flask as a chemical disinfectant at a dosage of 105 µL/kg mash. DEPC reacts with many enzymes containing amine, thiol and hydroxy groups in their sites, therefore the antimicrobial action of DEPC is based on their actions with

enzymes inside the microorganisms and with membranes. The flask containing mash was stored at 4°C for 72 h prior to fermentation in order to inhibit the microorganisms in the system and decompose DEPC completely.

3.3.2 Simultaneous Saccharification and Fermentation

After 72 h storage at 4°C, the mash was heated to 55°C in the incubator shaker at 200 rpm for 1 h. STARGEN^{TM} 002 (2.8 mL/kg of grain) was added to the mash of wheat, barley starch concentrate and barley flour, followed by a 1 h incubation at 55°C, 200 rpm. Urea (1 M) was added to the mash to obtain a final concentration of 16 mmol/kg. Sterile water was added to compensate the water loss during evaporation. When the temperature of the mash decreased to 30°C, 2.5 mL of hydrated yeast was added to the mash at an approximate initial viable cell concentration of 2×10^7 cfu/mL. The flask was then sealed with a gas trap, which had an S-lock filled with water to allow CO_2 escape during fermentation. All fermentations were carried out at 30°C, 200 rpm for 72 h.

3.4 Comparing enzymatic hydrolysis in barley starch concentrate, barley and wheat mashes

Enzymatic hydrolysis was carried out in a 500 mL Erlenmeyer flask in an incubator shaker (55°C, 200 rpm) for 72 h. Ground wheat flour (50.0 g; 0.5 mm) was mixed with 200 mL sterile water to get 20% (wt/wt) solids. Barley starch concentrate (37.9 g) and barley flour (45.3 g) were mixed with 200 mL sterile water to obtain the same starch concentration as the wheat mash. DEPC (105 µL/kg mash) was added into the mashes, which were then stored at 4°C for 24 h for decontamination purposes. Sterile urea (1 M) was added to the mash, after which the pH of all of mashes was adjusted to 4.2 using 4N HCl. This is the optimum pH for the enzymatic treatment using these enzymes. Then, the enzyme blends of 47.0 µL of FERMGEN^{TM} 2.5X (940 µL/kg of grain), 4 µL of OPTIMASH^{TM} TBG (80 µL/kg of grain), 22 µL of GC 626 (440 µL/kg of grain)

and 140 µL of STARGEN™ 002 (2.8 mL/kg of grain) were mixed in 1 mL sterile water and added immediately to the mash. Flasks were sealed with S-locks to avoid contamination and hydrolysis was conducted for 72 h. An aliquot of sample (1.5 mL) was removed aseptically at 0 (after adding enzymes), 2, 6, 12, 24, 48, and 72h. Samples were boiled for 5 min to inactivate the enzymes, followed by centrifugation at 12,396 x g for 10 min. Supernatant was passed through a 0.22-µm syringe filter into 1 mL vials for analysis using HPLC.

3.5 Effect of protease (FERMGEN™ 2.5X) on hydrolysis

The general hydrolysis strategy in these experiments is modified from the general fermentation strategy (Figure 3-1). The procedure of this hydrolysis experiment is shown in Figure 3-2.

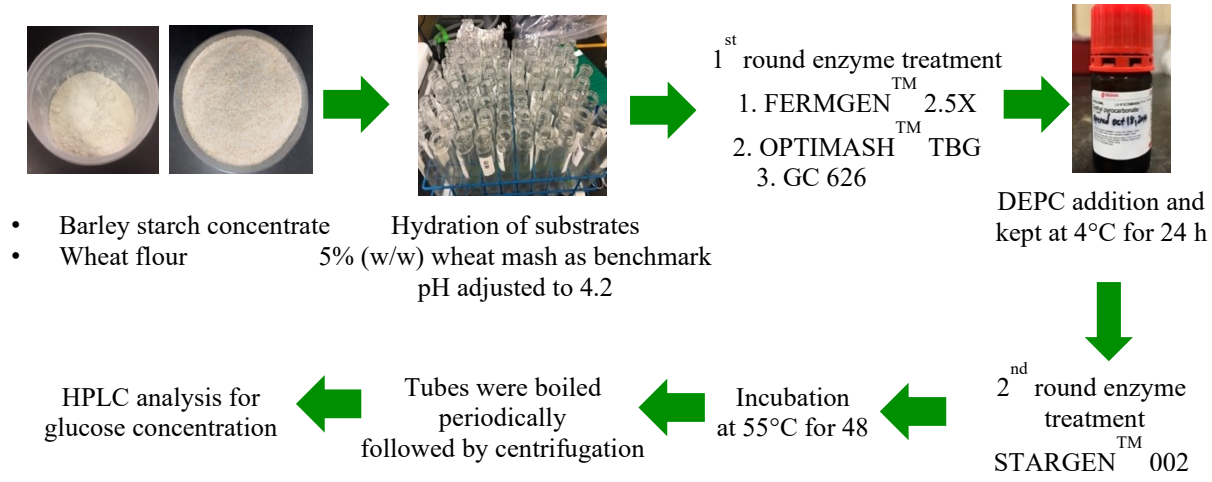


Figure 3-2 General hydrolysis strategy

3.5.1 Mash preparation

Enzymatic hydrolysis was carried out in 50 mL test tubes at 55°C, 200 rpm in an incubator shaker. Wheat (0.50 g) was measured into tubes and 10 mL sterile water was added to create a mash. Barley starch concentrate (0.44 g) was mixed with 10 mL sterile water containing the same mass of starch and water as wheat mash. Then, the pH of the slurries was adjusted to 4.2 using 4 N HCl.

3.5.2 Wheat benchmark hydrolysis

The wheat mash was pretreated with 0.47 µL of FERMGEN™ 2.5X (940 µL/kg of grain) at 55℃, 200 rpm for 1 h. After pretreatment, DEPC (105 µL/kg mash) was added to all the tubes and stored under 4℃ for 24 h. After storage, 160 µL of 1 M urea solution was added. Then, mashes were hydrolyzed using enzyme blends of 0.04 µL of OPTIMASH™ TBG (80 µL/kg of grain), 0.22 µL of GC 626 (440 µL/kg of grain) and 1.4 µL of STARGEN™ 002 (2.8 mL/kg of grain) at 55℃, 200 rpm for 48 h.

3.5.3 Enzymatic hydrolysis without FERMGEN™ 2.5X

In this experiment, the process variable is the requirement of FERMGEN™ 2.5X (a protease) during starch hydrolysis, and also the timing of adding it. Therefore, experiments were performed where FERMGEN™ 2.5X was omitted, or FERMGEN™ 2.5X was added after DEPC treatment.

3.5.4 Sampling

At 0 (after adding enzyme), 4, 12, 24, and 48 h time points, test tubes were boiled for 10 min to deactivate enzymes, followed by centrifugation at 6793 x g for 15 min in a 15-mL tube. Supernatant (1.5 mL) were transferred to O-ring tubes followed by centrifugation at 12,396 x g for 10 min. Supernatant was passed through a 0.22-µm syringe filter into 1 mL vials for analysis using HPLC.

3.6 Effect of enzyme dosage and timing on hydrolysis of wheat mash

Here, the hydrolysis strategy (Figure 3-2) was used. The wheat mash preparation and wheat benchmark hydrolysis were the same as described in sections 3.5.1 and 3.5.2, respectively. The second condition was the same as the benchmark, however, a 2X dosage of each enzyme was used. In the third condition, FERMGEN™ 2.5X, GC 626 and OPTIMASH™ TBG were added to the

mash in the 1st round treatment at 55°C, 200 rpm for 1 h. STARGEN™ 002 was added in the 2nd round treatment to hydrolyze substrate for 48 h. The sampling procedure in these experiments was the same as described in section 3.5.4.

3.7 Effect of omitting enzymes on hydrolysis of barley starch concentrate mash

Here, the hydrolysis strategy (Figure 3-2) was used. The mash preparation of barley starch concentrate in these experiments was the same as described in section 3.5.1. Hydrolysis was performed under seven conditions. The experimental design is shown in Table 3-2. For these experiments, the slurries were first pretreated with or without FERMGEN™ 2.5X at 55°C, 200 rpm for 1 h, followed by DEPC treatment for 24 h. Then, different combinations of enzymes (or no enzymes at all) were added to the substrate. The sampling procedure in these experiments was the same as described in section 3.5.4.

Table 3-2 Experiments for studying the omission of enzymes during hydrolysis

Condition	1st round enzyme treatment	2nd round enzyme treatment
1	FERMGEN™ 2.5X	STARGEN™ 002 + OPTIMASH™ TBG + GC 626
2	FERMGEN™ 2.5X	STARGEN™ 002
3	No enzymes	STARGEN™ 002
4	No enzymes	STARGEN™ 002 + OPTIMASH™ TBG + GC 626
5	FERMGEN™ 2.5X	No enzymes
6	FERMGEN™ 2.5X	OPTIMASH™ TBG + GC 626
7	No enzymes	OPTIMASH™ TBG + GC 626

3.8 Particle size measurement

The particle size distribution was determined using a Malvern Mastersizer 3000 laser diffraction particle size analyzer, which was equipped with an Aero dry dispersion unit (Malvern Instruments Ltd., Malvern, Worcestershire, UK). For analyses, the refractive indices used were as follows: 1.53 for wheat flour and barley flour (Angelidis *et al.*, 2016; Drakos *et al.*, 2017), and

1.52 for barley starch concentrate (Naguleswaran, *et al.*, 2013). An absorption index of 0.10 was used for all three feedstocks. Finally, the flour densities used were as follows: 1.48 g/cm^3 for wheat flour, and 1.50 g/cm^3 for both barley flour and wheat flour. Data were collected and analyzed using Malvern software (Version 2.01, Malvern Instruments Ltd, Malvern, UK).

3.9 Effect of lowering STARGEN™ 002 on hydrolysis of barley starch concentrate mash

The experiments described in this section were performed as described in section 3.5.1, but with the following modifications. The experimental design is shown in Table 3-3. Here, the hydrolysis strategy (Figure 3-2) was used.

3.9.1 Wheat benchmark and wheat negative control

FERMGEN™ 2.5X, OPTIMASH™ TBG, and GC 626 were added to slurries and incubated at 55°C, 200 rpm for 1 h. After 1st round treatment, DEPC was added as a chemical disinfectant with a dosage of 105 µL/kg mash. Tubes were stored at 4°C for 24 h before hydrolysis. Then, 160 µL of 1M urea solution was added. STARGEN™ 002 was added with standard dosage (2.8 mL/kg of grain) and hydrolysis was conducted for 48 h. A negative control was also performed in which a mock treatment with no enzymes was performed.

3.9.2 Effect of STARGEN™ 002 dosage on hydrolysis of barley starch concentrate mash

For these experiments, the procedure described in section 3.9.1 was performed. However, the 1st round treatment using FERMGEN™ 2.5X, OPTIMASH™ TBG, and GC 626 was omitted. Furthermore, the amount of STARGEN™ 002 used in this step was varied: 1X enzyme dosage, 0.5X enzyme dosage, 0.25X enzyme dosage, and 0.1X enzyme dosage.

3.9.3 Sampling

Test tubes were periodically sampled at 0 (after adding enzyme for the hydrolysis step), 5, 10, 15, 20, 30, 45, and 60 min, and 4, 12, 24 and 48 h. Immediately after sampling, tubes were

boiled for 10 min to inactivate enzymes. Three volumes of cold ethanol were added into one volume of hydrolysate supernatant. This was done to precipitate unhydrolyzed substrate and to decrease viscosity of the sample (Xu *et al*., 2014; Zhao *et al*., 2015). The treated samples were centrifuged at 12,396 x g for 10 min and then filtered before HPLC analysis.

Table 3-3 Experiments for studying the lowering of enzyme dosage on hydrolysis

Condition	Enzymes	
	1st round treatment	2nd round treatment
Wheat benchmark	With enzymes	1X
Wheat negative control	No enzymes	1X
BSC (1X enzyme dosage)	No enzymes	1X
BSC (0.5X enzyme dosage)	No enzymes	0.5X
BSC (0.25X enzyme dosage)	No enzymes	0.25X
BSC (0.1X enzyme dosage)	No enzymes	0.1X

NOTE: For the wheat benchmark, FERMGEN™ 2.5X, OPTIMASH™ TBG and GC 626 were added in the 1st round treatment.

3.10 Fermentation of barley starch concentrate

The benchmark ethanol production of wheat and barley starch concentrate were performed as described in section 3.3. Experimental groups of barley starch concentrate were performed as section 3.3, but with the following modifications. Firstly, the 1st round treatment using FERMGEN™ 2.5X, OPTIMASH™ TBG, and GC 626 was omitted. Secondly, the amount of STARGEN™ 002 used in this step was varied:1X enzyme dosage, 0.5X enzyme dosage, 0.33X enzyme dosage, and 0.25X enzyme dosage. Thirdly, different combinations of phosphate and trace minerals were added to the mash. Urea was added to all the samples. Phosphate buffer (5 M, pH 6.0) containing 2.61 mol/ L $Na_2HPO_4 \cdot 2H_2O$ and 2.38 mol/L $NaH_2PO_4 \cdot 2H_2O$ was prepared. Then 2.26 mL of 5 M phosphate buffer was added to the mash in order to establish a final carbon to

phosphorus ratio of ~100:1. Each liter of mash was supplemented with 1 mL of a trace metals solution with the composition (in grams per liter) shown in Table 3-4. The detailed experimental design is shown in Table 3-5.

Table 3-4 Composition of trace metals solution

Trace metals	$CaCl_2 \cdot 2H_2O$	H_3BO_3	$MnCl_2$	$FeCl_3$	$ZnCl_2$	$Na_2MoO_4 \cdot 2H_2O$	$CoCl_2$	$CuCl_2$
Concentration (g/L)	3.7	2.5	0.87	0.65	0.44	0.29	0.01	0.0001

Table 3-5 Experiments for studying the lowering of STARGEN™ 002 dosage and addition supplements on ethanol production

Substrate	Condition	Enzymes		Additives
		1st round	2nd round	
Wheat	Wheat Benchmark	With enzymes	1X	-
Barley starch concentrate	BSC Benchmark	With enzymes	1X	-
	1	No enzymes	1X	Phosphorus + Trace minerals
	2	No enzymes	0.5X	Phosphorus + Trace minerals
	3	No enzymes	0.33X	Phosphorus + Trace minerals
	4	No enzymes	0.25X	Phosphorus + Trace minerals
	5	No enzymes	0.25X	-
	6	No enzymes	0.25X	Phosphorus
	7	No enzymes	0.25X	Trace minerals

NOTE: For wheat and barley starch concentrate benchmark, FERMGEN™ 2.5X, OPTIMASH™ TBG and GC 626 were added in the 1st round treatment.

3.11 Analytical methods

3.11.1 Quantitative determination of glucose, lactic acid and acetic acid

The soluble products of fermentation (glucose, lactic acid and acetic acid) were analyzed by high performance liquid chromatography (HPLC). The HPLC (Agilent Technologies, Mississauga, ON, Canada) was equipped with refractive index detector (1100 series, Agilent Technologies, Mississauga, ON, Canada) using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). As a mobile phase, 5 mM (0.5 mL/min) of sulfuric acid was used at a working temperature of 60°C. Standard solutions of glucose, lactic acid and acetic acid were prepared to

generate the standard curve. 0 h fermentation samples (10 mL) were centrifuged at 6793 x g for 15 min in a 15-mL tube, and then 1.5 mL of supernatant were transferred to O-ring tubes and boiled for 5 min, followed by centrifugation at 12,396 x g for 10 min. Supernatant was passed through a 0.22-µm syringe filter into 1 mL vials for analysis. Residual glucose of 72 h fermentation samples was measured immediately at the end of fermentation using the glucose oxidase/peroxidase (GOPOD) method (Megazyme test kit Wicklow, Ireland). Briefly, GOPOD reagent (300 µL) was added to wells of microtiter plates (96 flat bottom well plate, Corning, NY, USA) along with glucose-containing samples (10 µL). The microplate was incubated in a microplate reader (Biotek, Winooski, VT, USA) at 50ºC for 20 min. After incubation, absorbance (510 nm) was measured and glucose levels were determined through comparison with the reagent blank and the glucose standard. Triplicates were done for each measurement. Fermentation samples (72 h) with a glucose concentration larger than 1 g/L were analyzed by HPLC.

3.11.2 Quantitative determination of ethanol

Ethanol was determined by gas chromatography (GC-FID) (7890A, Agilent Technologies, Mississauga, ON, Canada) and a flame ionization detector. A Restek Stabilwax–DA column (30 m length, 0.5 µm film thickness, and 0.53 mm ID) was used to separate compounds. The temperature of the inlet and detector were 170ºC and 190ºC, respectively. The GC oven temperature was programmed from 35°C, held for 3 min, and then increased to 190°C at a rate of 20°C/min (hold time: 1 min). The flow rate of the carrier gas (helium) through the column was 33 cc/min. Injections (1 µL) were made in split mode (20:1 split ratio). 10 mL of fermented mash sampled from flasks was centrifuged at 6793 x g for 15 min in a 15-mL tube. Then, 1 mL of supernatant was sampled and centrifuged at 12,396 x g for 10 min in a 2-mL microcentrifuge tube. An aliquot of 100 µL of the final supernatant was mixed thoroughly with 5 mL of high-

pressure liquid chromatography-grade water and 500 µL of a 1% 1-butanol internal standard solution. For standards, 100 µL of the final supernatant was replaced by the same volume of 10% (v/v) ethanol. The ethanol concentration was calculated based on the ratio of response factor of the sample to the response factor of the standard, according to the following equations:

$$\text{Response Factor}_{(\text{Standard})} = \text{Area}(\text{Ethanol}_{(\text{Standard})}) / \text{Area}(\text{Butanol})$$

$$\text{Response Factor}_{(\text{Sample})} = \text{Area}(\text{Ethanol}_{(\text{Sample})}) / \text{Area}(\text{Butanol})$$

$$\text{Ethanol \% (V/V)} = (\text{Rf}_{(\text{Sample})} / \text{Rf}_{(\text{Standard})}) \times 10$$

3.11.3 Ethanol yield and ethanol yield efficiency

At the end of fermentation, the total weight of the fermentation mash and flask were measured. Then, the fermentation mash was filtered using a funnel and pre-weighed dried filter paper. Then, the solids and filter paper were placed in a pre-dried and pre-weighted aluminum dish in an oven at a temperature of 105°C overnight until a steady mass was obtained (i.e. the mass of the residual solids). The density of liquid was measured by dividing the weight of 10 mL fermented mash supernatant by 10 mL of volume.

Ethanol yield refers to the weight of ethanol produced per 100 g of starch. Theoretical ethanol yields were calculated based on grain weight and total starch content. Theoretically, yeast can convert 1 mole of glucose to 2 moles of CO_2 and ethanol. Therefore, 1 g of starch can be hydrolyzed to 1.111 g of glucose (obtained by dividing the molar mass of glucose by the molar mass of one starch unit,180.16 g mol/162.16 g mol), and 1 g of glucose can be converted to 0.511 g of ethanol (Im *et al.*, 2016). Thus, 100 g of starch can be converted to 56.7 g of ethanol assuming that all the starch is completely converted to glucose. Actual ethanol yields were calculated based on the final ethanol concentrations and solid mass. Ethanol yield efficiencies were calculated as the ratio of actual ethanol yield over theoretical ethanol yield. The weight of fermented mash,

volume of liquid, actual ethanol yield and ethanol yield efficiencies were further calculated according to equations (1) (2) (3) (4), respectively.

$$\text{Weight of fermented mash} = \text{Weight}_{\text{fermentated mash + flask}} - \text{Weight}_{\text{empty flask}} \quad (1)$$

$$\text{Volume of liquid} = \frac{\text{Weight}_{\text{fermented mash}} - \text{Weight}_{\text{solid}}}{\text{Density}_{\text{liquid}}} \quad (2)$$

$$\text{Actual ethanol yield (in g/100g of starch)} = \frac{\text{Ethanol \%(v/v)} \times \text{Volume of liquid}}{\text{Density}_{\text{ethanol}}} \times \frac{100}{\text{Weight}_{\text{starch}}} \quad (3)$$

$$\text{Ethanol yield efficiency (\%)} = \frac{\text{Actual ethanol yield (g/100 g of starch)}}{\text{Theoretical yield (56.7 g of ethanol/100g of starch)}} \times 100 \quad (4)$$

where $\text{Weight}_{\text{fermented mash}}$ is the mass of the fermented mash (g); $\text{Weight}_{\text{solid}}$ is the solid mass (g) after drying overnight; $\text{Density}_{\text{liquid}}$ is the density of the fermented mash supernatant; $\text{Weight}_{\text{starch}}$ is the weight of total starch as is basis (g) present in the mash, which can be determined by the mass of flour used and its composition.

3.12 Statistical Analyses

All the experiments were carried out in triplicate. Outliers were evaluated by Q test (95% confidence). Statistical analyses were accomplished with SPSS Statistics version 24 (IBM, USA) using one-way analysis of variance (ANOVA) with Tukey test with 95% confidence ($\alpha = 0.05$).

4 Results and discussion

4.1 Batch fermentation of wheat, barley flour and barley starch concentrate

The project was about establishing a fermentation approach for bioethanol production from barley starch concentrate, which is a starch enriched by-product stream from the ACAPS process. These experiments were designed to examine the potential ethanol yield from barley starch concentrate in comparison to two well-studied benchmarks, wheat flour and barley flour.

Glucose and organic acid concentration of samples taken at 0 h and 72 h of fermentation were monitored. The quantities of glucose and organic acids present in the 0 h samples are presented in Table 4-1. The initial glucose concentration in the wheat, barley, and barley starch concentrate fermentation systems, followed the order: barley system > barley starch concentrate system > wheat system. This indicates that the three systems showed variable susceptibilities to enzymatic action. Meanwhile, both lactic acid and acetic acid were measured but not detected at the beginning of fermentation.

Table 4-1 Glucose and acid concentration of the mash at 0 h of fermentation**.**

Feedstock	Glucose (g/L)	Lactic acid (g/L)	Acetic acid (g/L)
Wheat	44.7 ± 1.7^{a}	BDL	BDL
Barley starch concentrate	64.2 ± 2.6^{b}	BDL	BDL
Barley flour	68.7 ± 0.6^{c}	BDL	BDL

Data are presented as means ± standard deviation of triplicate repeats for the mash at 0 h of fermentation. BDL, below detection limits. In the same column, means with different letters are significantly different ($P<0.05$).

A final fermentation efficiency was calculated after 72 h of yeast fermentation and is presented in Figure 4-1. It is noteworthy that all of the mashes had equivalent starch content in order to maintain the same fermentable carbon, but there were differences in ethanol efficiencies. It was observed that barley starch concentrate was efficiently converted to ethanol at 86.7 ± 3.5%,

which was similar to barley flour at 84.4 ± 0.5%. This reveals that barley starch concentrate may be a viable source for the ethanol industry when optimization of process conditions is applied in the future. However, ethanol production was observed to be higher with wheat flour than with barley starch concentrate or barley flour, using the same concentration of enzymes and experimental conditions. In this experiment, wheat is considered as the fermentation benchmark since it has been well-investigated in our lab (discussed in section 2.2.1). The value for ethanol efficiency of the wheat benchmark in this experiment (93.2 ± 3.2%) was within the conversion efficiency ranges previously reported (Jin *et al.*, 2016; Wang *et al*., 1999; Zhao *et al.*, 2009). In addition, Gibreel *et al.* (2009) reported that very high gravity (30% solids) STARGEN-based fermentation of Fibar barley demonstrated comparable performance (90.2% of ethanol efficiency) relative to that of CPS wheat (85.2%) under the same fermentation conditions. The likely reason for the lower ethanol efficiency of barley flour observed in the present study compared to that of Fibar barley from Gibreel *et al.* (2009) is due to the difference in substrates, fermentation processes and calculation methods. However, this is the first report showing successful fermentation of the ACAPS by-product.

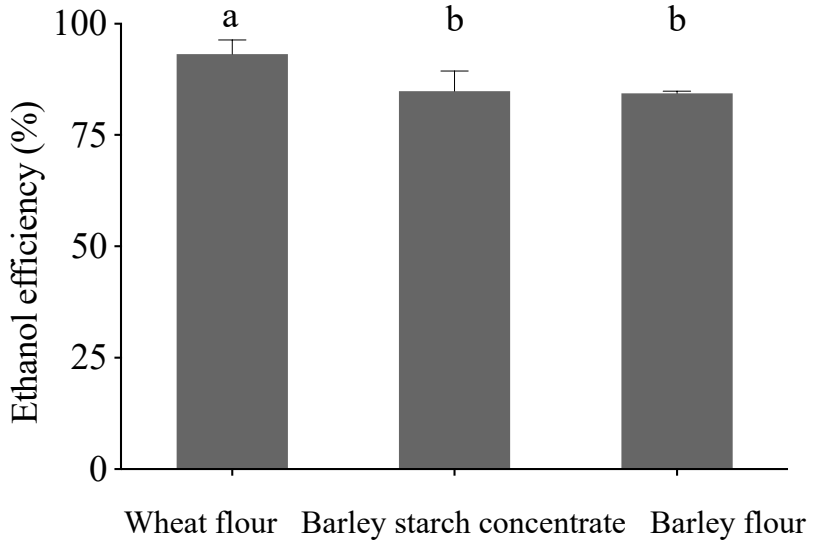


Figure 4-1 Ethanol yield efficiencies of the fermentations with various substrates. All the fermentations contained the same amount of fermentable carbon. Wheat flour was considered as the benchmark. Data are presented as means ± standard deviation of triplicate independent fermentations. Different letters indicate significant differences ($P<0.05$) between the mean values of fermentation efficiency.

Results obtained for the initial glucose concentration after starch hydrolysis by STARGEN^{TM} 002 may help to some degree to explain the difference in ethanol efficiencies. As shown in Table 4-1, the barley starch concentrate and barley flour fermentation systems showed higher initial starch hydrolysis by STARGEN^{TM} 002, which led to higher amounts of fermentable sugar. Additionally, the higher rates of hydrolysis will persist throughout the fermentation and thus it is possible that the sugar concentration reached extremely high levels. This higher amount of sugar in the mash most likely exerts higher osmotic stress on yeast cells, which may lead to underperforming yeast during fermentation. According to Panchal *et al*. (1980), increasing the initial sucrose concentration from 20% (w/v) to 40% (w/v) led to less sugar uptake (from almost complete sugar utilization to 55% sugar utilization) in parallel with a lower total ethanol produced in the medium. Panchal *et al.* (1980) also observed that the increase in osmotic pressure resulted in an increase in the proportion of intracellular ethanol to extracellular ethanol, and a corresponding remarkable decline in cell viability. This could explain why a lower ethanol yield efficiency was achieved using barley starch concentrate and barley flour compared to wheat flour. In general, lower sugar concentrations are recommended during ethanol fermentations as less osmotic stress is exerted on the yeast cells and also it retards the growth of competing microorganisms (Dickinson & Schweizer, 2004).

The end-products of fermentation were also measured and reported in Table 4-2. At the end of fermentation, a small amount of residual glucose was observed in all three fermentation

systems. According to Kaur *et al.* (2011), 0.5% (w/v) of residual glucose was found after 72 h simultaneous saccharification and fermentations of corn using STARGEN™ 001, indicating near complete fermentation. Similar residual glucose was observed in barley and wheat fermentation systems whereas barley starch concentrate fermentation system had the lowest. Other by-products (lactic acid and acetic acid) were observed for all the fermentations. It was observed that the barley starch concentrate fermentation system had significantly lower lactic acid than that of the barley and wheat fermentation systems. Similar amounts of acetic acid were produced at the end of fermentation for the three feedstock fermentation systems. Lactic acid production during fermentation was also detected in the barley fermentation studies of Nghiem *et al*. (2010).

Table 4-2 Residual glucose and acid concentration of fermented mash.

Feedstock	Residual glucose (g/L)	Lactic acid (g/L)	Acetic acid (g/L)
Wheat	0.22 ± 0.04^{a}	1.9 ± 0.2^{a}	1.5 ± 0.5^{a}
Barley starch concentrate	0.071 ± 0.060^{b}	0.25 ± 0.06^{b}	0.64 ± 0.23^{a}
Barley flour	0.24 ± 0.06^{a}	1.4 ± 0.9^{a}	1.1 ± 0.5^{a}

Data are presented as means ± standard deviation of triplicate repeats for the mash after 72 h of fermentation. Means with different letters within each column are significantly different ($P<0.05$).

4.2 Comparing enzymatic hydrolysis in barley starch concentrate, barley and wheat mashes

In the batch fermentation experiment described in section 4.1, differences in ethanol efficiencies were observed, possibly because of higher osmotic stress resulting from higher sugar levels in the barley starch concentrate and barley fermentation systems. It was of interest, therefore, to examine the hydrolysis efficiency of the barley starch concentrate, barley and wheat mashes. Thus, barley starch concentrate, barley and wheat mashes were hydrolyzed to evaluate their susceptibility towards enzyme cocktails, namely the blending of FERMGEN™ 2.5X, OPTIMASH™ TBG, GC 626 and STARGEN™ 002.

Figure 4-2 shows glucose production during 72 h of hydrolysis. For the three feedstock mashes, there was a sharp increase in glucose production during the first 2 h, and then a plateau region was reached. However, enzyme hydrolysis of barley starch concentrate mash was significantly more efficient compared to conventional wheat mash at the early timepoints, producing more than 2-fold greater glucose after only 2 h. It needs to be emphasized that the wheat mash always displayed the lowest hydrolysis during the 72 h. Enzymatic hydrolysis of barley starch concentrate, barley, and wheat mashes resulted in only 66.9%, 65.3% and 55.3% of hydrolysis efficiency after 72 h incubation, respectively, which indicated incomplete hydrolysis. According to industrial data, it is generally believed that approximately 90% hydrolysis efficiency can be obtained in terms of wheat mash after 72 h hydrolysis. For example, Textor *et al.* (1998) studied the effect of three types of barley α-Amylase on the hydrolysis of wheat starch granules using a mash with 30 g/L starch concentration, and obtained 98% hydrolysis efficiency. As reported by Naguleswaran *et al.* (2012), hydrolysis of wheat starch mixed with buffer by STARGEN^{TM} 002 showed 90.8-95.1% efficiency after a 1 h incubation at 55 °C, followed by a 72 h incubation at 30 °C, pH 4.0. Therefore, the low hydrolysis efficiencies observed for wheat mash in this hydrolysis study were not consistent with previous reports.

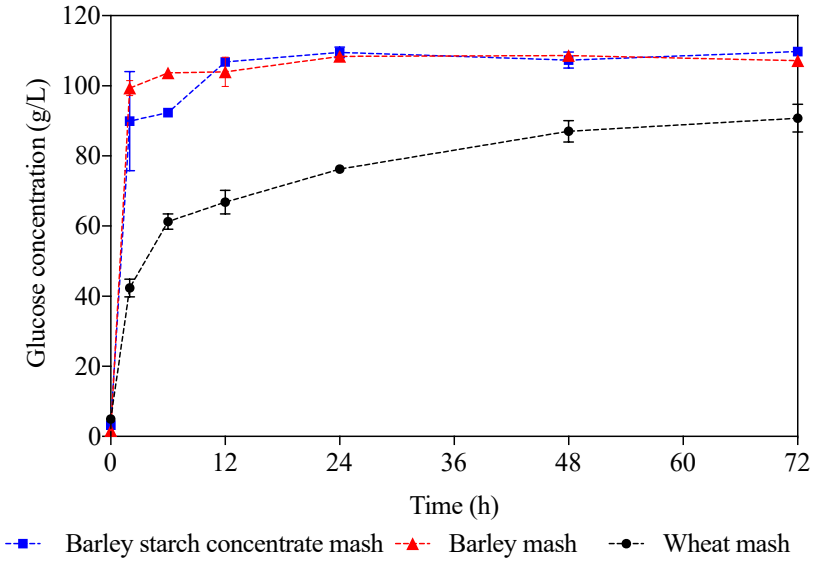


Figure 4-2 Hydrolysis of barley starch concentrate, barley and wheat mashes by enzyme blends. The glucose concentrations were plotted relative to time (h). Experiments were done in replicates (n=3).

4.3 Effect of protease (FERMGEN™ 2.5X) on hydrolysis

One possible explanation for the sub-optimum levels of hydrolysis observed in hydrolysis experiments (section 4.2) was the inclusion of FERMGEN™ 2.5X, which is a protease that is routinely used in industrial fermentations to degrade proteins in the feedstock. In the ethanol industry, several enzymes including FERMGEN™ 2.5X are used; however, it is added to mash at different time points. For example, in the standard fermentation procedure (Figure 3-1), FERMGEN™ 2.5X, OPTIMASH™ TBG and GC 626 were added in the 1st round of enzyme treatment to degrade the protein-matrix structure in wheat grains, thereby improving the starch susceptibility to hydrolysis. Following mash sterilization, a 2nd round of enzyme hydrolysis was performed using STARGEN™ 002. As shown in section 4.2, in order to simplify the experimental procedure for comparing hydrolysis efficiencies, an enzyme cocktail was prepared by mixing FERMGEN™ 2.5X, OPTIMASH™ TBG, GC 626 and STARGEN™ 002 together to use them all at the same time. However, the original data shown in section 4.2 indicated that this

methodology may have been problematic. FERMGEN™ 2.5X is the likely culprit as it is a protease and may degrade the other enzymes, including STARGEN™ 002. Therefore, the following experiments were designed to confirm this theory. The effect of both timing and the requirement of FERMGEN™ 2.5X for hydrolysis were tested. For these experiments, the following systems were employed as shown in Table 4-3. A fresh spring wheat (AC Andrew) feedstock was used as benchmark in all three of these systems.

Table 4-3 Experiments for studying the effect of FERMGEN™ 2.5X on hydrolysis

Condition	1st round treatment	2nd round treatment
Condition 1	FERMGEN™ 2.5X	OPTIMASH™ TBG + GC 626 + STARGEN™ 002
Condition 2	No enzymes	FERMGEN™ 2.5X + OPTIMASH™ TBG + GC 626 + STARGEN™ 002
Condition 3	No enzymes	OPTIMASH™ TBG + GC 626 + STARGEN™ 002

From Figure 4-3 it is observed that the glucose amount increased dramatically before 4 h in all three conditions, followed by a progressively decreased hydrolysis thereafter, reaching a plateau at 24 h. In condition 1 where FERMGEN™ 2.5X was added in the 1st round treatment, it was observed that both initial hydrolysis rate and extent increased, compared with the other two conditions. The glucose concentrations at 4 h and 12 h for the wheat mash were 18.4 g/L and 25.7 g/L, respectively, when FERMGEN™ 2.5X was added in the 1st round treatment, but only 8.27 g/L and 11.9 g/L, respectively, for condition 3 without FERMGEN™ 2.5X treatment. Thus, the production of glucose when hydrolysis was carried out with FERMGEN™ 2.5X in the 1st round treatment was around twice of the production in the case without FERMGEN™ 2.5X treatment. The efficiency of hydrolysis using FERMGEN™ 2.5X in the 2nd round treatment was significantly lower (49.0%) than that of FERMGEN™ 2.5X in the 1st round treatment (78.7%).

In conclusion, the experiments using wheat show that timing of FERMGEN™ 2.5X addition makes a huge difference in terms of hydrolysis. It is likely that when FERMGEN™ 2.5X was

mixed with OPTIMASH™ TBG, GC 626 and STARGEN™ 002, FERMGEN™ 2.5X likely degraded STARGEN™ 002, leading to a reduced STARGREN™ 002 activity and incomplete starch hydrolysis. According to Sattler *et al*. (1989), decreasing the dosage of cellulase from 100FPU/g to 5FPU/g was found to lower hydrolysis of pretreated poplar wood from 81.0% to 43.0%, A low hydrolysis degree for condition 1 (without FERMGEN™ 2.5X) was observed in our experiment. It is likely due to the protein-starch matrix which highly limited enzymatic action. This demonstrates the importance of protease pretreatment in hydrolysis of wheat mash.

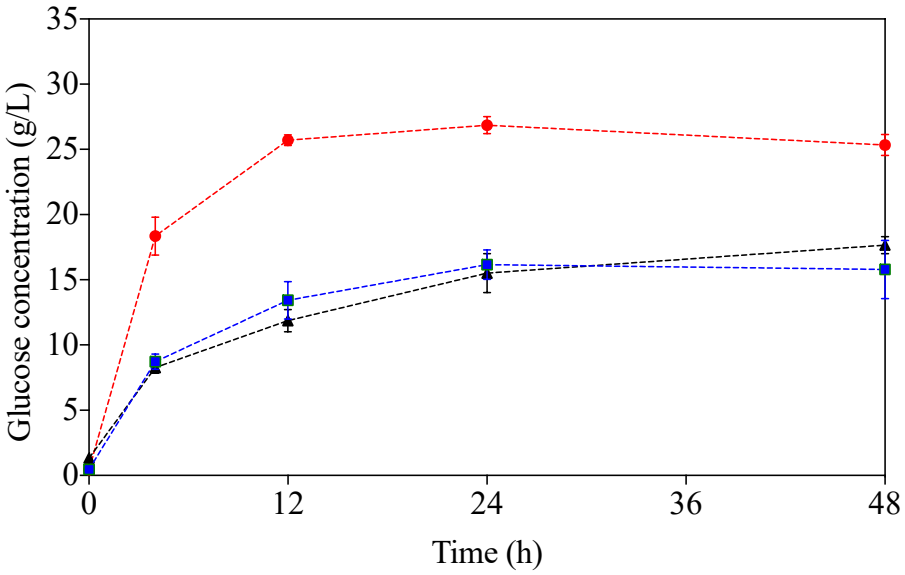


Figure 4-3 Effect of protease treatment on the hydrolysis of wheat mash. Experiments were done in replicate (n=3). Conditions of this figure are shown in Table 4-3.

The results of the wheat experiments agree with previous investigations where it was observed that the utilization of protease on maize and sorghum grains before conventional starch liquefication significantly increased the rate of starch hydrolysis by α-amylase. It was also reported that the protease treatment during liquefication resulted in higher initial glucose levels and bioethanol yields compared to untreated counterparts (Pérez-Carrillo & Serna-Saldívar, 2007). Many researchers concluded that the protein surrounding starch granules may act as barriers to starch hydrolysis. This is attributed to the findings that after protein hydrolysis, in vitro starch

digestibility increased significantly due to the clearance of passages for enzymes (Singh *et al*., 2010). Kim & Huber (2008) reported that the protease treatment removed both the surface and channel proteins exposing additional surface pores on starch granules from soft wheat. As a result, the higher degree of protein hydrolysis leads to a higher susceptibility to enzymes, producing more glucose. Similarly, Naguleswaran *et al*. (2011) found that the small internal channels in corn starch may have been blocked by minor components, such as protein and lipids, resulting in a lower hydrolysis.

Next, the hydrolysis of barley starch concentrate mash with the different FERMGEN^{TM} 2.5X treatments was examined as described in Table 4-3 (Figure 4-4). For all three conditions, there were sharp increases in glucose production during the first 4 h. Then, the hydrolysis reached a higher level at 12 h, and remained constant after this. At 4 h, a significantly lower glucose concentration was achieved for the condition without FERMGEN^{TM} 2.5X treatment (22.2 g/L), compared with the conditions of FERMGEN^{TM} 2.5X added in the 2nd round treatment (24.6 g/L) and in the 1st round treatment (24.9 g/L). However, other than the 4 h samples, there were no significant differences in glucose concentration for the three conditions at other time points. These observations indicated that the addition of FERMGEN^{TM} 2.5X had no overall improvement on the hydrolysis of barley starch concentrate mash. The seemingly higher accessibility of barley starch concentrate to enzymatic action may result from the ACAPS process. The β-Glucan isolation procedure could release granule-associated minor components, such as protein and lipid, resulting in a higher accessibility of barley starch concentrate and thus negating the need for FERMGEN^{TM} 2.5X addition.

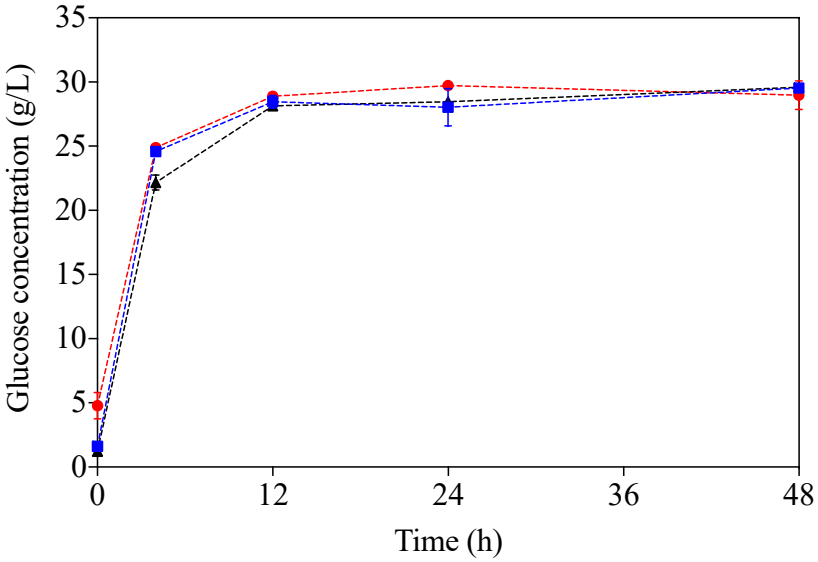


Figure 4-4 Effect of protease treatment on the hydrolysis of barley starch concentrate mash. Experiments were done in replicate (n=3). The 24 h point for condition 2 was based on duplicate results as an outlier was excluded. Conditions of this figure are shown in Table 4-3.

The results in this section confirm that hydrolysis of wheat mash using an enzyme cocktail containing all 4 enzymes is not the best practise to achieve complete conversion from starch to glucose. The condition of FERMGEN^{TM} 2.5X in the 1st round treatment, followed by hydrolysis with OPTIMASH^{TM} TBG, GC 626 and STARGEN^{TM} 002 was able to achieve a 78.7% hydrolysis efficiency of wheat mash. On the other hand, FERMGEN^{TM} 2.5X treatment did not have an effect on the overall hydrolysis of barley starch concentrate, indicating FERMGEN^{TM} 2.5X can be omitted for hydrolysis of barley starch concentrate mash in the future. However, even though FERMGEN^{TM} 2.5X is not necessary for the hydrolysis of barley starch concentrate mash, it is still possible that omitting this enzyme could impact the subsequent fermentation steps.

Based on these data, next steps were to: 1) establish the baseline procedure to use for wheat flour hydrolysis; 2) investigate the effect of omitting GC 626, OPTIMASH^{TM} TBG and STARGEN^{TM} 002 on hydrolysis of the barley starch concentrate mash.

4.4 Effect of enzyme dosage and timing on hydrolysis of wheat mash

The experiments described in section 4.3 above proved that the timing of FERMGEN™ 2.5X (protease) addition makes a huge difference on the hydrolysis of wheat mash. Now the question becomes: which order of enzyme addition in hydrolysis should be taken as the standard protocol for the wheat benchmark? To address this question, the effect of enzyme dosage and timing on hydrolysis of wheat mash was tested. For these experiments, the following systems were employed as shown in Table 4-4.

Table 4-4 Experiments for studying the effect of enzyme dosage and timing on the hydrolysis of wheat mash

Condition	1st round treatment	2nd round treatment
Condition 1	FERMGEN™ 2.5X	OPTIMASH™ TBG + GC 626 + STARGEN™ 002
Condition 2	Same as Condition 1, but using double the enzyme dosage	
Condition 3	FERMGEN™ 2.5X + OPTIMASH™ TBG + GC 626	STARGEN™ 002

The following experiment was designed to test whether increasing enzyme dosage would increase the hydrolysis efficiency of the wheat mash. Two conditions were investigated: FERMGEN™ 2.5X was added in the 1st round treatment, followed by OPTIMASH™ TBG, GC 626 and STARGEN™ 002 in the 2nd round treatment (condition 1); Condition 2 was the same as condition 1, but using double the enzyme dosage. As shown in Figure 4-5, with an increase of enzyme dosage, the hydrolysis of wheat mash increased during the first 12 h, but the same plateau was reached after 24 h of hydrolysis. At 48 h, the condition with standard dosage (Condition 1) and with 2-fold dosage (Condition 2) showed similar glucose yield. Thus, wheat mash showed an increasing hydrolysis rate with the increase of enzyme concentration at early time points, however, ultimately generated the same proportion of glucose. This indicated that 2-fold enzyme loadings did not favor greater starch conversion into glucose. Similar results were reported in the cellulose hydrolysis study by Mussatto *et al*. (2008) who showed that cellulase loading higher than 45 FPU/g did not affect the final glucose yield as the maximum glucose yield was constant for the cellulase

loading values ranging between 45 and 85 FPU/g. Enzymes represent a significant cost in the biomass conversion process, and the minimization of enzyme consumption should be taken into consideration (Chen *et al*., 2007). Therefore, considering the enzyme cost, the established enzyme dosage in subsequent experiments was used.

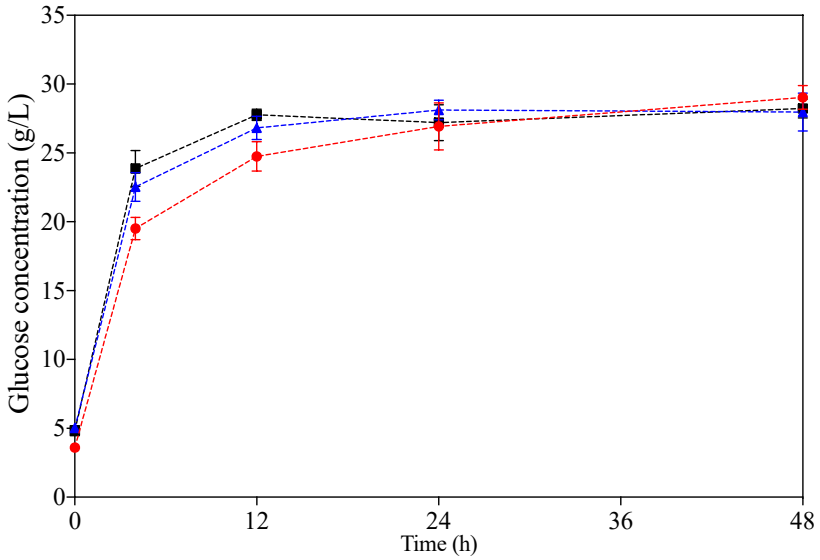


Figure 4-5 Effect of enzyme timing and dosage on the hydrolysis of wheat mash. Experiments were done in replicate (n=3). Conditions of this figure are shown in Table 4-4.

In addition, the following experiments were performed to examine the hydrolysis behaviour of wheat mash when the standard fermentation procedure was employed where FERMGEN^{TM} 2.5X, GC 626 and OPTIMASH^{TM} TBG were added in the 1st round treatment, followed by the addition of STARGEN^{TM} 002 in the 2nd round treatment (condition 3). During the first 12 h, this condition showed a higher hydrolysis than the condition of FERMGEN^{TM} 2.5X added in the 1st round treatment (using the same enzyme dosage) (Condition 1); however, they displayed similar release of glucose from 24 h to 48 h. Thus, the results here indicated that changing the timing of enzymes addition increased the hydrolysis at the early time points; however, did not make a difference in ultimate hydrolysis efficiency.

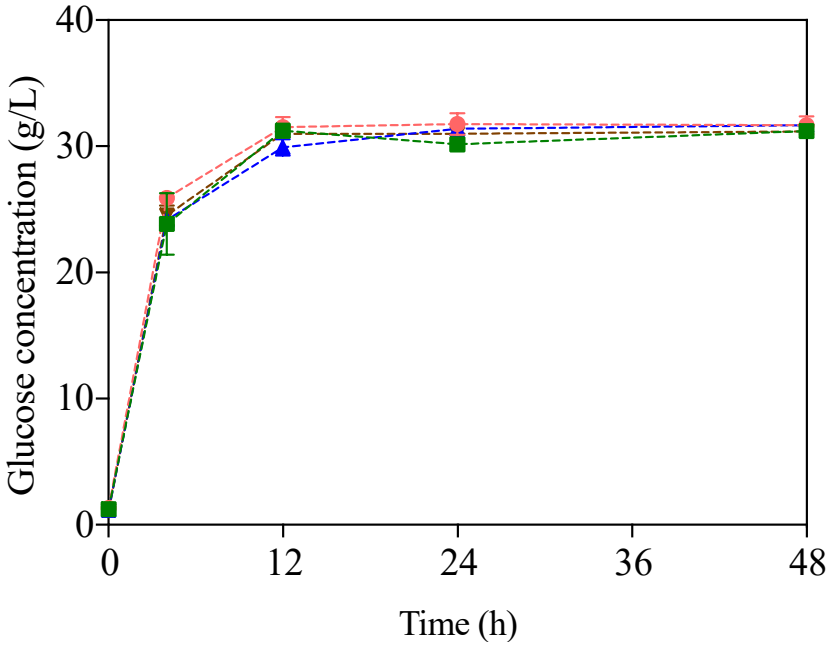
Thus, these investigations confirmed that, adding FERMGEN™ 2.5X, OPTIMASH™ TBG and GC 626 in the 1st round treatment, followed by the addition of STARGEN™ 002 in the 2nd round treatment was better than adding only FERMGEN™ 2.5X in the 1st round treatment followed by OPTIMASH™ TBG, GC 626 and STARGEN™ 002 in the 2nd round treatment (using the same enzyme dosage). Thus, in future experiments, the order of FERMGEN™ 2.5X, OPTIMASH™ TBG and GC 626 in the 1st round treatment followed by STARGEN™ 002 in the 2nd round treatment was established as the standard protocol for the wheat benchmark.

4.5 Effect of omitting enzymes on hydrolysis of barley starch concentrate mash

As observed in the experiment described in section 4.3, FERMGEN™ 2.5X is not necessary for the hydrolysis of barley starch concentrate mash. According to Naguleswaran *et al.* (2013a), barley starch samples hydrolyzed with STARGEN™ 002 (24 U/g starch) in 50 mM sodium acetate buffer (pH 4.0) at 55°C for 1 h followed by at 30°C for 72 h, ultimately achieved a 89.4 ± 0.7% hydrolysis efficiency. It is likely that barley starch concentrate does not need OPTIMASH™ TBG and GC 626 to open up the structure neither. In this regard, it is of interest to test if FERMGEN™ 2.5X, OPTIMASH™ TBG, GC 626, and STARGEN™ 002, are necessary for hydrolysis of barley starch concentrate. Here, the potential impact of different enzymatic combinations on hydrolysis were tested by evaluating glucose release during 48 h.

The benchmark treatment for the experiments described in this section, which consists of adding FERMGEN™ 2.5X in the 1st round treatment, followed by addition of STARGEN™ 002, OPTIMASHT™ TBG, and GC 626 in the 2nd round treatment is shown as Condition 1 (Figure 4-6). When OPTIMASH™ TBG and GC 626 were excluded (Condition 2), only the 24 h samples was statistically different from the benchmark (Condition 1). This indicated that for the hydrolysis of barley starch concentrate mash, OPTIMASH™ TBG and GC 626 are not required. However,

it should be pointed out that, it is possible that these enzymes can provide some nutritional benefits, i.e. minerals, during the fermentation process, even though they are not specifically required for the hydrolysis of barley starch concentrate.



Condition	1st round treatment	2nd round treatment
Condition 1	FERMGEN™ 2.5X	STARGEN™ 002 + OPTIMASH™ TBG + GC 626
Condition 2	FERMGEN™ 2.5X	STARGEN™ 002
Condition 3	No enzymes	STARGEN™ 002
Condition 4	No enzymes	STARGEN™ 002 + OPTIMASH™ TBG + GC 626

Figure 4-6 Effect of omitting enzymes on the hydrolysis of barley starch concentrate. Hydrolysis was performed with the 1st round enzymatic treatment, followed by DEPC treatment and then the 2nd round enzymatic treatment. Experiments were done in replicate (n=3). The glucose concentration at 0 h for condition 4 shows the result of duplicate samples.

The treatment where no enzyme was added in 1st round treatment, followed by addition of only STARGEN™ 002 is shown as Condition 3 (Figure 4-6). When FERMGEN™ 2.5X was

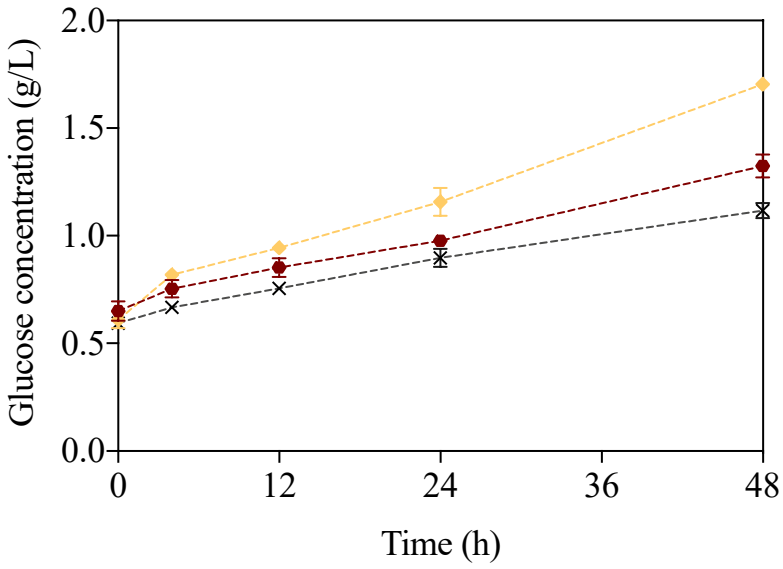
included in the 1st round treatment (Condition 2), only the 4 h samples were significantly different from Condition 3. These data indicated that FERMGEN™ 2.5X is not required to hydrolyze barley starch concentrate mash.

The treatment where no enzyme was added in the 1st round treatment, followed by addition of OPTIMASH™ TBG, GC 626 and STARGEN™ 002 is shown as Condition 4 (Figure 4-6). When FERMGEN™ 2.5X was included in the 1st round treatment (Condition 1), no difference in hydrolysis was observed. This confirmed again that FERMGEN™ 2.5X is not required for the hydrolysis of barley starch concentrate mash.

To conclude, complete hydrolysis of barley starch concentrate mash can be achieved with STARGEN™ 002 only, and there was no specific advantage of adding FERMGEN™ 2.5X, OPTIMASH™ TBG and GC 626. This is one of the most critical findings of this thesis as it suggested the possibility of using STARGEN™ 002 only for bioethanol production from barley starch concentrate. However, fermentation experiments need to be done to verify the requirement of FERMGEN™ 2.5X, OPTIMASH™ TBG and GC 626, as the conclusion above was based on hydrolysis experiments. If these enzymes were shown to be unnecessary, it would represent an important advancement for reducing production cost of bioethanol fermentation from barley starch concentrate.

In order to assess the hydrolysis levels of barley starch concentrate mash without adding STARGEN™ 002, hydrolysis was examined using different enzymatic combinations of FERMGEN™ 2.5X, OPTIMASH™ and GC 626 as shown in Figure 4-7. Since STARGEN™ 002 is primarily responsible for the conversion of starch to glucose, substantial glucose yields were not expected. Interestingly, when only FERMGEN™ 2.5X was used (Condition 5), there was still some release of glucose (albeit at very low levels), although FERMGEN™ 2.5X is a protease and

does not break down starch into glucose. Even though the exact reason for this observation is not clear, one possible explanation is that commercial enzyme preparations are not pure, and they will contain traces of other enzymes required for these complementary activities. Protease preparations may contain trace amounts of α-Amylase enzymes (Ndubisi *et al*., 2016). Another possible explanation is the presence of endogenous amylolytic enzymes within the barley starch concentrate mash as barley is a good source of α-amylase. The α-Amylase isolated from barley flour was used to hydrolyze amylose and the reaction products after 48 h were glucose and maltose in small amounts, traces of maltodextrins and much larger amounts of higher oligosaccharide (Greenwood & Macgregor, 1965). However, the enzyme isolated from germinated barley substrates permits more complete starch hydrolysis than from non-germinated ones (Tomasik & Horton, 2012).



Condition	1st round treatment	2nd round treatment
Condition 5	FERMGEN™ 2.5X	No enzymes
Condition 6	FERMGEN™ 2.5X	OPTIMASH™ TBG + GC 626
Condition 7	No enzymes	OPTIMASH™ TBG + GC 626

Figure 4-7 Effect of omitting enzymes on the hydrolysis of barley starch concentrate mash. Hydrolysis was performed with the 1st round enzymatic treatment, followed by DEPC treatment and then the 2nd round enzymatic treatment. Experiments were done in replicate (n=3).

Next, when OPTIMASH™ TBG and GC 626 were added after using FERMGEN™ 2.5X in the 1st round treatment (Condition 6), the glucose concentration increased slightly. However, since OPTIMASH™ TBG and GC 626 are not able to efficiently breakdown raw starch granules, the glucose yields were much lower than any of the experiments where STARGEN™ 002 was added (Condition 1-4) in Figure 4-6.

The treatment where no enzymes were added in the 1st round treatment, followed by OPTIMASH™ TBG and GC 626 in the 2nd round treatment, is shown as Condition 7. It displayed a significantly lower hydrolysis than the treatment where FERMGEN™ 2.5X was included in the 1st round treatment (Condition 6). One possible explanation for the above observation is that when FERMGEN™ 2.5X was present, it facilitated hydrolysis with α-amylase. The combined activity of α-Amylase plus the added protease may lead to the increase in glucose yield.

4.6 Particle size measurement

As shown in section 4.5, barley starch concentrate does not require FERMGEN™ 2.5X, OPTIMASH™ TBG and GC 626. The very high accessibility of barley starch concentrate to STARGEN™ 002 can be attributed to the ACAPS process, during which barley flour was fractionated using a micron-sized sieve. The fine particles passing through the micron-sized sieve were collected (i.e. barley starch concentrate). However, wheat flour was ground using a hammer mill equipped with a 0.5 mm-sieve. Thus, wheat flour and barley flour are likely coarser than the barley starch concentrate. In order to verify this assumption, the particle size distribution of wheat flour, barley flour and barley starch concentrate was investigated.

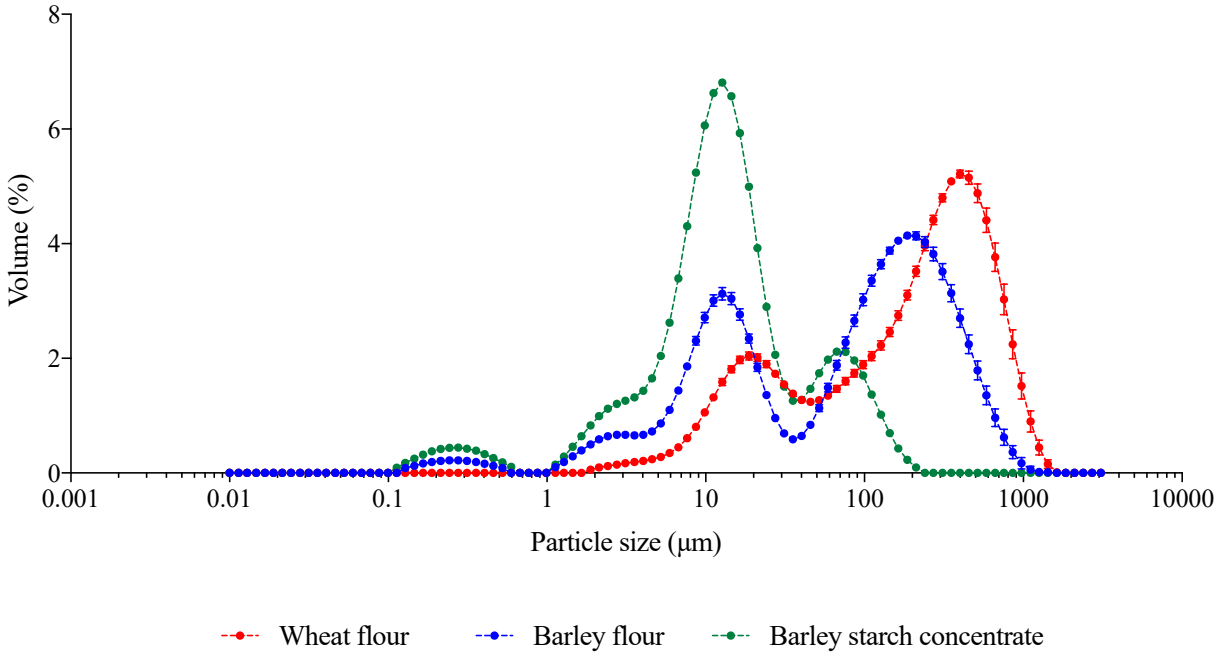


Figure 4-8 Particle size distribution of wheat flour, barley flour and barley starch concentrate. Experiments were done in analytical triplicates.

Table 4-5 Diameter of wheat flour, barley flour and barley starch concentrate

Feedstock	DV 10 (µm)	DV 50 (µm)	DV 90 (µm)
Barley flour	6.71 ± 0.21^{b}	102.8 ± 6.76^{b}	403.2 ± 20.1^{b}
Barley starch concentrate	3.16 ± 0.05^{c}	13.7 ± 0.12^{c}	72.7 ± 1.04^{c}
Wheat flour	16.9 ± 0.5^{a}	238.5 ± 13.4^{a}	704.5 ± 36.5^{a}

NOTE: DV 10 is the size of particle below which 10% of the sample is represented. DV 50 is the size at which 50% of the sample is smaller and 50% is larger. DV 90 is the size of particle below which 90% of the sample lies. Analysis was done in analytical triplicates. All data represent mean ± standard deviation. Means followed by a different letter, within the same column are significantly different ($P<0.05$).

As presented in Figure 4-8, the particle size distribution of the wheat flour and barley flour were broader than that of barley starch concentrate. Barley starch concentrate ended at 211 µm while the other two samples ended over 1000 µm. There are two major peaks for all three feedstocks. Barley starch concentrate has the first major peak at 12.7 µm, which was similar to

barley flour. The second major peak of barley starch concentrate and barley flour was at 66.9 μm and 186 μm, respectively. Wheat flour has the first and second major peak at 18.7 μm and 400 μm, respectively. This figure indicated that barley starch concentrate has a narrower range of particle size distribution, and a larger proportion of smaller particles than wheat and barley flour. Values of DV 10, DV 50 and DV 90, which indicate diameters of 10%, 50% and 90% of the volume of the particle group, respectively, were determined and shown in Table 4-5. As a result, values of DV 10, DV 50 and DV 90 for the three feedstock ranked as: barley starch concentrate < barley flour < wheat flour. This analysis provides strong corroborating evidence that larger surface area per volume contributed to the higher accessibility of barley starch concentrate to STARGEN^{TM} 002.

Lee *et al.* (2019) compared the starch hydrolysis of rice flour using hammer milling and jet milling. It was observed that jet milled flour had smaller particle size and starch hydrolysis increased with the decreasing particle size of rice flour. The hydrolysis rate of barley flour by α-Amylase showed a decrease with increasing particle size, likely attributed to a smaller surface area (Al-Rabadi *et al*., 2009). An alternative or additional reason is the disruption of the protein-starch matrix in barley starch concentrate during the ACAPS process. As can be seen in Table 4-6, barley starch contrate has lower protein content compared to barley flour, which is likely due to the removal of partial protein by ACAPS process. Wheat flour has the highest protein content among three feedstocks. Angelidis *et al*. (2016) presented the scanning electron micrographs of control wheat flour and jet milled wheat flour. Control wheat flour showed the large aggregates of protein matrix embedding starch granules. For the jet milled samples under intensive conditions, the aggregates were of smaller size and starch granules appeared to be defragmented, and released

from the protein matrix. Furthermore, barley starch concentrate contains waxy starch, which likely contributes to its high accessibility to enzymes (Wu *et al*., 2006; Zhao *et al.*, 2009).

Table 4-6 Protein content of three feedstocks

Feedstock	Wheat flour	Barley flour	Barley starch concentrate
Protein content (% dry weight)	14.4 ± 0.0^{a}	12.5 ± 0.3^{b}	11.1 ± 0.1^{c}

4.7 Effect of lowering STARGEN™ 002 on hydrolysis of barley starch concentrate mash

As described in section 4.5, FERMGEN™ 2.5X, OPTIMASH™ TBG, and GC 626 were proven to be unnecessary for the hydrolysis of barley starch concentrate mash. In addition, it was observed in section 4.2 that barley starch concentrate mash showed a significantly faster glucose release compared to wheat mash. Considering these two observations, experiments were performed to find out whether the hydrolysis kinetics of barley starch concentrate mash could be made to mimic that of wheat mash by decreasing the STARGEN™ 002 concentration. It is important for barley starch concentrate to meet the wheat benchmark interns of hydrolysis kinetics, because that helps generate lower sugar levels for reduced osmotic stress, thereby potentially improving ethanol yields with less enzyme costs.

Earlier fermentation studies on wheat flour in our laboratory employed a standard dosage at 2.8 mL/kg of grains (Gibreel *et al*., 2011; Jin *et al*., 2016). In the following experiments, the wheat benchmark involved addition of FERMGEN™ 2.5X, OPTIMASH™ TBG, GC 626 in the 1st round treatment, followed by addition of STARGEN™ 002. The glucose formed from barley starch concentrate using varying concentrations of STARGEN™ 002 including 1X, 0.5X, 0.25X and 0.1X enzyme dosage (with no FERMGEN™ 2.5X, OPTIMASH™ TBG, GC 626), are exhibited in Figure 4-9.

In all the treatments, the hydrolysis of barley starch concentrate mash by STARGEN™ 002 had a relatively rapid rate during the earlier stages, followed by a progressively decreased hydrolysis thereafter. Barley starch concentrate with 1X enzyme dosage showed the highest hydrolysis rate during the first 1 h. As anticipated, by decreasing the dosage of STARGEN™ 002, a lower hydrolysis rate of barley starch concentrate was observed during the first 1 h. This was because reducing enzyme dosage typically results in a longer reaction time (Pietrzak & Kawa-rygielska, 2014). It was notable that 0.5X enzyme dosage presented a statistically similar glucose production to the wheat benchmark at each time point during the 48 h experiment ($p>0.05$). Interestingly, although the 0.25X enzyme dosage resulted in a significant lower glucose concentration than the wheat benchmark ($P<0.05$) after 10 min of hydrolysis, similar glucose amounts were present in both systems after 24 h. For the 0.1X enzyme dosage, the lowest hydrolysis degree was observed during hydrolysis and 17.8 ± 0.5 g/L of glucose was achieved at 48 h. After 48 h hydrolysis, the wheat benchmark, 1X, 0.5X, and 0.25X enzyme dosages were hydrolyzed to the extent of 28.2 ± 0.8 g/L, 28.8 ± 0.9 g/L, 28.8 ± 1.3 g/L, of 28.6 ± 0.6 g/L, respectively.

The treatment for wheat flour, where no enzyme was added in the 1st round treatment, followed by the addition of STARGEN™ 002 is shown as the wheat negative control. Lower hydrolysis levels were observed from 1 h to 48 h ($P<0.05$) compared with wheat benchmark. Results from the wheat negative control further verified the necessity of using FERMGEN™ 2.5X, OPTIMASH™ TBG and GC 626 during wheat mash hydrolysis.

To conclude, hydrolysis kinetics of barley starch concentrate mash was successfully mimicked to that of wheat mash by decreasing the levels of STARGEN™ 002, and without the addition of FERMGEN™ 2.5X, OPTIMASH™ TBG, and GC 626. Of all the dosages examined

above, the 0.5X enzyme dosage was better than others. However, further optimization is truly needed since other amounts of STARGEN™ 002, such as a 0.33X enzyme dosage, were not examined. This successful demonstration in hydrolysis experiments indicates that it is promising to develop a strategy of applying less STARGEN™ 002 for the fermentation of barley starch concentrate. Granular starch hydrolysis enzymes (GSHE) are more expensive than conventional enzymes and the reduction in GSHE amount is important to improve the economics for bioethanol production (Wang *et al*., 2009). If it is confirmed that 0.5X dosage or even less of STARGEN™ 002 can yield similar ethanol efficiency of barley starch concentrate to wheat flour, the cost of production could be considerably reduced by using lower dosage of STARGEN™ 002.

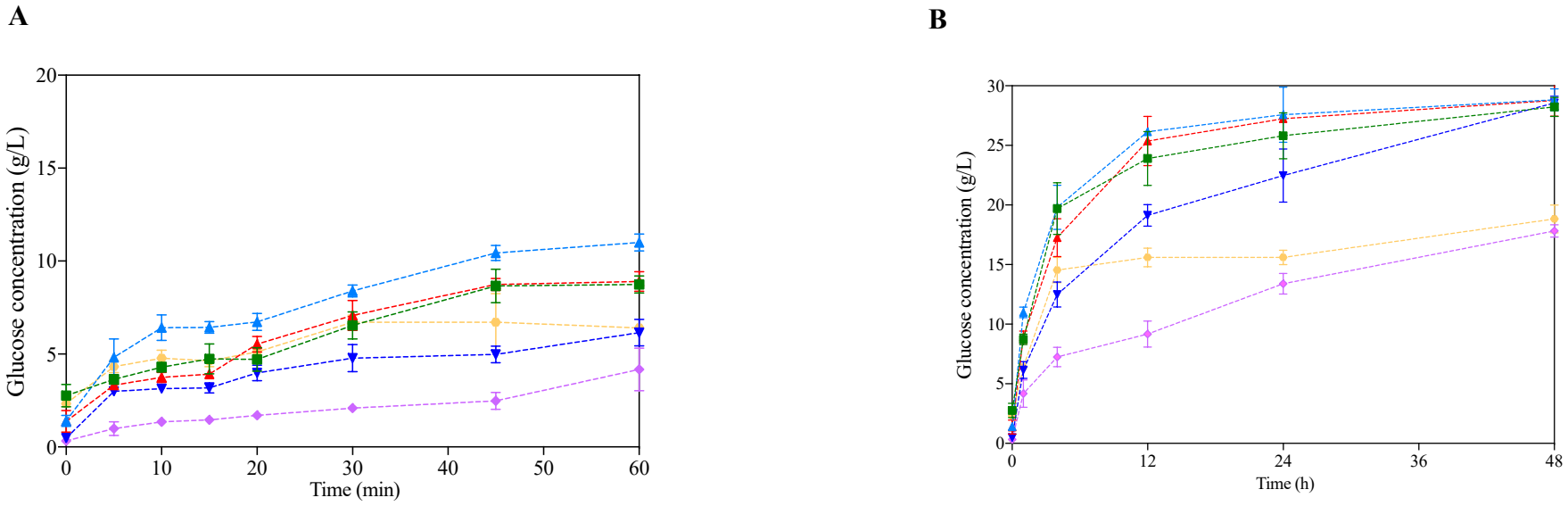
4.8 Fermentation of barley starch concentrate

To verify that the 0.5X dosage, or even lower STARGEN™ 002 loading, can yield similar ethanol efficiencies for barley starch concentrate and wheat flour, decreased dosages of STARGEN™ 002 were incorporated in the present study with simultaneous STARGEN™ 002 action and yeast fermentation. Parameters at 0 h and 72 h of fermentation were measured. The initial glucose concentrations and ethanol efficiencies are presented in Figure 4-10. Residual glucose and organic acid concentrations are shown in Table 4-6.

Firstly, benchmark fermentation experiments of both wheat flour and barley starch concentrate were set up. Briefly, the benchmark fermentation was performed where OPTIMASH™ TBG, FERMGEN™ 2.5X and GC 626 were added in the 1st round treatment, followed by DEPC treatment. Here, OPTIMASH™ TBG acts on the degradation of non-starch polysaccharides in cereal grains, the major of which present in barley and wheat are β-Glucan and arabinoxylan, respectively (Saulnier *et al.,* 1995). Those two non-polysaccharides are able to form viscous mash. Then, a short pre-saccharification step was employed where STARGEN™ 002 was

added to function for 1 h before the addition of yeast. Once the pre-saccharification step was completed, mashes were added with urea and then inoculated with yeast. As shown in Figure 4-10B, wheat flour and barley starch concentrate under benchmark fermentation conditions with the same standard enzyme concentrations resulted in 85.1 ± 0.7, and 4.01 ± 3.4 ethanol efficiency (%), respectively. Similarly, when OPTIMASH^{TM} TBG, FERMGEN^{TM} 2.5X and GC 626 were excluded, followed by the addition of 0.25X dosage of STARGEN^{TM} 002, which is shown as Condition 5, no ethanol was produced after 72 h of fermentation. One possible explanation for this observation is that phosphorus and trace minerals were limiting in barley starch concentrate. Phosphorus and trace minerals would be present in the wheat mash (Thomas & Ingledew, 1990); however, they may have been reduced below a threshold level in the barley starch concentrate fraction by the ACAPS process. In wheat, phytic acid acts as the primary phosphorus reserve accounting for up to 85% of the total phosphorus. Phytase is capable of hydrolyzing phosphate ester bond and yielding inorganic phosphate (Angel *et al.*, 2002). Majority of phytases are located at the aleurone layers of wheat grains and are inactive in dry grains due to lack of moisture (Oatway *et al*., 2001).

To boost the ethanol production, phosphorus and trace minerals were added to barley starch concentrate fermentation systems. Additionally, OPTIMASH^{TM} TBG, FERMGEN^{TM} 2.5X and GC 626 were excluded, followed by 1X STARGEN^{TM} 002, which is shown as Condition 1. This treatment presented similar initial glucose concentrations at 0 h of fermentation to barley starch concentrate benchmark (Figure 4-10A). More importantly, it was found that adding phosphate and



Substrate	Condition	Enzymes	
		1st round treatment	2nd round treatment
Wheat	Wheat benchmark	With enzymes	1X
	Wheat negative control	No enzymes	1X
Barley starch concentrate	BSC (1X enzyme dosage)	No enzymes	1X
	BSC (0.5X enzyme dosage)	No enzymes	0.5X
	BSC (0.25X enzyme dosage)	No enzymes	0.25X
	BSC (0.1X enzyme dosage)	No enzymes	0.1X

Figure 4-9 Effect of lowering STARGEN™ 002 on enzymatic hydrolysis kinetics of barley starch concentrate mash (A) Hydrolysis during the first 1 h; (B) Hydrolysis during 48 h. Hydrolysis was performed with the 1st round enzymatic treatment, followed by DEPC treatment and then the 2nd round STARGEN™ 002 treatment. For the wheat benchmark, FERMGEN™ 2.5X, OPTIMASH™ TBG and GC 626 were added in the 1st round treatment. Experiments were done in replicate (n=3). The glucose concentration at 1 h for the wheat negative control shows the result of duplicate samples.

trace minerals in the Condition 1 system remarkably enabled ethanol production similar to the wheat benchmark.

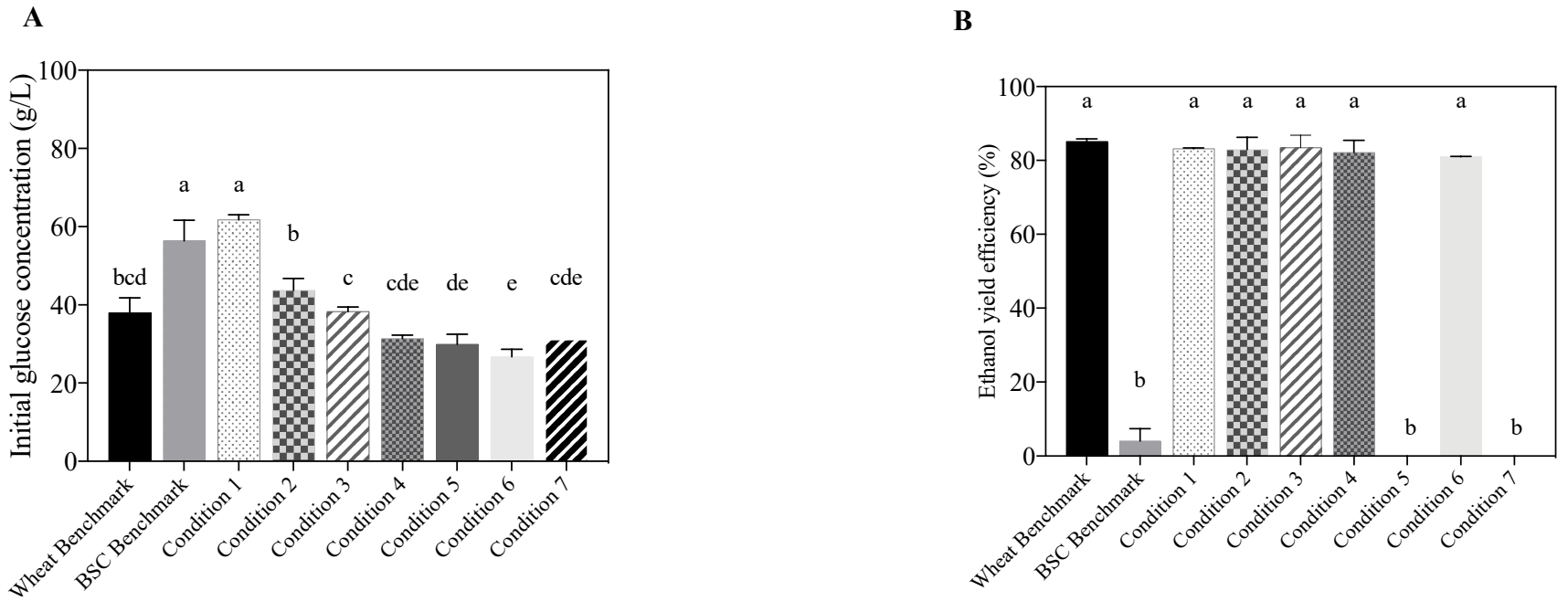
Moving forward, these experiments were performed to examine the impact of lowering STARGEN™ 002 dosage with the phosphate and trace mineral supplementation. Here, OPTIMASH™ TBG, FERMGEN™ 2.5X and GC 626 were excluded, then various dosages of STARGEN™ 002 including 0.5X, 0.33X, 0.25X, were applied to treatments Conditions 2, 3 and 4, respectively. It should be noted that these experiments included 0.33X enzyme dosage that was not in the hydrolysis experiments 4.7 as further optimization. In terms of parameters at 72 h of fermentation for Condition 1-4, not only were similar ethanol efficiencies observed (Figure 4-10B), but also the same amounts of residual glucose and lactic acid were detected (Table 4-6). To conclude, lowering STARGEN™ 002 dosage yielded similar ethanol efficiency of barley starch concentrate to the wheat benchmark. Of all the STARGEN™ 002 dosages examined above, the 0.25X enzyme dosage was better than the others.

It is worth noting that to study the medium supplementation needed, experiments were set up where 0.25X dosage of STARGEN™ 002 was applied, however with different combination of nutrient supplements. Here, the treatment with only phosphate added (Condition 6) enabled ethanol yields similar to the wheat benchmark and Condition 4, the latter of which was supplemented with both phosphate and trace minerals at a STARGEN™ 002 dose of 0.25X. Conversely, when only trace minerals were added (Condition 7), no improvement in ethanol yield was observed relative to Condition 5, and the residual glucose in these two fermentation systems remained high: 78.3 ± 9.8 g/L and 69.9 ± 5.7 g/L, respectively. The amount of organic acids in the Condition 4 and Condition 6 systems were very low, similar to that in the wheat fermentation

system. Thus, these data indicated that phosphorus was the limiting nutrient for bioethanol production of barley starch concentrate. Supplementing barley starch concentrate fermentation systems with phosphate maximized the ethanol production by yeast.

To summarize, results show that fermentation of barley starch concentrate achieved similar ethanol yields to the wheat benchmark commonly utilized in industry, but eliminated the need for FERMGEN^{TM} 2.5X, OPTIMASH^{TM} TBG, and GC 626, and required only a 0.25X dosage of STARGEN^{TM} 002, though phosphorus supplementation was required. The approach of applying less enzymes offers enormous potential benefits with regards to the reduction of bioethanol production costs. Therefore, the combined savings on feedstocks and enzymes could potentially raise the efficiency and competitiveness of bioethanol plants using barley starch concentrate from the ACAPS process.

Yeast has specific nutrient requirements and their limitation or deficiency will lead to incomplete or sluggish fermentation. Carbon, nitrogen, vitamins and minerals are included in these requirements (Walker & Stewart, 2016). Mineral ions play an important role in the maintenance of intracellular pH, osmotic stability, co-transport of solutes, and as co-factors in enzyme-catalyzed reactions (Priest & Campbell, 2003). The above-mentioned results reaffirmed that due to the lack of phosphorus in barley starch concentrate mash, yeast had difficulty in consuming the large amount of glucose for yeast growth and fermentation. Phosphorus is an essential component for structural molecules (e.g. phospholipids and nucleic acids) and phosphorylated metabolites (e.g. ATP and glucose-6-phosphate) (Jacques *et al*., 2003). According to Boer *et al*. (2010), during phosphate limitation in *S. cerevisiae*, ATP was a potential growth-limiting species due to the lack of phosphorylation of ADP to ATP. This can lead to the growth-limiting patterns of ATP products,



Substrate	Condition	Enzymes		Additives
		1st round treatment	2nd round treatment	
Wheat	Wheat Benchmark	With enzymes	1X	-
Barley starch concentrate	BSC Benchmark	With enzymes	1X	-
	Condition 1	No enzymes	1X	Phosphorus + Trace minerals
	Condition 2	No enzymes	0.5X	Phosphorus + Trace minerals
	Condition 3	No enzymes	0.33X	Phosphorus + Trace minerals
	Condition 4	No enzymes	0.25X	Phosphorus + Trace minerals
	Condition 5	No enzymes	0.25X	-
	Condition 6	No enzymes	0.25X	Phosphorus
	Condition 7	No enzymes	0.25X	Trace minerals

Figure 4-10 Glucose concentration (A) of the mash at 0 h of fermentation and ethanol yield efficiency (B) of the mash at 72 h of fermentation. Fermentation was performed with the 1st round enzymatic treatment, followed by DEPC treatment and then the 2nd round STARGEN™ 002 treatment. For wheat and barley starch concentrate benchmarks, FERMGEN™ 2.5X, OPTIMASH™ TBG and GC 626 were added in the 1st round treatment. Experiments were done in replicate (n=3). Duplicate data was presented for ethanol concentration and ethanol efficiency for BSC V6 as an outlier was excluded. Means with different letters are significantly different ($P<0.05$)

Table 4-7 Residual glucose and acid concentration of fermented mash.

Substrate	Condition	Residual glucose concentration (g/L)	Lactic acid concentration (g/L)	Acetic acid concentration (g/L)
Wheat	Wheat Benchmark	0.0194 ± 0.0011^{c}	0.385 ± 0.020^{b}	0.310 ± 0.060^{c}
	BSC Benchmark	97.6 ± 2.7^{a}	1.39 ± 1.24^{b}	0.0200 ± 0.0300^{bc}
Barley starch	Condition 1	0.0413 ± 0.0045^{c}	BDL	0.780 ± 0.140^{a}
concentrate	Condition 2	0.0376 ± 0.0015^{c}	BDL	0.410 ± 0.040^{b}
	Condition 3	0.0393 ± 0.0051^{c}	0.739 ± 1.280^{b}	0.380 ± 0.070^{bc}
	Condition 4	0.0601 ± 0.0033^{c}	0.00 ± 0.00^{b}	0.280 ± 0.060^{bc}
	Condition 5	69.9 ± 5.7^{b}	7.47 ± 0.63^{a}	0.450 ± 0.230^{ab}
	Condition 6	0.0534 ± 0.0021^{c}	0.536 ± 0.930^{b}	0.250 ± 0.110^{bc}
	Condition 7	78.3 ± 9.8^{b}	7.91 ± 0.65^{a}	0.300 ± 0.210^{bc}

Data are presented as means ± standard deviation of triplicate repeats for the mash at 72 h of fermentation. Means with different letters within each column are significantly different ($P<0.05$). BDL, below detection limits.

mostly nucleotide triphosphates, which are directly related to biopolymer synthesis. Markham & Byrne (1967) reported that phosphate-starved *S. cerevisiae* was unable to grow in a medium lacking phosphate; however, the yeast yield increased with the increasing phosphate concentration in the medium. Yu *et al* (2009) concluded that the most important factors that affect the bioethanol production of sweet sorghum juice included the initial amount of phosphorus and nitrogen. A maximum ethanol productivity for the optimized medium consisting of 0.77 g/L phosphorus and 2.15 g/L nitrogen was achieved.

5. Conclusions and future directions

To offset biofuel production costs, a biorefining approach that enables the co-production of high-value products was employed. The isolation of β-glucan, a high-value nutraceutical product, from Fibar barley using Air Currents Assisted Particle Separation (ACAPS) generates a starch concentrate, namely barley starch concentrate. Here, results showed that the barley starch concentrate may be an excellent feedstock for industrial fermentation applications.

This study demonstrated that hydrolysis of barley starch concentrate mash does not require FERMGEN™ 2.5X, OPTIMASH™ TBG and GC 626, which are currently employed in industrial ethanol fermentations using wheat and cellulosic feedstocks. Results also showed that hydrolysis of barley starch concentrate mash with a 0.5X dosage of STARGEN™ 002 showed similar enzymatic kinetics to that of the wheat benchmark using all four enzymes, which are FERMGEN™ 2.5X, OPTIMIASH™ TBG, GC 626 and STARGEN™ 002.

The fermentation experiments using barley starch concentrate showed that applying a 0.25X dosage of STARGEN™ 002 displayed a similar ethanol yield efficiency to the wheat benchmark, however, seemed to require phosphorous supplementation. Such an enhanced approach for barley starch concentrate fermentation could help substantially reduce the costs associated with commercial granular starch hydrolyzing (GSH) enzymes, thereby offering advantages to maintain the cost-effectiveness of ethanol production.

There are several recommendations regarding future work. In order to further decrease the production cost and at the same time maintain the ethanol efficiency of barley starch concentrate, dosages of STARGEN™ 002 lower than 0.25X dosage should be evaluated. Furthermore, in the present study, fermentation of barley starch concentrate (20% [wt/wt] solids) was carried

out. However, very high gravity fermentation technology (i.e. fermentations that contains 27 g or more of solids/100 g of mash) is commonly applied in the alcohol industry. Higher loadings of raw materials would make the process more profitable due to lower energy requirements for distillation when higher ethanol concentration is achieved (Thomas *et al*., 1996a). Therefore, future studies should consider application of very high gravity technology to fermentation of barley starch concentrate. Higher ethanol concentrations and less water utilization could be expected.

After the successful fermentation trial in batch experiments, the next step is to demonstrate the fermentability of barley starch concentrate in 5 L bio-reactors. Furthermore, in addition to ethanol, the distiller grains remaining post fermentation need to be isolated and chemically characterized with regards to protein and amino acid content, residual starch content, fatty acid content, mineral content and phenolic content, etc. The distiller grains are likely to consist of concentrated protein, lipid, minerals and thus may be used as high-value animal feed. This application could also help offset the cost of bioethanol production. It will also be of interest to explore the potential application of barley starch concentrate as a substrate for other microorganisms that can be used for the production of value-added materials.

Taken together, the research in this thesis demonstrated that barley starch concentrate is a promising bioethanol feedstock and is comparable to wheat, which is commonly used in bioethanol industry. This research provides the scientific evidence for utilizing the starch enriched by-product streams from the ACAPS process. Considerable cost savings are possible during production of bioethanol from barley starch concentrate by lowering enzyme consumption. Application of barley starch concentrate for bioethanol production will help valorize an underutilized by-product, making the ACAPS process more economically feasible and expanding the scope for utilizing starch enriched by-products in the bioethanol industry.

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