Co-production of ethanol and protein isolates from the by-product of pulse with Air-Currents Assisted Particle Separation (ACAPS) process

by

Elisabeth Kezia Widjaja

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Bioresource Technology

Department of Agricultural, Food and Nutritional Science
University of Alberta

ABSTRACT

Biorefinery approaches aimed at adding value to waste materials to support a sustainable bio-based economy have gained much more attention in the last decades. Bioethanol, as biofuel, stands out as a key product derived from the innovative processes that have been developed using various agricultural feedstocks. In this study, pulse starch concentrate obtained as a by-product of the Air-Currents Assisted Particle Separation (ACAPS) technology was used to produce bioethanol with a reduced environmental footprint, while concurrently generating a co-product in the form of protein-rich fermentation residue.

The conversion of starch-rich pulse materials starts with the hydrolysis of starch into glucose, facilitated by an enzyme cocktail containing α-amylase, glucoamylase, glucanase, and protease. Of all three types of feedstocks, the degree of hydrolysis of field pea, red lentil, and faba bean, was comparable to barley starch concentrate. The maximum conversion was achieved within 24 hours for field pea and red lentil, and within 48 hours for faba bean. The enzyme concentration used for hydrolysis was also studied and showed that lower enzyme concentrations than what was used do not produce the same glucose concentration within the same timeframe. The enzyme combination was also studied and it was found that the combination of α-amylase and glucoamylase only did not produce high glucose concentration, due to the presence of protein and fiber surrounding the starch granules. However, the addition of urea as a hydrogen bond breaker increased the starch conversion.

The starch hydrolysis behavior indicated partial hydrolysis, which was observed by quantifying residual starch after hydrolysis. The high amylose content of 28-32% for all pulse feedstock was one of the causes of partial hydrolysis, but it was also observed that the surface of pulse starch granules was smooth without any pinholes or fissures, decreasing the contact between enzyme and starch. However, with hydrolysis, physical changes in the starch granules were

observed as well as subsequent gelatinization behavior which combined appeared to support higher glucose production.

The final element of the starch conversion was fermentation in the bioreactor following an established Simultaneous Saccharification and Fermentation (SSF) protocol. At the end of fermentation, residual starch was observed, in accordance with the previously observed partial hydrolysis results. Improvement of ethanol conversion efficiency was achieved through enzyme combinations, the addition of urea, as well as supplementation of phosphate or trace minerals. Sodium chloride supplementation was also found to increase the ethanol conversion efficiency, due to its osmotic regulation properties. At the end of fermentation, a protein-rich Distiller's Dried Grains with Soluble (DDGS) was obtained.

To summarize, pulses, alternative feedstocks for ethanol production were examined in this study with a promising result. Pulses present different kinetics of amylolysis behavior compared to common feedstock such as corn, barley, and wheat due to the high protein content, but approaches taken were successful in producing both ethanol and protein-rich residue with improved conversion efficiency. It is expected that the hydrolysis and fermentation processes lowered the anti-nutritional content, making it more attractive for both human and animal consumption. As pulses are widely consumed for the protein and dietary fiber benefits, the nitrogen-fixing capabilities as well as the potential for fuel production are additional appeals for crop diversification. Thus, the bioconversion of ACAPS-treated pulse feedstock into bioethanol and protein residue using a biorefinery process can help address the bioeconomy challenges.

PREFACE

This thesis is an original work by Elisabeth Kezia Widjaja. There is no part of this thesis has been previously published. All the experiments in this thesis were conducted in the Biorefining Conversions and Fermentation Laboratory at the University of Alberta and were discussed with Dr. David C. Bressler.

ACKNOWLEDGEMENT

I would like to express my gratitude to my supervisor, Dr. David C. Bressler, who gave me the opportunity to work on this project. The freedom that he gave me to explore the intricacies of the subject was met with endless guidance and support. I also would like to thank Dr. Michael Chae and Dr. Justice Asomaning for giving their time and thoughts to this project. Although the time of us being together was short, their enthusiasm, patience, and dedication in this study were unmatched.

I would also like to thank Dr. Thava Vasanthan, my co-supervisor as well as supervisory committee member, for his valuable insights on this research. The completion of this project would not be possible without his expertise and contributions.

I was beyond grateful for the contribution of Jingui Lan, our beloved technician of the 2-38 laboratory, for giving me instrumental training and technical assistance with compassion. Jun Gao, the neighboring lab technician, was not exempt from my train of gratitude for his assistance specifically in handling the starch feedstock.

I would like to thank the Natural Sciences and Engineering Research Council of Canada (NSERC) for the funding provided, as well as GrainFrac and Dupont for providing the experimental materials. I would also like to thank various centers and facilities in the University of Alberta, for making the project possible.

I would like to thank all my lab mates for their support, making a long day in the lab much more fun and enjoyable. Also, to my friends I met both within and outside the laboratory, reminding me there is much more in life than an experiment gone wrong.

Finally, I would like to thank my family and soon-to-be husband, for their time and energy spent on trusting my ability when I did not feel capable.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACAPS	: air current assisted particle separation
ANOVA	: analysis of variance
ATP	: adenosine triphosphate
BCE	: before common era
BSC	: barley starch concentrate
CFS	: clean fuel standard
CFU	: colony forming unit
COVID-19	: coronavirus disease 2019
DDGS	: distiller's dried grains with soluble
DSC	: differential scanning calorimeter
EC	: enzyme class
FFV	: flexible fuel vehicle
g	: grams
g	: relative centrifuge force
g/mol	: grams per mole
GC	: gas chromatography
GOPOD	: glucose oxidase peroxidase
h	: hours
HPLC	: high performance liquid chromatography
IDF	: insoluble dietary fiber
L	: litres
M	: mol/L
mL	: millilitres
Mt	: million metric tonnes
NADH	: nicotinamide adenine dinucleotide hydrogen
SDF	: soluble dietary fiber
SEM	: scanning electron microscopy
SHF	: separate hydrolysis and fermentation
SSF	: simultaneous saccharification and fermentation
Rf	: response factor

RFO : raffinose family oligosaccharide

RID : refractive index detector

rpm : revolutions per minute

RS : resistant starch

TCA : tricarboxylic acid

TDF : total dietary fiber

TGA : thermogravimetric analysis

T_c : conclusion temperature

T_f : final temperature

T_g : gelatinization temperature

T_o : onset temperature

VHG : very high gravity

w/v : weight per unit volume percent

w/w : weight per unit weight percent

µm : micrometer

1. Introduction

1.1. Project background

Based on current predictions, global oil consumption is expected to increase by 1.6 million barrels per day in 2023 and by 1.7 million barrels per day in 2024 (EIA, 2023). Currently, the liquid production is still adequate to fulfill the global demand but the geopolitical situation can greatly affect this balance and will leave millions of people short on fuel. Bioethanol is one of the popular alternatives that is a renewable fuel and also has a positive outcome in terms of the life cycle energy balance compared to fossil fuel. Bioethanol is commonly used in a mixture with gasoline, with E5 (5% ethanol), E10 (10% ethanol), and E85 (85% ethanol) used on a midsize passenger vehicle (Winther et al., 2012). Currently, the USA is the biggest bioethanol producer with 55% of bioethanol production shares worldwide, followed by Brazil at 26%, and Europe at 5%. Canada is in sixth place with 2% of bioethanol production (RFA, 2023). Compared to fossil fuels, bioethanol is biodegradable and has less toxicity. In terms of production, agricultural biomass or edible crops such as sugarcane, corn, and cereals are the main feedstocks that are currently being used (Bušić et al., 2018).

Barley crops processed with Air-Currents Assisted Particle Separation (ACAPS) technology result in β-glucan as a dietary fiber supplement (Vasanthan, 2017). During the grain processing, a co-product of high fermentable sugar, barley starch concentrate (BSC), was obtained and then fermented to produce bioethanol as demonstrated by Lu et al., (2020). Recently, consumer interest in pulse grains such as field pea, lentil, and faba bean has been increasing, due to the high protein content as well as better starch quality in terms of more variety of minor components and lower glycemic index (Mitchell et al., 2021). The implementation of ACAPS processing has been studied on faba bean, a type of pulse, and resulted in coarse fractions high in both protein (35-39%) and dietary fibers (14-19%), as well as fine fractions high in starch (44-55%) as the co-product (Jeganathan et al., 2023).

The starch-rich fine fraction of the pulses from the ACAPS process then can be used to produce bioethanol through the simultaneous saccharification and fermentation (SSF) process. In addition to that, the fermentation residue is expected to have improved protein content and therefore can be further utilized as animal feed. It is hypothesized that this starch-rich fine fraction from the ACAPS process can be converted into bioethanol with high ethanol yields that are

comparable to conventional wheat or barley flour. 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.

1.2. Objectives

1) To understand the pulse starch concentrates physicochemical behavior in terms of morphology, hydrolysis pattern, and thermal behavior,
2) To achieve high conversion of starch-to-sugar of the pulse starch concentrates through enzyme treatments, and
3) To integrate the optimized enzyme treatments of starch concentrate into fermentation strategies.

2. Literature Review

2.1. Pulses

Pulse is the edible part of the legume. The word pulse itself originated from the Latin word "puls" or "pultis" meaning a thick soup. Examples of pulses include black bean (*Phaseolus vulgaris*), pinto bean (*Phaseolus vulgaris*), faba bean (*Vicia faba* L.), navy bean (*Phaseolus vulgaris*), chickpea (*Acer arietinum* L.), pea (*Pisum sativum* L.), and lentil (*Lens culinaris*). Pulses have been part of the human diet for the past 60,000 to 40,000 years and were domesticated during the Neolithic period and its wide cultivation began over the past 4,000 years. Consumption of pulses was highly common in the southern and western parts of Europe during the period of the Byzantine and Roman empires (Huebbe and Rimbach, 2020).

Today, pulses are commonly found in salad, soup, or mixed with meat, and consumed as a plant-based protein source by many cultures. With the high protein and high fiber content, pulses are being utilized more than direct human or animal consumption. Pulses are being used to produce bread, extrudates, pasta, and biscuits or other recent advances such as protein supplements as high-protein and gluten-free alternatives. However, using pulses presents challenges due to their distinct properties compared to common grains like wheat, resulting in variations in physical appearance and sensory characteristics of the final product". Another application of pulses is as a polymer or bioplastic due to their starch properties, low allergenicity, and compatibility with food. The starch and protein content of pulses is the key to achieving polymer materials with high elasticity and tensile strength (Salmoral et al., 2000).

In Canada, pulses are generally sown from April to early June and harvested in August to early October depending on the type of pulses. The pulses themselves have a preference for a low moisture environment and short planting season, thus making it a suitable summer crop. In 2022, Canadian pulse production of peas, lentils, beans, chickpeas, and faba beans are 3.4 million metric tonnes (Mt), 2.3 Mt, 0.3 Mt, 0.13 Mt, and 0.9 Mt, respectively (Statistics Canada, 2023). The trend of production and demand is predicted to be increasing, although in 2021 there was a general decrease in national crop production due to nationwide drought. However, investment and expansion in domestic pulse production are growing with a new pea-splitting facility of 60,000 metric tonnes annual capacity established in the summer of 2022 (GFI, 2022).

Another benefit of planting pulses is the low requirement of synthetic nitrogen fertilizer since pulses are able to fixate nitrogen by themselves through symbiosis with atmospheric nitrogen-fixing bacteria or fungi. This is marked by a significant increase in nitrogen fixation rate (Hossain et al., 2016; Reinprecht et al., 2020). After the 19th century, however, pulses were no longer commonly planted in rotation with other crops such as cereals due to consumer demand. Along with this shift, an increase in synthetic nitrogen fertilizer use was observed. Unfortunately, this practice resulted in negative impacts including soil acidification, an increase in potent greenhouse gasses from nitrous oxide, contamination of groundwater from nitrate leaching, and eutrophication that affected not only the surrounding land but also globally due to the matter being carried by water runoff as well as wind (Crews and Peoples, 2004).

The year 2016 was decided to be the International Year of Pulses by the United Nations with the highlight of the potential of pulses to be a cheap but nutritious food. Pulses are categorized as protein-rich foods, and the recommended daily intake of cooked pulses is 100 g per day to improve the nutrient density of a healthy diet (Marinangeli et al., 2017). In addition to protein, pulses are also a good source of complex carbohydrates including dietary fiber, iron, zinc, and vitamins. The current consumption of pulses is 21 g per person per day on average, with Latin America, the Caribbean, South Asia, and sub-Saharan Africa having the highest pulse consumption and Caucasus as well as Central Asia having the lowest (Semba et al., 2021). In comparison to cereals, pulses have a different amino acid composition. Pulses are rich in lysine while cereals are rich in sulfur-containing amino acids such as methionine. Thus, fortification of pulses in cereal-based foods can improve the functionality of food, however, change in dough properties should be considered with the addition of pulses (Subedi et al., 2022).

2.1.1. Pulses as feedstock for biofuel

In addition to the traditional pulse consumption for nourishments, some studies have looked into utilizing pulses for producing ethanol. The starch concentration of pea, lentil, and faba bean was reported to be 21-40%, 27-47%, and 39.9% (Hoover et al., 2010; Ren et al., 2021), and their protein concentration was approximately 7.9 ± 0.1%, 15.3 ± 0.1%, and 20.2 ± 0.4% after air classification (Li et al., 2019), providing sufficient raw materials for ethanol production. During the ethanol production from pulse, starch is converted into sugar then ethanol while the protein left can be used for feed purposes. The abundant starch content and the feed potential of the

fermentation residue, paired with the ability of legumes to improve nitrogen content in the soil make pulses a good crop to produce ethanol with lower environmental impact (Lienhardt et al., 2019).

A pin-milled and air-classified field pea with a starch concentration of 77.8 ± 0.6% w/w was previously studied to produce bioethanol through a simultaneous saccharification and fermentation (SSF) process (Nichols et al., 2005). Using a similar approach, dry common beans were also fermented into ethanol with high fermentation efficiencies above 90% (Nichols et al., 2011). In both studies, the conversion of starch started with hydrolysis involving amylolytic enzymes. However, pulses were known to exhibit different hydrolysis behavior compared to wheat or corn, due to their different proportions of starch types as well as the crystalline arrangements, resulting in different susceptibility to α-amylase, one of the most commonly used amylolytic enzymes (Hoover and Zhou, 2003).

2.1.2. Field pea

Field pea belongs to the genus *Pisum* and is consumed in different stages of maturity, such as snow pea and snap pea as examples of consumed forms of the immature pea. The domestication of field peas began in the Mediterranean between 7000 and 6000 BCE. In terms of planting, field peas can be planted in intercropping method with cereal and oilseed crops to increase land productivity. Field pea is naturally rich in iron and zinc, as well as relatively high in lysine as well as isoleucine, valine, and threonine content in comparison to other legumes (Duranti and Gius, 1997).

However, legume production requires a high level of phosphorous supplementation to support the nodule formation and function, increasing synthetic phosphorous fertilization. Phosphorous is taken up by the plant and is converted into phytic acid through the inositol phosphate pathway (Raboy, 2003). To overcome this, a variety of low-phytate field peas have been developed. However, this mutation came with a cost of lower biomass and yield as well as a decrease in stress tolerance (Warkentin et al., 2012).

2.1.3. Lentil

Lentil originated from East and Central Asia, where the temperate, subtropical, and high-altitude tropical regions suit them the best. Lentil prefers low temperatures and has a tolerance to

drought, thus is cultivated during the cooler season such as fall or early winter. Similar to pea, lentil is also intercropped with other plants, such as rice, maize, cotton, sorghum, and pearl millet. In terms of morphology, the lentil has a lens-shaped seed with a wide range of seed coat colors such as green, tan, brown, gray, white, and black, while the cotyledon can be yellow, green, or red (Khazaei et al., 2017).

Lentils are rich in protein, dietary fiber, complex carbohydrates, and essential micronutrients such as iron, zinc, and vitamin B complex. The protein of lentils is stored in the cotyledons with its amino acid profile relatively higher in comparison to other legume species. Depending on the cultivar, the anti-nutritional tannin content in lentils is relatively higher in comparison to other legume species, but a variant of zero-tannin lentils has been developed (Mirali et al., 2016).

2.1.4. Faba bean

The genus *Vicia* has many different species with faba bean or fava bean as one of the examples. Faba bean is highly consumed in Africa or the Middle East for both human consumption and animal feed, starting around 10,009 BCE in northwest Syria (Tanno and Willcox, 2006). Faba bean prefers cooler temperatures and can be grown in regions with a short growing season, thus is planted and harvested during the spring, fall, or winter season.

In general, faba bean has a molar ratio of phytate and zinc of more than 15, meaning it has a low bioavailability of zinc, although a cultivar of high zinc and iron bioavailability has been developed (Mayer Labba et al., 2021). Faba bean also contains anti-nutritional factors such as β-glucoside, vicine, and convicine that can cause favism, a hemolytic disorder, in an individual with hereditary glucose-6-phosphate dehydrogenase enzyme deficiency (Isbir et al., 2013).

2.2. Pulse nutritional component

The primary cell wall of pulses is composed of cellulose, hemicellulose, and pectin. The starch content of pulses is found in the cotyledon and is embedded in the protein matrix. This structural arrangement could restrict the swelling of starches due to the steric hindrance as well as the water availability. The thickness of the cotyledon cell walls may also prevent complete starch granule swelling (Singh et al., 2014).

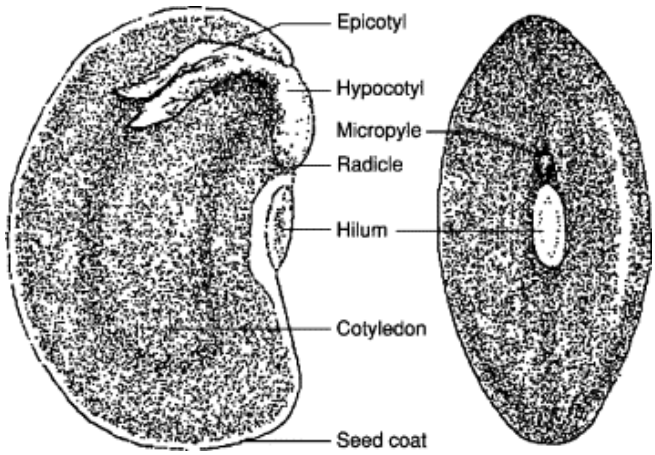


Figure 2-1 Anatomy of pulse. Republished with permission of ELSEVIER from [Encyclopedia of Food Sciences and Nutrition, Legumes – Legumes in the Diet (Uebersax and Occena, 2003)]; permission conveyed through copyright Clearance Center, Inc.

Pulses have a recognized highly valuable nutritional content of starch, protein, fiber, and others such as vitamins and minerals. The total ash content of pulses was found to be higher at 2.8-3.5% compared to wheat at 1% (Millar et al., 2019), indicating a high mineral content that can be beneficial to diet. However, the high content of ash can result in poor baking properties due to interference with protein and starch functionality. The fat content of pulses was found to be less than 2%, reducing the risk of oxidation of unsaturated fatty acid, and improving the flavor as well as the shelf-life of pulse-based food products (Millar et al., 2019).

2.2.1. Pulse starch

As a major component in pulses, around 22-45% in weight (Ratnayake et al., 2002), starch is accumulated in the seeds with an oval to irregular granule shape and 15-28 μm in size (Ren et al., 2021). Pulse starch contains a large proportion of resistant starch (RS) especially slowly digestible starch (SDS), therefore pulse starch is low in glycemic index and is beneficial for gut health (Fernando et al., 2010).

Raffinose family oligosaccharides (RFO) such as raffinose, stachyose, and verbascose are soluble carbohydrates that are abundant in the legume family as well as tubers. RFOs act as carbon storage and are accumulated in the endosperm, embryo, and seed coat of the plant seed. RFOs are not digested by humans due to the lack of α-galactoside enzyme, thus act as prebiotic where RFOs are utilized by the gut microbial bacteria during their fermentation, acting as prebiotics for the microbial gut population and providing beneficial effects of short-chain fatty acids such as immune

system improvement and decreasing the risk of obesity (Marinangeli and Jones, 2012) or colon cancer (Campos-Vega et al., 2010, 2009). This fermentation resulted in hydrogen, methane, and carbon dioxide, causing flatulence and may lead to abdominal discomfort (Dhaubhadel et al., 2022).

The total carbohydrate content for pulse flour ranges from 54.7% to 63.9% with a high portion of total dietary fiber (TDF) content that is insoluble. TDF consists of insoluble dietary fiber (IDF) and soluble dietary fiber (SDF). The difference in water solubility determines the digestibility and glucose absorption along the intestinal system. Dietary fibers that are not digested are passed to the large intestine, resulting in improved bowel function and prevention of gastrointestinal disorders. It was also studied that the consumption of dietary fiber can aid in the prevention of colorectal cancers, reduce inflammation, and regulate hormones (Millar et al., 2019; Ren et al., 2021).

Pulse starch is currently being utilized as a gluten-free substitute, especially for baked goods with the additional benefit of being high in protein and dietary fiber. Recent developments including bread from faba bean and pea (Hoehnel et al., 2019), and pasta from faba bean (Duta et al., 2019). And in the past, cowpeas, kidney beans, field peas, amaranth, and chickpea have been used to make muffins and crackers with comparable batter viscoelasticity of cereal-based products (Han et al., 2010; Shevkani et al., 2015; Shevkani and Singh, 2014). Another application of pulses in gluten-free products includes deep-fried foods and extruded snacks (Lazou and Krokida, 2010), bread (Aguilar et al., 2015), and pasta (Flores-Silva et al., 2014).

2.2.2. Pulse protein

Protein contributes 20-30% of pulse weight that is stored in the seed's cotyledon. Based on the solubility, there are a few types of protein including globulin (soluble in salt), albumin (soluble in water), prolamin (soluble in alcohol), and glutelin (soluble in dilute acid or base). Globulin and albumin are the two most abundant proteins, with an average content of 70-80% globulin and 10-20% albumin in pulses. Globulin is a major storage protein found in chickpeas, kidney beans, cowpea, mung bean, and red bean. Legumin is an example of globulin that has sulfur-containing amino acids such as methionine and cysteine. Albumin is primarily a metabolic protein that could be enzymatic and non-enzymatic with high content in cysteine and methionine compared to globulin. Few anti-nutritional proteins such as trypsin inhibitors, chymotrypsin inhibitors,

hemagglutinin, lectin, and amylase inhibitor belong to this group of proteins (Boye et al., 2010; Shevkani et al., 2019).

Protein in a starch structure can be found wrapped around the surface of a starch granule or embedded in the starch granule. The presence of this protein lowers the swelling and pasting ability of starch due to a lower hydration rate, resulting in lower digestibility. In addition, protein can also form a network that can restrict the expansion of starch and form a high-density structure that lowers the degree of hydrolysis (Lu et al., 2022). In terms of secondary structure, pulse protein is mainly comprised of β-sheets, β-strands, and β-turns, therefore is relatively more stable in higher temperatures (Shevkani et al., 2015; Shevkani and Singh, 2014). It is also been studied that β-structures have lower digestibility due to the lower accessibility of enzymes (Carbonaro et al., 2012).

Application of pulse protein includes fortification of cereal-based food with higher protein quality (based on amino acid composition), improving texture by increasing water absorption, and increasing viscoelasticity of gluten-free products (Giménez et al., 2013; Petitot et al., 2010). Pulse protein can also be found in non-dairy milk and alternative meat products. Interaction between protein and starch content enhances the stability of emulsion-based food such as dressings (Ma et al., 2016). And recently, a combination of pulse starch and protein is utilized to produce edible or biodegradable film as well as encapsulation material (Salmoral et al., 2000).

2.2.3. Antinutritional factor

Pulses are known to have limited content of iron, zinc, selenium, and sulfur-containing amino acids such as cysteine and methionine. The low content of some minerals is due to the presence of anti-nutrients which restricts the bioavailability and absorption of iron, zinc, calcium, and magnesium (Mayer Labba et al., 2021). A few examples of anti-nutrients include lectins, phytic acid, saponin, protease inhibitors, amylase inhibitors, and tannin. Lectins and phytate or phytic acid restrict the bioavailability of iron and zinc (Reinhold et al., 1973). Phytic acid can also chelate minerals, protein, and starch, reducing their bioavailability, while tannins can chelate iron, zinc, and copper (South and Miller, 1998).

Absorption of water by legume seeds during a process called germination has been found to reduce the antinutritional factor and increase the amino acid content. Germinated faba bean has been found to cause a significant increase in phytase activity by up to 147% and a reduction of

phytate content by up to 81% (Luo et al., 2012) and have an increase in protein solubility (Rahma, 1988). An increase in the emulsification capacity was also observed in germinated faba beans, possibly due to partial hydrolysis of the protein content by the protease enzyme that is activated during the germination process (Rahma, 1988). In terms of consumption, fortification of pulses with other grain products such as cereal can be an easy way to achieve all the essential amino acids as well as the protein fraction prolamins and glutelins (Bouchard et al., 2022).

2.3. Starch

Native starch granules are water-insoluble and non-structural carbohydrates comprised of two glucose polymers known as amylose (linear) and amylopectin (branched). In plants, starch is accumulated in the non-photosynthetic tissues such as seeds, stems, roots, or tubers as the major energy storage (Pfister and Zeeman, 2016). In pulses, starch is found in the cotyledon with minor components including cell wall fragments, protein, enzyme, amino acid, nucleic acid, and lipid. The size and shape of starch granules differ based on the plant species, with size varying from less than 1 to more than 100 μm (Jane et al., 1994; Lindeboom et al., 2004).

Due to its unique molecular arrangement, native starch remains as a semi-crystalline entity i.e., composed of a mixture of highly ordered crystalline regions and loosely packed amorphous regions. The double helical formation between amylopectin short chains and their packing is responsible for the formation of crystalline regions. Due to the tight packing of double helical conformation, crystalline regions resist hydrolysis/amylolysis. Based on the x-ray diffraction patterns, starch crystals are categorized into A-, B-, and C-types. A-type of starch can be found in cereals. B-type starch is mainly found in tubers. The mixture of both types is called C-type starch found in legumes and rhizomes. The rheological properties of starch differ based on the botanical origin (Guo et al., 2017; S. Wang et al., 2007).

2.3.1. Amylose and amylopectin

Amylose and amylopectin are the different polymers of starch that are different in terms of structure and biosynthesis. In general, plants contain around 20-30% of amylose and 70-80% of amylopectin (Ren et al., 2021). Amylose is a linear polymer of glucose that is linked mainly with α-1,4-glycosidic linkage that has a molecular weight between 10^5 and 10^6 g/mol with a degree of polymerization between 1,000-10,000 glucose units (K. Wang et al., 2014). Amylose has a helical

structure and is not soluble in cold water and is not stable in aqueous solution as it will form precipitate if left. The structure of amylose is a tight coil due to hydrogen bonding, making a semi-crystalline structure. With added water, the intramolecular hydrogen bond is replaced by the intermolecular hydrogen bond, opening up the structure and making the amylose more flexible. Amylose is used as a thickener, water binder, emulsion stabilizer, gelling agent, as well as biopolymer due to its film-forming properties (Bertoft, 2017).

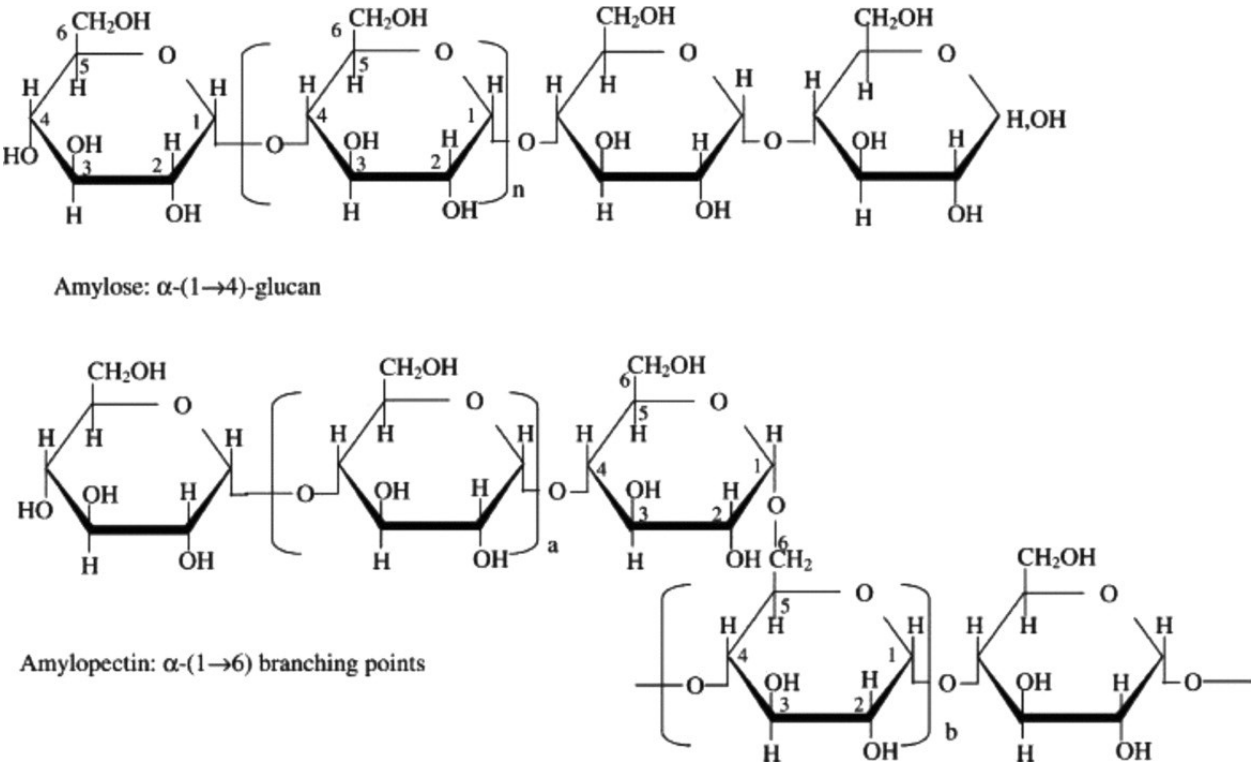


Figure 2-2 Structure of amylose and amylopectin. Republished with permission of ELSEVIER from [Journal of Cereal Science, Starch – composition, fine structure and architecture, Tester et al., 2004]; permission conveyed through copyright Clearance Center, Inc.

Amylopectin is the branched polymer of glucose with both α-1,4 and α-1,6 branch points. The branching pattern of amylopectin is random with an average molecular weight of 10^7-10^8 g/mol and a higher degree of polymerization compared to amylose (Chen and Bergman, 2007). Plants in general have 2-3 times the amount of amylopectin compared to amylose. Amylopectin structure consists of three chains; A chain being the outermost chain and is linked with α-1,6-glycosidic linkage to the B chain, which is the immediate branch point of the C chain as the backbone of the amylopectin structure.

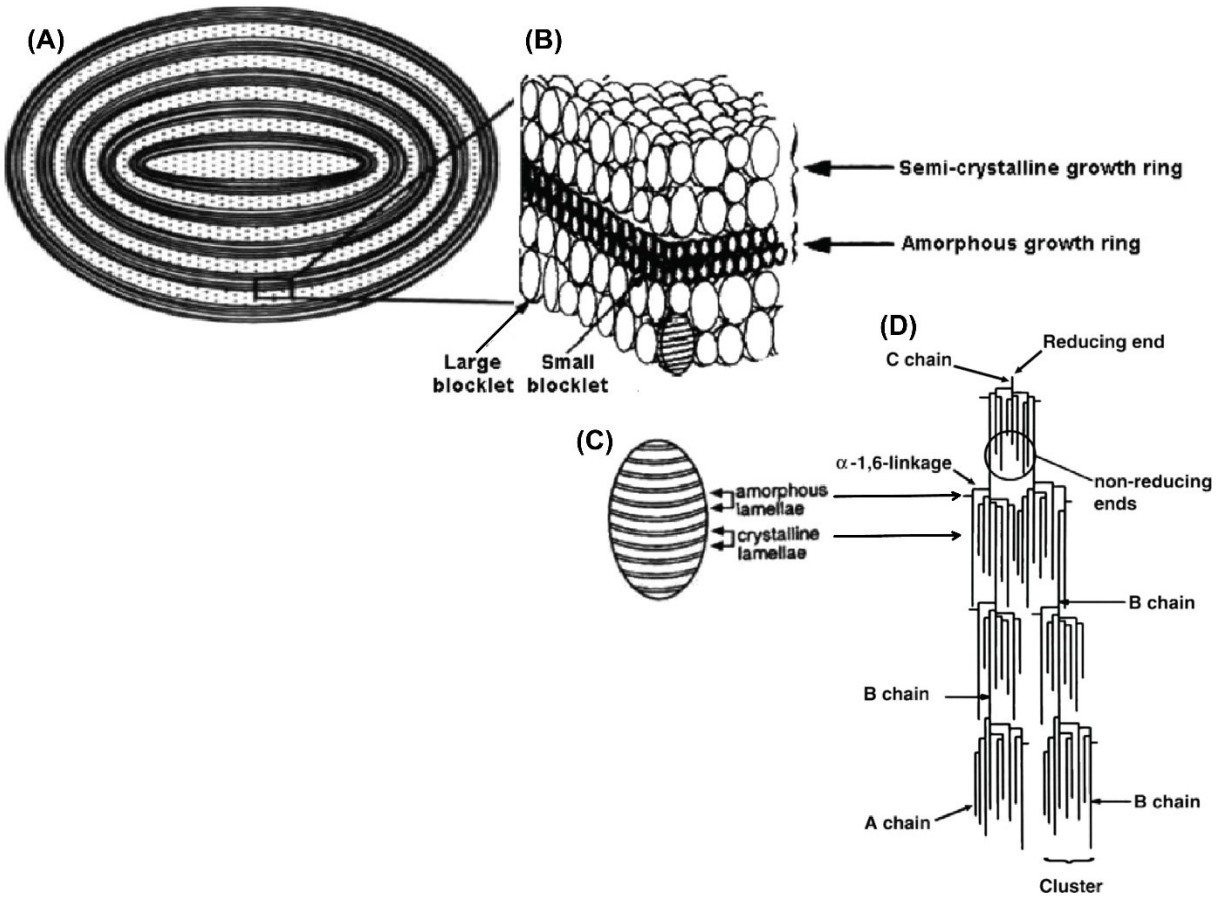


Figure 2-3 Schematic representation of a starch granule: (A) cross-section of a starch granule; (B) stacked starch blocklets; (C) starch blocklet with crystalline and amorphous lamellae; (D) aligned double helices with branched amylopectin. Republished with permission of Taylor & Francis from [Critical Reviews in Food Science & Nutrition, Application of Ultra High Pressure (UHP) in Starch Chemistry, H. S. Kim et al., 2011] with modification; permission conveyed through copyright Clearance Center, Inc.

The amylose chain is found to be intertwined with the amylopectin branches, creating a unique structure of starch with both amorphous and crystalline regions. The overlapping areas are the crystalline region while the amorphous region can be found in between the crystalline regions (Bertoft, 2017). The combination of amorphous and crystalline regions forms a blocklet structure, which collectively contributes to the alternating ring structure that can be observed in a starch granule (Gallant et al., 1997). These alternating structures create growth rings that can be prominently observed near the peripheral region of the starch granules. The width of the rings is influenced by the amylose content, as it primarily made up the amorphous region.

2.3.2. Starch hydrolysis

Due to the structure arrangement, different parts of amylopectin have different susceptibilities to enzymatic hydrolysis. The external chains have non-reducing ends and must be removed before the internal chains can be hydrolyzed. To completely hydrolyze amylopectin, de-branching enzymes such as isoamylase and pullulanase are often used to address the α-1,6-glycosidic linkage. The hydrolysis process also depends on the starch properties. The initial stages of hydrolysis are usually faster due to the presence of amorphous materials, followed by slower hydrolysis of the crystalline region. This process is also affected by the presence of pores on the surface of the granules, which gives the enzyme higher accessibility to the starch. In rhizomes, the C-type starch gradually transitions to A-type starch with the extent of hydrolysis, indicating preferential hydrolysis of B-type (S. Wang et al., 2007).

2.3.3. Starch gelatinization and retrogradation

Based on studies, amylopectin is more susceptible to hydrolysis, while amylose is found to resist hydrolysis better than amylopectin. This could be due to the compact structure of linear amylose, as well as the formation of an amylose-lipid complex that forms resistant starch type V (Seneviratne and Biliaderis, 1991). This process is influenced by the amylose and amylopectin ratio, molecular weight distribution, degree of branching, length of branch, as well as molecular conformation.

The hydrolysis process breaks down complex starch molecules and decouples both the crystallite and amorphous region, increasing the energy required to achieve the transition temperature. The study of these gelatinization parameters can be done with Differential Scanning Calorimeter (DSC) to measure the transition temperature of the melting of the starch crystals. Onset temperature (T_o) is the temperature at which the weaker crystals melt, whereas final or conclusion temperature (T_f or T_c) is the temperature at which the stronger crystals melt (Spies and Hoseney, 1982).

Granular starch requires energy to be converted to sugar. This process often involved high temperatures and water to aid the conversion process (Ellis et al., 1998). Starch in the presence of heat at a critical temperature range and excess water will undergo gelatinization. Gelatinization is the melting of the semi-crystalline region of amylopectin. During this process, water diffuses into

granules and is taken up by the amorphous region and causing swelling of structure, resulting in loss of crystalline order through uncoiling and dissociation of double helices arrangement. The amylose content was then released in a process called amylose leaching. If the heating stops, starch will undergo retrogradation which is the re-arrangement of starch structure that increases the degree of crystallinity and gel firmness, although not to the initial level prior to heating. The water content will also be released in a process called syneresis (Ratnayake et al., 2009).

2.3.4. Resistant starch

Types of resistant starch (RS) include starch that resists digestion, requiring more than 16 hours to be digested in the gut, or starch that is slowly digested, requiring more than 20 minutes to be digested (Englyst et al., 1982; Englyst and Hudson, 1996). In animals, resistant starch has a similar role to dietary fiber since it is not digested by the animal digestion system. Resistant starch can be used by gut bacteria as a substrate for fermentation to produce short-chain fatty acids such as acetic acid, propionic acid, and butyric acid. The role of resistant starch as a prebiotic gained lots of interest in recent years due to its health benefits such as reducing dietary caloric values, lowering the glycemic index of food, as well as decreasing the blood cholesterol level and risk of colon cancer by improving the level of *Firmicutes* as well as their enzymatic and metabolic activities in the gut (Maier et al., 2017).

Based on the characteristics, resistant starch can be classified into five major types; physically entrapped starch granules (RS1), high amylopectin crystallinity starch (RS2), gelatinized and retrograded amylose starch (RS3), chemically modified starch (RS4), and amylose-lipid complex that is formed during heating (RS5). The resistance of RS1 can be alleviated through the physical separation of starch granules from the surrounding protein matrix and other cell materials, while for RS2, a similar result can be achieved through cooking. Due to this processing of starch, RS1 and RS2 can be regarded as slowly digested, rather than truly resisting starch digestion like RS3, RS4, and RS5.

Resistance of starch is also influenced by other factors such as the presence of sugar or lipid, length of amylose chain, amylose and amylopectin ratio, and preparation method of the starch including drying time and temperature. Previous studies have shown that the digestibility of pulse is a third of the digestibility of maize by porcine pancreatic α-amylase in six hours. Many factors are involved in this low digestion level, including granule size, amylose and amylopectin

ratio, degree of crystallinity, crystalline polymorphic type, amylose-lipid complex, starch distribution in relation to dietary fiber, antinutrients, presence of α-amylase inhibitor, and drying as well as storage conditions (Hoover and Zhou, 2003). It is also studied that smaller granules are more resistant to hydrolysis compared to large granules due to their higher amylose content (Sanchez de la Concha et al., 2018).

2.4. Milling and fractionation

Milling is a process during grain processing in which the grain particles are reduced in size. Examples of milling technologies include hammer milling, pin milling, roller, and disc milling (Acar et al., 2020). While fractionation is the separation of the components such as starch, fiber, and protein. The separation process can be done with dry or wet methods to achieve a different level of yield and starch purity at the end of the process. Wet fractionation could yield starch with purity up to 92% while dry fractionation can only reach 65-80% with 8-20% impurities of protein (Li et al., 2019; Naguleswaran, 2013).

A recent development in the dry fractionation technique is a triboelectric separation that involves fluidizing and charging protein and starch particles to be separated under an electric field (Thomas et al., 2023). The milling process physically releases the starch granules from entrapment and can be separated based on size or density, with air classification, one of the most commonly used dry fractionation/separation methods (Li et al., 2019; Ren et al., 2021). Compared to the alternative wet fractionation, dry fractionation is considered more sustainable as it requires less water and energy. The efficiency of the dry fractionation process depends on the grain characteristic such as grain hardness and adhesion between the protein and starch. Pretreating the grains to lower the moisture content, soaking, freezing, and defatting are a few examples that can be done to improve the separation efficiency (Pelgrom et al., 2015).

During wet fractionation, the ground grains are soaked in solvents to separate fractions based on solubility in water, salt, acid, or base (Li et al., 2019). The soaking of the grains to solubilize some fractions are done to achieve higher starch purity at an extraction pH of 8.5-10. The addition of sulfur in the form of sulfur dioxide or sodium metabisulfite is to weaken the protein disulfide bonds, resulting in higher purification levels (Chávez-Murillo et al., 2018). Although producing high-purity starch, the process involves solvent, heating, and separation steps that make the processing more complicated and costly (Espinosa-Ramírez and Serna-Saldívar, 2019).

2.4.1. Air Current-Assisted Particle Separation (ACAPS)

ACAPS is a dry fractionation method developed by Dr. Thava Vasanthan from the University of Alberta. This process utilizes a vacuum and a high-pressure air pulsing to move the grains along the sieving apparatus (Vasanthan, 2017). Different than air classification, the ACAPS process separates grain fractions based on particle size, instead of particle density, where the milled grains are fluidized and the small particles can pass through a series of sieves and collected at the bottom of the apparatus. Similar to air classification, the ACAPS process requires fewer processing steps and less energy and or materials in comparison to wet fractionation (Li et al., 2019).

The ACAPS apparatus contains a chamber separated into two by a sieve. Vacuum suction is applied to pull milled grains from the inlet port into the chamber, and the finely ground particles are fluidized by vacuum and high pressure. The fine particles pass through the sieve and are collected as a starch-rich fraction, in contrast to the coarser particles that contain a higher number of fibers (Vasanthan, 2017). ACAPS technology was previously used to separate the β-glucan of barley that is located in the coarse fraction. The fine fraction that passed through the sieve was then collected and converted into ethanol through microbial fermentation (Lu et al., 2020).

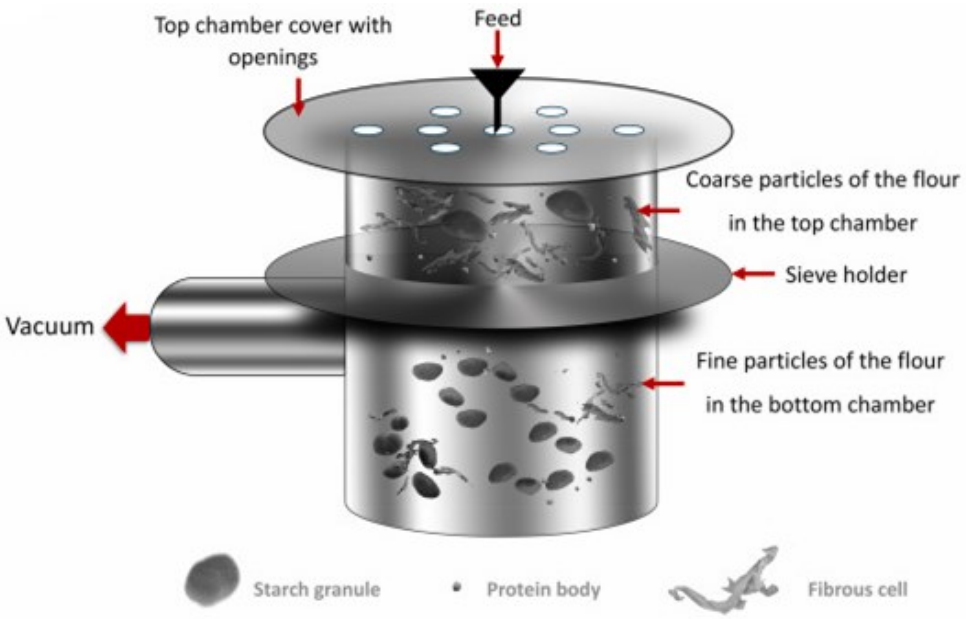


Figure 2-4 Simplified overview of air-currents assisted particle separation (ACAPS) of starch macro components. Republished with permission of ELSEVIER from [Journal of Food Engineering, Potential of air-currents assisted particle separation (ACAPS) technology for hybrid fractionation of clean-label faba bean (Vicia faba L.), Jeganathan et al., 2023]; permission conveyed through copyright Clearance Center, Inc.

Starch fractionation through the ACAPS process was studied by Jeganathan et al., (2023) and produced fine fraction with the size of less than 75 μm containing 40-61% of starch and 25-37% of protein. Proteins are present in the coarse fraction due to their strong attachment to the fibrous materials. However, the charge build-up during the milling process and the ACAPS process may also contribute to the difference in the charge density of the particles, leading to the attachment of protein bodies to non-fibrous materials (Jeganathan et al., 2023; Landauer and Foerst, 2018).

2.5. Grain processing

Grains and other agricultural products can be converted into biofuel through several processes involving pre-treatment, saccharification or hydrolysis, and fermentation. The extent of each step will depend on the structural complexity of the feedstock, as well as the degree of conversion required.

2.5.1. Pre-treatment

A pre-treatment process as the initial step is done to prepare the feedstock for conversion, by opening up the structure so it will be more accessible for the yeasts in the fermentation step. There are a few types of pre-treatments, including physical, chemical, biological, or a combination of the processes. In selecting a pre-treatment, the feedstock and yield balance during the process must be taken into consideration, as well as the formation of inhibitory compounds, flexibility of the feedstock used, and materials and energy requirement for upscaling the process (Duque et al., 2021).

Physical treatment is mechanically reducing the size of the feedstock which involves processes such as milling, grinding, and chipping. The goal of this process is to remove the outer layer of the grain (Zeng et al., 2007) as well as to lower the crystallinity of starch feedstock (Fan et al., 1980). The degree of hydrolysis will benefit greatly from the size reduction due to milling. Other than size reduction, irradiation of lignocellulosic biomass by gamma rays, electron beams, and microwaves could also improve the enzymatic hydrolysis of complex feedstock due to the dissociation of the glycosidic bonds (Bak, 2014).

Chemical treatment can use acid, alkali, or oxidizing agents to lower crystallinity as well as remove lignin content from lignocellulosic feedstock. Acids such as hydrochloric acid or sulfuric acid can hydrolyze the hemicellulose in the lignocellulosic materials to expose the cellulose. It must be kept in mind, however, that treatment of hexose and pentose sugar could produce toxic compounds such as furan (2-furaldehyde), 5-hydroxymethylfurfural, and phenolic compounds that could inhibit cell growth and reduce ethanol productivity (Świątek et al., 2020). Alkali pre-treatment involves sodium hydroxide, potassium hydroxide, or ammonium hydroxide, removing lignin in the plant tissues at a lower temperature and pressure (Zheng et al., 2018). Although the degree of lignin solubilization is high, the high cost of alkali is one of the major drawbacks (Talebnia et al., 2010).

Physiochemical treatment is a combination of physical and chemical methods including steam explosion (Pielhop et al., 2016), wet oxidation (Mcginnis et al., 1983), microwave (Jablonowski et al., 2022), and ultrasound to partially degrade hemicellulose content of the feedstock. Supercritical fluids that are compressed gaseous substances into liquid, is a non-toxic extraction solvent that also belongs to the physiochemical treatment group. This process disrupts the cellulose and hemicellulose content of the feedstock and improves the accessibility for the enzyme (Khanyile et al., 2022), while steam explosion, microwave, and ultrasonic treatment utilize heat and pressure differences to achieve the same result.

Lastly, biological treatment traditionally relies on fungi to rot the feedstock and lowers the hemicellulose as well as lignin content (Karunanandaa et al., 1995). The rate of natural fungi degradation is low, thus, these days the enzymes responsible to do the degradation such as α-amylase are extracted and produced outside the fungi and applied to feedstock independently, creating an easy-to-handle hydrolysis system (Balakrishnan et al., 2021). Compared to physical or chemical treatment, enzymatic hydrolysis is highly specific, requires lower energy input, as well as gives a high yield of glucose conversion.

2.5.2. Saccharification

Saccharification or hydrolysis is the step where feedstocks containing complex polysaccharides are broken down into fermentable sugars. This process often overlaps with the pre-treatment process. Acid hydrolysis is one of the oldest and most commonly used methods to break down complex carbohydrates into short-chain sugars. The combination of acid concentration

and reaction temperature is the key factor of successful conversion. While in enzymatic hydrolysis, specific enzymes are introduced to the feedstock with optimized reaction conditions including temperature, time, pH, and concentration. Unlike acid hydrolysis, the presence of enzymes does not impact the structural integrity of reactors. This process also resulted in less formation of inhibitory compounds such as furans as well as has a lower impact on the environment since no harsh chemicals are used. However, the use of enzymes contributes an additional cost to the process (Erdei et al., 2010).

2.5.3. Fermentation

Fermentation is the process of metabolism in which complex carbohydrate is converted into short-chain carbohydrate, carried out by yeast or bacteria. The short-chain carbohydrate is then catabolized into pyruvate, and the pyruvate produced during the glycolysis process is broken down into acetaldehyde and carbon dioxide, where the former is reduced to ethanol.

The fermentation process is influenced by temperature, concentration, pH, fermentation time, agitation rate, inoculum size, and many more parameters (Lin et al., 2012). Temperature affects the growth of yeast as well as enzyme activity, while pH influences the permeability of nutrients as well as yeast survival. Fermentation time depends on the sugar utilization and the yeast growth curve. The agitation rate is related to the mass transfer rate, where rigorous agitation improves the sugar concentration and lowers the ethanol inhibition, however, excessive stirring may cause limitation of cell metabolic activities. And lastly, a smaller inoculum size means it will take longer to produce the targeted concentration of the product. Feedstock concentration also plays a role in the fermentation process, where high sugar concentration can result in slower fermentation, due to the osmotic pressure (Basso et al., 2008).

The current method of bioethanol production from sugar or starch-based materials involves pre-treatment, saccharification, and fermentation. *Saccharomyces cerevisiae* is the most commonly used microorganism to ferment sugar into ethanol. It has an optimum condition of 30 °C, pH 4.0-5.0, as well as an agitation rate of 100-200 rpm, and does not tolerate high concentrations of sugar and salt in the medium (Basso et al., 2008).

2.6. Enzyme

An enzyme is a protein and biological catalyst that works by lowering the activation energy of a reaction converting substrate to products. Enzymes generally have a high specificity that can only catalyze the conversion of one type or a group substrate. The enzyme specificity was first proposed by Emil Fischer in 1894 who hypothesized the lock and key mechanism, where the substrate and the enzyme should be the correct size and shape to fit together. However, it has been further studied that the induced-fit model was a better fit due to the flexibility of enzyme molecules that can change in shape to accommodate the substrate (Robinson, 2015).

In addition to substrate, a large number of enzymes also require a non-protein component that could be an organic or inorganic molecule, or cofactor. The organic cofactor is called a coenzyme that could be permanently attached to the enzyme and is called the prosthetic group of the enzyme. An inorganic cofactor is typically a metal ion, such as iron, manganese, cobalt, copper, or zinc (Robinson, 2015).

Based on the mechanism of action, there are seven classes of enzymes; oxidoreductase (EC 1), transferase (EC 2), hydrolase (EC 3), lyase (EC 4), isomerase (EC 5), ligase (EC 6), and translocase (EC 7). Hydrolyzing enzyme belongs to the third class, where the catalytic site of the enzymes forms a complex with the substrate through hydrogen bonds, hydrophobic bonds, or Van der Waals and then cleaves the interaction between the molecules.

2.6.1. Starch hydrolyzing enzyme

There are four main groups of starch hydrolyzing enzymes; endoamylase, exoamylase, debranching enzyme, and transferase. Endoamylase works by hydrolyzing bonds in a random manner from its reducing end, producing both linear and branched oligosaccharides. Exoamylase produces small and well-defined oligosaccharides by its mode of attack from the non-reducing end. The debranching enzyme attacks the α-1,6-glycosidic linkages, producing chains of saccharides without branches, hence the name. This enzyme is capable of hydrolyzing such linkage in pullulan, thus often called pullulanase. Transferase catalyzes the transfer of a functional group from the donor to the acceptor (Bertoldo and Antranikian, 2001).

α-amylase enzyme (EC 3.2.1.1) or also known as α-1,4-glucan-4-glucanohydrolyase is an endo-acting enzyme that hydrolyzes the 1,4-glycosidic linkages randomly and produces linear and branched oligosaccharide. These branched oligosaccharides are often called limit dextrin due to

their 1,6-glycosidic linkage. A-amylase is a calcium-metalloenzyme, requiring metal cation as the cofactor and is found in Bacteria, Archaea, and Eukarya. Production of acid-stable α-amylase from *Aspergillus kawachii* was observed during the production of shochu, a Japanese spirit (Kajiwara et al., 1997), while bacteria such as *Bacillus amyloliquefaciens* is known to produce a thermostable α-amylase (Shareghi et al., 2007).

Glucoamylase enzyme (EC 3.2.1.3) or also known as 1,4-α-D glucan glucohydrolase, glucan 1,4-α-glucosidase, amyloglucosidase, or ϒ-amylase is an exo-acting enzyme that hydrolyzes the terminal α-1,4 glycosidic linkage from the reducing end to produce β-D-glucose. This enzyme is also able to hydrolyze a 1,6-glycosidic linkage if the next bond in the sequence is a 1,4-glycosidic linkage. Glucoamylase can also be found in Bacteria, Archaea, and Eukarya, but eukaryotic hosts such as molds and yeasts are the preferred method for industrial production due to glycosylation (Kumar and Satyanarayana, 2009).

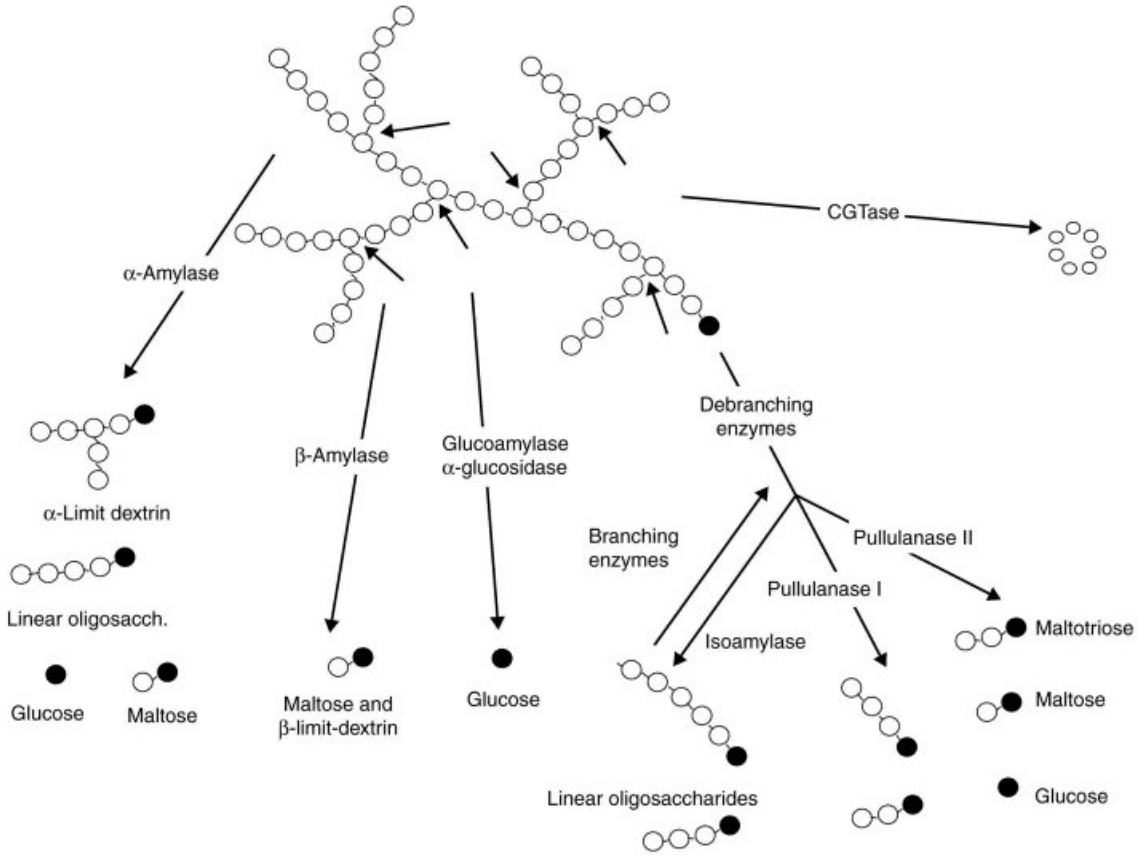


Figure 2-5 Starch hydrolysis by amylolytic and pullulytic enzymes. Each circle represents sugar monomer and black circles represent reducing sugar. Republished with permission of ELSEVIER from [Current Opinion in Chemical Biology, Starch-hydrolyzing enzymes from thermophilic archaea and bacteria, C. Bertoldo and G. Antranikian, 2001]; permission conveyed through copyright Clearance Center, Inc.

Processing of aqueous starch slurry with α-amylase and glucoamylase enzymes, under appropriate elevated temperatures, is called liquefaction and saccharification, respectively. During this treatment, the viscosity of the starch mixture is reduced due to the conversion of long-chain polysaccharides to oligosaccharides and finally converted to monosaccharides. There are a few factors affecting starch hydrolysis including starch processing, type of starch, and type of amylase used. Cooked starch has undergone the gelatinization process that make the starch more susceptible to hydrolysis, while the type of starch determined the granule size, shape, and structure, the ratio of amylose and amylopectin content, as well as the other minor component such as protein and lipid (Naguleswaran, 2013).

2.6.2. Raw starch hydrolyzing enzyme

Raw starch hydrolyzing enzymes are enzymes that are able to hydrolyze starch grains below the gelatinization temperature of starch. This system has been studied extensively in both the Bressler lab and the Vasanthan lab to produce ethanol from a starch-based material such as wheat, barley, corn, and triticale (Gibreel et al., 2011, 2009; Li et al., 2014; Lu et al., 2020; Naguleswaran et al., 2014, 2012). In addition, cassava, rice, corn, potato, sweet potato, and buckwheat also have been used to produce bioethanol (Krajang et al., 2021; P. Wang et al., 2007; Xu et al., 2016). Based on Robertson et al., (2006), the implementation of the raw starch hydrolysis process can reduce the energy required in the ethanol production process to up to 10-20%, in comparison to the traditional dry-grind method involving cooking the starch at a temperature above 90 °C for 1-2 hours. Examples of commercialized raw starch hydrolyzing enzymes are STARGEN™ 001 and STARGEN™ 002, which both contain α-amylase from *Aspergillus kawachii* expressed in *Trichoderma reesei* and glucoamylase enzyme from *Trichoderma reesei*. STARGEN™ 001 is optimized for the hydrolysis of corn and STARGEN™ 002 is optimized for the hydrolysis of barley, with fermentation efficiency of 90% and above (Lu et al., 2020; P. Wang et al., 2007).

2.6.3. Enzymatic hydrolysis with urea

Hydrogen bonding played a role in the starch gelation, by maintaining the tight helical conformation of amylose. Replacement of the intramolecular hydrogen bond with the

intermolecular hydrogen bond by the introduction of water molecules changed the rheological properties of starch, resulting in gel formation (McGrane et al., 2004). Urea is an organic compound containing nitrogen that is often used as a solid fertilizer. Urea also acts as a protein denaturant that acts by disrupting the non-covalent bond. In starch gelation, the introduction of urea causes the gel strength to be reduced.

Studies have shown, however, that urea did not disrupt α-amylase. The addition of urea to the starch mixture lowers the gelatinization temperature since the structure is disordered, but did not affect the degree of hydrolysis. At sub-gelatinization temperature, the starch is partially disrupted due to the swelling of the amorphous region and the expansion of the surface pinholes. The disrupted structure resulted in an increase in enzyme activity and a higher degree of hydrolysis (Li et al., 2012).

2.7. Bioethanol

Ethanol is an organic compound with a chemical formula of C_2H_5OH. Its physical properties include colorless volatile yet flammable liquid. Naturally, ethanol can be produced through the fermentation process of yeasts from sugars. Historically, ethanol was used as a form of anesthetic, but the usage has evolved to a broad spectrum including antiseptic, solvent, and fuel. The use of ethanol as a fuel started in the early 1800s, when ethanol was used to power an internal combustion engine. The popularity of ethanol as a fuel subsided but re-surfaced in the early 1900s. Its popularity was brought back in Brazil, where ethanol was produced through fermentation using sugarcane as the feedstock. The trend was followed by the United States and European countries and in 2021, the total annual fuel ethanol production surpassed the ethanol consumption (EIA, 2022).

Currently, ethanol is the most commonly used renewable material as fuel and is predicted to continue doing so through the implementation of sustainable energy policies especially in developed countries such as the United States, Canada, and European countries. Bioethanol at a volume fraction of 5% and 10% can be used in regular vehicles without engine modification, while 85% ethanol can be used in flexible fuel vehicles (FFV) (EPA, 2023). Compared to gasoline, ethanol has a more efficient combustion due to a higher octane number, broader flammability limit, higher flame speed, and increased heat of vaporization. And due to its production, it is less toxic,

more degradable, and produces fewer air-borne pollutants compared to traditional fossil fuels (Hill et al., 2006).

2.7.1. Canadian bioethanol

In Canada, 3 billion liters of ethanol was consumed in 2018 and 1.7 billion liters were produced within the country, leaving a gap of 1.3 billion liters of ethanol to be imported. The use of biofuels including bioethanol can contribute greatly to the Clean Fuel Standard (CFS) initiative, which targeted a 26 million tonnes reduction of greenhouse gasses by 2030. Currently, the renewable fuel mandate has at least an annual average of 5% of ethanol content in gasoline across the provinces (Government of Canada, 2023). To be able to meet the 2030 goal, it is estimated that 3.5 billion liters of ethanol must be produced with a 10-15% blend of clean fuels (Advanced Biofuels Canada, 2019).

2.7.2. Bioethanol feedstock

Currently, bioethanol is produced through the fermentation of biomass that is rich in glucose such as sugar cane, sugar beet, sorghum, and fruits, or rich in starch such as corn and barley, to produce a first-generation ethanol. In 2020, more than half of the world's ethanol production was produced in the United States from mainly corn feedstock with a dry grind process (Lee, 2015), followed by Brazil which produced ethanol from sugarcane, and European Union which uses cereals as the conversion feedstock (Vasić et al., 2021). Although the conversion from sugary feedstock to ethanol is relatively simple and easy to maintain, this process introduces a problem of prioritization of crops as food or as a fuel material.

Based on this problem, second-generation ethanol from lignocellulosic biomass is an alternative due to the low production cost, high availability, and lack of competition with food and feed (Tomás-Pejó et al., 2011). In this process, wastes from agricultural crops such as bagasse (Jugwanth et al., 2020), corn stover (Berchem et al., 2017), cereal straw (Zheng et al., 2018), rice hulls (Madu and Agboola, 2018), wood pulp (Beyene et al., 2017), and municipal solid wastes (Verhe et al., 2022) are treated physically, chemically, or biologically to produce sugar that can be utilized by yeasts for ethanol fermentation. The downside of this pathway is the cellulose, lignin, and hemicellulose content that poses a higher structural heterogeneity and recalcitrance, making it harder to convert into ethanol. Physicochemical treatment such as steam explosion or acid

hydrolysis is often employed to reduce the degree of polymerization and crystallinity of the substrate to improve the enzymatic conversion of second-generation biomass.

Another path of fuel production is by utilizing algae and its photosynthetic ability to produce fatty acids as the fuel backbone (Tanadul et al., 2014). The coupling ability with wastewater treatment is the main feature of this process, but the production of greenhouse gasses as a product of algae respiration and operation scale is the main drawback (Papadopoulos et al., 2023). Lastly, engineered microorganisms to improve biomass production or hydrolysis and fermentation efficiency is the fourth generation of fuel (Verhe et al., 2022). Although convenient, the environmental and health-related risks of the process are not to be dismissed. In addition, the high capital cost of producing these advanced types of bioethanol is still the main hindrance to commercialization.

2.7.3. Simultaneous Saccharification and Fermentation (SSF)

Hydrolysis and fermentation process that is done separately in a different time and location is called the separate hydrolysis and fermentation (SHF). In this process, each step is done independently from the other to achieve the different optimal conditions for hydrolysis and fermentation, since hydrolysis often uses higher temperatures than the tolerance limit of yeasts. However, this poses problems such as inhibitory action from accumulated glucose and other sugar as well as the risk of microbial contamination due to the long incubation period during hydrolysis.

The term simultaneous saccharification and fermentation (SSF) was first coined in the late 70s (Gauss et al., 1976). In the SSF process, the hydrolysis and fermentation are done in a single bioreactor at the same time. The sugar produced from the conversion of feedstock is immediately used by the microorganism for ethanol fermentation. The advantage of this process is the overall low glucose concentration during the fermentation which reduces the osmotic pressure on the microorganisms as well as shorten the process since the hydrolysis and fermentation step are combined. The immediate sugar utilization also lowers the enzymatic inhibition as well as decreases the risk of contamination due to low sugar concentration during the fermentation process, thus improving the bioethanol yield. However, the compatibility of enzymes and microorganisms used in terms of optimum conditions should be factored in, as both processes occur simultaneously (Olofsson et al., 2008).

Another variation of the SSF process is pre-hydrolysis SSF, where the pre-hydrolysis was carried out at a higher temperature prior to the SSF process. This is done to ensure the feedstock is homogenized and hydrolyzed into sugars at a higher degree. This process also helps to decrease the time of sugar consumption thus, resulting in higher ethanol production in a shorter amount of time (Khan Tareen et al., 2021).

2.7.4. Very High Gravity (VHG) fermentation

Very high gravity (VHG) fermentation is a type of fermentation that uses high substrate concentration, at least 270 g/L of dissolved solids (Thomas et al., 1993). Since the initial developments in 1980s (Casey et al., 1984), this process has been studied to have a significant increase in ethanol production from wheat (Thomas et al., 1993), rice (Chu-Ky et al., 2016), sorghum (Thangprompan et al., 2013), cassava (Lomthong et al., 2021) and other starchy materials while reducing the energy consumption during the distillation process.

In VHG, the total fermentable sugar content in the feedstock is proportional to ethanol production. However, the high presence of dissolved solids can be a hindrance to the heat and mass transfer rate. The addition of viscosity-reducing enzymes such as cellulase, xylanase, and glucanase becomes an important step to be included. In addition, the supplementation of nitrogen, as well as other trace elements such as zinc, magnesium, and manganese may be necessary to avoid sluggish fermentation due to the low content of nitrogen in comparison to carbon from the feedstock (Puligundla et al., 2019).

2.7.5. Distillers' Dried Grain with Solubles (DDGS)

At the end of grain fermentation, protein-rich residue or stillage is left behind, and when dried, it becomes the distillers' dried grain with solubles (DDGS) as a major co-product of fermentation. The protein content in DDGS comes from the accumulation of yeast or other microorganism used in the fermentation process. Other than protein, DDGS may also contain fat, resistant starch, and minerals. DDGS has been used to fortify baked products to improve the fiber and protein content (Li et al., 2020; Pourafshar et al., 2018), but is mainly used as animal feed (Kim et al., 2021; Larson et al., 2019). The DDGS yield depends on the digestibility of the feedstock itself, for example, the fermentation residue of barley was 41% compared to the 30% yield of corn (Wu, 1986).

Corn DDGS is one of the most abundant types of DDGS in the United States due to the high volume of bioethanol production from corn (Rho et al., 2018), while barley DDGS is commonly found as a by-product of the brewery industry at a smaller scale compared to corn DDGS (Naibaho and Korzeniowska, 2021). The composition of DDGS will vary based on the feedstock used, but in general, the protein content ranged from 13-33%, 1.5-14% of fat, and 11-40% dietary fiber. DDGS can also be used as a feedstock for further ethanol production, however, conversion of the fiber content through pre-treatment strategies is crucial to obtain fermentable sugars (Iram et al., 2020).

2.8. Yeast

Yeast is a single-cell microorganism that belongs to the Eukaryotic domain. Baker's yeast is the name of the common yeast strain, *Saccharomyces cerevisiae*, used for baking purposes as a leavening agent. The same yeast species can produce alcohol through the fermentation of sugar, producing alcoholic beverages such as beer, wine, and whisky. Compared to bacteria, yeasts are less nutritionally demanding, its requirements are basic compounds such as sugar, amino acid, vitamin, mineral, and oxygen. The main reproduction of yeast is asexual, forming a vegetative growth in the process known as budding (Joseph and Bachhawat, 2014). Initially isolated in 1883 by Emil C. Hansen, the use of *Saccharomyces cerevisiae* in ethanol fermentation has several benefits including resistance to ethanol, minimum contamination risk due to the fermentation of sugar at a low pH value, genetic characteristics are well explored, and generally recognized as safe (GRAS) in the food industry (Ishizaki and Hasumi, 2014).

Morphologically, *S. cerevisiae* has an ellipsoid shape with a diameter of 5-10 μm and a high water activity of 0.65. The optimum physiological condition for yeast growth is 20-30 °C and pH 4.5-6.5. It is categorized as a facultative anaerobe since it requires oxygen as a growth factor for the biosynthesis of membrane fatty acid and sterol. Yeasts metabolize organic substrate as energy and common carbon source used are maltose, sucrose, and lactose. Yeasts cannot fix atmospheric nitrogen, therefore require readily assimilable nitrogen such as amino acid or ammonium salt (Joseph and Bachhawat, 2014).

Active dry yeasts are yeasts that are grown then dried under high temperatures then ground into small granules. The heating process does kill a portion of yeasts which then acts as a coating layer for the living cells. Other benefits of using active dry yeast include low cost of production,

ease of storage since it does not need to be refrigerated, and long shelf-life. Active dry yeast can be activated by dissolving the outermost layer in warm water. Due to the preparation and rehydration process, the colony-forming unit (CFU) count obtained from active dry yeast can vary up to 4×10^3-fold. Its viability depends on the storage factors including temperature, time, and nutrients provided during the activation process (Joseph and Bachhawat, 2014).

2.8.1. Crabtree effect

Yeasts are chemoorganotrophic, able to metabolize organic substrate for energy with common carbon sources such as maltose, sucrose, and lactose. Sugars taken up are converted to pyruvate through glycolysis in the presence of oxygen to produce a high yield of adenosine triphosphate (ATP) through respiration. This pathway can produce approximately 18 ATP per glucose. Yeasts can also produce 2 ATP per glucose molecule through fermentation in the absence of oxygen. In the initial phase of growth, yeast will tend to do respiration, using sugars in the presence of oxygen. However, when the glucose concentration is sufficiently high, some yeasts use fermentation to produce energy even in the presence of oxygen. This strategy is known as the Crabtree effect, where organisms that perform fermentation in the presence of oxygen are called Crabtree-positive (Crabtree, 1929; Pfeiffer and Morley, 2014).

2.8.2. Yeast medium

In laboratory settings, yeasts are grown in a cultivation medium that reflects the elemental composition and biosynthetic capacity of specific yeast strains. Depending on the goal, the media may contain all the biosynthetic precursors required for anabolic pathways, eliminating the need to produce its precursor. On the other hand, a chemically defined media can be supplemented with specific nutrients to satisfy the auxotrophic requirements. In ethanol industries, the agricultural feedstock as media can account for at least 30% of the total production cost and highly fluctuate depending on the crop production (Irwin, 2022). Meaning the use of agricultural wastes as a fermentation medium will be very beneficial to the overall process since it will decrease the initial cost immensely.

In putting together a fermentation medium, factors such as utilization, byproduct, limitation, and presence of inhibitors should be considered. The presence of multiple substrates can lead to an incomplete substrate utilization or even catabolite repression, due to the preference

of substrate utilization (Hahn-Hägerdal et al., 2005). In ethanol production, using glucose as the substrate could lead to the formation of glycerol in up to 4% of the feedstock from the surplus of NADH formation (Nissen et al., 2000). Studies have shown that the presence of glycerol improves wine taste and mouthfeel (Jones et al., 2008) however, in bioethanol production, it takes away carbons that could form ethanol (Guo et al., 2011).

When using waste materials, the nutrient content may be incomplete. The absence or limited presence of nutrients can lead to slow fermentation. While the presence of inhibitors that could be released during the feedstock hydrolysis such as furan can hinder yeast growth and decrease the ethanol conversion efficiency. During fermentation, acidification due to the active transfer of proton may also happen and inhibit cell growth and metabolism. To maintain the pH at an optimum level, buffers such as citrate or phosphate are usually added to avoid growth inhibition (Hahn-Hägerdal et al., 2005).

2.8.3. Yeast metabolites

The primary metabolites of yeast are cell production and ethanol during fermentation. Ethanol fermentation is a way to maintain the redox balance of the glycolysis process as well as ATP production. The maximum ethanol production happens during the exponential phase of yeast growth, where the sugar uptake is at its highest point. In ethanol fermentation, sugar acts as an electron donor, electron acceptor, as well as carbon source at the same time. While in terms of enzymes, the synthesis of alcohol from pyruvate includes pyruvate decarboxylase and alcohol dehydrogenase enzyme with acetaldehyde as its transitional compound (Gambacorta et al., 2020).

Other than the primary metabolites, yeast also produces various secondary metabolites such as glycerol, succinic acid, lactic acid, and acetic acid. Glycerol is produced during the pyruvate fermentation as a redox-balancing agent or osmolyte, while succinic acid is produced during the citric acid cycle or tricarboxylic acid (TCA) cycle). Acetic acid is formed in the early stages of fermentation and can be metabolized in the absence of other carbon sources (Mira et al., 2010). Acetic acid production is increased with an abundance of amino acid asparagine or aspartate, through the dismutation of pyruvate into acetate and lactate (Palma et al., 2018). Some yeasts are able to undergo malolactic fermentation after the completion of the main ethanol fermentation. During this fermentation, malic acid is decarboxylated into lactic acid with the help of a malolactic enzyme (Ramon-Portugal et al., 1999).

3. Methods

3.1. Materials

Pulse starches of field pea, red lentil, and faba bean were obtained through the patented process of ACAPS (Vasanthan, 2017) belonging to GrainFrac Inc. (Edmonton, Alberta, Canada). In brief, pulse grains were milled and sieved through a 0.5 mm sieve before being passed through the ACAPS apparatus as described by Jeganathan et al., (2023). Starch concentrate samples were kept in an airtight plastic bag in the fridge at 4 °C until used for experiments. Active dry yeast SuperStart Distiller Yeast containing *Saccharomyces cerevisiae* was obtained from Lallemand Biofuels & Distilled Spirits (Duluth, Georgia, USA). Enzymes used were FERMGEN^{TM} 2.5X containing acid proteolytic enzyme, OPTIMASH^{TM} TBG containing thermostable endo-1,3-β-glucanase, GC 626 containing α-amylase enzyme, and STARGEN^{TM} 002 containing a blend of α-amylase and glucoamylase enzymes. All enzymes were provided by DuPont (Wilmington, Delaware, USA).

Chemicals used in this study including diethyl pyrocarbonate (DEPC; ≥97%), urea (≥98%), dimethyl sulfoxide (≥99.9%), lactic acid (88.0-92.0%), and acetic acid (≥99%) for HPLC were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Sodium phosphate dibasic dihydrate ($Na_2HPO_4.2H_2O$; 98.0-100.5%), sodium phosphate monobasic dihydrate ($NaH_2PO_4.2H_2O$; 99.5%), 1-butanol (≥99.4%) for GC, hydrochloric acid 37%, and sulfuric acid 4N, purchased from Fischer Scientific (Hampton, New Hampshire, USA). Trace metal supplement used includes calcium chloride dihydrate ($CaCl_2.2H_2O$, 96%, Fisher Scientific), boric acid (H_3BO_3, ≥99.5%, Sigma-Aldrich), manganese chloride tetrahydrate ($MnCl_2.4H_2O$, 98-101%, Fisher Scientific), iron(III) chloride hexahydrate ($FeCl_3.6H_2O$, (≥99%, Sigma-Aldrich), zinc chloride (ZnCl2, ≥97%, MP Biomedicals, Solon, Ohio, USA), sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$, 99.5-103.0%, Fisher Scientific), cobalt chloride hexahydrate ($CoCl_2.6H_2O$, 98.0-102.0%, Arcos Organics, Geel, Antwerp, Belgium), and copper chloride ($CuCl_2$, 99%, Sigma-Aldrich). Milli-Q water as solvent was produced using Milli-Q water system purification from MilliporeSigma (Burlington, Massachusetts, USA).

Sugar standards for HPLC used were D-(+)-glucose (≥95%), D-(+)-maltose monohydrate (≥99%), maltotriose (≥90%), D-lactose monohydrate (≥98%), D-(+)-trehalose dihydrate (≥99%), D-(+)-raffinose pentahydrate (≥99%), D-(+)-mannose (≥99%), D-ribose (≥99%) from Sigma-Aldrich, and D-(-)-arabinose (≥99%) from Fischer Scientific.

3.2. Proximate analysis

The proximate composition of the feedstocks was done quantitatively and reported on a dry weight basis.

3.2.1. Moisture analysis

The moisture content was determined using a moisture analyzer (Moisture Analyzer HE53, Mettler Toledo, Columbus, Ohio, USA). Starch concentrate samples weighing 2-2.5 g were placed on an aluminum pan and then heated until no significant weight change was observed. The difference between the initial weight and the final weight after heating was used to determine the initial water content.

3.2.2. Starch analysis

The starch content including resistant starch was determined using the glucose oxidase and peroxidase (GOPOD) method based on AOAC Method 996.11 and AACC Method 76-13.01 (Total starch assay procedure K-TSTA, Megazyme, Bray, Wicklow, Ireland). Starch concentrate samples of approximately 100 mg were precipitated with 80% v/v ethanol and then dispersed in 1.7 M sodium hydroxide. The starch content was hydrolyzed into glucose with the addition of α-amylase and amyloglucosidase enzymes. The glucose produced then reacted with glucose oxidase enzyme to produce D-gluconate and hydrogen peroxide. The latter then reacts with peroxidase enzyme as well as p-hydroxybenzoic acid and 4-aminoantipyrine to produce quinoneimine dye which has a pink color and absorbance at 510 nm (Megazyme, 2020).

3.2.3. Amylose/amylopectin analysis

The amylose and amylopectin content of pulse starch concentrate was determined through total starch content and amylose content using the GOPOD method (Amylose/Amylopectin assay procedure K-AMYL, Megazyme). A sample of approximately 20-25 mg was dispersed in dimethyl sulphoxide (DMSO) and the lipid content was removed by precipitating starch with 95% v/v ethanol. The amylopectin was specifically precipitated with Concanavalin A, a type of lectin, and removed by precipitation. The total starch and amylose content of the removed amylopectin were

then determined separately with the addition of α-amylase and amyloglucosidase enzymes, following the GOPOD determination method as discussed in 3.2.2 (Megazyme, 2018).

3.2.4. Crude protein analysis

The protein content determination was done using Elemental Analyzer (2400 Series II CHNS/O Elemental Analysis, PerkinElmer, Waltham, Massachusetts, USA). Approximately 1.5 to 2.5 mg of starch concentrate sample was placed in a tin foil before being combusted under oxygen gas. The nitrogen content measured was then converted to crude protein content with a factor of 6.25.

3.2.5. Crude lipid analysis

The fat content was determined using Bligh & Dyer's (1959) method for lipid analysis with modification. 500 mg of pulse starch concentrate was mixed with 1 mL of water followed by 3.75 mL of chloroform and methanol mixture (1:2 v/v) then vortexed for 10-15 minutes. After centrifugation, the pellet was removed and 1.25 mL of chloroform was added before vortexing for 1 minute. Finally, 1.25 mL of water was added and the mixture was vortexed. Centrifugation at 2,700 G for 10 minutes was done before the upper phase was removed using a pipette. The lower phase was collected and transferred to an aluminum dish to evaporate the remaining liquid and dried in the oven at 105 °C for 15 minutes. The final weight after liquid evaporation was recorded as the crude lipid content.

3.2.6. Ash analysis

The ash content determination was done using Thermogravimetric Analysis (TGA, TGA 8000, PerkinElmer) following the method developed by Poloz (2018). 30 mg of starch concentrate was placed on a sealed high-temperature platinum pan and then heated from 30 to 600 °C at a rate of 10 °C/min under a flow of nitrogen gas at 25 mL/min flow. The experiment was performed in triplicate and the analysis of the result was done using the Pyris software manager.

3.3. Scanning Electron Microscopy (SEM)

Microscopy imaging of the pulse starch concentrate was done at the Department of Earth & Atmospheric Sciences, Faculty of Science, University of Alberta. Pulse starch concentrate was

mounted on an aluminum stub and then coated with carbon using Leica EM SCD005 evaporative carbon coater. Observation of starch granules was done at 5-10 kV magnification in SEM apparatus (ZEISS Sigma 300 VP-FESEM).

3.4. Hydrolysis

Pulse starch concentrate was weighed in a 500 mL Erlenmeyer flask then sterile Milli-Q water was added to reach a concentration of 25% w/v. The pH of the mixture was adjusted to 4.0 using HCl 2.4 N. The mixture was heated at 55 °C for 1 hour then DEPC (105 µL/kg mash) was added before storing overnight at 4 °C. The flask was then heated to 55 °C and 1M of sterile urea was added to reach a concentration of 16 mmol/kg. 940 µL/kg grain of FERMGEN™ 2.5X, 80 µL/kg grain of OPTIMASH™ TBG, 440 µL/kg grain, and 2.8 mL/kg grain of STARGEN™ 002 was mixed with 1 mL sterile Milli-Q water before added to the mash. Enzymatic hydrolysis was then performed for 72 hours at 55 °C and 200 rpm in an incubator shaker (Innova 44 Incubator Shaker, New Brunswick Scientific, Edison, New Jersey, USA). Samples were taken prior to the enzyme addition as well as 6, 24, 28, and 72 hours for HPLC analysis.

The calculation of the degree of hydrolysis calculation was as follows:

$$\text{Degree of hydrolysis (\%)} = \frac{\text{Glucose concentration (g/L)} \times \text{Volume of liquid in hydrolysis (L)} \times 0.9}{\text{Feedstock mass (g)} \times \text{Starch concentration (\%)}} \times 100\% \qquad \text{Eq. 3-1}$$

0.9 = theoretical conversion of starch to glucose

For thermal behavior analysis and starch granule observation, the hydrolyzed sample taken was obtained after or without centrifugation at 7,000 rpm for 5 minutes. Samples were freeze-dried (Labconco Freeze 12, Kansas City, Missouri, USA) for at least 48 hours at vacuum condition then homogenized using mortar and pestle prior to analysis.

3.4.1. Hydrolysis with enzyme dosage variation

In this section, the experiment described in 3.6. was performed. However, the enzyme dosages used were varied to 0.5x and 2x enzyme dosage.

3.4.2. Hydrolysis with different enzyme combination

The hydrolysis was carried out as the strategy described in section 3.6. with 5% w/v feedstock concentration and different combination of enzymes were employed.

Table 3-1 Enzyme combinations used in hydrolysis

Condition	Enzyme treatment
FSOG	FERMGEN™ 2.5X + STARGEN™ 002 + OPTIMASH™ TBG + GC 626
SOG	STARGEN™ 002 + OPTIMASH™ TBG + GC 626
FSG	FERMGEN™ 2.5X + STARGEN™ 002 + GC 626
FSO	FERMGEN™ 2.5X + STARGEN™ 002 + OPTIMASH™ TBG
FS	FERMGEN™ 2.5X + STARGEN™ 002
S	STARGEN™ 002

3.4.3. Hydrolysis with urea addition

To observe the effect of the absence of urea, the hydrolysis strategy described in 3.6. was employed with a modification in the feedstock concentration to 5% w/v. In addition, the use of 1M sterile urea to achieve a concentration of 16 mmol/kg was omitted, along with the addition of any enzymes.

3.5. Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC 8500, PerkinElmer) was used to determine the temperature of onset (T_o), the temperature of gelatinization (T_g), and the final temperature (T_f) to complement the properties of pulse flour samples. Following the method described by Colussi et al., (2021), 5 mg of hydrolyzed starch sample was weighed directly on a hermetic aluminum pan and Milli-Q water was added to obtain a starch-water ratio of 1:3 (w/w). The pan was sealed and left to equilibrate at room temperature for 1 hour. The heating method was as follows; heating from 30 to 150 °C at a rate of 10 °C/min. As a reference, an empty pan was used and indium was used as the standard. The experiment was performed in triplicate and analysis of the result was done using Pyris software manager.

3.6. High Performance Liquid Chromatography (HPLC)

Samples from hydrolysis and SSF process underwent HPLC analysis to identify and quantify the sugar content as well as other metabolites. 2 mL of the samples obtained were boiled for 10 minutes to deactivate the enzymes. Boiled samples were then diluted with Milli-Q water at

a 1:1 ratio before being centrifugated at 12,400 g for 10 minutes. 1 mL of the supernatant then was transferred to a new 2 mL microtube and mixed with 1 mL Milli-Q water before being stored at 4°C overnight. To precipitate the remaining starch residue, the tube containing the diluted supernatant was centrifuged for the second time at the same condition. The final supernatant was then passed through a 0.22 µm filter into a glass vial for HPLC analysis. The analysis was done with HPLC Agilent 1100 system with a Refractive Index Detector (RID) (Agilent Technologies, Mississauga, Ontario, Canada). The column used for separation was the Aminex HPX-87H (Bio-Rad, Hercules, California, USA) and was heated to 60 °C. 5 mM sulfuric acid at a constant flow rate of 0.5 mL/min was used as the mobile phase. Sugar standards used include glucose, maltose, maltotriose, lactose, trehalose, raffinose, mannose, arabinose, and ribose, while other metabolites analyzed are lactic acid and acetic acid as a byproduct of fermentation.

3.7. Simultaneous Saccharification and Fermentation (SSF)

The fermentation mash was prepared at a concentration of 25% w/v with pulse starch concentrate and sterile Milli-Q water in a 500 mL Erlenmeyer flask. The mixture was then pre-hydrolyzed at 55 °C and the pH was adjusted to 4.0-4.5 with 2.4 N HCl. The first round of enzymes, containing FERMGEN (940 µL/kg of grain), OPTIMASH (80 µL/kg of grain), and GC (440 µL/kg of grain), was added to the mixture, and the flask was incubated at 55 °C for 1 hour. A sterilizing agent, DEPC, was added at a concentration of 105 µL/kg mash was added and the mixture was stored at 4 °C overnight. On the day of the fermentation, the mixture was brought to 55 °C, and a second round of enzyme, STARGEN (2.8 mL/kg of grain), was added. Hydrolysis was carried out for 1 hour in an incubator shaker (Innova 44 Incubator Shaker) at 55 °C 200 rpm. Urea 1 M was then added to achieve a concentration of 16 mmol/kg. When the temperature of the mash was 30 °C, hydrated active yeast with a concentration of at least 2×10^7 CFU/mL was added. A gas trap filled with sterile water was placed on top of the flask to keep the anaerobic condition. The fermentation was carried at 30 °C 200 rpm for 72 hours. Samples for the initial sugar concentration were taken prior to the addition of yeast, while samples for the final sugar and ethanol concentration were taken at the end of the fermentation. The initial pH of the fermentation mash was determined, as well as the final pH after the fermentation. Samples were taken periodically and grown in Sabouraud dextrose agar plates to check for contamination.

For DDGS analysis, the sample was obtained at the end of fermentation and was filtered through a Grade 2 Cytiva Whatman™ Qualitative Filter Paper, the filtrate was discarded and the retentate was dried at 60 °C. The dried solid was then homogenized using mortar and pestle before subjected to characterization analyses.

3.7.1. Fermentation with different enzyme combination

Similar to the hydrolysis, the fermentation was done with a different combination of enzymes, following the strategy described in 3.8.

Table 3-2 Enzyme combinations used in fermentation

Condition	Enzyme treatment
FSOG	FERMGEN™ 2.5X + STARGEN™ 002 + OPTIMASH™ TBG + GC 626
FS	FERMGEN™ 2.5X + STARGEN™ 002
S	STARGEN™ 002

3.7.2. Fermentation with addition of nutrient

The effect of addition of nutrient in the fermentation system was done with the addition of 2.26 mL of phosphate buffer 2.5 M pH 6.0 or 1 mL of trace mineral solution with composition as stated in Table 3-3.

Table 3-3 Composition of trace mineral supplement

Trace mineral		Concentration (g/L)
Calcium chloride dihydrate	$CaCl_2.2H_2O$	3.7
Boric acid	H_3BO_3	2.5
Manganese chloride tetrahydrate	$MnCl_2.4H_2O$	0.87
Iron (III) chloride hexahydrate	$FeCl_3.6H_2O$	0.65
Zinc chloride	$ZnCl_2$	0.44
Sodium molybdate dihydrate	$Na_2MoO_4.2H_2O$	0.29
Cobalt chloride hexahydrate	$CoCl_2.6H_2O$	0.01
Copper chloride	$CuCl_2$	0.0001

3.7.3. Fermentation with addition of sodium chloride

1% w/v and 4% w/v of sodium chloride was added prior to the yeast inoculation of the fermentation strategy described in 3.8.

3.7.4. Very High Gravity (VHG) fermentation

In this study, VHG fermentation was achieved by using feedstock concentrations of 30% w/v and 40% w/v, following the strategy described in 3.8.

3.7.5. Scaling Up to 5L Bioreactor

The SSF process was scaled up to a 5L Minifors bioreactor (Infors HT, Bottmingen, Basel-Landschaft, Switzerland) with a 2L working volume. The fermentation mash was prepared at a concentration of 25% w/v in a separate sterile carboy and then transferred into the bioreactor vessel using air pressure after sterilization and pre-hydrolysis. The system was devoid of aeration and no gas purging was done at the beginning of the fermentation to allow rapid growth of the yeasts. The condition of the fermentation was identical to the strategy described in 3.8. with a pre-hydrolysis temperature of 30 °C but on a larger scale. The fermentation was carried out at 30 °C and pH 4.0 by adding 0.1 M sodium hydroxide. The samples were taken aseptically at 0, 3, 6, 24, 27, 45, 48, 51, 69, and 72 hours of fermentation for ethanol and glucose concentration, as well as the yeast viability determination, plated on Sabouraud dextrose agar at 30 °C 48 h.

3.8. Gas Chromatography (GC)

GC was performed to quantify ethanol concentration at the end of fermentation. Samples were collected into a 2 mL microtube and then centrifuged at 12,400 G for 10 minutes. 100 µL of the supernatant was then transferred into a 15 mL glass tube then diluted with 5 mL of Milli-Q water. As an internal standard, 500 µL of 1% w/v 1-butanol was added. The mixture was then passed through a 0.22 µm filter into a glass vial. The analysis was done with GC Agilent A 7890A system with a Flame Ionization Detector (FID) (Agilent Technologies). The column used was a 30-meter Restek Stabilwax-DA with an inner diameter of 0.53 mm and a film thickness of 0.5 µm. The temperature was programmed at 170 °C for injection and at 190 °C for detection with helium as the carrier gas with a 10:1 split injection mode. The oven was programmed at 35 °C for start, held for 3 minutes, then increased to 190 °C at a rate of 20 °C/min, and final hold for 1 minute. 10% w/v ethanol was used as the standard.

The calculation of ethanol concentration was as follows:

$$\text{Response factor (Rf)} = \frac{\text{Ethanol concentration (\%)} \times \text{Butanol area}}{\text{Butanol concentration (\%)} \times \text{Ethanol area}} \quad \text{Eq. 3-2}$$

$$\text{Ethanol concentration (\%)} = \frac{\text{Butanol concentration (\%)} \times \text{Ethanol area} \times \text{Rf}}{\text{Butanol area}} \quad \text{Eq. 3-3}$$

The calculation of fermentation efficiency was as follows:

$$\text{Fermentation efficiency} = \frac{\text{Experimental ethanol concentration (g / 100 g starch)}}{\text{Theoretical ethanol (g / 100 g starch)}} \quad \text{Eq. 3-4}$$

$$\text{Experimental ethanol concentration (per 100 g starch)} = \text{Ethanol concentration (g/L)} \times \text{liquid volume in fermentation (L)} \times \frac{100\ \text{g}}{\text{mass of feedstock (g)} \times \text{starch content (\%)}} \quad \text{Eq. 3-5}$$

$$\text{Theoretical ethanol concentration (per 100 g starch)} = 56.7\ \text{g} \times \text{used starch (\%)} \quad \text{Eq. 3-6}$$

56.7 = theoretical conversion of starch to ethanol

3.9.Statistical analysis

Experiments were done in three replicates at the same time and each sample was measured three times. The statistical calculations were performed using SPSS Statistics version 25 (IBM, Armonk, New York, USA). Analysis was done using a one-way analysis of variance (ANOVA) and the Tukey test for the post hoc analysis with a confidence level of 95% ($\alpha = 0.05$). The results were reported as average ± standard deviation of the nine datasets unless stated otherwise.

4. Result and Discussion

4.1. Pulse starch composition

The focus of the project was to convert pulse starch concentrate into ethanol and protein-rich residue as a co-product. To obtain the maximum ethanol conversion yield from ACAPS-processed pulse starch through saccharification and fermentation, it is crucial to understand the physiochemical feature of pulse starch concentrate. Therefore, experiments were designed to examine the intrinsic behavior of the starch concentrate as well as the potential for ethanol conversion.

The proximate composition of pulse starch concentrate was determined separately and was expressed in percentage relative to each component as presented in Table 4-1. Moisture content was measured and the numbers are subtracted from the total mass. The field pea, red lentil, and faba bean starch concentrates obtained from the ACAPS process consisted of 52-53% starch, 25.5-32.2% protein, 7.7-13% ash, and 2.7-3.0% lipid. Similar to other dry fractionation methods, ACAPS processing of pulse starch resulted in the presence of non-starch components (Li et al., 2019).

Table 4-1 Proximate analysis of pulse starch concentrate (dry basis)

ACAPS Flour	Starch (%)	Protein (%)	Ash (%)	Lipid (%)
Field pea	52.7 ± 0.4^{a}	25.5 ± 0.4^{c}	13 ± 1^{a}	2.8 ± 0.3^{a}
Red lentil	52 ± 2^{a}	32.2 ± 0.9^{a}	7.7 ± 0.3^{b}	3.0 ± 0.4^{a}
Faba bean	53 ± 1^{a}	27.8 ± 0.3^{b}	10 ± 2^{ab}	2.7 ± 0.3^{a}

[a-c]Means with different letter are significantly different ($p < 0.05$) within each column.

The starch content of ACAPS flour was improved from the starch content of native pulses, 21-40%, 27-47%, and 39.9% for pea, lentil, and faba bean (Hoover et al., 2010). To further improve the starch content, starch concentrate could be exposed to further fractionation methods such as using salt or water to extract the protein followed by dialysis or micellization to achieve starch purity of 91-92% (Jeganathan et al., 2023) or using an alkaline solution to solubilize protein and achieve starch purity of 94-97% (Li et al., 2019). The low starch yield of ACAPS processing could result from the strong association of the starch with protein as well as fiber that can be found surrounding the starch granules (Li et al., 2019).

An increase of protein concentration was observed in the ACAPS flour, compared to the air-classified pulses with a protein concentration of 7.9 ± 0.1%, 15.3 ± 0.1%, and 20.2 ± 0.4% for

pea, lentil, and faba bean (Li et al., 2019). The majority of protein in field pea, red lentil, and faba bean is salt-soluble globulin (69.5-78.1%) and water-soluble albumin (12.0-18.4%), with high content of isoleucine and lysine amino acid (Martineau-Côté et al., 2022). The solubility characteristics of pulse protein can be utilized for further purification with water and/or salt as a low-cost method (Jeganathan et al., 2023). However, when purified, the protein yield could be lowered due to protein solubilization in an alkali solution as well as the mechanical scraping of the protein layer following the centrifugation (Li et al., 2019).

The mineral content of the ACAPS flour was reflected in the number of ash present. With the ACAPS process, the ash content was increased from 1.40 ± 0.01%, 1.78 ± 0.01%, and 2.29 ± 0.01% for pea, lentil, and faba bean (Li et al., 2019) to the numbers listed in Table 4-1. If lower impurities of starch are required, the wet pulse starch purification method by Hoover & Sosulski (1985) can be implemented to achieve an ash content of 0.01-0.04%. In general, compared to wheat, pulse has a higher ash content that can improve the nutritional quality of pulse starch, but is lower in terms of baking qualities (Millar et al., 2019).

Pulses contain a small amount of fat that is approximately 2-3% of its total mass (Table 4-1). Pulse starch concentrates have slightly higher lipid content compared to regular pulses that contain less than 2% fat (Millar et al., 2019). The type of fat contained are triacylglycerol and phospholipids with oleic, linoleic, and linolenic acid as the major fatty acids (Byrdwell and Goldschmidt, 2022).

As the two primary components in a starch granule, the ratio of amylose and amylopectin determines the starch behavior including its functionality and digestibility. The amylose content of native pulses ranges from 18.0-49.9% and is caused by the difference in cultivars, determination methods, as well as the formation of an amylose-lipid complex that can affect colorimetric analysis (Hoover et al., 2010). In general, pulses have higher amylose content than maize or tapioca starches (1.9-31.2%), resulting in higher resistance to digestion (Li et al., 2019). The amylose content of pulse starch concentrates obtained from the ACAPS process fit into the average amylose content of pulses (Table 4-2), suggesting the ACAPS process preserved most of the starch fraction.

Table 4-2 Amylose and amylopectin content of pulse starch concentrate

ACAPS Flour	Amylose (%)	Amylopectin (%)
Field pea	30.5 ± 0.3[a]	69.5 ± 0.3[a]
Red lentil	28.6 ± 0.1[a]	71.4 ± 0.1[a]
Faba bean	32.7 ± 0.1[a]	67.3 ± 0.1[a]

[a]Means with different letter are significantly different ($p < 0.05$) within each column.

Starch gelation under the presence of heat and excess water is highly related to amylose and amylopectin contents. Amylopectin generally has a less packed structure due to its branches, therefore, can be penetrated by water easily. The broken intramolecular hydrogen bonds allow the grain to swell and begin to leach amylose. The melting of the amylose crystal and the re-arranging of the amylopectin chains can form a gel-like structure (Zhu and Liu, 2020). The starch concentrate samples used in this study were not significantly different in terms of amylose and amylopectin content, suggesting there is no difference in their digestibility and thermal behavior.

4.2. Pulse starch hydrolysis

Previously, enzymatic hydrolysis of the ACAPS-processed grains was done using barley with 74.3% starch, resulting in approximately 150 g/L of glucose (Lu et al., 2020). Based on these numbers, the degree of hydrolysis of barley starch concentrate hydrolysis was calculated to be approximately 60%, similar to the degree of hydrolysis of pulses (Figure 4-1). The amylolytic enzyme used, STARGEN™ 002 containing α-amyslase and glucoamylase, was not optimized for hydrolysis of raw pulse starch, resulting in the lower hydrolysis efficiency of pulse starch concentrate compared to barley and in terms of the degree of hydrolysis as the feedstock for glucose production.

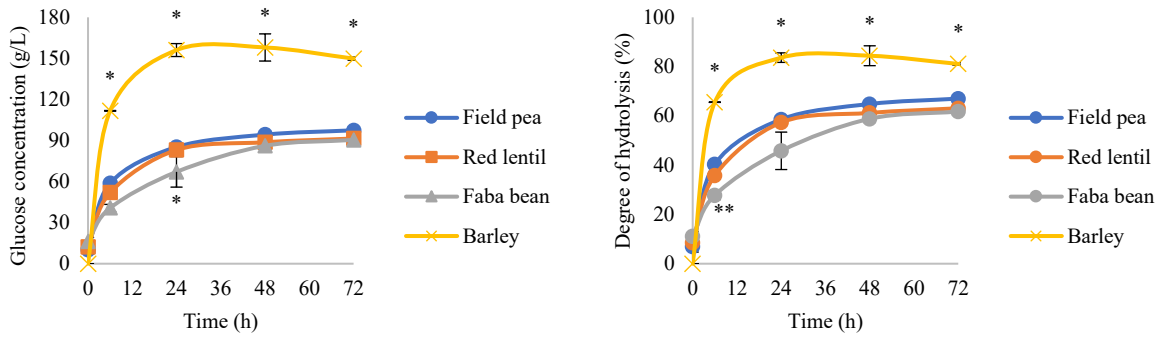


Figure 4-1 Glucose concentration of hydrolyzed pulse starch concentrate 25% w/v (left) and the degree of hydrolysis based on theoretically available starch (right). Degree of hydrolysis of 100% indicates complete hydrolysis of available starch. Asterisk (*) indicates significant difference at $p < 0.05$.

Glucose was in a very low concentration at the beginning of hydrolysis, prior to the addition of enzymes. At the sub-gelatinization temperature, a dramatic increase in glucose concentration was observed at 6 hours, indicating a rapid starch degradation. The maximum glucose concentration was achieved at 24 hours for field pea and red lentil, while faba bean did not reach its maximum until 48 hours. Based on the results, these time points of 0, 6, 24, and 48 hours were used as sampling points for starch behavior analysis during hydrolysis.

The different timing of maximum glucose production indicates a different mechanism in starch hydrolysis. Enzymatic hydrolysis of starch is influenced by factors including the morphology of starch, granule size, amylose content, as well as crystalline structure. Amylose content is known to have an inverse relationship with starch hydrolysis, where higher amylose content has a higher resistance to hydrolysis (Lin et al., 2018). Faba bean has a slightly higher amylose content in comparison to field pea and red lentil which can contribute to the longer time required to achieve maximum hydrolysis. In addition, when the starch are subjected to water and heat, it gelatinizes and undergoes recrystallization, where the amylose or amylopectin aggregates in a process called retrogradation (Bae et al., 2020).

The enzymes used during the hydrolysis process including protease, α-amylase, glucoamylase, and glucanase were selected based on previous studies with consideration of the grain composition (Gibreel et al., 2009; Lu et al., 2020). The protease was added to remove protein in the starch feedstock since the presence of protein can limit the starch hydration and lower the degree of hydrolysis (Kim et al., 2008). Protein may also form a network surrounding the starch granules (Kim et al., 2008; Rombouts et al., 2020) that can hinder starch digestion, therefore it is

important to address the presence of protein. With the removal of protein, the amylolytic enzymes will have greater access to the starch granules and penetrate easier as well as deeper (Bae et al., 2020). The presence of dietary fiber, a resistant starch, can also influence the hydrolysis efficiency. In addition to physically hindering the contact between enzyme and starch granules, it can also form gelatinized starch as previously discussed (Duta et al., 2019).

Hydrolysis of complex carbohydrates using α-amylase and glucoamylase will produce glucose, a sugar monomer. However, due to the nature of α-amylase, an endo-acting enzyme that randomly cleaves the α-1,4-glycosidic linkage of starch, other types of simple sugar can be produced. Mannose, maltose, and maltotriose are three examples of non-glucose sugar produced during the hydrolysis process in lower amounts than glucose (Ouellette and Rawn, 2015). In raw corn starch, for example, hydrolysis with STARGEN™ 002 resulted in a low concentration of maltose in comparison to glucose (Sakwa et al., 2018).

Pulses naturally contain other oligosaccharides that belonged to the raffinose family such as raffinose, stachyose, and verbascose. During the hydrolysis process, raffinose groups are hydrolyzed to their monomer, hexose and pentose sugars. Using the sugar analysis indicated in 3.6., the oligosaccharides were not well separated due to the overlapping retention time. However, no peaks indicating oligosaccharides were found after the hydrolysis process, suggesting the robustness of the amylolytic enzyme used in the hydrolysis.

4.2.1. Improving the degree of hydrolysis

Enzyme is one of the highest costs in the hydrolysis and fermentation process. Using the SSF process, where the hydrolysis and the fermentation process are done simultaneously, the final fermentation result is a liquid mash that contains solid residue. Within the solid residue are the yeast cells as well as the partially hydrolyzed feedstock. Although the enzymes theoretically can be re-obtained at the end of fermentation, separating the enzymes from the liquid mash and solid residue is a tedious process (Matano et al., 2013; Silva et al., 2016), even may cost more than using new enzymes (Chovau et al., 2013). Therefore, it is explored if fewer enzymes can be used to achieve the same amount of glucose at the same hydrolysis duration.

In Lu et al., (2020), the hydrolysis of barley starch concentrate with 0.5x enzyme and 1.0x enzyme reached the maximum glucose concentration at 48 hours of hydrolysis. In contrast to barley, pulse starch is lower in starch content and higher in other compounds such as protein. The

reduction of enzyme concentration used during the hydrolysis resulted in lower maximum glucose concentration as well as a longer time to achieve maximum glucose concentration (Figure 4-2).

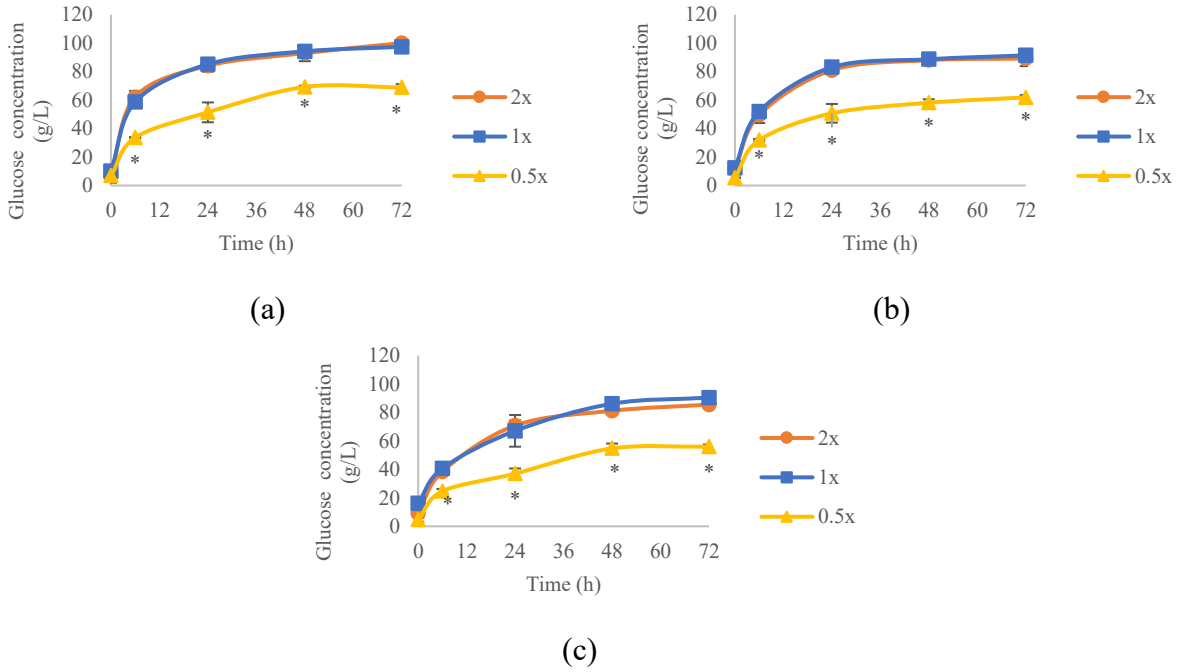


Figure 4-2 Glucose production over the course of 48 hours of hydrolysis of field pea (a), red lentil (b), and faba bean (c) starch concentrate (25% w/v) at different concentration of enzymes. Asterisk (*) indicates significant difference at $p < 0.05$.

In the raw starch hydrolyzing enzyme process, a combination of α-amylase and glucoamylase enzymes are used to convert the unprocessed starch to glucose as the feedstock for ethanol production. This system works well on corn and other cereal grains such as wheat and barley, producing ethanol with an efficiency of 85% and above (Lu et al., 2020; P. Wang et al., 2007). In a similar system, the sole use of α-amylase and glucoamylase, which are contained in the commercialized enzyme STARGEN™ 002, was tested on pulse starch feedstock. At a feedstock concentration of 5% w/v, it was found that the maximum glucose production from only using α-amylase and glucoamylase enzymes is not comparable to if other enzymes such as protease and glucanase were added (Figure 4-3). This is expected as pulse starch concentrate contained around 30% of protein and minerals that can interfere with the hydrolysis efficiency.

The addition of protease enzyme was also found to increase the crystallinity of corn and rice starches by improving the packing of amylopectin in corn and rice starches, instead of forming additional double helices structures, as suggested by Bae et al., (2020). Similarly, in corn, cassava,

and potato starch, the removal of protein and lipids increased the degree of hydrolysis and significantly reduced the amount of resistant starch left after hydrolysis (Zhang et al., 2022).

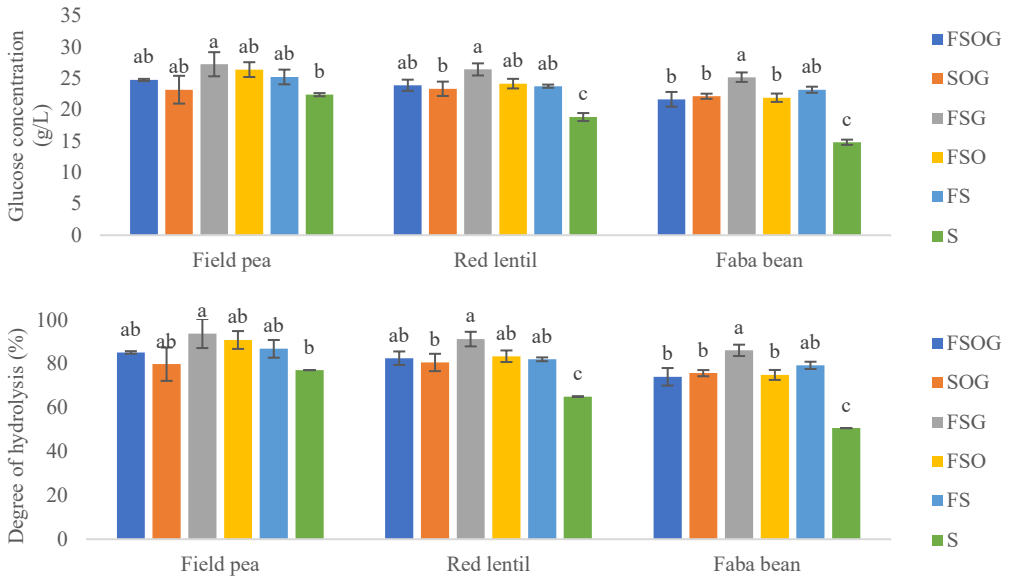


Figure 4-3 Maximum glucose concentration at 48 hours of hydrolyzed starch concentrate (5% w/v) (top) and the degree of hydrolysis (bottom) with different enzyme combinations (F = FERMGEN™ 2.5X (protease), S = STARGEN™ 002 (α-amylase and glucoamylase), O = OPTIMASH™ TBG (glucanase), G = GC 626 (acid amylase)). Degree of hydrolysis of 100% indicates complete hydrolysis of available starch. [a-c]Means with different letter are significantly different within each pulse type ($p < 0.05$).

The difference in the degree of hydrolysis of different feedstock concentrations can also be observed in Figure 4-1 and Figure 4-3. At 5% w/v feedstock concentration the degree of hydrolysis reached 80% or more while the degree of hydrolysis of 25% w/v feedstock is around 60%. In both treatments, the enzyme concentrations were kept at the same ratio and were done in a 500 mL Erlenmeyer flask, suggesting the system is susceptible to the mass transfer phenomenon. The high solid loading of hydrolysis could increase the viscosity of the mixture and could result in dead zones (Du et al., 2017). In addition, a higher pulse starch feedstock concentration also suffers from higher protein content, which is known to limit starch hydration and hydrolysis (Lu et al., 2022).

Urea is an intermolecular hydrogen bond breaker that breaks the hydrogen bonds between glucose monomers within the starch polymer (McGrane et al., 2004). The denaturation of protein has also been studied to be caused by urea addition, as the hydrogen bond is disrupted in the presence of urea (Derewenda et al., 1995). Generally, amylase enzymes are noncompetitively

inhibited or even denatured with the addition of urea (Shareghi et al., 2007; Weintraub et al., 1964). However, it has been demonstrated that the degree of hydrolysis of corn starch and triticale was not affected by the addition of up to 30% w/v urea instead, it lowers the gelatinization temperature (Li et al., 2012) which lowers the energy requirement for starch hydrolysis.

In this study, the effect of urea addition on the hydrolysis of pulse starch concentrate was studied. 6% w/v urea was added to the mixture prior to the enzymatic hydrolysis and compared to hydrolysis without urea addition. The addition of 6% w/v urea was able to achieve a higher glucose concentration in a shorter amount of time (Figure 4-4), indicating the melting of crystalline structure, and making the feedstock more accessible to enzyme penetration. Interestingly, the omission of enzymes in the hydrolysis process still generates a small amount of glucose that can result from starch polymer disruption by urea or native amylase that is contained by the feedstock itself.

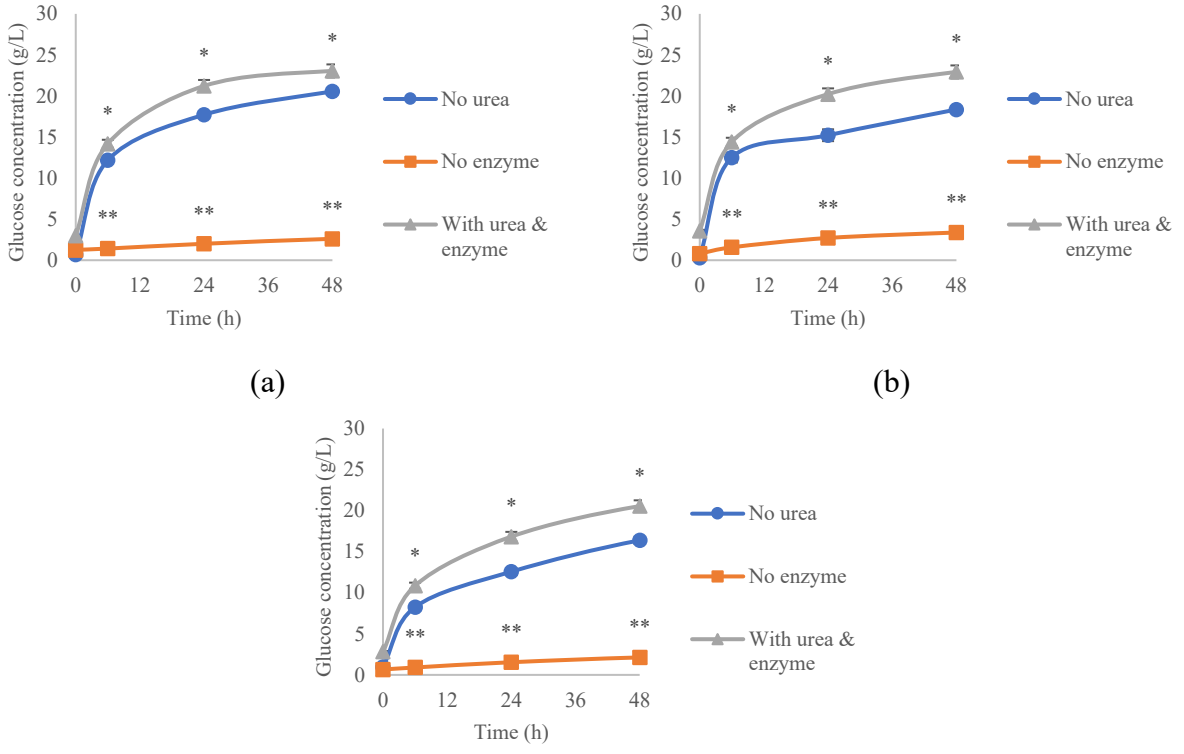


Figure 4-4 Glucose production over the course of 48 hours of hydrolysis of field pea (a), red lentil (b), and faba bean (c) starch concentrate (5% w/v) with 6% w/v urea addition. Asterisk (*) indicates significant different at $p < 0.05$.

4.3. Pulse starch hydrolysis behavior

Due to the native resistant starch content, the hydrolysis process of pulse starch concentrate was unable to fully utilize the starch content (Fabbri et al., 2016). During centrifugation, the fully hydrolyzed, partially hydrolyzed, and barely hydrolyzed pulse starch were separated based on the density, with the less hydrolyzed granule having higher density and will be found toward the bottom of the centrifuge column. With centrifugation, the starch content measured was of the partially and barely hydrolyzed granules. When measured, the starch content with centrifugation was significantly higher, meaning that there was a significant portion of the starch granules that is hydrolyzed differently. In hydrolysis without centrifugation, the starch content measured is the average of all the starch content of the hydrolyzed granules (Table 4-3).

In hydrolysis, partial hydrolysis usually referred to equal partial hydrolysis of all starch granules. However, in this study, it was suspected that only a portion of the starches are fully hydrolyzed while the rest was untouched. The result of the starch content measurement suggested that the degree of hydrolysis of the pulse starch concentrate was not equal for all starches, as observed in the partial hydrolysis of potato starch with amylase and pullulanase enzymes (Asiri et al., 2018). Some granules are more hydrolyzed than others, reflected by the different starch content between centrifuged and non-centrifuged samples.

Table 4-3 Starch content of pulse starch concentrate after hydrolysis

Starch concentrate	Starch content (%)	
	With centrifugation	Without centrifugation
Field pea	51 ± 1^{a}	27 ± 5^{b}
Red lentil	43 ± 3^{a}	22 ± 5^{b}
Faba bean	36 ± 3^{a}	25 ± 4^{b}

[a-b]Means with different letter are significantly different ($p < 0.05$) within each type of pulse.

The different preferential hydrolysis by enzyme was also reflected in the difference in amylose content (Table 4-4). STARGEN™ 002 was reported to have a preference for amylopectin during the hydrolysis of maize starch (Adams et al., 2012). Due to its branched structure, amylopectin is more susceptible to hydrolysis, meaning the unhydrolyzed granules will have reduced amylopectin content.

Table 4-4 Amylose content of pulse starch concentrate after hydrolysis

Starch concentrate	Amylose content (%)	
	With centrifugation	Without centrifugation
Field pea	54 ± 8^{a}	42.7 ± 0.6^{b}
Red lentil	48 ± 7^{a}	49 ± 2^{b}
Faba bean	59 ± 8^{a}	48.2 ± 0.9^{b}

[a-b]Means with different letter are significantly different ($p < 0.05$) within each type of pulse.

Studies have shown that α-amylase enzyme hydrolyzed both the amorphous and crystalline regions simultaneously, determined by the same level of crystallinity prior to and after the hydrolysis process (Zhou et al., 2004). Based on these results, it is apparent that amylopectin is the preferred type of starch to be hydrolyzed. However, in another study, long-chain amylopectin of corn starch has a lower degree of hydrolysis compared to its amylose counterpart (Zhang et al., 2022). These different results indicate the complexity of starch hydrolysis and how factors such as chain length and branch arrangements come into play.

4.4. Morphology of hydrolyzed pulse starch granules

Starch granules of field pea, red lentil, and faba bean all have a similar oval kidney shape with a size of 20-30 μm in diameter and smooth surface (Figure 4-5). The irregular shapes of fibers were observed at all sampling points of hydrolysis, while protein bodies surrounding the large granules are diminished with the extent of hydrolysis (Pelgrom et al., 2015). The presence of non-starch components indicates the limited ability of the ACAPS processing to disentangle and separate fibers and protein fractions from the starch granules, similar to the air classification process as demonstrated by Möller et al., (2021).

On a closer look, the surface of the starch granules has indentations that could form during the ACAPS processing, and based on references, the thin ridges along the surface are determined to be nitrogen-rich regions based on elemental map analysis (Möller et al., 2021). No obvious morphology change was observed with the extent of hydrolysis for 48 hours (Figure 4-5 a4, b4, c4). The surface of the starch granules also remained smooth. Partially hydrolyzed starch that does not show visible change on the surface often have a reduced size of the granules, indicating endo-corrosion activities (Asiri et al., 2018).

Unlike other starches such as corn, wheat, triticale, cassava, and potato, the granules of these pulse starch concentrates do not show the presence of pinholes or cavities due to amylolytic

activities (Asiri et al., 2018; Naguleswaran et al., 2012; Roy et al., 2013; Uthumporn et al., 2010; Xu et al., 2016). This indicates that the amorphous regions were mainly located at the center of the starch granules, a characteristic of type C starch. The presence of natural pores in corn starch granules make them more susceptible to hydrolysis (Uthumporn et al., 2010), thus the absence of those marks in pulse starch indicates the different starch granule structures that lead to different hydrolysis mechanisms.

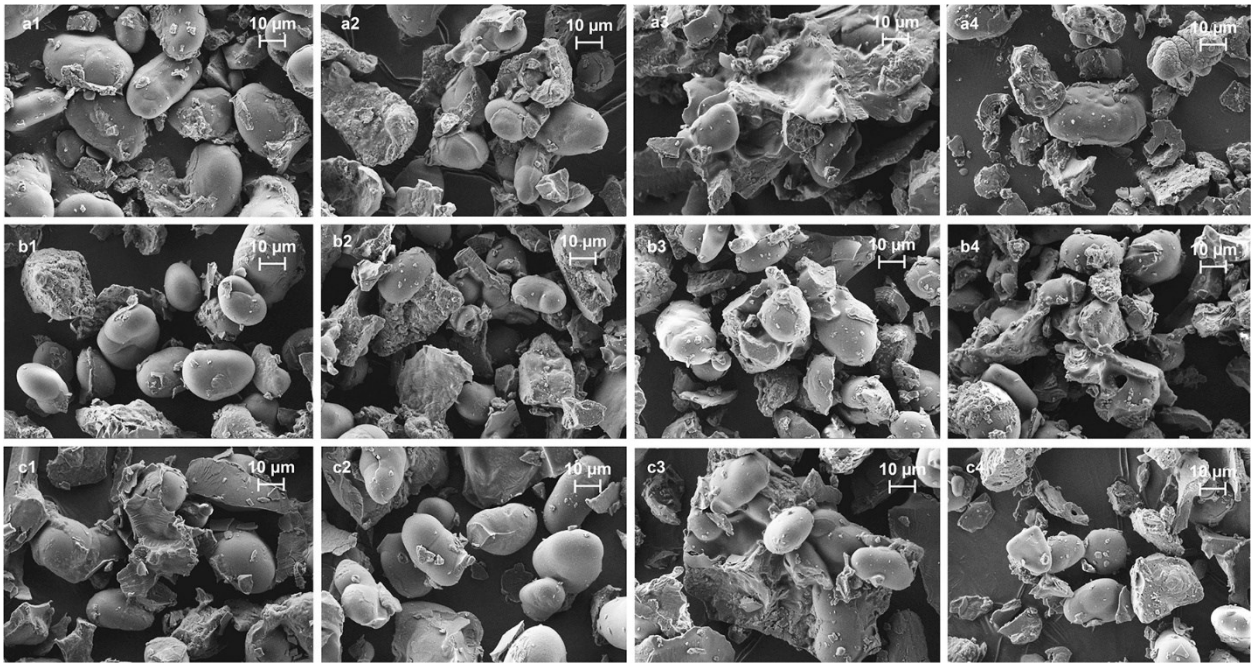


Figure 4-5 Scanning Electron Microscopy (SEM) images of starch granules of field pea (a), red lentil (b)), and faba bean (c) starch concentrate at 0 (1), 6 (2), 24 (3), and 48 (4) hours of enzymatic hydrolysis with 1000x magnification.

4.5. Thermal behavior of hydrolyzed pulse starch concentrate

The gelatinization pattern is unique for each starch, depending on the treatment condition. The gelatinization temperature of pulses is around 70 °C at a 1:3 ratio of starch and water (Hoover et al., 2010). The ACAPS process changed the composition as well as the structural integrity of the starches, resulting in different onset, peak, and final temperatures of gelatinization of pulse starch. And with the extent of hydrolysis, starch content is expected to diminish, resulting in higher gelatinization temperature.

The gelatinization temperature of ACAPS flour was found to be higher than that of the original pulse starch, and no significant differences in gelatinization parameters were found

between the different hydrolysis times, except for the final temperature of red lentil, and the onset and peak gelatinization temperatures of faba bean (Table 4-5). The presence of protein surrounding the starch granules can limit starch swelling and reduce starch solubility (S. Wang et al., 2014). The disruption of protein by the protease enzyme used is expected to increase the exposure of starch to water and result in lower gelatinization temperature. However, with the amylolytic enzymes, long starch chains were broken down into short-chain carbohydrates that can affect water activity and increase the energy required for gelatinization (Bresciani et al., 2022; Spies and Hoseney, 1982).

Table 4-5 Thermal stability result of hydrolyzed pulse starch concentrate analyzed using DSC

Pulse type	t (hour)	T_o (°C)	T_p (°C)	T_f (°C)	ΔH (J/g)
Field pea	0	86 ± 3^a	100 ± 10^a	110 ± 10^a	17 ± 5^a
	6	83 ± 4^a	95 ± 8^a	104 ± 5^a	20 ± 10^a
	24	87 ± 1^a	91 ± 2^a	100 ± 2^a	20 ± 10^a
	48	88 ± 5^a	91 ± 7^a	102 ± 2^a	15 ± 2^a
Red lentil	0	87 ± 7^a	95 ± 7^a	103 ± 2^b	16 ± 8^a
	6	85 ± 3^a	94 ± 0^a	103 ± 1^b	13 ± 0^a
	24	94 ± 4^a	115 ± 6^a	112 ± 2^a	19 ± 9^a
	48	96 ± 4^a	104 ± 5^a	112 ± 2^a	18 ± 5^a
Faba bean	0	70 ± 3^b	100 ± 10^a	110 ± 10^a	19 ± 4^a
	6	90 ± 10^a	110 ± 10^a	106 ± 6^a	13 ± 3^a
	24	85 ± 1^a	98 ± 2^b	106 ± 8^a	16 ± 1^a
	48	88 ± 5^a	100 ± 3^a	107 ± 6^a	17 ± 1^a

[a-b]Means with different letter are significantly different ($p < 0.05$) within each column of each pulse type. t: duration of hydrolysis, T_o: onset temperature, T_p: peak temperature, T_f: final temperature, ΔH: enthalpy of gelatinization.

An increase in the gelatinization temperature was also found in legumes and wheat as a result of acid hydrolysis (Li and Hu, 2021). On the contrary, in potato starch, the extent of hydrolysis did not influence the gelatinization temperature, however, retrogradation of the starch slurry occurs and lower the onset, peak, and final gelatinization temperature over time (Asiri et al., 2018). It was demonstrated that the gelatinization temperature was influenced by the distribution of amylopectin short chains in the crystalline region (Noda et al., 1998). The gelatinization temperature is also affected by the starch size, with smaller starch granules found to have a higher gelatinization temperature due to the larger surface area of the granules (Vasanthan and Bhatty, 1996).

Amylose chains were found to form double helices with amylopectin in the amorphous region, resulting in higher gelatinization temperature (Zhong et al., 2020). The increase or decrease of the gelatinization parameters could also indicate an increase or decrease in the length of amylopectin chains, with longer amylopectin double helices resulting in higher gelatinization temperature (Li and Gong, 2020). Shorter chains of amylose were also found to play a big role in the retrogradation process through recrystallization (Gong et al., 2019). The different gelatinization patterns between pulses could also be influenced by the different crystallite structures in terms of number and size within each pulse (Zhou et al., 2004).

In all three pulse samples, the different hydrolysis times do not significantly affect the enthalpy values. The difference between the onset temperature and the final temperature indicates the shift in crystallite stability (Zhou et al., 2004), while the enthalpy reflected the melting of double helices during the gelatinization (Li and Gong, 2020). Thus, the result suggested that field pea, red lentil, and faba bean all have similar molecular and granular starch characteristics as well as resistance (Ratnayake et al., 2009), due to molecular rearrangement during the retrogradation process (Wang et al., 2016).

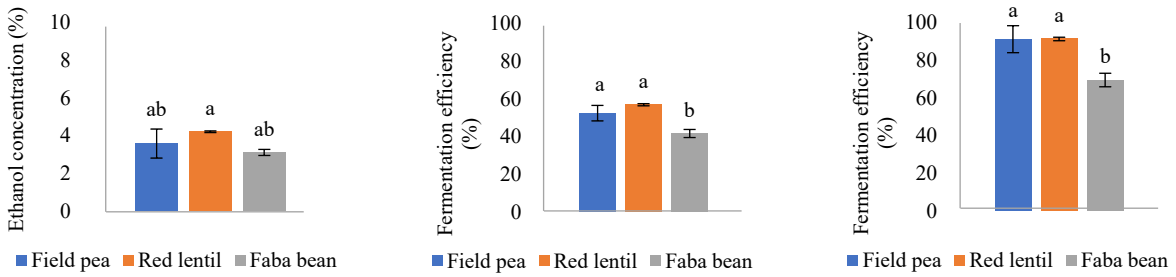
4.6. Ethanol production

As a standard for ethanol production from biomass, SSF was chosen for its advantages in avoiding end-product inhibition while keeping the osmotic pressure of sugar low during the fermentation process. The low risk of microbial contamination as well as the shorter process compared to separate hydrolysis and fermentation process was also the key to efficient ethanol production. To diminish the gap between the optimum condition of the hydrolysis and the fermentation process, a short time gap between the addition of enzyme and the yeast introduction was implemented to achieve a high starch conversion at the beginning of the fermentation (Gibreel et al., 2009; Ingledew et al., 1995).

With the simultaneous process, ethanol was obtained from pulse starches although the conversion efficiency was not maximized as observed from the presence of residual starch at the end of fermentation (Figure 4-6, Table 4-6), as half of the starch was not converted. Low ethanol concentration obtained during the fermentation could be a hurdle in the downstream processing as it requires a more rigorous distillation. Pre-treatment methods such as jet cooking or autoclaving, involving pressure and heat treatment to open up the starch structure, have been implemented and

improved the conversion efficiency greatly (Gibreel et al., 2009; Myat and Ryu, 2014; Rosa-Millan et al., 2020). Open or broken starch granules will allow yeast cells to access the converted starch (Strąk-Graczyk and Balcerek, 2020). However, such physiochemical methods require a high amount of energy and water that may not be feasible for low-cost ethanol production.

In raw corn starch, hydrolysis with STARGEN™ 002 containing α-amylase and glucoamylase resulted in a low concentration of maltose in comparison to glucose (Sakwa et al., 2018). During the fermentation with *S. cerevisiae*, glucose is consumed first by the yeast followed by maltose and maltotriose, based on the preferred order (Wang et al., 2007). Using raw starch hydrolyzing enzyme, however, the production of sugar other than glucose sugar is minimal due to the enzyme activity, thus leaving the yeast with mostly glucose for its consumption (Strąk-Graczyk and Balcerek, 2020). Similar to the hydrolysis results, oligosaccharides were not detected in the samples, both before and after 72 hours of fermentation.



(a) (b) (c)

Figure 4-6 Ethanol concentration at 72 hours of fermented starch concentrate (25% w/v) (a), fermentation efficiency without (b) and with (c) accounting for the residual starch at the end of fermentation. Fermentation efficiency of 100% indicate complete hydrolysis and fermentation of available starch. Starch residue content for field pea, red lentil, and faba bean were 38.7 ± 0.8%, 38.92 ± 0.07%, and 46.3 ± 0.9%, respectively. [a-b]Means with different letter are significantly different ($p < 0.05$).

Table 4-6 Residue analysis of SSF of pulse starch concentrate

	Residual starch (g)	Residual glucose (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Glycerol (g/L)
Field pea	5.58 ± 0.09^{b}	0.7 ± 0.1^{b}	4.4 ± 0.2^{b}	2.6 ± 0.7^{a}	3.0 ± 0.3^{a}
Red lentil	5.3 ± 0.1^{b}	1.9 ± 0.1^{a}	5.6 ± 0.6^{a}	4 ± 2^{a}	2.7 ± 0.6^{a}
Faba bean	6.1 ± 0.1^{a}	0.4 ± 0.4^{b}	6.2 ± 0.2^{a}	3.1 ± 0.9^{a}	2.1 ± 0.3^{b}

[a-b]Means with different letter are significantly different ($p < 0.05$) within each column of each pulse starch concentrate.

With high starch conversion within 24 hours of hydrolysis, it is expected that the highest ethanol productivity is found at a similar timeframe. In the hydrolysis of raw corn starch, there is rapid ethanol production observed during the first 18 hours of fermentation, with final ethanol conversion efficiency of 88.4 ± 0.3% (P. Wang et al., 2007). Ethanol is known to have an inhibitory effect on yeast metabolism, however, with low ethanol concentration, it is more likely that the residual starch was present due to the incomplete hydrolysis of the substrate (Wu & Lee, 1997). On the other hand, glucose concentration at the end of the fermentation was close to none (Table 4-6), indicating that the yeasts were able to use almost all the glucose present in the media. Thus, in this study, the fermentation was determined as complete before 72 hours by the minimum amount of glucose remaining at the end.

Lactic acid and acetic acid are two of the organic acids produced during the course of fermentation from the yeasts' intrinsic metabolism. Lactic acid is produced from the reduction of pyruvate instead of producing acetaldehyde which will be further reduced to ethanol while acetic acid is formed during the early phase of fermentation but is then later metabolized into acetyl-CoA (Whiting, 1976). During the fermentation, the pH dropped slightly from 4.0-4.5 to 3.8-4.0, contributing to the dismutation of pyruvate from the glycolysis process into lactate and acetate that could happen at a pH lower than 4. In addition, the fermentation was not done using autoclaved feedstock, thus contaminating lactic acid bacteria may present and contribute to the lactic acid level while the presence of acetic acid indicates stress and could lead to cell death and aging (Giannattasio et al., 2013).

The presence of contaminating microorganisms such as bacteria and yeast can lower the ethanol yield and increase the risk of contamination in multiple-cycle fermentation (Reis et al., 2018). Lactic acid and acetic acid can be produced by contaminating *Lactobacilli* in the fermentation broth. With the high concentration of organic acids found at the end of fermentation, it was feared that such contaminating agents were present. When the fermentation samples were cultivated on agar plates, no different colonies were found, indicating no microbial contamination present (Wang et al., 2007). However, the use of Sabouraud dextrose agar plate may not support the growth of lactic acid bacteria, thus making their presence undetectable. It was also studied that the fermentation broth with yeast contamination will leave more fructose at the end of fermentation due to the preferential consumption of glucose (Reis et al., 2018). The presence of fructose at the end of fermentation was not observed in this system, indicating the absence of contaminating yeast.

During the fermentation, glycerol as a byproduct of fermentation may be produced. This compound is produced during the glycolytic pathway as a response to osmotic pressure to maintain intracellular redox balance. Glycerol can be used as carbon storage for further metabolism but the presence of glycerol also indicates cellular stress of yeast and is typically to be found at less than 1% concentration (Hohmann, 2002). In raw corn starch hydrolysis, the presence of glycerol was lowered by the implementation of pre-hydrolysis and is determined to be caused by ethanol stress rather than sugar due to the low initial concentration of sugar (Strąk-Graczyk and Balcerek, 2020). In this study, glycerol was found at the end of fermentation with a concentration of less than 1% of the total fermentation volume, within the limit of yeast ethanol tolerance.

Similar to glycerol, trehalose is a stress protectant with a specific role of controlling protein denaturation and renaturation. In the brewing process, trehalose is found to have a protective effect from glucose concentration or osmotic pressure as well as heat stress, maintaining cell viability (D'amore et al., 1991). Trehalose is accumulated during the stationary phase and thus its presence indicates yeast stress (Hohmann, 2002). In pulse starch fermentation, trehalose was not observed at the end of 72 hours of fermentation, indicating the fermentation condition used was favorable for the yeasts.

4.6.1. Improving the fermentation efficiency

In raw starch hydrolysis, α-amylase and glucoamylase are the two enzymes that convert starch to glucose through a series of hydrolysis. α-amylase attacks the α-1,4 glycosidic bonds in the long-chain carbohydrates, producing oligosaccharides, α-dextrin, and maltose. Glucoamylase then hydrolyzes all the glycosidic bonds to produce glucose, hence the high glucose concentration at the end of hydrolysis (P. Wang et al., 2007). In this study, protease and glucanase enzymes are added to address the non-starch component of pulses as well as to reduce the viscosity of the mash (Gibreel et al., 2009). Although the omission of glucanase and acid amylase enzymes does not affect glucose production in the hydrolysis process, the omission of both enzymes resulted in significantly lower ethanol production in red lentil and faba bean (Figure 4-7). This result suggested that the presence of the additional enzymes contributes to higher ethanol production by lowering the viscosity of the mixture or conversion of starch to glucose (Jin et al., 2022).

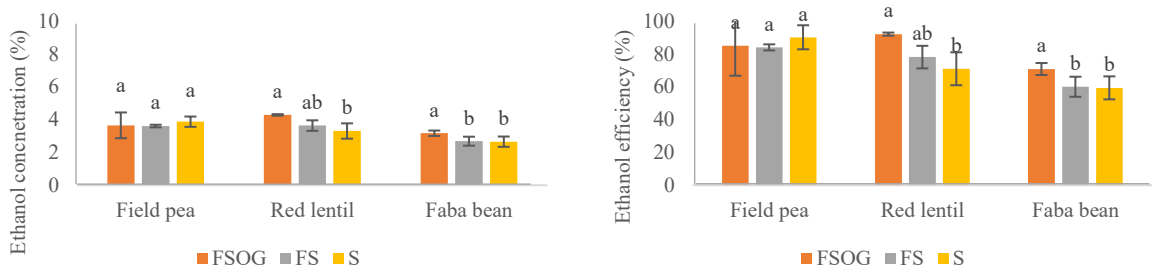


Figure 4-7 Ethanol concentration (left) and fermentation efficiency (right) with residual starch accounted for at 72 hours of fermented starch concentrate (25% w/v) with different enzyme combinations (F = FERMGEN™ 2.5X (protease), S = STARGEN™ 002 (α-amylase and glucoamylase), O = OPTIMASH™ TBG (glucanase), G = GC 626 (acid amylase)). Fermentation efficiency of 100% indicate complete hydrolysis and fermentation of available starch. [a-b]Means with different letter are significantly different ($p < 0.05$) within each pulse type.

The loss of sugar uptake from nitrogen deficiency is one of the causes of sluggish fermentation (Larsson et al., 1993; Varela et al., 2004). The addition of supplementary nitrogen is one of the most common strategies to overcome nitrogen deficiency. Pulse starch concentration has a high amount of protein and thus has a high concentration of nitrogen. Being said, the presence of nitrogen may or may not be accessible to the yeast, thus the addition of nitrogen in the form of urea is done.

In this study, the addition of 10% w/w urea did not result in higher ethanol production from red lentil and faba bean compared to the 6% w/w urea addition (Figure 4-8). This result suggested that the addition of excess urea negatively impacts the system, by targeting the enzymes instead of the protein surrounding the starch granules since urea is a protein denaturant that can disrupt noncovalent hydrogen bonds (McGrane et al., 2004). Interestingly, the omission of urea did not result in a significant difference in ethanol production in field pea, indicating there was adequate nitrogen amount in the feedstock to be used by the yeast during the fermentation.

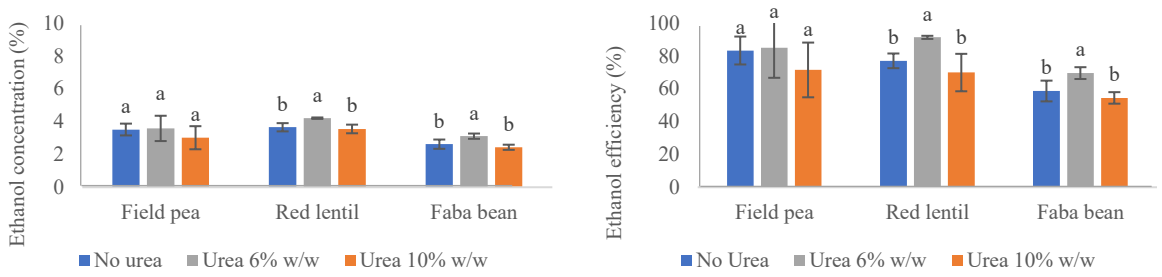


Figure 4-8 Ethanol concentration (left) and fermentation efficiency (right) with residual starch accounted for at 72 hours of fermented starch concentrate (25% w/v) with addition of urea. Fermentation efficiency of 100% indicate complete hydrolysis and fermentation of available starch. [a-b]Means with different letter are significantly different ($p < 0.05$) within each pulse type.

The supplementation of additional nutrients could increase ethanol production by increasing the yeasts' tolerance to alcohol, resulting in a lower residual solid at the end of the fermentation (Casey et al., 1984). Other than nitrogen, minerals required during fermentation are phosphate and trace minerals. In the fermentation of barley starch concentrate, phosphate is a limiting nutrient, which means the absence of phosphate addition results in very low ethanol concentration (Lu et al., 2020). Phosphate is required in the production of fatty acid esters or the reduction of short-chain fatty acids. When the phosphate concentration is lower than the required minimum, the yeast cell growth is negatively affected (Ribeiro-Filho et al., 2022). In pulse starch, the addition of phosphate resulted in higher ethanol efficiency (Figure 4-9).

Interestingly, the addition of either phosphate buffer or trace mineral also increased the fermentation efficiency (Figure 4-9). Both the phosphate buffer and the trace mineral solution contained sodium, therefore a second experiment by omitting the sodium in the trace mineral solution was carried out. Salt is known to influence the yeasts' tolerability to medium osmolarity by synthesizing metabolites such as trehalose and glycerol to maintain the balance (Logothetis and Walker, 2010). When salt was omitted, the ethanol conversion efficiency fell to the level of no added nutrient, confirming the requirement of sodium in this fermentation system.

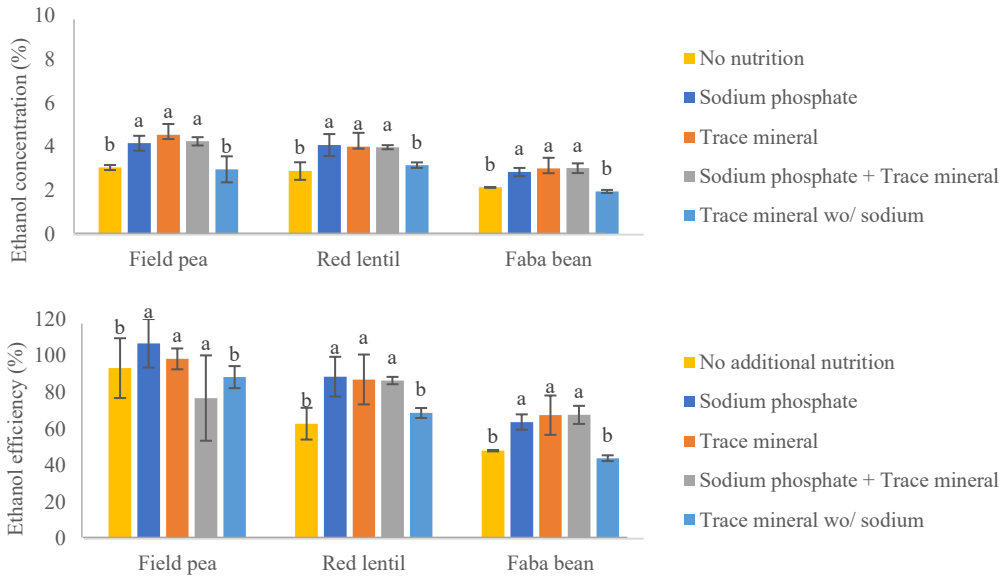


Figure 4-9 Ethanol concentration (top) and fermentation efficiency (bottom) with residual starch accounted for at 72 hours of fermented starch concentrate (25% w/v) with phosphate and trace mineral addition. Fermentation efficiency of 100% indicate complete hydrolysis and fermentation of available starch. [a-b]Means with different letter are significantly different ($p < 0.05$) within each pulse type.

To further investigate the effect of salt in this system, different concentration of sodium chloride was added to the fermentation broth. Exposure of yeast to sodium chloride in wine fermentation lowered the residual glucose concentration and improved ethanol concentration at the end of fermentation. A similar result was found in this study, where the addition of 1% and 4% w/v of sodium chloride (NaCl) resulted in improved ethanol production (Figure 4-10). Such results are expected since 1% NaCl is the physiological saline solution that is known to be tolerated by yeast while 4% NaCl is the high threshold of sodium addition before impacting the fermentation system negatively (Wei et al., 1982). Faba bean, however, behaves differently with the addition of 4% w/w of sodium chloride resulting in the highest ethanol concentration. Since the glucose production prior to the start of fermentation was at the same level for all sodium chloride concentrations, it can be concluded that salt plays a role in the fermentation step.

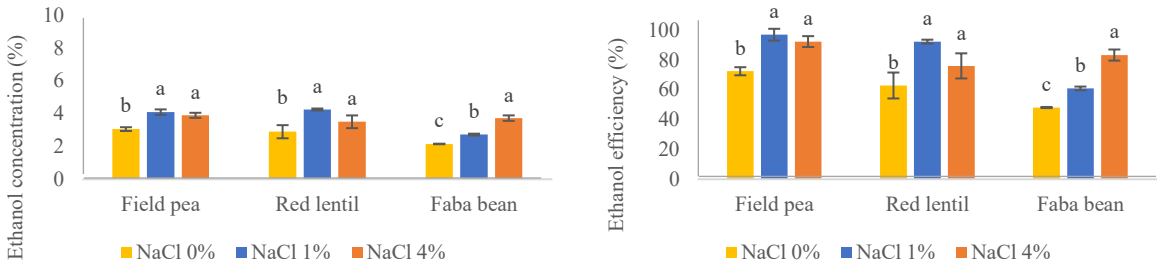


Figure 4-10 Ethanol concentration (left) and fermentation efficiency (right) with residual starch accounted for at 72 hours of fermented starch concentrate (25% w/v) with sodium chloride addition. Fermentation efficiency of 100% indicate complete hydrolysis and fermentation of available starch. [a-c]Means with different letter are significantly different ($p < 0.05$) within each pulse type.

It is known that salt negatively affects yeast growth, especially in hyperosmotic conditions or salt concentrations of more than 5%. However, the yeast viability in higher salt concentration was significantly higher after 88 hours to the end of fermentation at 208 hours (Morris et al., 1986). Salt also affects alcohol and glycerol production by increasing both productions. Prolonged exposure to salt has led the yeast to adapt through various measures such as retaining turgor pressure and cellular damage repair (Logothetis and Walker, 2010). When maltose is used as the substrate for fermentation instead of glucose, the inhibition effect of salt was observed and is determined to be caused by the different transport mechanisms. Glucose transport is carried through diffusion and thus is not affected by the Na^+/K^+ ion, while maltose is transported through transmembrane transporter that is influenced by the membrane potential (Trainotti and Stambuk, 2001).

Another way to improve the ethanol conversion efficiency is by using more than 25 g feedstock per 100 g of mash to achieve high gravity (HG) or very high gravity (VHG) fermentation (Thomas et al., 1993). The large volume of feedstock used was able to enhance ethanol production and minimize waste, resulting in a lower operational cost. However, a high substrate concentration increases the osmotic pressure which can lead to detrimental effects on the yeast cells as discussed before.

Using pulse starch concentrate at 20%, 25%, 30%, and 40% w/v of feedstock concentration, different results were achieved. The ethanol production was higher for 30% w/v and 40% w/v high gravity samples in comparison to 20% w/v and 25% w/v samples. The increase in ethanol concentration is aligned with the increase in the available substrate for fermentation.

However, if the amount of feedstock added to the mixture is accounted for, a significant efficiency improvement was only observed in red lentil at 40% w/v of feedstock and in faba bean at 30% w/v and 40% w/v of feedstock (Figure 4-11), indicating a maximum capacity of the fermentation system (Thangprompan et al., 2013).

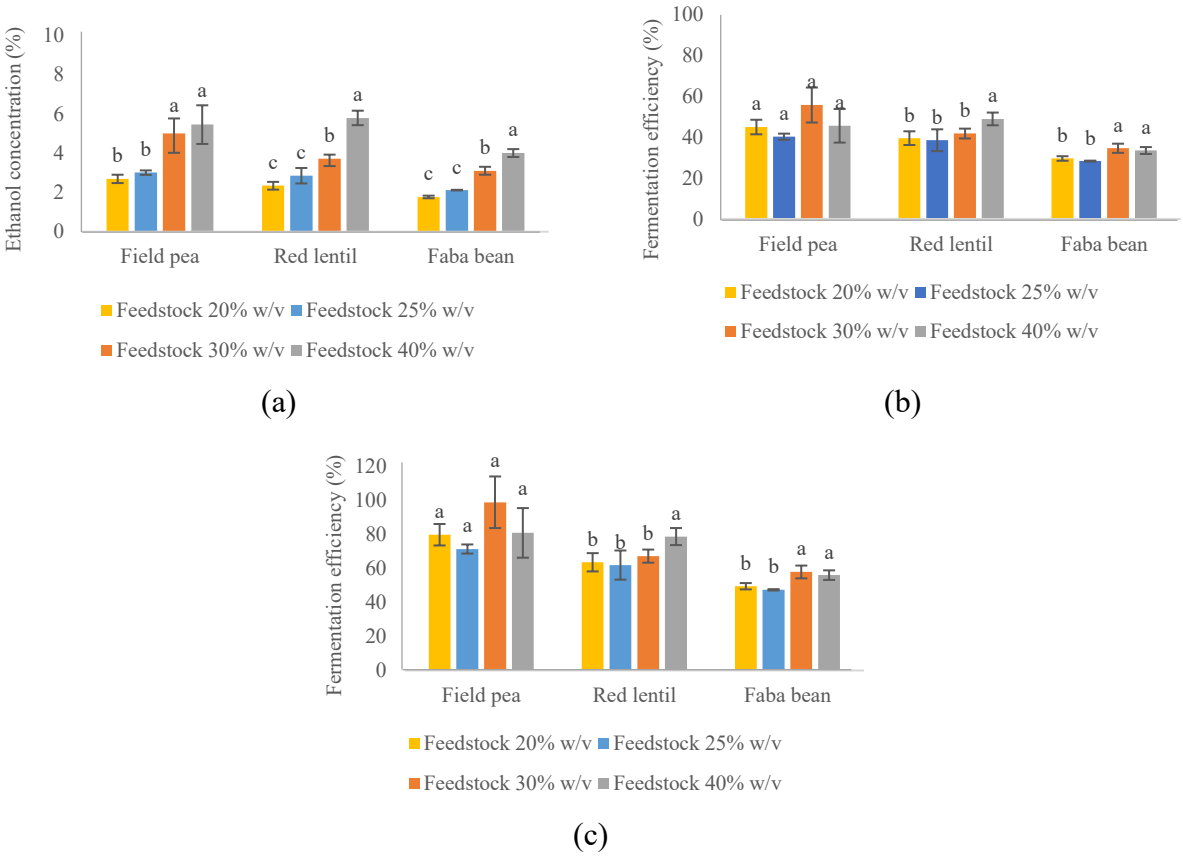


Figure 4-11 Ethanol concentration (a) and fermentation efficiency without (b) and with (c) the residual starch accounted for at 72 hours of fermented starch concentrate with different feedstock concentrations. Fermentation efficiency of 100% indicate complete hydrolysis and fermentation of available starch. [a-c]Means with different letter are significantly different ($p < 0.05$) within each pulse type.

4.7. Distillers' Dried Grains with Soluble (DDGS)

At the end of fermentation, solid residues that were partially hydrolyzed and unhydrolyzed were found (Table 4-7). These barley, corn, and other residues of cereal have been utilized for their protein-rich content, not only for animal feed but also for fortification of food for human consumption (Wu, 1986). The nutritional content of DDGS will vary based on the feedstock as well as the processing. The centrifuged solids also had higher protein content but lower in ash and

lipid than the original starch concentrate feedstock, as demonstrated in field pea (Nichols et al., 2005), common bean (Nichols et al., 2011), barley (Wu, 1986), corn (Filipe et al., 2023), and other commercial DDGS (Wang et al., 2018). In addition, in the proximate analysis, there was an unaccounted portion that could be contributed by fibers that were not dispersed by the addition of DMSO during the starch content determination.

Table 4-7 Proximate analysis of DDGS (dry basis)

Feedstock type	Starch (%)	Protein (%)	Ash (%)	Lipid (%)
Field pea	34 ± 8^{b}	25.6 ± 0.2^{b}	5 ± 1^{a}	1.9 ± 0.2^{b}
Red lentil	38.9 ± 0.1^{b}	34 ± 1^{a}	5.3 ± 0.9^{a}	2.5 ± 0.4^{a}
Faba bean	46 ± 1^{a}	34 ± 2^{a}	7 ± 1^{a}	2.6 ± 0.1^{a}

[a-b]Means with different letter are significantly different ($p < 0.05$) within each column.

The overall DDGS yield obtained were approximately 36 ± 2% w/w, 35.5 ± 0.5% w/w, and 45.1 ± 0.7% w/w of the mass of the initial feedstock for field pea, red lentil, and faba bean, higher than the DDGS yield of raw starch hydrolysis of corn of 30.3 ± 0.79% w/w (P. Wang et al., 2007) due to the presence of resistant starch. Compared to field pea and red lentil, faba bean has the highest DDGS yield, which indicates a low hydrolysis efficiency. This is in line with the high content of resistant starch contained in faba bean, 46.7% (Punia et al., 2019) that could be influenced by the physical entrapment of starch granules (Tovar et al., 1990) or starch retrogradation as reported in triticale, wheat, barley, and corn (Li et al., 2014). The high amylose content of faba bean also plays a role as STARGEN™ 002 was known to have a preference for amylopectin (Adams et al., 2012).

To increase the shelf life and ease of transportation, DDGS is often prepared by drying at a high temperature from 127 to 621 °C, triggering a loss of protein through the Maillard reaction. Other than protein loss, it also darkens the color of DDGS, leading to the use of color as an indicator of quality. However, factors such as water content, lipid content, and natural pigment play a big role in determining the final color of DDGS. Thus, energy potential, protein or amino acid content, and phosphorous bioavailability are better parameters to assign DDGS quality instead of color (Breitling and Herrick, 2017).

In corn DDGS, phenolic compounds such as xanthophyll, tocopherol, and tocotrienol are commonly found (Mohammadi Shad et al., 2021), while *p*-coumaric and ferulic acid are commonly found in field pea, lentil, and faba bean (Liu et al., 2020). However, pulses also contain

antinutritional factors that have a negative effect on health (Carbas et al., 2020). It was found that the antinutritional factors contained by pulses such as lectin and phytic acid decreased with heat treatment such as cooking, enzymatic hydrolysis (Saadi et al., 2022), and fermentation (Seo and Cho, 2016; Shi et al., 2017; Vlassa et al., 2022). The reduction of antinutritional factors such as phytic acid and improvement of the bioavailability of iron is important when the solid residues are going to be used for food supplementation or feed purposes.

4.8. Scaling up

The SSF process of field pea starch concentrate was successfully scaled up to ten times the initial volume, to a 2L working volume, proving the scalability of the system. The selection of field pea starch concentrate was due to its high starch-to-ethanol conversion, relative to the other two feedstock used. During the SSF process, glucose consumption and ethanol production can be followed (Figure 4-12). The glucose concentration was significantly reduced in the first 12 hours of fermentation, along with the exponential phase of the yeast growth. The ethanol production also increased significantly during this time, indicating sufficient nutrient was present to support the yeast metabolism. After this period, however, the ethanol production and glucose consumption slowed down, indicating maximum yeast growth was reached. The yeasts then start to decrease in population, entering the death phase, resulting in a constant ethanol and glucose concentration.

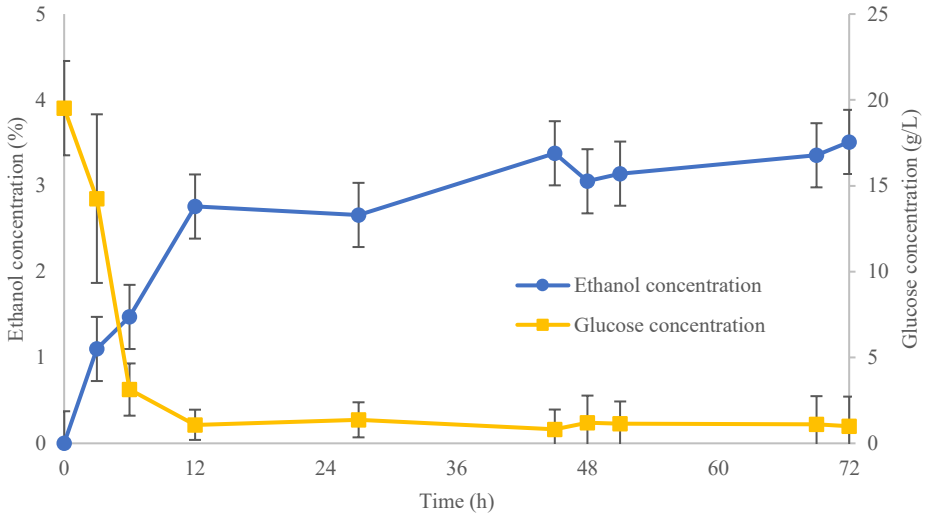


Figure 4-12 Glucose and ethanol concentration in Simultaneous Saccharification and Fermentation of field pea starch concentrate (25% w/v) in a 5L bioreactor. Data points are the mean of a triplicate run with error bars showing standard deviation.

5. Conclusion

Diversification of biofuel production through a biorefinery approach from agricultural products was demonstrated in this study. Starch concentrate of field peas, red lentils, and faba beans was generated as a co-product of protein and fiber production through the Air Currents Assisted Particle Separation (ACAPS) method. The pulse starch concentrates showed a high potential for bioethanol fermentation with protein-rich residue as a by-product.

The behavior of the starch concentrates was studied, revealing partial hydrolysis of the starches, particularly based on the residual starch at the end of enzymatic hydrolysis. Major structural damage was not observed at the surface of the starch granules and the gelatinization temperature did not significantly change with the extent of hydrolysis. In addition, the amylolytic enzyme used in this study, STARGEN™ 002 containing α-amylase and glucoamylase, has a preference to hydrolyze the amylopectin part of the starch.

The omission of protease and glucanase enzymes during the hydrolysis resulted in lower glucose production, which can be explained by the high protein and fiber content of the starch concentrate feedstock inherent to the plant's nature. Lowering the enzyme dosage to 0.5x showed a significant reduction of glucose production as well, thus was not employed in the following experiments. The addition of urea as an intermolecular hydrogen bond breaker improved the hydrolysis process instead of lowering the enzyme efficiency.

The fermentation of pulse starch concentrates resulted in a limited ethanol concentration, which could require a more rigorous distillation in the downstream processing. Residual starch was also found at the end of 72-hour fermentation, indicating the presence of resistant starches. Similar to the hydrolysis, the addition of protease and glucanase enzymes resulted in a higher fermentation efficiency, as well as the addition of urea. The nutrition requirement of the yeasts was addressed with the addition of sodium phosphate and trace minerals. It was found that the addition of sodium itself played the biggest role, possibly in regulating the osmotic pressure during the high ethanol fermentation process. Further optimization of the fermentation by higher feedstock concentration showed no significant benefit, due to the high viscosity of the mash.

Scaling up the SSF process to a 2L volume was successfully carried out and the Distillers' Dried Grains with Solubles (DDGS) were collected at the end of fermentation. Based on proximate analysis, the protein content of the DDGS was more than 34% for all feedstocks. The reduction of antinutritional factors content of the legume could also be expected. Although commonly used as

animal feed, DDGS could also be used to fortify the human diet. Thus, an offset of the bioethanol production cost could be expected from the implementation of this path. Further studies could explore the impact of anti-nutritional components on the *in vitro* and *in vivo* digestibility of DDGS for animal or human consumption.

To further improve the starch conversion, the physical or chemical treatment of the feedstock itself should be evaluated. Starch purification using low-cost materials such as salt can be explored to remove most of the protein and fiber content. A higher concentration of starch used would result in higher conversion to ethanol and make the process more profitable due to the improved efficiency. Purified starches would require little to no enzymes except for the amylolytic enzyme as well.

Overall, the conversion of pulse starch to ethanol through SSF with protein-rich co-product is possible. Improved strategy can be implemented in the grains and grain processing industry to achieve higher-value products while diversifying the feedstock for fuel production, gaining economic benefits from the valorization process.

Bibliography

Acar O, Izydorczyk MS, Kletke J, Yazici MA, Ozdemir B, Cakmak I, et al. Effects of roller and hammer milling on the yield and physicochemical properties of fibre-rich fractions from biofortified and non-biofortified hull-less barley. J Cereal Sci 2020;92:102907. https://doi.org/10.1016/j.jcs.2020.102907.

Adams JMM, Teunissen PJM, Robson G, Dunn-Coleman N, Donnison IS. Scanning electron microscopy and fermentation studies on selected known maize starch mutants using STARGEN^{TM} enzyme blends. Bioenergy Res 2012;5:330–40. https://doi.org/10.1007/S12155-011-9135-5/FIGURES/4.

Advanced Biofuels Canada. Clean Fuels Investment in Canada. 2019.

Aguilar N, Albanell E, Miñarro B, Capellas M. Chickpea and tiger nut flours as alternatives to emulsifier and shortening in gluten-free bread. LWT - Food Sci Technol 2015;62:225–32. https://doi.org/10.1016/j.lwt.2014.12.045.

Asiri SA, Ulbrich M, Flöter E. Partial Hydrolysis of Granular Potato Starch Using α-Amylase - Effect on Physicochemical, Molecular, and Functional Properties. Starch - Stärke 2018;71:1800253. https://doi.org/10.1002/star.201800253.

Bae J-E, Hong JS, Baik M-Y, Choi H-D, Choi H-W, Kim H-S. Impact of starch granule-associated surface and channel proteins on physicochemical properties of corn and rice starches 2020. https://doi.org/10.1016/j.carbpol.2020.116908.

Bak JS. Electron beam irradiation enhances the digestibility and fermentation yield of water-soaked lignocellulosic biomass. Biotechnol Reports 2014;4:30–3. https://doi.org/10.1016/J.BTRE.2014.07.006.

Balakrishnan M, Jeevarathinam G, Kumar SKS, Muniraj I, Uthandi S. Optimization and scale-up of α-amylase production by Aspergillus oryzae using solid-state fermentation of edible oil cakes. BMC Biotechnol 2021;21:1–11. https://doi.org/10.1186/S12896-021-00686-7/FIGURES/5.

Basso LC, De Amorim H V., De Oliveira AJ, Lopes ML. Yeast selection for fuel ethanol production in Brazil. FEMS Yeast Res 2008;8:1155–63. https://doi.org/10.1111/J.1567-1364.2008.00428.X.

Berchem T, Roiseux O, Vanderghem C, Boisdenghien A, Foucart G, Richel A. Corn stover as feedstock for the production of ethanol: chemical composition of different anatomical fractions and varieties. Biofuels, Bioprod Biorefining 2017;11:430–40. https://doi.org/10.1002/BBB.1755.

Bertoft E. Understanding starch structure: Recent progress. Agronomy 2017;7. https://doi.org/10.3390/agronomy7030056.

Bertoldo C, Antranikian G. Amylolytic enzymes from hyperthermophiles. Methods Enzymol 2001;330:269–90. https://doi.org/10.1016/S0076-6879(01)30382-8.

Beyene D, Chae M, Dai J, Danumah C, Tosto F, Demesa AG, et al. Enzymatically-Mediated Co-Production of Cellulose Nanocrystals and Fermentable Sugars. Catal 2017, Vol 7, Page 322 2017;7:322. https://doi.org/10.3390/CATAL7110322.

Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911–7. https://doi.org/10.1139/O59-099.

Bouchard J, Malalgoda M, Storsley J, Malunga L, Netticadan T, Thandapilly SJ. Health Benefits of Cereal Grain-and Pulse-Derived Proteins. Molecules 2022;27:1–26. https://doi.org/10.3390/molecules27123746.

Boye J, Zare F, Pletch A. Pulse proteins: Processing, characterization, functional properties and

applications in food and feed. Food Res Int 2010;43:414–31. https://doi.org/10.1016/J.FOODRES.2009.09.003.
Breitling BJ, Herrick KJ. 718 Factors affecting the color of dried distillers grains with solubles. J Anim Sci 2017;95:349–50. https://doi.org/10.2527/ASASANN.2017.718.
Bresciani A, Emide D, Saitta F, Fessas D, Iametti S, Barbiroli A, et al. Impact of Thermal Treatment on the Starch-Protein Interplay in Red Lentils: Connecting Molecular Features and Rheological Properties. Molecules 2022;27:1266. https://doi.org/10.3390/molecules27041266.
Bušić A, Mardetko N, Kundas S, Morzak G, Belskaya H, Šantek MI, et al. Bioethanol production from renewable raw materials and its separation and purification: A review. Food Technol Biotechnol 2018;56:289–311. https://doi.org/10.17113/ftb.56.03.18.5546.
Byrdwell WC, Goldschmidt RJ. Fatty Acids of Ten Commonly Consumed Pulses. Mol 2022, Vol 27, Page 7260 2022;27:7260. https://doi.org/10.3390/MOLECULES27217260.
Campos-Vega R, Guevara-Gonzalez RG, Guevara-Olvera BL, Dave Oomah B, Loarca-Piña G. Bean (Phaseolus vulgaris L.) polysaccharides modulate gene expression in human colon cancer cells (HT-29). Food Res Int 2010;43:1057–64. https://doi.org/10.1016/J.FOODRES.2010.01.017.
Campos-Vega R, Reynoso-Camacho R, Pedraza-Aboytes G, Acosta-Gallegos JA, Guzman-Maldonado SH, Paredes-Lopez O, et al. Chemical composition and in vitro polysaccharide fermentation of different beans (Phaseolus vulgaris L.). J Food Sci 2009;74. https://doi.org/10.1111/J.1750-3841.2009.01292.X.
Carbas B, Machado N, Oppolzer D, Ferreira L, Queiroz M, Brites C, et al. Nutrients, Antinutrients, Phenolic Composition, and Antioxidant Activity of Common Bean Cultivars and their Potential for Food Applications. Antioxidants 2020;9. https://doi.org/10.3390/ANTIOX9020186.
Carbonaro M, Maselli P, Nucara A. Relationship between digestibility and secondary structure of raw and thermally treated legume proteins: A Fourier transform infrared (FT-IR) spectroscopic study. Amino Acids 2012;43:911–21. https://doi.org/10.1007/S00726-011-1151-4/FIGURES/7.
Casey GP, Magnus CA, Ingledew WM. High-Gravity Brewing: Effects of Nutrition on Yeast Composition, Fermentative Ability, and Alcohol Production. Appl Environ Microbiol 1984;48:639–46. https://doi.org/10.1128/aem.48.3.639-646.1984.
Chávez-Murillo CE, Veyna-Torres JI, Cavazos-Tamez LM, de la Rosa-Millán J, Serna-Saldívar SO. Physicochemical characteristics, ATR-FTIR molecular interactions and in vitro starch and protein digestion of thermally-treated whole pulse flours. Food Res Int 2018;105:371–83. https://doi.org/10.1016/j.foodres.2017.11.029.
Chen MH, Bergman CJ. Method for determining the amylose content, molecular weights, and weight- and molar-based distributions of degree of polymerization of amylose and fine-structure of amylopectin. Carbohydr Polym 2007;69:562–78. https://doi.org/10.1016/j.carbpol.2007.01.018.
Chovau S, Degrauwe D, Van Der Bruggen B. Critical analysis of techno-economic estimates for the production cost of lignocellulosic bio-ethanol. Renew Sustain Energy Rev 2013;26:307–21. https://doi.org/10.1016/J.RSER.2013.05.064.
Chu-Ky S, Pham TH, Bui KLT, Nguyen TT, Pham KD, Nguyen HDT, et al. Simultaneous liquefaction, saccharification and fermentation at very high gravity of rice at pilot scale for potable ethanol production and distillers dried grains composition. Food Bioprod

Process 2016;98:79–85. https://doi.org/10.1016/j.fbp.2015.10.003.
Colussi R, Dias Antunes M, Gutkoski LC, Vanier NL, Zavareze E da R, Dias ARG. A- and B-type starch granules from wheat exhibiting weak, medium, and strong gluten: An investigation of physicochemical, morphological, and in vitro digestion properties. Cereal Chem 2021;98:547–56. https://doi.org/10.1002/cche.10395.
Crabtree HG. Observations on the carbohydrate metabolism of tumours. Biochem J 1929;23:536–45. https://doi.org/10.1042/bj0230536.
Crews T., Peoples M. Legume versus fertilizer sources of nitrogen: ecological tradeoffs and human needs. Agric Ecosyst Environ 2004;102:279–97. https://doi.org/10.1016/j.agee.2003.09.018.
D'amore T, Crumplen R, Stewart GG. The involvement of trehalose in yeast stress tolerance. J Ind Microbiol 1991;7:191–6.
Derewenda ZS, Lee L, Derewenda U. The occurrence of C - H ⋯ O hydrogen bonds in proteins. J Mol Biol 1995;252:248–62. https://doi.org/10.1006/JMBI.1995.0492.
Dhaubhadel S, Doria E, Singh SP, Elango D, der Laan VL, Haddad EN, et al. Raffinose Family Oligosaccharides: Friend or Foe for Human and Plant Health? 2022. https://doi.org/10.3389/fpls.2022.829118.
Du J, Cao Y, Liu G, Zhao J, Li X, Qu Y. Identifying and overcoming the effect of mass transfer limitation on decreased yield in enzymatic hydrolysis of lignocellulose at high solid concentrations. Bioresour Technol 2017;229:88–95. https://doi.org/10.1016/J.BIORTECH.2017.01.011.
Duque A, Álvarez C, Doménech P, Manzanares P, Moreno AD. Advanced bioethanol production: From novel raw materials to integrated biorefineries. Processes 2021;9:1–30. https://doi.org/10.3390/pr9020206.
Duranti M, Gius C. Legume seeds: Protein content and nutritional value. F Crop Res 1997;53:31–45. https://doi.org/10.1016/S0378-4290(97)00021-X.
Duta DE, Culetu A, Sozer N. Effect of dry fractionated hybrid protein ingredients on the structural, textural, thermal and sensory properties of gluten-free oat and faba pasta. Int J Food Sci Technol 2019;54:3205–15. https://doi.org/10.1111/ijfs.14297.
EIA. Short-Term Energy Outlook - U.S. Energy Information Administration (EIA) 2023. https://www.eia.gov/outlooks/steo/report/global_oil.php (accessed May 18, 2023).
EIA. Ethanol explained - supply of ethanol - U.S. Energy Information Administration (EIA) 2022. https://www.eia.gov/energyexplained/biofuels/ethanol-supply.php (accessed May 24, 2023).
Ellis RP, Cochrane MP, Dale MFB, Duffus CM, Lynn A, Morrison IM, et al. Starch production and industrial use. J Sci Food Agric 1998;77:289–311. https://doi.org/10.1002/(SICI)1097-0010(199807)77:3<289::AID-JSFA38>3.0.CO;2-D.
Englyst H, Wiggins HS, Cummings JH. Determination of the Non-starch Polysaccharides in Plant Foods by Gas = Liquid Chromatography of Constituent Sugars as Alditol Acetates. Analyst 1982;107:307–18.
Englyst HN, Hudson GJ. The classification and measurement of dietary. Food Chemisrry 1996;57:15–21.
EPA. Economics of Biofuels 2023. https://www.epa.gov/environmental-economics/economics-biofuels (accessed May 29, 2023).
Erdei B, Barta Z, Sipos B, Réczey K, Galbe M, Zacchi G. Research ethanol production from mixtures of wheat straw and wheat meal. Biotechnol Biofuels 2010;3:1–9.

https://doi.org/10.1186/1754-6834-3-16.
Espinosa-Ramírez J, Serna-Saldívar SO. Wet-milled chickpea coproduct as an alternative to obtain protein isolates. LWT 2019;115:108468. https://doi.org/10.1016/J.LWT.2019.108468.
Fabbri ADT, Schacht RW, Crosby GA. Evaluation of resistant starch content of cooked black beans, pinto beans, and chickpeas. NFS J 2016;3:8–12. https://doi.org/10.1016/J.NFS.2016.02.002.
Fan LT, Lee Y -H, Beardmore DH. Mechanism of the enzymatic hydrolysis of cellulose: Effects of major structural features of cellulose on enzymatic hydrolysis. Biotechnol Bioeng 1980;22:177–99. https://doi.org/10.1002/BIT.260220113.
Fernando WMU, Hill JE, Zello GA, Tyler RT, Dahl WJ, Van Kessel AG. Diets supplemented with chickpea or its main oligosaccharide component raffinose modify faecal microbial composition in healthy adults. Benef Microbes 2010;1:197–207. https://doi.org/10.3920/BM2009.0027.
Filipe D, Dias M, Magalhães R, Fernandes H, Salgado J, Belo I, et al. Solid-State Fermentation of Distiller's Dried Grains with Solubles Improves Digestibility for European Seabass (Dicentrarchus labrax) Juveniles. Fishes 2023;8:90. https://doi.org/10.3390/FISHES8020090.
Flores-Silva PC, Berrios JDJ, Pan J, Osorio-Díaz P, Bello-Pérez LA. Gluten-free spaghetti made with chickpea, unripe plantain and maize flours: functional and chemical properties and starch digestibility. Int J Food Sci Technol 2014;49:1985–91. https://doi.org/10.1111/IJFS.12529.
Gallant DJ, Bouchet B, Baldwin PM. Microscopy of starch: Evidence of a new level of granule organization. Carbohydr Polym 1997;32:177–91. https://doi.org/10.1016/S0144-8617(97)00008-8.
Gambacorta F V, Dietrich JJ, Yan Q, Pfleger BF. Rewiring yeast metabolism to synthesize products beyond ethanol HHS Public Access. Curr Opin Chem Biol 2020;59:182–92. https://doi.org/10.1016/j.cbpa.2020.08.005.
Gauss WF, Suzuki S, Takagi M. Manufacture of alcohol from cellulosic materials using plural ferments. 3,990,944, 1976.
GFI. GFI Announces first quarter 2023 results 2022. https://ir.gfiglobalfood.com/news/news-details/2022/GFI-ANNOUNCES-FIRST-QUARTER-2023-RESULTS/default.aspx (accessed May 29, 2023).
Giannattasio S, Guaragnella N, Ždralević M, Marra E. Molecular mechanisms of Saccharomyces cerevisiae stress adaptation and programmed cell death in response to acetic acid. Front Microbiol 2013;4. https://doi.org/10.3389/fmicb.2013.00033.
Gibreel A, Sandercock JR, Lan J, Goonewardene LA, Scott AC, Zijlstra RT, et al. Evaluation of value-added components of dried distiller's grain with solubles from triticale and wheat. Bioresour Technol 2011;102:6920–7. https://doi.org/10.1016/j.biortech.2011.03.075.
Gibreel A, Sandercock JR, Lan J, Goonewardene LA, Zijlstra RT, Curtis JM, et al. Fermentation of barley by using Saccharomyces cerevisiae: Examination of barley as a feedstock for bioethanol production and value-added products. Appl Environ Microbiol 2009;75:1363–72. https://doi.org/10.1128/AEM.01512-08.
Giménez MA, González RJ, Wagner J, Torres R, Lobo MO, Samman NC. Effect of extrusion conditions on physicochemical and sensorial properties of corn-broad beans (Vicia faba) spaghetti type pasta. Food Chem 2013;136:538–45.

https://doi.org/10.1016/J.FOODCHEM.2012.08.068.
Gong B, Cheng L, Gilbert RG, Li C. Distribution of short to medium amylose chains are major controllers of in vitro digestion of retrograded rice starch. Food Hydrocoll 2019;96:634–43. https://doi.org/10.1016/J.FOODHYD.2019.06.003.
Government of Canada. Clean Fuel Regulations 2023. https://www.canada.ca/en/environment-climate-change/services/managing-pollution/energy-production/fuel-regulations/clean-fuel-regulations.html (accessed May 24, 2023).
Guo Z, Jia X, Zhao B, Zeng S, Xiao J, Zheng B. C-type starches and their derivatives: structure and function. Ann N Y Acad Sci 2017;1398:47–61. https://doi.org/10.1111/nyas.13351.
Guo Z, Zhang L, Ding Z, Shi G. Minimization of glycerol synthesis in industrial ethanol yeast without influencing its fermentation performance. Metab Eng 2011;13:49–59. https://doi.org/10.1016/j.ymben.2010.11.003.
Hahn-Hägerdal B, Karhumaa K, Larsson CU, Gorwa-Grauslund M, Görgens J, van Zyl WH. Role of cultivation media in the development of yeast strains for large scale industrial use. Microb Cell Fact 2005;4:1–16. https://doi.org/10.1186/1475-2859-4-31.
Han J, Janz JA, Gerlat M. Development of gluten-free cracker snacks using pulse flours and fractions. Food Res Int 2010;43:627–33. https://doi.org/10.1016/j.foodres.2009.07.015.
Hill J, Nelson E, Tilman D, Polasky S, Tiffany D. From the Cover: Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proc Natl Acad Sci U S A 2006;103:11206. https://doi.org/10.1073/PNAS.0604600103.
Hoehnel A, Axel C, Bez J, Arendt EK, Zannini E. Comparative analysis of plant-based high-protein ingredients and their impact on quality of high-protein bread 2019. https://doi.org/10.1016/j.jcs.2019.102816.
Hohmann S. Osmotic Stress Signaling and Osmoadaptation in Yeasts. Microbiol Mol Biol Rev 2002;66:300–72. https://doi.org/10.1128/MMBR.66.2.300-372.2002.
Hoover R, Hughes T, Chung HJ, Liu Q. Composition, molecular structure, properties, and modification of pulse starches: A review. Food Res Int 2010;43:399–413. https://doi.org/10.1016/j.foodres.2009.09.001.
Hoover R, Sosulski F. Studies on the Functional Characteristics and Digestibility of Starches from Phaseolus vulgaris Biotypes. Starch - Stärke 1985;37:181–91. https://doi.org/10.1002/STAR.19850370602.
Hoover R, Zhou Y. In vitro and in vivo hydrolysis of legume starches by α-amylase and resistant starch formation in legumes - A review. Carbohydr Polym 2003;54:401–17. https://doi.org/10.1016/S0144-8617(03)00180-2.
Hossain Z, Wang X, Hamel C, Diane Knight J, Morrison MJ, Gan Y. Biological nitrogen fixation by pulse crops on semiarid Canadian prairies. Can J Plant Sci 2016;97:119–31. https://doi.org/10.1139/CJPS-2016-0185/ASSET/IMAGES/CJPS-2016-0185TAB6.GIF.
Huebbe P, Rimbach G. Historical Reflection of Food Processing and the Role of Legumes as Part of a Healthy Balanced Diet. Foods 2020;9. https://doi.org/10.3390/FOODS9081056.
Ingledew WM, Jones AM, Bhatty RS, Rossnagel BG. Fuel alcohol production from hull-less barley. Cereal Chem 1995;72:147–50.
Iram A, Cekmecelioglu D, Demirci A. Distillers' dried grains with solubles (DDGS) and its potential as fermentation feedstock. Appl Microbiol Biotechnol 2020;104:6115–28. https://doi.org/10.1007/S00253-020-10682-0/TABLES/4.
Irwin S. Ethanol Production Profits in 2021: What a Ride! Farmdoc Dly 2022;12.
Isbir T, Gormus U, Dalan AB. Favism. Brenner's Encycl Genet Second Ed 2013:22–3.

https://doi.org/10.1016/B978-0-12-374984-0.00520-9.
Ishizaki H, Hasumi K. Ethanol Production from Biomass. Res Approaches to Sustain Biomass Syst 2014:243–58. https://doi.org/10.1016/B978-0-12-404609-2.00010-6.
Jablonowski ND, Pauly M, Dama M. Microwave Assisted Pretreatment of Szarvasi (Agropyron elongatum) Biomass to Enhance Enzymatic Saccharification and Direct Glucose Production. Front Plant Sci 2022;12:3177. https://doi.org/10.3389/FPLS.2021.767254/BIBTEX.
Jane J -L, Kasemsuwan T, Leas S, Zobel H, Robyt JF. Anthology of Starch Granule Morphology by Scanning Electron Microscopy. Starch - Stärke 1994;46:121–9. https://doi.org/10.1002/STAR.19940460402.
Jeganathan B, Gao J, Temelli F, Vasanthan T. Potential of air-currents assisted particle separation (ACAPS) technology for hybrid fractionation of clean-label faba bean (Vicia faba L.) protein. J Food Eng 2023;339:111265. https://doi.org/10.1016/J.JFOODENG.2022.111265.
Jin Y, Ding F, Wang J, Yi Z, Gao Y, Yang L, et al. One-step conversion of sweet potato waste to butanol via fermentation by Clostridium acetobutylicum. Biomass Convers Biorefinery 2022;1:1–12. https://doi.org/10.1007/S13399-022-03314-2/FIGURES/3.
Jones PR, Gawel R, Francis IL, Waters EJ. The influence of interactions between major white wine components on the aroma, flavour and texture of model white wine. Food Qual Prefer 2008;19:596–607. https://doi.org/10.1016/J.FOODQUAL.2008.03.005.
Joseph R, Bachhawat AK. Yeasts: Production and Commercial Uses. vol. 3. Second Edi. Elsevier; 2014. https://doi.org/10.1016/B978-0-12-384730-0.00361-X.
Jugwanth Y, Sewsynker-Sukai Y, Gueguim Kana EB. Valorization of sugarcane bagasse for bioethanol production through simultaneous saccharification and fermentation: Optimization and kinetic studies. Fuel 2020;262:116552. https://doi.org/10.1016/J.FUEL.2019.116552.
Kajiwara Y, Takeshima N, Ohba H, Omori T, Shimoda M, Wada H. Production of Acid-Stable a-Amylase by Aspergillus kawachii during Barley Shochu-Koji Production. J Ferment Bioeng 1997;84:224–7.
Karunanandaa K, Varga GA, Akin DE, Rigsby LL, Royse DJ. Botanical fractions of rice straw colonized by white-rot fungi: changes in chemical composition and structure. Anim Feed Sci Technol 1995;55:179–99. https://doi.org/10.1016/0377-8401(95)00805-W.
Khan Tareen A, Punsuvon V, Niaz Sultan I, Waseem Khan M, Parakulsuksatid P. Cellulase Addition and Pre-hydrolysis Effect of High Solid Fed-Batch Simultaneous Saccharification and Ethanol Fermentation from a Combined Pretreated Oil Palm Trunk 2021. https://doi.org/10.1021/acsomega.1c03111.
Khanyile AT, Andrew JE, Paul V, Sithole BB. A comparative study of supercritical fluid extraction and accelerated solvent extraction of lipophilic compounds from lignocellulosic biomass. Sustain Chem Pharm 2022;26:100608. https://doi.org/10.1016/J.SCP.2022.100608.
Khazaei H, Podder R, Caron CT, Kundu SS, Diapari M, Vandenberg A, et al. Marker-Trait Association Analysis of Iron and Zinc Concentration in Lentil (Lens culinaris Medik.) Seeds. Plant Genome 2017;10. https://doi.org/10.3835/PLANTGENOME2017.02.0007.
Kim EHJ, Petrie JR, Motoi L, Morgenstern MP, Sutton KH, Mishra S, et al. Effect of structural and physicochemical characteristics of the protein matrix in pasta on in vitro starch digestibility. Food Biophys 2008;3:229–34. https://doi.org/10.1007/s11483-008-9066-7.

Kim HS, Kim BY, Baik MY. Application of Ultra High Pressure (UHP) in Starch Chemistry. Http://DxDoiOrg/101080/104083982010498065 2011;52:123–41. https://doi.org/10.1080/10408398.2010.498065.
Kim JH, Park GH, Han GP, Kil DY. Effect of feeding corn distillers dried grains with solubles naturally contaminated with deoxynivalenol on growth performance, meat quality, intestinal permeability, and utilization of energy and nutrients in broiler chickens. Poult Sci 2021;100:101215. https://doi.org/10.1016/J.PSJ.2021.101215.
Krajang M, Malairuang K, Sukna J, Rattanapradit K, Chamsart S. Single-step ethanol production from raw cassava starch using a combination of raw starch hydrolysis and fermentation, scale-up from 5-L laboratory and 200-L pilot plant to 3000-L industrial fermenters. Biotechnol Biofuels 2021;14:1–15. https://doi.org/10.1186/S13068-021-01903-3/TABLES/6.
Kumar P, Satyanarayana T. Microbial glucoamylases: Characteristics and applications. Crit Rev Biotechnol 2009;29:225–55. https://doi.org/10.1080/07388550903136076.
Landauer J, Foerst P. Triboelectric separation of a starch-protein mixture – Impact of electric field strength and flow rate. Adv Powder Technol 2018;29:117–23. https://doi.org/10.1016/J.APT.2017.10.018.
Larson QP, Maddock RJ, Neville BW. Effects of distillers dried grains with solubles supplementation on yearling heifers grazing Northern Great Plains rangeland: impacts on subsequent feedlot performance and meat quality. Transl Anim Sci 2019;3:1153. https://doi.org/10.1093/TAS/TXZ118.
Larsson C, Von Stockar U, Marison I, Gustafsson L. Growth and metabolism of Saccharomyces cerevisiae in chemostat cultures under carbon-, nitrogen-, or carbon- and nitrogen-limiting conditions. J Bacteriol 1993;175:4809–16. https://doi.org/10.1128/jb.175.15.4809-4816.1993.
Lazou A, Krokida M. Functional properties of corn and corn-lentil extrudates. Food Res Int 2010;43:609–16. https://doi.org/10.1016/j.foodres.2009.09.017.
Lee CY. Dry grind ethanol production process and system with front end milling method. US 9012191B2, 2015.
Li C, Gong B. Insights into chain-length distributions of amylopectin and amylose molecules on the gelatinization property of rice starches. Int J Biol Macromol 2020;155:721–9. https://doi.org/10.1016/J.IJBIOMAC.2020.04.006.
Li C, Hu Y. Effects of acid hydrolysis on the evolution of starch fine molecular structures and gelatinization properties. Food Chem 2021;353:129449. https://doi.org/10.1016/j.foodchem.2021.129449.
Li J, Vasanthan T, Bressler DC. Improved cold starch hydrolysis with urea addition and heat treatment at subgelatinization temperature. Carbohydr Polym 2012;87:1649–56. https://doi.org/10.1016/j.carbpol.2011.09.061.
Li J, Vasanthan T, Gao J, Naguleswaran S, Zijlstra RT, Bressler DC. Resistant Starch Escaped from Ethanol Production: Evidence from Confocal Laser Scanning Microscopy of Distiller's Dried Grains with Solubles (DDGS). Cereal Chem J 2014;91:130–8. https://doi.org/10.1094/CCHEM-05-13-0087-R.
Li L, Yuan TZ, Setia R, Raja RB, Zhang B, Ai Y. Characteristics of pea, lentil and faba bean starches isolated from air-classified flours in comparison with commercial starches. Food Chem 2019;276:599–607. https://doi.org/10.1016/j.foodchem.2018.10.064.
Li X, Wang C, Krishnan PG. Effects of corn distillers dried grains on dough properties and

quality of Chinese steamed bread. Food Sci Nutr 2020;8:3999. https://doi.org/10.1002/FSN3.1604.
Lienhardt T, Black K, Saget S, Costa MP, Chadwick D, Rees RM, et al. Just the tonic! Legume biorefining for alcohol has the potential to reduce Europe's protein deficit and mitigate climate change. Environ Int 2019;130:104870. https://doi.org/10.1016/J.ENVINT.2019.05.064.
Lin L, Zhang L, Cai X, Liu Q, Zhang C, Wei C. The relationship between enzyme hydrolysis and the components of rice starches with the same genetic background and amylopectin structure but different amylose contents. Food Hydrocoll 2018;84:406–13. https://doi.org/10.1016/j.foodhyd.2018.06.029.
Lin Y, Zhang W, Li C, Sakakibara K, Tanaka S, Kong H. Factors affecting ethanol fermentation using Saccharomyces cerevisiae BY4742. Biomass and Bioenergy 2012;47:395–401. https://doi.org/10.1016/J.BIOMBIOE.2012.09.019.
Lindeboom N, Chang PR, Tyler RT. Analytical, Biochemical and Physicochemical Aspects of Starch Granule Size, with Emphasis on Small Granule Starches: A Review. Starch - Stärke 2004;56:89–99. https://doi.org/10.1002/star.200300218.
Liu Y, Ragaee S, Marcone MF, Abdel-Aal ESM. Composition of Phenolic Acids and Antioxidant Properties of Selected Pulses Cooked with Different Heating Conditions. Foods 2020;9. https://doi.org/10.3390/FOODS9070908.
Logothetis S, Walker G. Influence of sodium chloride on wine yeast fermentation performance. Int J Wine Res 2010;2:35. https://doi.org/10.2147/IJWR.S10889.
Lomthong T, Netprasom P, Kancharu N, Jitmala K, Areesirisuk A, Trakarnpaiboon S, et al. Very high Gravity (VHG) Bioethanol Production Using Modified Simultaneous Saccharification and Fermentation of Raw Cassava Chips with Molasses by Kluyveromyces marxianus DMKU-KS07. Waste and Biomass Valorization 2021;12:3683–93. https://doi.org/10.1007/s12649-020-01257-1.
Lu X, Ma R, Zhan J, Wang F, Tian Y. The role of protein and its hydrolysates in regulating the digestive properties of starch: A review. Trends Food Sci Technol 2022;125:54–65. https://doi.org/10.1016/j.tifs.2022.04.027.
Lu Y, Chae M, Vasanthan T, Bressler DC. The potential of fiber-depleted starch concentrate produced through air currents assisted particle separation of barley flour in bio-ethanol production. Bioresour Technol 2020;303:122942. https://doi.org/10.1016/j.biortech.2020.122942.
Luo Y, Xie W, Luo F. Effect of Several Germination Treatments on Phosphatases Activities and Degradation of Phytate in Faba Bean (Vicia faba L.) and Azuki Bean (Vigna angularis L.). J Food Sci 2012;77. https://doi.org/10.1111/j.1750-3841.2012.02733.x.
Ma Z, Boye JI, Swallow K, Malcolmson L, Simpson BK. Techno-functional characterization of salad dressing emulsions supplemented with pea, lentil and chickpea flours. J Sci Food Agric 2016;96:837–47. https://doi.org/10.1002/JSFA.7156.
Madu JO, Agboola BO. Bioethanol production from rice husk using different pretreatments and fermentation conditions. 3 Biotech 2018;8:15. https://doi.org/10.1007/S13205-017-1033-X.
Maier T V., Lucio M, Lee LH, Verberkmoes NC, Brislawn CJ, Bernhardt J, et al. Impact of Dietary Resistant Starch on the Human Gut Microbiome, Metaproteome, and Metabolome. MBio 2017;8. https://doi.org/10.1128/MBIO.01343-17.
Marinangeli CPF, Curran J, Barr SI, Slavin J, Puri S, Swaminathan S, et al. Enhancing nutrition

with pulses: defining a recommended serving size for adults. Nutr Rev 2017;75:990–1006. https://doi.org/10.1093/nutrit/nux058.
Marinangeli CPF, Jones PJH. Pulse grain consumption and obesity: effects on energy expenditure, substrate oxidation, body composition, fat deposition and satiety. Br J Nutr 2012;108:S46–51. https://doi.org/10.1017/S0007114512000773.
Martineau-Côté D, Achouri A, Karboune S, L'Hocine L. Faba Bean: An Untapped Source of Quality Plant Proteins and Bioactives. Nutrients 2022;14:1–27. https://doi.org/10.3390/nu14081541.
Matano Y, Hasunuma T, Kondo A. Cell recycle batch fermentation of high-solid lignocellulose using a recombinant cellulase-displaying yeast strain for high yield ethanol production in consolidated bioprocessing. Bioresour Technol 2013;135:403–9. https://doi.org/10.1016/J.BIORTECH.2012.07.025.
Mayer Labba IC, Frøkiær H, Sandberg AS. Nutritional and antinutritional composition of fava bean (Vicia faba L., var. minor) cultivars. Food Res Int 2021;140. https://doi.org/10.1016/J.FOODRES.2020.110038.
Mcginnis GD, Wilson WW, Mullen CE. Biomass Pretreatment with Water and High-Pressure Oxygen. the Wet-Oxidation Process. Ind Eng Chem Prod Res Dev 1983;22:352–7. https://doi.org/10.1021/I300010A036/ASSET/I300010A036.FP.PNG_V03.
McGrane SJ, Mainwaring DE, Cornell HJ, Rix CJ. The role of hydrogen bonding in amylose gelation. Starch/Staerke 2004;56:122–31. https://doi.org/10.1002/star.200300242.
Megazyme. Total Starch Assay Procedure K-TSTA. Megazyme 2020;11.
Megazyme. Amylose/Amylopectin: Assay Procedure K-AMYL. Megazyme 2018;6:11.
Millar KA, Gallagher E, Burke R, McCarthy S, Barry-Ryan C. Proximate composition and anti-nutritional factors of fava-bean (Vicia faba), green-pea and yellow-pea (Pisum sativum) flour. J Food Compos Anal 2019;82:103233. https://doi.org/10.1016/j.jfca.2019.103233.
Mira NP, Palma M, Guerreiro JF, Sá-Correia I. Genome-wide identification of Saccharomyces cerevisiae genes required for tolerance to acetic acid. Microb Cell Fact 2010;9:1–13. https://doi.org/10.1186/1475-2859-9-79/FIGURES/6.
Mirali M, Purves RW, Stonehouse R, Song R, Bett K, Vandenberg A. Genetics and Biochemistry of Zero-Tannin Lentils. PLoS One 2016;11. https://doi.org/10.1371/JOURNAL.PONE.0164624.
Mitchell DC, Marinangeli CPF, Pigat S, Bompola F, Campbell J, Pan Y, et al. Pulse Intake Improves Nutrient Density among US Adult Consumers. Nutrients 2021;13. https://doi.org/10.3390/NU13082668.
Mohammadi Shad Z, Venkitasamy C, Wen Z. Corn distillers dried grains with solubles: Production, properties, and potential uses. Cereal Chem 2021;98:999–1019. https://doi.org/10.1002/CCHE.10445.
Möller AC, van der Padt A, van der Goot AJ. From raw material to mildly refined ingredient – Linking structure to composition to understand fractionation processes. J Food Eng 2021;291. https://doi.org/10.1016/j.jfoodeng.2020.110321.
Morris GJ, Winters L, Coulson GE, Clarke KJ. Effect of osmotic stress on the ultrastructure and viability of the yeast Saccharomyces cerevisiae. J Gen Microbiol 1986;132:2023–34. https://doi.org/10.1099/00221287-132-7-2023.
Myat L, Ryu GH. Effect of thermostable α-amylase injection on mechanical and physiochemical properties for saccharification of extruded corn starch. J Sci Food Agric 2014;94:288–95. https://doi.org/10.1002/JSFA.6252.

Naguleswaran S. Starch morphological and molecular structural relations to amylolysis. University of Alberta, 2013.
Naguleswaran S, Li J, Vasanthan T, Bressler D, Hoover R. Amylolysis of large and small granules of native triticale, wheat and corn starches using a mixture of α-amylase and glucoamylase. Carbohydr Polym 2012;88:864–74. https://doi.org/10.1016/j.carbpol.2012.01.027.
Naguleswaran S, Vasanthan T, Hoover R, Bressler D. Amylolysis of amylopectin and amylose isolated from wheat, triticale, corn and barley starches. Food Hydrocoll 2014;35:686–93. https://doi.org/10.1016/j.foodhyd.2013.08.018.
Naibaho J, Korzeniowska M. The variability of physico-chemical properties of brewery spent grain from 8 different breweries. Heliyon 2021;7:e06583. https://doi.org/10.1016/J.HELIYON.2021.E06583.
Nichols NN, Dien BS, Wu YV, Cotta MA. Ethanol fermentation of starch from field peas. Cereal Chem 2005;82:554–8. https://doi.org/10.1094/CC-82-0554.
Nichols NN, Sutivisedsak N, Dien BS, Biswas A, Lesch WC, Cotta MA. Conversion of starch from dry common beans (Phaseolus vulgaris L.) to ethanol. Ind Crops Prod 2011;33:644–7. https://doi.org/10.1016/j.indcrop.2010.12.029.
Nissen TL, Hamann CW, Kielland-Brandt MC, Nielsen J, Villadsen J. Anaerobic and aerobic batch cultivations of Saccharomyces cerevisiae mutants impaired in glycerol synthesis. Yeast 2000;16:463–74. https://doi.org/10.1002/(SICI)1097-0061(20000330)16:5.
Noda T, Takahata Y, Sato T, Suda I, Morishita T, Ishiguro K, et al. Relationships between chain length distribution of amylopectin and gelatinization properties within the same botanical origin for sweet potato and buckwheat. Carbohydr Polym 1998;37:153–8. https://doi.org/10.1016/S0144-8617(98)00047-2.
Olofsson K, Bertilsson M, Lidén G. A short review on SSF - An interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnol Biofuels 2008;1:1–14. https://doi.org/10.1186/1754-6834-1-7/FIGURES/4.
Ouellette RJ, Rawn JD. Carbohydrates. Princ Org Chem 2015:343–70. https://doi.org/10.1016/B978-0-12-802444-7.00013-6.
Palma M, Guerreiro JF, Sá-Correia I. Adaptive response and tolerance to acetic acid in Saccharomyces cerevisiae and Zygosaccharomyces bailii: A physiological genomics perspective. Front Microbiol 2018;9:274. https://doi.org/10.3389/FMICB.2018.00274/BIBTEX.
Papadopoulos KP, Economou CN, Stefanidou N, Moustaka-Gouni M, Genitsaris S, Aggelis G, et al. A semi-continuous algal-bacterial wastewater treatment process coupled with bioethanol production. J Environ Manage 2023;326:116717. https://doi.org/10.1016/J.JENVMAN.2022.116717.
Pelgrom PJM, Boom RM, Schutyser MAI. Method Development to Increase Protein Enrichment During Dry Fractionation of Starch-Rich Legumes. Food Bioprocess Technol 2015;8:1495–502. https://doi.org/10.1007/S11947-015-1513-0/FIGURES/6.
Petitot M, Boyer L, Minier C, Micard V. Fortification of pasta with split pea and faba bean flours: Pasta processing and quality evaluation. Food Res Int 2010;43:634–41. https://doi.org/10.1016/j.foodres.2009.07.020.
Pfeiffer T, Morley A. An evolutionary perspective on the Crabtree effect. Front Mol Biosci 2014;1:17. https://doi.org/10.3389/FMOLB.2014.00017/BIBTEX.
Pfister B, Zeeman SC. Formation of starch in plant cells. Cell Mol Life Sci 2016;73:2781–807.

https://doi.org/10.1007/s00018-016-2250-x.
Pielhop T, Amgarten J, Von Rohr PR, Studer MH. Steam explosion pretreatment of softwood: The effect of the explosive decompression on enzymatic digestibility. Biotechnol Biofuels 2016;9:1–13. https://doi.org/10.1186/S13068-016-0567-1/FIGURES/7.
Poloz I. Transformation of Wheat Starch During Baking and Storage of Bread. University of Guelph, 2018.
Pourafshar S, Rosentrater KA, Krishnan PG. Production of Barbari Bread (Traditional Iranian Bread) Using Different Levels of Distillers Dried Grains with Solubles (DDGS) and Sodium Stearoyl Lactate (SSL). Foods 2018;7. https://doi.org/10.3390/FOODS7030031.
Puligundla P, Smogrovicova D, Mok C, Obulam VSR. A review of recent advances in high gravity ethanol fermentation. Renew Energy 2019;133:1366–79. https://doi.org/10.1016/j.renene.2018.06.062.
Punia S, Dhull SB, Sandhu KS, Kaur M. Faba bean (Vicia faba) starch: Structure, properties, and in vitro digestibility—A review. Legum Sci 2019;1. https://doi.org/10.1002/LEG3.18.
Raboy V. myo-Inositol-1,2,3,4,5,6-hexakisphosphate. Phytochemistry 2003;64:1033–43. https://doi.org/10.1016/S0031-9422(03)00446-1.
Rahma EH. Functional and electrophoretic characteristics of faba bean (Vicia faba) flour proteins as affected by germination. Food / Nahrung 1988;32:577–83. https://doi.org/10.1002/food.19880320618.
Ramon-Portugal F, Seiller I, Taillandier P, Favarel JL, Nepveu F, Strehaiano P. Kinetics of Production and Consumption of Organic Acids during Alcoholic Fermentation by Saccharomyces cerevisae. Food Technol Biotechnol 1999;37:235–40.
Ratnayake WS, Hoover R, Warkentin T. Pea starch: Composition, structure and properties - A review. Starch/Staerke 2002;54:217–34. https://doi.org/10.1002/1521-379X(200206)54:6<217::AID-STAR217>3.0.CO;2-R.
Ratnayake WS, Otani C, Jackson DS. DSC enthalpic transitions during starch gelatinisation in excess water, dilute sodium chloride and dilute sucrose solutions. J Sci Food Agric 2009;89:2156–64. https://doi.org/10.1002/jsfa.3709.
Reinhold JG, Lahimgarzadeh A, Nasr K, Hedayati H. Effects of purified phytate-rich bread upon metabolism of zing, calcium, phosphorous, and nitrogen in man. Lancet 1973;301:283–8. https://doi.org/10.1016/S0140-6736(73)91538-9.
Reinprecht Y, Schram L, Marsolais F, Smith TH, Hill B, Pauls KP. Effects of Nitrogen Application on Nitrogen Fixation in Common Bean Production. Front Plant Sci 2020;11:1–19. https://doi.org/10.3389/fpls.2020.01172.
Reis VR, Paula Bassi AG, Cerri BC, Almeida AR, B Carvalho IG, Bastos RG, et al. Effects of feedstock and co-culture of Lactobacillus fermentum and wild Saccharomyces cerevisiae strain during fuel ethanol fermentation by the industrial yeast strain PE-2. AMB Express 2018;8. https://doi.org/10.1186/s13568-018-0556-9.
Ren Y, Yuan TZ, Chigwedere CM, Ai Y. A current review of structure, functional properties, and industrial applications of pulse starches for value-added utilization. Compr Rev Food Sci Food Saf 2021;20:3061–92. https://doi.org/10.1111/1541-4337.12735.
RFA. 2023 Ethanol Industry Outlook 2023:40.
Rho Y, Kiarie E, de Lange CKFM. Nutritive value of corn distiller's dried grains with solubles steeped without or with exogenous feed enzymes for 24 h and fed to growing pigs. J Anim Sci 2018;96:2352. https://doi.org/10.1093/JAS/SKY115.
Ribeiro-Filho N, Linforth R, Bora N, Powell CD, Fisk ID. The role of inorganic-phosphate,

potassium and magnesium in yeast-flavour formation. Food Res Int 2022;162:112044. https://doi.org/10.1016/j.foodres.2022.112044.
Robertson GH, Wong DWS, Lee CC, Wagschal K, Smith MR, Orts WJ. Native or raw starch digestion: A key step in energy efficient biorefining of grain. J Agric Food Chem 2006;54:353–65. https://doi.org/10.1021/JF051883M/ASSET/IMAGES/LARGE/JF051883MF00002.JPEG.
Robinson PK. Enzymes: principles and biotechnological applications. Essays Biochem 2015;59:1–41. https://doi.org/10.1042/BSE0590001.
Rombouts I, Wouters AGB, Lambrecht MA, Uten L, Van Den Bosch W, Vercruysse SAR, et al. Food protein network formation and gelation induced by conductive or microwave heating: A focus on hen egg white. Innov Food Sci Emerg Technol 2020;66:102484. https://doi.org/10.1016/j.ifset.2020.102484.
Rosa-Millan J de la, Orona-Padillla JL, Flores-Moreno VM, Serna-Saldivar SO. Effect of jet-cooking and hydrolyses with amylases on the physicochemical and in vitro digestion performance of whole chickpea flour. Int J Food Sci Technol 2020;55:690–701. https://doi.org/10.1111/ijfs/14338.
Roy JK, Borah A, Mahanta CL, Mukherjee AK. Cloning and overexpression of raw starch digesting α-amylase gene from Bacillus subtilis strain AS01a in Escherichia coli and application of the purified recombinant α-amylase (AmyBS-I) in raw starch digestion and baking industry. J Mol Catal B Enzym 2013;97:118–29. https://doi.org/10.1016/J.MOLCATB.2013.07.019.
Saadi S, Saari N, Ghazali HM, Abdulkarim MS. Mitigation of antinutritional factors and protease inhibitors of defatted winged bean-seed proteins using thermal and hydrothermal treatments: Denaturation/unfolding coupled hydrolysis mechanism. Curr Res Food Sci 2022;5:207–21. https://doi.org/10.1016/J.CRFS.2022.01.011.
Sakwa L, Cripwell RA, Rose SH, Viljoen-Bloom M. Consolidated bioprocessing of raw starch with Saccharomyces cerevisiae strains expressing fungal alpha-amylase and glucoamylase combinations. FEMS Yeast Res 2018;18:85. https://doi.org/10.1093/FEMSYR/FOY085.
Salmoral EM, Gonzalez ME, Mariscal MP. Biodegradable plastic made from bean products. Ind Crops Prod 2000;11:217–25. https://doi.org/10.1016/S0926-6690(99)00057-6.
Sanchez de la Concha BB, Agama-Acevedo E, Nuñez-Santiago MC, Bello-Perez LA, Garcia HS, Alvarez-Ramirez J. Acid hydrolysis of waxy starches with different granule size for nanocrystal production. J Cereal Sci 2018;79:193–200. https://doi.org/10.1016/J.JCS.2017.10.018.
Semba RD, Ramsing R, Rahman N, Kraemer K, Bloem MW. Legumes as a sustainable source of protein in human diets. Glob Food Sec 2021;28:100520. https://doi.org/10.1016/j.gfs.2021.100520.
Seneviratne HD, Biliaderis CG. Action of α-amylases on amylose-lipid complex superstructures. J Cereal Sci 1991;13:129–43. https://doi.org/10.1016/S0733-5210(09)80030-1.
Seo SH, Cho SJ. Changes in allergenic and antinutritional protein profiles of soybean meal during solid-state fermentation with Bacillus subtilis. LWT 2016;70:208–12. https://doi.org/10.1016/J.LWT.2016.02.035.
Shareghi B, Arabi M, Zargham M. Denaturation of Bacillus amyloliquefaciens α-amylase with urea. Pakistan J Biol Sci 2007;10:3154–7. https://doi.org/10.3923/PJBS.2007.3154.3157.

Shevkani K, Kaur A, Kumar S, Singh N. Cowpea protein isolates: Functional properties and application in gluten-free rice muffins 2015. https://doi.org/10.1016/j.lwt.2015.04.058.
Shevkani K, Singh N. Influence of kidney bean, field pea and amaranth protein isolates on the characteristics of starch-based gluten-free muffins. Int J Food Sci Technol 2014;49:2237–44. https://doi.org/10.1111/ijfs.12537.
Shevkani K, Singh N, Chen Y, Kaur A, Yu L. Pulse proteins: secondary structure, functionality and applications. J Food Sci Technol 2019;56:2787–98. https://doi.org/10.1007/s13197-019-03723-8.
Shi C, Zhang Y, Lu Z, Wang Y. Solid-state fermentation of corn-soybean meal mixed feed with Bacillus subtilis and Enterococcus faecium for degrading antinutritional factors and enhancing nutritional value. J Anim Sci Biotechnol 2017;8:1–9. https://doi.org/10.1186/S40104-017-0184-2/TABLES/3.
Silva VFN, Nakanishi SC, Dionísio SR, Rossell CEV, Ienczak JL, Gonçalves AR, et al. Using cell recycling batch fermentations to validate a setup for cellulosic ethanol production. J Chem Technol Biotechnol 2016;91:1853–9. https://doi.org/10.1002/JCTB.4778.
Singh J, Berg T, Hardacre A, Boland MJ. Cotyledon Cell Structure and In Vitro Starch Digestion in Navy Beans. Elsevier Inc.; 2014. https://doi.org/10.1016/B978-0-12-404610-8.00008-6.
South PK, Miller DD. Iron binding by tannic acid: Effects of selected ligands. Food Chem 1998;63:167–72. https://doi.org/10.1016/S0308-8146(98)00040-5.
Spies RD, Hoseney RC. Effect of sugars on starch gelatinization. Cereal Chem 1982;59:128.
Statistics Canada. Table: 32-10-0359-01 Estimated areas, yield, production, average farm price and total farm value of principal field crops, in metric and imperial units 2023. https://www150.statcan.gc.ca/t1/tbl1/en/tv.action?pid=3210035901 (accessed March 22, 2023).
Strąk-Graczyk E, Balcerek M. Effect of Pre-hydrolysis on Simultaneous Saccharification and Fermentation of Native Rye Starch. Food Bioprocess Technol 2020;13:923–36. https://doi.org/10.1007/s11947-020-02434-9.
Subedi S, Suttisansanee U, Kettawan A, Chupeerach C, Khemthong C, Thangsiri S, et al. Food Fortification of Instant Pulse Porridge Powder with Improved Iron and Zinc Bioaccessibility Using Roselle Calyx. Nutrients 2022;14:4070. https://doi.org/10.3390/NU14194070/S1.
Świątek K, Gaag S, Klier A, Kruse A, Sauer J, Steinbach D. Acid Hydrolysis of Lignocellulosic Biomass: Sugars and Furfurals Formation. Catal 2020, Vol 10, Page 437 2020;10:437. https://doi.org/10.3390/CATAL10040437.
Talebnia F, Karakashev D, Angelidaki I. Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. Bioresour Technol 2010;101:4744–53. https://doi.org/10.1016/J.BIORTECH.2009.11.080.
Tanadul O u. m., Vandergheynst JS, Beckles DM, Powell ALT, Labavitch JM. The impact of elevated CO2 concentration on the quality of algal starch as a potential biofuel feedstock. Biotechnol Bioeng 2014;111:1323–31. https://doi.org/10.1002/BIT.25203.
Tanno KI, Willcox G. The origins of cultivation of Cicer arietinum L. and Vicia faba L.: Early finds from Tell el-Kerkh, north-west Syria, late 10th millennium B.P. Veg Hist Archaeobot 2006;15:197–204. https://doi.org/10.1007/S00334-005-0027-5/TABLES/5.
Tester RF, Karkalas J, Qi X. Starch—composition, fine structure and architecture. J Cereal Sci 2004;39:151–65. https://doi.org/10.1016/J.JCS.2003.12.001.

Thangprompan P, Thanapimmetha A, Saisriyoot M, Laopaiboon L, Srinophakun P. Production of Ethanol from Sweet Sorghum Juice Using VHG Technology: A Simulation Case Study. Appl Biochem Biotechnol 2013;171:294–314. https://doi.org/10.1007/s12010-013-0365-1.
Thomas J, Gargari SG, Tabtabaei S. Tribo-electrostatic separation of yellow pea and its optimization based on milling types and screen sizes. Powder Technol 2023;415:118169. https://doi.org/10.1016/j.powtec.2022.118169.
Thomas KC, Hynes SH, Jones AM, Ingledew WM. Production of fuel alcohol from wheat by VHG technology. Appl Biochem Biotechnol 1993;43:211–26. https://doi.org/10.1007/bf02916454.
Tomás-Pejó E, Alvira P, Ballesteros M, Negro MJ. Pretreatment Technologies for Lignocellulose-to-Bioethanol Conversion. Biofuels 2011:149–76. https://doi.org/10.1016/B978-0-12-385099-7.00007-3.
Tovar J, Björck IM, Asp NG. Analytical and Nutritional Implications of Limited Enzymic Availability of Starch in Cooked Red Kidney Beans. J Agric Food Chem 1990;38:488–93. https://doi.org/10.1021/JF00092A034/ASSET/JF00092A034.FP.PNG_V03.
Trainotti N, Stambuk BU. NaCl stress inhibits maltose fermentation by Saccharomyces cerevisiae. Biotechnol Lett 2001;23:1703–7. https://doi.org/10.1023/A:1012456432280.
Uebersax M, Occena L. Legumes in the Diet. Encycl Food Sci Nutr 2003:3520–8. https://doi.org/10.1016/B0-12-227055-X/00693-3.
Uthumporn U, Zaidul ISM, Karim AA. Hydrolysis of granular starch at sub-gelatinization temperature using a mixture of amylolytic enzymes. Food Bioprod Process 2010;88:47–54. https://doi.org/10.1016/j.fbp.2009.10.001.
Varela C, Pizarro F, Agosin E. Biomass Content Governs Fermentation Rate in Nitrogen-Deficient Wine Musts. Appl Environ Microbiol 2004;70:3392. https://doi.org/10.1128/AEM.70.6.3392-3400.2004.
Vasanthan T. System and Method for Fractionating Grain. U.S. Patent No. 2017/0087596 A1, 2017.
Vasanthan T, Bhatty RS. Physicochemical properties of small- and large-granule starches of waxy, regular, and high-amylose barleys. Cereal Chem 1996;73:199–207.
Vasić K, Knez Ž, Leitgeb M. Bioethanol Production by Enzymatic Hydrolysis from Different Lignocellulosic Sources. Molecules 2021;26. https://doi.org/10.3390/MOLECULES26030753.
Verhe R, Varghese S, Thevelein JM, Nikroo JH, Lambrecht M, Redant E, et al. Production of Bio-Ethanol from the Organic Fraction of Municipal Solid Waste and Refuse-Derived Fuel. Biomass 2022, Vol 2, Pages 224-236 2022;2:224–36. https://doi.org/10.3390/BIOMASS2040015.
Vlassa M, Filip M, Țăranu I, Marin D, Untea AE, Ropotă M, et al. The Yeast Fermentation Effect on Content of Bioactive, Nutritional and Anti-Nutritional Factors in Rapeseed Meal. Foods 2022, Vol 11, Page 2972 2022;11:2972. https://doi.org/10.3390/FOODS11192972.
Wang C, Su W, Zhang Y, Hao L, Wang F, Lu Z, et al. Solid-state fermentation of distilled dried grain with solubles with probiotics for degrading lignocellulose and upgrading nutrient utilization. AMB Express 2018;8:188. https://doi.org/10.1186/S13568-018-0715-Z.
Wang K, Hasjim J, Wu AC, Henry RJ, Gilbert RG. Variation in amylose fine structure of starches from different botanical sources. J Agric Food Chem 2014;62:4443–53.

https://doi.org/10.1021/JF5011676/SUPPL_FILE/JF5011676_SI_001.PDF.
Wang P, Singh V, Xue H, Johnston DB, Rausch KD, Tumbleson ME. Comparison of raw starch hydrolyzing enzyme with conventional liquefaction and saccharification enzymes in dry-grind corn processing. Cereal Chem 2007;84:10–4. https://doi.org/10.1094/CCHEM-84-1-0010.
Wang S, Yu Jinglin, Yu Jiugao, Chen H, Pang J. The effect of acid hydrolysis on morphological and crystalline properties of Rhizoma Dioscorea starch. Food Hydrocoll 2007;21:1217–22. https://doi.org/10.1016/j.foodhyd.2006.08.002.
Wang Shujun, Li C, Zhang X, Copeland L, Wang Shuo. Retrogradation enthalpy does not always reflect the retrogradation behavior of gelatinized starch. Sci Rep 2016;6:1–10. https://doi.org/10.1038/srep20965.
Wang S, Luo H, Zhang J, Zhang Y, He Z, Wang Shuo. Alkali-induced changes in functional properties and in vitro digestibility of wheat starch: The role of surface proteins and lipids. J Agric Food Chem 2014;62:3636–43. https://doi.org/10.1021/JF500249W/ASSET/IMAGES/LARGE/JF-2014-00249W_0006.JPEG.
Warkentin TD, Delgerjav O, Arganosa G, Rehman AU, Bett KE, Anbessa Y, et al. Development and Characterization of Low-Phytate Pea. Crop Sci 2012;52:74–8. https://doi.org/10.2135/CROPSCI2011.05.0285.
Wei C-J, Tanner RD, Malaney GW. Effect of Sodium Chloride on Bakers' Yeast Growing in Gelatin. Appl Environ Microbiol 1982;43:757–63. https://doi.org/10.1128/aem.43.4.757-763.1982.
Weintraub BD, Hamilton GA, Henshaw C, Chase AM. An analysis of the inhibition of β-amylase by urea. Arch Biochem Biophys 1964;107:224–38. https://doi.org/10.1016/0003-9861(64)90324-8.
Whiting GC. Organic acid metabolism of yeasts during fermentation of alcoholic beverages - A review. J Inst Brew 1976;82:84–92.
Winther M, Møller F, Jensen TC. Emission consequences of introducing bio ethanol as a fuel for gasoline cars. Atmos Environ 2012;55:144–53. https://doi.org/10.1016/J.ATMOSENV.2012.03.045.
Wu YV. Fractionation and characterization of protein-rich mineral from barley after alcohol distillation. Cereal 1986;63:142–5.
Wu Z, Lee YY. Inhibition of the enzymatic hydrolysis of cellulose by ethanol. Biotechnol Lett 1997;19:977–9. https://doi.org/10.1023/A:1018487015129/METRICS.
Xu QS, Yan YS, Feng JX. Efficient hydrolysis of raw starch and ethanol fermentation: a novel raw starch-digesting glucoamylase from Penicillium oxalicum. Biotechnol Biofuels 2016;9:1–18. https://doi.org/10.1186/s13068-016-0636-5.
Zeng M, Mosier NS, Huang C-P, Sherman DM, Ladisch MR. Microscopic Examination of Changes of Plant Cell Structure in Corn Stover Due to Hot Water Pretreatment and Enzymatic Hydrolysis. Wiley Intersci (WwwInterscienceWileyCom) Biotechnol Bioeng 2007;97:265–78. https://doi.org/10.1002/bit.21298.
Zhang X, Wang L, Xu J, Yuan J, Fan X. Effect of Starch Chain Structure and Non-Starch Components on the Hydrolysis of Starch by α-Amylase. Starch/Staerke 2022;74. https://doi.org/10.1002/STAR.202100107.
Zheng Q, Zhou T, Wang Y, Cao X, Wu S, Zhao M, et al. Pretreatment of wheat straw leads to structural changes and improved enzymatic hydrolysis OPEN. Sci Rep 2018;8.

https://doi.org/10.1038/s41598-018-19517-5.
Zhong Y, Liu L, Qu J, Blennow A, Hansen AR, Wu Y, et al. Amylose content and specific fine structures affect lamellar structure and digestibility of maize starches. Food Hydrocoll 2020;108:105994. https://doi.org/10.1016/J.FOODHYD.2020.105994.
Zhou Y, Hoover R, Liu Q. Relationship between α-amylase degradation and the structure and physicochemical properties of legume starches. Carbohydr Polym 2004;57:299–317. https://doi.org/10.1016/j.carbpol.2004.05.010.
Zhu F, Liu P. Starch gelatinization, retrogradation, and enzyme susceptibility of retrograded starch: Effect of amylopectin internal molecular structure. Food Chem 2020;316:126036. https://doi.org/10.1016/j.foodchem.2019.126036.