Improving saccharification process by pre-swelling of normal maize starch granules for production of sugar and fermented chemicals

by

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B.S., Beihang University, 2013
M.S., University of Southern California, 2015

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
College of Agriculture

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Abstract

Partial swelling of granules above the gelatinization temperature was investigated as a strategy to enhance the enzymatic hydrolysis of normal maize starch to glucose. Native and partially swollen starches were hydrolyzed by a granular starch hydrolyzing enzyme (GSHE). After preheated at 70 °C for 30 min, enzyme kinetics study showed a 54% reduction in the Michaelis-Menten constant value (Km), suggesting that preheating increased the affinity of GSHE for the starch granules. Moreover, 94.8% of starch (2% in H_2O, w/w) was converted to glucose after a 24 h saccharification process. This relatively low-temperature process reduced the energy required to completely destroy the starch granules. Preheating at 70 °C, which resulted in partial swelling of starch granules, induced a greater degradation of large molecules, enzymatic erosion of crystallinity and granular structure. In addition, the enzyme resistant fraction could be converted to glucose after cooking. A full conversion of normal maize starch to glucose by GSHE could be achieved.

In the saccharification process with a high maize starch concentration (30% in H_2O, w/w), partial swelling starch granules would result in viscosity build-up problem. To overcome that, we used an α-amylase during heat pretreatment. The viscosity decreased greatly from 2.85×10^6 cP to 12 cP, which was preferable in later saccharification. The heat treatment with α-amylase at 70 °C partially destroyed crystalline lamellae and maize starch granule structure.

By combining α-amylase in the preheating process and saccharification by GSHE, a two-step enzymatic hydrolysis process was performed. Starch granules were pre-hydrolyzed by α-amylase at 70 °C for 6 h and followed the addition of GSHE and incubation at 62 °C for 72 h. The two-step enzymatic hydrolysis was more effective than the single hydrolysis at 62 °C and increased the conversion by 25%. More than 93% of total starch could be converted to glucose

and the enzyme resistant residue could be further hydrolyzed by conventional cooking method. The two-step enzymatic hydrolysis offered great advantages in the production of glucose syrups and other fermentable chemicals.

To further investigate the potential utilization of partially swollen maize starch with GSHE in the production of fermented chemicals, productions of citric acid and ethanol by low-temperature fermentations were studied in both lab-scale and large pilot scale. In the production of citric acid, maize starch (18% in H_2O, w/w) was fermented at 37 °C for 67 h. The initial substrate concentration (18%) was 2% greater than the starch concentration used in the conventional cooking process. The yield of the citric acid was 88%, which was 3% higher than that of conventional cooking production. For ethanol production, maize flour (30% in H_2O, w/w) was fermented at 32 °C for 72 h. The ethanol yield was 92.6%, which was 3.5% higher than that of ethanol produced by the cooking method.

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Chapter 1 - Overall Introduction

In conventional sugar production, starch is converted to glucose by liquefaction and saccharification. The jet cooking process in liquefaction comes with huge energy consumption which is not preferred by industry production.

To reduce the energy cost, granular starch hydrolyzing enzyme (GSHE) is used without cooking a starch. However, the maximum conversion of the normal maize starch to glucose was only 50% in our previous study. No work was done to understand why higher conversion was not achieved. In this study, partial swelling of granules above the gelatinization temperature was investigated as a strategy to enhance the enzymatic hydrolysis of normal maize starch to glucose. Native maize starch was heated at 70 °C to partially swell maize starch granules. Partial swelling of granules would increase enzyme efficiency by increasing the affinity of GSHE for the starch granules. It could enhance the conversion of starch to glucose at a relative low temperature. In this dissertation, a method of combining partial swelling starch granule with GSHE was studied to produce sugar and two fermented chemicals, citric acid and ethanol.

In the second chapter, the recent advances in citric acid production are reviewed. Substrates for fermentation and fermentation process are examined. Current industry production is summarized. Studies and patents in citric acid production in the past ten years are also reviewed.

In the third chapter, the effects of partial swelling on the saccharification of maize starch at a low concentration by GSHE were studied. The enzyme resistant residues were characterized. The conditions for glucose production were optimized.

In the fourth chapter, high-solid starch slurry saccharification process was examined. Partial swelling of starch was achieved in the presence of α-amylase. This mild heat treatment

reduced the viscosity during heating and helped hydrolyze crystalline regions as well as amorphous regions in starch granules.

In the fifth chapter, partial swelling in the presence of α-amylase was combined with GSHE saccharification at low temperature to enhance the bioconversion of granular maize starch. The saccharification efficiency and mechanisms were determined.

In the sixth chapter, partial swelling of maize starch was applied in the production of citric acid by fermentation. The approach was compared with the conventional method. The optimal substrate concentration, enzyme level, and preheating time were investigated.

In the seventh chapter, the method of partial swelling starch in maize flour was combined with GSHE in saccharification and production of ethanol by fermentation. Substrate concentration, pH, GSHE level, and extra glucoamylase level were optimized for ethanol production.

Chapter 2 - Citric acid production: a review on substrates and processes

Abstract

Citric acid is the world's largest consumed organic acid and the second largest fermented products after ethanol. It has high economic potential due to its numerous applications. The demand and production of citric acid are increasing gradually, along with higher yield efficiency and more advanced technologies. In this paper, we reviewed the most common two substrates used in citric acid fermentation - maize flour and maize starch and the related fermentation processes based on these two substrates. The new studies using alternative substrates and new citric acid fermentation methods in the past ten years are discussed. The latest patents on the production of citric acid are summarized. This review will help us to have a better understanding of the process of citric acid production, help design and improve current industrial citric acid production.

Introduction

Citric acid (2-hydroxy-propane-1,2,3-tricarboxylic acid) is a constituent from citrus plants and an intermediate of the tricarboxylic acid cycle with a molecular weight of 210.14 g/mol (Dhillon, Brar, Verma & Tyagl, 2011). It contains three carboxylic groups with three different pK_a values (3.13, 4.76, and 6.40). It is a common intermediate product of natural and physiological metabolism. Commercial citric acid is a colorless, transparent or translucent crystal or particle powder with strong acidity. It is widely used in the food industry (75%), the pharmaceutical industry (10%) and other industrial fields (15%). (Ates, Dingil, Bayraktar, & Mehmetoglu, 2002; Tran, Sly, & Mitchell, 1998). Citric acid has a pleasant acid taste, non-toxic

and been identified as a safe good additive by FAO/WHO (Soccol & Vandenberghe, 2003). In the beverage industry and brewing wine process, citric acid not only provide a fruit flavor but also enhance the soluble, antioxidant and antiseptic ability. In jam and jelly production, citric acid is mainly used for adding sour flavor and adjusting pH for pectin gelling. In frozen food, citric acid functions as chelating and regulating of pH, preventing oxidation and improving the stability of frozen food (Bal'a & Marshall, 1998; Chang & Holtzapple, 2000; Sommers, Fan, Handel, & Baxendale Sokorai, 2003). In the pharmaceutical industry, it can serve as a foaming agent, reacting with sodium carbonate to produce a large amount of carbon dioxide gas, which can help dissolve the active ingredients in drug and improve the tasting ability. In cosmetics, citric acid may serve as an ion chelating agent which can enhance the anticorrosion effect of wax (Yang, Webb, & Ameer, 2004). In other industrial areas, citric acid as a weak organic acid can effectively remove the metal surface oxides as a cleaning agent or as detergents (Ousmanova & Parker, 2007).

Citric acid is the world's largest consumed organic acid and the second largest fermented product after ethanol (Gurpreet Singh Dhillon, Brar, Kaur, & Verma, 2013). The global production capacity of citric acid has increased from 1.7 million tons in 2006 to 2.69 million tons in 2015. Table 2.1 summarizes the production status of the world citric acid bio-technology enterprises in 2017 (Zhou and Peng, 2018). In 2017, there were about 12 producers of citric acid in the world, with a total output of about 2.57 million tons. Companies including Tate & Lyle, ADM, Cargill, and Jungbunzlauer account for about 25% of the world's total citric acid production (Zhou and Peng, 2018). China is the world's largest producer of citric acid, accounting for 75 percent of all production (Zhou and Peng, 2018). The total industry output in China was about 1.92 million tons in 2017 (Table 2.1). More and more industrial factories were

built due to the huge demand for citric acid worldwide. According to the statistics of China Starch Industry Association in 2018, the ex-factory price of citric acid monohydrate is 4900-5100 yuan/ton, and the ex-factory price of anhydrous citric acid is 5400-5500 yuan/ton.

The production of citric acid by microbial fermentation has many advantages comparing with directly extracting from citrus fruits, which can be done at low temperature, low pH and high sugar conditions. At present, the types of microorganisms used for industrial production is *A. niger*, and the submerged fermentation is the mainstream fermentation technology in current industry production. The latest advances in citric acid strains and system metabolic engineering in citric acid fermentation have been reviewed (Tong et al., 2019), and will not be covered in this review.

In this review, we focus on substrates and fermentation process in the production of citric acid. Details about current industrial processing methods in citric acid production are discussed. The whole manufacturing path from maize grains to citric acid is summarized based on two main substrates: maize flour and maize starch. Recent lab studies of potential new substrates and new improved fermentation methods in lab scale are summarized as well. In addition, the latest patents on production of citric acid are reviewed and summarized.

Table 2.1 Production status of citric acid worldwide in 2017[A]

Number	Company	Regions	Production(ton)
1	YingXuan	China	60
2	Jinhe	China	36
3	Shenghua	China	40
4	Cofco	China	20
5	Xielian	China	20.9
6	Taihe	China	14.9
7	Jungbunzlauer	Austria and Canada	19
8	Tate&Lyle	Brazil, Columbia and America	12.6
9	Cargill	America and Brazil	12
10	CBT	Belgium	11

11	ADM	America	9
12	Ninglang	Thailand	2
		Total	257.4

[A] Adapted from Zhou and Peng (2018).

Substrates in citric acid production

Current industrial substrate

The main material could be used as the citric acid fermentation medium is starchy or raw materials containing glucose and sucrose, such as potato, tapioca, maize, rice, etc. Compared with other raw materials, maize is abundant, cheaper and easy to process. In citric acid industry production, maize grain has been the most common raw materials for fermentation of citric acid. With the development of technology and the pursuit of high value-added benefits of by-products, isolated starch has been successfully used as a substrate for fermentation production. This section focuses on the substrate process from maize grain to maize flour and maize starch in citric acid production. The important technical details and the advantages and disadvantages of both substrates are covered in this section. Other processes after liquefaction will be discussed in the next citric acid processing part.

Mazie flour in citric acid production

Maize grain used in citric acid production is divided into dry processing treatment and wet processing treatment. In maize flour process, mainly dry maize processing is used, consisting of impurity removal, maize grain pulverization, liquefaction, solid-liquid separation, and fermentation. The whole treatment process is summarized in Figure 2.1.

The raw maize grain contains impurities such as sand, metal, and fiber. These impurities often cause equipment damage, pipe blockage, and other damage to normal production processes, and therefore must be removed. Generally, the maize impurities are removed by

equipment such as maize cutting grid, primary cleaning sieve, permanent magnet barrel, etc., and clean maize is provided for the next pulverization process.

The pulverization further destroys the grain tissue, releases the starch, increases interactions between starch granules and water which are beneficial to the starch swell, gelatinization, and liquefaction and it could improve the heat treatment efficiency.

The pulverized particle size of maize grain has a great influence on gelatinization. In most cases, the finer the pulverization, the better. From the gelatinization perspective, the fine particle size gives good solubility and it is easy to gelatinize. If the particle size is too large, the steam is difficult to penetrate into the interior of the powder, which may cause incomplete gelatinization of starch. The resistance of the crystal structure of the starch granules to the enzyme is great when starch is not gelatinized, resulting in low fermentation efficiency. However, from the filterability perspective, if the particle is too small, the starch granules tend to block the filter cloth, which is not favorable in filtration. The bad filtration will result in loss of sugar due to the increased sugar content in the filter residue. In addition, if the pulverization is too small, and the energy consumption is also increased. Therefore, it is not necessary to over pulverize the grain. Gopinath (2013) studied different the influence of particle size on citric acid production. He ground and sieved different size grain chaff particles and further fermented for citric acid production. The results showed when particle size was about 4mm, the substrates reach its optimal conversion rate. There was also a distinctive yield decrease when the particle size is below 2 mm (Gopinath, 2013).

Maize flour is mixed with water and steeped in water prior to liquefaction. The purpose of steeping is to increase the absorption of water, soften tissue softening and make starch swell, thereby increasing the liquefaction effect of the raw materials. The steeping effect is dependent

on the steeping time, the quality of the raw materials, and the water temperature. The water temperature should not be too high. If the starch is genitalized and agglomerated at high temperatures, that would cause difficulty in transferring substrate and may result in incomplete liquefaction. In addition, it is necessary to prevent the long-time and low-temperature immersion, because the lactic acid bacteria are easily fermented at a low temperature which will influence the liquefaction due to the accumulation of lactic acid.

After the maize flour is evenly mixed with water, α-amylase is added. The pH of the liquid is adjusted to the specified range (5.8-6.2) with calcium hydroxide. Then the slurry is heated once (water heater), continuous laminar liquefaction, secondary heating (water heater), vapor-liquid two-phase separation (flashing), and then enter the maintenance tank to continue liquefaction. In the maintenance tank liquefaction, the iodine solution is used to determine the liquefaction endpoint.

After the liquefaction, the slurry is filtered through the plate frame to remove the insoluble residues such as enzyme resistant starch residue, insoluble protein, and fiber. The filtrate is transferred to the sugar liquid storage tank for the fermentation process. In addition, a small portion of the non-filtered liquefaction liquid is used as a nitrogen source for fermentation.

The insoluble residues are dried and recovered as starch slag feed. Liquified residue mixtures are dried through a heated metal tube, in which the water is vaporized.

Due to the nutrient-rich nature of the maize flour, the liquefied solution provides almost all the nitrogen, phosphorus and other metal ion required for citric acid seed growth and fermentation. As a result, using liquefied maize flour for fermentation requires almost no additional nutrients. However, the composition of maize grain is varied by the region and year, thus the stability of the medium is poor and fluctuates greatly. The fermentation conditions need

to be adjusted every time when using different maize grains. The amount of nitrogen and phosphorus is also hard to control due to the variations in raw materials and different pretreatments.

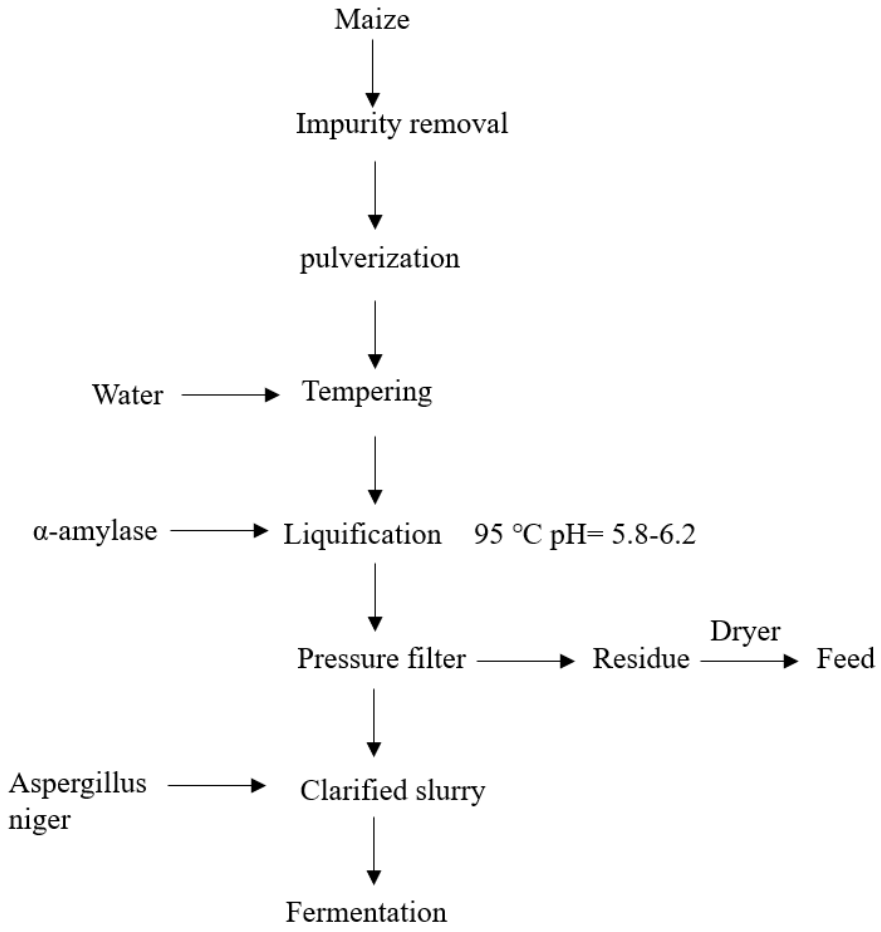

Figure 2.1 Process flow chart of maize flour for citric acid production

Maize starch in citric acid production

With the gradual increase in maize prices, the cost of maize grain for citric acid fermentation has gradually increased. Therefore, companies have studied various ways to reduce cost, increase starch utilization, and increase the production of high-value by-products. Thus, a process of using starch or starch milk in citric acid production has been developed. Chinese bio-manufacturing companies such as the China National Cereals, Oils and Foodstuffs Corporation

(COFCO), have successfully established citric acid production lines using starch milk recently (Zhou and Peng, 2018).

The starch production and isolation for citric acid production are the same methods as the wet milling process. That includes impurity removal, steeping, milling, centrifugation, and separation. The industrial flow chart of wet milling is summarized in Figure 2.2. The technical details of the maize starch production have been reviewed by Tong (2018) and BeMiller (1984).

The starch milk has some advantages over traditional maize flour slurry. Due to the rapid development of industrial technology for starch production, the starch recovery rate during starch production is significantly improved. For one ton of maize grain, 0.6248 tons maize starch can be recovered in industrial starch production. On the other hand, the recovery of maize starch in the maize flour fermentation is only about 0.61 tons.

Since starch milk is purer and only provide the carbon source, for different batches of raw material, nutrients formula does not need to be changed. Therefore, the composition of the medium is more controllable, and the performance of the medium is stable which was more preferable by industrial production.

In the medium, since the pH of the maize milk is low, in order to adapt to the pH requirement of the *A. niger*, it is necessary to adjust the pH by adding ammonia. Ammonia can both adjust the pH and provide the nitrogen source in the medium. In addition, in the citrate metabolism of *A. niger*, ammonium can influence ATP feedback inhibition of glycolysis EMP pathway and inhibit the synthesis of a-ketoglutarate dehydrogenase, which is beneficial to citric acid accumulation.

By separating maize starch from maize grain, high-value germ product and feedstock are produced that increases the overall profit of the whole production. At the same time, after the

fermentation and solid-liquid separation, pure mycelium from *A. niger* is obtained. There is no maize husk, fiber and other impurities in the high-protein mycelium, and the mycelium can be upgraded directly into feedstock.

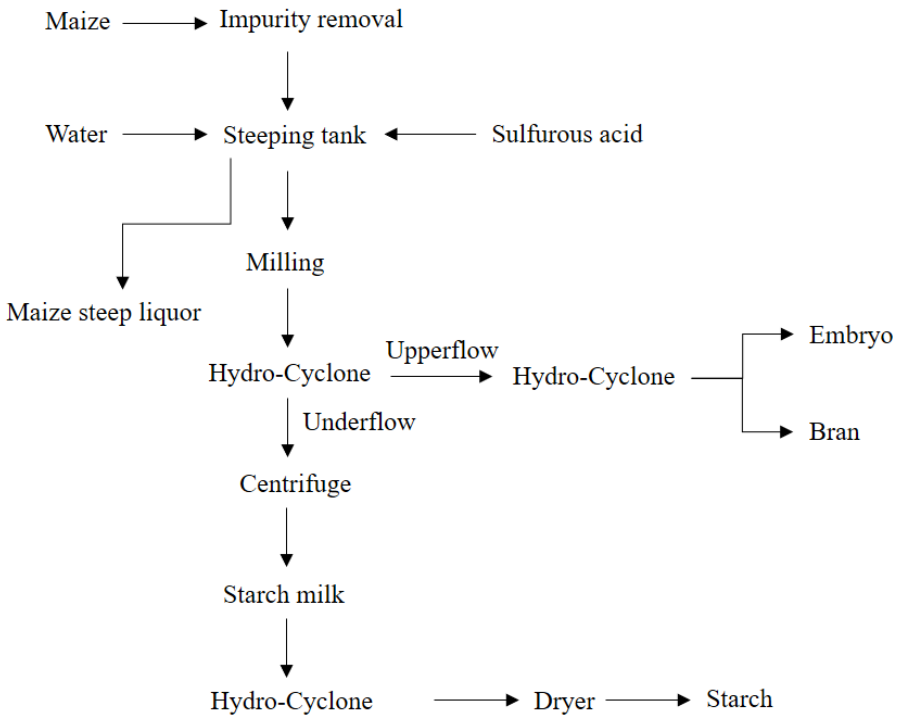

Figure 2.2 Process flow chart of maize starch production

Other potential substrates in citric acid production

Other feedstocks such as agro-industrial waste residues and industrial waste oils have been studied as potential carbon sources for the citric acid production (Ali, Anwar, Irshad, Mukhtar, & Warraich, 2016; Betiku & Adesina, 2013; Kamzolova, Lunina, & Morgunov, 2011). Table 2.2 lists different feedstocks and fermentation types used on citric acid fermentation in the recent ten years. Although other synthetic methods of citric acid production have been published, those chemical methods are uneconomical due to the feedstock usually expensive than the final products.

Agro-industrial waste has been studied as one of the best substrates for citric acid fermentation. This agro-industrial waste provides not only the carbon source but also the needed nutrients for yeast growth. Khosravi-Darani and Zoghi (2008) used different pretreatments of acid, alkali, urea on bagasse, and found that bagasse pretreated with urea could achieve the citric acid yield of 137.6 g/Kg (dry basis). The olive mill which is known as most polluted wastes with high phenolic content was used for citric acid production by Seda et al. (2016). By filtrating with charcoal, adjusting pH and centrifugation, they reduced harmful phenolic compounds by 70% and achieved citric acid of 8.18 g/L (Seda Karasu-Yalcin, Eryasar, Guler, Özdemir, & Baggul, 2016). Zhou and her colleagues provided a biorefining process which achieved a citric acid yield of 94.11% with maize stover (Zhou, Meng, & Bao, 2017). This method was comparable to sugar or starch-based citric fermentation with lower raw material cost.

Glycerol as an important byproduct of biofuel production is another potential substrate used for citric acid production. The increased production of biofuel leads to excess quantities of glycerol. Use the extra glycerol could not only add value to biofuel production but reduce citric acid production cost (Mitrea, Trif, Cătoi, & Vodnar, 2017). Raw glycerol was used as the feedstock for citric acid production as well. Under optimal conditions, using fed-batch cultivation and fermented with Yarrowia lipolytica strains, researchers achieved a 115 g/L citric acid solution product (Morgunov, Kamzolova, & Lunina, 2013). Soy-based glycerol was also used for citric acid fermentation with a new Candida parapsilosis. After growing in 10 g/L and 60 g/L glycerol culture medium, 3 g/L and 10.4 g/L citric acid yields were observed respectively (West, 2013).

Considering all the different substrates, starchy materials are still the best feedstock for large-scale citric acid production. While low-cost feedstock and industrial waste have broadened

the availability of raw materials for citric acid, offer a potential reduction in raw materials, and alleviate the environment pressure, but the complex procedure increases the extraction difficulty of products. The overall cost can be higher with the cheap feedstock. In addition, the rheological properties of raw materials (viscosity and particle size) need to be changed to improve the transmission of oxygen and mass transfer. Therefore, the application of new raw materials for industrial-scale production needs further study.

Table 2.2 Alternative substrates in citric acid production

substrate type	Fermentation type	Incubation temperature and time	References
sweet potato starch	surface fermentation	30 °C for 7 days	Betiku & Adesina, 2013
olive-mill wastewater		28 °C for 350 hours	Papanikolaou, Galiotou-Panayotou, Fakas, Komaitis, & Aggelis, 2008
wastewater		37 °C for 72 hours	Xu et al., 2016
corn steep	submerged fermentation	28 °C for 288 hours	Liu et al., 2015
extraction wastewater		36 °C for 72 hours	Xu et al., 2015
waste cooking oil		28 °C for 336 hours	Liu, Lv, et al., 2015
cane molass		30 °C for 10 days	Justin, Viateur, & Prudentienne, 2010
banana peel	solid state fermentation	28 °C for 72 hours	Karthikeyan & Sivakumar, 2010
beet molass		30 °C for 8 days	Guc & Erkmen, 2017
apple pomace		30 °C for 144 hours	Dhillon, Kaur, Sarma, & Brar, 2013
sugarcane		30 °C for 5 days	Khosravi-Darani & Zoghi, 2008
areca husk		30 °C for 120 hours	Narayanamurthy, Ramachandra, Rai, Manohara, & Kavitha, 2008
Carob pod		30 °C for 7 days	Lingappa, Pramod, & Ali, 2007
corn distillers grain		25 °C for 240 hours	Xie & West, 2006

glycerol	28 °C for 144 hours	Morgunov, Kamzolova, & Lunina, 2013
corn stover	33 °C for 8 days	Zhou, Meng, & Bao, 2017
agro-waste	30 °C for 5 days	Ali, Anwar, Irshad, Mukhtar, & Warraich, 2016
agro-industrial waste	30 °C for 7 days	Gurpreet S Dhillon, Brar, Kaur, & Verma, 2013
orange peel	33 °C for 72 hours	Hamdy, 2013
rapeseed oil	28 °C for 168 hours	Kamzolova, Lunina, & Morgunov, 2011
olive mill wastewater	28 °C for 187 hours	Sarris, Galiotou-Panayotou, Koutinas, Komaitis, & Papanikolaou, 2011
molasses	35 °C for 96 hours	Javed, Asgher, Sheikh, Nawaz, & Jamil, 2011
Potato waste	25 °C for 5 days	Afifi, 2011

Fermentation process to produce citric acid

Corn flour fermentation process

Clarified liquefied maize flour

In industrial citric acid production from maize flour, due to the high content of protein and other mineral ions, clarified liquefied maize flour instead of liquefied flour slurry is used. The clarified liquid is the filtered liquid obtained after liquefied flour slurry goes through a pressure filter.

In liquidized maize flour slurries, the ratio of carbon source and nitrogen source in maize flour is below 20, and the phosphorus content in maize flour is above 2500 ppm, resulting in too rich nutrients in the medium, and the bacteria grows fast which directly lead to low citric acid production. While in the clarified liquid, the ratio of carbon source and nitrogen source can be adjusted, so that the nutrient in the medium is more suitable for citric acid production. Other nutrients like phosphorus and minerals in the medium can be easily adjusted as well.

Clarified liquid fermentation is carried out by pressure filter, so that most of the solids in the sugar liquid are trapped which contain a large part of the protein and phosphorus and other insoluble residues, and are removed. By using the clarified liquid, the seed tank medium and the fermenter medium can be adjusted according to the optimum ratio, thereby achieving the nutrient requirement of the seed and obtaining the most suitable environment for *A. niger*.

Due to the nutrient-rich nature of maize liquefaction liquid, almost all the nitrogen, phosphorus and metal ions required for citric acid seed growth and fermentation are covered. Therefore, the use of maize clarified liquid fermentation requires very small additional nutrients.

Since the maize clarified liquid fermentation process removes most of the solid in the liquefied liquid, the viscosity in the material is lowered, the oxygen dissolution effect in the fermentation process is greatly improved, and the production energy consumption is greatly reduced.

Fermentation by clarified maize flour liquid

The fermentation process of maize flour is summarized in Figure 2.1. The liquefied maize slurry first passes through pressure filter to get clarified liquid which is served as fermentation medium. The purified air and seed (culture) are further added in the fermenter. With consistent stirring and oxygen input, the fermentation usually lasts for 60-80 h. There are important factors that need to be monitored and adjusted during citric acid fermentation including pH, aeration, temperature, and agitation. Table 2.3 summarizes different factors' influence on citric acid fermentation in recent studies.

In the fermentation process, when pH changes, enzyme and microorganism's efficiency is affected. Changes in pH may also lead to different products. In citric acid fermentation, *A. niger* produces citric acid when pH is around 3, but may produce oxalic acid when pH near 7.

With the accumulation of citric acid, the pH drops dramatically, particularly when pH is below 2.00. Consequently, saccharifying enzyme activity is inhibited and glucose formation rate is dropped as well. Synthesis rate decreases and fermentable sugar content remains very high. High residual sugar significantly reduces the conversion. At the same time, that further increases the difficulty of product isolation and purification (Lv et al., 2016). Membrane separation and column chromatography equipment may be used to solve these problems, but add costs. Because pH changes in a certain range during the fermentation process, monitoring pH change has become an important indicator for determining fermentation status. Effect of initial pH on citric acid fermentation has been studied under submerged fermentation. Sankpal et al. (2001) suggested that for carob pods, pH 5.5 was the optimum fermentation setting when *A. niger* MTCC 28 strain was used.

Temperature is also an important factor in citric acid fermentation. It may influence the reaction rate, microbial cell growth rate, and yeast metabolites. Extreme temperature may lead to the protein denaturation and inhibit microorganism function. As for citric acid fermentation, researchers usually conduct the fermentation under a temperature range of 25-37 °C. Karasu and his colleagues used a statistic method to obtain the optimal temperature in citric acid fermentation which is around 30 °C (Karasu-Yalcin, Tijen Bozdemir, & Yesim Ozbas, 2010). In the study, they observed a dramatic citric acid yield decreasing when the temperature was not optimal.

Agitation is another important environmental factor in citric acid fermentation. It helps transfer with heat and oxygen in the system. The proper agitation could help remove metabolic products, prevent agglomerates which may enhance the microbial growth. Agitation improves substrate and nutrient distribution which would lead to a higher product yield. Anita et al. (2012)

tested different agitation rates in citric acid production and found that when agitation rates set as 800 and 900 rpm, it could increase oxygen dissolution by around 40% which led to a great yield.

Aeration is used to adjust the amount of oxygen and carbon dioxide in the system. By proper aeration, heat and moister in the system can be transferred which is favored by the microorganism. Oxygen and carbon dioxide are the reactant and product in the citric acid production respectively. The ratio of oxygen to carbon dioxide concentration is an important factor in adjusting the reaction equations. Increasing oxygen concentration can enhance yeast metabolism. However, too high oxygen concentration would also enhance microorganism growth which inhibits citric acid production. In a recent study, Rodrigues et al. (2013) used an Erlenmeyer flask for citric acid ferment aeration and found that proper increasing carbon dioxide ratio in fermentation atmosphere would restrict microorganism growth and enhanced citric acid production.

By proper control of these environmental factors, a high yield may be achieved with few extra expenses. The automation technology during the fermentation process such as pH control, dissolved oxygen control, compensation methods can better enhance the strains performance and increase the yield. Those methods have been successfully applied to the amino acid and organic acid fermentation production (Cheng et al., 2013; Li, Lin, Chang, Jin, & Wang, 2015; Riaz et al., 2012; Sun et al., 2012; Zhang, Liu, Li, Du, & Chen, 2012). In short, the control technologies provide new solutions on how to limit the loss of glycosylase efficiency during the fermentation process. Though adjusting environment condition could enhance product yield, we still need to select right strain derivatives to achieve the optimal fermentation results.

Table 2.3 Different environment factors affecting citric acid production

Environment factors	Optimal settings	Effect on citric acid fermentation	Reference

pH	5.5	Enhance citric acid yield	Sankpal et al., 2001
Particle size	4mm	Increase reaction surface	Gopinath, 2013
Temperature	30 °C	Increase microbial cell growth and metabolites	Karasu-Yalcin, Bozdemir, & Ozbas, 2010
Aeration	Erlenmeyer flask	Restrict microorganism growth	Rodrigues et al., 2013
Agitation	800 or 900 rpm	Enhance oxygen dissolution	Anita, lzabela, Waldemar, Barbara, & Tomasz, 2012

Fermentation process using maize starch

Conversion of starch to sugar

Though the maize flour is widely used in citric acid production, it has some drawbacks which were discussed in Section 2. Researchers in China have developed new substrate and technology on citric acid production. In the new industrial production of citric acid, starch is the starting raw material for citric acid production. Starch is liquefied and saccharified to obtain fermentable sugar solution at the beginning and then other nutrients are added for fermentation.

One of the differences between maize starch and maize flour in citric acid production is that the new method usually has a saccharification process which would further convert dextrin to glucose or other fermentable sugar. Since the α-amylase and glucoamylase secreted by *A. niger* is very limited, and the hydrolysis efficiency cannot meet the needs of the synthesis of citric acid (Kubicek, Zehentgruber, Kalak, & Röhr, 1980). A separate saccharification step is used when starch is used as the substrate.

Fermentation by maize starch to produce citric acid

After ca. 24 h saccharification, a certain amount of nitrogen source and inorganic salt are added. The inorganic salt is added into the medium, and maize steep water is usually used as the nitrogen source. The fermentation medium is sterilized by high temperature and cooled before the spore suspension is connected. The batch fermentation cycle is about 60-70 h. After the

fermentation completes, citric acid is obtained by extraction and refining process. The flow chart of the fermentation process is shown in Figure 2.3.

Since there is almost no nitrogen in the starch solution (the protein content in the starch is about 0.6 g/100 g), additional nitrogen source and other ions are needed for the fermentation. In the starch fermentation process, maize steep water is normally used to provide the necessary nitrogen, phosphorus, potassium, and other minerals. It is very important to control these minerals in *A. niger* growth and maize starch fermentation.

Kristiansen & Sinclair (1979) suggested that nitrogen sources must be controlled in citric acid fermentation. The carbon to nitrogen ratio is a very important factor in *A.niger* growth control. When *A. niger* grows, phosphorus plays a vital role in energy metabolism, carbon metabolism, and substance transport. Shu and Johnson (1948) pointed out that citric acid would initiate accumulation when it reached a certain level and there is no need to limit the source of phosphorus in citric acid fermentation. Potassium ions have a great influence on the permeability of cell membranes (Dhillon, Brar, Verma, & Tyagi, 2011). When potassium ions are high, the permeability is enhanced, which facilitates the transport of nutrients to the cells. Due to the large external osmotic pressure, the accumulation of citric acid and transport of citric acid from intracellular to extracellular are hindered. Iron ions have the function of activating citrate dehydrogenase in citrate metabolism. The increase of iron ion concentration promotes the TCA cycle and it is not conducive to citric acid accumulation (Dhillon, Brar, Verma, & Tyagi, 2011).

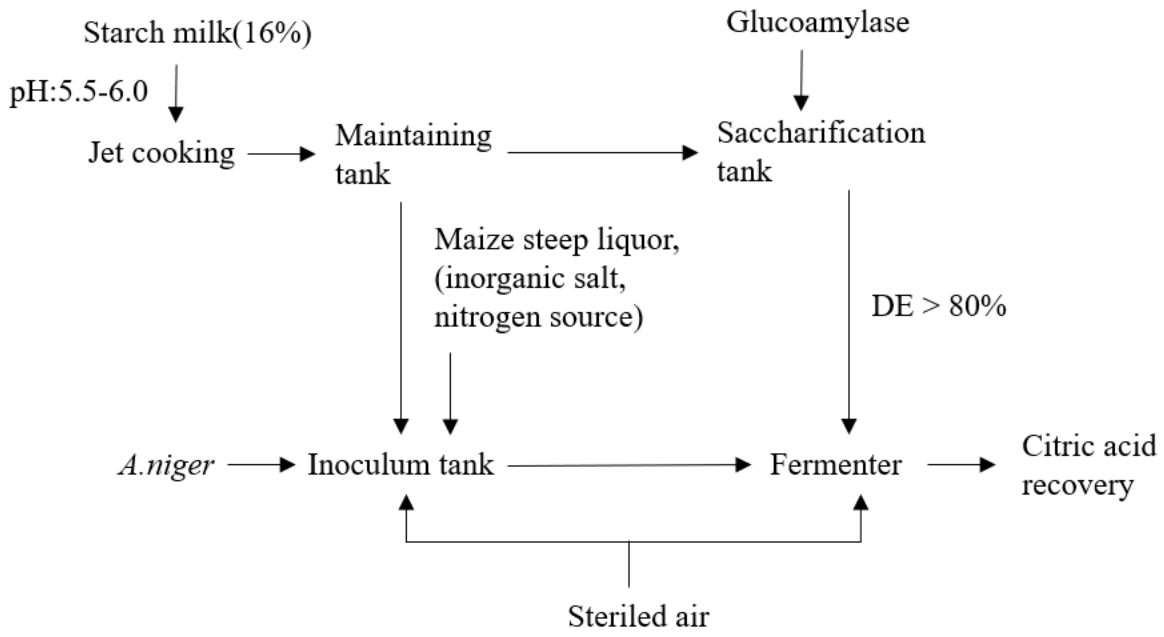

Figure 2.3 Process flow chart of maize starch for citric acid production

Potential improvements in citric acid fermentation process

Considering the high cost of obtaining citric acid from chemical synthesis, researchers are more into the fermentation process to produce citric acid especially with *A. niger* (Narayanamurthy et al., 2008). In recent studies, researchers have tried different processing methods to improve the yield of citric acid (Dhillon, Brar, Verma, & Tyagi, 2011). Although submerged fermentation is currently the dominated method for bulk production of citric acid, research has been conducted to explore different processing methods. There are new possibilities to increase productivity and decrease the cost of production by modifying the fermentation method (Dhillon et al., 2011) which will be discussed in the following sections.

Potential improvement in fermentation methods

There are three fermentation methods to produce citric acid- surface fermentation, submerged fermentation, and solid-state fermentation. In industry people mainly use submerged fermentation. However, researchers have put efforts into using other the two methods in citric

acid production. The detail introduction of the tree methods was reviewed by Dhillon, et al. (2011). The advantages and disadvantages of the three methods were summarized in Table 2.4.

Surface fermentation is superior to submerged fermentation in some cases. It has the advantages of low investment, simple and easy to operate (Darouneh et al., 2009). But it is labor-intensive, require a large area, low yield, and easy to pollute. Thus it is hard to replace submerged fermentation (Anastassiadis et al., 2018).

Solid fermentation is popular recently. Narayanamurthy et al. (2008) showed that solid-state fermentation produced the highest yield of critic acid and could be obtained in a shorter fermentation time compared with submerged fermentation and surface fermentation. It has advantages such as lower energy requirements and produces much less wastewater and thus less environmental concerns (Dhillon et al., 2011). The reaction happens in solid media, requires no free liquid and only needs a small amount of humidity (Krishna, 2005). This method has the advantages of low energy consumption and low wastewater production (Shojaosadati & Babaeipour, 2002). It can reduce the cost of raw material by using industrial waste as raw materials which in turn reduces the environmental pollution (Bari, Alam, Muyibi, Jamal, & Abdullah-Al-Mamun, 2009; Gurpreet Singh Dhillon, Brar, Verma, & Tyagi, 2011; Podgorski & Lesniak, 2000). Even though solid-state fermentation is a promising method, the shortage of automatic production, waste composition complexity makes it hard to isolate and extract citric acid and hard to be used in large-scale production. Submerged fermentation method which is less labor intensity, high production efficiency and a high degree of automation advantages is the main way in the industrial production of citric acid. More than 80% of the citric acid product is obtained with this method (Thompson & He, 2006).

Table 2.4 Advantages and disadvantages of three different citric acid fermentation methods

Fermentation type	Advantages	Disadvantages	Production usage	Reference
Surface fermentation	Less investment, easy to operate, less energy consumption	Intensive labor, long fermentation time, large space requirement	Use in small scale citric acid production	Darouneh et al., 2009
Submerged fermentation	Small space requirement, high production efficiency, controllable	High investment, contamination problem, waste water generation	80% of industrial citric production in the world	Max et al., 2010
Solid state fermentation	Less investment, easy to operate, short fermentation time	Intensive labor, large space requirement, by-product problem	High citric acid yields and value addition of waste	Narayanamurthy et al., 2008

Potential improvement in fermentation modes

There are many different modes of citric acid fermentation for industrial production, they have been reviewed by Krishna (2005). The major fermentation mode for citric acid is batch fermentation, which is time-consuming, poor in energy efficiency, and low in equipment utilization. These defections have been greatly hampered the expansion of citric acid production.

In comparison, new fermentation mode has been developed and offers advantages over the traditional batch fermentation. New fermentation modes including batch fermentation, fed-batch fermentation; repeated fed-batch fermentation was reviewed by Krishna (2005). Arzumanov and his colleagues found a repeat-batch (RB) cultivation to prolong synthesis of yeast. They separated the yeast cells from the fermentation broth and transferred them to a fresh culture medium. Such fermentation process was proved to be very steady (Arzumanov, Shishkanova, & Finogenova, 2000). Using online biosensor control method to facilitate the fermentation of citric acid, Moeller et al. (2010) further studied on the batch fermentation, fed-

batch fermentation, and repeated fed-batch fermentation. They concluded that repeated fed-batch fermentation is the optimal fermentation mode, which produces citric acid 100 g/L in three days. However, this fermentation mode could also accumulate a great amount of isocitric acid, which limited its large-scale application potential (Moeller, Grünberg, Zehnsdorf, Strehlitz, & Bley, 2010).

The studies on fed-batch and continuous production of citric acid mainly use yeast, while few researchers have studied the continuous production of citric acid with *A. niger*. Because *A. niger* is mycelium, the continuous production needs careful control of the level of dissolved oxygen. Otherwise, it may cause abnormal cell metabolism and abnormal citric acid synthesis (Thompson & He, 2006). Therefore, it is still challenging to continuously culture *A. niger* in a reliable way. Some researchers claim that *A. niger* should be fermented with immobilized cells, which controls the size of mycelial pellets (Garg & Sharma, 1992; Kim, Park, & Byun, 2002; Sankpal, Joshi, & Kulkarni, 2001). However, in this process, the accumulation of by-products may reduce the fermentation rate due to by-product inhabitation. Moreover, cell aging and failure of cell renewal may limit the growth of mycelial pellets. These limitations limit the application of this method. Therefore, knowing how to control the properties of mycelial pellets during the process of continuous fermentation is a key to the continuous production of citric acid with *A. niger*.

Recent industrial patents in production of citric acid

Recent patents in the past ten years have been reviewed and summarized in Table 2.5. Those patents mainly disclosed new strain development, substrates pretreatment, and pure product recovery. For new strains development, *A. niger* is still the major strain in recent studies. Researchers tried gene engineering methods to cultivate and select certain high yield strains

which may reduce fermentation time or cost (Chen, Lu, Zhong, Wu, & Xv, 2013; Nicaud, Beopoulos, Laou, Dulermo, 2016). As for substrate treatment, starch is the most common feedstock. Researchers mainly focus on pretreating starch for better liquification or proper treat broth to change reaction conditions which will increase the production (Lu, Yang, Cheng, & Miu, 2016; Ma et al., 2015; Qin et al., 2016). Methods for recycling product is another major focus in recent years. Different filters were used to increase the recycling of citric acids, including deep filtering method, nano-filter and membrane filter (Hu et al., 2017; Ma et al., 2015). Other patents disclosed using environmental conditions to facilitate the fermentation process, such as carbon dioxide adjustment in the fermentation process and salt content adjustment (Luo, Lu, Liu, Zhong, & Zhang, 2014; Zhou, Miu, Zhang, Zhang, & Cui, 2009). From all the patents we may find that strain, substrate and recovery are still the most common way to enhance yield and reduce the total cost.

Table 2.5 Recent patents on citric acid production by fermentation.

Improvement type	Patent name	Key technologies	Patents
Strain development	Aspergillus Niger, application of Aspergillus Niger and method for preparing citric acid by fermentation	A new Aspergillus Niger stain CGMCC5342	Chen, Lu, Zhong, Wu, & Xv, 2013
	Mutant Yeasts Having an Increased Production of Lipids and of Citric Acid	A mutant yeast strain, in which at least the expression or the activity of the 2-methyl-citrate dehydratase is inhibited	Nicaud, Beopoulos, Laou, & Dulermo, 2016
	Enhanced citric acid production in aspergillus with inactivated asparagine-linked glycosylation protein 3 (alg3), and/or increased laea expression	Certain gene fragment lead to an increasing production	Dao & Baker, 2015
	Genes Useful for the Industrial Production of Citric Acid	Newly identified genes that encode proteins that are involved in the (bio)synthesis of citric acid	Bauweleers, Groeseneken, & Peij, 2014
Substrate treatment	Preparation method of citric acid fermentation solution	preparation of fermentation broth with liquification enzyme	Zhou, Miu, Zhang, Zhang, & Cui, 2012

	Method for preparing fermentation liquor of citric acid by utilizing extrudate obtained after low-temperature extrusion and enzymolysis	Preparation the fermentation liquid by the extrudate obtained after the low-temperature extrusion and enzymolysis	Shen & Shen, 2016
	Preparation method for citric acid fermentation broth	A method of using corn as a raw material, continuous injection liquefaction is carried out twice for further fermentation	Kou et al., 2015
	Method for preparing citric acid through fermenting puffed dried sweet potato raw material by Aspergillus Niger	Fermentation of a puffed dried sweet potato raw material by Aspergillus Niger	Jin, 2013
	Method for preparing citric acid by fermenting	A four-stage citric acid fermentation process to increase production	Lu, Yang, Chen, & Miu, 2017
	Preparation method of citric acid	A method of CO2 utilized in the fermentation process to increase the product yield	Chen, 2017
	Fermentation tank applied to citric acid production	A fermentation tank which can be detached for cleaning to improve the stirring and ventilating effects of the fermentation	Zhu, Zhu, & Chang, 2017
Fermentation improvement	Method for improving citric acid fermenting level	A method of fermentation seeds inoculated into a fermentation medium	Qin et al., 2016
	Clean production method for extracting citric acid from citric acid fermented clear liquid	A clean production method for extracting citric acid from a citric acid fermented clear liquid	Gao et al., 2015
	The method of one kind of citric acid fermentation process stream plus protease	A method of protease added to the fermenter to flow during citric acid fermentation process	Kou et al., 2016
	A method of removing oxalic acid, citric acid fermentation process	A method of adding calcium carbonate slurry in the fermentation mash and reacted with oxalic acid	Kou et al., 2015
	Method for producing citric acid through continuous batch feeding fermentation	A method for batch fermentation production of citric acid plus continuous flow	Liu & Liu, 2015
	A citric acid producing strain screening method	A method of using an Aspergillus Niger spore space mutation means to obtain certain strain	Wang et al., 2016
	Method for producing citric acid by using high-strength fermentation technology	Production of citric acid by using a high-strength fermentation technology	Kou et al., 2014
	Method for culturing citric acid fermenting seeds and method for preparing citric acid by fermenting	A method of adding seed culture medium F into seed tank seed culture in citric acid fermentation	Luo, Lu, Liu, Zhou, & Zhang, 2014

	Citric acid fermented stock solution and preparation method for citric acid and starch sugar	A method of one kind of citric acid fermentation raw material solution	Xiong, Lu, Shao, Zhong, & Ma, 2013
	Process for the preparation of citric acid employing filamentous fungi in a culture medium comprising glycerol	A method for producing citric acid from glycerol with a yield of more than 70%	(Bauweleers & Groeseneken, 2011
	High yield method for extracting citric acid	A method of using alkali treatment and colchicine in citric acid recycle	Lan, 2017
	Deep filtering method for citric acid fermentation liquid	A deep filtering method for citric acid fermentation liquid to improve work efficiency and reduce cost	Hu et al., 2017
	Method for filtering and clarifying citric acid fermentation broth	A method of employing a filter aid and a centrifuge to conduct solid-liquid separation of the broth.	Kou et al., 2016
Broth filtration and product recovery	Method for extracting citric acid from citric acid fermentation liquid by utilizing nanofiltration membrane	Extraction of citric acid from citric acid fermentation liquid by utilizing a nanofiltration membrane	Li et al., 2015
	Citric acid fermentation solution pretreatment method	A method of going through an electric field cross-flow membrane filter device in order to carry out filtering and sterilizing treatment	Ma et al., 2018
	Method for purification of citric acid from citric acid fermentation liquid	A method of using of an anion exchange resin chromatography system for chromatographic separation of the citric acid fermentation liquid	Liu et al., 2015
	Method for screening high-yield citric acid strains by saccharifying enzyme	A method for screening high-yield citric acid strains by saccharifying enzyme	Li et al., 2013
	Method for extracting refined citric acid from citric acid fermentation liquid	A method for extracting refined citric acid from citric acid fermentation liquid	Shang, Zhang, Li, Xue, & Deng, 2013

Conclusions

In industrial production of citric acid, maize flour and maize starch are two main substrates. The pretreatment and fermentation on both substrates have been investigated in recent studies. The production based on maize starch shows more economic value, due to high-value germ and feeds produced during starch separation. Compared with the maize flour, the consistency of the starch milk can be ensured, and the filtration and slag removal can be omitted.

However, the isolation of starch requires more complicated separation process at the beginning. Therefore, using maize flour or maize starch depends on total values of varies products produced. Latest studies and patents showed that the modification of original citric acid production is viable. In conclusion, modification in substrate treatment and fermentation process provides great promise for achieving more economical and productive citric acid production.

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Chapter 3 - Partial swelling of granules enables high conversion of normal maize starch to glucose by granular starch hydrolyzing enzyme

Abstract

Normal maize starch (2.0% in 50 mM citrate butter) was partially swollen by heating at 62, 65 and 70 °C for 30 min, and the preswollen starch hydrolyzed by addition of a granular starch hydrolyzing enzyme (GSHE). After preswelling at 70 °C, enzyme kinetics study of the release of glucose showed a 54% reduction in the Michaelis-Menten constant (K_m), suggesting that preswelling increased the complexing of GSHE with the starch granules. Preswelling the starch at 62, 65 and 70 °C followed by digestion with 1.0% GSHE (starch basis) at 62 °C for 24 h in 50 mM citrate buffer (pH 4.5) goes 61.3, 76.0 and 94.5% conversion to glucose. The indigestible fraction compared to the untreated starch, contained less A-type starch crystals and probably less amylose-lipid complex, was more highly branched, contained a higher proportion of short chains, showed a higher gelatinization temperature but lower enthalpy of gelatinization, and contained a higher proportion of short chains. The indigestible fraction, after preswelling at 100 °C in citrate buffer, was converted practically quantitatively to glucose.

Keywords

granular normal maize starch, preheating, granular starch hydrolyzing enzyme, saccharification

Introduction

Maize starch is an abundant renewable biopolymer that can be processed into sugars and ethanol, or other fermented chemicals (Ellis et al., 1998; Manochio, Andrade, Rodriguez, &

Moraes, 2017; Zhou & Peng, 2018). Conventional processes for the production of sugars and fermentation products typically include gelatinization, liquefaction and saccharification. Jet cooking at 100 – 170 °C often is used to gelatinize and disperse maize starch slurries in water at approximately 30 - 35% (w/w) dry solids in the presence of a thermostable α-amylase (Myat & Ryu, 2013). After cooking followed by cooling to 60 °C, starch is converted to glucose by addition of glucoamylase (Robertson et al., 2006). The high temperature and the energy consumption during the gelatinization and liquefaction process constitutes the main drawback of the conventional process (Mandala & Bayas, 2004). The high thermal energy is used to destroy the ordered structure of starch in the maize starch granule.

Instead of jet-cooking starch, an alternative, low-temperature process, in which uncooked granular starch is directly converted to glucose below the gelatinization temperature, has been suggested (Uthumporn, Shariffa, & Karim, 2012). Using a granular starch hydrolyzing enzyme (GSHE) from Genencor International (Palo Alto, CA, USA), a subsidiary of DuPont, Inc., researchers have examined ethanol production from different starchy substrates of maize, cassava, wheat, and sweet potato (Sharma et al., 2007; Jin et al., 2016; Bunterngsook et al., 2017; Masiero et al., 2014). In addition, optimization of low-temperature ethanol production has been studied (Bialas et al., 2009; Shanavas et al., 2011). A limited number of studies have been reported on converting isolated starch to glucose using the commercial enzyme Stargen 001 which is an old version of GSHE from Genencor International (Johnson et al., 2009; Montalbo et al., 2010; Li et al., 2014; Li et al., 2016).

It is known that enzymatic hydrolysis of granular starch at low temperatures is not efficient (Uthumporn, Zaidul, & Karim, 2010; Shariffa, Karim, Fazilah, & Zaidul, 2009). Various treatments have been used to enhance the conversion of starch to sugars by GSHE.

Shariffa et al. (2009) reported that heat-treating starches (25%) at sub-gelatinization temperatures could improve enzymatic hydrolysis of granular potato and tapioca starches. After a heat treatment of maize starch (25%) in sodium acetate buffer at 60 °C for 30 min, the degree of hydrolysis increased up to 14% compared with the untreated sample (Shariffa et al., 2009). Defatting of granular starch has also been used to increase the susceptibility of maize, rice and wheat starches to GSHE (Uthumporn et al., 2013), and ultrasound pretreatment was used to enhance sugar release from cassava chips by GSHE (Nitayavardhana, Rakshit, Grewell, Leeuwen, & Khanal, 2008).

In our laboratory, we have used a different approach and reported that preswelling of starch granules in excess buffer enhances starch saccharification (Li et al., 2014). Alpha-amylase and glucoamylase molecules are several nanometers in size, so they have limited access to starch molecules inside granules (Payan et al., 1980; Netrabukkana et al., 1996). Swelling of starch granules increases the specific surface area of granules, and even slight swelling has a positive impact on enzymatic hydrolysis of granular starch (Li et al., 2014). Partial swelling of normal maize starch at 67.5 °C for 30 min in citrate buffer increased the conversion to glucose 3-fold after incubation with a GSHE (Stargen 001 from Genencor International) at 32 °C for 4 h. Enzyme kinetics showed that GSHE had a higher affinity for the partially swollen granules (Li et al., 2014). However, the maximum conversion of the normal maize starch to glucose catalyzed by GSHE was only 50% in our previous study (Li et al., 2014). No work was done to understand why high conversion was not achieved. In this study, we investigated the action of Stargen 002, a new GSHE from Genencor International, on normal maize starch granules. We also examined how partial swelling enhanced the starch saccharification process, and we characterized the indigestible residues and determined the optimal conditions for glucose production. The enzyme

kinetics of Stargen 002 was studied and compared with that of Stargen 001in the previous study (Li et al., 2014).

Materials and methods

Materials

Normal maize starch was obtained from Tate & Lyle (Hoffman Estates, IL). GSHE (Stargen 002, 570 GAU/g) and a mixture of pullulanase (390 ASPU/g) were obtained from Genencor International (Palo Alto, CA, USA). Stargen 002 is marketed as clear brown liquid with specific gravity 1.13 – 1.16 g/mL at room temperature. Quoting from Genencor's product brochure, one glucoamylase unit (GAU) of activity was defined as the amount of enzyme that liberates one gram reducing sugar equivalent to 5.6 mmol of glucose per hour (93.3 μmol/min) from 1 wt% soluble starch at pH 4.3 and a temperature of 30 °C. One ASPU of activity was defined as the amount of pullulanase that liberates reducing sugar equivalent to 0.45 μmole of glucose per minute from pullulan at pH 5.0 and a temperature of 40 °C. Isoamylase (240 U/mg) was purchased from Megazyme (Wicklow, Ireland). One unit of isoamylase activity was defined as the amount of enzyme required to release one μmole of reducing sugar per minute from oyster glycogen (10 mg/mL) in sodium acetate buffer (100 mM), pH 4.0 at 40 °C (Megazyme product brochure).

All other chemicals were analytical grade and purchased from Fisher Scientific (Santa Clare, CA, USA).

Light microscopy

Normal maize starch (20 mg, dry basis) was slurried in water (1.0 mL) and heated in a water bath at 65, or 70 °C for 30 min with constant shaking. The starch slurry without heat pretreatment was used as a control. Each sample was promptly examined by an Olympus BX51

microscope (Olympus Optical Co. Ltd., Shinjuku-ku, Tokyo, Japan) fitted with a polarized light filter and a digital camera. Observations were conducted under normal visible light and polarized light and captured by a SPOT 18.2 Color Mosaic camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

Enzyme kinetic studies

GSHE (Stargen 002) is reported to contain a mixture of fungal α-amylase and glucoamylase. Our enzyme kinetic analysis of the catalytic action of Stargen 002 was done with the Michaelis-Menten equation, which models the initial reaction rate of formation of D-glucose by the exo-action of glucoamylase. Enzyme kinetic studies was performed as described by Li et al (2014). Normal maize starch (5, 25, 50, 75, 100 µg) was mixed with 50 mM citrate buffer (pH = 4.5) to give five gram starch slurries at concentrations ranging from 0.05% to 1.0% (w/v). Starch slurries were heated in a water bath at 70 °C for 30 min with constant shaking. Maize starch slurry without heat-pretreatment was used as control. Stargen 002 (1.0 ml) was mixed with 99.0 ml distilled water to make the diluted enzyme solution, and 0.05 ml of the diluted enzyme reagent was mixed with 0.95 ml of a preswollen starch slurry at 32 °C. After 10 min, kinetic studies were performed by measuring the initial rate of glucose produced, and by plotting the reciprocal of the rate (mmol/min) versus the reciprocal of the starch concentration (Lineweaver-Burk plot).

Saccharification process of native and preheated starch

Normal maize starch slurry (100 g, 2% w/w, dry basis) in citrate buffer (pH = 4.5) was mixed in a water bath at 65 °C or 70 for 30 min. After cooling to 62 °C, GSHE (10, 20, or 30 mg) was added to each slurry to reach a final enzyme concentration of 0.5%, 1.0% or 1.5% (w/w, which were 2.85, 5.7,8.55 GAU/g starch) based on the weight of the starch. Maize starch without

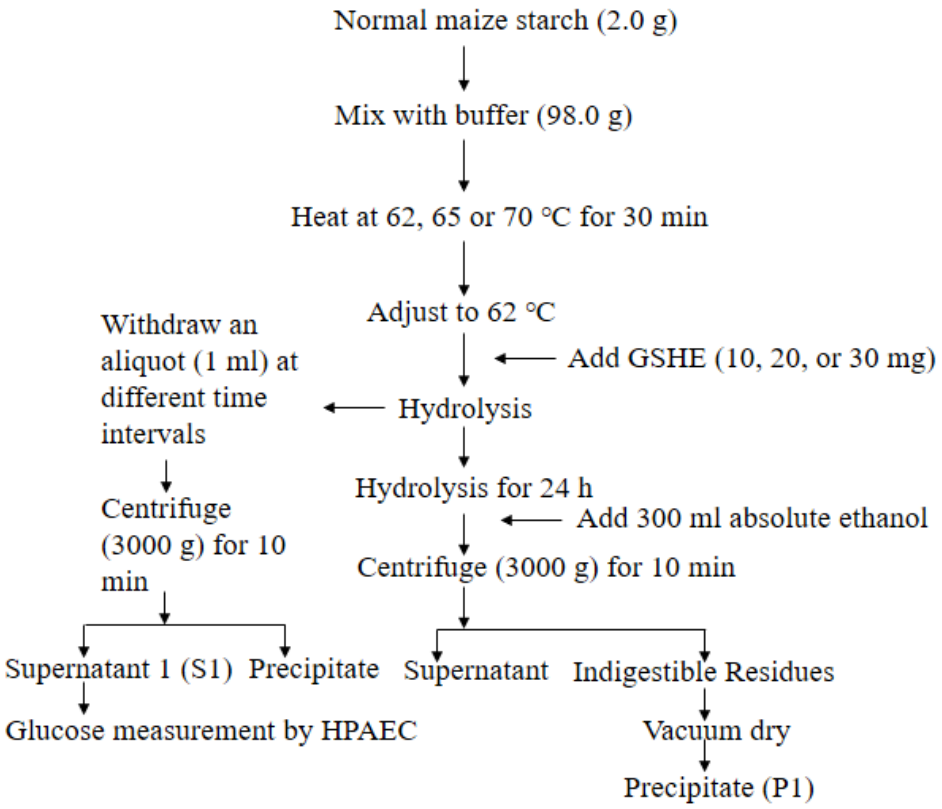
heat pretreatment was used as a control. The mixtures were incubated in an incubator shaker (FSSWB27, Fisher, 160 rpm) at 62 °C for 24 h. At different time intervals, starch hydrolysate (1 ml) was withdrawn and centrifuged. The glucose content in the supernatant was measured by high-performance anion exchange chromatography (HPAEC, Dionex ICS-3000, Dionex Corp., Sunnyvale, CA, USA). The mobile phase was 150 mM NaOH at a flow rate of 1 mL/min and glucose standard (1 mg/ml) was used for calibration. The saccharification process was stopped by adding 3-fold-volume absolute ethanol (300 ml). The mixture was centrifuged and the precipitate was vacuum dried for further analysis. The experiment procedure was shown in Scheme 1 (Figure 3.1).

To determine if the enzyme resistant residues from the saccharification process could be further converted to glucose, two approaches were used: (1) converting the residues with new fresh GSHE, and (2) cooking the residues followed by treatment with fresh GSHE.

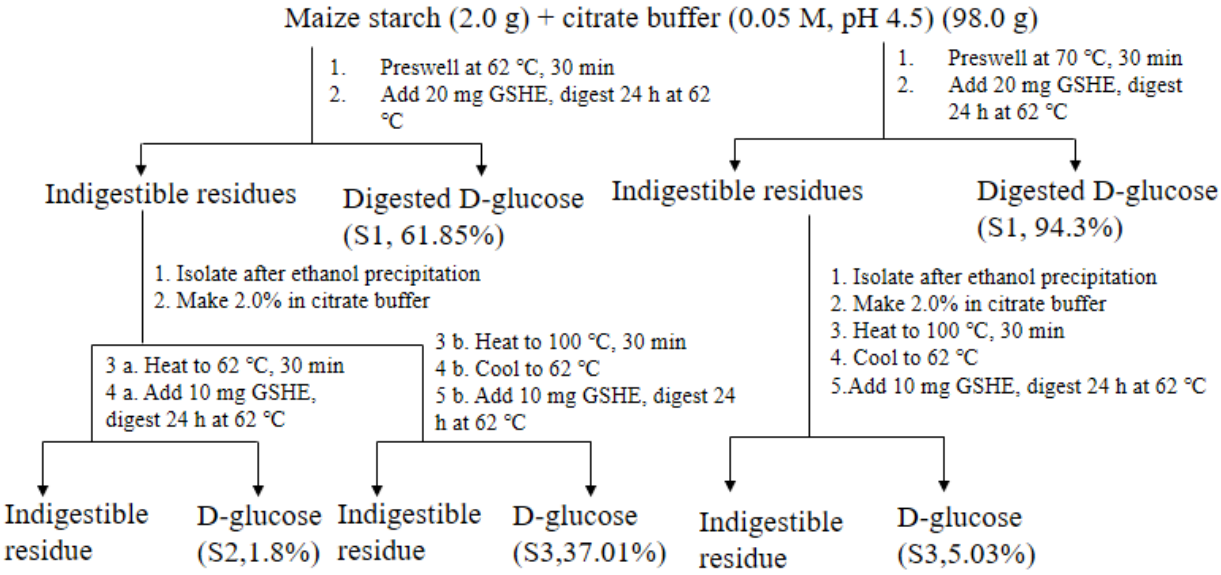
In the first approach, after the first hydrolysis by GSHE, the mixture was centrifuged and the precipitate (P1 in Scheme 2, Figure 3.1) was mixed with 62 °C fresh citrate buffers to have the total weight of 100 g. To hydrolyze the precipitate (P1), new fresh Stargen 002 (10 mg) was added to the mixture and held at 62 °C for 24 h with stirring. The re-hydrolyzed mixture was centrifuged and the glucose in the supernatant (S2 in Scheme 2, Figure 3.1) was determined by HPAEC.

In the second approach, after P1 mixed with fresh citrate buffer (total 100 g), the mixture was heated in a boiling water bath for 30 min with stirring. After the cooked mixture was cooled to 62 °C, Stargen 002 (10 mg) was added and the mixture was held at 62 °C for 24 h with stirring. The mixture was centrifuged and the glucose in the supernatant (S3 in Scheme 2, Figure

3.1) was determined by HPAEC. Maize starch slurry (2g, 2%, w/w) was cooked for 30 min and hydrolyzed for 24 h with 10 mg Stargen 002 as a control.



Scheme 1



Scheme 2

Figure 3.1 Experimental design of saccharification process of preheated starch (scheme 1), and two approaches (re-hydrolysis and cooking) on converting enzyme resistant residues from native and partially swollen maize starch (scheme 2).

Scanning electron microscopy (SEM)

A powdery sample was sprinkled sparsely onto double-sided sticky tape on a microscope mount. The sample was coated with gold-palladium in a sputter coater, and then viewed with a SEM (S-3500N, Hitachi Science System, Ltd, Japan) (Sittipod & Shi, 2016).

Differential scanning calorimetry (DSC)

The thermal property of each sample was determined by DSC using a TAQ5000 DSC (TA Instruments, New Castle, DE, USA). Each sample (ca. 8 mg) was weighed in a stainless-steel pan, water (24 μL) was added, and the pan was sealed. An empty pan was used as a reference. Scans were performed from 10 °C to 140 °C at a heating rate of 10 °C/min. The onset (To), peak (Tp) and conclusion (Tc) temperature and gelatinization enthalpy were calculated from the DSC thermogram.

X-ray diffraction (XRD)

The moisture of normal maize starch and enzyme resistant residues were adjusted to around 20% in a sealed container containing water at 25 °C. X-ray diffractograms of each sample were obtained by a wide-angle X-ray diffractometer (PANalytical, Almelo, the Netherlands) under 35 kV and 20 mA with theta-compensating slit and a diffracted beam monochromator. XRD patterns were acquired at room temperature within the 2θ range of 5 - 35°. The relative crystallinity was calculated by the ratio of the area under the fitted crystalline peaks to the total area (Komiya & Nara, 2010).

Chain-length distribution analysis

The chain-length distributions of starch samples were measured by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Dionex ICS-3000, Dionex Corp., Sunnyvale, CA, USA). Each sample (10.0 mg) was mixed with 2.0 ml

0.1 M sodium acetate solution (pH = 4.0), and the mixture boiled in a water bath for 30 min followed by cooling to 37 °C. After 10 min at 37 °C, isoamylase (100 U/g starch) was added, and the debranching reaction was allowed to proceed for 24 h. The enzyme was denatured by boiling for 15 min. After centrifugation at 13,000 × g for 10 min, a debranched sample was filtered through a 0.45 μm membrane filter. The filtrate was injected into an HPAEC-PAD system equipped with a CarboPacTM PA1 analytical column kept at 30 °C. Eluent A consisted of 150 mM sodium hydroxide, eluent B consisted of a mixture of 150 mM sodium hydroxide and 500 mM sodium acetate. The linear components were separated with gradient elution (40% eluent B at 0 min, 50% eluent B at 2 min, 60% eluent B at 10 min, and 80% of eluent B at 40 min) with a flow rate of 1 mL/min (Shi and Seib, 1992). The area under each peak was calculated as a percentage of the total peak area.

Gel permeation chromatography (GPC)

Unmodified normal maize starch, enzyme resistant residues from saccharification process, debranched maize starch and enzyme resistant residue were analyzed by GPC. Debranched maize starch and enzyme resistant residue were obtained from debranched samples in the previous section after 24 h freeze dry. For each sample, 4 mg powder was dissolved in 4 ml dimethyl sulfoxide (DMSO) (HPLC grade) containing 0.5% (w/w) lithium bromide (Vilaplana & Gilbert, 2010). The mixture was sealed and stirred in a boiling water bath for 24 h. After cooling to 25 °C, the solution was filtered through a 2.5 μm filter and injected into a PL-GPC 220 instrument (Polymer Laboratories, Inc., Amherst, MA, USA), equipped with three Phenogel columns (Brewer, Cai, & Shi, 2012) and a guard column (Phenomenex, Inc., Torrance, CA, USA). The mobile phase was DMSO containing 0.5% (w/w) lithium bromide, and the flow rate was 0.8 mL/min. The column oven temperature was set to 80 °C. Pullulan standards were

used for calibration (Vilaplana & Gilbert, 2010). The results of GPC were presented with the terms of hydrodynamic radius (R_h), with $V_h = 4/3\pi R_h^3$(Shi et al., 2018).

Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy

NMR spectra of starch samples were recorded using a Varian NMR spectrometer (Varian Inc., Palo Alto, CA) operating at a Larmor frequency of 400 MHz for ^{1}H. Prior to recording an NMR spectrum, each sample (20 mg) was dissolved in 1.0 mL of deuterium oxide (D_2O) and the mixture heated to 100°C for 1 h and then freeze dried. A freeze-dried sample (20 mg) was dissolved in 1.0 mL of D_2O again, and its ^{1}H NMR spectra was obtained at 25 °C. The ratio of α-1,4- and α-1,6-glucosidic linkage was determined from integration of the anomeric resonances at δ 5.4 and δ 5.0 ppm, respectively (Lopez-Rubio, Flanagan, Gilbert, & Gidley, 2008).

Statistical analysis

The analysis of variance (ANOVA) was conducted by Minitab 17 Statistical Software Program (Minitab Inc. State College PA, USA). Experimental results are reported as the average of triplicate measurements.

Results and discussion

Kinetic Parameters

Partial swelling of maize starch granules was achieved at 2.0 % starch solids in citrate buffer (0.05 M and pH 4.5) by warming to 62 – 70 °C for 30 min; the partial swelling greatly affected the kinetic properties of the glucoamylase in GSHE (Table 3.1). The Michaelis-Menten constant (K_m) value for GSHE acting on normal maize granules decreased after starch granules were partially swollen, suggesting an increased affinity of the GSHE for the starch. K_m decreased from 0.281 to 0.130, as starch granules was preswollen to 70 °C (Table 3.1). At the same time, after partial swelling, V_{max} values for the saccharification of granular starch increased

significantly compared with that for granular starch without preswelling (Table 3.1). Even when the starch granules were preswollen at 65 °C, a temperature at which the starch granules gained ~ 30% in volume (Li et al., 2014), V_{max} values were more than double than that of granular starch without preswelling. Moreover, after the starch granules were preswollen at temperatures above 65 °C, the increase in V_{max} was more dramatic.

Catalytic efficiency, which was expressed as the ratio of K_{cat} to K_m (Table 3.1), suggested that swollen starch granules were a better substrate than the untreated starch. For granular starch preswollen at 70 °C, the catalytic efficiency value was more than 4-times higher than that for granular starch without preswollen (Table 3.1).

Comparing the enzyme kinetic parameters we found for Stargen 002 in this study with those reported for Stargen 001(Li et al., 2014), the new Stargen 002 enzyme was more efficient (the k_{cat}/K_m was 1.3 - 1.6 times higher) and gave a V_{max} that was 6-7 times higher than Stargen 001. Those results suggest that Stargen 002 would be more efficient in the conversion of raw starch to glucose.

Table 3.1 Enzyme kinetic parameters for granular starch hydrolyzing enzyme (GSHE) acting on granular normal maize starch pretreated at a specific temperature for 30 min[A]

Pretreating Temperature (°C)	V_{max}[B] (mM/min)	K_m[B] (%)	K_{cat}[A] ($\times 10^4$)	K_{cat}/K_m ($\times 10^4$)
62	0.692±0.006 a	0.281±0.015 c	0.213±0.010 b	0.760
65	1.399±0.020 b	0.212±0.010 bc	0.432±0.023 a	2.036
70	1.696±0.018 c	0.130±0.019 ab	0.524±0.022 a	4.027

[A] The starch concentration in reaction mixtures ranged from 0.05% to 1.0%. Stargen 002 concentration [E_0] was expressed as enzyme activity units per milliliter of reaction mixture. Stargen 002 enzyme activity was 570 GAU/g. The standard Michaelis−Menten formula: $v = V_{max}[S]/(K_m + [S])$, where v is the rate of glucose formation, [S] is the substrate concentration, Vmax is the maximal velocity of the reaction, and Km is the Michaelis−Menten constant.

[B] Values represent Michaelis-Menten constants for GSHE acting on starches preheated at different temperatures. Values are means ± SD (n = 3). Means with different letters within the same column of are significantly different ($p < 0.05$).

Effect of partial swelling of starch granules on saccharification process

As shown in Figure 3.2, the higher the preheating temperature, the more starch was converted to glucose. Saccharification process was more efficient at the first 8 h of the reaction, during which period, more than 70% starch was converted. The conversion reached a plateau in the following 16 h (Figure 3.2).

The amylolysis of starch granules is a two-phase (solid-solution) reaction (Oates, 1997). The nano-sized α-amylase and glucoamylase must bind to the solid substrate and then cleave its glycosidic linkages (Bielecki, 2003). There is a limited number of pores on the surface of normal maize starch granules. Partial swelling of maize starch granules increased specific surface area of granules and weakened granular structure (Li et al., 2014). Thus, the GSHE was more accessible to the starch resulting in higher rate and extent of hydrolysis. Moreover, the crystalline lamellae in native starch granules is resistant to enzymatic erosion (Uthumporn, Shariffa, & Karim, 2012; Oates & Powell, 1996; Wang, Powell, & Oates, 1995). Partial swelling could cause a reduction in crystallinity and therefore significantly decreased resistance to enzymatic hydrolysis.

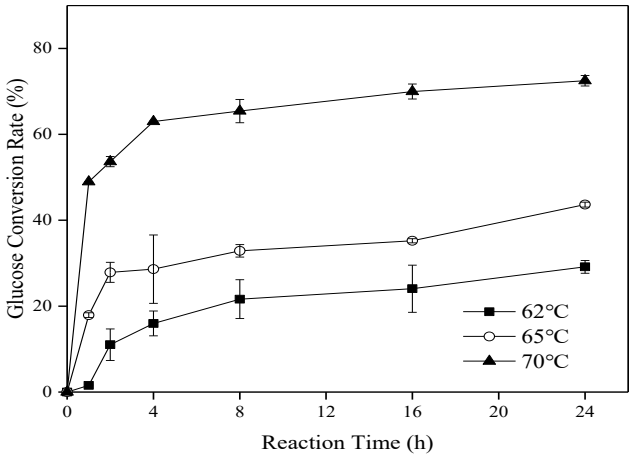


Figure 3.2 Glucose conversion rate during saccharification process of granular normal corn starch preheated at 62, 65 and 70 °C for 30 min with 0.5% GSHE (w/w) and constant shaking. The hydrolysis of all the starches by GHSE was conducted at 62 °C.

Effect of enzyme concentration on starch saccharification process

A higher degree of glucose conversion was achieved as the GHSE concentration was increased from 0.5% to 1.0% (w/w starch basis) in the digest at 62 °C for 24 h (Table 3.2). Nevertheless, there was no significant difference in glucose conversion when enzyme concentration was increased from 1.0% to 1.5%. The degree of granule swelling appeared more important compared to enzyme concentration. Preswelling at 70 °C resulted in partial swelling of starch granules (Figure 3.3), making them more accessible to the action of the GSHE, and enhanced amylolysis. When preswelling at 65 °C increasing the enzyme concentration from 0.5% to 1.0% led to an approximately 30% increase in conversion after 24 h of saccharification (Table 3.2). Preswelling at 70 °C resulted in a 94.5% starch conversion to glucose by 1.0% GSHE. Importantly, this relatively low temperature saccharification process reduces the energy required to deconstruct the crystalline lamellae in starch granules so as to reach a high degree of conversion (95%), matching that of traditional jet cooking of starch prior to saccharification (Kunamneni & Singh, 2005).

Table 3.2 Effect of granular hydrolyzing starch enzyme (GHSE) concentration on conversion of the normal maize starch to glucose.

Pretreating Temperature (°C)	Enzyme Concentration (%)	Starch Conversion[A](%)
62	0.5	30.1±1.08 e
	1.0	61.3±0.56 c
	1.5	64.1±0.42 c
65	0.5	45.1±0.75 d
	1.0	76.0±2.58 b
	1.5	77.9±0.52 b
70	0.5	74.8±0.89 b
	1.0	94.5±0.52 a
	1.5	95.0±0.23 a

[A]Values are means ± SD (n = 3). Means with different letters within the same column of are significantly different ($p < 0.05$).

Characterization of normal maize starch and enzyme resistant residues

Granule morphology

Granule morphologies of the untreated normal maize starch, preswollen starches, and indigestible residues were observed by light microscopy (Figure 3.3). Native normal maize starch granules had polygonal and spherical shapes. After preswelling at 65 °C in 50 mM citrate buffer pH 4.5, the granules began to swell slightly and most starch granules remained birefringent (Figure 3.3 B). When the preswelling temperature was increased to 70 °C, many starch granules were damaged and lost birefringence (Figure 3.3 D). The swelling factor which is the ratio of the volume of swollen granules to the volume of dry starch was reported (Li et al., 2014) to enlarge 1.2 for normal maize starch granules in water, but reached 4 after preheating at 65 °C and 8 at 70 °C (Li et al., 2014). When maize starch was preswollen at 70 °C in this work (Figure 3.3 D), the swollen granules were practically totally digested upon saccharification with 1.5 wt% GSHE at 62 °C for 24 h (Figure 3.3 E). The disappearance of granular in Figure 3.3 E is consistent with the high conversion (95%) of the preswollen (70 °C) starch given in Table 3.2.

When preswelling of starch was done at a temperature of 70 °C, the indigestible residues were small in size (Figure 3.3 E) compared to the residues (Figure 3.3 C) when preswelling was done at 65 °C.

The morphologies of starch granules were also observed by SEM (Figure 3.4). Untreated normal maize starch granules showed an especially smooth surface with a diameter around 10 - 15 μm (Figure 3.4 A). On the other hand, all the enzyme hydrolyzed granules showed a large number of bore holes (Figure 3.4 B - H). Both the number and diameter of those holes appeared to be positively correlated with the preswelling temperature (Figure 3.4 B - F). In addition, more fragments of granular were found in the residues from granular that had been preswollen at 70 °C (Figure 3.4 D - F). Compared with the indigestible residue without preswelling (Figure 3.4 B and 3.4 G), the residues from granules preswollen at 70 °C showed severe erosion by the enzyme (Figure 3.4 D - F).

The holes in indigestible residues we observed are consistent with the previous concept of "centripetal" hydrolysis starting from surface-to-core. That concept has been proposed to explain the enzymatic digestion of crystalline and amorphous regions in normal maize starch granules (Helbert, Schülein, & Henrissat, 1996). Enzymatic erosion is marginal at the surface of maize starch granules and more extensive inside a granule as observed in Figure 3.4 D - F. The increasing number of holes indicated that the swollen starch granules provided a better initial substrate for enzyme digestion. After the holes were formed, the crystalline and amorphous regions inside the starch granules would have been evenly digested (Zhang, Ao, & Hamaker, 2006). Finally, the "shell" of a granule remains (Figure 3.4 H).

Some granules showed an enlargement of holes into their interior regions (Figure 3.4 D & F). This was consistent with the previous study of surface pores leading into the interior by

channels (Zhang, Ao, & Hamaker, 2006). Compared to the indigestible residues isolated from starch that had undergone saccharification without preswelling (Figure 3.4 B), the 70 °C preswollen indigestible residue showing a severe erosion by the enzyme (Figure 3.4 F and 3.4 H).

For the starch granules preswollen at 65 °C (Figure 3.4 B and C), micropores on the surface were evident, but few granules were broken down into fragments after hydrolysis by the GSHE. Residues from the starch granules preheated at 70 °C showed more fragments, and the higher the enzyme concentration the more severe the erosion (compare Figure 3.4 E, 3.4 D, and 3.4 F). As shown in Figure 3.3, starch granules preheated in citrate buffer at 70 °C swelled to a high degree. Those swollen granules were more easily attacked and hydrolyzed by the GSHE with removal of their interior (Figure 3.4 D - F). After saccharification at 62 °C for 24 h, the maize starch hydrolyzed with a high concentration of GSHE showed more pores on the surface and a collapse of granular structure, indicating a high degree of internal hydrolysis (Figure 3.4 E and 3.4 F). Large holes and cavities in the indigestible residues were observed. Those recalcitrant residues appeared in pyramidal shape (Zhang, Ao, & Hamaker, 2006).

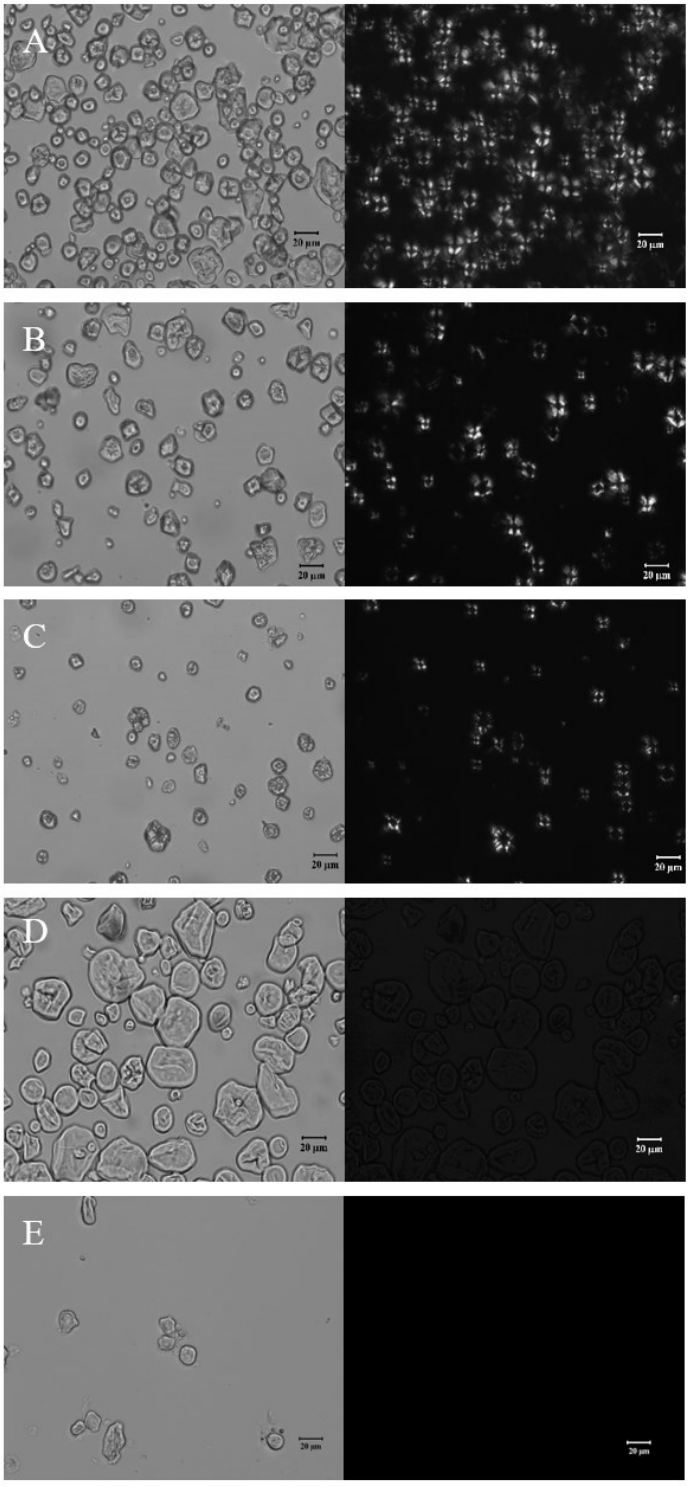


Figure 3.3 Light microscopic images of native normal maize starch (A), and preheated at 65 °C (B), 70 °C (D), the residue preheated at 65 °C and saccharified residue (C), and the residue preheated at 70 °C and saccharified (E). The scale bar indicates 20 µm.

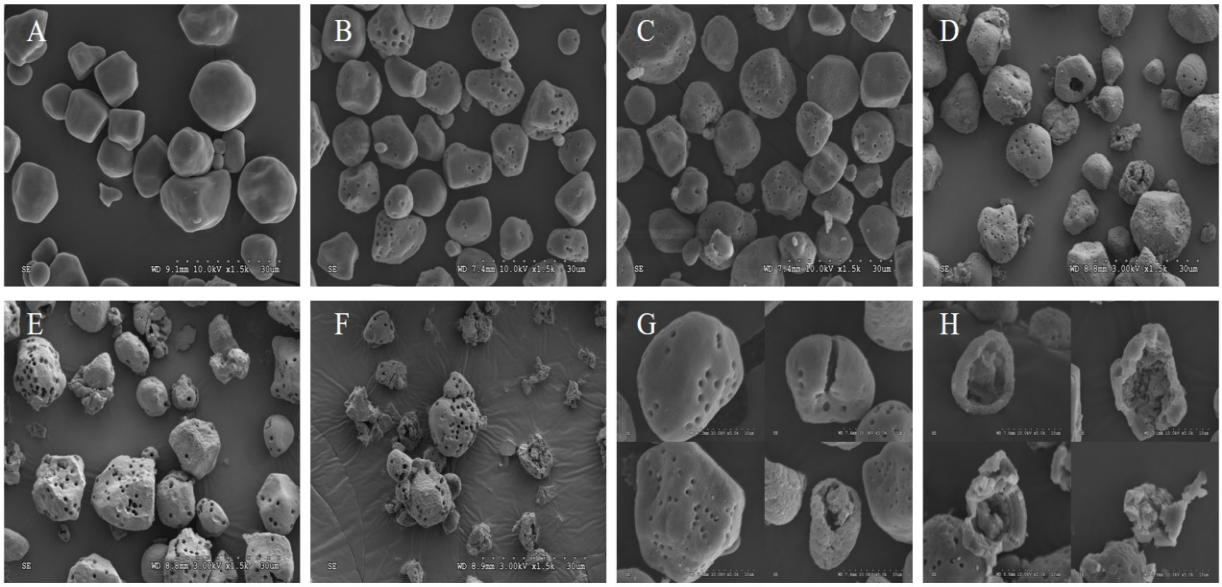


Figure 3.4 Scanning electron micrographs of normal maize starch (A) and enzyme resistant residues after hydrolyzed for 24 h by granular starch hydrolyzing enzyme (GSHE) on normal maize starch without preheating (GSHE = 0.5%, w/w) (B), preheated at 65 °C with 0.5% GSHE, w/w (C), preheated at 70 °C with 0.5% GSHE(D), preheated at 70 °C with 1.0% GSHE, w/w(E), preheated at 70 °C with 1.5% GSHE, w/w(F), single residues without preheating with 0.5% GSHE (G) and single residues preheated at 70 °C with 0.5% GSHE (H).

Thermal properties

Indigestible residues recovered after saccharification (0.5% GSHE, citrate buffer, 62 °C, 24 h), enzyme resistant residues showed a significant increase in gelatinization temperature (T_o, T_p and T_c) but a decrease in ΔH values compared to native normal maize starch (Table 3.3), Those data suggested that crystalline regions as well as the amorphous regions were attacked by GSHE. However, the temperature range ($T_c - T_o$) for gelatinization decreased after enzymolysis, indicating a higher homogeneity of crystallites in the indigestible residues. The lower ΔH values of the enzyme resistant residues suggested that the GSHE hydrolyzed both crystalline as well as amorphous regions. It is interesting to note that the ΔH decreased from 12.1 J/g for the native normal maize starch to 8.7 J/g for the residue hydrolyzed by the GSHE at 62 °C without preheat treatment (Table 3.3). Partial swelling caused that more starch was hydrolyzed (Table 3.2, Figure

3.2) so that less amount of enzyme resistant residues was left. The ΔH values of the enzyme resistant residues from the starch preheat treated at 65 and 70 °C were 8.0 J/g and 7.0 J/g, respectively (Table 3.3). These results suggest that the enzyme resistant residues were largely amorphous with a low degree of crystallinity.

Another endothermic peak was observed in the DSC curves at 94 - 115 °C (Table 3.3); the peak was assigned to the melting of amylose-lipid complex (Le et al., 1999). The ΔH of the melting of the amylose-lipid complex in the indigestible residue was reduced from 0.6 J/g to 0.2 J/g, indicating that two third of the complex was hydrolyzed during the saccharification at the preswollen starch and one third of the complex remained.

Table 3.3 Thermal properties of native normal maize starch and enzyme resistant residues after hydrolyzed by granular starch hydrolyzing enzyme (0.5%).

Starch	T_o[A] (°C)	T_p[A] (°C)	T_c[A] (°C)	ΔT[A] (°C)	ΔH[A] (J/g)
Native	68.6±0.4 b	72.6±0.4 b	84.3±0.2 b	20.6±0.7 a	12.1±0.3 a
62 °C	76.7±0.2 a	80.2±0.2 a	92.2±0.6 a	15.4±0.5 b	8.7±0.6 b
65 °C	77.6±0.4 a	82.6±0.5 a	94.2±0.4 a	16.6±0.6 b	8.0±0.1 b
70 °C	77.1±0.2 a	81.2±0.1 a	92.8±0.4 a	15.7±0.2 b	7.0±0.1 b
Melting of amylose-lipid complex					
Native	94.2±0.4 b	103.8±0.4 b	112.4±0.4 b	18.2±0.6 a	0.6±0.0 b
62 °C	100.4±0.2 a	106.5±0.6 ab	115.2±0.4 a	14.6±0.4 b	0.2±0.0 a
65 °C	101.3±0.3 a	108.1±0.64 a	115.4±0.3 a	14.2±0.3 b	0.2±0.0 a
70 °C	99.3±0.6 a	105.3±0.3 ab	111.7±0.6 b	12.4±0.1 b	0.2±0.0 a

[A] T_o=onset temperature, T_p=peak temperature, T_c=conclusion temperature, $\Delta T = T_c - T_o$, ΔH=enthalpy change. Values are means ± SD (n = 3). Means with different letters within the same column of are significantly different ($p < 0.05$).

Crystallinity

The XRD patterns and relative crystallinities of native and selected indigestible residues are shown in Figure 3.5. Both native maize and the indigestible residues showed the typical A-type starch diffraction pattern with prominent peaks at 2θ values of 15°, 17°, 18° and 23°.[40] After subjecting the starch, either preswollen at 62 °C or not, to the saccharifying enzyme (0.5%

GSHE, citrate buffer, 62 °C, 24 h), the relative crystallinities of hydrolyzed starch decreased. This trend is consistent with the gelatinization enthalpy results (Table 3.3). A decrease in crystallinity reflects the disruption of crystalline structure. The crystallinity of maize starch subjected to saccharification without preswelling showed a reduction of 10.9% compared to native normal maize starch. For the preswollen starches, the crystallinity reduction increased with preswelling temperature. In the case of the starch preswollen at 70 °C followed by the saccharification, relative crystallinity of the indigestible residue was reduced to 18.9% (Figure 3.5), indicating an approximate decrease of one-half of the starch's initial crystallinity.

It is of interest that the indigestible residues isolated here have relatively low crystallinity (Table 3.3). It has been suggested that non-crystalline but densely packed starch can decrease or prevent the accessibility and action of enzymes (Zhang, Dhital and Gidley, 2015). It is known that some almost amorphous starch material shows a high level of enzymatic resistance (Chanvrier et al., 2007; Htoon et al., 2009). The indigestible residues in this work may have amorphous matrices that resist amylolytic digestion similar to type 2 resistant starch (B-type polymorphic starch) found in potato and high-amylose maize starches (Dhital et al., 2010).

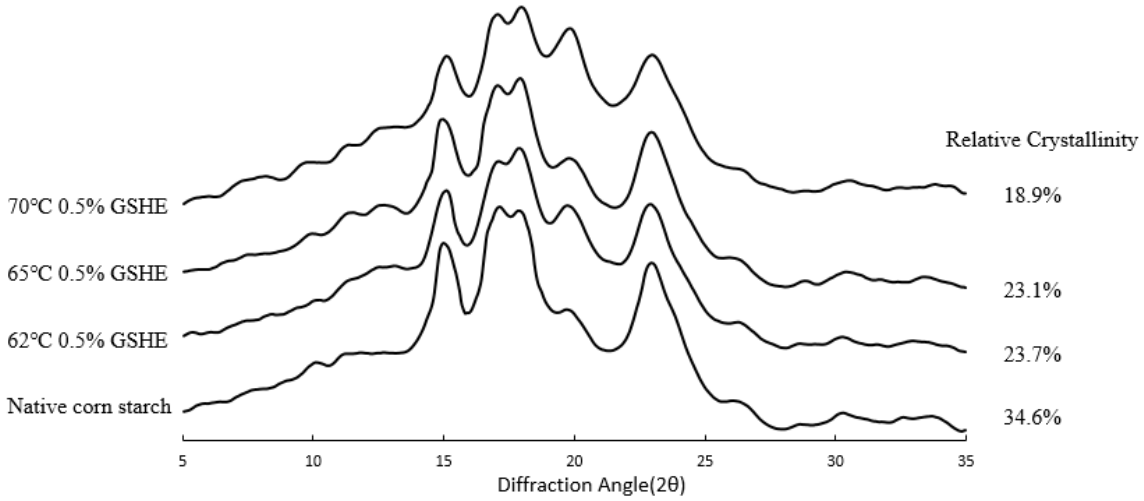
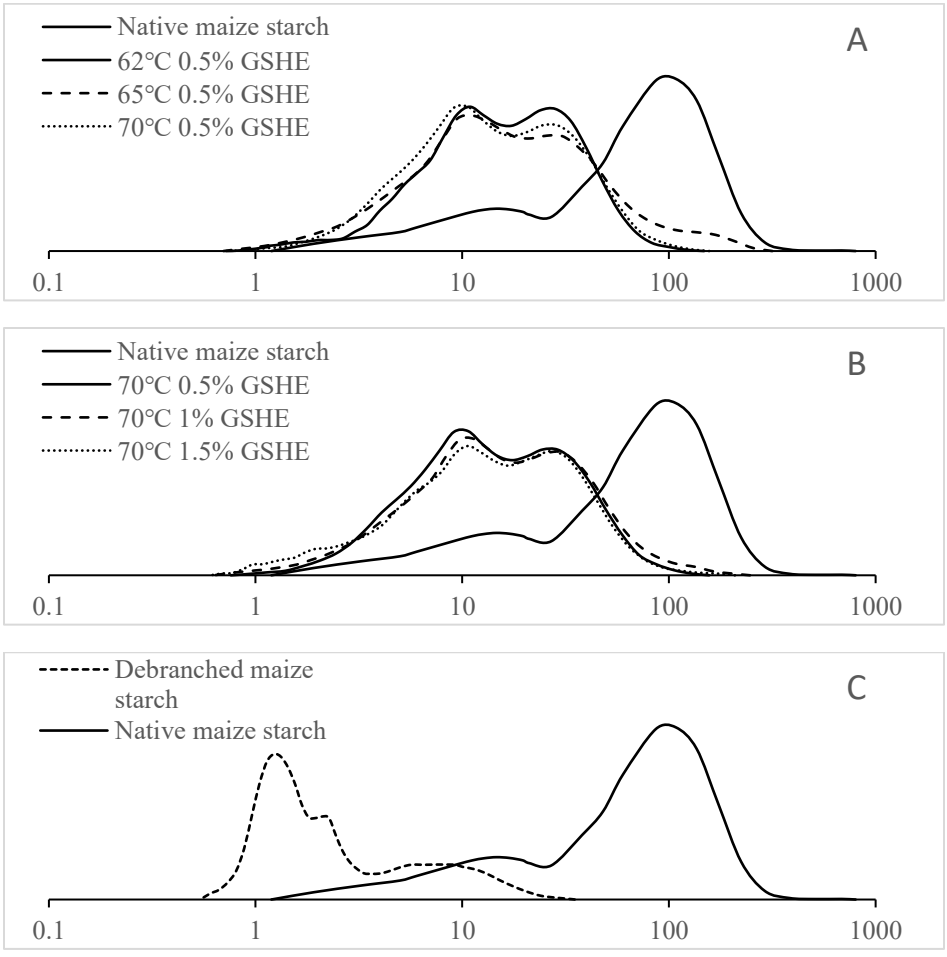


Figure 3.5 X-ray diffraction patterns of normal maize starch and residues from hydrolyzed normal maize starch preheated at 62, 65 and 70 °C for 30 min with constant shaking. Hydrolysis by the GHSE (0.5%, w/w) was performed at 62 °C.

Molecular size distribution

Figure 3.6 shows molecular size (MS) distributions of unmodified maize starch and indigestible residues isolated from preswollen and enzyme-treated maize starch. The size distribution of molecular in unmodified maize (Figure 3.6 A) shows two peaks. Peak 1 (Figure 3.6, right peak) represents the large molecules (amylopectin) while Peak 2 (left peak) represents the small molecules (amylose). When the starch was preswollen before enzyme-treatment, for example preswollen at 65 or 70 °C, the large indigestible molecules represented by Peak 1 were reduced in size to approximately the same size as in Peak 2 (Figure 3.6). That shift in size meant preswelling made the maize granules easier to be hydrolyzed which is consistent with the morphology study (Figure 3.3 and 3.4). Figure 3.6 B shows the effect of different enzyme concentrations on the samples preswollen at 70 °C. The increased enzyme concentration resulted in creation of the molecule with R_h values less than 5 nm.

Unmodified maize starch and indigestible residues were debranched by iso-amylase before determining molecular size distributions (Figure 3.6 C, D and E). For unmodified and indigestible residues, the large amylopectin molecules were completely debranched and converted to small molecules. The proportion of molecules between R_h values of 5 – 50 nm was higher in the indigestible residue of the starch preswollen to 70 °C (Figure 3.6 E) compared to 62 °C (Figure 3.6 D), indicating longer chains in the indigestible residues from the 70 °C swollen starch even though the yield of the indigestible residue from the 70 °C swollen starch was low (Table 3.2).



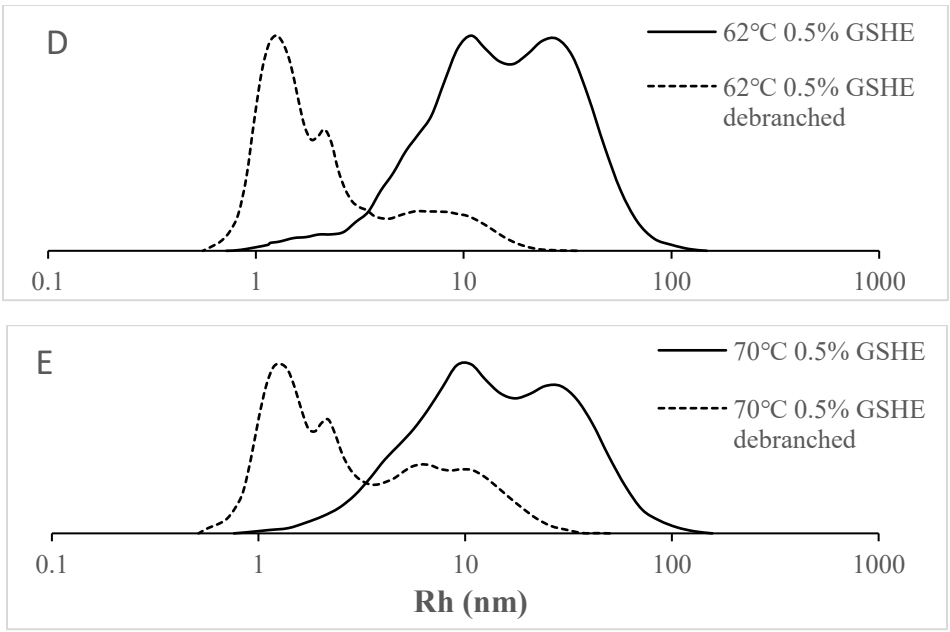


Figure 3.6 Molecular size distributions of normal maize starch, 62 °C, 65 °C and 70 °C pretreated enzyme resistant residue (A); enzyme resistant residue pretreated at 70 °C with different enzyme levels (0.5%,1% and 1.5%, w/w) (B) native maize starch before (solid line) and after debranching (broken line) (C), 62 °C pretreated enzyme resistant residue before (solid line) and after debranching (broken line) (D); 70 °C pretreated enzyme resistant residue before (solid line) and after debranching (broken line) (E). Enzyme resistant residue were from previous section.

Chain-length distribution

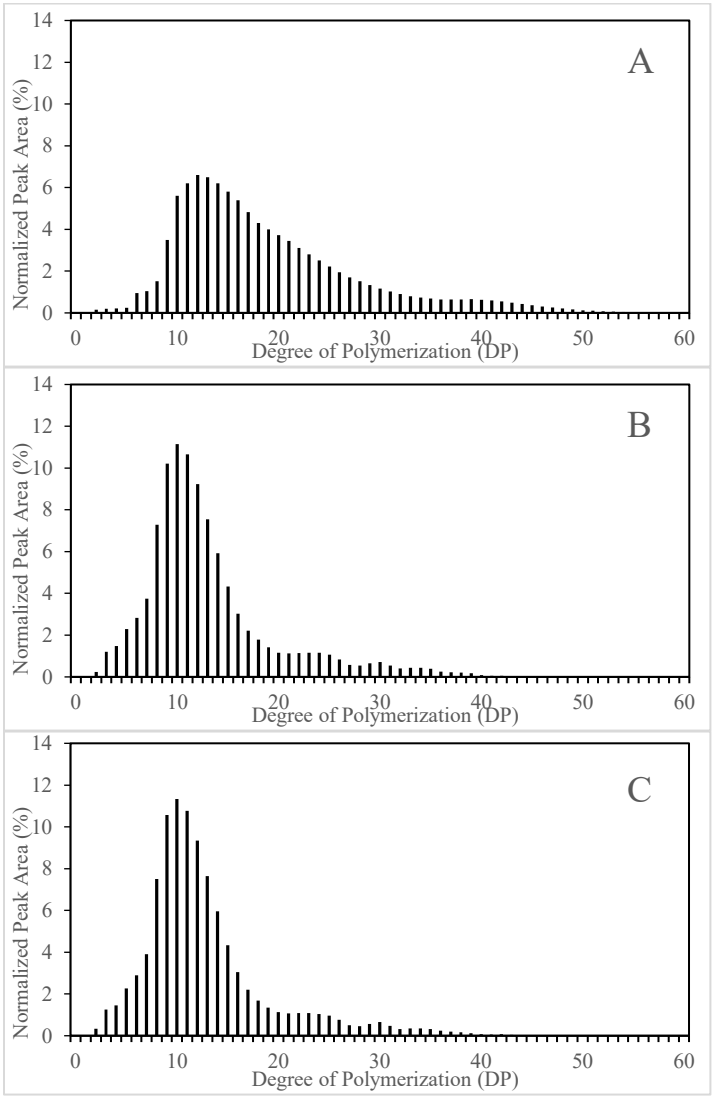
The chain-length distributions of untreated normal maize starch and indigestible residues are summarized in Table 3.4 and Figure 3.7. Hydrolysis of the maize starch by GSHE generally increased the proportion of short chains in the indigestible residue and caused a decrease in the long chains. This result can be explained by the α-amylase in GSHE cutting the long chains into short ones, while the glucoamylase "trims" the ends of the new chains to produce short chains.

Proton NMR showed the indigestible residues contained a higher and higher proportion of α-1,6 linkages with more and more digestion of maize starch with GSHE (Table 3.4). Table 3.4 shows that α-1,6 linkages of the maize starch increased from 4.7% to 8.7%. The increase in α-1,6 linkages is explained by the inaction of α-amylase on the branching points of starch, and the slow action of glucoamylase (Singh, Dartois, & Kaur, 2010).

Table 3.4 Chain length distributions and α-1,6 linkages of native normal maize starch and enzyme resistant residues after hydrolyzed by granular starch hydrolyzing enzyme

Pretreating Temperature (°C)	GSHE Concentration (%)	Branch chain length distribution/%				α-1,6 linkages (%)
		DP < 13	DP 13-24	DP 25-36	DP > 36	
Native	–	26.2±0.1d	52.6±0.2a	14.7±0.0a	6.5±0.2a	4.7±0.0d
62	0.5	60.3±0.8c	31.9±0.7b	6.9±0.1b	0.9±0.0b	6.0±0.3c
65	0.5	61.6±2.1c	31.6±1.3b	6.0±0.6b	0.8±0.1b	6.7±0.1bc
70	0.5	70.1±0.3b	23.2±0.5c	6.0±0.5b	0.7±0.2b	7.9±0.4ab
70	1.0	72.1±1.2b	21.6±1.0b	5.7±0.1b	0.6±0.1b	8.6±0.1a
70	1.5	83.9±0.8a	12.4±0.5d	3.3±0.2c	0.4±0.1b	8.7±0.2a

DP, degree of polymerization. All data are means ± SD (n = 3). Means with different letters within the same column of are significantly different ($p < 0.05$).



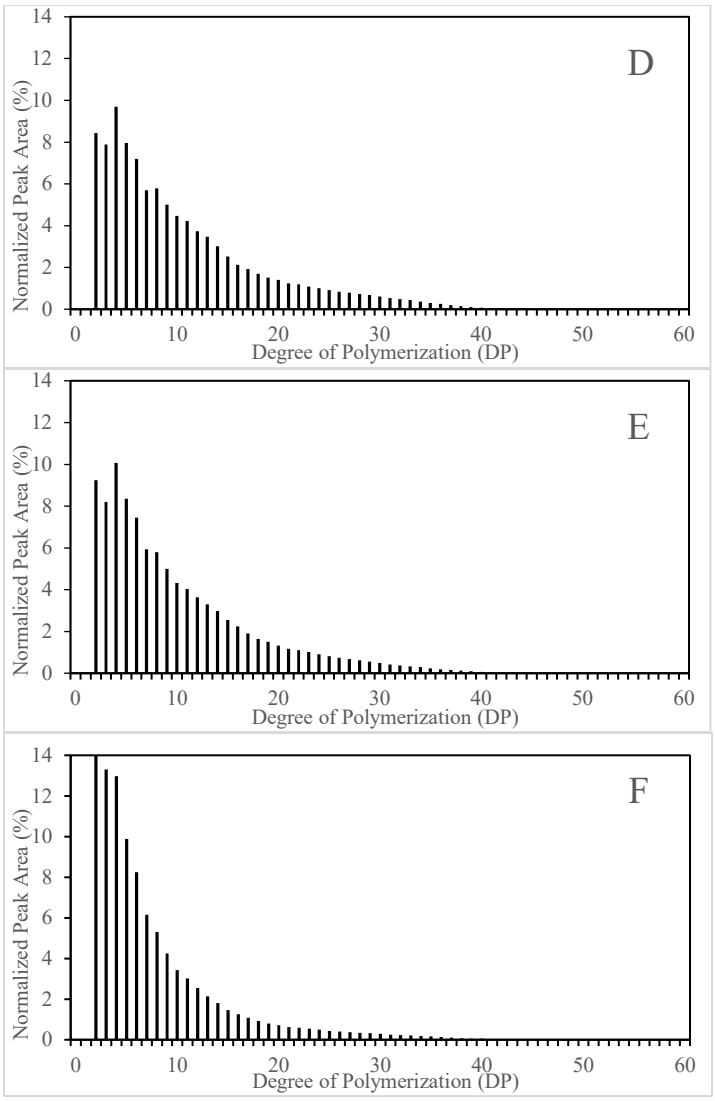


Figure 3.7 Chain length distribution of normal maize starch(A) and enzyme resistant residues after hydrolyzed for 24 h by granular starch hydrolyzing enzyme (GSHE) on normal maize starch without preheating (GSHE=0.5%, w/w) (B), preheated at 65 °C with 0.5% GSHE (C), preheated at 70 °C with 0.5% GSHE (D), preheated at 70 °C with 1.0% GSHE (E), preheated at 70 °C with 1.5% GSHE (F).

Additional process to convert enzymatic resistant residue to glucose

For the purpose of converting the indigestible residue to glucose, two approaches were used (Schemes 1 and 2 in Figure 3.1). In the first approach, we removed hydrolyzed product (i.e. glucose), and the indigestible residue (isolated from 2.0% starch treated with 0.5% GSHE 24 h at 62 °C) with fresh buffer, preswelled the starch at 62 °C for 30 min, and finally added 0.5%

GSHE and digested 24 h at 62 °C (left side Scheme 2, Figure 3.1). The additional conversion was only 1.8% (Table 3.5), indicating that the residue remained recalcitrant and that removing the glucose product from the system and adding fresh enzyme did not further the conversion.

The results from that first approach suggested that the reason that the indigestible residue was resistant to further enzymatic conversion was not caused by "kinetic control" but by "thermodynamic control." Considering that those enzyme indigestible residues have less than 20% crystallinity (Figure 3.5), the question arises as to how those enzyme-resistant residues persist against the action of α-amylase and glucoamylase in the GSHE. Dhital et al. (2017) proposed that there are two fundamental origins for resistance to α-amylase, namely (i) physical barriers that prevent access / binding of enzyme to starch or (ii) molecular features that prevent enzyme from hydrolyzing starch. Zhang et al. (2015) suggested that local molecular density of starch plays a major influence on digestion kinetics, and that dense amorphous packing may prevent / limit enzyme from physically binding to starch and initiating hydrolysis. In studying the mechanism for formation of starch granule ghosts (the insoluble remnant after low-shear cooking of maize and potato starches), Zhang et al. (2014) reported that the ghost structure is composed mainly of amorphous entangled amylopectin with limited reinforcement by a small fraction of single crystals (2 - 4% by weight), either V-type order based on amylose (for maize ghosts) or B-type order from amylopectin (for potato ghosts). Those researchers have illustrated that it is possible to have amylase-resistance from essentially amorphous (96 - 98%) starch. In this study, the indigestible residue from normal maize starch treated with GSHE was largely amorphous (ca. 82%) starch composed mostly of degraded amylopectin with the A-type polymorphic crystalline form and V-type crystallites.

In our second approach, depicted in Scheme 2 (right side Figure 3.1), we placed the isolated indigestible residue (P1, 2.0%) in citrate buffer. When that mixture containing indigestible residue was heated to 100 °C for 30 min, enzymolysis with 0.5% GSHE for 24 h at 62 °C gave 37.01% conversion to D-glucose based on the starting amount of maize starch (Figure 3.5). The two stage hydrolysis of maize starch by GSHE, where the second stage is the indigestible residue from the first, gave a total conversion of 98.86% of starch to D-glucose (61.85% + 37.01%) (Table 3.5).

Although we achieved ~ 95% conversion of granular maize starch by preswelling of normal maize starch at 70 °C in citrate buffer (Table 3.2), the approach 2 in Scheme 2 of Figure 3.1 provided a way to convert the indigestible residue to release the remaining 5% glucose. Therefore, we can achieve near 100% conversion of the starch to glucose. We should point out that we used a low starch concentration (2%, w/w) in this study, but we are currently designing a saccharification process to convert the maize starch at higher concentration (ca. 30%).

Table 3.5 Effect of re-hydrolysis and cooking on converting the enzyme resistant residues after native and partial swollen maize starch hydrolyzed by GSHE.

Pretreating Temperature (°C)	Processing temperature (°C) for P1[A]	Glucose content (%) in S1[B]	Glucose content (%) in S2[B] or S3[B]	Total Conversion (%)
62	62	62.01±0.23 b	1.8±0.01 d	63.81
62	100	61.85±0.53 b	37.01±0.25 b	98.86
70	100	94.3±0.5 a	5.03±0.19 c	99.33
100			99.57±0.81 a	99.57

[A]P1 was the precipitate from first time hydrolysis and the processing temperature was the heat treatment temperature in which Precipitate 1 (P1) mix with fresh buffer from Schema 2, Figure 3.1.

[B]S1, S2, S3 were the supernatant shown in Scheme 1 and 2, Figure 3.1 and all conversion rates were based on total glucose from initial maize starch (2 g, dry weight). Means with different letters within the same column of are significantly different ($p < 0.05$).

Conclusions

In conclusion, preswelling of maize starch granules at 70 °C in dilute citrate buffer (PH 4.5) enhanced starch saccharification by Stargen 002, a new version of GSHE. This relatively low temperature process reduces the energy required to destroy crystalline lamellae in starch granules so that the conversion of maize starch exceeded 95%. The reason that a portion of the starch was resistant to GSHE may be due to a dense starch structure which physically limits enzyme binding are thereby hinders enzymatic hydrolysis. The indigestible resistant residue, which was less than 5% of the starting normal maize starch was converted to D-glucose after heating to 100 °C for 30 min in citrate buffer. The total conversion of normal maize starch to glucose was practically quantitative in our low-temperature process using granular starch hydrolyzing enzyme.

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Chapter 4 - Partial Swelling Enhances Amylolysis of Granular Normal Maize Starch by α-Amylase

Abstract

Amylolysis of granular starch by α-amylase usually requires relatively long incubation time because granules are partially crystalline and densely packed. In this study, partial swelling was investigated as a strategy to enhance the enzymatic hydrolysis of granular normal maize starch at high solids content (30%, w/w). Starch granules were partially swelled at 70 °C and the amylolysis at 70 °C displayed an approximately 33% increment in degree of hydrolysis compared with the hydrolysis at 62 °C. Both thermal and degree of crystallinity analysis indicated that the reaction at 70 °C exposed crystalline lamellae to α-amylase hydrolysis. Microscopic analysis showed that many granules lost birefringence at 70 °C. Molecular size distribution further demonstrated a greater hydrolysis of amylopectin. Partial swelling of normal maize starch granules in the presence of α-amylase at 70 °C could enhance the enzymatic hydrolysis of crystalline lamellae in granular starch.

Keywords

granular normal maize starch, α-amylase, enzymatic hydrolysis, granule swelling

Introduction

Starch, the second most abundant biomass in nature, is biosynthesized as partially crystalline granules in higher plants, and is normally a mixture of two polysaccharides, amylose and amylopectin (BeMiller & Whistler, 1984). Normal maize starch is one of the most important raw material in industry, accounting for more than 80% of total starch production in the world (Bergthaller, 2003). Starch is commonly converted into fermentable sugars through liquefaction

and saccharification process by using α-amylase and amyloglucosidase (James & Lee, 2007; Sundrram & Murthy, 2014). However, the cooking process during liquefaction is a costly step due to high energy input and capital cost (Bothast & Schlicher, 2005; Sun et al., 2010).

Instead of cooking starch alternative approach is to enzymatically hydrolyze granular starch under sub-gelatinization temperatures. This granular starch hydrolysis has great potential benefits, e.g. reduction of energy consumption, capital savings (Uthumporn, Zaidul & Karim 2010; Li, Vasanthan & Bressler 2012). However, hydrolyzing starch granules normally result in a slow rate of hydrolysis (Oates, 1997) as a result of the structural heterogeneity and crystalline nature of starch granules (Robertson et al., 2006). Starch molecules are densely packed in starch granules that are partially crystalline, and are resistant to enzymatic hydrolysis depending on sources of starch (Bird et al., 2009).

In order to enhance the enzymatic hydrolysis of granular starch, our group has developed an approach to partially swell starch granules followed by conversion of starch using granular starch hydrolysis enzyme (GSHE) (Li et al., 2014). The conversion of normal maize starch to glucose could increase by 3 times and the enzyme kinetic study showed that GSHE had higher affinity for the partial swollen granules.

At high starch solids content (> 25%, w/w), swelling of granules would dramatically increase the viscosity of the normal maize starch slurry and lead to a gel formation. The objective of this study was to develop a process that would avoid the viscosity build up during swelling of starch granules. We added α-amylase during the swelling of normal maize starch granules at 30% solids. The effect of partial swelling at 70 °C on amylolysis were investigated and compared with the amylolysis at 62 °C without swelling at 70 °C. This approach of partial

swelling of starch granules in the presence of α-amylase may be followed by saccharification with granular starch hydrolyzing enzyme (GSHE), converting starch to glucose.

Materials and methods

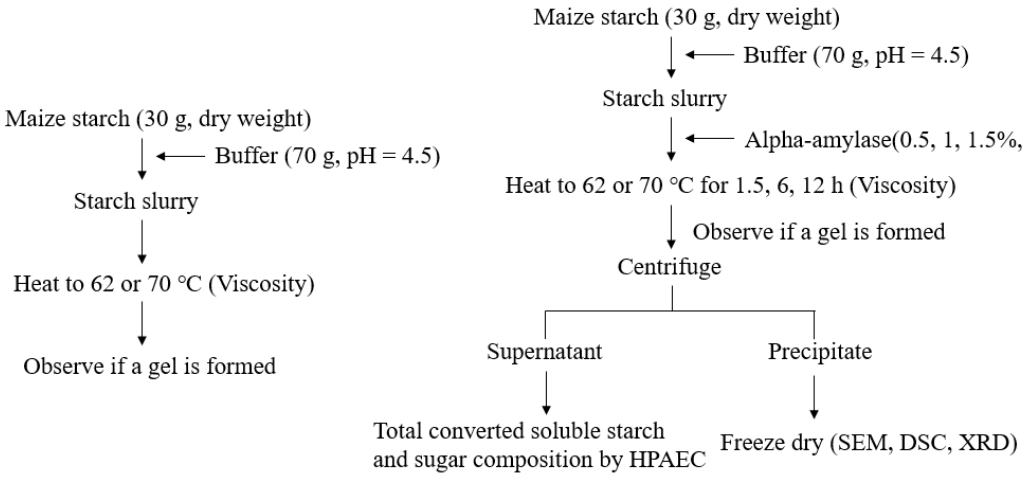
Materials

Normal maize starch was obtained from Tate & Lyle, Hoffman Estate, IL. Soluble starch was purchased from Zhanwang Chemical Reagent Co. (Huzhou, China). Acid-stable α-amylase (GC626) was obtained from Genencor International (Palo Alto, CA, USA). The activity of GC626 was 100,000 SSU/g (optimal temperature: 60 - 70 °C, optimal pH: 3.5 - 4.5). Based on information from supplier (Genencor), the soluble starch units (SSU) was determined by the reducing power of 1 mg of glucose released per minute of soluble starch at the specific incubation conditions (pH 4.5, 50 °C). One unit of α-amylase activity was defined as 1 ml enzyme liquefy 1mg soluble starch in 1 min at 70 °C, pH = 6.0. The unit is expressed in U/mL. All the other chemicals were purchased from Fisher Scientific (Santa Clare, CA, USA).

For α-amylase activity test, soluble starch (0.4 g) was dissolved in 25 mL of 50 mM sodium acetate buffer (pH 6.0). Mixed well and adjusted the slurry in 62 or 70 °C water baths for 8 min. Then added 1 mL of appropriately diluted enzyme solution and the mixture was incubated at 62 or 70 °C for 5 min. Then 1 mL of reaction solution was removed to a 5.5 mL solution which contained 5 mL iodine reagent (0.01% I_2 in 4% KI) and 0.5 mL of 0.1 M HCl, which stopped the enzymatic hydrolysis. Another mixture without enzyme was used as a control. The degradation of starch by the enzyme was determined by comparing the absorbance of the samples or the control at 660 nm.

Starch amylolysis by α-amylase

Amylolysis of normal maize starch by α-amylase is shown in (Scheme 2, Figure 4.1). Preheating the starch without α-amylase was used as a control (Scheme 1, Figure 4.1)). Normal maize starch slurry (100 g, 30%, w/w) was prepared in 50 mM citrate buffer (pH = 4.5). Acid-stable α-amylase (0.5%, 1.0% or 1.5% based on the weight of starch, w/w, which were 500, 1000, 1500 SSU/g starch) was added to the slurry. The mixture was incubated in a water bath at 62 or 70 °C for different time intervals (1.5, 6, 12 h). After centrifugation at 5000 g for 10 min, the supernatant was heated in a boiling water for 15 min to stop the hydrolysis. The concentration and composition of total sugar in the supernatant was separated and determine by a high-performance anion exchange chromatography. In addition, the starch residue in precipitate was collected and washed with absolute ethanol (200 ml), centrifuged at 1500 rpm for 10 min and repeated twice. After centrifugation, the starch residue was vacuum dried overnight and saved for further analysis. Preheating the starch slurry without α-amylase was used as control. The process was summarized in Scheme 2, Figure 4.1.



Scheme 1 **Scheme 2**

Figure 4.1 Experimental design of preheating 30% maize starch slurry with and without α-amylase hydrolysis

Rheological properties of preheated slurries

The rheological properties of the normal maize starch (preheated at 62 and 70 °C) were determined by an ATS rheometer (Canon Instrument Company, Bordentown, NJ). Each starch slurry (100 g, 30%, w/w) was added α-amylase (0.5%, w/w basing on starch weight) and heated at 62 or 70 °C for 30 min. After heat treatment in presence of α-amylase, the slurry was cooled to room temperature, and 1 g of starch mixture was taken out by a spoon for rheological property test. Starch slurry without adding α-amylase was used as a control. (Scheme 1, Figure 4.1).

The gel samples were loaded on a set of 25 mm surface parallel plate attached on an ATS rheometer (Canon Instrument Company, Bordentown, NJ) and pressed to 2 mm gap. Viscosity of the starch gel was determined at 1% strain at constant frequency of 1 Hz. Small deformation oscillatory measurements (10-1000 Pa) was carried out at 25 °C to test rheological properties. The liquid samples (scheme 2, Figure 4.1) were loaded in a concentric-cylinder and tested by the same system (Bohlin Rheometer System CVOR 150).

Total sugar in the supernatant

The total sugar content in the supernatant obtained from the previous section (scheme 2, Figure 4.1) was determined using AACC Method 76–13.01.

Composition of maltooligosaccharides (MOS) in the supernatant

The supernatant obtained from scheme 2 (Figure 4.1) was diluted with water (1:100, w/w) and filtered by a 0.22 μm syringe filter. The filtrate (10 μL) was analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Saccharide profile analysis of the diluted supernatants was analyzed using the procedure of Cai and Shi (2010). Eluents were (A) 500 mM NaOH and (B) 150 mM NaOH with 0.5 M sodium acetate. The gradient program was as follows: 85% eluent B for 0−0.4 min, 30% at 20

min, 25% at 30 min, 0% at 35 min, 0% at 40 min, 85% at 41 min, and 85% at 55 min. The separations were carried out at 25 °C at a flow rate of 1 mL/min. Quantification of maltooligosaccharides (MOS) were carried out by an external standard method using a mixture containing from glucose to maltoheptaose (G1–G7) in a concentration from 2 to 10 μg/mL (Grewal et al., 2015).

Differential scanning calorimetry (DSC)

The thermal properties of unmodified normal maize starch and each precipitated sample (scheme 2, Figure 4.1) were determined by differential scanning calorimetry (DSC) using a TAQ5000DSC machine from TA Instruments (New Castle, DE, USA). Each starch sample (8 mg, dry basis) was placed in an aluminum DSC pan, and deionized water was added to each sample to achieve a starch-water ratio of 1:3 (w/w). The sample pans were hermetically sealed, equilibrated at 4°C for 24 h and scanned from 10°C to 140°C at a heating rate of 10 °C/min. A sealed, empty DSC pan was used as a reference (Grewal et al., 2015).

X-ray diffraction

X-ray diffractograms of native maize starch and each precipitated sample (scheme 2, Figure 4.1) was obtained using a wide-angle X-ray diffractometer (PANalytical, Almelo, the Netherlands). The operation was under 35 kV and 20 mA, theta-compensating slit and a diffracted beam monochromator. X-ray diffraction (XRD) patterns were recorded within the 2θ range of 5-35°. The relative crystallinity was calculated by integrating the area under the fitted crystalline peaks using MDI Jade 6.0 software (Komiya & Nara, 2010).

Light microscopy

The normal maize starch and enzyme resistant residues (precipitates in Scheme 2, Figure 4.1) were examined under a light microscope (Olympus BX51TF, Olympus Optical Co. Ltd.,

Shinjuku-ku, Tokyo, Japan) which connected to a digital camera (SPOT 18.2 Color Mosaic camera, Diagnostic Instruments Inc., Sterling Heights, MI, USA) and a computer to visually observe the morphology and birefringence (scheme 2, Figure 4.1). Samples were viewed through a coverslip for images viewed with a 40× objective. Images were captured and analyzed using SPOT Insight camera and SPOT advance software (Diagnostic Instruments Inc., Sterling Heights, MI) (Sichaya and Shi 2016).

Scanning electron microscopy (SEM)

The normal maize starch and each precipitated sample (scheme 2, Figure 4.1) were coated with gold-palladium using a sputter coater (Denton Vacuum, LLC, Moorestown, NJ) and viewed at 1000× magnification with a scanning electron microscope (SEM) (S-3500N, Hitachi Science System, Ltd, Japan) operating at a voltage of 10.0 kV.

Gel permeation chromatography (GPC)

Molecular size (MS) distribution was performed on a PL-GPC 220 instrument (Polymer Laboratories, Inc., Amherst, MA, USA). The GPC instrument was determined with three Phenogel columns, a guard column (Phenomenex, Inc., Torrance, CA, USA) and a differential refractive index detector. Sample preparation and chromatographic conditions were followed by the method as described previously (Cai, Shi, Rong, & Hsiao, 2010).

Statistical analysis

Experimental results are reported as the average of triplicate experiments. Statistical analyses were carried out using Microsoft Excel 2010 (Microsoft, Seattle, WA).

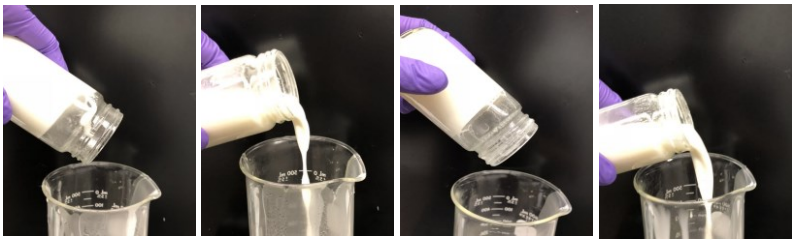
Results and discussion

Alpha-amylase activity and its effects on preventing starch from gelling during swelling of starch granules

The α-amylase activity of GC 626 was 3.8× 10^4 u/mL and 3.1× 10^4 u/mL at 62 and 70 °C, respectively (Table 4.1). The α-amylase activity at 70 °C was slightly lower at 62 °C.

Normal maize starch slurry (30% solids, w/w) formed a gel at 62 and 70 °C without α-amylase (Table 4.1). The viscosity of the starch heated at 62 °C and 70 °C was 2.05×10^6 and 2.85×10^6 cP, respectively. In contrast, the viscosity of the starch heated at 62 °C and 70 °C in the presence of α-amylase was only 15 or 12 cP. The α-amylase used during the heating process helped reduce the viscosity and kept the mixture in liquid form (Table 4.1) by hydrolyzing the swelling maize granules during the heating process. The slurry could be easily stirred and provided a substrate which could be further hydrolyzed by other enzymes. The α-amylase hydrolysis process degraded the maize granule into small fragments and maltooligosaccharides which would also be further discussed in following sections.

Table 4.1 Enzyme activity and viscosity of preheated starch slurry with or without α-amylase hydrolysis.

Temperature (°C)	62		70	
α-amylase (%)	0	0.5	0	0.5
Enzyme activity (×10^4 u/ml)	0	3.8±0.4b	0	3.1±0.2a
Viscosity (cP)	2.05×10^6±215c	15±1a	2.85×10^6±189b	12±0a
Appearance				

Means with different letters within the same column of are significantly different ($p < 0.05$).

Degree of hydrolysis

The granular normal maize starch was hydrolyzed by α-amylase to release soluble molecules (supernatant) with different degrees of polymerization (DP). Compared with the reaction at 62 °C, granular maize starch became more susceptible to α-amylase hydrolysis at 70 °C (Figure 4.2). The effect of reaction temperature was more evident at the lower level of α-amylase added (0.5%, w/w). Based on the yield of total converted soluble starch, after 12 h, the degree of hydrolysis at 70 °C was a nearly 33% greater compared to the hydrolysis at 62 °C. Holding the granular maize starch at 70 °C could cause partially swelling of the granules, leading to greater access for α-amylase to act on the starch (Li et al., 2014). It is also probable that during the amylolysis, the slight swelling of the granule resulted in expansion of the naturally present pores on the granule surface, facilitating the adsorption and subsequent penetration of enzyme molecules (Figure 6 A, D, E and F).

To determine whether it was swelling granule or the enzyme activity contributed to the enhancement of α-amylase hydrolysis. The enzyme activities at 62 and 70 °C were tested. When temperature increased from 62 to 70 °C, the enzyme activity decreased by 22% (Table 4.1). Therefore, the enhanced hydrolysis at 70 °C was due to the swelling of starch granules but not the α-amylase activity itself.

In addition, enzyme concentration played a positive role in granular starch enzymatic hydrolysis, especially at the reaction temperature of 62 °C. When the α-amylase concentration was doubled or tripled, the total soluble starch in starch hydrolysate consequently increased by approximately 35% or 50%, respectively (Figure 4.2). On the other hand, after 6 h of amylolysis at 70 °C, no further increase in soluble starch was observed. Similar trend was obtained when 0.5% α-amylase was used at 62 °C. However, the α-amylase increased to1.0% and 1.5%,

granular maize starch could be further hydrolyzed after 6 h, which might be caused by the improved access of enzyme and substrate. Moreover, increasing the reaction temperature from 62 °C to 70 °C caused a slightly 4% increment in the yield of total soluble starch after 12 h of amylolysis, suggesting that enzymatic hydrolysis at 62 °C could match that at 70 °C by increasing the level of α-amylase or prolonging the reaction time.

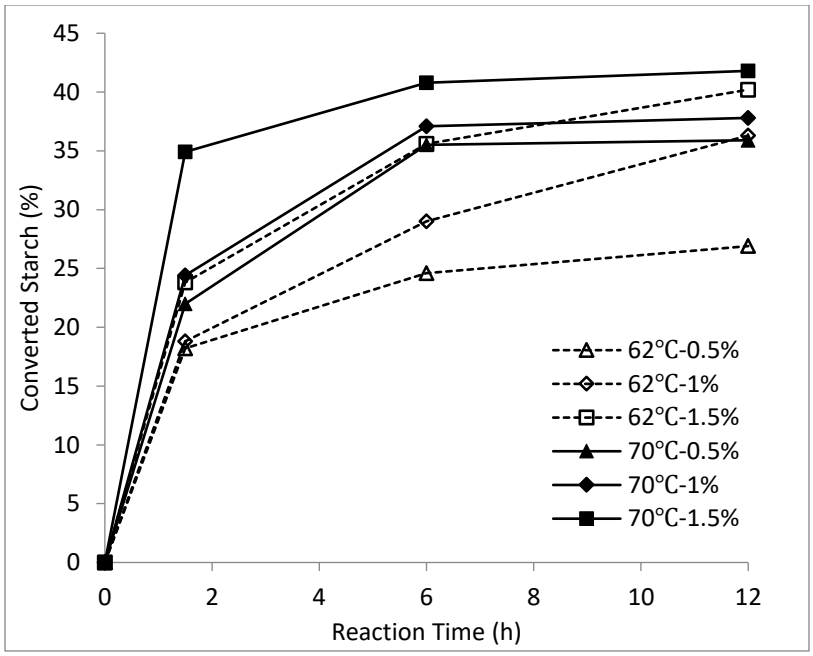


Figure 4.2 Yield of total converted starch during the amylolysis of normal maize starch.

Composition of soluble starch hydrolysate

To further analyze product profiles from the amylolysis of granular normal maize starch, the composition of MOS ranging from glucose (G1) to maloheptaose (G7) was determined by HPAEC-PAD and shown in Table 4.2. Glucose, maltose and maltotriose were major products in starch soluble hydrolysate and they made up more than 70% of the MOS. However, amylolysis at 62°C and 70°C showed significant difference in the composition of the soluble hydrolysate. Glucose was dominant in all hydrolysate samples hydrolyzed at 62 °C. In contrast, similar levels of glucose, maltose and maltotriose were observed in the hydrolysates hydrolyzed at 70 °C

As the incubation time prolonged, the composition of the soluble hydrolysate at 62 °C changed and the amount of glucose gradually increased. Interestingly, at the reaction temperature of 70 °C, the composition of the soluble hydrolysate almost stayed the same during the first 6 h, while the yield of total soluble starch increased rapidly. However, when total soluble starch hydrolysate reached a plateau value during the following 6 h of amylolysis, the changes in MOS composition was detected. These results suggested that α-amylase might primarily attack the swollen starch granules in the first 6 h of reaction at 70 °C, resulting in the increased yield of soluble hydrolysate but product composition remained the same. Subsequently, α-amylase molecules were more likely to hydrolyze on the soluble hydrolysate and form oligosaccharides shorter than DP3, but hardly affecting the amount of total hydrolysate in the supernatant. Throughout the incubation at 62 °C, hydrolysis possibly occurred on both insoluble granules and dissolved hydrolysate, causing the yield and composition of total hydrolysate changed simultaneously. In addition, after reaction for 6 h, the degree of hydrolysis at 70 °C was similar to that at 62 °C with tripled α-amylase addition (Table 4.2), but the composition of the hydrolysate produced at 62 °C and 70 °C for 12 h was different.

Table 4.2 Composition of maltooligosaccharides ranging from glucose to maloheptaose during the amylolysis of granular starch.

Temperature (°C)	Time (h)	CG 626 [A] (%)	Degree of polymerization (%)						
			1	2	3	4	5	6	7
62	1.5	0.5	35.2±0.1g	34.1±0.6a	19.1±0.8a	5.3±0.7cdef	2.7±0.7bc	1.9±0.7c	1.5±0.4ab
		1	48.0±0.2cd	33.6±0.9ab	8.1±0.8a	3.9±0.6ef	3.5±0.5bc	1.9±0.5c	0.9±0.5b
		1.5	50.0±0.3bc	30.16±0.6abc	8.5±0.7a	4.2±0.7ef	3.2±0.5bc	2.9±0.6abc	1.1±0.6ab
	6	0.5	38.9±0.9ef	29.6±1.0bcd	16.9±0.1ab	5.9±0.8cdef	3.6±0.4bc	2.9±0.3abc	1.9±0.5ab
		1	47.4±0.4cd	29.2±0.8cd	15.5±0.8ab	2.8±0.7f	2.2±0.2c	1.7±0.3c	1.0±0.2ab
		1.5	52.8±0.6b	25.9±0.5de	8.0±0.4ab	4.6±0.6def	3.9±0.7bc	3.0±1.0abc	1.6±0.2ab
	12	0.5	47.3±0.6cd	28.1±0.5cd	12.5±0.9abc	4.8±0.4def	3.3±0.6bc	2.6±0.3bc	1.3±0.7ab
		1	53.2±0.8b	27.1±0.7cde	7.7±1.0abc	5±1.0def	2.9±0.6c	2.1±0.6c	1.9±0.9ab
		1.5	58.4±0.4a	26.9±0.4cde	6.5±0.4abc	3.2±0.2f	2.1±0.5a	1.7±0.5c	0.9±0.7b
70	1.5	0.5	29.1±0.6h	23.2±0.2e	20.3±0.7abc	10.8±0.3a	7.3±0.5bc	5.3±0.1ab	3.9±0.2a
		1	36.0±0.2fg	28.3±0.7cd	19.4±0.1bcd	8.6±0.6abc	3.7±0.7bc	2.3±0.5c	1.6±0.2ab
		1.5	40.4±0.5e	28.4±0.8cd	18.8±0.3cd	5.4±0.5cdef	2.6±0.7a	2.3±0.2c	2.1±0.5ab
	6	0.5	29.8±0.2h	24.0±1.1e	20.2±0.2d	10.7±0.7a	7.3±0.5bc	5.5±0.2a	2.5±0.3ab
		1	36.3±0.4fg	28.5±0.4cd	19.5±0.8e	7.8±0.9abcd	3.6±0.9c	2.6±0.2bc	1.5±0.4ab
		1.5	40.5±0.7e	29.1±1.2cd	18.2±0.9e	5.5±0.5cdef	2.3±0.7c	2.4±0.2c	2.0±0.8ab
	12	0.5	33.3±0.8g	25.7±0.3de	20.6±0.7e	10.4±0.6ab	5.8±0.8ab	2.5±0.7c	1.7±0.1ab
		1	38.9±1.0ef	28.9±0.7cd	19.5±0.5e	7.1±0.2bcde	2.2±0.2c	1.9±0.3c	1.4±0.5ab
		1.5	45.2±0.d	29.4±0.9cd	16.2±0.6e	2.7±0.8f	2.4±0.4c	2.4±0.6c	1.7±0.9ab

[A] α-amylase (%) based on the weight of the starch (w/w).

Means with different letters within the same column are significantly different ($p < 0.05$).

Structure and properties of the enzyme resistant residues precipitate (scheme 2, Figure 1)

Thermal properties

Thermal properties of the native and normal maize starch and its enzyme resistant residues are shown in Table 4.3. Compared to the native starch, the onset gelatinization temperatures of the hydrolyzed starches increased ca 2-11 °C and enthalpy (ΔH) decreased from 12.15 J/g to 6.5 - 9.6 J/g (Table 4.3).

After the normal maize starch was hydrolyzed at 62 °C, the onset gelatinized temperature of enzyme resistant residues increased to 70.4 -73.8 °C, but the ΔH decreased to 8.9 - 9.6 J/g (Table 4.3). In comparison, at 70 °C, the onset gelatinization temperature of the enzyme resistant residues increased to 76.9 – 79.4 °C, and the ΔH decreased to 6.1 – 8.2 J/g. The reduction of ΔH largely occurred in the first 0.5 h. After 6 h, there was little change in the onset gelatinization temperature of the enzyme resistant residues.

Table 4.3 Thermal properties of native and hydrolyzed starches.

Temperature (°C)	CG 626[A] (%)	Time (h)	T_o (°C)	T_p (°C)	T_c (°C)	ΔT (°C)	ΔH (J/g)
Native			68.6±0.5f	71.6±0.4h	89.3±0.2a	20.6±0.4a	12.1±0.3a
62 °C	0.5	1.5	70.4±0.5ef	73.8±0.1gh	86.0±0.5cdef	15.5±1.0b	9.6±0.6a
		6	73.6±0.4cd	76.3±0.4fg	86.4±0.8bcde	12.7±1.2bcde	9.2±1.7a
		12	72.9±0.2cd	75.8±0.4fg	85.8±0.2def	12.9±0.0bcde	9.2±0.2a
	1.0	1.5	71.2±0.5de	74.6±0.5fg	85.5±0.9ef	14.3±0.5bc	9.4±0.4a
		6	73.5±0cd	76.2±0.1fg	86.4±0.8cdef	12.9±0.7bcde	9.2±0.5a
		12	73.8±0c	76.7±0.4def	87.0±0.4abcd	13.1±0.5bcde	8.9±0.4a
	1.5	1.5	71.6±0.2cde	74.9±0.6fg	85.1±0.6f	13.5±0.8bcd	9.5±0.4a
		6	73.2±0.4cd	76.2±0.4fg	85.8±0def	12.6±0.4bcde	9.1±0.4a
		12	73.7±0.2c	76.6±0.3ef	86.2±0.8cdef	12.5±0.6efg	9.2±0.1a
70 °C	0.5	1.5	77.3±0.6ab	80.8±0.8abc	88.6±0.2abc	11.4±0.9defg	8.2±0.8b
		6	79.0±0.5ab	81.7±0.3ab	89.3±0.2a	10.3±0.3defg	7.3±0.1b
		12	79.4±0.6a	81.8±0.4a	88.4±0.2abcd	9.0±0.4fg	6.1±0.2b
	1.0	1.5	76.9±0.7b	79.2±0.7bcd	89.0±0.3ab	12.1±1.0bcde	8.0±0.5b
		6	79.4±0.4a	82.0±0.3a	88.5±0.1abc	9.1±0.3fg	6.7±0.2b
		12	78.6±0.3ab	81.1±0.1abc	88.0±0.1abcd	9.4±0.3efg	6.5±0.3b
	1.5	1.5	77.0±0.8ab	79.0±0.8cde	89.5±0.4a	12.5±0.4bcde	7.9±0.2b
		6	78.5±0.2ab	80.6±0.1abc	87.3±0abcd	8.9±0.2g	6.6±0.2b
		12	78.7±0.1ab	80.8±0.1abc	88.3±0.2abcd	9.6±0.1efg	6.5±0.4b

[A] α-amylase (%) based on the weight of the starch (w/w).

[B] T_o=onset temperature, T_p=peak temperature, T_c=conclusion temperature, $\Delta T = T_c - T_o$, ΔH=enthalpy change. Values are means ± SD (n=3). Means with different letters within the same column are significantly different ($p < 0.05$).

Crystallinity

Native normal maize starch and enzyme resistant residues showed a typical A-type diffraction patterns with peaks at 2θ values of 15°, 17°, 18° and 23° (Figure 4.3). After hydrolyzing, the enzyme resistant residues had a significant loss in crystallinity (Figure 4.4), which is consistent with the ΔH results (Table 4.3).

It was worth noting that reaction temperature played a significant role in the relative crystallinity of hydrolyzed residues. Heating at 62 °C and 70 °C caused more than 20% and 33% (Figure 4.4) decrease in relative crystallinity, respectively, indicating that enzymatic hydrolysis at 70 °C resulted in more disruption of crystallite.

The reaction time was another important factor effecting digestion. Increased reaction time from 1.5 h to 6 h decreased the crystallinity greatly, but after 6 h, the relative crystallinity decreased very slowly, suggesting that the reaction was intense in the first 6 h then slowed down greatly. The further digestion after 1.5 h also indicated the decrease of crystallinity was caused by enzymatic erosion rather than persistent thermal destruction (Figure 4.4).

In addition, enzyme concentration positively affected the degree of hydrolysis at 62 °C (Figure 4.2) much greater than affected the relative crystallinity of hydrolyzed residues (Figure 4.4). Previous study had found that amylolysis occurred primarily in the amorphous regions of starch granules (Gallant, Derrien, Aumaitre, & Guilbot, 1973), which might explain the observations in Figure 4.4. Increased enzyme concentration from 1% to 1.5% could accelerate product released from amorphous regions, but hardly destruct the crystalline lamellae.

Swollen starch granules at 70 °C and proper increase the heating time could effectively destruct the crystalline structure and expose partial crystallites for enzymatic attack. Increased the enzyme amount could help accelerate the process but more α-amylase

did not enhance digestion. Alpha-amylase could only hydrolyze the granule starch at certain point and granule starch hydrolyze enzyme is still needed for further saccharification for low-temperature sugar production.

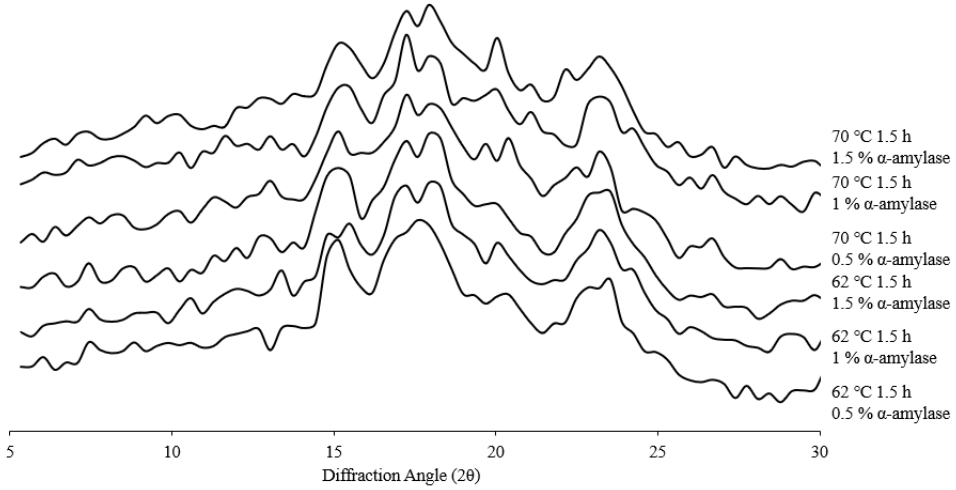


Figure 4.3 X-ray diffraction patterns of granules of native and hydrolyzed normal maize starch heated at different temperatures for 12 h with constant shaking.

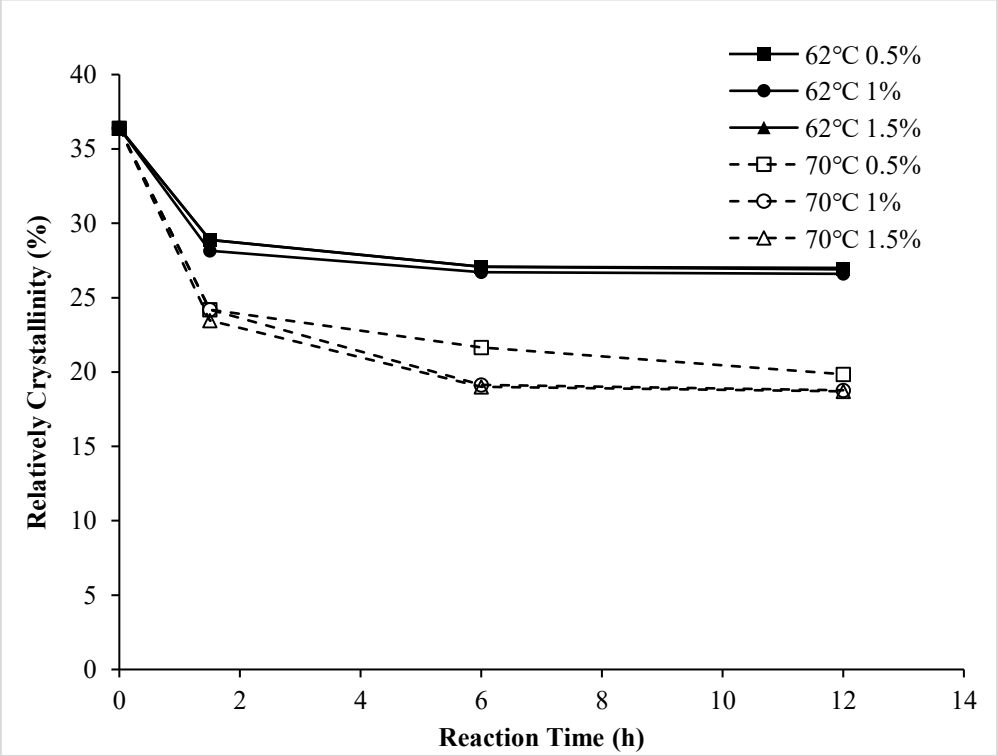


Figure 4.4 Relative crystallinity of granular starch residues during the amylolysis of normal maize starch.

Granule morphology

The shape, size, structure and surface characteristics of normal maize starch granules (native and hydrolyzed) were investigated using light microscope (Figure 4.5) and SEM (Figure 4.6).

The micrograph under normal light showed that the native normal maize starch was polygon and birefringent indicating that the orientation of the polymer chains was radial inside the granules (Figure 4.5 A). At 62 °C, few starches were disrupted and most of them remained birefringent, indicating that 62 °C heat treatment and α-amylase hydrolysis did not destruct crystalline structure of granule starch. On the other hand, many starch granules lost their birefringence when hydrolyzed at 70 °C, and the swollen of granules were very obvious after heating treatment. With the increase of enzyme concentration, the erosion of granules and loss of birefringence was increased at 70 °C.

SEM also shows that native starch granules displayed an irregular and mostly polygonal shape with randomly distributed visible pores and pits on surface (Figure 4.6 A). The effect of enzymatic treatment was readily visible in the hydrolyzed starches microstructure (Figure 4.6 B-F). As reported by Helbert, Schülein, and Henrissat (1996), enzymatic hydrolysis of starch granules starts mainly at the surface, penetrates into and finally disrupts the interior structure, forming porous starch granules.

When granular normal maize starch was hydrolyzed at 62 °C, few granules were broken down into fragments, indicating the slight disruption of crystalline lamellae, which was consistent with the inference from relative crystallinity (Figure 4.4). Contrarily, as shown in Figure 4.6 E-F, starch residues hydrolyzed at 70 °C could be eroded to a higher degree. The temperature of 70 °C caused the swelling of starch granules and exposed partial crystallites. As a consequence, the internal parts of granules were acted and degraded. After reaction of 12 h, starch granules hydrolyzed by higher concentrations (1% and 1.5%) of

enzyme showed more evident collapse of granular structure, indicating a higher degree of crystallinity degradation (Figure 6 E, F).

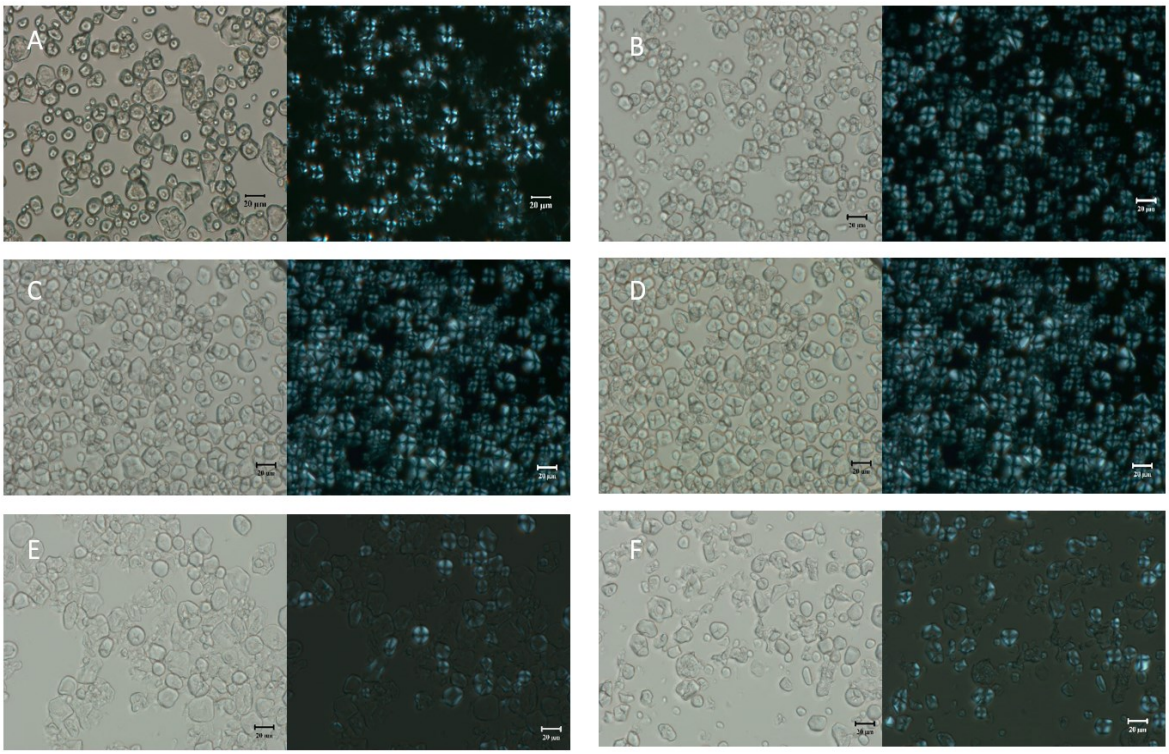


Figure 4.5 Light microscopic images of granules of maize starch native and hydrolyzed normal maize starch. The scale bar indicates 20 µm. (A) Native normal maize starch. (B) residues from a 1.5-h-amylolysis of normal maize starch at 62 °C (enzyme concentration=0.5%, w/w). (C) residues from a 6-h-amylolysis of normal maize starch at 62 °C (enzyme concentration=0.5%, w/w). (D) residues from a 12-h-amylolysis of normal maize starch at 62 °C (enzyme concentration=0.5%, w/w). (E) residues from a 1.5-h-amylolysis of normal maize starch at 70 °C (enzyme concentration=0.5%, w/w). (F) residues from a 12-h-amylolysis of normal maize starch at 70 °C (enzyme concentration=1.5%, w/w).

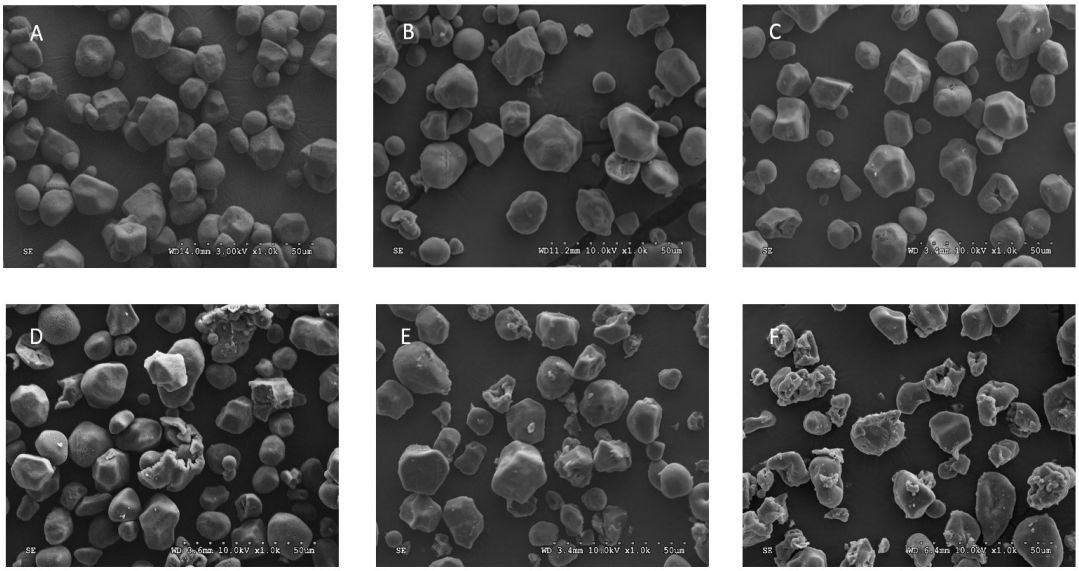


Figure 4.6 Scanning electron micrographs of native and hydrolyzed normal maize starch. The scale bar indicates 50 µm. (A) Native normal maize starch. (B) residues from a 1.5-h-amylolysis of normal maize starch at 62 °C (enzyme concentration=0.5%, w/w). (C) residues from a 6-h-amylolysis of normal maize starch at 62 °C (enzyme concentration=0.5%, w/w). (D) residues from a 12-h-amylolysis of normal maize starch at 62 °C (enzyme concentration=0.5%, w/w). (E) residues from a 1.5-h-amylolysis of normal maize starch at 70 °C (enzyme concentration=0.5%, w/w). (F) residues from a 12-h-amylolysis of normal maize starch at 70 °C (enzyme concentration=1.5%, w/w).

Molecular size distribution

The molecular size distribution (MSD) of normal maize starch and resistant residues is shown in Figure 4.7. As Li, Prakash, Nicholson, Fitzgerald, and Gilbert (2016) reported, Peak 1 (Figure 4.7, right peak) is amylopectin (AMP) with larger molecular sizes ($100 \leq R_h \leq 4000$), while Peak 2 (left peak) is amylose with smaller molecular sizes ($10 \leq R_h \leq 100$ nm). Compared with hydrolysis at 62 °C, amylolysis at 70 °C induced more significant reduction of Peak 1, implying a greater degradation of amylopectin. The peak in range of 0 – 10 nm is considered as starch hydrolysate with lower molecular weight. Evidently, amylolysis at 70 °C produced more smaller molecular dextrin. The crystalline lamellae in granules was affected to a greater extent when enzymatic hydrolysis was performed at 70 °C.

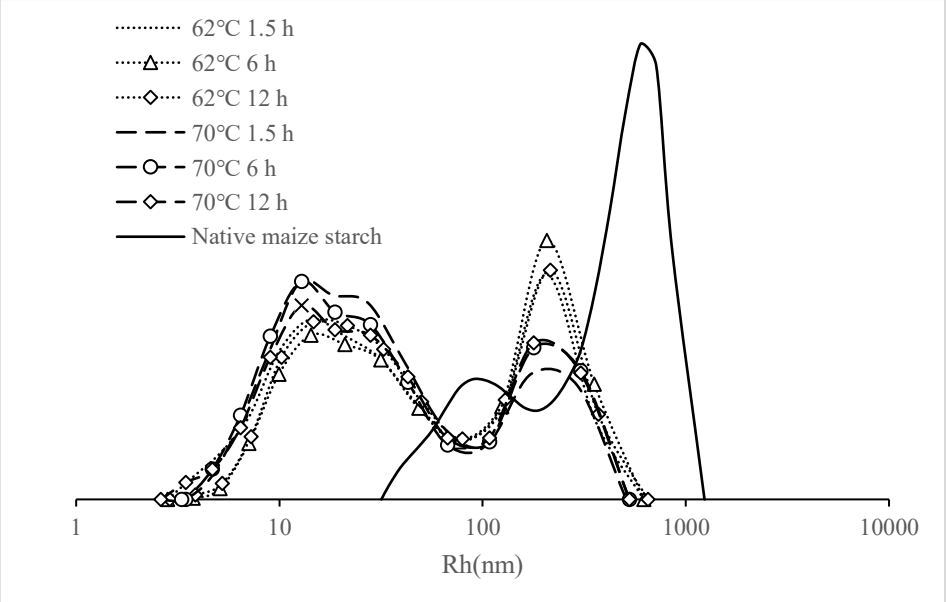


Figure 4.7 Molecular size distributions of granular starch residues during the amylolysis of granular starch (enzyme concentration=1.0%, w/w).

Conclusions

Partial swelling of normal maize starch granules (30% solids) at 70 °C in the presence of α-amylase enhanced amylolysis of the granular starch, and kept the viscosity of the starch slurry low. Without α-amylase, normal maize starch (30% solids) would swell at 70 °C and form a gel. Amylolysis at 70 °C was 33% greater than that at 62 °C. Partial swelling of granules allowed greater amylolysis and reduction in crystallinity. The approach of partial swelling of granules in the presence of α-amylase may be followed by hydrolysis with granular starch hydrolyze enzyme (GSHE), further converting starch to glucose.

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Chapter 5 - Two-Step Enzymatic Hydrolysis Enhances the Saccharification of Granular Normal Maize Starch at High Concentration

Abstract

The enzymatic hydrolysis of granular starch usually requires relatively long incubation times, as a result of the resistance of crystalline lamellae, restricting the application of granular starch hydrolyzing enzyme (GSHE). A two-step enzymatic hydrolysis, during which starch granules was pre-hydrolyzed using α-amylase for 6 h at 70 °C and followed the addition of GSHE and incubation at 62 °C for 72 h, was investigated as a strategy to improve the efficiency of saccharification process. The results of granule morphology analysis showed that during the amylolysis at 70 °C, α-amylase could partially destroy the crystalline lamellae and granular structure of normal corn starch. Hence, the first stage exposed loose crystallites to further enzymatic attack, which was beneficial for subsequent saccharification process by GSHE. As a consequence, the two-step enzymatic hydrolysis was more effective than a homothermal hydrolysis at 62 °C. After saccharification, the glucose convert rate was over 100% greater using the two-step enzymatic hydrolysis, compared with the homothermal hydrolysis. In summary, proper pretreatment could enhance the application of GSHE on granular starch and thus the two-step enzymatic hydrolysis offers great advantages in the production of glucose syrups in industry.

Keywords

granular normal corn starch, granular starch hydrolyzing enzyme, two-step enzymatic hydrolysis, saccharification

Introduction

Native starch granules are partially-crystalline and resistant to enzymatic hydrolysis (Cooke & Gidley, 1992). Due to the resistant of granular starch, most applications of amylases are carried out at high temperature for gelatinization of starch. During gelatinization, the granular structure and crystalline lamellae of the starch granule are disrupted, enhancing the susceptibility to enzymatic hydrolysis. However, a high amount of energy is required to break hydrogen bonds and gelatinize starch (Ellis et al., 1998).

With the view of reducing the energy requirement and effective utilization, currently, considerable interest in the use of granular starch hydrolyzing enzyme (GSHE) has generated (Li et al., 2014; Uthumporn, Shariffa, & Karim, 2012). With the activity of GSHE, granular starch could be hydrolyzed under the onset of gelatinization temperature. Unfortunately, the hydrolysis of starch granules by enzyme at low temperature is usually extremely slow (Oates, 1997), limiting the application of GSHE in sugar production or other fermented chemicals. Therefore, strategies have been undertaken to optimize the enzymatic hydrolysis of granular starch, e.g. heat treatment (Li et al, 2014; Kong et al., 2017; Shariffa, Karim, Fazilah, & Zaidul, 2009; Uthumporn, Karim, & Fazilah, 2013) and assistant addition (Faraj, 2005; Li, Vasanthan, & Bressler, 2012; Tester & Sommerville, 2003).

As previously reported, α-amylases can hydrolyze both amorphous and crystalline domains (Colonna, Buléon, & Lemarié, 1988; Gerard, Colonna, Buleon, & Planchot, 2001). Although the mechanisms involved in the disruption of crystalline lamellae are still not well known, the adsorption of α-amylase onto starch granule was seen as a prerequisite for hydrolysis. In addition, restricted heating is known to result in the swelling of starch granules, which increases the specific area of the granules and the number of enzyme adsorption sites (Li et al., 2014). Our previous work has shown that proper heating treatment could enhance

the enzymatic hydrolysis of crystalline lamellae in granular starch which provide a better substrate for GSHE digestion (Tong and Shi, unpublished).

According to our previous studies, we used α-amylase in high concentration maize starch hydrolyzation at 70 °C which was slightly higher than onset gelatinization temperature (Tong and Shi, unpublished) and we found the heat treatment could significantly improve crystallinity degradation and reduce viscosity build-up caused by maize starch swelling. The results showed that pre-hydrolyzation by α-amylase of granular maize starch could be an excellent pretreatment for GSHE saccharification since the degradation of the recalcitrant crystal region and the partial swelling starch had a higher affinity to GSHE (Tong and Shi, unpublished).

In this study, we combined α-amylase pre-hydrolyzation of granular maize starch and GSHE saccharification at low temperature, in order to enhance the bioconversion of granular maize starch. This process is summarized as two-step enzymatic hydrolysis, due to the two different temperature used in preheating treatment and saccharification process, which accompanied with the addition of α-amylase and GSHE. The two-step enzymatic hydrolysis was devised to improve saccharification efficiency and relative mechanisms were analyzed in this study. The results could offer a deep comprehension of the effect of this two-step procedure on the enzymatic hydrolysis and provide a strategy to promote the GSHE application for sugar or other fermented chemicals.

Materials and methods

Materials

Normal corn starch was obtained from Tate & Lyle (Hoffman Estates, IL). Granular starch hydrolyzing enzyme (GSHE) (Stargen 002, 570 GAU/g) and acid-stable α-amylase (GC626，100,000 SSU/g) were obtained from Genencor International (Palo Alto, CA, USA). All the other chemicals were purchased from Fisher Scientific (Santa Clare, CA, USA).

One Glucoamylase Unit (GAU) was the amount of enzyme that liberates one gram of reducing sugars equivalent to 5.6 mmole of glucose per hour, from soluble starch substrate at pH 4.3 and a temperature of 30 °C.

Soluble Starch Units (SSU) was determined by the reducing power of 1 mg of glucose released per minute of soluble starch at the specific incubation conditions (pH 4.5, 50° C).

Two-step enzymatic hydrolysis of starch

One hundred grams of slurry containing 30% (w/w, dry basis) starch was prepared in citrate buffer (pH = 4.5, 50 mM). Subsequently, acid-tolerance α-amylase (1.0% or 1.5%, w/w, which were 1000, 1500 SSU/g starch) was added to the slurry. The mixture was incubated in a water bath at 70 °C for 6 h. After cooling to 62 °C, different levels of GSHE (0.5% or 1.0%, w/w, which were 2.85, 5.7 GAU/g starch) were added to each slurry and incubated for 72 h. The control slurry was incubated at 62 °C with 1.0% α-amylase and 1.0% GSHE for 6 h and 72 h, successively. At different time intervals, starch hydrolysate was withdrawn for measurement of glucose content using high-performance anion exchange chromatography (HPAEC). The mobile phase was 150 mM NaOH at a flow rate of 1 mL/min. The reaction was stopped by adding 3-fold-volume absolute ethanol. The enzymolysis residues were freeze-dried and ground to powders. The experimental design schema was summarized in Figure 5.1.

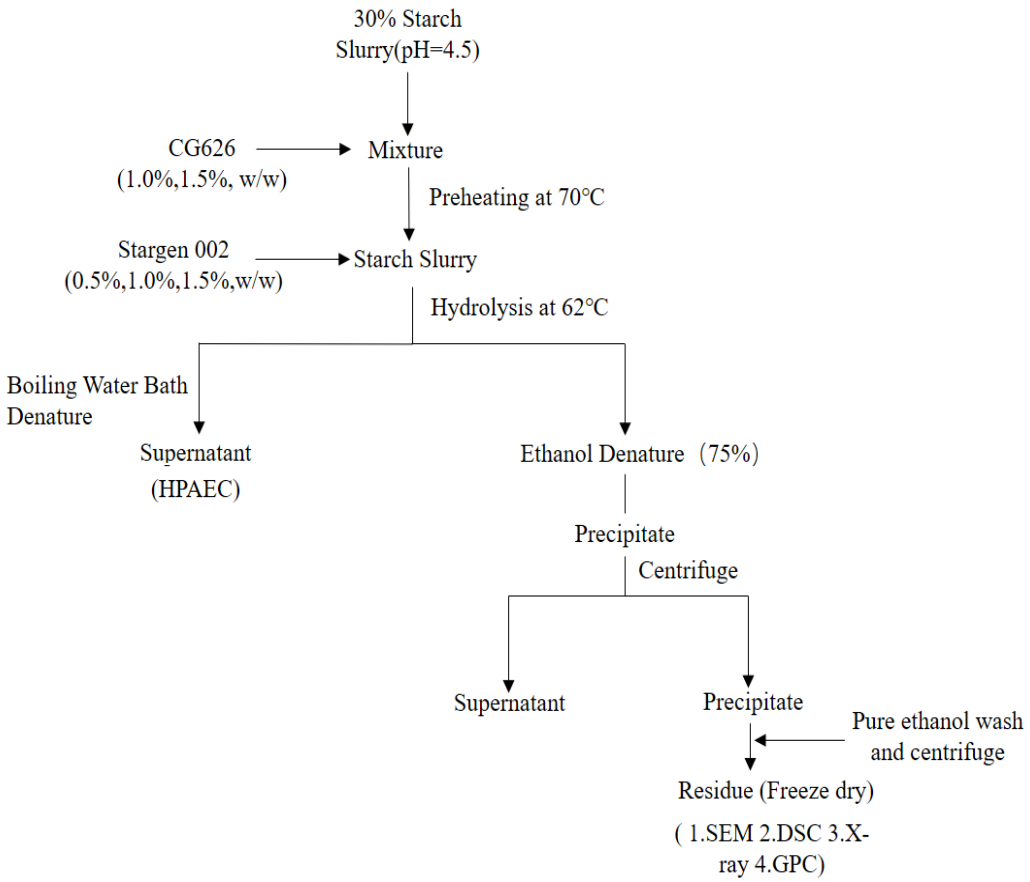

Figure 5.1 Experimental design of two-step enzymatic hydrolysis.

Light microscopy

A light microscope (Olympus BX51TF, Olympus Optical Co. Ltd., Shinjuku-ku, Tokyo, Japan) connected to a digital camera (SPOT 18.2 Color Mosaic camera, Diagnostic Instruments Inc., Sterling Heights, MI, USA) and a computer, was used to visually observe the morphology and birefringence of each sample. The images were examined and captured at the same magnification for all starch samples under both normal visible light and polarized cross-polarized light.

Scanning electron microscopy (SEM)

Each sample was coated with gold-palladium in a sputter coater (Denton Vacuum, LLC, Moorestown, NJ) and then observed using a scanning electron microscopy (SEM) (S-

3500N, Hitachi Science System, Ltd, Japan) which was operated at a voltage of 3.0 kV and 1500× magnification. (Sittipod & Shi, 2016)

Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) experiments of all samples were conducted on a TAQ5000 differential scanning calorimeter (TA Instruments, New Castle, DE, USA). Each sample (8 mg) was weighted in a stainless-steel pan and water (24 μL) was added then sealed the pan. Scans were performed from 10 °C to 140 °C at a constant rate of 10 °C/min. A sealed, empty crucible was used as a reference.

X-ray diffraction (XRD)

The moisture of samples was adjusted to around 20% in a sealed container at 25 °C. The crystalline structure of each sample was determined by a wide-angle X-ray diffractometer (PANalytical, Almelo, the Netherlands). The measurement was under 35 kV and 20 mA, theta-compensating slit and a diffracted beam monochromator. X-ray diffraction (XRD) patterns were recorded between the 2θ of 5° and 35°. Native corn starch was used as a control. (Komiya & Nara, 2010)

Statistical analysis

The statistical tests were carried out by Minitab 17 Statistical Software Program (Minitab Inc. State College PA, USA). The average and standard deviation of triplicate measurements were reported in this study.

Results and discussion

Effects of two-step enzymatic hydrolysis on the degree of saccharification

The degree of saccharification of granular normal corn starch was observed by measuring the glucose conversion rate over time and shown in Figure 5.2. The control sample was the native maize starch hydrolyzed at 62 °C instead of preheated partial swelling starch granules which could convert 58% of the starch to glucose. While for the partial swelling

starch samples we saw a great improvement in bioconversion. Six hours pretreated at 70 °C could end up with over 83% conversion of starch.

In addition, enzyme concentration played a significant role in the first stage. When the enzyme concentration of GC 626 in the first stage increased to 1.5% (w/w), the glucose conversion rate in starch hydrolysate consequently enhanced slightly by 2.4%. By comparison with 1.0% α-amylase, more amount of α-amylase in the first stage could reach a similar glucose conversion rate and we suggested keeping the same α-amylase dosage in the two-step hydrolysis. However, increasing the dosage of GSHE would benefit more on glucose conversion. When we increased the GSHE from 0.5% to 1.0%, we saw an 8-10% increment in glucose conversion. And when we used 1.5% GC 626 and 1% GSHE we could achieve 93.9% of starch converted to glucose.

Piacquadio, De Stefano, and Sciancalepore (2000) reported that heat treatment changed the internal structure of starch granules. The higher temperature in the first stage may cause greater access for both enzymes to the starch (Shariffa et al., 2009). During the first stage of the control and two-step enzymatic hydrolysis, the pretreatment to make maize starch partial swell showed important influence on starch saccharification which could enhance the affinity of starch to GSHE (Tong and Shi, unpublished).

Moreover, α-amylase played an important role in decrease viscosity and helped melt crystal region of granule maize starch (Tong and Shi, unpublished) and released a small amount of glucose which may be caused by the poorly efficient action of α-amylase on native starch granules (Sarikaya, Higasa, Adachi, & Mikami, 2000). Furthermore, the second phase was initiated by adding GSHE and subsequently incubated at 62 °C, the optimum temperature of GSHE. It's noticeable that the enzyme amount was important as well. However, once the enzyme over 1%, the influence of the enzyme was limited and for economic perspective, we should control the usage of both enzymes under 1%. In our

experiment, 1% GC 626 and 1% GSHE could achieve 93.7% conversion which was not significantly different from the best conversion of 93.9% and it was doubled compared with control.

Obviously, the two-step enzymatic hydrolysis showed a tendency to enhance the glucose conversion rate.

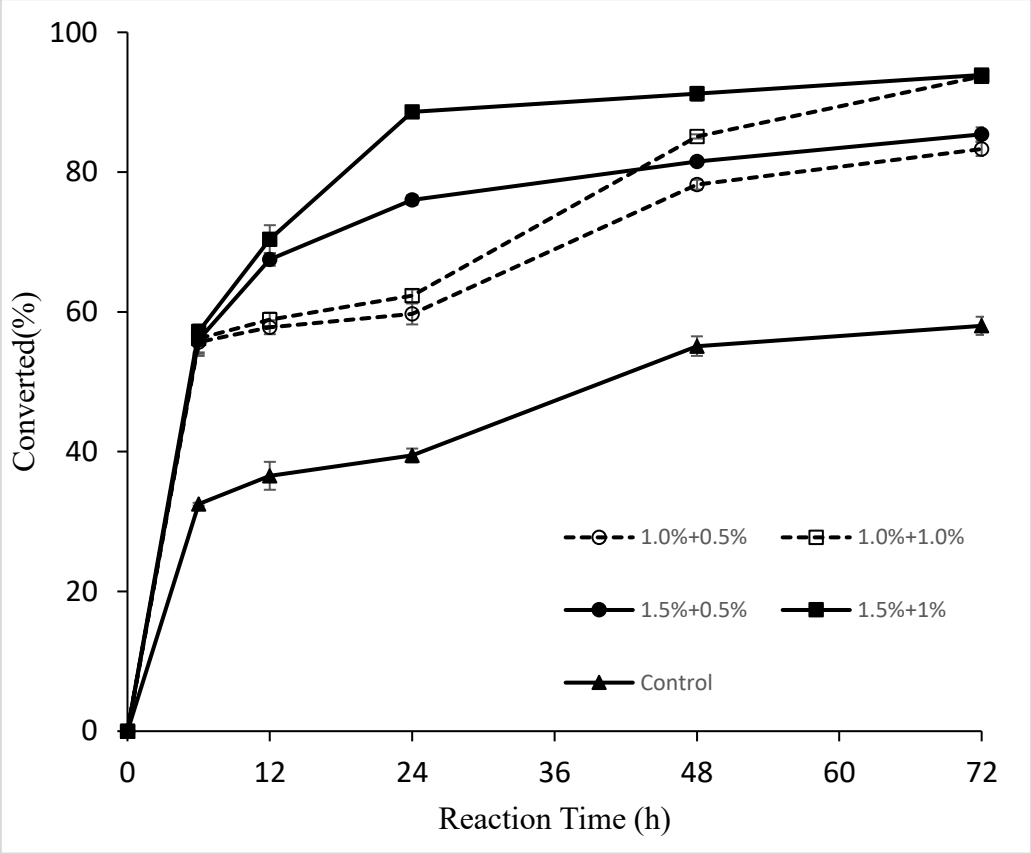

Figure 5.2 Effect of two-step enzymatic hydrolysis on the glucose conversion rate during saccharification process of granular normal corn starch

Light microscopy

Granule morphology of native, preheated maize starch, and its enzyme resistant residues was observed by a light microscope (Figure 5.3). The micrograph under normal light showed that the native normal corn starch was polygon and birefringence indicated that the average orientation of the polymer chains was radial (Figure 5.3 A). Granular starch might lose the "Maltese cross" under polarized light during a thorough enzymatic hydrolysis or heat treatment, which varied depending on the degree of hydrolysis and crystal packing

arrangement of starch. It was apparent that when starch slurry was heated at 70 °C (Figure 5.3 B) or hydrolyzed at 62 °C (Figure 5.3 C), few starch granules were disrupted and little loss of birefringence was observed. This suggested that neither heat treatment around gelatinization temperature nor enzymatic hydrolysis below gelatinization temperature could hardly cause evident destruction of crystalline structure of granular starch. In contrary, as shown in Figure 5.3 D-E, starch residues hydrolyzed at 70 °C could be broken down to a higher degree, leading to the extensive disappearance of their characteristic Maltese cross, indicating the widespread damage of crystalline lamellae in starch granules. Amylolysis at 70 °C could degrade the internal parts of granules, owing to the swollen starch granules and exposed partial crystallites. After the reaction of 6 h, the first stage in two-step enzymatic hydrolysis, residues showed an obvious collapse of granular structure, which might be beneficial to subsequent saccharification process by GSHE.

It was observed that, after saccharification by GSHE, qualitatively the damage of starch granules enhanced, resulting in a decrease in the birefringence. However, for starch with mere heat (Figure 5.3 F) or enzymatic treatment (Figure 5.3 G) before saccharification process, still many starch granules retained their birefringence, indicating the slight disruption of crystalline lamellae. On the other hand, as shown in Figure 5.3 H-J, the gradual vanishment of Maltese cross suggested that two-step enzymatic hydrolysis could destroy the starch granules to a higher degree. Moreover, keeping the same total dosage of enzyme in two-step hydrolysis, the more obvious collapse of granular structure in the first stage with higher α-amylase level (1.5%, w/w) caused a substantial disruption of crystalline lamellae in subsequent saccharification process, by comparison with 1.0% α-amylase. Furthermore, almost no birefringence was observed in polarizing microscope images, when increasing the addition of GSHE in the second stage, indicating that the crystalline structure of most starch granules was destroyed, to some extent, by the two-step enzymatic hydrolysis.

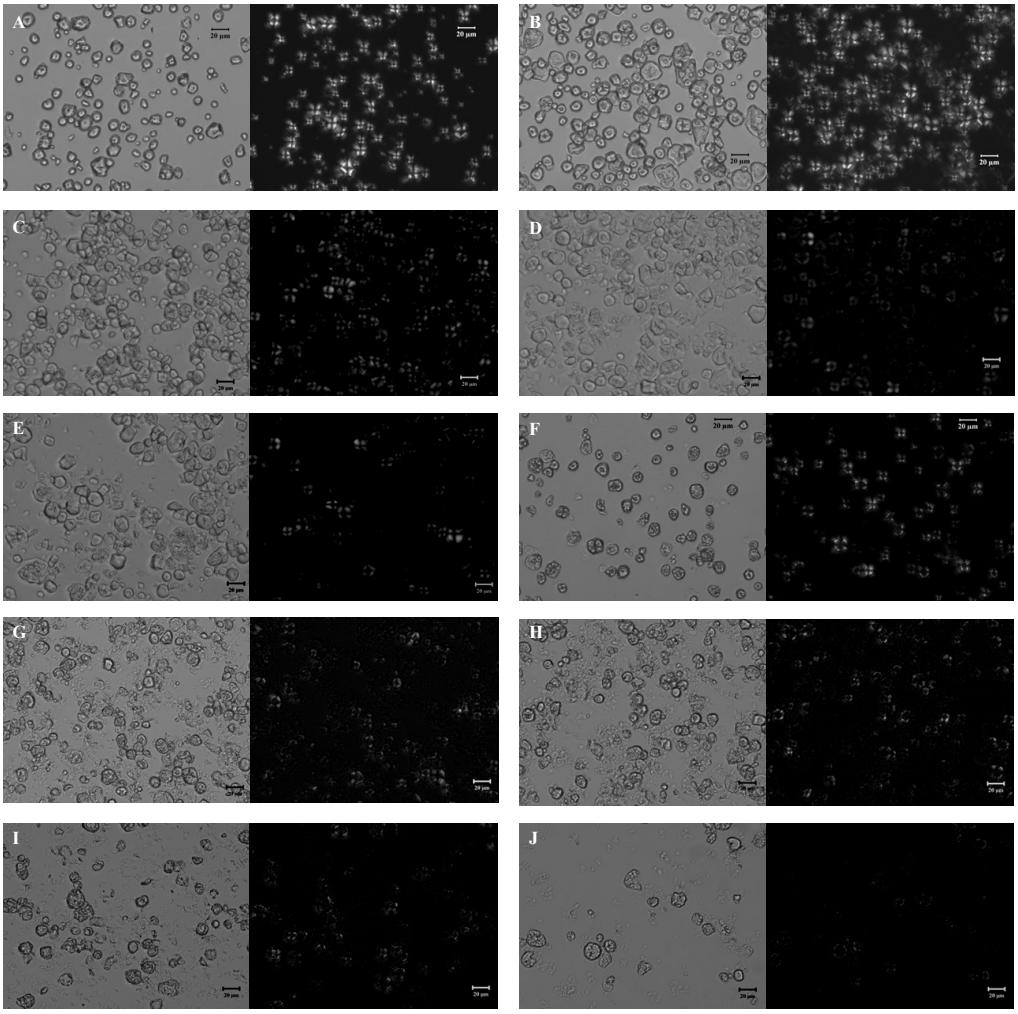

Figure 5.3 Light microscopic images of granules of corn starch native and hydrolyzed normal corn starch. The scale bar indicates 20 µm. (A) Native normal corn starch. (B) normal corn starch heated at 70 °C for 6 h. (C) residues from a 6-h-amylolysis of normal corn starch at 62 °C (α-amylase concentration=1.0%, w/w). (D) residues from a 6-h-amylolysis of normal corn starch at 70 °C (α-amylase concentration=1.0%, w/w). (E) residues from a 6-h-amylolysis of normal corn starch at 70 °C (α-amylase concentration=1.5%, w/w). (F) residues from a 72-h-saccharification of B at 62 °C (GSHE concentration=0.5%, w/w). (G) residues from a 72-h-saccharification of C at 62 °C (GSHE concentration=1.0%, w/w). (H) residues from a 72-h-saccharification of D at 62 °C (GSHE concentration=1.0%, w/w). (I) residues from a 72-h-saccharification of E at 62 °C (GSHE concentration=0.5%, w/w). (J) residues from a 72-h-saccharification of E at 62 °C (GSHE concentration=1.0%, w/w). Samples were freeze dried prior to imaging.

SEM

When hydrolyzed by enzymes, starch undergoes a collapse process during which the granular structure breaks down. The structural characteristics of native and hydrolyzed starch granules were shown in Figure 5.4. It was apparent that when starch slurry was not

hydrolyzed in the first stage, the residues show a large number of small pores on the surface of starch granules after saccharified by GSHE, but few starch granules were disrupted into fragments (Figure 5.4 B). Moreover, when granular normal corn starch was pre-hydrolyzed by α-amylase at 62 °C, a few parts of granules were broken down during saccharification process, while the other still retained almost intact granular structure (Figure 5.4 C). These images tallied with those from light microscopy analysis and indicated that mere heat treatment or enzymatic hydrolysis limitedly promote saccharification process in the next stage. On the other hand, as shown in Figure 5.4 D-F, two-step enzymatic hydrolysis evidently enhanced the collapse of starch granule. For starch granules pre-hydrolyzed at 70 °C in the first stage, the internal parts of granules could be acted and degraded to a higher degree, resulting in the improvement of subsequent amylolysis by GSHE. After incubation at 62 °C for 72 h, starch residues showed a higher degree of collapse in granular structure. Furthermore, the higher dosage of both enzymes in two-step hydrolysis led to a more substantial disruption of granules in the whole process.

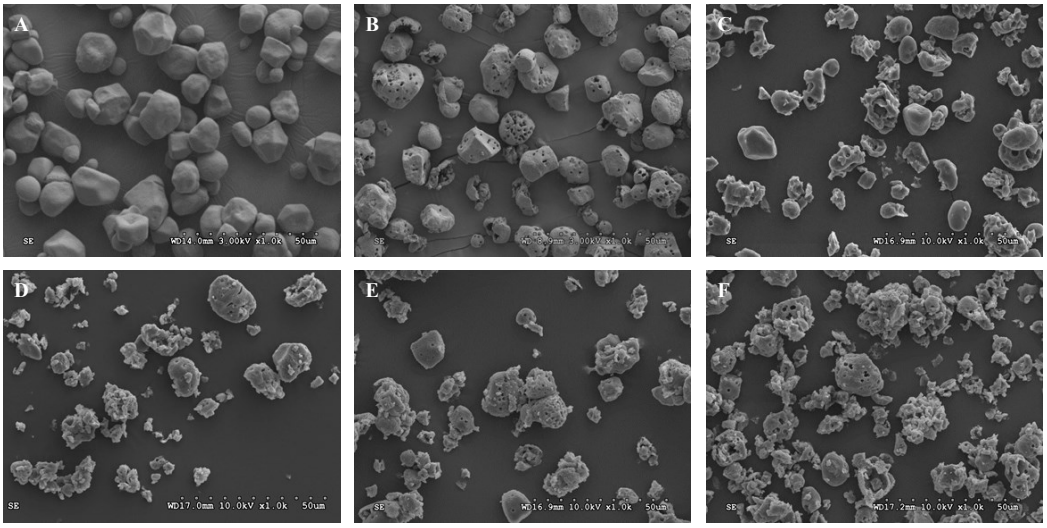

Figure 5.4 Scanning electron micrographs of native and hydrolyzed normal corn starch. The scale bar indicates 50 μm. (A) Native normal corn starch. (B) residues from a 72-h-saccharification of normal corn starch preheated at 70 °C (GSHE concentration=0.5%, w/w). (C) residues from a 72-h-saccharification of normal corn starch pre-hydrolyzed at 62 °C (α-amylase concentration=1.0%, GSHE concentration=1.0%, w/w). (D) residues from a 72-h-saccharification of normal corn starch pre-hydrolyzed at 70 °C (α-amylase concentration=1.0%, GSHE concentration=1.0%, w/w). (E) residues from a 72-h-

saccharification of normal corn starch pre-hydrolyzed at 70 °C (α-amylase concentration=1.5%, GSHE concentration=0.5%, w/w). (F) residues from a 72-h-saccharification of normal corn starch pre-hydrolyzed at 70 °C (α-amylase concentration=1.5%, GSHE concentration=1.0%, w/w).

Thermal properties

Thermal properties of native and its enzyme resistant residues were shown in Table 5.1. Compared to native starch, all hydrolyzed starched showed increased gelatinization temperatures (T_o, T_p and T_c) and decreased gelatinization temperature range (ΔT) and enthalpy change (ΔH), suggesting the relatively perfect crystalline structure, especially for the residual substrate present in two-step enzymatic hydrolysis. Hence, the amylolysis of granular starch could result in the degradation of amorphous areas and weaker crystallites. Compared with the control, the residues in two-step enzymatic hydrolysis exhibited significantly higher gelatinization temperatures as well as obviously lower ΔH, demonstrating the higher degree of granules destruction and the concomitant perfection of residual crystallites (Hoove & Vasanthan, 1993).

In addition, according to the light microscopy analysis, the combination of heat and enzymatic treatment in the first stage might enhance the action of GSHE on the crystalline lamellae in starch granules. The results of gelatinization enthalpy which is a measure of the loss of molecular order (Cooke & Gidley, 1992) and the gelatinization temperature which can be an indicator of crystalline quality relating to the double helix length (Hoover & Hadziyev, 1981) were consistent with the results in light microscopy analysis, higher addition of α-amylase in the first stage led to more evident hydrolysis in the second stage, reflect by higher gelatinization temperatures, even though total dosage of enzyme was kept the same.

Table 5.1 Thermal properties of native and hydrolyzed starches.

Preheat temperature (°C)	α-amylase [A] (%)	GSHE [B] (%)	T_o (°C)	T_p (°C)	T_c (°C)	ΔT (°C)	ΔH (J/g)
Native	0	0	68.6±0.5c	71.6±0.4c	89.3±0.2c	20.6±0.4a	12.1±0.3a
62	1	1	70.2±0.0bc	78.0±0.5b	88.3±0.3c	18.7±0.2a	6.6±0.2b

70	1	0.5	71.5±0.3ab	79.7±0.3b	89.7±0.3bc	19.6±0.3a	5.3±0.3bc
		1	71.6±0.2ab	80.0±0.4b	90.2±0.2bc	18.54±0.3a	5.3±0.3bc
	1.5	0.5	72.5±0.6a	83.3±0.4a	92.5±0.3ab	20.1±0.8a	5.2±0.1c
		1	72.6±0.4a	83.4±0.1a	91.4±0.6a	18.9±0.9a	5.2±0.1c
Melting of amylose-lipid complex							
Native	0	0	94.4±0.3c	103.6±0.3b	112.8±0.5b	18.4±0.3a	0.5±0.0a
62	1	1	101.2±0.3a	106.8±0.7a	115.8±0.2a	14.6±0.3b	0.3±0.0a
70	1	0.5	98.0±0.7b	102.3±0.8b	110.1±0.3c	12.1±0.2c	0.3±0.2a
		1	98.2±0.3b	102.6±0.6b	110.2±0.5c	12±0.3c	0.3±0.1a
	1.5	0.5	99±0.5ab	105.2±0.3ab	111.8±0.5bc	12.8±0.2c	0.3±0.1a
		1	99.5±0.5ab	105.3±0.3ab	111.9±0.5bc	12.4±0.1c	0.2±0.0a

[A] Ratio of α-amylase to dry starch (w/w).

[B] Ratio of GSHE to dry starch (w/w).

[C] T_o=onset temperature, T_p=peak temperature, T_c=conclusion temperature, $\Delta T = T_c - T_o$, ΔH=enthalpy change. Values are means ± SD (n = 3). Means with different letters within the same column of are significantly different ($p < 0.05$).

Crystallinity

The crystalline properties of starch granules and its enzyme resistant residues were studied by X-ray diffraction the relative crystallinity was shown in Table 5.2. After a 72-h enzymatic hydrolysis, the enzyme resistant residues showed a significant loss in crystallinity compared with native corn starch (Table 5.2), suggesting the damage of crystalline lamellae. The changes in crystallinity were similar to the trend in ΔH, and both of them could reflect the degree of granules degradation. In addition, the relative crystallinity after the first stage positively affected the degree of hydrolysis during the second step. The previous study had reported that α-amylase principally attacked the amorphous regions of starch granules (Gallant, Derrien, Aumaitre, & Guilbot, 1973), which might explain the high crystallinity of residues hydrolyzed by α-amylase at 62 °C for 6 h. In contrary, the temperature of 70 °C in the first stage enhanced the collapse of crystallinity, accelerating the erosion by GSHE in next step. Thus, partially disintegrated starch granules caused by α-amylase at 70 °C could

expose loose crystallites to further enzymatic attack, which was the reason for the improvement of two-step hydrolysis.

Table 5.2 Relative crystallinity of granular starch residues during the amylolysis of normal maize starch.

Preheat temperature (°C)	α-Amylase[A] (%)	GSHE[B] (%)	Relative Crystallinity (%)
Native	0	0	45.14±0.54a
62	1	1	17.82±0.41b
70	1	0.5	13.08±0.11c
		1	11.45±0.38cd
	1.5	0.5	12.21±0.15cd
		1	10.75±0.31d

[A] Ratio of α-amylase to dry starch (w/w).

[B] Ratio of GSHE to dry starch (w/w).

[C] Values are means ± SD (n = 3). Means with different letters within the same column of are significantly different ($p < 0.05$).

Molecular size distribution

GPC was used to determine the molecular size (MS) distribution of hydrolyzed residues and shown in Figure 5.5. A bimodal distribution of low and high Rh peaks was observed for all samples. Peak 1 (Figure 5.5, right peak) represents the larger MS distribution, while Peak 2 (left peak) represents smaller. Compared to low-level CG626 enzyme usage, 1.5% of α -amylase digestion showed a reduction of Peak 1 and increment of Peak 2, suggesting that a greater degradation of large molecular size particles. Moreover, Stargen 002 level showed the same impact on MS but less significant. The result indicates both enzymes contributed to the MS degradation and higher enzyme level was more powerful in degrading large polymer.

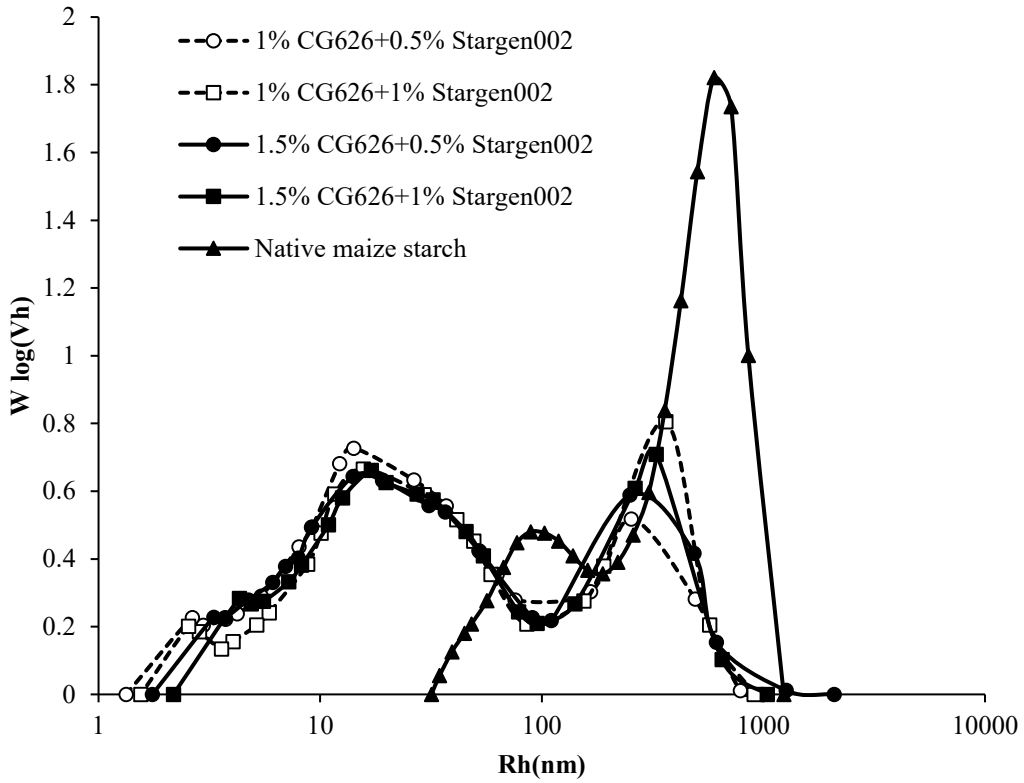

Figure 5.5 Molecular size distributions of hydrolyzed starches preheated at different temperatures for 90 min with constant stirring.

Conclusions

In this study, the saccharification process of granular normal corn starch was enhanced by two-step enzymatic hydrolysis, partial swelling granule increased the conversion by over 25% compared to normal maize starch. According to the evidence came from images of the light microscopy and SEM analyses, during the reaction at 70 °C, α-amylase could attack the swollen starch granules, leading to a great degradation of crystalline lamellae. At the end of the first stage, the obvious collapse of starch granules was beneficial to subsequent saccharification process by GSHE. When GSHE was added to the system and temperature was adjusted to 62 °C, the optimum temperature of GSHE, it was simpler to destroy the fragmentized granules and crystalline lamellae and released more amount of glucose. There was less than 7% enzyme resistant residue left based on our previous study, these residues could be completely converted by a cooking process (Tong and Shi, unpublished). In conclusion, the two-step enzymatic hydrolysis improves the application of GSHE which

could reduce the total cost by less energy input during liquification and saccharification. It provided a new method in industrial production of fermentable sugar.

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Chapter 6 - Optimization of partial swelling of starch for enhancement of saccharification and production of citric acid using granule starch hydrolyze enzyme

Abstract

Maize starch is the major substrate in citric acid production in the world. In current industrial production, citric acid was produced by using maize starch (16%, w/w) through liquefaction (cooking), saccharification and fermentation with final yield about 130 g/L. Cooking is the most energy consumption step during citric acid fermentation. The objective of this research was to optimize the citric acid production using a low-temperature cooking to achieve partially swelling maize starch and using granule starch hydrolysis enzyme (GSHE) to convert starch to reducing sugars at low temperature. Simultaneous saccharification and fermentation (SSF) using GSHE + Aspergillus niger system was used for citric acid production. The results showed that partial swelling starch enhanced GSHE efficiency and increased fermentation yield. In the pilot scale fermentation, 18% (w/w) starch slurry (pH 4.5) was preheated at 70 °C with 0.5% (w/w) α-amylase for 30 min, the partially swollen starch was saccharified by GSHE (1% W/W) at 62 °C, and over 99% of starch was hydrolyzed. After fermenting with GSHE and Aspergillus niger system for 67 h, the citric acid yield and conversion were 159 g/L and 88%, respectively. In this study, we not only increased the initial substrate level by 2%, but citric acid bioconversion increased by over 3% by SSF using uncooked starch. In addition, the GSHE + A. niger system also has a great potential for saving processing time and energy input.

Introduction

Citric acid (2-hydroxy-1, 2, 3-propanetricarboxylic acid) is a common intermediate product of natural and physiological metabolism. It is the intermediate of the tricarboxylic

acid cycle which has very high added value and it's widely used in the food industry (75%), the pharmaceutical industry (10%) and other industrial fields (15%) (Ates, Dingil, Bayraktar, & Mehmetoglu, 2002; Tran, Sly, & Mitchell, 1998). It is the world's largest consuming organic acid and the second largest fermentation products after alcohol (Dhillon, Brar, Kaur, & Verma, 2013). The demand for citric acid is increasing in recent years. The global production of citric acid has increased from 1.7 million tons from 2006 to 2.69 million tons in 2015 and the demand is increasing consistently (Zhou & Peng, 2018).

As a result of the increasing scale of global citric acid consumption, there has been an increasing trend toward efficient use of different substrates (Dhillon et al., 2013). Although substrates such as agro-industrial residues and by-products can offer advantages of managing waste material and reducing cost (Khosravi-Darani et al., 2008; Husseiny et al., 2010), the new substrates are still not commercialized in industry citric acid production. On the other hand, starch is abundant and have advantages of low cost and is environmentally friendly. For example, a large part of the industrial factories in China mainly uses maize starch as the raw materials for citric acid fermentation. In industrial production of citric acid, a process of cooked starch fermentation is used to produce citric acid (Zhou & Peng, 2018).

More than 90% of the world citric acid is produced by fermentation process (Khosravi-Darani et al., 2008). Many microorganisms can be used in citric acid fermentation, however, *A. niger* remains the best option in industrial citric acid production (Tong et al., 2019) which has the advantage of easy handling, high yield and cheap price (Alagarsamy and Nallusamy, 2010). More than 80% of citric acid fermentation is carried out by submerged fermentation which has advantages of lower investment and maintenance costs, and lower contamination risk (Gurpreet S. Dhillon, Brar, & Verma, 2012; Max et al., 2010).

The conventional way of citric acid production is summarized in Figure 6.1, in which maize starch is firstly liquefied and saccharified to obtain fermentable sugar solution with

high dextrose equivalent (DE). The saccharification process usually takes several hours until DE is higher than 80%, then a certain amount of nitrogen source and inorganic salt are added. The basal medium is sterilized by high temperature and cooled before the *A.niger* suspension is added. The batch fermentation cycle usually takes about 60 - 80 h. After the fermentation, the citric acid product is obtained through the extraction and refining process (Zhou & Peng, 2018).

The conventional process has several disadvantages. First, maize starch needs to be liquefied at high temperature with large energy consumption. Second, starch slurry cannot be directly used for citric acid fermentation after liquification, it needs to have a separate saccharification process to convert starch to fermentable sugars since large starch molecules need to be hydrolyzed into small monosaccharide before it can be used for the synthesis of citric acid (Förster, Aurich, Mauersberger, & Barth, 2007). Ideally, liquefied starch solution should be used directly for simultaneous saccharification and fermentation (SSF) as in the production of ethanol (Bai, Anderson, and Moo-Young, 2008; Li, Wang and Shi, 2019). *A. niger* could secrete saccharification enzyme during fermentation, the amount is limited and it does not work well in citric acid fermentation environment. The efficiency of the amylase and glucoamylase secreted by *A. niger* is limited, and the hydrolysis efficiency cannot meet the metabolism demand in citric acid fermentation. (Liu, Chi, Liu, Madzak, & Chi, 2013). Therefore, separate saccharification and fermentation processes still have to be used.

To overcome these drawbacks above, we used a low-temperature pretreatment on maize starch and an SSF method in a GSHE+ *A. niger* system to reduce the reduce energy consumption during liquefaction and increase the fermentation efficiency by enhancing glucose supply during fermentation. Our previous work showed that by using the granule starch hydrolyze enzyme (GSHE) and partial swelling maize starch, we could effectively

convert more than 95% starch to fermentable sugar in low-temperature (Tong and Shi, unpublished).

In this study, we partially swelled maize starch granules in the presence of α-amylase and followed by GSHE in the SSF process which could saccharify maize starch at low-temperature during fermentation. By using α-amylase in the pre-swelling process, we were able to perform the fermentation at a high starch concentration (18%, w/w). The α-amylase used during pre-swelling could reduce the viscosity of the swollen starch slurry and enhance saccharification process (Tong and Shi, unpublished). The GSHE could hydrolyze swollen starch granules more effectively. The new method we performed not only decrease the energy input in liquefaction process, but also increase the bioconversion and increase citric acid yield comparing to conventional cooking method of citric acid production.

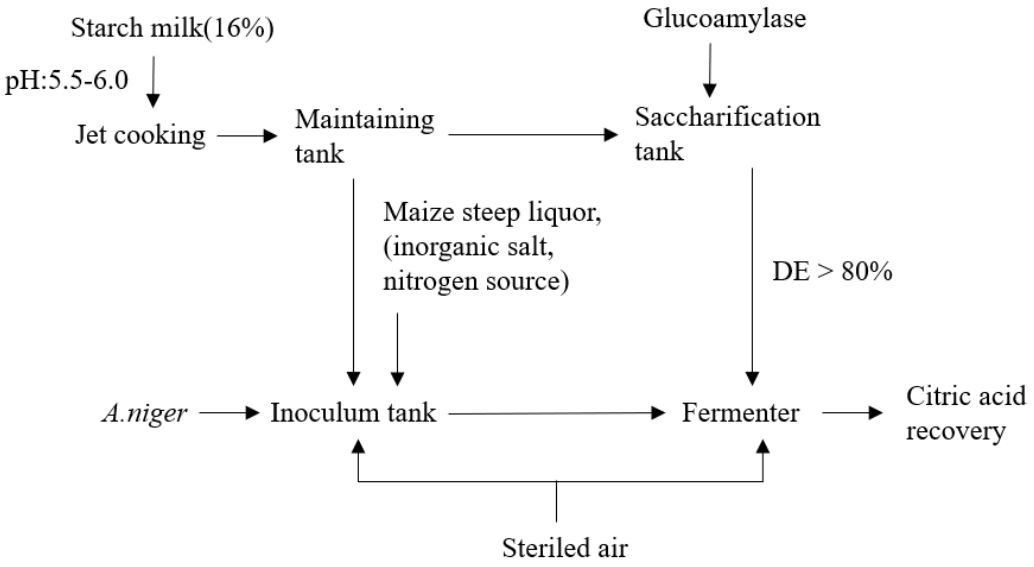


Figure 6.1 Production process of conventional citric acid batch fermentation

Materials and methods

Materials, enzymes and strain

Maize starch was provided by COFCO, Ltd. Aspergillus Niger used in this study was stored in COFCO, Ltd, which was an industrial strain for citric acid production. The inoculum of *A. niger* was performed in 250 ml Erlenmeyer flasks containing 50 ml of date

syrup (15% sugar and pH = 5.5). After inoculation, the flasks were incubated for 6 days at 30 °C and 150 rpm on a shaking incubator. The spore suspension contained 10^7 spores/ml.

Granular starch hydrolyzing enzyme (GSHE) (Stargen 002, 570 GAU/g) and acid-stable α-amylase (GC626, 100,000 SSU/g) were obtained from Genencor International (Palo Alto, CA, USA). All the other chemicals are purchased from Fisher Scientific (Santa Clare, CA, USA).

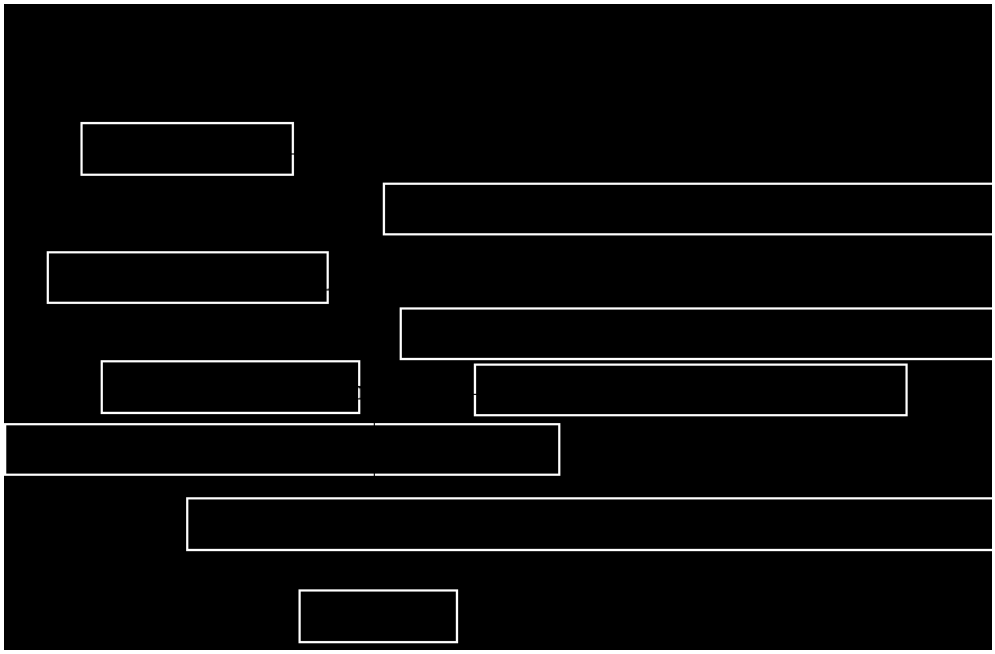
One Glucoamylase Unit (GAU) is the amount of enzyme that liberates one gram of reducing sugars equivalent to 5.6 mmole of glucose per hour, from soluble starch substrate at pH 4.3 and a temperature of 30 °C. Soluble Starch Units (SSU) was determined by the reducing power of 1 mg of glucose released per minute of soluble starch at the specific incubation conditions (pH = 4.5, 50 °C).

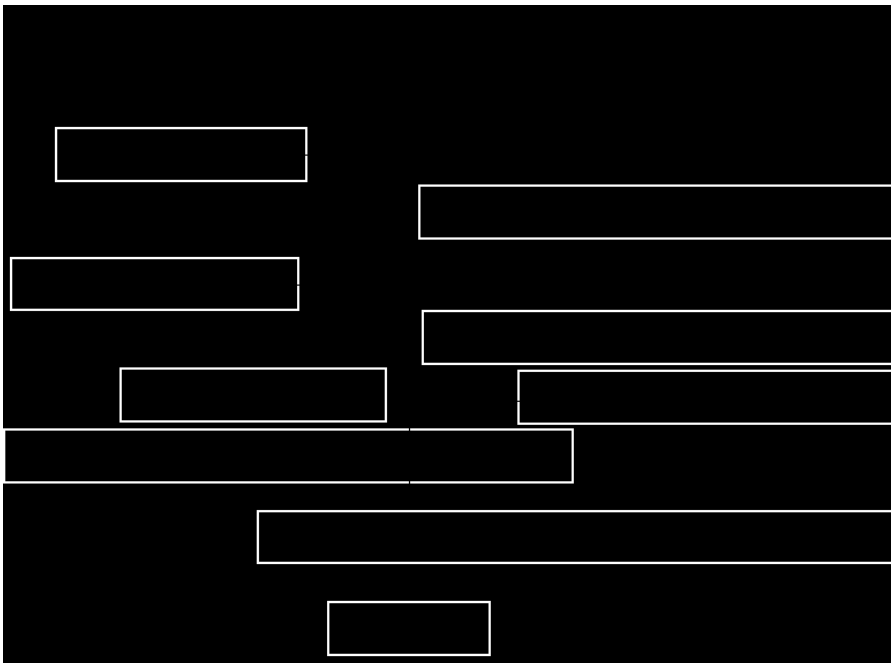
Effect of different substrate addition and different GSHE levels on citric acid fermentation

Maize starch (210, 240, 270, 300 g each) were prepared with distilled water (1290, 1260, 1230 and 1200 g) to form maize slurries with 14, 16, 18, and 20% solid contents, respectively, the slurries were adjusted to pH 4.5 by 1mM HCl, then 0.5% GC626 (w/w, which was 500 SSU/g starch) was added and the pre-swelling was carried out at 70 °C in a water bath (HH-8, Supple, Jiangsu) for 30 min with constant stirring. The mixture was cooled to 62 °C in a water bath and GSHE (0.5, 1, and 1.5%, w/w, or 2.85, 5.7, 8.55 GAU/g starch) was added for 24 hours with constant stirring. The reducing sugar released was quantified. The saccharified starch was cooled to 37 °C and poured into a 2 L fermenter (Biostat-B, Sartorius stedim, German). Thirty gram of corn steep liquor (30%, w/w) was added and 100 grams of the spore suspension were inoculated into the fermenter. The agitation was controlled at 500 rpm and the dissolved oxygen remained more than 30%. The reducing sugar and total sugar content of the saccharified starch slurries were determined before

fermentation. The reducing sugar concentration was examined every 8 h until the end of fermentation and the reducing sugar and total sugar concentration of the fermentation slurry were measured at the end of the fermentation. The experiment design was summarized in Figure 6.2, scheme 2.

$$Citric\ acid\ conversion\ = \frac{Citric\ acid\ produced(g)}{Total\ \text{theratical}\ glucose\ from\ starch(g)} \quad (6.1)$$





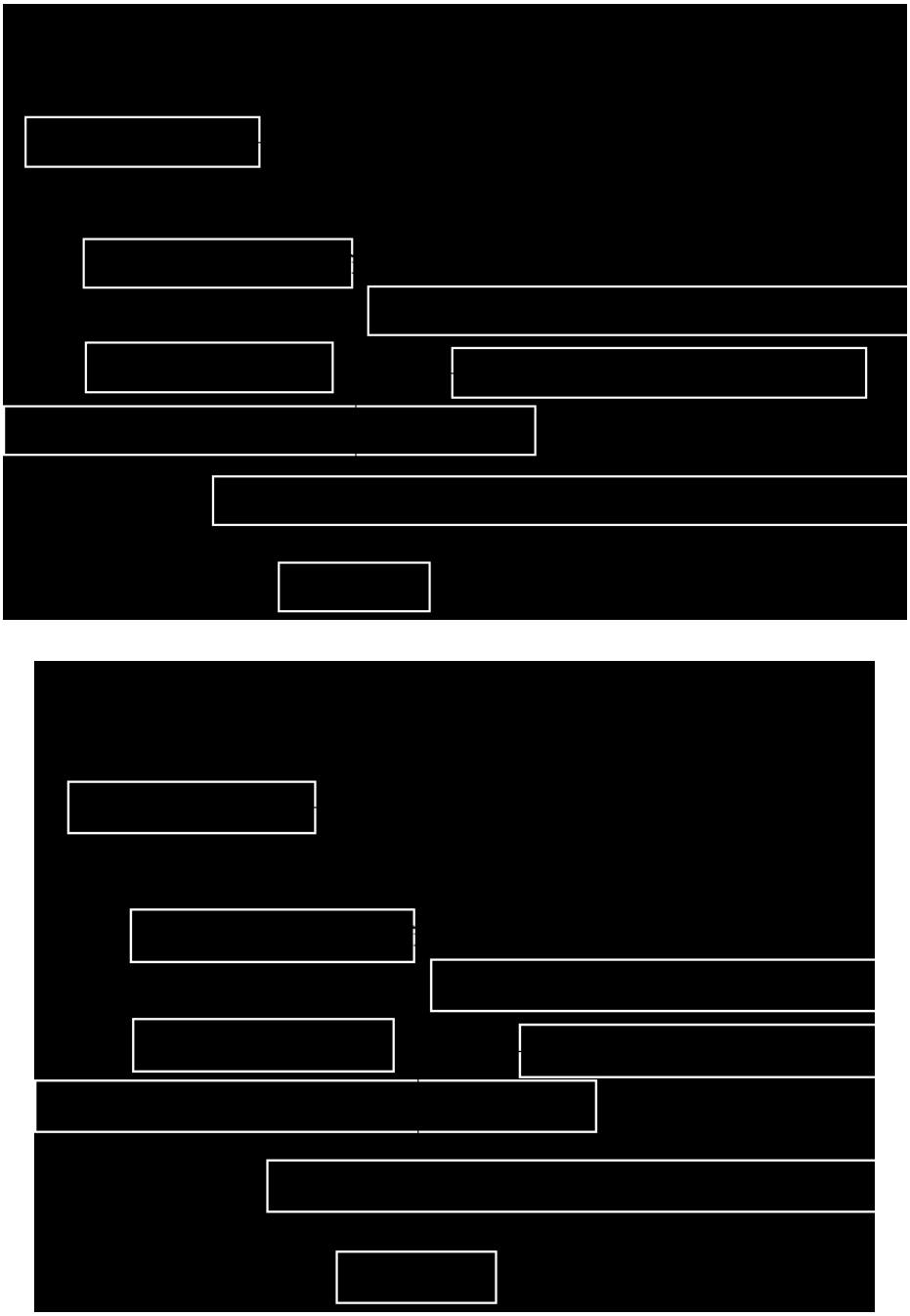


Figure 6.2 Experiment designs of conventional industrial cooking method of citric acid fermentation (scheme 1) and partial swelling starch fermentation basing on different starch concentration (scheme 2), different enzyme addition (scheme 2), different saccharification time (scheme 3) and pilot scale experiment (scheme 4).

Optimizing pre-saccharification time and simultaneous saccharification fermentation of citric acid

Maize starch slurries (1400 mL, 18%, w/w) were pre-swollen using the same method in section 2.2 and pre-swollen maize slurries were saccharified with 1% GSHE at 62 °C for

varied time intervals (0, 0.5, 1, 2, 4, 8, 16, 24 h). The reducing sugar of each slurry was tested when the saccharification was over.

The saccharified starch was cooled to 37 °C and poured into a 2 L fermenter (Biostat-B, Sartorius stedim, German). Thirty gram of corn steep liquor (30%, w/w) was added and 100 grams of the spore suspension were inoculated into the fermenter. The agitation was controlled at 500 rpm and the dissolved oxygen remained more than 30%. The reducing sugar and total sugar content of the saccharified starch slurries were determined before fermentation. The reducing sugar concentration was examined every 8 h until the end of fermentation and the reducing sugar and total sugar concentration of the solution were measured at the end of the fermentation. The experiment design was summarized in Figure 6.2, scheme 3.

Pilot scale enzymatic conversion and fermentation

Maize starch slurries (30 L, 18% w/w maize starch dry weight) were prepared in two 50 L fermenters.

In the first fermenter, the SSF was performed with the same procedure in previous section as the new method. After pre-swelling with 0.5% α-amylase at 70 °C for 30 min, partial swelling slurry was cooled to 37 °C and 1% GSHE (w/w) was added.

In the second fermenter, the slurry was fully cooked at 100 °C for 30 min then added 1% GSHE and pre-saccharified at 62 °C for 24 h. The reducing sugar of each slurry was tested when the saccharification was over. The saccharified slurry was sterilized at 108 °C for 20 min. The experiment design flow chart was summarized in Figure 6.2, scheme 4.

In each fermenter, seven hundred grams of corn steep liquor (30%, w/w) was added and 2 L of the spore suspension was inoculated into the fermenter (50JSA, Baoxing, China). The agitation was controlled at 500 rpm and the dissolved oxygen remained more than 30%. The reducing sugar and total sugar content of the saccharified starch slurries were determined

before fermentation. The reducing sugar concentration was examined every 8 h until the end of fermentation and the reducing sugar and total sugar concentration of the solution were measured at the end of the fermentation.

Analytical techniques

Reducing sugar analysis:

Ferrin reagent (10 ml) was put in an Erlenmeyer and pre-added 9.0-9.5 ml glucose standard solution (0.1%, w/w). The mixture was kept boiling and added the standard solution (2-3 drops/ second) until the blue color disappeared. The titration process must be carried out no more than 1 minute. The volume V_0 of the sample solution consumed by titration was recorded.

Sample supernatant (1 ml) was added to a 100 ml volumetric flask then added water to 100ml. Diluted sample solution (5 ml) was mixed with Ferrin reagent (10 ml) in a 50 ml Erlenmeyer. Glucose standard solution (0.1%, w/w) added to the Erlenmeyer at a speed of 2-3 drop/ second and kept the Erlenmeyer boiling until the blue color disappeared. The titration process must be carried out no more than 1 minute. The volume V_1 of the sample solution consumed by titration was recorded.

$$\text{Reducing sugar}(\%) = \frac{(V_0 - V_1) \times 0.1 \times 100}{1 \times 5} \quad (6.2)$$

The glucose content in the liquefication broth was measured by high performance anion exchange chromatography (HPAEC, Dionex ICS-3000, Dionex Corp., Sunnyvale, CA, USA). The mobile phase was 150 mM NaOH at a flow rate of 1 mL/min and glucose standard (1 mg/ml) was used for calibration.

Total sugar analysis

Ten milliliters of the sample were pipetted into a 250 mL Erlenmeyer flask, added 100 mL of distilled water, then added 7.5 mL of concentrated sulfuric acid (98%, w/w). A

condensing reflux tube was installed on the Erlenmeyer flask and heated the Erlenmeyer flask to boiling. The solution was boiling and hold for 10 minutes, then cool it to room temperature. The methyl red was used as an indicator and put in the Erlenmeyer. The concentrated sodium hydroxide solution (0.2%, w/w) was used to neutralize the mixture to pH = 7. The mixture was texted for its reducing sugar with the same method form last section and recorded the titration volume V_2.

$$\text{Total sugar}(\%) = \frac{(V_0 - V_2) \times 0.1 \times 500}{10 \times 2} \quad (6.3)$$

Citric acid analysis

One milliliter sample from supernatant was mixed with 99 ml distilled water in a 250 ml Erlenmeyer flask. Two drops of 1.0% phenolphthalein were added as an indicator. Sodium hydroxide standard solution (0.1429 mol/L) was used to titrate until the color of the solution changed from colorless to light pink/pink which was the end point of the titration. The volume of the sodium hydroxide standard solution consumed was recorded as V.

$$\text{Citric acid } (\%) = \frac{210.14 \times 0.5V}{3 \times 1000} \times 100\% \quad (6.4)$$

0.5: The concentration of sodium hydroxide standard solution, mol/l.

210.14：Molecular weight of citric acid monohydrate.

V: Volume of sodium hydroxide standard solution consumed by titration, ml.

Statistical analysis

The analysis of variance (ANOVA) was conducted by Minitab 17 Statistical Software Program (Minitab Inc. State College PA, USA). Experimental results are reported as the average of triplicate experiments.

Results and discussion

Effect of different starch concentrations on citric acid production

Four maize starch slurries with starch contents of 14, 16, 18, and 20% were used for citric acid production (Table 6.1). The slurry with 14% starch had the highest starch bioconversion, in which more than 90% of starch was converted to citric acid for all three GSHE levels. However, the higher citric acid yield (over 151 g/L) was achieve by maize starch slurry with 18% solid content among four different starch contents. The bioconversion decreased as starch content increased, while the citric acid yield increased as starch content increased except for 20% starch slurry. Partial swollen starch slurries with 16 - 18% starch contents in GSHE + *A. niger* system showed better performance in starch conversion (85 - 94.48%) and citric acid yield (136 - 157.0 g/L) comparing with the control with 16% starch content (83.02% and 133 g/L).

In the present industry citric acid production, 16% maize starch and a 2 h cooking liquefaction at 95 °C are used followed by 24 h saccharification. The fermentation usually takes 65 - 80 h and at 37 °C, around 83% of starch is converted to citric acid and the reducing sugar left in broth was less than 5 g/L. In this study, we used different substrate concentration of maize starch and firstly preheated with α-amylase GC626 at 70 °C for 0.5 h to make native maize starch granule partial swell which had higher affinity to GSHE and then the swollen starch was saccharified by GSHE for another 24 h. The decreasing trend of bioconversion when we increased the starting substrate concentration was due to high initial glucose concentration inhibited the fermentation which was consistent with the report that lower glucose concentration had a higher citric acid bioconversion. The high concentration substrate would result in feedback inhibition and decreased bioconversion (Bizukojc & Ledakowicz, 2003; Papagianni & Mattey, 2004).

Lower starting material showed a better bioconversion, 14% maize starch ended up with 93.13% citric acid conversion with only 0.15% reducing sugar left. However, the yield was close to industrial citric acid yield and it was not optimum since people would have more yield in industry production. Compared with the traditional cooking method, when we used same substrate level (16%, w/w), our method showed significant higher saccharification efficiency (86.5%) and fermentation yield (139.7 g/L) and less reducing sugar left (4 g/L).

In GSHE + *A. niger* system, since the initial reducing sugar content in broth was low (Table 6.2) and GSHE could release glucose consistently, we could increase the initial maize starch concentration to 18%, and still achieve a better bioconversion (85.25%) compared with traditional method (83%). When we further increased initial maize starch slurry to 20%, it showed a clear decline in both citric acids yield, bioconversion and more reducing sugar left in broth. In this study, the optimum starch content for citric acid fermentation was 18%. By using 18% maize starch slurry, the citric acid yield increased by 18% while the starch content increase is only 2% compared to the conventional method.

Table 6.1 Effects of starch concentration and enzyme level on citric acid production

Maize Starch (%)	GSHE (%)	Total Sugar (g/L)	End point reducing sugar (g/L)	Citric acid yield (g/L)	Conversion rate[A] (%)
Control	0	160.2	5±0.3a	133±1.2bc	83.02
	0.5	143.4	1.5±0.1a	130.4±1c	90.93
14	1	142.4	1.5±0a	132.2±0.8bc	92.83
	1.5	145	1±0a	137±0.3bc	94.48
	0.5	160	5.5±1.2a	136±1.5bc	85
16	1	160.8	6±0.3a	139.7±1.3b	86.88
	1.5	161	4.5±0.9a	140.1±2.1b	87.02
	0.5	180	4.9±0.8a	151.2±2.0a	83.89
18	1	183	5.3±0.1a	156.1±0.9a	85.25
	1.5	184	5.1±0.7a	157.3±0.1a	85.33
	0.5	203	9±1.7a	149±2.6a	73.4
20	1	200.3	7.7±0.1a	152.2±1.6a	75.89
	1.5	203.5	8.1±5.1a	157.1±2.1a	77.15

Means with different letters within the same column of are significantly different ($p < 0.05$).

$^{A}Conversion = \frac{Citric\ acid\ produced(g)}{Total\ \text{theratical}\ glucose\ from\ starch(g)}$

Table 6.2 Reducing sugar formation after liquefication

Starch Concentration (%)	Stargen 002 (%)	Reducing sugar(g/L)	Reducing sugar[A] (%)
14	0.5	139.2±0.3d	97.07
	1	140.1±0.3d	98.38
	1.5	141.2±0.3d	97.24
16	0.5	153±1.1c	95.63
	1	155.3±0.5c	96.39
	1.5	157.1±0.4c	97.52
18	0.5	173±2.1b	96.11
	1	176.3±1.9b	96.17
	1.5	178.1±2.5b	96.74
20	0.5	189.1±1.8a	93.15
	1	191.2±0.9a	95.46
	1.5	192.1±1.7a	94.40

Means with different letters within the same column of are significantly different ($p < 0.05$).

[A] Reducing sugar content was measured by reducing sugar measurement section, it equals reducing sugar measured (g) / weight of starch (g)×1.1.

Optimization of enzyme levels for maize starch fermentation

Different enzyme level of GSHE had been tested in the SSF and the results are summarized in Table 1. Increasing the enzyme level from 0.5% to 1% could cause 9.5% increment in citric acid yield (Table 6.1). However, no significant difference was found between 1% and 1.5% GSHE concentration, which might due to the limit of substrate concentration. With limited substrate, increasing enzyme level itself won't help increase the reaction chance between GSHE and maize starch. Our previous study showed that in GSHE saccharification process, once GSHE concentration greater than 1.0% (w/w), enzyme concentration had limited influence on the starch conversion (Tong and Shi, unpublished). Compared with traditional fermentation, the utilization of GSHE in citric acid fermentation could overall achieve higher starch bioconversion (86.88%) than cooking method due to the degradation of α-amylase in the preheating process and GSHE hydrolysis. This low-temperature treatment for partial swelling starch granular reduced the energy required to

destruct crystalline region in maize starch. It was reported that the partial swelling pretreatment could enhance the granule starch accessibility to the action of GSHE and accelerate the saccharification process by providing better affinity substrate (Li et al., 2014). Based on the results of different GSHE usage on citric acid yield, we found that 1% GSHE showed a very good bioconversion and it was comparable with 1.5% GSHE.

The effect of GSHE level on reducing sugar content of saccharified maize starch slurries was shown in Table 6.2. When the starch content was greater than 18%, the converted reducing sugar content in fermentation broth decreased even at high enzyme level, and the saccharification process was not complete within 24 h. The enzyme level showed a more significant impact on saccharification process when starch content in the slurry is less than 18%. When the substrate level was higher than 18%, longer saccharification time is needed to completely hydrolyze the starchy material before fermentation, which is not preferred for industrial production.

Optimizing the pre-saccharification time in citric acid fermentation

In this research, starch was firstly preheated and liquefied by α-amylase at 70 °C to make the maize granule partially swell and saccharified by GSHE at 62 °C for different time intervals (0, 0.5, 1, 2, 4, 8, 16, and 24 h) to investigate the effect of pre-saccharification time on citric acid fermentation at constant starch content (18%). The results showed the glucose conversion increased significantly from 21.7 to 92%, when the pre-saccharification time increased from 0 h to 24 h, (Figure 6.3). Moreover, the citric acid conversion decreased from 87.2 to 85.2% which indicated that the shorter pre-saccharification time was preferred by citric acid yield. In addition, saccharification also showed significant influence on fermentation when pre-saccharification time was less than 1 h. Especially, when the pre-saccharification time was zero, the citric acid conversion was 1.8% higher than that with 24 h pre-saccharification. There was no significant difference when pre-saccharification time over

1 h. Therefore, the fermentation was more thoroughly with less pre-saccharification time.

When the initial glucose conversion was over 70%, the citric acid conversion showed a decrement trend which was due to the inhibition of high glucose content on citric acid synthesis. This is the main reason why citric acid conversion decreased when the pre-saccharification time was over 1 h. The high glucose concentration environment would cause the feedback inhibition and stimulate the growth of *A. niger*, which decreased the citric acid fermentation efficiency (Tong et al., 2019). When the glucose was low enough, the *A. niger* would be kept in relatively small size and the synthesis of citric acid would be conducted more effectively. The SSF using GSHE and *A. niger* system not only reduced the saccharification time, but also showed a desirable citric acid yield (158.7 g/L) which is much higher than 133 g/L produced from conventional citric acid fermentation method.

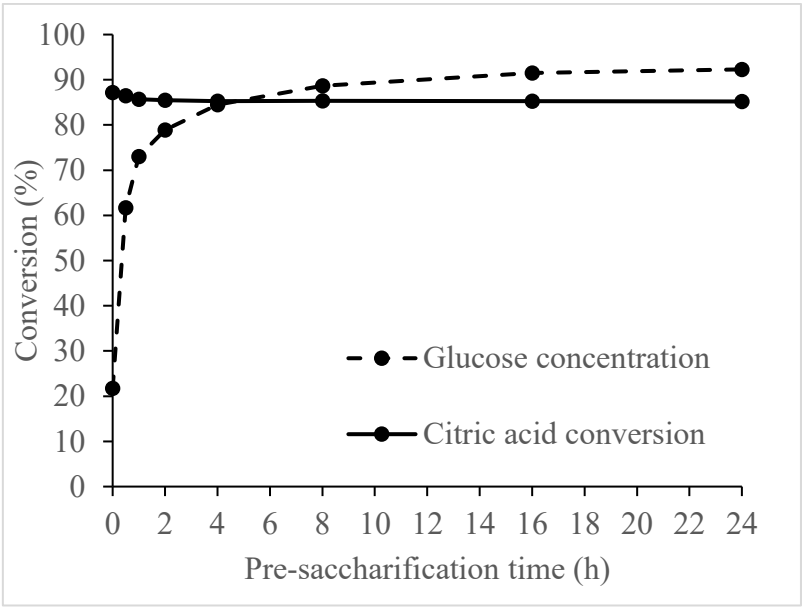


Figure 6.3 Glucose conversion rate and citric acid bioconversion at different pre-saccharification time (0, 0.5, 1, 2, 4, 8, 16, 24 h) at 62 °C for 30 min with 1% GSHE (w/w) with constant shaking. The fermentation after pre-saccharification conducted at 37 °C for another 67 hours.

Pilot scale production of citric acid

The lab-scale fermentation results showed that the partial swelling of normal maize starch with α-amylase provided initial fermentable broth with glucose concentration around

43 g/L (Figure 6.3). Followed by fermenting in using the GSHE + *A. niger* system in which GSHE would digest starch to glucose and consistently release glucose during fermentation. The low glucose concentration of fermentation medium and stable glucose supply during fermentation was favored by *A. niger* for citric acid production. To validate those finding from lab scale fermentation procedure, pilot scale SSF of citric acid using GSHE + *A. niger* system was conducted using 50 L fermenter. Conventional cooking fermentation at pilot scale was used as a control.

For conventional fermentation, citric acid yield of 154 g/L was obtained (Figure 6.4). While low-temperature fermentation showed a better result in both yield and bioconversion with 159 g/L citric acid yield and 88.2% bioconversion, respectively (Figure 6.4). The glucose content of uncooked fermentation broth was always lower than a cooked GSHE + *A. niger* system and the GSHE + *A. niger* system had a higher citric acid conversion, suggesting that consistent slow glucose releasing provided a better environment for citric acid fermentation. The low-glucose environment prevented the *A. niger* overgrowth and stimulated fermentation. In addition, the pilot-scale also showed a better result than the lab-scale experiments which was due to that the large fermenter had a higher oxygen pressure and better mixing system which made the dissolved oxygen level higher in a large-scale fermentation.

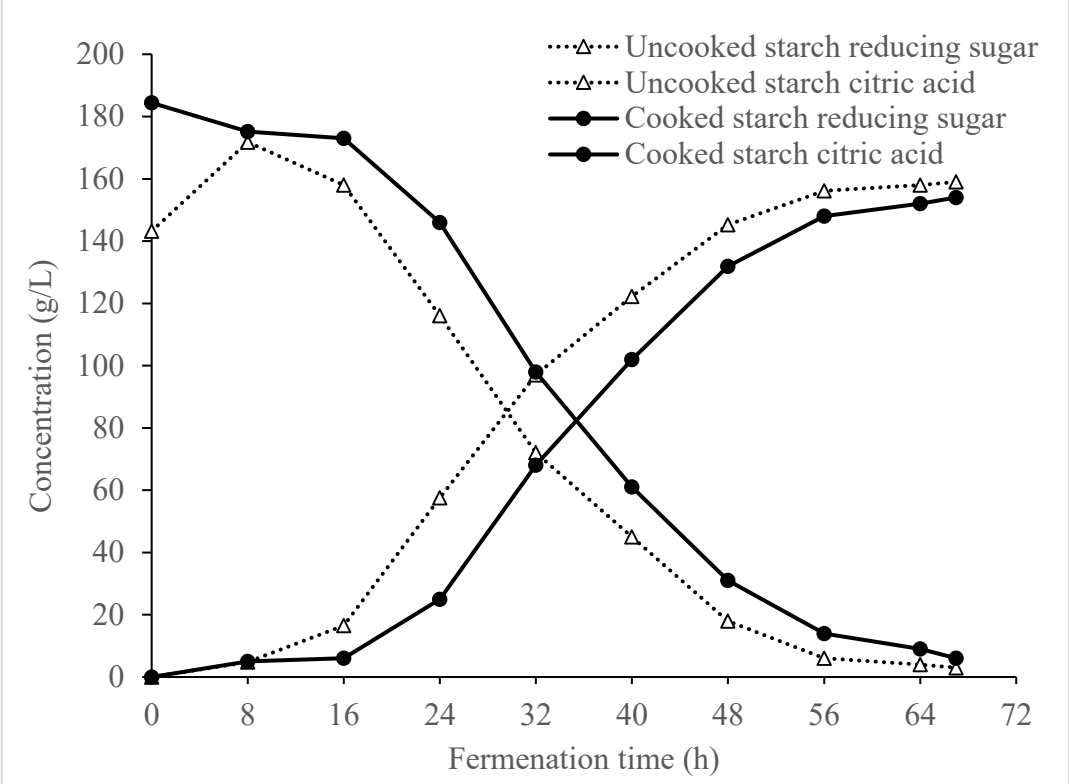


Figure 6.4 Reducing sugar and citric acid conversion during fermentation of large-scale pilot experiment of conventional cooked method and GSHE + *A. niger* uncooked method. The solid line indicated cooked method and the dotted line indicated uncooked method.

Conclusions

The use of partially swelling granule starch and GSHE had higher starch conversion and citric acid yield compared with conventional industry fermentation method. The maximum bioconversion reached 88% using GSHE + *A. niger* system at 37 °C within 67 h fermentation. The specific advantages of GSHE over previous industrial saccharifying enzymes are that the SSF approach can be applied to citric acid fermentation at room temperature and GSHE has desirable compatibility with *A. niger*. The swollen maize starch had a better affinity to the enzyme and could provide a consistent glucose supply during the fermentation. This low initial glucose and stable broth environment prevents *A.niger* from overgrowth and promotes the fermentation efficiency and increases the final bioconversion. In addition, the GSHE + *A. niger* system also has a great potential for saving processing time and energy input.

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Chapter 7 - Partial Swelling of Starch Enhances Bio-Conversion of Maize Flour to Ethanol

Abstract

In current industrial production, maize is the predominant crop used to produce ethanol. Using maize flour as substrate, a new dry grind ethanol fermentation process, during which maize slurry was pre-hydrolyzed by α-amylase at 70 °C for 30 min followed by simultaneous saccharification and fermentation, was investigated as a strategy to improve the efficiency of ethanol production. Granular starch hydrolysis enzymes (1.0%) and glucoamylase (0.4%) were used for starch saccharification. A simultaneous saccharification and fermentation (SSF) was conducted at 32 °C for 72 h. Results showed the proper heat treatment using α-amylase to partial swelling of starch enhanced starch bio-conversion and facilitated ethanol production for dry grind ethanol process. The new dry grind ethanol fermentation process significantly enhanced the ethanol production and increased ethanol yield by 4% compared with current industrial process in both small-scale and pilot-scales. In addition, the relatively low-temperature process would significantly reduce the energy input and provide a great economic potential in the industry.

Keywords

Maize flour, α-amylase, Granular starch hydrolysis enzyme, Glucoamylase, Ethanol fermentation

Introduction

Fuel ethanol is a clean energy source, providing a more environmentally friendly alternative to fossil fuels (Alvira et al., 2010; Bai et al., 2008; Hill et al., 2006; Falano et al., 2014; Frigon & Guiot, 2010; Soyeon and Jaewon, 2016). In traditional ethanol production by fermentation, a substrate containing 18-22% of total sugars is used to produce ethanol with

ethanol concentration around 14% (Tao et al., 2012). Maize is an abundant industrial raw material and the predominant crop used to produce ethanol in the US. Conventional processes for the production of ethanol typically includes gelatinization, liquefaction, saccharification, and fermentation. Starch is converted into fermentable sugars through a common liquefaction and saccharification process by using α-amylase and amyloglucosidase (Lin and Tanaka, 2006). However, a high amount of energy is required for starch gelatinization (Ellis et al., 1998), making liquefaction a costly step.

An alternative method using the enzymatic hydrolysis of granular starch under sub-gelatinization temperatures by granular starch hydrolyzing enzyme (GSHE) has generated (Li et al., 2014; Uthumporn et al., 2012), eliminating the cooking process. In addition, GSHE could simplify the process and equipment required to handle the high-viscosity starch slurries. GSHE has recently been proved to be associated with a simultaneous saccharification and fermentation (SSF) process for ethanol production (Amera et al., 2011; Sharma, et al., 2007).

In our group, we have used a partially swelling of starch granules to enhance starch saccharification (Li et al., 2014). The swelling of native maize starch increases the surface area and it has a positive influence on GSHE hydrolysis. In another paper, we successfully used GSHE on partial swollen starch and achieved high conversion of the normal maize starch to glucose > 95% (Tong and Shi, unpublished).

In this study, we applied the partial swelling maize starch and GSHE on ethanol fermentation to improve the efficiency of ethanol production. Maize flour was pre-hydrolyzed using α-amylases at a temperature slightly higher than onset gelatinization temperature, in order to enhance subsequent saccharification process by GSHE as well as simultaneous fermentation process. This process was systematically optimized to improve fermentation efficiency. It is expected that the partial swelling of starch to promote industrial

ethanol production with increased ethanol fermentation efficiency and yield as well as economic benefit.

Materials and methods

Materials

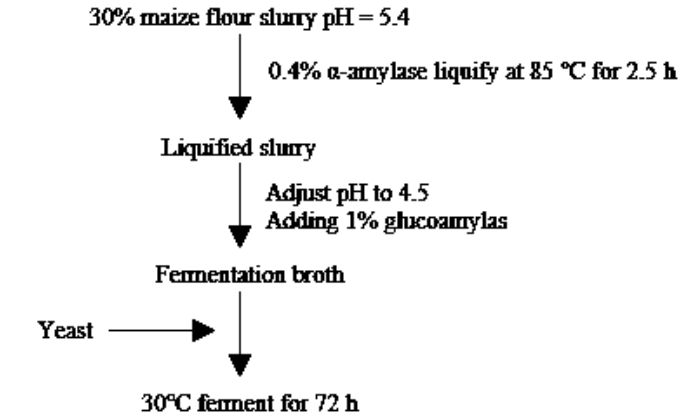
Maize flour was obtained from China National Cereals, Oils and Foodstuffs Corporation (COFCO, Beijing, China) and the chemical composition was shown in Table 7.1. Normal maize starch was obtained from Tate & Lyle (Hoffman Estates, IL). Yeast, used for the ethanol fermentation, was purchased from Angel Yeast Co., Ltd (Hubei, China. Granular starch hydrolyzing enzyme (GSHE) (Stargen 002, 570 GAU/g) and acid-stable α-amylase (GC626, 100,000 SSU/g) were obtained from Genencor International (Palo Alto, CA, USA). Glucoamylase (GA475, 160,000 AGU/mL) was purchased from Novozymes (Franklinton, NC, USA). All the other chemicals were purchased from Fisher Scientific (Santa Clare, CA, USA).

One Glucoamylase Unit (GAU) is the amount of enzyme that liberates one gram of reducing sugars equivalent to 5.6 mmol of glucose per hour, from soluble starch substrate at pH 4.3 and a temperature of 30 °C. Soluble Starch Units (SSU) was determined by the reducing power of 1 mg of glucose released per minute of soluble starch at the specific incubation conditions (pH 4.5, 50° C). One AGU is defined as the amount of enzyme that can hydrolyze 1 μm maltose/min under standard conditions (pH 4.3, 37 °C and 23.2 mM maltose).

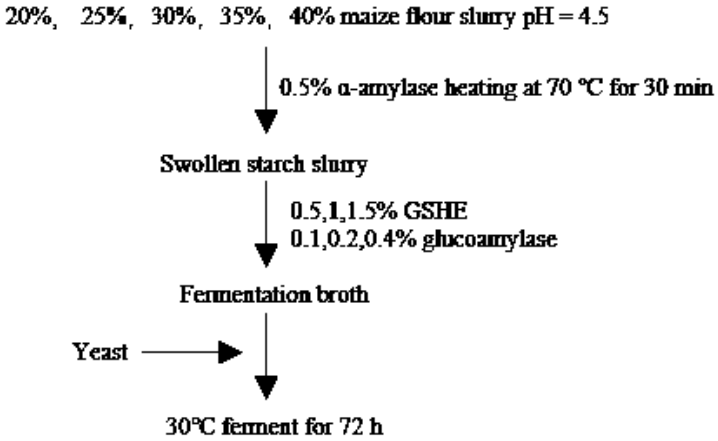
Table 7.1 Composition of the maize flour

Content	Percentage (%, db)
Moisture	6
Starch	87.0
Protein	4.8
Fiber	1.3
Fat	0.9

Ash	0.1



Scheme 1



Scheme 2

Figure 7.1 Schemes of traditional industry ethanol fermentation and experimental optimization process of ethanol fermentation

Light microscopy

Maize flour (20 mg, dry basis) was slurred in water (1.0 mL), mixed with 0.2 mg α-amylase and heated in a water bath at 70 °C for 30 min with constant shaking at 250 rpm. The starch slurry without heat and enzyme pretreatment was used as a control. Each sample was promptly examined by an Olympus BX51 microscope (Olympus Optical Co. Ltd., Shinjuku-ku, Tokyo, Japan) fitted with a polarized light filter and a digital camera.

Observations were conducted under normal visible light and polarized light and captured by a SPOT 18.2 Color Mosaic camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

Differential scanning calorimetry (DSC)

The thermal property of native maize starch and flour were determined by DSC using a TAQ5000 DSC (TA Instruments, New Castle, DE, USA). Each sample (ca. 8 mg) was weighed in a stainless-steel pan, water (24 μL) was added, and the pan was sealed. An empty pan was used as a reference. Scans were performed from 10 °C to 100 °C at a heating rate of 10 °C/min. The onset (To), peak (Tp) and conclusion (Tc) temperature and gelatinization enthalpy were calculated from the DSC thermogram.

Effect of substrate concentration and GSHE loading on ethanol fermentation

Maize flour (300, 375, 450, 525, 600 g) were mixed with distilled water (1200, 1125, 1050, 975, 900 ml) to form flour slurries with solid content 20%, 25%, 30%, 35% and 40%, respectively, and adjusted to pH 4.5 with 1 mol/l HCl. Subsequently, acid-stable α-amylase (0.5%, w/w, which was 500 SSU/g starch) was added to each slurry. The mixture was incubated in a water bath at 70 °C for 30 min with constant stirring (250 rpm, IKA RW-20, Staufen, Germany). The reducing sugars released in this process was quantified by the titration method. After adjusting water bath to 32 °C, certain amount of GSHE (0.5%, 1% or 1.5%, w/w, which were 2.85, 5.7, 8.55 GAU/g starch) was added to each slurry with constant stirring (250 rpm). The mixtures were used as fermentation broth.

For the preparation of inoculums, active dry yeast (100 g) was dispersed in 1900 mL of a preculture broth containing glucose (20 g/l), peptone (5 g/l), yeast extract (3 g/l), KH_2PO_4 (1 g/l), and $MgSO_4 \cdot H_2O$ (0.5 g/l) and incubated at 32 °C for 30 min with constant shaking.

The fermentation broth, containing above liquefied slurry (1500 g), activated yeast culture (15 ml), KH_2PO_4 (1 g/l), $CaCl_2$ (0.2 g/l) and a nitrogen source, were adjusted to pH

4.5 with 1 mol/l HCl. After added to flask, fermentation broth was subsequently sealed with an S-shaped airlock filled with about 5 mL of mineral oil. Ethanol fermentation was performed in an incubator shaker (250 rpm) at 32 °C for 72 h. The fermentation process was monitored by measuring the total weight of the fermentation flask to calculate the CO_2 loss, which is proportional to ethanol production. Ethanol yield was defined as the ethanol concentration in fermentation broth according to Equation (7.1).

$$\text{Fermentation efficiency} = \frac{\text{Ethanol production}}{\text{Total starch content} \times 1.11 \times 0.511} \times 100\% \qquad (7.1)$$

Effect of different pH on α-amylase pretreatment

Slurries, respectively containing 30% (w/w, dry basis) maize flour, were prepared in distilled water and adjusted to different pH (4.0, 4.5, 5.0, 5.5) with 0.1 mol/l HCl. Subsequently, α-amylase (0.5%, w/w dry starch) was then added to each maize starch slurry. The mixture was placed in a beaker in a water bath (70 °C) for 30 min with constant stirring. The reducing sugar conversion after pretreatment was tested and slurry viscosity were recorded by a SurgiFriend Medical viscometer (NDJ-5S, England). After adjusting water bath to 32 °C, 1% GSHE were added to each slurry with constant stirring (250 rpm). The mixtures were used as fermentation broth. Ethanol fermentation was performed in an incubator shaker (250 rpm) at 32 °C for 72 h. The fermentation process was monitored by measuring the total weight of the fermentation flask. Ethanol yield was calculated according to Equation (7.1). The yeast mortality was measured under an optical microscope (Model BX51, Olympus Co., Japan) by taking one drop of yeast dilution on the hemocytometer plate, added a drop of 0.1% methine blue, covered with a slide then observed the ratio of the number of stained yeast cells to the total number of cells.

Effect of glucoamylase addition on ethanol fermentation

Slurries containing 30% or 35% (w/w, dry basis) maize flour, were prepared in

distilled water and adjusted to pH 4.5 with 0.1 mol/l HCl. Subsequently, α-amylase (0.5%, w/w dry starch) was then added to each maize starch slurry. The mixture was placed in a flask in a water bath (70 °C) for 30 min with constant stirring. After adjusting water bath to 32 °C, certain amount of GSHE (1% or 1.5%, w/w dry starch) and glucoamylase (0.1%, 0.2% or 0.4%, w/w, dry starch) were added to each slurry with constant stirring (250 rpm). The mixtures were used as fermentation broth. Ethanol fermentation was performed in an incubator shaker (250 rpm) at 32 °C for 72 h. The fermentation process was monitored by measuring the total weight of the fermentation flask. Ethanol yield was calculated according to Equation (7.1).

Pilot-Scale fermentation to simulate industrial production

Based on above optimized process, the fermentation process with a 50 L fermenter (50JSA, Baoxing, China) was used to simulate industrial production. After an initial liquification of maize flour (30%, w/w, dry basis) by α-amylase (0.5%, w/w dry starch) at pH 4.5 and 70 °C for 30 min, the hydrolysate was cooled to 32 °C. Certain amount of GSHE (0.5%, 1% or 1.5%, w/w dry starch) were added to each slurry and mixed for another 2 h with constant stirring (250 rpm). The mixtures were used as fermentation broth. Yeast suspension (100 g/kg dry starch), urea (25.0 g/kg dry starch) and glucoamylase (0.4%, w/w dry starch) were added to above mixture and fermented for 72 h. The ethanol formed was distilled to obtain 99% purity and the yield from the various replicates was measured.

Slurry, containing 30% (w/w, dry basis) maize flour, was used to simulate traditional industry ethanol fermentation as a control. After adjusting pH to 5.4, α-amylase (0.4%, w/w dry starch) was added to each maize starch slurry. Liquification was carried on at 85 °C for 2.5 h. After liquification, 1 ml hydrolysate was withdrawn for analysis and the residue was cooled to 32 °C. After adjusting pH to 4.5, glucoamylase (1%, w/w dry starch) was added. Yeast suspension (100 g/kg dry starch) and urea (25.0 g/kg dry starch) were added to above

mixture and fermented for 72 h. The total weight of the fermenter was recorded during fermentation. Ethanol yield was calculated according to Equation (7.1).

Reducing sugar analysis

Ferrin reagent (10 ml) was put in an Erlenmeyer and pre-added 9.0-9.5 ml glucose standard solution (0.1%, w/w). The mixture was kept boiling and added the standard solution (2-3 drops/ second) until the blue color disappeared. The titration process must be carried out no more than 1 minute. The volume V_0 of the sample solution consumed by titration was recorded.

Sample supernatant (1 ml) was added to a 100 ml volumetric flask then added water to 100ml. Diluted sample solution (5 ml) was mixed with Ferrin reagent (10 ml) in a 50 ml Erlenmeyer. Glucose standard solution (0.1%, w/w) added to the Erlenmeyer at a speed of 2-3 drop/ second and kept the Erlenmeyer boiling until the blue color disappeared. The titration process must be carried out no more than 1 minute. The volume V_1 of the sample solution consumed by titration was recorded.

$$\text{Reducing sugar}(\%) = \frac{(V_0 - V_1) \times 0.1 \times 100}{1 \times 5} \quad (7.2)$$

Total sugar analysis

Ten milliliters of each liquefied flour slurry were pipetted into a 250 mL Erlenmeyer flask, added 100 mL of distilled water, then added 7.5 mL of concentrated sulfuric acid (98%, w/w). A condensing reflux tube was installed on the Erlenmeyer flask and heated the Erlenmeyer flask to boiling. The solution was boiling and hold for 10 minutes, then cool it to room temperature. The methyl red was used as an indicator and put in the Erlenmeyer. The concentrated sodium hydroxide solution (0.2%, w/w) was used to neutralize the mixture to pH = 7. The mixture was texted for its reducing sugar with the same method form last section.

$$\text{Total sugar}(\%) = \frac{(V_0 - V_2) \times 0.1 \times 500}{10 \times 2} (7.3)$$

Statistical analysis

The analysis of variance (ANOVA) was conducted by using Minitab 17 Statistical Software Program (Minitab Inc. State College PA, USA). Experimental results are reported as the average of triplicate experiments.

Results and discussion

Characterization of normal maize flour

Maize flour showed no significant difference in gelatinization temperature (T_o, T_p and T_c) and ΔH values (Table 7.2) compared to the normal maize starch, suggesting that the composition cause the curve on DSC was due to the starch in the maize flour. Preheating at 70 °C was little above the gelatinization temperature and caused the partial swelling of starch granule.

The micrograph under normal light showed that the native normal maize starch in flour was polygon and birefringence indicated that the average orientation of the polymer chains was radial (Figure 7.2). There were some impurities which were mainly composed of fibers and proteins. After preheating at 70 °C with α-amylase, few starch granules were disrupted and starch granule remained birefringent (Figure 7.2). Preheating with α-amylase at 70 °C could break down some of the granules and the significant swollen of granules were observed after heating treatment. There were visible pores and pits on the starch granule surface, suggesting the α-amylase erosion (Bai, Cai, Doutch, Gilbert, & Shi, 2014). Helbert, et al, (1996) had reported that enzymatic hydrolysis of starch granules started at the surface then penetrates into the interior structure, forming porous starch granules.

Table 7.2 Thermal properties of native normal maize flour and native normal maize starch

Material	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)

Maize starch	68.6±0.1b	72.4±0.2a	84.3±0.2b	12.1±0.2b
Maize flour	67.7±0.1a	73.8±0.1b	83.2±0.3a	11.8±0.3a

Means with different letters within the same column of are significantly different ($p < 0.05$).

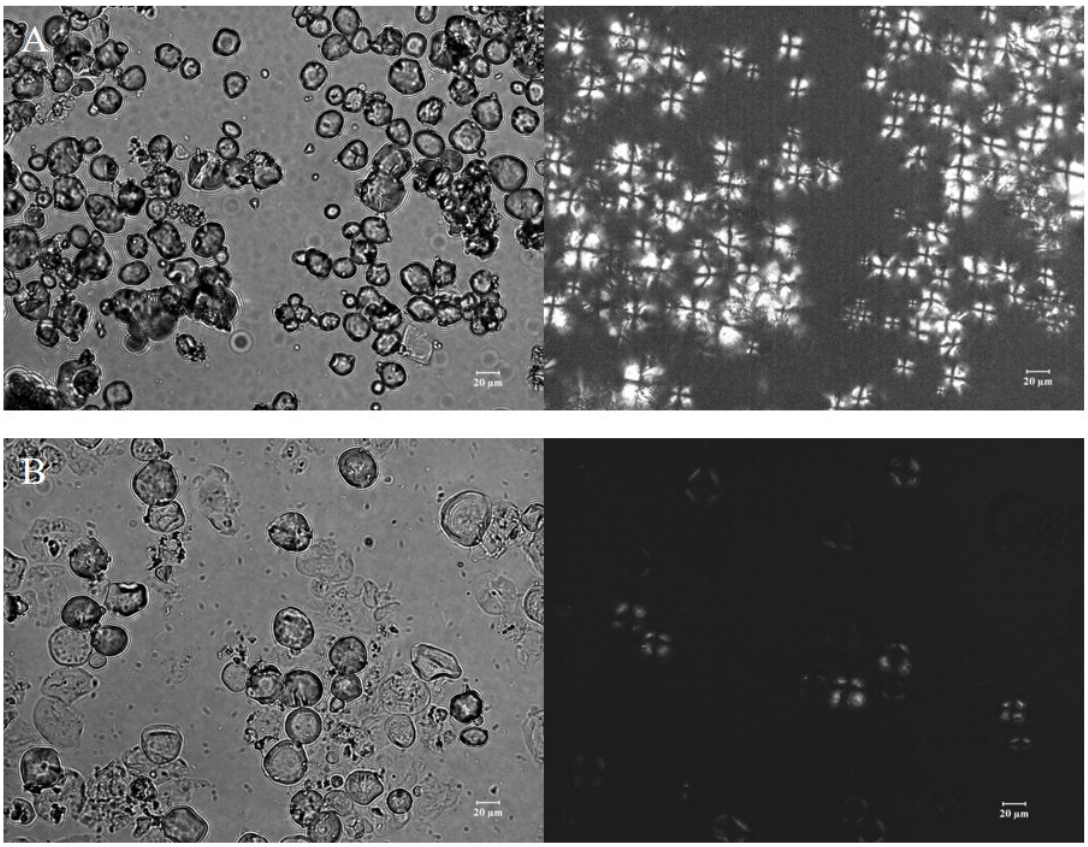


Figure 7.2 Light microscopic images of native normal maize flour (A), and native normal maize flour pretreated with 1% α-amylase at 70 °C for 30 min(B). The scale bar indicates 20 μm.

Effect of substrate concentration on ethanol fermentation

The influence of substrate concentration (20%, 25%, 30%, 35%, and 40%) on ethanol fermentation was investigated with maize flour as substrate (Table 7.3). The reducing sugar in maize hydrolysates increased significantly as substrate concentration increased, at a given liquefaction time. However, the reducing sugar as percent of total sugar decreased as solid content increased. It should be noted that, after 30 min liquefaction, low concentration maize flour slurry (20%) showed a higher reducing sugar conversion (78%). While in high concentration flour slurry (40%) there was only 49% starch had been converted to reducing sugar. It is probably due to that limited water in high concentration maize slurry would inhibit mobilities of enzyme and starch molecules during α-amylolysis, leading to incomplete

hydrolysis. Moreover, after ethanol fermentation, relatively low substrate concentration (20%, 25%, and 30%) resulted in an almost vanished sugar (~2%) which is consumed by yeast. Nevertheless, 40% of maize slurry had more sugar left, indicating that high solids content might inhibit yeast growth. Ethanol yield was consistent with the decrement of reducing sugar during the fermentation process. 25% and 30% flour slurry might provide an appropriate amount of carbon source for yeast, thus exhibited the highest fermentation efficiency (~90%). Considering the process productivity, a higher substrate concentration (30%) is preferable for subsequent research.

Table 7.3 Effect of substrate concentration and enzyme addition on ethanol fermentation

Corn flour (%)	Theoretical glucose from starch[A] (%)	GSHE (%)	Initial reducing sugar[B] (%)	Reducing sugar after fermentation (%)	Ethanol yield (g/kg)	Fermentation efficiency[C] (%)
20	16.58	0.5	12.38±0.02 g	1.38±0.04 ef	480.7±0.1 j	84.78
		1.0	12.68±0.04 fg	1.23±0.03 f	499±0.1 h	88.01
		1.5	12.97±0.05 f	1.20±0.05 f	503.4±0.2 1e	88.78
25	21.5	0.5	12.86±0.10 f	2.00±0.06 d	497.1±0.1 i	87.67
		1.0	13.88±0.06 e	1.90±0.02 d	510.7±0.1 c	90.07
		1.5	14.02±0.02 e	1.73±0.10 de	511.2±0.2 bc	90.16
30	27.00	0.5	14.76±0.03 d	2.70±0.08 c	507.6±0.1 d	89.52
		1.0	15.13±0.08 cd	2.65±0.08 c	511.7±0.2 b	90.25
		1.5	15.21±0.11 c	2.48±0.09 c	513.5±0.1 a	90.56
35	31.13	0.5	16.56±0.01 b	4.20±0.05 b	500.1±0.2 g	88.20
		1.0	16.73±0.08 ab	4.10±0.04 b	501.5±0.1 f	88.45
		1.5	16.91±0.09 ab	4.00±0.07 b	500.5±0.2 g	88.28
40	35.00	0.5	17.01±0.09 a	11.75±0.11 a	450.2±0.2 l	79.40
		1.0	17.06±0.08 a	11.70±0.12 a	451.5±0.2 k	79.63
		1.5	17.07±0.02 a	11.68±0.11 a	452.0±0.3 k	79.72

Means with different letters within the same column of are significantly different ($p < 0.05$).

[A] Theoretical glucose content from starch content×1.1.

[B] Reducing sugar content in fermentation broth after a 30 min-liquefaction.

[C] Fermentation efficiency was defined in previous section equation (7.1)

Effect of pH on α-amylase pretreatment

The influence of slurry pH (4.0,4.5, 5.0, 5.5) on α-amylase pretreatment and ethanol fermentation was investigated and summarized in Figure 7.3 and 7.4. The rheology and fermentation properties were tested after α-amylase pretreatment at 70 °C for 30 min. The lowest viscosity was 180 mpa.s at pH = 4 - 4.5 which was lower than the conventional cooking method (200 mpa.s) (Qi et al., 2013). The viscosity was increased with higher pH environment in α-amylase pretreatment. When the pH increased to 5, the viscosity was over 230 mpa.s which was unfavorable for stirring in ethanol fermentation. The reducing sugar content after pretreatment showed a small difference with the increase of pH. Only 14% to 14.9% starch was converted to reducing sugar after α-amylase pretreatment, while the conventional cooking method could convert 19% after cooking liquefication. What's more, the pH had an important influence on yeast mortality and ethanol yield during fermentation. With the increase of pH, the yeast mortality and final ethanol yield decreased. At pH 4 over 75% yeast was dead after 72 h fermentation and the yield was only 483 g/kg. When the pH over 4.5 the yeast mortality decreased to 65% and final production yield increased to 510 g/kg which was very close to the cooking method (60%, 505 g/kg).

During traditional cooking liquefaction process, starch in maize flour is usually gelatinized, which are far more susceptible to α-amylase and contribute to the liquefaction. Thus, after cooking liquefaction process, reducing sugar content (~19%) in hydrolysate is higher than that after pretreatment process (~14%). However, the pH difference didn't show much influence of reducing sugar conversion in -amylase pretreatment process.

The heat pretreatment would cause a high viscosity and an unconducive stirring condition, inhibiting the fermentation. Therefore, it's necessary to reduce the slurry viscosity during liquefaction process. An incubation at 70 °C only led to partial swelling of starch granules and a slurry with relatively high viscosity. From the results above, the slurry pH

showed an important influence on α-amylase pretreatment and yeast fermentation. The α-amylase showed high activity in low pH environment (4-4.5) in which the starch degraded more severe in acid environment which reduced slurry viscosity before fermentation. While the acid environment was no preferred by yeast in the fermentation process, the yeast mortality increased greatly when pH lower than 4.5 and the amount of yeast had a positive influence on ethanol yield. It's noticeable that when the pH was 4.5, the pH showed very limited influence in yeast and the α-amylase still in its optimal reaction condition. The slurry viscosity was below 200 mpa.s which was favorable by industrial production. The reducing sugar conversion and ethanol yield suggested both α-amylase and yeast showed high performance. Hence, we could perform the pretreatment and ethanol SSF at the same pH level.

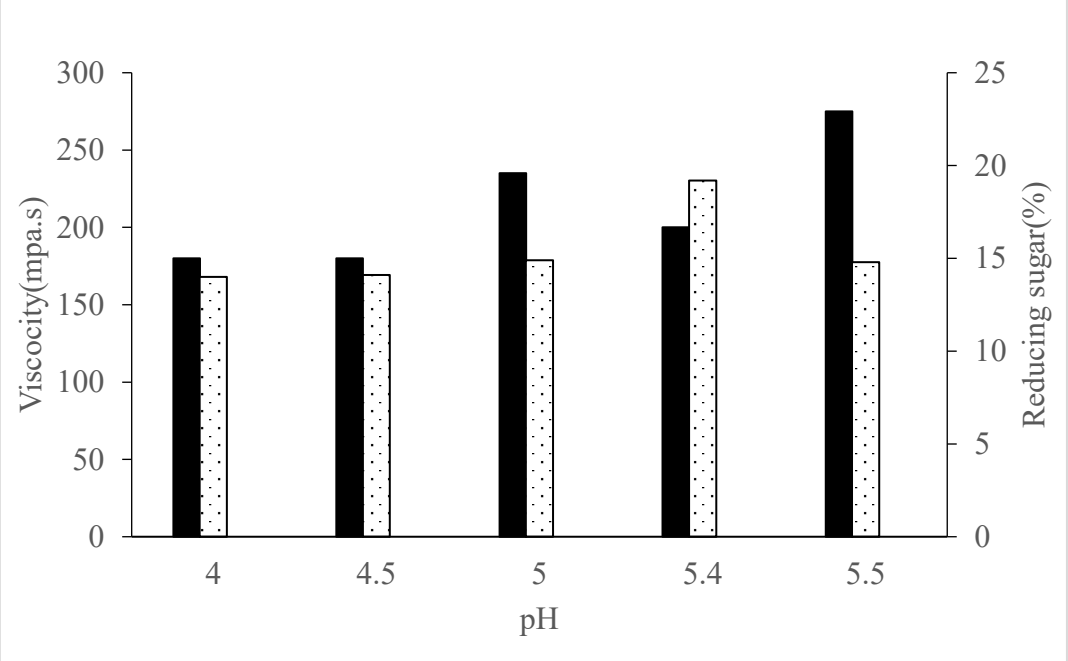


Figure 7.3 Viscosity and reducing sugar content of α-amylase pretreatment under different pH and conventional cooking liquification (control).

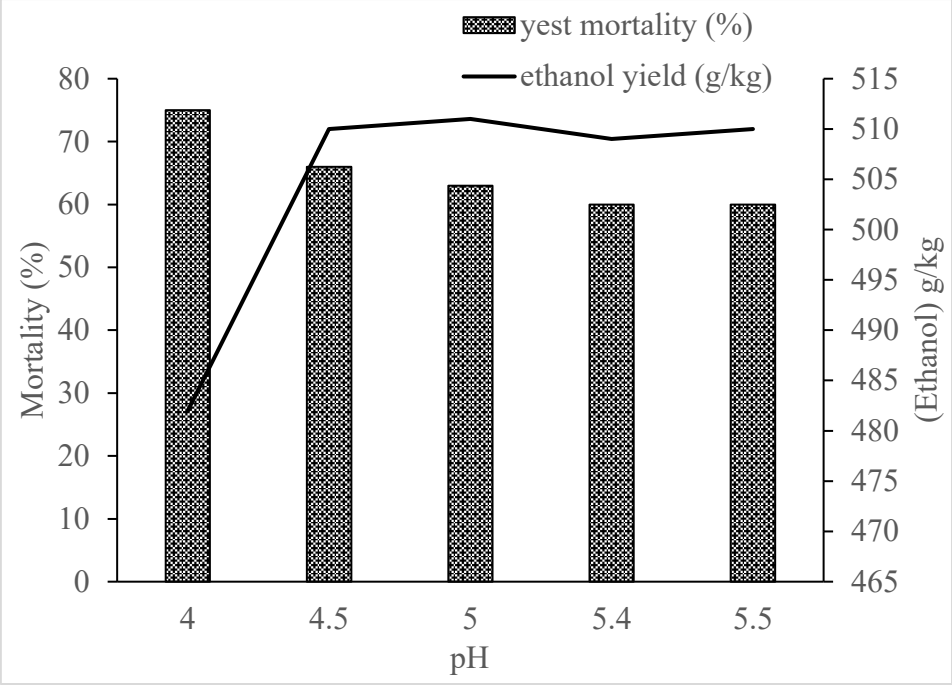


Figure 7.4 Yeast mortality and ethanol yield of fermented broth after α-amylase pretreatment under different pH or conventional cooking liquification (control).

Effect of GSHE addition on ethanol fermentation

During the liquefaction, α-amylase hardly released glucose which is the major carbon source for yeast (Pohu et al., 2004; Tawil et al., 2012). Therefore, GSHE was added to the maize flour hydrolysate to produce more glucose. The amylolysis of starch granules is a two-phase (solid-solution) reaction. The nano-sized enzymes must bind to the solid substrate and then cleave its glycosidic linkages (Genyi Zhang et al., 2006). Unfortunately, limited pores on the surface of starch granules inhibit binding with enzymes (Kong et al., 2017). The higher temperature in liquefaction stage may cause the looser surface structure and higher specific surface area of swollen granules. (Li et al., 2014). Thus, the GSHE was more accessible to the substrates resulting in extensive degradation of starch (Shariffa et al., 2009). However, the level of GSHE addition (0.5%, 1.0% and 1.5%) showed an unobvious effect on reducing sugar remnant and ethanol production, especially when the level was above 1.0% (Table 7.3). In addition, when the substrate concentration was over 30%, the amount of GSHE displayed very limited enhancement on ethanol yield. These results are in accordance with our unpublished research, which showed that an α-amylase hydrolysis could enhance the

subsequent glucose conversion by GSHE, independent of GSHE level.

Effect of glucoamylase addition on ethanol fermentation

Given the role of glucose in yeast fermentation, we additionally added glucoamylase for purpose of enhancing ethanol production. The effect of glucoamylase addition (0%, 0.5%, 1.0%, and 1.5%, which were 0, 800, 1600, 2400 AGU/g starch) on ethanol fermentation was investigated and shown in Table 7.4. After a 72h of simultaneous saccharification and fermentation, the additional glucoamylase significantly enhanced ethanol production, especially for slurry with 30% substrate concentration (Table 7.4). As glucoamylase dosage increased, the bioconversion rate increased, while reducing sugar content decreased, suggesting a more thorough fermentation process. Interestingly, however, when the substrate concentration increased to 35%, glucoamylase addition may result in an excessive concentration of fermentable sugar, which was unconducive to the fermentation. It is possible that exorbitant glucose concentration might inhibit yeast growth due to the osmotic pressure on the cell walls.

Based on the maize slurry with 30% substrate concentration, higher glucoamylase usage (0.4%) contributed to the improvement of ethanol production. Given conventional glucoamylase is cheaper than GSHE, 0.4% of glucoamylase addition was chosen for the pilot-scale experiment, attempting to reduce the usage of GSHE. Moreover, this level of glucoamylase addition is similar to industrial yeast fermentation (0.3-0.4%, w/w).

Table 7.4 Effect of glucoamylase addition on ethanol fermentation

Substrate concentration (%)	GSHE addition (%)	Glucoamylase addition (%)	Reducing sugar after fermentation (%)	Ethanol yield (g/kg)	Fermentation efficiency (%)
30	1.0	0	2.65±0.08 e	511.7±0.2 e	90.25
		0.1	2.03±0.04 f	510.8±0.2 f	90.09
		0.2	1.95±0.00 f	513.0±0.1 d	90.48
		0.4	1.92±0.05 f	517.7±0.0 b	91.31
	1.5	0	2.48±0.09 e	513.5±0.1 d	90.56
		0.1	1.96±0.07 f	514.6±0.2 c	90.76

35	1.0	0.2	1.95±0.00 f	517.6±0.0 b	91.29
		0.4	1.94±0.01 f	523.0±0.1 a	92.24
		0	4.10±0.04 b	501.5±0.1 g	88.45
		0.1	3.64±0.06 cd	494.1±0.3 j	87.14
		0.2	3.73±0.12 cd	495.4±0.2 i	87.37
		0.4	3.82±0.02 bcd	493.6±0.2 k	87.05
	1.5	0	4.00±0.07 abc	500.5±0.2 h	88.28
		0.1	4.00±0.10 abc	489.2±0.2 m	86.28
		0.2	4.05±0.01 ab	493.5±0.2 k	87.04
		0.4	4.15±0.00 a	491.9±0.2 l	86.75

Means with different letters within the same column of are significantly different ($p < 0.05$). Theoretical glucose content was the same as Table 7.1, 30% and 35% maize flour.

Pilot scale ethanol fermentation

In the pilot-scale fermentation experiment, the optimized process was used to simulate industrial ethanol production. Traditional cooking liquefaction process was set as a control. As compared with the industrial process, after a simultaneous saccharification and fermentation process, our experimental process yielded a significant improvement in ethanol production, independent of GSHE usage. Moreover, as GSHE addition raised from 0.5% to 1.5%, ethanol yield increased to 525 g/kg starch with a conversion rate of 92.6% (Table 7.5). Although slightly lower, 1.0% GSHE addition yielded a similar ethanol yield as 1.5% addition, which is 4% higher than that of control. Besides, a reduction of GSHE usage might significantly lower the ethanol production cost. Thus, 1.0% GSHE addition may be preferable in this process.

The new low-temperature treatment showed better performance over traditional cooking method may due to the lower sugar content after pretreatment, several studies have reported that excessive reducing sugar might inhibit yeast fermentation (Li et al., 2014; Wang et al., 2013). These reducing sugars were seen as a potential substrate for subsequent saccharification process and GSHE could consistently convert polysaccharides to glucose which was preferred by ethanol fermentation.

Table 7.5 Ethanol yield and fermentation efficiency after a pilot-scale ethanol fermentation

Substrate concentration (%)	GSHE addition (%)	Glucoamylase addition (%)	Reducing sugar after fermentation (%)	Ethanol yield (g/kg)	Fermentation efficiency (%)
30	Control	0.4	2.5±0.1 a	505.3±0.2 d	89.1
	0.5	0.4	2.0±0.1 a	510.5±0.1 c	90.0
	1.0	0.4	2.2±0.1 a	521.8±0.2 b	92.0
	1.5	0.4	2.3±0.1 a	525.3±0.1 a	92.6

Means with different letters within the same column of are significantly different ($p < 0.05$).

The control was using conventional jet cooking method.

Conclusion

This study optimized a new ethanol fermentation process, using maize flour as substrate. Maize slurry was liquefied at 70 °C by α-amylase to achieve a hydrolysate with appropriate reducing sugar content. Subsequently, the slurry was saccharified by 1.0% GSHE and 0.4% additional glucoamylase and simultaneously fermented by yeast. This process could effectively enhance ethanol production in both small-scale and pilot-scale fermentation as compared with the industrial process. The relatively low temperature (70 °C) liquefaction greatly reduced energy consumption and significantly increased the final ethanol yield by 4%. This new process provides a more efficient and economic alternate in ethanol production.

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Chapter 8 - Overall conclusions

Partial swelling of maize starch granules at 70 °C could effectively enhance starch saccharification by GSHE. This low-temperature saccharification could reduce the energy required to destroy crystalline lamellae in starch granules.

In high concentration starch saccharification, viscosity build up problem could be solved by adding additional α-amylase in the preheating process. This mild heat pretreatment enhanced the enzymatic hydrolysis of crystalline lamellae in granular starch and loosen the starch granule surface which was preferred by further GSHE saccharification. By combining α-amylase and GSHE in this two-step enzymatic hydrolysis, greater than 93% of starch were successfully converted to glucose. The enzyme resistant residue (less than 7%) was comprised of the local dense starch structure which limited enzyme binding, hindering the enzymatic hydrolysis. The enzyme resistant residue may be further cooked and converted to glucose.

The two-step enzymatic hydrolysis not only improves the application of GSHE in sugar production but provides a pretreatment for industrial production of citric acid and ethanol. In citric acid production, the swollen maize starch showed a better affinity to enzymes and provided a consistent glucose supply during the fermentation. Higher starch concentration (18%) could be used in a simultaneous saccharification and fermentation process comparing to conventional production (16%). The overall citric acid yield and bioconversion rate could reach 159 g/L and 88%, respectively. Partial swelling of starch was combined with GSHE in ethanol production using maize flour as substrate. The final product yield could achieve 525 g/kg in a simultaneous saccharification and fermentation process, which was 4% higher than conventional jet cooking method. This relatively low temperature process provides great economic potential in industry.

For future studies, we still need to investigate the fundamental origins for the enzyme resistant residues and determine whether it is because the barriers preventing the access of enzyme to starch or the structure features preventing the enzyme hydrolysis. If we could label the enzyme with fluorescence material, we could observe whether the enzyme bind on the starch under confocal microscope. By doing that we could have a better understanding and help us find a way to completely hydrolyze the starch at low temperature.

Moreover, we could further apply this partial swelling granule technique on other chemicals fermentation such as lactic acid and glutamic acid. They have very similar pretreatment process as citric acid. So we may use the similar method on fermentation of these chemicals. By doing that, we could broaden the utilization of this partial swelling method and make it more useful in industrial production of fermented chemicals.