Effect of Ultrasonic-assisted Pretreatment on Hydrolysis and Fermentation of Acorn Starch

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Acorn starch was used for ethanol production by separate hydrolysis and fermentation (SHF) in this study. The influence of tannins on hydrolysis and fermentation was investigated using ultrasonic-assisted extraction (UAE) to decrease the amount of tannin before SHF. The tannin was shown to have a negative role in hydrolysis and fermentation, and UAE can improve the two processes. The tannin content of acorn starch decreased from 6.19% to 1.91% with the UAE pretreatment time of 200 min. When the pretreatment time was 120 min, the glucose concentration increased from 78.08 to 98.76 g/L after 24 h of hydrolysis. The highest ethanol concentration was 42.22 g/L, which was obtained from the same pretreated acorn flour fermented for 12 h. However, the maximum ethanol yield was 88.06% of the theoretical yield, while pretreatment time was 80 min. Scanning electron microscope images indicated that protein was separated from the starch granules by UAE, as well as by the molecular weight of starch which decreased significantly based on the results from gel permeation chromatography (GPC) analysis.

Keywords: Acorn flour; Ultrasonic assisted ethanol extraction (UAE); Hydrolysis; Fermentation

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INTRODUCTION

Ethanol is being considered worldwide as a promising fuel due to a substantial rise in the price of oil and increasing environmental, social, and geographic issues. The so-called ‘‘bioethanol’’ is a fuel derived from renewable sources of raw materials, mainly plant biomass, and is used as an additive or a substitute for petroleum (Park et al 2011).

Bioethanol can be produced from different kinds of raw materials. The raw materials are classified into three categories of agricultural raw materials: sugar materials (*e.g.*, sugarcane, sugar beet, and sweet sorghum), starch materials (*e.g.*, corn, milo, wheat, rice, potatoes, and barley) and lignocellulosic materials (*e.g.*, wood, straw, and grasses) (Balat 2009, 2011; Bai *et al.* 2008; Man et al. 2013).

Bioethanol is produced on an industrial scale from sucrose and starch; however, these bioethanol production systems pose concerns about competition with food and feed supplies (Field *et al.* 2008; Hahn-Hagerdal *et al.* 2006). Currently, the focus is on bioethanol production from non-grain materials, such as cassava, sweet potatoes, as well as on highly abundant agricultural wastes. Ethanol production from starchy materials by the conventional fermentation method is a two-stage process, namely the saccharification of starch by amylolytic micro-organisms or amylase and the subsequent fermentation by using yeast (Kondo *et al.* 2002; Abegunde *et al*. 2013).

The nut of the Fagaceae family of plants, which is generally called an acorn, is an abundant source for various ingredients. There are seven genera and more than 900 species of acorn found all over the world, except for the tropics and South Africa. All seven of the genera and 294 species are found in China. Many species are important timber trees. Nuts of *Fagus*, *Castanea*, and most *Castanopsis* species are edible; oil is extracted from the nuts of *Fagus*. Most species of nuts contain copious amounts of water-soluble tannins (Huang *et al.* 1998).

Tannins in acorn have various potential uses. They can bind with proteins, and it is this property that makes them useful in the leather industry. The study of traditional Chinese medicine (TCM) takes advantage of a few biological activities of tannins, including decreased blood urea, psychotropic treatment, anti-allergic, angiotensin converting enzyme (ACE) inhibition, anti-pepsin, anti-ulcer, anti-virus, and anti-herpetic properties. Condensed tannins and aldehyde condensation compounds can be used for wood adhesives. This has been the main aspect of tannin-applied research in the 1990s (Sun 1992).

The literature contains several references to the inhibitory effect of tannins on enzymes, which is attributed to the ability of tannins to bind and precipitate protein. The investigations of Daiber (1975) and Kock *et al.* (1985) indicated that the polyphenols of birdproof grain sorghum can exert a severe inhibitory effect on amylolytic enzymes. Recently, the application of ultrasonics in the extraction and refining processes has received increasing attention (Mason *et al.* 1996).

Ultrasonication can significantly improve the extraction of pectin and the industrial production of medicinal tinctures from herbs (Valachovic *et al.* 2001). The effects of ultrasound on vegetal tissues during solvent extraction had been described (Toma *et al.* 2001), and the ultrasonically-assisted extraction of bioactive principles from herbs have been reviewed (Mircea 2001). Several phenolic compounds were extracted from *Polygonum* root by ultrasonic-assisted extraction technology, and multiple response surface methodology was used to optimize the extractions (Chen *et al*. 2012; Kuo *et al.* 2014).

The purpose of this study was to evaluate an ultrasonic-assisted extraction pretreatment of acorn as a method of removing enzyme inhibitors to increase the conversion efficiency during its enzymatic starch hydrolysis and fermentation of the ensuing sugars to ethanol.

EXPERIMENTAL

Materials

Quercus mongolica acorns obtained from Liaoning province in China were used as substrates for fermentation. Pre-milled acorn passed through a 0.425-mm screen was stored in the refrigerator (4 °C) until further use. The microorganism used for fermentation was *S. cerevisiae* in the form of dry yeast (Angel Yeast Company Ltd., Yichang, China). α-amylase (4 KU/g) and glucoamylase (100 KU/g) (Aoboxing Universeen Bio-Tech Company Ltd., Beijing, China) were used for acorn liquefaction and saccharification, respectively.

Methods

Pretreatment of acorn

The acorn was extracted using ultrasonic cycle extraction equipment (Xingzhi Bio-Tech Company Ltd, Ningbo, China) with 20-kHz ultrasonic frequency in a 70% ethanol solution for different times with a liquid-to-solid ratio of 15:1 (Wang *et al.* 2011). The extraction times were 40, 80, 120, 160, and 200 min.

Starch hydrolysis

Acorn flour was added to deionized water at a ratio of 1:5 and mixed with calcium chloride and *α*-amylase (30 U/g dry acorn) for 2 h at 87 °C. The liquefied mixture was saccharified with glucoamylase (150 U/g dry acorn) for 4 h at a pH of 4.0 at 60 °C (Tang *et al.* 2011). The saccharified mixture was used as the substrate for ethanol fermentation. In one of the experiments, acorn flour was treated as above for the liquefaction step, but the saccharification step was allowed to go for 24 h to completely convert all the starch to glucose. The glucose was measured to determine the total starch in the flour.

Fermentation

Fermentation experiments were conducted as described in a previous study (Tang *et al.* 2011). Fermentations were performed in a shaking incubator at 130 rpm, and the conditions were as follows: pH 5.5, 38 °C, and 3.6 g/L initial yeast cells.

Analysis methods

Tannin content was determined according to China National Standard GB/T 15686-2008. Glucose and maltose in cell-free samples were extracted by water at 45 °C for 1 h and quantitatively determined by high-performance liquid chromatography (Waters 2695e, USA) with an Aminex HPX-87P (300×7.8 mm, Bio-Rad, USA) column at 85 °C and a refractive index detection detector at 30 °C. The injection volume of the sample was 10 μL, and ultrapure water was used as the eluent at a flow rate of 0.6 mL/min. The nitrogen content of the acorn flour was quantified by Kjeldahl nitrogen determination (Kjeldahl 1883). Then protein content was obtained by multiplying the nitrogen content by the universal factor of 6.25 according to China National Standard GB 5009.5-2010.

The amount of reducing sugar was measured by the dinitrosalicylic acid method (Miller 1959). The liquid fractions from the supernatants of SHF were analyzed using a high-performance liquid chromatograph (Waters 2695e, USA) equipped with a refractive index detector. Ethanol and byproducts were separated with an Aminex HPX-87H (300 × 7.8 mm, Bio-Rad, USA) column at 65 °C. The injection volume of the sample was 10 μL, and 5 mM sulfuric acid solution was used as the eluent at a flow rate of 0.6 mL/min (Tang *et al.* 2013). Assuming that 1 g of starch present in the liquid theoretically provides 1.11 g of glucose and 0.568 g of ethanol, ethanol yield was expressed as a percentage of the theoretical yield.

SEM images of acorn flour at 5000 and 1000 times magnification were acquired using an S-3400N (HITACHI, Japan) SEM at 15 kV. The weight-average molecular weight (M_w) and number-average molecular weight (M_n) of acorn starch were analyzed by gel permeation chromatography (GPC) on a PL Aquagel-OH Mixed column (300 mm × 7.5 mm, Agilent Technologies, Inc., Santa Clara, CA, USA). The column was operated at 40 °C during measurements. The mobile phase consisted of 5 mM phosphate buffer at

pH 7.0 and 20 mM NaCl at a flow rate of 0.5 mL/min. Detection was achieved using a differential refractometer (Knauer, Berlin, Germany). The column was calibrated using polystyrene standards. The average relative error was found to be 3.8 to 5.6%, with a maximum error of 7.3%. The data was analyzed using the B.01.01 version of the Agilent GPC data analysis software. The M_n, M_w, M_z, M_p and polydispersity index could be obtained by the data analysis (Jiang *et al.* 2011; Jian *et al.* 2011). The acorn samples were dissolved in deionized water with a concentration of 0.002 g/L (w/v) at 90 °C and filtered through a 0.45-μm filter prior to injection into the column.

RESULTS AND DISCUSSION

Composition of Substrates

The chemical compositions of raw and pretreated materials are shown in Table 1. Ultrasonic-assisted extraction (UAE) was employed to decrease the tannin contents of acorn. The starch contents of pretreated materials were higher than that of raw feedstock. The starch content increased with the pretreatment time up to 120 min. The tannin levels were reduced from 6.19% to 1.91% with increasing the pretreatment time from 0 min to 200 min, and 69.1% of the tannins was removed by UAE at 200 min. This was mainly because most of the tannins in acorn of *Quercus mongolica* were hydrolysable and soluble in ethanol. The removed tannins can be used as a byproduct; however, this study did not focus on this constituent. Crude protein levels slightly decreased from 9.15% to 7.25% with increasing extraction time, which could be explained by the low solubility of proteins in 70% ethanol. As it can be seen in Table 1, UAE pretreatment sharply decreased the amount of soluble sugars in the materials. Glucose and maltose were not detected when the pretreatment time was over 120 min.

Table 1. Composition of Raw and Pretreated Materials (%)

Extracted time	Starch	Glucose[a]	Maltose[a]	Tannin	Crude Protein
Raw	43.57	7.82	5.19	6.19	9.15
40 min	45.70	1.26	3.11	3.28	8.68
80 min	46.09	0.30	0.78	2.90	8.36
120 min	51.71	-	-	2.80	7.69
160 min	50.40	-	-	2.01	7.48
200 min	50.70	-	-	1.91	7.25

[a] Glucose and maltose in cell-free samples were extracted by water at 45 °C for 1h

Saccharification of Acorn Flour

Figure 1 presents the glucose release during saccharification of acorn starch from the untreated and pretreated materials. In all cases, starch could not be converted completely before 8 h. De Jong *et al.* (1987) reported that the starch conversion efficiency was about 30% after 30 min of saccharification of high polyphenol birdproof grain sorghum. Figure 1 shows that the saccharification efficiency of the raw acorn flour was about 75%, which was higher than that of the raw lignocellulose. The initial rate of saccharification was increased with pretreatment time lasted. UAE also increased the final glucose concentration. When the pretreatment time was 120 min, the maximum concentration of glucose was 98.76 g/L at 24 h, compared with 78.08 g/L for the raw acorn flour. It should be noted that the raw acorn flour contained some soluble sugars

(about 25 g/L glucose equivalents). These observations indicated that the α-amylase and glucoamylase were inhibited by the tannins. The glucose concentrations at 160 min and 200 min were lower than that at 120 min, probably due to the reduction of low molecular weight proteins and accumulation of large molecular weight proteins. Generally, starch granules can be broken down to a gel in water under sufficient heat and readily converted to glucose. The gel consistency increases during the gelatinization process by large proteins, and the starch granules were broken down differently. The glucose content can therefore be lower. Mohamed and Rayas-Duarte (2003) suggested that wheat starch mixed with wheat protein/gluten increased protein amounts in the blend and increased the onset and the peak temperatures of the starch gelatinization.

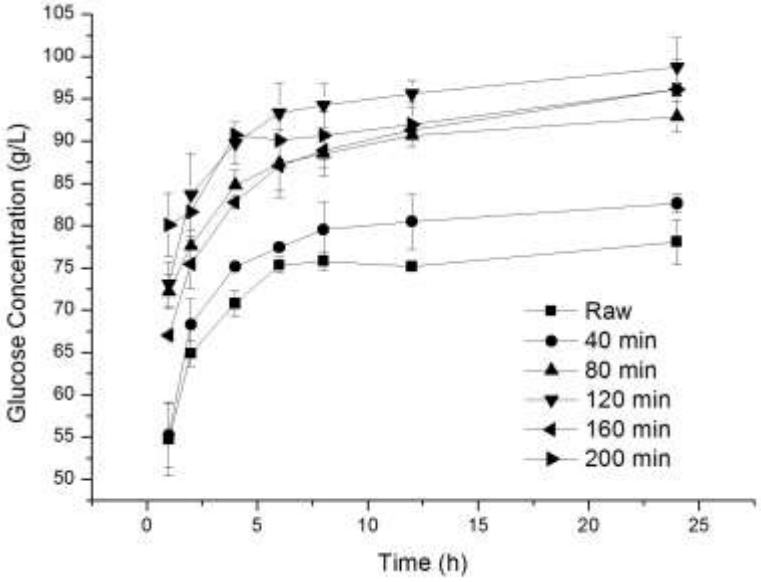

Fig. 1. Glucose concentration during saccharification of acorn and samples with ultrasonic-assisted extraction at different times

Scanning Electron Micrographs (SEM)

Micrographs of the raw acorn and pretreated acorn flours are compared in Figure 2. The shapes of acorn starch granules varied from spherical to ovoid shapes, which was in agreement with other investigators (Cho and Kim 2000; Jane *et al.* 1994; Soni *et al.* 1993; Stevenson *et al.* 2006). Acorn starch was found to have a continuous size distribution, with most of granules in the 3 to 8 μm diameter range, but with some granules as large as 10 to 15 μm in diameter. Cho and Kim (2000) reported that acorn granules are 5 to 20 μm in diameter. There are differences in granule size distribution among different species of acorn. Soni *et al.* (1993) observed that acorn starch granules from *Q. leucotrichophora* are 21 to 59 μm in diameter. Stevenson *et al.* (2006) suggested that acorn starch granules from *Q. palustris* are 3 to 17 μm in diameter. Starch granule sizes from different sources are also different. Jane *et al.* (1994) indicated that starch granules from tubers and roots are quite large, reaching sizes up to 75 μm in diameter for potatoes. Grain starch granules are quite small. Rice, normal maize, and wheat starch granules are less than 40 μm in diameter. Pea starch granules range in size from 10 to 45 μm. Starch granules of fruits and nuts such as banana and acorn are in diameter range of 15 to 45 μm and 5 to 20 μm, respectively (Jane *et al.* 1994; Buleon *et al.* 1998; Chiou *et al*. 2002; Correia *et al.* 2010).

Raw acorn starch granules were wrapped in colloidal material, which could be protein, and connected with each other (Fig. 2). After pretreatment with UAE, the protein was separated from the starch granules, which resulted in a material that was more easily hydrolyzed.

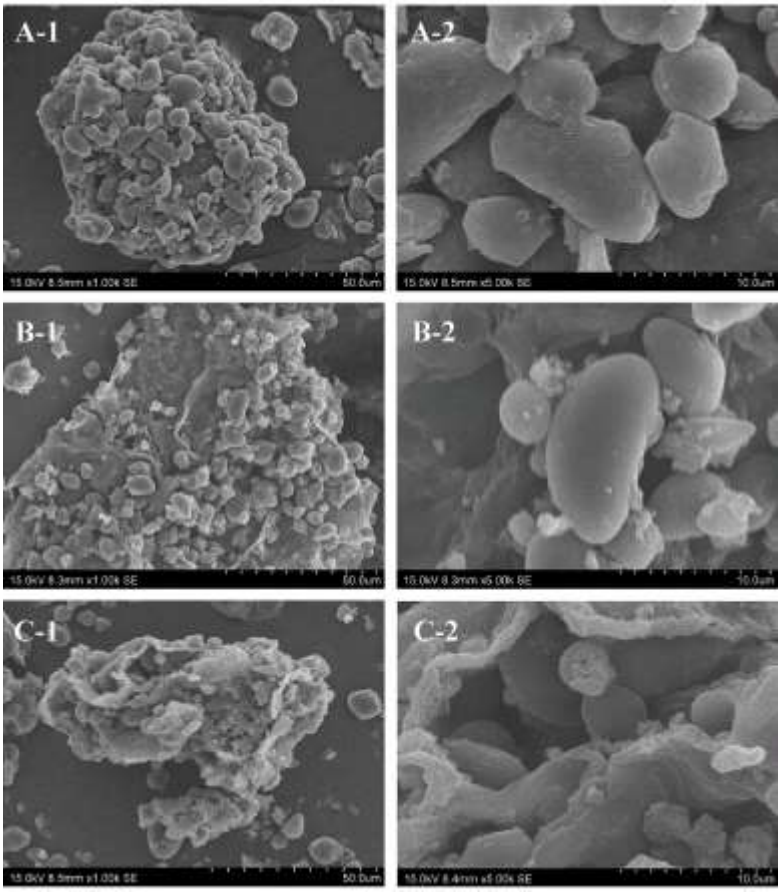

Fig. 2. Scanning electron micrographs of different acorn flours: (A) Raw acorn flours; (B) UAE 80 min. acorn flours; and (C) UAE 200 min. acorn flours. (1) Images taken at 1000× magnification; and (2) images taken at 5000× magnification.

Molecular Weight of Acorn Flour Starch

Table 2 summarizes data for the molecular weight of acorn flour starch as determined by gel permeation chromatography (GPC). The molecular weights were significantly influenced by UAE pretreatment in the following order: $M_z > M_w > M_p > M_n$; also, there were significant differences in the polydispersity index values. At different extraction times, the M_w values varied between 3.107×10^5 and 2.421×10^4 Da.

Table 2. Molecular Weight of Starch from Acorn Flours by GPC (Daltons)

Extracted time	M_n	M_w	M_p	M_z	PI (M_w/M_n)
Raw	89,133	310,665	122,319	1,274,551	3.485
40 min	42,074	77,584	57,641	142,259	1.844
80 min	25,918	41,490	35,262	72,421	1.601
120 min	22,778	34,782	28,473	50,111	1.527
160 min	20,074	28,023	24,089	36,133	1.396
200 min	18,403	24,209	21,851	28,958	1.315

Polydispersity index (PI) values of raw acorn were 2.2 and 2.6 times the flours UAE pretreated for 80 min and 200 min, respectively. Overall, the molecular weight was much lower than the reported values of *Quercus palustris* acorns (3.93×10^8 Da) (Stevenson *et al.* 2006). The reason may be the different oak species of the two samples.

Such difference in molecular weight could be explained as a consequence of the pretreatment method. This may be due to the smashing of starch grains by ultrasonic cavitation. Isono *et al.* (1994) suggested that the ultrasonic treatment degraded the waxy rice starch, as can be seen in the decrease in the number average molecular weight (M_n). The peak of GPC moved to the lower molecular weight values and became narrower as pretreatment time increased. The values of PI decreased with increased pretreatment time, as expected.

Fermentation of Acorn Saccharification Liquid

Figure 3 shows the effect of UAE pretreatment time on ethanol fermentation of acorn saccharification liquid. UAE increased the final ethanol concentration. Using 120 min of pretreatment time, the ethanol concentration reached the highest level, 42.22 g/L, which was increased 16% when compared with the raw flour. UAE slightly increased the fermentation rate when the pretreatment time was less than 80 min. Prolonging the pretreatment time was observed to have a negative effect on the fermentation rate. A lag at the beginning of ethanol fermentation was observed in the cases of 160 min and 200 min. Because the tannin levels were reduced from 6.19% to 1.91%, with the removal of 69.1% of the overall tannins by UAE at 200 min, it was thought that the tannins were not the main cause of the delay. Proteins with low molecular weight can be digested by the yeasts easily, and the reduction of proteins would lead to the shortage of nutrients, which likely caused the lag delay. Therefore, the optimum UAE pretreatment time for acorn flour fermentation is 120 min.

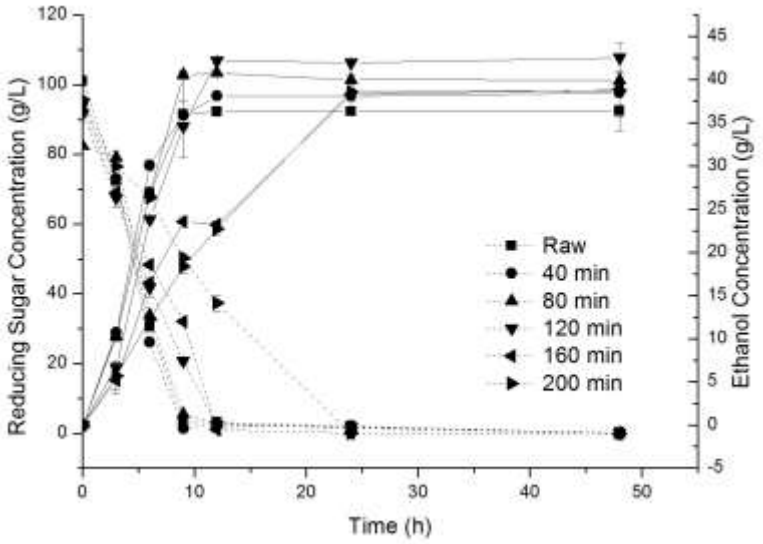

Fig. 3. Concentration of ethanol (solid line) and reducing sugars (dashed line) during the fermentation of saccharified acorn flour untreated and pretreated with ultrasonic assisted ethanol extraction at different times

Glycerol is the main byproduct made by the yeast during the fermentation process and is thought to partially inhibit the fermentation process. The molar ratio of ethanol-to-glycerol can be used to monitor glycerol inhibition of fermentation. Figure 4 compares the molar ethanol-to-glycerol ratios of different materials during fermentation. It can be seen that the molar ratios for UAE pretreatment of acorn flour were 12.52, 13.31, 13.37, 17.44, and 19.21 for extraction times of 40, 80, 120, 160, and 200 min, respectively. The ratios of UAE were apparently higher than that of raw acorn (7.97). The trend of UAE time less than 120 min was consistent with the fermentation results (Fig. 2), as all pretreated samples had the same level of tannins (more than 2.8%; Table 1) and the tannins are the main inhibitors of fermentation.

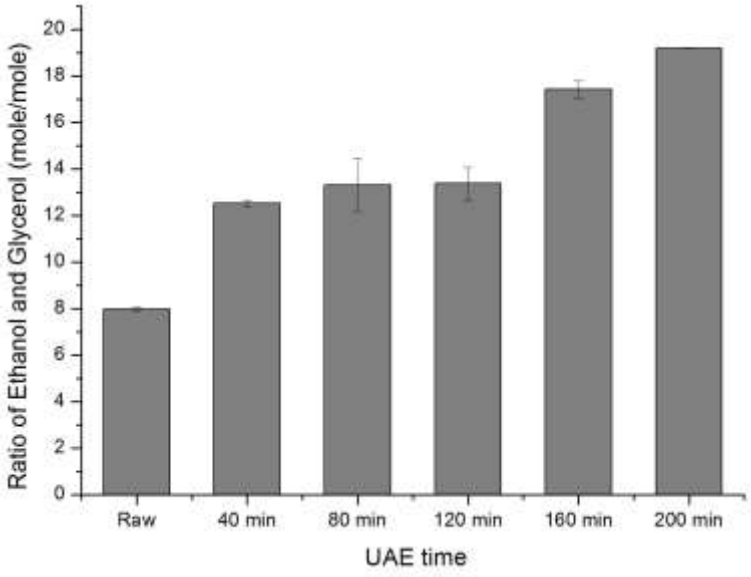

Fig. 4. The final molar ratio of ethanol-to-glycerol from fermentation of saccharified acorn flour untreated (raw) and pretreated with ultrasonic-assisted extraction at different times

Figure 5 shows the impact of UAE on the ethanol yield from the fermentation of saccharified acorn flour. Yields for pretreated acorn flour were higher than that for raw acorn flour. For pretreated materials, yields initially increased with increasing pretreatment time and then decreased. When the pretreatment time was 80 min., the maximum ethanol yield was 88.06% of the theoretical value, which was 28% higher than obtained from the fermentation of raw acorn flour.

Decreasing the tannin content could improve the ethanol yield of separate hydrolysis and fermentation (SHF). However the reduction of proteins with low molecular weight will slow the increasing trend of ethanol yield, particularly when extraction time was more than 120 min. Reducing low molecular weight proteins led to more glucose consumption by yeast to degrade large proteins during the fermentation process. Tannin in the acorn is another inhibitor. In the study by de Jong *et al.* (1987), pretreatment with dilute alkali gave slightly higher efficiency of starch conversion to ethanol by the fermentation of 20% grain sorghum slurry with high polyphenol; however, it was an advancement that NaOH pretreatment could get by using 35% slurry. The polyphenol content of acorn flour was about 4 times that of sorghum, and this may be the

reason why for the relatively low ethanol yield (69% of theoretical) at a low substrate loading of 20%. However, UAE pretreatment can effectively improve SHF of acorn flour.

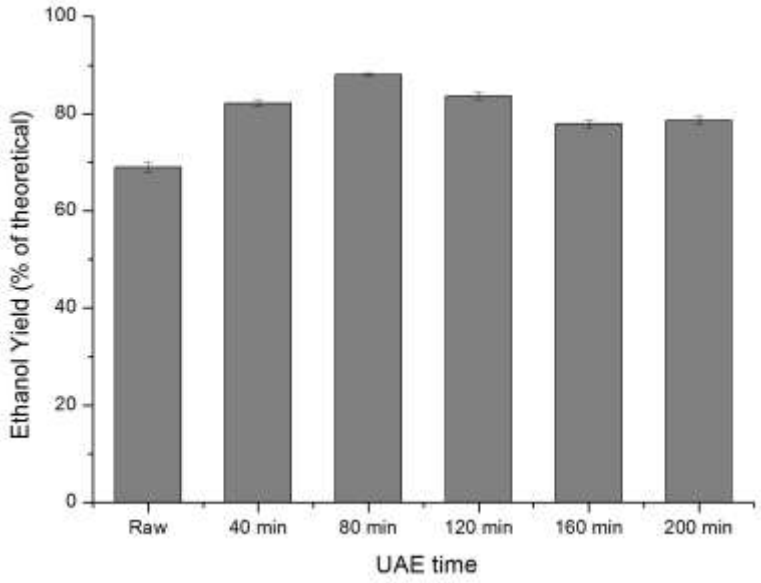

Fig. 5. The final ethanol yield from fermentation of saccharified acorn flour untreated (raw) and pretreated with ultrasonic-assisted extraction at different times

CONCLUSIONS

1. Raw *Q. mongolica* acorn flour is a natural biomass resource that contains 43.57% starch, 13.01% soluble sugar, 9.15% crude protein, and 6.19% tannins. Tannin levels were decreased to 1.91% by ultrasonic-assisted extraction (UAE) pretreatment; the extracted tannin could be used as a valuable co-product from the UAE pretreatment process.
2. The tannins from acorn flour have a negative effect on the hydrolysis and fermentation of this biomass starch; reducing the tannin levels could improve the ethanol yield of separate hydrolysis and fermentation (SHF). The maximum glucose concentration of 98.76 g/L was obtained when the flour was UAE pretreated for 120 min and then saccharified for 24 h. The maximum ethanol concentration of 42.22 g/L was obtained when the flour was pretreated by UAE for 120 min and then fermented for 12 h. The maximum ethanol yield was 88.06% of the theoretical value, and the optimum UAE pretreatment time was 80 min. The concentration and yield of ethanol from acorn starch decreased when the UAE pretreatment time was further prolonged.
3. The SEM images indicated that the protein was separated from the starch granules by UAE pretreatment, which resulted in starch granules that were more easily hydrolyzed during saccharification. Results from gel permeation chromatography analysis suggested that the molecular weights of acorn starches were significantly reduced by UAE pretreatment.

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