Enhancing the hydrolysis of corn starch using optimal amylases in a high-adjunct-ratio malt mashing process

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Abstract Incompletely degraded corn starch particles often seriously inhibit wort filtration and decrease a brewery's beer productivity. Herein, the inhibiting factors of starch hydrolysis and the application of amylases to degrade residual starch were evaluated. The results showed that resistant starch and the amylopectin of corn starch were not the inhibiting factors. Almost all residual starch left in the spent grain layer was proved to be degradable by amylases. Mesophilic α -amylase was selected through a comparison of nine amylases, which increased the wort filtration rate by 44%. However, >6% of corn starch was still left after mashing when a high ratio of corn starch to water (>1:3.5) was used in liquefaction. The low water content in liquefaction was proved to be the key inhibiting factor. Considering the existing equipment and brewing technology, the application of mesophilic α -amylases should be a simple and effective method for enhancing the hydrolysis of corn starch and accelerating the wort lautering process during a high-adjunct-ratio beer brewing process.

Keywords Amylase · Beer brewing · Corn starch · High-gravity fermentation · Low-water-content liquefaction

Introduction

Corn starch is widely applied in high-gravity fermentation processes such as the production of fuel ethanol, organic acid, and amino acid owing to its low cost, easy storage, and wide distribution [1, 2]. Owing to its high fermentability, corn starch is also used as an adjunct in high-gravity beer brewing [3, 4]. With the continuously increasing ratio of corn starch to malt, nowadays, a new “Three High” beer brewing technology is emerging in China that produces high-gravity wort, a high proportion of corn starch as the adjunct, and a high material–water ratio in the liquefaction process [5, 6]. However, when the proportion of corn starch increases up to 30–50%, new problems, e.g., extension of wort lautering, occur; this decreases the productivity of a brewery significantly. Thus far, only a few studies have investigated the filtration inhibiting factors in adjuncts; moreover, the lautering rate cannot be improved effectively by the routinely used viscosity-decreasing enzyme preparations. Our previous studies have proved that the key inhibiting factor in wort filtration is the incompletely degraded corn starch particles instead of wort viscosity or the special yellow floats on the surface after the liquefaction of corn starch [7]. The residual fine corn starch particles form a dense, viscous, and layered spent grain layer and decrease the wort filtration rate [6]. Therefore, to solve the problem of the extension of the lautering process, residual corn starch needs to be further degraded when a high adjunct ratio is used in the high-gravity beer brewing process.

In the ethanol and syrup industries, corn starch hydrolysis under low water conditions is achieved by using a two-stage technology including gelatinization in a twin-screw extruder under a very high temperature (109–130 °C) [8] and enzymatic liquefaction and

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saccharification in other reactors [2]. However, in the beer brewing industry, gelatinization and liquefaction occur in the same cereal cooker under a relatively low temperature (90–100 °C). Moreover, a hot liquefied corn starch mash (90–100 °C) needs to be merged with a low-temperature malt mash (45–48 °C) to heat the mixed mash to the exact mashing temperature of 63–65 °C; this ensures that the optimal energy efficiency is achieved in the mashing process. The problem of incomplete hydrolysis of starch cannot be solved by using the two-stage technology in most breweries owing to a lack of relevant equipment and consideration of energy efficiency. Therefore, application of amylase should be the alternative solution. Several types of amylases involving corn starch hydrolysis exist [9], including α -amylase, β -amylase, glucoamylase, and debranching amylases such as pullulanase and isoamylase. Note that α -amylase can be derived from several bacteria, yeasts, and fungi, which are composed of thermostable and mesophilic α -amylase, raw-starch-digesting α -amylase (raw-amylase) [10], and Taka-Diastase. In addition, β -amylases are derived from plants and microbes. To improve corn starch hydrolysis using a brewery's existing equipment and accelerate wort lautering when using the Three High beer brewing technology, nine amylases were compared and their combinational application was optimized in this study. Moreover, the factors causing an incomplete hydrolysis of corn starch under the low water condition were evaluated.

Materials and methods

Brewing materials and enzyme preparations

Corn starch was purchased from COCFO Biochemical Energy Co., Ltd (Jilin Province, China). The corn starch contained ~94% of dry starch, 0.3% of protein, and 0.5% of fat [7]. Canada Harrington barley was malted in Dalian malting (Liaoning Province, China). The malt was milled using a disk mill set at a 0.2-mm disk distance. The types of amylases used included thermostable α -amylase (TS α) (120 U/g, Novozymes, Copenhagen, Denmark), mesophilic α -amylase from *Bacillus licheniformis* (MS α) (6000 U/g, Boli, Jiangsu, China), isoamylase from *Pseudomonas* sp. (ISO) (280 U/mg protein, Megazyme, Wicklow, Ireland), barley β -amylase (Bar β) (1200 U/g, Genencor, USA), β -amylase from *B. cereus* (Bce β) (2400 U/mg protein, Megazyme, Wicklow, Ireland), raw-starch-digesting α -amylase (Raw α) (3000 U/g, Naijin, Shanghai, China), glucoamylase (GLU) (400 U/g, Novozymes, Denmark), pullulanase (PUL) (400 U/g, Novozymes, Denmark), and Taka-Diastase from *Aspergillus oryzae* (Taka) (100 U/mg, Sigma-Aldrich, CA,

USA). The routinely used brewing enzymes, such as β -glucanase (β GI) (320 U/g, Novozymes, Denmark) and xylanase (XYL) (225 U/g, Novozymes, Denmark), were also used.

Gelatinization and liquefaction of corn starch, its mashing, and the addition of enzyme preparations

The procedure for the gelatinization and liquefaction of corn starch was as follows. Fifty gram of corn starch was mixed with 125 mL of preheated water (55 °C) and stirred; then, thermostable α -amylase (0.6 g/kg corn starch of TS α or 72 U/kg) was added. The mixture was incubated at 75 °C for 10 min, followed by 95 °C for 30 min; it was then cooled to 63 °C for the next step. Evaporated water was supplemented according to its original weight. Except TS α , all the enzymes used in this study were added to the mashing process at 63 °C and incubated at 63 °C for 1.0 h.

The mashing procedure was as follows: 30 min at 48 °C, 40 min at 63 °C, 20 min at 72 °C, and 10 min at 78 °C. The heating rate for the temperature ramp-up was 1 °C per min; moreover, 50 g of barley malt was milled and mashed with a water-to-malt ratio of 3.5:1 in a mashing bath (BGT-8A, Bioer). After mashing for 30 min at 48 °C, the mash was mixed with the above liquefied corn starch mash and was heated to 63 °C. The enzymes were added after merging the two mashes when necessary.

Evaluation of the filtration rate

The filtration rate of the liquefied corn starch mash and the mixed malt mash were evaluated using a self-designed, transparent Buchner funnel with a filter paper at the bottom [6]. The corn starch mashes were filtered under the action of gravity at 63 °C using a thermostat. The mixed malt mashes were filtered at 78 °C. The filtrate was collected in a graduated cylinder. The first 50 mL of the filtrate was collected and refiltered; subsequently, the filtrate volume was recorded. Each filtration experiment was performed in triplicates.

Resistant starch and amylopectin proportion assay

The resistant starch content was analyzed using the Resistant Starch Assay Kit of Megazyme (Megazyme International Ireland Ltd., Wicklow, Ireland). The amylopectin proportion was analyzed using the Amylose/Amylopectin Assay Kit of Megazyme. The assay methods were performed according to the manufacturer's instructions. The pretreatments of different samples were performed as follows. (1) The raw material of corn starch: 100 mg of corn starch was directly used for assay. (2) The

yellow floats on the surface of the liquefaction mash: after the centrifugation of the corn starch mash ($5000\times g$, 10 min), the upper yellow floats were collected and washed twice with 95% alcohol; then, they were used for freeze-drying, and 100 mg of the drying yellow floats was used for assay. (3) Residual corn starch particles at the bottom of the liquefied corn starch mash: the corn starch mash was centrifuged at $10,000\times g$ for 10 min, the supernatant and yellow floats were removed, the sediment was washed twice with 95% alcohol and used for freeze-drying, and 100 mg of the drying sediment was used for assay. Each assay was performed in triplicates.

Assay of residual corn starch in the liquefaction mash

The liquefied corn starch mash was added in a 50 mL tube and centrifuged at $10,000\times g$ for 10 min. The sediment was washed twice with 95% alcohol and used for freeze-drying. The total starch content in the drying sediment was detected using the dinitrosalicylic acid (DNS) method [11]. The freeze-dried samples of sediment (0.1 g) were hydrolyzed in 10-mL 6 mol/L HCl at 100 °C for 30 min, and the concentration of glucose in the hydrolysate was determined using the DNS method. The obtained hydrolysate was adjusted with 6 mol/L NaOH to the neutral pH using phenolphthalein as an indicator and was then filtered using a filter paper. The filtrate was diluted by tenfold with deionized water for further analysis. Then, 1 mL of the diluted hydrolysate was added to 1 mL of the DNS solution (6.3% w/v) and 1 mL of water. The mixture was boiled for 5 min and then cooled in water for 2 min. Subsequently, 22 mL of deionized water was added, and the mixture was used to measure the absorbance at 540 nm. The calculated absorbance was used to measure the concentration of reducing sugar according to a standard curve of the glucose solution (0–0.6 mg/mL). The total starch content was calculated as follows: concentration of reducing sugar $\times 0.9$. The amount of residual corn starch in the liquefied corn starch mash was calculated based on the starch content in the drying sediment. Each sample was analyzed in triplicates.

Particle-size analysis

The particle-size distribution was measured through a Microtrac SDC lasers-diffraction analyzer (Microtrac Inc., USA) using a 300 mL flow-through reservoir. The liquefied corn starch mash was directly added to the reservoir until the concentration reached between 12 and 17%. The settings were optimized for the refractive index of starch in water, and the particle size was automatically

recorded by transferring light-scattering signals to the electrical signals.

Results and discussion

High ratio of corn starch as the adjunct decreased the wort filtration rate

Different proportions of corn starch (0–100%) in the total 150 g of raw material (malt and corn starch) were compared. The relative filtrate rate obtained after gelatinization and liquefaction using the routine procedure is shown in Fig. 1(A). The filtration rate declined with increasing proportion of corn starch. Even a low proportion of corn starch, e.g., between 10 and 20%, decreased the wort filtration rate. There were several residual fine corn starch particles in the spent grain layer (Fig. 1B), which greatly decreased the permeability of the spent grain layer, extended the wort lautering time, and decreased a brewery's productivity [6, 12]. These residual corn starch particles need to be degraded further.

Evaluation of the resistant starch content and amylopectin proportion upon incomplete hydrolysis of corn starch

The possible factors for the presence of residual corn starch particles in the spent grain layer include starch property, amylase performance, and technological parameters of gelatinization and liquefaction. The assay of starch property is shown Table 1, including the resistant starch content and amylopectin proportion in the upper yellow floats of the liquefaction mash (Fig. 2A), residual corn starch particles at the bottom, and corn starch material. The resistant starch content was very low (0.46–0.88%) in these samples, which means that residual corn starch particles were all hydrolysable by amylase. Comparing the molecular structure of starch with that of the corn starch material, the amylopectin proportion in both the yellow floats and residual corn starch particles reduced significantly ($p < 0.01$) (Table 1); this indicates that amylopectin was also not a factor responsible for the presence of residual corn starch particles in the spent grain layer.

The hydrolysable property of the residual corn starch particles was further evaluated by adding different types of amylase. As shown in Fig. 2, when the recommended amount of thermostable α -amylase (0.6 g/kg corn starch TS α or 72 U/kg) was used, the residual starch content reached to 12.6% and the filtration rate was 18 mL/2 h (Fig. 2B). When a tenfold recommended amount of TS α (6.0 g/kg corn starch), 50–100 U/kg of isoamylase (ISO) and 0.3–0.6 g/kg mesophilic α -amylase were used,

Fig. 1 Effect of different proportions of corn starch as adjunct on the filtration rate of wort. (A) The filtration rates of the mixed malt mash with different ratio of corn starch, evaluated using a self-designed, transparent Buchner funnel. (B) The mixed mash containing residual corn starch particles was filtrated using the transparent Buchner funnel

Table 1 Components of the raw material, yellow floats, and residual corn starch particles

	Total starch content (%)	Resistant starch content (%)	Amylopectin proportion in total starch (%)
Corn starch material	93.7 ± 1.8	0.46 ± 0.05	83.5 ± 0.2
Yellow floats	71 ± 4.8	0.79 ± 0.07	54.9 ± 1.8
Residual corn starch particles	65 ± 3.2	0.88 ± 0.06	48.3 ± 0.5

The contents are indicated by the percentage in the dry matter. Data are mean ± standard deviation of three replicates

Fig. 2 Amount of residual starch particles and their amylopectin proportion after the liquefaction of corn starch. (A) The liquefied corn starch mash and the yellow floats on the surface. The corn starch mash was centrifuged at 5000×g for 10 min in a 50-mL tube. (B) The amount of residual starch particles, their amylopectin proportion, and the filtration rate of the corn starch mash were compared when

different amylases were added to the liquefaction process. The filtrate volumes within 2 h were compared. 0.6 TSα: 0.6 g/kg of thermostable α-amylase; 6.0 TSα: 6.0 g/kg of TSα, tenfold usage; 50 ISO: 50 U/kg of isoamylase; 0.3 MSα: 0.3 g/kg of mesophilic α-amylase. (Color figure online)

respectively, the residual starch content decreased to 4–10% and the filtration rate increased to 50–70 mL/2 h. The addition of these enzymes also decreased the

amylopectin proportion from 48 to 32–39%. Therefore, the 12% residual corn starch particles could be further degraded by adding amylase to the process of wort preparation.

Optimal amylases degrading the residual corn starch particles

Eight types of amylase, except the routine TS α , were selected to optimize the degradation of residual corn starch. The recommended temperature range was 55–70 °C, and the recommended pH range was 5.0–7.0; this means that they could be used in the mashing process. Among the multiple α -amylases used, Taka-Diastase holds a wide working pH range and high resistance against low-pH conditions; raw amylase has the capability of performing efficient raw starch hydrolysis; the mesophilic α -amylase used in this study performs a highly effective hydrolysis of starch at 55–65 °C. However, the comparison between Taka-Diastase, raw amylase, and mesophilic α -amylase in this study would be based on their starch hydrolysis capability under low water conditions. When they were added to the liquefaction mash of corn starch (Fig. 3), mesophilic α -amylase obtained the highest filtration rate, followed by isoamylase, glucoamylase, raw amylase, barley β -amylase, and bacterial β -amylase. The addition of Taka-Diastase and pullulanase did not remarkably increase the filtration rate, which means that these two enzymes did not effectively perform corn starch liquefaction under the low water condition (the ratio of corn starch to water was 1:2.5).

Based on above performance of the addition of single amylase, four amylases, i.e., mesophilic α -amylase, isoamylase, glucoamylase, and raw amylase, were then

Fig. 3 Effect of the excess addition of single amylatic enzyme on the filtration rate of the liquefied corn starch mash. The curves of the filtrate volume were compared. Control: 0.6 g/kg of thermostable α -amylase (TS α); MS α : 1.2 g/kg of mesophilic α -amylase (7200 U/kg); Bar β : 1.0 g/kg of barley β -amylase (1200 U/kg); ISO: 100 U/kg of isoamylase; PUL: 1.0 g/kg of pullulanase (400 U/kg); GLU: 1.5 g/kg of glucoamylase (600 U/kg); Taka: 100 U/kg of taka-diastase; RAW α : 1.5 g/kg of raw-starch-digesting α -amylase (4500 U/kg); Bce β : 1500 U/kg of bacterial β -amylase

selected for further optimization. The filtration rate increased as the amount of each enzyme increased (data not shown). The saturated usages of mesophilic α -amylase, isoamylase, glucoamylase, and raw amylase were approximately 0.6 g/kg, 100 U/kg, 1.0 g/kg, and 0.8 g/kg, respectively.

Since these four enzymes could synergistically decompose corn starch, their usages were further optimized via an orthogonal experiment. The optimal combination was mesophilic α -amylase (0.6 g/kg), isoamylase (10 U/kg), glucoamylase (1.0 g/kg), and raw amylase (0.8 g/kg) (data not shown). To validate the optimized usages, three independent batches of the liquefied corn starch mash were investigated by using the optimal combinational enzymes. The filtration rate was 82.6 ± 3.1 mL/2 h, which was the highest filtration rate obtained; moreover, it confirmed the optimal effect of the combinational amylases. According to the range analysis, the effect of these amylases was ranked as follows: mesophilic α -amylase > glucoamylase > raw-amylase > isoamylase. Interestingly, the results of the orthogonal experiment indicate that increasing the usage of the isoamylase, decreased the filtration rate of the corn starch mash. When pullulanase was replaced with isoamylase, a similar result was obtained (date no shown). Therefore, isoamylase was not necessary in the synergistic liquefaction of corn starch particles for wort preparation. This result also confirmed that amylopectin proportion was not the key inhibiting factor for complete hydrolysis of starch. The optimal combination of amylases was determined to be mesophilic α -amylase (0.6 g/kg or 3600 U/kg), glucoamylase (1.0 g/kg or 400 U/kg), and raw amylase (0.8 g/kg or 2400 U/kg).

Effects of liquefaction time and the material–water ratio on corn starch liquefaction

Although the optimal combinational amylases were used, there was 6–8% of residual corn starch in the liquefied mash. Interestingly, the residual corn starch particles were fully degraded by using the Megazyme Kit. The main differences between the industrial liquefaction method and the starch-hydrolysis method involving the Megazyme Kit were the water content (the ratio of corn starch to water during liquefaction) and the enzymatic time. Therefore, these two factors evaluated, as shown in Fig. 4. When the liquefaction time was extended under the same ratio of corn starch to water (1:2.5 or 1:5), no significant decrease in the amount of residual corn starch was observed. In contrast, different ratios of corn starch to water produced considerably different amounts of residual corn starch. A low ratio (1:5) displayed a 2.5-fold decrease in the amount of residual corn starch when compared with the high ratio of 1:2.5. Moreover, comparing the ratios of 1:2.5, 1:3.5,

Fig. 4 Effect of the water content and liquefaction time on the liquefaction of corn starch. **(A)** The comparison of water content and liquefaction time. Two ratios of material to water (1:2.5 and 1:5) were compared in corn starch liquefaction; three liquefaction times (0.5, 1.0, and 16 h) were compared. Student's *t* test showed that different

ratios of material to water caused statistically significant differences ($p < 0.01$). **(B)** Different water contents in the liquefaction process were compared. The ratios of corn starch to water were 1:2.5, 1:5, 1:7, 1:10, and 1:20

1:5, 1:7, 1:10, and 1:20 (Fig. 4B), the amount of residual corn starch rapidly reduced to 2.2% when the ratio decreased from 1:2.5 to 1:7. When the ratio further dropped, the amount of residual corn starch changed in a small range. Therefore, water content was the key factor causing incomplete degradation of corn starch when a high ratio of corn starch to water was used. The particle-size distribution assay also confirmed that a low water content caused the accumulation of many small particles, which seriously inhibited mash filtration, whereas a high water content increased the hydrolysis of corn starch particles by decreasing the amount of the small particles of residual corn starch (data not shown). Low water content is reported to significantly affect the melting temperature of starch [13]; therefore, a high material–water ratio should inhibit the melting and gelatinization of starch at gelatinization temperatures of 90–95 °C and then inhibit the complete hydrolysis of corn starch.

Selected amylases enhanced the high-gravity wort lautering performance

The two key factors for inhibition of wort filtration from malt are soluble β -glucan and xylan; therefore, β -glucanase and xylanase are now commonly added in the mashing process. To evaluate the effect of the use of additional amylases on the lautering process of high-gravity wort, β -glucanase and xylanase were also used as controls (Fig. 5). When the proportion of corn starch was 50% and the ratio of material to water was 1:2.5, routine 0.6 g/kg TS α only obtained a filtration rate of 82 ± 3 mL/h. In contrast, the

Fig. 5 Evaluation of the effect of different enzyme preparations on the filtration rate of the mixed mash. Control: no enzyme was added in the mashing process except 0.6 g/kg of TS α , β Gln: 0.2 g/kg of β -glucanase, β Gln + XYL: 0.2 g/kg of β -glucanase, and 0.2 g/kg of xylanase. β Gln + OPT: 0.2 g/kg of β -glucanase and optimal complex amylases (0.6 g/kg of MS α , 1.0 g/kg of GLU, 0.8 g/kg of RAW α). β Gln + MS α : 0.2 g/kg of β -glucanase and 0.6 g/kg of mesophilic α -amylase. These enzymes were added when the liquefied corn starch mash and the malt mash were mixed. After mashing, the mashes were filtrated using the transparent Buchner funnel. The first 50 mL of the filtrate was collected and refiltrated; then, the volume of the filtrate within 1 h was measured. Each filtration experiment was performed in replicates

addition of β -glucanase increased the wort filtration rate by 24%. The addition of β -glucanase and xylanase increased the filtration rate by 34%. The addition of β -glucanase and optimal complex amylases increased the filtrate rate by 56%. Moreover, single mesophilic α -amylase resulted in a 44% increase in the filtration rate. Therefore, single mesophilic α -amylase also effectively improved the wort filtration rate.

Therefore, the factors causing incomplete degradation of corn starch in the high-adjunct-ratio mashing process were not the resistant starch content and amylopectin proportion but the low water content in liquefaction. Increasing the ratio of corn starch to water from 1:2.5 to 1:5 significantly decreased the amount of residual corn starch particles. However, based on the current Three High brewing technology, the material–water ratio of adjunct liquefaction can vary in a very small range (1:2.5–1:3.5). Therefore, the use of single mesophilic α -amylase should be a simple, effective, and practical solution for enhancing the lautering performance of high-corn-starch-ratio wort preparation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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