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Small-scale mashing procedure for predicting ethanol yield of sorghum grain☆

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ABSTRACT

A small-scale mashing (SSM) procedure requiring only 300 mg of samples was investigated as a possible method of predicting ethanol yield of sorghum grain. The initial SSM procedure, which was conducted similarly to the mashing step in a traditional fermentation test, hydrolyzed just 38.5–47.2% of total sorghum starch to glucose. The initial procedure was simplified to contain only one liquefaction step, which did not influence subsequent saccharification. Thereafter, parameters such as temperature, pH, enzyme dosage, and saccharification time were optimized. Results showed that 91.2–97.5% of the total starch in 18 sorghum hybrids had been hydrolyzed to glucose using the following conditions: liquefaction at 86 °C for 90 min, 20 μL of α-amylase per 30 g of sample; pH adjustment by adding 50 μL of 2 M acetate buffer at pH 4.2 to each microtube; saccharification at 68 °C for 90 min, 200 μL of amyloglucosidase per 30 g of sample. There were strong linear correlations between completely hydrolyzed starch (CHS) from SSM and ethanol yields from both traditional ($R^2 = 0.86$) and simultaneous saccharification and fermentation (SSF, $R^2 = 0.93$) procedures. CHS was a better indicator for predicting ethanol yield in fermentation than total starch.

1. Introduction

Promoting petroleum alternatives, including fuel ethanol, is an ongoing goal of U.S. energy policy. Federal incentives for ethanol use, such as tax incentives, the reformulated gasoline oxygenate standard, and the renewable fuels standard (RFS), promoted significant growth in the ethanol market. The new RFS schedule in law H.R. 6, the "Energy Independence and Security Act of 2007", calls for a minimum of 9 billion gallons of renewable and alternative fuels to be used nationwide in 2008, 20.5 billion gallons by 2015, and 36 billion gallons by 2022. U.S. ethanol production capacity reached 7.8 billion gallons by the end of 2007, a 44% increase from 2006. National production capacity will increase to 13.4 billion gallons at 139 facilities in 2008 if all existing projects are completed (RFA, 2008).

Grain sorghum is a reasonable feedstock for ethanol and could make a larger contribution to the nation's fuel ethanol requirements. Interest in using grain sorghum for bio-industrial applications is growing in the United States (Farrell et al., 2006). There is a large variation in fermentation quality among the hundreds of sorghum hybrids available commercially and under development; thus, it is important for the ethanol industry and sorghum producers to have proper methods that accurately predict sorghum ethanol yields and conversion efficiencies. Little research has been conducted to develop methods for evaluating sorghum fermentation quality.

Ethanol yield and conversion efficiency are major quality traits of cereal grains used as feedstocks to produce fuel ethanol. Grains with higher ethanol yield per unit will produce larger volumes of final product. Although laboratory fermentation is the most direct and reliable method of evaluating fermentation quality of cereal grains, current dry-grind procedures are time-consuming, tedious (Ingledew et al., 1995, 1999; Lee et al., 2000; Singh and Graeber, 2005; Singh et al., 2006; Thomas and Ingledew, 1990; Thomas et al., 1995; Wang et al., 1997, 1999; Wu et al., 2006a,b, 2007; Zhan et al., 2003), and require relatively large quantities of samples.

The dry-grind procedure is a biological process in which starch in whole grains is hydrolyzed to fermentable sugars such as glucose, maltose, and maltotriose, and sugars are converted into ethanol and carbon dioxide. Thus, the amount of total starch in grains is thought to be related to ethanol yield. Previous research in our group

Abbreviations: CHS, completely hydrolyzed starch; DMSO, dimethyl sulphoxide; DP, degree of polymerization; HPLC, high-performance liquid chromatography; LSD, least significant difference; SSF, simultaneous saccharification and fermentation; SSM, small-scale mashing.

☆ Names are necessary to report factually on available data; however, the U.S. Department of Agriculture neither guarantees nor warrants the standard of the product, and use of the name by the U.S. Department of Agriculture implies no approval of the product to the exclusion of others that may also be suitable.

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showed that starch content in sorghum is a good indicator of ethanol yield in the dry-grind process (Wang et al., 2008; Wu et al., 2007; Zhan et al., 2003). However, not all starch could be completely converted to fermentable sugars by enzymes. For example, the web-like protein matrix developed during mashing held not only starch granules but also some oligosaccharides or polysaccharides (Wu et al., 2007; Zhao et al., 2008). Incomplete starch gelatinization and inaccessibility of enzymes to the gelatinized starch also limit the ability to predict ethanol yield from total starch.

In AACC Approved Method 76-13 (AACC International, 2000), starch is hydrolyzed by sequential treatment with thermostable α-amylase and amyloglucosidase. This enzymic procedure requires high-purity enzymes, especially amyloglucosidase, free of contaminating activities from cellulase and catalase. Cellulase contamination contributes to false high starch values due to cellulose hydrolysis, and catalase reduces stability of chromogen formed in glucose assay methods based on the use of GOPOD reagent (McCleary et al., 1997). Megazyme (Megazyme International Ireland Ltd., Ireland) offers a total starch assay kit based on thermostable α-amylase and amyloglucosidase. However, widespread use of this approach in industry is limited by the prohibitive cost for per-sample analysis.

According to AACC Approved Method 76-13, complete solubilization and dextrinization for samples containing enzyme-resistant starch require pre-treatment with dimethyl sulphoxide (DMSO) at boiling temperature. Results from sorghum samples using Megazyme kits showed that total starch contents with DMSO pre-treatment were slightly higher than for samples without DMSO (unpublished data). Pre-treatment with DMSO is favorable and necessary to avoid underestimating starch content in sorghum samples. However, it is impossible for ethanol fermentation practices to have a DMSO pre-treatment before liquefaction and saccharification, which could be one reason that total starch content explained only 78% of the variability of ethanol yield among 70 sorghum samples (Wu et al., 2007). Completely converting starch to glucose in a short time requires enzyme levels much higher than those used in ethanol fermentation for the quantified amount of substrates. This could affect total starch content to explain the variability of ethanol yield among sorghum samples.

Research from Pioneer, a seed company, showed that the high total fermentables (HTF) trait of corn is a more accurate indicator of dry-grind ethanol production than total starch (Bryan, 2003). Additionally, Pioneer developed a point-of-sale assay using whole grain near-infrared (NIR) technology that allows ethanol plants to predict the value of corn for ethanol production by identifying HTF grain arriving at the plant.

The aim of this study was to develop a small-scale mashing (SSM) procedure that is similar to the mashing step in a fermentation test but requires only a few hundred milligrams of test samples. Industry-used enzymes including heat-stable α-amylase and amyloglucosidase would be employed in SSM as in fermentation procedures. We expected most of the starch in ground sorghum to convert to glucose with optimized liquefaction and saccharification parameters and hypothesized that ethanol yield from laboratory fermentation would be highly correlated to glucose yield from SSM. Samples would not be pre-treated with DMSO, and the effect of starch granules firmly trapped within the web-like protein matrix (Zhao et al., 2008) on glucose yield could be similar to their effect on ethanol yield. Glucose yield from SSM was anticipated to be a better indicator than total starch content for prediction of ethanol yield in fermentation.

A mini shaking incubator made it possible to develop the SSM procedure and was used in this study. The incubator was a kind of vortex mixer with accurate temperature control, and small samples could be simultaneously heated and mixed. Thus, we anticipated that the combination of an SSM procedure with rapid glucose determination in a mash would make prediction of ethanol yield more efficient. Moreover, the cost for per-sample analysis would be much lower than the total starch assay.

2. Experimental

2.1. Sorghum cultivars

Eighteen cultivars from a 2004 commercial winter breeding nursery were selected from a population of 70 proprietary sorghum genotypes and elite hybrids with a broad range of ethanol yields. Samples were hand cleaned to remove glumes, debris, and other impurities, packaged in plastic bags, and stored at 4 °C until testing.

2.2. Sample grinding

For SSM, samples were ground using a Udy mill (Udy Corp., Fort Collins, CO) through a 0.25-mm screen. For starch analysis, sorghum kernels were ground using the Udy mill with a 1.0-mm screen. Samples for ethanol fermentation were ground into fine meals in a Magic Mill III Plus grain mill (Magic Mill Products & Appliances, Monsey, NY) set at level III.

2.3. Enzymes and microorganisms

Liquozyme SC DC, a heat-stable α-amylase from *Bacillus licheniforms* was used for liquefaction. Enzyme activity was 240 KNU/g (KNU, the amount of enzyme which breaks down 5.26 g of starch per hour by Novozyme's standard method for determination of α-amylase). Spirizyme Fuel, an amyloglucosidase from *Aspergillus niger*, was used for saccharification. Enzyme activity was 750 AGU/g (AGU, the amount of enzyme which hydrolyzes 1 μmol of maltose per minute under specified conditions).

Saccharomyces cerevisiae (ATCC 24860) was used for traditional fermentation. Yeast cells were maintained on yeast extract/peptone/dextrose medium. The agar slant consisted of yeast extract (20 g/L), peptone (5 g/L), dextrose (5 g/L), agar (20 g/L), and distilled water. Yeast cells were precultured for 48 h at 30 °C in an aqueous solution containing glucose (20 g/L), peptone (5 g/L), yeast extract (3 g/L), KH_2PO_4 (1 g/L), and $MgSO_4 \cdot 7H_2O$ (0.5 g/L) with a shaking speed of 200 rpm. Yeast cell counts were determined by the direct microscopic method at a magnification of 400× using a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA) with a Micromaster phase-contrast microscope (Fisher Scientific, Fairlawn, NJ). Cell concentrations also were checked by measuring the optical density of the yeast precultures at a wavelength of 600 nm on a BioRite 3 spectrophotometer (Thermo Electron Corporation, Madison, WI). The A_{600} values of the 48 h precultures were 2.2–2.6 with cell counts of 2–2.8 × 10^8 cells/mL.

Active dry *S. cerevisiae*, Red Star Ethanol Red, was used for simultaneous saccharification and fermentation (SSF). Before inoculation, dry yeast was activated by adding 1.0 g of cells into 19 mL of the same preculture broth described previously and incubated at 38 °C for 25–30 min in an incubator operating at 200 rpm. The activated yeast culture had a cell concentration of 1 × 10^9 cells/mL.

2.4. Preparation of mashes for ethanol fermentation

For mashing, 30 g (db) of ground sorghum was dispersed in a 250-mL Erlenmeyer flask with an aliquot of 100 mL of fermentation solution, which was prepared by mixing 1 L of distilled water (60–65 °C) with 1.0 g of KH_2PO_4, 3.0 g of yeast extract, and 100 μL (for two-step liquefaction) or 200 μL (for one-step liquefaction) of Liquozyme. Flasks then were inserted into a water bath shaker (Amerex Instruments, Inc., Lafayette, CA) oscillating at 100 rpm. The

water bath had been preheated to 95 °C. In the beginning, flasks were shaken manually to prevent gel formation. Some grains had to be shaken intensively. This shaking process took several minutes depending on the number of flasks inserted. The water bath temperature decreased naturally to 82–87 °C at the end of shaking, and slurries in the flasks were well dispersed. Liquefaction proceeded in two different ways as follows:

2.4.1. One-step liquefaction

The temperature was brought to 86 °C, and slurries were incubated at this temperature for 90 min in the water bath shaker at 100 rpm.

2.4.2. Two-step liquefaction

The temperature was raised to 95 °C and held for 45 min with continuous shaking. Flasks then were taken out of the water bath and cooled to 80 °C, and another 10 μL of Liquozyme was added to each flask. Liquefaction continued for an additional 30 min at 80 °C.

At the end of liquefaction, flasks were taken out of the water bath, and the materials sticking on the inner surface of the flasks were scraped back into the slurries with a spatula and rinsed with 3–5 mL of deionized distilled water using a sterilized, fine-tipped polyethylene transfer pipette. Only slurries after two-step liquefaction were saccharified separately before fermentation.

2.4.3. Saccharification

The temperature of the liquefied slurries was lowered to 60 °C, and 100 μL of Spirizyme was added to each flask. Flasks were maintained at 60 °C for 30 min with the shaker running at 100 rpm.

2.5. Fermentation processes

All of the mashes after one-step liquefaction and the saccharified mashes were cooled to 25–30 °C and adjusted to pH 4.2–4.3 with 2 M HCl before inoculation.

2.5.1. Traditional fermentation

Saccharified mashes were inoculated with 5 mL of yeast preculture (*S. cerevisiae* ATCC 24860).

2.5.2. SSF

For one-step liquefied mashes, SSF started with addition of 1.0 mL of the activated dry yeast culture and 100 μL of Spirizyme to each flask.

Fermentation flasks were sealed with S-shaped airlocks filled with ≈2 mL of mineral oil. Ethanol fermentation was performed in an incubator shaker (Model I2400, New Brunswick Scientific Inc., Edison, NJ) at 30 °C for 72 h with continuous shaking at 200 rpm. The fermentation process was monitored by measuring the mass loss of the fermentation mash.

2.6. Distillation

At the final fermentation time (72 h), all materials in a flask were transferred to a 500-mL distillation flask with 100 mL of distilled water. Beers were distilled on a distillation heating unit, and the distillates were collected into a 100-mL volumetric flask that was dipped into ice water. Distillation was stopped when the collected distillates approached the 100-mL mark (≈99 mL). Collected distillates then were equilibrated to 25 °C, adjusted to 100 mL, and sampled for HPLC analyses.

2.7. Preparation of freeze-dried mashes

Ground sorghum (30 g, db) was liquefied by Liquozyme at 95 °C for 45 min and 80 °C for 30 min and then saccharified by Spirizyme at 60 °C for 30 min. After mashing, all materials in each flask were collected, frozen immediately, and then lyophilized. Enzyme dosages and mashing conditions were the same as preparation of mashes for ethanol fermentation, but yeast foods such as KH_2PO_4 and yeast extract were excluded from the mashes. All mashed grains were ground using the Udy mill with a 0.25-mm screen.

2.8. SSM experiments

A mini shaking incubator (Vortemp 1550, Labnet International, Inc., Edison, NJ) with a shaking rack for microtubes was used for SSM. Speed of the shaking platform was set at 900 rpm for all procedures. Before mashing, ground meal (300 ± 5 mg, as is basis, weighted accurately) was mixed with 1 mL of enzyme solution in a 2-mL pre-weighted microtube, and the microtube was vortexted to disperse the flour. Centrifuge microtubes were tolerant of high mashing temperatures (e.g., 100 °C) and tightly sealed during mashing. Enzyme solutions were prepared fresh by diluting Liquozyme or Spirizyme to a desired concentration in distilled water. For comparative purposes, the enzyme level in each microtube was calculated as the amount of an enzyme, Liquozyme or Spirizyme, which was used to liquefy or saccharify the substrate in 30 g of ground sorghum. Various dosages of Liquozyme (5, 10, and 20 μL per 30 g of sample) were used for liquefaction. Microtubes with slurries were inserted in the shaking rack, which then was put into the incubator at room temperature. Liquefaction proceeded in two different ways as follows:

2.8.1. One-step liquefaction

The incubator temperature was raised to 86 or 95 °C and held for 90 min.

2.8.2. Two-step liquefaction

The incubator temperature was raised to 95 °C and held for a period of time (45 or 60 min). The shaking rack together with the microtubes then was taken out and cooled for 5 min at room temperature while the incubator cooled naturally with its lid open. One hundred μL of an α-amylase dilution was added to each microtube with the second dosage equivalent to 10 μL of Liquozyme per 30 g of sample. Microtubes were vortexed and re-placed in the incubator. Incubator temperature was brought to 80 or 86 °C and held for 30 or 45 min.

After liquefaction, the shaking rack together with the microtubes was taken out, and the incubator cooled naturally with its lid open. In some cases, the pH of the liquefied mashes was adjusted by adding 50 μL of 2 M sodium acetate buffer at pH 3.5, 4.2, 4.5, or 5.5 to a microtube. For saccharification, 100 μL of a properly diluted amyloglucosidase solution was added to each microtube with the dosage equivalent to 15, 50, 100, 150, 200, or 250 μL of Spirizyme per 30 g of sample. Microtubes were vortexed and then put into the incubator.

2.8.3. Saccharification

The incubator temperature was raised to 60 or 68 °C and held for a period of time (30, 45, 60, 90, or 120 min).

After mashing, microtubes were cooled at room temperature for 20 min. During cooling, microtubes were vortexed and weighed. All mashes were centrifuged at 13,200 × g for 4 min, and the supernatants were filtered through syringe filters with a 0.45-μm membrane.

2.8.4. Mash dilution and inactivation of amyloglucosidase

The mash was diluted by mixing 200 μL of the filtrate with 480 μL of distilled water. In some cases, dilutions were sealed in 15-mL test tubes and cooked at 100 °C for 10 min to deactivate amyloglucosidase. In most cases, the mash was diluted by mixing 200 μL of the filtrate with 480 μL of 0.01 M phosphate buffer at pH 10.0 for inactivation of amyloglucosidase. The phosphate buffer was prepared by dissolving 59.8 mg of NaH_2PO_4 and 190.7 mg of

$Na_3PO_4 \cdot 12H_2O$ with distilled water to 1 L using the phosphate buffer calculator (Clymer, 2005). Some uncooked and cooked dilutions were kept at room temperature for 1–3 days. All dilutions were sampled for HPLC analyses.

2.9. Analytical methods

Moisture content was measured using AACC Approved Method 44-15A (AACC International, 2000). Total starch content was determined using a Megazyme total starch kit (Megazyme International Ireland Ltd., Wicklow, Ireland) according to AACC Approved Method 76-13. Method B was used, which involves pretreatment with DMSO at 100 °C. For glucose analysis in freeze-dried mashes, 0.5 g of samples was dispersed in 10 mL of H_2O. After vortexing for 5 min, the slurry was diluted to 250 mL, and glucose in the diluted solution was measured following the total starch method and analyzed by HPLC.

Sugars (glucose, maltose, and maltotriose) in diluted mashes from SSM and ethanol in distillate samples from fermentation were determined using a Shimadzu (Shimadzu Scientific Instruments, Inc., Columbia, MD) HPLC system equipped with a Rezex RCM 7.8 × 300 mm column (Phenomenex, Torrance, CA) with a security guard column. The mobile phase used was deionized distilled water at a flow rate of 0.6 mL/min. Injection volume was 20 μL. All components were detected with a refractive index detector (Model RID-10A, Shimadzu). Temperatures of the column and detection cell were maintained at 80 °C and 40 °C, respectively. To determine sugar and ethanol concentrations, HPLC data were processed using EZStart 7.4 software (Shimadzu).

Completely hydrolyzed starch (CHS) (%, db) was defined as a ratio of the mass of the starch that had been hydrolyzed to glucose in a mash to the sample mass (dry matter).

2.10. Experimental designs

As detailed in Table 1, four split-plot designs were used to investigate the effects of SSM conditions on CHS, and two completely random designs were used to optimize enzyme dosages and saccharification time.

2.11. Statistical analyses

All experiments were performed at least in duplicate. The tabular and diagrammatic results presented are the mean values of the repeated experiments. Analysis of variance, least significant difference (LSD), split-plot design, and linear regression were performed using SAS software version 9.1 (SAS institute, Cary, NC).

Table 1
Experimental designs[a].

Design no.	Design structure	Factors and treatment structure (one-way or two-way factorial)
1	Split-plot	Whole-plot factors: 2 samples (Sorghum II and VIII); one-way. Sub-plot factors: 7 treatments (I-0, I-3, II-3, III-0, III-1, III-2, and III-3); one-way
2		Whole-plot factors: 2 samples (Sorghum I and VII); one-way. Sub-plot factors: 3 treatments (I, II, and III); one-way
3		Whole-plot factors: 2 samples (Sorghum II and VIII); one-way. Sub-plot factors: 4 acetate buffers (pH 3.5, 4.2, 4.5, and 5.5); one-way
4		Whole-plot factors: 3 SSM procedures (1, 2, and 3); one-way. Sub-plot factors: 3 amyloglucosidase dosages (15, 50, and 50 μL) and 2 samples (Sorghum II and VIII); two-way
5	Completely random	3 Amyloglucosidase dosages (15, 50, and 100 μL) and 3 α-amylase dosages (5, 10, and 20 μL); two-way
6		3 Amyloglucosidase dosages (100, 150, and 200 μL) and 3 saccharification times (60, 90, and 120 min); two-way

[a] Results in the first, second, third, fourth, fifth, and sixth experimental designs were shown in Fig. 2, Fig. 3, Fig. 4, Table 3, Fig. 5,and Fig. 6, respectively.

3. Results and discussion

3.1. Calculation of the mass of glucose and the CHS in a microtube

To calculate the percentage of the starch which had been hydrolyzed to glucose in a sample, it was necessary and crucial to determine the mass of glucose in a microtube after SSM. Glucose concentration in a mash (expressed as mass of glucose per milliliter of solution) can be assayed conveniently by HPLC or the enzymic method (McCleary et al., 1997). However, measuring the total volume of the liquid part of the mash is difficult due to the concentrative properties of glucose aqueous solutions, in which the volume of solution is larger than that of water solvent. The most accurate way to obtain the mass of glucose is diluting all of the mash in a microtube to a known volume and then analyzing the glucose concentration in the dilution. This approach was time-consuming and was used only as a control for the measurement of glucose mass in this study.

Several sources of water including the water in a sample, diluted enzyme solutions, and the buffer for pH adjustment, contributed to the mass of water brought to a microtube after mashing. Mass of water in a mash was not equal to the sum of the sub-masses of all water sources due to evaporation, but it could be calculated using the following equation:

$$M_w = M_{tm} - M_s - M_t + M_s \times \frac{MC}{100} \quad (1)$$

where, M_w = mass of water (mg), M_{tm} = mass of a microtube together with mash after mashing (mg), M_s = mass of a sample (mg), M_t = mass of the empty microtube (mg), and MC = moisture content of the sample (%).

Looking at the concentrative properties of glucose aqueous solutions at 20 °C (Anonymous, 2004), we found that there was a strong, linear relationship ($R^2 = 1.00$, $p < 0.0001$) between the two concentrations, mass of glucose per milliliter of glucose solution (C_1, mg/mL) and mass of glucose divided by total mass of glucose solution (C_2, %). C_1 was transformed from the molarity (moles of glucose per liter of solution) by multiplying by 180. Thus, Eq. (2), the regression equation, was used to calculate mass percentage of glucose in a mash solution as follows:

$$C_2 = 0.0928 \times C_1 + 0.2352 \quad (2)$$

For simplicity, we assumed that the liquid part of the mash in a microtube was an aqueous solution of glucose (i.e., most of the starch had been hydrolyzed to glucose). This assumption was proved in SSM with optimum parameters and is discussed later. Based on the definition of mass percentage (C_2), the mass of glucose in a microtube at room temperature (22–24 °C) could be calculated using the following equation:

$$M_g = \left(\frac{C_2}{100 - C_2}\right) \times M_w \quad (3)$$

where, M_g = mass of glucose (mg).

CHS (%, db) was calculated using the following equation:

$$CHS = \left(\frac{M_g \times (162/180)}{M_s \times (100 - MC)}\right) \times 10,000 \quad (4)$$

where 162/180 = adjustment from free glucose to anhydro glucose (as occurs in starch) and 10,000 = factor to express CHS as a percentage of the sample mass.

3.2. Initial SSM procedure

At the beginning of this research, we tried an SSM procedure that followed the traditional fermentation process: liquefaction at 95 °C for 45 min and 80 °C for 30 min, α-amylase dosages equivalent to 2×10 μL of Liquozyme per 30 g of sample; saccharification at 60 °C for 30 min, amyloglucosidase equivalent to 100 μL of Spirizyme per 30 g of sample. A typical chromatogram of a mash analyzed right after diluting by distilled water is shown in Fig. 1. Results of sugar analyses by HPLC for the nine sorghum samples (Table 2) showed that 38.5–47.2%, 21.1–27.4%, and 1.7–6.7% of the total starch had been hydrolyzed to glucose, maltose, and maltotriose, respectively (data not shown). HPLC could not separate oligosaccharides and polysaccharides with glucose units greater than three, but a group of starch hydrolyzates with degree of polymerization (DP) > 3 were present in the mash and represented 21.2–35.4% of the total starch (calculated from the difference between total starch and sum of the starch which had been hydrolyzed to glucose, maltose, and maltotriose). Existence of maltose, maltotriose, and other hydrolyzates influences calculation of the mass of glucose in a mash. Using the earlier developed procedure, the mass of glucose was 5–10% lower than that in direct measurement after the mash in a microtube had been diluted to a known volume (data not shown), which was one reason the initial procedure needed to be modified further.

Glucose concentrations for the same mash dilution were slowly but continuously increased when measured repeatedly over time because amyloglucosidase in the diluted mash remained active throughout the room temperature setting. During a 3-day observation with the same mash sample, the peak of glucose increased while all other peaks decreased (Fig. 1). The hydrolysis of starch hydrolyzates with higher DP by amyloglucosidase to glucose was a dynamic process. However, this process seemed to cease after 3 days; chromatograms were unchanged and appeared similar to the one with a dotted line in Fig. 1. To improve experimental repeatability and guarantee the analysis results were not affected by the setting time after mashing, it was necessary to deactivate amyloglucosidase in the mash dilutions.

Table 2
Comparison of CHS in mashes from SSM and freeze-dried mashes from traditional laboratory fermentation.

Sample code	Total starch (%, db)	CHS (%, db)		
		SSM[a]	Laboratory fermentation[b,c]	
		Analyzed by HPLC	Analyzed by HPLC	Analyzed by Megazyme Kits
I	70.4 b,c [d]	37.8 c	40.0 b,c	39.3 d
II	71.5 a,b	35.8 d	38.6 c	37.8 e
III	68.0 d	37.5 c	41.3 b	40.5 c,d
IV	72.0 a,b	41.6 a	44.8 a	43.2 b
V	68.7 d	38.8 b,c	40.8 b,c	39.8 c,d
VI	71.1 b	41.7 a	40.8 b,c	40.8 c
VII	71.3 a,b	39.1 b,c	44.6 a	44.6 a
VIII	73.0 a	39.6 b	45.8 a	44.7 a
IX	68.8 c,d	34.3 d	35.2 d	34.4 f
Replications	2	2	2	2
Standard error	0.53	0.50	0.43	0.42
LSD (0.05)	1.69	1.61	1.38	1.33

[a] Liquefaction condition: 95 °C, 60 min and 80 °C, 30 min; 2 × 10 μL of Liquozyme per 30 g of sample. Saccharification condition: 60 °C, 45 min; 100 μL of Spirizyme per 30 g of sample.
[b] Liquefaction at 95 °C for 45 min and 80 °C for 30 min; saccharification at 60 °C for 30 min.
[c] Right after mashing, the liquid and solid parts of mashes were cooled, frozen, and lyophilized.
[d] Means followed by different letters in the same column are significantly different ($p < 0.05$).

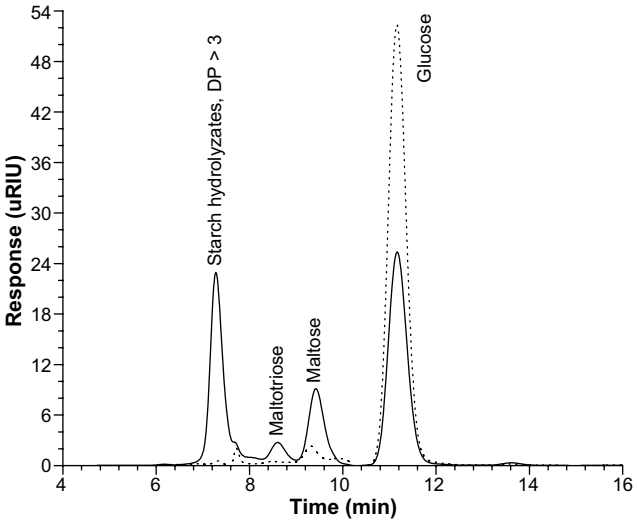

Fig. 1. Chromatograms of a mash analyzed right after diluting by distilled water (solid line) and re-analyzed after 3 days at room temperature for the same dilution (dotted line). Liquefaction condition: 95 °C, 60 min and 80 °C, 30 min; 2 × 10 μL of Liquozyme per 30 g of sample. Saccharification condition: 60 °C, 30 min; 100 μL of Spirizyme per 30 g of sample.

In SSM, slurries in microtubes were heated in air using a mini shaking incubator. In mashing procedures for fermentation, slurries in flasks were heated in a water bath shaker. Due to heat transfer, temperature of the slurries in the microtubes will lag behind that of the slurries in the flasks if both the incubator and the water bath are preheated to the same temperature (e.g., 95 °C). As listed in Table 2, it took extra liquefaction time (15 min at 95 °C) and an extra saccharification time (15 min at 60 °C) for mashes from SSM to have CHS similar to mashes prepared for laboratory fermentation. It was feasible for the SSM procedure to simulate the mashing process in a laboratory fermentation test. Freeze-dried mashes had slightly higher CHS than mashes from SSM, probably due to the continuous activity of amyloglucosidase in the process of cooling and freezing.

As shown in Table 2, only 49.8–58.6% of the total starch had been completely hydrolyzed to glucose for the nine sorghum samples with SSM (calculated by dividing the values in the third column by their counterparts in the second column). Moreover, there was no significant correlation between CHS from SSM and ethanol yield from traditional fermentation ($R^2 = 0.10$, $p = 0.41$). The difference in CHS among the samples could not explain the variability in ethanol yield, another reason the initially developed SSM procedure needed to be modified.

3.3. Inactivation of amyloglucosidase

According to manufacturer's instructions, the optimum temperature range for amyloglucosidase, Spirizyme Fuel, was 65–70 °C (Novozymes, 2004a), but surprisingly, this enzyme still worked well at room temperature even after being heated at 60 °C for 45 min and cooked at 100 °C for 10 min (Fig. 2). Amyloglucosidase was not sensitive to saccharification temperature and was active throughout fermentation. Thus, the heat-stability characteristic of amyloglucosidase is beneficial for ethanol production. The deactivating effect of boiling on amyloglucosidase was significant; CHS values after treatment II-3 were statistically lower ($p < 0.0001$) than those after treatment I-3. However, boiling was neither an efficient nor simple way to inactivate amyloglucosidase.

Amyloglucosidase was effectively deactivated by diluting 200 μL of mash supernatant with 480 μL of 0.01 M phosphate buffer at pH 10.0 (Fig. 2). CHS values of both samples in treatment III did not

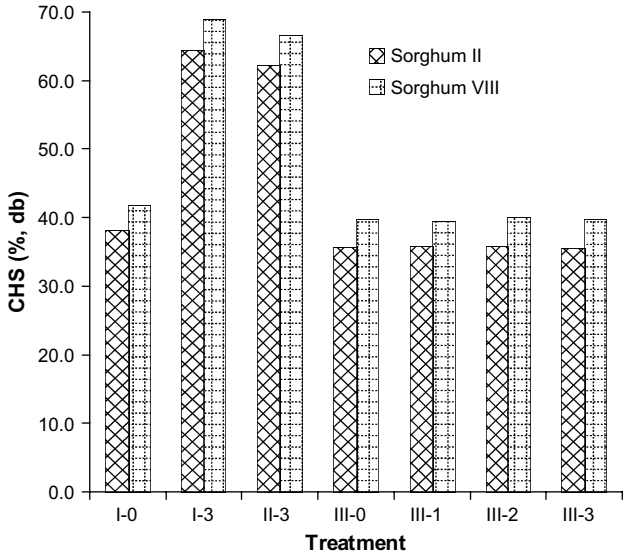

Fig. 2. Inactivation of amyloglucosidase in diluted mashes from SSM. The same liquefaction and saccharification conditions as used for the SSM in Table 2. Treatment I, no inactivation (i.e., diluted by distilled water); treatment II, inactivation by boiling the mash dilution at 100 °C for 10 min; treatment III, inactivation by 0.01 M phosphate buffer at pH 10.0. The numbers 0, 1, 2, and 3 following each treatment mean that the mash dilutions were set at room temperature for 0, 1, 2, and 3 days, respectively. Duplication for each combination of sample and treatment with standard error of 0.40 in the first experimental design.

change significantly over time (0–3 days) after mash dilution ($p > 0.46$). Again, CHS values in treatment I-0 (control) were slightly higher than those in treatment III ($p < 0.0001$) due to the short enzymic reaction time starting from preparation of mash dilution to sugar analysis by HPLC.

3.4. Effect of pH adjustment on SSM

The optimum pH range for efficient usage of Liquozyme was 5.7–6.0 (Novozymes, 2004b). The pH values of the slurries, mixtures of ground grains and water containing no backset stillage, ranged from 6.0 to 6.3 (data not shown). For liquefaction, we did not adjust pH in our fermentation procedures or in SSM.

In the traditional fermentation procedure, we adjusted the saccharified mashes to pH 4.2–4.3 with 2 M HCl before yeast inoculation. There was no pH adjustment for the separate saccharification, in which Spirizyme did not hydrolyze the substrates under its optimum pH of 3.5–4.5 (Novozymes, 2004a). Similar to the previously described results for SSM, about 51.2–62.7% of the total starch had been completely hydrolyzed to glucose in the freeze-dried mashes (Table 2). For the SSF procedure, we expected much lower levels of glucose in the liquefied mashes before inoculation. However, final fermentation results were not affected due to the continuous activity of amyloglucosidase during fermentation.

Compared with the fermentation procedure, it was not as convenient to insert a normal pH meter probe into a mash in SSM. Cleaning the electrode of a pH meter with minimal influence on the volume of a mash would be a challenge. In addition, titration with an acidic solution would be time-consuming and tedious, as found in preparation of fermentation broths. Considering these factors, the 2 M sodium acetate buffer at pH 4.2 was attempted, 50 μL of which was added directly to the microtube after liquefaction to adjust the pH of the mash for saccharification. This approach originated from the total starch assay (AACC International, 2000), in which 200 mM acetate buffer at pH 4.5 was used for pH adjustment. For each microtube, final ionic strength in the mash was about 80–90 mM of sodium acetate, which was similar to that in the total starch assay (80–110 mM).

Obviously in Fig. 3, amyloglucosidase was the most important determinant in converting starch hydrolyzates to glucose after liquefaction. Little glucose existed in the liquefied mashes (only 1.2% of CHS for both samples). The difference in CHS between treatment II and III was significant ($p < 0.0001$), suggesting that pH was important for saccharification by amyloglucosidase in SSM. Without pH adjustment (treatment II), the two samples had no statistical difference in CHS ($p = 0.08$), but they were significantly different in treatment III ($p = 0.002$). Thus, pH adjustment was expected to increase the resolution of SSM in differentiating samples.

3.5. Simplification of SSM procedures

Because they varied in the liquefaction step, three SSM procedures (1, 2, and 3) were tested further (Table 3). Both 95 °C and 86 °C were selected as the liquefaction temperature in consideration of starch gelatinization in sorghum grain and the optimum temperature range of 82–86 °C for Liquozyme (Novozymes, 2004b). Similar to the initially developed SSM procedure, the two-step liquefaction structure was kept in Procedure 2, but the temperature in the second step was increased to 86 °C. Liquefaction time for each step was 45 min. Procedures 1 and 3 were simplified from Procedure 2; they had the one-step liquefaction structure and a total 90 min of liquefaction time. Liquefaction temperatures were 95 °C and 86 °C in Procedures 1 and 3, respectively. In all procedures, saccharification temperature was raised to 68 °C in accordance with the optimum temperature of 65–70 °C for Spirizyme (Novozymes, 2004a), and saccharification time also was extended to 90 min for the purpose of obtaining higher CHS. The α-amylase dosage was equivalent to a total 20 μL of Liquozyme per 30 g of sample, except in Procedure 2, where half the dosage was used for each step. The pH of the liquefied mash was adjusted by adding

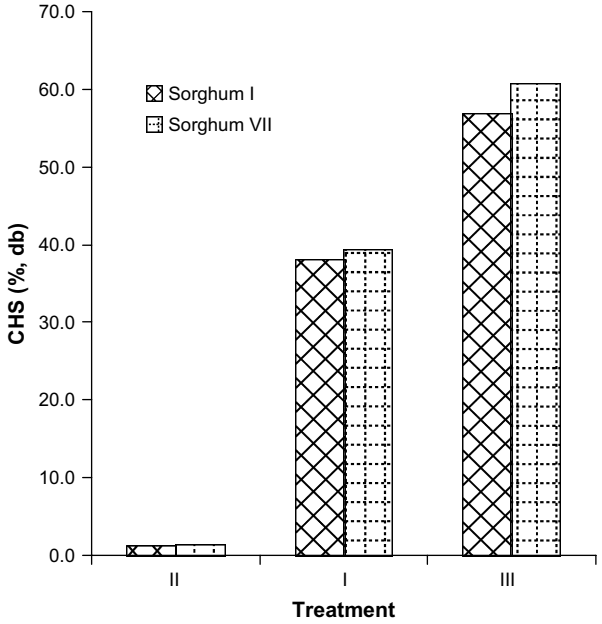

Fig. 3. Effect of pH adjustment on SSM. The same liquefaction and saccharification conditions as used for the SSM in Table 2, but no amyloglucosidase added in treatment I (i.e., distilled water as a substitute). Only in treatment III, pH adjusted after liquefaction by 50 μL of 2 M acetate buffer at pH 4.2. Amyloglucosidase was inactivated by 0.01 M phosphate buffer at pH 10.0. Duplication for each combination of sample and treatment with standard error of 0.39 in the second experimental design.

Table 3
Effects of SSM procedures and amyloglucosidase dosages on CHS (%, db) of mashes[a].

Sample code	Amyloglucosidase dosage (μL of Spirizyme per 30 g sample)	SSM procedure[b]		
		1[Ad]	2[B]	3[B]
II a[c]	15	34.1	35.4	35.3 a[e]
	50	54.7	56.4	55.5 b
	100	62.1	63.0	62.4 d
VIII b	15	37.0	36.7	37.1 a
	50	57.6	60.1	58.8 c
	100	66.4	66.5	66.8 e

[a] Duplication for each combination of sample, dosage, and procedure with standard error of 0.60 in the fourth experimental design.
[b] Procedure 1, liquefaction at 95 °C for 90 min; Procedure 2, liquefaction at 95 °C for 45 min, and at 86 °C for 45 min; Procedure 3, liquefaction at 86 °C for 90 min. For all procedures, the pH of liquefied mashes was adjusted by 50 μL of 2 M acetate buffer at pH 4.2, saccharification at 68 °C for 90 min, and the dosages of heat-stable α-amylase equivalent to 20 μL of Liquozyme per 30 g of sample. Amyloglucosidase was inactivated by 0.01 M phosphate buffer at pH 10.0.
[c] Samples are significantly different with their codes followed by different letters in the first column ($p < 0.05$).
[d] Procedures are significantly different with their codes followed by different capitals in superscript in the second row ($p < 0.05$).
[e] Means followed by different letters in the last column are significantly different ($p < 0.05$).

50 μL of 2 M sodium acetate buffer at pH 4.2 to each microtube, and amyloglucosidase in the mash dilution was deactivated with 0.01 M phosphate buffer at pH 10.0 before sugar analysis.

There was no significant difference in CHS between Procedures 2 and 3 ($p = 0.22$), indicating that one-step liquefaction would be feasible (Table 3). Procedure 1 had systematically lower CHS than the other two procedures ($p < 0.02$), more than likely due to the harsh conditions in Procedure 1 using a high temperature and long cooking time leading to extensive cross-linking of sorghum proteins (Zhao et al., 2008).

There was no interaction ($p = 0.60$) between sample and procedure (i.e., the effect of SSM procedures on CHS was independent of tested samples). The primary important factor influencing CHS was amyloglucosidase dosage ($p < 0.0001$). CHS values increased steadily and significantly as levels of amyloglucosidase increased (Table 3). In Procedure 3, the two samples had no statistical difference in CHS at low levels of amyloglucosidase ($p = 0.07$) but became distinct at higher levels ($p < 0.005$). Similar to pH adjustment, we expected a high level of amyloglucosidase to increase the resolution of SSM in differentiating samples.

When examining data in each procedure separately (Table 3), standard errors for Procedures 1, 2, and 3 were 0.83, 0.47, and 0.40, respectively. Therefore, Procedure 3 was selected, and its parameters were optimized further.

3.6. Optimization of SSM parameters

There was no significant difference ($p = 0.88$) in CHS between the two buffers at pH 3.5 and 4.2 (Fig. 4), suggesting that any 2 M sodium buffers at pH between 3.5 and 4.2 could be used for pH adjustment. The effect of mash pH on CHS was independent of tested samples (i.e., no interaction between sample and pH, $p = 0.34$). The CHS values adjusted by the buffer at pH 4.5 were slightly lower than those adjusted by the buffers at pH 3.5 and 4.2 ($p < 0.003$). Using the buffer at pH 5.5, CHS dropped significantly. The buffer at pH 4.20 was selected in this work, but was not indicative of the actual pH of the liquefied mashes. Checking the pH, the actual pH of the mashes was 4.4–4.6 at room temperature after adding 50 μL of 2 M sodium acetate buffer at pH 4.20 to each microtube.

The dosage range of Liquozyme was 0.013–0.015% (mass of enzyme to mass of corn 'as is'), equivalent to 3–6 μL of enzyme per

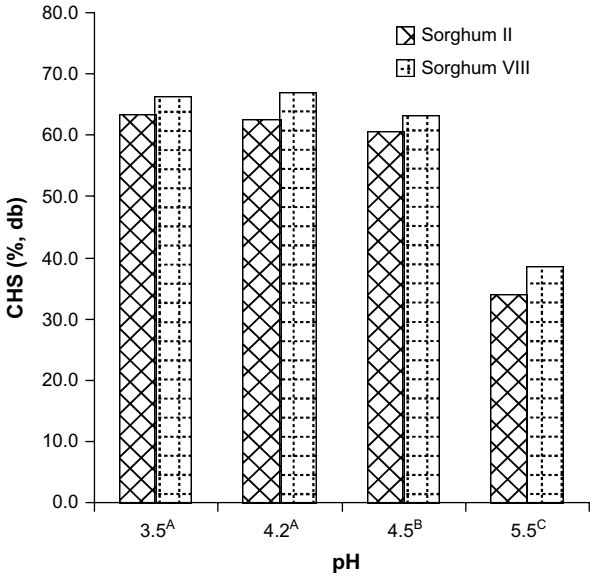

Fig. 4. Optimization of pH for saccharification in SSM. Liquefaction condition: 86 °C, 90 min; 20 μL of Liquozyme per 30 g of sample. Saccharification condition: 68 °C, 90 min; 100 μL of Spirizyme per 30 g of sample. The pH of liquefied mashes was adjusted by 50 μL of 2 M acetate buffers at pH 3.5, 4.2, 4.5, and 5.5, respectively. Amyloglucosidase was inactivated by 0.01 M phosphate buffer at pH 10.0. Different superscript letters after pH values indicate significant differences ($p < 0.05$) among pH adjustments. Duplication for each combination of sample and buffer with standard error of 0.66 in the third experimental design.

30 g of sample (Novozymes, 2004b). The dosage range of Spirizyme was 0.04–0.06% (mass of enzyme to mass of corn 'as is'), equivalent to 8–16 μL of enzyme per 30 g of sample (Novozymes, 2004a). The enzyme dosages (2 × 10 μL of Liquozyme and 100 μL of amyloglucosidase per 30 g of dry grains) used in the traditional fermentation procedure were determined via an orthogonal test design considering factors such as mashing properties of grains, conversion efficiency, repeatability (unpublished data). For development of SSM, enzyme dosages were anticipated to be as small as possible, and CHS was expected to be as high as possible within an acceptable period of time. As shown in Fig. 5, there was little difference in

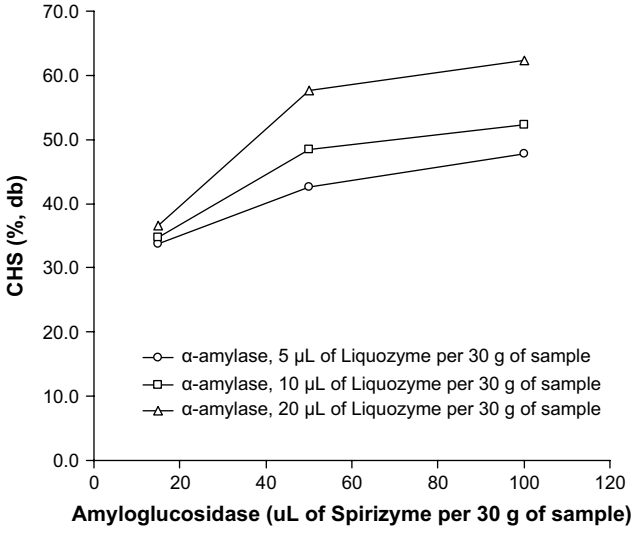

Fig. 5. Effect of enzyme dosages on CHS of a sample, sorghum II, in SSM. Liquefaction condition: 86 °C, 90 min. Saccharification condition: 68 °C, 90 min. The pH of liquefied mashes was adjusted by 50 μL of 2 M acetate buffer at pH 4.2. Amyloglucosidase was inactivated by 0.01 M phosphate buffer at pH 10.0. Duplication for each combination of enzyme dosages with standard error of 0.44 in the fifth experimental design.

CHS among the three dosages of α-amylase when amyloglucosidase dosage was low (15 μL of amyloglucosidase per 30 g of sample). At higher dosages of amyloglucosidase, CHS increased significantly with increased α-amylase dosages. The effects of both enzyme dosages on CHS were synergetic (i.e., an interaction between α-amylase and amyloglucosidase, $p < 0.0001$). A minimum dosage of α-amylase, equivalent to 10 μL of Liquozyme per 30 g of sample, was necessary. For some samples, especially sorghum hybrids with tannins, we observed some gelled particles in the bottom of some microtubes when α-amylase dosage was 5 μL of Liquozyme per 30 g of sample. To ensure all slurries were completely dispersed during liquefaction, α-amylase dosage was determined as 20 μL of Liquozyme per 30 g of sample in an SSM procedure. The highest CHS (62.4%) was achieved with the highest dosages of α-amylase and amyloglucosidase, and 87.3% of the total starch had been completely hydrolyzed to glucose in the sorghum II sample (Fig. 5).

There was no statistical difference ($p = 0.29$) in CHS between 90 and 120 min (Fig. 6). Thus, 90 min of saccharification could be used in SSM. The effect of amyloglucosidase dosage on CHS was independent of saccharification time (i.e., no interaction between dosage and time, $p = 0.77$). Increasing amyloglucosidase dosage was still an effective way to improve CHS, but the difference in CHS between 200 and 150 μL of Spirizyme per 30 g of sample was significantly ($p < 0.0001$) lower than that between 150 and 100 μL of Spirizyme per 30 g of sample. Additional experimentation verified higher levels of amyloglucosidase (e.g., 250 μL of Spirizyme per 30 g of sample) did not increase CHS significantly (data not shown). The highest CHS was 66.9%, which was approaching the theoretical value (71.5%) of the total starch of the sorghum II sample (Fig. 6).

The optimized SSM procedure (Fig. 7) was used to investigate the relationship between CHS and ethanol yield. All diluted mashes had very similar profiles to the chromatogram of a mash dilution set at room temperature for 3 days (the one with a dotted line in Fig. 1), except their glucose peaks differed mainly in the magnitude of height. There was little maltose or maltotriose left in the mashes. Calculated mass of glucose was not significantly different ($p = 0.11$) from that obtained through direct measurement after all of the mash in a microtube had been diluted to a known volume (data not shown). Using a mini shaking incubator in this procedure made it possible to analyze up to 50 microtubes in one test cycle, which would increase efficiency and reduce the cost of per-sample analysis.

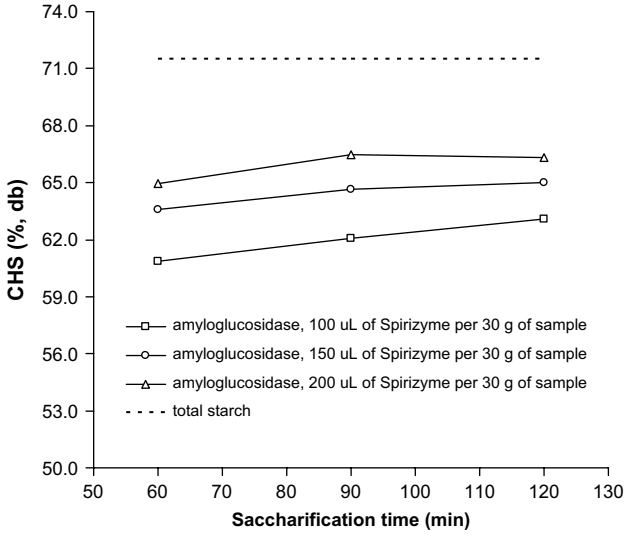

Fig. 6. Effect of amyloglucosidase dosage and saccharification time on CHS of a sample, sorghum II, in SSM. Liquefaction condition: 86 °C, 90 min; 20 μL of Liquozyme per 30 g of sample. Saccharification condition: 68 °C, 60–120 min. The pH of liquefied mashes was adjusted by 50 μL of 2 M acetate buffer at pH 4.2. Amyloglucosidase was inactivated by 0.01 M phosphate buffer at pH 10.0. Duplication for each combination of dosage and time with standard error of 0.46 in the sixth experimental design.

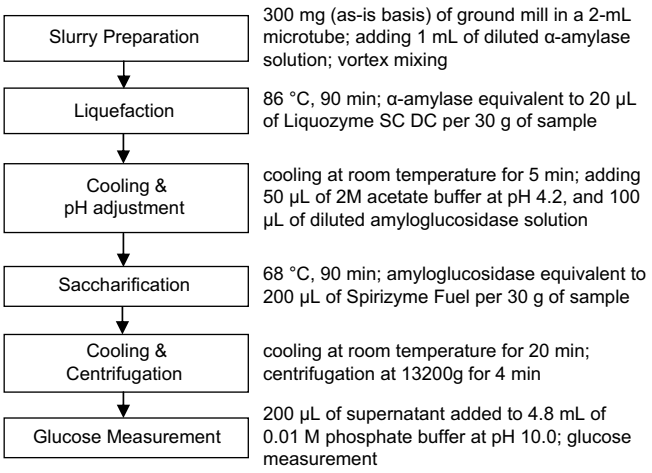

Fig. 7. The schematic flow diagram of the SSM procedure with optimum parameters.

3.7. Predicting ethanol yield with the SSM procedure

In addition to the nine primary samples, nine additional sorghum varieties were tested to evaluate their ethanol yields from the traditional fermentation and SSF procedures and their CHS values using the SSM procedure. The SSF procedure was used to mimic fuel ethanol production in the dry-grind industry. For all 18 sorghum hybrids, their ethanol yields (%, v/v) were 11.99–14.55 (13.35 on average) in traditional fermentation, and 12.38–14.77 (13.75 on average) in SSF. The ethanol yields in traditional fermentation were highly related to those in SSF ($R^2 = 0.97$, $p < 0.0001$). Ethanol yield improved significantly using the SSF procedure ($p < 0.0001$). The relative increases in ethanol yield were 0.6–4.9% (3.0% on average). Because amyloglucosidase and yeast were added simultaneously, a concentrated glucose solution was avoided and the initial osmotic stress of yeast was lowered, which could be one reason why ethanol yield increased in the SSF procedure (Bothast and Schlicher, 2005). Another reason could be that the active dry yeast of an industry strain, Ethanol Red, was used in SSF.

With all 18 sorghum hybrids in 3 replications, the optimized SSM procedure had an overall standard error of 0.28. A majority of the starch had been hydrolyzed to glucose in the saccharified mashes. The ratios of CHS to total starch ranged from 91.2% to 97.5% (94.1% on average). As shown in Table 4, total starch was highly correlated to CHS ($p < 0.0001$), but it explained only 82% of the variability in CHS.

As expected, total starch was correlated with ethanol yields ($R^2 = 0.78$, $p < 0.0001$ for traditional fermentation and $R^2 = 0.86$,

Table 4
Coefficients of determination (R^2) among ethanol yield, total starch, and CHS for 18 sorghum samples.

Parameters	Ethanol yield (%, v/v)		Total starch (%, db)
	Traditional fermentation[a]	SSF[b]	
Total starch (%, db)	0.78***	0.86***	–
CHS (%, db)[c]	0.86***	0.93***	0.82***

***Significant at 0.1% level.
[a] Liquefaction at 95 °C for 45 min, and at 80 °C for 30 min; saccharification at 60 °C for 30 min.
[b] Liquefaction at 86 °C for 90 min, no saccharification before fermentation.
[c] For SSM, the optimum liquefaction and saccharification conditions described in Fig. 7.

$p < 0.0001$ for SSF). Compared with total starch, CHS was more powerful at predicting ethanol yield (Table 4). There were strong, linear relationships between CHS and ethanol yields in both fermentation procedures ($R^2 = 0.86$, $p < 0.0001$ for traditional fermentation and $R^2 = 0.93$, $p < 0.0001$ for SSF). Relationships between total starch, CHS, and ethanol yields in the SSF procedure were stronger than those in traditional fermentation. Results of multiple regression showed that the role of CHS was dominant ($p = 0.007$ in traditional fermentation and $p = 0.0003$ in SSF), even when combined with total starch to predict ethanol yield.

4. Conclusions

This research investigated the feasibility of using SSM as a method for predicting ethanol yield of sorghum hybrids and developed an SSM procedure with optimum parameters. This procedure had advantages including small quantity requirement for grain samples, use of common industry enzymes, high repeatability, high efficiency, and low cost of per-sample analysis. The 18 sorghum hybrids tested showed strong, linear correlations between CHS from SSM and ethanol yields from both traditional and SSF procedures. CHS proved a reliable indicator for ethanol yield.

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