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Comparison of laboratory scale bourbon whiskey mashing and fermentation to pilot scale

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Abstract

Why the work was done: Laboratory scale methods are useful for optimising grain spirit production. While small scale methods exist, comparisons between conventional, simultaneous saccharification and fermentation, and distillery scale production are lacking. Further, there is limited understanding of how these methods affect the rheological properties of the mash and flavour compounds from fermentation. This study compared conventional and simultaneous saccharification and fermentation mashing methods to a pilot scale distillery to establish a standard laboratory method for simulating industrial bourbon production.
How the work was done: Two mashing methods for bourbon whiskey were compared: simultaneous saccharification and fermentation versus conventional mashing with a dedicated saccharification step. Laboratory scale (0.4 L) and pilot scale (1663 L) fermentations were compared. Analysis focused on mash rheological profiles, sugar composition, fermentation dynamics, and volatile flavour congeners.
What are the main findings: Ethanol yields from the laboratory and distillery methods were comparable. Simultaneous saccharification and fermentation offered advantages: lower lactic acid levels, lower viscosity during cooking, faster fermentation, and a flavour congener profile almost identical to that from the pilot distillery. While simultaneous saccharification and fermentation produced a sugar profile with higher glucose levels, total sugar concentrations were comparable using all approaches.
Why is the work important: This work provides insight into the use of laboratory scale methods for studying and optimising bourbon production. The findings demonstrate that simultaneous saccharification and fermentation, with lower complexity and process time, reliably reflect industrial scale processes, including ethanol yield and flavour compound profile. Accordingly, simultaneous saccharification and fermentation provides a reliable tool for optimising bourbon production parameters and investigating flavour development.

Keywords

bourbon, whiskey, small scale, mashing, fermentation, scale-up.

Introduction

A small scale method that simulates the spirit production process would support distillery experimentation with novel products, techniques, and efforts to improve efficiency in a competitive and evolving market. Such experiments are typically performed at the laboratory scale, to gain insights into fundamental principles which can be translated to industrial scale production with appropriate consideration of scale up effects. For small scale experiments to be of value, they should provide relevant and informative insights for optimising bourbon whiskey production at larger scale. However, it is not known whether it would be beneficial to use a laboratory scale process with a simpler and less time consuming simultaneous saccharification and fermentation (SSF) procedure over a dedicated saccharification step. A standard small scale method that simulates the cook, mash and fermentation processes would be of value when scaling up to pilot and production scale.

For bourbon whiskey - a distilled spirit made from a grain mash bill containing at least 51% corn - the initial process step involves cooking the milled grains to gelatinise the starch. Corn, rye and malted barley are commonly used grains in the mash bill. The gelatinised starch is converted to fermentable sugars by a saccharification step or 'mashing'. This process relies on enzymes from the malted grain, but exogenous enzymes can also be added. After mashing, yeast is added to ferment sugars into ethanol, producing 'distiller's beer'. Metabolites produced during the fermentation include glycerol, lactic acid, acetic acid, which contribute to the final flavour profile. Numerous factors affect the final ethanol yield obtained including milling, grain type, process temperature, enzyme loading, and yeast selection.

Previous work has outlined process steps for laboratory scale study of grain fermentations. For example, Agu et al (2008) developed an 81 mL procedure for corn, wheat, and other cereals using stainless steel mashing beakers. The cooking parameters need to consider the grain recipe, as wheat has higher yield at lower temperatures (85˚C) than corn, which has a similar yield at high (142°C) and low (85°C) temperatures (Agu et al. 2008). Previous work specific to bourbon, adapted the method at a 3 L scale using the lower cooking temp of 85°C (Arnold et al. 2019; Verges et al. 2023). Alternatively, other reports on spirit production utilise a SSF method using sorghum (Zhao et al. 2009) and corn (Weiss et al. 2023). Specifically, Zhao et al (2009) developed a small scale mashing procedure and correlated it to multi-step (conventional) and single step (SSF) small scale mashes using sorghum and found high repeatability, good predictability for ethanol yield, and completely hydrolysed starch.

To understand the impact of a small scale method on a real world production process, it is necessary to understand the rheological properties of the mash and predict the physical and chemical characteristics during cooking, mashing, mixing, pumping, and fermentation. Cereals exhibit unique rheological properties and have complex interactions within the mash, affected by grain composition and processing factors (Okolo et al. 2020). The Rapid Visco Analyser (RVA) determines the rheological properties of food materials, including grains. Previous research has shown RVA analysis to be a useful tool for assessing the viscous load during processing of individual cereals (Agu et al. 2006; 2008; 2009; 2012).

This study considers the rheological properties of multi-grain mashes used in bourbon whiskey production and considers the interactive effects of different grains and the influence of enzyme addition and malting. The bourbon production process is unusual in often including a step where a small amount of barley malt is added during the initial cooking stage to manage viscosity and altering mash rheology (Wright and Pilkington 2022). An understanding of mash rheology would help optimise industrial scale bourbon production that uses agricultural substrates which can vary over time. Variations in cereal composition, such as protein content and starch structure, can arise from environmental factors, such as elevated CO_2 concentration, higher temperature, and drought stress (Mariem et al. 2021). These compositional characteristics are known to influence mash viscosity and fermentation performance (Agu et al. 2009; 2014).

Here, a small scale method with a dedicated saccharification step is compared with a SSF method, to determine whether the SSF method reflects ethanol and flavour compound yields at

distillery scale.

By validating these laboratory scale methods against real world distillery practices, this work contributes data on predictability and reproducibility, supporting future process development.

Materials and methods

Grain

Bardstown Bourbon Company provided milled grains. Grain mashes represent a typical bourbon whisky recipe consisted of 75% corn, 15% rye, and 10% barley malt. Milled grains were characterised by the University of Kentucky Regulatory Services for macronutrients using infrared spectroscopy. Particle size distribution was cdetermined using a mechanical sieve shaker (Ro Tap, W.S. Tyler, Mentor, Ohio) with seven sieves of 2380, 1180, 425, 300, 250, 180, and 75 µm with a sieving time of 20 min (Supplementary information, Table S1). The starch content, which is assumed to correlate with the available carbohydrates content in grains, was calculated indirectly by difference (McCleary and McLoughlin 2021) using the following expression:

Starch (%) = 100% - Crude Protein (%) - Crude Fat (%) – Crude Fibre (%) - Acid Detergent Fibre (%) - Neutral Detergent Fibre - Ash (%) - Moisture (%)

Rheology analysis

Rheological properties were obtained using Rapid Visco Analysis (Perkin Elmer 4800). Corn, rye, and barley malt were characterised using an adapted method for the corn starch test profile (Supplementary information, Table S2) provided by the manufacturer and used for unmodified grain samples (Agu et al. 2006). Each analysis consisted of 6 g of sieved grain and 22 g of deionised water. Sieving ensured a fine, consistent particle size, necessary for obtaining accurate and repeatable RVA measurements (Crosbie and Ross 2007). While distillery practices utilise a wider range of particle sizes (Supplementary information, Table S1 from a collaborating distillery), this study focused on sieved flour to ensure consistent sample preparation and allow for a more focused comparison of the impact on grain physiological properties.

The RVA profile of a mixture of grains simulating a bourbon whiskey mash was analysed in duplicate using simultaneous saccharification and fermentation (SSF) and conventional bourbon mash temperature profiles (Supplementary information, Table S3). For all grain additions, the can was removed, and after stirring replaced in the RVA. Corn (6.83g, <250 µm particle size) was added to 23 mL deionised water and placed in the RVA and held at 93°C for 30 min. The mash was cooled to 77°C, 1.37 g rye (<425 µm particle size) added, held for 10 min, then cooled to 63°C. Barley malt (1.27 g, <180 µm particle size) was added, held for 15 min, then cooled to 30°C. Cooling rates were 3°C per minute.

The enzyme mixture contained an equal mix by volume of thermo stable alpha amylase (Termamyl SC DS from Novozymes North America, Franklinton, NC 27525, USA), glucoamylase (Saczyme Go 2x from Novozymes), and non-starch polysaccharide degrading enzymes (xylanase, beta glucanase, and cellulase and Viscoferm from Novozymes). The enzyme mix (1.5 µL) was added to each can and placed in the RVA set to 30°C for 30 minutes.

Simultaneous saccharification and fermentation bourbon mash

For the SSF mashing method, 475 g corn, 95 g rye, and 63.5 g barley malt (75, 15, 10%, respectively) were added to 1.6 L tap water split between two 2 L wide mouth glass Erlenmeyer flasks. The flasks were autoclaved using a 30 min cycle (121°C) on an aluminium tray to recover lost condensate. Flasks were weighed to measure any water loss, which was limited to 17 mL (1.1% of total volume).

Conventional bourbon mash

The conventional mash mimics the temperature and grain addition schedules were provided by the James B. Beam Institute for Kentucky Spirits (JBBI), and reflect commercial American spirits distilleries. The bourbon mash - 75% corn, 15% rye and 10% barley malt - was conducted in a 7.5 L stainless steel pot heated on an electric induction burner with temperature control (Eurodib Inc). A batch size of 3.2 L was used, later divided into enzyme and no enzyme treatment groups. Corn (950 g) and barley malt (34 g) was added to boiling water and maintained at 93°C for 30 min. The mash was cooled to 77°C, 190 g rye added and held for 10 min, then cooled to 63°C and 93 g barley malt added and held for 15 min. During the cooking and mashing

process, the mash was stirred every 3 to 5 minutes to prevent the grain sticking to the bottom of the pot. After mashing, water loss due to evaporation was measured by mass and was 724.5 mL or 22.6% loss. Makeup water of 720 mL deionised water was added to the mash.

Fermentation

The fermentation volume for both SSF and conventional mashes was 3.2 L of mixture per kg of grain. Fifteen 500 mL glass bottles were used for fermentation: five for the SSF mash and ten for the conventional mash. Each bottle was filled with 408 g ± 1% of mash and then cooled to 30°C. The enzyme mixture (100 µL) was added to the five bottles of SSF mash and to five of the conventional mash. Samples of the fermenting beer were collected at 0, 20, 46, and 91 h. The initial mash for conventional and SSF laboratory treatments had a specific gravity (SG) of 1.067 ± 0.4% and pH of 5.9 ± 2% (measured using a Thermo Scientific Orion Star pH meter with a Sensorel S350CD probe). Specific gravity for initial and final samples was determined using a hydrometer at 25°C. *Saccharomyces cerevisiae* (FSI 927, Ferm Solutions, Danville, KY, USA) in active dry form was suspended in 45 mL of deionised water, vortexed, and one mL added to each bottle of mash (5.4×10^6 cells/mL, determined via hemacytometer). Fermentation was for 91 hours in a temperature controlled water bath at 30°C in an automated Bioprocess Control Gas Endeavour system (BPC Instruments, Lund, Sweden). Each reactor was fitted with a motorised stirrer set at 150 rpm, alternating direction every 5 sec for 5 min of every hour. The Gas Endeavour system was used to automatically collect gas production data through pre-calibrated tip meters (Hockensmith et al. 2024).

Determination of sugars and fermentation products

Samples were centrifuged at 800 x g for 2 min and the supernatant passed through a 0.20 µm filter, and frozen at 20°C. The concentration of glucose, maltose, maltotriose, DP4+, ethanol, glycerol, lactic acid, and acetic acid were determined by High Performance Liquid Chromatography (HPLC; Agilent 1260 Infinity 2) equipped a Biorad Aminex HPX 87H column and a refractive index detector (RID), using 5 mM sulphuric acid as the mobile phase at a flow rate of 0.6 mL/min and a column temperature of 50°C. The concentration of DP4+ sugars could not be precisely quantified due to overlap with the negative water peak. The initial starch (w/w) concentration was calculated from the sum of the grain starch content and the mass loaded to each reactor, divided by the mash mass. The theoretical ethanol concentration was calculated using the initial starch content (Thomas et al. 1996). The conversion efficiency was calculated using the final ethanol concentration divided by the theoretical ethanol concentration. Fermentation time was determined based on when at least 90% of the measured cumulative gas production was produced.

Flavour compounds in bourbon whiskey distiller's beer were analysed using an Agilent 6890N Gas Chromatograph (GC) equipped with a Headspace Sampler (Agilent 7697A) and a Flame Ionisation Detector on a DB WAX Ultra Inert GC Column (Agilent; 30m x 0.25mm x 0.25µm). Seven volatile compounds were used as standards including acetaldehyde, ethyl acetate, propanol (2-propanol), isobutanol (2-methyl-1-propanol), isoamyl acetate, phenylethyl alcohol (2-phenylethanol), and isoamyl alcohol (3-methyl butanol).

Pilot scale fermentation

Eight batches of pilot scale mashing, fermentation, and distillation were performed at the JBBI Distillery in Lexington, Kentucky. Mash (SG 1.076 ± 1% and pH 5.6 ± 3%) was produced from a grain bill of 75 ± 1% corn, 15 ± 1% rye, and 10 ± 1% malted barley for 460 kg of total grain. Throughout the cooking process, sufficient water was added to achieve a final fermentation volume of 3.5 L of mixture per kg of grain. Corn was added with 12 ± 2 kg of malted barley at room temperature and heated using direct steam injection and agitation to 96°C and held for 30 min. Water cooling of the cooker was used to drop the temperature to 77°C, before adding the rye and holding the temperature for 10 min. Due to integration issues with rye, the protocol was revised to include 5.75 kg of malted barley during the rye addition step for the final five batches. The temperature was then reduced to 63°C and the remaining malted barley was added to the cooker and held for 15 min. Finally, the temperature was lowered to 32°C and transferred to fermenter. Cooling rates were between 0.3 and 1.5°C per

minute, depending on the difference in mash temperature to ambient. The volume of the fermenting liquid was 1663 ± 59 L. Active dry yeast, *S. cerevisiae* (FSI 927, Ferm Solutions, Danville, KY, USA) was pitched at 0.29 g/L at 32°C. Prior to pitching, the fermenter was held at 99°C for 15 min via steam injection and transfer lines, inlet, and outlet of fermenter were sanitised (Star San, Five Star, Arvada, CO, USA). The temperature of the fermentation was held below 29.4°C through a flow control valve on water cooled coils on a PID controller. Samples of the fermenting beer were collected after 0, 17, 41, 65, and 89 h. The cooker and fermenter at the JBBI are shown in Figure 1.

Statistical analysis

Laboratory scale data was compared to mashing and fermentation data from the JBBI distillery with the same grain mix, mashing temperature and times, and yeast strain. Data was analysed using R software, and Graphpad Prism 10. The effect of mashing method (4 levels) on the concentration of sugars, metabolites, and of fermentation time was examined using the lm() (linear regression) function in R. Variables were subjected to log transformation to address positive skew and to improve normality and homoscedasticity. Linear model residuals were checked for normality using the Shapiro Wilk test (*dplyr* R package). Homoscedasticity of the linear model residuals was checked using Levene's test (*car* R package) and did not reveal any deviation. Post hoc analysis was conducted using the Tukey HSD method. All statistical analyses were carried out at a significance level of $p < 0.05$. Principal component analysis (PCA) was used to compare the flavour compound profiles, to reduce the dimensionality of the congener concentrations data and visualise potential patterns.

Results and discussion

Grain characteristics

The composition of the corn, rye and barley malt grains is reported in Table 1. Based on the starch content of the grain mixture (53.5%), the starch content of the mash was 170 g/L with a theoretical ethanol yield of 96 g/L.

Grain and mash rheology

The viscosity of three types of grains were measured by RVA using low and high temperature profiles (Agu et al. 2006). This enabled the assessment of different grains by evaluating their intrinsic properties and suitability for different mashing regimes (Figure 2). The RVA profile of the corn sample exhibited a smooth viscosity curve with a broad pasting peak and a well-defined breakdown phase. Corn had a 15% lower peak viscosity at a higher temperature, similar final viscosity between temperatures, and the highest final viscosity among the grains studied. The RVA profiles of the corn exhibited a similar peak viscosity to those previously reported for whole corn flour; however, the presence of a defined breakdown phase suggests that the sieved material may have slightly different properties to whole flour or extracted starch (Agu et al. 2006; 2014).

The peak viscosity of rye was five times that of corn, which highlights the processing challenges from high amounts of pentosans (Ingledew et al. 1999). At the higher temperature profile, rye was opposite to that of corn, with an increase in both peak (12%) and final viscosity (32%) compared to the low temperature profile. The high rye starch pasting viscosity at higher temperatures has been attributed to increased solubilisation of amylose

Table 1.

Macronutrients (dry basis) using near infrared spectroscopy of grains used in laboratory scale mashes.
Starch content was calculated indirectly by difference.

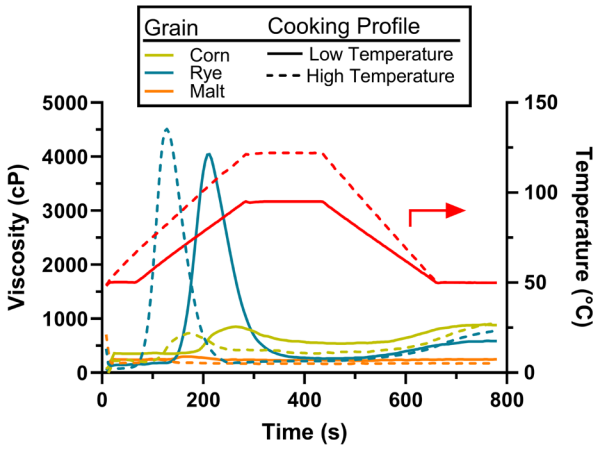
Grain	Crude Protein (%)	Crude Fat (%)	Crude Fibre (%)	ADF (%)	NDF (%)	Moisture (%)	Starch (%)
Corn	10.1	3.4	3.0	4.7	10.7	12.5	55.3
Rye	9.0	1.7	2.2	2.8	14.9	11.8	55.5
Barley malt	13.4	1.7	4.3	9.8	13.1	6.2	49.6

Figure 1.

Pilot scale equipment at the distillery of the James B. Beam Institute for Kentucky Spirits. The stainless steel cooker (left side images) **with grain auger, agitator, and water cooling jacket was used to cook and mash grains, while the conical closed top stainless steel fermenters** (right side images) **with water cooling coils was used for fermentation** (Vendome Copper & Brass Works, Inc, Louisville, KY, USA).

Figure 2.

Rapid visco analysis (RVA) profiles of grains used in bourbon whiskey production at low and high temperature profiles. Sample mass was sieved grain (6 g) added to deionised water (22 g). Each profile represents a single replicate.



Grain	Temp. Profile	Peak Visc. (cP)	Final Visc. (cP)	Pasting Temp (◦C)
Corn	Low	852	882	76.7
	High	733	909	78.4
Rye	Low	4044	586	66.1
	High	4511	772	67.2
Malt	Low	299	246	70.9
	High	253	176	75.4

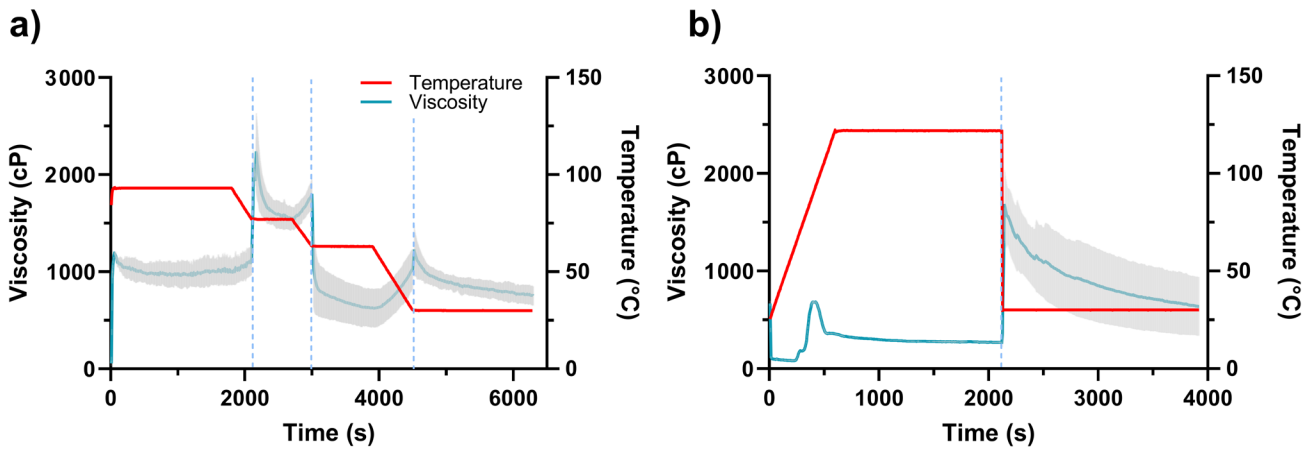
at temperatures above 100°C (Schierbaum and Kettlitz 1994). However, the width of the higher temperature profile pasting peak was also narrower, so it is unclear if this higher viscosity reflects the enhanced gelatinisation of rye starch or a more rapid pasting process. As a modified grain, barley malt behaved as expected and had the lowest viscosities.

The rheological properties of simultaneous saccharification and fermentation (SSF) and conventional mash were examined using RVA to predict processability during production (Figure 3). The conventional mash RVA profile exhibited a characteristic pattern with a rapid initial increase in viscosity following the addition of corn, followed by a significant peak upon the addition of rye. This high peak viscosity was then reduced with the addition of barley malt. The SSF profile showed a lower overall viscosity in the cooking step than the conventional profile. This may be due to the activity - during the initial heating - of barley malt amylase enzymes breaking down starch, similar to the technique used by distilleries to achieve partial liquefaction. After the addition of exogenous enzymes, the SSF process exhibited a more significant reduction in viscosity compared to the conventional mash. This is expected to be due to the higher concentration of soluble starch and other complex carbohydrates in the SSF mash, which have not been broken down during a dedicated saccharification step. The lower viscosity of enzyme treated mashes highlights the advantage for both laboratory scale and pilot scale distillery processing in making the mash easier to mix and transfer.

Previous research by Agu et al (2014) demonstrated that the addition of enzymes, particularly β-glucanase, may not result in consistent changes in viscosity. For extracted corn and wheat starch, viscosity may increase, but with whole flour it may decrease. As concluded in previous work by Agu et al (2006; 2014) the complex interactions between grain composition, particle size, starch granules, proteins, and enzyme addition within the grain matrix need to be considered when reviewing the results of mash viscosity analysis. Additionally, Agu et al (2012) cautioned that significant variations in modification levels can occur between barley malt batches due to differences in grain composition, even with the same varieties and when malted under identical conditions. These variations can impact on the rheological properties of the mash, leading to unreliable and unpredictable results. To minimise the impact of variability of barley malt batches, studies in the future should use well characterised sources of barley with consistent modification levels.

Figure 3.

Rapid visco analysis (RVA) profiles of bourbon whiskey mashes (6.83g of corn, 1.37 g rye, 1.27g malt, and 23 mL deionised water) using (a) conventional and (b) simultaneous saccharification and fermentation (SSF) mashing temperature profiles. Blue lines represent two replicates, with grey bands the standard deviation. Dashed blue lines indicate when the 'can' was removed for grain or enzyme additions, stirred, and replaced in the RVA. For (a) rye, malt, and 1.5 µL of the enzyme mixture (33% alpha amylase, 33% xylanase, beta-glucanase and cellulase, and 33% glucoamylase) was added. For SSF (b) only 1.5 µL enzyme mixture was added. Cooling rates were 3°C per minute.



Sugars and fermentation products

Table 2 shows the fermentation yield and efficiency of conventional and SSF mashing methods. These parameters directly influence yeast metabolic processes and the economics of spirit production. No significant differences were observed in ethanol or metabolic yield between the mashing methods or the distillery. Average conversion efficiency was 86% and did not differ with mashing method. This mirrors previous work using a high pressure and temperature cooking method with higher ethanol levels only found where hop antimicrobials were used (Pielech-Przybylska et al. 2017). The time to completion of fermentation was significantly reduced by the addition of enzymes to the conventional mash, with an average of 43 hours ($p = 0.002$, Supplementary information, Table S4). The SSF mash also demonstrated a faster fermentation completion time of 49 hours compared to the conventional mash (without added enzymes) of 55 hours, but the difference was not statistically significant. Data for sugar consumption at 46 hours paints a similar picture, with the conventional mash plus enzymes having the highest sugar consumption of 93 ± 1%, followed by the SSF mash of 88 ± 2%, and the conventional mash (without enzymes) of 82 ± 2%. These observations confirm that the addition of enzymes improves fermentation efficiency in bourbon production. Additionally, it hints at differences in fermentation time reflecting the amount of glucose and maltose present initially. Depending on the yeast strain, a higher level of glucose in the mash, can reduce the utilisation of maltose through carbon catabolite repression (Cason et al. 1987). However, Younis and Stewart (1998) did not observe differences in ethanol production by yeast using glucose or maltose as a sole carbon source, or a difference in fermentation time, although this may be due to the lower sugar loading or other experimental differences.

The SSF mash had a different sugar profile to the conventional mash and the pilot scale distillery, with significantly higher initial glucose levels (95 g/L) but correspondingly lower initial maltose (25 g/L) and maltotriose (9.5 g/L) (Figure 4a). With the conventional mash and the distillery, there were no differences in maltose but the conventional mash with exogenous enzymes had higher levels of glucose and lower maltotriose. The addition of commercial enzymes during mashing has been reported by Okolo et al (2020) to increase glucose levels while reducing maltotriose; however, the addition of malted barley can help restore a more balanced sugar profile by providing enzymes that

Table 2.

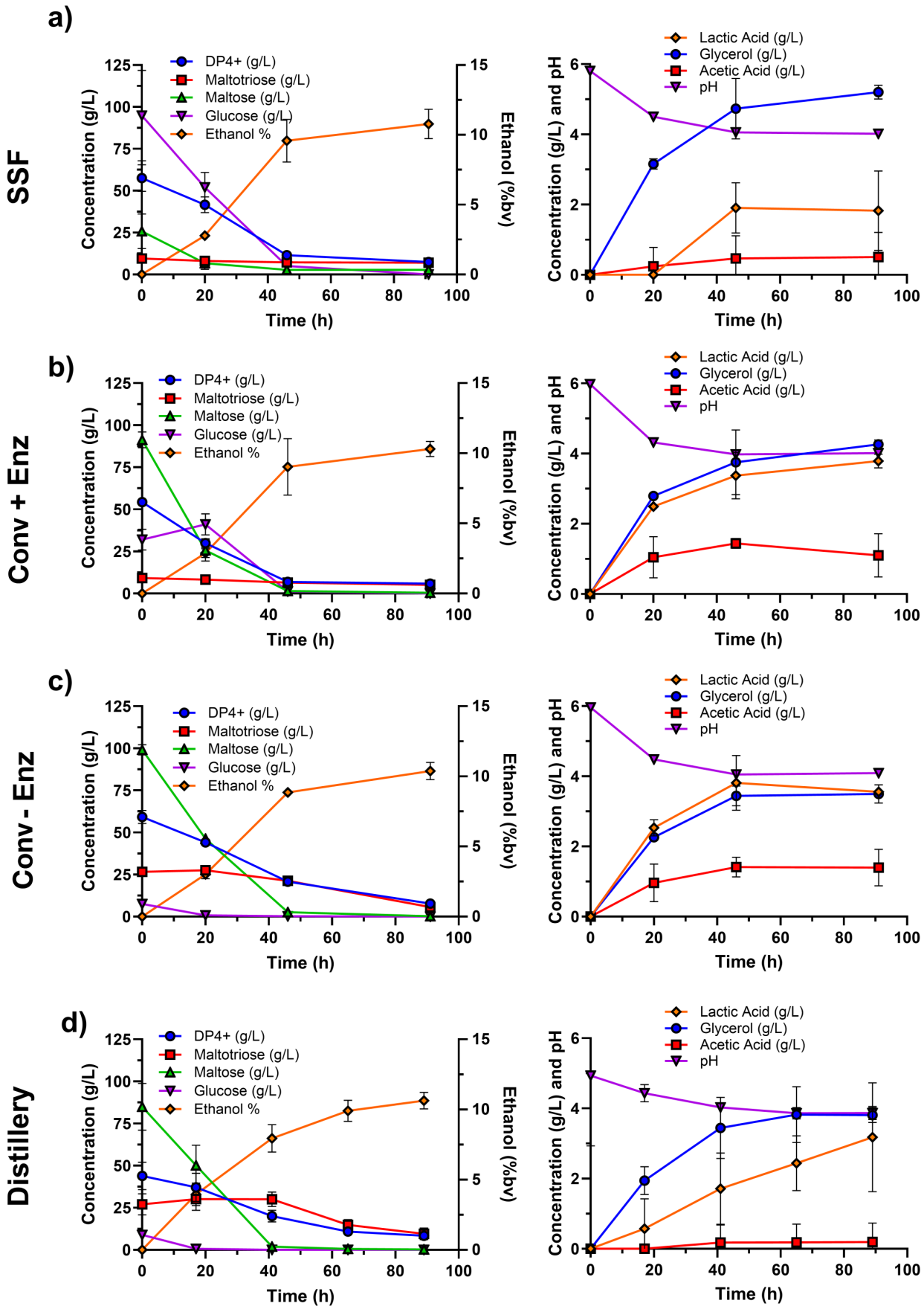
Effect of mashing method on ethanol production and conversion efficiency. Comparison of simultaneous saccharification and fermentation (SSF) mash and conventional (Conv) mash methods with added enzymes (Conv + Enz) and without (Conv).

Mashing	Ethanol (g/L)	Conversion efficiency (%)	Metabolic yield (g ethanol/g sugar)	Fermentation (h)
SSF	85.0 ± 8.4	88.5 ± 8.7	0.71 ± 0.08	49.4 ± 2.7
Conv	79.3 ± 7.2	82.6 ± 7.5	0.64 ± 0.03	55.4 ± 7.0
Conv + Enz	81.3 ± 4.3	84.7 ± 4.5	0.64 ± 0.05	42.6 ± 1.5
Distillery	83.9 ± 4.6	87.4 ± 4.8	0.79 ± 0.17	N/A

Values are the average of five replicates, except the 'distillery' data is from eight replicates. Errors are reported as standard deviation. Fermentation time is defined as the time when at least 90% of the measured cumulative gas production was produced. Gas production was measured continuously.

Figure 4.

Fermentation kinetics of bourbon whiskey. Laboratory scale mash using (a) simultaneous saccharification and fermentation (SSF), conventional (Conv) mashing with (b) and without (c) added enzymes, and (d) distillery pilot scale mash. All points represent five replicates, except distillery data which represents eight replicates. Error bars are the standard deviation.



contribute to maltose production. Between SSF and the conventional mashes, there was no difference in DP4+ sugars; however, these were significantly higher for laboratory scale mashes compared to the distillery. This is a limitation in using this method and comparing it to the pilot scale. The total level of sugars was comparable across all mashing methods and the distillery, which explains the similarity in final ethanol yield.

Markedly higher amounts of glucose (94.9 ± 26.9 g/L) were found in the SSF treatment (Figure 4a) than in previous research with corn and grain mashes. This is a likely consequence of the higher solids loading (2.5 L water per kg solids) used in this study. Arnold et al (2019) used a conventional mashing procedure (3.9 L/kg) with corn and added enzymes (alpha-amylase and glucoamylase), resulting in a lower glucose concentration of 78.8 g/L, but a similar proportion of total sugars in the mash. Similarly, Verges et al (2023), using a conventional mashing procedure (3.7 L/kg) with added alpha amylase and glucoamylase enzymes, observed 70 to 98 g/L glucose and 49.2 to 67 g/L maltose. Zhao et al (2009) reported that 51.2 - 62.7% of the total starch had been completely hydrolysed to glucose using a conventional laboratory fermentation method (3.3 L/kg) with sorghum. This compares to 56 ± 16% of the theoretical starch converted to glucose in the SSF treatment in the work reported here. Zhao et al (2009) also noted that glucose levels in SSF would be expected to be lower. Finally, higher glucose levels were found for a high temperature and pressure method compared to a conventional method, due to enhanced starch solubilisation and more efficient starch hydrolysis (Pielech-Przybylska et al. 2017). Accordingly, while the SSF mashing method yielded a final ethanol concentration and overall starch conversion efficiency comparable to conventional laboratory scale approaches, its primary advantage may lie in the process characteristics detailed elsewhere.

To understand the metabolic differences between SSF and conventional mashing methods, the production of lactic acid and glycerol was compared. Simultaneous saccharification and fermentation resulted in significantly higher glycerol levels than both conventional mashes and the distillery. In yeast, glycerol is produced to maintain the redox balance during fermentation and plays a role in osmoregulation (Wang et al. 2001: Hohmann 2002). The high glucose concentration and associated elevated osmotic pressure in SSF mashes may contribute to the increased glycerol production. Lactic acid is primarily produced by ubiquitous lactic acid bacteria and levels were elevated in the conventional mash (without exogenous enzymes) compared to the SSF mash. A potential source of lactic acid bacteria is the surface of cereal grains (Agu and Palmer 1999, Wilson 2022). However, the autoclaving step in the SSF process would reduce the viability of contaminating bacteria leading to the lower lactic acid levels. The difference in lactic acid between the conventional mash with added enzymes and the SSF mash was not statistically significant but further investigation would be warranted.

To compare the flavour compound profiles of SSF and conventional mashes to a distillery sample, the concentration of volatile flavour congeners was determined. The SSF mash exhibited an identical flavour compound profile to the distillery sample (Figure 5) with no significant differences apart from a marginally higher level of acetaldehyde ($p = 0.056$). This together with the increase in glycerol and fermentation time, may suggest osmotic stress from the higher glucose levels in the SSF mash, possibly leading to reduced yeast viability and lower activity of alcohol dehydrogenase (O'Sullivan et al. 1999; Pielech-Przybylska et al. 2017). Despite the process similarity, the conventional mash without enzymes showed significant differences to the pilot scale distillery sample, with lower levels of acetaldehyde, propanol, and isoamyl acetate, but higher levels of isobutanol. This may be due to improved starch solubilisation and hydrolysis from the autoclave step in the SSF method. The high pressure and temperature of sterilisation is used in the tequila industry to improve cooking efficiency (Jacques et al. 2003). Interestingly, the addition of enzymes to the conventional mash did not alter the profile of flavour compounds.

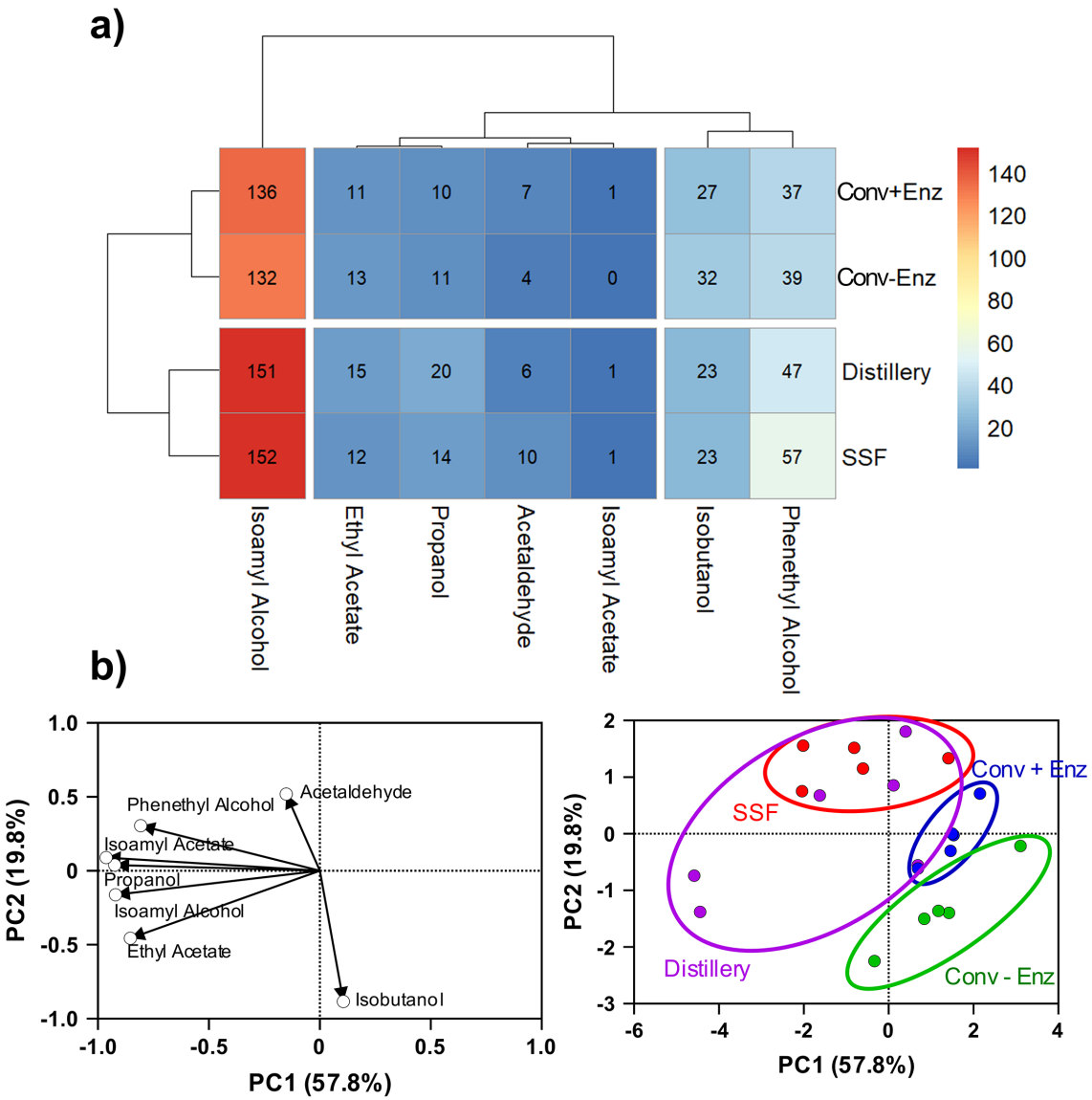
The initial composition of the fermentation medium determines the final flavour profile of the product (He et al. 2014). Compared to maltose, higher glucose levels can lead to increased production of volatile flavour compounds (Engan 1972; Younis and Stewart 1998). The results reported here show mashing methods to have an inconsistent effect on

higher alcohols. While SSF had the highest initial glucose level, it had lower levels of isobutanol but higher levels of phenylethyl alcohol compared to conventional mashes. This aligns with work which found elevated levels of isobutanol in distillates produced from barley malt using commercial enzymes and conventional mashing techniques, compared to those produced with barley malt and high temperature, high pressure methods (Pielech-Przybylska et al. 2017). While there is a metabolic association in *S. cerevisiae* between the production of glycerol, ethanol, and isobutanol (Wess et al. 2019), an explanation for the differing results in higher alcohol production reported here is not clear.

Simultaneous saccharification and fermentation is a potential tool for optimising bourbon production. The method requires the use of an autoclave for starch solubilisation which may limit its application. Further, the protocol for enzyme addition in conventional mashing method may not fully replicate industrial practices, where enzymes are

Figure 5.

(a) Heatmap of volatile flavour congener concentration (mg/L) in bourbon whiskey at laboratory scale using simultaneous saccharification and fermentation (SSF), conventional (Conv) mash methods with and without added enzymes (± Enz), and in distillery pilot scale mashes. The colour intensity of each cell represents the relative concentration of the flavour compound. Data from SSF and conventional mashes is from five replicates, with distillery data from six replicates, with two outliers removed, **(b) Principal Component Analysis (PCA) of volatile flavour congener concentration, including loadings plot (left) showing the contribution of variables to principal components and score plot (right) showing the distribution of samples in the PC space**.



typically added during the liquefaction stage. This simplified approach may influence the fermentation outcomes and limit the direct comparability of the method to industrial processes. Furthermore, this study was limited to seven key flavour congeners. These provide information about the potential flavour profile of the distillate but not the full sensory picture. However, the flavour profile of distilled spirits is complex and influenced by numerous compounds and their interactions. Future studies with a wider range of congeners, chemical analysis of the distillates, and sensory analysis with trained panellists would provide a more comprehensive understanding of the flavour differences between mashing methods.

Conclusions

This study demonstrates consistent ethanol yields from laboratory scale mashing methods, including simultaneous saccharification and fermentation (SSF) and conventional approaches, and from a pilot scale distillery. The SSF mashing method shows promise for predicting the distillery flavour profile, making it an ideal method for experimenting with variables such as yeast strain and fermentation conditions. Additionally, exploring high pressure thermal cooking techniques in the spirit industry could limit microbial contamination and enable greater control over the consistency of the flavour profile of the product. Such advancements can lead to consistent and high quality spirits, meeting the evolving demands of consumers.

Author contributions

Ryan Sarhan: conceptualisation, methodology, investigation, writing (original draft, review and editing), visualisation.
Brad J. Berron: resources, writing (review and editing), supervision.
Czarena Crofcheck: resources, writing (review and editing).
Glenna Joyce Welsko: investigation, writing (review and editing).
Tyler J. Barzee: conceptualisation, methodology, resources, writing (review and editing), visualisation, funding acquisition, supervision.

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Conflict of interest

The authors declare no conflict of interest.

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