Improved Production of ethanol using bagasse from different sorghum cultivars

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Abstract

For improved production of ethanol from whole sorghum residues, physico-chemical compositions and fermentation characteristics of the substrates are important factors to consider. In the present study, Nigerian sorghum cultivars SSV2, KSV8 and KSV3 were grown under rain-fed conditions without chemical fertilization in Kano state, Nigeria. On harvest, the whole sorghum residues (bagasse) comprising crushed stalks, leaves, panicles and peduncles were collected for further processing. Bagasse samples, which had different macromolecular composition and carbohydrate pasting properties, were pre-treated with dilute sulphuric acid at 75°C followed by enzymatic hydrolysis and sequential detoxification by $Ca(OH)_2$ over-liming and charcoal filtration. Hydrolysate samples were subsequently fermented with the yeasts, *Saccharomyces cerevisiae* and *Pachysolen tannophilus*. Sugar consumption, carbon dioxide evolution and ethanol production were shown to vary depending on the sorghum cultivar type. While KSV3 yielded most favourable biomass of 37 t ha^{-1} (dry basis), Bagasse from cultivar SSV2 yielded the most favourable level of sugars (69 g/100g) after enzymatic hydrolysis, and also consistently exhibited improved fermentation performance. Detoxification of pre-treated sorghum bagasse to remove potential yeast inhibitors resulted in improvement in ethanol yield, with 23 g L^{-1} ethanol (representing 72% of theoretical yield) being achieved from SSV2 bagasse following fermentation with *P. tannophilus*

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without exogenous nutrient supplementation. Our findings reveal that the choice of sorghum cultivar is important when converting bagasse to ethanol, and further that pretreatment with dilute acid at moderate temperature followed by detoxification improves fermentation kinetics and ethanol yield.

1.0   Introduction.

Plant biomass is the conventional sugar source for bioconversion to ethanol by yeast [1]. Stalk juice from sugarcane, starch from grains/tubers and lignocellulose from crop residues represent valuable fermentable sugar sources for bioethanol destined for use as a liquid transportation fuel [2,3].

Sorghum is a high biomass yielding cereal which is a water efficient crop that can be grown in 2-3 crop cycles per annum [4].Typical lignocellulose residues from sorghum harvest comprise crushed stalks (after juice extraction), panicles, peduncles and leaves. The sorghum lignocellulosic biomass usually comprises 25-27% hemicellulose, 34-44% cellulose and 18-21% lignin [5,6]. Cellulose and hemicellulose are polysaccharide polymers intertwined by tough lignin fibre [7]. Lignin acts as a barrier to efficient enzymatic hydrolysis of the cellulose and hemicellulose polysaccharides [8]. Previous studies [9,10,11,12,13,14] have investigated various pre-treatment methods designed to facilitate enzymatic hydrolysis of sorghum cellulose and hemicellulose polymers to fermentable sugars [6,15,16]. For example, sulphuric acidic pre-treatment appears economically attractive as a low cost pre-treatment option for commercial scale cellulosic bioethanol production [5,17]. However, the method typically requires high temperatures and/or extreme pH levels for effective degradation of lignin [15,18].

Consequently, a range of lignocellulose degradation by-products are generated including phenolic compounds from lignin and acetic acid derived from deacetylation of hemicellulose xylose side chains. In addition, formic acid is generated from the degradation of furfural or 5-hydroxymethyl furfural [19]. The individual or synergistic effects of these compounds on yeast include extended fermentation lag time and inefficient yeast metabolism resulting in reduced ethanol yield [19,20]. Dilute acid pre-treatment has the benefit of being less corrosive to handle and moderate hydrolysis temperatures will minimise cost of process energy requirement, in addition to preserving the substrate's nitrogenous content.

In this study, we investigated bioconversion of residues from different Nigerian sorghum cultivars for bioethanol production. SSV2, KSV8 and KSV3 are relatively high grain yielding sorghum cultivars that have similar numbers of crop cycles per year. Previous work has investigated bioconversion of crushed stalks and/or leaves of sorghum to ethanol under various pre-treatments and fermentation conditions [21,22]. However, the current study focused on the fermentation characteristics of whole sorghum residue (bagasse) after dilute acid pre-treatment at moderate temperature and enzymatic hydrolysis. We also investigated the effects of detoxifying the resultant bagasse hydrolysates on the fermentation performance with the yeast species *Saccharomyces cerevisiae* and *Pachysolen tannophilus*.

2.0   Materials and Methods

2.1   Sorghum crop cultivation and harvest

SSV2, KSV8 and KSV3 sorghum cultivars were cultivated in Kano (Nigeria) under rain-fed conditions and with only cow dung application as a fertilizer. For maximum extractible stalk juice yield, crops were harvested before grains reached

physiological maturation (i.e. when grains were at soft-dough stage). Thus, SSV2 cultivar was harvested 11 weeks after the planting date, when the grains were observed to have reached soft-dough maturation. However, KSV8 and KSV3 cultivars were harvested 16 weeks after planting date, because that was when their grains reached soft-dough maturation. The fresh bagasse (comprising crushed stalks, leaves, peduncles and panicles) were sun-dried for 2 days followed by oven drying at 60^oC for 72 h. The dried samples were hammer milled and sieved through 4 mm screen (Retsch, Germany). Moisture and total lignin contents of samples were determined according to National Renewable Energy Laboratory standard analytical procedure [23]. Proteins were determined by adding 2 g bagasse (dry wt.) into conical flasks containing 2M NaOH solution (50 mL). The mixtures were stirred at room temperature for 2 min followed by incubation in a rotary shaker at 120 rpm and 60^oC for 2 h. The final mixtures were centrifuged at 3800 rpm for 10 min. The supernatant (containing solubilised proteins) were filtered, diluted (1:10) and 1 mL of the solution transferred into 2 mL cuvettes. The protein concentrations were determined using Bradford™ reagent (Sigma-Aldrich, UK) according to manufacturer's standard protocol. Bagasse samples total starch content was determined using K-TSTA total starch kits (Megazymes®, Northern Ireland) according to manufacturer's standard procedure. Furthermore, the bagasse samples pasting properties were determined courtesy Scotch Whisky Research Institute Edinburgh (SWRI). A Rapid Visco-Analyzer equipment (Newport Scientific, Australia) was employed for the analysis.

Hammer milled sample of KSV8 bagasse (2.91 g) was added into canister containing distilled water (25.09 g). The suspension was homogenised using the canister paddle [24]. The paddle was placed into the canister and then inserted into the Rapid

Visco-Analyser for analysis. The typical RVA cycle profile is summarised in Table 1, while Fig. 1 shows the typical pasting profile for un-malted cereals. Important features of the RVA pasting profile shown in Fig. 1 include the peak viscosity which indicates the water binding capacity of the mixture being analysed and it correlates with final product quality. It is also indicative of the viscous load to be encountered by a mixing cooker. Also, at the hold temperature ($95^{o}C$), the ability of a sample to withstand the heating and shear stress of the RVA run is an important factor for many processes. It has been shown that RVA peak and final viscosities are highly correlated to ethanol yield [24,25]. The implications of these are discussed later in this paper.

2.2 Bagasse pre-treatment and saccharification

Bagasse (20 g dry wt.) was added into a conical flask containing 2%v/v dilute H_2SO_4 acid (80 mL). The mixture was incubated at $75^{o}C$ for 3 h with 150 rpm orbital shaking. This was followed by the addition of distilled water (30 mL) to the slurry and afterwards autoclaved at $121^{o}C$ for 15 min. Samples were withdrawn for sugar and free amino nitrogen analysis. The acidic hydrolysate was then adjusted to pH 5.5 with anhydrous sodium hydroxide crystals. An enzyme cocktail (Table 2) was added into the hydrolysate and the final volume adjusted to 200 mL with distiller water. The resultant hydrolysate was incubated at 150 rpm orbital shaking for 20 h at $50^{o}C$. Finally, the temperature was increased to $60^{o}C$ for 1 h incubation in order to complete the enzymatic hydrolysis.

2.3 Sorghum bagasse hydrolysate detoxification

The enzymatic hydrolysate was over-limed to pH 10.0 with anhydrous $Ca(OH)_2$ and incubated at $50^{o}C$ for 15 min with orbital shaking at 120 rpm [9]. Concentrated

sulphuric acid was used to adjust the hydrolysate pH to 6.0 and followed by centrifugation at 3800 rpm for 10 min. The supernatant (100 mL) was transferred into a conical flask and activated charcoal (2.5 g) added. The mixture was mixed at room temperature for 3 min followed by incubation with orbital shaking at 150 rpm for 30 min at 50^oC. Afterwards, the mixture was further centrifuged at 3800 rpm for 10 min and the supernatant (hydrolysates) filtered through vacuum pump equipped with GF/B Whatman glass microfiber filters. Samples (2 mL) were withdrawn from the filtrate for sugars, amino acids and FAN determination.

2.4   Sugars, free amino nitrogen (FAN) and amino acid analysis

1. Sugars analysis: glucose, xylose and arabinose were determined by HPLC. The hydrolysate (1.0 mL) at 1:10 dilution ratio were filtered through 0.22 μm micro syringe filters into 2.0 mL vials containing 1.0 mL *meso*-erythritol solution (internal standard sugar). The final solutions were vortexed and placed in an HPLC auto sampler (Spectra-physics, USA). Sugars are separated with a 300 mm × 7.8 mm REZEX RPM-monosaccharide Pb+2 (8%) column™ (Phenomenex, USA) and quantified using HPLC software (CSW32 version v.1.4 chromatogram software from DataApex®, USA).

2. Free amino nitrogen (FAN) analysis: FAN was determined by K-Large 02/11™ (yeast available nitrogen, YAN) and K-PANOPA 02/11™ (primary amino nitrogen, PAN) assay kits according to the manufacturer's standard protocols, respectively (Megazymes, Northern Ireland).

3. Amino acid analysis: Total free amino acids were determined courtesy of ICBD, Heriot-Watt University Edinburgh. Charcoal filtered hydrolysates (2 mL) were filtered through 0.22 μm filters into HPLC-grade vials. The analysis was performed by

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gradient elution, high performance liquid chromatography (HPLC), using fluorescence as a means of detection [26].

2.5 Yeast seed culture preparation

Yeast seed cultures were prepared separately by inoculating two loop full of strains *Pachysolen tannophilus* NCYC614 and *Saccharomyces cerevisiae* DCLM (courtesy of Kerry Biosciences, Menstrie, Scotland) into two separate 400 mL YEPD media respectively. The YEPD media comprised: of Bacteriological peptone (2.5% w/v), urea (2.5% w/v), yeast extract (1.0% w/v), glucose (3.0% w/v) and xylose (1.0% w/v) respectively. The cultures were incubated at $32^{\circ}C$ with orbital shaking at 150 rpm for about 28 h. Afterwards, the yeast pellets were washed by suspending in distilled water and vortexed, the water was decanted and the procedure repeated twice.

2.6 Hydrolysate fermentations

Fermentation progress was monitored by both CO_2 evolution and bioethanol production rates respectively.

i. CO_2 evolution monitoring: two sets of enzymatic hydrolysate samples (100 mL) were each added into a 250 mL ANKOM^{RF} glass bottle. *P. tannophilus* and *S. cerevisiae* (1.0×10^7 cell/mL) were inoculated into either of the fermentation media. The media were incubated at $32^{\circ}C$ with 130 rpm orbital shaking. Fermentation progress was monitored through automatic measurement of cumulative CO_2 gas pressure formation after every 20 min by the ANKOM^{RF} gas-production system (ANKOM Technology, USA). Fermentations were allowed to proceed undisturbed until CO_2 gas production rates were starting to decline.

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ii.   For Bioethanol monitoring was similar to (i) above, two sets of enzymatic hydrolysate samples (100 mL) were each added into a 250 mL Erlenmeyer flask. Either of *P. tannophilus* or *S. cerevisiae* (cultures) (1.0×10^7 cell/mL) was inoculated into each of the fermentation media. The substrates were incubated at 32^oC with 130 rpm orbital shaking. Samples were withdrawn after every 24 h from media for ethanol determination by FermentoFlash® equipment (Funke-Gerber[TM], Berlin). The fermentations were terminated after 72 h incubation.

Similar fermentation experimental set ups were replicated with the over-limed and charcoal filtered hydrolysates.

2.7   Ethanol concentration determination

Ethanol concentrations were determined using fermentoFlash® (Funke-Gerber[TM], Berlin). Fermentation broth (11 mL) was added into a 20 mL glass beaker. The broth sample (10 mL) was moved into the fermentoFlash® measuring cells by means of a suction pump (Funke-Gerber[TM], Berlin). The alcoholic content and density of the fermentation broth are automatically measured using thermal measuring effects. Constituents such as original and apparent wort extract and osmotic pressure are also determined but not reported in this study.

2.8   Statistical analytical method

Significant difference between means was tested by ANOVA using the Tukey method in Minitab™ 16 statistical software (MINITAB©, USA). Means that do not share a superscript letter (a-e) within the same rows are significantly different ($p \leq 0.05$), based on grouping information of the Tukey method at 95% simultaneous confidence interval.

3.0   Results and discussion

3.1   Compositional analysis

Table 3 shows that KSV3 sorghum grows significantly (p ≤0.05) taller and thicker than KSV8 while SSV2 has the least physical size. These results are consistent with a corresponding weight of bagasse obtained after harvest. However, SSV2 contains a significantly (p ≤0.05) higher starch level followed by KSV3. KSV8 has significantly (p ≤0.05) higher lignin content than both SSV2 and KSV3. With regards to pasting properties, there is very limited previous literature on sorghum bagasse pasting properties published. Pasting property is an important parameter in assisting to predict the hydrolysis properties of the substrate [27]. Thus, pasting property of substrates depends on their starch composition (i.e. amylose/amylopectin ratio), moisture content, degree of paste viscosity and pasting temperature [28,30].

Generally, sorghum bagasse comprises cellulose that is similar to amylose, in that they both consist of linear crystalline glucose homoploymers. Hemicelluloses are branch chained non-crystalline polysaccharides akin to amylopectin. Therefore, despite the relatively low starch contents of SSV2, KSV8 and KSV3 bagasse for pasting analysis [24], it is conceivable their cellulose and hemicellulose polysaccharides exhibit hydrolytic behaviour similar to those of amylose and amylopectin [27,31]. Table 3 shows that KSV8 has significantly (p ≤0.05) lower starch and higher lignin content than both SSV2 and KSV3. Hence, SSV2 and KSV3 show viscograms (Fig. 2) that are completely different from that of KSV8 (Fig. 3). Furthermore, data in Table 4 shows that KSV8 had significantly (p ≤0.05) the highest peak, setback and final viscosity values respectively Perhaps the combined effects of low starch content of KSV8 bagasse and higher lignin interacted with its carbohydrates resulting in significantly lower pasting peak times (Table 4). These

results suggest that SSV2 and KSV3 may be more favourable substrates for hydrolysis than KSV8 in terms of pasting profiles [30,31].

3.2 Bagasse hydrolysis and detoxification

The choice of mild sulphuric acid pre-treatment in this study was principally motivated by its economic benefits [15]. Dilute sulphuric acid is a relatively cheap chemical that can be used for efficient lignocellulose pretreatment [9]. Additionally, it is less corrosive compared to using concentrated acids. However, as previously mentioned, dilute or mild acid pretreatment of bagasse is usually accompanied with high temperatures, typically above 160°C, and this facilitates formation of furan derivatives and weak organic acids that are inhibitory to efficient yeast fermentation [32]. Furthermore, denaturation of proteins and Maillard reactions are most likely to occur at high hydrolysis temperatures, compromising the availability of amino nitrogen nutrients required by yeast for efficient growth and metabolism during fermentation [5,33]. This is the reason for the 75°C hydrolysis temperature employed in this study aimed to minimise generation of inhibitory compounds whilst maintaining efficient bagasse pretreatment.

Data in Table 5 indicates that acid pretreatment of sorghum bagasse from SSV2, KSV8 and KSV3 cultivars liberated more xylose and arabinose than glucose. This suggests effective degradation of the hemicellulose polysaccharides, which are primarily the sources of pentose sugars [6]. KSV8 sorghum bagasse hydrolysate had lower glucose, but higher xylose and arabinose levels, than both SSV2 and KSV3 (Table 5). In addition to degradation of the polysaccharides, proteins were also hydrolysed to liberate free amino nitrogen (FAN) compounds [33] as shown in Table 7. Table 5 shows levels of total sugars of 21 g/100g (KSV8 bagasse) and 26 g/100g

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(SSV2 and KSV3 bagasse) that compare favourably with previously reported sugar yield results of 18-29 g/100g in sorghum bagasse pretreated with both alkali and acid [5]. Similarly, Ban et. al. [34] reported total sugar yields of 30 g/100g of sorghum bagasse hydrolysed with 90% of 80 g L^{-1} phosphoric acid at 120°C for 80 min.

Following enzymatic hydrolysis, significant increases (p ≤0.05) in total sugar levels in the sorghum bagasse hydrolysates were observed, particularly glucose (see Table 5). This may be attributed to combined activities of cellulases and amylases during cellulose and starch hydrolysis. Hemicellulase action liberated xylose and arabinose [9,35], but at lower levels than those reported by Phuengjayaem and Teeradakorn [8] who employed ammonium explosion (AFEX) pretreatment of sorghum bagasse prior to prolonged (7days) enzymolysis. Table 6 compares glucose and xylose sugars obtained in this study to those of previously reported studies using different pre-treatment methods for sorghum stalks and/or leaves.

We investigated detoxifying sorghum bagasse with over-liming and charcoal filtration hydrolysates in an attempt to ameliorate the effects of chemical inhibitors on yeast fermentation. Weak organic acids and furan derivatives can be removed by over-liming to precipitate aliphatic acids as their corresponding salts [19,37]. However, we found that 5-7% of sugars and FAN were lost to precipitation following over-liming, as shown in Tables 5 and 7 [9,38]. Further de-toxification by charcoal filtration to remove phenolic compounds from hydrolysates showed additional 7-10% sugars and FAN nutrients were further lost (Tables 5 and 7) [15,17].

We analysed individual amino acid profiles in charcoal filtered sorghum bagasse hydrolysates (Table 8). Although certain amino acids will failed to be detected using the K-LARGE/K-PANOPA assay kit [40], total amino acid concentrations in the

hydrolysates nevertheless appeared to be higher than corresponding FAN levels. Sorghum bagasse hydrolysates prepared from SSV2, KSV8 and KSV3 cultivars contained Group 1, Group 2 and other group amino acids required for efficient yeast metabolism during fermentation [33,41,42].

4.0   Fermentation characteristics.

Pachysolen tannophilus used in this study was observed to be a xylose/glucose fermenting yeast while *Saccharomyces cerevisiae* is a glucose fermenting yeast. Generally, yeast fermentation performance depends on media and growth conditions including pH, temperature, sugar and FAN levels as well as alcohol and general stress tolerance of the cells [37,43]. Therefore, for effective comparison of fermentation performance of SSV2, KSV8 and KSV3 hydrolysates in this study, *P. tannophilus* and *S. cerevisiae* yeasts that exhibited favourable fermentation performance were selected out of five different yeast strains previously investigated (not reported here).

SSV2, KSV8 and KSV3 bagasse hydrolysates contain acetic acid, furan derivatives and phenols as by-products of the acid pretreatment [15-17]. Consequently, *S. cerevisiae* fermentation kinetics of these hydrolysates showed a yeast lag phase of over 12 h (Fig. 4). This may be associated with the synergetic effects of the inhibitory compounds on yeast physiology meaning that cells take time adapt to the relatively hostile growth environment. However, SSV2 sorghum bagasse hydrolysate comprised relatively higher glucose and FAN levels and this resulted in reduced yeast lag times and higher CO_2 formation compared with other sorghum cultivars (Tables 5 and 7). With *P. tannophilus* fermentation, bagasse hydrolysate produced from KSV8 sorghum cultivar showed the shortest lag time (Fig. 5). These results

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highlight the likely impact of toxic compound, liberated when bagasse is pretreated, on fermentation performance Higher nutrient contents for yeast may not necessarily reflect faster fermentation rates since the levels of inhibitory compounds in the media are of paramount importance [16,18,37].

Concerning ethanol production, *P. tannophilus* performed better than *S. cerevisiae* in sorghum bagasse hydrolysates (Figs. 6 and 7). SSV2 and KSV3 cultivars showed similar ethanol yields with *S. cerevisiae* but exhibited varied ethanol yields with *P. tannophilus* (Figs. 6 and 7), likely due to the ability of the latter yeast to ferment xylose. Consequently, while *S. cerevisiae* showed total sugar utilisations of 43-45% for SSV2 and KSV3 substrates, *P. tannophilus* showed corresponding higher total sugar utilisation of 54-57% (from Tables 5, 9 and 10). Thus, with SSV2 and KSV3 cultivars *P. tannophilus* fermentation exhibited ethanol of 12-13 g L^{-1} (Table 11). These compares favourably to the 14 g L^{-1} ethanol yield reported by Ban et al. [34], for sorghum bagasse pre-treated with phosphoric acid (80 g L^{-1} H_3PO_4) at 120°C for 80 min. However, KSV8 showed similar sugar utilisations of 35-37% by both *S. cerevisiae* and *P. tannophilus* fermentations respectively, this corresponds to 7-10 g L^{-1}. This is higher than 5-6 g L^{-1} ethanol yields reported by Ban et al. [34] and Cao et al. [11] for sorghum bagasse pre-treated with concentrated phosphoric acid and dilute NaOH/H_2O_2 solutions respectively.

Following the removal or reduction in the concentration of aliphatic acids in SSV2, KSV8 and KSV3 hydrolysates by over-liming [15,22], a notable reduction in yeast lag phase was observed after fermentation of the substrates. Particularly, SSV2 showed faster fermentation and a higher CO_2 evolution rate than KSV8 and KSV3 (Fig. 8). However, while KSV8 and KSV3 exhibits relatively similar lag phases by *S.*

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cerevisiae fermentations, the latter showed faster fermentation with *P. tannophilus* than the former (Figs. 8 and 9). Furthermore, SSV2 and KSV3 consistently exhibited similar total CO_2 yields after 60 h fermentation by either of the yeast cultures. With regards to yeast performance, *P. tannophilus* showed higher total CO_2 yields (Table 11) and faster fermentation rates than *S. cerevisiae.*

In terms of ethanol production, SSV2, KSV8 and KSV3 over-limed hydrolysates showed increased ethanol yields of 29%, 22% and 29% with *S. cerevisiae* fermentation relative to the corresponding non over-limed hydrolysates. Furthermore, *P. tannophilus* showed corresponding increased yields of 24%, 33% and 29% respectively (Table 11). Consistent with observed final total CO_2 yields of SSV2 and KSV3, they also show corresponding similar final ethanol yields by either *P. tannophilus* or *S. cerevisiae* fermentation (Figs. 10 and11). Compared to the non over-limed hydrolysates fermentations, total sugar utilisation of the over-limed hydrolysates has increased to 56-68% with the *P. tannophilus* fermentation (Table 9) and 48-57% with the *S. cerevisiae* fermentation (Table 10). Observed ethanol yields of about 17 g L^{-1} for SSV2 and KSV3 (Table 11) corresponds to 16-19 g L^{-1} ethanol yields previously reported for sorghum bagasse fermented by either co-culture of (*S. cerevisiae-P. stipitis*) or *S. cerevisiae* alone [11,35,44].

In addition to weak organic acid removal from SSV2, KSV8 and KSV3 sorghum bagasse hydrolysates, further removal of phenolics by charcoal filtration [6,9] show further improved fermentation performance of the substrates (Figs. 12 and 13). For example, SSV2, KSV8 and KSV3 show comparatively similar CO_2 evolution at the onset of fermentation and this reflects a robust exponential cell growth rate [44]. Sugar utilisation has further increased to 76-80% with *P. tannophilus* fermentation

and 62-74% with *S. cerevisiae* i.e. when compared to non-detoxified corresponding hydrolysates (Tables 9 and 10). Consistent with previous results, *P. tannophilus* showed the most favourable fermentation performance (in terms of observed CO_2 evolution). SSV2 followed by KSV3 bagasse, are the most favourable fermentation substrates. However, Gyalai-Korpos et al. [45] reported a relatively faster fermentation rate for detoxified sorghum bagasse hydrolysates (supplemented with exogenous yeast nutrients), maximum CO_2 evolution was achieved within 4 h of fermentation onset while in this study (without nutrient supplementation) maximum CO_2 evolution was achieved after 12 h of fermentation onset.

With regards to ethanol production, SSV2 and KSV3 show similar ethanol production rates at the onset of fermentation (Figs. 14 and 15). However, as fermentation progress beyond 24 h, SSV2 show higher ethanol yields. The faster fermentation characteristics of SSV2 and KSV3 is likely related to their having a higher Group 1 and 2 amino acid content than KSV8 as shown in Table 8 [41]. Furthermore, *P. tannophilus* ethanol yield has significantly increased by about 40-44% for SSV2 and KSV3 hydrolysates following charcoal filtration (relative to the non detoxified hydrolysates). However, *S. cerevisiae* shows corresponding 34-43% improved ethanol yield (Table 11). While *P. tannophilus* show 72-74% theoretical ethanol yield for SSV2 and KSV3 hydrolysates, *S. cerevisiae* shows corresponding 61-66% theoretical ethanol yield, respectively. Consequently, in this study, *P. tannophilus* is most favourable yeast compared to *S. cerevisiae*. Finally, previous studies have reported varied ethanol yields obtained for sorghum bagasse pre-treatment and fermentation under various conditions. The results obtained in this study are compared with other previous investigations and the results are summarised in Table 12.

4.4   Conclusion and recommendation

We investigated the physico-chemical composition and fermentation characteristics of whole sorghum residue (bagasse) as a bioethanol feedstock. Our findings suggest that sorghum cultivar KSV3 exhibited the most favourable biomass yield at 37 t ha^{-1} (dry basis) while bagasse from SSV2 cultivar provided the most favourable fermentation substrate. Dilute sulphuric acid hydrolysis at moderate temperatures was a favourable pre-treatment method with SSV2 yielding 69 g/100g bagasse of fermentable sugar after enzymatic hydrolysis. Detoxification of hydrolysates improved the fermentation kinetics with SSV2 and it exhibited faster fermentation kinetics and favourable ethanol yields of 23 g L^{-1} by *P. tannophilus* without exogenous nutrient supplementation. This represents over a 25% increase on non-detoxified hydrolysates. The moderately low temperature used for our technique also suggests low energy input and utilization in the conversion of the sorghum biomass to bioethanol that could reduce greenhouse gas emission. Further improvements in ethanol yield per hectare are envisaged through moderate application of agrochemicals during crop cultivation and the use of improved cellulolytic enzymes and exogenous yeast nutrient supplementation during fermentation.

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List of Tables

Table 1 The RVA run cycle profile

Cycle time profile	Parameter	Value
00:00:00	Temperature	50°C
00:00:00	Speed	960 rpm
00:00:10	Speed	160 rpm
00:00:30	Temperature	50°C
00:04:30	Temperature	98°C
00:09:00	Temperature	98°C
00:11:00	Temperature	65°C
00:15:00	Temperature	65°C

Note: Idle temp. = 50°C, total cycle time = 15 min, readings interval = 4 s.

Table 2 Composition of hydrolytic enzymes

Enzyme	Activity	Dosage	Source
Cellic® Ctec	(120 FPU/mL)[a]	1200 µL	Novozymes, Denmark
Cellic® Htec	(1090 FXU/mL)[b]	200 µL	Novozymes, Denmark
Promalt™ 295	(500 BGµ/mL-min)[c]	30 µL	Kerrys Biosciences, Ireland
Promalt™ 4TR	(300 BG µ/mL)	20 µL	Kerrys Biosciences, Ireland

[a]Filter paper unit.
[b]Fungal xylanan unit.
[c]betaglucanase unit/mL.

Table 3 Sorghum bagasse physico-chemical composition

Parameter	SSV2	KSV8	KSV3
Cultivation	11 weeks	16 weeks	16 weeks
Crop height (m)	1.80[a] ±0.05	3.20[b] ±0.07	3.60[c] ±0.04
Diameter (cm)	1.95[a] ±0.10	2.62[c] ±0.11	2.79[c] ±0.03
*Fresh bgs (t ha^{-1})	41.72[a] ±3.1	48.31[b] ±2.6	52.32[c] ±1.1
**Dry bgs (t ha^{-1})	28.60[a] ±1.1	32.72[b] ±0.8	36.83[c] ±1.5

Total starch: %	5.14^a ±0.54	1.09^b ±0.06	3.16^c ±0.21
Total lignin: %	18.40^a ±0.3	21.65^b ±0.2	18.70^a ±0.6
Total protein %	4.61^a ±0.2	3.53^b ±0.16	3.24^b ±0.12

SSV2, KSV8 and KSV3 sorghums were cultivated under rain-fed without chemical fertilizer application. *Fresh bgs: fresh bagasse (leaves, crushed stalks, peduncles and panicle). **Dry bgs: oven dried bagasse. Results are std. means of triplicate experiments. Means on the same row that do not share same superscript letter (a-c) are significantly different (p ≤0.05) by ANOVA using Tukey grouping method test.

Table 4   Sorghum bagasse pasting viscosities

Crop	Peak viscosity (cP)	Setback viscosity (cP)	Pasting Temp (°C)	Peak time (min)	Final viscosity (cP)
SSV2	1771^b ± 14	4541^b ± 21	49.90^a ± 0.2	6.93^a ± 0.3	7042^a ± 14
KSV3	1706^a ± 11	5861^a ± 19	50.45^a ± 0.1	7.00^a ± 0.4	5756^b ± 13
KSV8	19320^c ± 22	16549^c ± 20	49.95^a ± 0.1	1.20^b ± 0.1	22073^c ± 17

Bagasse pasting profile analyzed by Rapid Visco-Analyzer. Means in the same column that do not share same superscript letter (a-c) are significantly different (p ≤0.05) by ANOVA using Tukey grouping method test.

Table 5 Initial sugars of SSV2, KSV8 and KSV3 hydrolysates (g/100g bagasse)

Bagasse	Hydrolysates	Glucose	Xylose	Arabinose	Total sugars
	Acidic	8.82^a ±1.1	13.46^a ±0.4	3.49^a ±0.6	25.77^a ±0.8
SSV2	Enzymatic	46.46^{ab} ±1.1	17.29^{ab} ±0.5	5.45^b ±0.5	69.19^c ±1.1
	Over-limed	43.85^{af} ±1.0	15.06^{cd} ±0.9	5.27^b ±0.9	64.18^{ab} ±2.6
	Charcoal filtrate	42.88^{af} ±1.0	13.70^a ±0.2	5.08^b ±1.0	61.66^{bc} ±2.2

	Acidic	1.54^b ±0.2	15.35^c ±0.1	4.01^c ±0.6	20.89^b ±0.9
KSV8	Enzymatic	26.57^{ad} ±1.2	21.22^{ac} ±1.1	6.44^d ±0.4	54.22^e ±2.8
	Over-limed	23.25^{cf} ±0.9	17.87^{ab} ±0.9	6.34^d ±0.1	47.46^{ad} ±1.8
	Charcoal filtrate	22.84^{cf} ±1.0	15.80^c ±1.2	5.76^b ±0.2	44.40^{fe} ±0.3
	Acidic	8.36^a ±0.6	13.81^a ±0.7	3.47^a ±0.2	25.64^a ±0.9
	Enzymatic	44.62^{ac} ±0.8	16.94^{ab} ±1.1	5.23^b ±0.3	66.79^d ±1.2
KSV3	Over-limed	42.08^{af} ±0.9	15.20^c ±0.2	5.06^b ±0.6	62.34^{bc} ±1.7
	Charcoal filtrate	42.03^{af} ±0.3	14.01^c ±0.6	4.87^b ±0.7	60.88^{bc} ±1.6

Sorghum bagasse were pre-treated with dilute H_2SO_4 acid and followed by enzymatic saccharification, over-liming with $Ca(OH)_2$ and charcoal filtration. Sugars were determined by HPLC. Corresponding Means in the same column that do not share same superscript letter (a-f) are significantly different (p ≤0.05) by ANOVA using Tukey grouping method test.

Table 6 Comparison of this study bagasse sugar yields to previous literature.

Sorghum bagasse pre-treatment method	Sugar yields (g/100g substrate)	Reference
2% (v/v) H_2SO_4 digestion at 75^oC for 2 h followed by 24 h enzymatic hydrolysis	Glucose (27-47 g) & Xylose (17-20 g).	This study
3% CaOH digestion at 121^oC for 1 h followed by 24 h enzymatic hydrolysis.	Glucose (40 g) & Xylose (21 g).	Kim et. al. [32]

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Microwave assisted ammonium hydroxide digestion at 130°C for 1 h	Glucose (42 g).	Chen et. al. [16]
10% (w/w) NaOH digestion at 70°C for 4 h followed by 24 h enzymatic hydrolysis.	Glucose (31 g) & Xylose (14 g).	Panagiotopoulos et. al. [36]
3% H_2SO_4 digestion for 10 min followed by 96 h enzymatic hydrolysis.	Glucose (37 g) & Xylose (21 g).	Phuengjayaem and Teeradakorn [8]
10%(w/v) NaOH at 121°C for 25 min followed by 21% (v/v) H_2SO_4, digestion at 70°C for 73 min	Glucose (21 g).	Thanapimmetha et. al. [5]
2% NaOH digestion followed by 24 h enzymatic hydrolysis	Glucose (26 g).	Sathesh-Prabu and Murugesan [37]
Ammonium fibre explosion (AFEX) at 140°C for 30 min followed by 72 h enzymatic hydrolysis	Glucose (29 g) & Xylose (15 g).	Li et. al. [10]

Table 7 Initial free amino nitrogen (FAN) of sorghum bagasse hydrolysates (mg L^{-1})

Hydrolysates	SSV2	KSV8	KSV3
Acidic	130.3^a ±3.1	91.9^b ±1.9	123.2^c ±1.8
Enzymatic	251.8^a ±3.8	180.4^b ±2.1	248.0^c ±2.6
Over-limed	238.4^a ±3.6	168.0^b ±1.9	236.4^a ±2.8
Charcoal filtrate	205.8^a ±1.8	146.4^b ±2.1	188.0^c ±2.7

Bagasse were pre-treated with dilute H_2SO_4 acid followed by enzymatic saccharification, over-liming with $Ca(OH)_2$ and charcoal filtration. Means on the same row that do not share same superscript letter (a-c) are significantly different (p ≤0.05) by ANOVA using Tukey grouping method test.

Table 8 Initial amino acids of charcoal filtered hydrolysates (µmol mL^{-1})

Amino acid	SSV2	KSV8	KSV3
Group 1			
aspartic	1.492^a ±0.001	0.509^b ±0.006	0.753^c ±0.002
glutamic	0.240^a ±0.003	0.085^b ±0.007	0.176^c ±0.024
serine	0.234^a ±0.001	0.118^d ±0.008	0.135^c ±0.018
arginine	0.099^a ±0.001	0.027^c ±0.004	0.041^c ±0.011
threonine	0.157^a ±0.002	0.055^d ±0.007	0.091^c ±0.013

	lysine	0.113^a ±0.001	0.020^b ±0.003	0.051^c ±0.014
	asparagine	*ND	*ND	*ND
	glutamine	*ND	*ND	*ND
	Sub-total	**2.330 ±0.002**	**0.813 ±0.037**	**1.241 ±0.021**

Group 2

methionine	0.206^a ±0.002	0.081^d ±0.002	0.104^c ±0.010
valine	0.237^a ±0.001	0.095^b ±0.008	0.117^b ±0.011
isoleucine	0.110^a ±0.001	0.029^b ±0.006	0.052^c ±0.007
leucine	0.350^a ±0.000	0.067^b ±0.003	0.138^c ±0.002
phenylalanine	0.061^a ±0.002	0.016^b ±0.004	0.027^b ±0.013
histidine	0.077^a ±0.001	0.031^e ±0.001	0.040^b ±0.016
Sub-total	**1.039 ±0.003**	**0.319 ±0.003**	**0.477 ±0.020**

Other groups

glycine	0.335^a ±0.004	0.215^b ±0.008	0.174^c ±0.012
alanine	1.045^a ±0.003	0.279^b ±0.008	0.473^c ±0.076
proline	0.335^a ±0.001	0.114^b ±0.008	0.149^c ±0.015
tyrosine	0.104^a ±0.003	0.090^a ±0.004	0.065^b ±0.010
tryptophan	*ND	*ND	*ND
Sub-total	**1.818 ±0.003**	**0.698 ±0.004**	**0.860 ±0.054**

Grand Total	**5.186^a ±0.008**	**1.829^b ±0.044**	**2.577^c ±0.095**

SSV2, KSV8 and KSV3 Bagasse were pre-treated with dilute H_2SO_4 acid followed by enzymatic saccharification, over-liming with $Ca(OH)_2$ and charcoal filtration. Amino acids were determined by GC-MS. Means on the same row that do not share same superscript letter (a-e) are significantly different (p ≤0.05) by ANOVA using Tukey grouping method test. *ND = Not Detected.

Table 9   *P. tannophilus* fermentation residual sugars (g/100g bagasse)

Bagasse	Hydrolysates	Glucose	Xylose	Arabinose	Total sugars
	Enzymatic	13.25^a ±0.2	13.71^a ±0.5	4.93^a ±0.5	31.89^a ±1.2
SSV2	Ca(OH)$_2$ Overlimed	2.89^d ±0.9	12.57^a ±1.1	4.46^a ±0.4	19.92^b ±0.6
	Charcoal filtrate	*ND	8.76^{bc} ±0.9	3.65^b ±0.3	12.41^c ±1.1
	Enzymatic	10.42^b	17.67^e	5.49^c ±0.4	33.58^d

Bagasse	Hydrolysates	Glucose	Xylose	Arabinose	Total sugars
		±1.2	±1.1		±1.9
KSV8	Ca(OH)$_2$ Overlimed	*ND	14.51^c ±0.9	5.86^c ±0.1	20.37^b ±0.9
	Charcoal filtrate	*ND	7.30^b ±1.2	3.01^d ±0.2	10.31^f ±1.3
	Enzymatic	9.18^c ±1.2	16.14^f ±1.1	3.94^b ±0.5	29.26^{ab} ±1.7
KSV3	Ca(OH)$_2$ Overlimed	*ND	14.86^c ±0.3	4.72^a ±0.7	20.08^b ±1.0
	Charcoal filtrate	*ND	9.45^{bc} ±0.6	3.08^d ±0.1	12.53^c ±0.8

Residual sugars in sorghum bagasse hydrolysates after 72 h fermentation by *P. tannophilus* without exogenous nutrient supplementation and the sugars were determined by HPLC. Corresponding Means in the same column that do not share same superscript letter (a-f) are significantly different (p ≤0.05) by ANOVA using Tukey grouping method test. *ND = Not Detected.

Table 10   *S. cerevisiae* fermentation residual sugars (g/100g bagasse)

Bagasse	Hydrolysates	Glucose	Xylose	Arabinose	Total sugars
	Enzymatic	16.32^a ±1.2	16.33^a ±0.3	5.06^b ±0.5	37.71^a ±2.0
SSV2	Ca(OH)$_2$ Overlimed	9.40^b ±0.7	13.88^b ±1.2	4.63^a ±0.5	27.91^b ±1.3
	Charcoal filtrate	*ND	11.20^c ±1.1	4.71^a ±0.8	15.91^c ±1.9
	Enzymatic	9.58^b ±1.2	18.93^d ±1.8	6.29^d ±0.5	34.80^f ±2.5
KSV8	Ca(OH)$_2$ Overlimed	*ND	19.01^d ±1.2	5.73^e ±0.4	24.74^d ±1.6
	Charcoal filtrate	*ND	12.21^b ±1.1	4.40^a ±0.3	16.61^c ±1.3
	Enzymatic	17.42^a ±1.2	15.64^a ±0.9	5.04^b ±0.3	38.10^a ±0.6
KSV3	Ca(OH)$_2$ Overlimed	7.04^c ±0.6	14.96^e ±1.3	5.04^b ±0.1	27.04^b ±1.8
	Charcoal filtrate	*ND	11.37^c ±1.0	4.79^a ±0.8	16.16^c ±1.8

Residual sugars in sorghum bagasse hydrolysates after 72 h fermentation by *S. cerevisiae*, sugars were determined by HPLC. Corresponding Means in the same column

that do not share same superscript letter (a-f) are significantly different (p ≤0.05) by ANOVA using Tukey grouping method test. *ND = Not Detected.

Table 11   Fermentation ethanol and CO_2 yields

Bagasse	Hydrolysates	*P. tannophilus*		*S. cerevisiae*	
		Ethanol (g L^{-1})	CO_2 gas*	Ethanol (g L^{-1})	CO_2 gas*
SSV2	Enzymatic	13.03[a] ± 1.1	1423[a] ± 27	12.15[a] ± 0.88	1187[a] ± 23
	Ca(OH)$_2$ Over-limed	17.12[d] ± 0.9	2083[b] ± 31	16.81[b] ± 0.67	1930[b] ± 33
	Charcoal filtrate	23.12[ad] ± 0.5	3719[c] ± 24	20.99[ff] ± 0.94	3050[c] ± 26
KSV8	Enzymatic	9.81[b] ± 0.6	1142[f] ± 19	6.55[e] ± 0.59	754[cd] ± 22
	Ca(OH)$_2$ Over-limed	14.83[f] ± 0.8	1433[a] ± 23	8.60[f] ± 0.71	888[ca] ± 21
	Charcoal filtrate	16.89[ab] ± 0.3	2383[ab] ± 21	14.34[b] ± 0.48	2295[da] ± 25
KSV3	Enzymatic	11.84[c] ± 1.1	1382[ef] ± 31	12.03[a] ± 0.87	1124[ef] ± 21
	Ca(OH)$_2$ Over-limed	16.87[ab] ± 0.7	2093[b] ± 15	16.49[b] ± 0.64	1923[df] ± 20
	Charcoal filtrate	20.18[ef] ± 0.9	3118[df] ± 21	19.11ff ± 0.91	2647[ae] ± 23

Ethanol and CO_2 gas yields of SSV2, KSV8 and KSV3 sorghum bagasse hydrolysates at three treatment levels. Fermentations were by *P. tannophilus* and S. cerevisiae yeasts (without exogenous nutrients supplementation). Corresponding Means in the same column that do not share same superscript letter (a-f) are significantly different (p ≤0.05) by ANOVA using Tukey grouping method test. *C0$_2$ gas (mL/100g dry bagasse).

Table 12   Comparison of ethanol yields from this study to previous literatures

| Fermentation condition	Ethanol yield (g L^{-1})	Reference
Fermentation by *P. tannophilus* without nutrient supplementation.	17-23	This study
Fermentation by *S. cerevisiae* without nutrient supplementation.	16-20	This study
Fermentation by co-culture of *S. cerevisiae* and *Issatchenkia orientalis* and with nutrient supplements.	27	Wan et al. [35]
Fermentation by *P. tannophilus* with nutrient supplements.	16	Ballesteros et al. [13]
Fermentation by *S. cerevisiae* with nutrient supplementation.	23	Mehmood et al. [38]
Simultaneous saccharification and fermentation (SSF) with *S. cereviciae* (5 g L^{-1} cell density) and nutrient supplementation	23	Shen et al. [14]
Separate hydrolysis and fermentation (SHF) with *S. cereviciae* (3 g L^{-1} cell density) and nutrient supplementation	21	Shen et al. [14]
Fermentation by co-culture of S. cerevisiae and *Neurospora crassa* with nutrient supplementation.	28	Dogaris et al. [6]
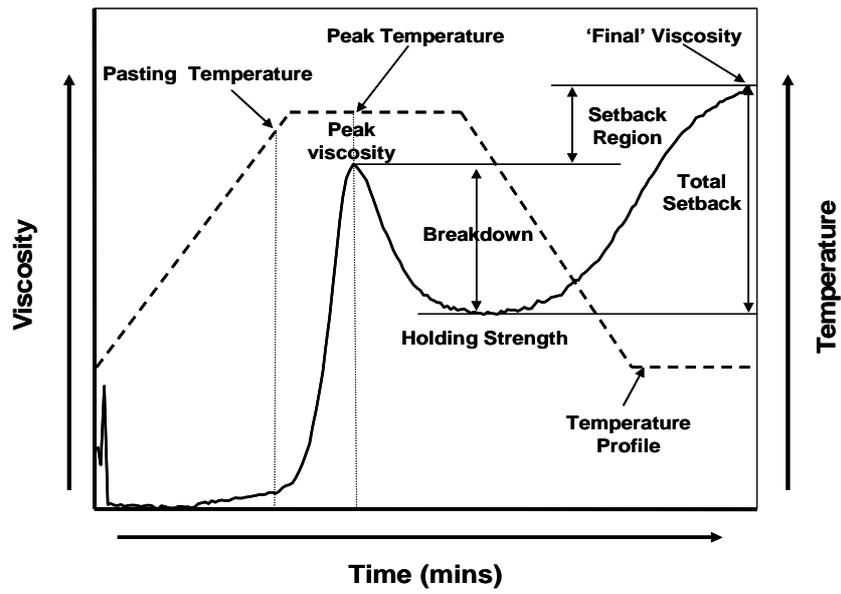
Fig. 1

Fig. 2

Fig. 3

Fig. 4

31
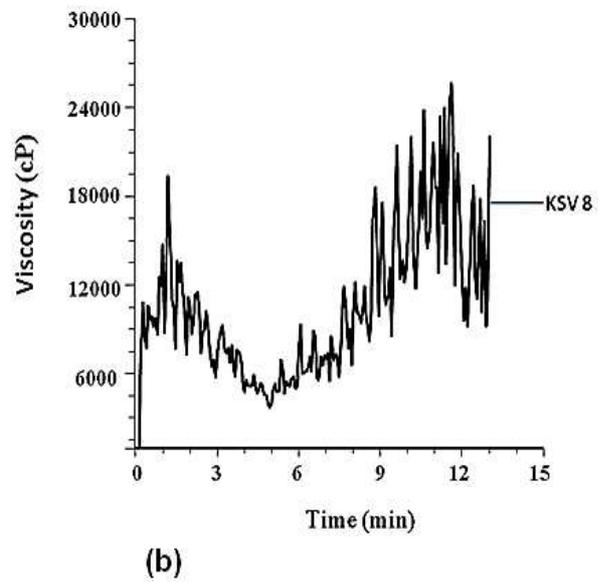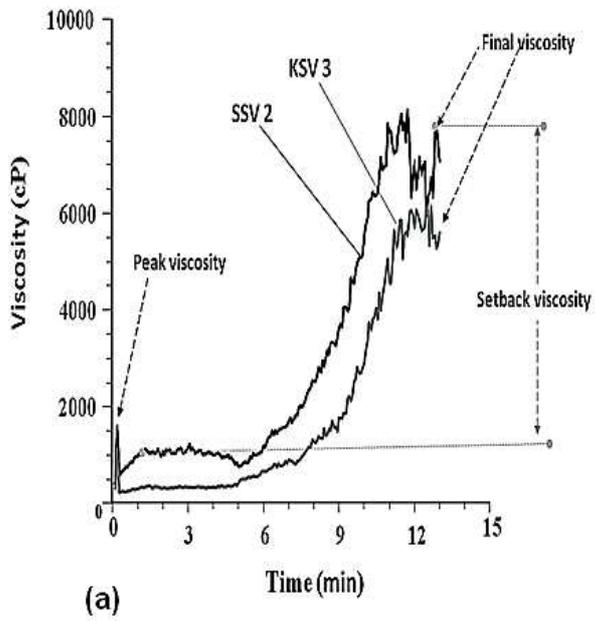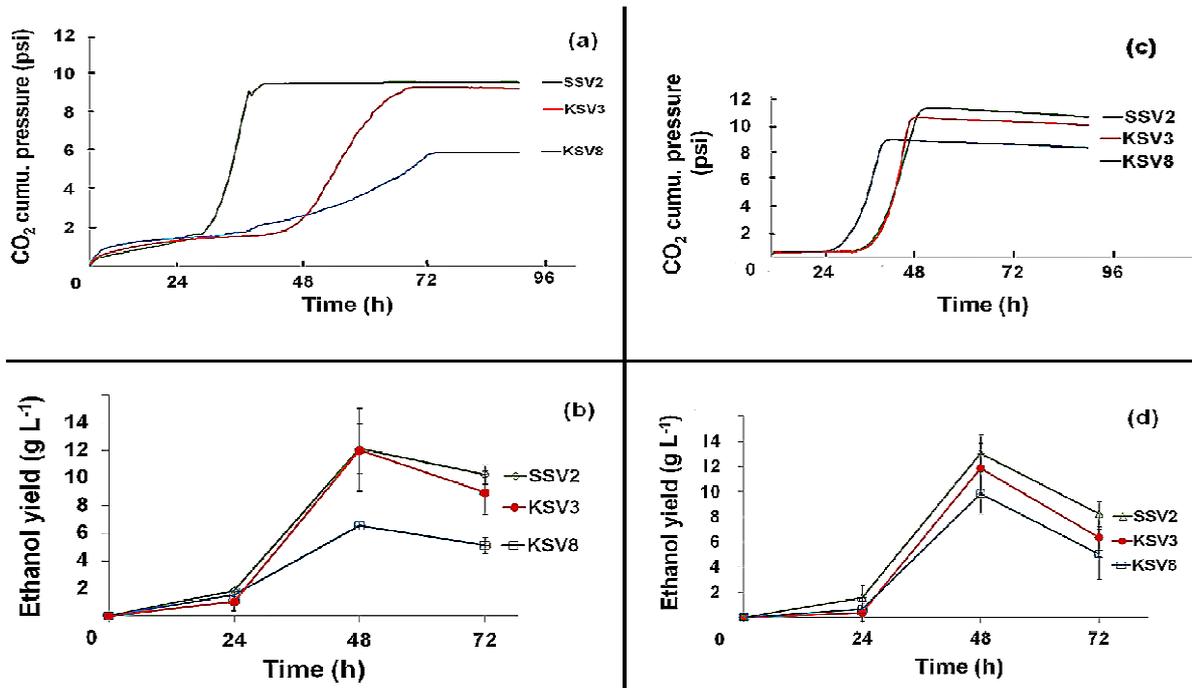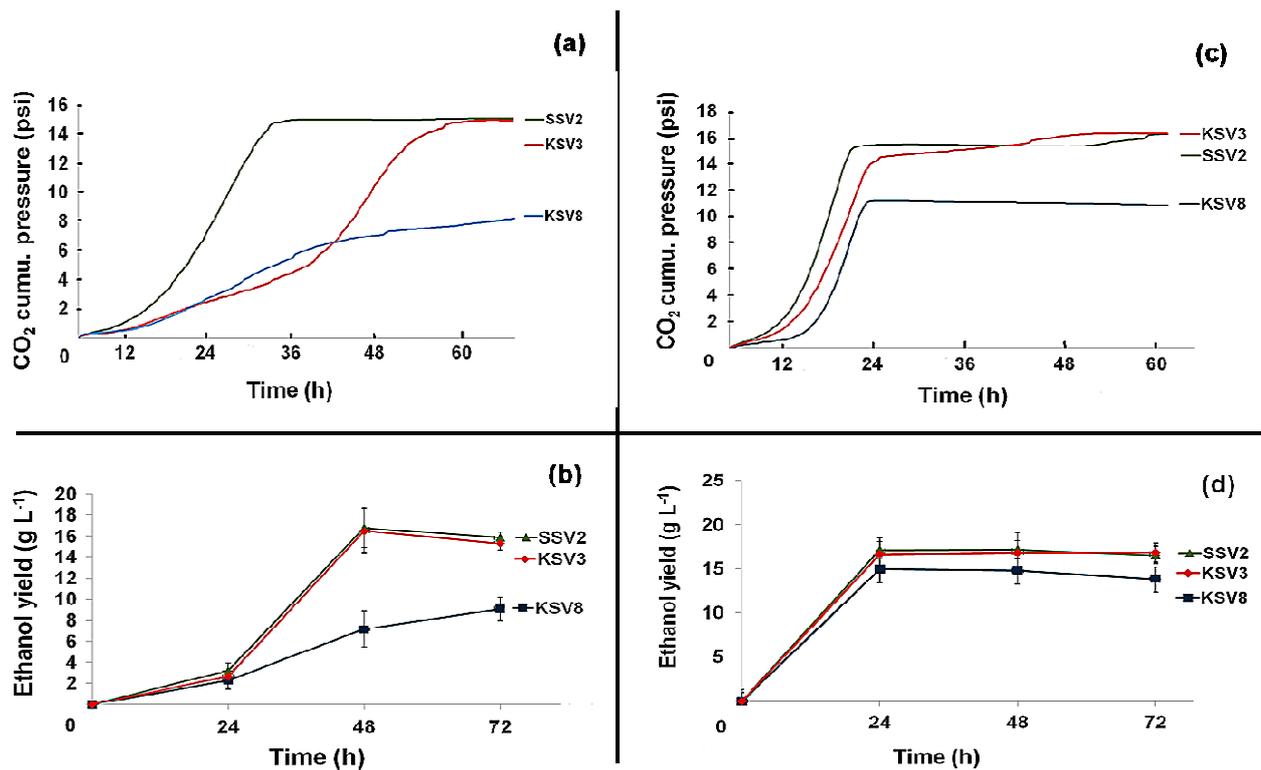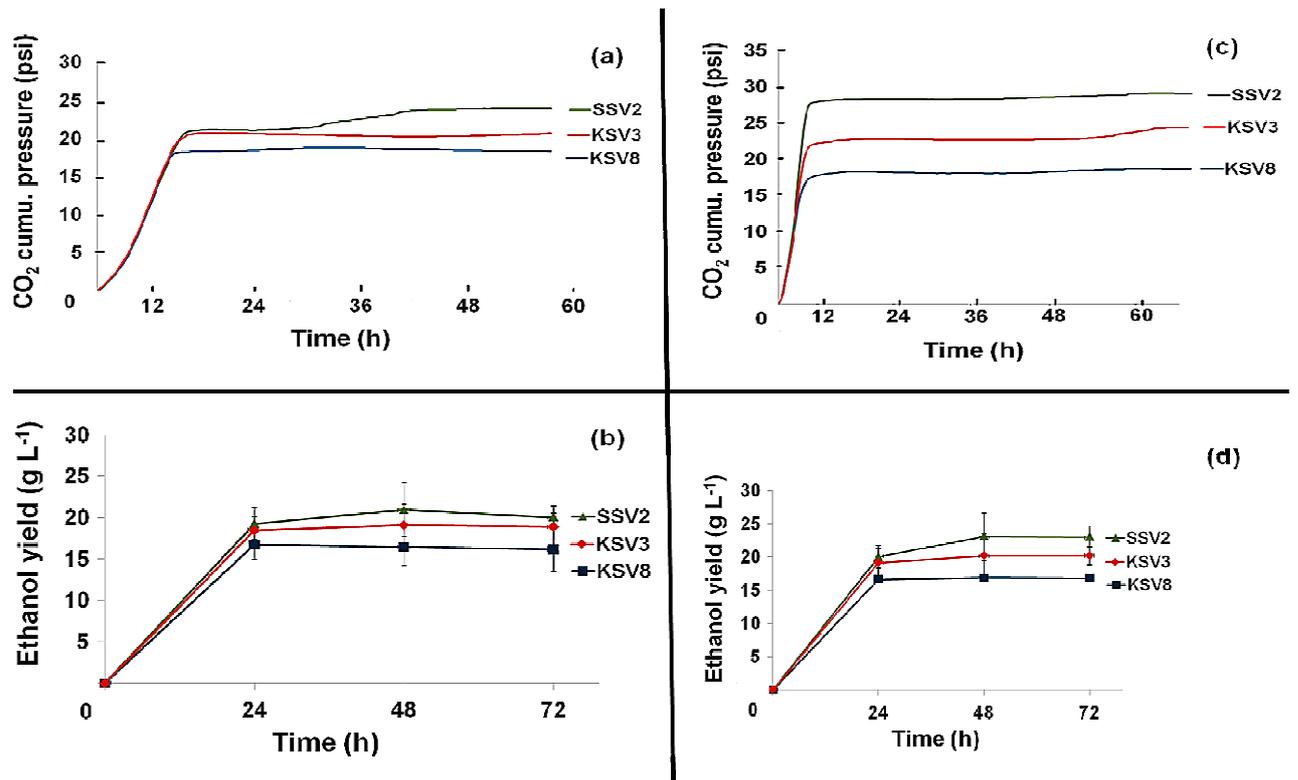
Fig. 5

Figures captions

Fig. 1 A typical RVA profile for un-malted cereals

Fig. 2 SSV2 and KSV3 sorghum bagasse viscograms. Pasting profiles were analysed using a Rapid Visco-Analyzer (RVA) in accordance to SWRI standard procedure (see materials & Methods). Table 1 provides the RVA cycle run profile. Data are std. means of duplicate experiments.

Fig. 3 KSV8 sorghum bagasse viscograms. Pasting profile were analysed by Rapid Visco-Analyzer (RVA) in accordance to SWRI standard procedure (see Materials & Methods). Table 1 provides the RVA cycle run profile. Data are std. means of duplicate experiments.

Fig. 4 SSV2, KSV8 and KSV3 sorghum bagasse fermentation kinetics. Bagasse was pre-treated with dilute H_2SO_4 followed by enzymatic hydrolysis. Hydrolysates are fermented with *S. cerevisiae* without nutrient supplementation. Fermentation progress was monitored by CO_2 formation rate using ANKOM[RF] system. Results are mean of duplicates.

Fig 5 SSV2, KSV8 and KSV3 sorghum bagasse fermentation kinetics. Bagasse was pre-treated with dilute H_2SO_4 followed by enzymatic hydrolysis. Hydrolysates are fermented with *P. tannophilus* without nutrient supplementation. Fermentation progress was monitored by CO_2 formation rate using ANKOM[RF] system. Results are mean of duplicates.

Fig 6 SSV2, KSV8 and KSV3 sorghum bagasse ethanol yields. Bagasse was pre-treated with dilute H_2SO_4 followed by enzymatic hydrolysis. Hydrolysates are fermented without nutrient supplementation by *S. cerevisiae* at $32^{o}C$ and 120 rpm orbital shaking. Results are std. means of duplicate experiments.

Fig 7 SSV2, KSV8 and KSV3 sorghum bagasse ethanol yields. Bagasse was pre-treated with dilute H_2SO_4 followed by enzymatic hydrolysis. Hydrolysates are fermented without nutrient supplementation by *P. tannophilus* at 32°C and 120 rpm orbital shaking. Results are std. means of duplicate experiments.

Fig. 8 SSV2, KSV8 and KSV3 sorghum bagasse fermentation kinetics. Bagasse was pre-treated with dilute H_2SO_4 followed by enzymatic hydrolysis and $Ca(OH)_2$ over-limed. Hydrolysates were fermented with *S. cerevisiae* without nutrient supplementation. Fermentation progress was monitored by CO_2 formation rate using ANKOM^RF system. Results are mean of duplicates.

Fig 9 SSV2, KSV8 and KSV3 sorghum bagasse fermentation kinetics. Bagasse was pre-treated with dilute H_2SO_4 followed by enzymatic hydrolysis and $Ca(OH)_2$ over-limed. Hydrolysates were fermented with *P. tannophilus* without nutrient supplementation. Fermentation progress was monitored by CO_2 formation rate using ANKOM^RF system. Results are mean of duplicates.

Fig. 10 SSV2, KSV8 and KSV3 sorghum bagasse ethanol yields. Bagasse was pre-treated with dilute H_2SO_4 followed by enzymatic hydrolysis and over-liming with $Ca(OH)_2$. Hydrolysates are fermented without nutrient supplementation by *S. cerevisiae* at 32°C and 120 rpm orbital shaking. Results are std. means of duplicate experiments.

Fig. 11 SSV2, KSV8 and KSV3 sorghum bagasse ethanol yields. Bagasse was pre-treated with dilute H_2SO_4 followed by enzymatic hydrolysis and over-liming with $Ca(OH)_2$. Hydrolysates are fermented without nutrient supplementation by *P. tannophilus* at 32°C and 120 rpm orbital shaking. Results are std. means of duplicate experiments.

Fig. 12 SSV2, KSV8 and KSV3 sorghum bagasse fermentation kinetics. Bagasse was pre-treated with dilute H_2SO_4 followed by enzymatic hydrolysis, $Ca(OH)_2$ over-liming and charcoal filtration. Hydrolysates are fermented with *S. cerevisiae* without nutrient supplementation. Fermentation progress was monitored by CO_2 formation rate using ANKOM^RF system. Data are mean of duplicates.

Fig 13 SSV2, KSV8 and KSV3 sorghum bagasse fermentation kinetics. Bagasse was pre-treated with dilute H_2SO_4 followed by enzymatic hydrolysis, $Ca(OH)_2$ over-liming and charcoal filtration. Hydrolysates are fermented with *P. tannophilus* without

nutrient supplementation. Fermentation progress was monitored by CO_2 formation rate using ANKOM[RF] system. Data are mean of duplicates.

Fig. 14 SSV2, KSV8 and KSV3 sorghum bagasse ethanol yields. Bagasse was pre-treated with dilute H_2SO_4 followed by enzymatic hydrolysis, over-liming with $Ca(OH)_2$ and charcoal filtration. Hydrolysates were fermented without nutrient supplementation by *S. cerevisiae* at 32°C and 120 rpm orbital shaking. Results are std. means of duplicate experiments.

Fig. 15 SSV2, KSV8 and KSV3 sorghum bagasse ethanol yields. Bagasse was pre-treated with dilute H_2SO_4 followed by enzymatic hydrolysis, over-liming with $Ca(OH)_2$ and charcoal filtration. Hydrolysates were fermented without nutrient supplementation by *P. tannophilus* at 32°C and 120 rpm orbital shaking. Results are std. means of duplicate experiments.