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Bioconversion of degraded husked sorghum grains to ethanol

Abstract: Efficient starch saccharification is an essential step towards achieving improved ethanol yields by fermentation. Sorghum grains are important starch sources for bioconversion to ethanol. In the present study, disease degraded (spoilt) husked grains from Nigerian sorghum cultivars were obtained from field sites and subjected to bioprocessing to ethanol. The crude husked grains (comprising husks, spikelet, awn, rachis and pubescence materials) were hammer milled and each meal separately mashed with enzyme cocktails comprising amylase, glucanase and protease enzymes. The saccharified worts obtained were then fermented with the yeasts, *Saccharomyces cerevisiae* and *Pichia stipitis* (aka *Scheffersomyces stipitis*), without exogenous nutrient supplementation. Sugars liberated during mashing were determined and it was found that enzymatic hydrolysis of milled sorghum grains was effective in yielding favourable levels of fermentable sugars up to 70g sugar/100g substrate with one particular cultivar (KSV8). Ethanol and carbon dioxide production was measured from subsequent trial fermentations of the sorghum mash and it was found that *S. cerevisiae* produced ethanol levels equating to 420 L/t that compares very favourably with yields from wheat and barley. Our findings show that crude degraded sorghum grains represent favourable low-cost feedstocks for bioconversion to ethanol with reduced energy input and without additional costs for nutrient supplementation during fermentation. Consequently, our results suggest some economic benefits could be derived from spoilt or degraded sorghum grains.

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1 Introduction

Continued use of fossil-based fuels as transportation energy sources significantly contributes towards emission of greenhouse gases worldwide [1]. Carbon dioxide is a highly potent greenhouse gas that is liberated from combustion of fossil fuels and is a precursor gas to global warming [2]. In contrast, bioethanol, a plant-based liquid fuel that is used as an additive or substitute to petroleum, is more environmentally sustainable. Bioethanol may be produced from plant stalk juices, starchy grains and lignocellulosic biomass [3]. The diversion of starch and sugar biomass for bioethanol production raises serious food security concerns worldwide. The technology for bioconversion of sugary plant juices and starch to ethanol is already well established and is commercially viable [4], whilst ethanol from lignocellulosic feedstocks has only recently progressed from laboratory and pilot scale to commercial plant operations [2].

Typical starch sources for commercial bioethanol production include corn, cassava and sorghum grain. Sorghum grains may come in various colours and shapes, depending on the crop cultivar type and growing conditions. Sorghum grain is the second most important cereal in Africa and 5th worldwide. It is cultivated on over 45 million hectares of global farmland with annual production estimated at over 60 million metric tonnes. It is a major source of staple food to over 500 million people in Asia and Africa [4,5]. Sorghum crops show high resistance to a variety of fungal and bacterial diseases but are susceptible to grain smut and mould infestation. Additionally, pests such as grasshoppers, rodents and

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quelea birds constitutes major sources of grain losses in farmland and/or in post-harvest storage facilities [6]. Nigeria is the world's second largest sorghum producer with over 10 million metric tonnes output annually [1], but over 15% of the crop may be lost to microbial disease infections and/or pest attacks [5]. Disease degraded grains, along with residues from pest invaded farmlands and storage facilities are not fit for either human or livestock consumption [7]. Farmers therefore suffer serious economic losses as a direct consequence of disease and pest attack on their farm produce.

In the present study, we envisaged that, if degraded sorghum grains could have some commercial value as a biofuel feedstock, they may serve as additional "economic bailout" for farmers (especially peasant farmers) whose produce are otherwise deleteriously affected.

Previous studies have investigated various mashing methodologies for improved liberation of sugars and free amino nitrogen from threshed/hulled sorghum grains [2,3,8]. These included steeping of grains with water, grain malting, and cooking with various enzyme cocktails. Additionally, previous studies have further investigated various fermentation techniques that included very high gravity fermentation (VHG), fermentation substrates supplementation with external nitrogen sources, and use of improved yeast strains [8-11]. However, there have been few studies into the potential for bioconversion of degraded husked sorghum grains to ethanol [5,7,12,13]. We investigated the potential of utilising degraded grains from different Nigerian sorghum varieties for bioethanol production, and focused on employing optimised methodologies for mashing and fermentation. The yeasts, *Saccharomyses cerevisiae* and *Pichia* (*Scheffersomyces*) *stipitis* are utilised in this study. This was because, while *S. cerevisiae* cells are only able to efficiently ferment glucose, *P. stipitis* can ferment both glucose and xylose. However, *S. cerevisiae* cells exhibit tolerance for high alcohol concentration during fermentation relative to *P. stipitis* [14].

2 Methods

Fresh crop heads of Nigerian SSV2, KSV8 and KSV3 sorghum varieties were obtained, courtesy of the National Horticultural Research Institute (NIHORT, Nigeria). Grains with smut/mold infections were observed on the grain samples. The crude husked grains (comprising husks, spikelet, awn, rachis and pubescence materials) were manually removed from the crop heads. Without further threshing or sorting, the husked grains were hammer milled (Ohaus®, Switzerland) and the resultant flours prepared for further analysis.

2.1 Crude flour compositional analysis

The total protein, lignin and starch contents of the crude floured samples were determined as discussed below. Pasting properties of the starch from the floured samples were also evaluated.

2.1.1 Protein determination

Crude protein was determined by digesting the crude floured samples with NaOH solution [15,16]. The albumin, globulin, glutelin and kafirin protein fractions of the samples were determined using a modified Osborne-Mendel method. The difference between the crude protein value and cumulative value of the albumins, globulins, glutelins and kafirins represents the "residual proteins" value in this study:

Residual proteins = Total crude proteins - Σ(albumins, globulins, glutelins, kafirins).

a. Albumins, globulins, glutelins and kafirins determination:

Crude flour samples (2 g dry wt.) of SSV2, KSV8 and KSV3 sorghum cultivars were separately added into Erlenmeyer conical flasks containing distilled water (30 mL). The slurries were thoroughly mixed followed by incubation in a rotating shaker at 120rpm and 65°C for 90 min. The resultant slurries were centrifuged at 3800 rpm for 10min. The suspended solution (containing solubilised albumin) were collected by decantation and refrigerated. The flask's bottom residues were washed by re-suspension in distilled water and the final residues retained in the respective conical flasks for globulin extraction. For globulin extraction, 0.5M NaCl solution (30 mL) was added into the retained flask's bottom residues. The mixtures were thoroughly stirred and incubated in a rotating shaker at 120 rpm and 65°C for 90 min. The final slurries were centrifuged at 3800 rpm for 10 min and the supernatants (containing solubilised globulin) decanted into flasks and refrigerated. The resultant bottom residues obtained were washed with distilled water and retained for analysis. These procedures were subsequently repeated twice to determine glutelin and kafirin protein contents. While 0.1 M NaOH solution (30 mL) was added to resultant flask bottom residues to obtain glutelin, 70%v/v ethanol solution (30 mL) was added to obtain kafirin. Subsequently the concentrations of albumins, globulins, glutelins and kafirins in each collected supernatant

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solution were determined by Bradford™ Coomassie reagent (Sigma-Aldrich, UK) in accordance with the manufacturer's protocol.

b. Crude proteins determination

The SSV2, KSV8 and KSV3 sorghum flours (2 g dry wt.) were added into 2M NaOH solution (50 mL), respectively. The mixtures were thoroughly stirred followed by incubation in a rotary shaker at 120 rpm and 60°C for 2 h. The final slurries were centrifuged at 3800 rpm for 10 min. The suspended solutions (containing solubilised crude protein) were collected by decantation and crude protein content in the supernatant determined by Bradford™ Coomassie reagent (Sigma-Aldrich, UK).

2.1.2 Total lignin

Total lignin refers to the sum of Klason lignin, also known as acid soluble lignin (ASL), and acid insoluble lignin (AIL). Lignin contents of the crude flour samples were determined by a modified Aldaeus and Sjöholm [17] method, as follows.

Acid soluble lignin (ASL) Crude flours from sorghum cultivars SSV2, KSV8 and KSV3 (1 g dry wt.) were each added into respective conical flasks containing 72%v/v H_2SO_4 acid solution (10 mL). The final slurries were incubated in a rotating shaker at 120 rpm and 30°C for 45 min. Afterwards, 250 mL distilled water was added into the resultant mixture and further incubated in a rotating shaker at 120 rpm and 65°C for 2 h. Finally, the mixtures were centrifuged at 3800 rpm for 10 min. The suspended solutions (containing acid soluble lignin i.e. ASL) were collected prior to transferring aliquots (2 mL) into quartz cuvettes for absorbance readings at 205 nm with a Genesys™ spectrophotometer (Thermo Spectronic®, USA).

The retained bottom residues (from above) were oven-dried at 60°C for 48 h. The weight of the dried residue was measured and the difference between the measured weight of residue and observed ash content is the acid insoluble lignin (AIL).

Thus, the total lignin is the sum of acid soluble lignin (ASL) and acid insoluble lignin (AIL) i.e. (Total lignin = ASL + AIL).

2.1.3 Total starch determination

Sorghum flour starch content was determined using a Megazyme™ K-TSTA kit, in accordance to the supplier standard protocol [18].

2.1.4 Starch pasting properties

The starch pasting properties of the crude flours were determined by RVA-4™ Rapid Visco Analyzer (Newport Scientific, Australia). Modified Scotch Whisky Research Institute Edinburgh (SWRI) standard procedure was followed.

Briefly, crude flour (2.9 g) was added into canisters containing distilled water (25.1 g). The suspension was homogenised by thoroughly stirring with a glass rod at room temperature. A paddle was placed into the canister and afterwards inserted into the Rapid Visco-Analyser for analysis. The instrument was allowed to pre-heat to 50°C. The total analysis cycle time was set at 15 min. Typical RVA cycle profile is provided in Table 1.

2.1.5 Starch enzymatic hydrolysis

Crude sorghum flour samples (30 g dry wt.) were mixed with distilled water (70 mL) in conical flasks and slurries cooked in a water bath at 80°C for 60 min with constant stirring. Additional 50 mL distilled water was added into the mixture and autoclaved at 121°C for 15 min. The final slurries were each divided into two equal halves respectively and prepared for enzymatic hydrolysis (mashing).

Two separate batches of enzyme cocktails were prepared from the commercially available enzymes shown in Table 2 (courtesy of Kerry Biosciences, Ireland). Batch-1 enzyme cocktail comprised promalt™ 295 (50 µL), bioglucanase™ ME1250L (50 µL) and hitempase™ 2XL (50 µL). Similarly, Batch-2 comprised promalt™ 295

Table 1: RVA run temperature 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.

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Table 2: Composition of hydrolytic enzyme supplements for mashing.

Notation	Enzyme	Activity	Description
E1	Promalt™ 295	500BG[a] μ/mL-min	*β-glucanase/β-amylase/protease*
E2	Bioglucanase™ ME1250L	750BG[a] μ/mL-min	*α-glucanase*
E3	Hitempase™ 2XL	4416 μ/mL-min	*α-amylase/β-glucanase*
E4	Promalt™ 4TR	300BG[a] μ/mL-min	*β-glucanse/β-amylase/protease*
E5	Termamyl® 120L	120 KNU[b]/g	*α,β-amylase*

Enzymes generously provided by Kerry Biosciences, Ireland.
[a]betaglucanase unit/mL
[b]Kilo Novo α-amylase Units (KNU)

(30μL), bioglucanase™ ME1250L (30 μL), hitempase™ 2XL (30 μL), promalt™ 4TR (30 μL) and termamyl® 120L (30 μL) respectively. The prepared enzyme cocktail was each added into mashing substrates and the final volume adjusted to 200 mL with distilled water. Finally, the mashes were incubated in a rotary shaker initially at 50°C (optimum glucanases enzymatic activities temp, according to supplier) for 120 min at 120 rpm. The, samples were further incubated at 60°C (optimum temperature for amylolytic enzymatic activities) for 60 min at 130 rpm (to further improve enzyme-substrate contact). Test samples (5 mL) were withdrawn from the final worts and filtered through Luer-Lok™ micro syringe (Chromacol, USA). For the analysis of liberated sugars, filtered samples (1 mL) were added into glass vials (Chromacol, USA) containing 1 mL of HPLC calibrated internal standard sugar (Meso-Erythritol solution). The vials were placed in a HPLC autosampler (Spectra-physics, USA) and sugars were separated with a 300 mm × 7.8 mm REZEX RPM-monosaccharide Pb+2 (8%) column™ (Phenomenex, USA). The sugars were detected using refractive index and quantified using HPLC software (CSW32 version v.1.4 chromatogram software from DataApex®, USA). Total free amino nitrogen (FAN) levels liberated in wort were determined by combination of K-LARGE kit (for determination of L-arginine/Urea/Ammonia) and K-PANOPA kit (for determination of total Primary Amino Nitrogen) respectively (Megazymes, Ireland), in accordance to the manufacturer's protocol.

2.2 Yeast seed culture preparation

Three sterile loopfuls of industrial yeast strain *Saccharomyces cerevisiae (DCLM* strain, courtesy of Kerry Biosciences, Menstrie, Scotland) and *Pichia* (*Scheffersomyces*) *stipitis* (NCYC1416) were each inoculated into separate 400 mL YEPD media respectively. Each prepared media comprised 4%(w/v) bacteriological peptone, 2%(w/v) yeast extract and 4%(w/v) glucose. The YEPD cultures were incubated at 32°C for about 20 h at 150 rpm in a shaking incubator.

2.3 Mash fermentation

Two fermentation experiments were set-up and ran in parallel. One employed the ANKOM™ system (ANKOM[RF] Technology, USA) monitors rate of CO_2 evolution as fermentation progress. The other employed shake flask fermentations to observe ethanol yields. Samples were withdrawn from the flasks every 24 h for ethanol determination using a FermentoFlash® (Funke-Gerber™, Berlin).

Total CO_2 gas volume liberated by fermentation was evaluated using cumulative CO_2 gas pressure and corresponding temperature data obtained from ANKOM[RF] system readings. Van der Waals gas law equation (i.e. $PV = nRT$) was used to calculate total CO_2 gas volume produced per substrate mass fermented (on dry basis). In this context, P = cumulative measured CO_2 gas pressure, T = corresponding measured temperature at peak of CO_2 pressure, R = Universal gas constant, V = ANKOM[RF] bottle rated volume, n = number of CO_2 gas moles liberated.

After mashing of the different sorghum cultivar's husked grains, filtered mash (10 mL) was added into a 250 mL ANKOM[RF] glass bottles and 250 mL conical flasks respectively. *S. cerevisiae* (DCLM) yeasts were inoculated at a pitching rate of $1.0{\times}10^7$ cells/mL into the ANKOM[RF] bottles and shaking flasks respectively. Both the ANKOM[RF] and shaking flask fermentation were conducted in a rotary shaking incubator (at 130 rpm and 32°C).

2.4 Statistical analyses

Significant differences between means was tested by ANOVA using the Tukey method by Minitab™ 16 statistical

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software (MINITAB©, USA). Means that do not share a superscript letter (a-f) within same rows are significantly different ($p \leq 0.05$) based on grouping information using the Tukey method at 95% simultaneous confidence interval.

3 Results and discussion

3.1 Sorghum grains composition

While SSV2 and KSV3 sorghum grains are brown coloured, KSV8 grain is white. Additionally, Table 3 shows that the chemical compositions of the husked grains were significantly different ($p < 0.05$). For example, KSV8 had significantly higher albumin and globulin proteins compared to KSV3 and SSV2. These proteins are highly amenable to proteolysis relative to glutelin and kafirin. Generally, proteins are the nitrogenous sources for effective yeast metabolism during fermentation [19,20]. Overall, KSV3 had the higher total protein and starch content compared with SSV2 and KSV8 substrates. Regarding pasting profiles, KSV3 and KSV8 showed similar viscogram profiles, but the former showed higher final and peak viscosities than the latter (Fig. 1). Starches with higher final and peak viscosities are normally considered to contain higher amylose contents compared to corresponding starch substrates with lower values [21]. Sorghum starch typically comprises 20-30% amylose and 80-70% amylopectin [22]. Finally, SSV2 starch exhibited comparatively different pasting profiles and low setback viscosities. The pasting characteristics of SSV2 may be due to lignin interference during pasting [16,23]. Table 3 shows that SSV2 had significantly higher lignin content than both KSV8 and KSV3 substrates.

Regarding SSV2, KSV8 and KSV3 sorghum cultivar mashings, the SSV2 control substrate showed significantly higher latent hydrolytic enzyme activity

Table 3: Physico-chemical composition of SSV2, KSV8 and KSV3 sorghum flours.

Parameter	SSV2	KSV8	KSV3
Moisture content (%)	$7.21^a \pm 0.55$	$9.86^b \pm 0.34$	$8.77^a \pm 0.41$
Ash content (%)	$2.81^a \pm 0.10$	$1.31^b \pm 0.07$	$2.25^c \pm 0.09$
Total lignin (g/100g flour)	$13.10^a \pm 0.13$	$10.58^c \pm 0.46$	$11.96^b \pm 0.51$
Total starch (g/100g flour)	$65.64^a \pm 1.67$	$69.87^b \pm 1.34$	$73.42^c \pm 1.86$
Total proteins (g/100g flour)	$\mathbf{15.57^a \pm 0.79}$	$\mathbf{14.31^a \pm 0.88}$	$\mathbf{16.38^b \pm 1.12}$
Albumins	$1.31^a \pm 0.74$	$1.99^b \pm 0.46$	$1.56^b \pm 0.17$
Globulins	$2.47^a \pm 0.31$	$3.50^b \pm 0.55$	$2.63^a \pm 0.67$
Glutelins	$2.78^a \pm 0.68$	$1.46^b \pm 0.39$	$2.70^a \pm 0.11$
Kafirins	$6.35^a \pm 0.53$	$5.08^b \pm 0.72$	$5.90^b \pm 0.52$
Residuals	$2.66^a \pm 0.87$	$2.29^a \pm 0.69$	$3.69^b \pm 0.88$

Means on the same row that do not share same superscript letter (a-c) are significantly different ($p \leq 0.05$) by ANOVA using Tukey grouping method test.

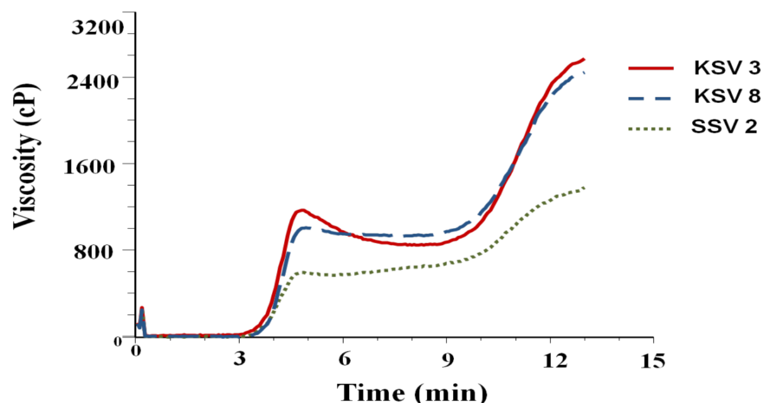

Figure 1: SSV2, KSV8 and KSV3 sorghum crude grains pasting profile. The crude grains comprise husks, awn, rachis and spikelet (Table 1 provides the RVA temperature profile). Values are mean of 2 replicates runs.

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by liberating more sugars [21], while KSV8 control substrate favoured higher proteolytic enzyme activity (Table 4). The significantly higher FAN level of KSV8 mash may largely be attributed to combinations of its relatively higher albumin and globulin protein contents [23]. However, on digestion of the substrates with Batch-1 enzyme cocktails, notable increases in levels of glucose and FAN were observed (Table 4). The KSV8 mash consistently showed significantly higher levels of liberated FAN and sugars compared to either SSV2 or KSV3 mashes. These results suggest that mashing of these substrates with exogenous enzymes may improve hydrolysis efficiency by over 50% over endogenous enzyme activities. Although supplementation with Batch-2 enzyme cocktail, that contains additional thermostable amylases and proteases, yielded higher levels of FAN and sugars (Table 4) the costs of using additional enzymes in this cocktail may have an adverse impact on the overall economics of the process. While amylases further degrade starch to monomeric sugars, proteases breakdown complex protein polymers to simple molecules such as free amino acids and peptone [16,23]. However, no significant increase was observed in concentration level of xylose, this may be due to the lack of exogenous hemicellulase enzymes in either of the prepared enzyme cocktails. Xylose sugars are considered to originate predominantly from husk and pubescence materials of the grain components.

3.2 Fermentation

Saccharomyses cerevisiae cells are efficient glucose fermenting yeast but limited in their ability to ferment xylose. However, *Pichia* (*Scheffersomyces*) *stipitis* are able to efficiently ferment both xylose and glucose [14]. Nevertheless, while *S. cerevisiae* cells exhibit tolerance for high alcohol concentration during fermentation, *P. stipitis* growth is restricted at alcohol levels beyond 33 g/L [14,24]. Thomas and Ingledew [25] suggested a minimum of 150 mg/L of FAN is required for efficient yeast metabolism during fermentation. The SSV2, KSV8 and KSV3 Batch-1 hydrolysates have observed FAN levels of over 150 mg/L. Thus, both *S. cerevisiae* and *P. stipitis* yeasts showed similar fermentation kinetics at commencement of fermentation. The fermentation progress appeared relatively sluggish during early stages. However, yeast cells appeared to reach steady growth phase about 4 h after start of fermentation (Fig. 2a). As fermentation progresses into later phases, *S. cerevisiae* appeared to out-perform *P. stipitis* in terms of cumulative CO_2 liberation, particularly with KSV8 as substrate (Fig. 2a). Perhaps this was due to the sensitivity of *P. stipitis* to higher ethanol concentrations, which may reduce the cells ability to efficiently utilise available xylose when glucose was depleted [26,27]. While both *S. cerevisiae* and *P. stipitis* produced similar volumes of CO_2 at end of fermentation with SSV2 as substrate, the former produced higher CO_2 than the latter with KSV8 as substrate (Table 5). This may be linked to the poor tolerance of *P. stipitis* to high ethanol concentration compared to *S.*

Table 4: SSV2, KSV8 and KSV3 substrate mashing with Batch-1 and Batch-2 enzyme cocktail supplementation.

Parameter	SSV2	KSV8	KSV3	SSV2	KSV8	KSV3	SSV2	KSV8	KSV3
	(Control sample)*			(Batch-1 enzyme cocktail)**			(Batch-2 enzyme cocktail)***		
FAN (mg/L)	78.52^{d} ± 1.8	88.35^{e} ± 1.61	60.62^{f} ± 0.99	147.28^{d} ± 1.8	159.09^{e} ± 1.9	154.20^{f} ± 2.1	159.35^{a} ± 1.88	173.34^{b} ± 1.25	167.29^{c} ± 1.41
Glucose (g/100g flour)	4.91^{d} ± 0.85	4.41^{d} ± 0.53	3.56^{f} ± 0.14	22.20^{a} ± 1.16	27.66^{c} ± 1.35	20.39^{a} ± 1.04	24.22^{a} ± 1.77	33.34^{b} ± 1.13	34.07^{b} ± 0.86
Maltose (g/100g flour)	10.60^{c} ± 1.1	10.10^{c} ± 0.87	8.78^{d} ± 0.69	18.89^{c} ± 1.23	21.06^{d} ± 1.07	23.20^{e} ± 2.01	33.63^{a} ± 2.03	32.75^{a} ± 1.89	29.19^{b} ± 1.11
Xylose (g/100g flour)	2.56^{c} ± 0.31	2.28^{c} ± 0.66	2.93^{a} ± 0.42	3.38^{b} ± 0.41	3.75^{b} ± 0.25	5.19^{c} ± 0.38	3.46^{a} ± 0.41	3.67^{a} ± 0.24	5.48^{b} ± 0.72
Total sugars (g/100g flour)	18.06^{d} ± 0.7	16.65^{e} ± 0.83	15.27^{e} ± 0.93	44.46^{c} ± 2.55	52.47^{d} ± 1.40	48.78^{e} ± 2.44	61.31^{a} ± 1.31	69.76^{b} ± 1.40	68.74^{b} ± 2.45

*Control samples are mashed without exogenous enzymes supplementation. **Batch-1 and ***Batch-2 enzyme cocktail comprises of freshly prepared commercial enzyme solutions presented in Table 3. Means on the same row that do not share same superscript letter (a-f) are significantly different ($p \leq 0.05$) by ANOVA using Tukey grouping method test.

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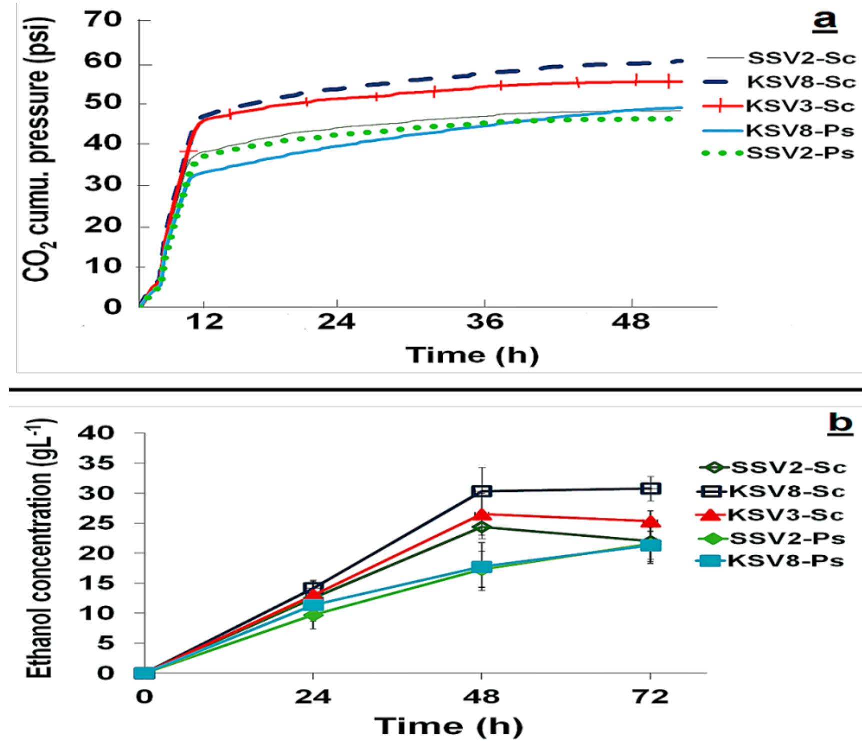

Figure 2: Fermentation profiles of SSV2, KSV8 and KSV3 substrates mashed with Batch-1 enzyme cocktail. **(a)** Cumulative CO_2 formation rate from fermentation of SSV2, KSV8 and KSV3 husked grains. **(b)** Represents corresponding observed ethanol yields of SSV2, KSV8 and KSV3 husked grains substrates. Data are mean of triplicate experiments. NOTE: **Sc** = *S. cerevisiae* yeast and **Ps** = *P. stipitis* yeast fermentations respectively.

Table 5: Batch-1 mashes fermentation yields.

Parameter	**SSV2**	**KSV8**	**KSV3**	**SSV2**	**KSV8**
	Fermented by *S. cerevisiae*			**Fermented by *P. stipitis***	
Ethanol yield (L/t)	$206.74^{a} \pm 2.0$	$260.12^{b} \pm 1.8$	$224.53^{c} \pm 1.7$	$185.89^{d} \pm 2.1$	$183.69^{d} \pm 1.2$
CO_2 gas (kL/t)	$41.45^{a} \pm 2.31$	$51.50^{b} \pm 3.56$	$46.81^{c} \pm 2.65$	$40.89^{d} \pm 3.14$	$39.77^{e} \pm 2.78$
Glucose (g/100 g flour)	*ND	*ND	*ND	*ND	*ND
Maltose (g/100 g flour)	*ND	*ND	*ND	*ND	*ND
Xylose (g/100 g flour)	$3.06^{a} \pm 0.65$	$2.87^{a} \pm 0.43$	$4.68^{b} \pm 0.27$	$2.15^{c} \pm 0.46$	$3.26^{d} \pm 0.61$
FAN (mg/L)	$16.83^{a} \pm 0.98$	$3.36^{b} \pm 1.12$	$17.87^{a} \pm 2.0$	$17.24^{a} \pm 1.01$	$19.53^{c} \pm 2.06$

SSV2, KSV8 and KSV3 sorghum husked grain mashed with Batch-1 enzymes cocktail and fermented by *S. cerevisiae*. However, SSV2 and KSV3 mash samples were also fermented by *P. stipitis* cells, respectively. Means on the same row that do not share same superscript letter (a-f) are significantly different ($p \leq 0.05$) by ANOVA using Tukey grouping method test. *ND = Not Detected.

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cerevisiae, particularly in the context of KSV8 hydrolysates having higher ethanol concentration potential than SSV2 [27]. Furthermore, despite the ability of *P. stipitis* to utilise all available glucose, maltose and the minute fraction of xylose, its corresponding ethanol concentration remained lower than that of *S. cerevisiae* fermentation (Table 6). This may be due to tendency of *P. stipitis* cells to re-assimilate portions of ethanol it produces in late fermentation phase. Hence, the cells may utilise more nutrients in cell maintenance and growth rather than ethanol production in the later fermentation phase [27,28]. From Fig. 2b, KSV8 followed by KSV3 substrates yielded most favourable ethanol by *S. cerevisiae* fermentation. Interestingly, in spite of observed residual FAN, *P. stipitis* was unable to appreciably utilise available xylose. This may be reflective of the cells impaired fermentative capacity as ethanol concentration in the media increases [28-30].

With regard to fermentation performance of Batch-2 enzyme generated worts, notable improvement was observed. This is largely because the Batch-2 substrates have improved levels of FAN and sugars over the Batch-1 substrates (Tables 4 & 5). For example, *S. cerevisiae* initial fermentation progress appeared faster with Batch-2 substrates than with corresponding Batch-1 substrates. In contrast, *P. stipitis* showed longer yeast lag phase with Batch-2 substrates relative to Batch-1 (Fig. 3a). This may be a reflection of the poor stress tolerance of *P. stipitis* compared with *S. cerevisiae*. Batch-2 substrates with higher sugars level tend to exert osmotic stress on cells than Batch-1 substrates [24,27,29]. Furthermore, the additional presence of lignin-derived phenolic compounds liberated during mashing (predominantly from husks materials) may further exacerbate environmental stress on *P. stipitis* cells [16,27,29,30]. KSV8 substrate consistently showed faster and higher fermentation rates in terms of CO_2 production by both *S. cerevisiae* and *P. stipitis* (Fig. 3a). Concerning sugar utilisation, Table 6 showed *S. cerevisiae* cells utilised all available glucose and maltose in media. However, *P. stipitis* cells were unable to utilise all available maltose. It is conceivable that as ethanol concentration approached 30 g/L (Fig. 3b), *P. stipitis* ability to biosynthesize maltase enzymes diminishes [24,27,29].

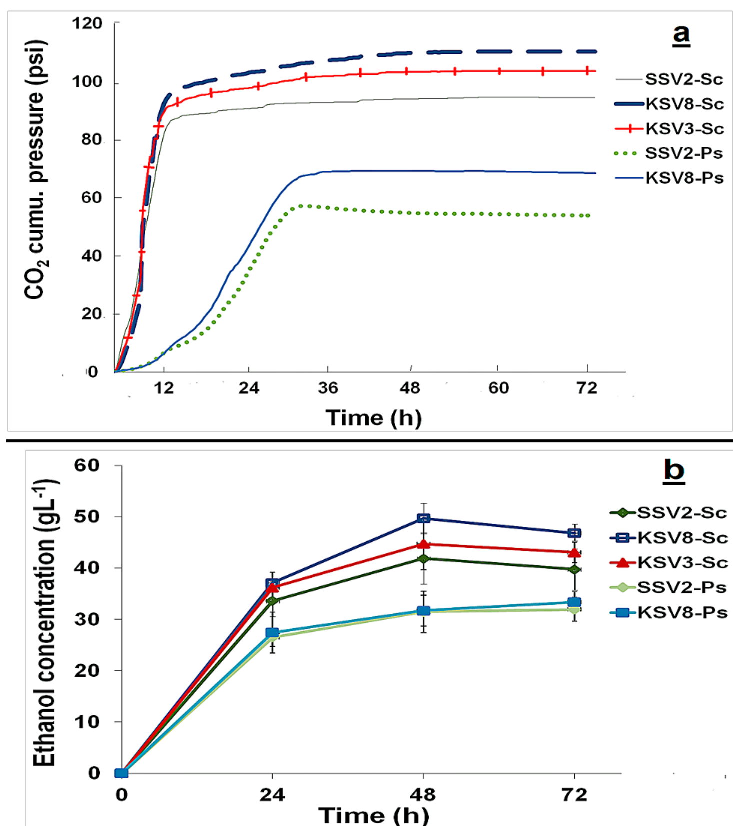

Figure 3: Fermentation profiles of SSV2, KSV8 and KSV3 substrates mashed with Batch-2 enzyme cocktail. **(a)** Cumulative CO_2 formation rate from fermentation of SSV2, KSV8 and KSV3 husked grains. **(b)** Represents corresponding observed ethanol yields of SSV2, KSV8 and KSV3 husked grains substrates. Data are mean of triplicate experiments. NOTE: **Sc** = *S. cerevisiae* yeast and **Ps** = *P. stipitis* yeast fermentations respectively.

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Table 6: Batch-2 mashes fermentation yields.

Parameter	SSV2	KSV8	KSV3	SSV2	KSV8
	Fermented by *S. cerevisiae*			Fermented by *P. stipitis*	
Ethanol yield (L/t)	354.67^{a} ± 1.8	420.89^{b} ± 2.9	378.49^{d} ± 2.0	270.66^{c} ± 1.9	272.11^{c} ± 2.3
CO_2 gas (kL/t)	80.70^{a} ± 2.34	93.90^{b} ± 1.72	88.40^{c} ± 2.34	48.60^{d} ± 2.45	58.90^{e} ± 1.98
Glucose (g/100 g flour)	*ND	*ND	*ND	*ND	*ND
Maltose (g/100 g flour)	*ND	*ND	*ND	6.85 ± 1.16	7.94 ± 1.02
Xylose (g/100 g flour)	2.74^{a} ± 0.71	2.81^{a} ± 0.53	4.59^{b} ± 0.86	2.18^{a} ± 0.51	3.11^{c} ± 0.69
FAN (mg/L)	10.31^{a} ± 0.6	8.01^{b} ± 1.0	10.43^{a}± 1.2	15.99^{c} ± 2.6	21.47^{d} ± 2.0

SSV2, KSV8 and KSV3 husked sorghum grain mashed with Batch-2 prepared enzymes cocktail and fermented by *S. cerevisiae*. However, SSV2 and KSV3 mash samples were also fermented by *P. stipitis* cells, respectively. Means on the same row that do not share same superscript letter (a-f) are significantly different ($p \leq 0.05$) by ANOVA using Turkey grouping method test. *ND = Not Detected.

The observed ethanol concentrations of SSV2 and KSV8 Batch-2 substrates were similar (at 32 g/L) with *P. stipitis* fermentation (Fig. 3b). These results correspond with previously reported ethanol concentrations of 30-35 g/L for *P. stipitis* fermentation with starch as substrates [23,31-33]. However, *S. cerevisiae* fermentation with SSV2, KSV8 and KSV3 as substrates yielded 42 g/L, 50 g/L and 45 g/L ethanol, respectively (Fig. 3b). These results corresponded to ethanol concentrations of 355 L/t, 421 L/t and 379 L/t respectively (Table 6). Wu *et al.* [32] reported similar ethanol concentration of 360 L/t for de-husked and un-malted sorghum grain. Furthermore, Sheorain *et al.* [33] reported ethanol concentrations of 380-390 L/t for various de-husked and un-malted sorghum grains. These results are similar to observed ethanol concentration of 379 L/t for KSV3 substrate reported in this study. However, previous studies have reported a favourable ethanol yield of 460-490 L/t with clean, sound and malted sorghum grains [8,34-37], which are higher values than those reported here for de-husked and un-malted KSV8 sorghum grain (421 L/t).

4 Conclusions

In this study, SSV2, KSV8 and KSV3 husked sorghum grains yielded 355, 421 and 379 L/t ethanol, respectively, following fermentation of saccharified mashes with *S. cerevisiae*. These results compare favourably with ethanol concentrations obtained from fermentation of de-husked sound sorghum grains reported in literature. Therefore, our findings indicate that spoilt/degraded sorghum grains could be utilised as a low-cost feedstock source for bioethanol production. Interestingly, the results suggest that residual or degraded grains sourced from fields or storage facilities could be utilised directly for bioethanol production without prior investment in pre-treatment process such as de-husking, threshing, steeping and malting. Exclusion of these processes prior to fermentation of substrates would not only save costs, it will reduce energy consumption that might otherwise be needed to carry out such processes. Less energy consumption is beneficial in both costs and GHG emission reductions. Finally, to achieve even greater fermentation performance of sorghum starch hydrolysates, from crude husked grains, further refinement of fermentation techniques such as high gravity fermentation, immobilised yeast fermentation and exogenous nitrogen supplementation may prove beneficial.

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Conflict of interest: Authors declare nothing to disclose.

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