The development of a biorefining strategy for the production of biofuel from sorghum milling waste

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ABSTRACT

The potential of using sorghum milling waste for the development of a biorefining strategy for the production of bioethanol was investigated. Both red and white sorghum were processed using a traditional Nigerian wet-milling process to sorghum flour. The sorghum milling waste, sorghum bran, was hydrolysed using both enzymatic and dilute acid hydrolysis to produce a generable fermentation feedstock. The hydrolysates were subsequently investigated for fermentative biofuel production. Following a hydrolysis step, a medium containing ~ 61 g/L glucose was obtained. Trace presence of inhibitors was detected in the hydrolysates and sufficient nitrogen content to support microorganism growth and bioethanol production. In test bioethanol production experiments using the sorghum milling waste derived hydrolysates only, 24.35 g/L bioethanol was produced by a yeast *Kluyveromyces marxianus*, equivalent to a yield of 0.15 gram bioethanol per gram of sorghum milling waste.

1 Introduction

In line with the desire to attain the sustainable development goal of renewable energy (SDG 7) renewable fuels are currently being used as alternatives to petroleum-based fuels in transportation. The introduction of renewable fuels decreases the negative environmental effects caused by the combustion of fossil fuels [1-3]. Bioethanol is currently the most commonly used and most important liquid biofuel. The annual production of bioethanol reached 51 billion liters in 2007 and increased to 85 billion litres in 2011 [4-5].

The first generation bioethanol is produced using glucose and starch [6-7]. However, the first generation biofuel production is unsustainable because it competes with the food cultivation uses of arable land, negatively impacts biodiversity, may contribute to deforestation and desertification, and has a high water footprint [8-9]. Consequently, efforts have been directed towards the development of second-generation bioethanol processes using feedstocks comprising agricultural, industrial and food processing wastes collectively known as lignocellulosic biomass. These feedstocks are rich in complex carbohydrates, are not considered to be food products, and have lower water footprint than energy crops and third generation biofuels from algae [8,10]. Biomass-based ethanol is the only liquid transportation fuel that does not exacerbate the greenhouse effect [4]. However, carbohydrates present in lignocellulosic biomass (i.e. cellulose and hemicellulose) are embedded within lignin, and are recalcitrant to hydrolysis into sugars due to their crystalline structure [11]. Thus a pretreatment process is required prior to enzymatic hydrolysis, such as dilute acid pretreament, alkaline pretreatment, or steam explosion pretreatments, to break lignocellulosic raw materials down to their monomeric

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sugars [12-14]. The generated sugars may then be fermented into bioethanol by several microorganisms including *Saccharomyces cerevisiae*, *Scheffersomyces stipites*, *Candida shehatae* and *Zymomonas mobilis* [4,15]. The pretreatment step increases the complexity and cost of the process, thus creating a need to develop simple, inexpensive processes to readily convert renewable feedstock into sugars for the production of higher-value products [16]. The pretreatment step can also lead to the release of compounds that can inhibit downstream chemical processes and/or inhibit the growth of microorganisms.

The wastes accumulated from the processing of cereals such as the brans of maize, rice, sorghum and wheat have also been widely used in bioprocesses [17-19]. These biomass types also contain less recalcitrant carbohydrates such as starch, which makes them potentially easier to break down into monomeric sugars. Sorghum (*Sorghum bicolor* (L.) Moench) is an annual cereal which belongs to the grass family *Poaceae*. It grows well in harsh environments where other crops grow poorly or produce poor yields and is the second most important cereal after maize in Africa [20]. Industrial dry-milling of sorghum for starch production produces waste bran, which has been explored as a feedstock, for bioethanol production [21,22]. The most common traditional processing method of sorghum in Nigeria involves steeping, wet-milling and sieving. The bran removed by this process is usually discarded or fed to animals as a low-value feed.

Nigeria is the second-largest producer of sorghum globally, with 6.5 million metric tonnes produced in the 2016/2017 agricultural year [23]. In 2018, the Federal Government of Nigeria announced plans to set up a $50 billion biofuel industry equity fund to develop the domestic biofuel sector [24], facilitating the global shift towards renewable fuels. The International Energy Agency reported that bioethanol would remain the dominant biofuel even by 2030 [25]. Hence, the use of sorghum bran as a cheap and widely available feedstock for ethanol production would considerably improve the sorghum value-chain and thus economic conditions in growing countries; in addition to other accruable benefits such as the reduction in wastes discarded into the environment and lowered greenhouse gas emissions.

Even though it has been utilised for enzyme production [26,27] and bioremediation [28], with the exception of the work of Corredor *et al.,* [22] the huge potential of sorghum bran (SB) for bioethanol production has not been sufficiently explored. Several sorghum varieties contain phenolic compounds such as tannins [29], and detannification significantly reduces peak and final viscosities of sorghum grain mashes. However, higher viscosities were shown to result in higher ethanol yields [30]. Thus, the presence of tannins in sorghum could potentially contribute to efficient ethanol production from its bran. In addition, since it has been reported that sorghum starch-based fermentation resulted in similar ethanol yields as maize [31], this work aims to investigate the suitability of sorghum bran to support yeast growth and metabolism by developing relatively inexpensive ways to produce a generic sugar-rich medium, sorghum bran hydrolysate, and subsequently determine the potential of this medium for ethanol production. Finally, the potential for bioethanol production using sorghum milling waste in Nigeria was assessed.

2 Materials and methods

2.1 Sorghum and sorghum-based biorefining process

There are several varieties of sorghum in Nigerian markets but they are generally classified as white or red sorghum with little and inconsistent variation in price. The red and white varieties of sorghum (*Sorghum bicolor)* grains were obtained from local

markets in Ilorin, Nigeria, and processed to obtain bran as described below, which is a typical processing technique for sorghum grain utilization in Nigeria.

Figure 1 shows a schematic diagram of a proposed sorghum based biorefining process. The sorghum bran is steeped and milled to separate the sorghum bran from starch. Starch is the main product while the waste sorghum bran is hydrolyzed by either enzyme or acid to generate a sugar-rich hydrolysate. The hydrolysate is then fermented to produce bioethanol via yeast fermentation. Waste yeast together with un-fermented solid residue can be used as high protein animal feed.

2.2 Bran preparation

The sorghum grains were washed twice with distilled water and steeped for two days at room temperature with a water to biomass ratio of 4:1 (v/v). They were washed again, then wet-milled in a conventional petrol-powered wet-mill until a smooth slurry was obtained. The slurry was sieved through muslin cloth to remove the starchy endosperm material. The bran residue was then air dried until a brittle, lumpy bran was obtained. These brans were comminuted in a planetary Ball Mill (Fritsch, Gmbh) to obtain a free-flowing powder. Twelve ceramic balls were added to each of two zirconium oxide pots and then filled with bran to about 75% capacity. The pots were closed and spun at 250 rpm for three cycles of 3 mins and two pauses of 2 mins (to prevent over-heating). The milled sorghum bran was stored in cool dry conditions pending use.

2.3 Sorghum bran compositional analysis

Total ash was determined according to the method of Sluiter *et al.* [32]. The total carbohydrates were determined by a two-step concentrated acid hydrolysis of the sorghum bran [33]. The reducing sugar component was determined by using the DNS method [34]. The starch content was determined using an enzymatic starch analysis kit (Megazyme®, Ireland). Structural carbohydrates were determined after the deduction of the starch's contribution to the carbohydrate content thus:

Starch glucan x 1.1 = glucose content attributable to starch   (Eqn. 1)
Glucose content attributable to cellulose = Total glucose estimated – glucose content attributable to starch   (Eqn. 2)
Cellulose = (Glucose attributable to cellulose + mannose) x 0.9   (Eqn. 3)
Hemicellulose = (Xylose + arabinose) x 0.88   (Eqn. 4)

where 1.1, 0.9 and 0.88 represent the conversion factors for starch, cellulose and hemicellulose respectively.

Lignin content was determined by the amended acetyl bromide method [35]. Protein content was determined using the Thermo Scientific FlashEA 1112 N/Protein Analyser (Thermo Fisher Scientific, UK), which estimates protein based on a complete combustion of the sample in a furnace, and then analyses the elemental gases produced.

Total lipid content was determined by gas chromatography (GC) via the direct fatty acid synthesis method. Briefly, 1.0 g of ball milled bran samples, 0.7 mL of 10 M KOH and 5.3 mL of methanol were added into a test tube. The tubes were incubated at 55ºC and vortexed at 20 min intervals for 90 min, before being transferred to an ice-water bath for 10 min to cool. Next, 0.58 mL of 12 M H_2SO_4 was added to the samples, vortexed and the incubation and cooling steps repeated once. Then, 3 mL of hexane was added to the samples and each tube vortexed for 30 sec then spun in a centrifuge (Eppendorf, U.K.) at 1,500 rpm for 5 min. The top fatty acid-hexane layer was then transferred to labelled LP4 tubes and dried by blowing nitrogen through them. The residual fatty acids were re-

suspended in 300 µL hexane, vortexed and transferred to solvent-resistant Eppendorf tubes from which 100 µL was transferred into vials with inserts and analysed by gas chromatography.

All solutions were prepared using reverse-osmosis (RO) water and all chemicals used were analytical grade purchased from either Fisher Scientific or Sigma-Aldrich. All analyses were performed in triplicate, and results are presented as mean values ± standard deviations

2.4 Sorghum Bran Hydrolyses
Two hydrolyses methods were investigated for hydrolysate production from both white and red sorghum brans.

2.4.1 Enzymatic hydrolysis
Enzymatic hydrolysis was carried out following the starch analysis method recommended by Megazyme®, Ireland with minor modifications. Briefly, a slurry of 200 g bran and 700 mL of water was prepared in duplicate in 1 L Duran bottles, followed by the addition of 100 mL dimethyl sulfoxide (DMSO). The mixture was mixed, then incubated in a shaking water bath at 95°C, 140 rpm for 15 min to gelatinize it. Next porcine pancreatic amylase (250 U/g sorghum bran, Sigma-Aldrich) in 0.2 M sodium acetate buffer (pH 6.9) was added and the slurry incubated for further 10 min. It was then cooled to 50°C. Then 250 U/g amyloglucosidase from *Aspergillus niger* (Sigma-Aldrich, UK) in 0.2 M sodium acetate buffer (pH 4.5) was added. The reaction was left to proceed for 40 min then the volume was adjusted to 1 L and reacted for a further 24 hr. Samples were collected at regular intervals, and the sugar content was analysed by HPLC.

$$\text{Glucose yield} = \frac{\text{Glucose content}}{\text{Weight of bran} \times \text{starch content} \times 1.11} \qquad \text{(Eqn. 5)}$$

2.4.2 Dilute acid hydrolysis
The dilute acid hydrolysis was carried out using either sulphuric acid or nitric acid at 1% or 3% (w/w) as follows. Exactly 500 mg of white bran (WB) or red bran (RB) was made up to a volume of 10 mL with the acids and vortexed, then autoclaved at 121°C for 15 or 30 min, respectively. The cooled slurry was vacuum-filtered through Whatman No. 1 filter paper and the sugar content was analysed by high performance liquid chromatography (HPLC).
Using the best conditions obtained from the experiment above, the effect of loading ratio on efficiency of sugar release was further investigated. Loading ratios of 5%, 10% and 20% bran (w/v) in dilute acid respectively were studied. The optimum conditions obtained were then combined for the preparation of large volumes of hydrolysates for fermentation.
All hydrolysates were stored at 4°C pending use, and filter sterilized using 0.2 μm Stericup® filters (Millipore, USA) before fermentation.

2.5 Microorganisms
Eight yeast strains with reported ethanologenic abilities [36][37] [38] were investigated in this study for the conversion of sorghum bran hydrolysate to bioethanol (Table 1).

2.6 Inoculum preparation

Frozen stock cultures of the yeasts were revived by streaking on YPD agar plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 15 g/L agar) and incubated at 30°C for 48 hr. Representative colonies were then used to inoculate 10 mL of YPD broth (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) and incubated at 30°C, 150 rpm for 48 hr. The cells were harvested by centrifugation at 17,000 x **g** for 5 min and the supernatant discarded, the pellets washed with sterile reverse osmosis water and centrifuged at 17,000 x **g**. Cells were washed three times to remove any traces of the medium before being re-suspended in 5 mL of sterile distilled water for use as inocula [36].

2.7 Yeast screening tests

Spot plate screening was performed according to the method of Greetham *et al.* [36] with the modification that in this work, sorghum bran hydrolysate replaced YNB and glucose in the original medium. A cell suspension with OD_{600} of 1.0 was serially diluted by ten-fold stages and a 5 µL aliquot of each strain was aseptically transferred on to a plate of sorghum bran agar. Four plates of each hydrolysate were prepared. The plates were left undisturbed until the spots were completely dry, two plates of each hydrolysate were then incubated aerobically or anaerobically at 30°C, for 72 hr. Anaerobic conditions were generated by placing each plate in a PM gas bag (Biolog, USA) which were then vacuum packed in an Audion VMS43 vacuum chamber (Audion Elektro BV, Netherlands). The plates were then photographed using a Bio-Rad-transilluminator (Bio-Rad, Cambridge, UK).

For phenotypic microarray (PM) analysis, the transmittance of the cell suspension was measured using a Biolog turbidimeter (Biolog, USA) and adjusted to 62% (approximately 5×10^6 cells/mL) with sterile RO water. For spot plate assay, suspensions were serially diluted in sterile RO water in cuvettes. They were covered with Nescofilm (Fisher Scientific, UK), inverted severally to mix and their absorbances were read at 600 nm with sterile RO water as the blank. The cell concentrations were adjusted with sterile RO water to an OD_{600} of 1.0 on a spectrophotometer (Bibby Scientific Ltd., UK) [36]. Next, 0.2 µL of a tetrazolium dye was measured into each well of a bespoke 96-well plate. Exactly 30 µL of the white bran enzyme hydrolysate (WBEH) was transferred into the first three wells. This was followed, also in triplicates, by the red bran enzyme hydrolysate (RBEH), white bran dilute acid hydrolysate (WBDAH) and red bran dilute acid hydrolysate (RBDAH) respectively in the succeeding wells. This was repeated for four more rows in the same arrangement, thus preparing 5 rows for the test organisms. Next, 125 µL of the yeast cell suspension were mixed with 2.65 mL of IFY^{TM} buffer (Biolog, USA) and adjusted to a final volume of 3 mL with sterile RO water followed by the addition of 90 µL of the cell suspension to each well. Each row of 12 wells was thus inoculated with just one organism. A final 15 wells were filled with YPD medium as controls for the hydrolysates and inoculated with the yeasts in triplicates in the same order [36].

2.8 Mini-fermentations

Four hydrolysates: WBEH, RBEH, WBDAH and RBDAH were fermented with the best-performing strains from the screening. Anaerobic fermentations were performed in 25 mL of sterile hydrolysate at pH 5.0 in Wheaton glass bottles (30 mL; Wheaton, USA) based on a method adapted from Powell *et al.* [39]. Cells were pitched at 1 x 10^7 cells/mL, the bottles were capped, and the set-up weighed to the nearest 0.1 mg. They

were then placed on magnetic stirrer plates set at 120 rpm and placed in an incubator (MIR-253, Sanyo Electric Co., Japan) set at 30ºC. Weight loss resulting from carbon dioxide release through the Bunsen valves in the cap was monitored periodically. The fermentation proceeded until constant weight was observed over several time points. For time-point experiments, triplicate samples were terminated at different times and analysed while other replicates were allowed to proceed.

Ethanol yields were expressed as a function (g/g) of the maximum possible based on the glucose consumed, and then this amount was expressed as a proportion (%) of the theoretical maximum yield of ethanol (0.51 g/g) as shown in Equation 6.

$$\text{Yield (\%) of theoretical maximum} = \left(\frac{\text{Actual Yield}}{0.51}\right) \times 100 \qquad \text{(Eqn. 6)}$$

2.9 Sugar and inhibitor content analysis by HPLC

The sugar contents of sorghum hydrolysates, residual sugar and ethanol yields in fermentation broths were determined by HPLC. The samples were centrifuged to remove solids, appropriately diluted then syringe-filtered (0.2 μm Minisart®) into HPLC vials. The modular HPLC setup comprised an AS-2055 auto-sampler, PU-1580 pump and RI2031 refractive index detector (Jasco International Co. Essex, UK). Sample injection volume was 10 μL and separation was achieved using a Hi-Plex H$^+$ column (7.7 mm x 300mm; Agilent Technologies, Inc., USA) at 45°C in a Series II 5890 GC oven (Hewlett Packard, CA, USA). The mobile phase was 0.005 N H_2SO_4 solution at a flow rate of 0.4 mL/min.

The presence of common inhibitors of ethanol fermentation was also investigated in the hydrolysates. The HPLC system (Waters Limited, UK) was fitted with a Techsphere ODS-2 column, photodiode array detector, pump and computer system. An injection volume of 10 μL of the samples was then run by a gradient method using a mixture of neat methanol and a 1:1 methanol:water mixture at a flow rate of 0.5 mL/min. Standard solutions containing 0.125-1 g/L of syringaldehyde, acetic acid, 5-hydroxymethylfurfural (5-HMF), furaldehyde, ferulic acid and vanillic acid were prepared in RO water and analysed by HPLC.

All analyses were performed in triplicate, and results are presented as mean values ± standard deviations

2.10 Statistical analyses

Student's T-test was performed to determine the significance of different experimental groups, while $p < 0.05$ was considered as significant difference.

2.11 Estimation of bioethanol production potential

The bioethanol production potential (*Bioethanol$_{SB}$*, g bioethanol per g sorghum bran) from sorghum bran was estimated using Equation 7:

$$Bioethanol_{SB} = Equivalent\ sugar\ content_{SB} \times Y_H \times Y_F \times 0.511 \qquad \text{(Eqn. 7)}$$

where, *Equivalent sugar content $_{SB}$* is the equivalent glucose component in sorghum bran (g/g); Y_H is the yield of hydrolysis (g/g); Y_F is the fermentation yield (% of theoretical yield, g/g), 0.511 is the theoretical bioethanol mass yield using glucose as the substrate.

The annual bioethanol production potential was estimated using Equation 8:

$$Bioethanol_Y = Sorghum\ production \times Y_B \times Bioethanol_{SB} \qquad \text{(Eqn. 8)}$$

where, Y_B is the yield of sorghum bran content obtained from sorghum milling process (g/g).

3 Results and Discussion

In this study, sorghum bran, an underutilised biomass in Nigeria was investigated for its potential use in the biosynthesis of value-added products, using the production of bioethanol as an example. The sorghum milling waste was found to have a favourable carbohydrate and other nutrient content, meaning that hydrolysates from the sorghum bran could be used successfully by certain yeasts to produce promising levels of bioethanol as described below.

3.1 Sorghum Bran Composition

The composition of the sorghum bran is presented in Table 2. The air-dried white bran (WB) had a moisture content (MC) of 11.6%, so it was further oven-dried at 37ºC to a MC of 6.8% to prevent spoilage during storage and to prevent interference by excess moisture with acid concentrations [33]. The sorghum bran was found to have a relatively high starch content of 49.7–53.0%, which contrasted with a lower value of 30 % reported by Corredor *et al.* [22]. This could be due to the bran layer in the sorghum grain kernel being not as brittle as that found in wheat [40], which is easily separated from starch granules during milling processing. The high starch content in sorghum bran makes the bran an ideal potential substrate for bioconversions. Similar high starch contents of 50-60% have been reported for cassava bagasse by Sriroth *et al.* [41] and Pandey *et al.* [42], and a starch content of 52.3% for oats bran by Bhatty [43]. The lignocellulosic material contents were around 20% in total. Lignocellulose is the main structural material found in the sorghum pericarp, testa and aleurone tissues [44] and these were retained in the bran. A high protein content (15.6-16.9%) was detected for both WB and red bran (RB), indicating that they are nutritionally balanced substrates with the potential to be used in bioethanol production.

3.2 Sorghum Bran Hydrolysis

3.2.1 Enzymatic Hydrolysis

Glucose was produced relatively quickly during the enzymatic hydrolysis of the brans, but the accumulation of glucose stopped after 4 hr (Figure 2). The glucose concentrations in the hydrolysate were 55 g/L and 61 g/L for WB and RB respectively, corresponding to a hydrolysis yield of 49.9% and 57.6%, respectively. The higher glucose content in RB correlated with RB having a higher starch content than WB. The yields were lower than the 75% reported by Corredor *et al.* [22] from sorghum bran where a combined hot water treatment (132ºC for 20 min) and enzymatic hydrolysis was utilised. By contrast, only a 9% yield was achieved using enzymatic hydrolysis without hot water treatment, [22], indicating the importance of sufficient gelatinization of the substrate material. The relatively low enzymatic hydrolysis yield in this study might therefore be attributed to insufficient gelatinization at 95ºC for 15 min.

3.2.2 Dilute Acid Hydrolysis

In addition to enzymatic hydrolysis, dilute acid hydrolysis of sorghum bran was tested as a potentially cost effective route and less complex technology for wider acceptability in developing countries. To determine suitable hydrolysis conditions, the effects of dilute sulphuric (at 1% and 3% concentrations) and nitric (at 1% concentration) acid treatment for 15- and 30-min reaction times were compared (Table 3).

Glucose was found to be the main monosaccharide in the hydrolysate, with no significant difference in glucose release by the different acids ($p>0.05$). The concentration of sulphuric acid used in WB hydrolysis had a significant effect on glucose release, with 3% acid releasing more sugar ($p = 0.0009$), with increased treatment time also leading to greater yield of glucose ($p = 0.0358$). For RB, increased sulphuric acid concentration also led to a significantly increased glucose yield ($p = 0.0202$), whereas the impact of residence time was here not significant ($p= 0.2957$). The highest glucose concentration achieved (34.53 ± 8.04 g/L) was with WB treated with 3% sulphuric acid for 30 min (Table 3). Residence time was also found to be not of great significance in the hydrolysis of microalgae (*Chlamydomonas reinhardtii*) [45]. It was discovered that the marginal increase in sugar recovered was not worth the economic implication of increasing the residence time above 30 min. Similar results were also reported in the hydrolysis of moso bamboo residues [12, 46].

To further the dilute acid hydrolysis investigation, the impact of Solid Loading Ratio (SLR) was investigated as it was reported to be an important factor to determine the concentrations of released sugars [47, 48]. Figure 3 shows the main sugar concentrations obtained at different SLR. It was observed that glucose concentrations increased from 35.4 g/L at 5% SLR to 41.2 g/L and 46.9 g/L at 10% and 20% SLR of WB, respectively. Similarly, 21.8, 41.6 and 52.1 g/L glucose was obtained at 5%, 10% and 20% SLR respectively for the RB. Statistical analyses revealed that these increases were significant in RB but not significant in WB.

By contrast, the relative sugar hydrolysis yields decreased with increasing SLR (Figure 3). For instance, the efficiency for WB was 112.5% at 5% SLR, 65.5% at 10% SLR, and 37.3% at 20% SLR. The reason that over 100% hydrolysis yield was observed was mainly due to the fact that a different batch of WB was used in the hydrolysis experiment to that used in the sorghum bran composition analysis (Table 2). The starch and cellulose composition varied slightly from batches to batches. A similar trend of hydrolysis yield decreasing along with increasing SLR was observed with RB. This phenomenon could be due to non-uniform contact of acids with the solids which sediment when present in high concentration. Recalcitrant oligomers may be formed due to the incomplete digestion of the carbohydrate polymers at high SLR [49], leading to a low hydrolysis yield. It is evident that the marginal increase in sugars released from 10% to 20% SLR does not justify the considerable waste in form of undigested bran due to decreased efficiency at the 20% SLR. In addition, since the highest increase in sugar release was observed moving from 5% to 10% SLR, the 10% level was selected for the subsequent production of sorghum bran hydrolysates. This figure is similar to the 13.1% SLR recommended for dilute acid hydrolysis of agave bagasse [50].

The overall yields from the dilute acid hydrolysis treatments were comparable with those from enzyme hydrolysis (Figure 1). This was an important finding given that sulphuric acid is a relatively cheap albeit strong acid [51], available at less cost than commercial enzymes. The dilute acid hydrolysis process is also arguably a more accessible process requiring less expertise.

3.2.3 Inhibitor Profile in the Hydrolysate

The presence of known inhibitory compounds of ethanol fermentation in the various sorghum bran hydrolysates was determined. The enzyme hydrolysates contained significantly lower levels of all of the inhibitor compounds than the dilute acid hydrolysates, except for acetic acid where levels were similar (Table 4). However, the inhibitory compounds were in general at considerably lower concentrations (mg/L) than reported previously for many lignocellulosic hydrolysates. For example, steam explosion of poplar led to the release of 2.1 g/L of acetic acid, 490 mg/L of furfural, 50 mg/L of syringaldehyde and 14 mg/L of vanillin [52]. The maximal 5-HMF concentration observed in this work was 5.9 mg/L, which was less than 0.15 g/L reported for the hydrolysis of potato starch using 2M HCl at 98°C [53], and is significantly less than the maximum 5-HMF concentration (0.2%) that the yeast *Saccharomyces cerevisiae* can tolerate [54].

3.3 Selection of Yeast Strains

Six yeast species and eight strains in total were employed in this study. These were selected based on favourable physiological characteristics including: (i) high potential ethanol yields (e.g. *Saccharomyces* spp.); (ii) the ability to ferment pentose sugars (e.g. *Candida aravinofermentans*, *Scheffersomyces stipitis*); and (iii) high tolerance to fermentation inhibitors [37]. Strain screening tests were performed to determine the best strain(s) to utilize the sorghum bran hydrolysates.

The spot plate test showed that all the yeasts successfully metabolized and grew on the sorghum bran hydrolysate under both aerobic and anaerobic conditions (Figure 4). Colonies on the dilute acid hydrolysates (DAH) were smaller than those on their enzyme hydrolysate (EH) equivalents. This was likely due to differences in the glucose content, which was about 33.6 g/L in the DAH batch used as compared to about 54 g/L in the EH and/or relatively higher inhibitor contents in DAHs.

The aerobic plates exhibited more typical yeast colonial growth, whereas the colonies on the anaerobically incubated EH plates showed a mat-forming, spreading tendency at every dilution. Yeast species show remarkably complex, strain-specific morphologies that are influenced by the environmental conditions including showing different morphologies under different conditions in the same media, and in different media under the same conditions [41]. It is likely that the absence of oxygen in the anaerobically incubated plates (Figures 4A and C) triggered the changes in growth phenotype in a similar way.

Phenotypic microarray (PM) analysis of the yeast strains, when grown on the various sorghum bran hydrolysates, was carried out which monitors respiration activity in a 96-well plate (Figure 5) [36,56]. *S. stipitis* showed the least metabolic activity while *C. arabinofermentans* and *S. cerevisiae* 1119 showed the highest activity (Figure 5). All strains showed typical respiration profiles except for *W. anomalous,* which had a relatively sharp decline in redox signal intensity at 10 hr in all the media, before plateauing into the log phase (Figure 5). The variation in the redox signal intensity (RSI) was considerable, ranging from 65.7 for *S. stipitis* to 116 for *S. pastorianus* at 70 h on RBEH and 58.7 (for *S. stipitis*) and to 106.7 (*S. cerevisiae* 1119), on WBEH. A similar trend was observed with the DAHs where the pattern of the growth signal was almost exactly the same for all species on both bran hydrolysates. The poor growth of *S. stipitis* was attributed to its sensitivity to inhibitors, and high oxygen demand [36]. Low RSI of

S. stipitis in PM analysis using glucose as a sole carbon source has also been reported on wheat straw and macroalgae hydrolysates [57].

Generally, the RSIs on EHs were about double that of DAHs, which was directly correlated with the concentration of sugars. The similarity in the metabolic profiles of strains on both bran types indicated that both bran types and both hydrolysate method could adequately support the growth of the yeasts that were tested.

3.4 Bioethanol Fermentation

Based on the strain selection experiment, *S. pastorianus* and *K. marxianus* were selected for models to assess possible bioethanol production when using sorghum bran hydrolysate as the fermentation substrate. Strain *S. cerevisiae* NCYC 2592 was also included as a reference control strain. In the fermentations a steady decline in weights of the growth vessels was observed, which correlated with sugar catabolism into CO_2 [39]. This indicates that sorghum bran hydrolysate contained sufficient nutrients for a microbial fermentation. Although PM assay showed a similar response for both brans, RB showed a greater weight loss (Figures 6B and D) which correlated with higher ethanol production (Table 5). The EHs had higher glucose contents than the DAHs, which explained the higher weight loss observed in the former due to the greater bioethanol formation and CO_2 release. RBEH showed the highest weight loss, losing 2.2% of the vessel weight (using *S. cerevisiae* NCYC 2592) compared to WBEH with 1.4%. This was higher than previous reports of the highest weight loss of 0.25-0.35 % by yeasts on the hydrolysates of two macroalgae *Chondrus crispus* and *Palmaria palmata* [57].

There were differences in the progression of fermentation among the three strains although the overall trends were similar. The weight loss shown by *S. cerevisiae* NCYC 2592 in all media was almost complete after 48 hr whereupon the culture entered the stationary phase. *S. pastorianus* had the longest lag phase and least weight loss (Figure 6). *S. cerevisiae* NCYC 2592 also showed the highest weight loss in all media except RBDAH, in which *K. marxianus* showed maximum weight loss.

The highest bioethanol titre of 24.35 g/L was produced by *K. marxianus* in RBEH (Table 5). This shows a direct correlation between metabolic activity as indicated by the weight loss and ethanol production. *K. marxianus* is a thermotolerant yeast which has been reported to be a good alternative to *S. cerevisiae* in bioethanol production [58] and produced over 90 g/L ethanol from a mixture of whey and molasses [59]. This study also confirmed that both types of sorghum bran hydrolysates provide a suitable growth substrate for *K. marxianus*.

There was no significant difference between the ethanol concentrations obtained with the white or red bran enzyme hydrolysates and between three yeasts. However, with the dilute acid hydrolysates, all strains produced significantly higher ethanol concentrations with RBDAH than WBDAH. Higher bioethanol concentrations and higher yields were obtained when using the enzyme hydrolysates than the dilute acid hydrolysates for all strains (Table 5). This is likely due to their relatively lower inhibitors content of the enzyme hydrolysates (Table 4). The highest ethanol yield of 89% of the theoretical maximum was obtained from RBEH, which was higher than the 78% reported yield obtained in fermentation of bagasse [14] and was comparable with the 91% yield reported by Erdei *et al.* from the fermentation of pre-saccharified wheat meal [60]. Statistical analysis showed no significant differences (p = 0.6825) in the performance of the three strains.

3.5 Estimation of bioethanol production potential

To calculate the bioethanol production yield from sorghum bran, the generic feedstock obtained from the dilute acid hydrolysis of red sorghum bran was used. The hydrolysis yield Y_H was 0.644 at a SLR of 10% and the fermentation yield Y_F was 70.9%. Based on Equation 7, the bioethanol yield achieved in this study was 0.151 g bioethanol per g sorghum bran. In 2016, sorghum production in Nigeria was 7.56 million tonnes [61]. The sorghum bran content Y_B was 64% (w/w, unpublished data) obtained from a traditional sorghum milling process that was commonly used in Nigeria. Therefore, there is a potential annual bioethanol production of 0.73 m ton (244 million US gallon) per year. Figure 7A shows a schematic diagram of the mass balance for the bioethanol production estimation.

For the estimation of bioethanol production potential from sorghum bran, the cellulose and hemicellulose content was considered for the calculation of equivalent sugar content, which was 70.2% (w/w, glucose equivalent). With an improvement of hydrolysis and fermentation efficiency, the hydrolysis yield and fermentation yield were estimated to be 90% (Y_H) and 95% (Y_F), respectively, the same as the targets set by NREL (National Renewable Energy Laboratory, USA) [62]. Based on this estimation, a bioethanol yield 0.307 g bioethanol per g sorghum bran could be achieved. The annual bioethanol production using all the sorghum bran available in Nigeria could therefore reach 1.48 m ton (497 million US gallon, 44.5 million GJ) annually (Figure 7B). It was estimated that the transportation fuel usage in Nigeria was 6.15 million ton (crude oil equivilant, 258 million GJ) in 2007 [63], the bioethanol production from sorghum bran could potentially provide 17% of transportation fuel in Nigeria (based on energy content). This would significantly contribute to the biofuel industry in Nigeria.

4 Conclusion

Sorghum bran is an under-utilized waste stream from the sorghum milling process in Nigeria which contains relatively high amounts of starch and proteins, indicating that it is a suitable substrate for fermentative conversion to value added products. Enzymatic hydrolysis of sorghum bran resulted in hydrolysates containing 55-61 g/L glucose, with a yield of around 50-58%. Dilute acid hydrolysis led to a relatively low sugar concentration of 26-35 g/L. The hydrolysates supported the growth and metabolism of different yeast genera, and ethanol production at high yields of 63-89% of the theoretical maximum. With a bioethanol production yield of 0.151 g bioethanol per g sorghum bran, it is estimated that if all the sorghum bran produced in Nigeria were utilized, the bioethanol produced could provide 17% of Nigeria's annual transportation fuel requirements. Thus, this work shows that fermentative bioethanol can be produced using low-cost sorghum bran hydrolysates, and it could potentially contribute significantly to the biofuel industry in Nigeria.

Acknowledgements

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List of Tables

Table 1: Yeast strains employed in this study

STRAIN	OTHER NAMES
Saccharomyces cerevisiae NCYC 2592	CBS 1200; ATCC 4126
Scheffersomyces stipitis	N/A
Wickerhamomyces anomalous	N/A
Kluyveromyces marxianus	N/A
Saccharomyces cerevisiae NCYC 1383	ATCC 44774; DBY 747
Saccharomyces pastorianus	N/A
Saccharomyces cerevisiae NCYC 1119	N/A
Candida arabinofermentans NCYC 2916	CBS 8468 NRRL YB-2248

Table 2

Table 2: Compositional analysis of sorghum brans (w/w)

Component	White sorghum Bran (%)	Red sorghum bran (%)
Moisture content	6.81 ± 0.21^a	6.29 ± 0.17^b
Ash	1.83 ± 0.25^a	1.45 ± 0.19^b
[j]Cellulose	7.48 ± 0.58^a	5.67 ± 0.43^b
^Hemicellulose	5.36 ± 0.05^a	5.21 ± 0.20^a
Starch	49.66 ± 0.86^a	52.96 ± 1.43^b
Lignin	6.73 ± 0.64^a	10.53 ± 0.8^b
Protein	15.61 ± 0.56^a	16.94 ± 0.13^b
Lipid	$2.86^j \pm 0.09^a$	$3.66^k \pm 0.13^b$
TOTAL	96.3	102.71
•**Monomeric sugars after strong acid hydrolysis**		
Glucose	57.96 ± 5.07^a	59.87 ± 2.14^a
Xylose	2.96 ± 0.08^b	3.05 ± 0.05^b
Arabinose	3.13 ± 0.89^c	2.88 ± 0.05^c
Mannose	4.98 ± 0.28^d	4.69 ± 0.21^d

•Determined from total carbohydrate analysis; [j]cellulose = (total glucose-starch glucose + mannose) x 0.9; ^hemicellulose = (xylose + arabinose) x 0.88.
Values are means (n = 3) ± SD; values within rows bearing different alphabet superscripts are significantly different (p<0.05).

Table 3: Sugars liberated from sorghum bran via dilute acid hydrolysis.

Sample	Time (min)	Treatment	Concentration (g/L)			
			Glucose	Xylose	Maltose	Arabinose
White sorghum bran	15	1% H_2SO_4	27.37 ± 4.09	1.2 ± 0.21	2.58 ± 0.08	2.04 ± 0.19
		3% H_2SO_4	32.24 ±7.27	1.1 ± 0.02	1.60 ± 0.04	1.96 ± 0.00
	30	1% H_2SO_4	28.07 ± 0.96	1.06 ± 0.10	1.45 ± 0.07	2.01 ± 0.10
		3% H_2SO_4	34.53 ± 8.04	1.15 ± 0.02	1.38 ± 0.02	1.92 ± 0.10
	15	1% HNO_3	25.77 ± 1.15	3.11 ± 0.41	1.12 ± 0.04	2.17 ± 0.40
	30	1% HNO_3	26.34 ± 1.05	2.15 ± 1.57	1.00 ± 0.02	2.13 ± 0.40
Red sorghum bran	15	1% H_2SO_4	26.48 ± 1.06	0.75 ± 0.05	2.22 ±0 .76	1.66 ± 0.10
		3% H_2SO_4	32.17 ± 6.05	0.79 ± 0.01	4.18 ± 0.16	2.13 ± 0.60
	30	1% H_2SO_4	27.11 ± 4.84	0.75 ± 0.00	1.30 ± 0.15	1.95 ± 0.00
		3% H_2SO_4	31.68 ± 5.71	4.53 ± 0.32	1.56 ± 0.08	3.63 ± 2.70
	15	1% HNO_3	28.25 ± 0.33	2.51 ± 0.04	1.04 ± 0.04	1.76 ± 0.00
	30	1% HNO_3	26.13 ± 1.52	2.46 ± 0.00	0.81 ± 0.00	1.98 ± 0.00

Values are means (n = 3) ± SD.

Table 4: Inhibitor concentrations in sorghum bran hydrolysates.

Sample	Concentration (mg/L)				
	HMF	Furfural	Vanillin	Ferulic Acid	Acetic Acid
WBDAH	5.05 ± 0.50^a	4.27 ± 0.11^a	5.62 ± 0.91^a	19.64 ± 1.47^a	22.37 ± 1.15
WBEH	1.18 ± 0.18^b	0.33 ± 0.06^b	ND	1.98 ± 0.21^b	39.94 ± 3.89
RBDAH	5.93 ± 1.01^c	5.95 ± 0.71^c	9.67 ± 1.13^b	17.12 ± 2.05^c	30.25 ± 2.76
RBEH	1.04 ± 0.23^b	ND	ND	ND	42.89 ± 5.42

[a,b] Values within rows bearing different alphabet superscripts are significantly different. HMF = Hydroxymethylfurfural; ND = not detected. WBEH = White bran enzyme hydrolysate, WBDAH = White bran dilute acid hydrolysate, RBEH = Red bran enzyme hydrolysate and RBDAH = Red bran dilute acid hydrolysate. Values are means (n = 3) ± SD.

Table 5: Ethanol concentrations obtained from yeast fermentation when using sorghum bran hydrolysates as a substrate

[a-k] Values within rows bearing different alphabet superscripts are significantly different. Ethanol yield was expressed as a percentage of theoretical maximum yield.

Strain		WBEH	WBDAH	RBEH	RBDAH
S. cerevisiae NCYC 2592	Ethanol (g/L)	23.08 ± 0.04^a	15.17 ± 0.25^b	23.75 ± 0.27^a	17.70 ± 0.30^c
	Yield	83.85 %	62.19 %	87.48 %	68.66 %
K. marxianus	Ethanol (g/L)	23.66 ± 0.24^e	15.38 ± 0.39^f	24.35 ± 0.22^e	18.29 ± 0.04^g
	Yield	85.94 %	63.07 %	89.06 %	70.94 %
S. pastorianus	Ethanol (g/L)	21.89 ± 0.60^i	14.65 ± 0.79^j	22.53 ± 0.24^i	18.32 ± 0.30^k
	Yield	79.51 %	60.06 %	88.88 %	71.08 %
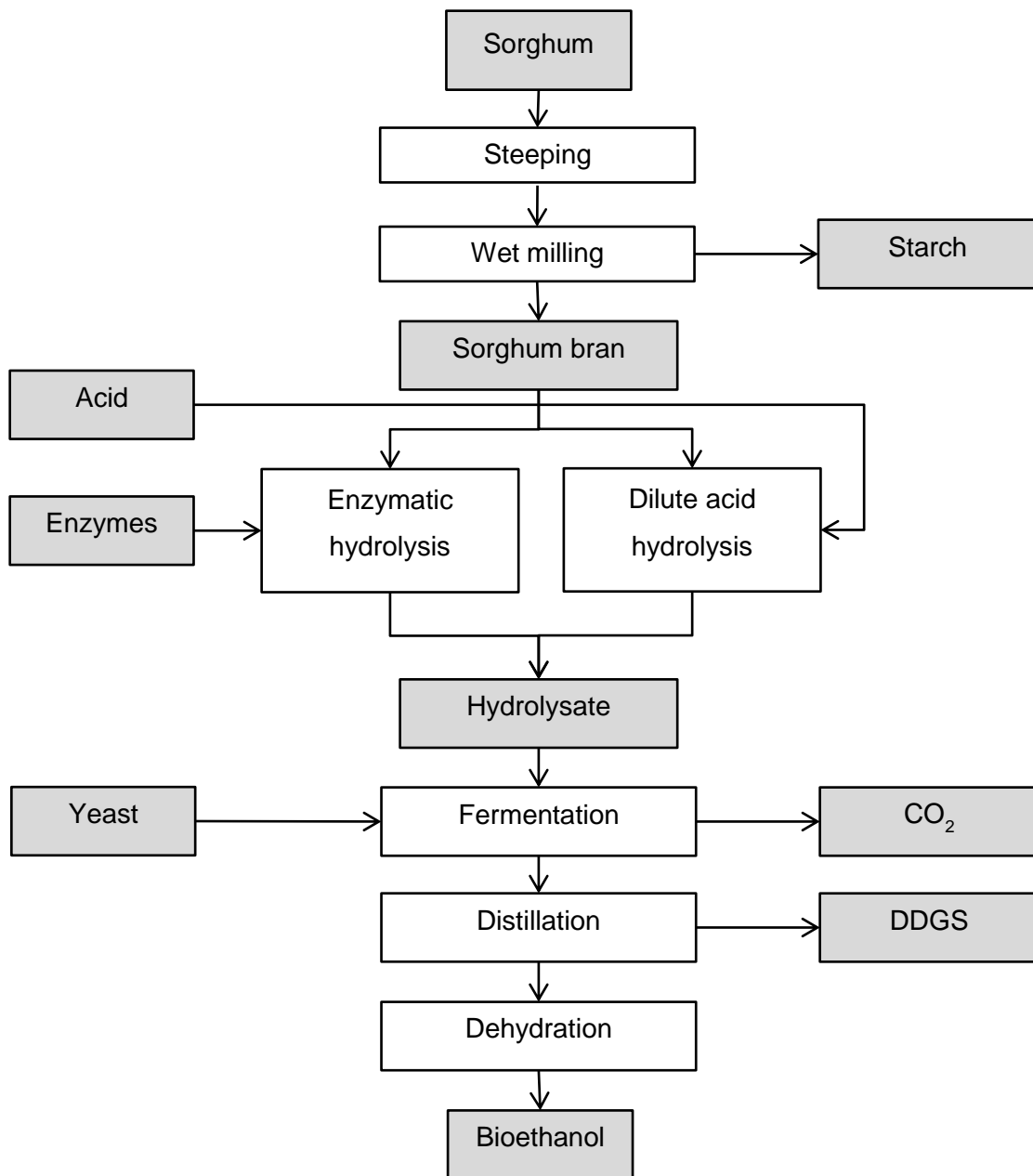
Figure 1

Figure 1: Schematic diagram of a sorghum based biorefining process. DDGS: Distiller's Dried Grains with Solubles.
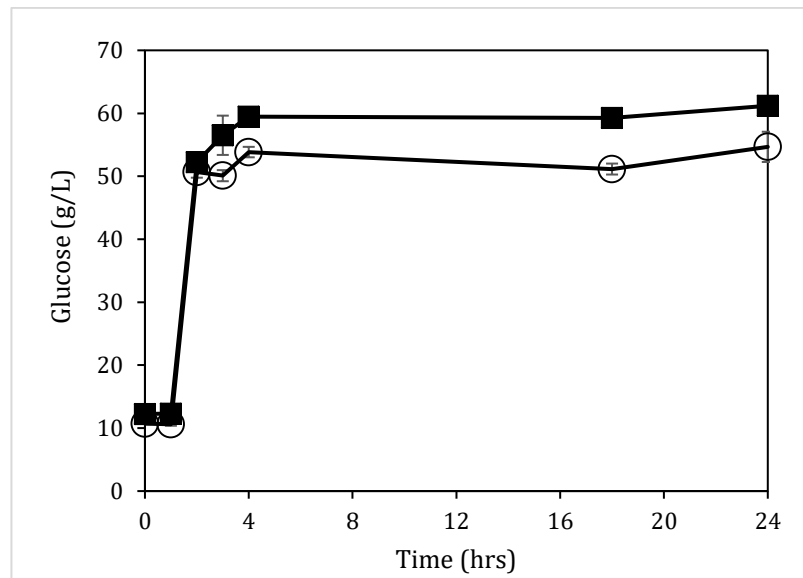
Figure 2

Figure 2: Glucose concentration of sorghum bran hydrolysate (filled square: red sorghum bran; open circle: white sorghum bran) from enzymatic hydrolysis. Values are means ± standard deviations (n = 3).
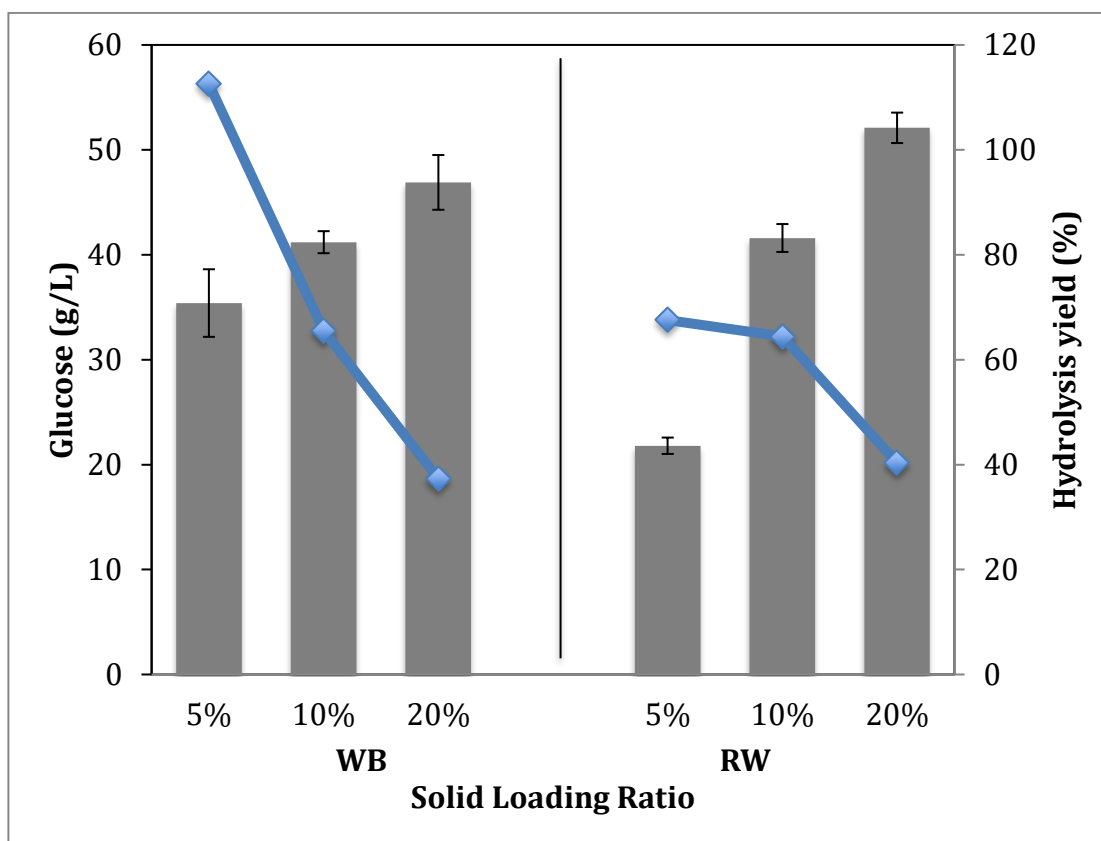
Figure 3

Figure 3: The effect of loading ratio on bran conversion efficiency in diluted H_2SO_4 hydrolysis at $121°C$ for 15 min. The blue lines represent glucose hydrolysis yield. Grey bars show glucose concentrations in the hydrolysate (g/L). Values are means ± standard deviations (n = 3).
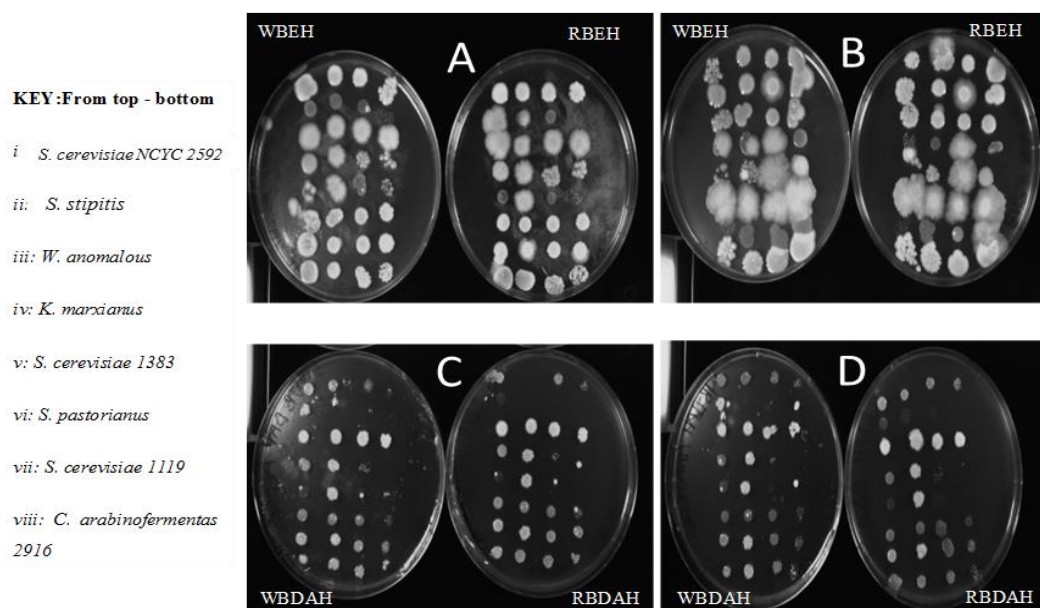
Figure 4

KEY:From top - bottom

i   *S. cerevisiae NCYC 2592*

ii: *S. stipitis*

iii: *W. anomalous*

iv: *K. marxianus*

v: *S. cerevisiae 1383*

vi: *S. pastorianus*

vii: *S. cerevisiae 1119*

viii: *C. arabinofermentas 2916*

Figure 4: Spot-plate screening tests showing typical growth pattern of eight yeast strains when grown on agar plates prepared from four different types of sorghum bran hydrolysates and viewed under UV light with a GelDoc Imaging system. A and C: Aerobic incubation; B and D: Anaerobic incubation. A and B: Enzymatic hydrolysis; C and D: Dilute Acid Hydrolysis. Spots comprise yeast cells in dilutions of $OD_{600} = 1$ stock from L-R: $10^{-1} - 10^{-4}$.
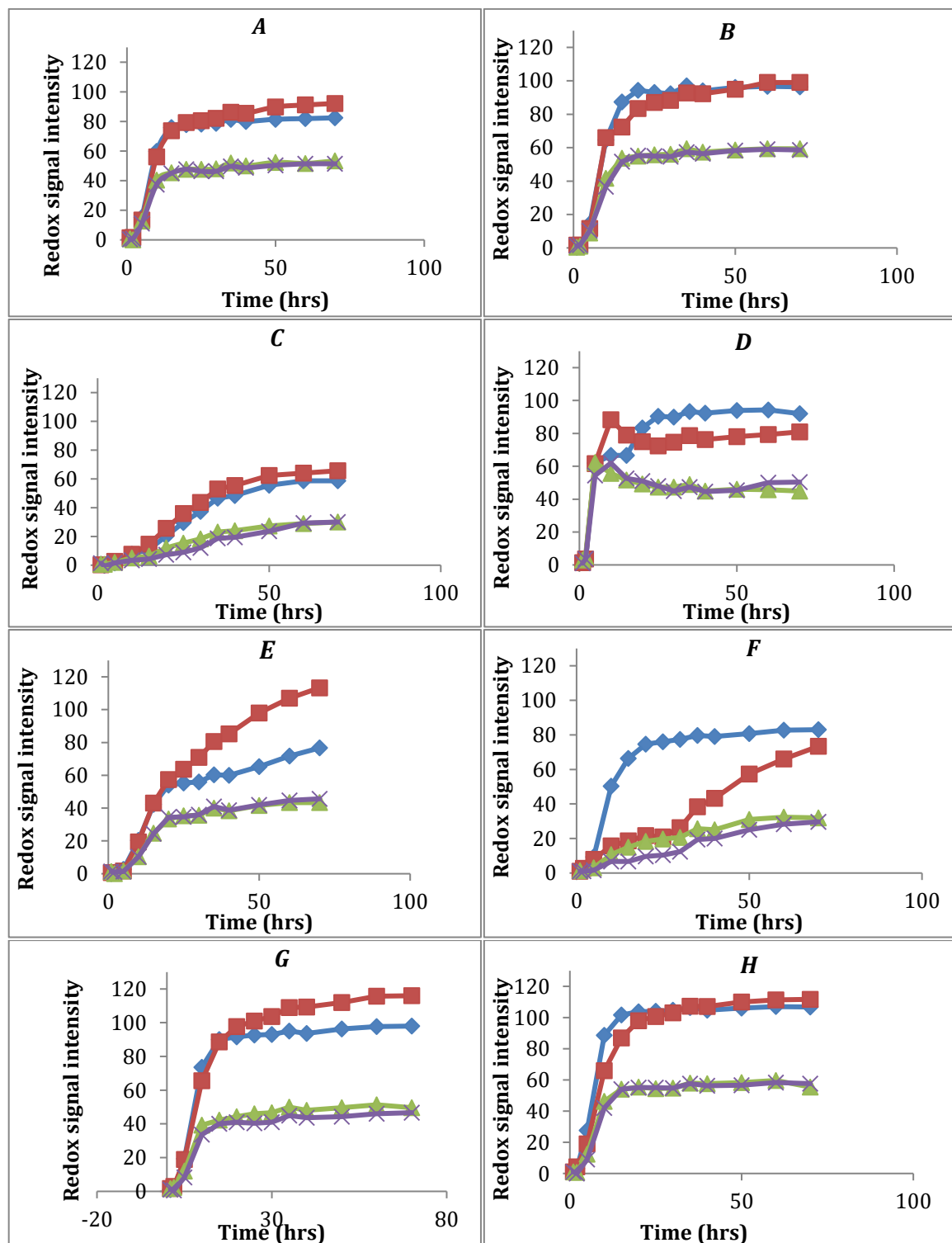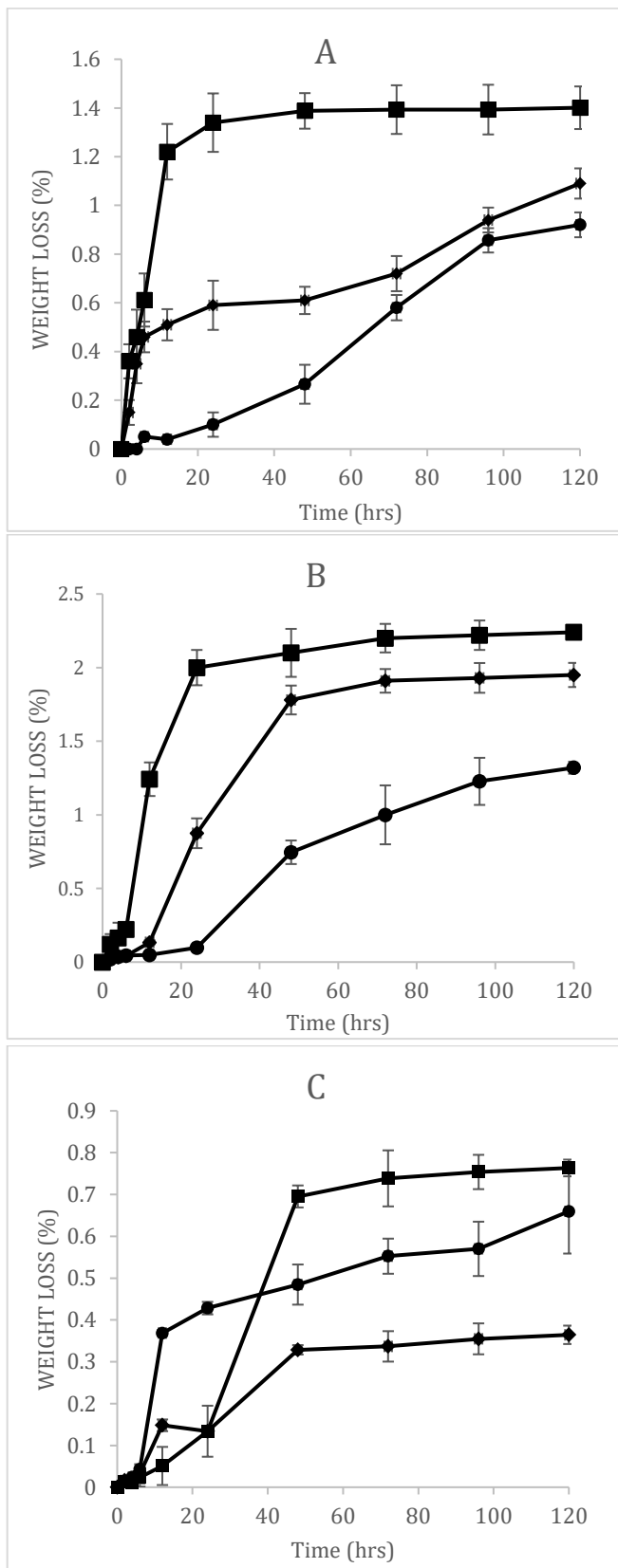
Figure 5.

Figure 5: Phenotypic microarray analysis showing metabolic activity of 8 yeast strains, as judged by redox signal intensity, when cultivated on four sorghum bran hydrolysates, anaerobically for 72 hr. A, *Saccharomyces cerevisiae NCYC 2592*; B, *Candida arabinofermentas*; C,

Scheffersomyces stipites; D, *Wickerhamomyces anomalous*; E, *Kluyveromyces marxianus*; F, *S. cerevisiae NCYC 1383*; G, *Saccharomyces pastorianus*; and H, *S. cerevisiae NCYC 1119.* Blue diamond: White sorghum bran enzymatic hydrolysate; Red square: Red sorghum bran enzymatic hydrolysate; Green triangle: White sorghum bran dilute acid hydrolysate; purple cross: Red sorghum bran dilute acid hydrolysate.
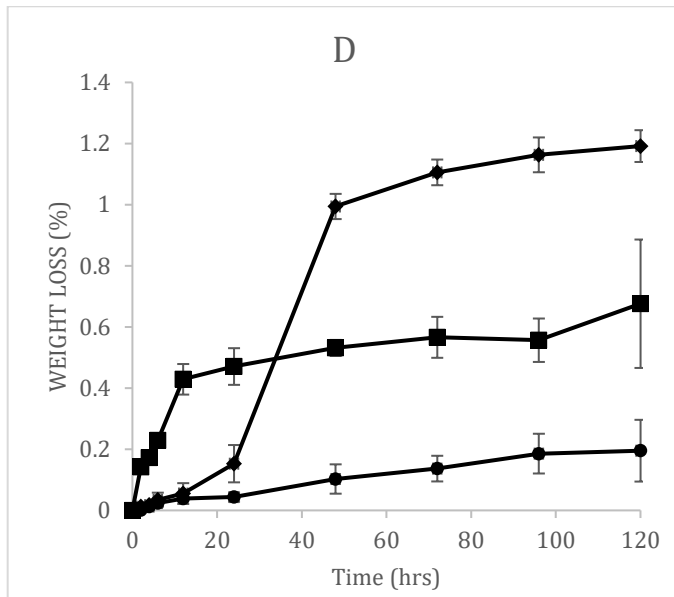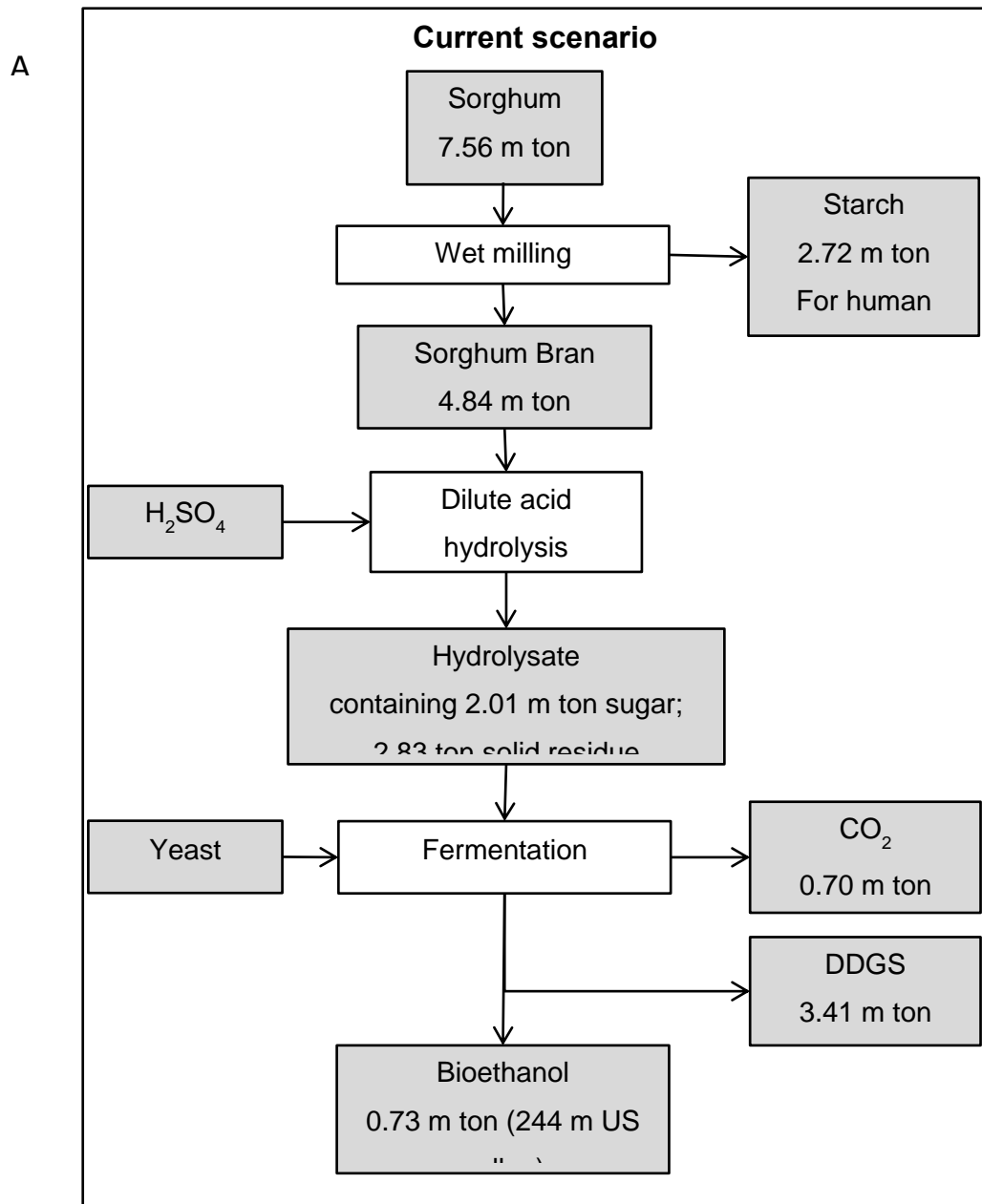
Figure 6

Figure 6: Weight loss trends for mini-fermentations of sorghum bran hydrolysates and a synthetic medium by three different yeast species. Square: *S. cerevisiae* NCYC2592; Diamond: *K. marxianus*; Circle: *S. pastorianus*. A = WBEH; B = RBEH; C = WBDAH; D = RBDAH. Values are means ± standard deviations (n = 3).

Figure 7

A

Current scenario

Sorghum
7.56 m ton

↓

Wet milling → Starch
2.72 m ton
For human

↓

Sorghum Bran
4.84 m ton

↓

H_2SO_4 → Dilute acid
hydrolysis

↓

Hydrolysate
containing 2.01 m ton sugar;
2.83 ton solid residue

↓

Yeast → Fermentation → CO_2
0.70 m ton

→ DDGS
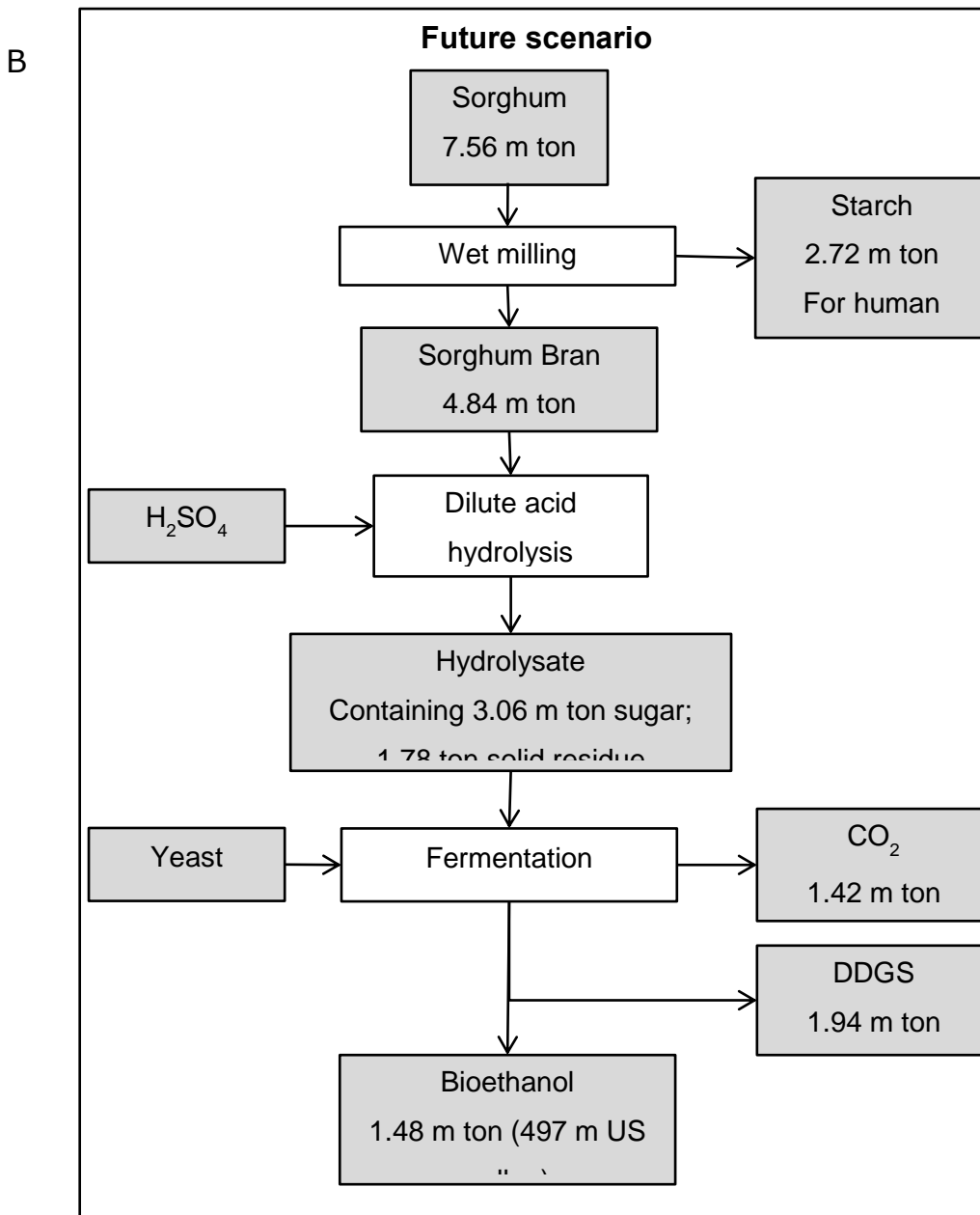3.41 m ton

↓

Bioethanol
0.73 m ton (244 m US
gallons)

Figure 7: A schematic diagram showing mass balance of bioethanol production potential in Nigeria using sorghum milling waste as the substrate. A: Current scenario, based on the current hydrolysis and fermentation yield obtained in this study. B: Future scenario, based on the assumption of potential hydrolysis (90%) and fermentation yield (95%). The mass introduced by H_2SO_4 and Yeast was not considered. The water contents in the sorghum, and DDGS were not considered.