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Abstract

Sorghum is the 5th most important cereal worldwide and is a major source of agricultural residues in tropical regions. Bioconversion of whole sorghum crop residues comprising stalks, leaves, peduncles and panicles to ethanol has great potential for improving ethanol yield per sorghum crop cultivated, and for sustainable biofuel production. Effective pretreatment of sorghum lignocellulosic biomass is central to the efficiency of subsequent fermentation to ethanol. Previous studies have focused on bioconversion of sorghum stalks and/or leaves only to bioethanol,

but the current study is the first report dealing with whole crop residues. We specifically focused on the impact of Nigerian sorghum cultivation location and cultivar type on the potential ethanol yield from whole sorghum crop residues. Efficient bioconversion of whole sorghum residues to ethanol provides a sustainable route for utilisation of crop residues thereby providing a non-food feedstock for industrial scale bioethanol production.

INTRODUCTION

Fermentation-derived ethanol, or ”bioethanol”, is a plant-based liquid biofuel that may be used in automobiles as an additive or substitute to petroleum as transport fuel.[1] Plant biomass such as grains (e.g. maize, wheat), tubers (e.g. cassava), stalk juices (e.g. sugarcane and sweet sorghum) as well as lignocellulosic materials are important feedstock sources for bioethanol production.[2] Food security concerns regarding use of food crops for bioethanol production favours the use of non-food lignocellulosic materials, which comprise inexpensive and abundant biomass in the form of agricultural and forestry residues.[3] Over ten billion metric tons of lignocellulose biomass is produced annually worldwide, of which Nigeria contributes over 83 million metric tons with an estimated 11 million metric tons being agricultural wastes.[4,5,6] For example, sorghum crop residues alone generate 2-3 million metric tons of lignocellulose biomass waste annually in Nigeria. Less than 40% of this material is utilised as livestock feed and fence thatching, while over 60% is left in the fields for burning.[7] Open field burning of sorghum residues (like other agricultural wastes) is considered a labour-saving and cost-saving strategy for green waste disposal by Nigerian farmers. However, the attendant environmental degradation consequences and health risks associated with such practices continue to raise serious concerns.[6]

Previous studies have investigated potential utilisation of sorghum stalks and/or leaves for bioethanol production,[8,9,10] but very little attention has been given to utilisation of the whole sorghum crop bagasse comprising crushed stalks, leaves, peduncles and panicles for bioethanol production.[8,10] Lignocellulosic biomass from sorghum crops comprises polysaccharides in the form of celluloses and hemicelluloses, which are structurally intertwined by tough lignin fibres for mechanical support and rigidity.[6] Typical composition of sorghum lignocellulosic biomass is cellulose (34-44%), hemicelluloses (27-25%) and lignin (18-21%).[9,10] The major constraint in bioconversion of sorghum bagasse to ethanol is the efficient hydrolysis of lignocellulose to liberate fermentable sugars, while ensuring minimum generation of yeast inhibitory compounds.[11] The following methods have been employed to maximise sugar liberation and to minimise formation of inhibitory chemicals when pretreating lignocelluloses; they are catalyzed steam explosion, ammonia fibre expansion (AFEX) and high energy radiation (e.g. ultrasound, microwave heating and electronic beam).[2,8,11-15] However, the successes of these technologies has been largely limited to laboratory and pilot scale applications, partly due to overall economic feasibility of scaling up the processes to an industrial scale.[3,15] Alternative chemical lignocellulose pretreatment methods such as acidic or alkaline hydrolysis have been widely investigated and reported to be efficient and cost-effective in terms of sugar liberation.[16,17] For example, the use of dilute sulphuric acid is considered to be effective and to be economically feasible for scaling up to industrial capacity. However, the challenges faced with this method include removal of the inhibitory compounds generated in the hydrolysates as a result of the degradation of lignin and hemicelluloses.[7,17] Such compounds include phenols (e.g. syringic and vanillic acid) from lignin, furan derivatives (e.g. furfural and

5-hydroxymethyl furfural) from hemicellulose and aliphatic acids (e.g. acetic and formic acids) from sugar decomposition.[16] Inhibitory compounds prolong yeast lag phases, which may then result in "sluggish" or "stuck" fermentations.[2,11] Consequently, various detoxification methods to remove these inhibitory compounds from fermentation media have been employed and include the following: organic based membrane filtrations, rotary-evaporations, extractions with ethyl acetate, ion exchange and alkaline over-liming.[7,18,19] Typical dilute acid hydrolysis temperatures range from 160-220^{o}C and substrate retention times vary from a few minutes to hours depending on the substrate type.[16] In spite of the benefits of high hydrolysis temperatures, whereby hemicellulose-lignin structures are effectively degraded thereby exposing cellulose for efficient hydrolysis, this decomposes the sugars resulting in the generation of higher aliphatic acids and furfurals[17,20]. In addition, proteins may be denatured, thereby limiting yeast available nitrogen sources in hydrolysates.

In this study, the potential to utilise whole sorghum residues (bagasse) consisting of crushed stalks, leaves, peduncles and panicles (left in the field after sorghum harvest) in bioethanol production was investigated. Previous studies have focused solely on sorghum stalks and/or leaves.[3,9,10,15] Also investigated was the benefit of choosing suitable sorghum cultivars and cultivation locations (to gain knowledge on how cultivation locations may affect ethanol production) for improved biomass yield and ethanol productivity. This study aimed to contribute towards harnessing whole sorghum crop residues for bioethanol production in Nigeria. The Nigerian 2007 biofuel policy identified cassava, sugarcane juice and sweet sorghum stalk juice as potential feedstock sources for the emerging bioethanol sector in the country.[1] However, use of cassava and sugarcane constitute food security risks,

therefore whole sorghum residue was envisaged as representing an alternative, less expensive and more sustainable feedstock source. Sorghum bagasse is abundantly available in Nigeria and has no economic value. Utilising it as a bioethanol feedstock therefore results in value addition to the Nigerian sorghum supply chain and contributes towards mitigating deleterious environmental impacts associated with greenhouse gas emission (GHG) and air pollution.[5,6]

MATERIALS AND METHODS

Sorghum cultivation and harvest

SSV2 and KSV8 sorghum crops were cultivated in Kano and Kaduna (Nigeria), respectively. The crops were grown under rain-fed conditions and with only cow dung application as fertilizer. For maximum extractible stalk juice yields, crops were harvested before the grains of each crop reached physiological maturation. Thus, SSV2 cultivar was harvested 11 weeks after the planting date, while KSV8 was harvested 16 weeks after planting.[21] The fresh bagasse samples (comprising crushed stalks, leaves, peduncles and panicles) were sun-dried for 2 days followed by oven drying at 60^{o}C 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.[22] 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^{o}C for 2 h. The final mixtures were centrifuged at 3800 rpm for 10 min. The supernatants (containing solubilised proteins) were filtered, diluted (1:10) and 1 mL of solution transferred into 2 mL cuvettes. The protein concentrations were determined using Bradford™ reagent (Sigma-Aldrich, UK) according to the

manufacturer's standard protocol. The total starch content of the bagasse samples was determined using the K-TSTA total starch kit (Megazymes®, Ireland), according to the manufacturer's standard procedure.

Bagasse pre-treatment and saccharification

Samples of sorghum bagasse (20 g dry wt.) were added into conical flasks containing 2%v/v dilute H_2SO_4 (80 mL). The mixtures were 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, followed by autoclaving 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 NaOH crystals. An enzyme cocktail was prepared and it was comprised of the following: Cellic® Ctec at 120FPU/mL activity (1200 µL), Cellic® Htec at 1090FXU/mL activity (200 µL) (Novozymes, Denmark), Promalt™ 295 at 500BGµ/mL-min activity (30 µL) and Promalt™ 4TR at 300BG µ/mL activity (20 µL) (Kerry Biosciences, Ireland), respectively. The freshly prepared enzyme cocktail was added to the hydrolysate and the final volume adjusted to 200 mL with distilled water. The resultant hydrolysate was incubated at 150 rpm orbital shaking for 20 h at 50^{o}C. Finally, the temperature was ramped to 60^{o}C for an additional 1 h incubation to complete the enzymatic hydrolysis.

Sorghum bagasse hydrolysate detoxification

The enzymatic hydrolysate was over-limed to pH 10.0 with anhydrous $Ca(OH)_2$ and afterwards incubated at 50^{o}C for 15 min with orbital shaking at 120 rpm.[12,19] Concentrated H_2SO_4 was used to adjust the hydrolysate pH to 6.0, followed by centrifugation at 3800 rpm for 10 min. The supernatant (100 mL), was transferred into conical flasks and activated charcoal (2.5 g) was added. The mixture was

swirled at room temperature for 3 min, followed by incubation with orbital shaking at 150 rpm for 30 min at 50^{o}C. Afterwards, the mixture was further centrifuged at 3800 rpm for 10 min, and the supernatant (hydrolysate) filtered through a vacuum pump equipped with GF/B Whatman glass microfiber filters. Samples (2 mL) were withdrawn from the filtrate for the determination of sugars, amino acids and free amino nitrogen (FAN) .

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

1. Analysis of sugars: glucose, xylose and arabinose were determined by HPLC. Hydrolysates (1.0 mL), at a 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 into an HPLC auto sampler (Spectra-physics, USA). Sugars were separated with a 300 mm × 7.8 mm REZEX RPM-monosaccharide Pb+2 (8%) column^{TM} (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 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 gradient elution, high performance liquid chromatography (HPLC), using fluorescence as a means of detection.[23]

Yeast seed culture preparation

Yeast seed cultures were prepared by inoculating a colony of *Pachysolen tannophilus* NCYC614 into 400 mL of yeast extract-peptone-dextrose (YEPD) media. The prepared YEPD media was 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 culture was incubated at 32^{o}C with orbital shaking at 150 rpm for about 28 h. Afterward, the yeast pellets were washed by suspending in distilled water and vortexed, the water was decanted, and the washing procedure repeated twice.

Hydrolysate fermentations

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

i. For CO_2 evolution monitoring: Enzymatic hydrolysate samples (100 mL) were each added into a 250 mL ANKOM^{RF} glass bottle. The yeast *P. tannophilus* (1.0×10^7 cell/mL) was inoculated into the fermentation media. The substrates were incubated at 32^{o}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 progress undisturbed until CO_2 gas production rate were observed to start declining.

ii. For bioethanol monitoring: Similar to (i) above, enzymatic hydrolysate samples (100 mL) were each added into a 250 mL Erlenmeyer flask. The yeast *P. tannophilus* (1.0×10^7 cell/mL) was inoculated into the fermentation

media. The substrates were incubated at 32^{o}C with 130 rpm orbital shaking. Samples were withdrawn after every 24 h from the media for ethanol determination using the FermentoFlash® equipment (Funke-Gerber^{TM}, Berlin). The fermentations were terminated at the end of 72 h.

Similar fermentation experimental setups were replicated with over-limed and charcoal filtered hydrolysates as substrates, respectively.

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 introduced into the FermentoFlash® measuring cells by means of a suction pump (Funke-Gerber^{TM}, Berlin). The alcoholic content and density of the fermentation broth were automatically measured using thermal measuring effects. Derived constituents as original wort, apparent extract and osmotic pressure were also determined, but are not reported in this study.

Statistical analyses

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

RESULTS AND DISCUSSION

Compositional analysis of bagasse

To assess the impact of cultivation location on sorghum bagasse composition, SSV2 and KSV8 sorghum cultivars were grown in Nigeria at Kano and Kaduna (i.e. sites B and Z respectively). Observed diurnal temperature and rainfall at Kano and Kaduna were 33.5^{o}C, 340 mm and 26.5^{o}C, 600 mm respectively (Fig. 1). The cultivation sites soil morphology and physical properties are summarised in Table 1. The results in Table 1 show that sorghum grown at Kano (site B) was more deficient in most of the minerals/elements required sorghum cultivation. The soil from this site was also more sandy, less silty and slightly more acidic. The SSV2 and KSV8 sorghum crops were harvested when their grains reached the soft-dough maturity stage in order to maximise extractible juice yield for syrup production, while the grains may be utilised as livestock feed or food.[21] SSV2 sorghum is a short season cultivar; hence, its grains reached soft-dough maturity at 11 weeks after planting while those of KSV8 grain took 16 weeks from date of planting to reach the soft-dough maturity stage.

From Table 2, it is observed that the physico-chemical composition of SSV2 and KSV8 varied significantly ($p < 0.05$) between the Kano and the Kaduna cultivation location. For example, Kano with drier and warmer climatic conditions appeared to favour not only higher starch and lignin formation, but also incorporated higher levels of protein. These observations support the fact that sorghum can thrive under harsh environmental conditions, which is an advantage for growing environments that are constantly changing. Furthermore, crops cultivated from Kano exhibited taller crop height and thicker stalk diameters, thereby favouring cumulative higher biomass yield relative to Kaduna cultivated crops. These observations were

consistent with reported C-4 agronomic features of sorghum crops.[7] C-4 plants have efficient photosynthetic characteristics. It is expedient to highlight that crops cultivated under limited rainfall and warm conditions are likely to develop thicker cell walls to minimise evaporation rates of water from their tissues. Hence, the crops cultivated in Kano showed a higher lignin content (which is associated with cell wall tissues) than Kaduna crops.[24]

Acid hydrolysis, enzymatic saccharification and detoxification

Sorghum crop leaves and panicles are reported to contain high monomeric leucoanthocyanidins compounds (such as flavan-3,4-diols) and dhurrin (cyanogenic glucoside). Cyanogen concentrations in sorghum bagasse can reach 750-790 mg HCN/kg, and these are rich sources of phenolic compounds liberated during acid hydrolysis of bagasse.[24,25] Thus, to minimise liberation of potentially toxic phenols (and other inhibitory chemicals), an optimised hydrolysis temperature of 75^{o}C was chosen, rather than the conventional 160-220^{o}C. Further benefits envisaged for subsequent yeast fermentation from using moderate hydrolysis temperatures (75^{o}C) are minimization of sugar decomposition and protein denaturation.[14,16,19]

The free amino nitrogen (FAN) content of the SSV2 and KSV8 pre-treated bagasse hydrolysates were observed to significantly vary with cultivation location as summarised in Table 3. It was also observed that acid hydrolysis of the respective sorghum bagasse samples liberated low levels of FAN, while subsequent enzymatic hydrolysis liberated higher FAN levels in corresponding hydrolysates. This is due to activities of the proteolytic enzymes (from Promalt^{TM} 295 and 4TR enzyme cocktails). However, over-liming, followed by charcoal filtration, resulted in sequential decreases in the levels of FAN by about 6% and 13%, respectively (Table 3). This may be due to the precipitation of amino acids and small peptides, along with

organic salts, as a result of over-liming; while the charcoal adsorbs amino acids and small peptides. It would therefore be desirable to minimise the loss of FAN and sugars during detoxification for improved fermentation performance. To achieve efficient fermentation performance, minimum FAN levels of 150mg/L are generally required by yeast in the fermentation media.[19] In spite of the Kaduna cultivated SSV2 bagasse hydrolysate having significantly higher FAN levels than the corresponding Kano SSV2 hydrolysates, the latter contained higher concentrations of total amino acids than the former. Such observations were due to the sensitivity limitations of K-LARGE/K-PANOPA assay kits in determining specific amino acids during FAN measurements.[26] Furthermore, asparagine and glutamine (Group 1 amino acids) as well as tryptophan were present in negligible concentrations in all of the sorghum hydrolysates analysed (Table 4). Group 1 amino acids are not synthesized by yeasts and are essentially required at the onset of fermentation to facilitate yeast adaptation. Group 2 amino acids may be synthesized by yeasts but are normally assimilated as the fermentation progresses and the other Group amino acids are assimilated as fermentation progresses into the latter phases.[21]

Regarding the sugar obtained from the acid pre-treatment of sorghum bagasse, Table 5 shows that most of the xylose and arabinose was liberated due to hemicellulose polymers being highly susceptible to acidic hydrolysis.[20] Cellulose requires enzymatic saccharification, and commercial cellulases were added together with amylases to facilitate degradation of cellulose and starch in the leaf fractions of the bagasse.[14,17,18] Significant increases in glucose levels after enzymatic hydrolysis were observed, while xylose and arabinose concentrations increased only marginally. Furthermore, a successive decrease in total sugar concentration (5-8%) was observed with sequential over-liming and charcoal filtration treatment of the

hydrolysates (Table 5). This corresponds to a loss of 10-12% of fermentable sugars, emphasising the necessity to optimise detoxification processes for lignocellulosic material. Despite employing mild acidic hydrolysis under moderate pre-treatment conditions, the total sugar yields of both the acidic and enzymatic hydrolysates compared favourably with those of previously reported literature (Table 6). The results from Table 6 suggested, despite the favourable sugar yields achieved in this study for whole sorghum bagasse, that there is scope to further improve the total sugar yields, for example, by increasing the hydrolytic enzyme dosage or by employing more efficient saccharification enzymes.

Fermentation

The yeast, *P. tannophilus* was employed to ferment the sorghum bagasse hydrolysates, as this yeast has been reported to be an efficient xylose-fermenting yeast.[12,15,20] Prior to the onset of fermentation, substrate utilisation kinetics exhibiting a lag phase, which was likely due to the effect of inhibitory compounds on *P. tannophilus* delaying yeast adaptation to the hostile fermentation media, can be seen (Fig. 2a). Previous studies on fermentation kinetics of sorghum bagasse have reported yeast lag phases of 5-8 h,[14,12,18,31] but Fig. 2a shows prolonged phases of yeast adaptation. This may be due to the deleterious effects on yeast physiology of monomeric leucoanthocyanidins (such as flavan-3,4-diols) and cyanogenic glycosides liberated from sorghum leaves and panicles during acid hydrolysis, in addition to other inhibitory compounds from the stalks.[11,13,16,18] Despite having lower levels of Group 1 amino acids (Table 4), Kaduna grown SSV2 and Kano grown KSV8 bagasse hydrolysates exhibited relatively shorter yeast lag times than the corresponding Kano grown SSV2 and Kaduna grown KSV8 bagasse hydrolysates,

respectively. This may be due to both the latter hydrolysates having higher concentrations of inhibitory chemicals than either of the former, because the levels of cyanogenic chemicals will be expected to vary from one crop to another.[24,25]

In spite of the lag times observed for the non-detoxified Kano grown SSV2 bagasse hydrolysates, its final ethanol concentration of 13 g/L is similar to the 14 g/L ethanol yield reported by Ban et al.[32] for non-detoxified sorghum bagasse hydrolysates pre-treated with phosphoric acid at 80^{o}C. Furthermore, evaluated mean ethanol yields of 10 g/L of Kaduna grown SSV2 and KSV8 as well as the Kano grown KSV8 bagasse substrates (Fig. 2b) were higher than the ethanol concentrations (6 g/L) reported by Cao et al.[12] for non-detoxified sorghum bagasse hydrolysates fermented for 96 h. However, Ballesteros et al.[33] reported a higher ethanol concentration of 16 g/L for non-detoxified but alkaline pre-treated sorghum bagasse hydrolysates. With regard to total fermentable sugar utilisation and theoretical ethanol yields, it was established that only about 40% of the fermentable sugars were utilised, resulting in theoretical ethanol yields of about 50% (see Tables 5 and 7). This suggests that some sorghum bagasse hydrolysate fermentations may result in "stuck fermentations"[16,18] with relatively high levels of unfermented sugars (Table 5). It is likely that the limiting factor dictating fermentation rates was the presence of yeast inhibitory compounds in the hydrolysates.

The removal of aliphatic and organic acids by over-liming significantly shortened yeast lag times (Fig. 2c). The reduced concentration of inhibitory chemicals in the hydrolysates presents a more favourable environment for yeast growth and efficient fermentative metabolism.[13,17,18,19] This is supported by the observed increase in CO_2 gas formation kinetics shown in Fig. 2c, and the

corresponding ethanol yields (Fig. 2d), which increased by 4-6% over non-detoxified fermentations

Generally, observed ethanol yields from fermentation of the over-limed bagasse hydrolysates were about 15-17 g/L (Table 7), and these represented less than 60% of theoretical yields. However, removal of polyphenols from the hydrolysates by charcoal filtration showed notable reductions in yeast lag times and improvements in cell growth, CO_2 gas production rates (Fig. 3a) and ethanol production (Fig. 3b). Gyalai-Korpos et al.[31] and Nichols et al.[34] reported similar fermentation kinetics for sorghum bagasse hydrolysates detoxified and supplemented with yeast nutrients. While SSV2 hydrolysate from both Kano and Kaduna contained similar initial FAN and sugar concentrations, the Kano substrate produced ethanol to about 23 g/L, which represented about 65% of the theoretical yield, which is a better yield compared to the figure of 48% obtained from KSV8 (see Table 7). Improved fermentation performance of Kano grown SSV2 hydrolysates over corresponding Kaduna grown SSV2 may reflect the former having a higher amino acid content than the latter (Table 4), providing a more nutritionally conducive environment for *P. tannophilus* metabolism. This yeast was generally able to utilise the available pentose sugars present in the sorghum bagasse hydrolysates. However, incomplete xylose fermentation (e.g. in the Kaduna grown SSV2 hydrolysate) may be due to deficiencies in other essential nutrients such as vitamins and/or minerals.[2]

Ethanol yields of 23 g/L were achieved from the fermentation of sorghum bagasse, which compares favourably with previous studies (see Table 8). The agronomic ethanol yields of sorghum bagasse cultivated at the two sites in Nigeria were evaluated on a per hectare basis. Yields of 8204 L/ha and 5304 L/ha for SSV2 6848

L/ha and 6450 L/ha for KSV8 were obtained. These results compare favourably with the 4560 L/ha reported by Gyalai-Korpos et al.[31] for sorghum bagasse (crushed stalks only). Serna-Saldívar et al.[3] reported similar result of 6375 L/ha as well as an improved result of 10184 L/ha.

CONCLUSIONS

The potential of utilising whole sorghum crop residues in bioethanol production was investigated. The findings suggest that the bagasse from Nigerian SSV2 and KSV8 sorghum cultivars residues represent favourable feedstock sources for bioethanol production. Furthermore, investigation into the impact of sorghum cultivation location and cultivar type on bioethanol yield showed that both cultivar type selection and favourable cultivation location could improve ethanol yields by over 15%. For example, both SSV2 and KSV8 sorghum crops produce higher biomass under warmer and drier climatic conditions. Mild acid pretreatment of sorghum bagasse at moderate temperatures, followed by detoxification, appeared to be a relatively cost-effective platform for the bioconversion of the whole sorghum crop to ethanol. Further improvements in ethanol yield per hectare are envisaged through application of agrochemicals during crop cultivation, use of improved cellulolytic enzymes, and exogenous yeast nutrient supplementation during fermentation.

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Table 1. Soil physical and morphological properties of Kano and Kaduna cultivation sites in Nigeria

Parameters	Kano (Site B)	Kaduna (Site Z)
pH	5.0	5.2
Org. C (g kg^{-1})	0.38	3.3
Total N (g kg^{-1})	0.08	0.53
Avail. P (mg kg^{-1})	0.56	1.8
Exchangeable bases (C mol kg^{-1})		
Ca	0.27	1.80
Mg	0.08	0.36
Na	0.30	0.05
K	0.19	0.33
Exch. Acidity (Al^{3+} H^{+})	0.24	0.10
CEC	1.08	4.0
Soil physical properties (g kg^{-1})		
Sand	78	46
Silt	12	40
Clay	10	14

Source: Nasidi et al. (2013).

Table 2. Sorghum bagasse physico-chemical composition

	Kano		Kaduna	
Parameter	**SSV2**	**KSV8**	**SSV2**	**KSV8**
Cultivation	11 weeks	16 weeks	11 weeks	16 weeks
Crop height (m)	1.80^{a} ±0.05	3.20^{b} ±0.07	1.62^{d} ±0.04	2.81^{e} ±0.04
Diameter (cm)	1.95^{a} ±0.10	2.62^{c} ±0.11	1.80^{d} ±0.10	2.51^{e} ±0.02
*Fresh bgs (t/ha)	41.72^{a} ±3.1	48.31^{b} ±2.6	37.06^{d} ±2.8	45.78^{e} ±1.4
**Dry bgs (t/ha)	28.60^{a} ±1.1	32.72^{b} ±0.8	24.31^{d} ±0.9	30.49^{e} ±1.2
Total starch: %	5.14^{a} ±0.54	1.09^{b} ±0.06	4.17^{d} ±0.14	0.78^{e} ±0.05
Total lignin: %	18.40^{a} ±0.3	21.65^{b} ±0.2	16.86^{d} ±0.4	19.41^{e} ±0.3
Total protein %	4.61^{a} ±0.2	3.53^{b} ±0.16	5.23^{c} ±0.16	2.69^{d} ±0.21

Bagasse properties of SSV2, KSV8 and KSV3 sorghums cultivated in Kano and Kaduna under varied climate conditions. *Fresh bgs: fresh bagasse (leaves, crushed stalks, stover 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-e) are significantly different ($p \leq 0.05$) by ANOVA using the Tukey grouping method test.

Table 3. Initial free amino nitrogen of bagasse hydrolysates (mg/L)

	Kano		Kaduna	
Hydrolysates	**SSV2**	**KSV8**	**SSV2**	**KSV8**
Acidic	130.3^{a} ±3.1	91.9^{b} ±1.9	122.7^{c} ±2.5	83.4^{d} ±1.7
Enzymatic	251.8^{a} ±3.8	180.4^{b} ±2.1	254.4^{a} ±3.2	163.5^{d} ±1.3
$Ca(OH)_2$ Over-limed	238.4^{a} ±3.6	168.0^{b} ±1.9	240.5^{c} ±3.1	151.2^{e} ±2.0
Charcoal filtrate	205.8^{a} ±1.8	146.4^{b} ±2.1	211.4^{d} ±2.2	139.5^{e} ±1.6

Milled oven-dried sorghum bagasse pre-treated with dilute H_2SO_4 acid followed by enzymatic saccharification and over-limed with $Ca(OH)_2$ and finally filtered with activated charcoal. FAN was determined by K-PANOPA™/K-LARGE™ Megazymes® kits. Means on the same row that do not share the same superscript letter (a-e) are significantly different ($p \leq 0.05$) by ANOVA using the Tukey grouping method test.

Table 4. Initial amino acid concentrations of charcoal filtered sorghum bagasse hydrolysates (µmol/mL)

Parameter	**Kano**		**Kaduna**	
Amino acids	**SSV2**	**KSV8**	**SSV2**	**KSV8**
Group 1				
aspartic	1.492^{a} ±0.001	0.509^{b} ±0.006	0.618^{d} ±0.004	1.279^{e} ±0.010
glutamic	0.240^{a} ±0.003	0.085^{b} ±0.007	0.186^{c}±0.007	0.221^{d} ±0.005
serine	0.234^{a} ±0.001	0.118^{d} ±0.008	0.095^{e} ±0.005	0.216^{b} ±0.007
arginine	0.099^{a} ±0.001	0.027^{c} ±0.004	0.025^{c} ±0.005	0.072^{b} ±0.005
threonine	0.157^{a} ±0.002	0.055^{d} ±0.007	0.061^{d} ±0.005	0.123^{b} ±0.005
lysine	0.113^{a} ±0.001	0.020^{b} ±0.003	0.036^{d} ±0.005	0.092^{e} ±0.005
asparagine	*ND	*ND	*ND	*ND
glutamine	*ND	*ND	*ND	*ND
Sub-total	***2.330 ±0.002***	***0.813 ±0.037***	***1.019 ±0.033***	***2.000 ±0.006***
Group 2				
methionine	0.206^{a} ±0.002	0.081^{d} ±0.002	0.086^{d} ±0.004	0.186^{b} ±0.006
Valine	0.237^{a} ±0.001	0.095^{b} ±0.008	0.102^{c} ±0.005	0.222^{d} ±0.008
isoleucine	0.110^{a} ±0.001	0.029^{b} ±0.006	0.040^{c} ±0.004	0.094^{d} ±0.006
leucine	0.350^{a} ±0.000	0.067^{b} ±0.003	0.118^{d} ±0.003	0.226^{e} ±0.005
phenylalanine	0.061^{a} ±0.002	0.016^{b} ±0.004	0.029^{b} ±0.007	0.050^{a} ±0.006
histidine	0.077^{a} ±0.001	0.031^{e} ±0.001	0.026^{c} ±0.002	0.075^{a} ±0.006
Sub-total	***1.039 ±0.003***	***0.319 ±0.003***	***0.400 ±0.009***	***0.853 ±0.015***
Other Groups				
glycine	0.335^{a} ±0.004	0.215^{b} ±0.008	0.154^{d} ±0.006	0.254^{e} ±0.006
alanine	1.045^{a} ±0.003	0.279^{b} ±0.008	0.343^{d} ±0.005	0.889^{e} ±0.008
proline	0.335^{a} ±0.001	0.114^{b} ±0.008	0.105^{b} ±0.006	0.271^{d} ±0.007
tyrosine	0.104^{a} ±0.003	0.090a ±0.004	0.173^{d} ±0.004	0.072^{b} ±0.005
tryptophan	*ND	*ND	*ND	*ND
Sub-total	***1.818 ±0.003***	***0.698 ±0.004***	***0.775 ±0.009***	***1.485 ±0.014***
Grand Total	**5.186^{a} ±0.008**	**1.829^{b} ±0.044**	**2.1925^{d} ±0.05**	**4.338^{e} ±0.035**

SSV2, KSV8 and KSV3 sorghum bagasse comprising crushed stalks, leaves, peduncles and panicles were cultivated in Kano and Kaduna (Nigeria) hydrolysed by dilute H_2SO_4 acid followed by enzymatic saccharification. The hydrolysates were over-limed with $Ca(OH)_2$ and filtered with charcoal. The amino acids were determined by GC-MS. Means on the same row that do not share same superscript letter (a-e) are significantly different ($p \leq 0.05$) by ANOVA using the Tukey grouping method test. *ND = Not Detected.

Table 5. Sugar concentrations in sorghum bagasse hydrolysates (g/100g bagasse)

		Initial sugars (before fermentation)				**Residual sugars** (after fermentation)			
Bagasse	**Hydrolysates**	**Glucose**	**Xylose**	**Arabinose**	**Total**	**Glucose**	**Xylose**	**Arabinose**	**Total**
SSV2B*	Acidic	8.82^{a} ±1.1	13.46^{a} ±0.4	3.49^{a} ±0.6	25.77^{a}±0.8				
	Enzymatic	46.46^{ab}±1.1	17.29^{ab}±0.5	5.45^{b} ±0.5	69.19^{c} ±1.1	13.25^{a}±0.2	13.71^{a}±0.5	4.93^{a} ±0.5	31.89^{a}±1.2
	$Ca(OH)_2$ Over-limed	43.85^{af}±1.0	15.06^{cd}±0.9	5.27^{b} ±0.9	64.18^{ab}±2.6	2.89^{d}±0.9	12.57^{a}±1.1	4.46^{a} ±0.4	19.92^{b}±0.6
	Charcoal filtrate	42.88^{af}±1.0	13.70^{a} ±0.2	5.08^{b} ±1.0	61.66^{bc}±2.2	*ND	8.76^{bc}±0.9	3.65^{b} ±0.3	12.41^{c}±1.1
SSV2Z**	Acidic	9.82^{a} ±1.0	12.35^{b} ±0.4	3.22^{a} ±0.2	25.39^{a}±0.8				
	Enzymatic	44.03^{ac}±2.1	16.86^{ab}±1.1	5.19^{b} ±0.1	66.07^{d}±0.8	14.17^{a}±2.0	14.70^{c}±1.1	4.58^{a} ±0.1	33.45^{d}±1.0
	$Ca(OH)_2$ Over-limed	42.07^{af}±0.5	14.14^{c} ±1.5	4.96^{b} ±0.9	61.16^{bc}±2.9	2.87^{d}±0.5	12.56^{a}±1.5	4.46^{a} ±0.9	19.89^{b}±1.1
	Charcoal filtrate	41.76^{af}±1.0	12.11^{e} ±0.2	4.03^{c} ±0.1	57.88^{cd}±1.2	*ND	11.08^{d}±0.2	3.21^{b} ±0.1	14.29^{e}±0.2
KSV8B*	Acidic	1.54^{b} ±0.2	15.35^{c} ±0.1	4.01^{c} ±0.6	20.89^{b}±0.9				
	Enzymatic	26.57^{ad}±1.2	21.22^{ac}±1.1	6.44^{d} ±0.4	54.22^{e}±2.8	10.42^{b}±1.2	17.67^{e}±1.1	5.49^{c} ±0.4	33.58^{d}±1.9
	$Ca(OH)_2$ Over-limed	23.25^{cf}±0.9	17.87^{ab}±0.9	6.34^{d} ±0.1	47.46^{ad}±1.8	*ND	14.51^{c}±0.9	5.86^{c} ±0.1	20.37^{b}±0.9
	Charcoal filtrate	22.84^{cf}±1.0	15.80^{c} ±1.2	5.76^{b} ±0.2	44.40^{fe}±0.3	*ND	7.30^{b}±1.2	3.01^{d} ±0.2	10.31^{f}±1.3
KSV8Z**	Acidic	2.61^{c} ±0.7	14.54^{c} ±0.7	3.62^{a} ±0.2	20.75^{b}±1.2				
	Enzymatic	24.38^{bc}±0.8	20.37^{ac}±1.7	5.38^{b} ±0.3	50.14^{f}±1.2	11.15^{b}±0.8	18.04^{e}±1.7	4.74^{a} ±0.3	33.93^{d}±1.2
	$Ca(OH)_2$ Over-limed	22.13^{cf}±0.9	16.91^{ab}±0.4	5.33^{b} ±0.6	44.37^{fe}±1.9	*ND	15.85^{f}±0.4	4.52^{a} ±0.6	20.51^{b}±0.9
	Charcoal filtrate	21.80^{cf}±0.1	14.09^{c}±0.7	5.03^{b} ±0.2	40.91^{ce}±0.9	*ND	7.45^{b}±0.7	2.88^{e} ±0.1	10.33^{f}±0.8

Milled and oven-dried sorghum bagasse pre-treated with dilute H_2SO_4 acid followed by enzymatic saccharification then over-liming with $Ca(OH)_2$ and finally filtered with activated charcoal. Sugars were determined by HPLC. Corresponding Means in the same column that do not share same superscript letter (a-f) are significantly different ($p \leq 0.05$) by ANOVA using the Tukey grouping method test.

NOTE: B* = Kano (site B), Z** = Kaduna (site Z).

Table 6. Comparison of sugar yields from sorghum bagasse using different pre-treatments.

Sorghum pre-treatment method	Sugar yields*	Reference
2% (v/v) H_2SO_4 digestion at 75^oC for 2 h followed by 24 h enzymatic hydrolysis	24-47 g (glucose) & 17 to 20 g (xylose)	This study
3% CaOH digestion at 121^oC for 1 h followed by 24 h enzymatic hydrolysis.	40 g (glucose) & 21 g (xylose)	Kim et al.[10]
Microwave assisted ammonium hydroxide digestion at 130^oC for 1 h	42 g (glucose)	Chen et al.[13]
10% (w/w) NaOH digestion at 70^oC for 4 h followed by 24 h enzymatic hydrolysis.	31 g (glucose) & 14 g (xylose)	Panagiotopoulos et al.[27]
3% H_2SO_4 digestion for 10 min followed by 96 h enzymatic hydrolysis.	37 g (glucose) & 21 g (xylose)	Phuengjayaem and Teeradakorn[28]
10% (w/v) NaOH at 121^oC for 25 min followed by 21% (v/v) H_2SO_4, digestion at 70^OC for 73 min	21 g (glucose)	Thanapimmetha et al.[29]
2% NaOH digestion followed by 24 h enzymatic hydrolysis	26 g (glucose)	Sathesh-Prabu and Murugesan[30]
Ammonium fibre explosion (AFEX) at 140^oC for 30 min followed by 72 h enzymatic hydrolysis	29 g (glucose) & 15 g (xylose)	Li et al.[15]

*Sugar yield = (g/100g bagasse).

Table 7. Ethanol and CO_2 production in sorghum bagasse fermentations with the yeast *P. tannophilus.*

Bagasse	**Hydrolysates**	**Ethanol** (g/L)	**CO_2 gas***
	Enzymatic	$13.03^{a} \pm 1.1$	$1423^{a} \pm 27$
SSV2B*	$Ca(OH)_2$ Over-limed	$17.12^{d} \pm 0.9$	$2083^{b} \pm 31$
	Charcoal filtrate	$23.12^{ad} \pm 0.5$	$3719^{c} \pm 24$
	Enzymatic	$10.53^{b} \pm 1.0$	$1237^{d} \pm 26$
SSV2Z**	$Ca(OH)_2$ Over-limed	$15.86^{e} \pm 0.4$	$1817^{e} \pm 22$
	Charcoal filtrate	$17.44^{d} \pm 1.0$	2546 ± 21
	Enzymatic	$9.81^{b} \pm 0.6$	$1142^{f} \pm 19$
KSV8B*	$Ca(OH)_2$ Over-limed	$14.83^{f} \pm 0.8$	$1433^{a} \pm 23$
	Charcoal filtrate	$16.89^{ab} \pm 0.3$	$2383^{ab} \pm 21$
	Enzymatic	$9.36^{b} \pm 0.8$	$1125^{f} \pm 22$
KSV8Z**	$Ca(OH)_2$ Over-limed	$14.52^{f} \pm 0.3$	$1395^{ad} \pm 20$
	Charcoal filtrate	$16.97^{ab} \pm 0.3$	$2217^{ae} \pm 22$

Ethanol and CO_2 gas yields of SSV2, KSV8 and KSV3 sorghum bagasse hydrolysates at three treatment levels. Fermentations were by *P. tannophilus* (without exogenous nutrient supplementation). Corresponding Means in the same column that do not share same superscript letter (a-f) are significantly different ($p \leq 0.05$) by ANOVA using the Tukey grouping method test. *****$C0_2$ gas (mL/100g dry bagasse). NOTE: B* = Kano (site B), Z** = Kaduna (site Z).

Table 8. Comparison of ethanol yields from sorghum bagasse fermentations

Fermentation condition	Ethanol yield (g/L)	Reference
Fermentation by *P. tannophilus* without nutrient supplementation.	17-23	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 supplemented.	16	Ballesteros et al.[33]
Fermentation by *S. cerevisiae* with nutrient supplementation.	23	Mehmood et. al.[36]
Simultaneous saccharification and fermentation (SSF) with *S. cerevisiae* (5 g/L cell density) and nutrient supplementation	23	Shen et al.[37]
Separate hydrolysis and fermentation (SHF) with *S. cerevisiae* (3 g/L cell density) and nutrient supplementation	21	Shen et al. [37]
Fermentation by co-culture of *S. cerevisiae* and *Neurospora crassa* with nutrient supplementation.	28	Dogaris et al.[38]

LIST OF FIGURES

Fig. 1. Map of Nigeria showing sorghum cultivation location, mean precipitation and diurnal temperatures. SSV2 and KSV8 sorghum cultivars were grown in Nigeria at Kano (site B) and Kaduna (site Z) under rain fed conditions without chemical fertilizer application.

Fig. 2. Fermentation kinetics and corresponding ethanol yields from sorghum bagasse hydrolysates. (**a**) Kinetics of enzymatic hydrolysed substrate. (**b**) Corresponding ethanol yield. (**c**) Kinetics of over-limed hydrolysate, after enzymatic hydrolysis. (**d**) Corresponding ethanol yield. SSV2B & KSV8B are sorghums cultivated in Nigeria at Kano and SSV2Z & KSV8Z at Kaduna, respectively. Crops were grown under rain fed conditions without chemical fertilizer application.

Fig. 3. Fermentation kinetics and corresponding ethanol yields of charcoal filtered hydrolysates, after sequential enzymatic hydrolysis and over-liming of sorghum bagasse substrates. (**a**) Kinetics of substrates. (**b**) Ethanol yield. SSV2 and KSV8 sorghums were cultivated in Nigeria at Kano (site B) and Kaduna (site Z) under rain fed conditions.

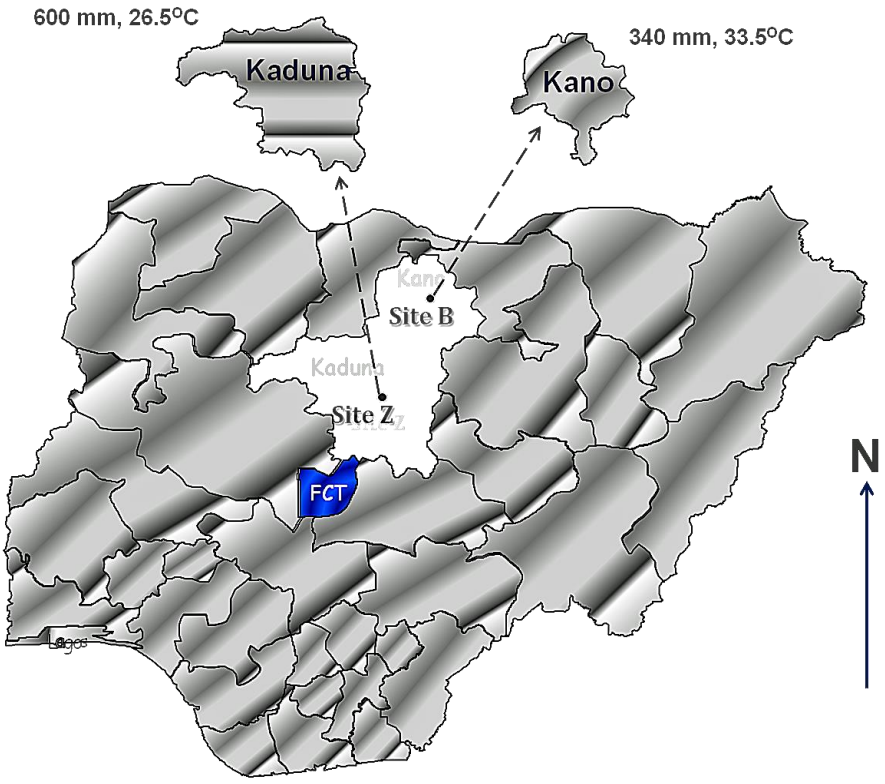

Fig.1

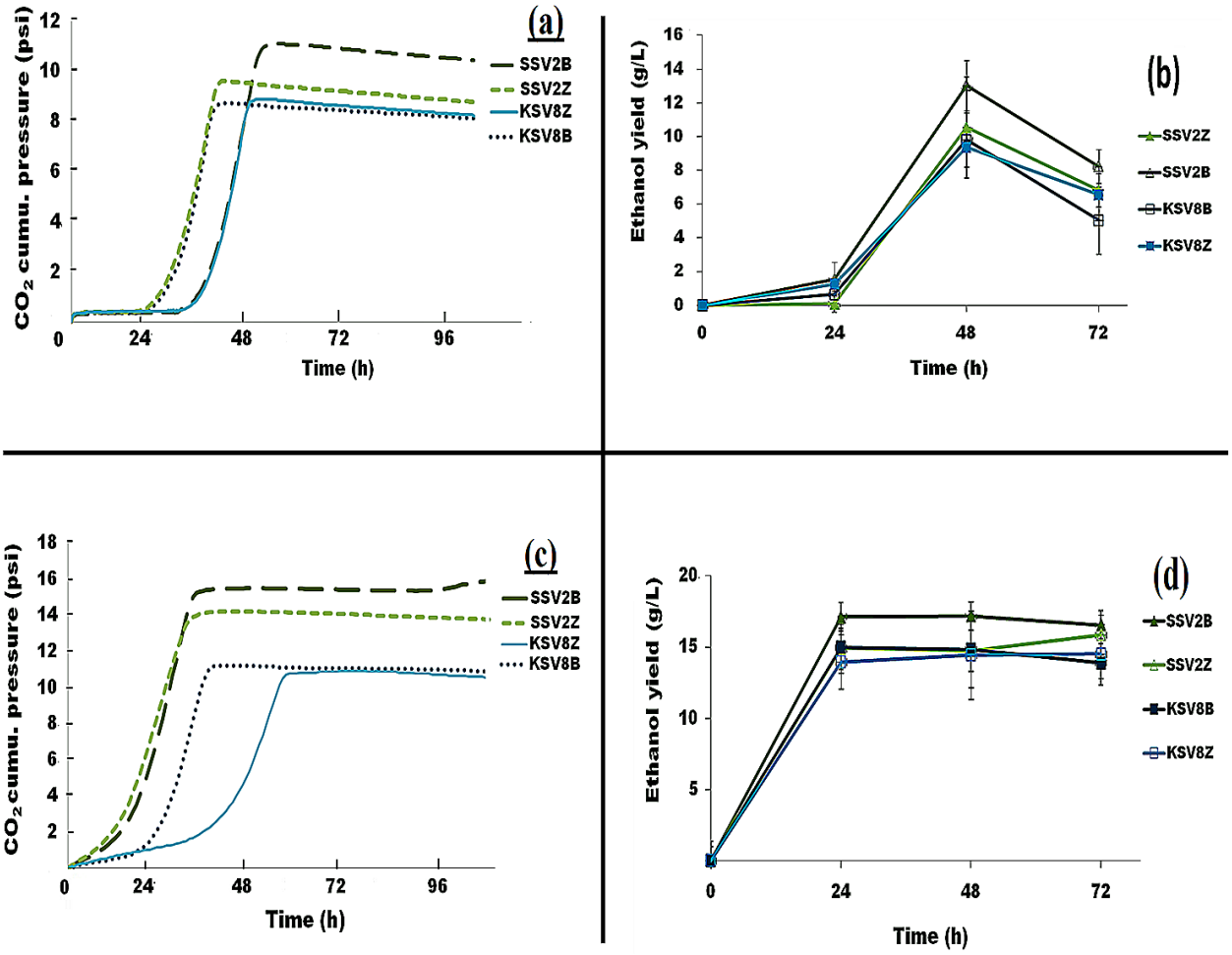
(a)
CO2 cumu. pressure (psi)
Time (h)
SSV2B
SSV2Z
KSV8Z
KSV8B
(b)
Ethanol yield (g/L)
Time (h)
SSV2Z
SSV2B
KSV8B
KSV8Z
(c)
CO2 cumu. pressure (psi)
Time (h)
SSV2B
SSV2Z
KSV8Z
KSV8B
(d)
Ethanol yield (g/L)
Time (h)
SSV2B
SSV2Z
KSV8B
KSV8Z

Fig. 2

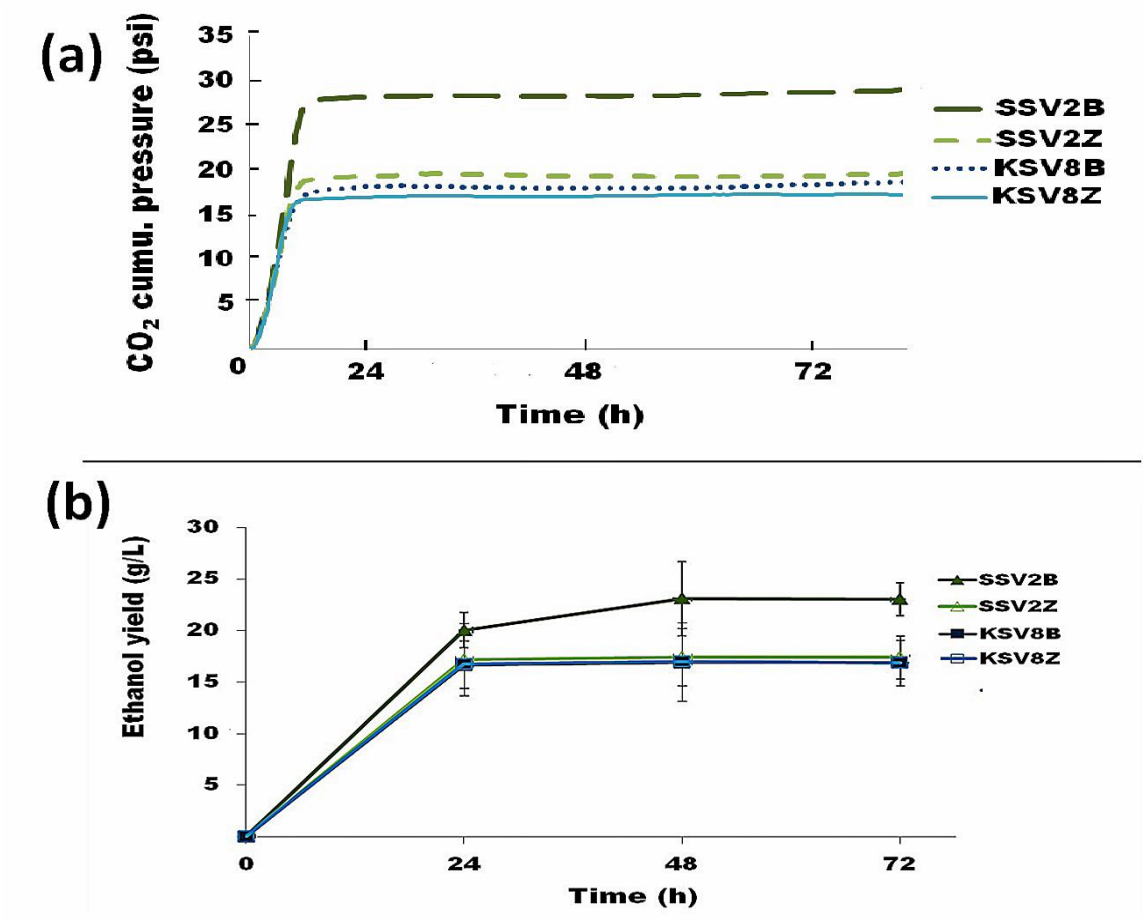
(a)
CO_2 cumu. pressure (psi)
35
30
25
20
15
10
5
0
24
48
72
Time (h)
SSV2B
SSV2Z
KSV8B
KSV8Z
(b)
Ethanol yield (g/L)
30
25
20
15
10
5
0
24
48
72
Time (h)
SSV2B
SSV2Z
KSV8B
KSV8Z

Fig. 3