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RESEARCH ARTICLE

# Fermentation of Pretreated High-Biomass Sorghum Hydrolysates to Biohydrogen by Mixed Consortia

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**Abstract** In the present study, hydrolysate generated during pretreatment of high-biomass sorghum as a carbon source for biohydrogen production was investigated. The high-biomass sorghum bagasse (HBS) was pretreated using acid ($H_2SO_4$) and alkali (NaOH) at various concentrations (0.5–5 % w/v) for the residence time of 30 min at 121 °C, 15 lbs pressure at 10 % (w/v) solid loading. At the optimal acid load of 2 % (w/v) $H_2SO_4$ yielded 78.0 g COD/L of hydrolysate. The hydrolysate generated during this pretreatment was analyzed and noticed to contain glucose 10 g $L^{-1}$, xylose 23 g $L^{-1}$, arabinose 2.0 g $L^{-1}$, HMF 1.9 g $L^{-1}$, furfural 3.5 $g^{-1}$, acetic acid 9.3 g $L^{-1}$, formic acid 5.0 g $L^{-1}$, and phenols 1.9 g $L^{-1}$. The fermentation studies were conducted in dark conditions using all the hydrolysates by heat-treated mixed microbial consortia. Maximum $H_2$ production rate (HPR), cumulative $H_2$ production (CHP), and specific $H_2$ yield (SHY) were measured. Maximum CHP (328 mL) and SHY (4.68 mol/kg CODr) were registered with acid treatment-resulted hydrolysate, and volatile fatty acid analysis indicated higher acetic acid concentration (1.6 g $L^{-1}$) showing acidogenic microenvironment directing fermentation toward acetate pathway. The present study assumes importance in safe disposal and simultaneous production of value-added byproducts during lignocellulosic biorefinery.

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**Keywords** Sorghum · Pretreatment · Hydrolysates · Mixed microbial consortia · Fermentation · Biohydrogen

## Introduction

During the last few years, the utilization of lignocellulosic biomass for biofuel production has conquered more attention due to the rapid increase of energy consumption throughout the globe. In the biorefinery concept, biomass can be converted to useful biomaterials and/or energy shippers in an integrated manner, by this means maximizing the economic value of the feedstock used while reducing the waste streams produced (Thomsen 2005). The cellulose and hemicellulose, which typically comprise 2/3rd of the dry lignocellulosic materials, are polysaccharides. Both hemicellulose and lignin provide a protective scabbard around the cellulose that must be removed prior to efficient utilization of the embedded polysaccharides (Mosier et al. 2005). Biomass pretreatment technologies change the accessibility of plant cell wall polysaccharides to hydrolytic enzymes. The sustainability of a biorefinery depends on the comprehensive exploitation of the biomass feedstock so as to stretch diverse products. The viable biomass-derived energy has a great potential to keep the environment green. The lignocellulosic biorefineries for the production of bioethanol or biobutanol involve the pretreatment process to dislocate lignin barrier from holocellulosic complex, this stage of biorefinery generates high amounts of effluent based on sort of treatment, and these effluents need to be recycled to make the process economically feasible (Yang and Wyman 2008). The operations for treating the solid and liquid residues from cellulosic ethanol production are capital intensive; they

have a major impact on profit margins needed to realize acceptable rates of return on investment (Aden et al. 2002).

Hydrogen is considered as a specialized energy resource in view of its high energy yield (122 kJ/g) and energetic density compared to other fuels (Antonopoulou et al. 2010). However, raw material cost is one of the major limitations for biohydrogen production. Utilization of some carbohydrate-rich or fermentable sugar-rich lignocellulosic biorefinery industry effluents is an attractive approach for biohydrogen production. The present study is intended to utilize the hydrolysate streams generated from acid or alkali pretreatments of high-biomass sorghum for biohydrogen production.

## Materials and Methods

### Sorghum Biomass and Its Pretreatment

The high-biomass sorghum bagasse material (IS27206) used in this study was obtained from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India. The selected sorghum biomass material has considerable amounts of structural polysaccharides which was analyzed and provided in our previous work (Nagaiah et al. 2012). The biomass material was air dried and shredded followed by sieving through a laboratory mesh (0.5 mm) to obtain uniform particle size before subjecting it to the pretreatment. Dilute acid ($H_2SO_4$) and alkali (NaOH) pretreatments were carried out to their effects on the release of fermentable sugars. Both the treatments were performed with different chemical (acid/alkali) loadings, i.e., 0.5–5 % (w/v) for the residence time of 30 min at 121 °C, 15 lbs pressure in a laboratory autoclave. Pretreatment with simple water was selected as control. 10 % (w/v) of substrate loading was considered in all the treatments. After the pretreatment reaction, the undigested solids were separated from the sugar hydrolysates by simple filtration process, and the obtained filtrates were subsequently neutralized using the respective agents (1 N NaOH/1NHCl). Then hydrolysate resulted from each treatment was analyzed for various products such as sugars, furfurals, and volatile fatty acids.

### Inoculum Preparation

Inoculum for $H_2$ production was developed according to Prakasham et al. (2009). In brief, hydrogen-producing mixed consortia that originated from buffalo dung compost was collected in Hyderabad, Andhra Pradesh, India, and the hydrogenotrophic methanogens were deactivated by heat treatment at 100 °C for 30 min. The developed inoculum was stored under anaerobic environment for further use.

### Biohydrogen Production

Batch experiments were carried out to evaluate $H_2$ production using sorghum biomass pretreatment-generated hydrolysates. All the experiments were executed using a series of 250-mL conical flasks (working volume of 200 mL). Nutrient solution comprises (in g $L^{-1}$) $KH_2PO_4$, 1.24; $MgSO_4 \bullet 7H_2O$, 0.1; $NH_4HCO_3$, 80; $Na_2MoO_4 \bullet 2H_2O$ 0.01; $CaCl_2 \bullet 2H_2O$, 0.01; NaCl, 0.01; $MnSO_4 \bullet 7H_2O$, 0.015; and $FeCl_2$, 0.0278. The size of inoculum used was maintained at 4 % (v/v) and the initial pH of the media was 6.0. After inoculation, anaerobic condition was achieved by purging the flasks with oxygen-free nitrogen gas for 5–10 min followed by capping tightly with a rubber septum (suba seals, sigma) and placing in an orbital shaker (150 rpm). All the experiments were performed at a constant mesophilic temperature of 37 ± 2 °C, and hydraulic retention time (HRT) was maintained at 72 h. During the fermentation process, the generated $H_2$ gas in the head space (115 mL) of the conical flasks was analyzed by hydrogen sensor at predetermined time intervals. All the experiments were carried out in triplicate, and the mean values were furnished.

## Analytical Methods

The concentration of sugar (glucose, xylose, and arabinose) and byproducts such as furfurals, HMF, and organic acids (acetic and formic) of pretreatment-resulted hydrolysates was analyzed in HPLC (Waters Corp) using Luna-$NH_2$ column (sugars) connected to RI detector and Luna-C18 column (furfurals and organic acids) connected to PDA detector. The mobile phases used were water/acetonitrile (20: 80) and MQ water (pH 2.5), respectively. In both the cases, the flow rate was maintained at 1 mL/min.

Total phenol content (TPC) was analyzed spectrophotometrically using UV–visible spectrophotometer (UV-2450, Shimadzu) by FolineCiocalteau reagent using gallic acid (GA) as the standard, according to the method of Singleton et al. (1999). Basically, the method is grounded on the redox reaction and the absorbance was measured at 725 nm against a reagent blank.

Microprocessor-based pre-calibrated $H_2$ sensor (electrochemical sensor, FMK satellite 4–20 mA version, ATMI GmbH Inc., Germany) was used to estimate the produced $H_2$ gas. Substrate removal during $H_2$ production coupled with anaerobic fermentation was monitored by the chemical oxygen demand (COD) based on potassium dichromate

closed refluxing titrimetric method, while pH and volatile fatty acids (VFA) were measured according to APHA.

## Results and Discussion

### Composition of Sorghum Biomass Hydrolysates

The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India, has recently identified several high-biomass sorghum varieties. Among the selected high-biomass sorghum traits (IS22868, IS27206, CSH22SS, IS15957, ICSV93046, and IS16529), IS27206 was identified as the best source in terms of its structural carbohydrate content (Nagaiah et al. 2012). Hence, the biomass of this trait was considered in this study for understanding the role of pretreatment in utilization of this high biomass as a substrate for biohydrogen production under anaerobic fermentation. Table 1 displays the composition of hydrolysates resulted when the high-biomass sorghum was pretreated at different concentrations of $H_2SO_4$ and NaOH. Glucose and xylose were the principal sugars sensed in the acid treatments. Arabinose was found in small amounts. Glucose may be consequent from the cellulose, major structural homopolysaccharide present in lignocellulosic biomass (Klinke et al. 2002). Glucose is the fundamental carbon source for any cell. The highest glucose concentration observed was 3.0 g $L^{-1}$ when 0.5 % $H_2SO_4$ was employed to treat the biomass at fixed conditions of 121 °C, 15lbs pressure for about 30 min (Table 1). A diverse increment in glucose release was observed when $H_2SO_4$ concentration was raised from 0.5 % (3 g $L^{-1}$) to 1 % (6 g $L^{-1}$) and 2 % (10 g $L^{-1}$). However, the 2 % acid concentration caused the degradation of released sugars to other spinoffs such as HMF and furfurals to some extent. Further, it was observed that glucose concentration decreased (8.5 g $L^{-1}$) when the acid concentration is increased to 5 %.

Xylose was observed as the chief sugar in the sorghum hydrolysates and 2 % $H_2SO_4$ contributed 23 g $L^{-1}$ of xylose (Table 1). The concentration profile of the xylose was analogous to that of glucose. The maximum concentration of arabinose sugar was observed to be 6 g $L^{-1}$ when 1 % acid concentration was employed. The xylose and arabinose are derived from the hemicellulosic components such as xyloglucans and arabinoxylans (Bercier et al. 2007). The high concentrations of acid in the process of biomass hydrolysis could facilitate a strong reaction for the deconstruction of holocellulosic polymers yielding sugars (Song and Lee 1984). However, when the acid concentration becomes excess, further conversion of released sugars to special compounds (furfurals and HMF)

**Table 1** Effect of acid and alkali pretreatments on the release of sugars and byproducts

| Treatment | Sugars released | | | | Byproducts formed | | | | | COD (g $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| | Glucose (g $L^{-1}$) | Xylose (g $L^{-1}$) | Arabinose (g $L^{-1}$) | TRS (g $L^{-1}$) | HMF (g $L^{-1}$) | Furfural (g $L^{-1}$) | Acetic acid (g $L^{-1}$) | Formic acid (g $L^{-1}$) | TPC (g $L^{-1}$) | |
| 0.5 % (w/v) $H_2SO_4$ | 3.0 | 11 | 2.0 | 16 | 0.25 | 0.7 | 2.3 | 1.2 | 0.34 | 20.79 |
| 1.0 % (w/v) $H_2SO_4$ | 6.0 | 15 | 6.0 | 27 | 0.7 | 1.2 | 5.0 | 2.0 | 0.81 | 36.71 |
| 2.0 % (w/v) $H_2SO_4$ | 10 | 23 | 2.0 | 35 | 1.9 | 3.5 | 9.3 | 5.0 | 1.9 | 56.6 |
| 5.0 % (w/v) $H_2SO_4$ | 8.5 | 18 | 3.0 | 29.5 | 4.4 | 7.9 | 21 | 12.5 | 2.7 | 78.0 |
| 0.5 % (w/v) NaOH | – | – | – | – | – | – | – | – | 3.2 | 3.2 |
| 1.0 % (w/v) NaOH | – | 0.2 | – | 0.2 | – | – | – | – | 5.0 | 5.4 |
| 2.0 % (w/v) NaOH | 0.2 | 0.4 | 0.1 | 0.7 | – | – | 0.2 | 0.18 | 13 | 14.78 |
| 5.0 % (w/v) NaOH | 0.3 | 1.2 | 0.1 | 1.6 | – | – | 0.6 | 0.4 | 25 | 29.2 |
| Control[a] | 0.1 | 1.1 | 0.2 | 1.4 | – | 0.1 | 0.1 | – | 0.1 | 3.1 |

All the chemical treatments were performed at 121 °C and 15 lbs pressure with 30-min duration

*TPC* total phenolic content, *COD* chemical oxygen demand

– Not observed

[a] Biomass + $H_2O$ was used as a control

takes place (Fan et al. 1982). Furfural and HMF could be generated from the dehydration reaction of pentose and hexose sugars which occurs regularly in the presence of acidic environment (Fan et al. 1982). Our results showed the similar trend in the release of furfurals when increasing the acid concentration (Table 1).

Other degraded products found in the hydrolysates were acetic acid, formic acid, and phenols, and these compounds are inhibitory to any microbial fermentation process (Thomsen et al. 2006). Results showed that phenol concentration was not affected by $H_2SO_4$ concentration (Table 1). The acetic acid formed in the hydrolysates was removed through neutralization process if not it can diffuse through the microbial cell membranes and reduce the pH of the intracellular compartment leading to the contrary effects on the microbial metabolism (Passos et al. 1993).

## Biohydrogen Production

Pretreatment of any lignocellulosic biomass is considered as a key process step toward increasing the microbial access to structural polymeric carbohydrates. However, during this process step based on the methodology adopted, the deconstruction of lignocellulosic biomass structure differs. Furthermore, these pretreatment agents tend to depolymerize the biomass structural carbohydrate polymers (cellulose and hemicellulose) into monomeric sugar moieties. Since the aim of the present investigation is to study the impact of pretreatment on biohydrogen production by hydrogen-producing bacterial consortia, initially the release of soluble structural components of selected sorghum high-biomass material (IS27206) was monitored as COD after pretreatment. The experimental results showed that each pretreatment method affected the total carbohydrate solubilization to a different extent (Table 1). At solid-to-liquid loading ratio (S:L) of 1:10, maximum COD (78.0 g $L^{-1}$) was noticed up on 5 % $H_2SO_4$ (w/v) treatment. The NaOH pretreatment did not significantly contribute to the sugars' COD. Similar trend has been noticed by Karunanithy and Muthukumarappan (2010) where pretreatment process mediated the variation of released cellulose, hemicellulose, and lignin oligomeric components. The observed variation in COD values up on treatment of selected high-biomass sorghum with alkali and acid may be attributed to the reactivity nature of pretreatment conditions as under acid environment; hemicellulose is more reactive compared to alkali environment. Similar observations were noticed by Cui et al. (2009) who reported higher COD values attributed to hydrolysis of hemicellulose to xylose.

Hydrogen production rates (HPR), cumulative hydrogen production (CHP), and specific hydrogen yields (SHY) were monitored with respect to time during the fermentation experiments. All the above parameters HPR, CHP, and SHY varied with fermentation time in above experimental conditions indicating the imperative role of pretreatment in the availability of carbon source to fermenting anaerobic hydrogen-producing microbial consortium. Pretreated conditions showed higher HPR, CHP, and SHY than untreated ones. Among the pretreated operating conditions, acid pretreatment-resulted hydrolysate produced higher HPR, CHP, and SHY (Fig. 1) than BTSB and UTSB. In addition, pretreatment also influenced the lag phase on CHP where it started much earlier in ATSB-supplemented conditions over the other two conditions (Fig. 1). In case of ATSB as a substrate source, CHP was noticed after 4 h of fermentation initiation, while in case of BTSB it was noticed after 8 h of initial fermentation. Higher prolonged lag phase of CHP was observed with UTSB as a substrate (Fig. 1). This initial difference in CHP continued till 48 h of fermentation period and thereafter in all studied environments CHP remained constant. This observed variation in CHP with pretreatment condition may be attributed to easily fermentable sugar availability. This observation may be further strengthened with noticed variation in COD noticed under ATSB, BTSB, and UTSB (Table 1) which may be attributed to the release of monomers as the resulting products of cellulosic content of high-biomass sorghum bagasse produced during pretreatment processes. These available monomeric compounds in the pretreated conditions influenced the process efficiency leading to more $H_2$ production. Maximum HPR was observed with ATSB (11.3 mL/h) followed by BTSB (10.7 mL/h) and UTSB (9.6 mL/h). Critical analysis revealed that comparatively less HPR was noticed with BTSB and UTSB. A gradual increase in HPR was noticed in all experimental conditions from the startup of the experiment and attained maximum after 40th h of operation. A sudden drop in HPR was observed with UTSB at 44th h and this continued till the end (72 h) of the experimental study. CHP also followed the same trend with the respective experimental conditions. Higher CHP was observed in the ATSB (328.2 mL) followed by BTSB (277 mL) and UTSB (238 mL/5 g sorghum biomass). $H_2$ production ceased at 32nd h in the case of UTSB condition but at 44th h in the case of BTSB. Whereas, in ATSB condition $H_2$ production was registered till 48th h of operation which might be due to the availability of simple sugars and more accessibility of pretreated biomass to microbial consortia for metabolism because of acid hydrothermal treatment-associated changes on the surface of high-biomass sorghum bagasse.

Specific $H_2$ yield (SHY, mol/kg CODr) is an integral expression of total $H_2$ produced (in moles) and total substrate degraded (in kg of COD) during the process. SHY with respect to three different conditions is depicted in

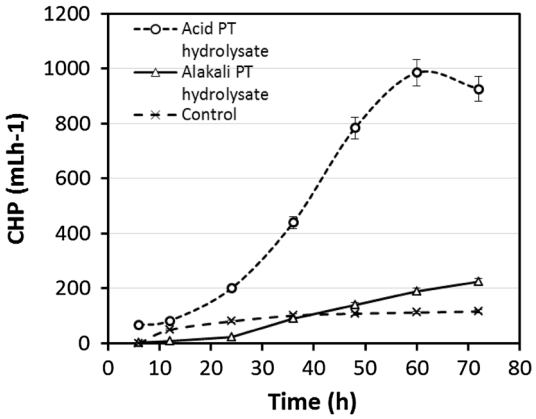


**Fig. 1** Cumulative hydrogen production at different time intervals (final data)

Fig. 2. Monitoring of SHY at the end of cycle operation with all individual conditions denoted that ATSB showed maximum SHY (3.68 mol/kg COD) followed by BTSB (1.2 mol/kg COD) and UTSB (0.92 mol/kg COD). SHY correlated well with the substrate degradation in terms of $COD_r$, and the pattern of $COD_r$ varied with the condition. These observations further confirmed that substrate accessibility is one of the key regulatory factors which favor the $H_2$ yield during the experimental study. Variation in the HPR, CHP, and SHY yields in all the respective experimentations might be attributed to the availability of easily fermentable sugar moieties and/or increased surface availability of high-biomass sorghum for effective hydrolysis of substrates which can be readily consumed by microorganisms for their metabolic activities and subsequent product production.

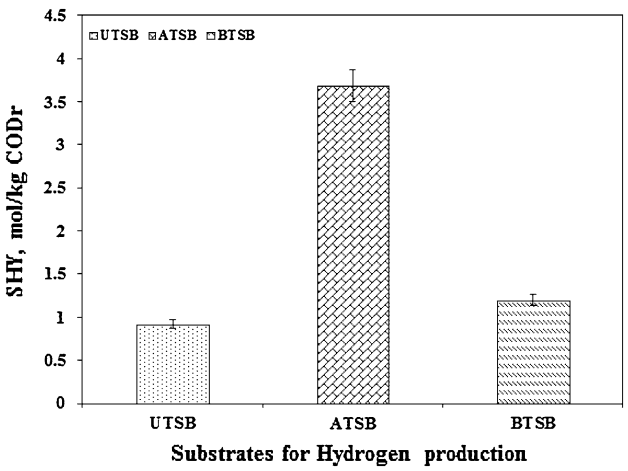


**Fig. 2** Specific hydrogen yield (SHY) in all experimental conditions

## Substrate Utilization

The organic fraction present in the substrate accounts for the total COD of the sorghum bagasse. In the present study, removal of COD was observed in all the experimental variations studied. Maximum COD removal efficiency was noticed with ATSB (56 %) than the other operated conditions, followed by BTSB (47 %) and UTSB (36 %). The observations confirm that the residual organic matter in the substrate was degraded successfully by acidogenic bacteria and might have used for the efficient $H_2$ production. Higher substrate degradation was noticed in ATSB than other experimental conditions which might be due to the availability of simpler substrates released during the pretreatment of high-biomass sorghum bagasse.

## pH Versus Time

pH of the system plays a crucial role in carrying out the metabolic reactions in all the living systems. Initial pH influences the efficiency of substrate metabolism, protein synthesis, synthesis of storage material, and metabolic byproducts' release. Depending on organisms and growth conditions, changes in external pH can bring subsequent alterations in several primary physiological parameters, including internal pH, concentration of other ions, membrane potential, and proton motive force. In the present study, all the operational conditions were operated at acidic pH 6. Variation in the pH profiles was observed in all the conditions with respect to substrate degradation and correlated well with $H_2$ production (Fig. 3). At the initial stage of operation, a sudden drop in the pH was noticed but subsequently got stabilized thereafter without further decrease. This might be due to the utilization of available organic substrates in the metabolic process which further converted to intermediate volatile fatty acids (VFA) and finally to $H_2$. Acidogenic environment prevailed in all the conditions studied indicated the favorable conditions for $H_2$ production. Acidic conditions prevailed during experimentation in all conditions might be due to the presence of $H_2$-producing acidogens. During experimentation, initial pH of all conditions was found to be around $6.0 \pm 0.2$ (Fig. 3). At the end of fermentation (72 h), the pH of ATSB was 3.5 followed by BTSB (3.9) and UTSB (4.7). Change in the pH of the system might be due to the generation of acid metabolic intermediates in the form of VFA, during the acidogenic fermentative process of the microbial population in the reactor Fig. 4.

## Soluble Metabolic Intermediates and Composition

Under acidophilic microenvironment, $H_2$ generation is generally accompanied by acid and solvent production due

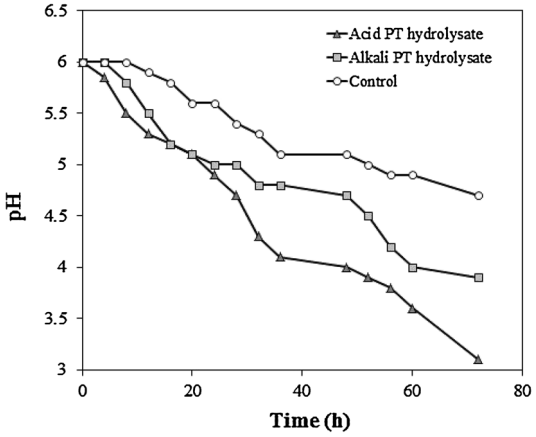


**Fig. 3** Incubation time versus pH drop

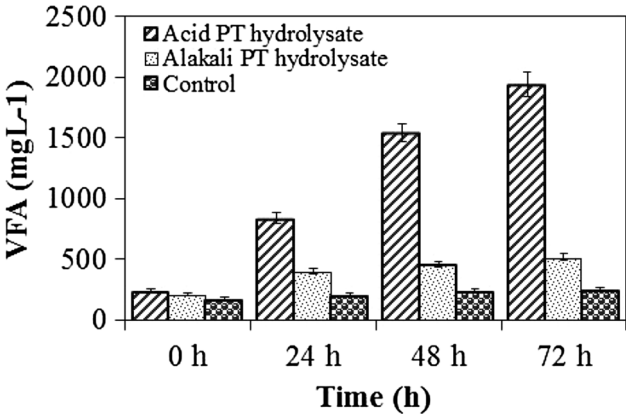


**Fig. 4** VFA (acetic acid) production changes in all experimental variations

to acidogenic metabolism. Acidic intermediate generation reflects changes in the metabolic pathway of the microorganisms involved and provides information on regulatory parameters which can be used to improve the conditions favorable for $H_2$ production. VFA production was always associated with conversion of organic fraction to acid intermediates in the anaerobic microenvironment with the help of specific group of bacteria. Relatively higher VFA concentrations were recorded in ATSB condition (1942 mg $L^{-1}$) at the end of cycle operation. In the case of BTSB, VFA production was slightly decreased (520 mg $L^{-1}$) followed by UTSB condition (250 mg $L^{-1}$) to that of ATSB. Increment in VFA generation directly indicates more substrate degradation which supports higher $H_2$ production. Analysis of correlative VFA generation with system's oxidation–reduction conditions indicated a steady drop in pH along with the increase in VFA production. This may be due to the generation of metabolic intermediates by biocatalyst during fermentative $H_2$ generation which was often considered as a critical factor to understand the metabolic pathway. In general, production of acetate and butyrate favors the production of $H_2$, while production of propionate consumes $H_2$. Determination of the composition of VFA by HPLC revealed the presence of acetate, butyrate, and propionate in the experimental studies. Among total volatile fatty acids, acetic acid showed the highest concentration in all modes of operations, i.e., ATSB (1.6 g $L^{-1}$), BTSB (0.8 g $L^{-1}$), and UTSB (0.5 g $L^{-1}$). Followed by acetic acid, propionic acid showed the higher concentration in ATSB condition (0.67 g $L^{-1}$) to that of BTSB (0.51 g $L^{-1}$) and UTSB (0.3 g $L^{-1}$) conditions. This trend varied with respect of another VFA component, i.e., butyric acid. Higher butyric acid concentration was noticed in BTSB (0.49 g $L^{-1}$) followed by UTSB (0.34 g $L^{-1}$) and ATSB (0.15 g $L^{-1}$). Formic acid was detected to be higher at 6 h of operation in all conditions. In the case of ATSB (1.8 g $L^{-1}$) and UTSB (0.7 g $L^{-1}$) conditions, the formic acid concentration was registered higher than the BTSB (1.4 g $L^{-1}$) and ATSB (1.3 g $L^{-1}$). In the entire operating conditions, rapid drop in the concentration of formic acid was detected at the end of 48 h. These observations related to higher production of metabolic intermediates correlate well with the $H_2$ production. Overall, higher generation of acetate in case of ATSB condition compared to other cases of operation supports the system following acidogenic microenvironment directing toward acetate pathway for higher $H_2$ production.

## Conclusions

The present study infers the impact of acid pretreatment on hydrolysis of high-biomass sorghum bagasse and subsequent biological hydrogen production using heat-treated mixed microbial consortia as a biocatalyst. ATSB was observed to be the best substrate for efficient biohydrogen production. Maximum substrate degradation was observed using ATSB. Observed variation in HPR, CHP, and SHY yields was associated with effective hydrolysis of substrates and consumption by microorganisms for metabolic activities. Acetic acid was observed to be the highest in all modes of operations which supports the system following acidogenic microenvironment directing toward acetate pathway for higher $H_2$ production.

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