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RESEARCH ARTICLE

Sweet Sorghum Juice as an Alternate Substrate for Fermentative Hydrogen Production: Evaluation of Influencing Parameters Using DOE Statistical Approach

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Abstract Biohydrogen production using agro-industrial by-products has considerable practical importance. Sweet sorghum is an ideal feedstock for biofuel production in the semi-arid and arid climatic regions. In the present investigation, the juice of SSV 74, a sweet sorghum variety, was examined as a novel substrate for biohydrogen production. The impact of medium pH, substrate, inoculum level and incubation temperature were analyzed at individual and interactive levels on biohydrogen production. Substrate level concentration and pH of the fermentation medium played a critical role on overall biohydrogen production at individual level, and indicated >90 % influence on product yield. On the other hand at interactive level; pH of the fermentation medium, inoculum and substrate concentrations revealed maximum severity index of 78 % (43 % for medium pH vs inoculum concentration and 35 % for inoculum vs substrate concentration). Overall, biohydrogen production yield was enhanced from 283 to 546 mL/3.25 g glucose equivalents of juice upon statistical optimization leading to a >190 % of H_2 yield. Along with the H_2 production, various acid intermediates were produced with acetate in maximum concentration indicating the occurence of acetogenic fermentation.

Keywords Biohydrogen production · Volatile fatty acids · Sweet sorghum · Fermentation · DOE · Optimization

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Introduction

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). In addition, when this gas is burned for energy recovery, it produces water as a byproduct, the cleanest effluent of any reaction (Lee et al. 2011; Wang and Wan 2009) hence, this gas is viewed as a viable alternative fuel and "energy carrier" of the future. Several hydrogen producing processes, including physical, chemical and biological ones have been developed by the scientific community. Among these processes, the biological methods are advantageous. Since they can be carried out at ambient temperature and pressure, these bioprocesses are less energy intensive (Cohen et al. 1984).

Glucose is considered as the most suitable substrate for any microbial fermentation. In fermentation-based biohydrogen production using glucose as substrate revealed that hydrogen is always produced as co-product during pyruvic acid metabolism under anaerobic environment. The theoretical yield of 4 moles hydrogen per mole of glucose is achieved and any deviation from above is associated with co-production of other organic acids and/or alcohols during metabolic conversion (Turker et al. 2008).

Since, glucose is an integral chemical component of any biomass material, biomass is considered as the one of the

oldest and the most promising energy substrates of the future. It is estimated that biomass provides approximately 14 % of the world energy needs suggesting that it is an important contributor to the world economy. A variety of renewable biomass materials like agricultural and industrial wastes and energy crops have been evaluated as feedstocks for biohydrogen production. However, most of the studies indicated that utilization of biomass material is inefficient mostly due to its recalcitrant nature. Because of this, a novel substrate, sweet sorghum was used for biohydrogen production in this study.

Sweet sorghum is an annual C_4 plant of tropical origin and is an extraordinarily promising multifunctional crop due to its sustainable productivity of grains, and lignocellulosic biomass. In addition, this plant is considered as a potential biomaterial to provide a very wide range of renewable energy products, industrial commodities and animal feed products (Srinivasa Rao and Kumar 2013). Also, it is well-adapted to sub-tropical and temperate regions; it does not need much fertilizer, pesticides or irrigation; has high photosynthetic efficiency (about 2–3 % higher compared to other varieties); and high productivity (Lee et al. 2011; Wang and Wan 2009). Recently, a great deal of research has been undertaken in the EU and other countries to explore its biomass productivity and energy potential under various environmental conditions and cultural practices (Lee et al. 2011; Wang and Wan 2009). The sugar content has been found to vary from 8 to 12 % depending on the harvest time. It consists of sucrose, glucose, and fructose, which are considered as good substrates for biohydrogen and ethanol production. Using sorghum juice, several investigators have reported direct fermentation of its sugars to ethanol and biohydrogen using either mixed cultures or pure cultures (Ariyajaroenwong et al. 2012).

Sweet sorghum exhibits great genetic variability and the genes for sweetness and lignin content have been identified (Semra et al. 1995). The University of Agricultural Sciences, Dharwad under the National Agricultural Research System has developed a sweet sorghum variety with high biomass and sugar content designated as SSV 74. The syrup of this variety contains a mixture of many sugars which could be converted into simple fermentable sugars after heat treatment. Sucrose is the dominant sugar present in SSV 74 at maturity, and is at the highest concentration amongst all the sweet sorghum varieties (Anonymous 2010, 2011) which is favorable for biohydrogen production. Biohydrogen production by mixed microbial consortia is preferred with this type of sugar; hence, there is notable potential when sweet sorghum is used as substrate for bio-based products and biohydrogen production (Prakasham et al. 2009b; Sreenivasrao et al. 2006). In view of the above, the SSV 74 juice was evaluated for biohydrogen production in the present investigation.

Materials and Methods

Inoculum Preparation

Cattle dung obtained from Hyderabad suburbs was used as the source of anaerobic mixed cultures. Prior to use, the cattle dung was heat-treated at 100 °C for 30 minutes to inactivate methanogenic bacteria and hydrogen consumers. For inoculum preparation, the culture was cultivated by supplementing 20 mL per liter sweet sorghum syrup as a carbon source with the supplementation of mineral salt solution according to Prakasham et al. (2009b) and Saraphirom and Reungsang (2010). The composition of mineral salt solution (per liter) is 5 g NH_4Cl, 2.5 g KH_2PO_4, 2.5 g K_2HPO_4, 3 g $MgCl_2{\cdot}H_2O$, 0.25 g $FeCl_3$, 0.16 g $NiSO_4$, 0.25 g $CoCl_2$, 0.115 g $ZnCl_2$, 0.105 g $CuCl_2$, 0.05 g $CaCl_2$, and 0.15 g $MnCl_2$. The culture was incubated at 150 rpm for 24 hours before being used as the inoculum in batch experiments.

Sweet Sorghum Syrup

Sweet sorghum (SSV 74) used in this study was obtained from the field experiment of the Directorate of Sorghum Research, Hyderabad, India. Sweet sorghum syrup that was extracted using mechanical pressure according to (Sreenivasrao et al. 2006; Saraphirom and Reungsang 2010) and sterilized at 110 °C for 20 min to prevent the contamination. The sugars in the stalks are mainly sucrose and glucose (Billa et al. 1997; Cunningham et al. 1988).

Biohydrogen Production

Batch experiments for biohydrogen production were performed in 250 mL serum bottles with a working volume of 150 mL according to (Prakasham et al. 2009b). The hydrogen production experiments were performed using juice as carbon source and by supplementing the mineral salts. Nitrogen gas was flushed deeply into the serum bottles remove oxygen and then they were capped with suba seals to keep them under anaerobic conditions. The bottles were incubated at room temperature and kept on an orbital shaker at 150 rpm to provide better mass transfer. At each predetermined time interval, the total gas volume was measured by releasing the pressure in the bottles using a 50 mL glass syringe. Replicates in thrice were used for all experiments. Finally, the setups were allowed to ferment until biogas production was stopped.

Analytical Methods

Biogas composition was estimated using a gas chromatograph (GC, agilent 4890D) equipped with a thermal conductivity detector (TCD) and a 6 feet stainless column packed with Porapak Q (80/100 mesh). The data obtained from the GC were compared and analysed using individual standards of pure components. The operational temperatures of the injection port, the oven and the detector were 100, 80, and 150 °C, respectively. Nitrogen gas was used as the carrier at a flow rate of 20 mL/min. Total sugars in the fermentation medium before and after fermentation were measured using standard protocol (APHA, AWWA, WPCE 1995). Hydrogen gas production was calculated according to (Prakasham et al. 2009b).

Taguchi Experimental Method

Four critical fermentation parameters viz., initial pH, initial substrate level, inoculum and temperature were selected at four levels (Table 1). L-16 Orthogonal Array was selected for the above control parameters with four levels of factor variation. In the case of substrate variation, sweet sorghum juice was concentrated so as to reach a sugar concentration of 9.5 %. This was done by boiling it at 100 °C to evaporate water present in the juice, then cooling it to room temperature. The resulting syrup was used as a stock solution. For inoculums development cattle dung was heat-treated for 30 min at 100 °C and 100 mL distilled water was added for every 80 g (dry weight) cattle dung. Qualitek-4 software from Nutek, Inc. was used for analysing the data according to (Prakasham et al. 2010).

Analysis of Experimental Data and Prediction of Performance (AEDPP)

Qualitek-4 software (Nutek Inc., MI) for automatic design of experiments using the Taguchi approach was used in the study. The experimental data obtained was processed using Qualitek-4 software with bigger-is-better quality characteristic for the determination of the optimum culture conditions for the fermentation. Also, the analysis was used to identify the influence of individual factors on biohydrogen production and to determine the optimum fermentation conditions.

Validation (V)

To validate the optimized conditions from the software, further confirmation experiments were performed thrice with aliquots using the optimized culture conditions obtained from analysis of the results.

Results and Discussion

Selection of Significant Factors for Biohydrogen Production

Growth and growth-associated biohydrogen production are influenced by metabolic processes which in turn regulated by physiological, nutritional and biological growth factors (Prakasham et al. 2009a, b; Antonopoulou et al. 2011). In mixed acid fermentation processes, the predominance of different fermentative pathways are based on sugar degradation mainly regulated by fermentation parameters like pH, incubation temperature, incubation time, etc. (Prakasham et al. 2009b). Hence, in the present investigation the role of medium pH, inoculum level, incubation temperature and incubation time on biohydrogen production was investigated using sweet sorghum (SSV 74) juice as carbon source inoculated mixed anaerobic consortia. The biohydrogen production was observed in all experimental conditions indicating that SSV 74 stalk juice is a favorable substrate for biohydrogen production. This finding is in accordance with previous studies where biohydrogen production was observed using sweet sorghum juice as a carbon source (Saraphirom and Reungsang 2010). The maximum biohydrogen production (283.0 mL of H_2/25 mL) was observed in a pH 6.0 medium incubated at 40 °C for 18 hours (results not shown). This observation differs from literature in terms of the pH of the medium wherein pH 5.0 was found to be the optimum for biohydrogen production using synthetic substrates as carbon sources (Prakasham et al. 2010). However, Antonopoulou et al. reported that pH 5.2 was the optimum for quality biohydrogen production with sweet sorghum extract as carbon source (Billa et al. 1997).

Design of Experiments and Their Influence on Biohydrogen Production

The metabolism of any biological entity is a coordinated activity of genetical, environmental, physiological, and nutritional parameters. Among these nutritional and physiological factors influence metabolism through genomics and proteomics. In fact, understanding of interactive behavior of each fermentation parameter would an effective solution in designing the bioprocess for economic yield. Therefore, based on the preliminary results, the critical biohydrogen production parameters such as medium pH, incubation temperature, substrate levels and inoculum levels were selected at four levels (Table 1) for understanding both their interactive as well as individual contributions during the biohydrogen production process.

Table 1 Experimental design of selected factors and cumulative biohydrogen production

Run #	pH	Substrate level (mL)	Inoculum level (mL)	Temperature (°C)	Cumulative hydrogen production (mL/g)
1	L1 (5.0)	L1 (10)	L1 (5)	L1 (30)	30.8
2	L2 (5.5)	L2 (15)	L2 (10)	L2 (35)	230.6
3	L3 (6.0)	L3 (20)	L3 (15)	L3 (40)	120.4
4	L4 (6.5)	L4 (25)	L4 (20)	L4 (45)	168.7
5	L2 (5.5)	L1 (10)	L2 (10)	L3 (40)	210.3
6	L2 (5.5)	L2 (15)	L1 (5)	L4 (45)	250.6
7	L2 (5.5)	L3 (20)	L4 (20)	L1 (30)	192.1
8	L2 (5.5)	L4 (25)	L3 (15)	L2 (35)	248.0
9	L3 (6.0)	L1 (10)	L3 (15)	L4 (45)	240.2
10	L3 (6.0)	L2 (15)	L4 (20)	L3 (40)	308.7
11	L3 (6.0)	L3 (20)	L1 (5)	L2 (35)	210.1
12	L3 (6.0)	L4 (25)	L2 (10)	L1 (30)	190.9
13	L4 (6.5)	L1 (10)	L4 (20)	L2 (35)	174.0
14	L4 (6.5)	L2 (15)	L3 (15)	L1 (30)	186.3
15	L4 (6.5)	L3 (20)	L2 (10)	L4 (45)	190.5
16	L4 (6.5)	L4 (25)	L1 (5)	L3 (40)	96.8

An experimental set up was developed using Qualitek-4 software for the above four critical factors considering four levels. The factor fixing was performed based on the one-at-a-time method, wherein optimum value is set as central level (0) and an incremental value on either side of it is set using for other levels. The obsolete values of each selected parameter and predicted and wet lab experimental data are presented in Table 2. Analysis of this data revealed a maximum and a minimum of 308.7 mL H_2/15 mL juice (1.425 g total sugars) and 30.8 mL H_2/10 mL juice respectively, with SSV 74 juice as substrate (Table 1). Comparative evaluation of experimental and predicted biohydrogen production under selected experimental conditions revealed very little variation. This observed variation in biohydrogen production suggested that the selected critical factors and their concentrations play a significant role on overall microbe mediated anaerobic biohydrogen production.

Influence of Bioprocess Parameters on Biohydrogen Production

Evaluation of biohydrogen production data with respect to selected fermentation parameters showed that among all parameters, medium pH was the most critical factor at all studied levels. The selected pH range (5.0–6.5) revealed the maximum (237 mL H_2) and minimum (138 mL H_2) production per 1.425 g glucose equivalents at level 3 (pH 6.0) and level 1 (pH 5.0), respectively (Fig. 1). However, H_2 yield decreased drastically from 237 to 162 mL of H_2 per g glucose equivalent as the pH increased from 6.0 to 6.5 (Fig. 1) whereas a minimal variation in product yield (225–237 mL H_2/1.425 g glucose equivalent) was noticed from level 3 (pH 6.0) to level 2 (5.5). These variations in biohydrogen denote the regulatory role of pH on product yield. In fact, pH is considered as one of the most important process parameters in any bioprocess as this factor influenced the metabolic pathways. This especially holds true for biohydrogen production using mixed acid fermentation where the involved microbial strains typically produce a spectrum of metabolic compounds rather than only acetic acid (Prakasham et al. 2009b). Figure 1 indicated that though biohydrogen content varied with medium of the pH from pH 5.0 to 6.5, the maximum yield was observed at 6.0 pH, A 0.5 pH unit variation towards neutral pH resulted in drastic reduction in overall yield. Similar change in medium pH towards acidic side (pH 5.5) did not show much variation while further change of pH to 5.0 resulted in >40 % reduction on biohydrogen production. This is very interesting because in a similar study using a synthetic substrate as carbon source, the optimum pH was found to be pH 5.0—indicating the dependence of biohydrogen yield on the nutrient (carbon) source itself (Sreenivasrao et al. 2004).

Differences in substrate concentration in the fermentation medium also resulted in variation of H_2 yield; however, the pattern observed is different from those made with pH variation (Fig. 1). The maximum H_2 yield of 244 mL per 1.425 g glucose equivalents was observed at substrate concentration of level 2 (15 mL). Further changes in substrate concentration, both increases and decreases

Table 2 Interaction influence, ANOVA and optimized conditions for selected factors for biohydrogen production using mixed anaerobic consortia

	pH	Substrate level (mL)	Inoculum level (mL)	Temperature (°C)	Others/error	Total
Interactions [severity index (%)]						
pH	NA	28.69	43.19	25.89		
Substrate level	28.69	NA	35.89	17.79		
Inoculum level	43.19	35.89	NA	25.11		
Temperature	25.89	17.79	25.11	NA		
ANOVA table DOF (f)*	3	3	3	3	3	15
Sum of squares (s)	28,111.683	15,743.779	10,383.036	11,190.361	−0.003	65,709.381
Variance (V)	9,370.561	5,247.926	3,461.012	3,730.12	−0.001	
F-ratio (F)	6.212	5.623	2.013	3.819		
Pure sum (s*)	27,831.162	23.532	15.374	16.603		
Percent contribution [p (%)]	42.354	23.532	15.374	16.603	2.317	100 %
Optimum conditions and performance						
Level description	6	15	20	35		
Level	3	2	4	2		
Contribution	46.912	53.487	20.312	25.112		
Total contribution from all factors				145.823		
Current grand average of performance				190.562		
Expected result at optimum condition				336.385		

DF degrees of freedom

resulted in decreased product yield. The data showed that product yield decreased in almost the same rate as substrate concentration, as shown by the observed yield (per gram of glucose equivalents) of 164 mL at substrate level 1 (10 mL, 0.95 g glucose equivalents) and 174 mL at substrate level 3 (20 mL, 1.9 g glucose equivalents). Further increase in substrate concentration to 25 mL (2.375 g) did not show any significant variation in product yield compared to a substrate concentration of 20 mL (1.9 g). The lower yield of H_2 at substrate level 1 may be attributed to the limited carbon source for microbial metabolism. Associated biohydrogen production, while the same at higher substrate concentration, may be due to the limitations with other fermentation factors as observed in other studies (Prakasham et al. 2007). This may also be attributed to other influences as initial biomass concentration is known to play a significant role in bioprocesses, since as inoculum concentration regulates the succeeding growth phases and its associated product yields. The data generated on H_2 yield against inoculum concentration suggested that the product yield is also influenced by inoculums concentration (Fig. 1). An increase in H_2 yield from 147 to 206 mL was observed with increase of inoculum from level 1 (5 mL) to level 2 (10 mL); however, further increase in inoculum concentration did not show significant improvement in H_2 yield. A similar trend was observed with incubation temperature in the range studied (Fig. 1).

The H_2 yield at the 2nd level of substrate concentration with mixed microbial cultures (Fig. 1) and observed consistency in product yield with any further increase in substrate concentration indicated that substrate is not the limiting factor under these fermentation conditions. Such substrate concentration mediated variation in biohydrogen production also noticed earlier (Saraphirom and Reungsang 2010; Antonopoulou et al. 2011). Among all studied factors and their concentration levels, it was found that the pH of the medium at level 3, substrate concentration at level 2, inoculum concentration at level 4, and incubation temperature at level 3 revealed the greatest influence (283, 274, 260 and 251 mL/15 mL, 1.425 g glucose equivalents) on biohydrogen production, respectively.

Individual and Interactive Influences of Parameters on H_2 Bioprocess

At individual level, pH of the medium revealed maximum contribution (42 %) to overall biohydrogen production, followed by substrate concentration, inoculum concentration, and incubation temperature (Table 2). Since the pH of the medium has the largest contribution, then the process of biohydrogen, production is mainly regulated by this factor. Substrate concentration, though one of the essential requirements for microbial growth and metabolism—associated biohydrogen production, showed a maximum of

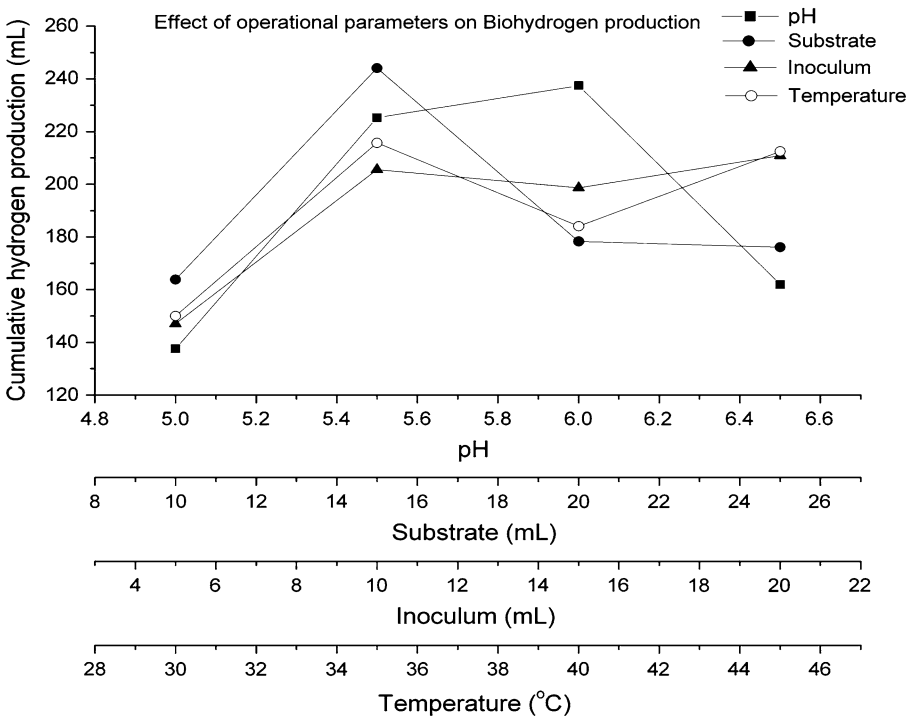

Fig. 1 Influence of different selected fermentation parameters levels on biohydrogen production using sweet sorghum juice as substrate material under anaerobic fermentation with mixed consortia

24 % contribution at individual level. Finally, the selected inoculum and incubation temperature influenced the H_2 yield by approximately 16 %.

Analysis of the data for interactive influences among the selected parameters showed major interaction between medium pH and substrate concentration (Table 2). This interactive pair (pH vs. substrate concentration at levels 3 and 4, respectively) revealed the highest interactive severity index (ISI) at 43.19 %, suggesting H_2 yield is more than 40 % dependent on interaction between these factors. The other major interaction at 29.69 % ISI was observed between substrate and inoculum at level 2 and 4, respectively (Table 2). The weakest interaction at 17.79 % ISI was observed between substrate concentration and incubation temperature at levels 2 and 3, respectively. Overall, pH interacted effectively with substrate concentration, inoculum level, and incubation temperature as shown by the higher ISI and the weakest interaction was observed among incubation temperature, inoculum, and substrate concentration (Table 2).

Optimum Conditions and Validation Experiments

A maximum of 336 mL of H_2 was predicted by the software using a fermentation medium consisting of 15 mL (1.425 g glucose equivalents) of SSV 74 sorghum juice inoculated with 20 mL mixed microbial inoculum at 40 °C and pH 6.0 (Table 2). As per predicted data, the total contribution from all the fermentation parameters would be 145 mL/g glucose equivalents, while the grand average of anaerobic process

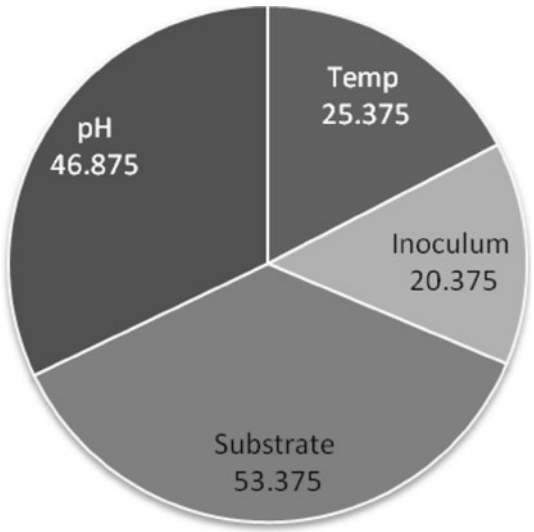

Fig. 2 Fermentation parameter percent contribution on overall biohydrogen yield with sweet sorghum (SSV 74) juice as substrate material

would contribute 190 mL/g glucose equivalents, (Table 2). Though pH of the medium revealed maximum contribution (43 %) at ANOVA, at overall performance of the bioprocess, substrate concentration was found to provide a greater contribution (37 %) compared to pH (32 %) (Fig. 2). This suggests that a parameter may influence overall performance differently at individual, interactive, as well as performance level. Confirmation and validation under an optimized environment indicated maximum biohydrogen yield of 324 mL/g glucose equivalents, showing overall improvement of more

than 190 % compared to that of maximum biohydrogen yield observed before optimization (Table 1).

Conclusions

In this study, batch fermentative hydrogen production was investigated using a promising sweet sorghum variety, SSV 74. The influence of pH, substrate level, inoculum level, and temperature on H_2 production was analyzed. Substrate level was observed to be the most significant parameter over all. However, at the individual and interactive level, other parameters such as pH, inoculum level, temperature were also found to play a significant role on H_2 yield. The maximum H_2 yield of 328 mL/3.25 g glucose equivalents was observed at the following optimum condition: substrate level at 15 mL (1.95 g), inoculum level at 20 mL (2.6 g), initial pH of the medium at pH 6.0, and incubation temperature at 35 °C. Overall the studied fermentation parameters, the contribution of substrate concentration to H_2 yield was observed to be highest at 53 %. After optimization of selected parameters H_2 yield was improved by 190 %. The present investigation suggests that the exploitation of sweet sorghum would be beneficial as it has the capability to influence and improve the rural livelihoods in India due to its potential industrial use for both biohydrogen and biofuel production.

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