Biohydrogen Production Through Dark Fermentation of Food Wastes by Anaerobic Digester Sludge Mixed Microbial Consortium

Adiba Fatima, Bikram Basak, Amit Ganguly, Pradip K. Chatterjee and Apurba Dey

Abstract Food waste is a promising renewable feedstock, which contains a significant amount of fermentable carbohydrate for biohydrogen production. The present research was conducted to investigate the effects of pH (5–7), chemical oxygen demand (COD) in food waste (8–10.8 g/L), and different substrate pretreatment methods on biohydrogen production from food waste. Inoculum enrichment was done by treating anaerobic digester sludge with 2-bromoethanesulfonate (BES) to inhibit methanogenic microorganisms. Dark fermentation was carried out at an initial pH of 7 for 48 h at 37 °C. Total protein before and after fermentation is estimated to be 646.5 μg/mL and 0.1 μg/mL, respectively while total carbohydrate is found to be 5.38 mg/mL and 3.885 mg/mL, respectively. Volatile fatty acids and ethanol production accompanied biohydrogen production which was detected through HPLC. Under optimized conditions of pH 7, temperature 37 °C for 48 h and without any substrate pretreatment around maximum 52% H_2 was obtained in the produced biogas.

Keywords Biohydrogen · Food wastes · Dark fermentation · Pretreatment

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1 Introduction

The world is facing a major climate change caused due to the exploitation of fossil fuels by man. Combustion of fossil fuels results in the release of carbon, nitrogen, and sulfur oxides with trace amounts of soot, ash, tar droplets, and other organic compounds that contribute to the greenhouse effect. Therefore, the development of eco-accommodating fuel is the need of the hour. This is essentially important for the conservation of biodiversity and self-preservation of human life [1, 2]. Global warming and depletion of nonrenewable fuels are no longer a new issue to mankind and ceaseless efforts are being made to mitigate it [3]. This has driven researchers worldwide to develop renewable bioenergy technologies which on combustion does not endanger the environment. Biohydrogen (bioH_2), biomethane, bioethanol, and biodiesel contribute to energy-efficient biofuels produced by various chemical and biological processes but considering the present scenario which among the above can be used in the long run as a sustainable energy, fuel is still a question that is yet to be addressed by researchers [4, 5].

Hydrogen (H_2) is a fascinating alternative as it is clean, nonpolluting, easily recyclable, lighter than air, and has a high heating value (142 kJ/g) which is 2.75 times greater than hydrocarbon fuels [6]. It has the highest gravimetric energy density of 141 MJ/kg and low volumetric energy density at only 12 MJ/m^3 (under normal temperature and pressure) [7]. High gravimetric and low volumetric energy density are the two critical factors which facilitate it being used as a transportation fuel [8]. Its combustion produces high amounts of energy and water as the only reaction product and leaves no net carbon footprint. If bioH_2 technology as a sustainable fuel is to be realized relatively in the long term, further advances have to be made so that it can be called a versatile fuel both from technological and economical points of view [9–11].

Commercially hydrogen is being produced from fossil fuels, biomass, and water by steam reforming of natural gas, biomass gasification, water electrolysis, etc., which are driven by high energy input and subsequent increase in carbon dioxide emissions. This has drastically affected our greenhouse layer of the atmosphere. Biological hydrogen production methods stand out among a plethora of other processes because it is economical, an eco-friendly process in which microorganisms produce hydrogen from renewable biomass or wastewater through dark fermentation [12]. Among the various processes employed for biological hydrogen production, dark fermentation serves as the most promising and judicious technology than photofermentation due to its high production rate, yield, the utilization efficiency of various organic wastes and feedstock as substrate and its ability to catalyze the reaction without the presence of light. Thereby, manifesting no external energy input making it reliable and cost-effective [13, 14].

The magnitude of waste generated depends on lifestyle, the extent of commercialization, eating habits, and season. According to the United Nations Food and Agriculture Organization, approximately one-third of the total global food produced is wasted, costing the world economy about $750 billion. Globally, India currently

ranks seventh in terms of overall food wastage of agricultural produce, poultry, and milk. It has been evaluated that around 67 million tonnes of food is wasted from India amounting to 92,000 crores; which is capable enough to feed all of an Indian state of Bihar for a year. Food waste generated by Mumbai alone amounts to 9,400 metric tonnes of solid waste per day of which 73% is food, vegetable, and fruit waste, while only 3% is plastic [15]. With numbers such as these current systems in the country are unable to cope up with this burden, subsequently leading to a negative impact on the environment and public health. Open landfills release various harmful gases such as methane which is an add-on to global warming. It also causes the formation of toxins and/or black leachate which oozes from the waste and is absorbed by the soil leading to groundwater contamination. These brim-full landfills have become the root cause of gridlocked drains, soil, and water pollution [15].

In view of economic consideration, bioH_2 production from waste is constructive as it reduces the cost to manage waste thereby increasing revenue from agricultural produce via its waste to energy conversion route. This has led to mitigating CO_2 emissions owing to the utilization of renewable biomass which has efficiently superseded fossil fuels. In the quest for the development of bioH_2 production, a major challenge that needs to be conquered is the increasing cost of feedstock if the substrates for hydrogen dark fermentation typically employ sucrose or starches which are not practically possible on an economic scale and also edible crops which will ultimately lead to food shortages. Biomass particularly waste and/or wastewater containing high concentrations of carbohydrates, discarded from textile and food processing industries, agricultural processes, municipal solid waste, and restaurant food waste are preferred for pecuniary reasons.

Food wastes are copious in nature and profusely dominated by carbon skeleton. It is a waste comprising of underdone and prepared food removed before or during food preparation from various domestic sources like a restaurant, canteens, etc. It has a high energy content and easily biodegradable because it comprises 85–95% of volatile solids and 75–85% of moisture favoring microbial fermentation. If managed properly it will thwart the leaching of pollutants into the groundwater [16]. Therefore, the biotransformation of food waste to bioenergy is a topic pursued by many scholars worldwide. Enhanced bioH_2 capability by anaerobic digestion of food waste is favored due to high moisture content, substrate concentration and carbon to nitrogen ratio [17]. pH, temperature, partial pressure of hydrogen, pretreatment of substrates, and inoculum enrichment are important physicochemical parameters that need to be monitored in order to uplift bioH_2 production [14]. To stimulate the degradation rate of food wastes, they have coalesced with water. Higher the degradation of food waste higher is the rate of hydrogen liberation [11–13]. Food waste is chemically composed of carbohydrate, protein, triglycerides, and polysaccharides such as starch, cellulose, and hemicelluloses. The major limiting step is the rate of hydrolysis of different components of food waste for energy inception. By far carbohydrate is considered to be a supreme substrate; however, fats and protein can also be exploited for energy liberation. It has been reported that there is a 20-fold increase in bioH_2 production rate utilizing carbohydrate-rich substrate

compared to protein and lipid-rich substrates [15]. This paper envisages bioH_2 production by dark fermentation of food wastes utilizing mixed microbial consortia.

2 Materials and Methods

2.1 Enrichment of Mesophilic Hydrogen-Producing Microorganism

Anaerobic digester sludge was collected from biomethanation plant located in CSIR-CMERI colony, Durgapur, India. Prior to its usage, it was pretreated with 200 mM of 2-bromoethane sulfonic acid (2-BES) to inhibit the hydrogen consumers like methanogens and enrich hydrogen-producing bacteria.

2.2 Preparation of Food Waste

The collected food waste used as substrate was comprised of rice 40% (w/w), vegetable 35% (w/w), and lentils 25% (w/w). Renewable food waste was obtained from two cafeterias in and around NIT Durgapur, India. The food wastes were milled in an electric blender without the addition of water in order to make a homogeneous sample and stored in a sealed glass bottle at 4 °C until used.

2.3 Pretreatment of Substrate

Combination of enzymatic and heat treatment 80μL of amylase was added to the milled substrate and subjected to heating in a water bath at 90 °C for 1 h. After pretreatment, the substrate was cooled down and added to serum bottles along with mixed microbial consortia, 2-BES, and phosphate buffer. The mixed solution was adjusted to pH 7 by the addition of NaOH. This was kept in incubation at 37 °C for 72 h.

Combination of enzymatic, heat, and sparging of nitrogen gas 80μL of amylase was added to the milled substrate and subjected to heating in a water bath at 90 °C for an hour. After pretreatment, the substrate was cooled down and added to serum bottles along with mixed microbial consortia, 2-BES, and phosphate buffer. A swarm of nitrogen gas was sparged for 2–3 min to create anaerobic environment in the reactor. This was kept in incubation at 37 °C for 48 h.

Combination of physical and enzymatic treatment The food wastes were subjected to microwave heating for 10 min at 400 W followed by sonication for 20 min at 40 °C and 80 µL of glucoamylase was added and heated at 40 °C for 2 h.

2.4 Batch Acidogenic Fermentation of Food Waste

Pretest experiments were performed in 125 mL serum bottle as batch reactors with a working volume of 80 mL comprising the mixed microbial consortium and pretreated food waste. In the latter part of this study bioreactor with 2.5L working volume operating in batch mode was used in order to scale-up the process. The temperature and mixing of the system were maintained at 37 °C and 150 rpm, respectively, using a magnetic stirrer with heater. The pH of the system was adjusted to 7 prior to fermentation. The volume of biogas produced was measured in a wet gas meter using the water displacement method (Fig. 1).

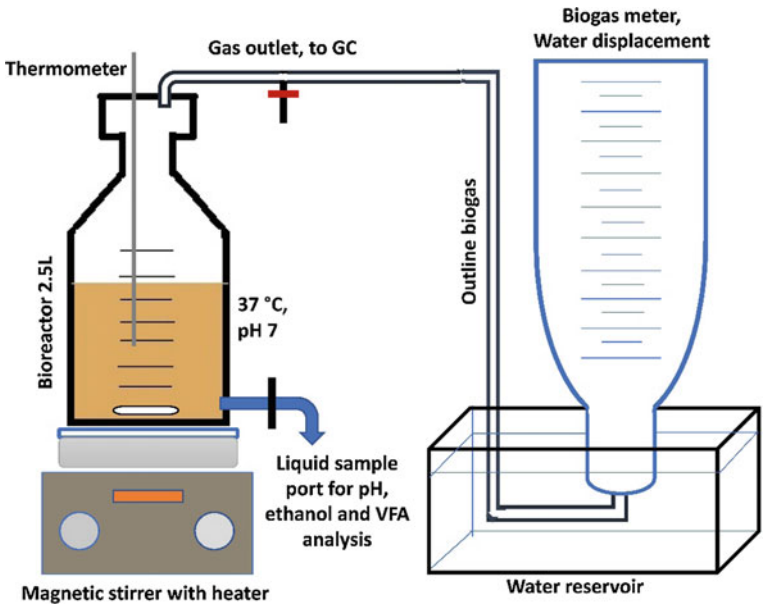

Fig. 1 Schematic representation of batch fermentation set up for bioH_2 production from food waste

2.5 Analytical Methods

Cumulative H_2 production was plotted against fermentation time over the course of fermentation and is analyzed using the modified Gompertz equation

$$H(t) = H_{\max}\ exp\left[-exp\left\{\frac{R_m \times e}{H_{\max}}(\lambda - t) + 1\right\}\right] \quad (1)$$

where H defines cumulative hydrogen production (mL) at time t, $H_{\max}$ is the H_2 production potential (mL), R_m (mL/Lh) stands for the maximum H_2 production rate (mL H_2/Lh), λ is the lag phase time (h), and e is 2.718 [12]. The percentage of H_2 in biogas was measured using a gas chromatograph (Chemito GC 1000, Chemito Instruments Pvt. Ltd., India) equipped with Thermal Conductivity Detector (TCD) and stainless steel packed molecular sieve column. The injector, oven, and detector of the GC were set at an operational temperature of 130 °C, 50 °C, and 150 °C respectively at a voltage of 5 V. Argon was used as a carrier gas. For the determination of volatile fatty acids (VFAs) in the liquid sample, they were first centrifuged at 10,000 rpm for 10 min and the supernatant was subjected to filtration through a 0.22 μM cellulose acetate membrane before being analyzed by HPLC. The concentrations of VFAs (acetate, butyrate, and propionate) and ethanol were analyzed by HPLC (Waters™600, Milford USA) equipped with Cosmosil 5 NH_2-MS column and UV/Visible and Refractive Index detector. Sulphuric acid (0.005N) served as the mobile phase at a flow rate of 0.5 mL/min.

The pH and Chemical Oxygen Demand (COD) were determined using standard methods. Phenol-sulfuric acid method was used to determine the total carbohydrate,

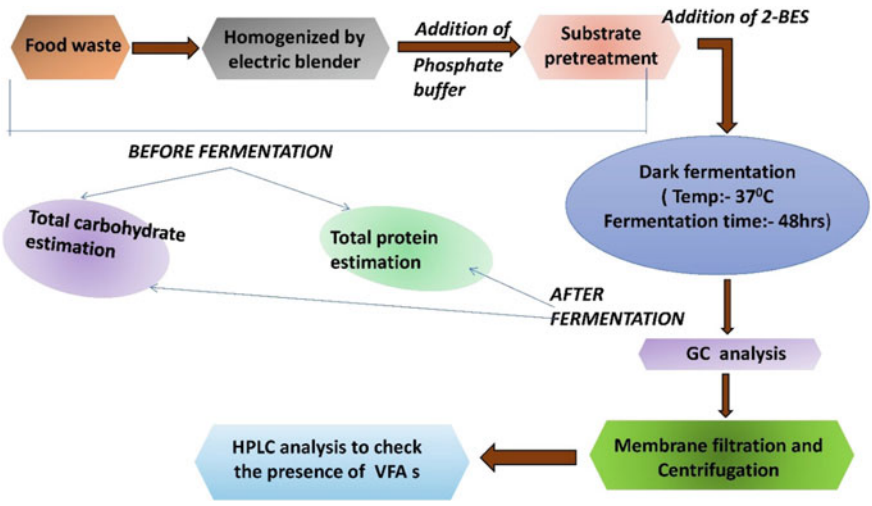

Fig. 2 Schematic representation of experiments performed

while soluble protein was measured by the Lowry's assay using a UV/Visible spectrophotometer (Thermo Scientific™ UV 10, USA). The overall steps of all experiments performed are summarized in Fig. 2.

3 Results and Discussion

3.1 *Amount of BioH$_2$ in the Produced Biogas*

The amount of hydrogen in the produced biogas was measured with respect to standard hydrogen (100% pure) using gas chromatography equipped with TCD. The retention time for the standard was 1 min. Chromatogram of the gas produced from the fermentation of food waste shows hydrogen content to be around maximum 52.2% with no detectable methane because 2-BES is an efficient suppressor for methanogens. 2-BES functions by inhibiting the activity of coenzyme M reductase which is a key enzyme involved in methanogenesis. The other 47.8% comprises traces of nitrogen, carbon dioxide, and water vapor (Fig. 3). The mixture of H_2 and CO_2 can be stripped off from the gas stream with 30% potassium hydroxide (KOH) solution.

3.2 *Effect of PH on Kinetic Parameters for BioH$_2$ Production by Dark Fermentation of Food Waste*

The regulation of pH is critical to the dark fermentative bioH_2 production as it directly affects the activity of enzyme hydrogenase and metabolic pathways. It has been found that when the pH of the fermentation media is below the optimum pH

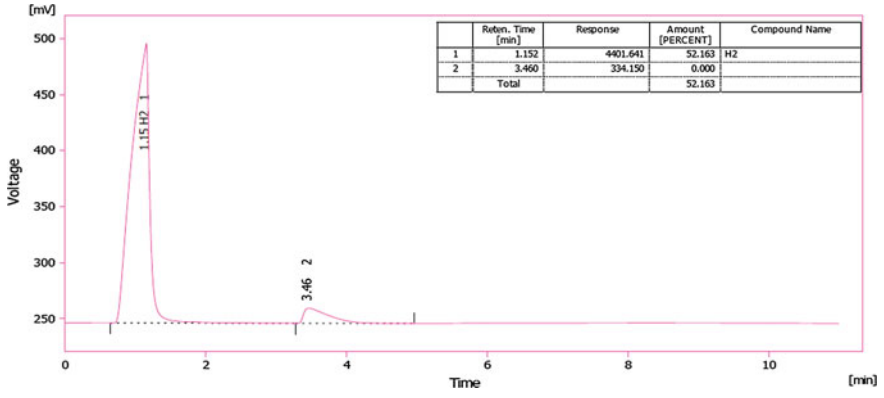

Fig. 3 Chromatogram showing a maximum content of 52.2% bioH_2 from fermentation of food wastes

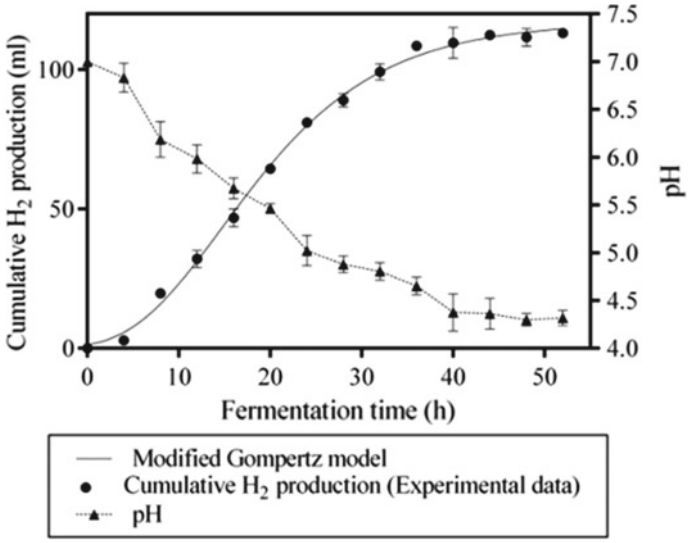

Fig. 4 Kinetic parameters for bioH_2 production are hydrogen production potential (H_{max}) 117.2 mMol/L; maximum hydrogen production rate (R_{max} 4.44 mMol/L/h); and lag phase time (λ) 4.99 h

for bioH_2 production either of the two consequences arose, first, the metabolic activity of hydrogen favoring microorganisms was stalled and second, a shift in the metabolic activity of hydrogen producers would occur resulting in cessation of bioH_2 generation. In this study, the effect of different initial pH and fermentation time on cumulative bioH_2 production during anaerobic fermentation of food wastes in the mesophilic batch reactor was evaluated (Fig. 4). It is observed that with a decrease in pH and increase in the duration of fermentation the cumulative hydrogen production decreases [12]. Experimental results showed that a relatively insignificant amount of bioH_2 was detected in the biogas with an initial pH of 5 or 6, while an initial pH of 7 substantially augmented bioH_2 generation from food waste. This could be possibly due to a swap in the metabolic pathway of microorganisms resulting in the production of organic acids such as acetate, butyrate, and propionate thereby hindering the hydrogenase activity, which contributes to enhancing bioH_2 yield. At pH beyond 7, the cumulative hydrogen accumulation in the biogas declines due to the generation of a little amount of methane. This suggests that pH should be kept between 6 and 7 during the acidogenic fermentation process. Similar findings have been carried out by other researchers [6]. The lag phase for mixed microflora persisted for 4.99 h after which they entered the exponential growth phase. The maximum hydrogen production potential (H_{max} = 117.2 mMol/L) and maximum hydrogen production rate (R_{max} = 4.44 mMol/L/h) is achieved at pH 7 when the reactor is operated for 50 h in batch mode.

3.3 *Effect of Different Pretreatment Methods on the Hydrogen Content of Produced Biogas*

Pretreatment is a biochemical process employed to ensure complete hydrolysis of complex carbohydrate polymers to simple fermentable sugars which can be readily consumed by the mixed microbial consortia. An ideal pretreatment process should bear the following salient features: (a) improve saccharification by hydrolysis; (b) inhibit the degradation of carbohydrate whilst the formation of inhibitory products; (c) lucrative. If biomass is the choice of the substrate to be used in dark fermentation, the cellulose, and hemicellulose that obstruct enzymatic attack and crystallinity of cellulose needs to be decreased by appropriate pretreatment processes. The number of pretreatment steps is dependent on the type of feedstock being used. Biomass pretreatment is carried out by physical, chemical and/or biological processes. Physical treatment is associated with a decrease in size or involvement of physical forces to degrade and/or alter the cellulose structure in such a form that the enzymes that breakdown cellulose into fermentable sugars have an easy access. Chemical treatment is carried out under high temperature and severe alkali/alkaline conditions. Conversely, biological or enzymatic pretreatment can be achieved at ambient conditions with lower conversion rates. The main principle underlying all the pretreatment steps is to facilitate the access of microorganisms to fermentable sugars within the biomass.

In the present study, food wastes underwent enzymatic and physical treatment while inoculum was enriched by a 2-bromoethane sulfonic acid (2-BES). In the first case, amylase followed by heat treatment was done at 90 °C for an hour to alter the cellulose structure associated with the food waste. The maximum hydrogen content in biogas was found to be 40.1% when compared to the untreated food waste having 52% hydrogen (Fig. 5). The reason behind a decrease in bioH_2 production

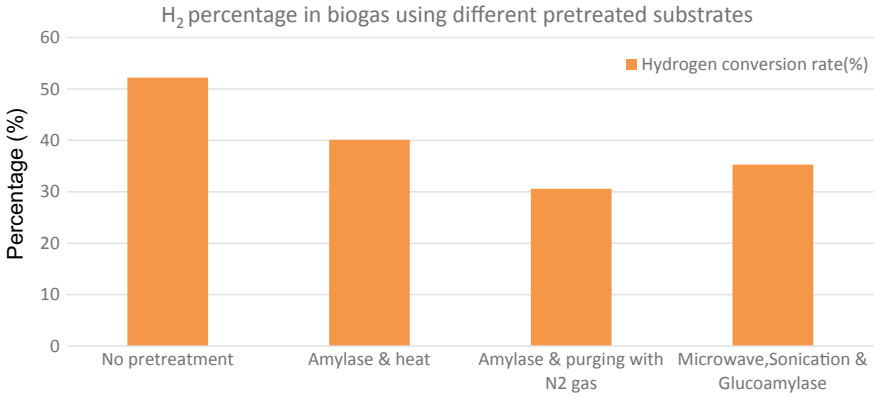

Fig. 5 Different substrate pretreatment and their corresponding H_2 content (%) in produced biogas

could possibly be due to the formation of fermentation inhibitors associated with heat treatment. In order to enhance bioH_2 production, the formation of furfural and 5-hydroxymethyl furfural associated with carbohydrate degradation of pentoses (C_5) and hexoses (C_6) should be prevented by keeping the process temperature and residence time as low and as petite as possible.

In the second case, amylase treatment followed by 2–3 min of sparging with nitrogen gas was performed. The production of bioH_2 via the dark fermentation route are extremely sensitive to the partial pressure of hydrogen, which is a rate-limiting parameter, particularly due to its inhibitory effect on the enzyme hydrogenase, which is involved in the transfer of an electron from an intracellular carrier to H^+. The decrease in enzyme activity is particularly because of feedback inhibition. Hence, sparging with inert gases such as nitrogen, argon in the reactor headspace will lower the hydrogen partial pressure whilst increasing the hydrogen yield. In combination with partial pressure-lowering post-amylase treatment, hydrogen conversion rate subsequently lowered to 30.6%. The probable reason behind this could be lower conversion rate of feedstock into fermentable sugars as reported by Chen et al. that pretreatment of wheat straw by fungi for a continuous period of 10 days resulted in high amounts of fermentable sugars and diminution in fermentation inhibitors [16]. Although enzymatic pretreatment is highly enthralling, the rate of hydrolysis is too slow and is practically not feasible on an industrial scale. In order to make enzymatic pretreatment at par with other pretreatment, a combination of either physical or chemical pretreatment should be followed post enzyme treatment. This has been reported in various studies such as that of Wang et al. (2012), he combined biological with liquid hot water treatment for enhanced saccharification of *Populustormentosa*. This combination resulted in maximum hemicellulose removal leading to an increase in glucose yield by 2.66-fold compared to pretreatment carried out with liquid hot water alone [17].

Structural disruption of organic matter present in food waste can be achieved with microwave irradiation and ultrasonication. When organic material is subjected to microwaves, the organic molecules and water within the cells grasp this wave energy resulting in tremendous heat generation and consequently cell wall is degraded. Conversely, when biomass is subjected to ultrasonication, a prodigy known as bubble collapse occurs by a method involving cavitation which causes an increase in pressure and heat formation resulting in the collapse of the cell wall structure.

As is observed in the third case of the present study where food wastes were subjected first to microwave heating for 10 min at 400 W followed by sonication for 20 min at 40 °C and subsequent addition of glucoamylase followed by heated at 40 °C for 2 h. It was found that only 35.2% of hydrogen is liberated. This study demonstrates that the power, duration of sonication, and time of heating should be kept as low as possible. High sonication power is known to adversely affect the pretreatment process with the formation of bubbles near the tip of the ultrasound transducer, which thwarts the transfer of energy to liquid medium [18]. Similar

studies were carried out by Chen et al. (2006) with poplar wood cellulose powder suspension, which turned viscous on treatment with a high-power of 1200 W [19].

It was found that when no substrate pretreatment is carried out hydrogen conversion rate in the biogas is around maximum 52.2% as shown in Fig. 5, which could be possibly due to the presence of cellulose-degrading microorganisms in the mixed microbial consortia. Nevertheless, an imperative analysis of the aforementioned pretreatment methods brings us to an inference that pretreatment step is a "custom-made" process for every individual biomass which requires scrupulous selection and plan based on the characteristic features that are solely associated with that biomass. A comparison of different pretreatment methods on bioH_2 content (%) in the produced biogas is shown in Table 1.

Table 1 A comparison of different pretreatment methods and respective biohydrogen content (%) in the produced biogas

Substrate	Pretreatment method	Inoculum	Hydrogen content in biogas (%)	References
Food waste	–	Anaerobic sludge treated with 2-BES	52.2	This study
Glucose	–	Digested sludge boiled at 100 °C for 15 min	44.5	[20]
Glucose	–	Anaerobic sludge baked at 104 °C for 2 h	24.2	[21]
Food waste	Amylase and heat treatment at 90 °C for 1 h	Anaerobic sludge treated with 2-BES	40.1	This study
Food waste	Amylase treatment followed by purging with nitrogen gas	Anaerobic sludge treated with 2-BES	30.6	This study
Food waste	Microwave heating for 10 min at 400 W followed by Sonication for 20 min at 40 °C and Glucoamylase treatment at 40 °C for 2 h	Anaerobic sludge treated with 2-BES and incubated for 1 h	35.3	This study
Co-digestion of cow manure and waste milk	Waste milk was treated with cefazolin	–	48.7	[22]
Co-digestion of pig manure with fruit and vegetable waste	–	The initial inoculum was formulated by digesting glucose and fruit wastes	42 ± 0.5	[23]

*2-BES = 2-bromoethanesulphonic acid

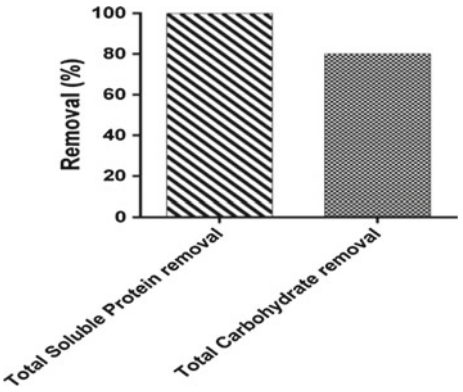

Fig. 6 Total carbohydrate and soluble protein removal (%)

3.4 Evaluation of Nutrients Utilized by the Microflora

The food wastes upon hydrolysis release carbohydrate and protein as the main carbon and nitrogen source for bacteria to readily utilize it and release bioH_2 as the major reaction product. Total carbohydrate and soluble protein were determined before and after fermentation to calculate removal (%). It is clearly observed that total carbohydrate removal is 80.05% while total soluble protein removal is 99.98% respectively. Thus, it can be inferred that the microbial population present in the pretreated anaerobic sludge has efficiently utilized protein and carbohydrate (Fig. 6).

3.5 Volatile Fatty Acids (VFAs) and Ethanol Production

The production of BioH_2 is accompanied by VFAs and ethanol production, thereby lowering the pH of the system. VFAs mainly include acetate and butyrate with acetate being the major by-product. The overall equation of bioH_2 production from food wastes is as follows:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (2)$$

This gives a clear notion that pretreatment procedure such as heat, chemical, or pH shock of the seed culture is a prerequisite to reduce the inhibitory effects of lactic acid-producing bacteria and other hydrogen consumers during dark fermentation [12]. The choice of formation of organic acids depends exclusively on the dominance of respective bacteria favoring their production.

4 Conclusion

The most economical and viable process for producing renewable energy is the utilization of organic waste. H_2 production employing acidogenic dark fermentation represents an exciting new arena of technology for bioenergy production. The lower yield of H_2 obtained with acidogenic fermentation compared to other production methods of hydrogen remains the main issue to be addressed before the process can be commercialized. There is an effort to find ways to enhance both the production rates of hydrogen and its ultimate yield. In this study, if no substrate pretreatment is done, we obtained a high bioH_2 content (52%) in the biogas produced by the rich H_2-producing microflora obtained from anaerobic digester sludge with subsequent consumption of carbohydrate and protein.

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