



Thermo-acidophilic biohydrogen production from rice bran de-oiled wastewater by Selectively enriched mixed culture

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Abstract

The present study focuses on the biohydrogen production in an anaerobic batch reactor operated at thermophilic (57°C) and acidophilic conditions (pH 6) with rice bran de-oiled wastewater (RBOW) as substrate. The hydrogen generating mixed microflora was enriched from slaughter house sludge (SHS) through acid treatment (pH 3-4, for 24h) coupled with heat treatment (1h at 100°C) to eliminate non-spore forming bacteria and to inhibit the growth of methanogenic bacteria (MB) prior to inoculation in the reactor. The hydrogen production rate was maximum at 57°C ($1861 \pm 14 \text{ml/L-WW/d}$) compared to 37°C ($651 \pm 30 \text{ml/L-ww/d}$). The Hydrogen yield increased with temperature from 1.1 to 2.2 molH₂/mol of substrate respectively. The optimum pH range for hydrogen production in this system was observed in between 5.5 to 6. Acid-forming pathway with butyric acid as a major metabolite dominated the metabolic flow during the hydrogen production.

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Keywords: Biohydrogen, Rice bran de-oiled wastewater, Pre-treatment, Thermophilic temperature, PH control.

1. Introduction

Today, most of the energy demands were met by nonrenewable energy sources, resulting in resource depletion, environmental deterioration, and public health problems. Therefore, a demand to develop novel renewable energy-harvesting technologies and introduce sustainable energy carrier exists [1]. Hydrogen was expected to be cleanest energy source of the future, since its sole product is water, and it does not release CO₂ and other harmful gases into the natural environment when it is used for energy production. Recently, great progress has been achieved in the technological development of fuel cells and in the storage and transportation of hydrogen [2]. Currently, hydrogen is produced by chemical, thermal, and electrical processes, which are neither sustainable nor cost-effective. Hydrogen production by biological methods is attractive because it is an energy-saving production process compared with chemical process. Recently, dark fermentative hydrogen production has been reported to have great potential for development as a practical biohydrogen system [3]. Few studies on dark fermentative hydrogen production have been reported with authentic wastewaters, such as cheese processing waste water [4], Olive Pulp [5] and food processing wastewaters [6]. Wastewaters show great potential for

economical production of hydrogen because producing a product from a waste could reduce waste treatment and disposal costs. Rice Bran De-oiled Wastewater (RBOW) is the carbohydrate rich and easily hydrolysable wastewater. Wastewater has a high chemical oxygen demand (COD) value and is therefore suitable for anaerobic treatment process. Early investigations on converting those organic wastes to hydrogen mainly focused on pure cultures aiming at achieving a high yield [7, 8, 9, 10, 11], while in some cases, mixed cultures were also used to produce hydrogen from organic wastes [12]. When mixed cultures were utilized in biohydrogen production, the reaction pathways and the resulting byproducts can change unpredictably depending on reactor conditions such as pH, temperature, pretreatment of inoculum and feedstock concentration as well as the nature of the microbial community. Fundamental understanding of dark fermentative hydrogen production was still not complete and suggested optimal conditions seem to differ from one study to the next. Moreover, most studies have been performed mesophilic (35°C) and only a few have applied thermophilic temperatures (57°C). In the case of thermophilic condition, however it has been recently demonstrated that stable continuous hydrogen production can be achieved [13]. Furthermore, in the thermophilic range, reaction rates proceed faster than under mesophilic conditions, so that the organic loading potentials of the anaerobic reactors were substantially higher [14]. Therefore, in the present study, the hydrogen production from RBOW as substrate and enriched Slaughter House Sludge (SHS) as microflora was examined under thermophilic conditions, and also the operating conditions of the reactor were optimized (pretreatment, pH, temperature and nitrogen source).

2. Materials and methods

2.1 Rice bran de-oiled wastewater

Rice bran de-oiled wastewater was collected from local oil producing company located in and around Vijayawada. The RBOW was preserved at temperature lower than 4°C but above freezing to prevent it from undergoing biodegradation due to microbial action. The characteristics of wastewater are shown in Table 1.

2.2 Anaerobic mixed inocula

Inocula for the experiments were obtained from the pilot scale, upflow anaerobic sludge blanket reactor at local slaughterhouse sludge (SHS) manure treatment plant. Raw seed sludge was filtered through a screen (pore size, 2mm) to remove any fiber-like, undigested materials before use. These inocula were stored at 4°C prior to use for hydrogen production. The characteristics of sludge are shown in Table 2.

2.3 The experimental setup

The anaerobic batch experiments were carried out in magnetically stirred 5L batch reactor (Figure 1) with a working volume capacity of 3L. The reactor was provided with a cork containing inlets for loading feedstock and bubbling nitrogen gas required for unloading and also, an outlet nozzle with stop cork for removing effluent and venting biogas. The reactor was placed on magnetic stirrer provided with heating mantle for continuously mixing and maintaining a constant temperature. The gas outlet was connected through Teflon tube to the liquid displacement system. The entire system was checked for gas leaks and protected by a black cover to avoid the growth of photosynthetic bacteria. Nitrogen gas was sparged in to the reactor for 2 to 3 min to create strict anaerobic conditions prior to seeding of the active anaerobic sludge. The influent was prepared by using the raw wastewater as the sole carbon source, supplemented with balanced nutrients and buffering chemicals. The pH of the mixed liquor in the reactor was adjusted by using 2N HCl and 2N NaOH solutions. The batch reactor was routinely monitored for pH, gas production and gas composition, volatile fatty acids (VFA) composition and volatile suspended solids (VSS). Each series consisted of 3 to 4 runs, when steady-state conditions are reported.

2.4 Analytical methods

The hydrogen gas percentage was calculated by comparing the sample biogas with a standard of pure hydrogen using a gas chromatograph (GC, Agilent 4890D) equipped with a thermal conductivity detector (TCD) and 6 feet stainless column packed with porapak Q (80/100 mesh). The operational temperatures of the injection port, the oven and the detector were 100°C, 80°C and 150°C respectively. Nitrogen was used as the carrier gas at a flow rate of 20ml/min. The concentrations of the volatile fatty acids (VFAs) and the alcohol were analyzed using another GC of the same model with a flame ionization detector (FID) and a 8 feet stainless column packed with 10% PEG-20M and 2% H₃PO₄(80/100). The

temperatures of the injection port, the detector and the oven were 220°C, 240°C and a programmed column temperature of 130-175°C, respectively. Nitrogen was the carrier gas at a flow rate of 20ml/min. The pH values inside the digesters were measured by a microcomputer pH- vision 6071.

Table 1. Characteristics of rice bran de-oiled waste water

S.No.	Parameter	Value (mg/l)
1	pH	5.8
2	suspended solids	720
3	Total dissolved solids	1,630
4	Total solids	2,120
5	Chemical Oxygen Demand	11,720
6	Biological Oxygen Demand	5,200

Table 2. Characteristics of selectively enriched hydrogen producing mixed consortia

S.No.	Parameter	Value (mg/l)
1	pH	7.2
2	Total solids	19,200
3	Volatile suspended solids	8,200



Figure 1. Experimental setup

3. Results and discussions

3.1 Effect of pretreatment on biohydrogen production

Several types of seed sources are used for anaerobic hydrogen fermentation in previous studies, such as anaerobic municipal sewage sludge [15] Composts [16] and agricultural soil [17]. However, little work has been done using SHS. Hence, in the present study, SHS was used as the source of selectively enriched mixed microorganisms for hydrogen production. Analysis showed that the biogas produced from the anaerobic fermentation contained only hydrogen and carbon dioxide, without detectable methane.

Initially, the SHS was subjected to two pretreatments-acid treatment and heat treatment. The advantage in pretreating the sludge is inactivating non-sporing hydrogen consumers like methanogens and accelerating hydrogen producers like *Clostridium* species [18]. In heat treatment process, the production of hydrogen mainly depends on the duration of heat treatment of sludge. From Figure 2, it can be observed that, after 60min of duration time, the sludge produced maximum hydrogen production of 430ml after which there was a sudden decrease in the hydrogen production. This may be due to the inactivation of enzymatic nature of certain hydrogen producing organisms at high temperatures [19]. However, during this heat treatment certain amount of methane is produced indicating the presence of some heat resistant methanogens in sludge. In the acid treatment method, the collected sludge was treated with 0.1 N HCl to maintain the pH range 3-4 and then sustained for 24h. The acid-treated sludge showed the minimal amount of hydrogen production (330ml). In addition, it also showed less efficiency with respect to substrate removal than the heat treatment method. But the advantage in this method is that no methane is detected. The main disadvantage in heat treatment was the presence of methanogens whereas low efficiency in substrate removal and hydrogen productions were the major drawbacks of acid treatment. The combined use of heat treatment and acid treatment is a way of solving this problem (Figure 3). Fewer studies have been devoted to the production of hydrogen by using both treatments simultaneously [20, 21].

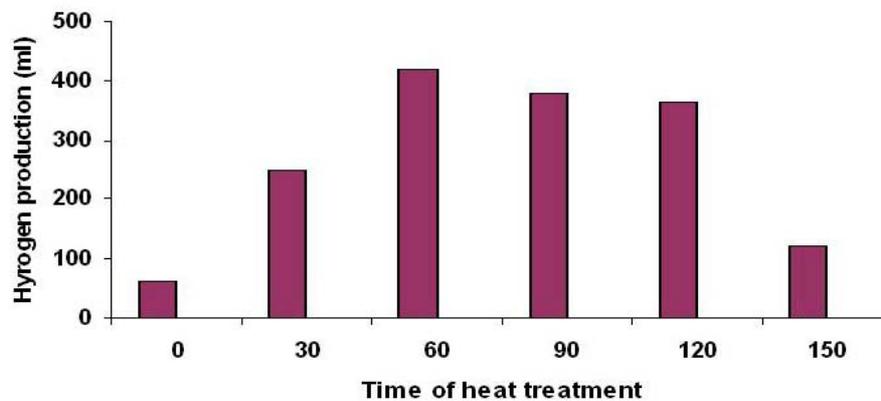


Figure 2. Effect of heat treatment on hydrogen production

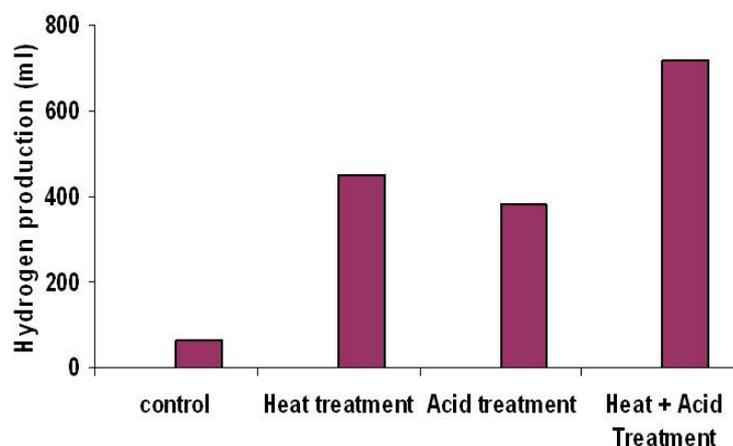


Figure 3. Comparison methods of pretreatment

3.2 Effect of pH on hydrogen production

In dark anaerobic fermentation, the control of pH is crucial to the hydrogen production, due to the effects of pH on the hydrogenase activity and on the metabolism pathways. In the present experiments, the effect of the initial pH of the medium on hydrogen production was investigated by varying the pH between 5 to 7. As shown in Figure 4, maximum hydrogen production of 670ml was observed at pH 6. This may be due to the suppression of methanogenic activity under acidic conditions. At higher or lower than this pH accumulation of acids causes a sharp drop of culture pH and subsequent inhibition of

bacterial hydrogen production. The poor hydrogen production at pH lower than 5.5 could be due to the increased formation of acidic or alcoholic metabolites, which destroys the cells ability to maintain internal pH [22]. It might have resulted in lowering of intracellular level of ATP, there by inhibiting substrate uptake. Complete inhibition in H₂ production was reported in the pH range of 4-5 [23]. Initial pH values of 5-7 were used in hydrogen fermentation by microflora, which are believed to be suitable against methanogens in various fermentation systems [24].

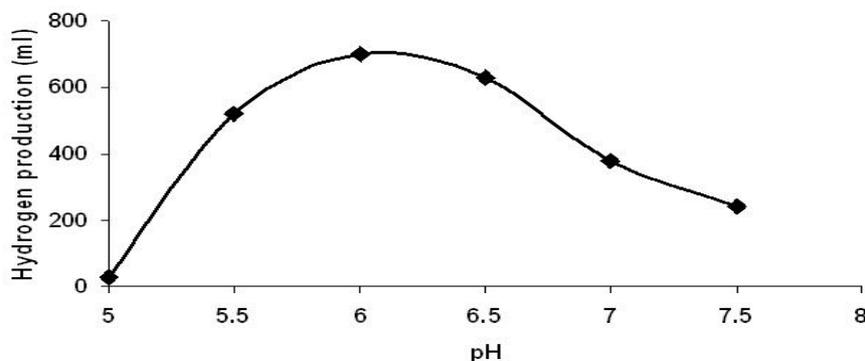


Figure 4. Effect of pH on hydrogen production

3.3 Effect of nitrogen source

Nitrogen is an essential nutrient for hydrogen production by dark fermentation under anaerobic conditions [25]. In the present experiment, different nitrogen sources were used to study their impact on hydrogen production (Table 3). The organic nitrogen sources used were yeast extract, tryptone and, the inorganic nitrogen sources used were Ammonium chloride, urea and ammonium sulphate. From the experiments, it can be observed that fermentation with organic nitrogen sources showed better hydrogen production compared to inorganic nitrogen sources.

Inorganic nitrogen sources are able to assimilate ammonium and reduce to nitrate [26]. However, these inorganic nitrogen sources probably contain only the nutrients that satisfy no more than the minimal requirement for increased hydrogen production. Of all the organic nitrogen sources used, fermentation with yeast extract showed maximum hydrogen production (920ml). This may be due to the presence of amino acids and peptides, water soluble vitamins and carbohydrates [27, 28]. Moreover, several authors suggested [29] that yeast extract is a good substrate for many microorganisms as it is easily utilized, which inturn helps in the reduction of the duration of lag phase.

Following this investigation, the amount of yeast extract was optimized for hydrogen production over a range of 0.25 – 2.5g/l of total nitrogen (Figure 5). Increasing the amount of yeast extract enhanced both the hydrogen production and the yield. The highest yield (1.61 mol/mol) was obtained with 2g/l yeast extract. Therefore, subsequent experiments were conducted with yeast extract (2 g/l total nitrogen) as the sole source of nitrogen.

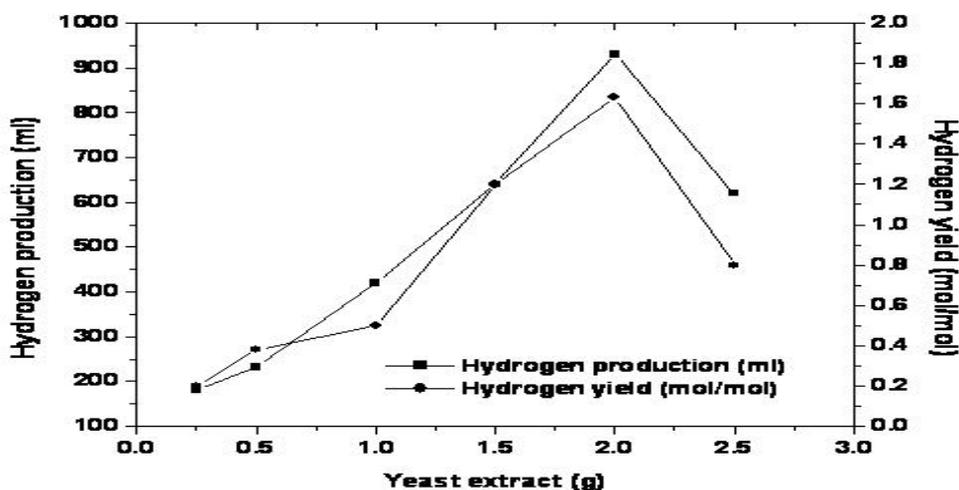


Figure 5. Effect of yeast extract concentration on hydrogen production

Table 3. Effect of Nitrogen source on Hydrogen production

Nitrogen Source	λ (h)	Hydrogen production (ml)
Yeast extract	13.4	920 \pm 20
Tryptone	17	430
Ammonium sulphate	22	280 \pm 20
Ammonium chloride	34	480 \pm 16
Urea	36.6	82 \pm 20

3.4 Effect of temperature on hydrogen production

Temperature, one of the most important ecological factors, influences all kinds of physiological activities of microorganisms and conversion rate of fermentation products [30]. As the characteristics of hydrogen producers varied, optimal temperatures for hydrogen production was diverse. In this study, the experiments were carried out at four different fermentation temperatures (37-67°C). Figure 6 illustrates the total biogas production, hydrogen percentage, hydrogen production and hydrogen yield over incubation time at each temperature. Maximum hydrogen production and yield were observed at 57°C than at the other temperature conditions. This may be due to the reduction of the solubility of hydrogen at higher temperatures. Moreover, at this temperature higher degradation rate of organic substances is observed compared to mesophilic temperature and also alleviate inhibition from hydrogen partial pressure [31]. Simultaneously, the hydrogen percentage versus incubation time has the similar trend at each time at 22h, for example, at 57°C, hydrogen percentage reached maximum of 42% at 22h. After that, as the temperature increased from 57°C to 67°C, the hydrogen percentage gradually decreased to 32%. This may be due to the inactivation of some essential enzymes and proteins associated with cell growth or hydrogen production. Obtained results indicated that the sensitivity of mixed bacteria in SHS to temperature was significantly high-and the optimal –temperature for hydrogen production was around 57°C.

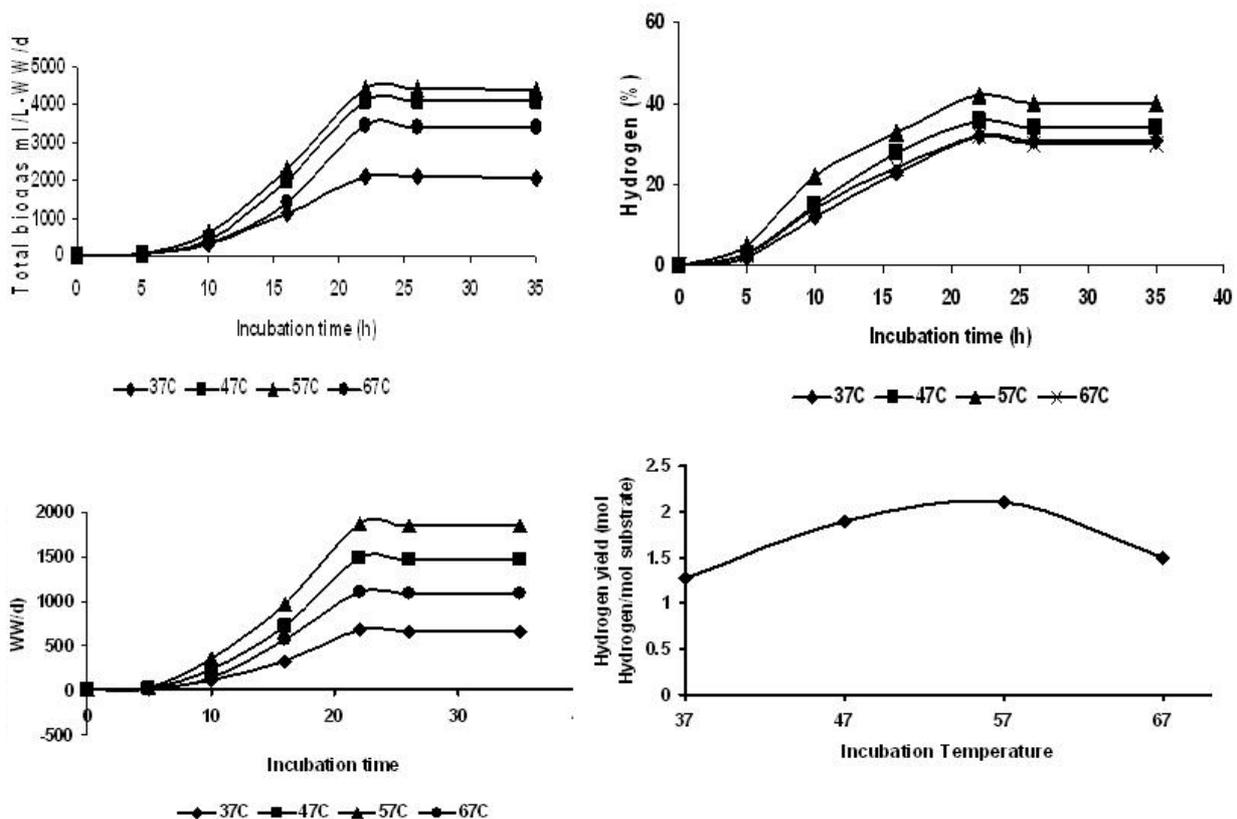


Figure 6. Effect of temperature on biogas, Hydrogen percentage, Hydrogen production and Hydrogen yield

H₂ production is normally accompanied with the acid production coupled with solvent production due to the acidogenic metabolism where generation of these acidic intermediates reflects changes in the metabolic pathway of the microorganisms [32]. Volatile fatty acids (VFA) production was always associated with organic fraction of acid intermediates in the anaerobic microenvironment with the help of specific group of bacteria. The production of VFA by microorganisms was affected by various factors, such as pH, temperature, finds of inoculum, and treatment of seed sludge. Table 4 illustrates VFA production during reactor operation and it is interesting to note that VFA production varied consistently with the temperature variations. the major soluble metabolites during hydrogen fermentation were butyric acid and acetic acid, accounting for 41% and 32% of total VFA along with minute amounts of ethanol and propionic acid. These results are in coincidence with the previous findings which show that hydrogen production with anaerobic bacteria is often through HBU-type fermentation that preferably produces HBU and HAC as major soluble metabolites [33]. In contrast, the production of Ethanol and propionate are unfavorable for hydrogen production due to consumption of hydrogen and more electrons from NADH [34]. More over, at higher temperatures the specific VFA/alcohol production rates in the thermophilic condition were slightly greater than the corresponding values in the mesophilic conditions. This confirms that increased operating temperature accelerated the acidogenic reaction rate and substrate removal. Therefore, the VFA concentration, distribution and their fractions have been successfully used as indicators for monitoring hydrogen production.

Table 4. Production of soluble metabolites during Hydrogen fermentation operated at different temperatures

Temperature (°C)	Ethanol (%)	Acetate (%)	Propionate (%)	Butyrate (%)	HAc+HBU (%)	Total VFA(mg/l)
37	21.2	21	10±1.4	31±0.2	53	2430±18
47	17.2±0.6	28±0.3	7.1±0.4	34±0.3	62	3200±20
57	11.2±0.1	32±1.2	3.8±0.3	41±1.4	73	4200±42
67	11±0.3	22±2.4	9±0.5	32±1.2	54	3140±30

4. Conclusion

The batch experiments demonstrated the feasibility of H₂ generation from RBOW wastewater as primary substrate using selectively enriched mixed consortia by heat treatment and acid treatment. Thermophilic temperature showed positive influence on the hydrogen production and hydrogen yield at 57°C. pH is the key factor affecting fermentation pathway for hydrogen production stage. The optimum pH for hydrogen production in this system was found at 6. Therefore, The study proved the feasibility of the fermentative method of biohydrogen production from RBOW at thermophilic range by selectively enriched mixed microflora.

Acknowledgements

The authors gratefully acknowledge the financial support from the Ministry of New Renewable Energy (MNRE) [102/9/2004-NT], Government of India for carrying out this research work.

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