Experimental Investigation on Biological Hydrogen Production Using Different Biomass

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Abstract

Hydrogen is a clean and efficient fuel, considered as a potential and more sustainable energy substitute for fossil fuels. Biological hydrogen production stands out as an eco friendly process carried out under mild operating conditions with renewable resources. In the current work laboratory scale production of hydrogen using phototrophic purple non-sulphur bacteria *Rhodobacter sphaeroides*, anaerobic dark fermentative bacteria's *Clostridium pasteurinum, Bacillus licheniformis* and *Enterobacter clocae* with different substrates was investigated. The study mainly emphasized on assessing the potential of biological conversion of different substrates to hydrogen by studying various experimental parameters like temperature, pH and cell density. It was found that the *Rhodobacter sphaeroides* took relatively longer duration (48 hrs) for hydrogen production. The optimum temperature and pH for maximum production of hydrogen in case of *Rhodobacter sphaeroides* was found to be 32° C and 7.5 respectively, 32°C and 7 for *Clostridium pasteurinum* respectively, 30°C and 6.8 respectively for *Bacillus licheniformis* and *Enterobacter clocae*. Results of the batch tests depicted that *Rhodobacter sphaeroides* produced maximum amount of hydrogen (35%) as compared to 21% by *Clostridium pasteurinum,* 16% by *Bacillus licheniformis* and 8% by *Enterobacter clocae*. However the quantity of hydrogen production in case of *Rhodobacter sphaeroides* was relatively lower compared to *Clostridium pasteurinum.*

Keywords: Hydrogen; Biomass; dark fermentation; light fermentation; Non-sulphur bacteria; Anaerobic.

1.0 Introduction

During the last decade, much attention has been paid to the hydrogen gas and its potential use as fuel for transportation purposes (automobiles) and electricity generation. This happened mainly because hydrogen is a clean and renewable energy source, possessing a high-energy yield (122 $kJ.g^{-1}$), not contributing to the green house effect as reported by Benemann J [1].

Hydrogen offers tremendous potential as a clean, renewable energy currency. It can be produced by various methods (reforming of hydrocarbons, coal gasification, electrolysis, photochemical process etc.,) and one of it is via purely biological routes through the fermentation of biomass using microorganism, or directly from cyanobacteria and micro-algae. Biological systems provide a wide range of approaches to generate hydrogen, and include direct biophotolysis, indirect biophotolysis, photofermentations and dark fermentations [2]. All biological hydrogen production process depends on the presence of a hydrogen-producing enzyme.

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The production of hydrogen by two important routes namely dark and light fermentation has attracted many investigators do solely because of their high yield and economic feasibility.

1.1 Dark Fermentation

Dark hydrogen fermentation is a ubiquitous, natural phenomenon under anoxic or anaerobic conditions. The yield is reported to be approximately 83-100% of the maximal theoretical value of 4 mol hydrogen/mol glucose, in contrast to the strict anaerobic *Clostridia* which produce hydrogen with an approximate yield of 2 mol/mol and the facultative anaerobes which show a H_2 yield of < 2. Besides the conversion to fermentable feedstock, it is also of prime importance to devise a strategy for a profitable utilization of the residual biomass which can not be fermented. The optimization of dark hydrogen fermentation can not be executed without addressing the constraints or potentialities of the complete biomass on offer. In short: dark hydrogen fermentation is the first step in achieving biological hydrogen production; optimization of dark hydrogen fermentation is dependent on increasing hydrogen productivity and availability of fermentable feedstock. For an economically sound bioprocess for hydrogen production, the end-products of dark hydrogen fermentation have to be utilized as well.

On of the major limitations of the dark fermentation is the production of secondary metabolites that are produced during the process. These secondary metabolites inturn inhibit the hydrogenase enzyme thereby decreasing the yield of the process. Hence several investigators are working on the use of co-cultures for the fermentation process, to avoid the inhibitory effect of the secondary metabolites.

1.2 Light Fermentation (Photo Fermentation)

Light energy is the driving force for photobiological hydrogen production. Energy from absorbed solar irradiation is converted into chemical energy stored in hydrogen in biocatalytic processes. In photo fermentations phototrophic bacteria are grown heterotrophically and used to convert organic substrates like organic acids or alcohols (from biomass) into hydrogen and carbon dioxide according to the reaction. The photosynthetic bacteria are acquatic gram-negative organisms. They utilize solar energy for the fixation of CO_2 and nitrogen.

$$2\ CH_3COOH + 4\ H_2O + \text{light} \longrightarrow 8\ H_2 + 4\ CO_2$$

The basic biological principle through which solar energy is stored is photosynthesis. The basic principles and processes for photobiological hydrogen production that are under development are outlined below.

The microorganisms that can be applied in such a process are purple bacteria that produce hydrogen mainly due to the nitrogenase enzyme present in the cells. The nitrogenase is used by the organisms for nitrogen fixation i.e. reduction of N_2 from the air to NH_3 which is a nitrogen source required for growth. The nitrogenase enzyme also catalyses the evolution of H_2, particularly in the absence of N_2. The nitrogenase enzyme is also sensitive to oxygen (O_2). In this case, however, this is not a problem because no oxygen is produced during the process (anoxygenic photosynthesis). A disadvantage of the process is, that the nitrogenase enzyme requires extra metabolic energy in the form of ATP, which somewhat lowers the H_2

yield per unit absorbed light. Photoheterotrophic bacteria are able to use light between 400 and 950 nm. It can be calculated that under ideal circumstances 10 % of the solar energy can be stored as hydrogen gas by these bacteria. In this scenario the energy content of the organic acid is neglected. It should be noted that the gas produced in this process is a clean product consisting of only hydrogen (80-90 vol %) and carbon dioxide (10-20 vol %).

Photofermentations are mostly applied in 2-stage processes as the second step following a dark fermentation process. Suitable substrates for the process are organic compounds originating from diluted waste products (e.g. molasses; industrial residues) or biomass hydrolysates. In the first step the sugars are converted to hydrogen and acetate (dark fermentation). The acetate can be further converted into hydrogen using a phototrophic fermentation step.

Hydrogen production in photo-biological systems is presently limited by low energy conversion efficiencies. Solar conversion efficiencies are normally less than 1% in cultures of micro-algae, and a ten-fold increase is required before hydrogen production from micro-algae can become practical. Another difficulty is the fact that hydrogenase enzymes are inhibited by oxygen concentration above 0.1%. The most serious problem in the photo-biological systems is the "light saturation effect", in which the cells near outside of the culture medium absorb all the available sunlight. In the laboratory this problem can be overcome, but in large-scale plants it severely reduces the yield of hydrogen. In this view biological production of hydrogen by fermentative bacteria has advantages since hydrogen can be produced continuously in the fermenter without light [4]. Several researchers have investigated the possibility of hydrogen production by continuously operated bioreactors [5] but substantial hydrogen production still remains a major challenge.

The theoretical maximum yield of hydrogen fermentation is reported to be four moles of hydrogen per mole of glucose [6] or eight moles of hydrogen per mole of sucrose [7] if all the substrate are converted to acetic acid. These values correspond to a theoretical maximum yield of 0.467 l-H_2/g-COD. If all the substrates were converted to butyric acid, these values are two and four moles of hydrogen per mole of glucose and sucrose respectively [8]. Considering the high theoretical yields, several researchers have continuously explored different approaches to increase hydrogen production. Though the literature indicated feasibility of producing hydrogen from wastewater sludge, the hydrogen formed during the first 16-24 h of fermentation was consumed in later stage [9]. However hydrogen production by dark fermentation using municipal waste is not pertinent particularly in places where the quantity and composition of the municipal sludge varies from day to day. The presence of suspended particles in the sludge will decrease the process efficiency and hence the hydrogen yield.

The present study focusses on assessing potential of biological conversion of bagasse to hydrogen using different microorganisms (*Bacillus licheniformis*, *Clostridium pasteurianum* and *Enterobacter cloacae)* under anaerobic conditions (dark fermentation) and *Rhodobacter sphaeroides* under anaerobic condition (Photofermentation). Based on the batch test data the process efficiency and practical yield under different experimental conditions will be presented. This fermentation process is much less dependent on the biomass parameters and will be addressed elsewhere.

2.0 Materials & Method

2.1 Substrate

Waste biomass containing high percentage of cellulose content like vegetable waste fruit waste, sugarcane waste (bagasse) was selected as substrates. The substrate was pretreated by washing with deionized water and dried (120°C) to remove moisture from the substrate. The dried substrate was macerated into small pieces of approximately 1 to 2 mm in particle diameter.

2.2 Media

Minimal media was prepared as a nutrient source for the inoculum. Table 1. Provides the composition of various chemicals used for the preparation of 1l of the solvent. The minimal media consisted of a mixture of ammonium chloride, di-sodium hydrogen phosphate, potassium di-hydrogen phosphate, along with salts of magnesium and sodium. The minimal media thus prepared had a pH of 7 and was known as natural pH. This minimal media was used for all the experiments.

Table 1 Chemical composition of minimal media.

Chemical	Quantity (g/l)
Potassium di-hydrogen phosphate	3.0
Di-sodium hydrogen phosphate	6.0
Sodium chloride	5.0
Ammonium chloride	2.0
Magnesium sulphate	0.1

2.3 Inoculum

The microbial strains were obtained from culture collection center (IMTECH Chandigarh, India). The selected strain in the present investigation was identified as *Clostridium pasteurianum* (MTCC No. 116), *Enterobacter cloacae* (MTCC No. 509), and *Bacillus licheniformis* (MTCC No. 429). *Rhodobacter sphaeroides* (ATCC No. 17023).

2.4 Batch Experiment

Batch experiments were conducted by using 500 ml serum bottles with an active volume of 400 ml. A predetermined quantity of substrate and minimal media was taken in three different 500ml serum bottles and was autoclaved (120°C and 15lb/in^2) separately. After autoclaving, the minimal media and inoculum was transferred to the substrate in a laminar flow chamber. The experiments in the present work were conducted in dark at 32°C for *Clostridium pasteurianum, Enterobacter cloacae, and Bacillus licheniformis.* The photofermentation or photobiological hydrogen production using *Rhodobacter sphaeroides* was carried ouy in a 500ml volume serum bottle photobioreactor, the reactor was illuminated by using a tungsten lamp (40W) from a distance of 15cm.The temperature was maintained at 32°C.

Biogas production was measured by water displacement technique using airtight water displacement jar of 5 l capacity. Each experimental condition studied was duplicated or triplicated and average gas productions were analyzed and reported.

2.5 H_2 Analysis

The biogas composition was measured using gas chromatographs (Chemito 8610, India) equipped with thermal conductivity detector (TCD), poropaq Q column. Argon was used as carrier gas at a flow rate of 30 ml/min. The hydrogen gas percentage was calculated by comparing the sample biogas under investigation with a standard pure gas (hydrogen, methane, carbon di-oxide). The temperatures of the injection port, oven and detector were maintained at 80°C, 60°C, and 100°C respectively.

3.0 Results and Discussion

Experiments were conducted with different substrates at varying temperatures, optical density of inoculum and pH. The cultures of hydrogen producing anaerobes (i.e. *Clostridium pasteurianum*, *Bacillus licheniformis*, and *Enterobacter cloacae, Rhodobacter sphaeroides)* obtained from the natural inocula typically displayed a lag period indicating the spore suspensions transformation into vegetative cells.

3.1 Effect of Substrate

Dark Fermentation

The percentage yield of hydrogen for different substrates on *Clostridium pasteurianum* is as shown in Fig 1. It can be seen from the figure that the sucrose and enriched corn fiber waste yields hydrogen respectively. Since sucrose consists only carbohydrates and also it is in the pure form the yield of hydrogen will normally be high. The hydrogen yield from sweet lime and fruit waste was found to be less, this can be attributed due to the acidic (pH 4 – 3) nature of the substrate, which will affect the survival of *Clostridium pasteurianum*. The hydrogen yield in the case of bagasse was found to be relatively less when compared to corn fiber waste. The yield of hydrogen is mainly dependent upon the breakdown of carbohydrates. Vegetable waste and bagasse consists of large amounts of lignin and cellulose, which has to be broken down to simpler molecules of glucose, and will be further reduced to H_2, CO_2, and traces of CH_4.

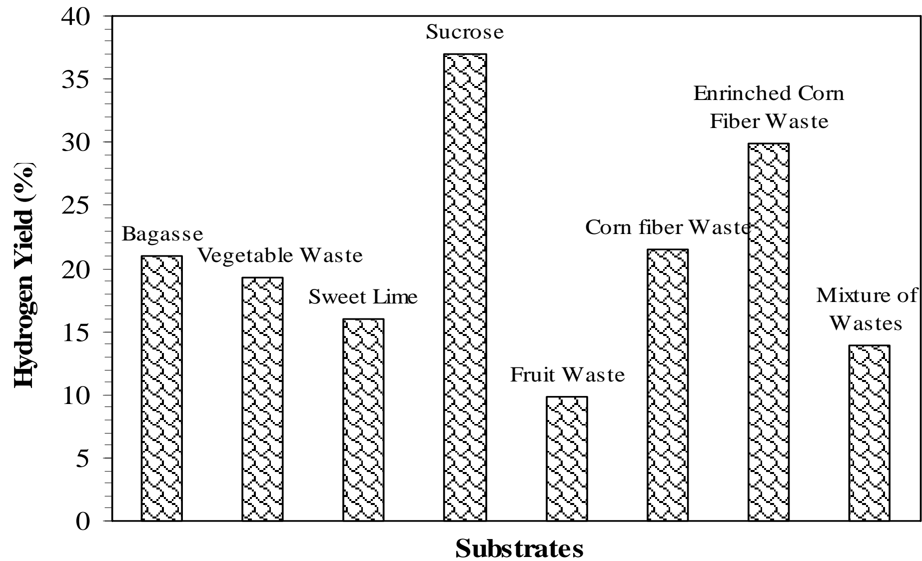

Figure 1 Percentage of hydrogen yield from different substrate using *Clostridium pasteurianum* at room temperature and natural pH.

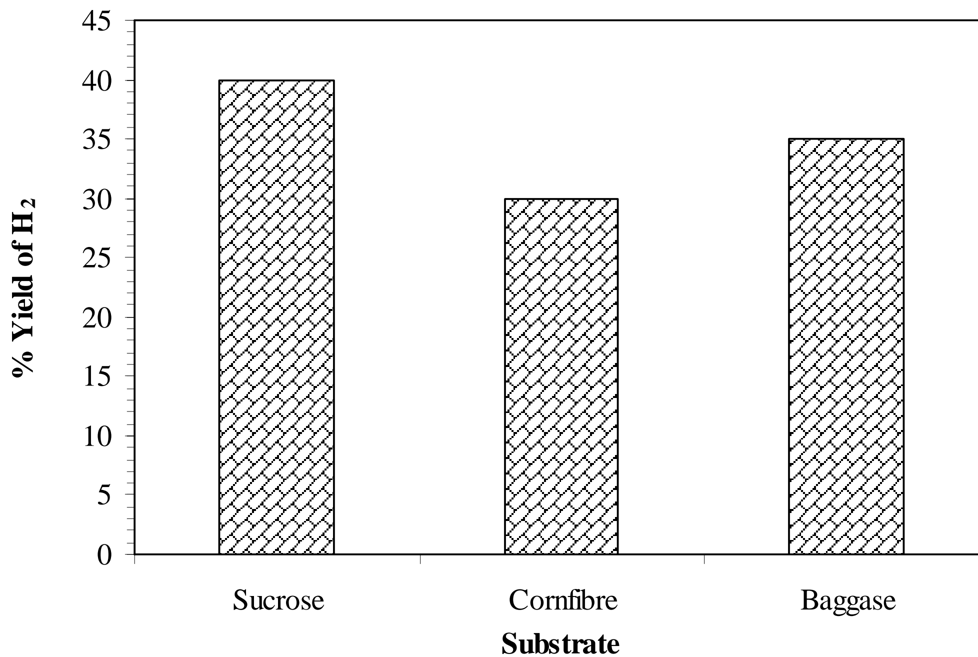

Figure 2 Percentage of hydrogen yield from different substrate using *Rhodobacter sphaeroides* at room temperature and natural pH..

Clostridium pasteurianum contains enzyme cellulase, which helps in breaking down cellulose to glucose, however it does not contain enzyme lignases, which is essential for breaking lignin. Though the yield of hydrogen is more in corn fiber waste compared to bagasse, the desired composition of vegetable waste is very difficult to obtain. For all future experiments bagasse was used as substrate since it is abundant and easily available.

The percentage yield of hydrogen for different substrates on *Rhodobacter sphaeroides* is as shown in Fig. 2. It can be seen from the figure that the sucrose yields relatively high (40%) percentage when compared to cornfiber (30%) and bagasse (35%). Since sucrose contains only carbohydrates and also it is in the pure form the yield of hydrogen will normally be high.

3.2 Effect of pH on microorganisms

The effect of pH on hydrogen production enzymes, *Clostridium pasteurianum*, *Bacillus licheniformis*, and *Enterobacter cloacae, Rhodobacter sphaeroides* is as shown in Fig 3. and 4. The results depicts that the number of colonies of the microorganism will decrease with increase in acidity of the fermentation broth. The optimum pH was found to be in the range of 6.8 – 7 for dark fermentative bacteria where as for photofermentaive bacteria it was found to be in the state of neutrality. Clearly, during hydrogen production, the solution's pH decreased from 7 with time until the pH became 4.2, after which the pH did not decrease further. This phenomenon can be attributed due to the production of secondary metabolites, butyrate and acetate which decrease the pH of the broth. The acidic environment created as a result of this will inhibit the production of hydrogen.

This indicates that the production of a by-product which is acidic in nature. It was also observed that with decrease in pH of the broth media the relative yield of hydrogen decreased, due to the inhibitory effect of the secondary metabolites. Suitable experiments have to be conducted to ascertain the decrease in the pH of the broth.

From SEM studies, aging temperatures not play an important role in the level of degradation of natural fiber. At low aging temperature, fibers were well embedded in matrix and relatively, the push-out fibers were longer than composite heated with higher aging temperatures as shown in Figure 3 and 4. Composites aged with 27^0C, it can be seen that push-out holes were bottomless and as a result longer push-out fibers were observed. This will increase higher maximum sustained loads and higher maximum elongation as revealed in Figure 3a and 3b through fiber bridging that preventing the composites to break and therefore increasing the interfacial friction between matrix and fibers. As aging temperature increases, material stiffness increased but it was strongly dependent with fiber loading as shown in Figure 3c. This behavior can be correlated with fracture mechanisms as depicted in Figure 5. Shorter push-out fibers were observed and it can be seen that the fibers were well griped by the polyester matrix and at certain location, interfacial debonding observed especially at matrix/fiber interfaces. Fiber morphology not affected by the thermal treatment and the fiber still intact as Figure 4. Apparently, as suggested by most available data, temperature has no effect on altering the mechanisms of fracture mechanisms and crack growth [5].

3.3 Effect of Temperature

Figure 3 represents the effect of temperature on yield of hydrogen for the three different anaerobic bacteria. After repetitive experiments it was found that the gas yield was high at 32°C. The yield of gas was higher for *Rhodobacter sphaeroides* as compared to *Clostridium pasteurianum, Bacillus* and *Enteroba*cter. Similar results have been reprted by various researchers [4,7,10,11].

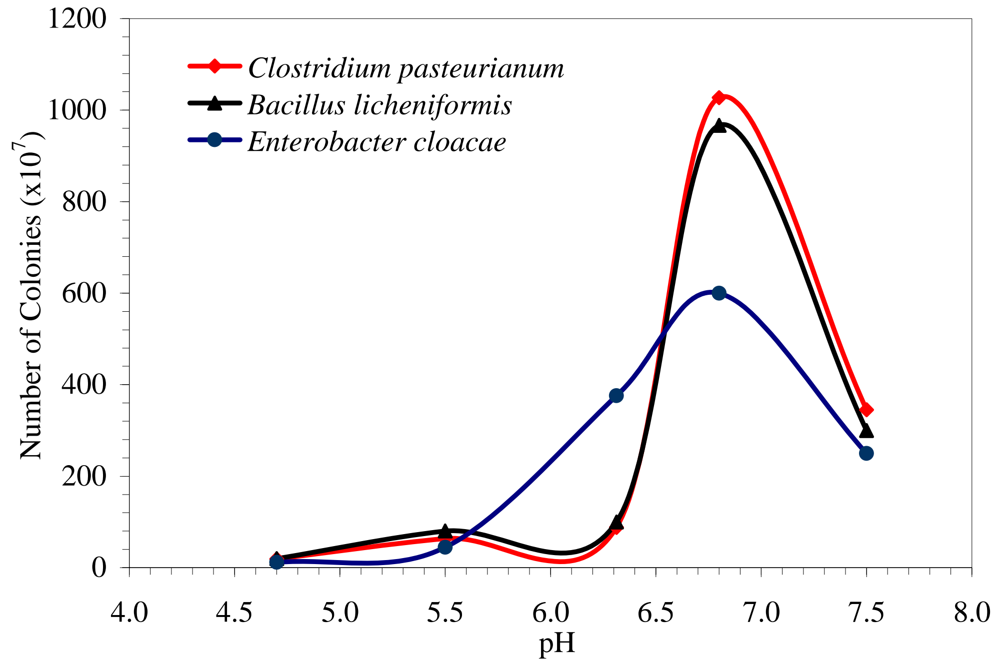

Figure 3 Effect of pH on the number of colonies of microorganism for bagasse, at 30 ± 2°C.

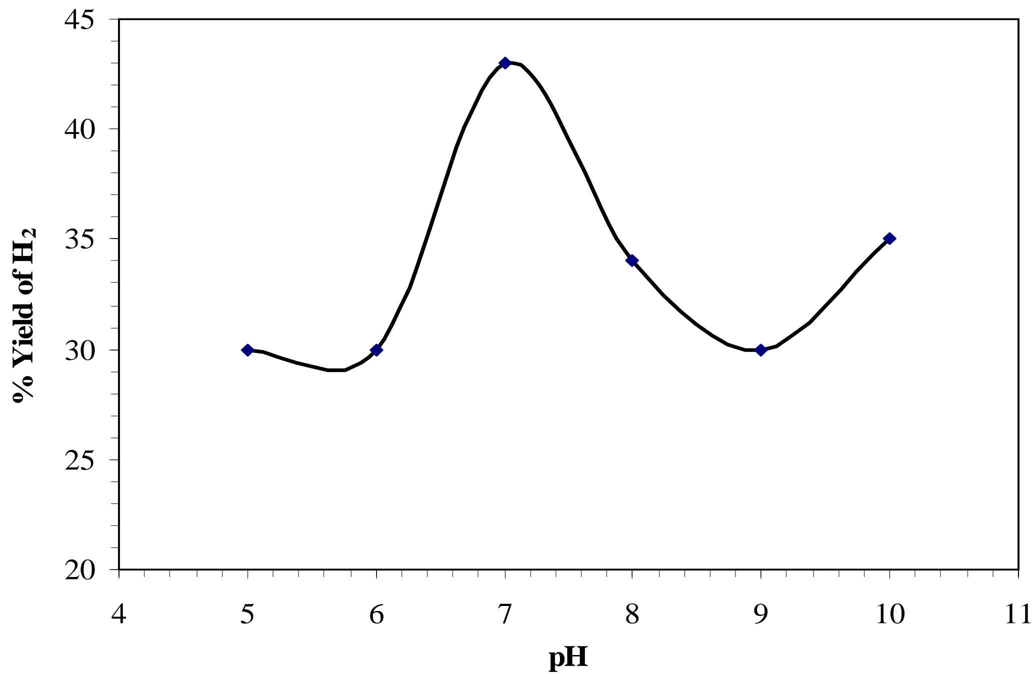

Figure 4 Effect of pH on the number of colonies of state and the microorganism for bagasse, 30 ± 2°C.

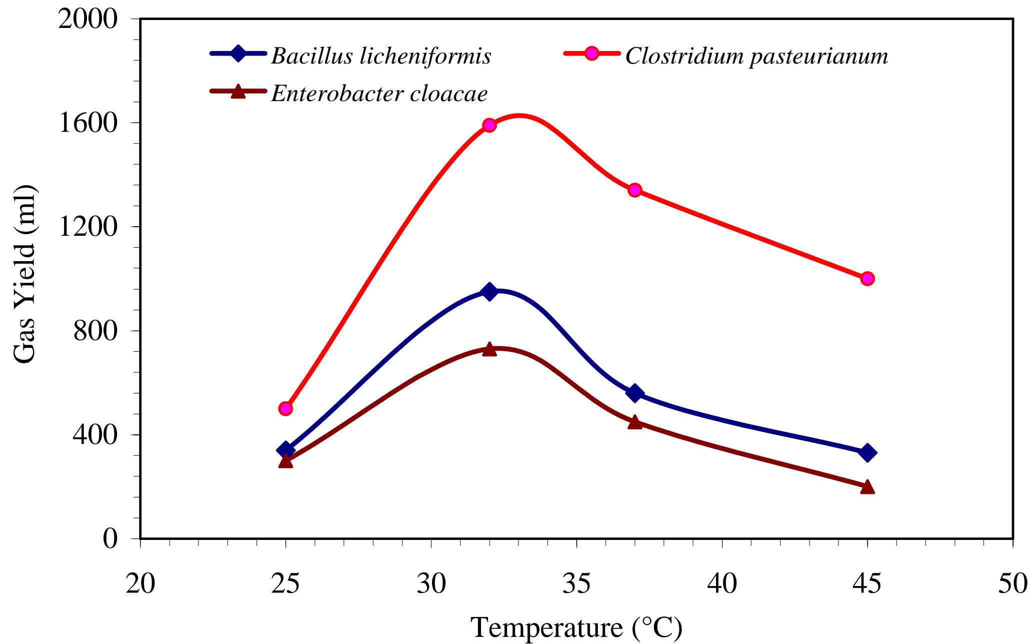

Figure 5 Quantity of gas yield as a function of temperature for bagasse, pH 7.

3.4 Effect of Cell Density

Experiments were also conducted to ascertain the effect of cell density on gas yield. As shown in Fig 4, production of hydrogen increases with increase in cell density to a maximum of 0.5 and thereafter it decreases with increase in cell density. This is due to the consumption of hydrogen by the anaerobic species. Increase in the cell density above an optimum value will inhibit the process of carbohydrates breakdown as the organisms will compete with each other for the utilization of the substrate available. At higher cell density the amount of media requirement will increase, which occupies more volume in the reactor.

Table 2 Hydrogen yield for various substrates for *Clostridium pasteurianum*
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Substrate (105 gm)	Gas Yield (ml)	Hydrogen (%)	Hydrogen Gas (ml)	Yield (mol/kg sub.)
Bagasse	5330	21	938	0.472
Vegetable waste	3630	19.26	699.14	0.297
Fruit waste	2952	9.78	288.71	0.123
Corn fiber waste	5400	21.54	1163.16	0.495
Enriched corn fiber waste	3150	29.91	942.17	0.400
Mixture of wastes	3336	13.96	465.71	0.194

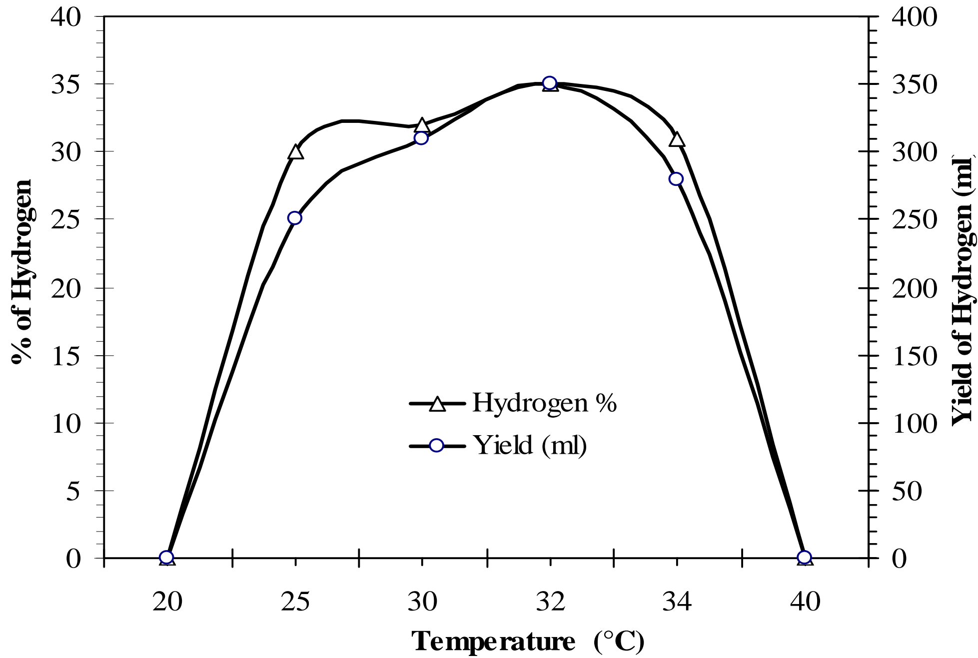

Figure 6 Quantity of gas yield as a function of temperature for Rhodobacter sphaeroides, pH = 7.

Table 2. Provides a comparative data of hydrogen yield for various substrates for *Clostridium pasteurianum* at neutral pH and at a temperature of 32 ±1°C. It was observed that the yield of hydrogen was relatively more in the case of enriched corn fiber but the generation of gas stopped after 36 hrs indicating an inhibition effect probably due to generation of secondary metabolites. The amount of hydrogen yield in case of corn fiber and bagasse was relatively similar. However needs to be conducted to understand the technical viability and economics of this process.

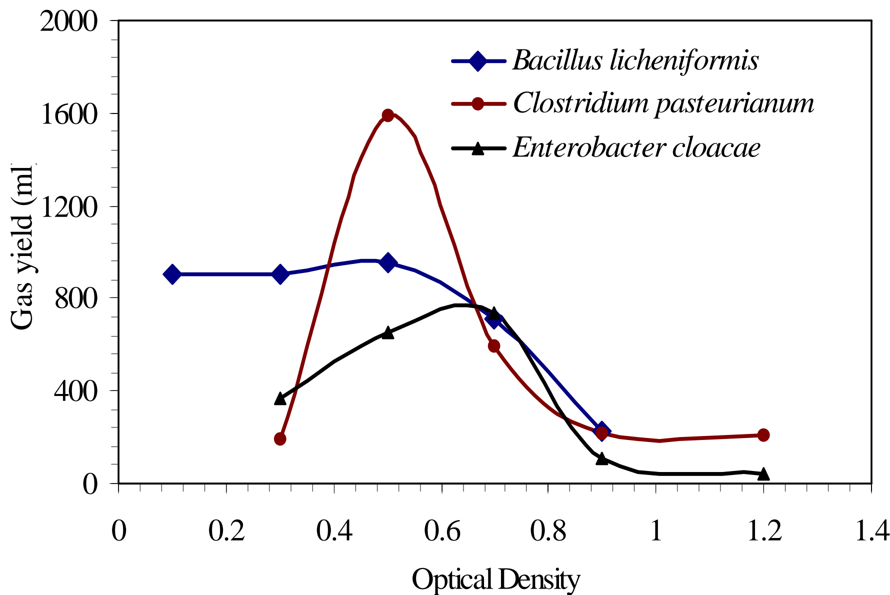

Figure 6 Gas yield as a function of optical density (cell density) for pH = 7, bagasse at 32°C.

4.0 Conclusion

Naturally obtained, spore forming, hydrogen producing anaerobic bacteria i.e. *Clostridium pasteurianum*, *Bacillus licheniformis*, and *Enterobacter cloacae* has considerable potential in the transformation of naturally available biomass (carbohydrates) into hydrogen. The results of the present investigation revealed that hydrogen was generated for 5 days and the maximum rate of production was attained after 24 hrs of incubation. In the case of photofermentative bacteria using *Rhodobacter sphaeroides the* hydrogen was generated for 7 days and the maximum rate of production was attained after 48 hrs of incubation and the optimum conditions were pH 7 and at 32°C. In the present work we observed that the percentage yield of hydrogen of different microorganisms are of the order *Rhodobacter sphaeroides > Clostridium pasteurianum > Bacillus licheniformis > Enterobacter cloacae.* However the volume of hydrogen collected for different microorganisms are of the order *Clostridium pasteurianum > Bacillus licheniformis > Enterobacter cloacae > Rhodobacter sphaeroide.* Also hydrogen generation by photofermentation took nearly 7days as compared to 5 days in case of dark fermentation. This indicates that though the hydrogen percentage is more in photofermentation the total amount of gas collected is relatively low as compared to dark fermentation. Hence more experimental trials varying detail operating parameters needs to be investigated in selecting economically feasible method for production of hydrogen. In both cases decease in pH resulted in decreased hydrogen generation rate mainly because of poor metabolic activity of the organisms in the acidic environment and due to the inhibition of the microorganisms by the production of secondary metabolites. In practice, during the fermentation process both butyrate and acetate is formed, but the ratio may vary with growth conditions within thermodynamically determined limits, pH, and intermediate products (byproducts) especially volatile fatty acids drive the hydrogenase reaction during the hydrogen fermentation. During dark fermentation, besides pH control, it is also important to suppress hydrogen consumers and obtain an enriched culture of hydrogen producing *Clostridia*. Future optimization should address, besides increased performance of microorganisms, removal or conversion of by-products, and system efficiency, research and development of process parameters such as bioreactor design capable of handling of 5 kg of biomass.

Acknowledgements

The authors gratefully acknowledge the financial support by the TIFAC (DST, Govt. of India) for the project work.

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