

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 were investigated. The study investigated the potential of biological conversion of different substrates to produce hydrogen by studying various experimental parameters like temperature, pH and optical density (OD) of cells. 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* were 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 showed 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, microbial bioconversion, dark fermentation, photofermentation, 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 is mainly because hydrogen is a clean and renewable energy source, possessing a high-energy yield (122 kJ.g⁻¹) and does not contribute to the green house effect [1].

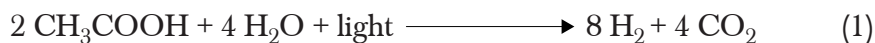
Hydrogen offers tremendous potential as a clean and renewable energy currency. It can be produced by various methods such as reforming of hydrocarbons, coal gasification, electrolysis, photochemical process and biological routes through fermentation of biomass using microorganism. Biological systems provide a wide range of approaches to generate hydrogen that include direct biophotolysis, indirect

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biophotolysis, photofermentations and dark fermentations [2]. All biological hydrogen production process depends on the presence of a hydrogen-producing enzyme. 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.

Dark hydrogen fermentation is a ubiquitous, natural phenomenon under aerobic or anaerobic conditions. The yield is reported to be approximately 83 – 100% of the maximal theoretical value of 4 mol of hydrogen/mol of glucose, in contrast to the strict anaerobic *Clostridia* which produces hydrogen with an approximate yield of 2 mol of hydrogen/mol of glucose and the facultative anaerobes which show a hydrogen 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 cannot be fermented. The optimization of dark hydrogen fermentation cannot be executed without addressing the constraints or potentialities of the complete biomass 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. One of the major limitations of the dark fermentation is the production of secondary metabolites that are produced during the process. These secondary metabolites in turn 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.

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 (Equation (1)). The photosynthetic bacteria are aquatic gram-negative organisms. They utilize solar energy for the fixation of CO₂ and nitrogen.



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 N₂ from air to NH₃ which is a nitrogen source required for growth. The nitrogenase enzyme also catalyses the evolution of H₂, particularly in the absence of N₂. 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). However, disadvantage of this process is that, the nitrogenase enzyme requires extra metabolic energy in the form of ATP, which lowers the H_2 yield per unit absorbed light. Photoheterotrophic bacteria are able to use light between 400 and 950 nm.

Photofermentations are mostly applied in two 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. Another difficulty is the fact that hydrogenase enzymes are inhibited by oxygen concentration above 0.1%. The most serious problem in photo-biological systems is “light saturation effect”, in which, cells near outside of the culture medium absorb all the available sunlight. In 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 would be converted to acetic acid. These values correspond to a theoretical maximum yield of 0.467 l- H_2 /g-COD. If all the substrates would be 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 exploring 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].

The present study is emphasized on assessing potential of biological conversion of bagasse to hydrogen using three 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 of under different experimental conditions are presented.

2.0 MATERIALS AND METHODS

2.1 Substrate

Waste biomass containing high percentage of cellulose content like vegetable waste, fruit waste, sugarcane waste (bagasse) were 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 1000 ml 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 dihydrogen phosphate	3.0
Disodium hydrogen phosphate	6.0
Sodium chloride	5.0
Ammonium chloride	2.0
Magnesium sulphate	0.1

In the batch experiments 35 g of the substrate were taken to fill the serum bottle till the neck and 500 ml of minimal media were used along with the inoculums for each different experimental setup.

2.3 Inoculum Preparation

The selected strain for the experimental investigation were identified as *Clostridium pasteurianum* (MTCC No. 116), *Enterobacter cloacae* (MTCC No. 509), and *Bacillus licheniformis* (MTCC No. 429). The inoculum for small and large batch studies was obtained from Microbial Type Culture Collection (MTCC), Gene Bank, India, in the freeze-dried form. Standard microbiological and safety procedures were followed while handling cultures. Once the culture is ready, its Optical Density (OD) was checked, the required OD is adjusted using a Spectrophotometer (Model-Sytronics, India). The culture is now said to be ready for inoculation. The bacterial cultures so prepared are sub cultured once in every 30 days.

2.4 Batch Experiments

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 500 ml serum bottles and were autoclaved (120°C and 15lb/in²) separately. After autoclaving, the minimal media and inoculum were transferred to the substrate in the laminar flow chamber and the serum bottle were flushed with nitrogen for sufficient duration to achieve anaerobic condition. The experiments in the present work were conducted in dark at 32°C for *Clostridium pasteurianum*, *Enterobacter cloacae*, and *Bacillus licheniformis*. The photofermentation or hydrogen using *Rhodobacter sphaeroides* was carried in a 500 ml volume serum bottle photo bioreactor. The reactor was illuminated by using a tungsten lamp (40 W) from a distance of 15 cm. The temperature was maintained at 32°C.

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

2.5 Analytical Analysis

The biogas composition was measured using gas chromatographs (Model-Chemito 8610, India) equipped with thermal conductivity detector (TCD), poropak 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 dioxide). The temperatures of the injection port, oven and detector were maintained at 80°C, 60°C, and 100°C respectively.

3.0 RESULTS AND DISCUSSIONS

Experiments were conducted with different substrates at various 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. It was observed 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 pH 7, temperature 32°C.

3.1 Effect of Substrate

The percentage yield of hydrogen for different substrates on *Clostridium pasteurianum* at neutral pH (7) and a temperature of 32°C is shown in Figure 1. It can be seen from

the figure that sucrose shows a relatively higher percentage yield of hydrogen whereas enriched corn fiber waste yields comparably low percentage of hydrogen. Since sucrose consist only carbohydrates and being present in the pure form (>99.5%), the yield of hydrogen will normally be high. The hydrogen yield from sweet lime and fruit waste was found to be significantly less, this can be attributed due to the acidic (pH 4 – 3) nature of the substrate, that affects the survival of *Clostridium pasteurianum*. The hydrogen yield in the case of bagasse was found to be relatively less compared with corn fiber waste. The yield of hydrogen is mainly dependent upon the breakdown of carbohydrates. Vegetable waste and bagasse consist large amounts of lignin and cellulose, which have to be broken down to simpler molecules of glucose, and were further reduced to H₂, CO₂, and traces of CH₄.

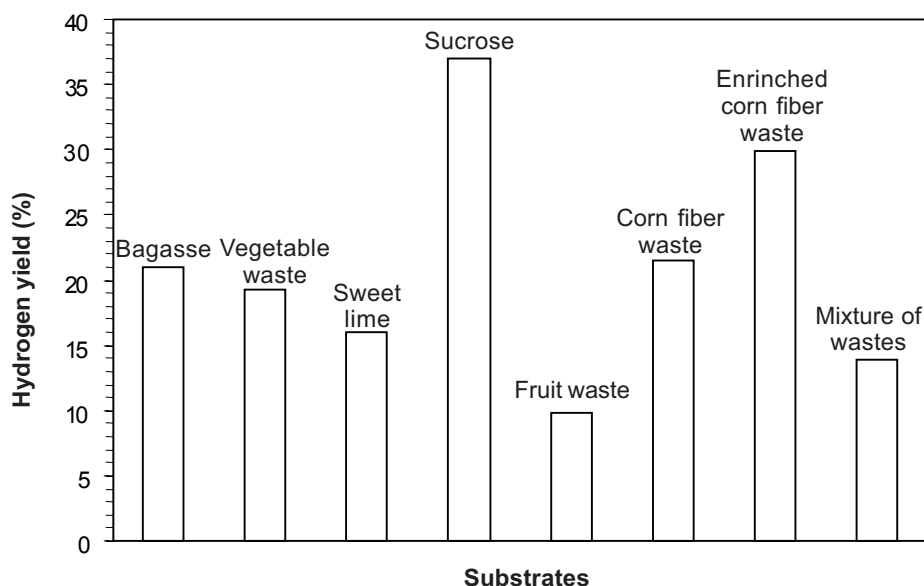


Figure 1 Percentage of hydrogen yield from different substrate using *Clostridium pasteurianum* at room temperature and natural pH

Clostridium pasteurianum contains enzyme cellulose, that helps in breaking down cellulose to glucose but it does not contains enzyme lignases that, is essential for breaking lignin. While the yield of hydrogen is more in corn fiber waste when compared to bagasse, the desired composition of vegetable waste is very difficult to obtain.

The percentage yield of hydrogen for different substrates on *Rhodobacter sphaeroides* is as shown in Figure 2. It can be seen from the Figure 2 that the sucrose yields relatively high (40%) when compared to cornfiber (30%) and bagasse (35%). Since sucrose consists only carbohydrates and also it is in pure form, the yield of hydrogen is normally high. To study the effect of temperature, pH, optical density of microbial culture, bagasse was used as a substrate because it was easily available and found abundantly in biomass.

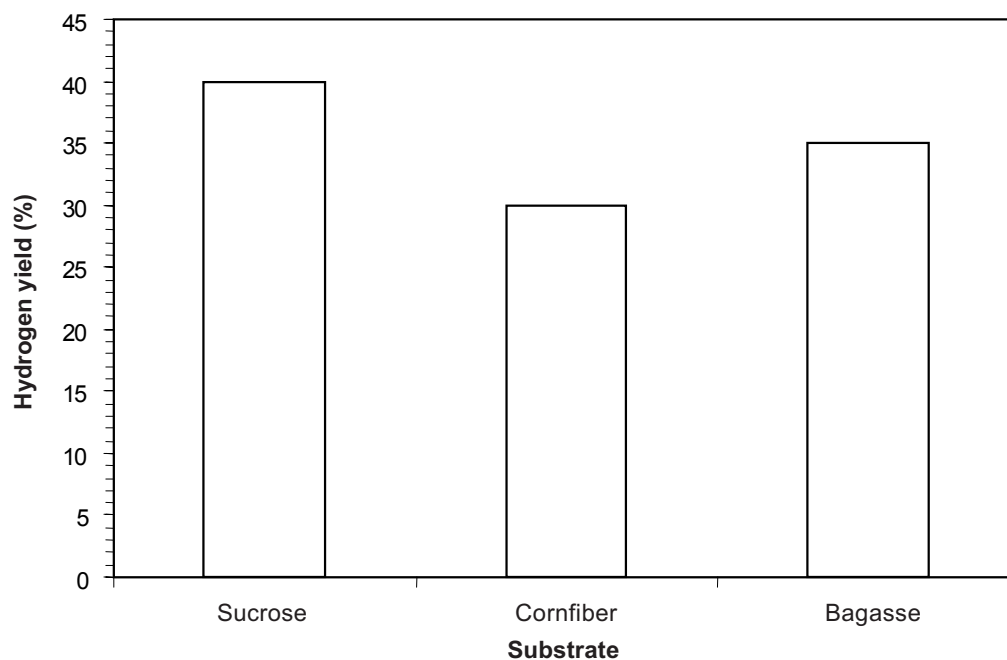


Figure 2 Percentage of hydrogen yield from different substrate using *Rhodobacter sphaeroides* at room temperature (32°C) and neutral pH (7)

Table 2 Hydrogen yield for various substrates for *Clostridium pasteurianum*

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

Table 2 provides a comparative data of hydrogen yield for various substrates for *Clostridium pasteurianum* at neutral pH and at a temperature of $32 \pm 1^\circ\text{C}$. It was observed that the yield of hydrogen was relatively more in case of enriched cornfiber 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 same for similar experimental conditions

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 Figures 3 and 4. The initial pH is 7 and the results depict that the number of colonies of microorganism decreases with an increase in acidity of the fermentation broth. The

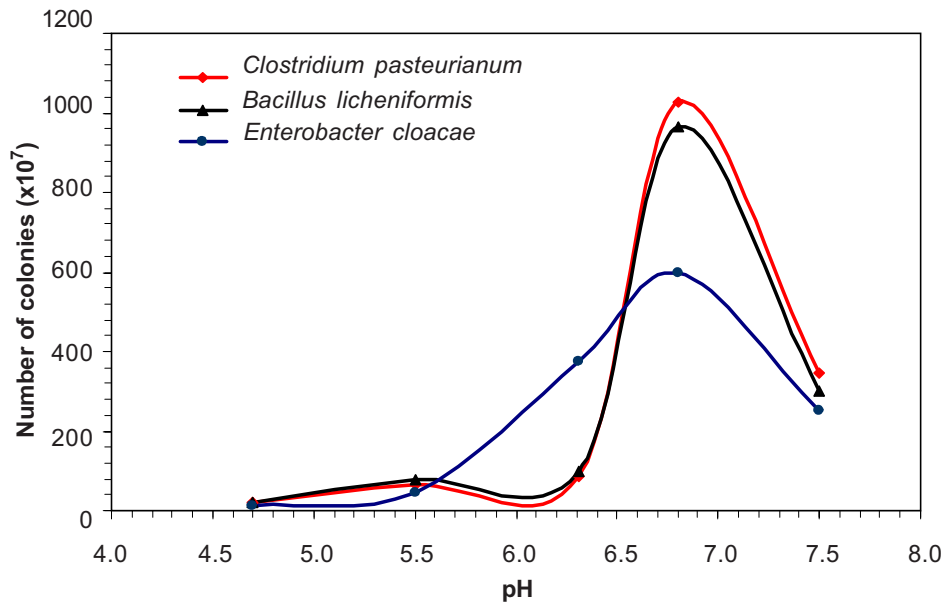


Figure 3 Effect of pH on the number of colonies of microorganism for bagasse

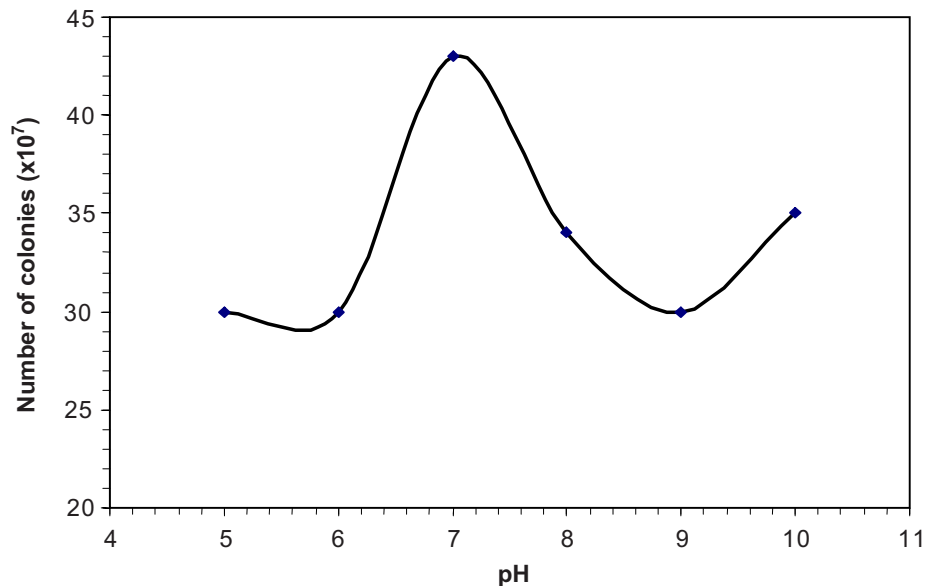


Figure 4 Effect of pH on the percentage yield of hydrogen for *Rhodobacter sphaeroides* with bagasse

optimum pH was found to be in the range of 6.8 to 7 for dark fermentative bacteria whereas for photofermentative bacteria it was found to be in the range of 6.5 to 7. During hydrogen production, the pH decreased from 7 to 4.2 in dark fermentation and 7 to 3.5 in photofermentation, at the end of the fermentation the pH were 4.2 and 3.5 for dark and photofermentation respectively. This phenomenon can be attributed due to the production of secondary metabolites, butyrate and acetate which increases the pH of the broth. The acidic environment created as a result of this would inhibit the production of hydrogen. This provides an insight on to the production of a by-product which is acidic in nature. It was also observed that as pH decreases, relatively yield of hydrogen also decreased, due to the inhibitory effect of the secondary metabolites.

3.3 Effect of Temperature

Figures 5 and 6 represent the effect of temperature on yield of hydrogen for three different anaerobic bacteria and photofermentative bacteria respectively. After repetitive experiments it was found that the gas yield was highest at 32°C. The yield of gas in case of *Rhodobacter sphaeroides* was found to be high as compared to *Clostridium pasteurianum*, *Bacillus* and *Enterobacter*. Similar results are reported by various researchers [4, 7, 10-12].

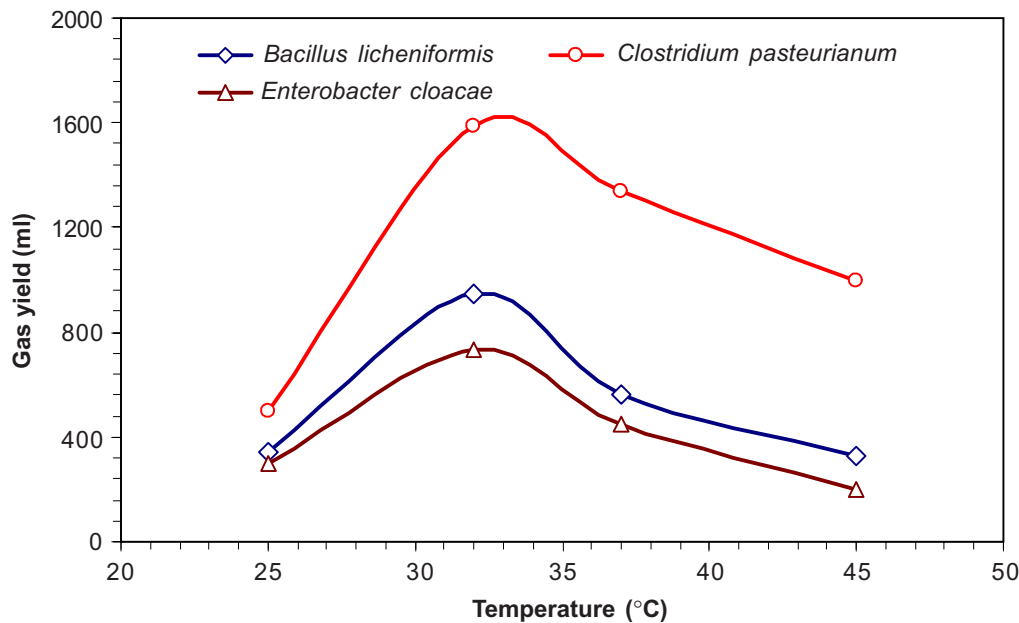


Figure 5 Quantity of gas yield as a function of temperature for bagasse, pH 7

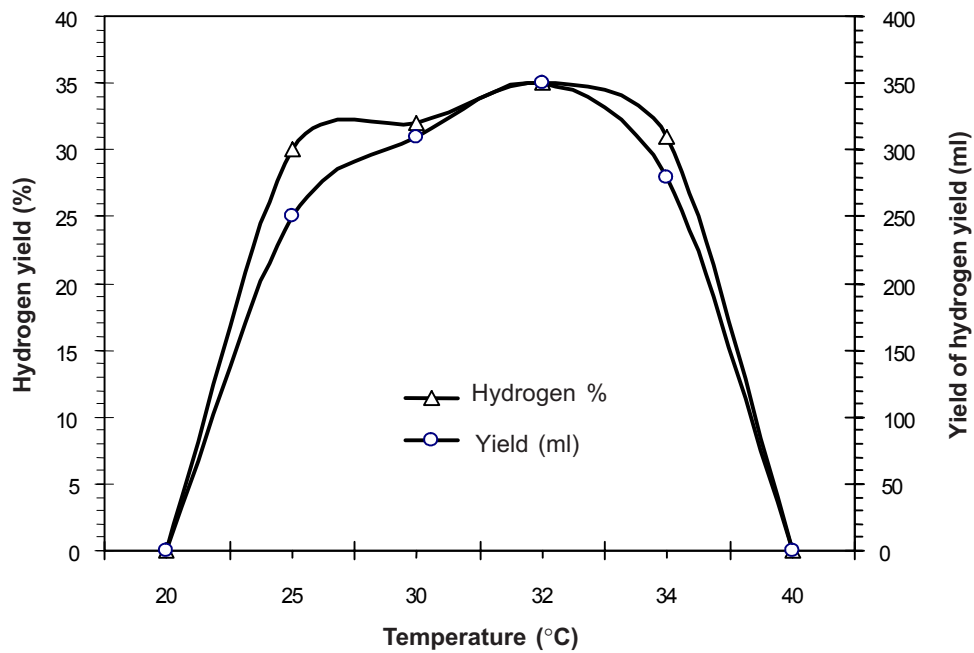


Figure 6 Quantity of gas yield as a function of temperature for *Rhodobacter sphaeroides* at pH 7

4.0 EFFECT OF CELL DENSITY

Experiments were also conducted to ascertain the effect of cell density on gas yield. As shown in Figure 7, production of hydrogen increases with increase in cell density to

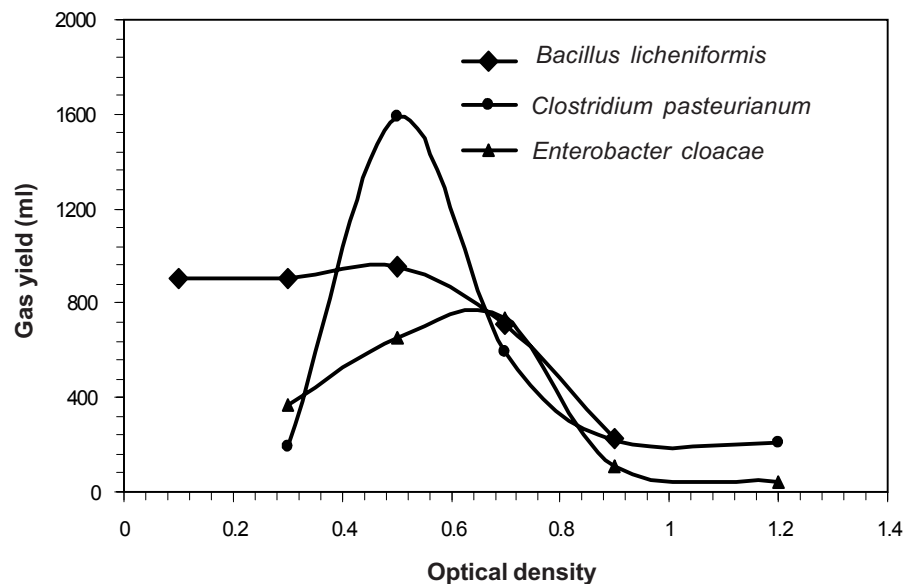


Figure 7 Gas yield as a function of optical density (cell density) for pH 7, bagasse at 32°C

a maximum at 0.5 for *Clostridium pasterianum* and *Bacillus licheniformis* and 0.7 for *Enterobacter cloacae* and thereafter it decreases with increase in cell density. This is due to the consumption of hydrogen by anaerobic species. Increase in cell density above the optimum value will inhibit the process of carbohydrates breakdown as the organisms will compete with each other for utilization of the substrate available. At higher cell density, the amount of media requirement will increase, which occupies more volume in reactor.

5.0 CONCLUSIONS

Naturally obtained, spore forming, hydrogen producing anaerobic bacteria i.e., *Clostridium pasteurianum*, *Bacillus licheniformis*, and *Enterobacter cloacae* has considerable potential in transformation of naturally available biomass (carbohydrates) into hydrogen. In the present work, it was observed that *Rhodobacter sphaeroides* produced maximum amount of hydrogen (35%) as compared to 21% by *Clostridium pasteurianum*, 16% by *Bacillus licheniformis* and 8% by *Enterobacter cloacae*. However, the volume of hydrogen collected for different microorganisms varied in the following order; *Clostridium pasteurianum* > *Bacillus licheniformis* > *Enterobacter cloacae* > *Rhodobacter sphaeroides*. It was also seen that hydrogen generation by photofermentation took nearly 7 days 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 very less when compared to dark fermentation. In both, the process (dark and photo fermentation) drop in pH resulted in decreased hydrogen generation rate mainly because of poor metabolic activity of organisms in acidic environment and also due to inhibition of microorganisms by 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. Hence more experimental trials varying detail operating parameters needs to be investigated in selecting economically feasible method for production of hydrogen.

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