Improvement of Hydrogen Production by Immobilized *Rhodopseudomonas palustris CGA009* Using Reverse Micelles as Microreactor

Anita Singh* and Krishna Misra**

* Department of Biochemistry and Cell Biology, Rice University, Houston, TX. anita2singh@gmail.com
**Indo-Russian Center for Bioinformatics, Indian Institute of Information Technology, Allahabad.

ABSTRACT

In the present study, the bacterium *Rhodopseudomonas palustris CGA009* has been immobilized separately in an aqueous pool of the reverse micelles fabricated by various surfactants (AOT, CBAC and SDS) and apolar organic solvents (benzene and isooctane). All reverse micellar systems of bacterial culture gave encouraging hydrogen production (rate as well as yield) compared to the aqueous system. An average of 50 fold increase in hydrogen production rate was observed in case of AOT/isooctane reverse micellar system as compared to the aqueous culture. CBAC/isooctane reverse micellar system is used for the first time for hydrogen production and is as promising as AOT/isooctane reverse micellar system. All these reverse micellar media were screened to reduce the inhibitory effect of NH_4^+ on hydrogen production. Studies of the effects of pH, temperature and light intensities on hydrogen production in reverse micelles show optimal hydrogen production at 37°C temperature, 600 lx light intensity and 7 pH.

Keywords: *Rhodopseudomonas palustris CGA009*, Reverse micelles, AOT, Biological hydrogen production.

1 INTRODUCTION

Hydrogen production from biological sources is gaining major emphasis recently since these processes are less energy intensive and comparatively cost effective than any other thermochemical or electrochemical processes. Among different alternative routes of biological hydrogen production microbial hydrogen production has already been investigated with both photosynthetic [1,2] and fermentative microorganisms [3,4].

Various groups of investigators have attempted to look for the possibility to improve hydrogen production through immobilization techniques using polysaccharide gels like agar, alginate and chitosan combined with agar etc. [5,6]. The major disadvantage of polysaccharide gel immobilization technique is inhomogeneous entrapment of bacteria, so that few bacterial cells suffer from nutritional exhaustion as well as low light penetration and this results in decreased hydrogen production.

In a reverse micellar system, bacterial cells are fully exposed to the light owing to the transparency of the solutions [7]. Therefore, a reverse micellar system is preferred over other immobilization techniques to produce hydrogen. Reverse micelles are essentially spheroid aggregates formed by dissolving a surfactant in a polar organic solvent with a limited amount of water to produce a macro-homogenous transparent solution, which provides an artificial system that mimics many life systems. AOT/ isooctane and SDS/benzene reverse micellar system for hydrogen producing bacteria have shown encouraging results [8-10].

The scope of this paper is to show the effects of various reverse micelles using bacteria on hydrogen production. For this purpose, we have used photosynthetic bacteria *CGA009* immobilized within AOT/isooctane, CBAC/isooctane and SDS/benzene reverse micelles. To the best of our knowledge, one of the reverse micellar systems CBAC/isooctane has not been reported for hydrogen production by this bacterial culture. All these reverse micellar systems were also screened to reduce the inhibitory effect of NH_4^+ on hydrogen production. Furthermore, effects of pH, temperature and light intensities on hydrogen production in reverse micelle have also been studied.

2 MATERIAL AND METHODS

2.1 Chemicals

All chemicals used in the experiments were of AR Grade, purchased from Merck India Ltd., L-malic acid and vitamins were purchased from Himedia Laboratories Pvt. Ltd., India and used without further purification.

2.2 Microorganism and Culture Conditions

The photosynthetic bacteria *R. palustris CGA009* was cultivated photosynthetically at 30^0C in Sistrom's growth media [11]. In order to increase hydrogen production, the growth medium was modified by adding 30 mM of malate as a carbon source, 7 mM of glutamate as a nitrogen source, and by removing succinic acid, aspartic acid, ammonium sulphate and nitrilotriacetic acid. Furthermore, sodium molybdate ($Na_2MO_4.2H_2O$) was used in place of ammonium molybdate. Batch cultivations for *CGA009* was performed at 30^0C in a gyratory incubator with a shaking speed of 250 rpm. An anaerobic condition was developed in a serum bottle of 155 ml by filling it with the media and flushing with argon gas (99.99%) till the working volume of 50 ml was achieved. A tungsten lamp was placed to

maintain a luminance intensity of 6000 lx. The inoculum was cultivated in the bottle and transferred anaerobically at the late-exponential phase by a sterile disposable syringe. The culture was centrifuged at 10,000 rpm for 15 min. The pellets were resuspended in the growth medium and used for entrapment studies.

2.3 Preparation of reverse micelles for hydrogen production

The surfactants were added in powder form gradually to the organic solvents taken in a flask and homogenized. sodium lauryl sulfate (SDS) (1.2 g) was added to benzene (250 ml); sodium bis-2-ethylhexyl-sulfosuccinate (AOT) (8.86 g) and cetylbenzyldimethyl-ammonium chloride (CBAC) (0.95 g) were added to isooctane (250 ml) at 37^0C. 2.0 ml of the bacteria *CGA009*, grown during the late-log phase, was injected into the homogenates to fabricate reverse micellar system. The reverse micellar medium containing entrapped bacterial cells was transferred to hydrogen producing air tight setup. The setup was placed on a magnetic stirrer in an upright position fitted with a clamped stand and illuminated with a tungsten lamp of 600 lx intensity at 37^0C temperature. Hydrogen gas produced was collected in the upper portion of the setup by displacement of the reverse micellar organic medium.

2.4 Determination of optimum temperature, pH and light intensity for maximum hydrogen production

To study the effect of temperature on reverse micellar systems for the hydrogen production 5 sets of experiments were conducted. 2.0 ml late-log phase culture was entrapped within each set of AOT/isooctane reverse micelles at 600 lux light intensity. Using hot plate cum stirrer and water bath the temperature of the systems was maintained at 25, 30, 35, 37 and 40°C and the hydrogen production rate was measured every 30 min. For determining the pH dependence of the H_2 production, bacterial culture was suspended within several different buffer solutions. These were as follows (all in 100 mM): acetate buffer (pH 4.0 and 5.0), phosphate buffer (pH 6.0 to 7.0), and trizma base buffer (pH 8.0 and 9.0). 2.0 ml volume of suspended culture was entrapped within the reverse micelles formed by AOT/isooctane and the set-ups were exposed to 600 lux intensity and maintained at 37°C, the hydrogen production rate was measured every 30 min. To determine the effect of light intensity, late-log phase culture was entrapped within AOT/Isooctane reverse micelles at different light intensity from 100 to 2500 lux. Temperature of the micellar solution was maintained at 37°C and hydrogen production in each case was recorded every 30 min. Dry weight of the organisms was taken to express the results.

2.5 Determination of the effect of NH_4^+ on hydrogen production by *CGA009*

To determine the effect of NH_4^+ on hydrogen production, culture was grown in modified sistrom's medium by replacement of glutamate with ammonium sulphate. Five sets of experiments were conducted. In each set culture was grown in above media with varying concentration of ammonium sulphate i.e. 3mM, 6mM, 9mM, 12mM and 15mM. 2.0 ml volume of suspended culture and 1.0 ml of above media was entrapped within the various reverse micellar system of AOT/isooctane, CBAC/isooctane and SDS/benzene. The set-ups were exposed to 600 lux light intensity and maintained at 37°C temperature. The hydrogen production rate was measured every 30 min.

2.6 Measurement of protein content

Protein content of the bacterial cells was measured using Lowry method. In this method, a bovine serum albumin (BSA) is used as standard [12].

2.7 Gas analysis

The concentration of the evolved molecular hydrogen was measured by a gas chromatograph (Nikon) equipped with a thermal conductivity detector and a stainless-steel column (Carbosieve II). In the equipment argon gas is served as carrier and pure hydrogen gas is used as standard. The column temperature was maintained at 60^0C and the temperature of detector and injector was set to 70^0C. The volume of hydrogen was converted into the value at 37^0C and 0.1 MPa.

Stoichiometric maximum evolution of hydrogen was calculated by assuming that hydrogen was evolved by complete conversion of the carbon source in the catabolic process. The yield of hydrogen evolution was calculated as a percentage of the stoichiometric maximum. For example, the stoichiometric maximum of hydrogen evolution from 30 mM malate was 4.58 ml/ml-medium at 37^0 C and 0.1 MPa.

3 RESULTS AND DISCUSSION

3.1 Hydrogen production by entrapped *CGA009* in reverse micelles

Table 1 show the rates of hydrogen production, total volume of hydrogen evolved and hydrogen yield by the bacterial cells of *CGA009* entrapped within different reverse micellar systems of AOT/isooctane, CBAC/isooctane and SDS/benzene at 37^0C temperature, pH 7 and 600 lx light intensity. The bacterial cells produce more hydrogen in the AOT/isooctane reverse micellar medium in comparison with the other reverse micellar media. The hydrogen production rates of the *CGA009*

entrapped within all reverse micellar systems (i.e. AOT/isooctane, CBAC/isooctane and SDS/benzene) were 30–50 times higher when compared with the hydrogen production rates of the aqueous culture The hydrogen yield increased by a factor of 2 in the reverse micellar systems when compared with the aqueous culture. The evolved hydrogen gas was found to be 99.99% pure.

All reverse micellar systems used in the study were compatible with the photosynthetic bacterial cells. Solvents and surfactants in the reverse micellar systems did not affect the viability of the bacterial cells much and gave an appropriate microenvironment within the water pool for hydrogen production. Reverse micelles act as small microreactors in which the activity of cellular enzymes is increased by bringing the reactants together [13]. Thus, the enzymes responsible for carbohydrate degradation and hydrogen production become more active in the anaerobic water pool of reverse micellar microreactor owing to compartmentalization of the cells resulting in enhanced hydrogen production. Furthermore, nitrogenase and hydrogenase enzymes are active only in anaerobic environment and reverse micelles provide such environment to these enzymes as the reverse micellar aqueous pool itself has a very low concentration of dissolved oxygen and the surrounding organic medium helps in providing anaerobic condition to the aqueous pool. Free bacterial cells in aqueous culture, however, are more exposed to oxygen. In this study it was observed that AOT/ isooctane reverse micellar system was the most transparent and SDS/benzene system was the least transparent [14]. Therefore, increased light availability for nitrogenase enzyme of the bacterial cells in AOT/isooctane reverse micelles resulted in increased hydrogen production rate [Table 1]. Hydrogen evolving systems, AOT/isooctane and CBAC/isooctane gave very similar results.

3.2 Effect of temperature, pH and light intensity on hydrogen production in reverse micellar system

The results in Fig. 1 show that, by increasing the temperature from 25 to 37°C, hydrogen production rate gradually increases. At temperatures higher than 37°C, the rate decreased. At pH 7 optimal hydrogen production was observed. At higher or lower pH the rate decreased significantly [Fig. 2]. Also note that hydrogen was not produced at pH 4 . The evolution of hydrogen was affected significantly by the light intensity. The evolution rate of hydrogen increased exponentially with the light intensity from 100 lux to 600 lux. Hydrogen production rate remains constant up to 1500 lux light intensity [Fig. 3]. Further increase in light intensity caused a decrease in the rate of hydrogen production.

3.3 Effect of NH_4^+ on hydrogen production in reverse micellar system

The results shown in Fig. 4 indicate that at the initial concentration of ammonium sulphate i.e., 3 mM, larger amount of hydrogen was evolved by all the reverse micelles (AOT/isooctane, CBAC/H_2O/isooctane and SDS/benzene) than by the normal aqueous culture. Comparison within the reverse micelles indicates that the maximum hydrogen evolution was obtained by AOT/isooctane and lowest hydrogen production by SDS/benzene. However, when ammonium sulphate concentration was increased to 15 mM, hydrogen evolution rate was much depressed. In all the 3 systems, maximum evolution appears at 3mM concentration of NH_4^+ ion.

4 CONCLUSIONS

Under the experimental conditions during the present work, reverse micelle system AOT/isooctane at 37°C, pH of 7.0, light intensity of 600 lux and an optimal concentration of 3 mM NH_4^+ shows highest hydrogen evolution by bacterial cells. CBAC/isooctane reverse micellar system also gave an encouraging amount of hydrogen by bacterial cells.

On the basis of above results, we believe that the entrapment of whole cells within these reverse micellar systems can be applied to the construction of bioreactor for hydrogen production

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6 TABLE AND FIGURES

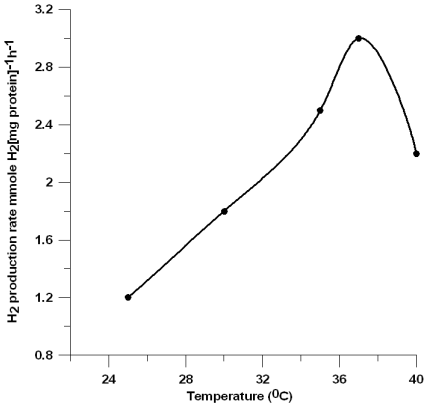

Figure 1: Effect of temperature on hydrogen production by *CGA009* in AOT/isooctane reverse micelle at pH 7 and 600 lux light intensity.

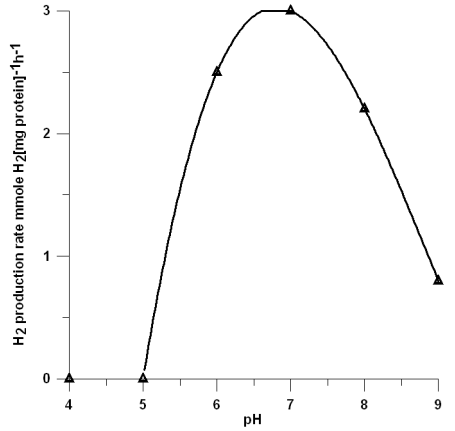

Figure 2: Effect of pH on hydrogen production by *CGA009* in AOT/isooctane reverse micelle at 37ºC and 600 lux light intensity.

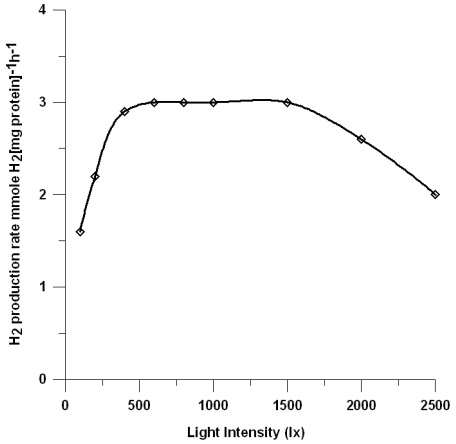

Figure 3: Effect of light intensity on hydrogen production by *CGA009* in AOT/isooctane reverse micelle at 37ºC and pH 7.

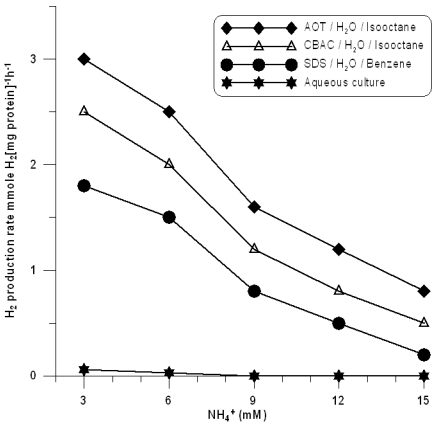

Figure 4: Effect of NH_4^+ concentration on hydrogen production in different reverse micellar systems and in aqueous culture by *CGA009* at temperature 37ºC, pH 7 and 600 lux light intensity**.**

Table 1: H_2 production in various reverse micellar systems and in aqueous medium using *CGA009*.

Reverse Micellar Systems	H_2 production rate [mmol of H_2 (mg protein)$^{-1}$ h^{-1}]	Fold enhancement in rate w.r.t. aqueous culture	Total amount of H_2 evolved [ml/ml-media]	Yield (%)
AOT/ isooctane	3.00 ± 0.80	~50	1.15 ±0.01	25.20
CBAC/ isooctane	2.50 ± 0.50	~42	1.05 ±0.03	23.00
SDS/ benzene	1.80 ± 0.40	~30	0.80 ±0.01	17.50
Aqueous culture	0.06 ± 0.02	1	0.64 ±0.01	14.00

Note: Carbon source: 30 mM DL-malate. Temperature: 37^{0}C, pH: 7 and light intensity: 600 lx. Each value is mean of at least three times replication (±standard deviation).

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