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# Optimization of hydrogen production by *Halobacterium salinarium* coupled with *E coli* using milk plasma as fermentative substrate

**Brijesh Rajanandam K S*, Siva Kiran R R**

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## Abstract

Batch experiments were conducted to investigate the fermentative hydrogen production by coupled system of *Halobacterium salinarium* and *E. coli.* Increase in the light intensity from 6000 lux to 12000 lux and changing the inoculums level of *E coli* resulted in 10 fold increase in the rate of hydrogen production using the coupled system. Statistical based design of experiments was applied to optimize the rate of hydrogen production using milk plasma, popularly known as cheese whey, a dairy industry byproduct. An optimal rate of hydrogen production of 56.7 ml/l h was achieved with 14.42 % (by volume) of milk plasma and an initial pH of 6.6. The investigations provided information on achieving higher yields with milk plasma as substrate, its optimal concentration, and importance of media pH for producing higher rate of hydrogen.

**Key words:** *Halobacterium salinarium, E. coli,* hydrogen, milk plasma, central composite design

## Introduction

Hydrogen is regarded as a potential energy carrier fuel for the future. Hydrogen is produced mainly from natural gas, a finite resource, through steam reforming, a process that generates large quantities of carbon dioxide ($CO_2$) which is a principal cause of global warming. Cleaner technologies like Fermentative Hydrogen Production (FHP) from renewable feedstocks must be developed. FHP can be produced using direct biophotolysis by green algae, indirect biophotolysis by cyanobacteria, photo-fermentation by anaerobic photosynthetic bacteria and dark fermentation by anaerobic fermentative bacteria. A large number of fermentative bacterial consortium have been investigated for biohydrogen production. One of the major disadvantage of the bacterial systems is the expenditure incurred in maintenance of the process parameters like pH, temperature and sterility in the photo-bioreactor.

**Brijesh Rajanandam K S*,**

Department of Chemical Engineering, MS Ramaiah Institute of Technology, MSR Nagar, Bangalore-560054

**Siva Kiran RR**

Department of Biotechnology, MS Ramaiah Institute of Technology, MSR Nagar, Bangalore-560054

Tel.: 0091 8023600822, Fax: 0091 80 23603124,
Email: ksrajanandam@gmail.com

Taqui khan et al. (1989) reported photosensitized production of hydrogen by *Halobacterium halobium* (also known as *Halobacterium salinarium*) coupled to *Escherichia coli* (Taqui Khan and Bhatt, 1989). Interest in this process renewed due to the high salt concentration media, reducing the risk of contamination and handling problems. The authors modified the system by using photo electrochemical method (Taqui Khan and Bhatt, 1990) to increase the efficiency of the system and later used *E. coli* in reversed micelles of sodium lauryl sulfate (Taqui Khan and Bhatt, 1991) to enhance the yield. The media for hydrogen production by this coupled system constituted mainly peptone and salt (greater than 20% w/v). The main objective of present investigation was to achieve higher rate of hydrogen production at optimum process parameters used in the coupled system reported by Taqui Khan et al. (1989), and also to produce hydrogen with renewable raw material source.

Milk plasma is the lactose-rich (6%-6.5%) watery by-product of cheese manufacturing industry. According to the Food and Agricultural Organization (FAO) of the United Nations, over 18 million metric tons of cheese was produced worldwide in 2004 contributing to 14.29 million tons of milk plasma. Even though there are a number of technological developments in the transformation of milk plasma to other useful products, utilization of milk plasma is one of the significant problems in the dairy industry. Mongi et al. (2005) investigated the potential of using milk plasma as a fermentative substrate and effect of initial pH for hydrogen generation using *Clostridium* sp. in photo-bioreactor and achieved a maximum rate of 28.3 ml/hr.

The protein composition in milk plasma is about 0.5% - 0.7%, and the peptone concentration used in media for hydrogen production by Taqui khan et al. (1989) was 1%. The present paper demonstrates that peptone in the Taqui Khan et al. media can be replaced by milk plasma for hydrogen production. *E. coli* consumes lactose and acts as a source of hydrogenase enzyme to generate electrons. The

electrons donated by hydrogenase to the protons released from *Halobacterium salinarium* results in the evolution of molecular hydrogen. The detailed mechanism is explained by Taqui khan et al. (1989). The preliminary studies by Taqui khan et al. states that *Halobacterium salinarium* requires protein source for producing hydrogen.

Statistically based experimental designs like response surface methodology are more efficient in experimental biology when compared with traditional one-factor-at-a-time studies. The main advantage is that the variables can be tested simultaneously. The total number of experiments can be minimized by the application of these experimental designs. The response surface methodology, central composite design is applied for achieving higher rate of production of hydrogen using synthetic dairy wastes (milk plasma) as a substrate by *Halobacterium salinarium* and *E. coli* at 20% (by weight) salt concentration.

## Materials and Methods

*Culture and maintenance media*

Bacterial cultures, *Halobacterium salinarium* MTCC 1626 and *E. coli* K12 MTCC 729, were procured from Microbial type culture collection, Chandigarh, India. *E. coli* K12 MTCC 729 was made salt tolerant. The cultures were maintained in a medium containing 250 g NaC1, 20 g $MgSO_4.7H_20$ 3.0 g Trisodium citrate.$2H_20$, 2 g KCl, 10.0 g Peptone and 1000 ml distilled water, pH 7.0 and nutrient broth (+20% NaC1) respectively. The organisms were sub-cultured every 6-8 weeks.

*Experimental setup and culture conditions*

Hydrogen production experiments were carried out in a 1 liter standard flat bottom flask photo-bioreactor (Figure 1). The experimental set up was similar to the one reported by Zabut et al (2005) with few modifications. The reactor had two necks with adapted lids. One opening was for product gas outlet, which was connected to the collection system, the second opening was for purging Argon. The reactor had a flattened bottom, which has allowed the use of 3 cm magnetic bar. The stirring speed was 120 rpm. The temperature was maintained constant using magnetic stirrer with thermostat. Manipulation of the flow of liquid sample or gas through the tubing was made by ratchet tubing clamps available in the Bangalore city local market, India. The reactor was illuminated by using four tungsten lamps (60 W) from a distance of 23 cm in all four directions. The distance between the lamps was maintained constant. The intensity of the light was measured by using lux meter. The intensity was measured at ten points round the reactor and tried maintaining constant by moving the tungsten lamps.

The volumetric hydrogen production rate was calculated based on the gas collected for 30 minutes. Argon was purged initially to the culture medium and the hydrogen produced was collected by water displacement method in an inverted micro burette with a septa. The gas was analyzed for its hydrogen content using Gas chromatography (Neon Ashco Pvt Ltd, India) with thermal conductivity detector (TCD). The oven, TCD and injection port temperatures were 40°C, 150°C, and 80°C respectively. The carrier gas used was Argon and the column was packed with molecular sieve (13X, 80/100, Alltech, USA). The optical density was determined by UV Spectrophotometer (UV Mini -1240, Shimadzu). The photo-bioreactor was maintained at 40°C and 6000 lux. The photo-bioreactor medium contained, 15 ml of 24 hours grown *H. salinarium* MTCC 1626 ($OD_{540}$:0.178) and 15 ml of overnight grown *E. coli* ($OD_{540}$:1.46) suspended in 25 g NaC1,

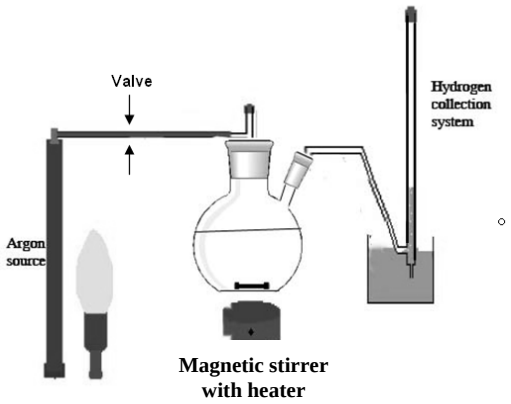


Figure 1: A schematic diagram of the experimental setup with few modifications (Zabut et al. 2006)

2 g $MgSO4.7H_2O$, 0.3 g trisodium citrate $2H_2O$, 0.2 g KCl, 1 g Peptone. The final volume was made up to 100 ml using distilled water. Initial pH of 7.0 was adjusted using NaOH/ HCl (Taqui Khan and Bhatt, 1989).

*Experimental design*

A $2^2$-factorial central-composite-experimental-design with four star-(a)-points (α-1.414), two replicates at the centre point and four cube points, all in duplicate, leading to 10 sets of experiments, were used to optimize the production of hydrogen. The levels of the independent variables, viz., milk plasma concentration and pH chosen for this study are given in Table 1.

**Table 1:** Independent variables and the levels studied in the optimization design

| Variables | Factors | -α | -1 | 0 | +1 | +α |
|---|---|---|---|---|---|---|
| $X_1$ | Milk plasma (%) | 6 | 10 | 20 | 30 | 35 |
| $X_2$ | pH | 6 | 6.5 | 7 | 7.5 | 8 |

The average rate of hydrogen production was taken as the dependent variable or response, $Y_i$. Regression analysis was performed on the data obtained. The results of central composite design were used to fit a second-order polynomial equation as it represents the behavior of such systems more appropriately than first-order designs. A second order polynomial of the following form was fitted:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2$$

where Y = predicted response (Rate of $H_2$ production), $\beta_0$ = offset term, $\beta_1$, $\beta_2$= linear effect, $\beta_{11}$, $\beta_{22}$= squared effect, $\beta_{12}$= interaction effect. Statistica software (Version 6.0, by StatSoft Inc., Tulsa, USA) was used for the study (Nair et al, 1997).

## Results and Discussion

An experiment was conducted using the above medium and the hydrogen production was found to be 5 ml/l-h at 30 minutes. It is observed that light intensity plays a major role in the production of hydrogen. Hydrogenase enzyme supplied by *E. coli* species react with the light activated NH=CH group present in the protein compound. Light plays a major role in the production of hydrogen and it is also observed that the inoculums level of *E. coli* plays a major role in the production of hydrogen. The Taqui et al (1989)

medium was modified by increasing the light intensity to 12000 lux. The inoculum level was increased to 15 ml of 24 hours grown *H. salinarium* MTCC 1626 ($OD_{540}$:0.178) and 50 ml of overnight grown *E. coli* ($OD_{540}$:1.46). The other medium components were 25 g NaC1, 2 g MgSO4.7H20 0.3 g trisodium citrate $2H_20$, 0.2 g KCI, 1 g Peptone. The final volume was made up to 100 ml using distilled water. Initial pH of 7.0 was adjusted using NaOH/HCl. The maximum rate of hydrogen production was achieved to be 102.283 ml/l-h at 4 minutes and constant rate of 49.040 ml/l-h at 30 minutes (Figure 2). Modification of the Taqui et al (1989) medium resulted in 10 fold increase in the rate of hydrogen production using the coupled system.

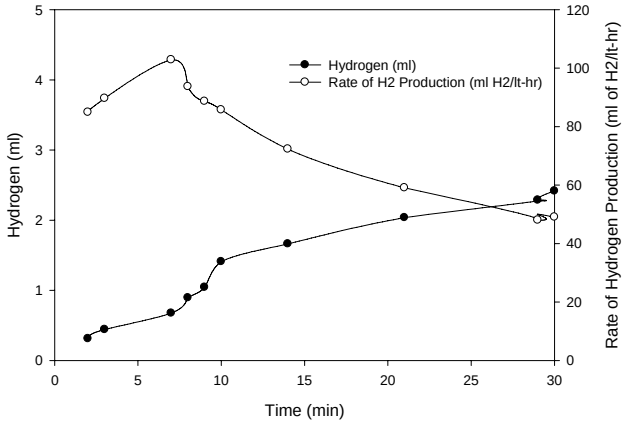


Figure 2: Effect of time on the rate of hydrogen production

The above optimized medium was again modified by replacing peptone with dairy industry waste milk plasma. The exact proportion of milk plasma concentration and pH was determined using response surface methodology, central composite design. The experimental design matrix with predicted and observed response is shown in Table 2. The model has a regression coefficient of 0.96746. The second order polynomial equation generated from the model is given below.

$$Y = -182.540 + 1.553X_1 + 68.317X_2 - 0.048X_1^2 - 5.108X_2^2 - 0.027X_1X_2$$

The contour plot given in figure 4 shows the relative effects of milk plasma percentage and pH variables. The contours correspond the to the rate of hydrogen production expressed in ml/l h. The contour plot suggests the approximate range of the two factors, which could result in maximum rate of production of hydrogen under these conditions. The coordinates of the central point within the highest contour level of the Figure 3 will correspond to the optimum rate of hydrogen production.

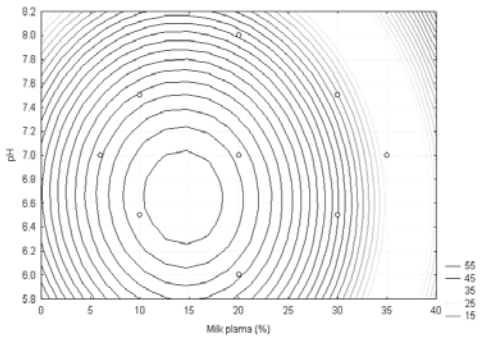


Figure 3: Contour plot on the effect of milk plasma concentration and pH for hydrogen production

**Table 2:** Experimental plan of the optimization design with the experimental and the predicted values

| S. No | Milk plasma (Volume %) | pH | Rate of $H_2$ production (ml/l h) | |
|---|---|---|---|---|
| | | | Experimental | Predicted |
| 1 | 10 | 6.5 | 54.24 | 54.73106 |
| 2 | 10 | 7.5 | 50.41 | 51.26772 |
| 3 | 30 | 6.5 | 45.37 | 44.17644 |
| 4 | 30 | 7.5 | 41.01 | 40.18311 |
| 5 | 6 | 7.0 | 52.94 | 51.86065 |
| 6 | 35 | 7.0 | 33.55 | 34.78875 |
| 7 | 20 | 6.0 | 51.99 | 52.25729 |
| 8 | 20 | 8.0 | 44.9 | 44.80063 |
| 9 | 20 | 7.0 | 51.47 | 53.63717 |
| 10 | 20 | 7.0 | 55.46 | 53.63717 |

Maximum volumetric rate of hydrogen production predicted by the model equation using the optimized values (milk plasma concentration, 14.42% and pH, 6.6) was 55.79 ml/l-h. The experimental rate at the predicted responses was 56.7 ml/l-h, confirming the validity of the optimized results.

## Conclusions

The results show that dairy industries waste has potential to be utilized as a media for bio-hydrogen generation in the coupled system of *Halobacterium salinarium* and *E. coli*. A significantly higher volumetric hydrogen production rate of 56.7 ml/l h was achieved experimentally using milk plasma as substrate at the optimum conditions predicted by statistical analysis. The change in the medium composition from peptone to milk plasma resulted in 1.15 folds increase in the rate of hydrogen and 10 folds increase when compared with preliminary studies. It is also observed that the coupled system is more robust and has the distinct advantage of low operating cost owing to less contamination problems as it grows in high salt concentrations. This could be of major advantage in commercial scale up of this process.

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