

STATISTICAL OPTIMIZATION OF PROCESS PARAMETERS FOR HYDROGEN PRODUCTION IN *Halobacterium salinarium* IMMOBILIZED WITH CALCIUM ALGINATE AND *Escherichia coli*

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ABSTRACT

The individual and mutual effects of the key parameters for hydrogen production in a photo-bioreactor containing *Halobacterium salinarium* MTCC 1626 immobilized in calcium alginate and *E. coli* MTCC 40 were investigated through factorial and the Box-Behnken experimental design. A full factorial design was utilized to determine the optimal ratio of *H. salinarium* and *E. coli* for maximum hydrogen production. The Box-Behnken design was used to optimize temperature, pH, light intensity, and stirrer speed in a photo-bioreactor. The quadratic model adequately predicted the individual and interaction effects of the variables. The optimal rate of hydrogen production of 53.22 mL/L.h was obtained with 50% *E. coli* and 15% immobilized *H. salinarium* with calcium alginate. The maximum experimental rate of hydrogen production of 75.8 mL/L.h was obtained at an initial pH of 7.38, a temperature of 35.8 °C, a light intensity of 12186 lux, and a stirring speed of 104 rpm.

Keywords: Hydrogen, photo-bioreactor, Box-Behnken design, immobilization, *Halobacterium salinarium*, calcium alginate

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INTRODUCTION

The energy demand is increasing globally with the increase in population and improvements in living standards. Indiscriminate usage of fossil fuels has seriously deteriorated the delicate balance between energy, environment, and sustained development of mankind. The need to explore environment-compatible renewable energy sources is significant. The dependence of the world on hydrogen is continuously increasing¹. Several studies on hydrogen production in recent years are reported in the literature.^{2,3,4} Among various methods of hydrogen production, the biological route has gained increased attention over the years due to its less energy-intensive nature and the ability to process varied substrates⁵. Production of hydrogen from the biological route reduces the dependency on fossil fuels and also has the potential for waste treatment.^{6,7} Biological hydrogen production has been reported in several strict or facultative anaerobes through dark fermentation, photo fermentation, and bio-photolysis.^{8,9} *Halobacterium salinarium* and its habitat in high concentrated salt solutions preserve its structure. It is primarily aerobic but also grows anaerobically in the presence of light, containing retinal protein bacteriorhodopsin, or in the dark with a medium containing arginine. In the system of *H. salinarium* and *Escherichia coli*, the protons liberated from *H. salinarium* in the presence of light combine with electrons released by the hydrogenase of *E. coli*, causing the evolution of molecular hydrogen.¹⁰

Immobilization increases the number of cells per unit volume, improves conversion, and prevents the inhibition of cell growth. The process can be made to be continuous as the loss of cells is reduced. Washout of cells is a serious issue in continuous reactors.¹¹ Vedat (2008)¹² carried out studies on immobilized cells of *H. halobium* in cellulose acetate. In this paper, *Halobacterium salinarium* MTCC 1626 immobilized with calcium alginate beads and *E. coli* MTCC 40 were used for the production of hydrogen, and important

process variables were optimized using the full factorial design and the Box-Behnken design. The current combination proved to be effective and was rarely attempted based on the literature review.

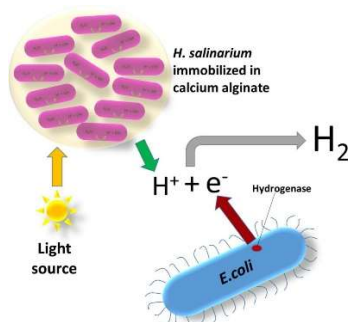


Fig.-1: Graphical illustration of Immobilized *H. salinarium* MTCC 1626 and *E. coli* MTCC 40 Coupled System for Hydrogen Production

EXPERIMENTAL

Materials and Methods

H. salinarium MTCC 1626 cultures were obtained from the Microbial Type Culture Collection Centre (MTCC), Chandigarh, India. Nutrient agar at $\text{pH } 7.0 \pm 0.2$ was used to maintain the bacterial cultures at 37°C in an incubator. The growth medium for *H. salinarium* MTCC 1626 consisted of 250g sodium chloride, 10g peptone, 20g magnesium sulphate heptahydrate, 2g potassium chloride, 3g tri-sodium citrate dihydrate, and 1000 mL distilled water. The nutrient broth was used to grow the *E. coli* MTCC 40.

Photo-bioreactor

The experiments for hydrogen generation were conducted in a 1L photo-bioreactor flask with a flat bottom. The flask had three adapted lids for the gas outlet, for the temperature sensor, and for purging argon (Fig.-2). The photo-bioreactor was fed with a medium containing an overnight grow salt-tolerant *E. coli* and *H. salinarium* that was grown for 24 h. The final volume of the medium was made up to be 500 mL using the media with 23% salt. 1 N NaOH/HCl was used to maintain an initial pH of 7.0 for the media. The photo-bioreactor temperature was maintained by a thermostat, and the stirrer speed was controlled by a magnetic stirrer with a controller.

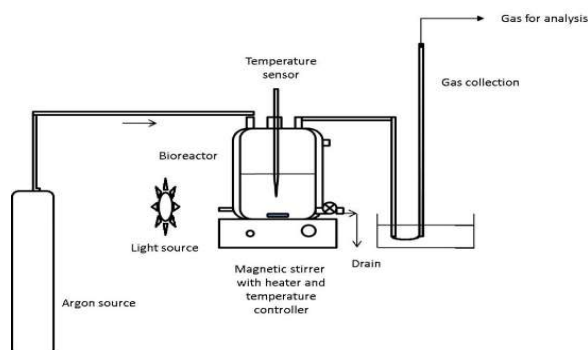


Fig.-2: Photo-bioreactor with *H. salinarium* Immobilized with Calcium Alginate and *E. coli* Connected to a Hydrogen Collection Arrangement

Three 60 W tungsten lamps were used to illuminate the bioreactor, and the light intensity was measured by a lux meter at ten different points around the reactor. Hydrogen generated in the process was collected by displacement of water in an inverted 50 mL burette.¹³ The burette had a threaded cap and septa sealed at the top for withdrawing the gas sample by a syringe.

Analytical Methods

The volume of hydrogen produced per unit batch volume per unit time (mL/L.h) was calculated based on gas collected for the first 30 min.¹⁰ Analysis of the hydrogen content of the gas was carried out by gas chromatography (Neon Ascho, India) with a thermal conductivity detector. The oven temperature was 40

°C, the injection port was maintained at 80 °C and the thermal conductivity detector at 150 °C. Argon was used as carrier gas with a molecular sieve column (13X, 80/100, Alltech, USA). The light intensity was measured by lux meter (MexTech, LX-1010B). A UV-Visible spectrophotometer (Shimadzu UV Mini 1240) was used to determine the optical density of the cell suspension.

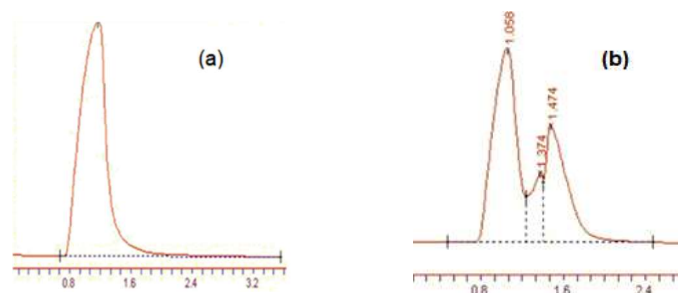


Fig.-3: Gas chromatograph Showing Pure Hydrogen (a) And Hydrogen with Carrier Gas (b)

Preliminary experiments were carried out on batch cultures of both microorganisms to determine the optimum growth conditions. The optimal growth parameters determined experimentally for *H. salinarium* MTCC 1626 were an initial pH of 7.5, 37 °C, 10% (v/v) inoculum level, 15% (w/w) salt concentration, 24 h inoculum age, and a light intensity of 6000 lux. For *E. coli* MTCC 40, the conditions were an initial pH of 7.0, 40 °C, 10% (v/v) inoculum level, and 12 h inoculum age. A 50 mL suspension of *H. salinarium* grown for 24 h was added to a mixture of 50 mL of 3% (w/v) sodium alginate in water. This mixture was added using a syringe into 100 mL of a 2% (weight/volume) CaCl_2 solution to form immobilized cell beads of 1 to 3 mm diameter. Preliminary studies showed that the use of the coupled system for hydrogen generation has the advantage of lower cost of operation due to reduced contamination problems in high salt concentrations. It was verified that the rate of hydrogen production followed a trend that initially increased for a short time of about 30 min and then started decreasing.¹³ Therefore, the hydrogen production studies were carried out for the first 30 min, after which the rate of hydrogen generation declined. A statistical method was applied to optimize the variables and enhance the rate of hydrogen production. The ratio of immobilized *H. salinarium* and *Escherichia coli* for increasing the rate of production of hydrogen in the photo-bioreactor was determined by factorial design. The levels of the factors were established from preliminary experiments and are shown in Table-1. Immobilized cells of *H. salinarium* grown for 24 h were represented in terms of mass/volume percent, and *E. coli* grown for 12 h was represented in terms of volume percent.

Table-1: Levels of the Independent Variables to Determine the Culture Proportion

Variables	Strains	Levels of Variables		
		-1	0	1
A	Immobilized <i>H. salinarium</i> (%)	10	20	30
B	<i>E. coli</i> (%)	25	50	75

A 2^2 -fractional factorial experimental design with two levels and two factors, resulting in 9 experimental sets, was used to optimize the inoculums to produce hydrogen. The average volumetric hydrogen production during the first 30 min was considered as the response, Y_i .

Box-Behnken Design

In this study, a 3-level, 4-factorial Box-Behnken design was used to study the effect of the factors, initial pH (6.5, 7, 7.5), temperature (30°C, 35°C, and 40°C), light intensity (9000 lux, 11000 lux, and 13000 lux), and stirrer speed (100 rpm, 120 rpm, and 140 rpm), on hydrogen production. Preliminary experiments were conducted to decide the levels of the parameters for the statistical study. A total of thirty experimental trials were fixed. The center point was replicated six times to predict the error in the experiments. A response surface methodology was applied to the experimental data, and a second-order polynomial was fitted to determine the regression equation. The quadratic response model including all linear terms, square terms, and interaction terms can be described as:

$$Y = \alpha_0 + \alpha_1 A_1 + \alpha_2 A_2 + \alpha_3 A_3 + \alpha_4 A_4 + \alpha_{11} A_1^2 + \alpha_{22} A_2^2 + \alpha_{33} A_3^2 + \alpha_{44} A_4^2 + \alpha_{12} A_1 A_2 + \alpha_{13} A_1 A_3 + \alpha_{14} A_1 A_4 + \alpha_{23} A_2 A_3 + \alpha_{24} A_2 A_4 + \alpha_{34} A_3 A_4$$

Where, Y is the predicted response (hydrogen production rate), α_0 is the offset term, $\alpha_1, \alpha_2, \alpha_3, \alpha_4$ are the linear effects, $\alpha_{11}, \alpha_{22}, \alpha_{33}, \alpha_{44}$ are the squared effects, and $\alpha_{12}, \alpha_{13}, \alpha_{14}, \alpha_{23}, \alpha_{24}, \alpha_{34}$ are the interaction effects.

RESULTS AND DISCUSSION

The design of the experiments minimizes the number of trials required in determining the optimum values of the parameters affecting the process. Table-2 gives the predicted response from the factorial design along with the experimental data. Analysis of variance (ANOVA) of the response surface quadratic model shows high significance ($p > 0.05$). The model predicts the actual response to a satisfactory extent with a correlation coefficient of 0.996. This denoted model was satisfactory. The model for the rate of H_2 production (Y) is given below:

$$\text{Hydrogen Production Rate} = 51.16889 - 6.515A_1 - 1.91833A_2 - 3.0225A_1A_2 - 5.90833A_1^2 - 8.31833A_2^2$$

Table-2. Full Factorial Design Showing Rate of Hydrogen Production

S. No.	Inoculum level (%)		The H_2 production rate in mL/L.h	
	Immobilized <i>H. salinarium</i>	<i>E. coli</i>	Experimental	Predicted
1	10	25	42.14	43.10306
2	10	50	52.32	52.10889
3	10	75	44.23	43.47806
4	20	25	47.12	44.10222
5	20	50	49.23	51.83556
6	20	75	41.52	41.93222
7	30	25	28.23	30.28472
8	30	50	39.14	36.74556
9	30	75	25.23	25.56972

The contours in Fig.-4 show the relative effects of the two variables, the inoculum of immobilized *Halobacterium salinarium* and *E. coli*, on hydrogen production. In this case, the contour had a circular curvature without any saddle point. The central point coordinates in the region of the maximum contour level correspond to the optimum inoculum percentage of the respective components for the maximum hydrogen production rate. The maximum contour level in this fig. is 53.83 mL/L.h.

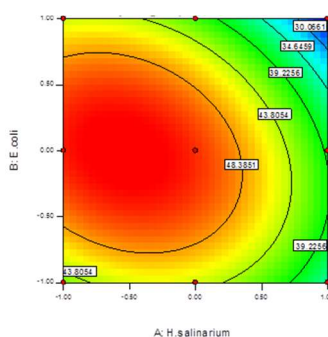


Fig.-4. Effect of the Inoculum (%) of Immobilized *Halobacterium salinarium* and *E. coli* on the Rate of Hydrogen Production. The Maximum Value of the Hydrogen Production Rate is 53.83 mL/L.h.

The contour level of 53.83 mL/L.h is situated close to 50% *E. coli* and 15% *Halobacterium salinarium* in the immobilized form. Experimental investigations gave an idea about the range of these parameters for optimum growth, and these values were used for hydrogen production in a photo-bioreactor. An experiment was conducted at the optimal parameters, and the hydrogen production for 30 min was found to be 53.22 mL/L.h. Table-3 shows the experimental and predicted responses for the Box Behnken design.

ANOVA of the fitting model indicated linear, quadratic, and effect of interactions of the initial pH, temperature, light intensity, and stirrer speed. The model was highly significant with $p < 0.0001$, indicating its effectiveness in describing the effect of the parameters on the rate of hydrogen production.

Table-3: Box-Behnken design for optimizing the Process Variables

S. No	pH (A ₁)	Temp. (°C) (A ₂)	Light Intensity (lux) (A ₃)	Stirrer Speed (rpm) (A ₄)	H ₂ production (mL/L.h)	H ₂ production (mL/L.h)
					Experimental	Predicted
1	7.0	35	13000	140	63.67	59.33
2	7.0	35	11000	120	56.87	56.88
3	6.5	40	11000	120	49.25	45.70
4	7.5	40	11000	120	69.96	67.24
5	7.0	35	9000	100	43.64	42.09
6	7.0	35	13000	100	72.68	70.11
7	7.5	30	11000	120	42.77	40.42
8	7.0	35	9000	140	37.08	33.75
9	6.5	30	11000	120	33.83	30.64
10	7.0	35	11000	120	56.88	56.88
11	7.0	30	9000	120	25.02	27.03
12	7.0	40	13000	120	75.53	74.77
13	7.0	35	11000	120	56.88	56.88
14	7.0	30	13000	120	46.58	46.85
15	7.0	40	9000	120	39.99	40.99
16	7.5	35	11000	100	66.72	66.73
17	7.0	35	11000	120	56.88	56.88
18	7.5	35	11000	140	47.64	48.75
19	6.5	35	11000	100	42.47	42.65
20	6.5	35	11000	140	40.24	41.51
21	7.0	40	11000	140	48.63	51.95
22	7.5	35	9000	120	43.15	43.75
23	7.0	30	11000	140	26.87	28.83
24	7.0	35	11000	120	56.85	56.88
25	7.0	35	11000	120	56.87	56.88
26	6.5	35	13000	120	50.86	54.89
27	7.0	30	11000	100	39.27	40.57
28	6.5	35	9000	120	32.48	33.75
29	7.0	40	11000	100	56.66	59.33
30	7.5	35	13000	120	72.87	76.21

The coefficient of determination of the model for the rate of hydrogen production was determined to be 0.9916. The quadratic model for the response is:

$$\begin{aligned} \text{Hydrogen production rate} = & 56.88 + 7.83 A_1 + 10.47 A_2 + 13.40 A_3 - 4.78 A_4 + 2.94 A_1 A_2 + 2.83 A_1 A_3 \\ & - 4.21 A_1 A_4 + 3.49 A_2 A_3 + 1.09 A_2 A_4 - 0.6125 A_3 A_4 - 3.07 A_1^2 - 7.81 A_2^2 \\ & - 1.66 A_3^2 - 3.90 A_4^2 \end{aligned}$$

The rate of hydrogen production estimated from the equation was 76.092 mL/L.h at the initial pH of 7.38, the temperature of 35.85 °C, the light intensity of 12186 lux, and the stirrer speed of 103.5 rpm. The experimental value at these conditions was determined to be 75.8 mL/L.h. The light intensity needs to be increased for this operation, which could be due to the reduced photon excitation of the purple membrane required for proton transfer.

CONCLUSION

Immobilization of *H. salinarium* in calcium alginate coupled with *E.coli* has the potential for continuous biohydrogen generation. The factorial method determined the optimal proportion of immobilized *H.*

salinarium and *E. coli* for hydrogen production. The Box-Behnken design with the Response Surface Methodology was effective in optimizing the initial pH, temperature, light intensity, and stirrer speed. The optimal temperature was obtained in the mesophilic range (35–40°C), the initial pH was close to 7.4, light intensity was 12186 lux, and stirrer speed was 104 rpm. The quadratic model obtained was verified by the experiments. The results predicted from the design (76.092 mL/L.h) were compared with the experimental values (75.8 mL/L.h) and were determined to be a reasonable fit.

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