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Optimization of Process Variables for Biohydrogen Production from Glucose by *Enterobacter aerogenes*

P. Karthic*, Shiny Joseph and Naveenji Arun

Department of Chemical Engineering, National Institute of Technology Calicut, Kozhikode, India

Abstract

The individual and interactive effects of significant process parameters were investigated for the optimization of biohydrogen production using glucose as a substrate. Response surface methodology was applied to optimize the process parameters for maximum hydrogen production using *Enterobacter aerogenes* MTCC 111. The important factors influencing hydrogen production such as glucose, initial pH, inoculum size, tryptone, yeast extract, and ferric chloride were screened using Plackett-Burman design. Based on the Plackett-Burman design, significantly influencing process variables identified were glucose, initial pH and ferric chloride. 3-dimensional (3-D) response surface and 2-dimensional (2-D) contour analysis were adopted to further investigate the mutual interaction between the parameters and to determine the optimal values for maximum hydrogen yield. The optimal values estimated using the statistical design to achieve maximum H_2 yield of 1.69 mol H_2/mol glucose were glucose 16.56 g/l, initial pH 6.15 and ferric chloride 213.13 mg/l.

Keywords: Biohydrogen; Modified gompertz model; Box-Behnken design; *Enterobacter aerogenes*; Dark fermentation.

Introduction

Biological hydrogen production is an eco-friendly, harmless process carried out under mild operating conditions, using renewable sources as substrates. Fermentative hydrogen production is a very complex process and is influenced by many factors. *Clostridium butyricum* and *Enterobacter aerogenes* have been known to be strong and efficient producers of hydrogen [1]. *Enterobacter aerogenes* is a representative of facultative anaerobes can rapidly consume oxygen and recover the activity of Fe-hydrogenase under anoxic condition in contrast to strict anaerobes which are sensitive to oxygen inhibition. The disadvantage of the dark fermentative process is its lower achievable yield compared to photosynthetic route, appears too low to be economically attractive as an alternative to the existing conventional methods. It was reported that the conversion of pyruvate to solvent and acids were the main reasons for obtaining lower yields than the theoretical value [2]. Microorganisms are capable to change their metabolic pathway according to metabolites (volatile fatty acids) concentration which is greatly influenced by environmental factors such as initial pH, temperature and nutritional requirements [3]. The optimization of nutritional and environmental conditions plays a vital role in developing bioprocesses and improving their performance [4]. It is very tedious and time-consuming to perform the operation using one-factor-at-a- time method [5]. This method may lead to unreliable results and inaccurate conclusions. Moreover, it does not depict the interactive effects among the variables and guarantee the determination of optimal conditions. On the other hand, the statistically based experimental design is a time-saving method, which minimizes the error in determining the interactive effect of process parameters [6]. Statistical optimization design on biohydrogen production has recently been reported in literatures [7-9]. Optimization studies were carried out using *Enterobacter aerogenes* with respect to hydrogen production rate [10-12]. Some studies proved that the process parameters such as pH, temperature and iron concentration had significant influence on biohydrogen production [13,14]. Although many studies have been done on the effect of various environmental factors on hydrogen production, the information on the statistical optimization of factors such as yeast extract, tryptone and ferric chloride using *Enterobacter aerogenes* are still lacking. Therefore, this present study aims to investigate the parameters significance and optimize the nutritional and environmental factors affecting hydrogen production from glucose using *Enterobacter aerogenes*.

Materials and Methods

Micro-organism and pre-cultivation

Facultative anaerobe *Enterobacter aerogenes* MTCC 111 was obtained from Microbial type culture collection, Chandigarh. Pure culture of the cells was maintained on nutrient agar slants at 4°C and sub-cultured once in a month.

Experimental procedure

All batch experiments were conducted in 250 ml conical flask. 1 L of synthetic medium was prepared at various pH values, glucose concentrations (g/l) and iron concentrations (mg/l). The synthetic medium consisted of (in g/l): KH_2PO_4, 0.75; K_2HPO_4, 0.75; $MgSO_4.6H_2O$, 0.8; $MnSO_4.4H_2O$, 0.2; sodium chloride, 0.2; yeast extract, 4; $(NH_4)2SO_4$, 2; L-cysteine hydrochloride monohydrate, 0.5 in the conical flask. The initial pH of the medium was adjusted using 3N HCl and/or 3N NaOH solution. The room temperature (30°C) was maintained throughout the batch experiment. The flasks were then flushed with nitrogen gas before the start up to remove oxygen in the headspace of the flasks to ensure anaerobic condition. These flasks were immediately air-sealed with butyl rubber stopper and covered with aluminium seal cap. The flasks were covered with aluminium foil to prevent the contact of sunlight. These were agitated in an orbital shaker kept at 120 rpm to provide better contact among the components. The evolved gas was collected and determined by the water displacement method in graduated cylinders prefilled with water which was adjusted to pH 3.0 or less in order to prevent dissolution of the gas [15]. Each batch test was conducted in triplicate and their average was taken.

***Corresponding author:** P. Karthic, National Institute of Technology Calicut, Kozhikode - 673601, India, Tel: 0495 2520950; Fax: 0495 2287250; E-mail: apkarthic@nitc.ac.in

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Analytical methods

Hydrogen gas generated during experiments was estimated using a microprocessor based pre-calibrated H_2 sensor (electrochemical sensor, ExTox Gas Detector 4–20 mA version, GmBH Inc., Germany)[16]. The output signal displayed % volume of H_2 in the headspace of flasks, which was further converted to mmol. The sensor has a measuring range of 0–30% H_2 with 5s response time in a temperature range of 20–50°C. The system was calibrated once in a week using calibration cap provided with the instrument. pH values were determined by a pH meter (Systronic Instruments Ltd., India).

Response surface methodology

Plackett–Burman design: A 2^k factorial Plackett–Burman design [17] is used to reduce the number of ingredients and the medium components required for the production of hydrogen and are given based on the first order model:

$$Y = \beta_0 + \sum(\beta_i X_i) \quad (1)$$

where, Y is the response (maximum H_2 yield), β_0 is the model intercept and β_i is the linear coefficient and X_i is the level of the independent variable. Each factor in the design was prepared in two levels: -1 for low level and +1 for high level and screened in twelve experimental designs. Table 1 illustrates the levels of each factor and their statistic analysis in the experimental design. Experiments were conducted based on the Plackett-Burman design and the corresponding H_2 yield was presented in the Table 2.

Box-Behnken design: In order to optimize the critical factors for enhanced hydrogen production, a three-variable Box–Behnken design [18] with three replicates at the centre point was applied. For statistical calculations, the relation between the coded and actual values are used and is given by

$$X_i = (A_i - A_0)/\Delta A \quad (2)$$

Where X_i is a coded value of the variable; A_i the actual value of variable; A_0 the actual value of the A_i at the centre point; and ΔA, the step change of variable. Based on the Box–Behnken design, a minimum of 15 combinations of all the three factors including 3 replicates at the centre point was prepared and the experimental design with respective H_2 yield obtained was represented in Table 3. For predicting the optimal condition, the quadratic polynomial equation was fitted to correlate the relationship between variables and response (i.e., hydrogen yield), and estimated with the following equation:

$$Y = \alpha_0 + \sum_{i=1}^{3} \alpha_i X_i + \sum_{i=1}^{3} \alpha_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{i<j=2}^{3} \alpha_{ij} X_i X_j \quad (3)$$

Where X_i are the input variables, which influence the response variable Y; α_0 is the offset term; α_i is the i^{th} linear coefficient; α_{ij} is the ij^{th} interaction coefficient. The input values of X_1, X_2 and X_3 corresponding to the maximum value of Y were solved by setting the partial derivatives of the functions to zero.

Kinetic analysis

The modified Gompertz equation (Equation (4)) was used to determine the cumulative hydrogen production [19].

$$H = P \exp\left\{-\exp\left[\frac{R_m e}{P}(\lambda - t) + 1\right]\right\} \quad (4)$$

Where, H is the cumulative volume of hydrogen produced (mL), R_m is the maximum hydrogen production rate (mL H_2/lh), λ is the lag-phase time (h), t is the incubation time (h), P is the hydrogen production potential (mL H_2) and e is 2.718. Parameters (P, R_m and λ) were determined by best fitting the hydrogen production data for Equation (4) using the Matlab 7.3.0 (R2006b) version with curve fitting toolbox [20]. H_2 yield could be determined by dividing the cumulative hydrogen produced by the amount of glucose added.

Results and Discussion

Screening of culture parameters

The combined effect of initial pH, glucose, peptone, yeast extract, tryptone and ferric chloride for hydrogen production were investigated using Plackett–Burman design. In the Table 2, the main effect of each variable upon hydrogen yield was estimated as the difference between both averages of measurements made at the high level (+1) and at the low level (-1) of that factor. The positive sign of the effect, E_{xi} of the tested variable implies that the influence of the variable on

Code	Variable	Low level	High level	Effect	t-Values	p-Values
X_1	Glucose (g/l)	10	20	59.33	7.94	0.001
X_2	Initial pH	5	7	24.67	3.33	0.021
X_3	Inoculum (%v/v)	5	15	-4.67	-0.62	0.561
X_4	Tryptone (g/l)	5	7	-0.00	-0.00	1.000
X_5	Yeast Extract (g/l)	2	4	6.67	-0.89	0.413
X_6	Ferric chloride (mg/l)	100	300	19.33	2.59	0.048

Table 1: Levels and statistic analysis of variables for Plackett-Burman Design.

Run	X_1	X_2	X_3	X_4	X_5	X_6	Hydrogen yield (molH_2/mol glucose)
1	1	-1	-1	-1	1	1	1.34
2	1	1	-1	1	1	-1	1.26
3	1	-1	1	-1	-1	-1	1.05
4	-1	-1	-1	-1	-1	-1	0.67
5	-1	-1	1	1	1	-1	0.75
6	1	1	1	-1	1	1	1.59
7	-1	1	1	-1	1	-1	0.79
8	-1	-1	-1	1	1	1	0.84
9	-1	1	1	1	-1	1	0.97
10	1	1	-1	1	-1	-1	1.43
11	-1	1	-1	-1	-1	1	0.97
12	1	-1	1	1	-1	1	1.16

Table 2: Evaluation of variables influencing hydrogen yield using Plackett-Burman design.

Run	Glucose (g/l)		Initial pH		$FeCl_3$ (mg/l)		Hydrogen yield (molH_2/mol glucose)	
	X_1	Code	X_2	Code	X_3	Code	Experimental	Predicted
1	-1	10	-1	5	0	200	0.54	0.51
2	-1	10	1	7	0	200	1.07	1.05
3	0	15	1	7	-1	100	1.08	1.09
4	0	15	-1	5	1	300	0.97	0.96
5	-1	10	0	6	-1	100	0.72	0.74
6	1	20	0	6	-1	100	1.23	1.19
7	0	15	0	6	0	200	1.66	1.69
8	1	20	-1	5	0	200	1.26	1.29
9	0	15	1	7	1	300	168	166
10	-1	10	0	5	1	300	0.79	0.83
11	1	20	0	5	1	300	1.38	1.36
12	1	20	1	7	0	200	1.23	1.26
13	0	15	0	6	0	200	1.68	1.69
14	0	15	-1	5	-1	100	0.93	0.95
15	0	15	0	6	0	200	1.71	1.69

Table 3: The Box-Behnken experimental design with three independent variables.

hydrogen yield is greater at a high level; the negative sign shows that the influence of the variable is greater at a low level. From the multiple linear regression analysis, it was observed that the main effect and the corresponding t-values are negative for the variables X_3 (inoculum size), X_4 (yeast extract) and X_5 (tryptone), whereas positive for X_1 (glucose), X_2 (initial pH) and X_6 (ferric chloride) (Table 1). Variables X_3, X_4 and X_5 had confidence levels below 95% and hence were considered to be insignificant. The rest of variables X_1, X_2, and X_6 having confidence levels above 95% were considered significant and were used in the next optimization using Box-Behnken design. Variables with insignificant effect were not considered for further optimization, but used in all trials at their (-1) level and (+1) level, for the negatively and the positively contributing, respectively.

Regression analysis

An analysis of variance was performed to evaluate the quadratic model (Equation. (5)). By applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to give the hydrogen yield:

$$Y = 1.683 + 0.25X_1 + 0.12X_2 + 0.06X_3 - 0.35X_1^2 - 0.3X_2^2 - 0.3X_3^2 - 0.14X_1X_2 + 0.02X_1X_3 + 0.05X_2X_3 \quad (5)$$

Where Y is the predicted hydrogen yield; X_1, X_2 and X_3 are the coded values of glucose (g/l), initial pH and ferric chloride (mg/l). The regression coefficients were estimated for the model and the corresponding P-values were shown in the Table 4. ANOVA analysis showed that the linear and quadratic effect of glucose, initial pH and ferric chloride, and the interactive effect of glucose and initial pH and initial pH and ferric chloride on hydrogen yield were highly significant ($p < 0.05$). This indicates that these terms had great impact on hydrogen production and yield. However, the interactive effect between ferric chloride and glucose concentrations on hydrogen yield was not significant ($p > 0.05$). The Model F-value of 104.6 implies the model is significant (Table 5). There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Lack-of-fit F-value is another evidence to confirm the model significant. There is only 18.7% chance that a "Lack of Fit F-value" this large could occur due to noise. These investigations confirm that the Equation. (5) correlated reasonably well with the experimental data and demonstrated well the effect of each independent variable on the hydrogen yield.

Factor	Hydrogen yield (Y)	
	Coefficient estimate	Probability (p-Value)
Intercept	1.683	0.000
X_1	0.25	0.000
X_2	0.12	0.000
X_3	0.06	0.008
X_1^2	-0.35	0.000
X_2^2	-0.30	0.000
X_3^2	-0.30	0.000
X_1X_2	-0.14	0.001
X_1X_3	0.02	0.396[a]
X_2X_3	0.05	0.048

[a]not significant at 5% level (p>0.05)

Table 4: Model coefficients estimated by multiple linear regressions.

Source	Degrees of Freedom	Sequential Sum of Square	Mean Square	F value	P value
Regression	9	1.74	0.19	104.06	0.000
Linear	3	0.65	0.22	116.78	0.000
Square	3	0.98	0.33	178.37	0.000
Interaction	3	0.092	0.03	17.04	0.005
Residual Error	5	0.008	0.0016	-	-
Lack of fit	3	0.007	0.0023	4.51	0.187
Pure fit	2	0.001	0.0006		
Total	14	1.74			

Table 5: ANOVA results of the experimental response at different factor levels.

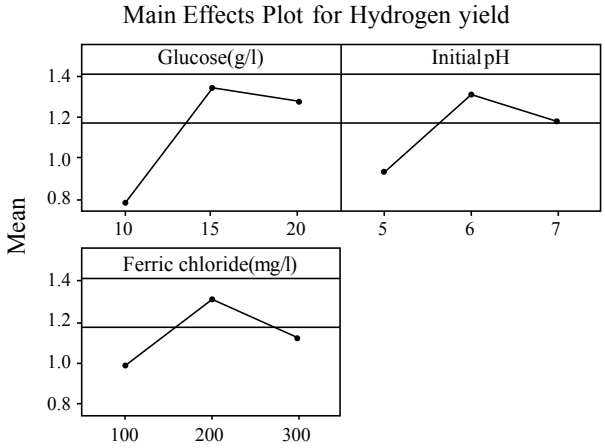

Figure 1: Main effects plot of three experimental factors on H2 yield.

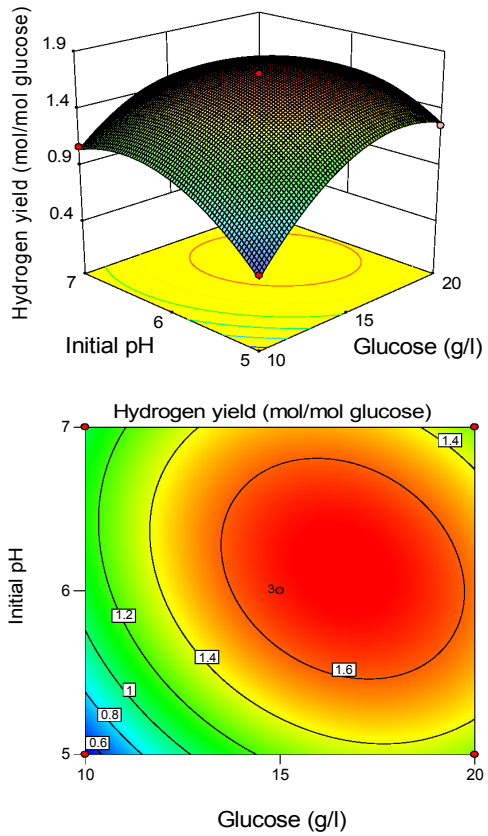

Figure 2: 3-D surface plot and 2-D contour plot on H_2 yield (Initial pH; Glucose).

Effect of independent variables

Figure 1a represented the effect of three factors on the H_2 yield. Increasing H_2 yields were observed with increasing initial pH from 5.0 to 6.15, and then declined with further increase to 7.0. In many studies with pure cultures, increasing H_2 yields have been observed at lower pH values [21,22]. The observations in this study are in agreement with the findings that increasing pH results in a decrease in H_2 yield [20,23-25]. It was evident from the Figure 1b, that increased glucose level from 10 g/l to 16.56 g/l had significant effect on H_2 yield. Further increase from 16.56 g/l led to decrease in H_2 yield. It has already been reported that substrate inhibition gets predominant at higher glucose concentration because this modifies the metabolic pathways [10,26]. An increase in substrate concentration could lead to a partial pressure in the fermentation system. Increased partial pressure level in the headspace of the system will switch the process from acidogenesis to solventogeneis, thus inhibiting the hydrogen production [27]. The generation of hydrogen by fermentative bacteria accompanies the formation of volatile fatty acid as metabolic products. Since alcohol production involves the consumption of hydrogen in the form of reducing equivalents such as NADH, it is inevitable that fermentation conditions that favor the metabolism of sugar to alcohols reduce hydrogen production. Solvent production would cause a drop in the culture pH and subsequent reduction in the hydrogen production [28]. Similarly, in Figure 1c, increasing H_2 yields have been observed at increased $FeCl_3$ concentration from 100 mg/l to 213.13 mg/l, and then declines. These results were in agreement with Yang and Shen [29] and Lee et al. [30]. The addition of external iron concentration promoted the bioactivity of hydrogen producing microbe [31]. Iron is the important micronutrient to form Fe-hydrogenase or other enzymes which almost all biohydrogen production needs fundamentally [32]. Fe-hydrogenase is an iron containing enzyme that catalyzes the reversible oxidation of molecular H_2 from protons and electrons [33]. All these indicate that H_2 yield increases significantly up to the optimal conditions of initial pH, glucose level and iron concentration.

3 D response surface plot and 2 D contour plots were constructed using the Design expert 8.0 and were produced in Figures 2-4. here, each contour plot represents the effect of two independent variables taking the third variable at its centre level. The shape of the contour plot explicitly demonstrates the mutual or combined effect of the independent variables on the response variable. It was obvious from the Figures 2-4, the entire response surface plot had a clear peak and their corresponding contour plots had a clear highest point. This confirms that the maximum hydrogen yield was achieved inside the design boundary. As can be seen from Figure 2, the relative effect between initial pH and glucose concentration (X_1X_2) was highly significant. It means the change in initial pH and glucose level led to the change in hydrogen yield. The inclination angle of the principal axis indicates that the positive effect of increased glucose level on yield was more pronounced as initial pH increased. The 2-D contour plot with respect

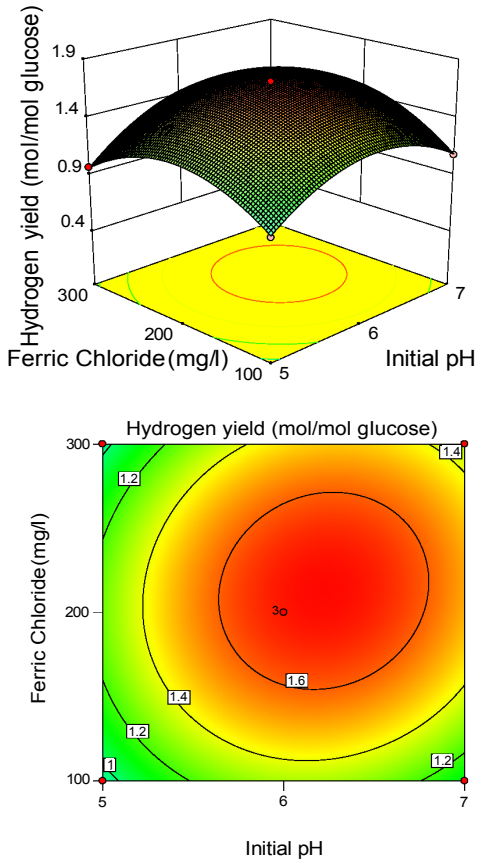

Figure 3: 3-D surface plot and 2-D contour plot on H_2 yield (Ferric chloride; Initial pH).

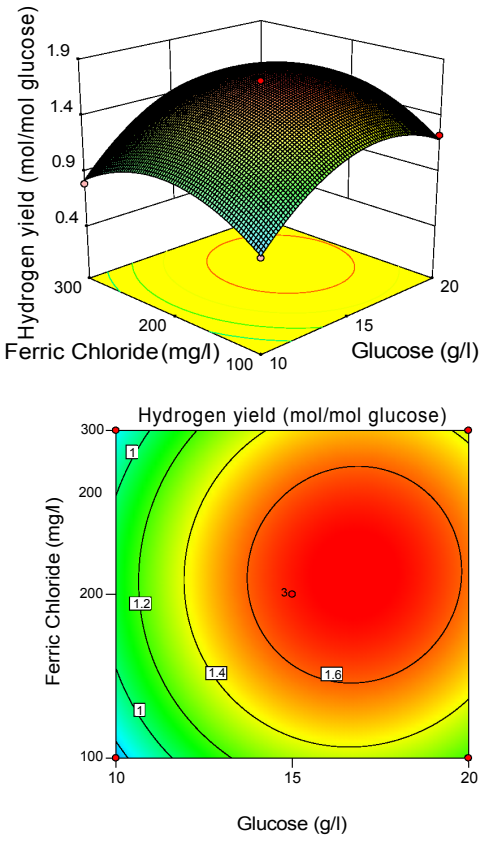

Figure 4: 3-D surface plot and 2-D contour plot on H_2 yield (Glucose; Ferric chloride).

to glucose and initial pH showed a clear elliptical diagonally on plot, suggesting that glucose and initial pH were interdependent.

Figure 3 illustrated the effect of initial pH (X_2) and $FeCl_3$ (X_3) concentration for the hydrogen production with glucose concentration (X_1) kept constant. Hydrogen yield increased with increasing $FeCl_3$ and initial pH to optimum conditions, and then decreased with a further increase. It was obvious that yield of *Enterobacter aerogenes* was sensitive, when $FeCl_3$ concentration was subjected to small alteration above 213.13 mg/l. The model showed that the interactive effect of $FeCl_3$ and initial pH was most significant with p-value<0.05, indicating that this effect has great impact on H_2 yield. Presence of Fe^{3+} in the fermentative medium would facilitate the iron supply for survival of bacteria [30,34-37]. The elliptical nature of the contour plots indicates that the mutual interactions between the two independent variables (X_2, X_3) are significant. These significant interaction effects mean that the effect of initial pH on yield is dependent on the level of Fe^{3+} used. Figure 4 illustrated the effects of glucose level (X_1) and $FeCl_3$ (X_3) level on biohydrogen production with initial pH (X_2) at the centre level. The response H_2 yield showed a peak at 16.56 g/l of glucose and 213.13 mg/l of $FeCl_3$. The angle of inclination of the principle axis was slight in Figure 4 explaining that the hydrogen yield was nearly less dependent than the other two interactive effect (Figure 2 and 3).

Model verification and validation

The optimal factor setting was identified by the D-optimality analysis and their values in the actual were: glucose - 16.56 g/l, initial pH – 6.15 and ferric chloride – 213.13 mg/l respectively. At these optimal conditions, the maximum predicted value of hydrogen yield calculated was 1.74 mol H_2/mol glucose. In order to confirm the predicted results of the model, an experiment in triplicate was carried out at an optimal condition and was found that a maximum hydrogen yield of 1.69 mol H_2/mol glucose was obtained. The experimental response (1.69 mol H_2/mol glucose) was approximately 2.8% lesser than the predicted maximum response. From the Equation (5), computed response evaluated are correlated reasonably well with the experimental values with the coefficient of determination (R^2) 0.9967 (Figure 5).

Cumulative hydrogen production by modified Gompertz model

Cumulative hydrogen produced from glucose was plotted in Figure 6. This curve was fitted by using modified Gompertz equation at an optimal condition obtained by the D-optimality analysis. The values of P, R_m and λ were evaluated by best fitting the cumulative hydrogen production data in the Equation (4) using the Matlab program (Table 6). R^2 value of 0.9876 indicated a strong correlation between the experimental data and the fit. Table 7 illustrated the comparison of hydrogen yield of the present study with the other cited in literatures. The results obtained in this study were higher than that reported by Tanisho et al. [38]. Moreover, the results were in good agreement with Lin et al. [39].

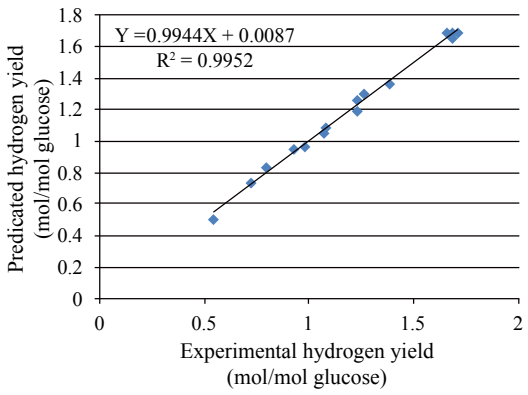

Figure 5: Plot for model predicted and the experimental values for hydrogen yield.

Conclusions

The response surface design was employed for the optimization

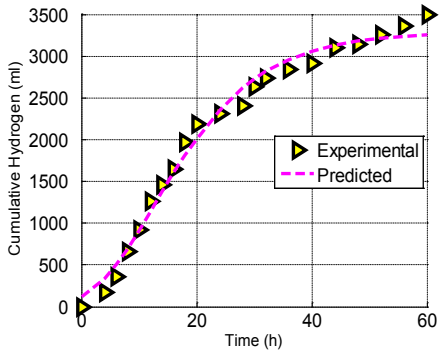

Figure 6: Effect of time on cumulative hydrogen production at the optimal conditions (glucose=16.56g/l; initial pH=6.15 and $Fecl_3$=213.13mg/l).

Substrate concentration (g/l)	Initial pH	$FeCl_3$ (mg/l)	P	R_m	λ	H	R^2
16.56	6.15	213.13	3285.03	116.42	2.19	1.69	0.9876
P - Hydrogen production potential, ml							
R_m - Maximum hydrogen production rate, ml/lh							
λ - Lag phase time, h							
R^2 – Coefficient of determination							
H - hydrogen yield, mol H_2/mol glucose							

Table 6: Modified Gompertz model kinetic parameters for hydrogen production at an optimal condition.

Microorganism	Initial pH	Substrate (g/l)	Iron concentration (mg/l)	H_2 yield (mol H_2/ mol glucose)	References
Citrobacter sp.Y19	7.0	1.0	na	2.49	[27]
C. butyricum EB6	5.6	15.7	390	2.21	[35]
Ethanoligenens Harbinense B49	6.0	14.5	180	2.21	[36]
Enterobacter sp. CN1	6.0	16.15	250	2.0a	[37]
Enterobacter sp. CN1	6.0	16.15	250	0.64	[37]
E. aerogenes	5.8	10.0	na	1.0	[38]
C.butyricum ATCC 19398	7.2	3.0	270	1.8	[39]
C. beijerinckii L9	7.2	3.0	270	2.81	[39]
E.aerogenes MTCC111	6.15	16.5	213	1.69	This study

na not available
[a]mol/ mol xylose

Table 7: Comparison of biohydrogen production obtained in this study with other reports cited in literature.

of hydrogen yield from glucose by *Enterobacter aerogenes*. Plackett–Burman design and Box–Behnken design were applied to screen the significant process variables and to identify the optimal values for the maximum hydrogen production. The R^2 value of 0.9947 confirms the accuracy of model fitness with the experimental data. The linear, quadratic and interactive effects of glucose, initial pH and ferric chloride had been explained significant influence on biohydrogen production. Maximum H_2 yield of 1.69 mol H_2/mol glucose was achieved under the optimal factor setting of three factors using *Enterobacter aerogenes*. Based on the experimental conditions, the response model can accurately predict the H_2 yield and therefore the model is said to be valid over the factor space under consideration. The above results explicitly indicate that the statistical design methodology could be able to offer an efficient and feasible approach for hydrogen production optimization.

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