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## Optimization of key factors for enhanced fermentative biohydrogen production from water hyacinth by RSM

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**This communication discusses the optimization of key factors for the enhanced bio-hydrogen production from water hyacinth. Three critical factors inoculum age (18–24 h), inoculum volume (20–80 ml/l) and concentration of sulphuric acid (0.5–2.0%) were optimized by response surface methodology (RSM) with central composite design (CCD) for better production. RSM analysis showed that all three factors significantly influenced hydrogen production. The optimum hydrogen production was 705 ml/l obtained with 21 h old bacterial culture, 50 ml/l inoculum with 1.25% sulphuric acid pre-treatment. The hydrogen concentration produced by *Clostridium acetobutylicum* NCIM 2877 was enhanced after using RSM. The results obtained indicate that RSM with CCD can be used as a technique to optimize culture conditions for enhancement of hydrogen production by pre-treatment of low-cost organic substrate; water hyacinth using dark fermentation methods may be one of the most promising approaches.**

**Keywords:** Central composite design, *Clostridium acetobutylicum* NCIM 2877, hydrogen production, response surface methodology, water-hyacinth.

BIOHYDROGEN has high energy density (122 kJ/g) and also does not produce any harmful combustion products. These characteristics make it the most promising and advanced biofuel when compared to other biofuels. Processes such as steam reformation and water electrolysis are efficient methods for hydrogen production but require high energy input, whereas biological processes are operated at ambient temperature and mild operational conditions. Certain bacterial species like clostridium and enterobacter are used in the process of dark fermentation for biohydrogen production. However, there are two bottlenecks in the biological hydrogen production process. They are, low hydrogen yield and high-cost substrates. Conventional substrates like glucose, sucrose and starch used for biological hydrogen production are expensive which restricts the application and development of biohydrogen<sup>1</sup>.

Water hyacinth (*Eichhornia crassipes*) is a free-floating aquatic plant. In India it was first observed in West Bengal, in the beginning of 1890, and is now found

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throughout the country. It grows very fast and leads to problems such as navigation and irrigation<sup>2</sup>. Compared to other bio-energy plants annual production of the yield of water hyacinth is 39.5 tonne/ha-year (ref. 3). It mainly consists of cellulose (18–31%), hemicellulose (18–43%), and lignin (7–26%)<sup>4</sup>. It will be a great promotion for the development of clean biological energy and reduction of environment pollution if the biomass from water hyacinth is utilized as a substrate. The above-mentioned components of water hyacinth can be hydrolysed easily into fermentable sugar. In most research studies, conversion of lignocellulosic materials to hydrogen consists of two steps: firstly, hydrolysis of lignocellulosic materials to monosaccharides by acidic, alkaline or enzymatic pre-treatments followed by fermentation process for the production of bio-hydrogen. During the pre-treatment process solid lignin is destroyed and removed, which tightly surrounds the hemicelluloses and cellulose. These are then further hydrolysed into reducing sugars, which can further be utilized by the anaerobic bacteria for the production of bio-hydrogen<sup>5</sup>. Researchers are using various pre-treatment methods such as mechanical, biological, acid or alkali, steam explosion, hot water, supercritical CO<sub>2</sub> treatment, microwave irradiation, for the hydrolysis of complex framework of biomass<sup>6–9</sup>. Alkali treatment is inefficient for hemicelluloses and water hyacinth contains high hemicelluloses. Acid pretreatment is more favourable for the disruption of lignocellulosic material and in degradation of hemicelluloses. Therefore acid pretreatment is considered as one of the suitable and efficient methods for pretreatment of water hyacinth<sup>10,11</sup>.

In this study, effects of three different factors (inoculums age, inoculums volume and concentration of sulphuric acid) and their interactions on H<sub>2</sub> fermentation by *Clostridium acetobutylicum* NCIM 2278 using RSM with CCD, at room temperature and conditions for maximized H<sub>2</sub> production were studied.

Freshwater hyacinth was collected from Kharun River, Raipur, Chhattisgarh (India). It was washed with tap water for the removal of adhering dirt. Then it was chopped into small pieces and air dried. Further, the sample was milled and stored at room temperature. Bacterial culture of *Clostridium acetobutylicum* NCIM 2877 was purchased from NCIM (National collection of Industrial Microorganism) Pune, India. The culture was grown in cooked meat broth medium (beef extract 4.5 g, dextrose 0.2 g, peptone 2.0 g, NaCl 0.5 g pH 7).

The batch experiment of bio-hydrogen production was carried out in a 250 ml conical flask. 10 g of water hyacinth (leaves, stems or roots) pre-treated with concentrated sulphuric acid was inoculated with a specific volume of bacterial broth (ml) with specific age (h). CCD was applied to find out the effects of these variables. A solid residue was separated from the hydrolysate by filtering through a cheese cloth. The pH of acid hydrolysate was adjusted to 6 with 1 N NaOH and 1 N HCl and the

hydrogen production was operated at 37°C for 3 days. The biohydrogen and CO<sub>2</sub> released from the fermentor headspace ran through a liquid filter (20% KOH) where CO<sub>2</sub> was absorbed and collected in a graduated cylinder.

For the assessment of factors affecting hydrogen production from water hyacinth hydrolysate, a factorial central composite experimental design (CCD) and RSM (Response Surface Methodology) using minitab 16 (Minitab, Inc., State College, PA)<sup>12</sup> were conducted to evaluate the key variables in influencing hydrogen production potential from three selected factors, inoculums age ( $X_1$ ), inoculums volume ( $X_2$ ) and concentration of sulphuric acid ( $X_3$ ). RSM is a statistical technique used for multiple regression analysis using the quantitative data obtained from an experimental design for solving multivariate equations. The response surface is a graphical representation of these equations. The range and levels of variables are presented in Table 1. Output dependent variable was hydrogen production. The relation between the coded and real values is described by following equation for statistical analysis

$$x_i = (X_i - X_0)/\Delta X_i, \quad (1)$$

where  $x_i$  is a coded value of the variable  $X_i$  (Table 1),  $X_i$  is the real value of the  $i$ th independent variable,  $X_0$  the real value of  $X_i$  at the center point and  $\Delta X_i$  is the step change of the value. The polynomial quadratic equation was used to correlate each response variable ( $X_1$ ,  $X_2$  and  $X_3$ ) to independent variables. The mathematical equation is

$$Y_i = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2, \quad (2)$$

where  $Y_i$  is the predicted response,  $\beta_0$  the offset term,  $\beta_1$ ,  $\beta_2$  and  $\beta_3$  are linear coefficients,  $\beta_{11}$ ,  $\beta_{22}$  and  $\beta_{33}$  are square coefficients,  $\beta_{12}$ ,  $\beta_{23}$  and  $\beta_{13}$  are interaction coefficients. The model was evaluated using ANOVA with a significance level ( $P \leq 0.05$ ) and statistically fitting ( $R^2$ ).

Inoculums age, inoculums volume and concentration of sulphuric acid were selected to investigate their interactive effects for enhanced bio-hydrogen production, using RSM and CCD (Table 1).

Following second order polynomial equation was established by applying multiple regression analysis to the experimental data

$$\begin{aligned} YH_2 = & 698.638 + 11.063 \times X_1 + 19.021 \times X_2 + 7.742 \\ & + 13.875 \times X_1 \times X_2 + 4.125 \times X_1 \times X_3 \\ & + 1.375 \times X_2 \times X_3 - 89.467 \times X_1^2 \\ & - 65.249 \times X_2^2 - 42.091 \times X_3^2, \end{aligned}$$

**Table 1.** Experimental range and levels of independent variables

Independent variables	Range and level-coded value $x_i$				
	$-x$	$-1$	$0$	$+1$	$+x$
$X_1$ age of inoculums (h)	15.95	18	21	24	26.04
$X_2$ size of inoculums (ml/l)	0.454	20	50	80	100.454
$X_3$ conc. of sulphuric acid (%)	0.011	0.050	1.25	2.0	2.51

**Table 2.** Central composite experimental design with three independent variables

Run	Independent valve						Hydrogen production	
	Coded values			Real values			Observed (ml)	Expected (ml)
	$X_1$	$X_2$	$X_3$	$X_1$	$X_2$	$X_3$		
1	+1	+1	+1	24	80	2.0	555	558.00
2	+1	+1	-1	24	80	0.50	522	531.53
3	+1	-1	-1	24	20	0.50	460	468.48
4	+1	-1	+1	24	20	2.0	493	486.84
5	-1	+1	-1	18	80	0.50	483	489.90
6	-1	+1	+1	18	80	2.0	505	499.89
7	-1	-1	-1	18	20	0.50	482	482.36
8	-1	-1	+1	18	20	2.0	493	489.47
9	+x	0	0	15.95	50	1.25	472	463.23
10	-x	0	0	26.04	50	1.25	422	426.01
11	0	+x	0	21	100.454	1.25	552	545.11
12	0	-x	0	21	0.454	1.25	479	481.13
13	0	0	+x	21	50	2.51	583	591.64
14	0	0	-x	21	50	0.011	579	565.60
15	0	0	0	21	50	1.25	701	698.64
16	0	0	0	21	50	1.25	699	698.64
17	0	0	0	21	50	1.25	695	698.64
18	0	0	0	21	50	1.25	700	698.64
19	0	0	0	21	50	1.25	705	698.64
20	0	0	0	21	50	1.25	701	698.64

where  $Y$  is the predicted yield of hydrogen,  $X_1$ ,  $X_2$  and  $X_3$  are the coded values for inoculums age, inoculums volume and concentration of sulphuric acid respectively. Analysis of variance (ANOVA) was conducted for significance of the fit of the second order polynomial equation for the observed experimental data (Table 2).

To check the significance of each variable,  $P$ -values were used as tools which also indicate the strength of interaction between each independent variable. If the  $P$  value is smaller, the significance of the corresponding variable is higher. The corresponding  $P$  value is  $<0.0001$  for model  $F$  value of 216.28 suggesting that the model is significant (Table 3).

Here the  $R^2$  value is 99.49% suggesting that the model could explain 99.49% of the variability of response which implies that the selected model is reliable for good hydrogen production for the present study. The result also shows that the quadratic model in terms of inoculums age ( $X_1$ ), inoculums volume ( $X_2$ ) and concentration of sulphuric acid ( $X_3$ ) have significant influences on hydrogen production potential ( $P$ -value  $< 0.0001$ ).

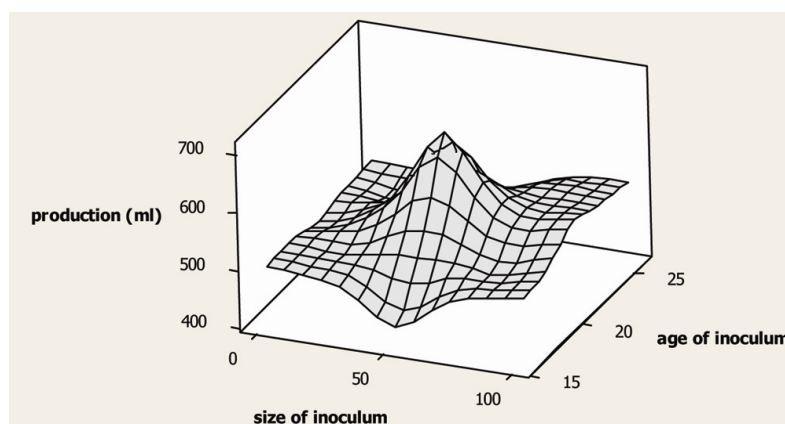
Figures 1–3 show response surface plots which depict the interactions between pairs of variables when the other variable is kept at zero level for hydrogen production.

Figure 1 and Tables 2 and 3 show response surface plot, the interaction effect of inoculums age and volume, on bio-hydrogen production, with inoculums age ranging from 18 to 24 h, volume (20–80) ml/l. The  $P$  values for each factor are less than 0.001, suggesting that these factors are helpful in enhancing the bio-hydrogen production. Interaction of these two factors also affected bio-hydrogen production in a positive way, because the  $p$ -value ( $P < 0.013$ ) was less than 0.05 (Tables 3 and 4).

Figure 1 and Table 3 shows that hydrogen production decreased when inoculums age and volume were at  $-1$  (18 h inoculums age, 20 ml/l inoculums volume) the volume was about 482 ml/l. With higher inoculums age (24 h) and volume (80 ml/l) level  $+1$  the yield increased to 522 ml/l. At the central points (level 0 with 21 h and 50 ml/l), the total gas production was 552 ml/l and 479 ml/l for inoculums volume at level  $-x$  (0.454 h) and  $+x$  (100.454 h) respectively. The production further

**Table 3.** Analysis of variance for hydrogen production

Sources	DF	Seq. SS	Adj. SS	Adj. MS	F	P value
Regression	9	182,317	182,317	20,257	216.28	0.000
Linear	3	7431	7431	2477	26.45	0.000
$X_1$ , age of inoculum	1	1672	1672	1672	17.85	0.002
$X_2$ , size of inoculum	1	4941	4941	4941	52.76	0.000
$X_3$ , Conc. of sulphuric acid	1	819	819	819	8.74	0.014
Square	3	173,195	173,195	57,732	616.38	0.000
$X_1^2$	1	93,381	115,353	115,353	1231.60	0.000
$X_2^2$	1	54,281	61,354	61,354	655.07	0.000
$X_3^2$	1	25,532	25,532	25,532	272.60	0.000
Interaction	3	1691	1691	564	6.02	0.013
$X_1X_2$	1	1540	1540	1540	16.44	0.002
$X_1X_3$	1	136	136	136	1.45	0.255
$X_2X_3$	1	15	15	15	0.16	0.696
Residual error	10	937	937	94		
Lack of fit	5	697	697	139	2.91	0.133
Pure error	5	240	240	48		
Cor total	19	183,254				
$R^2$ 99.49%		$R^2$ (pred.) 96.91%		$R^2$ (adj.) 99.03%		

**Figure 1.** Interactive effects of inoculums age and volume on biohydrogen production from water hyacinth.

increased to 583 ml/l and 579 ml/l with inoculums age and volume at level 0, 21 h and 50 ml/l respectively.

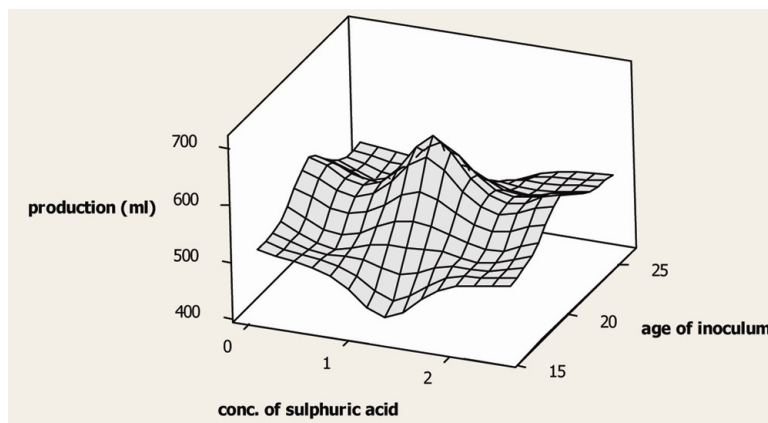
Age of inoculums is directly related to the healthy growth of bacterial culture and also with hydrogen production. Therefore many workers have optimized inoculums age and volume for successful biohydrogen production. Kotay and Das<sup>13</sup> achieved the best production of hydrogen from 14 h old culture with 10% inoculums volume. Japaar *et al.*<sup>14</sup> achieved maximum production of 138.05 ml/g with 24 h bacterial culture. Prakashan *et al.*<sup>15</sup> by comparing all ANOVA data amongst all the selected factors, concluded that inoculums size showed the most significant effect on bio-hydrogen production followed by inoculums age. Inoculums age and volume contributed its regulatory role more than 70%.

The interactive effect of inoculums age and concentration of sulphuric acid are shown in Figure 2 and Tables 3 and 4. The *P*-value for  $X_2X_3$  is 0.255, showing that this

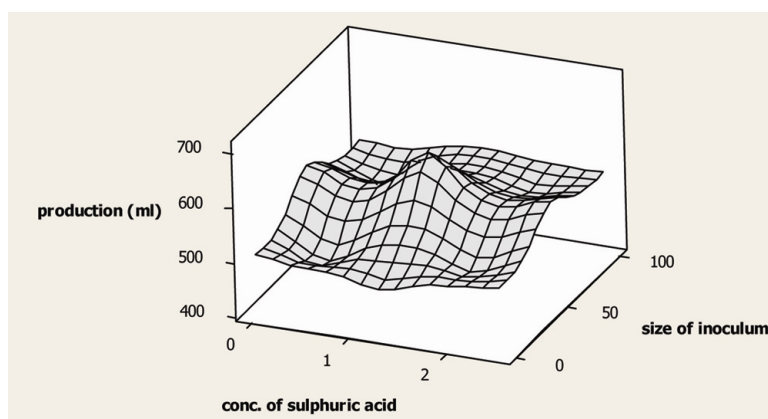
interaction is not so significant for bio-hydrogen production. At level-1 ( $X_2$  is 20 ml/l and  $X_3$  is 0.50%), the production was 482 ml which increased to 505 ml at +1 level. At central point 0 ( $X_2$  is 50 ml/l and  $X_3$  is 1.25%), a sharp decrease in production was observed, i.e. 472 and 422 ml.

Ferchichi *et al.*<sup>16</sup> worked with *Clostridium saccharoperbutylacetonicum* ATCC 27021 and got maximum production of 2.36 mm/h with 10% inoculums volume. Mannikadan *et al.*<sup>17</sup> worked on various parameters affecting biohydrogen production from sugarcane bagasse using *Bacillus* sp. and achieved maximum biohydrogen yield of 0.23 mol H<sub>2</sub>/mol of the substrate at 4% (v/v) inoculums size.

The interactive effect of inoculums volume and concentration of sulphuric acid is shown in Figure 3 and Tables 3 and 4. The *P*-value for  $X_1X_3$  is 0.636, showing that this interaction is not so significant for bio-hydrogen



**Figure 2.** Interactive effects of age of inoculums and concentration of sulphuric acid (pre-treatment) on biohydrogen production from water hyacinth.



**Figure 3.** Interactive effect of inoculums size and concentration of sulphuric acid (pre-treatment) on biohydrogen production from water hyacinth.

**Table 4.** Model coefficient estimated by multiple linear regression

Intercept	Coefficient estimate	P value
$X_1$	411.201	0.02
$X_2$	4.57001	0.00
$X_3$	155.837	0.014
$X_1^2$	-9.94079	0.00
$X_2^2$	-0.0724985	0.00
$X_3^2$	-74.8283	0.00
$X_1X_2$	0.154167	0.002
$X_1X_3$	1.83333	0.256
$X_2X_3$	0.061111	0.696
95%		

production. At level-1 ( $X_1$  is 18 h and  $X_3$  is 0.50%), the production was 482 ml which increased to 493 ml at +1 level ( $X_1$  is 24 h and  $X_3$  is 2.0%), At central point 0 ( $X_2$  is 50 ml/l and  $X_3$  is 1.25%), a sharp increase in production was observed, i.e. 552 ml.

Cheng *et al.*<sup>18</sup> carried out microwave assisted dilute acid pre-treatment of water hyacinth at 1% sulphuric acid and achieved production of 134.9 ml/g total volatile

solids (TVS). Pattra *et al.*<sup>19</sup> optimized various factors affecting acid hydrolysis of water hyacinth using RSM and CCD and achieved maximum hydrogen production of 127.6 mmol/H<sub>2</sub>/l with 1.31% (v/v) concentrated sulphuric acid pre-treatment. Acid pre-treatment methods are the most effective for dissolving cellulose and hemicelluloses of water hyacinth.

Critical factors affecting bio-hydrogen production has been optimized by RSM in many studies. However to the best of our knowledge three selected factors (inoculums age, volume and concentration of sulphuric acid) in the present study has not been studied before by RSM. Age and volume of inoculums are factors linked with the performance of the culture; aged cultures are responsible for poor production of bio-hydrogen. Hydrogen production is basically affected by the yield of reducing sugars from hydrolysis. High hydrogen yield corresponds to an efficient pretreatment method which results in high reducing sugar yields.

The present study focused on optimization of factors for enhancement of bio-hydrogen production using RSM with CCD. The obtained results show that three

independent variables (inoculums age, volume and concentration of sulphuric acid) and their interactions significantly influenced the hydrogen production. Optimum conditions optimized during the study were inoculums age, 21 h, inoculums volume, 50 ml/l and concentrations of sulphuric acid, 1.25%. Maximum bio-hydrogen production obtained with these optimum conditions was 705 ml. Results show that RSM (CCD) was a useful statistical tool for optimization of the key factors influencing the biohydrogen production.

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Author's contribution: Veena Thakur and Mona Tandon were involved in modelling and designing of the experiment. Final manuscript drafting and compiling was done by S. K. Jadhav.

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## Ionospheric precursors observed in TEC due to earthquake of Tamenglong on 3 January 2016

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**Ground-based GPS data show the presence of earthquake precursor in the form of perturbation in TEC of the ionosphere. The analysis of data for Tamenglong Earthquake ( $M = 6.7$ , 3 January 2016) from the stations at Lhasa, China (29.65°N, 91.10°E), Hyderabad, India (17.41°N, 78.55°E), and Patumwan, Thailand (13.73°N, 100.53°E) for the duration of 5-days before and after the main shock of the earthquake show large enhancement and decrease in TEC. The results for Lhasa station which lies in the Earthquake preparation zone showed a decrease in TEC on 29 December (–37%) and 30 December (–9%) which is followed by an enhancement in TEC (47%) on 31 December, i.e. 3 days before the main shock. After the**

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