Optimization of Microbial Hydrogen Production from Maize Stalk Using an Isolated Strain

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ABSTRACT: *Experimental designs were applied for optimizing media and process parameters for hydrogen production from maize stalk hydrolyzate by a newly isolated facultative strain. Plackett-Burman design was used to identify the significant components and using this method the media components - glucose, yeast extract, malt extract, peptone, and NaCl were identified as significant variables influencing the production of hydrogen. The concentrations of these components were optimized using Central Composite Design (CCD) and were found to be: glucose, 19.25g/L; peptone, 5.64g/L; malt extract, 1.64g/L; yeast extract, 3.16g/L and NaCl, 4.312g/L. For further maximizing the production of hydrogen, the process parameters including pH, temperature and fermentation time were optimized by adopting Box-Behnken design. A maximum hydrogen yield of 0.91 mol H_2/mol substrate was achieved under the optimum conditions of pH, 7.0; temperature, 34.5°C and fermentation time, 42.5h.*

KEYWORDS: *Hydrogen production; Facultative strain; Hydrolysate; Maize stalk.*

INTRODUCTION

Hydrogen energy is now considered as the most promising alternative to fossil fuels, as it has fewer side effects and has less environmental damage [1]. It is preferred to biogas or methane because hydrogen is not chemically bound to carbon and combustion provides only water [2]. Besides, hydrogen has a high energy yield of 122 kJ/g which is 2.75 times greater than the hydrocarbon fuel [3,4]. It has been reported that 50 million tons of hydrogen are traded annually worldwide with a growth rate of nearly 10% per year [5].

Currently, 90% of commercially usable hydrogen is obtained by steam reformation of natural gas apart from coal gasification and water electrolysis which are not eco friendly [6,7]. The other methods of hydrogen production are photocatalytic and biological routes. For a sustainable hydrogen economy, hydrogen production should be from renewable source and the production process has to be energy intensive. This is possible only through biological routes of hydrogen production, which can be achieved either via photo fermentation [8,9] or dark fermentation [10]. Though hydrogen production through photo fermentation is high, it is not applicable, as it needs continuous supply of light energy and the difficulties associated with the design of the reactor [11]. Hence production of hydrogen by exploiting renewable biomass via dark fermentation seems to gain more

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1021-9986/2017/3/173-181 *9/$/5.90*

prominence. Utilization of biomass for hydrogen production through biological means will be a dual solution for renewable source and less carbon emission process [12]. The major problem associated with dark fermentative hydrogen production is the low yield and the high production cost involved. A better approach for cost reduction is the utilization of carbohydrate rich waste biomass as substrate [13]. Agricultural residues, which is rich in fermentable lignocellulosic substrate constitutes a promising substrate for the growth of microorganism. Utilization of sugarcane bagasse, rice straw, barley straw, corn stalk, etc has been studied in the recent years [14-17]. Being a complex substrate, the lignocellulosic biomass has to be pretreated prior to fermentation [18].

Like any other bioprocess, hydrogen production efficiency is also influenced by the environmental factors such as pH, temperature and media composition. Maintaining these factors at an optimum level is required for a better hydrogen yield [19]. The classical one-factor at a time approach is time consuming and unable to predict the interactive effect between the variables. Thus a more effective and time saving statistical design of experiment such as Plackett-Burman and Response Surface Methodology (RSM) has been adopted by many researchers for the optimization of media composition, pH and temperature for hydrogen production [20-22]. In this work, we isolated a facultative bacterial strain from the soil sample of maize stalk storage yard, which produces hydrogen. Maize stalk hydrolyzate was used as the sole carbon sources for the production of hydrogen. An attempt was made to improve the hydrogen production by optimization of media components and process parameter using statistical experimental design.

EXPERIMENTAL SECTION

Microorganism and culture conditions

The strain used in this study was isolated from the soil samples of maize stalk storage yard. The media consisting of glucose, 1% (w/v); malt extract 0.1% (w/v); yeast extract, 0.2% (w/v); peptone, 0.5% (w/v); and NaCl, 0.5% (w/v)) at pH 7 and temperature 30^{o}C was used for the growth and agar slants with 1.5% (w/v) of agar was used for the maintenance of the organism. Sub culturing was carried out once in 2 weeks and the culture was stored at 4°C.

Acid hydrolysis

5g of the powdered maize stalk is hydrolyzed with one hundred milliliters of 1% sulfuric acid for 75 minutes at 121°C and 15psi in an autoclave (Hi-tech equipment, India). After hydrolysis, the hydrolysate was filtered through ordinary filter paper followed by filtration through Whattman No.1 filter paper. The filtrate was mixed thoroughly with the media and was neutralized with concentrated NaOH solution to attain a neutral pH. Maize stalk hydrolysate (42.8 % (v/v) equivalent to 1% (w/v) glucose) was used as the carbon source throughout the experiment and wherever a higher concentration was demanded which cannot be met by 100% hydrolysate, additional glucose was added.

Batch experiments

One hundred milliliters of sterile medium with pH 7 was taken in 250ml Erlenmeyer flask. The medium was cooled and inoculated with one day pre grown culture 5% (v/v). The fermentation was allowed to take place in a fermentation jar, which is kept in a constant temperature water bath in order to maintain constant fermentation temperatures. The released gas during the fermentation was collected in a separate jar by water displacement method. The gas sample was taken in a syringe and was loaded into gas chromatography for the qualitative assay of hydrogen. All the experimental runs were carried out in triplicate and the average value was taken.

Analytical methods

The gas produced during the fermentation was collected in a graduated aspirator bottle by water displacement method at regular time intervals. The percentage of hydrogen constituted in the total gas was analyzed by a gas chromatograph (AIMIL- NUCON 5765, Mumbai, India) equipped with a thermal conductivity detector (TCD) and 2.0 m (1/4 in. inside diameter) steel column filled with Porapak Q (50/80 mesh) using nitrogen as carrier gas at a flow rate of 20 mL/min. Injector, oven and column temperature was set at 150°C, 80°C and 200°C respectively.

Experimental design and optimization

Plackett Burman design was used to screen the fifteen media components (Glucose, Peptone, Beef extract, Malt extract, Yeast extract, KCl, NaCl, NH_4Cl, $ZnCl_2$,

Table 1: Twenty run Plackett-Burman design matrix for fifteen variables with the experimental and predicted H_2 yields.

Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	Hydrogen yield (mol H_2/mol glucose)	
																Exp.	Pred.
1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	0.670	0.7229
2	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	0.023	0.0170
3	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	0.262	0.2919
4	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	0.719	0.6671
5	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	0.232	0.2720
6	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	0.673	0.6560
7	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	0.638	0.6550
8	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	0.432	0.4069
9	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	0.673	0.6411
10	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	0.021	0.0150
11	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.564	0.5939
12	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	0.696	0.6512
13	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	0.139	0.1641
14	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	0.720	0.6949
15	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	0.531	0.5629
16	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	0.464	0.4192
17	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	0.022	0.0759
18	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	0.494	0.4641
19	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	0.696	0.7279
20	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	0.464	0.5020

Glucose(X_1); Peptone(X_2); Beef extract(X_3); Malt extract(X_4); Yeast extract(X5); KCl(X6); NaCl(X_7); NH4Cl(X_8); $ZnCl_2$(X_9); KH2PO4(X_{10}); K2HPO4(X_{11}); $MnSO_4.7H_2O$(X_{12}); $MgSO_4.7H_2O$(X_{13}); $ZnSO_4.7H_2O$(X_{14}); and $FeSO_4.7H_2O$(X_{15})

KH_2PO_4, K_2HPO_4, MnSO4.7H_2O, $MgSO_4.7H_2O$, $ZnSO_4.7H_2O$ and $FeSO_4.7H_2O$) in 20 experimental runs. The screened medium components affecting hydrogen production were optimized using Central composite design (CCD) [23,24]. 3^3 factorial Box-Behnken design was adopted to optimize the process parameters pH, temperature and fermentation time. The statistical software package Minitab version 15.0 was used to analyze the experimental data.

RESULT AND DISCUSSION

Identification of important medium constituents using Plackett Burman design

A total of 15 medium components including carbon, nitrogen and mineral sources were screened using Plackett Burman design through 20 experimental runs. The experimental design matrix for screening of important variables for hydrogen production is shown in Table 1. The resulting effects of the variables on the responses, the associated t-value and significant levels are shown in Table 2. A p-value less than 0.05 for the five variables: glucose, peptone, yeast extract, malt extract and NaCl indicate that they were significant. These variables had a confidence level above 95% in comparison to other variables and thus were considered to be significant for hydrogen production. Other than carbon, nutrients like nitrogen, phosphate and iron are reported to have an influencing effect on hydrogen production [25-27]. Maize stalk, which is used as the substrate is also a source of various macro and micro

Table 2 Estimated effects and coefficients of the Plackett–Burman design

Terms	Variable	Low level -1(g/l)	High level +1(g/l)	Effect	t	P
Constant					25.01	0.000
X_1	Glucose	20	40	-0.2627	-7.19	0.002
X_2	Peptone	1	5	0.2067	5.66	0.005
X_3	Beef Extract	0.5	2.5	0.0625	1.71	0.162
X_4	Malt extract	1	3	-0.2257	-6.18	0.003
X_5	Yeast extract	2	10	-0.1131	-3.10	0.036
X_6	KCl	1	9	-0.0381	-1.04	0.356
X_7	NaCl	1	5	0.1535	4.20	0.014
X_8	NH_4Cl	1	9	-0.0195	-0.53	0.622
X_9	$ZnCl_2$	0.5	5	0.0533	1.46	0.218
X_{10}	KH_2PO4	0.05	0.5	0.0469	1.28	0.269
X_{11}	K_2HPO4	.5	2.5	0.0855	2.34	0.079
X_{12}	$MnSO_4.7H_2O$	0.5	5	-0.807	-2.21	0.092
X_{13}	$MgSO_4.7H_2O$	0.1	1	-0.0533	-1.46	0.218
X_{14}	$ZnSO_4.7H_2O$	0.1	5	-0.0013	-0.04	0.973
X_{15}	$FeSO_4.7H_2O$	0.1	1	-0.0881	-2.41	0.073

nutrients (N, P, K, Ca, Mg, Fe etc) [28]. A higher amount of nitrogen source is reported to be essential for biohydrogen production [29], which were satisfied by peptone and yeast extract. Some previous studies have reported an inhibitory effect of NaCl on biohydrogen production [30]. On the contrary, this system showed no such inhibition similar to the work reported by *Bakonyi et al.* [29].

The nutrient components glucose, peptone, malt extract, yeast extract and NaCl were selected for further optimization by RSM. A CCD experimental design with 52 experimental runs was used for studying the interaction of these variables. The range of the significant variables was decided by considering the effect value for each variable in Table 2. With a higher effect, the variable has a greater influence on hydrogen yield at a higher level and with a lower effect, the variable has a greater effect on hydrogen yield at a lower level. The real and coded values of the variables are given in Table 3.

The results obtained from the CCD experiments were fitted to the following second order polynomial equation to represent the hydrogen production adequately.

$$Y= 0.789 - 0.0038X_1 + 0.001X_2 + 0.015X_3 + 0.0038X_4 - 0.009X_5 - 0.027X_1^2 - 0.018X_2^2 - 0.033X_3^2 - 0.019X_4^2 - 0.021X_5^2 - 0.018X_1X_2 + 0.006\ X_1X_3 + 0.012\ X_1X_4 + 0.005\ X_1X_5 + 0.014\ X_2X_3 + 0.014\ X_2X_4 - 0.005\ X_2X_5 - 0.008\ X_3X_4 - 0.006\ X_3X_5 + 0.0068\ X_4X_5 \quad (5)$$

where (Y) is the predicted response, (X_1) concentration of glucose, (X_2) peptone, (X_3) malt extract, (X_4) yeast extract and (X_5) NaCl. The analysis of variance of the quadratic regression model calculated are listed in Table 4, which contain one constant, five linear, five quadratic and ten interaction terms. The significance of each coefficient was determined by p-values and is also listed in Table 4. The analysis of variance of the regression model demonstrates that the model is highly significant. The goodness of the fit of the model was confirmed by the determination coefficient (R^2). The correlation coefficient, R^2, was 0.987, revealing that 98.7% of experimental data of the hydrogen production were compatible with the data predicted by the model. The predicted optimum values obtained for the maximum production of hydrogen as determined from the second degree polynomial equation were: glucose, 19.25g/L; peptone,

Table 3: Coded and uncoded value of medium components used for central composite design.

Independent variables	Symbols	Coded levels				
		-2	-1	0	+1	+2
Glucose, (g/L)	X_1	10	15	20	25	30
Peptone, (g/L)	X_2	1	3	5	7	9
Malt extract, (g/L)	X_3	0.5	1.0	1.5	2.0	2.5
Yeast extract, (g/L)	X_4	1	2	3	4	5
NaCl, (g/L)	X_5	1	3	5	7	9

Table 4: Results of the regression analysis of second order polynomial model for the optimization of hydrogen production obtained in the screening experiment.

Term constant	Regression coefficient	Std. deviation	t-statistics	p-value
Intercept	0.788605	0.002844	277.299	<0.001
X_1	-0.003651	0.001375	-2.655	0.012
X_2	0.001536	0.001375	1.117	0.272
X_3	0.015171	0.001375	11.035	<0.001
X_4	0.003857	0.001375	2.805	0.009
X_5	-0.009275	0.001375	-6.746	<0.001
$X_1 X_1$	-0.027689	0.001182	-23.433	<0.001
$X_2 X_2$	-0.018950	0.001182	-16.038	<0.001
X_3X_3	-0.033250	0.001182	-28.140	<0.001
X_4X_4	-0.019656	0.001182	-16.635	<0.001
X_5X_5	-0.021863	0.001182	-18.503	<0.001
X_1X_2	-0.018875	0.001600	-11.799	<0.001
X_1X_3	0.006250	0.001600	3.907	0.001
X_1X_4	0.012125	0.001600	7.579	<0.001
X_1X_5	0.005250	0.001600	3.282	0.003
X_2X_3	0.014438	0.001600	9.025	<0.001
X_2X_4	0.014938	0.001600	9.337	<0.001
X_2X_5	-0.005437	0.001600	-3.399	0.002
X_3X_4	-0.008313	0.001600	-5.196	<0.001
X_3X_5	-0.006188	0.001600	-3.868	<0.001
X_4X_5	0.006812	0.001600	4.258	<0.001

Table 5: Three level Box-Behnken design matrix for the optimization of significant process parameters.

Run No.	pH	Temperature (°C)	Time (hr)	Hydrogen yield (mol H_2/mol glucose)	
				Exp.	Pred.
1	7.0	34	42	0.889	0.888
2	6.5	34	40	0.700	0.701
3	6.5	34	44	0.685	0.683
4	7.0	34	42	0.886	0.888
5	7.5	34	44	0.812	0.812
6	7.0	36	44	0.747	0.748
7	7.5	36	42	0.785	0.785
8	7.0	36	40	0.715	0.713
9	7.0	34	42	0.888	0.888
10	6.5	34	42	0.680	0.681
11	6.5	32	42	0.660	0.660
12	7.5	34	40	0.764	0.766
13	7.5	32	42	0.752	0.751
14	7.0	32	44	0.698	0.700
15	7.0	32	40	0.707	0.706

5.64g/L; malt extract, 1.64g/L; yeast extract, 3.16g/L and NaCl, 4.312g/L. At the optimum medium concentration maximum hydrogen production of 0.791mol H_2/mol substrate was obtained.

Optimization of process parameter using Box-Behnken design

The Box-Behnken design was applied to optimize the important process parameters, namely, initial pH of the medium, temperature and fermentation time. The design matrix which consists of 15 experimental runs was constructed, in order to arrive at a second order polynomial equation to predict the hydrogen fermentation system shown in Table 5. The polynomial equation derived from the multiple regression analysis of the data is as follows:

$$Y=0.887 + 0.049A + 0.0137B + 0.007C + 3.25AB + 0.015AC + 0.01BC - 0.07A^2 - 0.09B^2 - 0.07C^2 \quad (6)$$

where, Y, predicted hydrogen potential: A, pH; B, time and C, temperature respectively.

The goodness of fit of the model depends on the multiple correlation coefficients, R^2. The R^2 value obtained from the ANOVA was 0.9998, which implies that the variation of 99.98% hydrogen production is attributed to the independent variables. The parameter estimate and the corresponding p-value ($p<0.5$) (Table 6) suggest that all the linear, square and interactive terms of pH, time and temperature have a significant effect on hydrogen production. R^2 value closes to 1 denotes better correlation between the observed and predicted value. Three dimensional surface plots are drawn to determine the optimum values and the interactive effect of the three process parameters. Fig. 1(a) represents the interaction between time and pH. The shape of the response surface curves showed a maximum hydrogen production obtained when both the factors lies between -0.5 and 1 level. Fig. 1(b) represents the interaction between temperature and pH. Maximum hydrogen production was recorded at pH 7 and temperature of 34.5°C. The optimal values of pH and temperature obtained here are in accordance with that obtained for Souparno et al for a defined microbial consortium producing hydrogen from molasses [31]. A low pH is reported to associate with low hydrogen production due to low hydrogenase activity [32]. *Xiao et al.*, has reported the highest hydrogen evolving hydrogenase

Table 6: Results of the ANOVA for the quadratic model.

Source	Sum of Squares	Degrees of Freedom (DF)	Mean Square	F Value	P-value Prob > F
Model	0.12	9	0.013	3331.77	< 0.0001
pH (A)	0.019	1	0.019	4878.74	< 0.0001
Temperature (B)	1.513×10^{-3}	1	1.513×10^{-3}	392.13	< 0.0001
Time (C)	3.920×10^{-4}	1	3.920×10^{-4}	101.63	< 0.0001
AB	4.225×10^{-5}	1	4.225×10^{-5}	10.95	0.0129
AC	9.923×10^{-4}	1	9.923×10^{-4}	257.25	< 0.0001
B C	4.202×10^{-4}	1	4.202×10^{-4}	108.95	< 0.0001
AA	0.022	1	0.022	5678.61	< 0.0001
BB	0.039	1	0.039	9981.91	< 0.0001
CC	0.023	1	0.023	6079.10	< 0.0001
Residual	2.700×10^{-5}	7	3.857×10^{-6}		
Lack of fit	1.900×10^{-5}	3	6.333×10^{-6}	3.17	0.1473
Pure error	8.000×10^{-6}	4	2.000×10^{-6}		
Total	0.12	16			

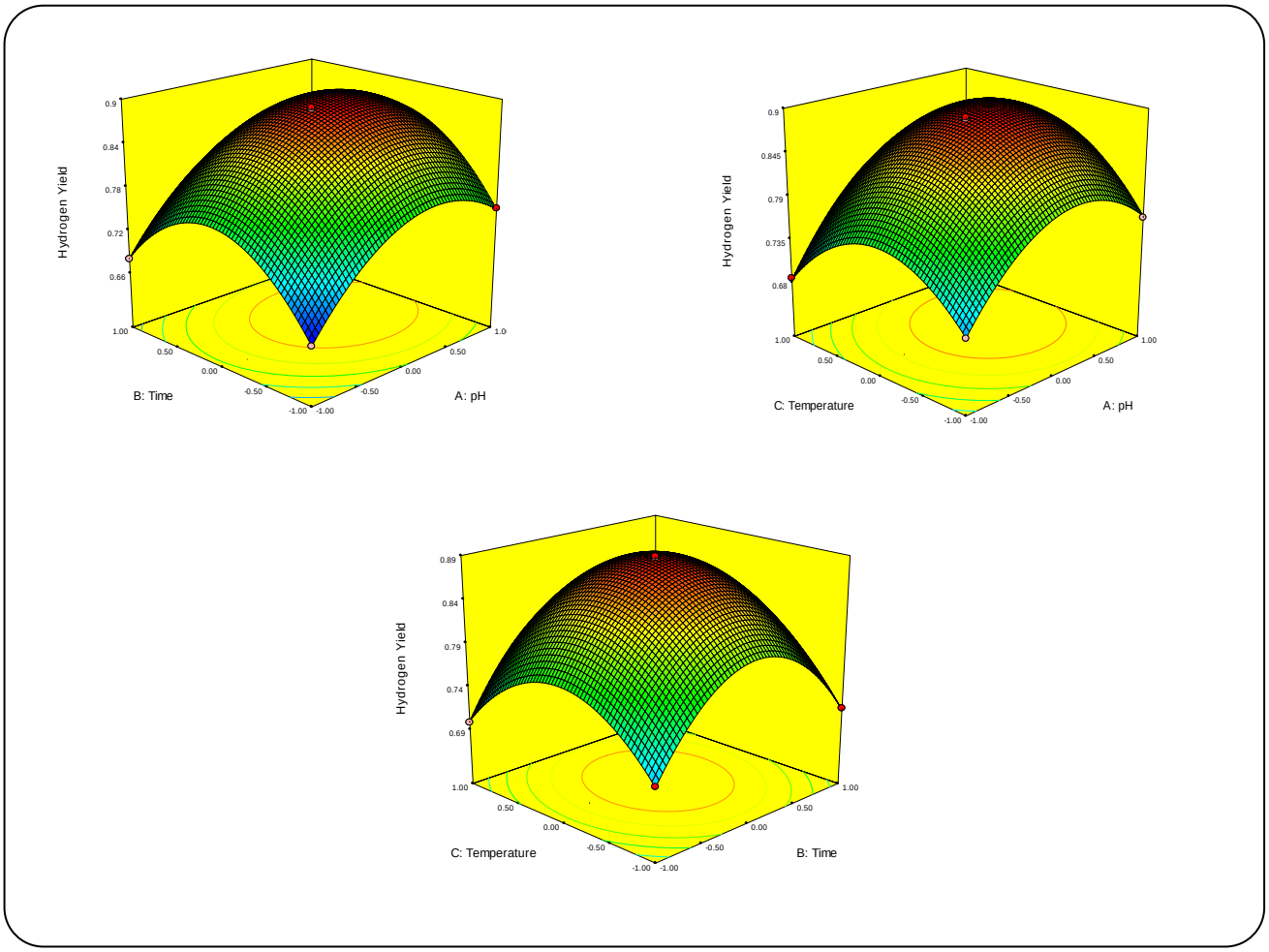

Fig. 1 (a-c) 3D plots showing the interactive effects between the significant process parameters on hydrogen yield in batch fermentation

activity at a temperature of 40°C but, the highest production was observed at a lower temperature due to the activity of uptake hydogenase [33]. Further increase or decrease of pH or temperature negatively affects the yield of hydrogen. Fig. 1(c) represents the interaction between temperature and time and it follows the same trend obtained previously. The experimental results suggest that the maximum values of hydrogen yield (0.89 mol H_2/mol glucose) were obtained for the runs with the central points. The optimum values obtained by solving the second degree polynomial equation are as follows: pH, 7.0; temperature, 34.5°C and fermentation time, 42.5h.

CONCLUSIONS

The present study optimized the media components and process parameters for the production of hydrogen using the newly isolated facultative strain. A suitable maize stalk hydrolysate in the fermentation medium is essential to get higher hydrogen yields; however, an excessively high or low concentration of maize stalk hydrolysate will affects the growth of the organism which will reduce the yield. The results also showed the use of cheaper lignocellulosic substrate for fermentation, thus contributing to the reduction in the cost of production medium. Five variables were identified by Plackett Burman design on significant for hydrogen production. These variables were further optimized using CCD. The optimum medium constituents of the fermentation medium are as follows: glucose, 19.25g/L; yeast extract, 3.046g/L; malt extract, 1.64g/L; peptone, 5.640 and NaCl, 4.312g/L. Box-Behnken design was adopted to identify optimum values of process parameters that bring maximum hydrogen production. The optimized values of process parameters for hydrogen production were as follows: pH, 7.0; temperature, 34.5°C and fermentation time, 42.5h. At these conditions highest hydrogen yield of 0.91 mol H_2/mol glucose was achieved, when maize stalk hydrolysate (equivalent to 1.5% (w/v)) was used as the carbon source. This showed that the microorganism used in this study possessed a high hydrogen producing capacity under optimal conditions.

Received : Mar. 20, 2015 ; Accepted : Mar. 6, 2017

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