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Microbial production of hydrogen from sorghum stalk

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Abstract : An aerobic strain of *α-Proteobacteria*AUChE 103, isolated from sorghum stalk storage yard has been identified as a potential hydrogen producer. In the present study, the media components and process parameters were optimized for enhanced hydrogen production. The significant media components namely glucose, malt extract, yeast extract, peptone andNaCl were determined using Plackett-Burman design. These significant variables were then optimized using central composite design. The optimum conditions were found to be : 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 employed to optimize the process parameters. Under the optimum conditions a maximum hydrogen yield of 0.91 mol H_2/mol glucose was achieved.

1. Introduction

The worldwide energy demand has increased due to rapid growth in population and industrial development. To meet out the energy demand has become difficult due to fast depletion of fossil fuel reserves. Also combustion of these fossil fuel increases the green house gas emission resulting in global warming and pollution. Hydrogen is now considered one of the alternatives to fossil fuels [1]. It is preferred to biogas or methane because hydrogen is not chemically bound to carbon and therefore, combustion does not contribute to green house gases or acid rainproducing 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]. It has been reported that 50 million tons of hydrogen are traded annually worldwide with a growth rate of nearly 10% per year [4]. Currently, 90% of commercially usable hydrogen is obtained by steam reformation of natural gas apart from coal gasification and water electrolysis. But these processes are expensiveenergy intensive, require high operating temperatures and are detrimental to the environment [5, 6].The other methods of hydrogen production are photocatalytic and biological routes. Hence production of hydrogen by exploiting alternative renewable source seems to gain more prominence. Biomass and water can be used as renewable resources for hydrogen gas production where biomass is one of the energy sources [7]. Utilization of biomass for hydrogen production through biological means will be a dual solution for renewable source and less carbon emission process [8]. In the present study, hydrogen production has been attempted from the renewable lignocellulosic biomass, sorghum stalk using a newly isolated strain of *α-Proteobacteria*AUChE 103.

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2. Materials and methods

2.1 Isolation of hydrogen producing strain

The strain used in this study was isolated from the soil samples of maize storage yard. Pure culture was obtained by serial dilution and plating on nutrient agar medium. The following composition: Malt 0.1% (w/v); yeast extract, 0.2% (w/v); peptone, 0.5% (w/v); NaCl, 0.5% (w/v); glucose, 1.5% (w/v) and agar, 1.5% (w/v) at pH 7 and temperature of 34^{o}C was used for the growth and agar slants with 1.5% (w/v) of agar was used for the maintenance of organism.

2.2 Acid hydrolysis

Hydrolysis was carried out by treating 5g of the powdered sorghum stalk with 100ml of 1% H_2SO_4 for 75 minutes at 121℃ and 15psi in an autoclave. After hydrolysis, the hydrolysate was filtered through ordinary filter paper followed by filtration through Whattman No.1 filter paper. The filtrate [63.75 % (v/v) (equivalent to 1.5% (w/v) glucose)] was mixed thoroughly with the above mentioned media composition and was neutralized with concentrated NaOH solution to attain neutral pH.

2.3 Batch studies

The pH of the medium was adjusted to 7.0 and was sterilized in an autoclave (121^{o}C and 14 psi) for 20 minutes. 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 the 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 in to gas chromatography for the qualitative assay of the hydrogen. All the experimental runs were carried out in triplicate and the average value was taken.

2.4 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 determined using a gas chromatograph (AIMIL- NUCON 5765, Mumbai, India) equipped with a thermal conductivity detector 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.

2.5 Media optimization

The variables which significantly affect the hydrogen yield were screened using Plackett-Burman design. Fifteen variables were screened in 20 experimental runs and the insignificant ones were eliminated. The statistical software package Minitab version 15.0 was used to analyze the experimental data. Once the critical factors were identified through screening, the central composite design (CCD) was used to study the effect of screened variables and their interactive effects on hydrogen production. The effect of significant medium components such as glucose, yeast extract, peptone, malt extract, beef extract and sodium chloride were tested for their significance on the production of hydrogen.

3. Result and discussion

3.1 Isolation and identification of hydrogen producing strain

Four morphologically different colonies were picked from the agar plates inoculated with serially diluted soil samples. They were further analyzed for hydrogen production and the strain with the highest production was chosen for further studies. The nucleotide sequence obtained by the sequencing of PCR amplified 16S rDNA gene was compared with the available database in Genebank. Sequence analysis of 16S rDNA gene demonstrates that the bacterial isolate is a new clone of *α-Proteobacteria* and designated as *α-Proteobacteria*AUChE 103.

3.2 Acid hydrolysis

Acid hydrolysis reduces the complexity of the complex substrate and the high yields of sugar after treatment is necessary for hydrogen fermentability [9, 10]. The batch hydrolysis was carried out for sorghum stalk in order to obtain an optimum yield of glucose. Sulphuric acid and hydrochloric acid of different concentrations ranges between 0.5% and 5% (v/v) were used in order to find an optimum acid concentration. All hydrolysis studies were carried out in an autoclave at 121^{o}C and 15psi. Sulphuric acid has resulted the highest glucose yield (0.34 g glucose/g of substrate) where as the hydrochloric acid resulted a slightly decreased yield (0.30 g glucose/g of substrate). For the case of sulphuric acid the yield was increased steeply when the acid concentration was increased from 0.5% (v/v) to 1.0% (v/v). But for the case of hydrochloric acid, the yield was increased continuously up to an acid concentration of 4% (v/v). In both cases the yield was decreased drastically to the minimum at higher levels of acid concentration. The decrease in yield may be due to the decomposition of glucose at higher level of acid concentrations. A higher acid concentration was also reported to increase the formation of acetic acid and furfural, which are known for microbial growth inhibition [11]. Among the two acids sulphuric acid with 1% concentration could be the best choice for the hydrolysis of sorghum stalk and was used throughout the experimentation.

In order to get an optimum time required for the hydrolysis of sugarcane bagasse, the hydrolysis was carried out with 1% (v/v) sulphuric acid with different hydrolysis times varied in the range of 15 to 120 minutes. The maximum glucose yield of 0.39 g/g of sugarcane bagasse was obtained at seventy five minutes hydrolysis time after which there observed a constant decrease in the yield. The decrease in glucose yield at higher exposure times may be due to the decomposition of the converted glucose at prolonged hydrolysis. The optimum hydrolysis conditions of 1.0% (v/v) sulphuric acid and seventy five minutes of hydrolysis time were used for all further experimentation.

3.3 Media optimization

The medium constituents namely, carbon, nitrogen and mineral sources used in the fermentation were screened using a two-level fractional factorial experiment. Fifteen media components were investigated for their influencein the yield of hydrogen and their coded values and the corresponding actual values are given in Table3.1. The design was subjected to factorial analysis. Experimental and the predicted values of hydrogen are presented in Table 3.2.Parity plot between the experimental and the predicted values showed the effects of the variables and their significance on the hydrogen yield (Fig. 1). The P values of the variables are shown in Table 3.3 and those with P values < 0.05 are considered to be significant. The Pareto chart showed that the variables like, glucose, peptone, yeast extract, malt extract and NaCl have significant effect on hydrogen yield and are selected for further optimization to attain a maximum yield (Fig. 2).

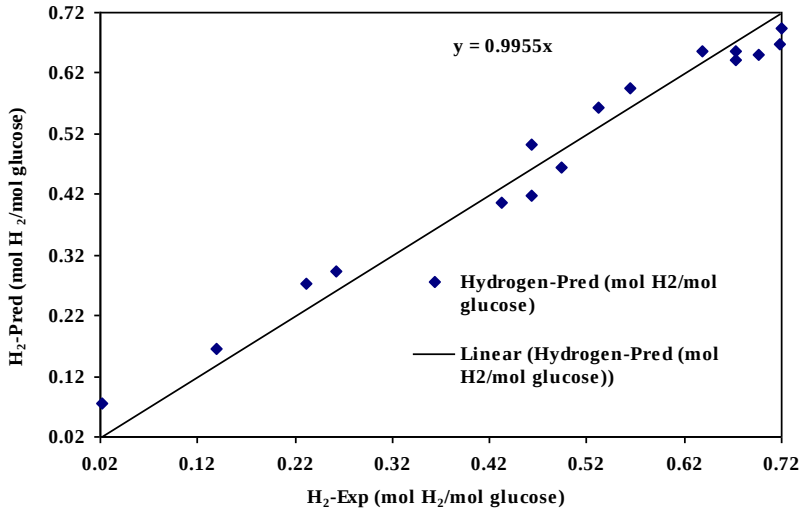


Fig. 1 - Parity plot between the experimental and predicted values of hydrogen yield (*α-Proteobacterium*AUChE 103*)* from Plackett–Burman design

Table 3.1 Variables showing medium components used in Plackett-Burman design for the production of hydrogen using*α-Proteobacterium*AUChE 103

Variables	Low level (–) values (g/L)	High level (+) values (g/L)
Glucose	20	40
Peptone	1	5
Beef Extract	0.5	2.5
Malt extract	1	3
Yeast extract	2	10
KCl	1	9
NaCl	1	5
NH_4Cl	1	9
$ZnCl_2$	0.5	5
KH_2PO4	0.05	0.5
K_2HPO4	.5	2.5
$MnSo_4.7H_20$	0.5	5
$MgSo_4.7H_20$	0.1	1
$ZnSo_4.7H_20$	0.1	5
$FeSo_4.7H_20$	0.1	1

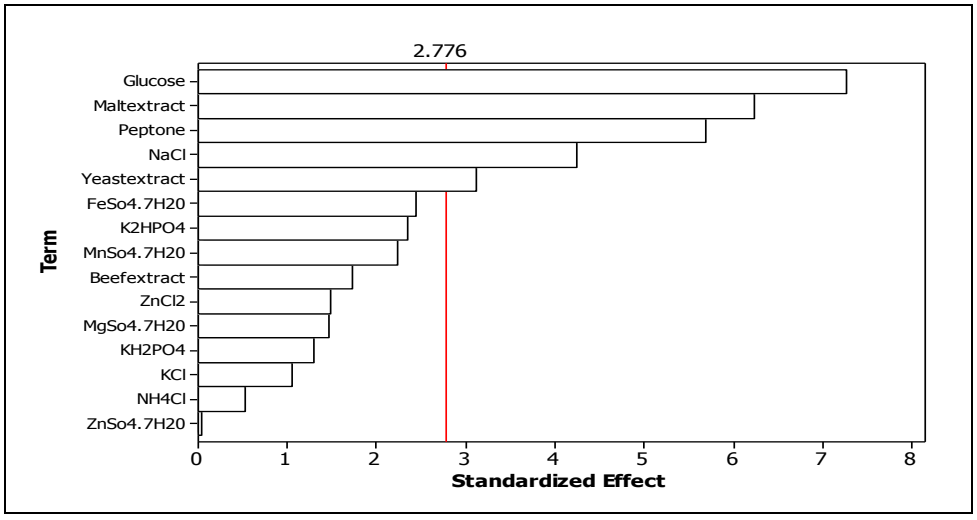


Fig. 2 - Pareto's Chart for (*α-Proteobacterium*AUChE 103) screening of media constituents by Plackett-Burman design

Table 3.2Twenty run Plackett-Burman design matrix (*α-Proteobacterium*AUChE 103*)* for fifteen variables with the experimental and predicted H_2 yields

Runs	A	B	C	D	E	F	G	H	J	K	L	M	N	O	P	Hydrogen yield (mol H_2/mol	
																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(A); Peptone(B); Beef extract(C); Malt extract(D); Yeast extract(E); KCl(F); NaCl(G); NH_4Cl(H); $ZnCl_2$(J); KH_2PO_4(K); K_2HPO_4(L); MnSO4.7H2O(M); $MgSO_4.7H_2O$(N); $ZnSO_4.7H_2O$(O); and $FeSO_4.7H_2O$(P)

Table 3.3 Estimated effects and coefficients of the Plackett–Burman design for α-*Proteobacterium*AUChE 103

Terms	Effect	Coeffi.	SE Coeffi.	T	P
Constant		0.4569	0.01827	25.01	0.000
A.	-0.2627	-0.1313	0.01827	-7.19	0.002
B.	0.2067	0.1033	0.01827	5.66	0.005
C.	0.0625	0.0312	0.01827	1.71	0.162
D.	-0.2257	-0.1129	0.01827	-6.18	0.003
E.	-0.1131	-0.0566	0.01827	-3.10	0.036
F.	-0.0381	-0.0190	0.01827	-1.04	0.356
G.	0.1535	0.0768	0.01827	4.20	0.014
H.	-0.0195	-0.0097	0.01827	-0.53	0.622
J.	0.0533	0.0267	0.01827	1.46	0.218
K.	0.0469	0.0234	0.01827	1.28	0.269
L.	0.0855	0.0428	0.01827	2.34	0.079
M.	-0.807	-0.0403	0.01827	-2.21	0.092
N.	-0.0533	-0.0267	0.01827	-1.46	0.218
O.	-0.0013	-0.0007	0.01827	-0.04	0.973
P.	-0.0881	-0.0440	0.01827	-2.41	0.073

Following screening, response surface methodology using CCD was applied to determine the optimal levels of the significant variables. The coded and the actual values of the five significant medium components used in the design are presented in Table 3.4.The data obtained from the five level central composite design matrix were used to develop models in which the dependent variable Y (hydrogen yield, mol H_2/mol glucose) was obtained as the sum of the contributions of the independent variables through second order polynomial equation and interaction terms. The hydrogen yield obtained from experiments and from the model predictions are given in Table 3.5. The correlation coefficient, R^2, between the experimental and predicted data was 0.987, revealing that 98.7% of experimental data of the hydrogen production was compatible with the data predicted by the model. The parity plot shows a satisfactory correlation between the experimental and predicted values of the hydrogen production (Fig.3). The experimental results suggest that the minimum and maximum values of hydrogen yield obtained were0.551mol H_2/mol glucose and 0.791mol H_2/mol glucose for Run No.50 and Run No.34 respectively.

Regression analysis of the second order polynomial model for hydrogen production isgiven in Table 3.6. ANOVA indicated that, the linear and quadratic effects of glucose, peptone, malt extract and NaCl, and interaction effects of glucose-peptone, glucose-malt extract, peptone-sodium chloride and yeast extract-sodium chloride concentration were significant. The production of hydrogen could be predicted by the model:

$$Y= 0.789 - 0.0038X_1 + 0.001X_2 + 0.015X_3 + 0.0038X_4 - 0.009X_5 – 0.027X_1^2 – 0.018X_2^2$$

$$– 0.033X_3^2 – 0.019X_4^2 – 0.021X_5^2 – 0.018X_1X_2 + 0.006\ X_1X_3 + 0.012\ X_1X_4 + 0.005X_1X_5 + 0.014\ X_2X_3 + 0.014\ X_2X_4 – 0.005\ X_2X_5 –0.008X_3X_4 – 0.006\ X_3X_5 + 0.0068X_4X_5 \ldots\ldots\ldots \quad (3.1)$$

where X_1, the glucose concentration (g/L); X_2, peptone (g/L); X_3, malt extract concentration (g/L); X_4, yeast extract concentration (g/L) and X_5, the sodium chloride concentration (g/L).

Hydrogen yield for different levels of variables was predicted from the respective contour plots (Fig. 4 (a-j)). Each contour plot represents an infinite number of combinations of the two test variables with the other three maintained at their respective zero levels. There was a relative significant interaction between every two variables and the maximum predicted yield was indicated by the surface confined in the smallest ellipse in the contour plot.

The optimum values obtained by solving the second degree polynomial equation for α-*Proteobacterium*AUChE 103 are glucose, 19.25g/L; peptone, 5.64g/L; malt extract, 1.64g/L; yeast extract, 3.16g/L and NaCl,4.312g/L.

Table 3.4Codes and actual levels of the independent variables for design of experiment for α-*Proteobacterium*AUChE 103 H_2 fermentation

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 3.5Five level factorial central composite design with experimental and predicted values of hydrogen yield (mol H_2/mol glucose) for α-*Proteobacterium*AUChE 103 H_2 fermentation

Run No.	**Coded values**						**Hydrogen yield (mol H_2/mol glucose)**	
	X_1	**X_2**	**X_3**	**X_4**	**X_5**		**Exp.**	**Pred.**
1	1.00	1.00	-1.00	-1.00	1.00		0.571	0.566
2	1.00	1.00	1.00	-1.00	1.00		0.643	0.637
3	0.00	0.00	0.00	2.38	0.00		0.696	0.686
4	0.00	0.00	0.00	0.00	0.00		0.790	0.789
5	1.00	1.00	-1.00	-1.00	-1.00		0.584	0.581
6	1.00	-1.00	1.00	1.00	1.00		0.668	0.683
7	-1.00	1.00	1.00	-1.00	1.00		0.678	0.683
8	0.00	0.00	0.00	0.00	0.00		0.790	0.789
9	-1.00	-1.00	1.00	1.00	-1.00		0.629	0.622
10	-1.00	1.00	1.00	1.00	1.00		0.702	0.694
11	-1.00	-1.00	-1.00	-1.00	-1.00		0.673	0.681
12	0.00	0.00	0.00	0.00	0.00		0.789	0.789
13	-1.00	-1.00	1.00	-1.00	-1.00		0.705	0.699
14	-1.00	1.00	1.00	1.00	-1.00		0.728	0.732
15	0.00	2.38	0.00	0.00	0.00		0.694	0.685
16	1.00	-1.00	1.00	-1.00	-1.00		0.701	0.707
17	0.00	0.00	0.00	-2.38	0.00		0.672	0.668
18	-1.00	-1.00	1.00	1.00	1.00		0.598	0.605
19	1.00	-1.00	-1.00	-1.00	1.00		0.662	0.665
20	1.00	-1.00	-1.00	1.00	-1.00		0.672	0.669
21	0.00	0.00	0.00	0.00	-2.38		0.695	0.687
22	-1.00	1.00	1.00	-1.00	-1.00		0.740	0.749
23	2.38	0.00	0.00	0.00	0.00		0.633	0.623
24	1.00	-1.00	-1.00	-1.00	-1.00		0.652	0.664
25	-1.00	-1.00	1.00	-1.00	1.00		0.643	0.654
26	1.00	-1.00	1.00	1.00	-1.00		0.680	0.679
27	1.00	-1.00	1.00	-1.00	1.00		0.691	0.684

Run No.	Coded values						Hydrogen yield (mol H_2/mol glucose)	
	X_1	X_2	X_3	X_4	X_5		Exp.	Pred.
28	-2.38	0.00	0.00	0.00	0.00		0.644	0.640
29	1.00	1.00	1.00	-1.00	-1.00		0.678	0.682
30	0.00	0.00	0.00	0.00	0.00		0.790	0.789
31	1.00	-1.00	-1.00	1.00	1.00		0.701	0.698
32	-1.00	1.00	-1.00	1.00	-1.00		0.680	0.690
33	-1.00	-1.00	-1.00	1.00	1.00		0.643	0.645
34	0.00	0.00	0.00	0.00	0.00		0.791	0.789
35	1.00	1.00	1.00	1.00	-1.00		0.701	0.714
36	1.00	1.00	-1.00	1.00	1.00		0.645	0.653
37	0.00	0.00	0.00	0.00	0.00		0.790	0.789
38	1.00	1.00	-1.00	1.00	-1.00		0.648	0.646
39	0.00	0.00	0.00	0.00	0.00		0.788	0.789
40	-1.00	-1.00	-1.00	-1.00	1.00		0.674	0.661
41	0.00	0.00	2.38	0.00	0.00		0.643	0.636
42	-1.00	-1.00	-1.00	1.00	-1.00		0.626	0.637
43	-1.00	1.00	-1.00	-1.00	1.00		0.623	0.632
44	0.00	0.00	0.00	0.00	0.00		0.789	0.789
45	0.00	0.00	0.00	0.00	0.00		0.789	0.789
46	1.00	1.00	1.00	1.00	1.00		0.695	0.696
47	0.00	-2.38	0.00	0.00	0.00		0.682	0.678
48	-1.00	1.00	-1.00	-1.00	-1.00		0.685	0.674
49	0.00	0.00	0.00	0.00	2.38		0.648	0.643
50	0.00	0.00	-2.38	0.00	0.00		0.551	0.554
51	-1.00	1.00	-1.00	1.00	1.00		0.676	0.676
52	0.00	0.00	0.00	0.00	0.00		0.790	0.789

Table 3.6Results of the regression analysis of second order polynomial model for theoptimization of hydrogen production using α-*Proteobacterium*AUChE 103

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_1X_1	-0.027689	0.001182	-23.433	<0.001
X_2X_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 3.7Analysis of the variance (ANOVA) for the fitted quadratic polynomial model for the production of hydrogen using α-*Proteobacterium*AUChE 103

Sources of variation	**Sum of squares**	**Degrees of freedom (DF)**	**Mean square (MS)**	**F- value**	**P-value**
Regression	0.191106	20	0.191106	116.68	<0.001
Linear	0.015025	5	0.015025	36.69	<0.001
Square	0.138167	5	0.138167	337.42	<0.001
Interaction	0.037915	10	0.037915	46.30	<0.001
Residual Error	0.002539	31	0.002539		-
Lack-of-Fit	0.002534	22	0.002534	230.39	<0.001
Pure Error	0.000004	9	0.000004	-	-
Total	0.193645	51	-	-	-

Table 3.8Optimum values of the variables obtained from regression equation

Independent variables	**Optimum value (coded)**	**Optimum value (real) (g/L)**
Glucose	-0.1683	19.25
Yeast extract	0.0240	3.160
Malt extract	0.3125	1.640
Peptone	0.3125	5.640
NaCl	-0.3125	4.312

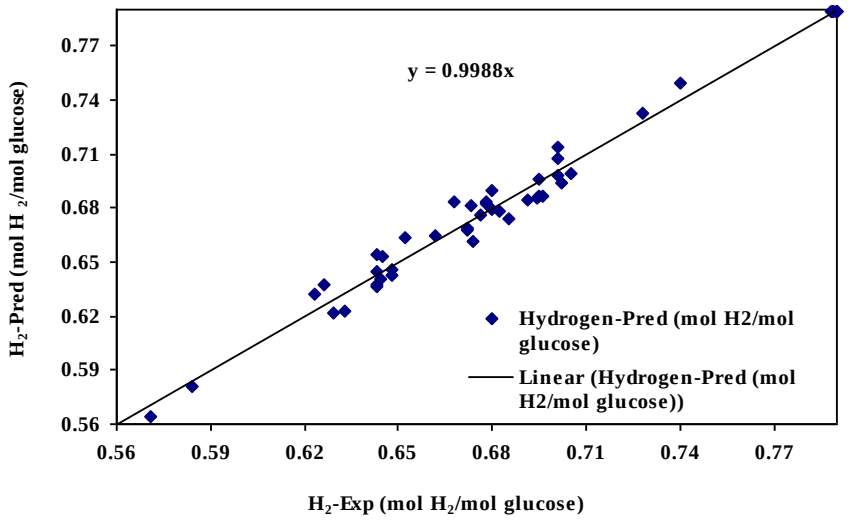
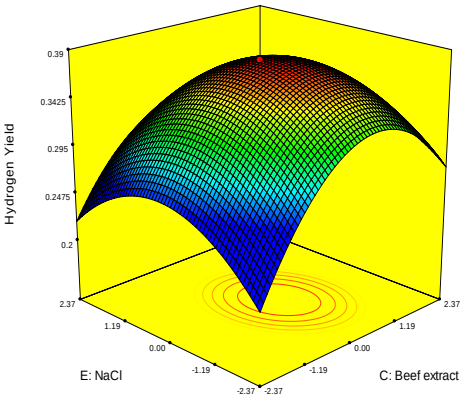
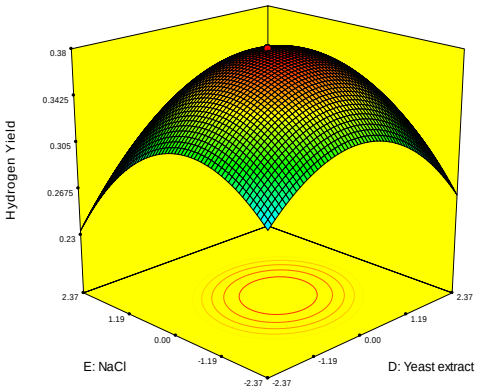


Fig.3 - Parity plot showing the distribution of experimental versus predicted values of hydrogen yield (α-*Proteobacterium*AUChE 103) by RSM

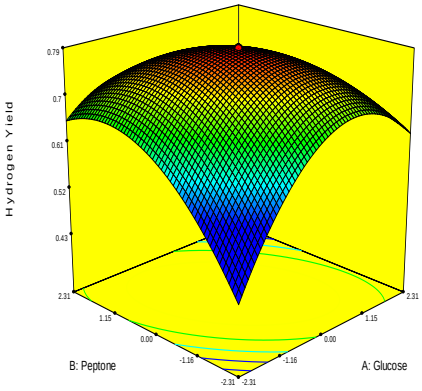
(a)


Hydrogen Yield
0.39
0.3425
0.295
0.2475
0.2
E: NaCl
C: Beef extract
2.37
1.19
0.00
-1.19
-2.37

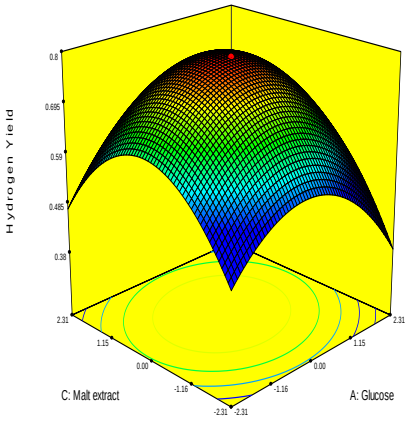
(b)


Hydrogen Yield
0.38
0.3425
0.305
0.2675
0.23
E: NaCl
D: Yeast extract
2.37
1.19
0.00
-1.19
-2.37

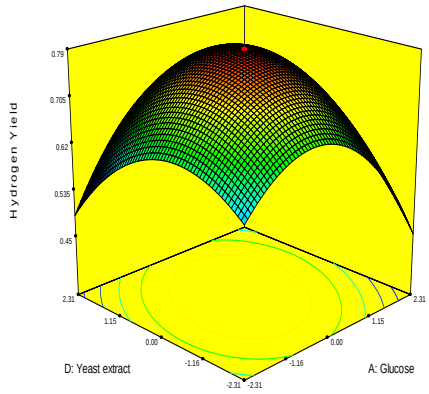
(c)


Hydrogen Yield
0.79
0.7
0.61
0.52
0.43
B: Peptone
A: Glucose
2.31
1.15
0.00
-1.16
-2.31

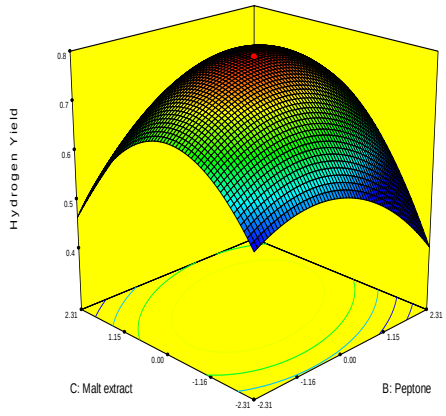
(d)


Hydrogen Yield
0.8
0.695
0.59
0.485
0.38
C: Malt extract
A: Glucose
2.31
1.15
0.00
-1.16
-2.31

(e)


Hydrogen Yield
0.79
0.705
0.62
0.535
0.45
D: Yeast extract
A: Glucose
2.31
1.15
0.00
-1.16
-2.31

(f)


Hydrogen Yield
0.8
0.7
0.6
0.5
0.4
C: Malt extract
B: Peptone
2.31
1.15
0.00
-1.16
-2.31

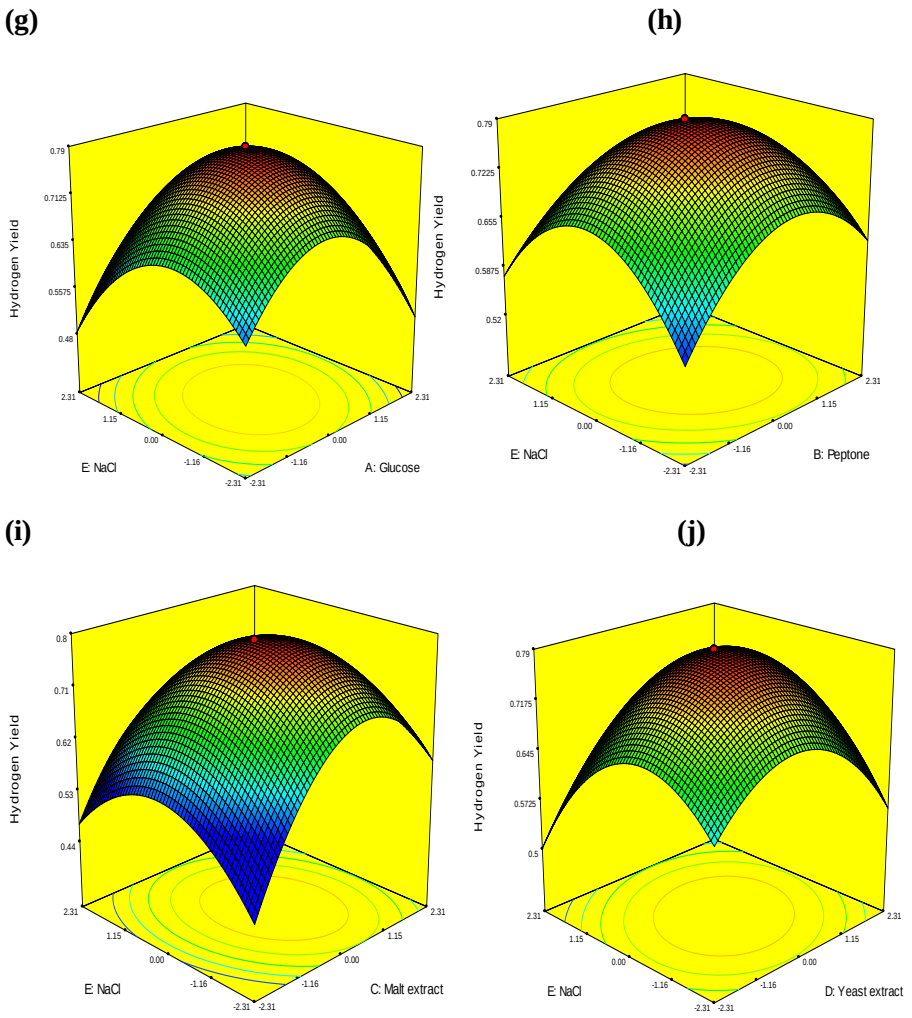


Fig. 4 (a-j) - 3D plots showing the interactive effects between significant variables on hydrogen yield in α-*Proteobacterium*AUChE 103 batch fermentation

3.4 Process parameter optimization using Box-Behnken design

The Box-Behnken design was used to optimize the important process parameters namely, initial pH of the medium, temperature and time of fermentation.The coded and the actual values of the parameters used in the design are presented in Table 3.9. 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. The design matrix and their corresponding experimental and the predicted values are given in Table 3.10. The highest hydrogen yield of 0.89 mol H_2/mol glucose was obtained for the experimental runs of 1, 4 and 9. The results were analyzed using the analysis of variance (ANOVA) and the estimated coefficients are presented in Table 3.11. The hydrogen yield using α-*Proteobacterium*AUChE 103can be expressed in terms of the following regression equation;

$$Y=0.887 + 0.049A + 0.0137B + 0.007C + 3.25AB + 0.015AC + 0.01BC - 0.07A^2 - 0.09B^2 - 0.07C^2 \quad \ldots\ldots\ldots (3.2)$$

where, A, pH; B, time and C, the temperature.

The multiple correlation coefficient, R^2, obtained from the ANOVA was 0.9998, which indicate that the model is capable of explaining 99.98% of the variation in response. This is supported by the parity plot between the experimental and predicted hydrogen yield (Fig. 5).Three dimensional surface plots are drawn to determine the optimum values and the interactive effect of the three process parameters (Fig. 6 (a-c)). 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 obatained by solving the second degree polynomial equation are as follows: pH, 7.0; temperature, 34.5°C and fermentation time, 42.5h.

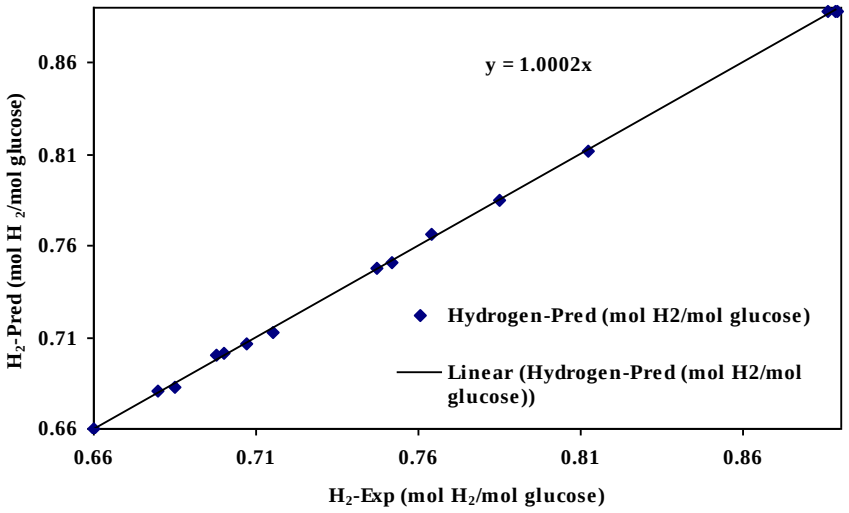


Fig. 5 - Parity plot between the experimental and predicted values of hydrogen yield (α-*Proteobacterium*AUChE 103) by Box-Behnken design

Table 3.9The coded and actual values of the variables used in the design

Parameters	**Coded values**		
	-1	**0**	**1**
pH (A)	6.5	7.0	7.5
Time (h) (B)	40	42	44
Temperature (°C) (C)	32	34	36

Table 3.10 Three level Box-Behnken design matrix for the optimization of significant process parameters in α-*Proteobacterium* AUChE 103 H_2 fermentation

Run No.	**pH**	**Temperature**	**Time**	**Hydrogen yield (mol H_2/mol glucose)**	
				Exp.	**Pred.**
1	0.00	0.00	0.00	0.889	0.888
2	-1.00	0.00	-1.00	0.700	0.701
3	-1.00	0.00	1.00	0.685	0.683
4	0.00	0.00	0.00	0.886	0.888
5	1.00	0.00	1.00	0.812	0.812
6	0.00	1.00	1.00	0.747	0.748
7	1.00	1.00	0.00	0.785	0.785
8	0.00	1.00	-1.00	0.715	0.713

9	0.00	0.00	0.00	0.888	0.888
10	-1.00	1.00	0.00	0.680	0.681
11	-1.00	-1.00	0.00	0.660	0.660
12	1.00	0.00	-1.00	0.764	0.766
13	1.00	-1.00	0.00	0.752	0.751
14	0.00	-1.00	1.00	0.698	0.700
15	0.00	-1.00	-1.00	0.707	0.706

Table 3.11 Results of the ANOVA of the process parameter optimization data in α-*Proteobacterium*AUChE 103 H_2 fermentation using Box-Behnken design of experiments

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	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	4.202×10^{-4}	1	4.202×10^{-4}	108.95	< 0.0001
A^2	0.022	1	0.022	5678.61	< 0.0001
B^2	0.039	1	0.039	9981.91	< 0.0001
C^2	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			

Table 3.12 Estimated coefficient values for α-*Proteobacterium*AUChE 103 H_2 fermentation

Factor	**Coefficient Estimate**	**Degrees of freedom (DF)**	**Standard error**	**95% CI Low**	**95% CI High**	**VIF**
Intercept	0.89	1	8.783×10^{-4}	0.88	0.89	
pH (A)	0.049	1	6.944×10^{-4}	0.047	0.050	1.00
Temperature (B)	0.014	1	6.944×10^{-4}	0.012	0.015	1.00
Time (C)	7.000×10^{-3}	1	6.944×10^{-4}	5.358×10^{-3}	8.642×10^{-3}	1.00
AB	3.250×10^{-3}	1	9.820×10^{-4}	9.280×10^{-4}	5.572×10^{-3}	1.00
AC	0.016	1	9.820×10^{-4}	0.013	0.018	1.00
BC	0.010	1	9.820×10^{-4}	7.928×10^{-3}	0.013	1.00
A^2	-0.072	1	9.571×10^{-4}	-0.074	-0.070	1.01
B^2	-0.096	1	9.571×10^{-4}	-0.098	-0.093	1.01
C^2	-0.075	1	9.571×10^{-4}	-0.077	-0.072	1.01

(a) **(b)**

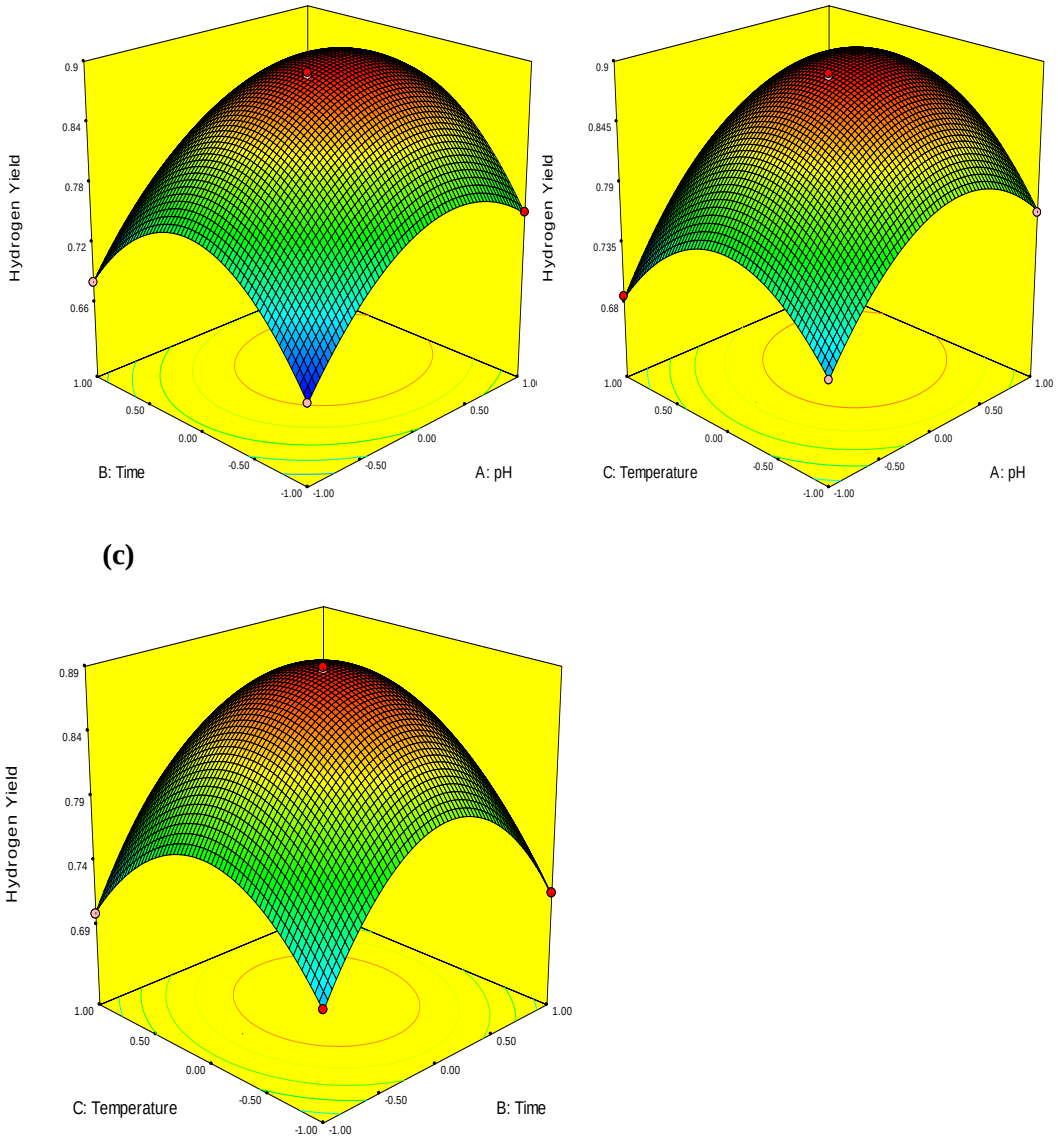


Fig. 6 (a-c)- 3D plots showing the interactive effects between the significant process parameters on hydrogen yield in α-*Proteobacterium*AUChE 103 batch fermentation

3.5 Effect of different substrates

Different substrates such as, glucose, sucrose, sugarcane bagasse hydrolysate, sorghum stalk hydrolysate and cellulose were tested for α-*Proteobacterium*AUChE 103 for its ability to utilize variety of sugars and its hydrogen synthesizing ability. The cheaper lignocellulosic substrate, sorghum stalk hydrolysate has produced a better hydrogen yield (0.91 mol H_2/mol glucose) compared to all synthetic carbon and the other lignocellulosic substrate (sugarcane bagasse hydrolysate) (Fig. 7). In order to understand the kinetics of α-*Proteobacterium*AUChE 103 hydrogen fermentation under different initial substrate concentration, the experiments were performed at different initial hydrolysate concentrations ranging between 0.5% and 3% (w/v) (glucose equivalent). High initial substrate concentration may play an important role in hydrogen production rates and yields [12-15]. However, fermenting microorganisms can also have limited tolerance to increased substrate loading [16-17]. The rate of total gas collection was almost constant throughout the fermentation periods. The percentage hydrogen content in the gas mixture almost doubles during the period of 30 hours to 36 hours. The rate of hydrogen production increases linearly up to 30 hours of fermentation, after that, almost it doubles up (Fig. 8). The maximum volumetric hydrogen productivity of 8.10 ml/Lh was obtained at a fermentation period of 42 hours with 1.5% (glucose equivalent) initial substrate concentration.

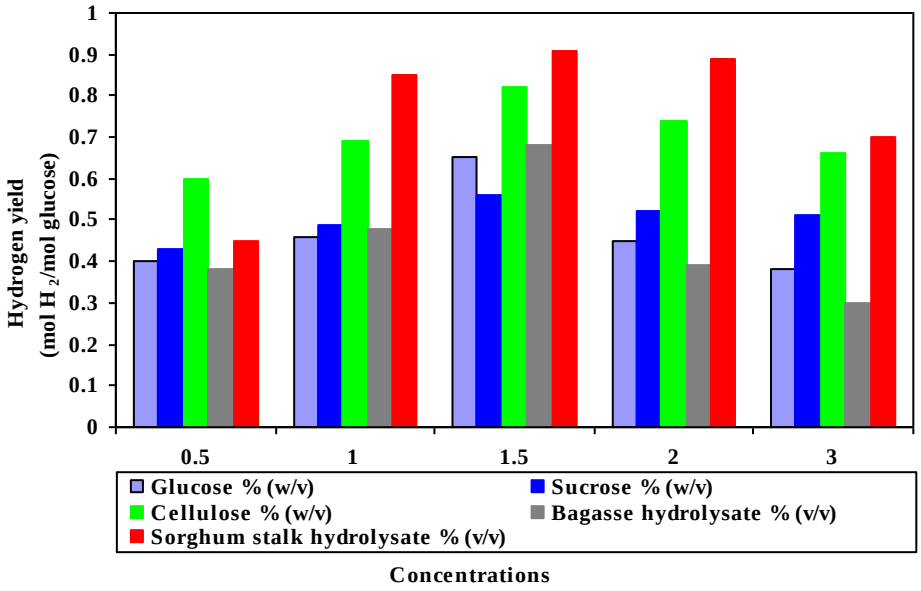


Fig. 7 - Effect of different substrates on hydrogen yield in *α-Proteobacterium*AUChE 103 batch cultures

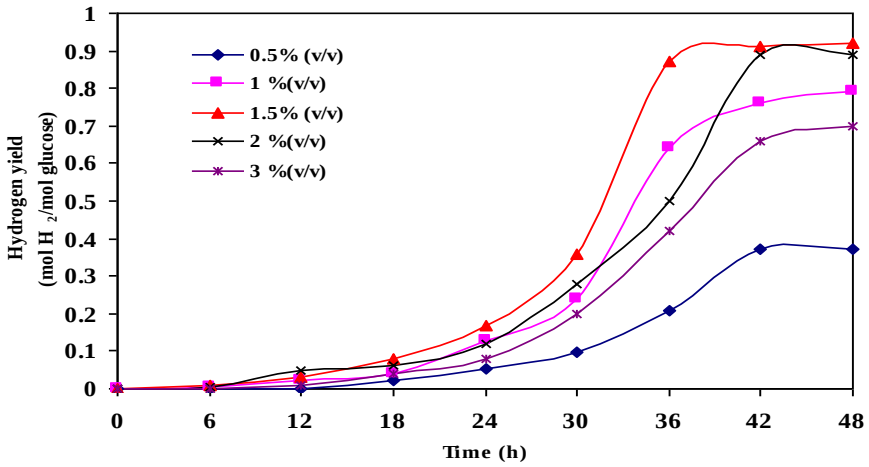


Fig. 8 - Time profile of hydrogen yield for different substrate concentrations in*α-Proteobacterium*AUChE 103 batch culture

4. Conclusions

The study demonstrated the optimization of media components and process parameters for the production of hydrogen using the isolated strain, *α-Proteobacterium*AUChE 103.An optimum hydrolysis time and acid concentration is required for the proper hydrolysis of sorghum stalk, above and below which the glucose yield was low. A suitable sorghum stalk hydrolysate in the fermentation medium is essential to get higher hydrogen yields; however, an excessively high or low concentration of sorghum stalk hydrolysate will affects the growth of the organism which will reduce the yield.The optimum concentration of medium components for *α-Proteobacterium*AUChE 103 hydrogen production was found to be: 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 used to test the importance of process parameters on hydrogen production. The optimized values of process parameters for hydrogen production were as follows: pH, 7.0; temperature, 34.5^{o}C and fermentation time, 42.5h. A highest hydrogen yield of 0.91 mol H_2/mol glucose was achieved, when sorghum stalk hydrolysate (equivalent to 1.5% (w/v)) was used as the carbon source.

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