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 and NaCl were determined using Plackett-Burman design. These significant variables were then optimized using central composite design. The optimum conditions were found to be: glucose, 19.25 g/L; yeast extract, 3.046 g/L; malt extract, 1.64 g/L; peptone, 5.640 g/L and NaCl, 4.312 g/L. Box-Behnken design was employed to optimize the process parameters. Under the optimum conditions a maximum hydrogen yield of 0.91 mol H₂/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 rain producing 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 expensive, energy 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°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% H₂SO₄ for 75 minutes at 121°C and 15psi in an autoclave. After hydrolysis, the hydrolysate was filtered through ordinary filter paper followed by filtration through Whatman 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°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-ProteobacteriaAUChE 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°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 influence in the yield of hydrogen and their coded values and the corresponding actual values are given in Table 3.1. 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).

![Parity plot between the experimental and predicted values of hydrogen yield](image-url)
Table 3.1 Variables showing medium components used in Plackett-Burman design for the production of hydrogen using \textit{a-ProteobacteriumAUChE 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$_4$Cl           | 1                           | 9                          |
| ZnCl$_2$           | 0.5                         | 5                          |
| KH$_2$PO$_4$       | 0.05                        | 0.5                        |
| K$_2$HPO$_4$       | .5                          | 2.5                        |
| MnSo$_4$.7H$_2$O   | 0.5                         | 5                          |
| MgSo$_4$.7H$_2$O   | 0.1                         | 1                          |
| ZnSo$_4$.7H$_2$O   | 0.1                         | 5                          |
| FeSo$_4$.7H$_2$O   | 0.1                         | 1                          |

Fig. 2 - Pareto’s Chart for \textit{(a-ProteobacteriumAUChE 103)} screening of media constituents by Plackett-Burman design
Table 3.2 Twenty run Plackett-Burman design matrix (α-ProteobacteriumAUChE 103) for fifteen variables with the experimental and predicted H₂ yields

| Runs | A  | B  | C  | D  | E  | F  | G  | H  | J  | K  | L  | M  | N  | O  | P  |   | Hydrogen yield (mol H₂/mol) |
|------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|---|------------------------------|
|      | Exp.| Pred.|    |    |    |    |    |    |    |    |    |    |    |    |    |   |------------------------------|
| 1    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.670                        |
| 2    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.7229                       |
| 3    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.023                        |
| 4    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.0170                       |
| 5    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.262                        |
| 6    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.2919                       |
| 7    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.719                        |
| 8    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.6671                       |
| 9    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.232                        |
| 10   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.2720                       |
| 11   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.673                        |
| 12   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.6560                       |
| 13   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.638                        |
| 14   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.6550                       |
| 15   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.432                        |
| 16   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.4069                       |
| 17   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.673                        |
| 18   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.6411                       |
| 19   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.021                        |
| 20   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   | 0.0150                       |

Glucose(A); Peptone(B); Beef extract(C); Malt extract(D); Yeast extract(E); KCl(F); NaCl(G); NH₄Cl(H); ZnCl₂(J); KH₂PO₄(K); K₂HPO₄(L); MnSO₄·7H₂O(M); MgSO₄·7H₂O(N); ZnSO₄·7H₂O(O); and FeSO₄·7H₂O(P)
Table 3.3 Estimated effects and coefficients of the Plackett–Burman design for α-
ProteobacteriumAUCHe 103

| Terms | Effect | Coeff. | SE Coeff. | 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 H₂/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, R², 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 were 0.551 mol H₂/mol glucose and 0.791 mol H₂/mol glucose for Run No.50 and Run No.34 respectively.

Regression analysis of the second order polynomial model for hydrogen production is given 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:

\[ Y = 0.789 - 0.0038X_1 + 0.001X_2 + 0.015X_3 + 0.0038X_4 - 0.009X_5 - 0.027X_2^2 - 0.018X_3^2 - 0.033X_1X_2 - 0.021X_1X_3 - 0.018X_1X_4 + 0.006X_1X_5 + 0.012X_2X_3 + 0.005X_2X_4 + 0.014X_2X_5 + 0.014X_3X_4 - 0.005X_3X_5 - 0.008X_4X_5 - 0.006X_2X_5 + 0.0068X_2X_5 \ldots \ldots (3.1) \]

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-
ProteobacteriumAUChE 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 3.4 Codes and actual levels of the independent variables for design of experiment for α-ProteobacteriumAUChE 103 H₂ fermentation

| Independent variables | Symbols | Coded levels |
|-----------------------|---------|--------------|
| Glucose, (g/L)        | X₁      | -2  10  15  20  25  30 |
| Peptone, (g/L)        | X₂      | -1  1   3   5   7   9  |
| Malt extract, (g/L)  | X₃      | 0.5 1.0 1.5 2.0 2.5 |
| Yeast extract, (g/L) | X₄      | 1   2   3   4   5   |
| NaCl (g/L)           | X₅      | 1   3   5   7   9   |

Table 3.5 Five level factorial central composite design with experimental and predicted values of hydrogen yield (mol H₂/mol glucose) for α-ProteobacteriumAUChE 103 H₂ fermentation

| Run No. | Coded values | Hydrogen yield (mol H₂/mol glucose) |
|---------|--------------|------------------------------------|
|         | X₁  X₂  X₃  X₄  X₅ | 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 |
Table 3.6: Results of the regression analysis of second order polynomial model for the optimization of hydrogen production using \( \alpha \)-ProteobacteriumAUChE

| 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         | 2.38   | 0.00   | 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         | -2.38  | 0.00   | 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 |

| 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\(_1\)X\(_2\) | -0.018950              | 0.001182      | -16.038      | <0.001  |
| X\(_1\)X\(_3\) | -0.033250              | 0.001182      | -28.140      | <0.001  |
| X\(_1\)X\(_4\) | -0.019656              | 0.001182      | -16.635      | <0.001  |
| X\(_1\)X\(_5\) | -0.021863              | 0.001182      | -18.503      | <0.001  |
| X\(_2\)X\(_2\) | -0.018875              | 0.001600      | -11.799      | <0.001  |
| X\(_2\)X\(_3\) | 0.006250               | 0.001600      | 3.907        | 0.001   |
| X\(_2\)X\(_4\) | 0.012125               | 0.001600      | 7.579        | <0.001  |
| X\(_2\)X\(_5\) | 0.005250               | 0.001600      | 3.282        | 0.003   |
| X\(_3\)X\(_3\) | 0.014438               | 0.001600      | 9.025        | <0.001  |
| X\(_3\)X\(_4\) | 0.014938               | 0.001600      | 9.337        | <0.001  |
Table 3.7 Analysis 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 3.8 Optimum 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
|   | C: Beef extract | E: NaCl |
|---|----------------|---------|
| 1 | -2.37          | -1.19   |
| 2 | 0.00           | 1.19    |
| 3 | 2.37           | 2.37    |

| Hydrogen Yield |
|----------------|
| A: Glucose     |
| B: Peptone     |
| C: Malt extract|
| D: Yeast extract|

|   | C: Malt extract | E: NaCl |
|---|----------------|---------|
| 1 | -2.31          | -1.16   |
| 2 | 0.00           | 1.15    |
| 3 | 2.31           | 2.31    |

| Hydrogen Yield |
|----------------|
| A: Glucose     |
| B: Peptone     |

|   | C: Malt extract | E: NaCl |
|---|----------------|---------|
| 1 | -2.31          | -1.16   |
| 2 | 0.00           | 1.15    |
| 3 | 2.31           | 2.31    |

| Hydrogen Yield |
|----------------|
| A: Glucose     |
| B: Peptone     |

|   | C: Malt extract | E: NaCl |
|---|----------------|---------|
| 1 | -2.31          | -1.16   |
| 2 | 0.00           | 1.15    |
| 3 | 2.31           | 2.31    |

| Hydrogen Yield |
|----------------|
| A: Glucose     |
| B: Peptone     |

|   | C: Malt extract | E: NaCl |
|---|----------------|---------|
| 1 | -2.31          | -1.16   |
| 2 | 0.00           | 1.15    |
| 3 | 2.31           | 2.31    |

| Hydrogen Yield |
|----------------|
| A: Glucose     |
| B: Peptone     |

|   | C: Malt extract | E: NaCl |
|---|----------------|---------|
| 1 | -2.31          | -1.16   |
| 2 | 0.00           | 1.15    |
| 3 | 2.31           | 2.31    |

| Hydrogen Yield |
|----------------|
| A: Glucose     |
| B: Peptone     |

|   | C: Malt extract | E: NaCl |
|---|----------------|---------|
| 1 | -2.31          | -1.16   |
| 2 | 0.00           | 1.15    |
| 3 | 2.31           | 2.31    |

| Hydrogen Yield |
|----------------|
| A: Glucose     |
| B: Peptone     |
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 H₂/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 AUCe 103 can be expressed in terms of the following regression equation;

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

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

The multiple correlation coefficient, R², 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)).
results suggest that the maximum values of hydrogen yield (0.89 mol H₂/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.

Table 3.9 The 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 H₂ fermentation

| Run No. | pH    | Temperature | Time | Hydrogen yield (mol H₂/mol glucose) |
|---------|-------|-------------|------|-------------------------------------|
|         | Exp.  | Pred.       |      |                                     |
| 1       | 0.889 | 0.888       | 0.00 |                                     |
| 2       | 0.700 | 0.701       | -1.00|                                     |
| 3       | 0.685 | 0.683       | 1.00 |                                     |
| 4       | 0.886 | 0.888       | 0.00 |                                     |
| 5       | 0.812 | 0.812       | 1.00 |                                     |
| 6       | 0.747 | 0.748       | 1.00 |                                     |
| 7       | 0.785 | 0.785       | 0.00 |                                     |
| 8       | 0.715 | 0.713       | -1.00|                                     |
Table 3.11 Results of the ANOVA of the process parameter optimization data in α-ProteobacteriumAUChE 103 H<sub>2</sub> 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<sup>-3</sup> | 1                      | 1.513×10<sup>-3</sup> | 392.13  | <0.0001        |
| Time (C)   | 3.920×10<sup>-3</sup> | 1                      | 3.920×10<sup>-3</sup> | 101.63  | <0.0001        |
| AB         | 4.225×10<sup>-4</sup> | 1                      | 4.225×10<sup>-4</sup> | 10.95   | 0.0129         |
| AC         | 9.923×10<sup>-4</sup> | 1                      | 9.923×10<sup>-4</sup> | 257.25  | <0.0001        |
| B          | 4.202×10<sup>-5</sup> | 1                      | 4.202×10<sup>-5</sup> | 108.95  | <0.0001        |
| A<sup>2</sup> | 0.022          | 1                       | 0.022      | 5678.61 | <0.0001        |
| B<sup>2</sup> | 0.039          | 1                       | 0.039      | 9981.91 | <0.0001        |
| C<sup>2</sup> | 0.023          | 1                       | 0.023      | 6079.10 | <0.0001        |
| Residual   | 2.700×10<sup>-5</sup> | 7                      | 3.857×10<sup>-6</sup> |         |                |
| Lack of fit| 1.900×10<sup>-7</sup> | 3                      | 6.333×10<sup>-3</sup> | 3.17    | 0.1473         |
| Pure error | 8.000×10<sup>-9</sup> | 4                      | 2.000×10<sup>-9</sup> |         |                |
| Total      | 0.12           | 16                      |            |         |                |

Table 3.12 Estimated coefficient values for α-ProteobacteriumAUChE 103 H<sub>2</sub> fermentation

| Factor     | Coefficient Estimate | Degrees of freedom (DF) | Standard error | 95% CI Low | 95% CI High | VIF |
|------------|----------------------|-------------------------|----------------|------------|-------------|-----|
| Intercept  | 0.89                 | 1                       | 8.783×10<sup>-4</sup> | 0.88       | 0.89        |     |
| pH (A)     | 0.049                | 1                       | 6.944×10<sup>-4</sup> | 0.047      | 0.050       | 1.00|
| Temperature(B) | 0.014               | 1                       | 6.944×10<sup>-4</sup> | 0.012      | 0.015       | 1.00|
| Time (C)   | 7.000×10<sup>-3</sup> | 1                      | 6.944×10<sup>-4</sup> | 0.047      | 0.050       | 1.00|
| AB         | 3.250×10<sup>-3</sup> | 1                      | 9.820×10<sup>-4</sup> | 0.013      | 0.018       | 1.00|
| AC         | 0.016                | 1                       | 9.820×10<sup>-4</sup> | 0.013      | 0.018       | 1.00|
| BC         | 0.010                | 1                       | 9.820×10<sup>-4</sup> | 0.013      | 0.018       | 1.00|
| A<sup>2</sup> | -0.072               | 1                       | 9.571×10<sup>-4</sup> | -0.074     | -0.070      | 1.01|
| B<sup>2</sup> | -0.096               | 1                       | 9.571×10<sup>-4</sup> | -0.098     | -0.093      | 1.01|
| C<sup>2</sup> | -0.075               | 1                       | 9.571×10<sup>-4</sup> | -0.077     | -0.072      | 1.01|
Fig. 6 (a-c)- 3D plots showing the interactive effects between the significant process parameters on hydrogen yield in α-ProteobacteriumAUChE 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-ProteobacteriumAUChE 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 H₂/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-ProteobacteriumAUChE 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 $\alpha$-Proteobacterium AUChE 103 batch cultures

Fig. 8 - Time profile of hydrogen yield for different substrate concentrations in $\alpha$-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, $\alpha$-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 affect the growth of the organism which will reduce the yield. The optimum concentration of medium components for $\alpha$-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°C and fermentation time, 42.5h. A highest hydrogen yield of 0.91 mol H₂/mol glucose was achieved, when sorghum stalk hydrolysate (equivalent to 1.5% (w/v)) was used as the carbon source.
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