Optimization of photo-fermentative hydrogen production using volatile fatty acids

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ABSTRACT

A combination of photofermentation with dark fermentation is an ideal option for efficient hydrogen production. In the present study, the optimum concentration of substrates for the hydrogen production was found by response surface methodology. The optimum combination of three individual fatty acids was determined by Box Behnken design and Central Composite design. The influence of a mixture of volatile fatty acids was evaluated using the Phototrophic Hydrogen-Producing Mixed Bacterial Consortium obtained from bio-electrochemical system used to produce hydrogen. For these experiments, 100 mg VSS L^{-1} were used. The serologic bottles were then sparged with argon for a period of 30 s to remove dissolved oxygen and to create an oxygen free microenvironment prior to closing with rubber septum**.** The tests were placed on the shaker plate at 100 rpm and operated at 30-35 °C and illuminated under a constant radiation of 5000 lux. It was observed an inhibition of hydrogen production by the propionic acid. Also was presented the decrease in hydrogen production with the increase pH that occurred in the tests during the experimentation. The ammonium formation was responsible for pH changes. The concentrations of ammonia nitrogen formed at the tests ranged between 0.4 and 10.8 mg L^{-1}. From the ANOVA analysis the both experimental designs resulted significant, as it is evident from the Fischer's F-test with a high F-values ($p<0.001$-$p<0.017$). The R^2 values near to 1 (0.9766-0.9089) in both cases suggests good correlation between experimental and predicted values. The results showed that a maximum hydrogen production rate of 6.5 mmol d^{-1} L^{-1} and maximal amount of hydrogen produced of 108.8 mmol L^{-1} were obtained when the concentrations of acetic acid, propionic acid and butyric acid were 1200 mg L^{-1}, 715 mg L^{-1} and 1571 mg L^{-1}, respectively.

Keywords: Photofermentation, hydrogen, volatile fatty acids

1. Introduction

At present, fossil fuels are considered the main source of energy supply. However, because of its growing demand and limited availability an alternative source of energy is needed [1]. Hydrogen (H_2) is considered to be one of the most promising fuels of the future due to high energy content (122 kJ g^{-1}) which is 2.72 times that for gasoline [2-3]. Hydrogen gas is also a clean fuel with no CO_x, SO_x and NO_x emissions. Besides, H_2 is an important energy carrier and can be used in fuel cells for generation of electricity [4-5]. However, hydrogen gas is not readily available in nature like fossil fuels and natural gas, but can be produced from renewable materials such as biomass [2] and water [6]. The biological production of hydrogen is considered environmentally friendly. In particular, this process can use a variety of organic substrates as carbon sources, including wastes [7]. So, there exists a double benefit: the waste reduction and the energy production.

The combination of photosynthetic bacteria with fermentative bacteria can provide a system for hydrogen photo production from residual carbohydrates such as organic wastes. Photosynthetic non-sulfur (PNS) bacteria have the ability to convert volatile fatty acids (VFAs) to H_2 and CO_2 under anoxygenic conditions [5, 8]. PNS bacteria also have the ability to use carbon sources like glucose, sucrose, succinate rather than VFA for H_2 production [9-11]. The most widely known PNS bacteria used in photo-fermentative H_2 production are *Rhodobacter sphaeroides O.U001*, *Rhodobacter capsulatus*, *R. sphaeroides-RV*, *Rhodobacter sulfidophilus*, *Rhodopseudomonas palustris* and *Rhodospirillum rubrum* [12]. Both hydrogenase and nitrogenase enzymes were detected in PNS bacteria [5, 13]. However, nitrogenase is the main enzyme responsible in molecular H_2 production under anoxygenic conditions [13-14]. The photofermentation culture is very promising because of the purity of the generated biogas (H_2:80%) [15]. However, it must be considered the different potential of substrates. For example, during dark fermentation are produced acetate, propionate and butyrate mainly [16].

The one factor-at-a-time design has two main drawbacks, one is it does not take into consideration the interactions among different factors; it involves a relatively large number of experiments, which makes it laborious and time-consuming to carry out the experiments, especially when the number of factors is large [17]. On the contrary, factorial design is able to study the effects of more than one factor at two or more levels. The experimental design generally includes various combinations of different factor levels, which enables it to depict the interactions among different factors and to be more efficient to deal with a large number of factors. Factorial design can be classified into two categories: full factorial design and fractional factorial design.

The number of runs for a full factorial design increases geometrically as the number of factors increases, when the effects of a large number of factors are to be studied simultaneously, a great many runs of experiment are required. Generally, this will constitute a larger experiment that is not economically and practically feasible [18]. Fractional factorial design provides an alternative when the number of runs for a full factorial design is too large to be practicable. Taguchi design, Plackett–Burman design, central composite design and Box–Behnken design are fractional factorial designs that were used a lot for fermentative hydrogen production processes provides an economical alternative [19].

The use of the consortium photofermentative can to be better than a pure strain because it isn't need sterilized and the operation conditions are easier of controller. Also the consortium mixed obtained can present good resistance to inhibitions produced for variations in the system. In the present study, Box-Behnken design and Central Composite design were used for evaluate the effect of a mixture of acetic, propionic and butyric acids on the hydrogen production rate using photobacteria. The individual maximum

substrate concentration for acetic acid, propionic acid and butyric acid were already found by literature review. The optimum concentration of volatile fatty acids for maximum hydrogen production is found by applying response surface methodology.

2. Experimental

2.1 Inoculating and enriching the microbial consortium

The Phototrophic Hydrogen-Producing Mixed Bacterial Consortium (PHPMBC) was obtained from bio-electrochemical system used to produce hydrogen. Firstly, the sludge was the growth tests, bacteria plus 150 mL of medium and the organic acid and glutamate were placed in 250 mL glass bottles. Then, argon gas was purged in the headspace to ensure the anaerobic conditions. The culture medium was supplemented with acetate (2.46 g L^{-1}), butyrate (3.30 g L^{-1}) and sodium glutamate (0.37 g L^{-1}). The initial pH was adjusted to 6.8 with NaOH (1 N). The bottle was incubated about at 30-35 °C illuminated by 4 fluorescent lamps (20 W) and 4 tungsten lamps (60 W). After growing for 100 hours, the biomass with the purple non-sulfur photosynthetic bacteria was harvested.

2.2. Operation of the batch reactors

The basal medium [20] was constituted of 0.75 gL^{-1} K_2HPO_4, 0.85 gL^{-1} KH_2PO_4, 0.2 gL^{-1} $MgSO_4$, 11.78 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 2.8 mg L^{-1} H_3BO_3, 0.75 mgL^{-1} $Na_2MoO_4 \cdot 2H_2O$, 0.24 mg L^{-1} $ZnSO_4 \cdot 7H2O$, 2.1 mg L^{-1} $MnSO_4 \cdot 4H_2O$, 0.04 mg L^{-1} $CuCl_2 \cdot 2H_2O$, 0.75 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 2.0 mg L^{-1} EDTA-Na, 3.78 mg L^{-1}, B1 vitamin and 3.57 mg L^{-1} de biotin. For this stage a C/N ratio of 16. The influence of a mixture of VFAs was evaluated using the PHPMBC. For these experiments, 100 mg VSS L^{-1} were used. The serologic bottles were then sparged with argon for a period of 30 s to remove dissolved oxygen and to create an oxygen free microenvironment prior to closing with rubber septum. All the bottles were placed on the shaker plate at 100 rpm. Was operated at 30-35 °C and illuminated under a constant radiation of 5000 lux.

2.3 Box-Behnken design

Box–Behnken design (BBD) is a three-level fractional factorial design developed by Box and Behnken. The design can be thought of as a combination of a two-level factorial design with an incomplete block design. In each block, a certain number of factors are put through all combinations for the factorial design, while other factors are kept at the central levels. BBD provides an economical alternative to the central composite design, because it has less factor levels than the central composite design and does not contain extreme high or extreme low levels. BBD matrix was used to investigate the effect of VFAs concentration on photofermentative hydrogen production. The variables designated were acetic acid concentration (X_1), butyric acid concentration (X_2) and propionic acid concentration (X_3) on hydrogen production. The factors X_1, X_2 and X_3 were considered as an independent variables and maximal amount of hydrogen produced (H_{max}) and maximum hydrogen production rate (R_{max}) were the response (dependent variable). The low, middle and high level of each variable were coded as −1, 0 and +1 respectively. The coded and actual values were given in Table 1.

The following second order polynomial equation was adopted to study the effects of variables to the response.

$$H_{max}, R_{max} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where H_{max} and R_{max} are the response, β_0 is the constant term; β_1, β_2 and β_3 are the coefficient of linear terms; β_{11}, β_{22} and β_{33} are the coefficient of quadratic terms; β_{12}, β_{13} and β_{23} are the coefficient of cross product terms respectively. A statistical design package, Minitab 15 is used for regression analysis of the data obtained and to estimate the coefficients of the second-degree polynomial equation.

The estimated second-order polynomial model can be displayed as a surface plot and a contour plot, by varying only two factor levels, while keeping other factor levels constant. The surface plot and contour plot will visually show the response over a region of interesting factor levels. In addition, they will indicate how sensitive the response is to the change of each factor levels and to what degree the factors interplay as they affect the response. Based on the analysis of variance (ANOVA) of the estimated model, terms which have significant effects on the response can be determined. It is important to note that all interactions higher than second order have been neglected in Eq. (1). A total of 15 experiments were needed to estimate of the model.

Table 1. Experimental range, level and code of independent variables for the optimization of hydrogen production

Independent variables	Symbol coded	Range and levels		
		-1	0	1
Acetic acid concentration (mg L^{-1})	X_1	125	1235	2345
Butyric acid concentration (mg L^{-1})	X_2	50	160	270
Propionic acid concentration (mg L^{-1})	X_3	175	672.5	1170

2.4 Central composite design

The BBD presented only the optimized value of propionic acid. A two-level central composite design (CCD) was used to construct second-order models for hydrogen production (Table 2). The CCD consisted of three types of experimental points: cube points, axial points, and center points. The center points were used to calculate the experimental error, and the distance of axial points from the center points in this work was fixed at 1.414 [21]. The experimental factors taken into consideration were the acetic acid concentration and butyric acid concentration. The maximal amount of hydrogen produced (H_{max}) and maximum hydrogen production rate (R_{max}) were selected as the model response. The experimental runs were analyzed to fit second-order polynomial equation, as shown in Eq. (2).

$$H_{max}, R_{max} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (2)$$

where H_{max} and R_{max} are the response, β_0 is the constant term; β_1 and β_2 are the coefficient of linear terms; β_{11} and β_{22} are the coefficient of quadratic terms; β_{12} is the coefficient of cross product terms respectively. A statistical design package, Minitab 15 is used for regression analysis of the data obtained and to estimate the coefficients of the second-degree polynomial equation. The adequacy of the proposed model was verified using analysis of variance (ANOVA), R^2 and adjusted R^2.

New concentrations of acetic acid and butyric acid (Table 2) were proposed based on data Nan-Qi [22]. The propionic acid concentration remained fixed at 715 mg L^{-1} for the 13 tests.

Table 2. Experimental range, level and code of independent variables for the optimization of hydrogen production

Independent variables	Symbol coded	Range and levels				
		-1.414	-1	0	1	1.414
Acetic acid concentration (mg L^{-1})	X_1	85.8	500	1500	2500	2914.2
Butyric acid concentration (mg L^{-1})	X_2	85.8	500	1500	2500	2914.2

2.5 Analytical methods

Hydrogen, carbon dioxide and methane were analyzed with a gas chromatograph (Agilent 6890 N) equipped with a thermal conductivity detector and a 30 m long (0.53 mm id) Carboxen 1010 Plot column. The temperature of the injection port, column and detector, were 200, 100 and 230 °C, respectively. Nitrogen was used as the carrier gas at a flow rate of 4 mL min^{-1}. Liquid samples were taken at the end of the run for the analysis of VFAs and solvents. To analyze VFA, alcohols and acetone, 1 mL of sample was centrifuged at 600 g during 10 min. Concentration of these by-products was determined using a chromatograph (Varian 3300) fitted with a FID detector and a 15 m long (0.53 mm id) Zebron ZB-FFAP column. Injector and detector temperatures were maintained at 190 and 210 °C, respectively. The temperature of the column was maintained at 45 °C for 1.5 min; then, it was increased to 135 °C at a rate of 8 °C min^{-1}. The carrier gas was nitrogen at 9.5 ml min^{-1}. Biogas was monitored daily using a pressure transducer. Hydrogen content, from the head space of the bottles, was analyzed twice a week, VSS concentration, COD and N–NH_3 were determined according to standard methods [23].

2.6 Kinetic analysis

The cumulative hydrogen volume in the tests followed he modified Gompertz Eq. (3) was used to fit the kinetics of biohydrogen production using Microsoft's software Excel 2010. This equation has been widely used to model gas production data [24]:

$$H = H_{max} exp\left\{-exp\left[\frac{R_{max} \times e}{H_{max}}(\lambda - t) + 1\right]\right\} \quad (3)$$

where, H(t) (mL) is the total amount of hydrogen produced at culture time t (h); H_{max} (mL) is the maximal amount of hydrogen produced. R_{max} (mL h^{-1}) is the maximum hydrogen production rate; λ (h) is the lag time before the exponential hydrogen production.

3. Results and discussion

3.1 Box-Behnken design approach for optimization of hydrogen production

Multiple regression analysis of the experimental data yielded the following regression equations for the hydrogen production (Eqs. 4 and 5).

$$H_{max} = -0.2017 + 4.526 \times 10^{-4} X_1 - 6.845 \times 10^{-3} X_2 + 1.146 \times 10^{-2} X_3 - 2.203 \times 10^{-7} X_1^2 + 1.721 \times 10^{-5} X_2^2 - 6.51 \times 10^{-6} X_3^2 + 2.285 \times 10^{-6} X_1 X_2 + 2.427 \times 10^{-7} X_1 X_3 + 2.056 \times 10^{-7} X_2 X_3 \quad (4)$$

$$R_{max} = 1.002 + 2.096 \times 10^{-4} X_1 + 8.421 \times 10^{-4} X_2 - 8.201 \times 10^{-4} X_3 + 5.675 \times 10^{-8} X_1^2 - -7.714 \times 10^{-6} X_2^2 + 1.279 \times 10^{-8} X_3^2 - 1.009 \times 10^{-6} X_1 X_2 + 2.852 \times 10^{-8} X_1 X_3 + 2.695 \times 10^{-6} X_2 X_3 \quad (5)$$

where H_{max} is the maximal amount of hydrogen produced, R_{max} is the maximum hydrogen production rate, X_1 is the initial acetic acid concentration, X_2 is the initial butyric acid concentration and X_3 is the initial propionic acid concentration.

BBD matrix of independent variables in coded units along with experimental and predicted values for hydrogen production was shown in Table 3. According to BBD, batch experiments were carried out with different combinations of the independent variables in order to find the combined effects of these factors toward hydrogen production.

Table 3. The Box-Behnken experimental design with Gompertz values.

Run	X_1	X_2	X_3	H_{max} of H_2			R_{max} of H_2		
				Observed (mmol)	Predicted (mmol)	Error (%)	Observed (mmol/d)	Predicted (mmol/d)	Error (%)
1	0	0	0	5.00	4.81	3.9	0.84	0.85	-2.1
2	0	0	0	5.00	4.81	3.9	0.99	0.85	16.3
3	-1	1	0	4.06	4.16	-2.3	0.57	0.61	-6.4
4	-1	0	-1	1.38	1.06	23.4	0.96	0.88	9.6
5	0	1	1	5.13	5.11	0.5	0.64	0.63	2.6
6	-1	0	1	3.88	3.82	1.7	0.54	0.51	4.4
7	0	0	0	4.42	4.81	-8.7	0.73	0.85	-14.3
8	0	1	-1	1.83	2.06	-12.5	0.62	0.67	-6.9
9	0	-1	-1	1.70	1.72	-1.7	1.18	1.20	-1.3
10	0	-1	1	4.96	4.73	4.6	0.61	0.57	8.1
11	1	0	-1	1.70	1.76	-3.9	1.29	1.31	-1.7
12	1	0	1	4.73	5.06	-6.8	0.92	1.01	-8.4
13	1	1	0	5.98	5.69	4.9	0.89	0.82	8.3
14	1	-1	0	4.87	4.77	2.0	1.34	1.30	3.0
15	-1	-1	0	4.06	4.36	-7.3	0.53	0.59	-11.5

The statistical significance of the polynomial equation was checked by F-test. Analysis of variance (ANOVA) for response surface quadratic model is given in Table 4. Significance of model terms is checked by their respective p values. P-value less than 0.05 suggests model terms are significant and less than 0.0001 are highly significant. The goodness of fit of the model was checked by determination of coefficient (R^2) and adjust R^2. The R^2 value varies from 0 to 1.0 and R^2 value close to 1.0 implies better accuracy of the model. But incorporation of large number of insignificant variables in the model may result high R^2 value but model become erroneous and there by predict poor response. So the term adj R^2 was introduced which corrects R^2 value according to the sample size and number of terms in the model. Ideally adjust R^2 should be close to R^2 value. Large difference between R^2 and adj R^2 gives a warning that model contain too many insignificant terms [25].

Table 4. Analysis of variance (ANOVA) for the hydrogen production.

Source of variation	H_{max}					R_{max}				
	DF[a]	SS[b]	MS[c]	F-value	P	DF[a]	SS[b]	MS[c]	F-value	P
Regression	9	30.925	3.436	23.16	0.001	9	0.960	0.010	7.98	0.017
Linear	3	20.243	6.812	45.92	0.000	3	0.757	0.252	18.88	0.004
Square	3	10.104	3.368	22.71	0.002	3	0.054	0.018	1.36	0.357
Interaction	3	0.383	0.123	0.86	0.518	3	0.148	0.049	3.71	0.096
Residual	5	0.741	0.148			5	0.066	0.013		
Lack-of-fit	3	0.517	0.172	1.54	0.417	3	0.032	0.011	0.62	0.664
Pure error	2	0.224	0.112			2	0.034	0.017		
Total	14	31.667				14	1.027			

H_{max}:R^2=0.9766, Adjust R^2=0.9344 R_{max}:R^2=0.9349, Adjust R^2=0.8178

[a] Degrees of freedom

[b] Sum of squares

[c] Mean square

For H_{max} (maximal amount of hydrogen produced) the model was found to be significant, as it is evident from the Fischer's F-test with a high F-value 23.16 ($p<0.001$). The high R^2 value (0.9766) suggests good correlation between experimental and predicted values and 97.66 % variability of the response could be explained by the model and about only 2.34 % of the total variation cannot be explained by this model. Adjust R^2 value (0.9344) was found to be very close to R^2. The lack of fit test, measures the failure of the model to represent experimental data in the experimental domain at point which are not included in regression analysis. Lack of fit was found to be non-significant (P value 0.417) and it suggests that the model equation was adequate to predict the maximal amount of hydrogen produced under any sets of combination of the variables. In the case of R_{max} (maximum hydrogen production rate) the model was found to be significant, as it is evident from the Fischer's F-test with a high F-value 7.98 ($p<0.017$). The high R^2 value (0.9349) suggests good correlation between experimental and predicted values and 93.49 % variability of the response could be explained by the model and about only 6.51 % of the total variation cannot be explained by this model. Adjust R^2 value (0.8178). Lack of fit was found to be non-significant (p value 0.664) and it suggests that the model equation was adequate to predict the maximum hydrogen production rate under any sets of combination of the variables.

P-value serves as a tool for checking the significance of each coefficient. P-value less than 0.05 indicate model terms are significant. It can be concluded from Table 5 that, for H_{max} the coefficient of linear effect of initial acetic acid concentration (X_1) and initial propionic acid concentration (X_3) were significant on amount of hydrogen produced. The coefficient of quadratic term of initial propionic acid concentration (X_3*X_3) was found to be highly significant. On R_{max} the coefficient of linear effect of each model terms (X_1, X_2 and X_3) were significant on hydrogen production rate. 3D plot represents response surface of two independent variables where remaining variables are fixed at their respective zero level. It helps to understand the main and their interaction effect and also to determine optimum level of each variable [26]. The response surface 3D plots for H_{max} and R_{max} have been shown in Fig. 1(a–c) and Fig. 2(a-c), respectively.

Table 5. Significance of regression coefficient with codified values (BBD)

Model term	H_{max}			R_{max}		
	Parameter estimate (coefficients)	T	P	Parameter estimate (coefficients)	T	P
Constant	4.806	21.616	0.000	0.853	12.790	0.000
X_1	0.485	3.564	0.016	0.230	5.633	0.002
X_2	0.178	1.312	0.247	-0.116	-2.853	0.036
X_3	1.512	11.107	0.000	-0.167	-4.095	0.009
$X_1 * X_1$	-0.271	-1.354	0.234	0.069	1.162	0.298
$X_2 * X_2$	0.208	1.039	0.346	-0.093	-1.551	0.182
$X_3 * X_3$	-1.611	-8.038	0.000	0.003	0.053	0.960
$X_1 * X_2$	0.279	1.449	0.207	-0.123	-2.132	0.086
$X_1 * X_3$	0.134	0.696	0.518	0.015	0.272	0.796
$X_2 * X_3$	0.011	0.058	0.956	0.147	2.552	0.051

Applying an optimization model was performed with the program Minitab 15. The optimum values of test variables corresponding to the maximum hydrogen production (4.9 mmol y 1.27 mmol d^{-1}) in uncoded units X_1=2345 mg L^{-1}, X_2=50 mg L^{-1} y X_3=715 mg L^{-1}. A maximal amount of hydrogen produced of 4.9 mmol and maximum hydrogen production rate of 1.27 mmol d^{-1} were obtained under optimum conditions. However an optimal concentration of 715 mg L^{-1} of propionic acid was found. This behavior suggests an inhibition of hydrogen production by the propionic acid.

The maximum hydrogen production rate and amount of hydrogen produced obtained was 1.34 mmol H_2 d^{-1} and 5.98 mmol H_2 respectively. Ammonia nitrogen concentrations formed during the experimentation ranged between 0.63 and 2.62 mg L^{-1}. The COD removal varied, the best removal was 87.80 % and the lowest was 41.63 %, have been reported COD removals over 80 % were obtained using purple non-sulfur bacteria named ZX-5 [27]. The pH remained stable throughout the test in 7 approximately. The amount of biomass was measured indirectly by optical density at 660 nm and the results were in the range of 541 and 913 mg VSS L^{-1}. The 15 different bottles were incubated during 400 hours.

3.3 Central Composite design approach for optimization of hydrogen production

CCD matrix of independent variables in coded units along with experimental and predicted values for H_{max} and R_{max} were shown in Table 6. According to CCD, batch experiments were carried out with different combinations of the independent variables in order to find the combined effects of these factors toward hydrogen production. The second order quadratic model expressed by the following equation represents maximal amount of hydrogen produced (H_{max}) and maximum hydrogen production rate (R_{max}) as a function of initial acetic acid concentration (X_1) and is the initial butyric acid concentration (X_2).

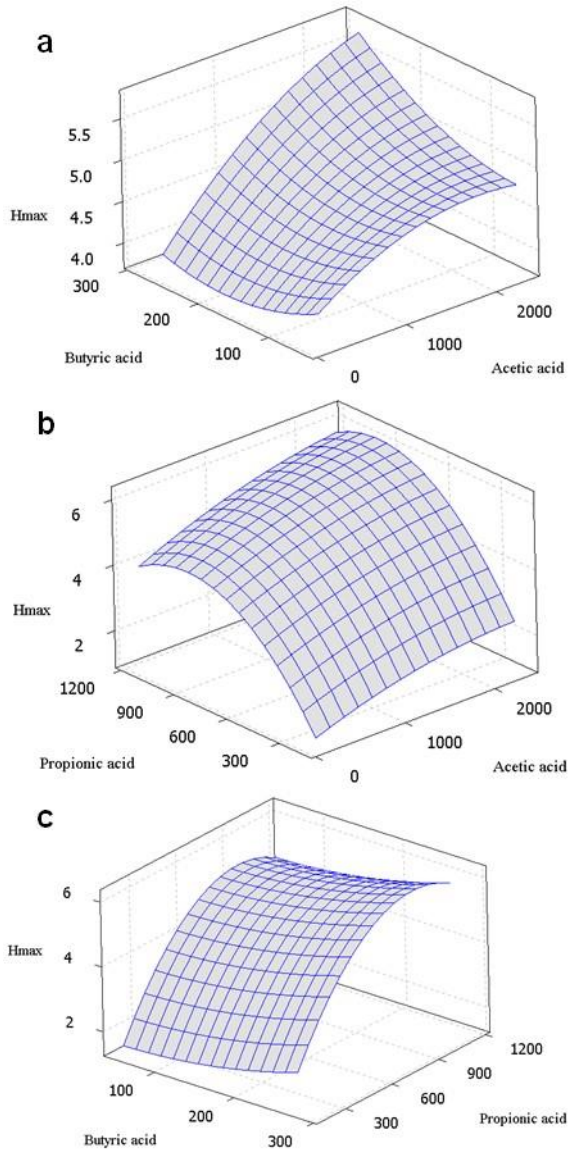

Fig. 1. (a) Response surface plot showing the effect of two variables acetic acid concentration (X_1) and butyric acid concentration (X_2) on amount of hydrogen produced. (b) Response surface plot showing the effect of two variables acetic acid concentration (X_1) and propionic acid concentration (X_3) on amount of hydrogen produced. (c) Response surface plot showing the effect of two variables butyric acid concentration (X_2) and propionic acid concentration (X_3) on amount of hydrogen produced.

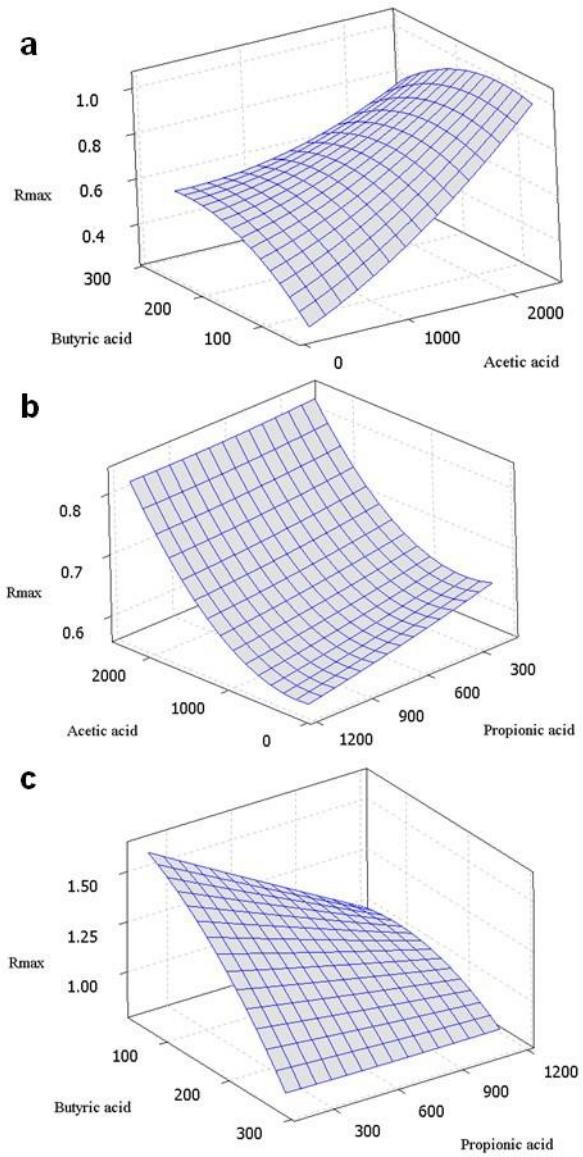

Fig. 2. (a) Response surface plot showing the effect of two variables acetic acid concentration (X_1) and butyric acid concentration (X_2) on hydrogen production rate. (b) Response surface plot showing the effect of two variables acetic acid concentration (X_1) and propionic acid concentration (X_3) on hydrogen production rate. (c) Response surface plot showing the effect of two variables butyric acid concentration (X_2) and propionic acid concentration (X_3) on hydrogen production rate.

$$H_{max} = 5.074 + 3.108 \times 10^{-3}X_1 + 5.038 \times 10^{-3}X_2 - 1.156 \times 10^{-6}X_1^2 - 1.681 \times 10^{-6}X_2^2 - 1.116 \times 10^{-6}X_1X_2 \quad (6)$$

$$R_{max} = 0.428 + 4.598 \times 10^{-5}X_1 + 2.205 \times 10^{-4}X_2 - 4.781 \times 10^{-8}X_1^2 - 7.192 \times 10^{-8}X_2^2 + 2.679 \times 10^{-8}X_1X_2 \quad (7)$$

Table 6. The Central Composite experimental design with Gompertz values.

Run	X_1	X_2	H_{max} of H_2			R_{max} of H_2		
			Observed (mmol)	Predicted (mmol)	Error (%)	Observed (mmol/d)	Predicted (mmol/d)	Error (%)
1	0	-1.414	7.72	7.55	2.2	0.39	0.41	-6.8
2	1	-1	7.68	7.70	-0.3	0.44	0.37	15.6
3	0	0	11.21	10.88	2.9	0.66	0.62	6.8
4	-1	1	8.13	8.41	-3.6	0.57	0.57	-1.3
5	0	1.414	7.63	7.49	1.9	0.50	0.54	-7.0
6	-1.414	0	9.46	9.11	3.8	0.63	0.60	4.3
7	1	1	7.63	7.64	0.0	0.51	0.51	12.8
8	0	0	10.22	10.88	-6.5	0.58	0.62	-7.0
9	0	0	11.47	10.88	5.1	0.65	0.62	5.2
10	0	0	10.63	10.88	2.4	0.61	0.62	-15.6
11	-1	-1	8.13	8.43	-3.8	0.53	0.54	-2.6
12	1.414	0	7.99	8.04	-0.6	0.44	0.44	-25.2
13	0	0	10.89	10.88	0.1	0.66	0.62	6.8

Table 7. Analysis of variance (ANOVA) for the selected quadratic model.

Source of variation	H_{max}					R_{max}				
	DF[a]	SS[b]	MS[c]	F-value	P	DF[a]	SS[b]	MS[c]	F-value	P
Regression	5	27.011	5.402	28.77	0.000	5	0.094	0.018	13.97	0.002
Linear	2	1.144	0.572	3.05	0.112	2	0.031	0.015	11.67	0.006
Square	2	25.866	12.933	68.87	0.000	2	0.062	0.031	23.18	0.001
Interaction	1	0.001	0.001	0.00	0.960	1	0.0002	0.0002	0.17	0.696
Residual	7	1.314	0.188			7	0.009	0.001		
Lack-of-fit	3	0.360	0.120	0.50	0.700	3	0.003	0.001	0.74	0.581
Pure error	4	0.954	0.238			4	0.006	0.001		
Total	12	28.325				12	0.103			

H_{max}:R^2=0.9536, Adjust R^2=0.9204 R_{max}:R^2= 0.9089, Adjust R^2=0.8439

[a] Degrees of freedom

[b] Sum of squares

[c] Mean square

Analysis of variance (ANOVA) for response surface quadratic model is given in Table 7. F-values for H_{max} and R_{max} the model were found to be significant, as it is evident from the Fischer's F-test with a high F-value 28.77 and 13.97 ($p<0.000$, $p<0.002$), respectively. Because the R^2 values ($R^2_{Hmax}=0.9536$, $R^2_{Rmax}=0.9089$) and adjust R^2 values ($R^2adj_{Hmax}=0.9204$, $R^2adj_{Rmax}=0.8439$) are in reasonable agreement, the quadratic models (Eqs. 6 and 7) satisfactorily describe the process behaviors within the proposed experimental range. The model adequacy was tested through lack of fit F-tests. The lack of fit, which had a F-value of 0.50 and 0.74 and a P-value of 0.700 and 0.581 in sets H_{max} and $_{Rmax}$, respectively, showed that they were not significant. This implies that all the models had a good fit for prediction.

Table 8. Significance of regression coefficient with codified values (CCD)

Model term	H_{max}			R_{max}		
	Parameter estimate (coefficients)	T	P	Parameter estimate (coefficients)	T	P
Constant	10.883	56.161	0.000	0.633	38.568	0.000
X_1	-0.377	-2.465	0.043	-0.051	-3.993	0.005
X_2	-0.021	-0.139	0.893	0.035	2.718	0.030
$X_1 * X_1$	-1.156	-7.037	0.000	-0.043	-3.136	0.016
$X_2 * X_2$	-1.680	-10.230	0.000	-0.089	-6.401	0.000
$X_1 * X_2$	-0.011	-0.052	0.960	0.007	0.407	0.696

It can be concluded from Table 8 that, for H_{max} the coefficient of linear effect of initial acetic acid concentration (X_1) was significant on amount of hydrogen produced. The coefficient of quadratic terms of all initial substrate concentration (X_1*X_1, X_2*X_2) was found to be highly significant. On R_{max} the coefficient of linear effect of each model terms (X_1 and X_2) were significant on hydrogen production rate. The coefficient of quadratic terms of all initial substrate concentration (X_1*X_1, X_2*X_2) was found to be significant. The response surface 3D plots for H_{max} and R_{max} have been shown in Fig. 3(a–b).

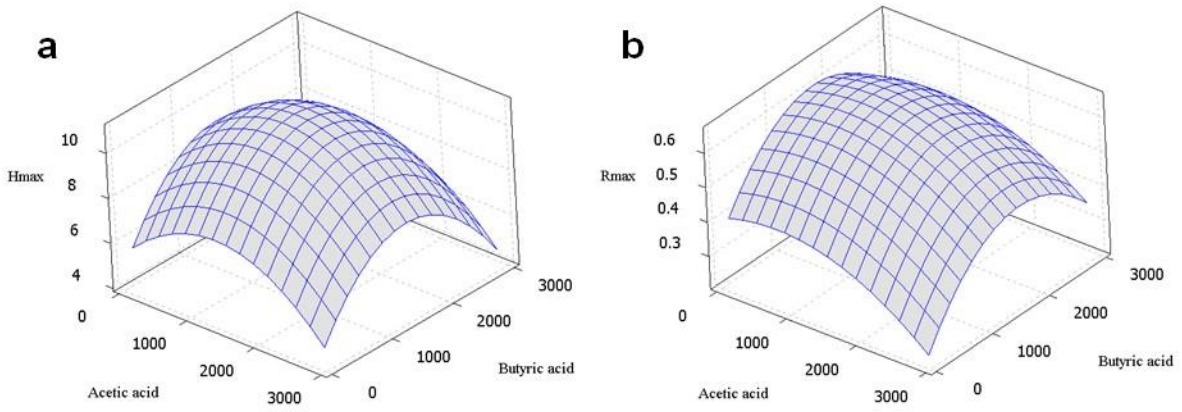

Fig. 3. (a) Response surface plot showing the effect of two variables acetic acid concentration (X_1) and butyric acid concentration (X_2) on amount of hydrogen produced. (b) Response surface plot showing the effect of two variables acetic acid concentration (X_1) and butyric acid concentration (X_2) on maximum hydrogen production rate.

The optimum conditions for the selected parameters were predicted by Minitab 15 software. The maximum hydrogen production was predicted could be achieved with the following concentration conditions; acetic acid of 1200 mg L^{-1} and butyric acid of 1571 mg L^{-1}. Under these conditions, the predicted H_{max} was 10.88 mmol H_2 and R_{max} was 0.65 mmol H_2d^{-1} with a desirability value of 0.99. The optimum concentrations corresponding to the maximum hydrogen production were acetic acid 1200 mg L^{-1}, propionic acid 715 mgL^{-1} and butyric acid1571 mg L^{-1}.

Have been reported that the optimum concentrations of VFAs in the hydrogen production found between 1800 and 2500 mg L^{-1} [28-30]. During the experimentation were obtained the maximum hydrogen yields were to average concentrations at 3500 mg L^{-1} of VFAs. Shi and Yu [31] reported its optimal condition at 1800, 200 and 1000 mg L^{-1} of acetic acid, propionic and butyric respectively using *Rhodopseudomonas capsulata*.

The 13 different bottles were incubated during 700 hours. Hydrogen content in the biogas was comprised from 77 to 87 %; hydrogen purity from 95 % were obtained using a pure culture of *Rhodobacter sphaeroides* O.U.001 when malate was used as the substrate [32-33]. On the other hand have been reported hydrogen purities between 80 and 90 % using a phototrophic sludge [20]. The amount of biomass was measured indirectly by optical density at 660 nm and the results were in the range of 1.19 and 3.76 mg VSS L^{-1}. The COD removal varied, the best removal was 69.67 % and the lowest was 37.58 % and total VFAs concentration in the effluent was 58-21% after photofermentation.

Hydrogen yields were obtained ranging between 76.3 and 114.7 mmol H_2 L^{-1} culture, Nan- Qi et al. [22] reported hydrogen yields that varied between 26.8 and 84.8 mmol H_2 L^{-1} culture using a pure strain of *Rhodopseudomonas faecalis RLD-53* when remained constant acetate concentration at 2051 mg L^{-1} and the concentration of butyrate varied at intervals of 1.38, 2.75, 5.50, 8.26 and 11.0 g L^{-1}. Other studios [34] reported hydrogen yields in a range of 5.6 to 13.9 mmol H_2 L^{-1} culture using various organic acids (acetate, butyrate, citrate, lactate and malate) with Phototrophic Hydrogen-Producing Bacterial Consortium. Hydrogen production rates obtained ranging between 0.35 to 1.34 mmol H_2 d^{-1}; similar range to reported by Fang et al. [35] (0.03 to 1.37 mmol H_2 d^{-1}). The maximum hydrogen production rate obtained was 6.6 mmol H_2 d^{-1} L^{-1}, the specific hydrogen production rate was 0.66 mmol H_2 d^{-1} g-VSS^{-1}.

3.4 Influence of pH in the hydrogen production

The decrease in hydrogen production rates is related to the long lag phase that occurred during the tests; for all the cases the activation time "lambda" was comprised between 1 and 177 h. The longer latency phases were associated with pH changes that occurred during the experimentation, these changes pH depended on the formation of ammonium in the medium. The concentrations of ammonia nitrogen formed at the end of the test ranged between 0.4 and 10.8 mg L^{-1}, inhibition for this compound is reported from concentrations of 0.36 mg L^{-1} [14,32]. Have been reported to excess of ammonia and other nitrogen compounds usable repress the synthesis of nitrogenase in purple non sulfur bacteria [36]. The first increase pH occurred at 144 h after the test started. The pH increased in average of 6.8 at 7.1 -8.7. A very high pH values was decrease

and even inhibited hydrogen production. Optimal pH reported for the production of hydrogen is 6.8-7.5 [12, 13] thus pH was adjusted to 6.8 with 1N HCl twice at 144 and 288 h after test started.

4. Conclusions

Effect of volatile fatty acids on hydrogen production by Phototrophic Hydrogen-Producing Mixed Bacterial Consortium was investigated in batch tests and the optimization was done by response surface methodology with a Box-Benhken and Compost Central designs. It was observed an inhibition of hydrogen production by the propionic acid. Also was presented the decrease in hydrogen production with the increase pH that occurred in the tests during the experimentation. The ammonium formation was responsible for pH changes. The concentrations of ammonia nitrogen formed at the tests ranged between 0.4 and 10.8 mg L^{-1}. The results showed that a maximum hydrogen production rate of 0.65 mmol H_2 d^{-1} and maximal amount of hydrogen produced of 10.88 mmol H_2 were obtained when the concentrations of acetic acid, propionic acid and butyric acid were 1200 mg L^{-1}, 715 mg L^{-1} and 1571 mg L^{-1}, respectively.

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