Optimization of biohydrogen production by the novel psychrophilic strain N92 collected from the Antarctica

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Abstract

In this study, the response surface methodology (RSM) and central composite design (CCD) were employed to improve the hydrogen production by the psychrophilic N92 strain (EU636058) isolated from Antarctica, which is closely related to *Pseudorhodobacter* sp. (KT163920). The operational conditions such as temperature (4.7-55.2°C), initial pH (3.44-10.16), and initial glucose concentration (4.7-55.23 g/dm^3), as well as the initial concentrations of $(NH_4)_2SO_4$ (0.05-3.98 g/dm^3), $FeSO_4$ (0.02-1.33 g/dm^3) and $NaHCO_3$ (0.02-3.95 g/dm^3) were evaluated. The linear effect of glucose concentration, along with the quadratic effect of all the six factors were the most significant terms affecting the biohydrogen yield by N92 strain. The optimum conditions for the maximum hydrogen yield of 1.7 mol H_2/mol glucose were initial pH of 6.86, glucose 28.4 g/dm^3, 29°C and initial concentration of $(NH_4)_2SO_4$, $FeSO_4$ and $NaHCO_3$ of 0.53, 1.55 and 1.64 g/dm^3, respectively. Analysis of the metabolites produced under the optimum conditions showed that the most abundant were acetic acid (0.8 g/dm^3), butyric acid (0.7 g/dm^3) and ethanol (2.1 g/dm^3). We suggest that the bioprocess established in this study using the strain N92 could be an alternative for hydrogen production with the advantages of constituting low energy costs in fermentation.

Keywords: Biohydrogen; Central composite design; Dark fermentation; Psychrophilic bacteria; Response Surface Methodology.

1. Introduction.

Hydrogen is considered as an attractive future energy carrier and it is preferred over biogas or methane because hydrogen is not chemically bound to carbon, and therefore, its burning does not contribute to greenhouse gases or acid rain [1]. There are several approaches to produce hydrogen, among them; biological methods offer different advantages in contrast to chemical methods, which are energy-intensive and expensive. These approaches mainly include photosynthetic and dark fermentative hydrogen production. However, dark fermentation has advantages over other processes because of its ability to continuously produce hydrogen from a number of renewable feedstocks [2]. Nowadays most of the fermentative hydrogen production processes are focused on the use of mesophilic and thermophilic microorganisms and there are few reports available addressing psychrophilic bacteria [3-5]. The use of psychrophilic hydrogen producing microorganisms could be an economical advantage due to its operation temperatures. These microorganisms have high enzymatic activities and catalytic efficiencies in the 0-20°C temperature range in which homologous mesophilic enzymes are less active, and allow to renounce on expensive heating/cooling systems, thus constituting a considerable progress towards the saving of energy [6]. Therefore, the aim of this experimental work was the production of hydrogen using a newly psychrophilic N92 strain isolated from Antarctica [7]. Since there is insufficient information about the operational conditions for psychrophilic hydrogen production, we have applied the response surface methodology to set the optimal operation conditions and media composition to reach the maximum hydrogen production. In this context, temperature, pH and substrate concentration are important factors influencing the activity of bacteria towards hydrogen production.

Moreover, temperature is a key factor since it might alter process efficiency, hydrogen production activity, and liquid product distribution by influencing the bacterial enzymatic activity. Kumari and Das [8] reported that an initial pH in an inadequate range affects the activity of the hydrogenase enzymes as well as an inadequate initial substrate concentration affects metabolic pathways decreasing the production of biohydrogen. On the other hand, the media composition is of primary importance, particularly the concentrations of nitrogen and iron, essential nutrients for hydrogen production, as well as buffer supplementation [9]. Low or high concentration of these nutrients may cause low hydrogen yields. Therefore, in this work the effects of these operational factors (temperature, pH and substrate concentration) and mineral nutrient concentration (ammonia, carbonate and ferrous ion) on hydrogen production were studied using two central composite designs to obtain optimal hydrogen production conditions by the psychrophilic N92 strain.

2. Material and Methods.

2.1 Microorganism and growth media.

In this work, the strain N92 (EU636058) highly related to *Pseudorhodobacter* sp. (KT163920) according to NCBI blast was used. It was isolated from samples of glacier sediment from Antarctica [7]. The strain was grown in YPG agar plates in g/dm^3 (2.75 of Bacto-tryptone, 0.25 of yeast extract, 25 of glucose and 15 of Bacto-agar) and maintained at 4°C [4].

2.2 Experiment designs

The first central composite design with two center points was implemented to optimize the temperature, initial pH and initial glucose concentration to maximize biohydrogen yield by batch cultures fermentations of N92 strain (Table 1) [10] .

A second order polynomial mathematical model (Equation 1) was proposed to describe the effects of several factors on the response based on experimental results.

$$Y_i = \beta_0 + \sum_{i}^{\beta} x_i + \sum_{ii}^{\beta} x_i^2 + \sum_{ij}^{\beta} x_i x_j \quad (1)$$

Where Y_i is the corresponding response, x_i and x_j are the independent variables, β_0 is the model intercept, βi are the linear coefficients, βii are the squared coefficients and βij are the interaction coefficients [9]. In addition, the analysis of variance (ANOVA) was used to obtain the relationship between independent variables and the response, as well as to describe the effects of several factors on the response based on the experimental results by using a second order polynomial model. The statistical software, Design-Expert 7.0.0 version (Stat-Ease, Inc., Minneapolis, MN, USA) was used to performance the regression analysis and the response surface analysis [11].

Furthermore, a second central composite design with two center points was used to optimize the culture medium with the objective of increasing the biohydrogen production by dark fermentation using N92 strain (table 2). ANOVA was used to obtain the relationship between independent variables and to describe the effects of various factors on the response based on experimental results of a second order polynomial model (Equation 1) [12-14].

2.3 Batch Fermentation Experiments

The batch fermentations were carried out in 0.120 dm^3 serum bottles. Silicone rubber stoppers were used to avoid gas leakage from the bottles [3]. The mineral medium used in the first experimental design to evaluate the influence of initial pH, temperature and initial glucose concentration consisted of the following composition in g/dm^3: 3 of KH_2PO_4, 7 of K_2HPO_4, 1 of $MgSO_4$, 0.39 of $FeSO_4\,7H_2O$, 3 of yeast extract and 0.5 of bacto-tryptone[14].

In the second experimental stage, in order to evaluate the effect of the concentration of $FeSO_4{\cdot}7H_2O$, $(NH_4)_2SO_4$ and $NaHCO_3$, the medium used for hydrogen production experiments was the same as the one used in the first experimental design without the addition of $FeSO_4{\cdot}7H_2O$, since this was tested in the experimental design. All bottles in both experimental designs were inoculated with 0.5 OD_{600nm} of N92 strain [12, 15].

2.4 Analytical Methods

The biogas produced was determined at room temperature (25°C) by displacement of acid water (pH=2) [16]. The percentage of hydrogen in the biogas accumulated in the headspace of serum bottles was measured by Gas Chromatography as described elsewhere [16]. The pH value was obtained by Thermo Orion 8103BN, Waltham, MA. Remaining glucose and fermentation end products (succinic acid, lactic acid and acetic acid) were analyzed by High Performance Liquid Chromatography (HPLC, Infinity LC 1220 Agilent Technologies, Santa Clara, CA, USA) using a column Phenomenex Rezex ROA (Phenomenex, Torrance, CA, USA) at 60°C and using 0.0025 M H_2SO_4 as mobile phase at 0.55 cm^3/min. ethanol, acetoin, propionic acid and butyric acid were analyzed in a Gas Chromatograph 6890N (Agilent Technologies, Wilmington, DE, USA) using a capillary column HP-Innowax with the following dimensions (30 m X 0.25 mm i.d. X 0.25 m film thickness; Agilent Technologies, Wilmington, DE, USA). Temperatures of the injector and flame ionization detector (FID) were 220 and 250°C respectively. Helium was used as carrier gas at a flow rate of 25

133 cm^3/min. The analyses were performed with a split ratio of 5:1 and a temperature program of
134 25°C for 10 min to 280°C, and was maintained at this temperature for a final time of 10 min
135 [3].
136
137 **3. Results and discussion**
138 *3.1 Optimization of operational conditions*
139 Response surface methodology was adopted to investigate and optimize the effect of process
140 variables on biohydrogen production yield. Applying multiple regression analysis to the
141 experimental data, the following mathematical second order model was established to explain
142 the biohydrogen yield as a function of the independent variables within the region under
143 investigation, expressed by the equation 2.
144 $$Y = 0.66 - 0.020X_1 + 8.515e^{-3}X_2 - 0.049X_3 - 0.011X_1X_2 + 0.017X_1X_3 -$$
145 $$4.374e^{-3}X_2X_3 - 0.23X_1^2 - 0.23X_2^2 - 0.17X_3^2 \quad (2)$$
146 The code of the variables of model equation corresponds to temperature (X_1), initial pH (X_2),
147 and initial glucose concentration (X_3) along with the experimental values of the biohydrogen
148 yield. In table 3 is shown the ANOVA conducted to test the significance of the fitting model
149 along with the linear, quadratic, and interactive effects of the variables. The *p*-values were
150 used to check the significance of each variable, also to indicate the strength of the interaction
151 between each independent variable. The *p* values (probability > F) lower than 0.05 indicate
152 that model terms are significant, while *p* values greater than 0.05 indicate that the model terms
153 are insignificant. The model *p* value of 0.0011 implies that the model was significant. Table 3
154 shows the model F value of 18.1, which indicates an adequate description of the variation

about its mean. The coefficient of determination R^2 was 0.9645, indicating that the model could explain 96.45% variability of the response variable and that the mathematical model is reliable to estimate the predicted values.

Figure 1 shows 3D response surface plots and 2D contour plots depicting the interactions between pairs of variables keeping the third variable at its optimum level for biohydrogen yield. The shape of the contour plot explicitly demonstrates the mutual or combined effect of the independent variables on the response. A clear peak point can be found in each response surface plot, which indicates that the maximum biohydrogen yield could be achieved inside the design boundary of all three variables.

The effect of temperature in dark fermentation on the production of biohydrogen was analyzed according to the ANOVA, showing that only in the quadratic terms of the polynomial mathematical model showed significant effect with a lower *p* value of 0.05 (table 3). In the figures 1b and 1d the contour plots of temperature with respect to the initial pH and initial glucose concentration showed that the temperature in both variables has an interactive effect on the biohydrogen yield due to the circular shape that is shown in the plots. The response surface plots of the figures 1a and 1c show that at low temperatures of 15°C both variables have a negative effect on the anaerobic fermentation using the strain N92, showing the lowest yield of biohydrogen. The gradual increase of the temperature in a range from 15 to 30°C resulted with gradual increase in the production of biohydrogen reaching the maximum production of biohydrogen at a temperature of 29.3°C, from this value the gradual increase in temperature caused a gradual decrease in the biohydrogen yield having the lowest of value at 45°C. This behavior exhibited by the N92 strain can be attributed to psychrophilic nature of bacteria which has the ability to ferment sugars at low temperatures and produce biohydrogen,

however the biohydrogen production is low, this is due to the fact that it has been shown that incubation temperature dramatically affects the growth rate of bacteria, since it affects the rates of all cellular reactions, the metabolic patterns, the nutritional requirements and the composition of bacterial cells [17]. The increase in biohydrogen yield corresponds to the increase in temperature, which can be explained as a positive effect on the hydrolysis of the complex particles. It has also been demonstrated that an increase in temperature produces an increase in the hydrogen production because the increment of temperature doubles the enzymatic activity every 10°C until reaching the optimum temperature [17]. Above this value the enzymatic activity decreases rapidly. Other studies performed by Niu et al. [18] concluded that higher temperature, such as 37°C, could inhibit the expression of the uptake hydrogenase, as well as stimulate the expression of H_2 evolving hydrogenase.

The pH is often one of the most important factors influencing the performance of the fermentation process for the biohydrogen production. In this study regarding to the mathematical model, the linear effect and the interaction between the variables of temperature and initial concentration of glucose according to the ANOVA showed no significant effect since values of *p* are greater than 0.05. In terms of the quadratic model, this variable showed a significant effect according to the ANOVA (table 3).

The response surface plots (figures 1a and 1e) show that the biohydrogen yield increases with the increment of initial pH from 4.8 to 6 in both variables. Reaching the highest increase in biohydrogen yield at a value of pH of 6.8, the decrease in biohydrogen yield is shown from higher values of pH. Changes in external pH values also affect several physiological parameters in cells such as the proton motive force and membrane potential [19]. In this study at pH values below 4.8 the lack of hydrogen production may be due to the extremely acidic

201 microenvironment pH <4.5 was detrimental to the ability of the bacteria to produce
202 biohydrogen as reported in other studies [20].

203 While at a value of pH 8.8 alkaline microenvironment is presented and fermentative pathways
204 are prone to solventogenesis [21]. Other studies mention that hydrogenase enzyme activity
205 gets inhibited by maintaining low or high pH beyond the optimum range [22]. The optimal pH
206 value of 6.8 obtained in this study is in the optimal range for other biohydrogen producing
207 bacteria that is between 6.0-6.8. In this pH range it has been reported that it might be
208 beneficial due to the prevention of solventogenesis [23, 24].

209 The initial glucose concentration was evaluated by the ANOVA showing that linear and
210 quadratic effects were significant, since values of p <0.05 were obtained (table 3). In figures
211 1c and 1e the response surface plots show that at low glucose concentrations of 15 g/dm^3,
212 temperature of 15°C and initial pH of 4.8 the yield of biohydrogen had the lowest level. As
213 the glucose concentration increased, the biohydrogen yield increased reaching its maximum
214 value at an initial glucose concentration of 28.4 g/dm^3. The increase in biohydrogen yield
215 with the increase in initial glucose concentration may be due to the fact that it has been
216 reported by Wu and Lin [25], that in an appropriate range, increasing of substrate
217 concentration could increase the ability of bacteria to produce biohydrogen. In our study, it is
218 shown that from the optimal concentration of 28.4 g/dm^3, the increase in the glucose
219 concentration caused a decrease in the yield of biohydrogen. Furthermore, studies show that
220 high substrate concentrations become inhibitory to the microorganisms as a result of a pH
221 drop and hydrogen pressure increase [26, 27]. Prakasham et al. [28], also reported that higher
222 concentrations of glucose can also negatively impact on biohydrogen production.

223 *3.2 Optimization of nutrient formulation for biohydrogen production by strain N92*

The effect of the nutrients concentration levels added to the formulation was evaluated using a central composite design with two center points. From regression analysis of the experimental results, a second order polynomial model for biohydrogen yield Equation 3 was obtained.

$$Y = 1.51 + 0.092X_4 + 0.075X_5 + 0.13X_6 + 0.050X_4X_5 + 5.732e^{-3}X_4X_6 + 0.057X_5X_6 - 0.39X_4^2 - 0.47X_5^2 - 0.36X_6^2 \quad (3)$$

Where Y is the biohydrogen yield, X_4 is the initial $FeSO_4$ concentration, X_5 is the initial $(NH_4)_2SO_4$ concentration and X_6 is the initial $NaHCO_3$ concentration.

In table 4 the ANOVA demonstrates that the second order model for biohydrogen yield is highly significant as evident from the calculated F value of 7.05 and a very low probability value p model <F=0.05.

In the table 4 the p values for each factor $(NH_4)_2SO_4$ and $FeSO_4$ concentration and their corresponding interaction were greater than 0.05 indicating that these factors have no significant effect on biohydrogen yield. However, in the quadratic terms of the model, both factors showed that p <0.05 have a significant effect on the biohydrogen yield.

In figures 2b, 2d and 2f the contour plots of both factors show elliptical shapes indicating the mutual interactions between $NaHCO_3$ and $FeSO_4$.

In figure 2a, response surface plot shows that the biohydrogen yield decreases when the $(NH_4)_2SO_4$ and $FeSO_4$ concentrations are presented in the lowest level being these 0.05 g/dm^3 and 0.02 g/dm^3 respectively.

The hydrogen yield increased as the $(NH_4)_2SO_4$ and $FeSO_4$ concentration increased, reaching their maximum yield at a concentration of 1.57 and 0.56 g/dm^3 respectively. From this

concentration, the increase in concentration caused a significant decrease in biohydrogen yield.

According to the results obtained at the concentration of $FeSO_4$ 0.02 g/dm^3, this condition did not favor the dark fermentation by strain N92 for biohydrogen production, since the yield showed the lowest value. However, the gradual augmentation of $FeSO_4$ favored the fermentation as the biohydrogen yield increased until reaching the maximum.

The increase in biohydrogen production may be attributed to the fact that Fe^{+2} increases the activity of hydrogenases, since Fe^{+2} is the metal in the catalytic center of hydrogenases which are responsible to catalyze the oxidation of hydrogen or the reduction of proton [29].

Others studies carried out by Wang et al. [30] showed that the cumulative hydrogen quantity in batch tests increased with increasing Fe^{+2} concentrations from 0 to 300 mg/dm^3, however, when the Fe^{+2} concentrations were higher than 300 mg/dm^3, the cumulative hydrogen quantity tended to decrease with increasing Fe^{+2} concentrations. Several studies have shown that suitable concentration of Fe^{+2} ranges were able to enhance the biohydrogen yield by the mixed cultures, while much lower or much higher Fe^{+2} concentrations than the suitable one are not favorable to raise the biohydrogen yield [30].

From the optimum Fe^{+2} concentration, the increase in concentration caused an inhibition during dark fermentation since the biohydrogen yield decreased significantly, it has been reported that in an excess concentration of ferrous iron exerts a slight inhibitive influence on hydrogen production.

Related reports carried out by Ding et al. [31] studied the effect of the Fe^{+2} concentrations ranging from 0 to 1473.7 mg/dm^3 on the fermentative hydrogen production from glucose by

mixed cultures, obtaining the maximum hydrogen yield at the Fe^{+2} concentration of 200 mg/dm^3.

The results obtained in this study show that the addition of the lower concentration levels of $(NH_4)_2SO_4$ does not increase the biohydrogen yield. However, the increase in $(NH_4)_2SO_4$ concentration showed a positive effect on the fermentation by strain N92, as the biohydrogen yield increased to reach the maximum. But from this ammonium concentration, the increase caused a gradual decrease of the yield until reaching the lowest levels of biohydrogen production.

This behavior is similar to that described by several studies, demonstrating that in an appropriate concentration range, ammonia nitrogen is beneficial to fermentative biohydrogen production, while at a much higher concentration, ammonia nitrogen could inhibit fermentative hydrogen production, for it may change the intracellular pH of hydrogen producing bacteria, increase the maintenance energy requirement for hydrogen producing bacteria or inhibit specific enzymes related to fermentative biohydrogen production [32].

Table 4 shows *p* values for the carbonate and ferrous iron and their interaction, only the linear and quadratic terms of the model for the $NaHCO_3$ concentration had a significant effect since the values of $p > 0.05$ for the linear interaction between the two factors has no significant effect ($p>0.05$) on the biohydrogen yield. The response surface plot in figure 2c shows the interactive effect of these two factors on biohydrogen yield.

With the $FeSO_4$ and $NaHCO_3$ concentration at levels -1 (0.02 g/dm^3 and 0.02 g/dm^3, respectively), the biohydrogen yield decreased below 0.52 mol H_2/mol glucose .The maximum biohydrogen yield obtained in the optimum condition was 1.52 mol H_2/mol glucose

289 in $NaHCO_3$ and $FeSO_4$ concentrations of 1.65 g/dm^3 and 0.5 g/dm^3 respectively, from this
290 concentration the increase in ferrous iron and carbonate concentration did not favor the
291 fermentation by N92 to increase the biohydrogen yield, conversely caused the biohydrogen
292 yield gradually decreased reaching the minimum at concentrations obtained in the level -1.
293 Regarding the results obtained, the carbonate in suitable concentrations has a significant effect
294 on biohydrogen production since it has been shown in several studies that the addition of
295 carbonate is used to maintain the pH of 6.8, by neutralizing organic acids formed during
296 fermentation and maintaining the necessary pH conditions in microorganisms environment,
297 and increasing the biohydrogen production [33]. Other studies mention that the addition of
298 carbonates restored the growth of the bacteria [34].
299 The increase in carbonate concentration, followed by the optimum concentration showed a
300 decrease of biohydrogen since it has been mentioned that an increase in carbonate
301 concentration in the feed increases the carbon dioxide concentration because of carbonate
302 dissolution and therefore decreased the hydrogen content in the gas phase [12].

303 The interaction between carbonate and ammonium on biohydrogen production is shown in the
304 table 4. The *p* value on interaction of both factors was greater than 0.05 indicating that both
305 factors had no significant effect on biohydrogen yield. The effect of both factors is shown in
306 figure 2e, showing that at the extreme levels -1 and +1 (table 2) the biohydrogen yield
307 decreased below 0.62, while at level 0 this increased to reach the maximum biohydrogen yield
308 using $NaHCO_3$ and $FeSO_4$ 1.73 g/dm^3 and 1.43 g/dm^3 respectively.

309 *3.3 Metabolites produced during dark fermentation by strain N92*

310 Biohydrogen production is accompanied by the production of metabolites such as volatile
311 fatty acids (VFAs) and solvents during anaerobic digestion. The analysis of the metabolic

products of the second experimental design of optimization is shown in figures 3 and 4. The values average concentration in (g/dm^3) of VFAs and solvents of different experimental treatment were: 1.19 acetic acid, 1.06 butyric acid, 0.27 succinic acid, 0.27 propionic acid, 1.57 ethanol and 0.43 acetoin.

The formation of VFAs obtained from the acidogenic pathway of pyruvate showed that the metabolic activity presented by the N92 bacterium is oriented to two metabolic reactions for biohydrogen production, which are that of acetic acid and butyric acid.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$$ Acetic acid pathway

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$$ Butyric acid pathway

Studies report that the accumulation of both acetic and butyric acid caused the greatest decrease in biohydrogen yield [35]. Hüsemann et al. [36] found that the undissociated acetic acid concentration did not correlate with the initiation of solventogenesis but undissociated butyric acid did. However, there is no general agreement on why butyric acid is more toxic than acetic acid, but likely it is a consequence of NAD^+ regeneration [35].

Studies show that accumulation of VFAs such as succinic acid and propionic acid beyond a certain level inhibits cell growth, since it has been shown that the presence of these acids are able to cross the cell membrane at a low pH and then dissociate in the cell at the higher cytoplasm pH releasing a proton inside the cell [37, 38]. The uptake of protons in this way uncouples the proton motive force, which causes an increase in maintenance of energy requirements to maintain the intracellular pH near to neutrality. The uptake of acid also causes a decrease in the available coenzyme A and phosphate pools which decreases the flux of glucose through glycolysis [39].

334 The accumulation of ethanol and acetoin produced by N92 strain in our study can affect the
335 production of biohydrogen since studies have reported that the appearance of these solvents
336 produced during the dark fermentation can cause the inhibition of the enzyme hydrogenase by
337 carbon monoxide diverting reducing equivalents from H_2 a major electron sink to solvent
338 production [40, 41]. Other studies have reported that the presence of alcohols on bacteria
339 causes chaotropic effects on the membrane structure due to perturbation of the orderly array
340 of the fatty acid side chains of the phospholipids, that affect the ability of the cells to retain
341 and exclude electrolytes and nonelectrolytes [42] .

342 *3.4 Experimental validation*

343 An experimental validation was conducted to check the effectiveness of optimal conditions of
344 pH, temperature, concentration of glucose and compound formulation obtained. The
345 experiments were performed in duplicate showing the results in table 5. The results showed
346 that the maximum biohydrogen yield of 1.7 mol H_2/mol glucose was obtained under
347 optimization conditions.

348 The table 6 show hydrogen yields from others fermentation processes using cultures of
349 microorganisms mesophilic and thermophilic are reported in a range between 0.85 and 4.0
350 mol H_2/mol hexose [43-49]. Comparing the yields obtained in this study with respect to those
351 reported (table 6), the yield obtained from our study is in a mean value of the reported range,
352 however these studies were performed at temperatures above the optimum of fermentation
353 process constituting for our fermentation process an energetic advantage since the reactor can
354 be operable at room temperature reducing the operational costs of biohydrogen production
355 process, and making up an alternative process for those cold countries.

4. Conclusions

In this study, operational conditions initial pH, glucose concentration levels, temperature and initial nutrients in growth medium for enriching biohydrogen produced by N92 strain were optimized using a central composite design with two center points. Optimal conditions for biohydrogen production were estimated to be a temperature of 29.0 °C, initial pH of 6.86 and glucose concentration of 28.4 g/dm^3. The optimum fermentation medium of nutrients were 1.64 g/dm^3 $(NH_4)_2SO_4$, 0.53 g/dm^3 $FeSO_4$ and 1.55 g/dm^3 of $NaHCO_3$. The maximum biohydrogen yield obtained under these optimum conditions was 1.7 mol H_2/mol glucose comparable to those reported for mesophilic and thermophilic microorganisms. Therefore, the results of our research indicate that this dark fermentation process with N92 strain has the potential to be used with agroindustrial residues as carbon source for biohydrogen production, with the advantage that it can be carried out at room temperature constituting a greater energy efficiency of the process.

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Figure caption

Figure 1. Response surface plots and contour plots showing the interactive effect of operational conditions on biohydrogen yield.

Figure 2. Distribution of final VFAs produced in different treatments.

Figure 3. Solvents released from anaerobic fermentation by N92 strain at different treatments.

Figure 4. Response surface plots and contour plots showing the interactive effect of different concentrations of $(NH_4)_2SO_4$, $NaHCO_3$ and $FeSO_4$ on biohydrogen yield.

Figure 5. Organic acid produced in the dark fermentation by N92 strain by effect of the different concentrations of $(NH4)_2SO_4$, $NaHCO_3$ and $FeSO_4$.

Figure 6. Ethanol and acetoin produced in the dark fermentation by N92 strain by effect of the different concentrations of $(NH_4)_2SO_4$, $NaHCO_3$ and $FeSO_4$.

Table 1. Experimental design showing the operational factors and their levels.

Independent variables	Levels		
	-1	**0**	**1**
X_1**-Temperature (°C)**	15	30	45
X_2**-pH (-)**	4.8	6.8	8.8
X_3**-Glucose concentration (g/dm^3)**	15	30	45

Table 2. Experimental design showing independent variables corresponding to the medium composition and their levels.

Independent variables	Levels		
	-1	**0**	**1**
X_4-$FeSO_4$ (g/dm^3)	0.02	0.51	1
X_5-$(NH_4)_2SO_4$ (g/dm^3)	0.05	1.515	2.98
X_6-$NaHCO_3$ (g/dm^3)	0.02	1.485	2.95

573 **Table 3**. ANOVA of the fitting model of the experimental response at various levels of

574 temperature, pH and glucose concentration.

Source	Sum of squares	df	Mean Square	*F* value	*p*-value
Model	0.75	9	0.083	18.1	0.0011
X_1-Temperature	5.25E-03	1	5.25E-03	1.14	0.3263
X_2-pH	9.90E-04	1	9.90E-04	0.22	0.6589
X_3-Glucose concentration	0.033	1	0.033	7.15	0.0368
X_1X_2	1.05E-03	1	1.05E-03	0.23	0.6495
X_1X_3	2.20E-03	1	2.20E-03	0.48	0.5152
X_2X_3	1.53E-04	1	1.53E-04	0.033	0.8612
X_1^2	0.49	1	0.49	106.62	< 0.0001
X_2^2	0.49	1	0.49	106.62	< 0.0001
X_3^2	0.28	1	0.28	60.05	0.0002
Residual	0.028	6	4.60E-03		
Lack of Fit	0.028	5	5.51E-03	634.15	0.0301
Pure Error	8.69E-06	1	8.69E-06		
Total	0.78	15			

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589 **Table 4.** ANOVA of the fitting model of the experimental response at various levels of

590 $FeSO_4$, $(NH_4)_2SO_4$ and $NaHCO_3$ concentrations.

Source	Sum of Squares	df	Mean Square	*F* value	*p*-value
Model	3.12	9	0.35	7.05	0.0137
X_4-$FeSO_4$	0.11	1	0.11	2.33	0.1778
X_5-$(NH_4)_2SO_4$	0.077	1	0.077	1.57	0.2567
X_6-$NaHCO_3$	0.25	1	0.25	5.01	0.0664
X_4X_5	0.02	1	0.02	0.41	0.5436
X_4X_6	2.63E-04	1	2.63E-04	5.35E-03	0.9441
X_5X_6	0.026	1	0.026	0.53	0.4959
X_4^2	1.42	1	1.42	28.81	0.0017
X_5^2	2.09	1	2.09	42.48	0.0006
X_6^2	1.21	1	1.21	24.67	0.0025
Residual	0.3	6	0.049		
Lack of Fit	0.29	5	0.059	215.33	0.0517
Pure Error	2.74E-04	1	2.74E-04		
Total	3.42	15			

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Table 5. Biohydrogen yield coefficients and metabolic products of the fermentation at the optimal conditions.

		Volatile fatty acids (g/dm^3)				Solvent (g/dm^3)	
Run	**Biohydrogen yield (mol H_2/mol glucose)**	**Acetic acid**	**Propionic acid**	**Butyric acid**	**Succinic acid**	**Ethanol**	**Acetoin**
CE-1	1.66	0.856	0.31	0.715	0.297	2.121	0.497
CE-2	1.67	0.832	0.302	0.691	0.289	2.252	0.51

622 **Table 6.** Comparison of biohydrogen production with respect to other mesophilic and
623 thermophilic fermentative anaerobic processes in batch mode using glucose as substrate.

Microorganism	T (°C)	Maximum Biohydrogen yield (mol H_2/mol glucose)	Reference
Thermotoga maritima	80	4	(Schröder et al., 1994)
Thermotoga elfii	65	3.3	(Van Niel et al., 2002)
***Escherichia coli* MC13-14**	37	1.2	(Ishikawa et al., 2006)
Sewage sludge	40	1.75	(Wu et al., 2005)
Soybean meal	35	0.85	(Mizuno et al., 2000)
Clostridium tyrobutyricum	35	1.47	(Lin et al., 2007)
Klelsiella oxytocin	35	1	(Minnan et al., 2005)
N92	29	1.7	This study

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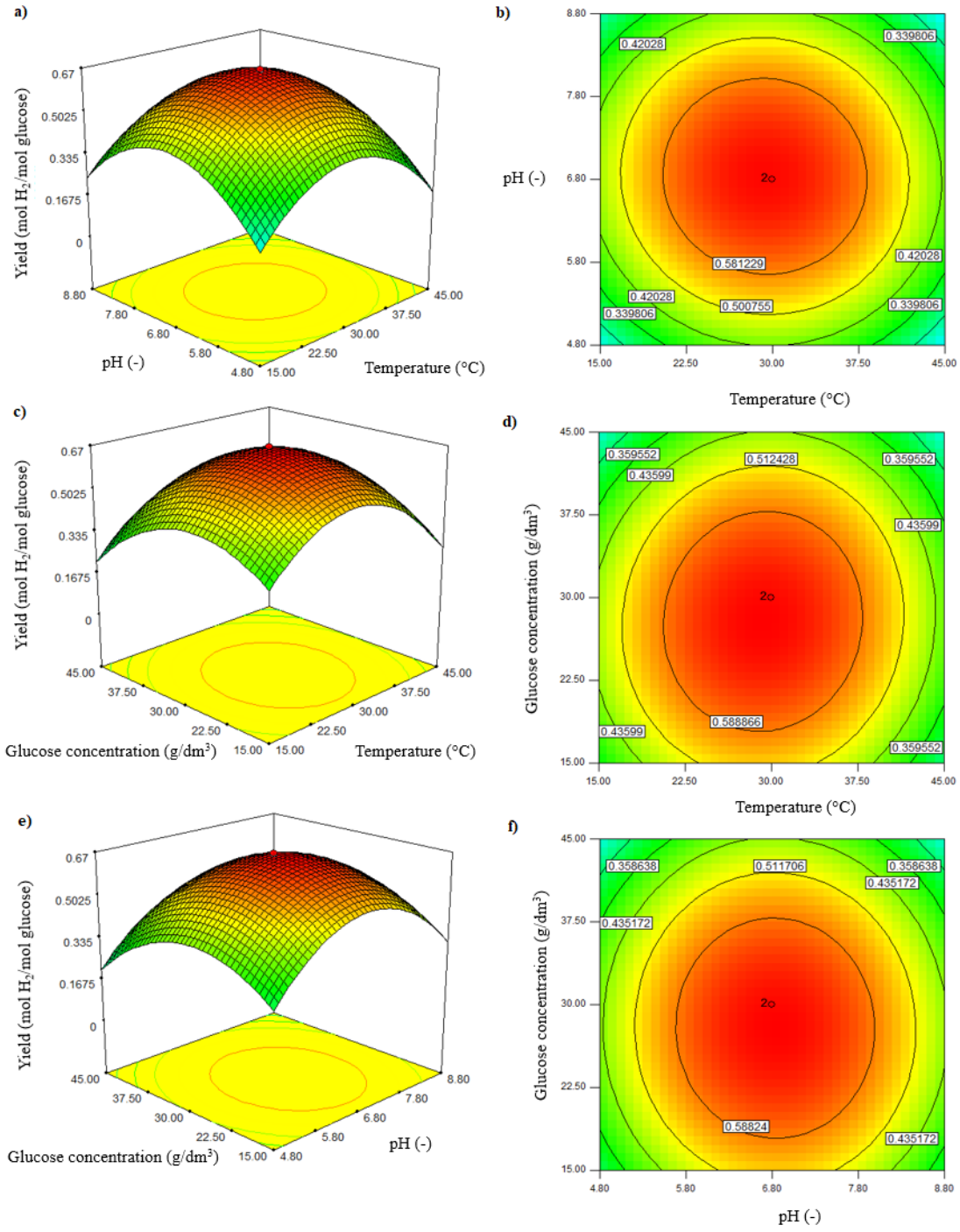
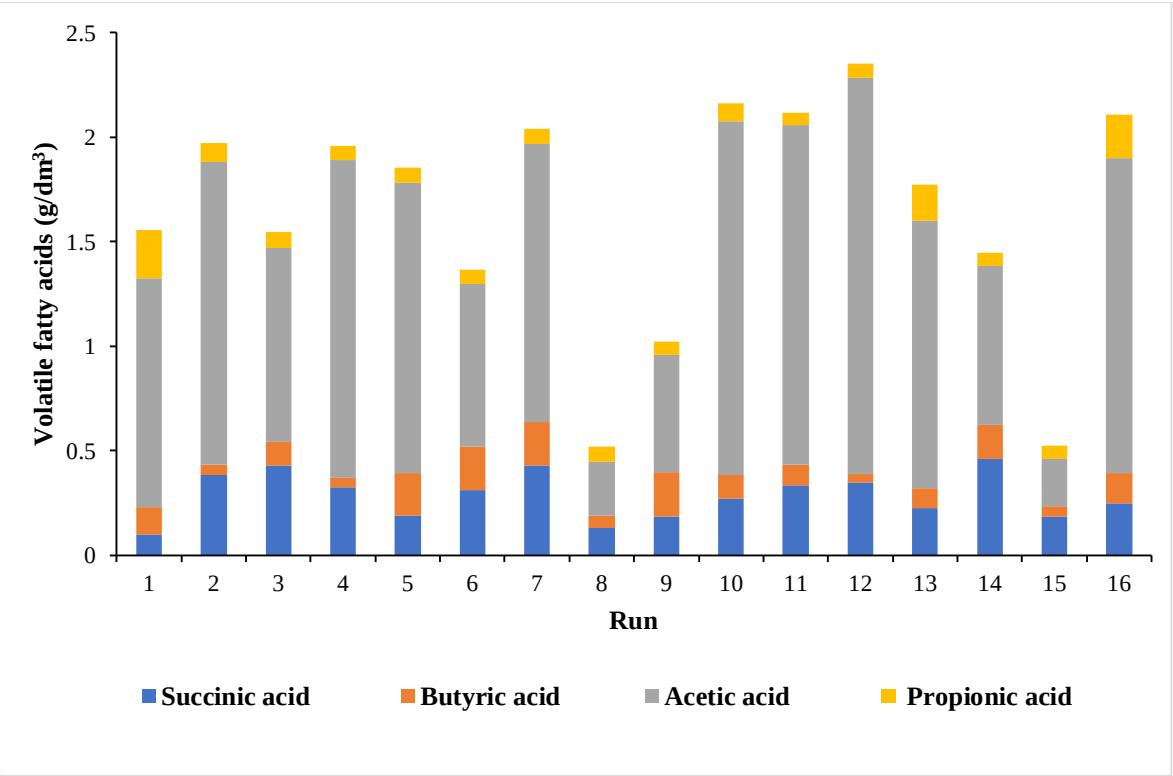

a)
Yield (mol H2/mol glucose)
0.67
0.5025
0.335
0.1675
0
pH (-)
8.80
7.80
6.80
5.80
4.80
Temperature (°C)
15.00
22.50
30.00
37.50
45.00
b)
pH (-)
Temperature (°C)
0.42028
0.339806
0.581229
0.500755
2o
c)
Glucose concentration (g/dm3)
d)
Glucose concentration (g/dm3)
0.359552
0.512428
0.43599
0.588866
e)
pH (-)
f)
0.358638
0.511706
0.435172
0.58824

Fig. 1


2.5
2
1.5
1
0.5
0
Volatile fatty acids (g/dm³)
1
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4
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15
16
Run
Succinic acid
Butyric acid
Acetic acid
Propionic acid

Fig. 2

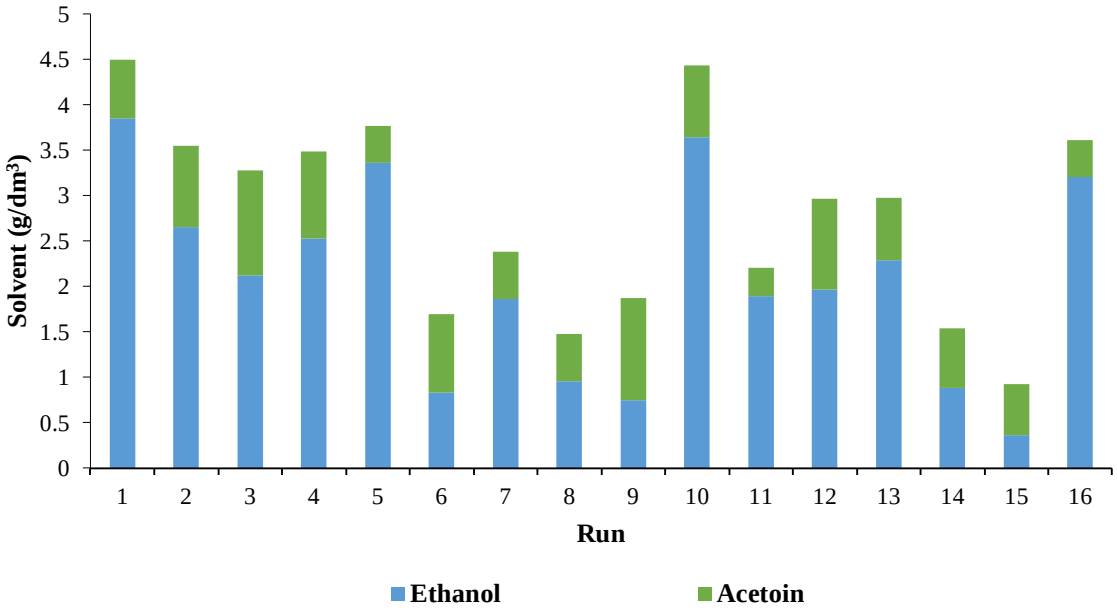
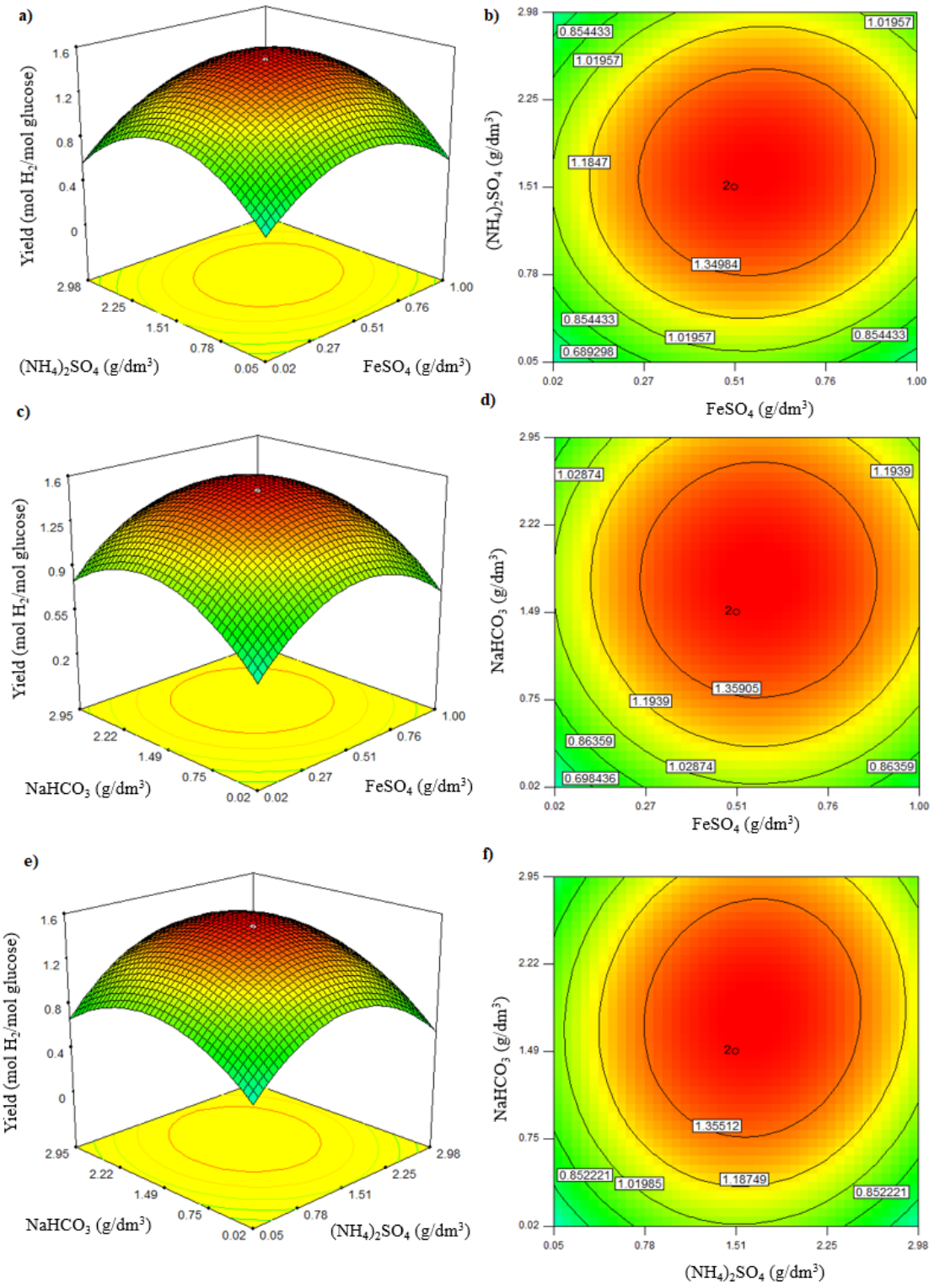

Solvent (g/dm3)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Run
Ethanol
Acetoin

Fig. 3


a)
Yield (mol H2/mol glucose)
1.6
1.2
0.8
0.4
0
2.98
2.25
1.51
0.78
0.05
(NH4)2SO4 (g/dm3)
0.02
0.27
0.51
0.76
1.00
FeSO4 (g/dm3)
b)
2.98
2.25
1.51
0.78
0.05
(NH4)2SO4 (g/dm3)
0.854433
1.01957
1.1847
2o
1.34984
0.689298
0.02
0.27
0.51
0.76
1.00
FeSO4 (g/dm3)
c)
Yield (mol H2/mol glucose)
1.6
1.25
0.9
0.55
0.2
2.95
2.22
1.49
0.75
0.02
NaHCO3 (g/dm3)
0.02
0.27
0.51
0.76
1.00
FeSO4 (g/dm3)
d)
2.95
2.22
1.49
0.75
0.02
NaHCO3 (g/dm3)
1.02874
1.1939
2o
1.35905
0.86359
0.698436
0.02
0.27
0.51
0.76
1.00
FeSO4 (g/dm3)
e)
Yield (mol H2/mol glucose)
1.6
1.2
0.8
0.4
0
2.95
2.22
1.49
0.75
0.02
NaHCO3 (g/dm3)
0.05
0.78
1.51
2.25
2.98
(NH4)2SO4 (g/dm3)
f)
2.95
2.22
1.49
0.75
0.02
NaHCO3 (g/dm3)
2o
1.35512
0.852221
1.01985
1.18749
0.05
0.78
1.51
2.25
2.98
(NH4)2SO4 (g/dm3)

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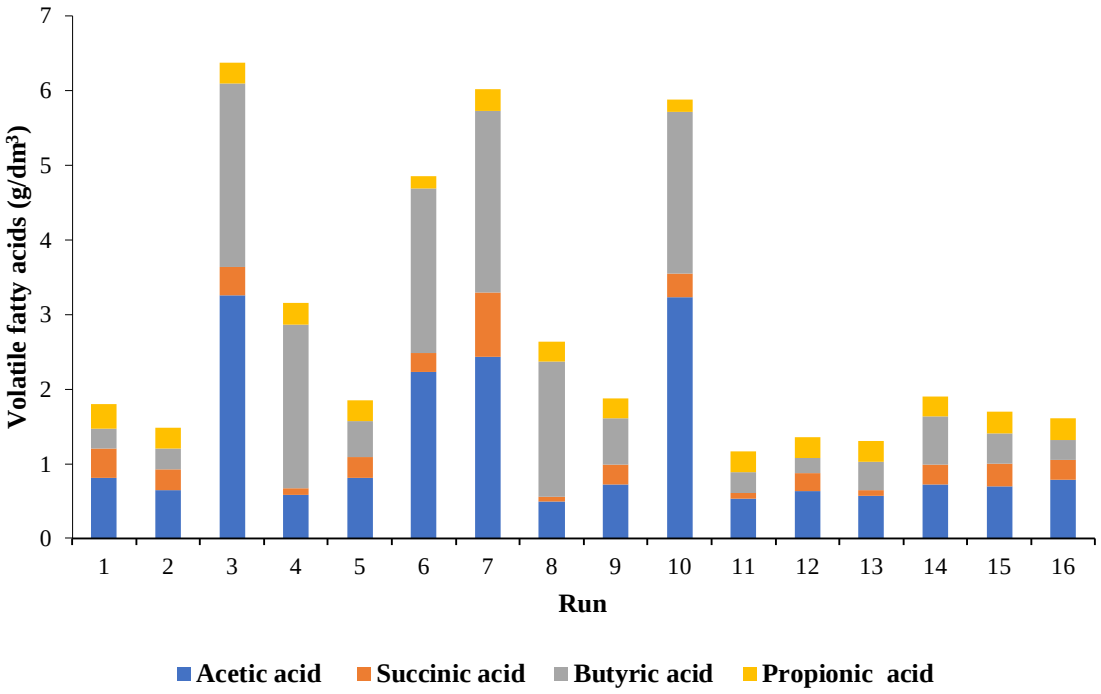
673 Fig. 4


Volatile fatty acids (g/dm³)
0
1
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4
5
6
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1
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5
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8
9
10
11
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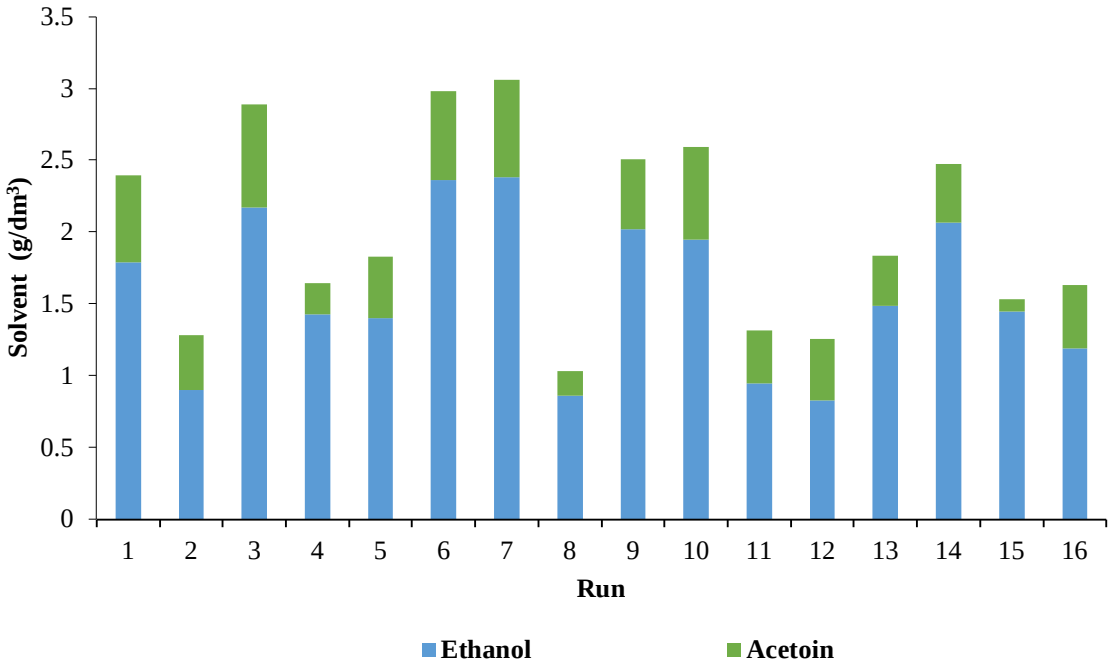
13
14
15
16
Run
Acetic acid
Succinic acid
Butyric acid
Propionic acid

Fig. 5


3.5
3
2.5
2
1.5
1
0.5
0
Solvent (g/dm3)
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Run
Ethanol
Acetoin

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689 Fig. 6