Biohydrogen production from mixtures of agro-industrial wastes: chemometric analysis, optimization and scaling up

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Abstract

Cheese whey (CW) and wheat straw hydrolysate (WSH) were used to produce biohydrogen by anaerobic co-digestion of multiple substrates. In this work, the influence of pH, temperature, substrates concentrations on the biohydrogen production was explored with the application of the principal component analysis (PCA) and the hierarchical clustering analysis (HCA), allowing the identification of the main clusters and the uniqueness of some experiments. Response surface methodology (RSM) was used to evaluate the individual and interactive effects of pH, temperature, CW concentration and WSH concentration in the fermentation. Optimal operational conditions obtained by RMS were 5 g L^{-1} WSH, 25 g L^{-1} CW, 26.6ºC and pH 7.25. With these conditions was expected 5,724.5 mL H_2 L^{-1}. When optimal conditions were tested using 0.11-L anaerobic serological bottles, 1-L and 4-L bioreactors the results obtained for biohydrogen production were 4,554.5 ± 105, 3,685 ± 305 and 4,132.3 ± 151 mL H_2 L^{-1}, respectively; on the other hand, the biohydrogen production rate was improved from 66.6 to 89.5 mL H_2 L^{-1} h^{-1}. Results demonstrate that it is possible to use WSH and CW, both individually and in combination, as a substrate for the production of biohydrogen.

Keywords: Biohydrogen, Co-digestion, Dark Fermentation, Cheese whey, Wheat straw hydrolysate, Chemometric analysis

1 Introduction

Exhaustion of fossil fuel resources and environmental damages owing to petroleum production and its consumption highlight the importance of a shift to renewable sources for fuels. Bioenergy production from organic waste is becoming an essential component in the overall development of sustainable energy sources. The biological processes to produce hydrogen are environmentally friendly and can convert a wide variety of abundant organic biomass at low cost. In particular, biological production of hydrogen by dark fermentation can be emphasized for its large use of sustainable substrates, the high hydrogen production rates, and its simplicity of operation [1,2]. The dark fermentation can be defined as the partial oxidation of carbohydrates without external electron acceptor. This process also produces by-products such as fatty acids and solvents, thus there is an opportunity for further combination with other processes that yield more bioenergy. Dark fermentation can be carried out by mixed cultures of bacteria, like *Prevotella*, *Lactobacillus, Clostridium*, *Selenomonas*, *Megasphaera,* and *Enterobacter* genera [3–5].

Organic wastes are abundant sources of renewable and low cost substrate that can be efficiently fermented by microorganisms. The main criteria for the selection of waste materials to be used in biohydrogen production are the availability, cost, carbohydrate content and biodegradability. Simple sugars such as glucose, sucrose and lactose are readily biodegradable and preferred substrates for hydrogen production. However, pure carbohydrate sources are expensive raw materials for hydrogen production [6]. The advantages of using organic wastes for biohydrogen are: reduction of CO_2 and other pollutants emissions, added value agricultural wastes, partial substitution of fossil fuels with sustainable biomass fuels, and reduction of environmental and economic costs for diverging the disposition of municipal solid wastes [2]. The production of renewable

energy, a reduction of waste and prevention of environmental pollution promote the industrial application of anaerobic co-digestion for the treatment of agro-industrial organic wastes. Co-digestion is defined as the anaerobic treatment of a mixture of at least two different waste types with the aim of improving the efficiency of the anaerobic digestion process [7]. Due to the ability of dark fermentation to use complex substrates as livestock, crop residues, wastes and wastewater, there are several opportunities to develop co-digestion of two or more substrates with supplementary characteristics. Co-digestion can be used to enhance the dark fermentation process due to a better carbon and nutrient balance; in addition, it has other potential benefits such as dilution of toxic compounds, synergistic effect of microorganisms and better biogas yield. Furthermore, in the research about co-utilization of different carbon sources by bacteria is important to reveal the role of each carbon in bacterial physiology and how it enhances biohydrogen production [8–11].

According to FAO, in 2013 there was reported a production of 71.6×10^7 ton of wheat, whose waste contains approximately 8.73×10^6 ton of nutrients, and 2.5×10^6 ton of cheese whey [12]. In Mexico, SIACON-SIAP reported in 2011 a production of 4.4×10^6 ton of wheat straw and 1.9×10^5 ton of milk of which it is estimated that 4.4×10^3 are cheese whey approximately [13]. The wheat straw is rich in cellulose (35-45%), hemicellulose (20-30%) and lignin (18-15%) [14], its pretreatment is necessary to break down the lignocellulose into the three major polymeric constituents [15]. The thermal pretreatment of biomass results in two major streams: the solid fraction mainly consisting of cellulose (hexose: glucose) and liquid phase (hydrolysate) mainly constituted of hemicellulose (pentose: xylose and arabinose) [16]. Meanwhile cheese whey (CW) is a liquid that separates from the milk coagulation during cheese manufacture and corresponds to around 85–90% of the total volume of processed milk. This residue is one of the polluting residues in the dairy

93 industry that can negatively affect the environment and biological processes during
94 wastewater treatment. [17]. In a dry basis, bovine whey contains 70-80% of lactose, 9% of
95 proteins, 8-20% of minerals and other minor components, such as some hydrolyzed
96 peptides of k-casein and lipids [18]. Therefore, the treatment of the degradable fraction of
97 solid wastes, allows the generation of carbon-neutral bioenergy, nutrients and other
98 resources or valuable products [2].
99 Since initial pH, temperature, substrate concentration, inoculum type, macronutrients and
100 micronutrients impacts the biohydrogen production; the optimization of the operating
101 conditions of bioreactors stills is a key parameter to improve the production of this energy
102 carrier [19–21]. The optimization of operational conditions can be achieved by using
103 chemometrics approaches through the application of experimental design, response surfaces
104 methodology and multivariate data analysis. Response surface methodology consists of a
105 group of mathematical and statistical techniques that are based on the fit of empirical
106 models to the experimental data obtained in relation to the experimental design. The
107 procedures are based on the simultaneous variation of numerous factors (independent
108 variables) to a specific number of levels and possible combinations of these levels are used
109 to evaluate the response (dependent variable) in order to determine the effect of individual
110 factors and their interactive influences. The optimization is simple to perform and enables
111 the optimum conditions to be found with a reduced number of experiments. The
112 multivariate data analysis, such as principal component analysis (PCA) and hierarchical
113 cluster analysis (HCA) are used to process a large number of data, assisting the
114 interpretation of the results [22–24].
115 As mentioned before, co-utilization of different organic material in the fermentation
116 complements the bacterial nutritional requirements, for instance typical wheat straw

hydrolysates (WSH) are nitrogen and mineral deficient and they can be obtained from the cheese whey. Therefore, the goal of this work was to study the biohydrogen production by dark fermentation by using two typical agroindustrial wastes as carbon sources with a chemometric approach through the application of a response surface methodology and multivariate data analisys.

2 Material and Methods

2.1 Substrates and inoculum

CW was purchased from Land O'Lakes Inc. (Arden Hills, Minnesota) and WSH was obtained from CUCBA (University of Guadalajara, Jalisco, Mex). The lactose content of CW solution was 6.9 g L^{-1}. To obtain WSH, wheat straw was slurred in dilute H_2SO_4 (0.75% v/v) at 4% (w/v) and pre-treated at 121°C for 1 h in a steam sterilizer with heating and cooling ramps of 30 min each. The liquid fraction was recovered and the samples were taken, it was centrifuged at 10,000 rpm and concentrated by evaporation at 70°C [25]. WSH contained per litter: total reducing sugars (TRS) 21 g, glucose 1.54 g, xylose 13.96 g, arabinose 1.93 g, furfural 0.12 g, formic acid 1.01g, and acetic acid 3.6 g. Anaerobic granular sludge was obtained from a wastewater treatment plant in San Luis Potosi, Mexico. The granular sludge was washed with three volumes of tap water and then boiled for 40 minutes to inactivate methanogenic microflora according to Davila-Vazquez et al. [26] and stored at 4°C before use.

138 *2.2 Experimental design*

139 A Central Composite experimental design with six central points (Table 1) was used to find
140 the optimal conditions for biohydrogen production using mixtures of CW and WSH as
141 substrate. The independent variables were pH, temperature and concentration of CW and
142 WSH. Three levels for each variable were included and 2 star points. The response variable
143 was biohydrogen production (H_2). The experiments were performed in 120 mL anaerobic
144 serological bottles with a working volume of 110 mL, all bottles containing medium B [26]
145 and 2.75 g L^{-1} yeast extract. The temperature and initial pH, as well as CW and WSH
146 concentrations used in each experiment were determinate by the central composite
147 experimental design. The cultures were shaken at 175 rpm during the period of experiment
148 until no generation of biohydrogen was observed. Consequently, the data was analyzed by
149 the response surface methodology (RSM). Analysis of variance (ANOVA), RSM and the
150 optimum conditions were performed using Design-Expert® Version 7.0 (Stat-Ease, Inc.).
151 The ANOVA *F* test was used to assess the adjusted models. The significance of each
152 coefficient was determined with the *t*-test with a *P* value less than 0.05.

153

154 *2.3 Batch cultures on bioreactor*

155 Batch fermentations were performed using a mixture of WSH and CW (25 g L^{-1} and 5 g L^{-1}
156 , respectively) in 1-L and 4-L bioreactors (Applikon, Foster City, CA) equipped with two
157 six-blade Rushton turbines, pH was monitored using an autocleavable electrode (Applikon)
158 and controlled at 6.5 by a Bioconsole ADI 1035/Biocontroller 103 (Applikon). BioXpert
159 1.3 software (Applikon) was used for data acquisition. The experiments were performed at
160 26.6ºC with a initial pH of 7.25 and stirred at 175 rpm. Culture samples of 1 mL were taken
161 every 4 h from the bioreactors and centrifuged at 600 rpm. The supernatant was filtered

through a 0.22 μm syringe filter (Millipore, Bedford, MA, USA) before analysis of fermentation products.

2.4 Analytical methods

Total reducing sugars (TRS) analysis was performed by the dinitro-salicylic acid (DNS) method, with some modifications as follows: 0.25 mL of WSH with 0.75 mL of DNS reagent (10 g L^{-1} NaOH, 200 g L^{-1} $KNaC_4H_4O_6 \cdot 4H_2O$, 0.5 g L^{-1} $Na_2S_2O_5$, 2g L^{-1} C_6H_6O, 10 g L^{-1} 3,5-Dinitrosalicylic acid) were heated for 15 min in a boiling water bath and then cooled to room temperature. For the calibration curve, glucose (0.1-1.0 g L^{-1}) was used as the reference standard. The absorbance was measured at 550 nm (Varian's Cary 50 Bio UV–Visible Spectrophotometer) [25]. The gas production was measured by the 1N NaOH displacement in an inverted burette connected to the bioreactor or to serological bottles with rubber tubing and a needle. Hydrogen content in the gas phase was measured by the Gas Chromatograph model 6890N (Agilent Technologies, Wilmington, DE) as described elsewhere [27]. Remaining substrates and fermentation end products (glucose, succinic acid, lactic acid, formic acid, acetic acid, methanol, propanol, and butanol) were analyzed by High Performance Liquid Chromatography (HPLC, Infinity LC 1220, Agilent Technologies, Santa Clara, CA, USA) using a Refraction Index Detector (Agilent Technologies, Santa Clara, CA, USA), and column Phenomenex Rezex ROA (Phenomenex, Torrance,CA, USA) at 60ºC, and using 0.0025 M H_2SO_4 as mobile phase at 0.55 mL min^{-1} flow rate. Ethanol, acetoin, propionic acid, and butyric acid were analyzed by injecting a 1 μl sample in a Gas Chromatograph 6890N (Agilent Technologies) equipped with capillary column Innowax (30 m x 0.25 mm i.d. x 0.5 μm film thickness;

185 Agilent, Wilmington, USA). Helium was used as carrier gas at a flow rate of 25 mL min^{-1}.
186 Temperatures for the injector and flame ionization detector (FID) were 220 and 250ºC,
187 respectively. The analyses were performed with a split ratio of 5:1 and a temperature
188 program of 25ºC for 10 min, 175ºC for 1 min increased at 5ºC min^{-1} to 280ºC, and
189 maintained at this temperature to a final time of 10 min.
190
191 *2.5 Chemometric techniques*
192 Principal Component Analysis (PCA) and Hierarchical Clustering Analysis (HCA) were
193 employed to investigate the similarities and dissimilarities between the studied biohydrogen
194 production from mixtures of agro-industrial wastes at different conditions (T, pH, substrate
195 concentrations). All chemometric analyses were performed with MATLAB® Software
196 version R2015a. PCA is the chemometric technique most commonly applied in the
197 exploratory analysis of multivariate data sets. It enables to reduce data dimensionality, to
198 visualize it and to interpret relationships between objects and parameters. HCA, on the
199 other hand, allows investigating the similarities (or dissimilarities) between experiments in
200 the variables space, or similarities (dissimilarities) between variables in the experiments
201 space. It is characterized by the similarity measure used and the way the resulting sub-
202 clusters are merged. The results of HCA are presented in the form of dendrograms where
203 on the *x*-axis the indices of clustered experiments or studied variables are presented, and the
204 *y* axis corresponds to the linkage distances between the two experiments or variables
205 linked. The visualization method may be applied to the studied data sorted according to the
206 order of objects and parameters used in the HCA, so that the similarities between objects in
207 terms of the original parameters could be followed.
208

3 Results and discussion

3.1 Optimization of the culture conditions to improve biohydrogen production

The effect of substrates concentrations, temperature and pH over the biohydrogen production was evaluated with a Central Composite experimental design (Table 1). Central points attained a production average of 3,592.3 ± 167.6 mL H_2 L^{-1}. The highest production was obtained in experiment 11 with 5,359.1 mL H_2 L^{-1}. Experiments with pH ≤ 5.5 and temperatures < 28°C or ≥ 46°C, obtained less than 70 mL H_2 L^{-1}. With the ANOVA it was established that biohydrogen was affected significantly ($p < 0.05$) by temperature and pH (Table 2), whereas, the concentration of the substrates does not have a statistically significant effect ($p < 0.05$) on response variables that are studied. In Table 1 it is possible to observe that if the concentration of substrates is higher then, the biohydrogen production will be higher too, this can be seen by comparing the following pairs of experiments 15 and 23, 1 and 11, 25 and 29, in which the substrate concentration was 20, 30 and 40 g L^{-1}, respectively. Nevertheless, if the substrate concentration is doubled the increase in H_2 is not the double, to notice this we can consider that the biohydrogen production in experiment 5 was just 38% higher than the one obtained in experiment 6; the concentration of substrates is 20 and 10 g L^{-1}, respectively. This phenomenon can be explained as an inhibitory effect of the substrates, an excessive amount of substrate increases osmotic pressure and hence inhibits H_2-producing bacteria growth, besides when the substrate is in excess, it is rapidly converted to hydrogen and this leads to the accumulation of H_2, thus the hydrogen partial pressure increases [28]. It can also be noted that temperature and pH have a more important role on biohydrogen production, because an optimum pH helps to maintain the surface charge on the cell membrane which facilitates nutrient uptake and hence sustains growth of H_2-producing bacteria; while temperature determinates the physiological activities of H_2-

producing bacteria [28,29]. In the results of experiments 1, 2 and 24 all these had the same concentration of substrates, but the temperature in the experiment 2 and pH in the experiment 24 produced less biohydrogen. These findings were consistent with the typical dark fermentation using solely one carbon source [30–33]. Therefore the use of a mixture of two carbon sources can be carried out without affecting the performance of the dark fermentation.

The second-order-polynomial representing the variable response as a function of the evaluated variables in the experimental region is expressed by the following equation:

$$H_2\ (mL\ H_2\ L^{-1}) = -71{,}174.9 - 161.6 \cdot WSH + 839.5 \cdot CW + 1{,}586.2 \cdot T + 11{,}918.3 \cdot pH - 14.7 \cdot WSH \cdot CW + 2.8 \cdot WSH \cdot T + 67.9 \cdot WSH \cdot pH - 6.5 \cdot CW \cdot T - 36.3 \cdot CW \cdot pH - 102 \cdot T \cdot pH - 5.1 \cdot WSH^2 - 2.9 \cdot CW^2 - 13.1 \cdot T^2 - 592 \cdot pH^2$$

With the RSM, contour and response surface plots for biohydrogen production (Figs. 1 and 2) were obtained. From the plots it can be revealed that temperature and pH have great influence on biohydrogen production. Maximum biohydrogen production was found to be approximately in a range of 5,200-5,700 mL H_2 L^{-1} at range of concentration of substrates of 5-10 g TRS L^{-1} WSH and 20-25 g L^{-1} CW, incubation temperature of 25-31°C and initial pH of 6.5-8.5. As noted in the Figs. 1, the reduction of biohydrogen production with the increasing of WSH concentration can be explained by the increment of the fermentation inhibitors becoming from the lignocellulosic hydrolysate such as furfural, formic acid, acetic acid, and others [34,35]. These inhibitors affect to microorganisms in three distinct modes of action: organic acids penetrate microbial cells and decrease the intracellular pH, furan derivatives interfere with glycolytic and/or fermentative enzymes, while phenolic compounds cause damage to the microbial cellular membranes [36–39].

Hence, from these results, biohydrogen production was optimized to get the optimum values of WSH concentration, CW concentration, temperature and pH for maximum values of H_2. According to the second-order-polynomial, maximum biohydrogen production of 5,724.5 mL H_2 L^{-1} (95% CI: 3,375.53-6,722.02 mL H_2 L^{-1}) can be attained at WSH 5 g TRS L^{-1}, CW 25 g L^{-1} CW, 26.6°C and initial pH 7.25. To verify the predicted results, additional experiments were performed by triplicate using these optimized conditions and the biohydrogen production attained was 4,554.55 ± 10.9 mL H_2 L^{-1} (Fig. 3). The optimization of operational conditions using RSM was successful because the result that was obtained is within the confidence interval. Davila-Vazquez *et al*. reported 2,133.8 [26] and 3,812.5 [27] mL H_2 L^{-1} using anaerobic granular sludge and CW as substrate, the biohydrogen production obtained by us in optimal conditions increased in 113.5% and 19.5% in comparison with these two works. In a previous work from our group, we evaluated the use of a waste residue wheat straw as a substrate for biohydrogen production obtaining 3,277.7 mL H_2 L^{-1} [25], this value is lower than the obtained in the present work. In other studies in which mixed culture was used for biohydrogen production and rice straw hydrolysate, sucrose, kitchen wastes, fruit-vegetable waste and rotten wheat straw were employed as substrates, the reported H_2 biohydrogen was between 2 to 1,500 mL H_2 L^{-1} [40–44], lower values than the obtained in the present study. Wu *et al.* [45], reported a higher biohydrogen production using bagasse as substrate, 8,105 mL H_2 L^{-1} and the incubation temperatures used in these studies were between 35-60°C, which are higher compared to the ones found by us (26.6°C). However, this value is within the range of 25 to 55°C reported as an optimal temperature for mixed cultures [46].

3.2 Chemometric description of the biohydrogen production from mixtures agro-industrial wastes

Several studies have applied the analysis of variance (ANOVA) to determine the effects of different variables on biohydrogen production [47–50]. Alternatively, the multivariate analysis, such as a hierarchical cluster analysis (HCA) and principal component analysis (PCA) could be used to summarize and explain large datasets statistically and visually. Multivariate analysis is superior to other bivariate statistical techniques, since it describes the interrelationships among a set of variables and it expresses the data by highlighting their similarities and differences [51,52].

The PCA model with three significant principal components described 99.35% of the total data variance. Score plots and loading plots obtained as a result of the analysis are presented in Fig. 4. PC1, which described 61.83% of the total data variance, is constructed mainly due to the differences in H_2 obtained by objects of the experimental design (Table 1). Furthermore, along the PC1 the objects can be divided into three clusters and one non-grouped object corresponding to experiment 20 (experiment conducted at 28°C, pH of 5.5, 20 g L^{-1} of WSH and 20 g L^{-1} of CW). The first cluster is composed of the experiments 1, 3, 5, 6, 10, 11, 13, 15, 16, 18, 21, 23, 24, 25, 26, 29. The second cluster is composed of the experiments 17, 19, 22. The third cluster is composed of the experiments 2, 4, 7, 8, 9, 12, 14, 27, 28, 30. According to the interpretation of the plots (Fig. 4) it can be observed that a) the first cluster was characterized by an accumulated biohydrogen production (H_2ac)>300 mL H_2, a biohydrogen production (H_2) ranged in 2900 mL H_2 L^{-1}<H_2<5400 mL H_2 L^{-1} and a biohydrogen production rate (r_{H_2}) ranged in 5.0 mL H_2 L^{-1} h^{-1}< r_{H_2}< 20 mL H_2 L^{-1} h^{-1}; b) the experiments included in the second cluster are characterized by relatively

middle values of H_2ac (<10 mL H_2), H_2 (<50 mL H_2 L^{-1}) and r_{H_2}(<5.0 mL H_2 L^{-1} h^{-1}); and c) the third cluster is characterized by lower values of H_2ac, H_2 and r_{H_2} than the obtained by the experiments of the second cluster. As for the PC2, it described the 36.14% of the total data variance and it was constructed due to the high accumulated biohydrogen production obtained by the experiments from cluster one and the low accumulated biohydrogen production obtained by clusters two and three. The PC3, describing 1.38% of the total variance, revealed that the experiments 10, 11, 15, 16, 18, 21, 23, 24, 25, 26 and 29 were characterized by pH≥6.5 and 28°C<T<37°C.

The detailed conclusions drawn on the basis of the final PCA are useful proofs that the data compression was effective. Therefore, the alternative method of the Hierarchical Clustering Analysis was applied for an in-depth investigation of the biohydrogen production by anaerobic granular sludge from mixtures of agro-industrial wastes. The dendrograms constructed with the application of the Ward's linkage method are presented in Fig. 5. Euclidean distance was employed as the similarity measure. The dendrogram presenting the 30 experiments from the Central Composite experimental design in the space of four measured parameters (Fig. 5A) revealed two main clusters. Cluster A grouped experiments with biohydrogen production lower than 70 mL H_2 L^{-1}. Cluster B collected the experiments incubated at temperatures lower than 46°C but higher than 19°C and with a biohydrogen production exceeding 2950 ml H_2 L^{-1}.

Furthermore, in cluster A two sub-clusters could be distinguished:

- Sub-cluster A_1 including experiments 2, 4, 7, 8, 9, 12, 14, 17, 19, 22, 27, 28 and 30, and
- Sub-cluster A_2 collecting the experiment 20.

Also, in cluster B two sub-clusters could be observed:

- Sub-cluster B_1 composed of experiments 1, 5, 25, 29 and11, and
- Sub-cluster B_2 collected of experiments 3, 6, 10, 13, 15, 16, 18, 21, 23, 24 and 26.

The dendrogram constructed for the independent and response variables (Fig. 5B) reveals two main classes:

- Class C containing WSH, CW, T, pH, H_2ac and r_{H_2}; and
- Class D enclosing H_2.

The HCA was complemented with a color map of the studied data, showing the measured values, arranged in accordance with the order of objects and the parameters shown in Fig. 6. Analysis of the colour map allowed determining the relationships between the experiments in the variables space, and between the variables in the experiments space. A simultaneous interpretation of the dendrogram presenting the experiments in the space of studied variables with the color map of studied data allowed concluding that all experiments collected in cluster A differed from the remaining ones mainly because they showed H_2ac<10 mL H_2, H_2<70 mL H_2 L^{-1} and r_{H_2}<5.0 mL H_2 L^{-1} h^{-1}. Moreover, the uniqueness of the experiments 17, 19, 20 and 22 was observed resulting from the highest H_2ac, H_2 and r_{H_2} in comparison with the remaining experiments included in the cluster A. Experiments included in cluster B were characterized by the high values of H_2ac, H_2 and r_{H_2} when compared to the remaining experiments. The color map analysis enabled discovering the uniqueness of sub-cluster B_1 because it showed 4500 mL H_2 L^{-1} < H_2 < 5400 mL H_2 L^{-1} at initial pH of 6.5 or 7.5. Furthermore, the experiment 11 was characterized by the highest biohydrogen production at 28 °C and pH of 7.5. Sub-cluster B_2 was unique due to their experiments which obtained biohydrogen productions levels

between 2970 and 3750 mL H_2 L^{-1} at temperature of 28 °C or 37 °C and initial pH of 5.5, 6.5, 7.5 and 8.5.

3.3 Biohydrogen production from a mixture wheat straw hydrolysate with cheese whey under optimal operating conditions

To identify if the biohydrogen production by anaerobic sludge culture using a mixture of WSH and CW could be scale up, the optimal operating conditions were tested in 1-L and 4-L bioreactors. In 1-L and 4-L bioreactors (Fig. 3) the biohydrogen production started at 8 h reaching 3,685 ± 305 and 4,132.1 ± 37.8 mL H_2 L^{-1}, respectively. In 1-L bioreactor the carbohydrates were totally consumed at the end of the fermentation (164 h) while in 4-L bioreactors the carbohydrates were totally consumed in 96 h. As it is observed, the lag-time was reduced when the cultures were scaled-up from 0.11 to 4 L, which is result of the use of the final culture cells as inoculum for the subsequent experiment indicating that microbial community has been adapted to the mixture of substrates. Therefore, the biohydrogen production rate was improved from 66.6 to 89.5 mL H_2 L^{-1} h^{-1}. Table 3 summarizes a comparison of production, production rate and yield of biohydrogen between the results reported by other authors and those achieved by us. These experiments (Table 3) were performed using agro-industrial wastes or analytical grade carbohydrates as substrate. The temperature and pH used were in the ranges of 30-70°C and 4.7-7, respectively. The results obtained under these conditions were 1000 mL H_2 L^{-1} < H_2 < 4100 mL H_2 L^{-1}, 60 mL H_2 L^{-1} h^{-1} < r_{H_2} < 520 mL H_2 L^{-1} h^{-1} and 95 mL H_2 g^{-1} < Y_{H_2} < 600 mL H_2 g^{-1}. Our biohydrogen production results are higher than those presented in Table 3, but not so, for the production rate and yield of biohydrogen.

371 Using a Student's *t*-test we obtained that the difference was not statistically significant ($p <$
372 0.05) in the results of biohydrogen production that was achieved in 0.11-L serological
373 bottles, 1-L and 4-L bioreactors, therefore it is possible to conclude that the optimal
374 operating conditions can be scaled successfully. Nevertheless, to maximize the production
375 rate and yield more experiments it is necessary to find the conditions that permit high
376 production as well as high production rate and high yield.
377
378 *3.4 Production of soluble metabolites*
379 Biohydrogen production is typically accompanied by the generation of organic acids and
380 ethanol during dark fermentation processes. Hence, the composition and concentration of
381 the produced soluble metabolites are useful indicators for monitoring the biohydrogen
382 production process [43]. The investigation of the soluble metabolites at the end of the
383 hydrogenogenic process is shown in Table 4. We can observe that the acetic acid is the
384 main organic acid produced; other organic acid and ethanol are also produced. Similar
385 results for metabolic products were reported by using sweet sorghum and indigenous micro
386 flora; 5.55 g L^{-1} butyric acid, 3.5 g L^{-1} acetic acid and others metabolites with values lower
387 than 1.55 g L^{-1} (propionic acid, ethanol, lactic acid) were produced [53]. In a study in which
388 two thermophilic bacteria were used, the most abundant byproduct was butyric acid with a
389 concentration of 1.06 g L^{-1} at the fermentation end [44]. Another work reported that the
390 dominant byproducts in fermentation were butyric acid (9.5 g L^{-1}) and acetic acid (3.8 g L^{-1})
391 by anaerobic granular sludge when the substrate was kitchen waste [42]. Also a work in
392 which "piggery anaerobic digested residues" was used as inoculum the formation of butyric
393 and acetic acids were favored, fruit-vegetable waste was used as substrate; ethanol,
394 propionic and lactic acids were detected at lower values of 0.5 g L^{-1} [43]. Additionally, by

using *Klebsiella oxytoca ΔadhE* HP1, Wu et al. [45] reported that the byproducts on the fermentation of bagasse were acetic acid, lactic acid and ethanol. As noted the metabolite profile in the present work was different especially comparing the serological bottles (0.11-L) with respect to those found in the bioreactors (1-L and 4-L), this could be explained as result of the adaptation and natural selection of the microbial community to the mixture of substrates.

4 Conclusion

The PCA enabled an in-depth analysis of the influence of temperature, pH and substrates concentrations on biohydrogen production. The HCA method was also applied to analyze the clustering tendency of the studied data set and to trace the similarities between the studied experiment in the independent and response variables space and the independent and response variables in the experiments space. Biohydrogen production using the co-digestion of two different sources of carbohydrates by anaerobic granular sludge was successful. Through ANOVA analysis we observed that temperature and pH are the most important variables in the biohydrogen production. Also the proposed mathematical model proved to be valuable for optimizing the biohydrogen production with the optimal conditions of 5 g L^{-1} WSH, 25 g L^{-1} CW, 26.6ºC and pH 7.25. The results obtained in this work demonstrate that it is possible to use mixtures of agro-industrial wastes to generate biofuels through a cheap process that it is also industrially scalable.

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611

612 **Table captions**

613

614 **Table 1** Central Composite experimental design and corresponding results by anaerobic

615 granular sludge using mixtures of wheat straw hydrolysate and cheese whey as substrate.

616

617 **Table 2** Analysis of variance for biohydrogen production.

618

619 **Table 3** Comparison of production, production rate and yield of biohydrogen from different
620 microorganisms and substrates.

621

622 **Table 4** Soluble metabolite concentrations accumulated during biohydrogen production
623 process.

624

Figure captions

Fig. 1 Contour and response surface plots of biohydrogen production by anaerobic granular sludge under optimized conditions. Temperature was fixed at 26.6°C and pH adjusted to 7.25 in (a) and (b), concentration of CW was fixed at 25 g L^{-1} and pH adjusted to 7.25 in (c) and (d), concentration of CW was fixed at 25 g L^{-1} and temperature fixed at 26.6°C in (e) and (f).

Fig. 2 Contour and response surface plots of biohydrogen production by anaerobic granular sludge under optimized conditions. Concentration of WSH was fixed at 5 g L^{-1} and temperature was fixed at 26.6°C in (a) and (b), concentration of WSH was fixed at 5 g L^{-1} and pH adjusted to 7.25 in (c) and (d), concentration of WSH was fixed at 5 g L^{-1} and concentration of CW was fixed at 25 g L^{-1} in (e) and (f).

Fig. 3 Biohydrogen production in batch culture of anaerobic sludge at optimal conditions (5 g L^{-1} WSH, 25 g L^{-1} CW, 26.6ºC and pH 7.25) in 0.11L, 1L and 4L bioreactors.

Fig. 4 Score plots (A) and loading plots (B) as a result of PCA for centered and standardized data X (30 x 7).

Fig. 5 Dendrograms of (A) 30 experiments in the space of independent and response variables and (B) variables in the experiments space show the similarity of the studied objects and parameters.

649 **Fig. 6** Color map of the studied data sorted according to the Ward linkage method

650

651 **Table 1**

Experiment	WSH[a] (g L^{-1})	CW[b] (g L^{-1})	Temperature (ºC)	pH	H_2ac[c] (mL H_2)	H_2[d] (mL H_2 L^{-1})	r_{H_2}[e] (mL H_2 $L^{-1}h^{-1}$)
1	10	20	28	7.5	546.0	4,963.6	11.4
2	10	20	46	7.5	0.5	4.5	0.4
3	15	15	37	6.5	408.0	3,709.1	10.3
4	20	10	28	5.5	0.0	0.0	0.0
5	20	20	28	7.5	520.5	4,731.8	7.9
6	10	10	28	7.5	376.8	3,425.5	10.3
7	20	10	46	7.5	0.0	0.0	0.0
8	20	20	46	7.5	0.0	0.0	0.0
9	15	15	37	4.5	1.0	9.1	0.8
10	15	15	37	6.5	393.0	3,572.7	7.6
11	20	10	28	7.5	589.5	5,359.1	10.6
12	15	15	19	6.5	0.5	4.5	0.4
13	15	15	37	6.5	409.0	3,718.2	12.8
14	20	20	46	5.5	0.5	4.5	0.2
15	5	15	37	6.5	327.0	2,972.7	12.8
16	15	15	37	6.5	398.0	3,618.2	8.6
17	15	15	55	6.5	5.0	45.5	3.8
18	15	15	37	6.5	403.3	3,666.4	11.6
19	10	20	46	5.5	4.0	36.4	3.0
20	20	20	28	5.5	7.0	63.6	0.1
21	15	15	37	6.5	359.6	3,269.1	15.1
22	10	10	28	5.5	3.0	27.3	0.0
23	15	5	37	6.5	379.5	3,450.0	12.0
24	10	20	28	5.5	412.5	3,750.0	6.1
25	15	25	37	6.5	497.0	4,518.2	18.8
26	15	15	37	8.5	396.2	3,601.8	14.3
27	20	10	46	5.5	1.0	9.1	0.8
28	10	10	46	7.5	0.0	0.0	0.0
29	25	15	37	6.5	501.5	4,559.1	12.1
30	10	10	46	5.5	0.0	0.0	0.0

652 [a]Wheat straw hydrolysate. [b]Cheese whey. [c]Accumulated biohydrogen production. [d]Biohydrogen
653 production. [e]Biohydrogen production rate.
654

655 **Table 2**

Source	SS[a]	DF[b]	MS[c]	F-value	*p*-value
Model	9.963E+007	14	7.117E+006	4.77	0.0024
WSH	53657.13	1	53657.13	0.036	0.8521
CW	1.966E+006	1	1.966E+006	1.32	0.2687
T	2.051E+007	1	2.051E+007	13.76	0.0021
pH	2.050E+007	1	2.050E+007	13.75	0.0021
WSH×*CW*	2.154E+006	1	2.154E+006	1.44	0.2480
WSH×*T*	2.462E+005	1	2.462E+005	0.17	0.6902
WSH×*pH*	1.845E+006	1	1.845E+006	1.24	0.2834
CW×*T*	1.358E+006	1	1.358E+006	0.91	0.3550
CW×*pH*	5.266E+005	1	5.266E+005	0.35	0.5611
T×*pH*	1.348E+007	1	1.348E+007	9.04	0.0088
WSH^2	4.419E+005	1	4.419E+005	0.30	0.5941
CW^2	1.434E+005	1	1.434E+005	0.096	0.7607
T^2	3.094E+007	1	3.094E+007	20.76	0.0004
pH^2	9.612E+006	1	9.612E+006	6.45	0.0227
Residual	2.236E+007	15	1.491E+006		
Lack of Fit	2.222E+007	10	2.222E+006	79.07	< 0.0001
Pure Error	1.405E+005	5	28099.41		
Cor Total	1.220E+008	29			

656 [a]Sum of squares, [b]Degree freedom, [c]Mean square

657

Table 3

Microorganisms	T (°C)	pH	Substrate	Concentration	H_2 (mL H_2 L^{-1})	r_{H_2} (mL H_2 L^{-1} h^{-1})	Y_{H_2} † (mL H_2 g^{-1})	Reference
Escherichia coli EGY, *Clostridium acetobutylicum* ATCC	30	7.3	Rotting date palm fruits	10 g L^{-1} sucrose	1,023* (2 L accumulated, 1.95 L V_w)	63.7* (2.56 mmol H_2 L^{-1} h^{-1})	218* (3 mol H_2 mol $sucrose^{-1}$)	[54]
				2.5 g L^{-1} sucrose	NR	87.2* (1.2 mmol H_2 L^{-1} h^{-1})	443.3* (6.1 mol H_2 mol $sucrose^{-1}$)	
Mixed culture	35	4.7 – 5.5	Sweet sorghum	0.45 g L^{-1} glucose	NR	212.5* (2550 mL H_2 d^{-1}, 0.5 L V_w)	98.2* (0.70 mol H_2 mol $glucose^{-1}$)	[53]
				0.47 g L^{-1} glucose	NR	122.5* (1740 mL H_2 d^{-1}, 0.5 L V_w)	120.7* (0.86 mol H_2 mol $glucose^{-1}$)	
Thermoanaerobacterium aotearoense SCUT27/*Δldh*	55	6.5	Sugarcane bagasse (SCB)	2 L of nonsterilized SCB hydrolysate	4,017.5* (298.4 mmol accumulated, 2 L V_w)	520	278* (1.86 mol H_2 mol $hexose^{-1}$)	[55]
Caldicellulosiruptor saccharolyticus DSM 8903, *C. kristjanssonii* DSM 12137	70	6.7	Glucose/xylose	NR	NR	135.2* (4.8 mmol H_2 L^{-1} h^{-1})	578.3* (3.7 mol H_2 mol $hexose^{-1}$)	[56]
Mixed culture	37	6 – 7	Glucose	20 g L^{-1}	2,327* (24.8 L accumulated gas, 56.3 % H_2 content, 6 L V_w)	212.2	213.1	[57]
Anaerobic granular sludge (*Citrobacter freundii* JCM, Uncultured *Lachnospiraceae* bacterium MS146A1 E12, *Clostridium perfringens* W11, *Enterobacter cloacae* GH1[26])	26.6	7.25	Wheat straw hydrolysate	5 g TRS L^{-1}	4,132.1 ± 37.8 (4L V_w)	89.5	199 **	This work
			Cheese whey	25 g L^{-1}				

† Biohydrogen yield. * Converted units from the original data; ** Substrate is the mixture of CW and WSH; V_w: Working volume. NR: Not reported.

660 **Table 4**

Metabolite	Concentrations (g L^{-1})		
	0.11-L Serological Bottles	1-L Bioreactor	4-L Bioreactor
Lactic acid	-	0.47 ± 0.06	1.14 ± 0.18
Formic acid	-	1.03 ± 0.07	0.41 ± 0.08
Acetic acid	6.09 ± 1.11	2.84 ± 0.18	3.58 ± 0.33
Propionic acid	0.60 ± 0.02	1.76 ± 0.11	0.57 ± 0.08
Butyric acid	3.73 ± 1.21	1.20 ± 0.11	0.41 ± 0.06
Ethanol	0.42 ± 0.04	0.59 ± 0.04	0.56 ± 0.04
Propanol	-	-	0.70 ± 0.13

661

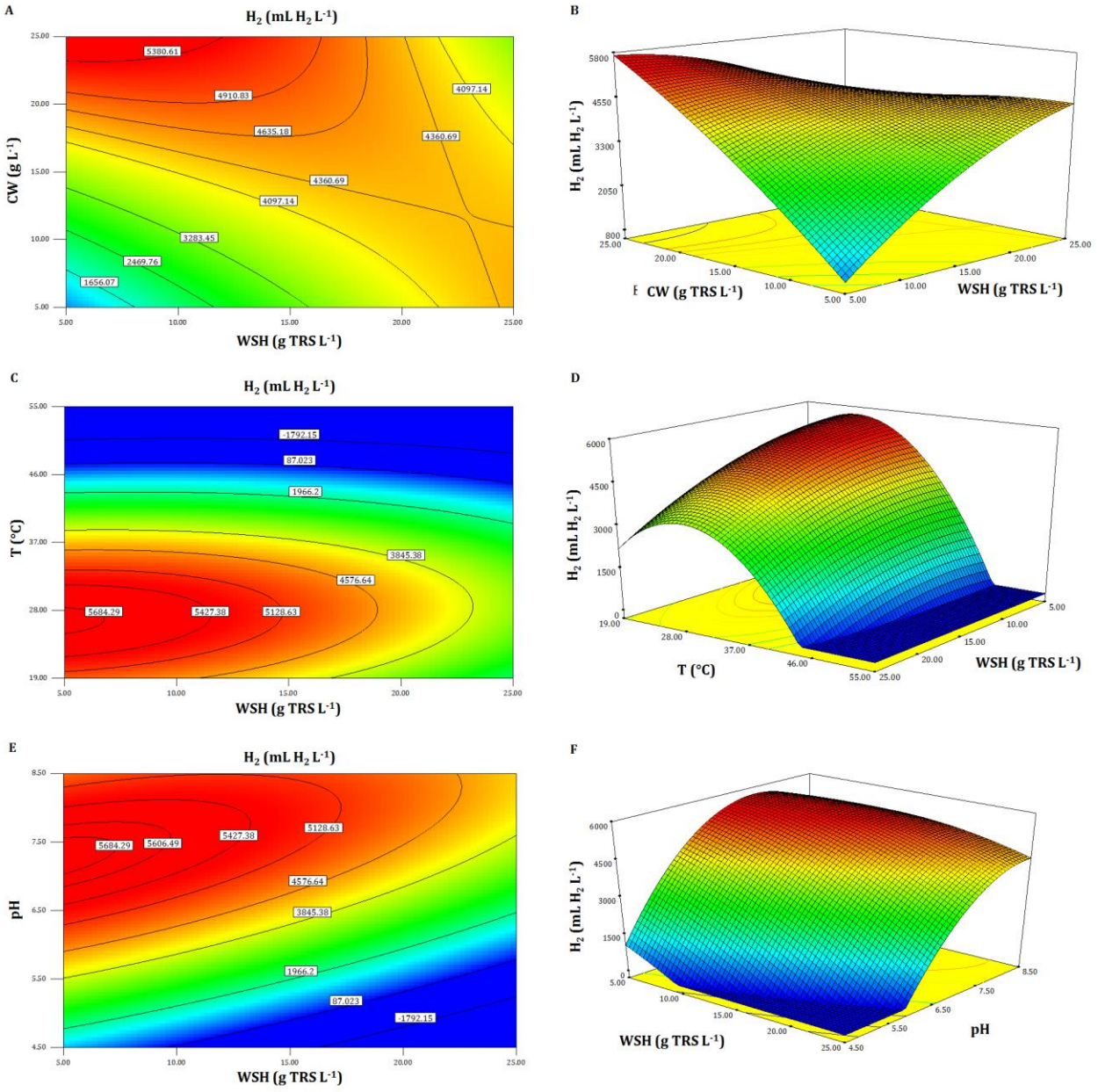
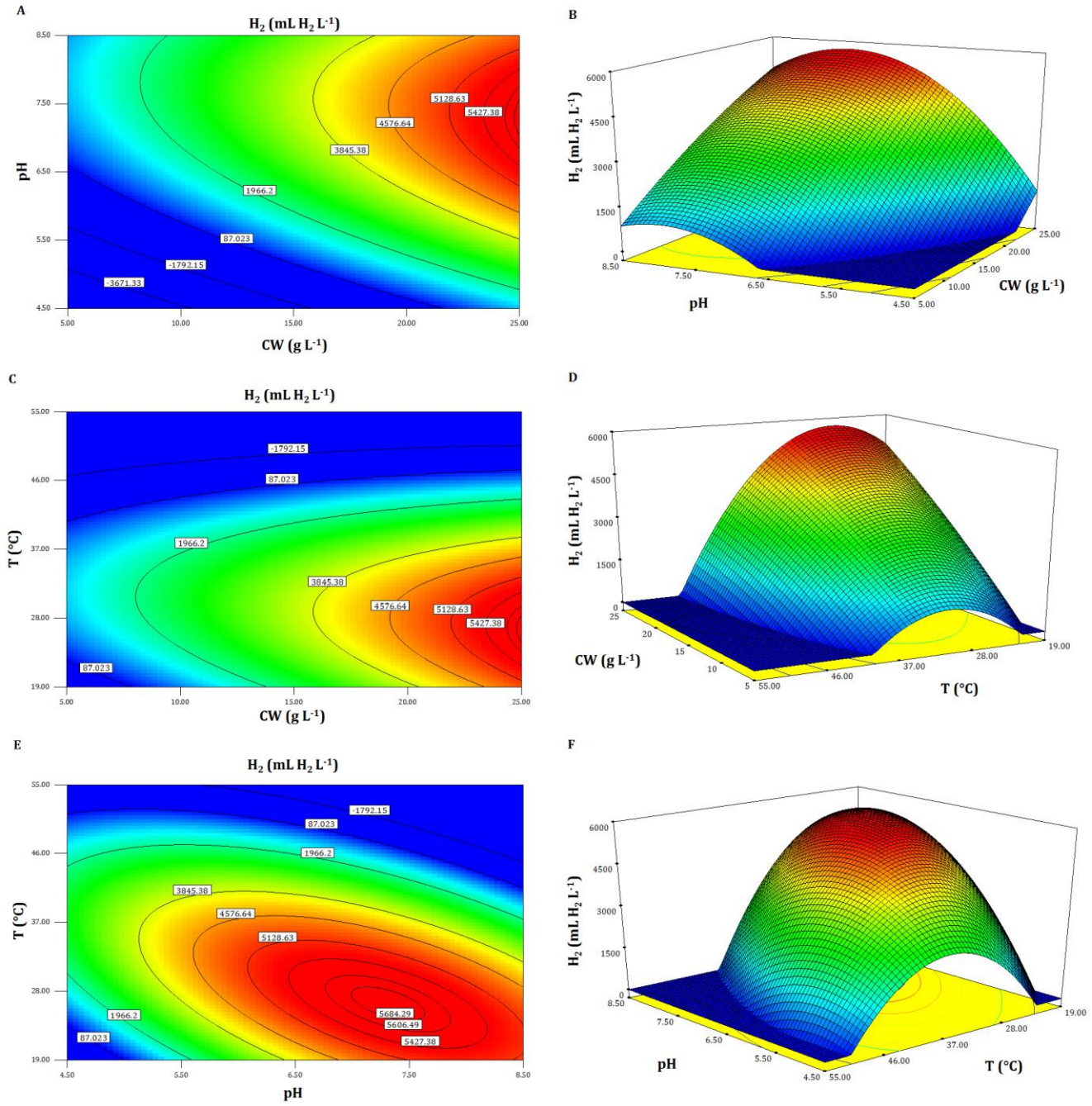
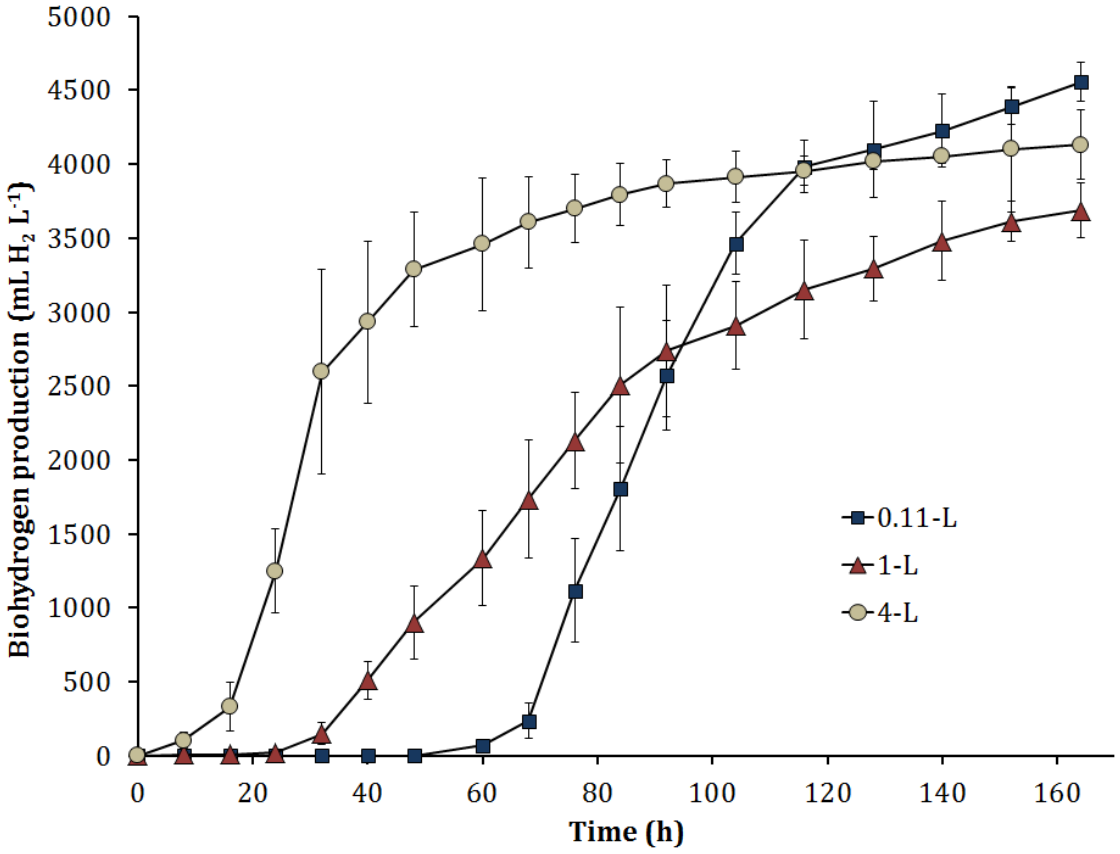

A
H2 (mL H2 L-1)
CW (g L-1)
WSH (g TRS L-1)
5380.61
4910.83
4635.18
4360.69
4097.14
3283.45
2469.76
1656.07
B
H2 (mL H2 L-1)
CW (g TRS L-1)
WSH (g TRS L-1)
C
H2 (mL H2 L-1)
T (°C)
WSH (g TRS L-1)
-1792.15
87.023
1966.2
3845.38
4576.64
5684.29
5427.38
5128.63
D
H2 (mL H2 L-1)
T (°C)
WSH (g TRS L-1)
E
H2 (mL H2 L-1)
pH
WSH (g TRS L-1)
5606.49
F
H2 (mL H2 L-1)
WSH (g TRS L-1)
pH

Fig. 1


A
H2 (mL H2 L-1)
pH
CW (g L-1)
-3671.33
-1792.15
87.023
1966.2
3845.38
4576.64
5128.63
5427.38
B
H2 (mL H2 L-1)
pH
CW (g L-1)
C
H2 (mL H2 L-1)
T (°C)
CW (g L-1)
D
H2 (mL H2 L-1)
CW (g L-1)
T (°C)
E
H2 (mL H2 L-1)
T (°C)
pH
5684.29
5606.49
F
H2 (mL H2 L-1)
pH
T (°C)

665

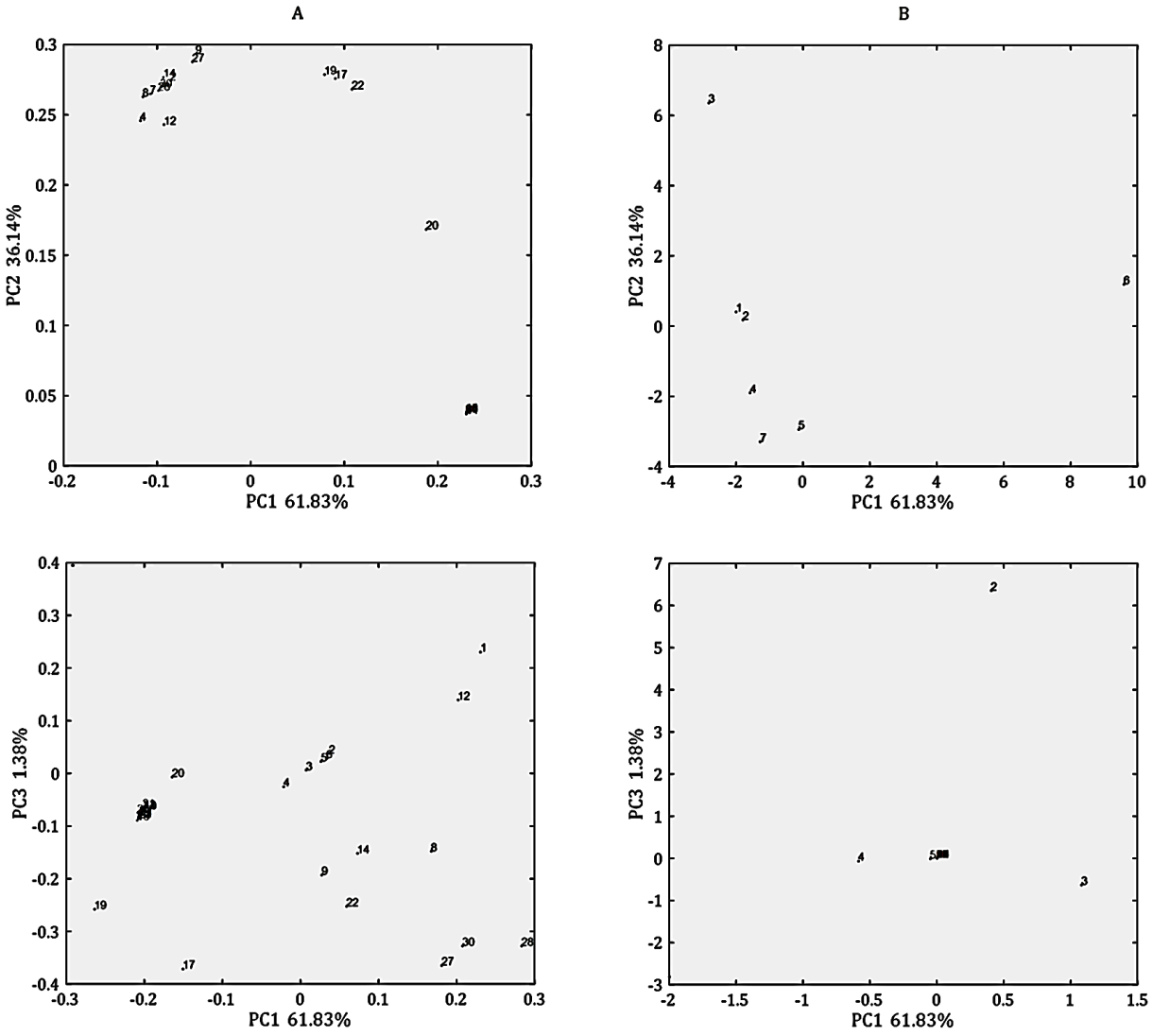
666 **Fig. 2**



667

668 **Fig. 3**

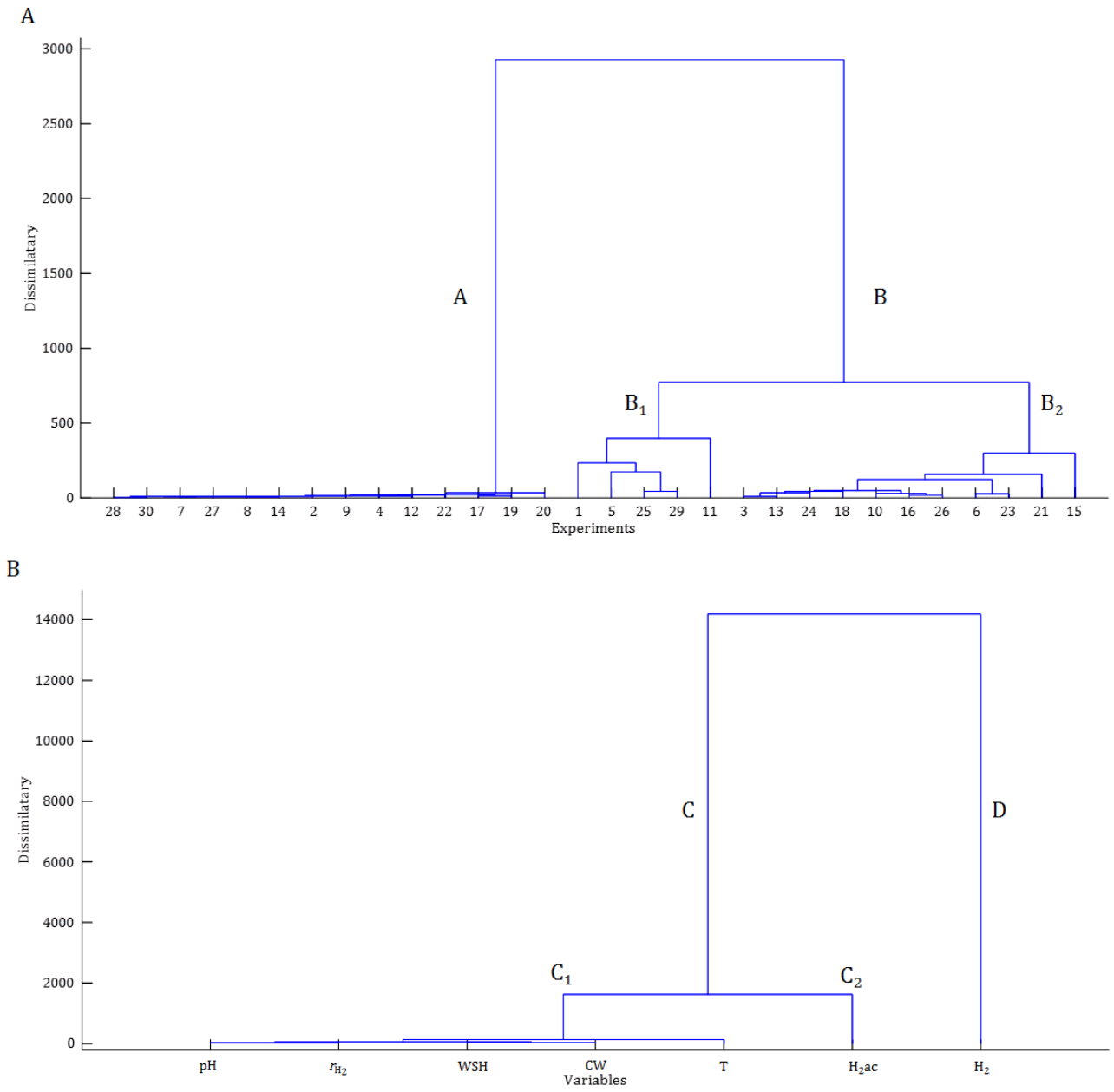
669



670

671 **Fig. 4**

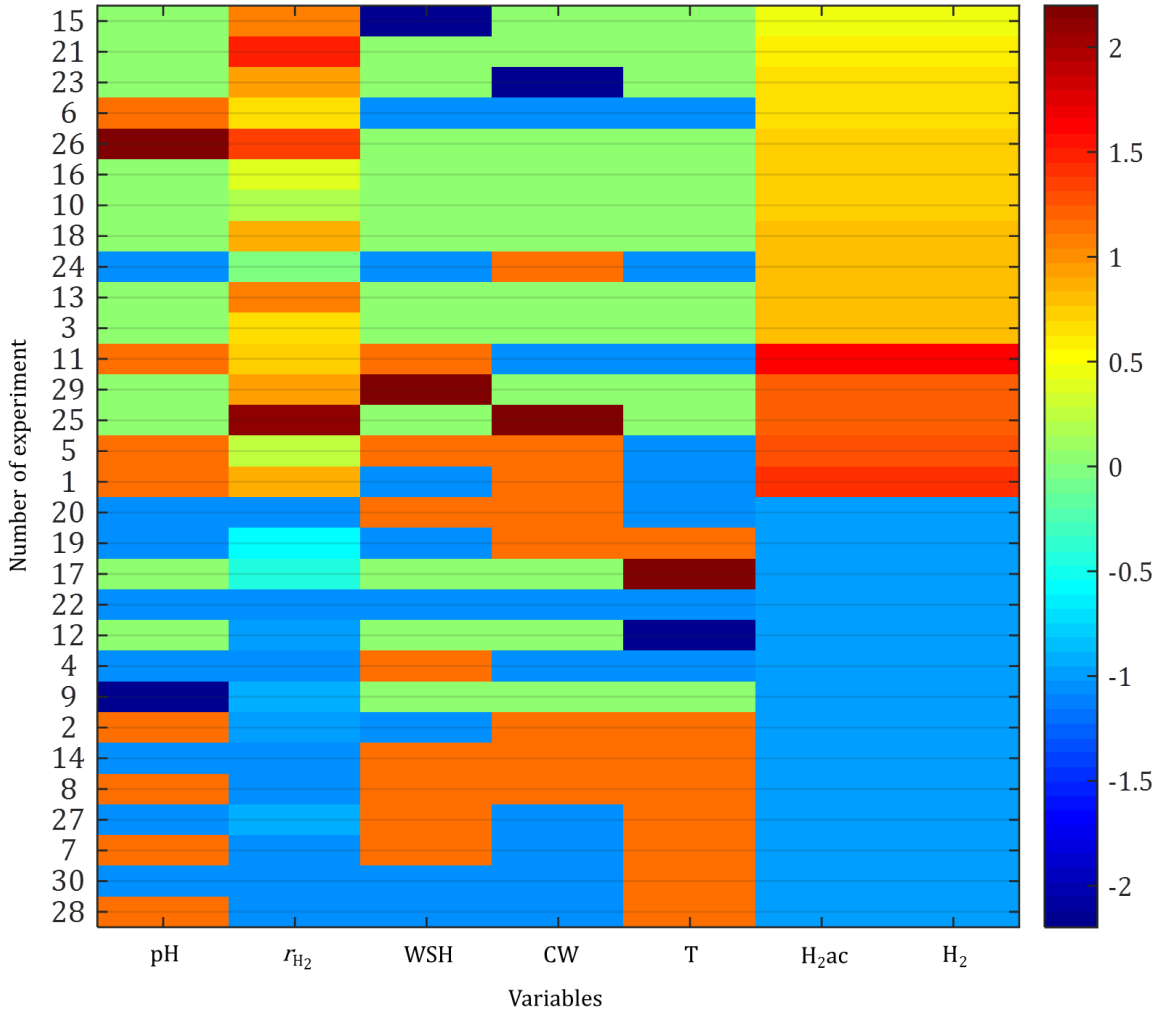
672



673

674 **Fig. 5**

675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12
4
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2
14
8
27
7
30
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pH
r_{H_2}
WSH
CW
T
H_2ac
H_2
Variables
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-1
-1.5
-2

676

677 **Fig. 6**