Estimation of hydrogen production in genetically modified *E. coli* fermentations using an artificial neural network

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Abstract

Biological hydrogen production is an active research area due to the importance of this gas as an energy carrier and the advantages of using biological systems to produce it. A cheap and practical on-line hydrogen determination is desired in these processes. In this study, an artificial neural network (ANN) was developed to estimate the hydrogen production in fermentative processes. A back propagation neural network (BPNN) of one hidden layer with 12 nodes was selected. The BPNN training was done using the conjugated gradient algorithm and on-line measurements of dissolved CO_2, pH and oxidation-reduction potential during the fermentations of cheese whey by *Escherichia coli* Δ*hycA* Δ*lacI* (WDHL) strain with or without pH control. The correlation coefficient between the hydrogen production determined by gas chromatography and the hydrogen production estimated by the BPNN was 0.955. Results showed that the BPNN successfully estimated the hydrogen production using only on-line parameters in genetically modified *E. coli* fermentations with or without pH control. This approach could be used for other hydrogen production systems.

Keywords: back propagation neural network, dissolved CO_2, hydrogen, redox potential, pH, cheese whey.

1. Introduction

Hydrogen is considered as a good choice as future energy carrier since it has the highest energy content per weight unit and its utilization either via combustion or fuel cells results in pure water [1]. Among the hydrogen production processes, the biological production is an attractive method because it is carried out at ambient pressure and temperature, therefore consumes less energy than chemical or electrochemical processes [2]. The fermentative hydrogen production is a promising method since it has the higher production rate; it does not need light and utilizes a wide range of carbon sources [2-5]. In the dark fermentation, several microorganisms can use carbohydrate rich substrates. From the enterobacteria, *Escherichia coli* is the main microorganism used for studies of hydrogen production, since its genetic and metabolism are well documented [6-12]. Under anaerobic conditions and in absence of external electron acceptors *E. coli* converts sugars to pyruvate that may be converted to lactate or broken into formate and acetyl-coenzyme A (acetyl-CoA), which is converted to acetate or ethanol, whereas formate is metabolized to hydrogen and CO_2 (Fig. 1).

The on-line hydrogen determination is strongly desired to establish feedback or feed forward control algorithms. However, the most common method to determine hydrogen is by gas chromatography (GC) off-line [13-19]. This method is very useful, accurate and sensitive to determine hydrogen, but requires equipment and specific installations. Another method used is the gas displacement using a solution of NaOH, however the solution could be saturated and confirmation by GC is still needed [20-24]. Massanet-Nicolau *et al.* [25] measured the composition of the gas produced by the fermentation of sewage biosolids with hydrogen, CO_2 and CH_4 sensors. Ferchichi *et al.* [26] used a solution of 30% of KOH to remove CO_2, and the

residual gas was channeled into a bubble counter for the measurement of hydrogen and it was confirmed by a specific hydrogen sensor. The counter was linked to a computer and the on-line hydrogen production was recorded.

Until now, there are few parameters for on-line monitoring in bioreactors, the most frequents are temperature, pH, oxidation-reduction potential, dissolved oxygen and dissolved CO_2. Therefore, a useful approach is the use of mathematical models with these on-line determinations for the estimation of the fermentative products. For this purpose, the Artificial neural networks (ANNs) have been successfully used, since they are based on the connectivity of biological neurons that have an incredible capability for emulation, analysis, prediction, association and adaptation [6, 27]. For instance, Poirazi *et al.* [28] used pH, temperature and NaCl concentration to predict the maximum specific growth rate and bacteriocin production using feed-forward ANNs in *Streptococcus macedonicus* ACA-DC 198 cultures. Chen *et al.* [27] used the dissolved oxygen, feed rate and liquid volume to determine the biomass concentration in *Saccharomyces cerevisiae* cultures using a recurrent neural network. Escalante-Minakata *et al.* [29] used the oxidation-reduction potential and a back propagation neural network to estimate the ethanol and biomass production in non-axenic cultures.

The aim of this work is to develop an ANN to estimate the hydrogen production in genetically modified *E. coli* fermentations based on the on-line measurements of the oxidation-reduction potential, pH, and dissolved CO_2.

2. Materials and methods

2.1 Strain and culture media

Escherichia coli Δ*hycA* Δ*lacI* (WDHL) a hydrogen overproducing strain was used in this study. A complete description of this strain can be found elsewhere [14]. For hydrogen production, inocula were grown overnight in Luria Bertani (LB) medium at 37°C and shaken at 200 rpm, afterwards added to fresh LB medium and cultured in closed twist cover bottles at 37°C for 48 h. Fermentations were done in hydrogen production (HP) medium described elsewhere [14]. HP medium was pasteurized at 65°C during 25 min and chilled 20 min on ice. Cheese whey powder (Land O´Lakes, Arden Hills, Minnesota) at 20 g L^{-1} was used as carbon source.

2.2 Batch cultures in bioreactor

Pre-inocula was harvested, washed once and inoculated into 1 L bioreactor (Applikon, Foster City, CA) equipped with two six-blade Rushton turbines. Oxidation-reduction potential (ORP), pH and dissolved CO_2 (DCO_2) were monitored using autoclavable electrodes (Applikon) connected to Bioconsole ADI 1035/Biocontroller ADI 1030 (Applikon). The ORP and DCO_2 electrodes were calibrated according to the manufacturers at 215 mV using the reference solution HI7020 (Hanna Instruments, Armazem, Portugal) and using 100% of CO_2 gas saturation at atmospheric pressure, respectively. BioXpert 1.3 software (Applikon) for data acquisition was used. The cultures were performed at 37°C and stirred at 175 rpm. Culture samples were periodically taken from the bioreactor, and centrifuged at 11,500 x *g* for 5 min. The supernatants were filtered through a 0.22 µm filter (Millipore) before the analysis of fermentation products.

2.3 Analytical methods

The gas was measured by water displacement in an inverted burette connected to the bioreactor with rubber tubing and a needle. The hydrogen content in the gas phase, was determined in a Gas Chromatograph 6890 N (Agilent technologies, Wilmington, DE) as described elsewhere [30]. Ethanol was measured by GC as described by De Leon-Rodriguez *et al* [31]. Organic acids and carbohydrates were analyzed by isocratic liquid chromatography using a Waters 600 HPLC system and UV-Vis 2487 detector (Waters) at wavelenght-190 nm. Samples of 20 µL were separated on a Rezex ROA H^+ column (300 mm x 7.8 mm, 8 µm) from Phenomenex (Torrance, CA) at 60ºC and using 0.005N H_2S0_4 at 0.6 mL/min as mobile phase.

2.4 Structure of ANN

To predict the hydrogen production through the on-line measurements of pH, dissolved CO_2 and ORP, a back propagation neural network (BPNN) was chosen. The model was structured as follows:

H_2= F (pH, DCO_2, ORP, W)

Where ORP is the oxidation-reduction potential in mV, DCO_2 is the % of dissolved CO_2, pH is the H^+ potential and W is the vector of adjustable parameters of the network or weight. The variable of response H_2 is the hydrogen produced in mL. The selected architecture was a standard network of one hidden layer with 12 nodes [32]. The structure of the BPNN is shown in Fig. 2. The output layer had a node that predicted the value of hydrogen production whereas the input layer consisted on 3 nodes for pH, DCO_2 and ORP. All the neurons of hidden layer

were non-linear with sigmoid activation function. The output layer neuron had a lineal activation function. The BPNN was trained on a Matlab platform R2008 (MathWorks, Inc.).

2.5 BPNN Training

One hundred and two data of 7 different experiments were used for the BPNN training. The characteristics of the experiments are shown in Table 1. The data of the input variables were scaled in the range (-1, +1) and the output variable was scaled in the range (0, +1). The training was made by minimal squares methodology with respect to error function as follow:

$$Error = (1/(2p))\sum_i^p \left((H_2)_{\exp}^i - (H_2)^i \right)^2$$

Where $(H_2)_{exp}^{\ i}$ is the experimental value for the *i*-point, $(H_2)^i$ is the value estimated by the network, *p* is the number of data. The network training was done using the conjugated gradient algorithm [33]. The BPNN parameters W were randomly assigned in the range of (-0.5, +0.5). 25 full cycles of conjugated gradient were needed to reach convergence and the error was 0.0016.

3. Results and discussion

3.1 Hydrogen production by *E. coli*

A typical batch culture of *E. coli* WDHL at pH 5.5 is showed in Fig. 3. Cultures at other operational conditions showed similar trends as those in Fig. 3, although rates of the various

parameters measured, their maximum concentrations, and times to reach them were different in each case. Lactose was consumed quickly and was undetectable after 18 h of fermentation (Fig. 3A). Only a slight increment on the biomass was observed and the maximum concentration was 1.16 g/L and dropped gradually after 10 h of culture (Fig. 3A). In the Fig. 3B the production of organic acids and ethanol are shown. Lactate was the main organic acid produced essentially in the first 12 h and reached a maximum of 5 g/L in this fermentation. Succinate, propionate and acetate were also produced and each acid reached around 1.6 g/L at 30 h. Only slight amount below of 0.2 g/L of formate was detected in the experiment, because it was rapidly used to produce hydrogen and CO_2 as soon as is produced. Ethanol was also produced and the final concentration was 0.75 g/L. Fig. 3C shows the hydrogen and the DCO_2 profile. A fast increase on DCO_2 was observed on the first 10 h as result of metabolically activity, reached 90% and then remained constant at this value. Since inoculation, the hydrogen production was observed and became slow according the lactose concentration decreased. For this culture, the maximum hydrogen production was 745 mL. The hydrogen and DCO_2 trends are similar (Fig. 3C) and it is explained because the production of hydrogen and CO_2 are linked, formate is broken down to give one mole of hydrogen per mole of CO_2 (Fig. 1). The relation should be direct if no other reactions involve CO_2 production or degradation, but oxaloacetate is formed by the condensation of phosphoenolpyruvate and CO_2 [34]. The initial pH was 7.5 and dropped to 5.5 at 2.5 h because the accumulation of organic acids then it was automatically controlled at this value with NaOH (Fig. 3D). The pH is one of the most important parameters in hydrogen production by different microorganisms. For instance, Li *et al.* [35] reported a direct relationship between initial pH of 5-7 and hydrogen production rate using glucose in non-axenic cultures. Davila-Vazquez *et al.* [30] reached the highest hydrogen molar yield at pH of 7.5 and 6.5 using lactose and cheese whey respectively. Working with axenic cultures, the

highest hydrogen production rate was attained at initial pH of 6 by *Clostridium saccharoperbutylacetonicum* using cheese whey as substrate [26], whereas the maximum hydrogen production was reached at initial pH of 6.5 and 7.5 by metabolically engineered *E. coli* strains using glucose [36] and CW [14] respectively. The role of the pH on the hydrogen production in *E. coli* is explained because the metabolism and the import-export of formate are pH-dependent. Moreover, the transcription of the FHL complex which converts formate to hydrogen and CO_2 depends on the acidic pH of the growth medium [37]. The fermentative metabolism had an effect on ORP and its drops at the beginning of fermentation and then remained constant around -500 mV (Fig. 3D). The global measured ORP corresponds to the sum of the all redox species. Table 2 shows the standard reduction potentials of main redox pairs involved in the hydrogen metabolism by *E. coli*. The ORP has been considered as a variable related to hydrogen production. For instance, Hussy *et al.* [19] reported that ORP was negatively related to hydrogen production rate in a continuous process with non-axenic cultures. Ren *et al.* [38] found that ORP and pH determined to fermentation type in a continuous flow reactor with non-axenic cultures and the best condition for hydrogen production occurred in the alcoholic fermentation at ORP and pH below of -217 mV and 4.5, respectively. Rosales-Colunga *et al.* [14] related the ORP with the cell-growth in a batch processes using a hydrogen over-producer *E. coli* strain.

ORP, dissolved CO_2 and pH are important parameters in hydrogen production as discussed above and can be easily measured on-line. By these reasons the three parameters were chosen to estimate the hydrogen production by the BPNN.

3.2 Prediction of hydrogen production using a BPNN

The final parameters of the BPNN after training are shown in table 3. The weights between the input layer and the hidden layer are represented by the W1 values, whereas W2 represents the weights between the hidden layer and the output layer. The BPNN was used with theses parameters to estimate H_2 for the new values of pH, CO_2 and ORP. The comparison between experimental hydrogen values and predicted values based on the BPNN for the cultures at pH 5.5 and 6 is shown in Fig. 4. In both cultures, there is a good fit in the trends between the predicted and the experimental data. Similar behavior was observed for the cultures without pH control (data not shown). BPNN application was in off-line mode, however, the BPNN can be applied on-line mode by the incorporation of a subroutine on the acquisition software. The Fig. 5 shows the correlation between the hydrogen production determined experimentally by GC and the hydrogen estimated by the BPNN for all experiments with or without control of pH. The R^2 value of 0.955 confirms that the model can predict the hydrogen production well. ANNs have been used in another hydrogen production processes (Table 4). For instance, Nikhil *et al.* [39] reported a BPNN to predict the hydrogen production rate in a Continuous Stirred Tank Reactor (CSTR) using sucrose as substrate. Shi *et al.* [40] reported a similar system but using kitchen wastes as substrate. Mu and Yu [41] used a neural network and genetic algorithm to predict the hydrogen production and the steady-state of an Upflow Anaerobic Sludge Blanket (UASB) reactor at various sucrose concentration and hydraulic retention times. Guo *et al.* [42] estimated hydrogen yield and the chemical oxygen demand through a BPNN in an Expanded Granular Sludge Bed (EGSB) reactor using starch as substrate. Therefore, BPNNs are useful for prediction of hydrogen production, since their ability to learn complex non-linear input-output relationships, use sequential training procedures and adapt themselves to data [39-43].

Aforementioned works were for non-axenic cultures and they used off-line data such as alkalinity, substrate or metabolites concentration as input variables, and only when the BPNNs were chosen, additional on-line variables were included. To our knowledge, this is the first report on the use of BPNN to estimate the hydrogen production by genetically modified microorganisms and using only on-line variables.

4. Conclusions

There are few methods for hydrogen determination. The on-line determination can be performed using expensive devices. Thus, cheap and practical approaches for hydrogen determination are necessary. According to the results, the BPNN predicted successfully the hydrogen production using only on-line parameters in *E. coli* fermentations with or without control of pH. This approach could be used for other hydrogen production systems. The BPNN can be applied in off-line mode as showed here and in on-line mode by incorporation a subroutine in the acquisition software.

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Legends of Figures

Fig. 1. Schematic representation of the fermentative pathways in *Escherichia coli*. Final products are framed.

Fig. 2. Structure of the Artificial Neural Network used in this work. A standard network of one hidden layer with 12 nodes was selected. The continuous lines represent adjustable parameters W; dashed lines are for W<0. The ANN training was done using on-line measurements of ORP, DCO_2 and pH during the fermentations of cheese whey by *Escherichia coli* WDHL strain.

Fig. 3. Typical batch culture of *E. coli* WDHL during the hydrogen production using cheese whey as substrate at pH 5.5. A) Lactose and biomass concentration; B) Metabolites; C) Hydrogen production and dissolved CO_2; D) ORP and pH.

Fig. 4. Comparison between the experimental data of hydrogen production measured experimentally by gas chromatography (closed symbols) and the prediction based on the BPNN model (continuous line). A) Culture at pH 5.5. B) Culture at pH 6.

Fig. 5. Correlation between the hydrogen measured experimentally and the values estimated by the BPNN. The lineal regression is $y = 0.9005x+189.85$ and $r^2 = 0.955$.