INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

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"Hydrogen production by *Escherichia coli* genetically modified"

Tesis que presenta

M. en C. Luis Manuel Rosales Colunga

Para obtener el grado de

Doctor en Ciencias en Biología Molecular

Codirectores de la Tesis:

Dr. Antonio De León Rodríguez

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DOCTOR EN CIENCIAS EN BIOLOGÍA MOLECULAR

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Luis Manuel Rosales Colunga

sobre la Tesis intitulada:

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que se desarrolló bajo la dirección de

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Dr. Elías Razo Flores

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Resumen

Producción de hidrógeno por *Escherichia coli* genéticamente modificada

Palabras Clave: Suero de leche, formiato, galactosa, lactato, red neuronal artificial *hycA*, l*acI.*

El hidrógeno es considerado como el acarreador energético del futuro debido a su alto contenido energético y a que sólo genera agua como subproducto. Entre los métodos de producción de este gas, los procesos biológicos son una alternativa atractiva ya que requieren menos energía y se pueden utilizar subproductos agroalimentarios como sustratos. El suero de leche es el principal subproducto de la industria quesera y por lo tanto se usó como sustrato para la producción de hidrógeno por medio de *Escherichia coli*. La deleción de los genes *hycA* y *lacI* en la cepa WDHL, permitió un rápido consumo de lactosa y un incremento del 22% en la producción de hidrógeno comparado con la cepa silvestre. La velocidad específica de producción de hidrógeno se incrementó 78.7 % al utilizar las condiciones óptimas de pH, temperatura y concentración de suero de leche.

De acuerdo a los resultados obtenidos, el pH mostró ser unos de los factores más influyentes en la producción de hidrógeno. Al controlar el pH a 6.5 se alcanzó la mayor producción de hidrógeno y el rendimiento más alto.

Lactosa, glucosa y galactosa son fuentes de carbono que se encuentran comúnmente en desechos de la industria agroalimentaria, por lo tanto la producción de hidrógeno a partir de estos sustratos también fue evaluada. El rendimiento de hidrógeno a partir de glucosa (0.19 mol de H_2/ mol de glucosa) fue menor que el que se obtiene a partir de galactosa (1.15 mol de H_2/ mol de galactosa). El bajo rendimiento de hidrógeno a partir de glucosa se debe a que el metabolismo se desvía hacia la producción de lactato, en comparación con la fermentación de galactosa donde se favorece la producción de formiato, que es convertido rápidamente en hidrógeno. Además se desarrolló una red neuronal artificial que predice satisfactoriamente la producción de hidrógeno a partir exclusivamente de parámetros en línea.

Abstract

Hydrogen production by genetically modified *Escherichia coli*

Keywords: Cheese whey, formate, galactose, lactate, artificial neural network, *hycA*, l*acI.*

Due to its high energy content and because its use only result in water as sub-product, hydrogen is seen as the energetic carrier for the future. Among the methods of production of this gas, the biological processes are an attractive choice since are less energy intensive and can use agro-food by-products as substrates. The cheese whey is the main by-product of the cheese manufacturing industry, and by this reason it was used in this work for hydrogen production by *Escherichia coli.* The deletion of *hycA* and *lacI* genes in the strain WDHL resulted in a faster consumption of lactose and an increase of 22% in hydrogen production compared with the wild type strain. The hydrogen specific production rate was increased by 78.7% when optimized conditions of pH, temperature and cheese whey concentration.

According to the results the pH is one of the most important factors is hydrogen production. Controlling the pH at 6.5 resulted in the highest hydrogen production and yield.

Lactose, glucose and galactose are carbon sources commonly present in wastes of the agro-food industry, the hydrogen production from these substrates was also evaluated. The hydrogen yield from glucose (0.19 mol of H_2/ mol of glucose) was lower than that from galactose (0.19 mol of H_2/ mol of galactose). The low hydrogen yield from glucose is due to the large production of lactate, whereas in the galactose fermentation the formate production is increased and it is converted to hydrogen. Moreover an artificial neural network to estimate the hydrogen production using only on-line parameters was developed.

Chapter 1

General Introduction

1.1 Background.

Depletion of fossil fuels, environmental issues and the growing demand of energy have lead to the search of sustainable technologies based on renewable raw materials. Hydrogen has been considered a viable alternative energy carrier, since its high energy content per unit of mass and its utilization either on combustion or fuel cells results in pure water [1]. Unfortunately, the conventional methods for hydrogen production, such as electrolysis of water and reformation of natural gas, are energy intensive because they use high temperatures or pressures. The energy used in these processes is usually obtained from non renewable resources [2]. The biological hydrogen production (biohydrogen) is an attractive alternative method because it is carried out at ambient temperature and pressure. Furthermore, organic industrial wastes can be used as substrate for the hydrogen production. There are a great variety of microorganisms that can produce hydrogen like algae, cyanobacteria, non-sulfur purple bacteria and enterobacteria. Each kind of microorganism presents some advantages and disadvantages for hydrogen production. Among all the diversity of microorganism, *Escherichia coli* has a principal role in studies with axenic cultures. Moreover this bacterium is still the lab workhorse for metabolic engineering directed to improve the hydrogen production. *E. coli* has been used in the majority of studies because its metabolic

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pathways and genomic sequence are known and for the availability of molecular tools for its manipulation.

1.2 Hydrogen production by *Escherichia coli*.

The metabolic pathway for biohydrogen production by *Escherichia coli* is shown in Figure 1.1 Under anaerobic conditions a fraction of pyruvate can be transformed to lactate by the lactate dehydrogenase enzyme (LDH), but most of it is hydrolyzed by the pyruvate formate lyase (PFL) into acetyl-CoA and formate. PFL cleaves pyruvate only when cell grow fermentatively, while pyruvate dehydrogenase (PDH) descarboxylates pyruvate under aerobic conditions [3]. Both enzymes are active under oxygen limiting conditions. The acetyl-CoA is partially converted into ethanol and acetate. Formate is the electron donor in anaerobic metabolism for nitrate reduction or can be transformed into hydrogen by the formate-hydrogen-lyase complex (FHL).

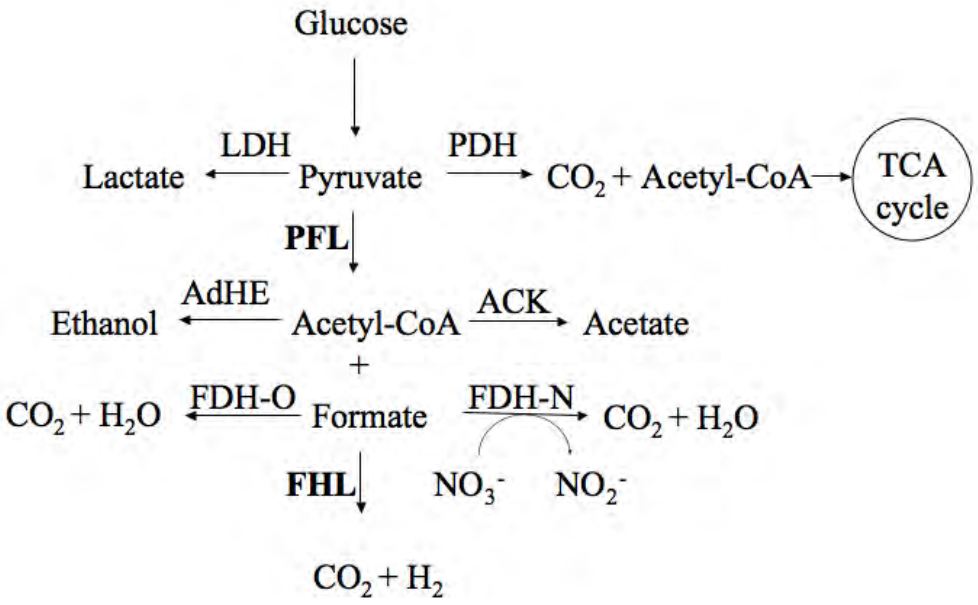

Figure 1.1 Metabolic routes of pyruvate and formate in *E. coli*. Key reactions in the generation of hydrogen are shown in bold.

In *E. coli* there are three formate dehydrogenases (FDH) denominated O, N and H. The FDH-H (encoded by the *fdhF* gene) forms part of the FHL complex.

The enzymes required for formate metabolism are encoded in the formate regulon which includes genes *hycB-I*, *hypA-E*, *hycA* and *hypF*. Hyc proteins are the structural proteins forming the FHL and Hyp proteins are involved in the maturation of the FHL, whereas HycA is the negative transcriptional regulator for the formate regulon and FhlA (encoded by *fhlA* gene) is the positive transcriptional regulator for the expression of *fdhF* gene (Figure 1.2). A complete description of formate regulon has been published elsewhere [4] [5].

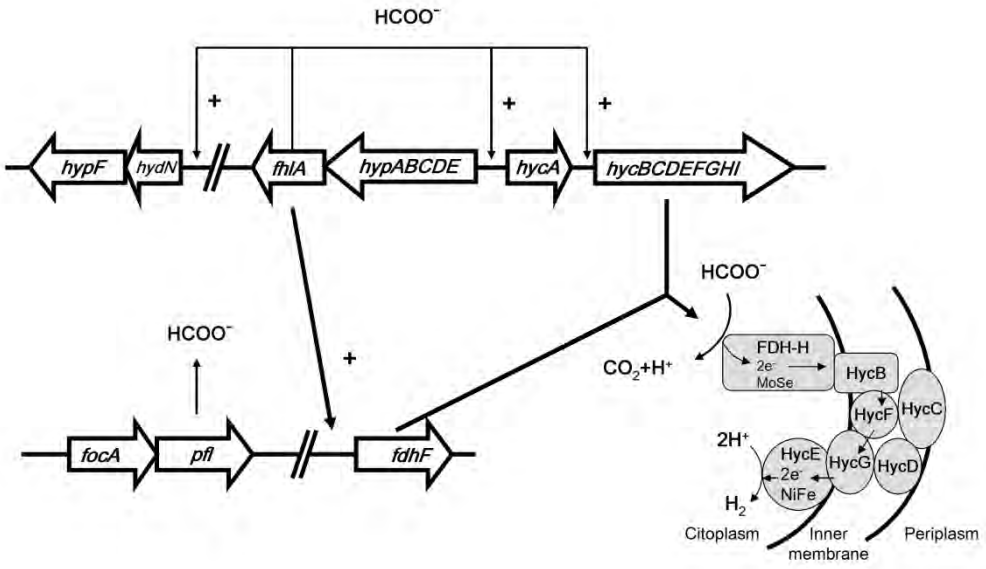

Figure 1.2 The formate regulon of *E. coli*: formate is generated by the *pfl* gene product. Genes or operons positively regulated by formate through the action of the transcriptional regulator FhlA are designated by + (Modified from [5]).

1.3 Improvement of hydrogen production and yield by genetic manipulations.

The biohydrogen production needs to be improved to be competitive to the conventional methods. The genetic manipulation has proved to be useful improving the hydrogen production (Table 1.1).

Table 1.1 Examples of genetically modified *Escherichia coli* strains and the strategies used to improve the hydrogen production

Strain	Strategy	Maximum yield (mol H_2/mol substrate)	Substrate	Ref.
SR13	Inactivation of *hycA* and overexpression of *fhlA.* High density cultures.	1	Formate	[6]
FTD701	Inactivation of *hycA* and *tatC*	NR	Glucose	[7]
DJT135	Deletion of uptake hydrogenases, mutation of *ldhA* and constitutive expression of *fhl.* Use of limiting concentrations of ammonia and glucose.	2	Glucose	[8]
DJT135	Use of optimal conditions of pH (6.5) and temperature (35°C).	1.51	Glucose	[9]
DJT135	Continuous culture under limitation of nitrogen, glucose, sulfur and phosphate.	2	Glucose	[10]
BW25113	Deletion of *hyaB, hybC, hycA, fdoG, frdC, ldha and aceE*	1.35	Glucose	[11]
SR15	Deletion of *ldhA* and *frdBc* High cell density cultures.	1.82	Glucose	[12]
FTD701/ pUR400	Inactivation of *hycA* and *tatC* and expression of the genes necessary for transport and metabolism of sucrose.	NR	Sucrose	[13]
BL-21/ PGEX4T-1/*hydA*	Expression of the Fe-hydrogenase from *Enterobacter cloacae*	NR	LB	[14]
BL21(DE3) Δ*iscR* pAF pYdbK	Construction of a synthetic hydrogen pathway, medium supplemented with thiamine pyrophosphate.	1.88	Glucose	[15]

NR: Not reported

The FHL activity is increased when *hycA* is mutated [4], thus HycA mutants are hydrogen overproducing strains [6, 16]. For instance, Yoshida *et al.* [6] constructed an *E. coli* strain overexpressing FHL by combining *hycA* inactivation with *fhl*A overexpression. With these genetic modifications, the transcription of *fdhF* (large-subunit formate dehydrogenase) and *hycE* (large-subunit hydrogenase) increased 6.5 and 7-fold, respectively, and hydrogen production increased 2.8-fold compared with the wild-type strain. The hydrogenases 1 and 2 and formate dehydrogenase N and O are located on the periplasmic space, and they must be transported by Twin arginine translocation (Tat) protein system to be active. Therefore, Tat mutant do not take formate up needed for hydrogen production, whereas hydrogenase 3 and FDH-H are located on cytoplasm and hence are not to be transported. Penfold *et al.* [7] reported that mutant strains defective of Tat transport (Δ*tatC* and Δ*tatA*-*E*) showed a hydrogen production comparable to *E. coli* strain carrying a Δ*hycA* allele. However, Δ*tatC* Δ*hycA* double mutant strain did not increase hydrogen production. Thus, it is possible that hydrogen production by *E. coli* could be increased by discarding activities of the uptake hydrogenases, which recycle a portion of hydrogen, and the formate hydrogenases N and O, which oxidize the formate without hydrogen production. Indeed the effect of mutations in uptake hydrogenases, in lactate dehydrogenase gene (*ldhA*) and *fhlA* was studied by Bisaillon *et al.* [8]. They reported that each mutation contributed to a modest increase in hydrogen production and the effect was synergistic. Then the same strain was used in batch [9] and continuous mode [10].

Maeda *et al.* [11] performed multiple stable mutations to direct the metabolic flux toward hydrogen production. The best strain involves mutations on *hyaB hybC hycA fdoG frdC ldhA aceE* genes. The *hyaB hybC* were deleted to abolish the uptake activity of hydrogenases 1 and 2. The *fdoG* and *aceE* genes code for the α subunit of formate dehydrogenase O and the pyruvate dehydrogenase respectively. The inactivation of *frdC* abolishes the succinate synthesis pathway. Yoshida *et al.* [12] using a strain *ΔldhA*, *ΔfrdBC* enhanced the hydrogen yield from 1.08 with the wild type to 1.82 mol H_2/mol of glucose.

In other studies genes from other microorganisms were cloned into *E. coli*. Penfold & Macaskie [13] transformed *E. coli* HD701, a hydrogenase-upregulated strain and FTD701, a derivative of HD701 that has a deletion of the *tatC* gene, with the plasmid pUR400 carrying the *scr* regulon. This regulon contains the genes of *Salmonella thompson* to metabolize sucrose. The resulted *E. coli* strains produced hydrogen from sucrose. The parental strains did not produce hydrogen, whereas recombinant strains produced 1.27 and 1.38 ml H_2/mg dry weight-$L_{culture}$. Mishra *et al*. [14] overexpressed a [Fe]-hydrogenase from *Enterobacter cloacae* (obtained with degenerate primers designed from the conserved zone of *hydA* gene) in a non-hydrogen producing *E. coli* BL21. The resultant recombinant strain showed the ability to produce hydrogen. Akhtar *et al* [15] constructed a synthetic hydrogen pathway by co-expression of *ydbK* from *E. coli*, [4Fe–4S]-ferredoxin from *Clostridium pasteurianum* and *hydF*, *hydE*, *hydG*, and *hydA* from *Clostridium acetobutylicum*. The hydrogen pathway worked, and the pathway was enhanced

with the deletion of *iscR*, the gene that codes for the negative regulator of the *isc* operon (Fe-S cluster).

1.4 Scope and structure of the thesis.

Escherichia coli is a valuable microorganism as model for the hydrogen production as discussed above. The aim of this thesis was to produce and improve the hydrogen production of *E. coli*.

In Chapter 2, the hydrogen production by *E. coli* using cheese whey as substrate was evaluated. To improve the biohydrogen production, a Δ*hycA* Δ*lacI* strain was constructed. A Box-Behnken experimental design was used to optimize pH, temperature and substrate concentration.

The influence of pH control on hydrogen production using cheese whey as substrate was studied in Chapter 3.

In Chapter 4 experiments were conducted in order to study the hydrogen production using lactose, glucose and galactose as substrates.

In Chapter 5 an Artificial Neural Network was developed to estimate the hydrogen production. Finally, the results obtained in this research are discussed in Chapter 6.

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Chapter 2

Hydrogen production by *Escherichia coli* Δ*hycA* Δ*lacI* using cheese whey as substrate

Abstract

This study proposes a fermentative hydrogen production by *Escherichia coli* using cheese whey as substrate. To improve the biohydrogen production, an *E. coli* Δ*hycA* Δ*lacI* strain (WDHL) was constructed. The absence of *hycA* and *lacI* genes had a positive effect on the biohydrogen production. The strain produced 22% more biohydrogen and in less time than the wild-type (WT) strain. A Box-Behnken experimental design was used to optimize pH, temperature and substrate concentration. The optimal initial conditions for biohydrogen production by WDHL strain were pH 7.5, 37°C and 20 g/L of cheese whey. The specific production rate was improved from 3.29 mL H_2/optical density at 600nm ($OD_{600\ nm}$) unit-h produced by WDHL without optimized conditions to 5.88 mL H_2/$OD_{600\ nm}$ unit-h under optimal conditions. Using optimal initial condition, galactose can be metabolized by WDHL strain. The maximum yield obtained was 2.74 mol H_2/mol lactose consumed, which is comparable with the yield reached in other hydrogen production processes with *Clostridium* sp. or mixed cultures.

Rosales-Colunga LM, Razo-Flores E, Ordoñez LG, Alatriste-Mondragón F, De León-Rodríguez A. **"Hydrogen production by Escherichia coli Δ*hycA* Δ*lacI* using cheese whey as substrate"** International Journal of Hydrogen Energy 2010;35:491-99.

2.1 Introduction.

Hydrogen has been considered a viable alternative energy carrier. It has a high-energy yield of 122 kJ/g, which is 2.75 fold greater than hydrocarbon fuels [1]. The main advantage of hydrogen is the absence of polluting emissions, since its utilization either via combustion or fuel cells, results in pure water [2]. Although the hydrogen storage is a challenge, efficient adsorption-desorption system are being developed [3-6]. The biological hydrogen production or biohydrogen is an attractive method because it is carried out at ambient temperature and pressure. Despite photosynthetic and fermentative processes can produce biohydrogen, the fermentative hydrogen production utilizes a wide range of carbon sources, does not need light and generally yields higher rates than the photosynthetic processes [1, 7-9]. In addition, it can be coupled to the use of organic industrial wastes [9-14]. Among the fermentative microorganisms, *Escherichia coli* has been the main microorganism genetically modified to improve the biohydrogen production. This is because its metabolic pathways and genomic sequence are known [10]. Glucose has been the main substrate used for biohydrogen production by *E. coli* modified strains [15-22] and few works reported biohydrogen production from formate [23, 24]. There are few reports on biohydrogen production using mutant *E. coli* strains and industrial wastes as a raw material [25, 26].

Cheese whey (CW) is the by-product from cheese production and represents an 85-90% of the total volume of processed milk. Only a minor proportion is used in the food industry and for animal feeding and the rest has the risk of being a

pollutant. However, CW is an inexpensive potential raw material for fermentative process considering its high content of lactose [27]. The aim of this work is to produce hydrogen from CW by *E. coli*. To improve the hydrogen production an *E. coli* W3110 Δ*hycA,* Δ*lacI* strain was constructed. Hydrogen is produced from formate in *E. coli* and the required enzymes are encoded in the formate regulon [10]. The *hycA* gene codes for the negative regulator of the formate regulon and strains with defective *hycA* gene are hydrogen overproducers' strains [24, 25]. In *E. coli*, the genes necessary to metabolize lactose are coded by the *lac* operon. The *lacI* gene was deleted to express constitutively the *lac* operon and increase the lactose consumption rate. This is the first work showing the biohydrogen production by a genetically engineered *E. coli* using cheese whey as substrate.

2.2 Experimental procedures.

2.2.1 Construction of mutant strains.

Strains, plasmids and primers used for the construction of the mutant strains are shown in Table 1. *E. coli* W3110 (WT) [28] strain was used because it grows well using CW as carbon source [27]. Mutant strains were constructed according to the method of Datsenko and Wanner [29] as follows: to generate the *E. coli* W3110 Δ*hycA*, WT strain was transformed with pKD46 plasmid and grown at 30°C to a 0.6 $OD_{600\ nm}$ in SOB medium (Invitrogen) plus 1 mM L-arabinose (Sigma, St Louis, MO) and ampicillin 200 μg/mL. These cells were transformed by electroporation with the PCR product obtained from the plasmid pKD3 as template with HYCF and HYCR primers. Afterward 900 μL SOC medium (Invitrogen) were added to

shocked cells and incubated 3-4 h at 30°C, 200 µL of this culture were spread on LB agar with 25 µg/ml of chloramphenicol, and incubated again at 30°C.

Table 2.1 Strains, primers and plasmids used in this work.

	Name	Description (Genotype) or sequence 5´-3´	Ref
Strain	WT	*Escherichia coli* W3110 (lac^+, gal^+, F^-, λ^- IN (rrnD-rrnE)1, rph-1)	[28]
	WDH	WT Δ*hycA*	This study
	WDHL	WT Δ*hycA* Δ*lacI*	This study
Plasmid	pKD46	Helper plasmid expressing the λ Red genes (*bla*(Amp^R) pBAD-λ -Red (*γ β exo*), pSC101 ori TS)	[29]
	pKD3	Template plasmid carrying the *cat* gene (*bla*(Amp^R) *oriR6Kγ,* FRT- *cat*-FRT	[29]
	pCP20	FLP recombinase expression plasmid (*bla* (Amp^R) *cat* (Cm^R) λp_R *FLP*$^+$, λ cI857$^+$, pSC101 ori TS)	[29]
Primers	HYCF	GCCTGCAAAACGGGCAAAGCCTCAGCTCATGCTGCCG GGCTTTGTCCCTGTGTAGGCTGGAGCTGCTTCG	This study
	HYCR	GCATCTCTGTTAAACGGGTAACCTGACAATGACTATTTG GGAAATAAGCGCATATGAATATCCTCCTTAG	This study
	LACF	CTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTT CCAGTCGGGTGTGTAGGCTGGAGCTGCTTCG	This study
	LACR	AGAGAGTCAATTCAGGGTGGTGAATGTGAAACCAGTAA CGTTATACGATGCATATGAATATCCTCCTTAG	This study
	OGHF	CACCAAGGCATTCCTCAGG	This study
	OGHR	GTCGAAATGACACGTCGA	This study
	OGLF	CGCAGGCTATTCTGGTGGCCG	This study
	OGLR	AGGGTTTTCCCAGTCACGACG	This study

The deletion of *hycA* was verified by colony PCR with OGHF and OGHR primers, which bind upstream and downstream of *hycA* gene. The mutants were incubated at 42°C to induce the loss of pKD46 plasmid and then tested for ampicillin sensitivity. The Δ*hycA* strain was transformed by electroporation with the pCP20 plasmid, and selected by both chloramphenicol and ampicillin resistant at 30°C. Transformed cells were incubated overnight in LB medium without antibiotic at 42°C, and then tested for sensitivity for both antibiotics. Colonies sensible to both antibiotics were PCR tested to confirm the lost of *hycA* or *cat* genes with OGHF and OGHR primers. This strain was named as WDH. Then, WDH strain was transformed with pKD46 and the deletion of *lacI* gene was done in the same way described above but using the LACF and LACR primers to obtain the PCR product to delete *lacI* gene and the OGLF and OGLR primers were used to verify the deletion. The Δ*hycA* Δ*lacI* resultant strain was named as WDHL.

2.2.2 Culture media.

Strains were maintained in LB plates. Hydrogen production experiments were done in HP medium, which contains per liter 0.8 g NaCl, 0.2 g KCl, 1.43 g Na_2HPO_4, 0.2 g KH_2PO_4, 1 ml of trace elements solution (0.015 g/L $FeCl_2.4H_2O$, 0.00036 g/L $Na_2MoO_4.2H_2O$, 0.00024 g/L $NiCl_3.6H_2O$, 0.0007 g/L $CoCl_2.6H_2O$, 0.0002 g/L $CuCl_2.2H_2O$, 0.0002 g/L Na_2SeO_3, 0.01 g/L MgSO4, 0.05 g/L rezasurine as redox indicator) and the concentration of CW powder (Land O´Lakes, Arden Hills, Minnesota) specified in each experiment. The pH was fixed at 6.8 for general

purpose or according to the Box-Behnken design described below. The HP medium was pasteurized during 25 min at 65 °C and chilled 20 min on ice.

2.2.3 Comparative growth kinetics of mutant strains using glucose or lactose.

WDH and WDHL strains were aerobically cultured (37 °C; 175 rpm) in HP medium plus 1 g/L NH_4Cl, 40 µg/L thiamine (Sigma), but using 5 g/L glucose or 5 g/L lactose instead CW. Samples were taken and the optical density at 600nm ($OD_{600\ nm}$) was measured as described in the section 2.7. Inoculum of each strain was pregrown overnight using the HP medium with glucose plus 5 g/L yeast extract (BD, Le Pont-de-Claix, France).

2.2.4 Comparative hydrogen production ability by the WDH and WDHL mutant strains.

To evaluate the hydrogen production by the WT, WDH and WDHL strains, each strain was cultured in 120 mL anaerobic serological bottles containing 110 ml of HP medium with 16.5 g/L CW. The cultures were started with 1.5 $OD_{600\ nm}$, pH of 6.8 and were incubated at 37°C and 175 rpm. Inocula were pregrown 48 h in LB medium in anaerobic conditions. Cells were harvested, centrifuged, washed and inoculated into the serological bottles. Nitrogen gas was sparged into the bottles to ensure the anaerobic condition.

2.2.5 Experimental design.

A Box-Behnken experimental design (Table 2) was used to find the optimal conditions for the hydrogen production using CW as substrate. The independent variables were: pH, temperature and CW concentration. Three levels for each variable were included. The response variables were volumetric hydrogen production (VHP) and hydrogen production rate (HPR). The experiments were done in 120 mL anaerobic serological bottles; the cultures were adjusted to initial $OD_{600\ nm}$ of 1.5 and were shaken at 175 rpm. Data were analyzed according to response surface methodology (RSM). Analysis of variance (ANOVA), RSM and the optimal conditions were performed using Statgraphics Plus *v* 5.0 software (Statistical Graphics Co). *F*-test from ANOVA was used to evaluate the adjusted models. The significance of each coefficient was determined with the *t* test with a *P*-value smaller than 0.05.

2.2.6 Batch cultures on bioreactor.

Batch cultures were performed using HP medium plus 20 g/L of CW in a 1 L bioreactor (Applikon, Foster City, CA) equipped with two six-blade Rushton turbines. Redox potential, pH and dissolved oxygen were monitored using autocleavable electrodes (Applikon) and connected to the Bioconsole ADI 1035 (Applikon) controlled by the ADI 1030 Biocontroller (Applikon). The redox electrode was calibrated at 215 mV using the reference solution HI7020 (Hanna Instruments, Armazem, Portugal) and was corrected using the pH modified Nerst equation [30]. BioXpert 1.3 software (Applikon) for data acquisition was used. The cultures were

maintained at 37°C and stirred at 175 rpm. Culture samples were periodically taken from the bioreactor, and centrifuged. The supernatant was filtered through a 0.22 µm filter (Millipore) before analysis of fermentation products. Inocula were pregrown overnight in 25 mL of LB medium at 37°C and shaken at 200 rpm, afterwards added to 900 mL of fresh LB medium in closed twist cover bottles and were incubated at 37°C for 48 h. Cells were harvested, washed and inoculated into the bioreactor.

2.2.7 Analytical methods.

The gas produced was measured by water displacement in an inverted burette connected either to the bioreactor or to serological bottles with rubber tubing and a needle. The hydrogen content in the gas phase, sugars and organic acids were determined as described elsewhere [31]. Ethanol was determined as described elsewhere [32]. Cell growth was monitored at $OD_{600\ nm}$ using a spectrophotometer Cary BIO-50 (Varian, Palo Alto, CA).

2.3 Results and discussion.

2.3.1 Hydrogen production using CW as substrate by WDH and WDHL mutant strains.

E. coli W3110 Δ*hycA* strain (WDH) was constructed to improve the hydrogen production. In the first experiment, WDH strain was grown on lactose or glucose in aerobic condition to measure the lag phase duration on these substrates (Figure. 2.1). It can be observed that the lag time using lactose was 1.5 h higher that the culture using glucose. Since this behavior can be more dramatic under anaerobic conditions a WDHL strain was constructed deleting the *lacI* gene in the WDH strain. As expected, the resultant WDHL strain showed the same lag-time using glucose or lactose as substrate. No effect of the *lacI* deletion was observed on the specific growth rate or overall biomass yield (Figure. 2.1).

The hydrogen production by the WT, WDH and WDHL strains was evaluated using CW as substrate. Since, the experiment was done in serological bottles and the

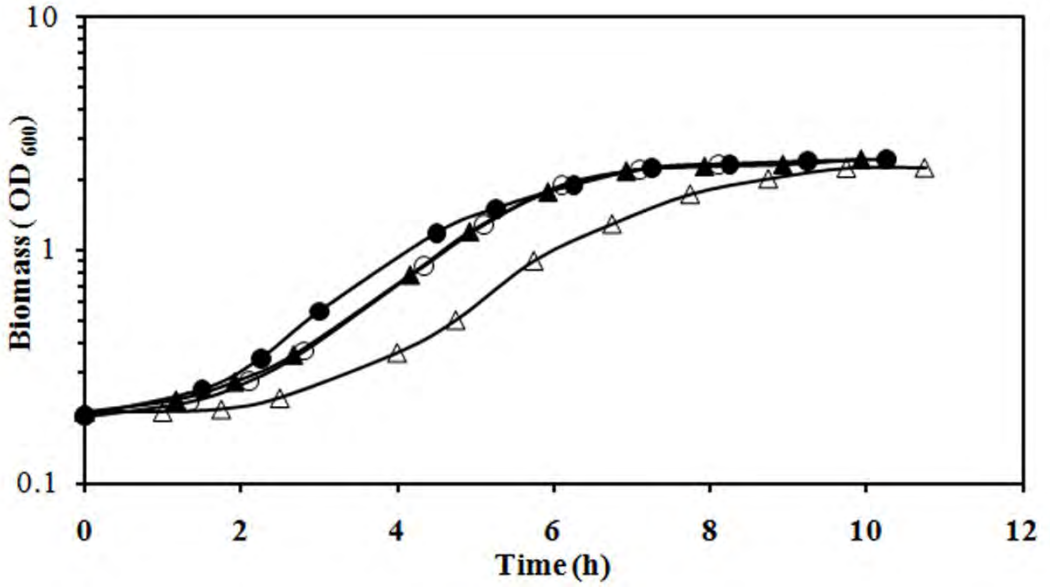

Figure 2.1 Growth kinetics of the *E. coli* WDH (W3110 Δ*hycA*) in triangles and WDHL (W3110 Δ*hycA,* Δ*lacI*) in circles using lactose (open symbol) or glucose (filled symbol) as carbon source.

gas volume was measured periodically by water displacement, hydrogen production could be affected by the high partial pressure as reported for other microorganism [7]. Figure. 2.2 shows that the WT strain produced 94.7 mL of hydrogen. The deletion of the *hycA* gene had no effect on the final hydrogen production, whereas the WDHL strain produced 22% more hydrogen than the WT, leading to a final hydrogen production of 115.5 mL. In WDHL strain the *lac* operon transcription becomes constitutive and the induction of the formate regulon is constant. Moreover WDHL produces almost 110 mL of hydrogen in 170 h, which is 95% of the final production, whereas the WT and WDH strains only produced 72% and 76% of the final production. Therefore the WDHL strain was selected for subsequent experiments.

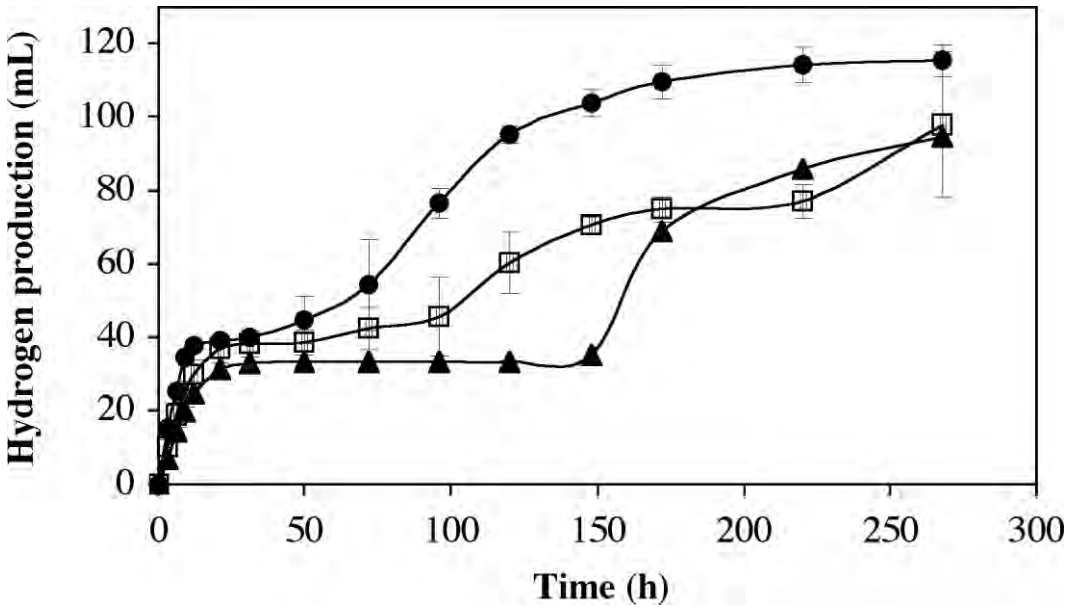

Figure 2.2 Hydrogen production kinetics by WT (▲), WDH (□) and WDHL (●) strains using CW as carbon source. The error bars indicate standard deviations.

2.3.2 Hydrogen production by the WDHL strain in bioreactor.

Kinetic behavior of *E. coli* WDHL batch culture conducted in bioreactor is shown in Figure. 2.3. Only a slight biomass concentration increment from 1.3 to 2.35 $OD_{600\ nm}$ was attained (Figure. 2.3A). Cell growth was observed on the first 6 h and between 50 and 66 h. The lactose from the CW was consumed quickly and galactose accumulated to a maximum concentration of 22 mM. For this culture, lactose decreased from 42 to 15 mM (Figure 2.3A). A rapid production of hydrogen was observed from the beginning of the culture and it attained a maximum production of 983.8 mL (Figure 2.3B). Besides the hydrogen production *E. coli* WDHL produced succinate, acetate, lactate and ethanol. Only a slight amount of formate was detected (less than 5 mM); hence this was used immediately to produce hydrogen instead of the formate exportation to the medium (Figure 2.3C). A notorious decrease of pH was observed due to the accumulation of these organic acids (Figure 2.3D). This behavior continued until 40 h of fermentation, but the rate of hydrogen and acids production decreased and the pH dropped slowly. This strain began to metabolize acetate and lactate due to the low pH. The amount of acetate was almost 12.5 mM at 42 h and dropped to almost 3.5 mM at 120 h and this amount was constant until the end of fermentation. The amount of accumulated lactate was 13.5 mM at 42 h, achieving 1.7 mM at 100 h and became undetectable at 163 h of fermentation. The acetate and lactate consumption increased the pH from 4.65 to 4.9 at 65 h, and then began to decrease slowly to 4.75 at the end of fermentation (Figure. 2.3D). Succinate was also produced during

the culture and it attained a maximum concentration of 30 mM and then it remained constant until the end of fermentation. Ethanol was produced at the beginning of fermentation and then remained constant at 13 mM.

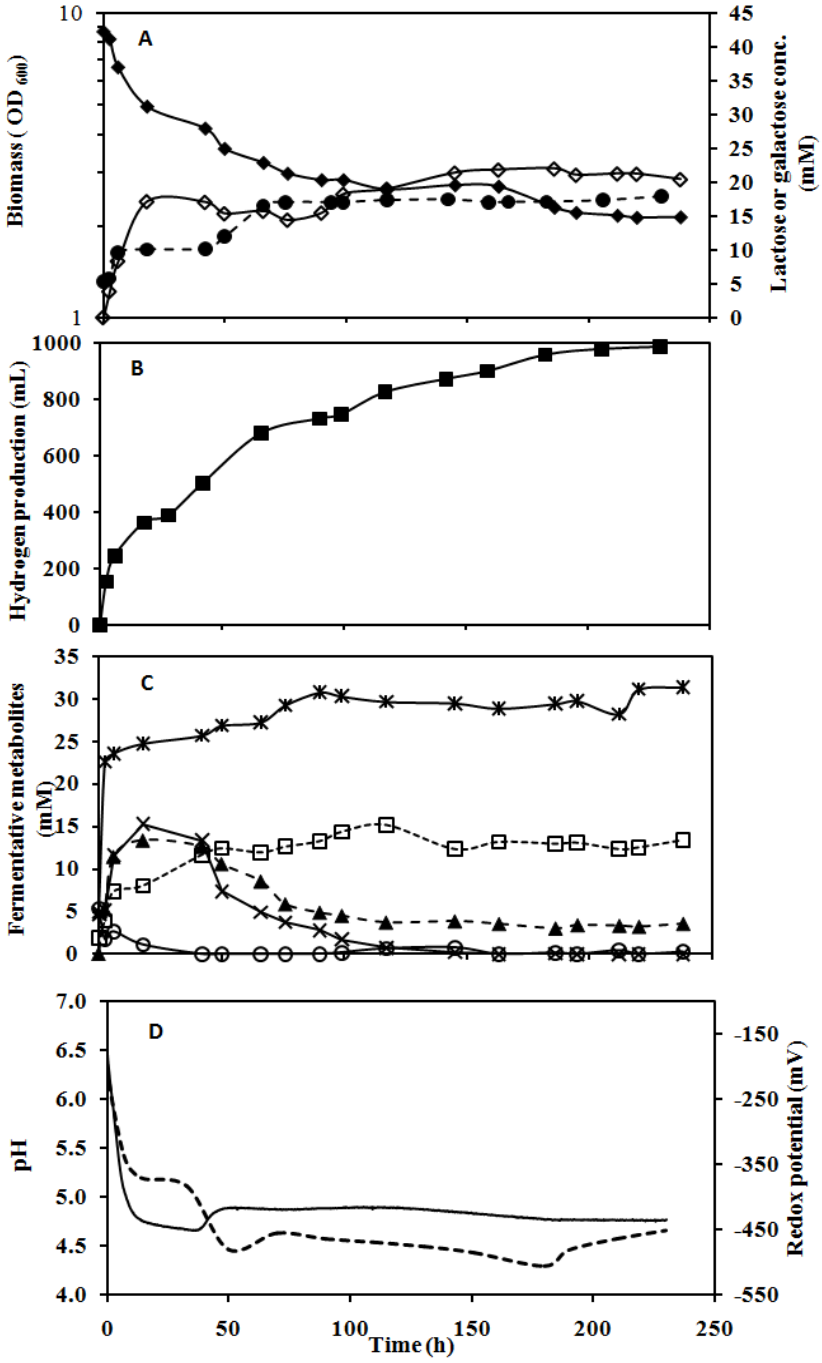

Figure 2.3 Batch culture of the E. coli WDHL strain using 20 g/L of cheese whey, initial pH 6.5 and 37°C. (**A**) Biomass (●), lactose (◆) and galactose conc.(◇). (**B**) Hydrogen production. (**C**) Production of organic acids; succinate (✳), lactate (×), acetate (▲), formate (O), and ethanol (□). (D) pH (—) and redox potential (– –).

The redox potential decreased from -104 to -450 mV as a result of the metabolic activity. The decrease was related to the cell growth, since the redox potential went down dramatically when the cell concentration increased. The specific production rate was 3.29 mL $H_2/OD_{600\ nm}$ unit-h respect to the initial $OD_{600\ nm}$ and the yield was 1.21 mol H_2/mol lactose consumed or 0.97 mol H_2/ mol hexose consumed.

2.3.3 Optimization of the culture conditions to improve the hydrogen production.

In order to find the best conditions for the hydrogen production by the WDHL strain using CW as substrate, an experimental Box-Behnken design was done. The effect of the substrate concentration, pH and temperature on the hydrogen production was evaluated. The experimental design used and results obtained from these 15 experiments are shown in Table 2.2. The maximum hydrogen production and hydrogen production rate were reached by experiment 2 (pH 7.5, 37°C, 20 g/L of CW).

Table 2.2 Box-Behnken experimental design and corresponding results for volumetric hydrogen production (VHP) and hydrogen production rate (HPR) by WDHL strain in HP medium.

Experiment	pH	Temperature (°C)	Conc. of CW (g/L)	VHP (mL H_2)	HPR (mL H_2/h)
1	6.5	37	10	119.6	1.63
2	7.5	37	20	175.7	2.76
3	6.5	37	20	136.7	1.46
4	7.5	37	10	121.6	1.79
5	7	28	20	95.5	0.90
6	7.5	28	15	97.4	0.59
7	6.5	28	15	77.5	0.65
8	7	46	10	51.3	0.16
9	7	37	15	121.5	1.42
10	7.5	46	15	87.4	0.27
11	7	46	20	94.3	0.28
12	7	28	10	68.1	0.53
13	6.5	46	15	51.0	0.26
14	7	37	15	131.1	1.29
15	7	37	15	119.4	1.33

The mathematical model representing the hydrogen production as a function of the evaluated variables in the experimental region studied is expressed by the following equation:

(1) Hydrogen production (mL) =

$$1272.17 - 496.992*A + 40.3002*B - 33.5917*C + 30.85*A^2 + 0.916667*A*B + 3.7*A*C - 0.659105*B^2 + 0.0866667*B*C + 0.2675*C^2$$

Where A is the pH, B is the temperature in °C and C is the CW concentration. The standard error was 5.47 and the R^2 value was 99.07%. These values indicate a good fit between the model and the experimental data indicating that the treatment was highly significant. The analysis of variance (ANOVA) for the adjusted model showed the hydrogen production was significantly affected by A, B, C, AA, AC and BB (Table 2.3).

Table 2.3 Analysis of variance for the adjusted model for hydrogen production in HP medium.

Source[a]	Sum of Squares	Df[b]	Mean Square	*F*-Ratio[c]	*P*-Value[d]
A	1183.41	1	1183.41	39.46	0.0015
B	371.281	1	371.281	12.38	0.0169
C	2506.32	1	2506.32	83.58	0.0003
AA	219.628	1	219.628	7.32	0.0425
AB	68.0625	1	68.0625	2.27	0.1923
AC	342.25	1	342.25	11.41	0.0197
BB	10523.9	1	10523.9	350.93	0.0000
BC	60.84	1	60.84	2.03	0.2136
CC	165.13	1	165.13	5.51	0.0658
Total error	149.943	5	29.9885		
Total (corr.)	16094.5	14			

[a] A = pH, B = Temperature, C = CW concentration; [b] Degrees of freedom; [c] Fisher test;
[d] Probability distribution value; *P*-value less than 0.05 indicates the term was significant.

The response surface plots for hydrogen production are shown in Figure. 2.4A to 2.4C. CW concentration had a positive effect on the hydrogen production, *i.e.* high CW concentration produced more hydrogen. A similar effect but less intense was observed with the pH, whereas the temperature presents a maximum value of hydrogen production around 37°C.

The effect of the temperature, pH and CW concentration on the hydrogen production rate was also evaluated. The mathematical model representing the hydrogen production rate is represented by the following equation:

(2) Hydrogen production rate (mL/h)=

$$47.0488 - 16.5747*A + 1.04193*B - 1.06736*C + 1.07667*A^2 + 0.00388889*A*B + 0.114*A*C - 0.0144856*B^2 - 0.00138889*B*C + 0.0117667*C^2$$

In this case the R^2 was 95.8% and the standard error was 0.2513. Table 2.4 shows that the hydrogen production rate was significantly affected only by BB.

Table 2.4 Analysis of variance for the adjusted model for hydrogen production rate in HP medium.

Source[a]	Sum of Squares	Df[b]	Mean Square	*F*-Ratio[c]	*P*-Value[d]
A	0.248513	1	0.248513	3.94	0.1041
B	0.36125	1	0.36125	5.72	0.0622
C	0.208012	1	0.208012	3.29	0.1292
AA	0.26751	1	0.26751	4.24	0.0946
AB	0.001225	1	0.001225	0.02	0.8947
AC	0.3249	1	0.3249	5.15	0.0726
BB	5.08324	1	5.08324	80.51	0.0003
BC	0.015625	1	0.015625	0.25	0.6400
CC	0.31951	1	0.31951	5.06	0.0743
Total error	0.315692	5	0.0631383		
Total (corr.)	7.51877	14			

[a] A = pH, B = Temperature, C = CW concentration; [b] Degrees of freedom; [c] Fisher test; [d] Probability distribution value; *P*-value less than 0.05 indicates the term was significant.

The response surface plots of Figure 2.4D to 2.4F were obtained based on this equation. Figures 2.4D and 2.4F show that the amount of CW also affects hydrogen production rate; at high concentrations the production rate also increased. The best parameters for the hydrogen production rate were 20 g/L of cheese whey, pH 7.5 and 36°C. The best conditions for improving both the hydrogen production and hydrogen production rate were 20 g/L of cheese whey, pH of 7.5 and 37°C, since similar hydrogen production rate was observed at 36 and 37°C according to response surface plot.

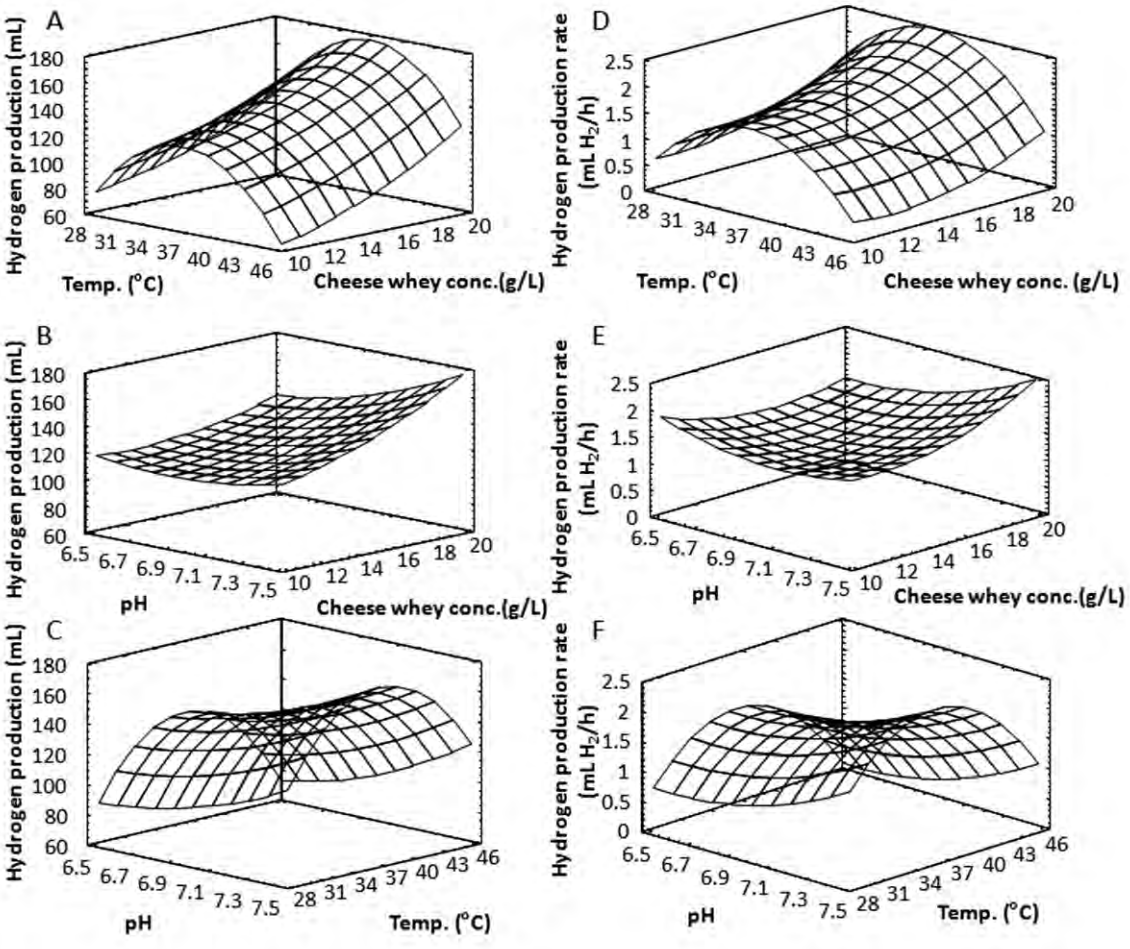

Figure 2.4 Response surface plots of the hydrogen production (**A** to **C**) and hydrogen production rate (**D** to **F**) by WDHL strain in HP medium. pH fixed to 7.5 in **A** and **D**, temperature fixed at 37°C in **B** and **E**, concentration of CW fixed at 20 g/L in C and F.

Similar to the results obtained here, Li *et al.* [28] found a direct correlation between the initial pH in the range 5 to 7 with the hydrogen production rate and the hydrogen yield in batch cultures based on natural sludge and using glucose as substrate. The possible reason for this is that the higher initial pH could buffer the acid production that accompanied the hydrogen production. This could also explain that the higher pH tested on the Box-Behnken design resulted in the better condition. The extrapolation of Figure 4B suggests that a pH value up of 7.5 should improve the hydrogen production, however Ghosh and Hallenbeck [33] reported that initial pH values up of 7.5 reduce the hydrogen production by a metabolically engineered *E. coli*. Yoshida *et al.* [24] established the maximum hydrogen production rate at 42°C and pH around 6.5, with *E. coli* W3110, using sodium formate as substrate. Ferchichi *et al.* [34] reported that the hydrogen production rate from cheese whey peaked at an initial pH 6 with *Clostridium saccharoperbutylacetonicum*. Davila-Vazquez *et al* [31] found the highest hydrogen molar yield at pH of 7.5 and 6.5 using lactose and CW powder respectively in mixed cultures. Therefore, the pH is one of the most important parameters that affect the hydrogen production on different microorganisms.

2.3.4 Hydrogen production under the best conditions.

The best initial conditions were tested in bioreactor experiments and the results are shown in Figure. 2.5. Lactose was quickly consumed at the beginning of fermentation and galactose began to accumulate but interestingly in this case, the initial pH of 7.5 allowed galactose consumption. The residual concentration of lactose was 4 mM (Figure 2.5A) and glucose was not detected during the whole

fermentation. Hydrogen was produced from the beginning of fermentation and its production showed a similar behavior than the non-optimized fermentation, but the hydrogen production was increased. At 200 h of fermentation the hydrogen production was 2488 mL. At this time it seemed that the fermentation process was halted, like in the previous fermentation. However, 630 mL of hydrogen were further produced between 250 and 300 h of fermentation (Figure. 2.5B) therefore the cumulative hydrogen production was 3245.4 mL. Organic acids and ethanol production are shown in Figure 2.5C. The initial pH of 7.5 allowed a slight increment of the initial organic acid production. Ethanol reached a concentration of 10 mM in the first 12 h and then remained around 12 mM until the end of fermentation. The concentration of formate was 5 mM at the beginning of the fermentation and after 9 h of fermentation became less than 0.5 mM. This low amount indicates that it was used to produce hydrogen as soon as it was produced. Succinate, lactate and acetate were produced since the beginning; afterwards cells metabolized these acids, mainly lactate, and to a lesser extent acetate and succinate. It is very likely that the initial conditions used in this experiment caused the succinate consumption. Ren *et al.* [35] reported that redox potential and pH are related with changes on the fermentation type in continuous flow reactor and mixed cultures. Also, Hussy *et al* [36] reported that redox potential is negatively related to the rate of gas production with mixed cultures in a continuous process. As in the case of the previous fermentation under non optimal conditions, the organic acids production and consumption caused changes on pH, and the drop of redox potential was mainly related with the cell-growth (Figure. 2.5).

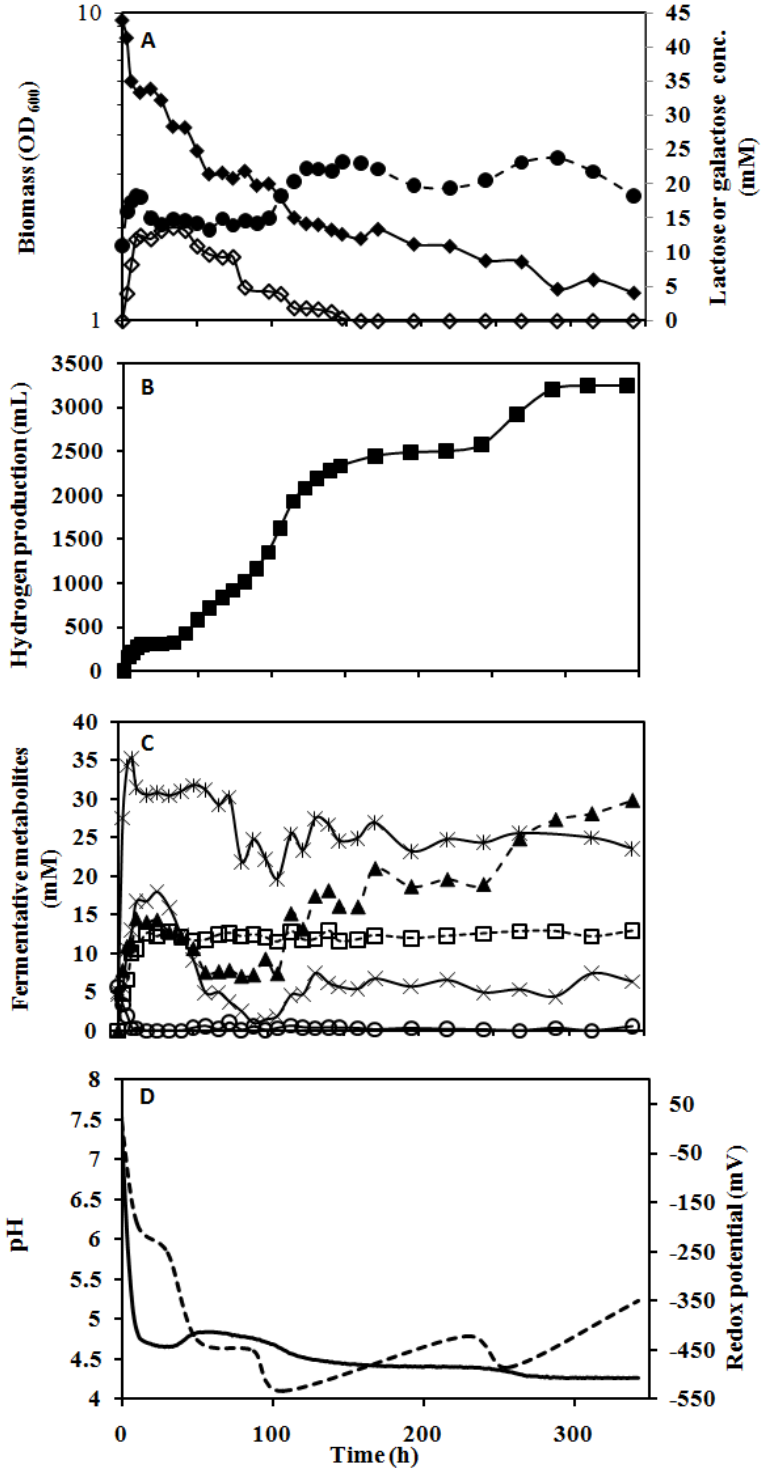

Figure 2.5 Batch culture of the *E. coli* WDHL strain at optimal initial conditions: 20 g/L of cheese whey, pH 7.5 and 37 °C. (**A**) Biomass (●), lactose (◆) and galactose conc. (◇). (**B**) Hydrogen production. (**C**) Production of organic acids; succinate (✳), lactate (×), acetate (▲), formate (○), and ethanol (□). (D) pH (—) and redox potential (– –).

According to the data obtained from the optimization experiments, higher CW concentrations should improve the hydrogen production and hydrogen production rate (Figure. 2.4). For this reason, an additional experiment was carried out using 40 g/L instead of 20 g/L of CW. No improvement of the hydrogen production was observed at the higher CW concentration (data no shown). The specific production rate at optimized conditions was 5.88 mL $H_2/OD_{600\ nm}$ unit-h respect to the initial $OD_{600\ nm}$ and it was 1.8-fold higher than that attained under non-optimal conditions. The hydrogen yield in the optimized fermentation was 2.74 mol H_2/mol lactose consumed or 1.37 mol H_2/mol hexose consumed. The yield obtained in this work using a double mutant strain was similar to the value reported using a multi-gene deleted *E. coli* strain by Maeda *et al* [16]. They obtained a yield of 1.3 mol H_2/mol glucose using an *E. coli* BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*. The *hyaB* and *hybC* genes were deleted to remove the hydrogen uptake activity, whereas *fdoG* and *aceE* were deleted to redirect the glucose metabolism to formate. The succinate and lactate synthesis were inactivated by the deletion of *frdC* and *ldhA* genes. Higher yields have been reported, Yoshida *et al.* [20] obtained a maximum yield of 1.82 mol H_2/mol glucose, using an *E. coli* with *hycA*, *frdC* and *ldhA* genes deleted and *fhlA* overexpressed. Bisaillon *et al.*[15] used an *E. coli* JW135 strain carrying deletions on the two uptakes hydrogenases and mutations on *ldhA* and *fhlA* genes, and the hydrogen yield reported approaching 2 mol H_2/mol glucose. In those works glucose was the substrate for the hydrogen production. Although the main way to reduce the cost of hydrogen production is by increasing the yield from glucose, another way is by converting inexpensive feedstock into hydrogen [13]. Few works have reported the use of *E. coli* mutant

strains consuming industrial wastes as a raw material to produce hydrogen. Penfold *et al.* [25] reported the production of hydrogen by *E. coli* HD701 (a hydrogenase upregulated strain) at expense of glucose and fructose, the compounds of sucrose, which is a major constituent of many waste materials. In that work, industrial nougat waste (containing sucrose, fructose and glucose) was used to produce 31.63 mL H_2/h-$OD_{600\ nm}$ unit-$L_{culture}$. *E. coli* HD701 and FTD701 (an isogenic strain of the HD701 that has a deletion of the *tatC* gene) were transformed with the plasmid pUR400 by Penfold and Macaskie [26]. This plasmid carries the genes necessary for sucrose transport and metabolism to produce hydrogen from sucrose. The parental strains did not produce hydrogen, whereas the recombinant strains produced 1.27 and 1.38 mL H_2/mg dry weight-$L_{culture}$. In our study, CW was used as substrate and 2.74 mol H_2/ mol consumed lactose was obtained, this yield is similar to the highest yield of 2.7 mol H_2/ mol lactose reached by Ferchichi *et al* [34] in a pure culture of *Clostridium saccharoperbutylacetonicum* using CW, and it is comparable with 3.1 mol H_2/ mol lactose reported by Davila-Vazquez *et al* [31] with CW powder and mixture cultures.

2.4 Conclusions.

This study showed that the CW can be used to produce hydrogen by *E. coli* W3110 mutant strains. The deletion of *lacI* gene led to a lag-time reduction using lactose as carbon source. Meanwhile, deletion of *lacI* and *hycA* genes on strain WDHL improved hydrogen production by 22% in a shorter time than with the WT. The optimal culture conditions were found by RSM. The best initial conditions for

hydrogen production were pH 7.5, 37°C and 20 g/L of cheese whey. The specific production rate was improved from 3.29 mL H_2/OD_{600nm} unit-h produced by WDHL under non-optimal conditions to 5.88 mL $H_2/OD_{600\ nm}$ unit-h at optimal conditions. The hydrogen yield was improved from 1.21 mol H_2/mol lactose consumed to 2.74 mol H_2/mol lactose consumed under the best conditions. The results showed that the pH is an important variable on the hydrogen production and that the control of pH could improve the hydrogen production. This work enriches the information on hydrogen production using genetically improved *E. coli* strains and provides the basis for further studies.

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Chapter 3

Influence of pH on hydrogen production by *Escherichia coli* Δ*hycA* Δ*lacI* using cheese whey as substrate

Abstract

The pH is one of the most important factors on hydrogen production by *Escherichia coli*. In this work the influence of pH control on the hydrogen production by *E. coli* Δ*hycA,* Δ*lacI* (WDHL) strain using cheese whey as substrate was studied. The bioreactor was operated at values of 5.5, 6 and 6.5. From the range of pH evaluated, 6.5 was the best condition and the highest hydrogen production and yield were obtained. Moreover all carbon sources from the cheese whey were consumed. At this pH a mix of ethanol and acids mainly lactate are produced from glucose, whereas galactose yields acetate ethanol and succinate. Operating the reactor at 5.5 resulted in high production rate but smaller yield because only the glucose of the lactose was metabolized. At this pH a mix of ethanol and acids mainly lactate are produced from glucose, the metabolism of galactose yields other acids than lactate and ethanol. Controlling pH at 6 not all the carbohydrates of cheese whey were consumed and was not favorable for hydrogen production. Lactose consumption and growth kinetics were not affected by the pH.

The results show the importance of controlling the pH to improve the hydrogen production and the galactose consumption using cheese whey as substrate.

Rosales-Colunga LM, Razo-Flores, Alvarado-Cuevas Z, De León-Rodríguez A. **"Influence of pH on hydrogen production by *Escherichia coli* Δ*hycA* Δ*lacI* using cheese whey as substrate"** In preparation to be submitted to Biomass and Bioenergy.

3.1 Introduction.

Hydrogen has been recognized as a clean substitute of fossil fuels because of its higher energy yield of 122 kJ/g, which is 2.75-fold greater than hydrocarbon fuels [1]. Moreover its use is environmentally benign because the combustion or use on fuel cells only produce water [2]. The biological hydrogen production by fermentative way is an attractive method because it is carried out at ambient temperature and pressure. In addition a widely type of substrates can be used [3-7].

To be economically competitive the fermentative hydrogen production must use carbohydrate rich wastes or by-products. The cheese whey (CW) is the major by-product from cheese production and represents an 85-90% of the total volume of processed milk. Disposal of the CW is a major problem of the dairy industry [8]. Most of this by-product is discharged to the environment [9] and only a minor proportion is used in the food industry and for animal feeding. Therefore, the CW disposed without treatment is considered a source of environmental pollution due to its bulk quantities and high organic content [10].

The main components of CW are lactose (70-72% dried extract), proteins (8-10%) and mineral salts (12-15% dried extract) [11]. Considering its components CW is an inexpensive potential raw material for fermentative process [12, 13].

There are two kinds of whey; the by-product of the production of hard, semi hard and soft cheese is known as sweet whey. Whereas the manufacture of mineral-acid precipitated casein yields acidic whey [14]. The pH of sweet whey and acidic

whey is 5.9-6.6 and 4.3-4.6 respectively. This parameter is important if the use of this by-product as substrate is desired for hydrogen production.

The fermentative hydrogen production and proportions of end products are strongly affected by the pH of the culture medium [15]. The initial pH is one of the most important parameters that influences the fermentative hydrogen production with axenic [16-18] and non axenic cultures [19]. The fermentative pathway in *Escherichia coli* is linked to the production of organic acids such as acetic, formic, lactic and succinic. These products are accumulated and can affect the hydrogen production if the pH is not controlled in a favorable range. Although the effect of initial pH on the hydrogen production has been widely described using a variety of inocula [16-19], few works described the influence of on line control of pH in non axenic cultures [20, 21]. To our knowledge, the effect of control of pH on *E. coli* fermentations has been poorly studied. In this work, the influence of on-line control of pH on the hydrogen production by *Escherichia coli* WDHL [16] using sweet cheese whey as substrate was studied.

3.2 Experimental procedures.

3.2.1 Strain and culture media.

Escherichia coli WDHL strain [16] was used in this work. Inocula were pre-grown overnight in 25 mL of LB medium at 37°C and shaken at 200 rpm, afterwards added to 900 mL of fresh LB medium in closed twist cover bottles and were incubated at 37°C for 48 h. Cells were harvested, washed and inoculated into the

bioreactor. Cultures on bioreactor were done using HP medium (a complete description of the medium was reported elsewhere [16]) with 20 g/L of cheese whey powder (Land O´Lakes, Arden Hills, Minnesota).

3.2.2 Cultures on bioreactor.

Batch cultures were performed in a 1-L bioreactor (Applikon, Foster City, CA.). The pH was monitored on-line using an autoclavable electrode (Applikon) connected to the ADI 1035 Bioconsole (Applikon). The initial pH was 7.5 in all the experiments and was allowed to decrease to the desired value. Once the pH reached the value indicated in each experiment, was automatically controlled by the ADI 1030 Biocontroller (Applikon) at the set point indicated and using 2.5 N NaOH and HCl solutions. The control parameters were a dead zone of 0.1 and hysteresis of 1. BioXpert 1.3 software (Applikon) was used for data acquisition. The cultures were maintained at 37°C and stirred at 175 rpm with two six-blade Rushton turbines. The fermentations at pH of 5.5 and 6 were done by triplicate.

3.2.3 Analytical methods.

Cell growth was monitored at $OD_{600\ nm}$ using a spectrophotometer Cary BIO-50 (Varian, Palo Alto, CA). Culture samples were periodically taken from the bioreactor, centrifuged and the supernatant was filtered through a 0.22 µm filter (Millipore). The gas produced 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, sugars and organic acids were determined as described elsewhere [19]. Ethanol was determined as described elsewhere [22].

3.3 Results and discussion.

3.3.1 Influence of on line pH control on hydrogen production.

The pH is one of the most important factors on hydrogen production by *Escherichia coli* [16, 23, 24]. In a previous work, it was observed that the hydrogen production by WDHL (Δ*hycA,* Δ*lacI*) strain was better with respect the WT strain and the initial pH is an important factor over the hydrogen production using cheese whey as substrate [16]. In order to study the effect of the control of pH on the hydrogen production a set of experiments were conducted at values of 5.5, 6 and 6.5. The experiments at 5.5 and 6 were done by triplicate; data from a representative experiment of each value are showed in figures.

Figure 3.1 shows the hydrogen production and cell growth at pH values of 5.5 (A), 6 (B) and 6.5(C). The growth kinetics showed a similar behavior in the 3 cases. A slight increment in biomass concentration was observed during the first 12 h, and then the biomass decreased slowly. The highest increase on biomass was observed at pH of 6.5.The control of pH at 5.5 resulted in a maximum hydrogen amount of 868 mL (Figure 3.1A) and it was the lowest hydrogen production of the conditions tested. Hydrogen was only produced in the first 20 hours, and then the hydrogen production stopped. At pH of 6 two phases of hydrogen production were

observed; 1157 mL were produced in the first 56 h and 689 mL were produced in 164 h to yield a maximum amount of 1846 mL.

The highest hydrogen production of 2402 mL was attained at 6.5 (Figure 3.1C).

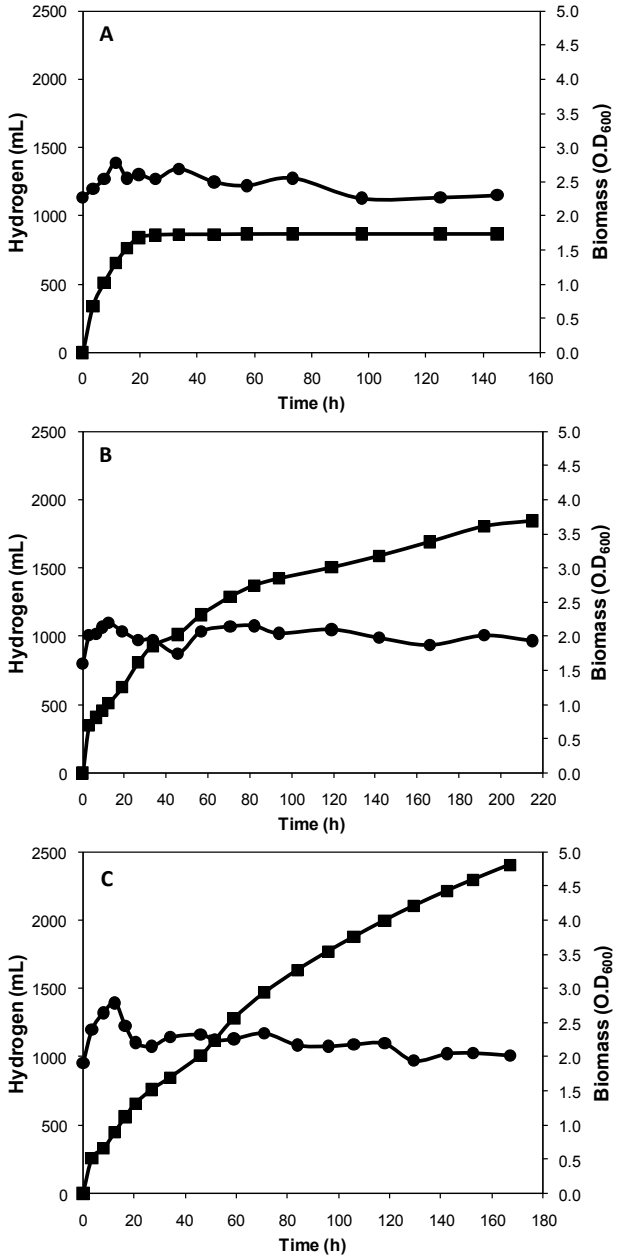

Figure 3.1 Biomass (●) and hydrogen production (■) of the cultures at pH of 5.5 (**A**), 6 (**B**) and 6.5 (**C**).

3.3.2 Effect of pH on substrate consumption.

Lactose is the main component of the cheese whey, which is hydrolyzed by the β–galactosidase enzyme giving glucose plus galactose. The amount of this carbohydrate was analyzed in the samples of the fermentations to study the effect of pH in the up-take of this sugar. The Figure 3.2 shows the lactose (A) and galactose (B) consumption at pHs of 5.5, 6 and 6.5. Lactose was quickly consumed during the first hours of fermentation at pH of 5.5 and 6.5 and after 20 h of cultivation it was not detected. At pH of 6, the lactose was completely consumed after 45 h. This could be due to the lowest initial O.D used in this experiment. As lactose decreased, galactose accumulated in the three conditions whereas glucose was not detected. Galactose was completely consumed when the pH value was maintained at 6.5 and partially consumed at 6. Interestingly, when the pH was 5.5, the galactose was not metabolized even after 150 h of fermentation.

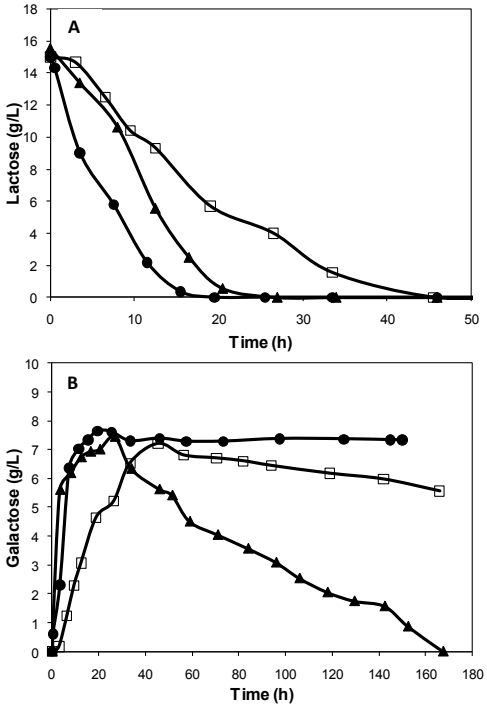

Figure 3.2 Lactose (**A**) or Galactose (**B**) consumption at pH of 5.5 (●), 6 (□) and 6.5 (▲).

The response of *E. coli* to the pH of the culture media is important to survive. The increase or decrease of the expression of specific genes to adapt to high or low pH has been studied before in cultures of *E. coli* [25, 26]. For instance Yohannes *et al* [27] found high pH induction of glycolitic enzymes under anaerobic conditions and it was suggested that an increment of the fermentation rate and acids production helps to neutralize the high alkalinity. The accumulation of galactose observed at pH of 5.5 in the present work, could be explained by a low expression of the genes related to the galactose catabolism caused by the low pH.

The effects of pH on the hydrogen production, yield and hydrogen production rate are showed on Table 3.1. When the pH was 5.5, high hydrogen production rate but smaller hydrogen production and yield per mol of lactose were obtained. This is because at pH 5.5 only glucose was metabolized and all the galactose was accumulated driving the yield and hydrogen production to low values. The pH of 6.5 resulted in the best condition for hydrogen production and yield, but the maximum specific hydrogen production rate was the lowest. At pH of 6 the hydrogen production and rate were slightly lower than 6.5, but were the double as that the obtained at 5.5. The production rate also showed an intermediate value.

Table 3.1 Comparison of hydrogen production at different pH.

pH	Hydrogen (mL)	Yield (mol H_2/mol lactose)	MSHPR (mL/L h $O.D_{600}$)
5.5*	835.5 (63.6)	0.66 (0.05)	17.07 (0.74)
6.0*	1788.6 (53.4)	1.38 (0.04)	15.36 (2.71)
6.5	2402.0	1.78	11.9

MSHPR- Maximum Specific Hydrogen Production Rate. It was calculated by dividing the maximum slope of hydrogen production kinetics by the $O.D_{600}$.

*Experiments were done by triplicate, average values are showed and standard deviations are in ().

3.3.3 Effect of pH on the production of metabolites.

The hydrogen production pathway in *E. coli* involves the conversion of sugars to pyruvate that is broken into formate and acetyl-coenzyme A. Formate is metabolized to hydrogen and CO_2 whereas acetyl-coenzyme A is converted to acetate or ethanol [28]. However, lactate can be produced from pyruvate and succinate from phosphoenolpyruvate and CO_2 [29]. Therefore, formate, acetate and ethanol are desirables metabolic by-products in the hydrogen fermentations whereas lactate and succinate must be avoided.

The production of organic acids is related to the pH. The Table 3.2 shows the acids produced in the fermentative pathway and their pKas. The pKa is an important parameter because determine the amount of dissociated and undissociated acid present at a specific pH. The undissociated form of the acids is able to cross the membrane and it can affect the hydrogen production [30]. The fermentative metabolites were analyzed to evaluate the effect of pH on the metabolites ratio.

Table 3.2. Organic acids involved in *E. coli* fermentative pathway.

Acid	pKa
Lactic	3.5
Formic	3.74
Succinic	4.2, 5.6
Acetic	4.76

The profiles of the pH and metabolites produced at pH 5.5 fermentation are shown in Figure 3.3. The accumulation of acids was very strong during the first 20 h. Although the pH was controlled, during the first 20 h oscillated between 5.4 and 5.5. Then the acids production stopped and the pH remained at 5.5.

The main product was lactate and it reached a concentration of 6.5 g/L followed by succinate, which reached a maximum concentration of 2.5 g/L. Acetate and ethanol were produced at a final concentration of 1.4 and 0.6 g/L. Only a slight amount of formate was detected, with a maximum concentration of 0.2 g/L at 7.5 h. The low pH and the high amount of lactate means a high concentration of undissociated form of the lactic acid which could affect the hydrogen production and the inhibition of metabolic functions of the cell [31] like the sugars metabolism.

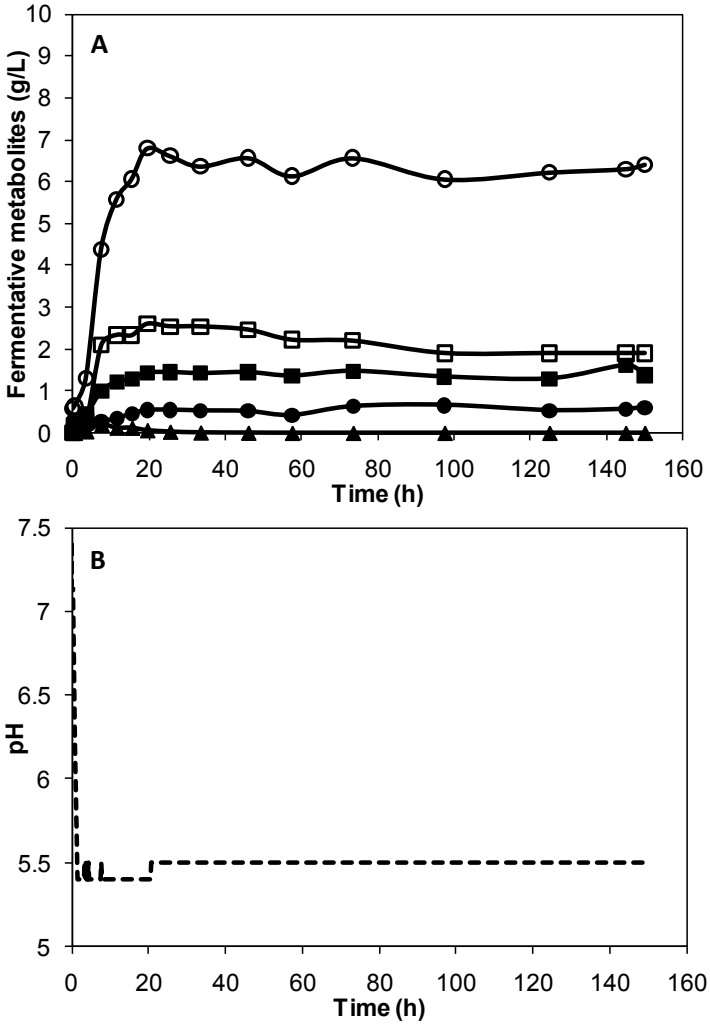

Figure 3.3 A Production of fermentative metabolites: formate (▲), succinate (□), acetate (■), lactate (○) and ethanol (●) and **B** pH (--), controlling the pH at 5.5.

The Figure 3.4 shows the profiles of the pH and metabolites produced at pH 6 fermentation. Similar to pH of 5.5 it can be observed a variation in the pH between 5.9 and 6 in the first 20 h due to the production of organic acids, mainly lactate. This acid reached a concentration of 4 g/L at 33 h and then remained constant until the end. However, the concentration of lactate was lower than that observed at pH 5.5. The lactate production seems to be the principal factor that affects the pH. Among the acids produced in *E. coli* fermentations, the pKa of this acid is the lowest (Table 3.2), and then is the strongest acid. By this reason, oscillations on pH were observed when lactate is produced (Figures 3.3B and 3.4B).

Succinate is the other product that must be avoided in hydrogen fermentations, in this case it was produced and reached a maximum value of 1.66 g/L at 142 h, and then a slightly decrement was observed. This concentration also was lower than in the case of pH 5.5. Besides to the differences on the substrate consumption caused by the pH, the production of metabolites that are not involved in the hydrogen production is different, at pH of 6 the production of lactate and succinate was diminished. Acetate and ethanol were produced to a maximum concentration of 1.25 and 1.89 g/L respectively. Interestingly in this case an accumulation of formate was observed, its concentration reached 0.78 g/L at 58 h and then decreased to a final concentration of 0.43 g/L at 215 h.

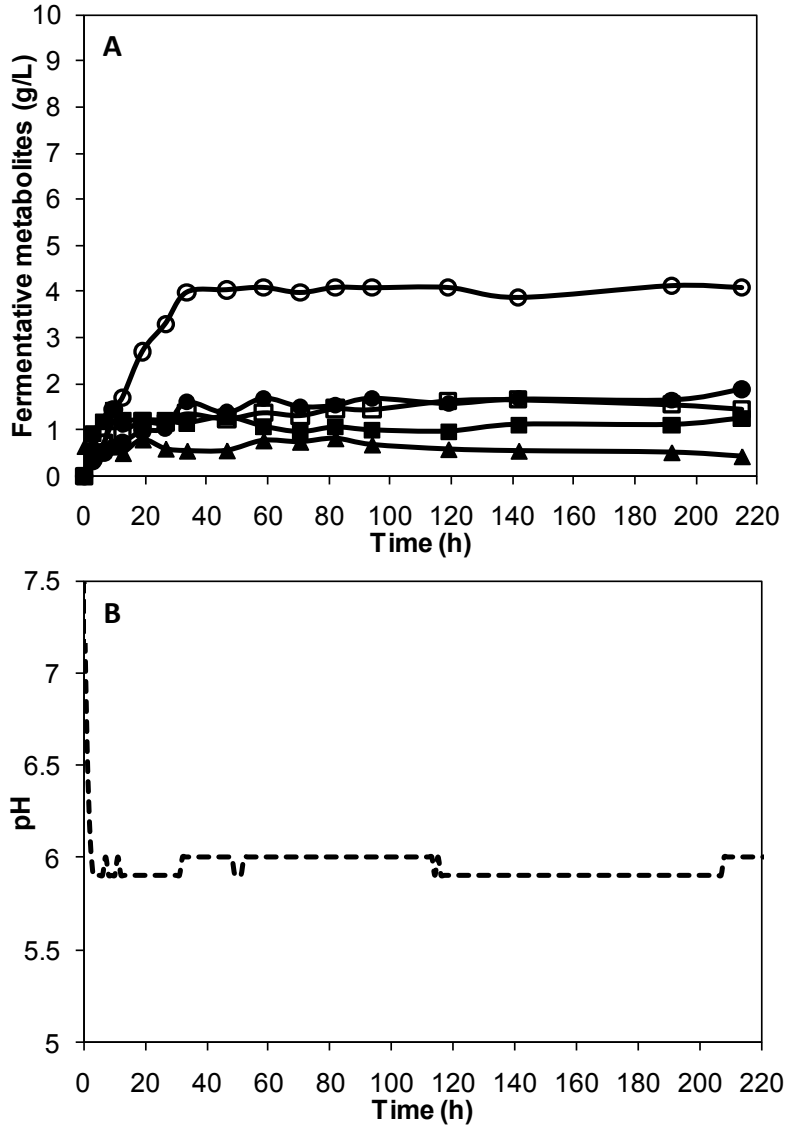

Figure 3.4 A Production of fermentative metabolites: formate (▲), succinate (□), acetate (■), lactate (○) and ethanol (●) and **B** pH (--), controlling the pH at 6.

The profiles of the pH and metabolites produced at pH 6.5 fermentation are showed in Figure 3.5. Like in the pH of 5.5 and 6, the pH oscillated between 6.4 and 6.5 when the metabolites production was very active. In this case the main metabolites were lactate and ethanol with a final concentration of 3.33 and 3.26 g/L respectively. It can be noted that the lactate production was the lowest of the

conditions tested, and its production was only on the first 27 h when the maximum concentration was 3.57 g/L and then remained constant. The production of the other metabolites was constant during the fermentation. Acetate reached a maximum concentration of 2.5 g/L whereas the highest concentration of succinate was 1.88 g/L. Propionate was detected at this pH and reached a concentration of 2.7 g/L. As observed in Figure 3.5 the concentration of final products from alternative pathways that do not involve hydrogen production were low and by this reason, high hydrogen yield was observed at this pH

Formate was accumulated and reached 1.34 g/L at 84 h and then its concentration decreased becoming undetectable at the end of the experiment. This metabolite is initially exported out of the cells to avoid the acidification of cytoplasm by the protein FocA [32, 33]. The import of formate depends on the pH of culture media and at pH below 6.8 formate is re-imported [33]. A possible explanation of the accumulation of formate observed in the present work is a balance between export-import of formate. The formate metabolism and subsequent hydrogen production is affected by alkaline pH in *E. coli*, and by this reason pH higher than 6.5 was not tested. For instance Bagramyan *et al.*[34] observed that a inclusion of 30 mM formate in the growth medium did not increase hydrogen production rates at pH 6.5 or 7.5.

The optimal pH for hydrogen production depends on the inocula and substrate. A pH of 5 and 5.3 were reported as optimal for hydrogen production using xylose or lactose respectively with a mixed culture at 55°C [21]. Li *et al* [20] reported an optimal constant pH of 6 using 7.5 g/L of glucose with a natural sludge. Using

axenic cultures, Masset *et al* [35] reports the maximum yield for glucose when the pH was maintained at 5.2 and a maximum yield and production using starch at pH 5.6 with *Clostridium butyricum* CWBI1009. Liu *et al*. [36] evaluated the effect of pH on hydrogen production by three *Clostridium* species using glucose. The maximum hydrogen yield for *Clostridium butyricum* CGS2 was achieved at pH 6, whereas a high hydrogen production with *Clostridium beijerinckii* L9 and *Clostridium tyrobutyricum* FYa102 could be achieved under uncontrolled pH conditions. In the present work, using cheese whey and *E. coli* WDHL, the optimal pH was 6.5 for maximize the yield and 5.5 to attain the higher rate.

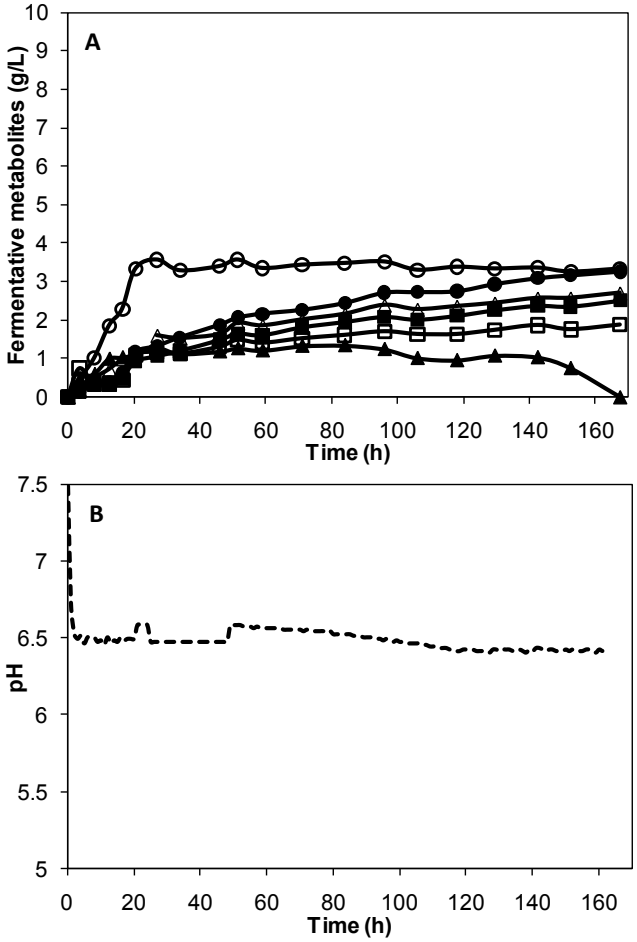

Figure 3.5 A Production of fermentative metabolites: formate (▲), succinate (□), acetate (■), lactate (○) propionate (△) and ethanol (●) and **B** pH (--), controlling the pH at 6.5.

3.4 Conclusions.

The pH has an important effect on the fermentative metabolism of *Escherichia coli* and it includes hydrogen production because it influences the formate metabolism. In this work, the effect of operating the reactor at controlled pH values of 5.5, 6 and 6.5 on the hydrogen production was evaluated.

Controlling the pH at 6.5 resulted in the best condition since higher production yield was obtained and all the sugars of the cheese whey were metabolized. At this pH a mix of ethanol and acids, mainly lactate, were produced from glucose; the metabolism of galactose yielded other acids than lactate and ethanol.

Operating at pH of 5.5 resulted in high production rate but smaller yield because only glucose was metabolized. At pH of 6 not all the carbohydrates of cheese whey were consumed and this was not favorable for hydrogen production.

The results show the importance of controlling the pH to improve the hydrogen production and the substrate consumption. It could be interesting to determine how the pH is affecting the galactose catabolism in this system.

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Chapter 4

Fermentation of lactose and its constituent sugars by *E. coli* WDHL: Impact on hydrogen production

Abstract

Hydrogen is an attractive future substitute of fossil fuels since its high energy density and low or non-generation of pollutants. The biological hydrogen production is less energy intensive than the chemical and electrochemical processes. Furthermore, wastes or by-products of agro-industry can be used as substrate for fermentative hydrogen production. In this study, the fermentations of lactose, glucose and galactose using *Escherichia coli* WDHL, a hydrogen overproducer strain, were performed. The results showed that the pyruvate is mainly routed to the lactate pathway using glucose as substrate. Thus the formate and consequently hydrogen production was diminished. The hydrogen production and yield obtained with glucose were 1037 mL and 0.19 mol H_2/mol of glucose, respectively. The galactose catabolism was slower than the glucose one. Using galactose, the pyruvate formate lyase pathway was the main route for pyruvate, thus the hydrogen and ethanol production was favored. The galactose fermentation yield 1.15 mol H_2/mol of galactose and the hydrogen production was 2080 mL. The fermentation of lactose or glucose plus galactose showed similar behavior. Lactose yield was 1.02 mol H_2/mol of lactose. The maximum hydrogen production rate was high when glucose is present as substrate whereas galactose yielded the lowest maximum production rate. This work provides valuable information of the hydrogen production using lactose or its components, and can be used for the improvement of hydrogen production using lactose, glucose or galactose rich wastes.

Rosales-Colunga LM, Razo-Flores E, De León-Rodríguez A. **"Fermentation of lactose and its constituent sugars by *E. coli* WDHL: Impact on hydrogen production"** In preparation to be submitted to Bioresource Technology.

4.1 Introduction.

The biofuels production is a very active research area due to the future depletion of fossil fuels and the environmental problems associated with the use of them [1]. Among the biofuels, biohydrogen is an attractive future substitute of fossil fuels due to its potentially higher efficiency of conversion to usable power, low or non-generation of pollutants and high energy density [2-4]. The biological hydrogen production is carried out at ambient temperature and pressure, by this reason is less energy intensive than the chemical and electrochemical processes [5, 6]. But to be competitive to the other methods of production, the biological production must use wastes or by-products rich in carbohydrates to reduce costs of production and, at the same time, to dispose pollutant wastes.

The dark fermentation is a promising biological process to obtain hydrogen because it could use organic wastes from agricultural and food-producing industry as substrates [7] and only a relatively simple equipment is necessary [8]. *Escherichia coli* produce hydrogen by dark fermentation under anaerobic conditions when no external electron acceptors are present [9, 10]. The hydrogen yield can be improved by genetic engineering and *E. coli* is one of the microorganisms most used because of its genetics and metabolism are well documented [11]. The substrate more extensively studied for hydrogen production had been glucose [12-15].

Lactose, and the sugars released from its hydrolysis glucose and galactose are commonly present in some agro-industrial wastes. Lactose is found in the cheese and dairy industry wastewater [16, 17]. Glucose is currently obtained from the

hydrolysis of molasses, cellulose and other agricultural wastes [18]. Besides the release of galactose from lactose hydrolysis, this sugar is a component of hemicellulose [19]. Thus, it is interesting to study the use of these carbohydrates as single substrates or in mixture for hydrogen production. Despite the use of glucose by *E. coli* has been extensively studied as mentioned above, the use of lactose and galactose as substrates have not been sufficiently studied.

Consequently, the fermentation of lactose, glucose and galactose using *Escherichia coli* WDHL was studied; in this strain the *hycA* and *lacI* genes were deleted to improve the hydrogen production [20]. The *hycA* gen codes for the negative regulator of the hydrogen pathway whereas the deletion of *lacI* lead to the constitutive expression of the lac operon.

4.2 Materials and methods.

4.2.1 Strain and culture media.

Escherichia coli WDHL strain, a hydrogen over producer strain, which lacks *hycA* and *lacI* genes, was used. A complete description of the strain has been published [20]. Preinocula were grown overnight in 25 mL of LB medium at 37°C and shaken at 200 rpm, afterwards added to 900 mL of fresh LB medium in closed twist cover bottles and were incubated at 37°C for 48 h. Cells were harvested, washed and inoculated into the bioreactor. Cultures on bioreactor were done using HP medium reported elsewhere [20] with 15 g/L of sugars (lactose, glucose, galactose or a mixture of 7.5 g/L of glucose and 7.5 g/L of galactose).

4.2.2 Batch Cultures.

Cultures were performed in batch mode using a 1-L bioreactor (Applikon, Schiedam, The Netherlands). The pH, oxidation-reduction potential, dissolved oxygen and dissolved carbon dioxide were monitored using autoclavable electrodes (Applikon) connected to the ADI 1035 Bioconsole (Applikon). The initial pH was 7.5 in all the experiments and then automatically controlled to 6 using 2.5 N NaOH and HCl solutions. BioXpert 1.3 software (Applikon) was used for data acquisition. The cultures were maintained at 37°C and stirred at 175 rpm with two six-blade Rushton turbines.

4.2.3 Analytical methods.

Cell growth was monitored at OD_{600nm} using a spectrophotometer Cary BIO-50 (Varian, Palo Alto, CA). Culture samples were periodically taken from the bioreactor, centrifuged and the supernatant was filtered through a 0.22 μm filter (Millipore) for the analysis of sugars, organic acids and ethanol. The gas produced 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, sugars and organic acids were determined by gas chromatography and capillary electrophoresis as described elsewhere [21]. Ethanol was determined by gas chromatography as described elsewhere [22].

4.3 Results.

4.3.1 Growth of *E. coli* using different sugars as substrate.

The fermentation of lactose, glucose, galactose or glucose plus galactose was studied in this work. The cell growth in these sugars was measured; an initial OD_{600} of 2.03 ± 0.02 was used in all the experiments. The OD_{600} decreased in all the cases, and the final OD_{600} was 1.53 ± 0.06. Since the culture medium is nitrogen source free to decouple growth from hydrogen production, a decreasing of cell density was observed. The growth kinetics was similar in all the experiments, and any effect due to differences on cell density can be discarded.

4.3.2 Fermentation of Lactose.

Lactose is commonly present in wastes of food industry and it can be used as substrate for the production of hydrogen [16, 18, 23]. A typical batch culture using lactose as substrate is shown in Figure 4.1. It can be noted three phases of hydrogen production (Figure 4.1A). In the first 60 h the hydrogen specific production rate was 0.34 mmol H_2/L h OD_{600}unit and 980 ml were produced. In the next 35 h, the hydrogen production rate was 0.02 mmol H_2/L h OD_{600}unit and only 35 ml were produced. After 95 h and onwards, hydrogen was continuously produced reaching 2092 ml in 576 h. The maximum hydrogen production rate was 15.41 mL/L h.

When lactose was consumed galactose and glucose were accumulated. Glucose reached a maximum of 3.2 g/L at 9 h and became undetectable after 34 h. The

maximum galactose concentration was 7.45 g/L at 27 h, after that the concentration decreased to 1.2 g/L at the end of the experiment. Lactose was not detected after 12 h (Figure 4.1B).

The soluble metabolites produced are shown in Figure 4.1C. Lactate was the main soluble product and its maximum concentration was 5 g/L at 27 h and remained constant until the end of the experiment. The production of acetate and ethanol showed a similar profile and two phases were observed for both metabolites. In the first phase, a rapid increment was observed, acetate reached 1 g/L and ethanol 0.7 g/L at 27 h, after that the concentration of both metabolites steadily increased to a maximum concentration of 2.6 and 2.5 g/L, respectively. The succinate production also showed two phases, 0.4 g/l were produced in the first 27 h and the maximum concentration was 1 g/L. Only a slight amount of formate was detected on the culture medium. Formate reached a maximum of 0.2 g/L at 12 h, and it was undetectable at 70 h. Thus this fermentation was mainly lactic.

It is clear that after the lactose was hydrolyzed, it was consumed in two phases. In the first one the glucose was consumed and galactose was accumulated, in the second phase galactose was slowly used and the metabolites produced were different in the two phases. In order to study both phases, independent experiments using either glucose or galactose as substrates were conducted.

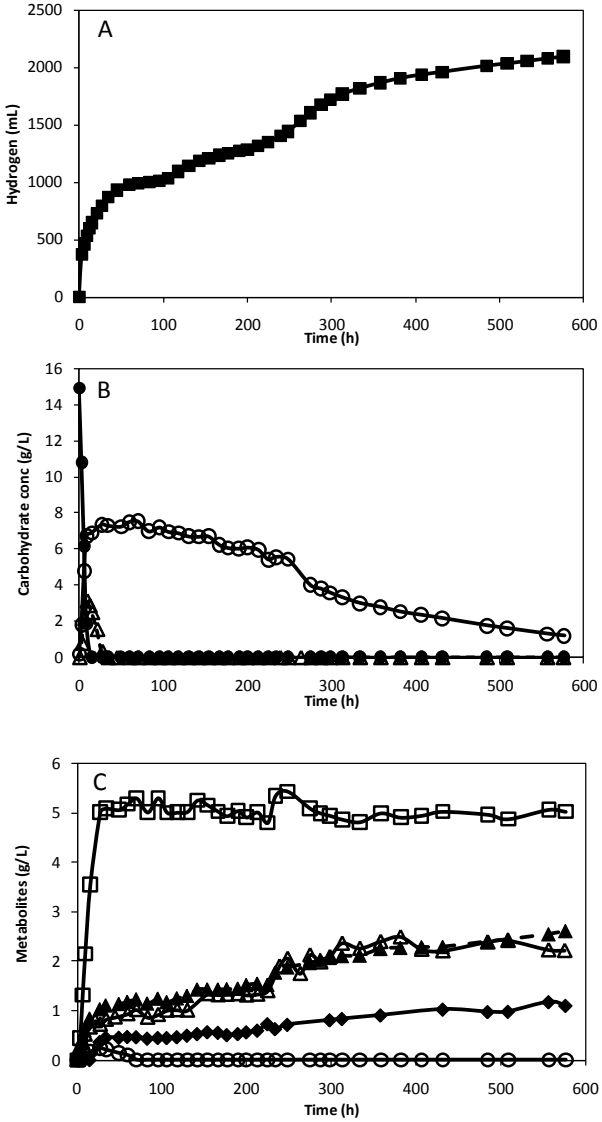

Figure 4.1 Batch culture of *E. coli* WDHL using lactose as substrate. **A** Hydrogen production (■). **B** Sugars consumption: lactose (●), galactose (○) and glucose (△)**. C** Production of fermentative metabolites: succinate (◆), lactate (□), formate (○), ethanol (△) and acetate (▲).

4.3.3 Fermentation of glucose.

A typical batch culture using glucose as substrate is shown in Figure 4.2. As expected, this carbohydrate was used immediately and was not detected after 44 h. The hydrogen production also started since the beginning of culture, with a final

hydrogen production of 1037 mL in about 60 h (Figure 4.2A). In this case the maximum hydrogen production rate was 18.61 mL/L h.

The production of metabolites is shown in the Figure 4.2B. Lactate was the main soluble product of the fermentation with a maximum concentration of 10.1 g/L whereas the other products were produced in minor concentrations. The maximum concentration of succinate, acetate, and ethanol were 1.6, 1.5 and 1.2 g/L respectively. The highest formate concentration was 0.6 g/L at 12 h and then decreased. This fermentation was lactic similar as when lactose was used as substrate.

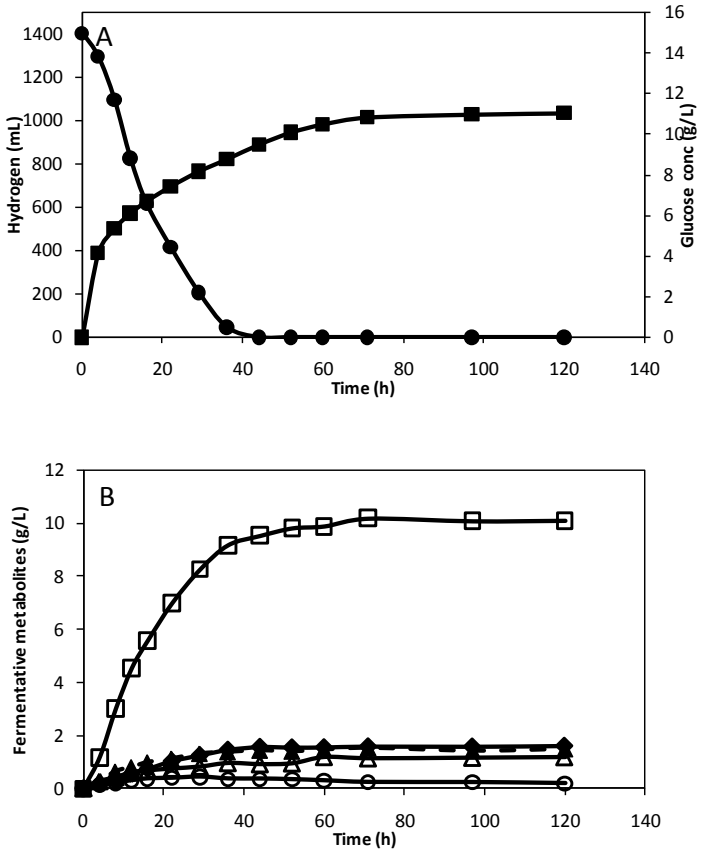

Figure 4.2 Batch culture of *E. coli* WDHL using glucose as substrate. **A** Hydrogen production (■) and glucose consumption (●). **B** Production of fermentative metabolites: succinate (◆), lactate (□), formate (○), ethanol (△) and acetate (-▲-).

4.3.4 Fermentation of galactose.

A typical batch culture using galactose as substrate is shown in Figure 4.3. In this case, a lag phase of 18 h was observed. Other marked difference was the time for the galactose up-take; whereas the glucose was completely consumed at 44 h (Figure 4.2A), galactose required a longer time and at 356 h of culture, 4.2 g/L of galactose still remained in the culture medium (Figure 4.3A). Since the galactose consumption became asymptotic, the culture was stopped. The hydrogen production from galactose is showed in Figure 4.3A. Due to the lag phase in the galactose consumption the hydrogen production began 18 h after the experiment started. The hydrogen production attained 2080 mL in 356 h. This production represents two-fold the hydrogen produced from glucose despite no total galactose was consumed. The maximum hydrogen production rate (13.21 mL/L h) using galactose was lower than using glucose.

The production of metabolites from galactose is presented in Figure 4.3B. In this case, the main soluble metabolite was ethanol with a final concentration of 5.3 g/L. Acetate and succinate were also produced and reached 2.7 and 1.7 g/L, respectively. In contrast, with the fermentation of glucose, galactose produced only 0.4 g/L of lactate. The formate concentration was less than 0.2 g/L during the fermentation. In this case, the fermentation was ethanolic.

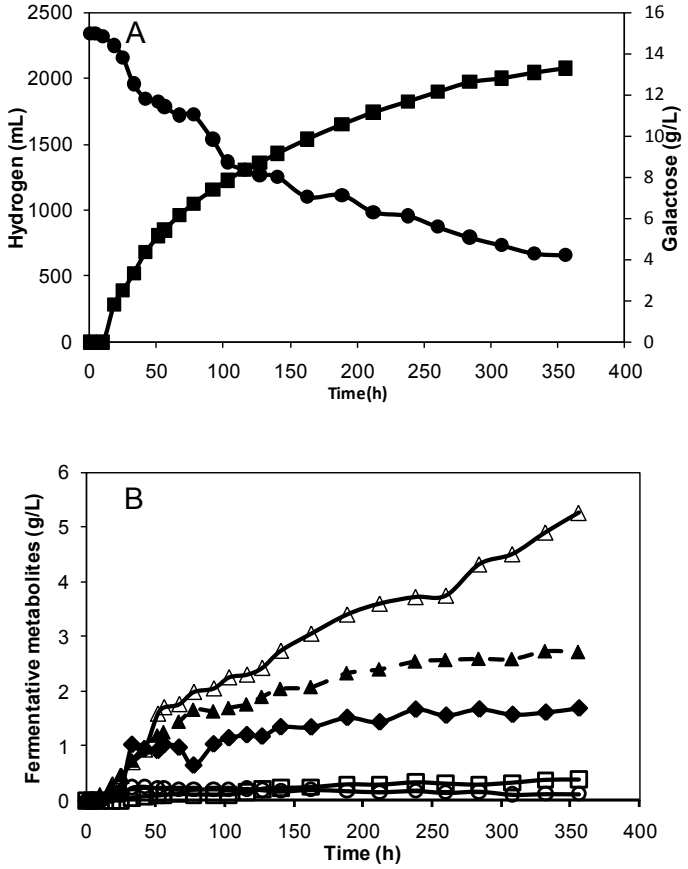

Figure 4.3 Batch culture of *E. coli* WDHL using galactose as substrate. **A** Hydrogen production (■) and galactose consumption (●). **B** Production of fermentative metabolites: succinate (◆), lactate (□), formate (○), ethanol (△) and acetate (-▲-).

4.3.5 Fermentation of a mixture of glucose and galactose.

To investigate the differences observed in the production of hydrogen and other metabolites when glucose or galactose were used as single substrates, an experiment using glucose plus galactose was carried out. The Figure 4.4 shows the sugars consumption and production of hydrogen and soluble metabolites. This culture clearly shows two phases. In the first phase glucose was quickly consumed in the initial 25 h and the hydrogen production reached 983 mL (Figure 4.4A). Lactate was the main soluble product in this phase and attained a concentration of 6.7 g/L at 25 h. The concentration of succinate, acetate and ethanol in the first 25 h were 0.5, 1.5 and 1.2 g/L respectively. Formate peaked at 19 h with a concentration of 0.5 g/L and then decreased (Figure 4.4B). After glucose was

depleted, a lag phase of 120 h was necessary to start the galactose consumption. In this case the lag-phase was 6.7-times higher than the culture started with galactose. Interestingly, galactose was consumed completely at 320 h of culture and 1467 mL of hydrogen were produced, which is nearly 50% more hydrogen than the produced from the glucose (Figure 4.4A). Acetate, ethanol and succinate were produced in this second phase and reached a maximum concentration of 4, 4 and 1.6 respectively, whereas lactate remained constant. Formate also showed a peak in its concentration and reached 0.5 g/L at 200 h and then decreased and was undetectable at the end of fermentation. The maximum hydrogen production rate was 24.45 mL/L h during this experiment.

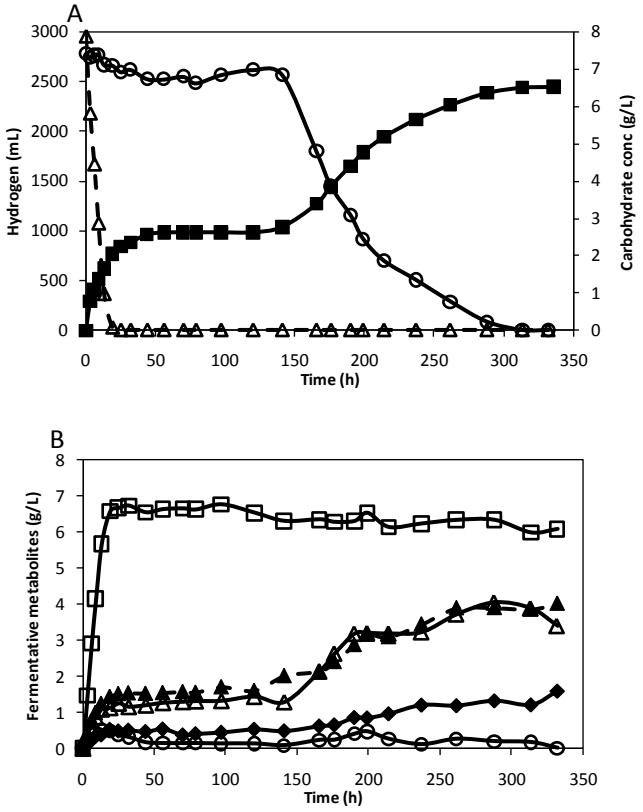

Figure 4.4 Batch culture of *E. coli* WDHL using a mix of glucose plus galactose as substrate. **A** Hydrogen production (■) and sugars consumption: glucose (△), galactose (○). **B** Production of fermentative metabolites: succinate (◆), lactate (□), formate (○), ethanol (△) and acetate (-▲-).

4.4 Discussion.

Measures on hydrogen and soluble metabolites produced by the fermentation of lactose, glucose and galactose showed differences. Table 4.1 shows that using glucose as substrate results in a low hydrogen production of only 1037 mL and poor yield of 0.19 mol H_2/mol of glucose, the main soluble product was lactate. Whereas galactose gave the highest hydrogen yield (1.15 mol H_2/mol galactose) and the main product was ethanol. The lactose fermentation produced 2092 mL and like in the glucose fermentation, the main product was lactate, but in this case the lactate was produced only when the glucose was consumed. Interestingly in the fermentation of glucose plus galactose, a higher production of hydrogen of 2450 mL was attained and the main products were lactate, acetate and ethanol. The yield of this fermentation was the same as the lactose one (1.02 mol H_2/mol hexose). The maximum hydrogen production rate using glucose plus galactose of 24.45 ml/L h was higher than the 15.41 ml/L h attained with lactose. Galactose yielded the lowest maximum production rate (13.21 ml/L h).

Table 4.1 Comparative hydrogen production using different substrates.

Substrate	Production (mL)	Yield (mol H_2/mol hexose consumed)	Maximum hydrogen production rate (mL/L h)	Main product
Lactose	2092	1.02	15.41	Lactate
Glucose	1037	0.19	18.61	Lactate
Galactose	2080	1.15	13.21	Ethanol
Glucose + galactose	2450	1.02	24.45	Lactate, ethanol and acetate

Although the fermentation of glucose to hydrogen is straightforward, the main drawback is that only a small fraction of the electrons in the starting substrate ends

up in hydrogen [24]. In *E. coli,* glucose is transported into the cell by the phosphotransferase system and then catabolized to phosphoenolpyruvate and this is the first branch of the fermentative pathway because it can be converted to oxaloacetate, and at last produces succinate. In the other branch most of phosphoenolpyruvate is transformed to pyruvate, which is cleaved to formate and acetyl-CoA by the pyruvate formate lyase complex. The formate is converted to hydrogen and CO_2, whereas the latter yields acetate or ethanol [25]. But, during conditions of high pyruvate accumulation or at low pH, pyruvate may be converted to lactate by lactate dehydrogenase enzyme (LDH) coded by the *ldhA* gene [26]. The glucose uptake rate was 210.83 mg of glucose/L h OD_{600} unit and that means that the glucose is quickly converted to pyruvate, and therefore the concentration of pyruvate must be high. Then, the lactate pathway must be very active since the LDH activity increases with increased pyruvate concentration [26] and it has been showed that the addition of exogenous pyruvate increased the expression of *ldhA* [27], and this might be the reason why lactate was the main soluble metabolite and the hydrogen yield was low using glucose.

The hydrogen yield from glucose found in the present work, 0.19 mol H_2/mol of glucose, is similar to the yield of 0.17 mol H_2/mol of glucose consumed predicted by metabolic flux analysis reported by Manish *et al* [28]. They also predicted an increment of 35% in hydrogen yield in a strain lacking *ldhA* gene. Other studies found higher yield, Bisaillon *et al* [29] reported highest yield of 2 mol H_2/mol of glucose, using a strain with mutations on uptake hydrogenases, *ldhA* and *fhlA*, in batch cultures and limiting concentrations of glucose. Similar yields were reported

by Turcot et al. [30] with the same strain and nutrient limitations in continuous cultures, whereas Ghosh and Hallenbeck [31] attained 1.51 mol H_2/mol of glucose using the same strain. Maeda *et al* [14] reached a hydrogen yield of 1.3 mol H_2/mol of glucose using a strain with mutations on *hyaB hybC hycA fdoG frdC ldhA aceE* genes. Yoshida *et al* [15] enhanced the hydrogen yield to 1.82 mol H_2/mol of glucose with a strain *ΔldhA*, *ΔfrdBC*. It is important to notice that in all the works discussed above a mutation in the *ldhA* gene was included, and the lactate pathway abolished, contributing to increase the hydrogen yield. Other study performed by Penfold *et al* [12] did not involve a mutant of *ldhA*, they used a *hycA* mutant strain and they found that the amount of hydrogen decreased as the concentration of glucose increased, it could be that the lactate pathway is not very active due to the low pyruvate concentration caused by the low glucose concentration used

The galactose is important for *E. coli* not only as energy source but also as building block in complex polysaccharide synthesis. The transport of galactose unlike glucose is by two specific transporting systems, one of high affinity and one of low affinity, but it can also be transported by LacY permease and other non-specific transporters [32]. In the galactose fermentations a lag phase is observed because the *gal* operon is not activated immediately despite the high galactose concentration and the constitutive presence of LacY permease due to the lack of *lacI* gene in the strain used here. After this lag phase galactose catabolism began but the galactose consumption rate was slower than glucose (26.47 mg of galactose/L h OD_{600} unit). Thus the pyruvate concentration is low, the lactate

pathway is poorly activated and the hydrogen pathway is strongly favored. Also it was suggested that the expression of *ldhA* gene might be affected by the nature of carbon source, and with the PTS system [27]. The difference on the uptake rates between glucose and galactose must be due to the transportation or the enzymes related to the metabolism of galactose before it can be converted to glucose 6-P. If the galactose transportation caused the slow consumption, then the galactose can be metabolized quickly in the fermentation of lactose. Since, in the case of lactose, which is transported by the lactose permease and then intracellular lactose is split into glucose and galactose by β-galactosidase [33], the transportation of galactose is not involved. Since, in this case the galactose was slowly consumed suggesting that it is due to inefficient expression of *gal* regulon or low activity of the enzymes coded by this regulon. Finally, in the experiment of glucose plus galactose as substrates, the lag phase for galactose uptake is longer and it could be caused by catabolic repression, since both glucose and galactose were present in the culture medium [34]. The hydrogen yields from galactose and lactose were 1.15 and 1.02 mol H_2/mol hexose consumed using a strain lacking *hycA* and *lacI* genes. These yields are higher than the 0.69 and 0.73 mol H_2/mol reported by Ghosh and Hallenbeck [31], for galactose and lactose respectively using a strain with mutations on uptake hydrogenases, *ldhA* and *fhlA*. It seems that the mutation on *lacI* improve the hydrogen yield from those sugars.

4.5 Conclusions.

The study of fermentation of sugars present in wastes of agro-industry as substrates is important for biofuels production such as hydrogen. The fermentation of lactose, glucose and galactose was performed in this work. These sugars are commonly found in the cheese whey and the hydrolysis of starch, cellulose and hemicellulose. The two phases of production of metabolites using lactose are due to the initial consumption of glucose and after that, the consumption of galactose. The yield of fermentative metabolites is different in both phases. Using glucose as substrate, pyruvate is mainly channeled to the lactate pathway and only a small proportion to formate pathway and therefore hydrogen production is diminished. Whereas, using galactose as substrate, its catabolism is slower than the glucose and the formate and acetyl Co-A pathway is the main route, which turns on the metabolism to produce hydrogen and ethanol as the main products. The maximum hydrogen production rate is high when glucose is present as substrate whereas galactose yields the lowest maximum production rate. Finally the presence of glucose in the culture medium produces a longer lag phase of galactose than the lag phase of galactose as a sole carbon source. This work provides valuable information to improve the hydrogen production using carbohydrates found in agro industry wastewaters.

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Chapter 5

On-line hydrogen estimation in genetically modified *E. coli* fermentations using an artificial neural network

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* 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.

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5.1 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 (Figure 5.1).

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*. [25] 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.

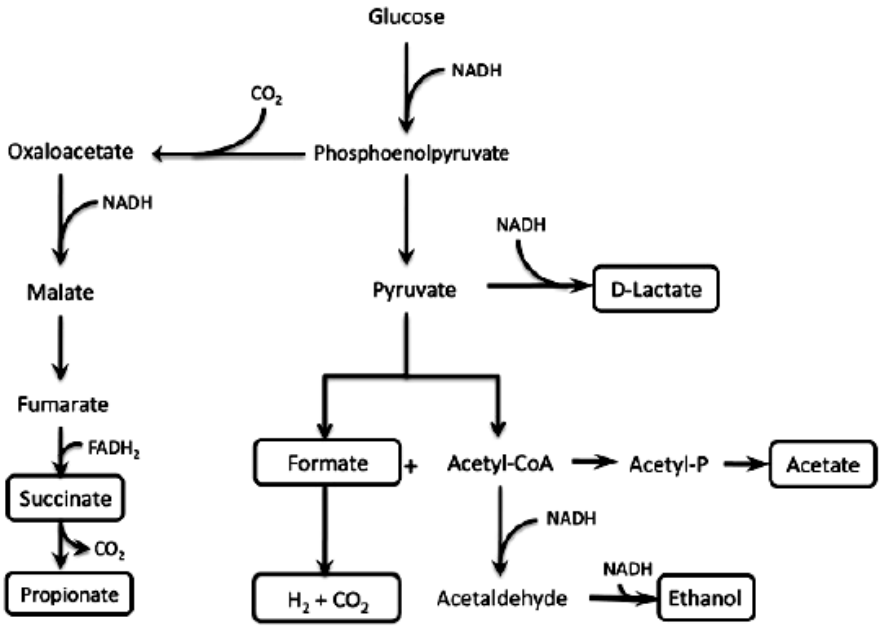

Figure 5.1 Schematic representation of the fermentative pathways in *Escherichia coli*. Final products are framed.

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 a.l* [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.

5.2 Materials and methods.

5.2.1 Strain and culture media.

Escherichia coli WDHL hydrogen overproducing strain was used in this study. In this strain *hycA* and *lacI* genes were deleted. 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 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 was used as carbon source.

5.2.2 Batch cultures in bioreactor.

Pre-inocula was harvested, washed 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.

5.2.3 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.

5.2.4 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:

(1) 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. 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 Figure 5.2. 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.) and the use of the BPNN on-line was done trough a subrutine programed in Microsoft Visual Basic for Applications, which imported the data from the acquisition software.

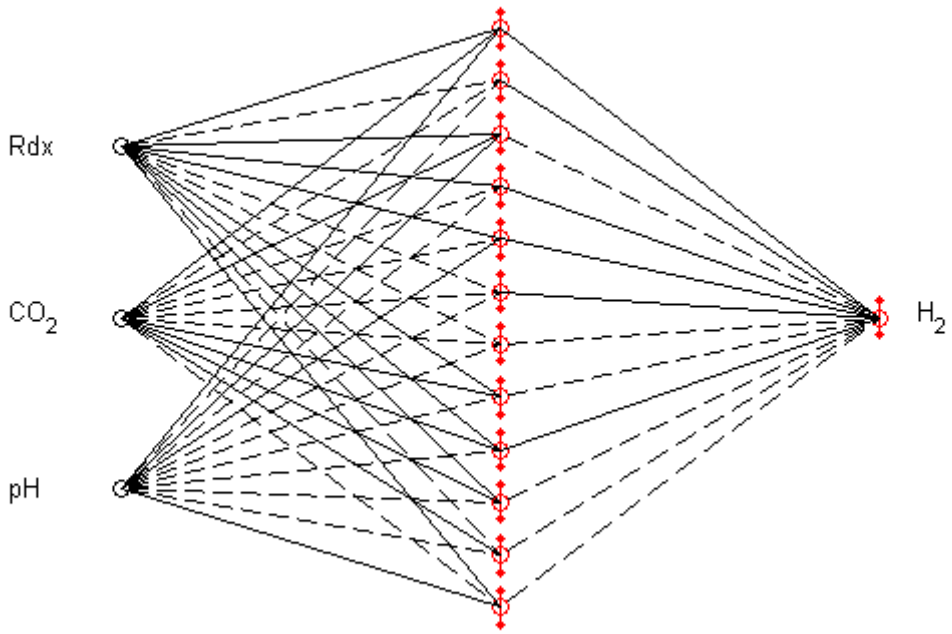

Figure 5.2 Structure of the Artificial Neural Network used in this work. A standard network of one hidden layer with 12 nodes was selected. The continuous lines represent adjustable parameters W; dashed lines are for W<0. The ANN training was done using on-line measurements of ORP (Rdx), DCO_2 (CO_2) and pH during the fermentations of cheese whey by *Escherichia coli* WDHL strain.

5.2.5 BPNN Training.

One hundred and two data of 7 different experiments were used for the BPNN training in static mode. The characteristics of the experiments are shown in Table 5.1. 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:

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

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.

Table 5.1 Characteristics of the experiments used for the BPNN training.

Experiment	Initial pH	pH controlled	Number of experimental data	Time (h)
1	6.5	N.C.	12	249
2	7.5	N.C.	27	358
3	7.5	5.5	7	54
4	7.5	6.5	16	122
5	7.5	5.9	9	76
6	7.5	6.0	16	143
7	7.5	6.0	15	215

N.C= Not controlled

5.3 Results and discussion.

5.3.1 Hydrogen production by *E. coli.*

A typical batch culture of *E. coli* WDHL at pH 5.5 is showed in Figure 5.3. Cultures at other operational conditions showed similar trends as those in Figure 5.3, 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 (Figure 5.3A).

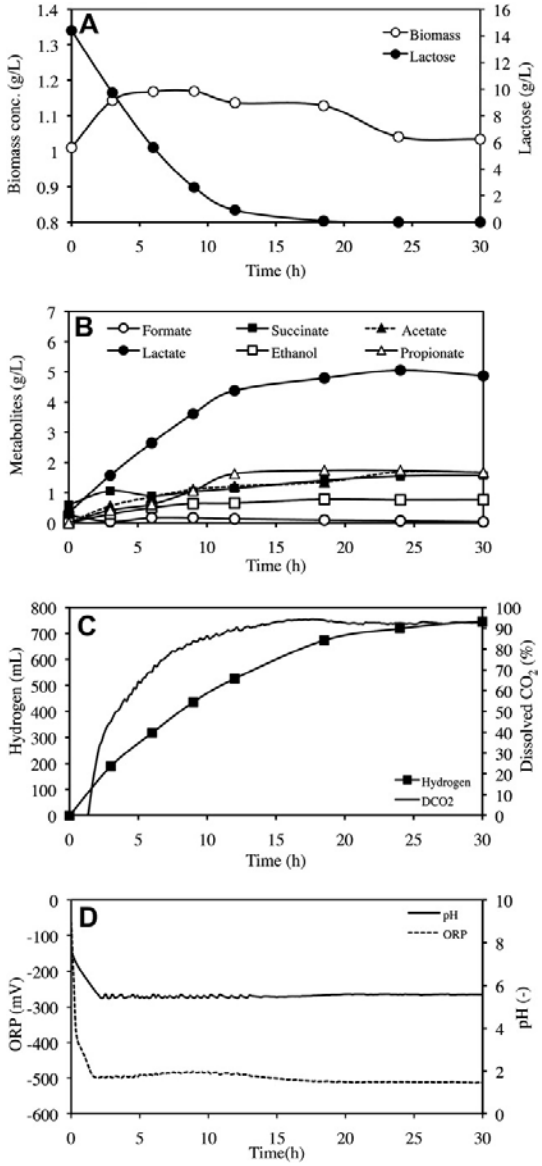

Figure 5.3 Typical batch culture of *E. coli* WDHL during the hydrogen production using cheese whey as substrate at pH 5.5. **A** Lactose and biomass concentration. **B** Metabolites. **C** Hydrogen production and dissolved CO_2. **D** ORP and pH.

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 (Figure 5.3A). In the Figure 5.3B 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. Figure 5.3C shows the hydrogen and the DCO_2 profile. A fast increase on DCO_2 was observed on the first 10 h as result of metabolic activity, reached 90% and then remained constant at this value. Hydrogen production started at the beginning of the fermentation and became slowly while the lactose was consumed. The maximum hydrogen production was 745 mL. The similitude between hydrogen and DCO_2 trends observed in Figure 5.3C 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 (Figure 5.1). The relation would 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 (Figure 5.3D).

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 H_2 and CO_2 depends on the acidic pH of the growth medium [37].

The fermentative metabolism had an effect on ORP and it drops at the beginning of fermentation and then remained constant around -500 mV (Figure 5.3D). The global measured ORP corresponds to the sum of the all redox species. Table 5.2 shows the standard reduction potentials of main redox pairs involved in the hydrogen metabolism by *E. coli*.

Table 5.2. Standard reduction potentials of redox pairs in *Escherichia coli* mixed acid fermentation.

Oxidant	Reductant	ΔE° (volts)
Acetate	Acetaldehyde	-0.60
$2H^+$	H_2	-0.42
NAD^+	$NADH^+ + H^+$	-0.32
FAD (free)	$FADH_2$	-0.22
Acetaldehyde	Ethanol	-0.20
Pyruvate	Lactate	-0.19
Oxaloacetate	Malate	-0.17
Fumarate	Succinate	+0.03

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.

5.3.2 Prediction of hydrogen production using a BPNN.

Table 5.3 shows the final parameters of the BPNN after training. The parameters between the input layer and the hidden layer are represented by the W1 values whereas W2 represents the values between the hidden layer and the output layer. The BPNN was used with these parameters to estimate H_2 for the new values of pH, CO_2 and ORP.

Table 5.3 BPNN Parameters after training.

Weights between the input and the hidden layer (W1)			**Threshold for W1**	**Weights for the hidden layer and the output (W2)[a]**
ORP	**DCO_2**	**pH**		
-0.1469	0.377	-0.0104	0.0038	-0.6307
0.473	-0.4577	0.4635	-0.0506	-7.7839
0.1751	0.0864	0.1671	-0.3108	14.7107
-0.4807	0.3112	0.1203	-0.139	-0.8163
-0.2688	0.1513	0.4748	-0.4161	-13.68
-0.2422	-0.2316	-0.378	-0.0965	15.8115
-0.3783	-0.152	-0.3478	-0.1683	0.1201
-0.101	0.43	-0.4057	0.3842	3.3378
0.2947	0.236	-0.1576	-0.4526	-2.7498
-0.4452	0.3936	0.1862	0.0449	9.4509
0.2202	-0.3045	-0.4538	-0.1963	17.6505
-0.4293	0.0824	0.3778	0.2218	12.4244

[a] Threshold for W2 was -7.3080

The comparison between experimental hydrogen values and predicted values based on the BPNN for the culture at pH 5.5 and 6 is shown in Figure 5.4. There is a good fit in the trends between the predicted and the experimental data. Similar behavior was observed for the cultures with or without pH control (data not shown).

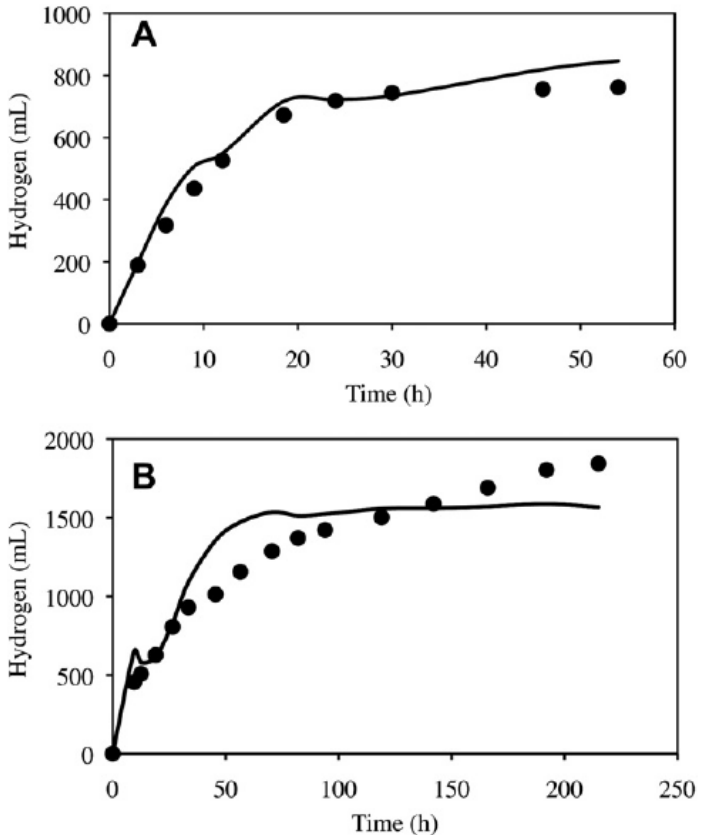

Figure 5.4 Comparison between the experimental data of hydrogen production measured by gas chromatography (closed symbols) and the predictions based on the BPNN model (continuous line). **A**) Culture at pH 5.5. **B**) Culture at pH 6.

The Figure 5.5 shows the correlation between the hydrogen production determined 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.

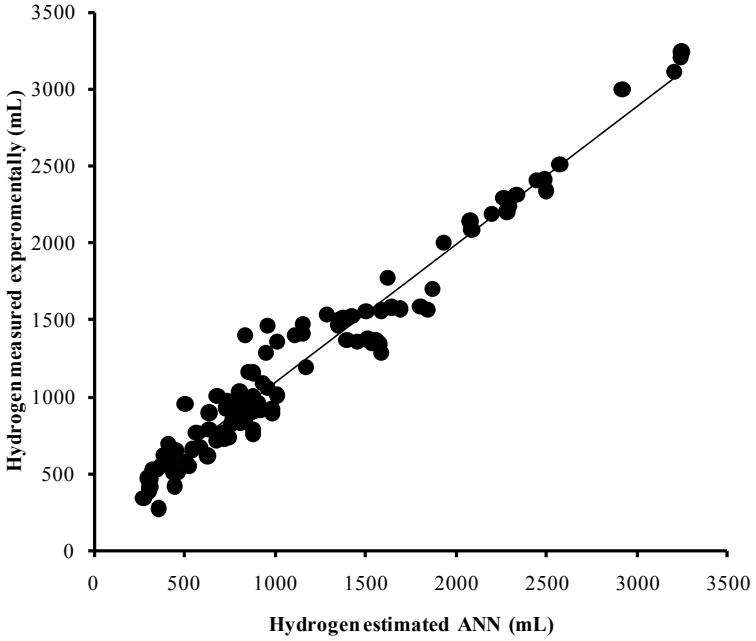

Figure 5.5 Correlation between the experimental hydrogen production values and the predicted calculated by the BPNN. The lineal regression is y = 0.9005x+189.85 and r^2 = 0.955

ANNs have been used in another hydrogen production processes (Table 5.4). 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.

Table 5.4 Some ANN reported in hydrogen production processes.

Input	Output	Type of Reactor	Substrate	Inoculum	Type of ANN	Ref.
ORP, pH, dissolved CO_2	H_2P	Batch	CW	*E. coli* WDHL	BPNN	This work
HRT, sucrose, biomass, ethanol, organic acids conc., ORP, pH, recycle ratio and alkalinity	H_2PR	CSTR	Sucrose	Sludge	BPNN	[39]
OLR, ORP, alkalinity, pH	H_2 P	CSTR	Kitchen wastes	Sludge	BPNN	[40]
OLR, HRT, influent alkalinity	Percent of H_2, H_2PR, H_2Y, total organic carbon in effluent, products conc.	UASB	Sucrose rich synthetic waste water	Sludge	NN and GA	[41]
OLR, pH, HRT, starch conc. in influent	H_2Y, effluent COD	EGSB	Starch containing waste water	Sludge	BPNN	[42]

H_2P= Hydrogen Production; CSTR= Continuous Stirred Tank Reactor; CW= Cheese Whey; BPNN= Back Propagation Neural Network; HRT= Hydraulic Retention Time; ORP= Oxidation-Reduction Potential; H_2PR= Hydrogen Production Rate; OLR= Organic Loading Rate; H_2Y= Hydrogen Yield; UASB= Upflow Anaerobic Sludge Blanket; NN= Neural Network GA= Genetic Algorithm; COD= Chemical Oxygen Demand; EGSB= Expanded Granular Sludge Bed Reactor.

5.4 Conclusions.

There are few methods for hydrogen determination; most of them are carried out off-line, while the on-line determination can be performed using expensive devices. Thus, cheap and practical approaches for on-line hydrogen determination are strongly needed. According to the results, BPNN was successfully applied to predict the hydrogen production using only on-line parameters in genetically modified *E. coli* fermentations with or without control of pH. This approach could be applied for other hydrogen production systems.

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Chapter 6

Concluding remarks

This study shows *Escherichia coli* as an excellent model for the hydrogen production. The extensive knowledge of its metabolism and genetics lead to a better understanding of the hydrogen production process. Most studies with *E. coli* are focused in the use of model substrates, but these substrates are expensive and the production of biofuels must use wastes or sub-products to be economically competitive and to dispose of pollutant wastes. The results presented in this work shows that the cheese whey, a problematic by-product of cheese industry, can be used as a cheap substrate for hydrogen production by *E coli*.

Besides the use of sub-products, the hydrogen production by biological processes must be improved to be competitive to the conventional methods. One way to improve the hydrogen production is by the development of mutant strains by genetic manipulation. From the results of this study it was confirmed that the genetic engineering is a useful tool to improve both the substrate consumption and the hydrogen production. The deletion of *lacI* gene reduced the lag-time using lactose as carbon source. Using cheese whey as substrate, the hydrogen production was improved by 22% by the deletion of *lacI* and *hycA* genes on WDHL strain.

Other approach to improve the hydrogen production is to optimize the culture conditions. According to the results, the response surface methodology shows to be accurate to find the optimal culture conditions using cheese whey as substrate. The specific production rate was improved from 3.29 mL H_2/OD_{600nm} unit-h produced by WDHL under non-optimal conditions to 5.88 mL $H_2/OD_{600\ nm}$ unit-h at optimal conditions. The hydrogen yield was improved from 1.21 mol H_2/mol lactose consumed to 2.74 mol H_2/mol lactose consumed under the best conditions. The approach used here to improve the hydrogen production was the culture conditions, particularly pH, substrate concentration and temperature. However there are many other factors that could improve the production such as the culture medium composition.

Among the culture conditions studied, the initial pH and its control are the most important variable on the *E. coli* metabolism and hence in the hydrogen production. The control of pH had an effect over the consumption of galactose and in the production of metabolites. The hydrogen production in *E. coli* and formate metabolism are acidic pH dependent, but using cheese whey as substrate, the more acidic pH tested (5.5) was unfavorable for both the galactose catabolism and the hydrogen production, whereas the highest pH tested (6.5) was the best condition. The lactate production is the main route at more acidic pH, therefore it could be interesting to investigate the cheese whey fermentation at acidic pH with a strain defective on lactate production.

The biological hydrogen production must use wastes or by-products rich in carbohydrates to reduce the cost of production and to dispose off pollutant wastes as discussed above. Lactose, glucose and galactose are sugars commonly present in some agro-industrial wastes, the use of these sugars as substrate from hydrogen production was studied. Despite glucose and galactose are catabolized by the same pathway, with exception of transport and phosphorylation, the hydrogen yield was different. Galactose is the better substrate because it produces the highest hydrogen yield, whereas the use of glucose resulted in the lowest yield. This low yield is because pyruvate is mainly channeled to the lactate pathway and only a small proportion to formate pathway, therefore hydrogen production is diminished. Whereas, using galactose as substrate, its catabolism is slower than the glucose, possibly due to inefficient expression of *gal* regulon or low activity of the enzymes coded by this regulon. By this way the pyruvate concentration is low and the lactate pathway is poorly active. Thus the formate and acetyl Co-A pathway is the main route, which turns on the metabolism to produce hydrogen and ethanol as main by-product, respectively. The two phases of production of metabolites observed in the fermentation of lactose are due to the initial consumption of glucose and after that, the consumption of galactose. From the results of the assay using a mix of glucose plus galactose it can be concluded that the presence of glucose in the culture medium produces a longer lag phase of galactose than the lag phase of galactose as a sole carbon source. These results provide valuable information to improve the hydrogen production using carbohydrates found in alimentary industry wastewaters. For example, the deletion of the repressor of the *gal* operon could reduce the fermentation time using

galactose. The deletion of lactate pathway could improve the hydrogen yield from glucose and the combination of these mutations possibly will improve the use of lactose as substrate.

One of the most important issues in the study of the hydrogen production is actually the determination of this gas. The most common methods are carried out off-line despite the on-line hydrogen determination is strongly desired to establish feedback or feed forward control algorithms. The on-line determination can be performed using expensive devices. Thus, unexpensive and practical approaches for on-line hydrogen determination are strongly needed, and an Artificial Neural Network was developed to predict the hydrogen production based in on-line parameters. According to the results, Back Propagation Neural Network can be applied to predict the hydrogen production using only on-line parameters in genetically modified *E. coli* fermentations with or without control of pH. This approach could be applied for other hydrogen production systems and can be used in control algorithms.

According to the results presented here, *Escherichia coli* is an important model to study the hydrogen production, however improvement of this process is still needed. Here it was showed the positive effect of some gene deletions over the hydrogen production and consumption of substrate, but there are many possibilities to improve the hydrogen production by genetic manipulations. For example it can be introduced new genes that code for catabolic enzymes for

specific sugars and by this way the bacteria can use many kinds of carbohydrates as substrate. On the other hand, in this study only gene deletions were done, but the overexpression of some other genes like *pflB* which codes for pyruvate formate lyase could improve the hydrogen rate and yield. However these genetic manipulations only improve the hydrogen yield to the maximum of 2 mol/mol hexose which is the theoretical maximum yield in *E coli*. Among the wild-type hydrogen producer microorganisms, the theoretical hydrogen yield of *E. coli* is low. Other hydrogen producing pathways can be introduced in *E coli* to improve the hydrogen production and obtain higher yield. Finally the overall yield could be increased by a two stages strategy, taking the organic acids produced by the dark fermentation and using them as a substrate by photofermentation.