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

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Submitted to: Bioresource Technology

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Abstract

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. Thus the formate and consequently hydrogen production ~~was~~ were diminished. The hydrogen production and yield obtained with glucose were 1037 mL and 0.30 mol H_2/mol of glucose, respectively. The galactose catabolism was slower than ~~the~~ that of glucose ~~one~~. Using galactose, the pyruvate formate lyase pathway was the main route for pyruvate; the ethanol production was also favored. The galactose fermentation yield 1.12 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. This work provides valuable information which can be used for the improvement of hydrogen production using lactose, glucose or galactose rich wastes.

Keywords: glucose, galactose, lactate, hydrogen yield, *Escherichia coli* WDHL

1. Introduction.

~~The biofuels~~Biofuel 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~~their use (Luque et al., 2008). ~~-~~Among ~~the~~ biofuels, biohydrogen is an attractive future substitute ~~of~~ for fossil fuels due to its potentially higher efficiency of conversion to usable power, low or non-generation of pollutants and high energy density (Antonopoulou et al., 2011; Hallenbeck and Ghosh, 2009; Sinha and Pandey, 2011). ~~The b~~Biological hydrogen production is carried out at ambient temperature and pressure~~, by this reason~~; therefore it is less energy intensive than ~~the~~ chemical and electrochemical processes (Nath and Das, 2004; Rosales-Colunga et al., 2010a). ~~But~~ However, to be competitive ~~to the~~with other methods of production, ~~the~~ biological production must use ~~wastes~~ waste or by-products rich in carbohydrates, thus ~~and then~~ reducing the cost of production and, at the same time, disposing ~~off~~ of pollutant wastes.

~~The dark~~Dark fermentation is a promising biological process to obtain hydrogen because it could use organic ~~wastes~~ waste from the agricultural and food-producing ~~industry~~ industries as substrates (Liu et al., 2008), and only ~~a~~ relatively simple equipment is necessary (Hallenbeck, 2005). *Escherichia coli* produce hydrogen by dark fermentation under anaerobic conditions when no external electron acceptors are present (Leonhartsberger et al., 2002; Sawers, 2005). 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 (Davila-Vazquez et al., 2008a). The ~~substrate more~~most extensively studied substrate for hydrogen production ~~had~~ has been glucose (Maeda et al., 2007; Maeda et al., 2008; Penfold et al., 2003; Yoshida et al., 2006).

Lactose, and the sugars released from its hydrolysis~~:~~, glucose and galactose, are commonly present in some agro-industrial ~~wastes~~waste. Lactose is found in ~~the~~ cheese and dairy industry wastewater (Calli et al., 2008; Chong et al., 2009). Glucose is currently obtained from the hydrolysis of molasses, cellulose, and other agricultural wastes (Kapdan and Kargi, 2006). Besides the release of galactose from lactose hydrolysis, this sugar is a component of hemicellulose (Ren et al., 2009). Thus, it is interesting to study the use of these carbohydrates as single substrates or in mixture for hydrogen production. Despite the fact that the use of glucose by *E. coli* has been extensively studied as mentioned above, the use of lactose and galactose as substrates ~~have~~ has 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 (Rosales-Colunga et al., 2010b). The *hycA* ~~gen~~ gene codes for the negative regulator of the hydrogen pathway whereas the deletion of *lacI* lead to the constitutive expression of the lac operon.

2. Materials and methods

2.1 Strain and culture media.

Escherichia coli WDHL strain, a hydrogen ~~over producer~~overproducer strain, which lacks *hycA* and *lacI* genes, was used. A complete description of the strain has been published (Rosales-Colunga et al., 2010b). ~~Preinocula~~ Preinnocula 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 the bioreactor were done using HP medium reported elsewhere (Rosales-Colunga et al., 2010b) 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). A duplicate using glucose in M9 medium was ~~done~~ made to validate the reproducibility of the experiments.

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.

2.3 Analytical methods

Cell growth was monitored at OD_{600nm} using a Cary BIO-50 spectrophotometer ~~Cary BIO-50~~ (Varian, Palo Alto, CA). Culture samples were periodically taken from the bioreactor, and 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 (Davila-Vazquez et al., 2008b). Ethanol was determined by gas chromatography as described elsewhere (De Leon-Rodriguez et al., 2006).

3. Results

3.1 Fermentation of lactose

Lactose is commonly present in ~~wastes of~~ food industry waste, and it can be used as substrate for the production of hydrogen (Chong et al., 2009; Kapdan and Kargi, 2006; Keskin et al.). A typical batch culture using lactose as substrate is shown in Fig. 1. ~~It can be noted t~~Three phases of hydrogen production can be noted (Fig. 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~~ following 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 ~~the~~ 27 h, after ~~that~~ which the concentration decreased to 1.2 g/L at the end of the experiment. Lactose was not detected after 12 h (Fig. 1B).

The soluble metabolites produced are shown in Fig. 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~~ increase was observed. ~~,~~ A~~a~~cetate reached 1 g/L and ethanol 0.7 g/L at 27 h, after ~~that~~ which the concentration of both metabolites steadily increased to a maximum concentration of 2.6 and 2.5 g/L, respectively. ~~The s~~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.

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.

3.2 Fermentation of glucose.

A typical batch culture using glucose as substrate is shown in Fig. 2. As expected, this carbohydrate was used immediately and was not detected after 44 h. ~~The hydrogen~~Hydrogen production also started ~~since~~ from the beginning of the culture. ~~,~~ T~~t~~he final hydrogen production was 1037 mL in mainly in 60 h (Fig. 2A). In this case the maximum hydrogen production rate was 18.61 mL/L h. The d~~D~~uplicate attained a final production and a maximum hydrogen production rate of 965 mL and 15.3 mL/L h, respectively.

The production of metabolites is shown in ~~the~~ Fig. 2B. Lactate was the main soluble product of the fermentation and attained a maximum concentration of 10.1 g/L, whereas the other products were produced in ~~a~~ minor concentration. The maximum concentrations of succinate, acetate, and ethanol were 1.6, 1.5 and 1.2 g/L, respectively. The highest formate concentration ~~was~~ reached 0.6 g/L at 12 h and then decreased.

3.3 Fermentation of galactose.

A typical batch culture using galactose as substrate is shown in Fig. 3. In this case, a lag phase of 18 h was observed. ~~Other~~ Another marked difference was the ~~time for the~~ galactose up-take time; whereas the glucose was completely consumed at 44 h (Fig. 2A), galactose required a longer culture time and, at 356 h of culture, 4.2 g/L of galactose still remained in the culture medium (Fig. 3A). Since the galactose consumption became asymptotic, the culture was stopped. The hydrogen production from galactose is ~~showed~~ shown in Fig. 3A.~~,~~ D~~d~~ue 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~~two times 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 when using glucose.

The production of metabolites from galactose is presented in Fig. 3B. In this case, the main soluble metabolite was ethanol with a final concentration of 6.1 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.

3.4 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~~ Fig. 4 shows the sugar~~s~~ 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 (Fig. 4A). Lactate was the main soluble product in this phase and attained a concentration of 6.7 g/L at 25 h. The concentration~~s~~ 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 (Fig. 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~~ that produced from ~~the~~ glucose (Fig. 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.

3.5 Comparison of hydrogen production.

Measures ~~on~~ of hydrogen and soluble metabolites produced by the fermentation of lactose, glucose and galactose showed differences. Table 1 shows the differences ~~on~~ in hydrogen production. Using glucose as substrate resulted in low hydrogen production and poor yield (0.30 mol H_2/mol of glucose), with the main soluble product ~~was~~ being lactate,~~.~~ ~~Whereas~~ whereas galactose gave the highest hydrogen yield (1.12 mol H_2/mol galactose) and the main product was ethanol. Lactate was the main product using lactose, but in this case ~~the~~ lactate was produced only when the glucose was consumed. Interestingly in the fermentation of glucose plus galactose, the highest production of hydrogen was attained, and the main products were lactate, acetate and ethanol. The yield of this fermentation was the same as that of ~~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.

4. Discussion

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 (Rittmann, 2008). In *E. coli*, glucose is transported into the cell by the phosphotransferase system and then catabolized to phosphoenolpyruvate. T~~and t~~his 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 the phosphoenolpyruvate is transformed ~~to~~ into

pyruvate, which is cleaved to formate and acetyl-CoA by pyruvate formate lyase complex. The formate is converted to hydrogen and CO_2, whereas the latter yields acetate or ethanol (Clark, 1989). ~~But~~However, ~~during~~ under 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 (Tarmy and Kaplan, 1968). The glucose uptake rate was 210.83 mg of glucose/L h OD_{600} unit, ~~and that means~~meaning that the glucose is quickly converted to pyruvate., ~~T~~ T~~and t~~herefore the concentration of pyruvate must be high. Then, the lactate pathway must be very active since the LDH activity increases with increased pyruvate concentration (Tarmy and Kaplan, 1968) and it has been showed that the addition of exogenous pyruvate increased the expression of *ldhA* (Jiang et al., 2001), 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, 0.30 mol H_2/mol of glucose, is higher than the yield of 0.17 mol H_2/mol of glucose ~~consumed~~ consumption predicted by metabolic flux analysis reported by- Manish et al., 2007. They also predicted an ~~increment~~ increase of 35% in hydrogen yield in a strain lacking the *ldhA* gene. Other studies that found higher yield include ~~,~~ Bisaillon et al., 2006, which reported the 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., 2008, with the same strain and nutrient limitations in continuous cultures, whereas Ghosh and Hallenbeck, 2009 attained 1.51 mol H_2/mol of glucose using the same strain. Maeda et al., 2007, 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., 2006

enhanced the hydrogen yield to 1.82 mol H_2/mol of glucose with a strain of *ΔldhA*, *ΔfrdBC*. Mathews et al., 2010 obtained the same hydrogen yield with the strain GW16 (*ΔhyaAB, ΔhybABC, ΔhycA, ΔfrdBC ΔldhA*) using rich defined media., ~~T~~they also observed an increment in the acetate concentration. In the results presented here the acetate and hydrogen production were low. 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 the increase ~~the~~ in hydrogen yield. ~~Other~~ Another study performed by Penfold et al., 2003 did not involve a mutant of *ldhA*., ~~T~~they used a *hycA* mutant strain and ~~they~~ found that the amount of hydrogen decreased as the concentration of glucose increased., ~~I~~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 an energy source but also as a building block in complex polysaccharide synthesis. The transport of galactose, unlike that of 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 (Weickert and Adhya, 1993). 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 that of 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. I was also suggested ~~Also it was suggested~~ that the expression of the *ldhA* gene might be affected by the nature of carbon source, and ~~with~~ by

the PTS system (Jiang et al., 2001). 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 is 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 (Kremling et al., 2007), the transportation of galactose is not involved. I~~Since, i~~n this case the galactose was slowly consumed suggesting that it is due to the 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, which ~~and it~~ could be caused by catabolic repression, since both glucose and galactose were present in the culture medium (Adhya and Echols, 1966). The hydrogen yields from galactose and lactose were 1.12 and 1.02 mol H_2/mol hexose consumed using a strain lacking the *hycA* and *lacI* genes. These yields are higher than the 0.69 and 0.73 mol H_2/mol reported by Ghosh and Hallenbeck, 2009, for galactose and lactose, respectively using a strain with mutations on uptake hydrogenases, *ldhA* and *fhlA*. It seems that the mutation on *lacI* improves the hydrogen yield from those sugars.

5. Conclusions

The two phases of metabolite~~s~~ production using lactose are due to the consumption of glucose and after that, ~~the~~ galactose consumption. Using glucose, pyruvate is mainly channeled to the lactate pathway and hydrogen production is diminished. Using galactose,

the formate and acetyl Co-A pathway is the main route, hydrogen and ethanol are the main products. The maximum hydrogen production rate is high when glucose is present,whereas galactose yields the lowest maximum production rate. 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.

Acknowledgements

L.M. Rosales thanks ~~to~~ CONACyT for his scholarship number 174494. Partial financial support of REDFE-CONACyT. We thank to Leandro G. Ordoñez-Acevedo, Dulce M. Partida and Guillermo Vidriales for the technical assistance. We thank to Jennifer Eckerly Goss for the English revision.

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Figure captions:

Fig. 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 (▲).

Fig. 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 (-▲-).

Fig. 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 (-▲-).

Fig. 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 (-▲-).