I COBBIND
I CONGRESSO BRASILEIRO DE BIOTECNOLOGIA INDUSTRIAL
XXIV SINAFERM ·· XV SHEB ·· XV ENZITEC FLORIANÓPOLIS ·· 2024
Creating connections between biotechnology and industrial sustainability
August 25 to 28, 2024
Costão do Santinho Resort, Florianópolis, SC, Brazil
ENVIRONMENTAL BIOTECHNOLOGY

BIOHYDROGEN PRODUCTION BY *Enterobacter cloacae* ATCC 13047 USING MILK WHEY AS FEEDSTOCK FOR PHOTOFERMENTATION

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ABSTRACT

Hydrogen is being studied as a highly promising renewable energy source to address global warming intensified by the use of fossil fuels. In this context, the biological production of hydrogen (biohydrogen) has advantages, as it allows the use of renewable raw materials and is considered carbon-neutral. Therefore, the objective of this study was to evaluate the biohydrogen production via photofermentation by *Enterobacter cloacae* bacterium, using whey as a carbon source. Fermentation assays were conducted in the presence of light, evaluating the lactose concentrations from whey at 9, 18, and 36 g/L. The highest lactose concentration resulted in the greatest hydrogen production in mL, reaching 258 ± 16 mL with 72 h. However, the bioprocess using 9 g/L lactose showed a higher efficiency than the other conditions, achieving 5.7 ± 0.6 mol of H_2 per mol of lactose. The production of ethanol and acetic acid was also observed throughout the fermentation, along with the acidification of the fermentative medium. Thus, this work demonstrated that the studied microorganism is capable of converting whey lactose into hydrogen through the acidogenic and alcoholic pathways.

Keywords: Hydrogen. Photofermentation. *Enterobacter cloacae*. Lactose. Substrate concentration.

1 INTRODUCTION

One of the great challenges of contemporary society is to develop and incorporate clean and renewable energy sources**,** facing the critical scenario of climate change and global warming[1]. In this sense, hydrogen (H_2) is one of the clean energy sources that has shown to be very promising for land and air transport, with the advantage of high energy density[2]. Hence, researchers are trying to develop more efficient and sustainable approaches to hydrogen production and to help establish full utilization of this fuel[3]. Faced with the search for new methods of sustainable hydrogen production, its production from biomass and organic waste has attracted attention, since this fuel gas is considered carbon neutral[4].

The biological production of hydrogen (biohydrogen, $BioH_2$) has been studied. This bioprocess can be carried out in the presence or absence of light, photofermentation, or dark fermentation, respectively[5]. In these processes, raw materials can be used as feedstock, resulting in energy savings and excellent sustainable performance[6].

The raw material for this production can be obtained from various sources, such as industrial waste[6]. Whey is an example of a byproduct generated in the food industry, which can be used as a substrate for $BioH_2$ production. This waste is mainly obtained from cheese manufacturing, in which 1 kg of cheese generates approximately 9 kg of whey[7]. Brazil is a large consumer of cheese, having consumed 818 thousand tons of cheese in 2021[8]. Then, research are being developed to apply this waste;

Despite the advantages of H_2 production through bioprocesses, challenges are still faced in their application, such as low hydrogen yield and low substrate degradation rate[9]. Therefore, this work aims to explore the biohydrogen production by *Enterobacter cloacae* ATCC 13047 in the presence of light using milk whey as a substrate.

2 MATERIAL & METHODS

Commercial whey powder was pretreated following established methodology[9]. Afterward, the lactose concentration was measured by High-Performance Liquid Chromatography (HPLC) using a Thermo Finnigan Surveyor system (Thermo Fisher Scientific) equipped with a refractive index detector and a Supelco 610 H column. The whey was diluted to obtain lactose concentrations of 9, 18, and 36 g/L, and the pH of the medium was adjusted to 6.8 ± 0.2. The whey solutions were used as culture medium in the hydrogen production assays.

The microorganism used was the anaerobic *Enterobacter cloacae* bacterium under code ATCC 13047, purchased from André Tosello Foundation. The inoculum was prepared using a culture medium containing 3 g/L meat extract and 5 g/L peptone and the cells were incubated at 37 °C under anaerobic conditions and light restriction. After, *E. cloacae* was then stored in cryovials (80% inoculum and 20% glycerol) and kept under refrigeration. For the hydrogen production assays, the strain was reactivated at 32 ºC for 16 h in the same medium previously described.

The experiments were carried out in 250 mL sealed reactors, with a reaction volume of 200 mL, composed of 20 mL (10%) of inoculum and 180 mL (90%) of deproteinized whey with pre-established lactose concentrations. The bioprocess was conducted at 32 ± 2 ºC and 130 rpm in the light presence (using an incandescent lamp of 150 W with 2000 lux). Anaerobic conditions were obtained by injecting nitrogen gas into the medium, at a flow rate of 0.5 L/min for 5 min. The liquid and gas phases were analyzed during fermentation. For this purpose, samples of 1 mL of liquid phase were collected at predetermined intervals. Microbial

biomass (MB) growth was assessed by measuring optical absorbance at 600 nm (OD_{600}). Samples were centrifuged at 3000 rpm for 5 min and filtered with 0.2 µm filters. The concentrations of lactose and formed metabolites were monitored by HPLC. The biogas generated was stored during fermentation in gas sampling bags. Its composition was determined by gas chromatography (Shimadzu Chromatograph, model GC-2010 ProAF), composed of a thermal conductivity detector, using a Carboxen 1010 capillary column (30 m of lenght and an internal diameter of 0.53 mm). Argon was employed as carrier gas at a rate of 6 mL/min. The temperatures of the injector, column and detector were conserved at 200, 30 and 230 ºC, respectively. The volume of gas produced was measured using graduated syringes.

3 RESULTS & DISCUSSION

The highest cell density reached during the bioprocesses was 0.396 g/L at 32 h using 18 g/L of lactose (Figure 1-B). *E. cloacae* showed exponential growth until 32 h of fermentation, except for the 9 g/L condition: this process obtained the lowest cell concentrations, reaching a maximum concentration of 0.122 g/L (Figure 1-A). In this case, the cell growth was limited by substrate availability. In total, 39.2% of lactose were consumed in the process conduct using 9 g/L of lactose in the medium, 76.4% using 18 g/L and 54.2% with 36 g/L.

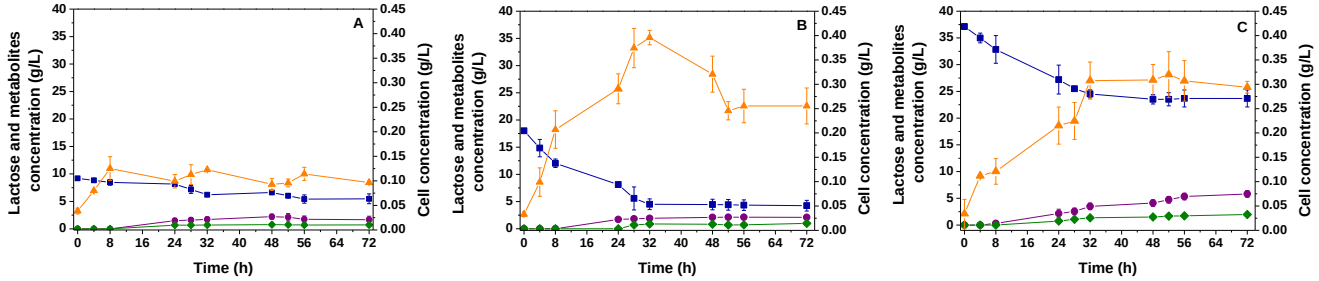

Figure 1. Lactose (■), ethanol (♦), acetic acid (●) and cell (▲) concentrations profiles using 9 g/L (A), 18 g/L (B) and 36 g/L (C) of lactose from whey during photo-fermentation by *E. cloacae* conducted at 32 ± 2 ºC and agitation of 130 rpm.

The fermentative medium containing 36 g/L of lactose showed greater hydrogen production with 258 ± 16 mL of the gas at 72h (Figure 2-A). The use of 18 g/L and 9 g/L of lactose produced 109 ± 17 mL and 74 ± 11 mL of hydrogen, respectively, in the same period (Figure 2-B). Consequently, the increase in lactose concentration (in the evaluated range) also increased hydrogen production in mL. However, the highest $BioH_2$ yield (in produced moles of hydrogen per consumed mole of lactose) (Figure 2-C) was obtained using only 9 g/L of lactose, reaching a yield of 5.7 ± 0.6. In the bioprocess employed disaccharides (i.e. lactose) as the carbon source, *E. cloacae* can exhibit a better hydrogen yield[11], showing a maximum theoretical yield of 8 moles of H_2 per mole of lactose[12]. The lowest $BioH_2$ yields were obtained using 18 g/L and 36 g/L lactose, and it can be attributed to the increased consumption of this carbohydrate, which is coupled with a low hydrogen production that did not correspond to the level of intake. However, it is possible to notice a drastic decrease in yield when only 9 g/L of lactose was used (Figure 2-C). This decrease occurred because there was a considerable production of hydrogen in the first 24 hours, and after this period, the production decreased. Nevertheless, there was still lactose consumption, indicating that the metabolism of the sugar was directed towards other pathways besides hydrogen production.

Furthermore, the highest cumulative hydrogen was obtained in the process using 36 g/L lactose, achieving 56.1 ± 4.8 mmol $_{H2}/L_{medium}$, and in the processes using 18 g/L and 9 g/L of lactose were obtained 24.9 ± 4.0 mmol and 9.5 ± 0.1 mmol of H_2 per liter of whey, respectively (Figure 2-A).

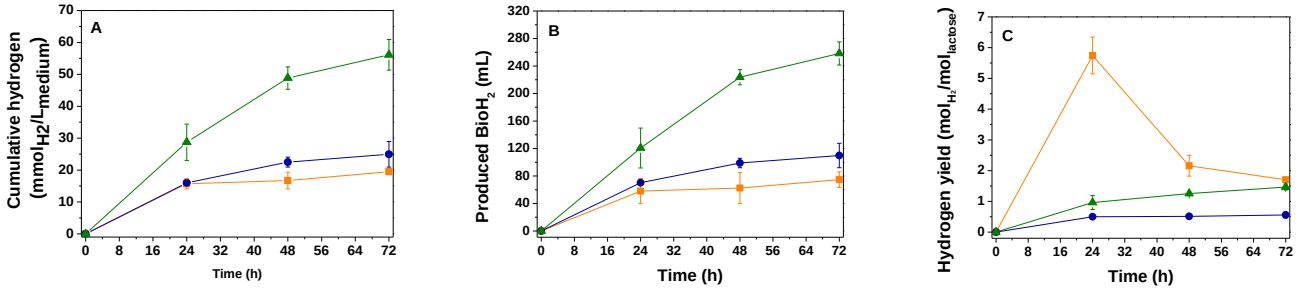

Figure 2. Cumulative $BioH_2$ (A), produced $BioH_2$ in mL (B) and $BioH_2$ yield (C) obtained in the processes using 9 g/L (■), 18 g/L (●) and 36 g/L (▲) of lactose from whey during photo-fermentation by *E. cloacae* conducted at 32 ± 2 ºC and agitation of 130 rpm.

The production of acetic acid was observed in the three conditions evaluated (Figure 1). The theoretical maximum yield for hydrogen production is achieved by fermentative bacteria during the production of acetic acid[10]. The redirection to acidogenic routes results in a decrease in pH, as observed in the work, in which there was a gradual reduction in pH throughout the fermentation, and the final pH for the three conditions reached values around 4.9 ± 0.2, demonstrating a considerable decrease in relation to the initial pH of 7.0. The acidic condition in the fermentation system can decrease the production of biohydrogen, as it can make bacterial cells unviable[11]. Furthermore, bacterial growth was affected after 24 h. It reached stationary growth phase

even in the presence of sugar. Ethanol production was also observed after 24 h of fermentation in three evaluated conditions (Figure 1), indicating that the culture directed its metabolism towards alcohol production.

4 CONCLUSION

The conducted study demonstrated that the *Enterobacter cloacae* ATCC 13047 is capable of converting lactose from whey into hydrogen via acidogenic and alcoholic pathways, producing also acetic acid and ethanol. The ethanol pathway is associated with a reduction in hydrogen yield. When comparing three different lactose concentrations, the bioprocess using the highest concentrations of the disaccharide (36 g/L lactose) obtained the highest production of $BioH_2$, reaching 258 ± 16 mL of gas. However, the highest $BioH_2$ yield was observed using only 9 g/L of lactose, achieving 5.7 ± 0.6 mol of H_2 per mol of lactose, which corresponds to 71.25% of the theoretical maximum yield. This production profile may result from inhibition by high substrate concentrations, as indicated by low consumption. Gas production decreased throughout the fermentation, which could have been affected by the acidification of the medium, given that low pH inhibits both bacterial growth and hydrogen production. The study of new forms of hydrogen production, as carried out in this work, is very important since this fuel is considered a promising fuel due to its high energy density and clean combustion. Furthermore, this study shows that agricultural residues such as whey can be utilized for biohydrogen production through fermentation.

REFERENCES

1 ALNOUSS, A.; MCKAY, G.; AL-ANSARI, T. 2020. Applied Energy, 266. 114885.
2 CHENG, D. et al. 2022. Bioresour. Technol. 357. 127341.
3 HALLENBECK P. 2009. Water Sci. Technol. 52.
4 HASSAN, N. S. et al. 2023. Int. J. Hydrogen Energy. 52. 420-441.
5 RAO, R.; BASAK, N. 2022. Appl. Biochem. Biotechnol. 194 (9).
6 KUMAR, R.; KUMAR, A.; PAL, A. 2020. . Mater. Today Proc. 46 (11).
7 LEÓN-LÓPEZ, A. et al. 2022. Polymers.14. (6). 1258.
8 SEBRAE. 2022. O mercado de queijos finos. Potencial de mercado.
9 LIMA, F. et al. 2013. Process Biochem. 48 (3).443–452.
10 M, A. et al. 2023. Renew. Sustain. Energy Rev. 188. 113791.
11 SOUZA, I. et al. 2019. Int. J. Hydrogen Energy. 44 (31).16190–16198.
12 XUE YAN SIM et al. 2023. Renew. Sustain. Energy Rev. 187. 113755–113755.
13 WANG, S. et al. 2020. Biotechnol. J. 15 (1).
14 MOREIRA, F. S. et al. 2022. Energy. 239.
15 PENG, K. et al. 2017. Int. J. Agric. Eng.10 (6).192–200.
16 SARANGI, P. K.; NANDA, S. 2020. Chem. Eng. Technol. 52. 335-357
17 XU, X.; ZHOU, Q.; YU, D. 2022. Int. J. Hydrogen Energy. 47 (79). 33677–33698.

ACKNOWLEDGEMENTS

The authors gratefully recognize the support from Brazilian and Ceará Government, *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq – grant number 405840/2022-5 and 408071/2022-2) and *Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico* (FUNCAP), respectively.