# Biohydrogen Production From Agro-industrial Wastes Using Clostridium Beijerinckii and Isolated Bacteria as Inoculum

**Walter José Martinez-Burgos**
Universidade Federal do Paraná - Campus Centro Politécnico: Universidade Federal do Parana - Campus Centro Politecnico

**Jair Rosário do Nascimento Junior**
Universidade Federal do Paraná - Campus Centro Politécnico: Universidade Federal do Parana - Campus Centro Politecnico

**Adriane Bianchi Pedroni Medeiros** ( adrianebpm@ufpr.br )
Universidade Federal do Paraná - Campus Centro Politécnico: Universidade Federal do Parana - Campus Centro Politecnico https://orcid.org/0000-0002-8804-0407

**Leonardo Wedderhoff Herrmann**
Universidade Federal do Paraná - Campus Centro Politécnico: Universidade Federal do Parana - Campus Centro Politecnico

**Eduardo Bittencourt Sydney**
Federal Technological University of Paraná: Universidade Tecnologica Federal do Parana

**Carlos Ricardo Soccol**
Universidade Federal do Paraná - Campus Centro Politécnico: Universidade Federal do Parana - Campus Centro Politecnico

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# Abstract

The search for renewable and sustainable sources of energy has been one of the main goals of society in recent years, especially to reduce the environmental impacts of fossil fuels. One promising alternative is the production of hydrogen, which does not emit greenhouse gases and can be produced from agro-industrial wastes. The *Clostridium* genus is recorded as having high hydrogen yields compared to other genus, with several producing species. The objective of this work was to evaluate biohydrogen production potential of four agro-industrial residues, which were soft drink wastewater, corn steep liquor, cheese whey, and expired Guaraná soft drink, using one model strain *Clostridium beijerinckii* ATCC 8260 and newly isolated *Clostridium butyricum* DEBB-B348. The agro-industrial wastes were characterised in terms of monosaccharide, organic acid, amino acid, cation, and anion concentrations and compared to the literature. After performing subsequent experimental designs, the significant factors were cheese whey concentration, corn steep liquor concentration, and fermentation time for *C. beijerinckii,* and corn steep liquor concentration and fermentation time for *C. butyricum* ($p \leq 0.05$), with an $R^2$ of 0.950 and 0.895, respectively. The maximum hydrogen volume production was 18.5 ± 1.68 mL and 27.4 ± 1.84 mL for each strain, respectively. The *C. butyricum* 16s rRNA gene phylogenetic tree and the carbohydrate, organic acid, and amino acid kinetics of the optimum medium are also presented. These results indicate a potential hydrogen production process utilising less expensive substrates, proposing more proper disposal for agro-industrial wastes and using an isolated strain with high yield.


# Introduction

Alternative renewable energies are a transversal area that encompass many of the sustainable development goals, such as renewable energy, innovation and infrastructure, sustainable cities and communities, responsible consumption, climate action, and life on land. The unacceptable environmental impacts caused by the large use of fossil fuels have boosted research on sustainable biofuels with reduced environmental impacts [1]. Among the existing biofuels, hydrogen ("bio$H_2$") stands out for not producing greenhouse gases during its combustion, for being produced from a variety of energy sources, for being safely stored and transported, and for being converted into electricity [2]. Hydrogen may play an important role in building a more sustainable society, especially in the transportation sector where $CO_2$ capture from vehicles is virtually impossible.

The renewable production of hydrogen is derived from the dissociation of $H_2O$ (commercial technology), the thermochemical processing of biomass, and the anaerobic fermentation of organic raw materials. Fermentative hydrogen production ("bio$H_2$") is generated under milder conditions and using (agro)industrial byproducts.

Several types of microorganisms are capable of producing biohydrogen, such as cyanobacteria (*Synechocystis sp.*, *Desertifilum sp.*, *Synechococcus sp.*, *Phormidium corium*, *Synechocystis sp.*, *Oscillatoria sp.*, *Anabaena sp.*) [3], microalgae (*Scenedesmus obliquus*, *Chlamydomonas reinhardtii*, *garis*) [4], and facultative and anaerobic

bacteria [1]. Producing genres involve *Bacillus*, *Citrobacter*, *Enterobacter*, *Escherichia*, and *Rhodopseudomonas* [5, 6]. *Clostridium* deserves special attention due to the highest hydrogen yields when compared to other genera and its tendency to dominate dark fermentation in cases of co-culture [7]. Many species of this genus are capable of producing biohydrogen, such as *C. acetobutylicum*, *C. butyricum*, *C. beijerinckii*, and *C. tyrobutyricum* [8].

The use of agro-industrial residues as a substrate for bio$H_2$ production significantly reduces the costs of production and provides more rational disposal for these wastes. Recent studies have proven that the use of substrates such as cassava processing wastewater, corn steep liquor (CSL), beverage wastewater, mushroom farm waste and others are suitable for biohydrogen production in significant quantities [9–12]. The possibility of using regional wastes for the production of a renewable source of energy has a great impact on local communities, economies, and the environment. The objective of this work was to evaluate the biohydrogen production potential of four agro-industrial residues (soft drink wastewater, corn steep liquor, cheese whey, and expired Guaraná soft drink) using one model strain of *Clostridium beijerinckii* (ATCC 8260) and newly isolated *Clostridium butyricum* (DEBB-B348).

## Materials And Methods

# Physico-chemical characterisation of wastewaters

Soft drink wastewater (SDW) and expired Guaraná were donated by the company Ambev (Almirante Tamandaré, Paraná, Brazil). Cheese whey (CW) was provided by the company Anila (Fernandes Pinheiro, Paraná, Brazil), and CSL was provided by the company Ingredion (Balsa Nova, Paraná, Brazil). The industrial wastewaters were stored at − 20°C until use.

Cations and anions were analysed by ion chromatography (761 Compact IC, Metrohm AG) using Metrosep C 3250/4.0 and Metrosep A Supp5 250/4.0 columns, respectively. For the quantification of cations, a mobile phase composed of 3.5 mM $HNO_3$ at a flow rate of 0.9 mL $min^{-1}$ was used, while for anions a mobile phase containing 3.2 mM $Na_2CO_3$ and 1.0 mM $NaHCO_3$ at a flow rate of 0.7 mL $min^{-1}$ was used. Run times were 25 and 30 min, respectively.

The determination of the chemical oxygen demand (COD) and the total nitrogen content were done using Standard Methods for the Examination of Water and Wastewater 1992 [13].

The free amino acid content of CSL and CW was analysed by an automated SYKAM S433 amino acid analyser (Eresing, Germany) using the ninhydrin method. Three solutions were used in the identification reactions: buffer A, B, and a regeneration solution. Buffer A was composed of tri-sodium citrate dihydrate, citric acid, methanole, hydrochloric acid, and phenol. Buffer B was of composed of tri-sodium citrate dihydrate, sodium hydroxide, and boric acid, and the regeneration solution was composed of sodium hydroxide and boric acid. The operating conditions were the following: the flow rates in the gradient and in the amino module were 0.45 mL $min^{-1}$ and 0.35 mL $min^{-1}$, respectively, and the reaction temperature

in the reactor was 130°C. This method was also used to determine the amino acid kinetics during the fermentation of the best bio$H_2$ production condition.

### Inoculum

*Clostridium beijerinckii* ATCC 8260 and an isolated strain from a consortium obtained from sugarcane cultivation soil (LPBAH3) [14] were first compared for their ability to produce biohydrogen from a combination of the industrial wastewaters. Isolation was carried by successive cultivation in Man, Rogosa, & Sharpe (MRS) medium by the pour plate method and incubation in an anaerobic jar. Upon isolation, the strain was submitted to identification. The 16S rRNA gene sequences of the reference strains retrieved from NCBI (National Center for Biotechnology Information, MA, USA) were aligned using the online version of MAFFT program, version 7, with the option Auto (FFT-NS-1, FFT-NS-2, FFT-NS-i or L-INS-i). A neighbour-joining phylogenetic tree was constructed using the MEGA X 10.1 computer [15] based on the MSA file by MAFFT. The evolutionary distances were computed by the maximum composite likelihood method [16] and maximum-parsimony [17]. The robustness of individual branches was estimated by bootstrapping with 1000 replicates [18]. The isolated strain was identified as *Clostridium butyricum* (DEBB-B348). *Clostridium beijerinckii* and *Clostridium butyricum* (DEBB-B348) were kept in a medium composed of soft drink wastewater containing 3% CSL (v/v). The pH of the medium was adjusted to 7.0 using 35% NaOH solution. Procedures according to Balch et al. [19] were adopted to guarantee an anaerobic environment. Oxygen was removed from the medium by boiling (100–105°C) under anoxic conditions under an argon atmosphere. Reduction of redox potential was ensured by the addition of $NaHCO_3$ (1.0 g $L^{-1}$) and Cysteine-HCl (0.5 g $L^{-1}$) at 85 ºC and 65 ºC, respectively (for inoculants only). All experiments were carried in Hungate tubes with a working volume of 6 mL and a total volume 16 mL. The tubes were sealed with bakelite caps and autoclavable rubber stoppers. To keep the strains active, they were subcultured weekly by transferring 1 mL of inoculum to fresh medium and incubated at 37°C.

### Experimental Design, Optimisation, And Statistical Analyses

A Plackett-Burman design for six parameters with a total of 12 runs was used for each strain. The parameters studied were (i) glucose 0–5 g $L^{-1}$, (ii) fermentation time 24–48 h, (iii) expired soft drink 0–20% (v/v), (iv) inoculum 10–20%, (v) corn steep liquor 0–10% (v/v), and (vi) cheese whey 0–40% (v/v). The Pareto diagram was used to determine the effects of significance in the experiments performed.

Upon significant effects determination, Box-Behnken design and rotational central composite design (RCCD) were carried for *Clostridium beijerinckii* ATCC 8260 and *Clostridium butyricum* DAEBB-B348, respectively (Table 1). The Box-Behnken design contained 3 factors, 3 central points, and 15 runs; RCCD contained 2 factors, with 5 central points, 4 axial points, and 13 runs. The statistical analyses were performed using Minitab software. The optimisation provided an adjusted quadratic model Eq. 1 shown below.

Table 1
Rotational central composite design (RCCD) and the Box-Behnken planning matrix.

| *Clostridium beijerinckii* (Box-Behnken) | | | | | *Clostridium butyricum* (RCCD) | | | |
|---|---|---|---|---|---|---|---|---|
| **Run** | **%CSL (Real value)** | **t (h) (Real value)** | **% CW (Real value)** | **V $H_2$ (mL)** | | | | |
| **1** | **-1 (6)** | **1(48)** | **0(40)** | **4.87** | | | | |
| **2** | 0 (10) | 0(36) | 0(40) | **11.35** | **Run** | **% CSL (Real value)** | **t (h) (Real value)** | **V $H_2$ (mL)** |
| **3** | 1()14 | 0(36) | -1(35) | **13.23** | **1** | 1 (14) | -1(24) | **15.5** |
| **4** | -1(4.34) | 0(36) | -1(35) | **7.65** | **2** | 0(10) | -1.41(19.029) | **15.3** |
| **5** | 1(14) | -1(24) | 0(40) | **5.91** | **3** | -1(6) | 1(48) | **16.7** |
| **6** | 0(10) | -1(24) | -1(35) | **6.90** | **4** | 0(10) | 0(36) | **19.9** |
| **7** | 0(10) | 1(48) | -1(35) | **12.95** | **5** | 0(10) | 0(36) | **20.5** |
| **8** | 1(14) | 0(36) | 1(45) | **16.41** | **6** | 0(10) | 0(36) | **21.5** |
| **9** | 0(10) | 1(48) | 1(45) | **10.32** | **7** | -1.41(4.34) | 0(36) | **13.17** |
| **10** | -1(6) | -1(24) | 0(40) | **3.78** | **8** | 1.41(15.65) | 0(36) | **21.9** |
| **11** | 0(10) | -1(24) | 1(45) | **8.10** | **9** | 1(14) | 1(48) | **22.3** |
| **12** | -1(6) | 0(36) | 1(45) | **6.86** | **10** | -1(6) | -1(24) | **8.23** |
| **13** | 0(10) | 0(36) | 0(40) | **12.33** | **11** | 0(10) | 0(36) | **22.34** |
| **14** | 0(10) | 0(36) | 0(40) | **12.22** | **12** | 0(10) | 1.41(52.97) | **25.9** |
| **15** | 1(10) | 1(48) | 0(40) | **17.14** | **13** | 0(10) | 0(36) | **20.05** |

$$H2 = \beta o + \sum_{i=1}^{k} \beta iXi + \sum_{i=1}^{k} \beta iiXiXi + \sum_{i=1} K - 1 + \sum_{j=1+i}^{k} \beta ijXiXj \quad \textbf{(Eq. 1)}$$

### Kinetics Of Bioh And Volatile Fatty Acids

The kinetics of hydrogen and volatile fatty acid production were carried out in optimised media under anaerobic conditions. All experiments were conducted in Hungate tubes with a working volume of 6 mL that were sealed with bakelite caps and autoclavable rubber stoppers. The pH of the medium was adjusted to 7.0 using 35% NaOH solution. The inoculum rate was 10%, and the temperature of incubation was 37 ºC.

Quantification of hydrogen production and volatile fatty acids was carried periodically at 8 h and 16 h intervals for *Clostridium butyricum* and *Clostridium beijerinckii*, respectively. A glass syringe was used to collect the produced gas, which was analysed in a 490 Micro GC System gas chromatograph (Agilent) equipped with two columns (Molsieve 5Å and PoraPLOT U) and a thermal conductivity detector (TCD). In the Molsieve 5 Å column, the injection temperature was 110°C, with an injection time of 20 ms, column temperature of 90°C, and an initial pressure of 190 kPa. In the PoraPLOT U (PPU) column, the injection temperature was 110°C, with a column temperature of 90°C and an initial pressure of 150 kPa. The time for each run was 1.2 min. The mobile phase used was argon gas with a purity of 99.999%.

The qualitative and quantitative analysis of volatile fatty acids and substrate consumption were carried in a HPLC Agilent 1260 Infinity Quaternary LC with RI detector and Hi-Plex-H column. The detector and column temperature were 50°C and 60°C, respectively. The mobile phase used was 5 mM $H_2SO_4$ at a flow rate of 0.6 mL $min^{-1}$, and the injection volume was 10 µL. Prior to injection (1 mL), the liquid samples were submitted to centrifugation at 6000 rpm for 10 min and microfiltration in cellulose acetate membranes (0.22 µm).

## Results And Discussion

# Physicochemical characterisation of effluents

The physicochemical composition of the three studied agro-industrial residues (CSL, SDW, and CW) was different in terms of carbohydrates, organic acids, nitrogen, cations, and anions (Table 2). The expired refrigerant “Guaraná” was not characterised since it was not significant. The CW was the only waste that presented lactose in detectable quantities (4.55 g $L^{-1}$), while glucose, fructose, and maltose were undetectable. Similar results has been documented in whey used for hydrogen production by *Escherichia coli*, but with a higher content of lactose (15 g $L^{-1}$) [20, 21]. Both CSL and soft drink wastewater contained glucose and fructose; however, CSL exhibited much higher concentrations (> 35 g $L^{-1}$ for each carbohydrate, versus 0.7 and 3.98 g $L^{-1}$ of glucose and fructose, respectively, in SDW). The CSL used in this study is richer in the cited carbohydrates than the used by Martinez-Burgos et al. (2021b) for the production of bio$H_2$. It is important to highlight that CSL also presented an elevated concentration of lactic acid (92.5 g $L^{-1}$) which can be used as a second carbon source for some lactic acid bacteria and species of the *Clostridium* genus [23, 24].

Table 2
Physicochemical characterisation of corn steep liquor, soft drink wastewater, and cheese whey.

| Substance | CSL | SDW | CW | Substance | CSL | CW |
|---|---|---|---|---|---|---|
| Glucose (g $L^{-1}$) | 36.31 | 0.7 | - | Thr (µmol $mL^{-1}$) | 147 ± 12 | 75 ± 12.5 |
| Fructose (g $L^{-1}$) | 35.49 | 3.998 | - | | | |
| Maltose (g $L^{-1}$) | 1.493 | - | - | Cys (µmol $mL^{-1}$) | 116.4 ± 18.0 | 35 ± 14.8 |
| Lactose (g $L^{-1}$) | - | - | 4.55 | | | |
| Lactic Acid (g $L^{-1}$) | 92.55 | 0.5676 | - | Val (µmol $mL^{-1}$) | 135.3 ± 22.9 | 58.8 ± 5.4 |
| Citric Acid (g $L^{-1}$) | - | 1.182 | - | | | |
| Acetic Acid (g $L^{-1}$) | 0.3617 | 0.3071 | - | Met (µmol $mL^{-1}$) | 211.8 ± 28.4 | 15 ± 7.4 |
| Total nitrogen (g $L^{-1}$) | 10.8 | 0.5 | 3.6 | | | |
| Phosphorus $P_2O_5$ (g $L^{-1}$) | < 1.0 | < 1.0 | 12 | Ile (µmol $mL^{-1}$) | 69.5 ± 18 | 47.9 ± 8.9 |
| Na(mg $L^{-1}$) | 1.412 | 268.6 | 805 | | | |
| K (mg $L^{-1}$) | 38.00 | 38.3 | 1650 | Phe (µmol $mL^{-1}$) | 77.9 ± 22.2 | 25 ± 5.8 |
| Ca (mg $L^{-1}$) | 1.170 | 43 | 819 | | | |
| Mg (mg $L^{-1}$) | 2.875 | 32.45 | 131 | His (µmol $mL^{-1}$) | 72.5 ± 15.5 | 17.89 ± 6.5 |
| Fe (mg $L^{-1}$) | - | 3 | - | | | |
| $NH_4$ (mg $L^{-1}$) | 1.412 | 5.85 | 217 | Trp (µmol $mL^{-1}$) | 49.1 ± 17.6 | 17.5 ± 8.7 |
| $NO_3$ (mg $L^{-1}$) | - | 16.4 | - | | | |
| F (mg $L^{-1}$) | 25.057 | - | - | Lys (µmol $mL^{-1}$) | 64.8 ± 12.7 | 100 ± 15.4 |
| Cl (mg $L^{-1}$) | 36.576 | 87.8 | - | | | |
| pH | 4.32 | 7.56 | 4.76 | Arg (µmol $mL^{-1}$) | 124.8 ± 19.7 | 17.35 ± 5.35 |
| COD (g $L^{-1}$) | 245.4 | 4.27 | 47.3 | | | |

According to the organic matter composition, all effluents showed high COD. The COD of CW and CSL were similar to that described in the literature [25–28]. SDW, however, presented lower values for COD in comparison to the scientific literature [11, 29, 30] and was more similar to beverage wastewater [31].

The presence and concentration of Mg, Fe, Na, and K have a significant effect on hydrogen productivity, especially the first two [32]. Mg, Na, and K were observed in high concentrations in all wastewaters. The concentration of Mg observed in SDW, although lower than in CW and CSL, is enough to stimulate biohydrogen production metabolism. Fe was not detected, and its supplementation could enhance the productivity obtained in this study [33–35]. Ca was another cation observed at high concentrations for all the three wastewaters. Mn and Cu, which are widely recognised as enzymes cofactors, were not identified. Supplementation with Mn and Cu could be considered in future studies.

CSL and CW showed up as important sources of nitrogen (10.5 and 3.6, respectively), values comparable to other research [21, 36]. This content is sufficient to support biomass production without the need to supplement with other nitrogen sources, such as yeast extract, meat extract, and ammonium sulphate [20, 37], which have a significant impact on the process economy. The amino acid composition of CSL and CW showed that L-cysteine, a recurring supplement for hydrogen production fermentations [35, 38, 39], is present in CW and CSL in significant amounts. The presence of methionine, alanine, histidine, and lysine in ranges from 1.0 to 10.0 g $L^{-1}$ may also have a positive impact on biohydrogen production. The synergetic effect of these amino acids with ferric oxide was described to increase the volumetric hydrogen produced 1.3 times [40, 41]. The higher amino acid concentrations found in this work were methionine and valine for CSL and histidine and arginine for CW. According to Hofer et al. (2018), phenylalanine and isoleucine should be primarily free in the effluents, while arginine, histidine, and lysine are essentially bound in proteins or peptides. Thus, arginine, histidine, and lysine concentrations in CW may be underestimated.

The initial pH is relevant for biohydrogen production. The optimum pH for Clostridium is approximately 6.0 [42, 43], while fermentation is severely impaired under pH 4.0 or over pH 12.0 [12]. Although all the fermentations were adjusted to pH 7.0, it is interesting to consider the amount of alkali necessary to correct the pH of CSL and CW. Despite the chemical composition of SDW not being remarkably interesting in terms of carbohydrates, cations, and anions, it could be used as a dilutant to facilitate pH correction.

Clostridium butyricum **identification**

According to the BLAST analysis and phylogenetic tree (Fig. 1), the isolate strain was identified as *Clostridium butyricum* with 99% similarity. The phylogenetic tree constructed with the neighbour-joining method revealed that the *C. butyricum* was included in the same subcluster with *C. saccharoperbutylacetonicum*, C. puniceum, *C. saccharobutylicum*, *C. chromiireducens*, *C. diolis*, *C. beijerinckii*, and *C. neonatale*.

## Variable Selection, Optimisation, And Mathematical Models

The Pareto diagram (Figure not shown) showed that among the five variables tested, only %CSL and fermentation time significantly affected the production of hydrogen ($p \leq 0.05$) for the isolated strain. For *Clostridium beijerinckii*, CW, %CSL, and fermentation time were significant ($p \leq 0.05$). Previous works

demonstrate that the concentration of CSL, whey, and the fermentation time affect hydrogen production via dark fermentation [22, 27]. The inoculum rate was not significant ($p \leq 0.05$), which may have been because large inoculum volumes were tested; an inoculum rate of 10% was then used for all experiments.

It was expected that due to its large amount sugar the expired soft drink would be significant in all tests performed. However, this variable was not significant ($p \leq 0.05$). Soft drinks were added to the medium in a concentration of 20%, and even though it was diluted, the antimicrobial properties of the finished product were still outstanding. According to Kregiel [44], soft drinks contain chemical preservatives and acids that play an important role in avoiding microbial growth. Sorbates and benzoates present in the soft drink act together to enhance antimicrobial effectiveness against bacteria and other microorganisms. Notwithstanding, the author still emphasises that this combination causes the inhibition of amino acid uptake and destroys the internal proton level of microbial cells.

On the other hand, the contour surface graphs (Figs. 2A and 2B) showed that the optimal hydrogen production is reached with 12–14% CSL, at 37.5–48 h time of fermentation and 38.5–45 % CW for *Clostridium beijerinckii.* In the case of *Clostridium butyricum*, the optimum hydrogen production was 10–14 % CSL and 36–52 h (Fig. 2C).

## Mathematical Models

Two second-order polynomial models that simulate the production of biohydrogen under the conditions described are presented in equations 2 (*Clostridium beijerinckii*) and 3 (*Clostridium butyricum*). The mathematical models presented an $R^2$ of 0.95 and 0.895 for equations 2 and 3, respectively, which indicates that 95% and 89.5% of hydrogen production is governed by the variables selected. According to Hye et al. and Martinez-Burgos et al. [23, 45], mathematical models with $R^2 \geq 80$ % are adequate to simulate the behaviour of the response variables.

$$H_2 = 11.96 + 3.7CSL + 2.58t - 1.29CSL^2 - 2.74t^2 + 2.54CSL * t + 0.13CW \quad \textbf{(Eq. 2)}$$

$$H_2 = 20.76 + 4.68CSL + 5.13t - 4.76CSL^2 \quad \textbf{(Eq. 3)}$$

According to the mathematical model (Eq. 2), the optimal conditions for the production of biohydrogen with *Clostridium beijerinckii* were CSL = 12%, t = 48 h, and CW = 40%. The experimental and predicted hydrogen volumes at the optimum condition were 18.5 ± 1.68 mL and 16.87 mL, respectively. In the case of *Clostridium butyricum*, the conditions were CSL = 12 % and t = 48 h. The experimental and predicted biohydrogen volumes for this strains were 27.4 ± 1.84 mL and 26.5 mL, respectively. In both cases, there was no significant difference between the experimental and modelled volumes.

## Evaluation Of Biohydrogen Production Under Optimum Conditions

Under the optimum conditions selected, the maximum volume of hydrogen produced by the bacteria *Clostridium butyricum* and *Clostridium beijerinckii* was 27.47 ± 1.8 mL and 19.05 ± 0.70 mL, respectively *. butyricum* and *C. beijerinckii*, respectively, the

production of hydrogen did not improve so the fermentation process could be stopped at this time. It is noteworthy that the yield of hydrogen produced with *C. butyricum* (310 mL $H_2$/g $_{COD\ removed}$) was approximately 40% greater than that obtained as *C. beijerinckii* (235 mL $H_2$/g $_{COD\ removed}$). These yields are higher than those reported in other studies that had been supplemented with some compounds. [46] supplemented the effluent with $Fe^{2+}$, peptone, and $Na_2HPO_4 + 2H_2O$ and obtained a yield of 215 mL $H_2$/g $COD_{removed}$. Zampol et al. [47] supplemented the medium with $KH_2PO_4$, $K_2HPO_4$, $MgSO_4$, NaCl, $CaCl_2$, yeast extract, iron citrate and reported a maximum yield of 137 mL $H_2$/g COD $_{removed}$. Ozkan et al. [48] supplemented the effluent with Basal medium and obtained a yield of 115.6 mL $H_2$/g COD $_{removed}$.

On the other hand, it was observed that for both microorganisms studied no significant difference (p-value $\leq 0.05$) between the hydrogen production between the cysteine-HCl-supplemented and not supplemented media (Fig. 3B). This indicates that the CSL can supply the amino acid demands of the microorganisms and its use could decrease the costs of the culture media destined for the production of hydrogen via biological production.

In both cases, the main carbon sources such as glucose and fructose were consumed almost completely (Figs. 3C and 3D). In addition, it was observed that the microorganisms could use lactic acid as an alternative carbon source. The metabolites produced in the fermentation were mainly butyric and acetic acid, with predominance of the first. It is noteworthy that the production of the latter was greater with *C. butyricum* which could be an explanation of the greater production of hydrogen with this strain, acetate generates 2× more $H_2$ than butyrate [49]. Also, it was observed that part of the acetic acid and the butyric acid produced was consumed in the same fermentation, as these can also be used as alternative carbon sources. [23, 50] showed that acetic and butyric acid can be used as carbon sources by some microorganisms in dark fermentation. Likewise, both microorganisms produced traces of propionic acid. However, the production of this metabolite is not desirable in dark fermentation because stoichiometrically hydrogen is needed for its production [8, 9].

The amino acid profile during the *C. butyricum* fermentation (Fig. 3E) show a slight consumption in the amino acids cysteine and threonine available in the medium. The methionine and valine concentrations were maintained above the concentration of the other amino acids, and most of them showed similar concentrations in the beginning and end of the fermentation time. Sharma and Melkania [40, 41] tested the effect of methionine, alanine, histidine, cysteine and lysine in the production of hydrogen by co-culture of *E. coli* and *Enterobacter aerogenes* in municipal solid waste. The best productivities were with the addition of alanine and cysteine, and the butyrate concentration was increased during the fermentation, favouring the butyrate hydrogenogenic pathway. A similar increase in the butyric acid occurred in this work, possibly explaining why cysteine was consumed. Several authors indicate that cysteine supplementation in the medium enhance biohydrogen production [34, 35, 38, 39]. The bioavailability of each amino acid also indicates which can be used and directed for the metabolic pathways. Hofer et al. [24] found that the content of threonine, tryptophan, and phenylalanine were more than 60% free in CSL,

while cysteine and valine were around 35% free in the solution. The cysteine and threonine found in this work were also likely available for *C. butyricum* dark fermentation.

# Conclusion

Biohydrogen is a promising alternative to the fossil fuels since it emits no greenhouse gases and its production from agro-industrial wastes significantly reduces the production costs. This work showed that the species *Clostridium beijerinckii* ATCC 8260 and the identified strain *Clostridium butyricum* DEBB-B348 are capable of producing hydrogen from soft drink wastewater, corn steep liquor, and cheese whey. The mathematical models reached by the RCCD showed an $R^2$ of 0.950 and 0.895, with fermentation time and corn steep liquor being the most significant factors. The carbohydrate consumption and organic acid formation kinetics also indicate that the butyrate metabolic pathway was favoured. The maximum hydrogen volume production was 18.5 ± 1.68 mL and 27.4 ± 1.84 m L for *C. beijerinckii* and *C. butyricum*, respectively. The results show an interesting alternative to the production of hydrogen through dark fermentation, giving a sustainable alternative for agro-industrial waste disposal and generating an eco-friendly fuel.

# Declarations

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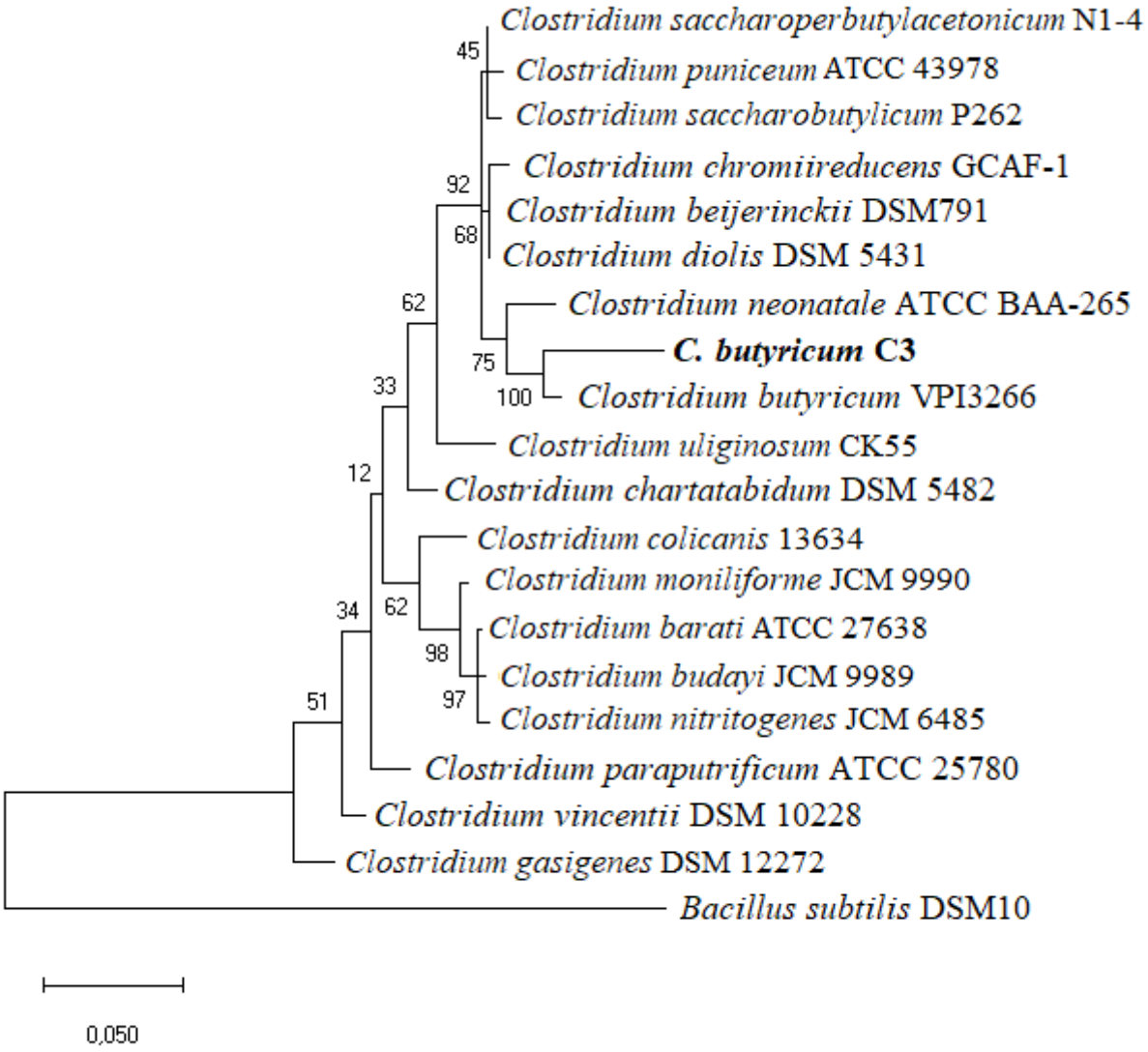
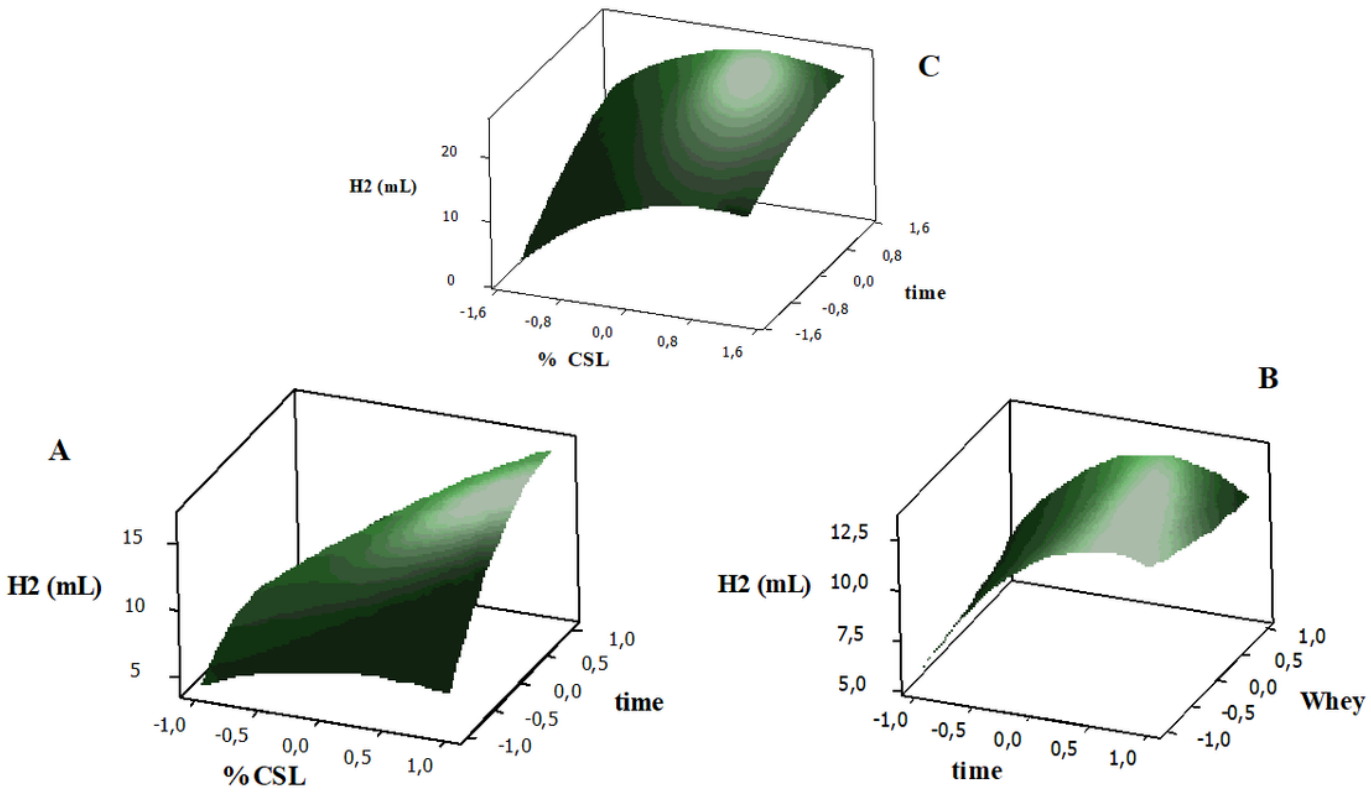
# Figures



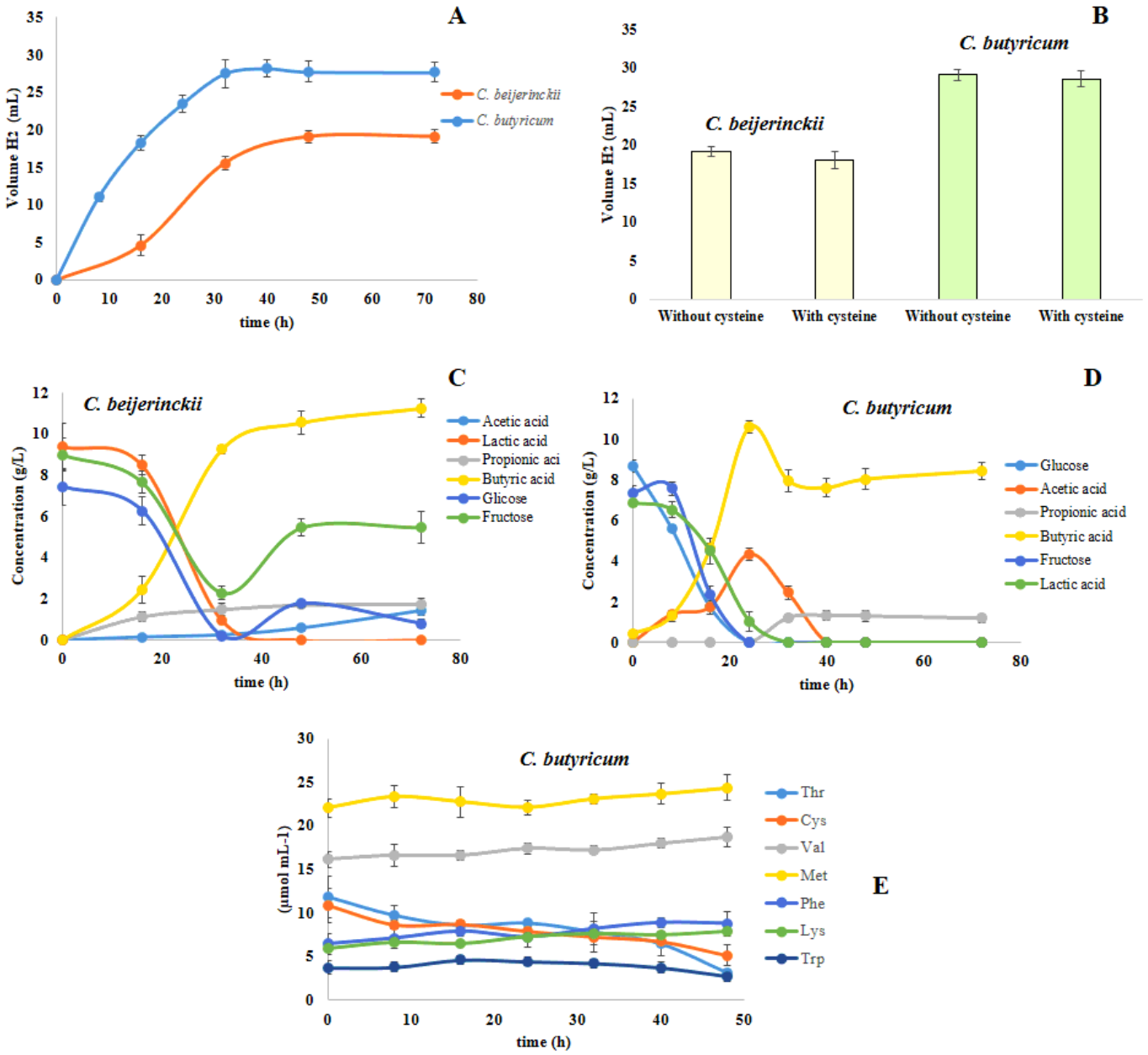

## Figure 1

Maximum-likelihood tree based on 16s rRNA gene sequences showing the phylogenetic relationships. Bootstrap values (%) based on 1000 replications are shown at branch points. The substitution model used was kimura 2-parameter model bar, with 0.05% sequence divergence.




## Figure 2

Response surfaces for bio hydrogen production using C. beijerinckii and C. butyricum. A and B) C. beijerinckii. C) C. butyricum.




## Figure 3

Behaviour of substrate consumption and metabolite production in dark fermentation