



The use of the carbon/nitrogen ratio and specific organic loading rate as tools for improving biohydrogen production in fixed-bed reactors[☆]



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ABSTRACT

This study assessed the effect of the carbon/nitrogen (C/N) ratio on the hydrogen production from sucrose-based synthetic wastewater in upflow fixed-bed anaerobic reactors. C/N ratios of 40, 90, 140, and 190 (g C/g N) were studied using sucrose and urea as the carbon and nitrogen sources, respectively. An optimum hydrogen yield of 3.5 mol H₂ mol⁻¹ sucrose was obtained for a C/N ratio of 137 by means of mathematical adjustment. For all C/N ratios, the sucrose removal efficiency reached values greater than 80% and was stable after the transient stage. However, biogas production was not stable at all C/N ratios as a consequence of the continuous decreasing of the specific organic loading rate (SOLR) when the biomass accumulated in the fixed-bed, causing the proliferation of H₂-consuming microorganisms. It was found that the application of a constant SOLR of 6.0 g sucrose g⁻¹ VSS d⁻¹ stabilized the system.

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1. Introduction

Over the last few decades, the fast population growth has increased the global energy demand, leading to the excessive use of fossil fuels. Hydrogen gas is a clean alternative energy carrier due to its high energy yield (122 kJ g⁻¹), and it can be converted into electricity using a fuel cell. In addition, the hydrogen combustion process is attractive because it generates only water vapor as waste [12,43,54]; thus, hydrogen is an environmentally friendly, combustible source of energy.

The lack of pure hydrogen (H₂) in the environment and its high production cost are the main barriers to using hydrogen as a fuel source [4]. Processes such as electrolysis and thermal decomposition of water are more expensive than the standard methods that are used for the production of fossil fuels [9]. Nonetheless, the Hydrogen National Program of the United States estimates that in 2025, hydrogen will account for 10% of the total global energy market [43].

Currently, hydrogen is produced primarily by reformation of natural gas, high-density fuels, and naphtha. However, it can also be produced biologically by phototrophic organisms or through a fermentation process [11]. Biological hydrogen production is sustainable when the energy source is obtained from organic compounds that are present in industrial or domestic wastewater or solid waste. These sources are widely available and inexpensive, and it is necessary to treat them to control environmental pollution [6]. Furthermore, fermentative hydrogen production can be performed under non-sterile conditions at ambient temperatures and pressures without light, and there are no oxygen limitation problems [2,8,25].

Through the fermentative process, complex organic compounds such as carbohydrates are broken into simpler compounds, e.g., organic acids and alcohols; this process is accompanied by hydrogen release from both facultative and strict anaerobic microorganisms [52]. In this process, several factors such as the type of reactor [6,22,46,57], hydraulic retention time (HRT) [5,13], organic loading rate (OLR) [21,26,50], inoculum and pre-treatments [45,47], degree of back-mixing [34], and pH can affect the hydrogen production [57]. Furthermore, the characteristics of the wastewater are extremely important because the hydrogen yield depends on the organic compounds and nutrients that are available to the microorganisms.

Macronutrients (N, P, and S) and micronutrients (K, Mg, Ca, Fe, Mn, Co, Cu, Mo, and Zn) are essential for biological metabolism

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[41]. Often, the nutritional needs of a microorganism are defined by an analysis of the cell's chemical composition, and the substances necessary for the vital functions of a microorganism are called limiting factors [38]. Thus, excess or insufficient levels of nutrients in the medium might affect the biological activity and microbial diversity, causing variations in the predominant fermentation products [43].

According to Wang and Wan [57], nitrogen concentration plays an important role in fermentative hydrogen production because nitrogen is part of the proteins, nucleic acids, and enzymes that are responsible for the growth of hydrogen producers [57]. Excess nitrogen may inhibit hydrogen production because of changes in the microbial structure and the consequent shifting of the metabolic pathway [29,42]. In addition, low nitrogen concentrations might compromise cell growth [35].

Several papers have been published regarding adequate nitrogen concentrations for improving hydrogen production, but it is not possible to define an optimal carbon/nitrogen ratio due to the differences between the configuration and operation of the fermentative process. For example, Lin and Lay [35] showed that the best hydrogen yield of $4.8 \text{ mol H}_2 \text{ mol}^{-1}$ sucrose was reached with a C/N ratio of 47, and Cheong and Hansen [9] observed a maximum hydrogen rate of $25 \text{ mL H}_2 \text{ h}^{-1} \text{ g}^{-1}$ at a C/N ratio of 30 [9,35]. The aforementioned authors concluded that a high concentration of nitrogen is necessary to improve the hydrogen production.

In contrast, Argun et al. [4] observed that an adequate nitrogen concentration depends on the phosphorus concentration in the medium. That is, systems with a low phosphorus concentration require a low nitrogen concentration and vice versa. However, in their research, the best hydrogen yield of $281 \text{ mL H}_2 \text{ g}^{-1}$ starch was obtained at a C/N ratio of 200 and a C/P ratio of 1000, namely, for lower concentrations of nutrients [4].

Peixoto et al. [44] showed a similar example when added urea (COD:N of 100:0.7) was used as the nitrogen source in one of their upflow fixed-bed reactors. Under that condition, the hydrogen production ceased completely after eight days of operation. In contrast, the reactor with a COD:N ratio of 100:0.3 produced hydrogen continuously for seventy days with an average hydrogen yield of $3.5 \text{ mol H}_2 \text{ mol}^{-1}$ substrate. These authors suggested that the excessive cell growth caused by the addition of nutrients affected the reactor hydrodynamic pattern, hindering the liquid–gas transfer mass of hydrogen. In addition, the decrease of the HRT increased the production of non-reduced compounds Peixoto et al. [44].

Wang and Wan [57] summarized six studies in which the optimal nitrogen concentration varied from 0.01 to 7 g NL^{-1} in batch reactors with various substrates, inocula, and nitrogen sources. However, the differences among the hydrogen measurement parameters were the main source of difficulty in comparing the performance of those systems. These authors suggested that additional research on the effects of the nitrogen concentration in continuous systems must be performed [57].

The published data in the literature indicate the importance of the C/N ratio in the fermentative process. However, on one hand, low nitrogen concentrations may lead to nutritional deficiency, affecting hydrogen yield and productivity. On the other hand, high nitrogen concentrations tend to improve the assimilative metabolism, thus resulting in a high cellular concentration, which in turn could lead to adverse changes in the microbial pathways. Furthermore, it is noted that the biomass accumulation, mainly in fixed-bed reactors, leads to low specific organic load rates (SOLRs), which could give rise to improved homoacetogenic activity, thus resulting in a decrease in the overall hydrogen production. In this context, the existence of an optimum C/N ratio for fixed-bed reactors is hypothesized in this study.

Based on both the literature data and the stated hypothesis, this paper aimed to identify the C/N ratio that maximizes the hydrogen production of a fermentative process in a continuous system. C/N ratios of 40, 90, 140, and 190 were used in a continuous upflow fixed-bed anaerobic reactor to produce hydrogen from synthetic wastewater. A second objective, namely, to estimate the SOLR as a function of time in upflow fixed-bed anaerobic reactors, was achieved through this research.

2. Materials and methods

2.1. Reactors

Experiments were carried out in upflow fixed-bed anaerobic reactors, as depicted in Fig. 1. Each reactor consisted of an acrylic tube with an internal diameter of 80 mm, an external diameter of 88 mm, and a length 750 mm, with a total volume of 3.8 L. Each tube had four compartments: feeding (100 mm), fixed-bed (500 mm), effluent outlet (100 mm) and headspace for gas collection (50 mm). The reactors were sealed to avoid gas leakage during the experiments.

2.2. Support for biomass attachment

Cylinder-shaped particles of recycled low-density polyethylene with diameters between 7.1 mm and 17.5 mm and a length of approximately 30 mm were used as support for biomass attachment. The material provided a surface area of $7.9 \text{ m}^2 \text{ g}^{-1}$ with no porosity. Each bed contained $374 \text{ g support L}^{-1}$ bed with a uniformity coefficient of 1.20, resulting in a bed porosity of 60%.

2.3. Lab-made wastewater

The synthetic wastewater with a COD of 2 g L^{-1} was mainly composed of sucrose (1789.2 mg L^{-1}) and urea (40.6 mg L^{-1} , 17.9 mg L^{-1} , 11.5 mg L^{-1} , and 8.5 mg L^{-1} for C/N ratios of 40, 90, 140, and 190, respectively). The C/N ratios were calculated based on the percentages of carbon and nitrogen by mass in sucrose ($\text{C}_{12}\text{H}_{22}\text{O}_{11}$) and urea ($\text{CH}_4\text{N}_2\text{O}$). Micronutrients were added according to Peixoto et al. [44]: $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ (0.5 mg L^{-1}),

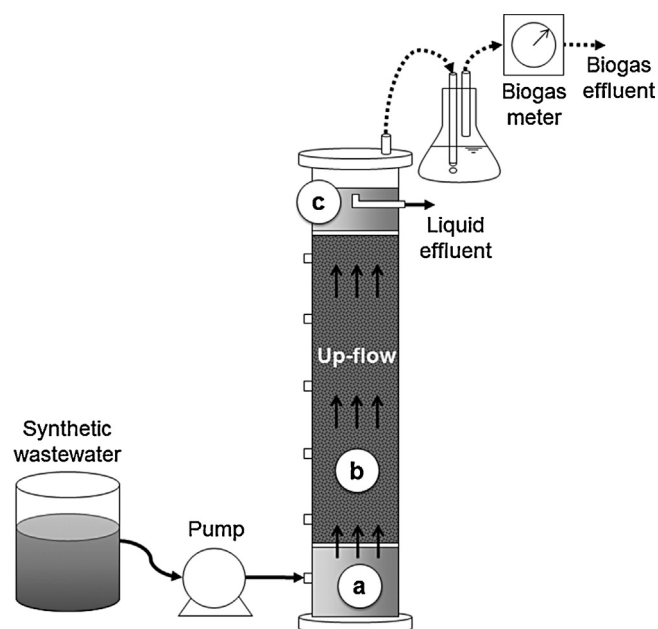


Fig. 1. Upflow fixed-bed anaerobic reactor for biological hydrogen production.

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (2.5 mg L^{-1}), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.25 mg L^{-1}), $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$ (0.04 mg L^{-1}), $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (2.06 mg L^{-1}), SeO_2 (0.036 mg L^{-1}), KH_2PO_4 (5.36 mg L^{-1}), K_2HPO_4 (1.3 mg L^{-1}), and $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (2.7 mg L^{-1}). The pH was maintained at approximately 6.5 by the addition of NaHCO_3 and HCl.

2.4. Inoculum

The natural inoculum was obtained according to procedures described by Leite [33] and Peixoto et al. [44]. Inoculum was generated during fermentation of the synthetic wastewater. Natural fermentation of 40 L of synthetic wastewater occurred after five days of exposure to the atmosphere at approximately 25°C for each C/N ratio. The fermented solution was re-circulated through the reactor with a flow rate of 1.5 L h^{-1} for one week to promote biomass attachment. After that period, the fermented solution was discarded and unfermented synthetic wastewater was added, starting the continuous feeding.

2.5. Systems operation

The C/N ratios of 40, 90, 140, and 190 were assayed separately. The reactors were operated continuously for sixty days with an HRT of 2 h and $25 \pm 1^\circ\text{C}$ using a temperature-controlled chamber. The ORL applied was $21.4 \text{ g sucrose L}^{-1} \text{ d}^{-1}$. Synthetic wastewater was prepared every day to avoid fermentation in the storage tank. Monitoring of the reactor's performance consisted of collecting and analyzing gas and liquid (effluent) samples four times a week. At the end of each operation, the added and suspended biomass inside the reactor was quantified, and a sample was used for the molecular biology analysis.

2.6. Physicochemical analysis

The flow rate of the produced biogas was measured by a type TG1 gas meter, Ritter Inc., Germany. The biogas composition (H_2 , CH_4 , and CO_2) was determined according to the method of Stenerson [53] by gas chromatography (GC-2021, Shimadzu) using a thermal conductivity detector (TCD) and Supelco Carboxen 1010 plot column ($30 \text{ m} \times 0.32 \text{ mm}$). Argon was used as the carrier gas. The temperatures of the injector, detector, and column were kept at 30°C , 200°C , and 230°C , respectively.

The concentrations of volatile acids (acetic, propionic, isobutyric, isovaleric, valeric, and caproic acids) and alcohols (ethanol, methanol, and *n*-butanol) were measured according to Adorno et al. [1] using a Shimadzu GC-2010 gas chromatography system equipped with a flame ionization detector (FID). Samples were introduced using COMBI-PAL headspace vials (AOC 5000 model and HP-INNOWax column of $30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \mu\text{m}$ of film thickness).

The sucrose analysis was performed as proposed by Dubois et al. [16]. The analyses of the chemical oxygen demand (COD), volatile suspended solids (VSS), total volatile solids (TVS), and pH were performed according to standard methods [3].

2.7. Molecular techniques

2.7.1. DNA extraction

DNA extraction from the sludge sample was performed using a protocol based on those of Grobkopf et al. [20] and Neria-González et al. [40], as described previously by Silva et al. [51].

2.7.2. 16S rRNA gene library

To construct a 16S rRNA gene library, DNA amplification was performed using the bacterial primer set 27f and 1100r [31]. The $50 \mu\text{L}$ reaction mixtures contained 50–100 ng of total DNA, 2 U of

Taq DNA polymerase (Invitrogen®), 1X Taq buffer, 1.5 mM MgCl_2 , 0.2 mM dNTP mix (GE Healthcare), and 0.4 μM primer. The PCR amplification was carried out in triplicate using an initial denaturation step of 2 min at 95°C ; 30 cycles of 1 min at 94°C , 1 min at 55°C , and 3 min at 72°C ; and a final extension step at 72°C for 3 min in an Eppendorf thermal cycler.

2.7.3. Cloning and sequencing of the 16S rRNA PCR products

The PCR products were pooled, purified using the GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare), and concentrated in a 5301 Eppendorf speed vacuum concentrator with an A-2-VC rotor. The purified PCR products (200 ng) were ligated into a pGEM-T Easy Vector (Promega) according to the manufacturer's instructions and transformed into *Escherichia coli* JM109 competent cells. Approximately 200 positive clones were selected for subsequent sequencing. The 16S rRNA inserts were amplified from the plasmid DNA of the selected clones using the universal M13 forward ($5'\text{-CGC CAG GGT TTT CCC AGT CAC GAC-3'}$) and reverse primers ($5'\text{-TTT CAC ACA GGA AAC AGC TAT GAC-3'}$). PCR was performed in a $50 \mu\text{L}$ reaction volume containing 1–2 μL of an overnight culture, 0.4 μM primer, 0.2 mM dNTP mix, 2 U Taq DNA polymerase (Invitrogen®), 1X Taq buffer, and 1.5 mM MgCl_2 . The amplification program consisted of an initial denaturation step at 94°C for 3 min and 30 cycles of $94^\circ\text{C}/20 \text{ s}$, $60^\circ\text{C}/20 \text{ s}$, and $72^\circ\text{C}/90 \text{ s}$. The PCR products were purified as previously described for automated sequencing in the MegaBace 500 DNA Analysis System (GE Healthcare). The sequencing was carried out using the 10f and 1100r primers [31] and the DYEnamic ET dye terminator cycle sequencing kit (GE Healthcare), according to the manufacturer's recommendations.

2.7.4. Sequence analysis

Partial 16S rRNA sequences obtained from the clones were assembled into contigs using the Phred/Phrap/CONSED software package [17,19]. Identification was performed by comparing the obtained 16S rRNA contigs with 16S rRNA sequence data for reference strains, type strains, and environmental clones available in public databases—GenBank (www.ncbi.nlm.nih.gov) and RDP (Ribosomal Database Project – Release 9; <http://rdp.cme.msu.edu/>)—using the BLASTn and Classifier routines, respectively. The sequences were aligned using the CLUSTAL X program and analyzed with MEGA software v.4 [55,56]. Evolutionary distances were derived from sequence-pair dissimilarities calculated in MEGA using Kimura's DNA substitution model [27]. The neighbor-joining (NJ) algorithm was used for the phylogenetic reconstruction. The bootstrap values were calculated from 1000 replicate runs, and the routines included in the MEGA software program were used.

2.8. Calculation of the specific organic loading rate (SOLR) throughout the experiments

The biomass was measured as the dried mass. Eqs. (1)–(3) were used to estimate the produced biomass and the consumed substrate during the continuous operation:

Total produced biomass (x_T):

$$x_T(g) = x_a + x_s + x_d \quad (1)$$

where x_a is the attached biomass and x_s is the suspended biomass; both of these quantities were measured at the end of the experimental uptime, i.e., after 90 days of continuous operation. Both the attached and suspended biomass were measured using the gravimetric method [3]. To quantify the attached biomass, approximately 100 g of support were collected from the bed. The sample was washed with deionized water, and then the support and biomass were dried separately. In the same manner, 50 mL of

total liquid drained from the reactor was used to quantify the suspended biomass. The discharged biomass (x_d) was estimated according to Eq. (2):

$$x_d(g) = Q[VSS_1 t_1 + VSS_2(t_2 - t_1) + VSS_3(t_3 - t_2) + \dots VSS_n(t_n - t_{n-1})] \quad (2)$$

where Q is the liquid flow, VSS_n is the concentration of VSS in the effluent, and t_n is the time.

Consumed substrate (s_c):

$$s_c(g) = Q[s_{c1} t_1 + s_{c2}(t_2 - t_1) + s_{c3}(t_3 - t_2) + \dots s_{cn}(t_n - t_{n-1})] \quad (3)$$

where s_c is the concentration of substrate consumed.

Therefore, the biomass growth per consumed substrate factor ($Y_{x/s}$) was calculated according to Eq. (4):

$$Y_{x/s} = \frac{x_T}{s_c} \quad (4)$$

Next, Eq. (5) was used to estimate the concentration of biomass inside the reactor in a given time (Cx_n):

$$Cx_n(g \text{ VSSL}^{-1}) = \left[\frac{S_c \times Y_{x/s}}{V_u} \times \left(\frac{x_a + x_s}{x_T} \right) \right] + Cx_{n-1} \quad (5)$$

where V_u is the useful volume of the reactor.

Finally, the SOLR in a given time ($SOLR_n$) was calculated by Eq. (6):

$$SOLR_n(g \text{ sucrose g}^{-1} \text{ VSS d}^{-1}) = \frac{Cx_n}{OLR} \quad (6)$$

where OLR is the organic loading rate.

3. Results and discussion

3.1. Hydrogen production

Hydrogen gas was produced at all C/N ratios in an upflow fixed-bed reactor. Biogas production and the hydrogen concentration reached the average values as shown in Table 1. The C/N ratio of 140 produced the highest biogas volume, containing an average of 61.0% hydrogen. The biogas was primarily composed of hydrogen and carbon dioxide; methane was not detected. The sucrose conversion efficiency was similar for all C/N ratios, reaching values above 88%.

The observed maximum hydrogen yields were 1.7, 3.1, 3.5, and 2.9 mol H₂ mol⁻¹ sucrose at C/N ratios of 40, 90, 140, and 190, respectively. Therefore, if all of the sucrose was converted into acetic acid, the maximum hydrogen yield achieved at a C/N ratio of 140 would be equivalent to 43.5% of the theoretical maximum value based on the stoichiometry [39].

The maximum hydrogen yield increased 3.5-fold when the C/N ratio was changed from 40 to 140, but the yield was 17% less under a C/N ratio of 90 compared with a C/N ratio of 140. Thus, in Fig. 2, maximum hydrogen yields versus the C/N ratio were fit to a polynomial function, and the optimum C/N ratio was estimated by taking its derivative. The optimum C/N ratio has been found to be 137, which produces a maximum yield of 3.5 mol H₂ mol⁻¹ sucrose. The polynomial fit is empirical, and it was only used to search for

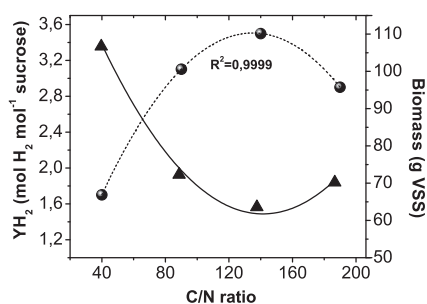


Fig. 2. Maximum hydrogen yield versus C/N ratio. (●) Maximum hydrogen yield, (---) polynomial curve, (▲) final biomass, and (---) final biomass tendency. Final biomass is the amount of sludge accumulated into the reactor (attached and suspended) at the end of the continuous operation (90 days).

the maximum value. Such a polynomial fit was also used in other papers for the same purpose [18,34].

The hydrogen yield variation among the C/N ratios could be related to the cell growth rate when the nitrogen availability changed. Fig. 2 shows that the biomass accumulated in the reactor at the end of the operation, i.e., after 90 days, was inversely related to the hydrogen yield. Hence, when nitrogen was in excess (a C/N ratio of 40), energy was mainly employed for assimilation and cell growth [4,43,44]. Thus, non-producers and/or consumers of hydrogen most likely grew along with hydrogen-producing microorganisms, affecting the hydrogen yield.

As soon as the C/N ratio increased up to 140, the cell growth tended to be lower owing to nutritional deficiencies, favoring hydrogen release. Nevertheless, with a C/N ratio of 190, the biomass growth was 10% greater than with a C/N ratio of 140. The decrease in the hydrogen yield at the same C/N ratio suggested that a nitrogen deficiency could have limited the enzymatic activity [43]. The small difference in the final biomasses for C/N ratios of 140 and 190 indicated an alteration of the balance among hydrogen-producing and non-producing microorganisms [37].

In other studies, the C/N ratio influenced the hydrogen production because of the variation of the amount of carbon causing shifts in the pathways, primarily for formation of fermentative soluble products. However, in this study, variations in nitrogen affected the cell growth and thus the hydrogen release. Moreover, the concentrations of the soluble products did not exhibit significant changes as the nitrogen concentration was altered. Lin and Lay [35] did not report augmentation of the biomass when the C/N ratio changed. However, they observed that increasing the C/N ratio from 40 to 130 by varying the substrate concentration caused a decrease in the fraction of butyrate from 51% to 39%. In contrast, for the same C/N ratio range, the fractions of acetate and propionate increased from 19% to 32% and from 8% to 15%, respectively. In that study, the optimum C/N ratio reported was 47.

Similarly, when Liu and Shen [37] varied the concentration of urea from 0.56 to 11.28 g L⁻¹ in 15 g L⁻¹ of starch, the distribution of the main fermentative products was nearly unchanged, whereas the best hydrogen yield occurred with 5.64 g L⁻¹. However, when they fixed the content of urea to 5.64 g L⁻¹ and varied the concentration of starch from 2 to 32 g L⁻¹, the fraction of acetic acid

Table 1

Average values of the upflow fixed bed reactor performance from the 25th day of continuous operation.

C/N ratios	Sucrose conversion efficiency (%)	Biogas flow rate (mL h ⁻¹)	H ₂ in biogas (%)	CO ₂ in biogas (%)
40	88.4 ± 5.3	131.0 ± 92.1	52.7 ± 7.9	28.3 ± 6.6
90	92.7 ± 7.1	286.5 ± 172.4	48.8 ± 10.1	28.5 ± 7.2
140	88.5 ± 5.1	423.4 ± 102.9	61.0 ± 6.3	34.8 ± 5.2
190	89.5 ± 9.7	371.2 ± 122.7	52.1 ± 10.1	30.5 ± 6.6

Table 2

Average concentration of soluble fermentation products.

C/N ratios	Et ^a (mg L ⁻¹)	H-Ac ^b (mg L ⁻¹)	H-Bu ^c (mg L ⁻¹)	H-Pr ^d (mg L ⁻¹)	TSFP ^e (mg L ⁻¹)
40	476.8 ± 110.9	324.6 ± 113.9	213.3 ± 138.7	40.0 ± 33.1	1195.8
90	322.0 ± 125.1	314.2 ± 119.1	245.5 ± 137.5	21.1 ± 13.8	971.7
140	475.2 ± 130.8	346.7 ± 95.1	210.5 ± 72.2	34.6 ± 18.3	1133.9
190	333.8 ± 112.2	374.0 ± 118.9	217.2 ± 106.9	27.7 ± 19.5	1046.7

^a Ethanol.^b Acetic acid.^c Butyric acid.^d Propionic acid.^e Total soluble fermentation products.

increased markedly as the substrate concentration was increased. It was observed that the best hydrogen yield and specific hydrogen production occurred at 2 g L⁻¹ and 24 g L⁻¹ of substrate, respectively [37]. Accordingly, varying the C/N ratio by altering either the carbon or nitrogen concentration resulted in different optimum C/N ratios for enhancing the hydrogen production.

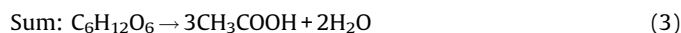
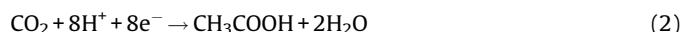
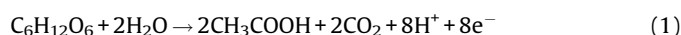
Moreover, because the nutritional needs of an organism depend on the cell composition, the optimum C/N ratio also depended on the culture involved in the process [10]. Apparently, mixed cultures dominated by *Clostridium pasteurianum* and from wastewater treatment plants required high nitrogen concentrations to enhance hydrogen production [35,37,43]. In contrast, mixed cultures from natural inoculation of sugar, as were used in this research, required low nitrogen concentrations to improve hydrogen production Peixoto et al. [44]. Some dominant cultures included microorganisms related to the families Ruminococcaceae, Veillonellaceae and Clostridiaceae, which are described below through molecular biology analyses.

3.2. Soluble fermentation products

The primary metabolic pathways for hydrogen production from sucrose are fermentation patterns of the acetate, butyrate, and ethanol types [26,60]. Table 2 presents the average concentrations of acids and solvents produced during the fermentation process. Ethanol, acetic acid, and butyric acid were the main intermediate products, and the concentrations were similar at all C/N ratios. Thus, the C/N ratio did not have a clear influence on the metabolic pathways.

Acetic acid accounted for approximately 30% of the total soluble fermentation products, and butyric acid accounted for approximately 20%. The H-Ac/H-Bu ratio was greater than 1.0; according to several authors, such a ratio is favorable for hydrogen production [23,26,29]. However, this ratio must be evaluated with caution because acetic acid can be produced via heterotrophic or

autotrophic metabolism, and this statement refers only to the acetate produced by hydrogen-producing microorganisms. During the growth of heterotrophic microorganisms on hexoses, two-thirds of the acetate is formed directly from the sugars according to Reaction (1), whereas one-third is synthesized via the acetyl-CoA (Wood-Ljungdahl) pathway according to Reaction (2) [15,39]:



Therefore, in this study, some of the acetic acid could have been produced by homoacetogens, which used the biogas as a substrate (Reaction (3)). As reported by Oh et al. [42], the same microorganisms can shift from hydrogen production to acetic acid production when biogas—H₂ and CO₂—is available. Then, since the beginning of the continuous operation, there was most likely a loss of hydrogen due to the acetic acid production by the homoacetogenic pathway.

Ethanol-type fermentation could be favorable for hydrogen production [48]. Following Reaction (4), 2 moles of hydrogen can theoretically be produced from sugars [60]. Here, ethanol accounted for approximately 35% of the total soluble fermentation products at C/N ratios of 90 and 190 and for 45% at C/N ratios of 40 and 140, suggesting that a portion of the hydrogen could have been produced by this metabolic pathway.



Regarding propionic acid, which is associated with low hydrogen production, Table 2 showed average concentrations less than 50 mg L⁻¹ at all C/N ratios. In other words, propionic acid accounted for less than 4% of the total metabolites, and it exhibited

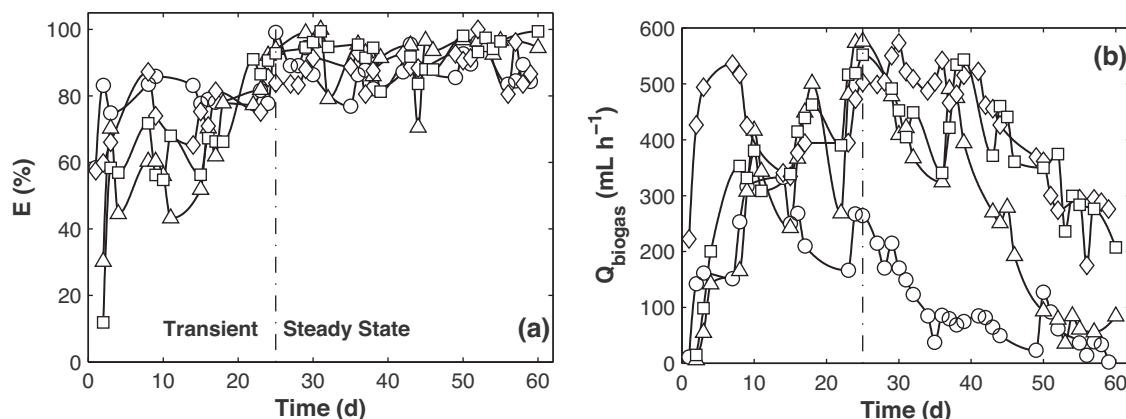
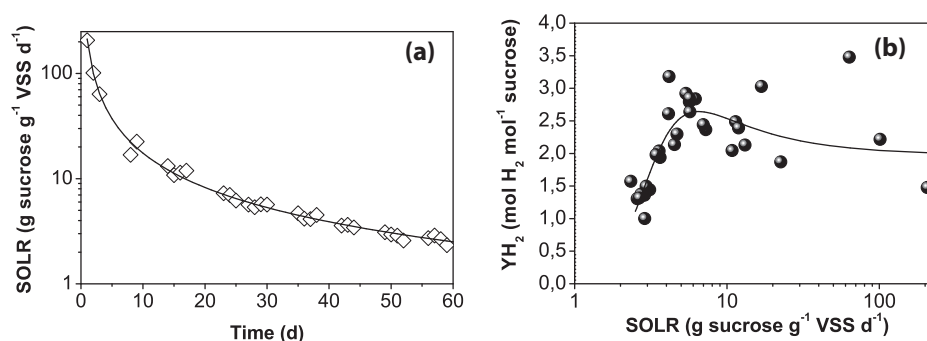


Fig. 3. (a) Sucrose conversion efficiency – E; (b) biogas production – Q_g. C/N ratio of 40 (○); C/N ratio of 90 (△); C/N ratio of 140 (◇); and C/N ratio of 190 (□).

Table 3

Biomass and consumed substrate amount at the end of the continuous operation.

C/N ratio	40	90	140	190
Attached biomass – x_a (g)	23.7	22.5	12.9	13.6
Suspended biomass – x_s (g)	4.4	6.6	6.4	7.2
Dragged biomass – x_d (g)	78.5	43.1	44.3	49.4
Total produced biomass – x_T (g)	106.7	72.3	63.6	70.2
Total consumed sucrose – s_c (g)	2555.8	2598.8	2294.4	2527.3
Cell growth per consumed substrate factor – $Y_{x/s}$	0.04	0.03	0.03	0.03

**Fig. 4.** SOLR behavior at a C/N ratio of 140 (a) SOLR as a function of time, (b) hydrogen yield relative to SOLR; ($\diamond$) SOLR ($\bullet$) hydrogen yield (–) tendency.

no significant alterations during the experimental uptime. These results indicated that the C/N ratio did not effect propionic acid production.

3.3. Instability of biogas production

The C/N ratio was determined to be an important parameter for hydrogen production; hence an adequate amount of nitrogen, related to the carbon increases the hydrogen yield (C/N of 137). However, although the fermentative process in a liquid medium seems to be steady from approximately the 25th day (Fig. 3a), the volumetric biogas production showed a notorious instability as a function of time, directly affecting the hydrogen yield. At all C/N ratios, the biogas increased continuously until it reached its maximum peak, followed by a constant decline, until production ceased completely at a C/N ratio of 40 (Fig. 3b).

Soluble fermentation products did not show any alteration throughout the period of continuous operation. Namely, the formation of more reduced compounds or metabolites not related to hydrogen production was not observed. Furthermore, the chromatography gas analyses detected the absence of methane during the experimental uptime. Thus, such behavior is thought to be related to the growth and accumulation of biomass in the fixed-bed reactor as a function of time. The continuous decrease of the SOLR from an optimum value could have generated shifts in the microbial structure.

SOLR was calculated from the estimation of the total biomass and consumed substrate after 60 days of continuous operation, as presented in Table 3. It is possible to note that, at all C/N ratios, the percentage of biomass naturally washed out x_d from the reactor was approximately 70%, and the cell growth per consumed substrate factor $Y_{x/s}$ was approximately 0.03.

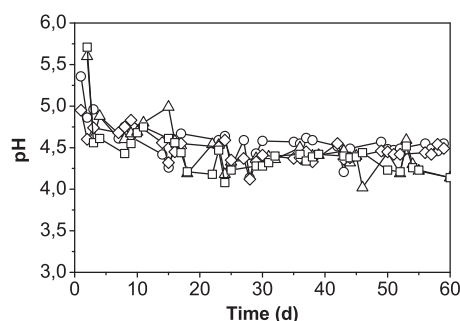
Fig. 4a shows the continuous decrease of the SOLR as a function of time, and Fig. 4b shows the hydrogen yield related to the SOLR at a C/N ratio of 140. It should be noted that higher hydrogen yields tended to occur at an adequate SOLR, in this case, approximately $6.0 \text{ g sucrose g}^{-1} \text{ VSS d}^{-1}$; this value is within the range suggested in the literature ($4.4\text{--}6.4 \text{ g DQO g}^{-1} \text{ VSS d}^{-1}$) as the most appropriate for hydrogen production [21]. The high values of SOLR during the first 25 days of continuous operation coincident with the best

hydrogen yields indicate that there was high hydrogen activity, although the sucrose conversion was low [32].

It has been stated that the SOLR is another factor controlling the fermentative process [58]. High or low values of the SOLR ratio are adverse for hydrogen production [21]. Whereas higher values could cause inhibition owing to substrate overload, lower values increase the substrate competition, causing shifts in the metabolic pathways. Lin and Lay [35] observed interference in the electron flow at high carbon concentrations related to the amount of biomass, which reduced the hydrogen production due to the formation of more reduced products such as alcohols.

In this study, changes in the metabolic pathways seemed to be associated with the establishment of homoacetogenic bacteria when the SOLR was less than the optimum value. Biogas consumption, namely, that of hydrogen concomitantly with carbon dioxide, led to this inference. In addition, the low pH observed in Fig. 5 and mesophilic conditions could have caused the competitiveness of the homoacetogens to increase [7,15]. Furthermore, according to Drake et al. [15], these organisms can become even more competitive for H_2 when positioned close to H_2 -producing bacteria.

Homoacetogens can grow autotrophically on a gas mixture of H_2 and CO_2 by employing the acetyl-CoA pathway. Acetyl-CoA can be used for anabolic syntheses of biomass, or it can be converted to acetate [14,15,39]. Here, acetyl-CoA could have been assimilated

**Fig. 5.** pH of the effluent at a C/N ratio of 40 ($\circ$), C/N ratio of 90 (Δ), C/N ratio of 140 ($\diamond$), and C/N ratio of 190 ($\square$).

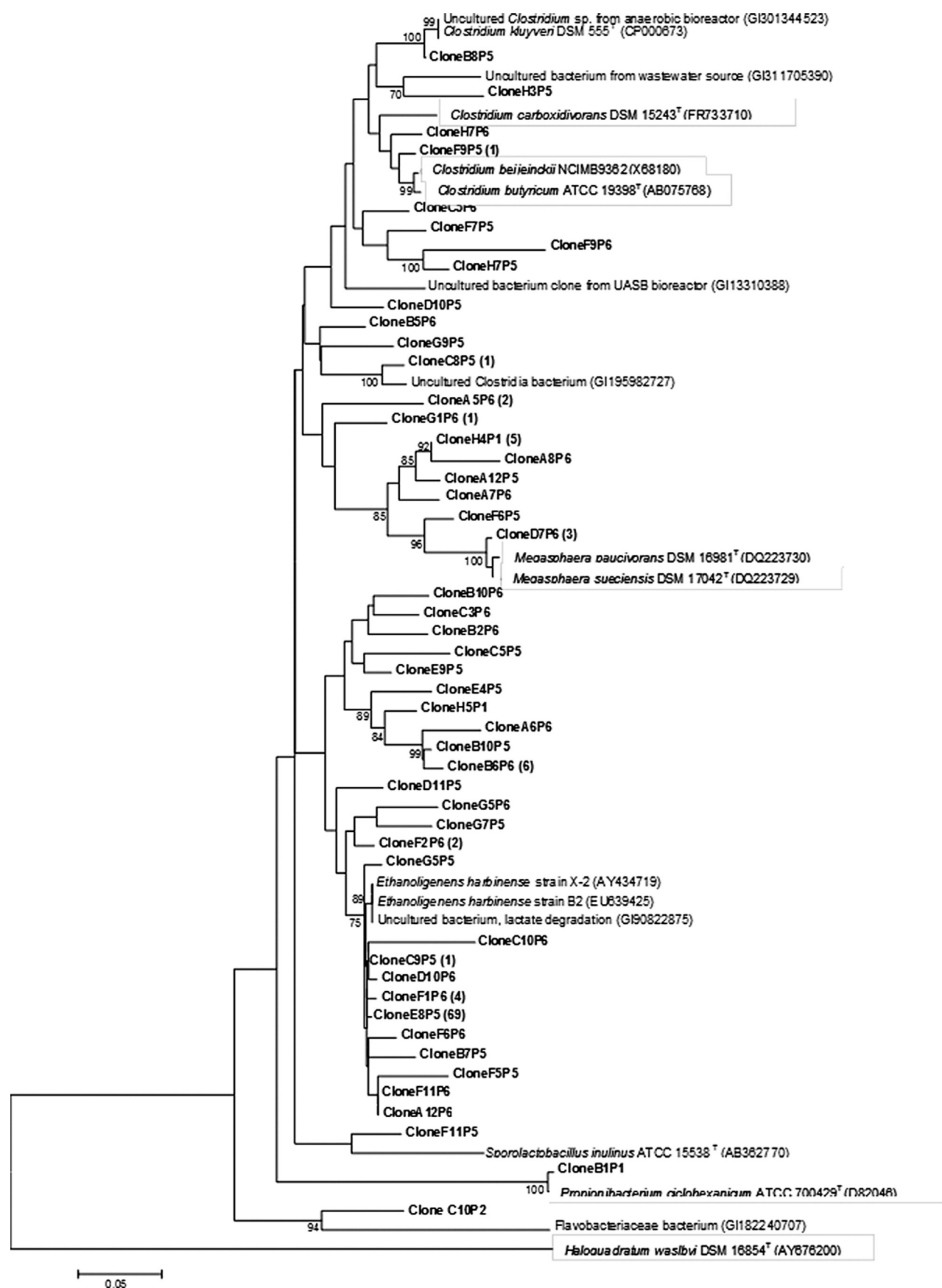


Fig. 6. Phylogenetic analysis based on the partial 16S rRNA sequences of the clones derived from sludge with a C/N ratio of 90 and the related species. Bootstrap values (1000 replicate runs, shown as percentages) greater than 70% are listed. GenBank accession numbers are listed after the species names. *Haloquadratum walsbyi* was used as an outgroup.

into biomass once the acetic acid concentration did not show an increase as SOLR was decreased. Molecular biology analyses revealed similar microorganisms with homoacetogenic features; however, it was not possible to determine the exact moment at which these bacteria began to proliferate or at which the homoacetogenic activity began [42].

3.4. Molecular biology

A total of 153 clones containing inserted 16S rRNA genes were successfully sequenced. The sequences were compared with sequences from reference and type strains and from environmental clones available in the GenBank and RDP II databases, such as those presented in Fig. 6. Ten sequences were not included in the phylogenetic analysis due to their short sequence lengths, but BLASTn analysis revealed five of the sequences matched family Veillonellaceae and five matched family Clostridiaceae.

The overwhelming majority of clones (98.7%) were related to the phylum Firmicutes. Of these, 107 clones (70% of all clones) were related to the family Ruminococcaceae, with *Ethanoligenens harbinense* being the closest species. A few clones showed 99% sequence similarity with *Ethanoligenens harbinense*. This bacterium is Gram-positive and a strict anaerobe. It produces ethanol, acetate, H₂, and CO₂ as the final products of glucose fermentation. Here, its growth may have been favored by the low pH of the system (Fig. 5) [59]. The other clones related to the family Ruminococcaceae had 16S rRNA sequences similar to uncultured bacterial clones.

24 clones (15.7%) were related to the family Veillonellaceae. 4 clones were closely related (99% sequence similarity) to the species *Megasphaera paucivorans* and *Megasphaera sueciensis*. These species grow at temperatures between 15°C and 37°C and at pH between 4.1 and 4.5. Both *M. paucivorans* and *M. sueciensis* are strict anaerobes. They generate butyric and isovaleric acids from fructose, glucose, and sucrose and beer production wastewater. Other intermediate products, such as acetic, isobutyric, valeric, and caproic acids and H₂S, can be produced in low quantities [24].

The other main group, comprising 15 clones (10%), was related to the family Clostridiaceae. One clone grouped with the type strain of *Clostridium kluyveri* and presented a high bootstrap value (100%). 3 clones clustered with *Clostridium carboxidivorans*, *Clostridium beijerinckii*, and *Clostridium butyricum*, but species-level identification was not possible. 5 clones formed clearly distinct clusters and most likely represent a new genus in the family Clostridiaceae.

Both *C. butyricum* and *C. beijerinckii* are saccharolytic microorganisms; they generate mainly butyrate, acetate, CO₂, and H₂ as fermentation products [39]. *C. kluyveri* is not saccharolytic, and its main fermentative products are butyrate, caproate, and H₂ from ethanol and acetate as substrates [39,49].

C. carboxidivorans is an acetogenic and strictly anaerobic species that is able to grow autotrophically using H₂/CO₂ or CO and chemiorganotrophically using several sugars, including xylose, fructose, glucose, and sucrose. It grows at temperatures between 24°C and 42°C and at pH levels between 4.4 and 7.6. Its final products of metabolism via the autotrophic pathway are acetic acid, ethanol, and butanol [36]. *C. carboxidivorans* could have been responsible for the biogas decrease once the pH and temperature conditions for its growth were optimum. Furthermore, the H₂ and CO₂ were available to be consumed easily.

4 clones were recovered in a distinct cluster that was not related to any other family of the phylum Firmicutes. 2 of these clones grouped with high bootstrap values (100%) with an uncultured *Clostridium* bacterium.

One clone was related to the family Sporolactobacillaceae, with high sequence similarity to *Sporolactobacillus inulinus*. This microorganism is mesophilic and microaerophilic. It can produce lactic acid without gas release from several sugars, such as glucose, fructose, and sucrose [28].

Finally, one clone showed 99% sequence similarity with *Propionibacterium cyclohexanicum* (phylum Actinobacteria, family Propionibacteriaceae) and was grouped in a tight cluster with the type strain of this species in the phylogenetic tree. *P. cyclohexanicum* is aerotolerant, and it is able to produce mainly lactic and propionic acids from the glucose fermentation. It grows at pH values between 3.2 and 7.5 (optimum value between 5.5 and 6.5) and at temperatures between 20°C and 40°C [30]. Likely, the growth of microorganisms related to the Sporolactobacillaceae and Propionibacteriaceae families in this study can be attributed to the micro-aeration from the feeding system (silicone tube) and the initial pH of approximately 6.5.

4. Conclusions

The C/N ratio was shown to have an influence on hydrogen production in an upflow fixed-bed anaerobic reactor. Between C/N ratios of 40 and 190, an optimal C/N ratio of 137 was calculated by mathematical estimation, producing 3.5 mol H₂ mol⁻¹ sucrose. High nitrogen concentrations (C/N < 137) led to excessive cell growth, whereas low nitrogen levels (C/N > 137) suggested enzymatic activity inhibition. The C/N ratio did not influence the fermentation patterns; ethanol, butyric acid, and acetic acid were the main products observed.

Although the results incontestably indicated the strong influence of the C/N ratio on hydrogen yield, the generation of biogas was not stable as a function of time for all of the experiments. Biomass accumulation in the fixed bed, leading to continuous decrease of the SOLR, was shown to be the main cause of this behavior. The variation of SOLR could have promoted changes in the microbial structure and/or biological activity, favoring the proliferation of adverse microorganisms for hydrogen production, such as homoacetogens, which were observed in the microbial analyses. The analysis suggests an SOLR of 6.0 g sucrose g⁻¹ VSS d⁻¹ is needed to maintain the stability of the system.

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