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Mixotrophic co-cultures fermentation of C1-gases and carrot discard hydrolysate by *C. carboxidivorans* and *C. beijerinckii* in stirred tank bioreactor and gas-lift bioreactor

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ABSTRACT

The current study represents the bioconversion of C1 gases (CO and CO_2) using co-cultures of *Clostridium carboxidivorans* and *Clostridium beijerinckii*, with hydrolysates from carrot discard as a co-substrate and supplemental Fe^0. The performance of two distinct bioreactor configurations, a stirred-tank bioreactor (STB) and a gas-lift bioreactor (GLB) was compared under various gas flow rates. The GLB technology, operating at a gas flow of 50 mL/min, proved more efficient, yielding up to 12.0 g/L of butanol, 2.7 g/L of acetic acid, and 7.4 g/L of butyric acid within a 47 h fermentation period. This process achieved complete consumption of sugars and CO, alongside a maximum CO_2 uptake of 82.4 % at t = 33 h. Consequently, the gas-lift bioreactor represents a promising strategy for the co-fermentation of C1 gases and carrot discard hydrolysate by *C. carboxidivorans* and *C. beijerinckii*, offering low energy requirements, cost-effectiveness, and simplicity in design and operation.

1. Introduction

In a circular economy, the valorization of fruit and vegetable residues (such as carrot discard) to generate diverse products (for instance, high value-added products, fine chemicals, platform chemicals, and/or biofuels) is of paramount importance [1]. For example, 25–30 % of global carrot production (36 Mt, worldwide [2]; and 0.4 Mt, in Spain [3] in 2020) is discarded due to physical flaws, rendering it unsuitable for sale [2]. The significant composition of carrot discard in free sugars and structural carbohydrates is particularly relevant for bioconversion [4]. Therefore, the biological valorization of this vegetable residue into biofuels and other by-products warrants considerable attention.

On the other hand, C1-gases (such as CO and CO_2), recognized as major contributors to climate change, are primarily generated through anthropogenic activities, including fossil fuel combustion and deforestation [5]. In 2023, the anthropogenic CO_2 emissions into the atmosphere were over 36 billion tons [6]. For example, a residual gas from the industrial combustion process could be composed of CO (20–35 %), CO_2 (20–30 %), and N_2 (50–60 %) [7]. Given these figures, C1-gas capture strategies, such as low cost and environmentally friendly biological processes [8], could be crucial for reducing atmospheric C1-gas levels [9]. In addition, these biological processes could be framed within a biorefinery concept, facilitating the generation of various products including butanol, ethanol, acetic, lactic, formic, and butyric acids, and 2,3-butanediol [10].

A key microbial strain used in these biological processes to capture C1-gases is *Clostridium carboxidivorans* [11,12], which is able to produce anaerobically organic acids (mainly acetic and butyric acids) in a first step of the process, followed by their conversion into alcohols (ethanol and butanol) [13], primarily via the Wood-Ljungdahl pathway (WLP) [14]. Moreover, it is worth mentioning that, in mixotrophic fermentation, the bacteria can grow larger and faster because there is a heterotrophic carbon source (for example, fructose) in the fermentation broth, employing a mixture of heterotrophic and autotrophic substrates that

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bring together the WLP and glycolysis pathways [15]. The strategy of mixotrophic fermentation, simultaneously using fructose and C1-gases as substrate is essential, as it benefits the growth and metabolic activity of *C. carboxidivorans*, improves the generation of intermediates (such as pyruvate and acetyl-CoA), and overcomes problems concerning the low solubility of gas in liquids, thus enhancing the overall efficiency of the fermentation [16]. It is worth highlighting that an external energy source (such as H_2 gas) is essential to the capture of CO_2 by *C. carboxidivorans*, the CO_2 being reduced to formate (in the first step of the WLP methyl branch) or to CO (in the WLP carbonyl branch) [17]. Nevertheless, the use of other alternatives that are less dangerous, more environmentally friendly and more energy efficient, as well as having a lower cost, such as Zero Valent Iron (ZVI (Fe^0). This could be very interesting, since under anaerobic conditions, Fe^0 can act as a reductant, facilitating both the generation of H_2 and direct electron donation for CO_2 bioconversion [18]. Another interesting microorganism within the *Clostridium* spp. Strains is *C. beijerinckii*, which, although it is not able to capture C1-gases, can employ sugars to produce organic acids in a first stage and then turn them into acetone-butanol-ethanol (ABE) in a second stage [19], these being their main fermentation products. In this way, *C. carboxidivorans* and *C. beijerinckii* could be used in co-cultures as a very interesting strategy, the C1-gases being captured by *C. carboxidivorans* and organic acids are then turned into alcohols (ethanol and butanol) by *C. beijerinckii*.

Despite the advancements in utilizing C1-gases and valorizing agro-industrial wastes, the effective integration of these diverse carbon streams into a single, high-performing bioprocess remains a significant scientific and engineering challenge. While C1-gas fermentation by acetogens shows promise, achieving consistently high titers and productivities is often hindered by limitations in electron donors, nutrient availability, or suboptimal mass transfer [16]. Concurrently, the bioconversion of complex agro-hydrolysates often necessitates tailored microbial strategies. Current approaches predominantly focus on monoculture fermentations or employ bioreactor technologies that may not be optimally suited for the dual demands of efficient gas-liquid mass transfer and complex liquid substrate handling, particularly when considering cost-effectiveness and industrial scalability [20–22]. Consequently, studies exploring the synergistic potential of co-cultures to simultaneously utilize C1-gases and complex agro-hydrolysates within an efficient and robust bioreactor configuration are notably scarce in the literature.

Mechanical agitation is a standard configuration employed in biological bioreactors (for instance, in stirred-tank bioreactors (STB)), that introduce efficient mixing and easy to control [23]. However, due to its high cost, low energy efficiency and hard operation, other alternatives, such as gas-lift column bioreactors (GLB), could be a promising option. GLB is considered a very interesting alternative, since it has lower energy requirements, cost effective, as well as an easier design and operation [20–22]. In this way, for instance, Riegler et al. [22] reported an 88 % drop in operational costs with GLB compared to STB. In GLB, agitation is carried out through gas circulation, the bioreactor being pneumatically agitated [24].

The main aim of the current study is to analyze the use of a gas-lift bioreactor in co-cultures by *C. carboxidivorans* and *C. beijerinckii*, using co-substrates (C1-gases and carrot discard enzymatic hydrolysate) in the presence of Fe^0. In addition, two different fermentation technologies, stirred-tank and gas-lift bioreactors, were comparatively evaluated. The influence of different gas flows rates was also studied for both fermentation strategies. The bioconversion of these co-substrates was assessed in terms of fermentation efficiency for the production of alcohols (butanol and ethanol) and organic acids (acetic and butyric acids), among other by-products. To achieve these objectives, a series of fermentation experiments were conducted in stirred-tank and gas-lift bioreactors under controlled conditions, monitoring substrate consumption and product formation using high-performance liquid chromatography and gas chromatography. This study represents the first investigation to evaluate gas-lift bioreactor technology -an innovative fermentation strategy characterized by low energy requirements, cost-effectiveness, and ease of design and operation-for the bioconversion of a complex co-substrate system (C1-gases and enzymatic hydrolysate derived from carrot discard) by a co-culture of *C. carboxidivorans* and *C. beijerinckii*, particularly in the presence of Fe^0. Furthermore, the application of gas-lift column bioreactors for butanol production, even by monocultures, has rarely been reported [20–22,25].

2. Materials and methods

2.1. Raw material

Carrot discard (moisture content of about 80 %), from the vegetable industry "Horcaol Cooperative Society" (Olmedo, Valladolid, Spain), was milled to a particle size of 1–3 mm with a household grinder for use in the enzymatic hydrolysis process. The composition was (% w/w dry matter) [4]: galacturonic acid, 11.2 ± 0.2; cellulose, 11.2 ± 0.1; hemicellulose, 5.5 ± 0.3 (galactose + fructose, 4.2 ± 0.2; and arabinose, 2.0 ± 0.2); acid-insoluble lignin (AIL), 0.3 ± 0.0; acid-soluble lignin (ASL), 1.6 ± 0.0; extractives, 58.8 ± 0.4 (water extractives, 42.6 ± 0.3); (galacturonic acid in water extractives, 1.2 ± 0.0; glucose in water extractives, 15.3 ± 1.9; galactose + fructose in water extractives, 12.6 ± 1.3; arabinose in water extractives, 0.7 ± 0.3); ethanol extractives, 16.1 ± 0.4); ash, 7.5 ± 0.4; and acetyl groups, 0.6 ± 0.0.

2.2. Enzymatic hydrolysate of carrot discard

Enzymatic hydrolysis of the carrot discard was carried out (according to López-Linares et al. [4]) in order to obtain an enzymatic hydrolysate, for later use as the fermentation medium in mixotrophic co-culture fermentation tests with C1-gases.

The enzymatic hydrolysis was performed in 1000 mL Erlenmeyer flasks, at 10 % w/v substrate loading (25 g substrate and 250 mL enzymatic solution), 50 °C, 150 rpm, and pH 4.8 (adjusted with 10 M KOH solution) for 24 h, using an orbital shaker (Optic Ivymen Systems, Comecta, Barcelona, Spain) and water as solvent. 10 FPU/g substrate of Cellic CTec2 and Viscozyme L enzymes mixture (enzymatic activity of 90 and 54.5 filter paper units (FPU)/mL, respectively), kindly donated by Novozymes A/S (Bagsvaerd, Denmark), was used. The conditions of enzymatic hydrolysis were selected on the basis of previous results. Once the enzymatic hydrolysis had been completed, the final enzymatic hydrolysate was vacuum filtrated, analyzed for its sugar content, and then used as the fermentation medium in mixotrophic co-culture fermentation tests with C1-gases.

2.3. Microorganism and culture media

C. carboxidivorans DSM 15243 and *C. beijerinckii* DSM 6422, from the German collection of microorganisms (DSMZ, Leibniz, Germany), were the strains utilized in the current study. DSMZ liquid medium was used to reactivate lyophilized cells, in an orbital shaker (Optic Ivymen Systems, Comecta, Spain) at 35 °C and 150 rpm for 48 h. Once the cells had grown, they were stored as glycerol stock (40 % v/v) at – 80 °C until further use.

To prepare the inocula, 50 mL of appropriate growth medium (modified DSMZ medium for *C. carboxidivorans* and Reinforced Clostridial Medium (RCM) for *C. beijerinckii*) was anaerobically dispensed into sterile 100 mL serum bottles (previously sterilized at 121 °C for 15 min), which were then sealed with sterile butyl rubber septa and crimped aluminum caps. To establish and maintain strict anaerobic conditions, the headspace of bottles designated for *C. carboxidivorans* was flushed with a sterile C1-gas mixture ($CO:CO_2:N_2$, 20:20:60), while bottles for *C. beijerinckii* were flushed with sterile pure N_2. Subsequent incubations were performed in a rotary shaker at 35 °C and 150 rpm for 48 h (*C. carboxidivorans*, OD_{600} = 2.7–2.8) or 24 h (*C. beijerinckii*, OD_{600}

= 4.5–4.6).

The composition of the modified DSMZ medium for *C. carboxidivorans* was (per liter distilled water): 10 g yeast extract, 5 g Trypticase peptone (BD BBL), 5 g meat peptone (pepsin-digested), 0.5 mL resazurin (from 0.5 g/L stock solution), 40 mL salt solution, and 50 mL secondary solution. The salt solution was composed of: 0.25 g/L $CaCl_2.2H_2O$, 0.5 g/L $MgSO_4.7H_2O$, 1 g/L K_2HPO_4, 1 g/L KH_2PO_4, 2 g/L NaCl, and 10 g/L $NaHCO_3$. However, the secondary solution was made up of: 150 g/L glucose, 20 g/L Na_2CO_3, and 10 g/L cysteine $HCl.H_2O$.

Both modified DSMZ (except salt and secondary solutions) and RCM media were sterilized at 121 °C for 15 min in septum bottles, while salt and secondary solutions were prepared separately and sterilized by filtration using 0.2 μm cellulose nitrate filters (Sartorius 254 stedim Biotech, Göttingen, Germany).

2.4. Mixotrophic *C. carboxidivorans* and *C. beijerinckii* co-cultures in stirred tank bioreactor (STB)

The fermentation of mixotrophic co-cultures by *C. carboxidivorans* and *C. beijerinckii* was carried out in a 2 L stirred tank bioreactor (STB) (Biostat B Plus bioreactor (Sartorius®)), equipped with a 2 L borosilicate glass vessel, at 35 °C and 50 rpm, containing 1 L of modified DSMZ medium, as described for *C. carboxidivorans* in Section 2.3. (at pH 7 and previously sterilized as described in Section 2.3). Previously, the bioreactor had been sterilized at 121 °C for 15 min. Agitation in STB was provided by dual Rushton turbine impellers (5 cm diameter each) at 50 rpm, with four equally spaced baffles. Sterile gases were sparged through a ring sparger located beneath the lower impeller, with flow rates controlled by a rotameter (10 and 50 mL/min). 10 % (v/v) of inoculum loading was used for both *C. carboxidivorans* and *C. beijerinckii* microorganisms (ratio 1:1), *C. carboxidivorans* and *C. beijerinckii* being inoculated at t = 0 and t = 24 h of fermentation, respectively. 12.5 g/L Fe^0 (Iron reduced, powder (fine); 99 % purity; Sigma-Aldrich (Burlington, Massachusetts, United States)) (at t = 0) and 30 g/L fructose (at t = 0 or t = 24 h) were also added in the process. The conditions of fermentation (for instance, pH of medium (pH 7), *C. carboxidivorans*: *C. beijerinckii* ratio (1:1) and Fe^0 concentration (12.5 g/L)) were selected on the basis of previous results [26]. pH was not controlled during the fermentation, but it was tracked continuously throughout process.

To establish and maintain strict anaerobic conditions, initially (t = 0), the headspace of bioreactor was flushed with a sterile C1-gas mixture ($CO:CO_2:N_2$, 20:20:60). In addition, continuous gas feeding operation mode was utilized, continuously introducing a mixture of $CO:CO_2:N_2$ (20:20:60) in the bioreactor headspace and different gas flows (10, 25, 35 and 50 mL/min).

On the other hand, the fermentation of mixotrophic co-cultures by *C. carboxidivorans* and *C. beijerinckii* was also carried out in a 2 L stirred tank bioreactor, but using the carrot discard enzymatic hydrolysate (instead of 30 g/L synthetic fructose solution) and continuous gas feeding (of $CO:CO_2:N_2$ (20:20:60) mixture) as co-substrates (both added at t = 0). Previously, the carrot discard enzymatic hydrolysate was supplemented with modified DSMZ medium, as described for *C. carboxidivorans* in Section 2.3. (except fructose), with its pH adjusted to 7 (with 10 M NaOH solution) and sterilized by filtration using 0.2 μm cellulose nitrate filters (Sartorius 254 stedim Biotech, Göttingen, Germany). The co-culture fermentation test was carried out under sterile and anaerobic conditions as described previously, using 10 mL/min of the gas mixture.

Liquid samples were withdrawn at different times of fermentation, centrifuged (at 13,500 rpm for 10 min) and their composition was analyzed in terms of sugars and fermentation products (ethanol, butanol, and acetic and butyric acids). Gaseous samples (1 mL) were also collected, and analyzed for their content of CO, CO_2, and N_2 concentration. It is worth mentioning that samples were taken taking care of maintaining anaerobic (through continuous gas feeding operation mode) and sterile (using flame near of sampling port) conditions of the bioreactor.

2.5. Mixotrophic *C. carboxidivorans* and *C. beijerinckii* co-cultures in gas-lift bioreactor (GLB)

The fermentation of mixotrophic co-cultures by *C. carboxidivorans* and *C. beijerinckii* was also carried out in a 7.5 L gas-lift bioreactor (GLB) (custom-made), with internal loop, under the same conditions as in the process previously described for co-cultures in the stirred tank bioreactor (Section 2.4.), but using 5.5 L of fermentation medium (DSMZ medium, previously sterilized, as described for *C. carboxidivorans* in Section 2.3.) and 50, 75 and 200 mL/min as gas flows of the $CO:CO_2:N_2$ (20:20:60) mixture. This difference in working volumes used in GLB (5.5 L), compared to STB (1 L) (Section 2.4), was necessitated by the inherent design limitations of the available bioreactor systems. Similarly, the distinct gas flow rates used in GLB (50, 75 and 200 mL/min vs 10, 25, 35 and 50 mL/min for STB) were selected to ensure adequate mixing and to achieve comparable gas-liquid mass transfer characteristics relevant to each bioreactor type. GLB consisted of a concentric draft tube design. The vessel had a jacketed glass column (9.5 (i.d.) x 115 cm) with a draft tube (riser) (6 (i.d.) x 95 cm) inside, resulting in a riser-to-downcomer area ratio of 36:90.25 (0.4). Gas was introduced at the base of the riser through a perforated plate sparger, with flow rates regulated by a rotameter (50 and 200 mL/min). Previously, the bioreactor had been sterilized at 121 °C for 15 min. To establish and maintain strict anaerobic conditions, initially (t = 0), the headspace of bioreactor was flushed with a sterile C1-gas mixture ($CO:CO_2:N_2$, 20:20:60). Moreover, in this case, *C. beijerinckii* was added after 10 h of fermentation by *C. carboxidivorans*. As it was described in Section 2.4., 10 % (v/v) of inoculum loading was used for both *C. carboxidivorans* and *C. beijerinckii* microorganisms (ratio 1:1). pH was not controlled during the fermentation, but it was tracked continuously throughout process.

On the other hand, the fermentation of mixotrophic co-cultures by *C. carboxidivorans* and *C. beijerinckii* was also carried out in a 7.5 L gas-lift bioreactor, but using the carrot discard enzymatic hydrolysate (instead of 30 g/L synthetic fructose solution) and continuous gas feeding as co-substrates (both added at t = 0). Previously, the carrot discard enzymatic hydrolysate was supplemented with modified DSMZ medium, as described for *C. carboxidivorans* in Section 2.3. (except fructose), with its pH adjusted to 7 (with 10 M NaOH solution) and sterilized by filtration using 0.2 μm cellulose nitrate filters. The co-culture fermentation test was carried out under sterile and anaerobic conditions as described above, using 50 mL/min of the gas mixture.

Liquid samples were withdrawn at different times of fermentation, centrifuged (at 13,500 rpm for 10 min) and their composition was analyzed in terms of sugars and fermentation products (ethanol, butanol, and acetic and butyric acids). Gaseous samples (1 mL) were also taken and analyzed for their content of CO, CO_2, and N_2 concentration. It is worth mentioning that samples were taken taking care of maintaining anaerobic (through continuous gas feeding operation mode) and sterile (using flame near of sampling port) conditions of the bioreactor.

2.6. Analytical methods

High-Performance Liquid Chromatography (HPLC) was the method conducted to determine the content of sugars (glucose, fructose and arabinose) and fermentation products (such as ethanol, butanol, and acetic and butyric acids, among others) in the liquid phase, using a refractive index detector (Waters 2414), an Aminex HPX-87H column (300 × 7.8 mm) (at 30 °C (solvents) or 60 °C (sugars, organic acids)) operating in stationary phase, and 0.01 N H_2SO_4 (0.6 mL/min) as the mobile phase. Injection volume of sample used was of 20 μL. Individual calibration curves for glucose, fructose, arabinose, butanol, ethanol, acetic acid, and butyric acid were established using five external standards, prepared from analytical grade reagents, across a concentration range of 0.25–6 g/L. All curves exhibited linearity with $R^2 > 0.999$. The

limit of detection (LOD) for all analyzed compounds was approximately 0.1 g/L, determined as three times the standard deviation of the blank.

The gas composition (CO, CO_2, and N_2) in gaseous samples was analyzed using an 8860 GC gas chromatograph (GC, Agilent Technologies, Spain) equipped with a thermal conductivity detector (TCD), using helium as the carrier gas. The GC was fitted with a 15-m HP-PLOT Molecular Sieve 5 A column (ID, 0.53 mm; film thickness, 50 μm) at 45 °C, with 250 °C as the detector temperature. Injection volume of gas used was of 100 μL, using a Hamilton syringe. Calibration curves for CO, CO_2, and N_2 were generated using a series of certified gas mixtures (Carburos Metálicos, industrial grade) across a linear range of 0–100 % (v/v). A minimum of five calibration points were utilized, and all regressions yielded R^2 values greater than 0.998. The limit of detection (LOD) for each gaseous compound was determined to be approximately 0.1 % (v/v). The gas uptake efficiency was calculated as the difference between the initial gas concentration (t = 0) in the bioreactor and the gas concentration along of fermentation process in the bioreactor.

All analytical determinations were carried out in triplicate, and the average results were recorded.

2.6.1. Determination of kinetic rates

To evaluate process efficiency, volumetric rates of gaseous and liquid substrate consumption, and metabolite production were calculated from the concentration profiles obtained over the fermentation time.

The gas uptake rate (CO, CO_2) was determined using the following equation (Eq. (1)), considering the mass balance in the bioreactor headspace:

$$r_{gas} = \frac{\left(F_{M,gas,t0} - F_{M,gas,t1}\right) x\, PM}{V_{liquid}} \quad \text{Eq. 1}$$

Where r_{gas} is the volumetric rate of gas uptake or production (g/L·h), $F_{M,gas,t0}$ and $F_{M,gas,t1}$ are the molar flow rate of CO or CO_2 (mol/min) at time t_0 and t_1, respectively, PM is the molecular weight of CO or CO_2 (g/mol), and V_{liquid} is the working volume of the culture (L). Molar flow rate of CO or CO_2 were calculated from total molar flow and the percentage of CO or CO_2 in the C1-gas. Total molar flow was determined as the ratio between the gas volumetric flow (L/min) and molar volume (L/mol), which was calculated using the ideal gas law at the operating pressure and temperature conditions of the bioreactor.

The sugar consumption rates (glucose, fructose, and galactose) and metabolite production rates (ethanol, butanol, acetic acid, and butyric acid) were calculated as the ratio between the sugar consumption or metabolite production and fermentation time, obtained during the phase of the highest butanol production, using the following expression (Eq. (2)):

$$r_{compound} = \frac{\Delta C_{compound}}{\Delta t} \quad \text{Eq. 2}$$

Where $r_{compound}$ is the volumetric consumption or production rate (g/L·h), $\Delta C_{compound}$ is the change in the compound's concentration in the liquid (sugar consumption or metabolite production) (g/L), and Δt is the time interval (h). The rates were reported for the phase of the highest butanol production.

2.7. Data analysis

The statistical differences were determined using variance analysis (ANOVA) at a confidence level of 95 % ($p < 0.05$). The Tukey multiple range test was used to find significantly different means. All statistical calculations were performed using Microsoft Excel.

3. Results and discussion

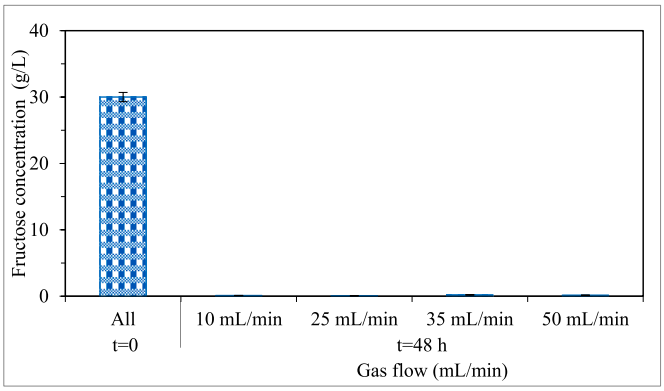
3.1. Mixotrophic C. carboxidivorans and C. beijerinckii co-cultures in stirred tank bioreactor (STB)

Bioprocesses based on co-cultures by *C. carboxidivorans* and *C. beijerinckii* could be considered as an innovative and effective fermentation process, since *C. carboxidivorans* is able to metabolize C1-gases (CO and CO_2) producing organic acids (such as acetic and butyric acids), which in turn can be turned by *C. beijerinckii* into butanol without using gases.

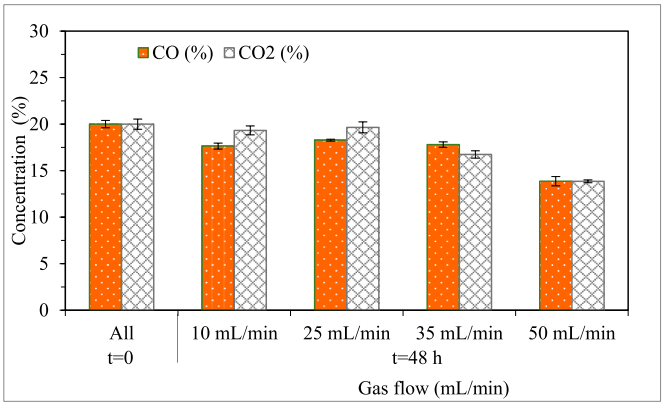
Mixotrophic (fructose (30 g/L)/C1-gases ($CO:CO_2:N_2$ (20:20:60)) co-culture fermentation by *C. carboxidivorans* and *C. beijerinckii* was carried out in a 2 L stirred tank bioreactor (STB), using different gas flow (10, 25, 35 and 50 mL/min) of $CO:CO_2:N_2$ (20:20:60) mixture in order to study the influence of the gas flow on $CO:CO_2$ uptake by *C. carboxidivorans* in this type of bioreactor, as well as the production of organic acids (acetic and butyric acids), ethanol and butanol, among other products, in the co-culture fermentation. Fig. 1 shows the results obtained at the time of maximum production of butanol (t = 48 h). Regarding the fructose consumption (Fig. 1a), the total sugar (30 g/L) was metabolized in all cases at fermentation times as short as 48 h, with a kinetic rate of −0.625 g L/h (Table S4). Most of this consumption (about 67 %) took place in the first 24 h of the process (Fig. S1a) in all cases. Then, this consumption was exclusively due to the presence of *C. carboxidivorans* (as *C. beijerinckii* was added in the fermentation after this time), which justifies the use of mixotrophic (fructose/C1-gases) co-culture in this work. Mixotrophic fermentation using C1-gases was previously analyzed in a previous work [27], using 100 mL sealed bottles (50 mL as working volume), where was explored this type of fermentation to improve the production of target compounds, focusing on *C. aceticum* and *C. carboxidivorans*. This previous work got to overcome the limitations of conventional gas fermentation (autotrophic fermentation) and assessed the potential of mixotrophic substrates for enhancing yields. So, mixotrophic fermentation with fructose and C1-gases by *C. carboxidivorans* significantly boosted microbial growth and metabolic activity, increasing butanol (1600 vs 0 mg/L) and butyric acid production, compared to autotrophic fermentation.

On the other hand, CO and CO_2 consumptions obtained for each gas flow in STB are shown in Fig. 1b at the time of maximum butanol production (t = 48 h). Considering CO consumptions and as can be seen, except for 50 mL/min of gas flow (CO consumption = 30.7 %), CO consumption was similar (8.6–11.8 %), independently of the gas flow used, at this time of fermentation. However, the specific speed of CO consumption (between $-2.697{\cdot}10^{-4}$ and $-3.514{\cdot}10^{-3}$ g/L·h, Table S4) increased with increasing gas flow. Om the other hand, observing the CO consumption kinetics for the fermentations with the four different gas flows studied (Fig. S1b), it can be seen that the highest CO consumptions were obtained at the shorter fermentation times (t = 30–32 h) in all cases studied, except for gas flow = 50 mL/min, where the highest CO consumption was obtained at 48 h of process. Higher CO consumptions are obtained when lower gas flows are used, a maximum CO consumption of up to 55.9 % (t = 30 h) being achieved for a gas flow of 10 mL/min (followed by 41.2 % (t = 32 h), 36.4 % (t = 30 h) and 30.7 % (t = 48 h) for gas flows of 25, 35 and 50 mL/min, respectively). This behavior is coherent, since when lower CO flows is introduced in bioreactor, *C. carboxidivorans* has greater ease to consume it, as lower amount of CO is available in bioreactor and then, the CO consumptions by this microorganism will be higher. In this way, the use of low gas flows (e.g., 10 mL/min) has been also successfully tested in previous works; for example, in fermentations by *C. carboxidivorans P7* DSM 15243, also using 2 L STB with continuous gas feeding (10 mL/min), but employing only CO (100 %) as the sole gaseous substrate [28].

As for CO_2 consumptions at the time of maximum butanol production (t = 48 h) (Fig. 1b), concentrations similar to the initial CO_2 level of feeding (20 %) were attained at this fermentation time when lower gas



a)



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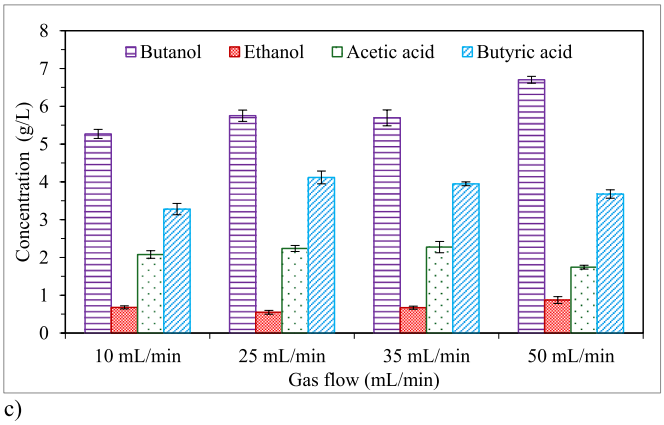


c)

Fig. 1. Mixotrophic *C. carboxidivorans* (t = 0) and *C. beijerinckii* (t = 24 h) co-cultures (Fe^0 = 12.5 g/L) in stirred tank bioreactor (STB) for different gas flows (10, 25, 35 and 50 mL/min). (a) Fructose concentration (g/L), (b) Gas concentrations (CO and CO_2) (%), (c) Product concentrations (Butanol, Ethanol, Acetic Acid, and Butyric Acid) (g/L). The results are shown at the time of maximum butanol production obtained (t = 48 h).

flows were used (10 and 25 mL/min). However, for higher gas flows (35 and 50 mL/min), as for CO, CO_2 was also metabolized by *C. carboxidivorans*, achieving a CO_2 consumption of 30.8 % for 50 mL/min of gas flow (t = 48 h). As for CO, as can be seen in Table S4, CO_2 specific speed increased when gas flow increased (from $-1.220{\cdot}10^{-4}$ to $-5.535{\cdot}10^{-3}$ g/L·h). The observed partial CO_2 consumption can be attributed to the presence of Fe^0, which functions as an electron donor for CO_2 conversion. Unlike direct H_2 supplementation, Fe^0 offers a distinct advantage by generating electrons *in situ* through its corrosion, typically via cathodic depolarization reactions that produce H_2:

$$Fe^0 + 2\,H_2O \rightarrow Fe^{2+} + H_2 + 2\,OH^- \quad \text{Eq. 3}$$

This direct or indirect electron supply positions Fe^0 as a potentially more sustainable and safer alternative to gaseous H_2. Its solid-state nature mitigates the hazards associated with handling flammable H_2, while potentially lowering bioprocessing costs and improving overall process sustainability, as extensively discussed in the literature [18,29]. On the other hand, observing the CO_2 kinetics for the fermentation with the four different gas flows studied (Fig. S1c), it can be seen that higher CO_2 concentrations than the initial CO_2 level of feeding (20 %) are obtained at short fermentation times (t = 26–32 h) in all cases studied. Consequently, a CO_2 concentration of up to 41.3 % (t = 30 h) was attained when a gas flow of 10 mL/min was used, followed by 34.6 % (t = 32 h), 30.4 % (t = 30 h) and 25.4 % (t = 26 h) for gas flows of 25, 35 and 50 mL/min, respectively. This could be due to *C. beijerinckii* producing CO_2 within its metabolic pathway [30]. Therefore, the CO_2 consumptions at short fermentation times are much higher than those mentioned before for 48 h (Fig. 1b), as the CO_2 has really been consumed from values higher than the initial 20 % (CO_2 level of feeding), the CO_2 consumptions then being about 76–114 %. This fact could explain why the use of Fe^0 has a positive influence. In addition, the fermentation efficiency in the presence and absence of Fe_0 was studied in a previous work [26], where the bioconversion of C1 gases (CO and CO_2), using mixotrophic co-culture fermentation by *C. carboxidivorans* and *C. beijerinckii*, was evaluated, analyzing the influence of the presence (12.5, 25 and 50 g/L) or not of Fe_0, among other factors. In this case, the co-culture fermentation tests were carried out in 100 mL sealed bottles equipped with a rubber septum, using 50 mL as working volume. As a result, the presence of Fe_0 was successful, resulting in complete fructose and CO consumptions, much higher butanol concentrations (increasing from 0.6 g/L –absence of Fe_0-to approximately 7–8 g/L –in presence of Fe_0) and lightly higher levels of acetic acid (ranged from 1.2 g/L -without Fe_0-to 2.1 g/L -with 50 g/L of Fe_0) and butyric acid (from 3.5 g/L (without Fe_0) to 4.4 g/L (with 50 g/L of Fe_0)). On the other hand, in this previous work, although the highest butanol concentration of 8 g/L was obtained with 50 g/L of Fe^0 in the medium, showing significant differences ($p < 0.05$) compared to the other two Fe^0 concentrations studied, the use of a lower Fe^0 concentration (for instance, 12.5 g/L) could help to enhance the process feasibility, being able to achieve up to 7.1 g/L butanol. Therefore, the chosen Fe^0 concentration to supplement the fermentation medium was 12.5 g/L in this previous work.

The gas flow rate significantly influenced (p-value <0.05) the distribution of key fermentation products (butanol, ethanol, and acetic and butyric acids) in the stirred-tank bioreactor (STB) at 48 h, the point of maximum butanol production (Fig. 1c). Butanol concentrations varied from 5.3 to 6.7 g/L across the tested conditions (kinetic rates of production from 0.110 to 0.140 g L/h (Table S4)), peaking significantly (p-value <0.05) at the highest flow rate of 50 mL/min. Notably, this production peak occurred at 48 h for all flow rates evaluated (Fig. S1d). In contrast to butanol, ethanol concentrations remained low (0.6–0.9 g/L) across all conditions at this time point (Fig. 1c) (production kinetic rates: 0.011–0.018 g L/h (Table S4)), no being observed differences significant in this case (p-value >0.05).

Analysis of the acid precursors revealed an inverse relationship between acid accumulation and butanol production. The highest residual concentrations of acetic acid (2.3 g/L) and butyric acid (4.0–4.1 g/L) were observed at intermediate flow rates (25–35 mL/min), with a production kinetic rate of 0.047 and 0.086 g L/h, respectively (Table S4), being observed significant difference (p-value <0.05). Conversely, the conditions that yielded the most butanol (50 mL/min) resulted in lower residual concentrations of acetic (1.7 g/L) and butyric (3.8 g/L) acids. This trend can be attributed to the effect of the gas sparging rate on mass transfer and mixing within the bioreactor. Increasing the gas flow likely enhances turbulence, which improves the transfer of gaseous substrates (e.g., CO, H_2) to the liquid phase for consumption by *C. carboxidivorans*

and simultaneously facilitates better contact between *C. beijerinckii* and the acid substrates. The superior butanol production at 50 mL/min suggests that the enhanced mixing promoted a more efficient conversion of acetic and butyric acids into solvents. In contrast, at lower flow rates, while acidogenesis was robust, the subsequent solventogenesis may have been limited by suboptimal mass transfer, leading to the observed accumulation of acid intermediates.

This interpretation is further supported by the continued production of acetic and butyric acids observed after 48 h in cultures with lower gas flow rates (Fig. S1e and S1f), which reached final concentrations as high as 3.2 g/L and 5.4 g/L, respectively. These observations are consistent with a hypothesis of metabolic decoupling, where acidogenesis by *C. carboxidivorans* from C1 gases appears to persist even after the solventogenic activity of *C. beijerinckii* - which typically consumes these acids for butanol synthesis [31] - had significantly diminished or ceased. While our macroscopic data provide indirect support for this concept, direct molecular evidence (e.g., transcriptomic or metabolomic profiling) would be necessary to fully substantiate the mechanistic basis of such decoupling.

In addition to these main products which have been described before, mixotrophic co-culture fermentation by *C. carboxidivorans* and *C. beijerinckii* in STB generated some by-products, such as formic acid (0.6–0.7 g/L), acetoin (0.9–1.3 g/L) and 2,3-butanediol (0.2 g/L) (Table S1). The formation of by-products low concentrations from C1-gases has been previously reported from the literature [32–34]. This is very interesting, since it promotes the fermentation of mixotrophic co-cultures in STB using this type of microorganisms and it can be considered profitable from an economic view point [35,36].

On the other hand, in order to avoid the partial consumption of fructose by *C. carboxidivorans*, STB mixotrophic co-culture fermentation by *C. carboxidivorans* and *C. beijerinckii* (at gas flow = 10 mL/min) was also carried out, adding the co-substrate (fructose) together with *C. beijerinckii* (at t = 24 h). However, in this case, no butanol was generated, while low concentrations of some organic acids (1.5 g/L acetic acid and 0.9 g/L butyric acid), ethanol (0.3 g/L) and acetoin (1.1 g/L) were produced, with a maximum CO consumption of 40 % (at 29 h of fermentation). *C. carboxidivorans* probably needs the use of both fructose and C1-gases at the start of the fermentation, as fructose could benefit from its growth and metabolic activity, thus improving the generation of intermediates (such as pyruvate and acetyl-CoA), as well as overcoming problems of low gas solubility in liquids [16].

As can be seen in Table 1, in general, the results obtained in the current study are much better than those reported in the literature. For instance, Fernández-Naveira et al. [28] achieved much lower butanol concentrations (2.7 g/L vs 5.3–6.7 g/L obtained in this work), in fermentations by only *C. carboxidivorans P7* DSM 15243, also using 2 L STB with continuous gas feeding (10 mL/min), but using only CO (100 %) as the sole gaseous substrate and pH 5.75. Concentrations of acetic acid (1.5 g/L) and butyric acid (0.2 g/L) were also lower than those obtained in the current study (1.7–2.3 and 3.3–4.2 g/L, respectively), at the time of maximum butanol production. However, in this case, higher ethanol concentrations (5.6 vs 0.6–0.9 g/L produced in our work) were obtained. Vees et al. [16] also reported lower butanol concentrations (2.6 g/L) and butyric acid (0.7 g/L) than those obtained in the current study, but higher levels of ethanol and acetic acid (5.7 and 3.1 g/L, respectively), also using a mixotrophic substrate (20 % CO, 10 g/L glucose) in 2 L STB with continuous gas-feed and only *C. carboxidivorans P7* DSM 15243. Much lower butyric acid concentrations (0.66 g/L) and no

Table 1
Comparative studies about metabolizing C1 gases and/or lignocellulosic residues by *C. carboxidivorans* and/or *C. beijerinckii*.

Microorganism	Fermentation equipment	Substrate	By-products conc. (g/L)				Reference
			Acetic acid	Butyric acid	Ethanol	Butanol	
C. carboxidivorans P7 (DSM 15243)	STB	CO (100 %): continuous feeding (10 mL/min)	1.5	0.2	5.6	2.7	[28]
C. carboxidivorans P7 (DSM 15243)	STB	20 % CO + 10 g/L glucose: continuous feeding (0.25 vvm)	3.1	0.7	5.7	2.6	[16]
C. carboxidivorans P7 (DSM 15243)	STB	$CO:CO_2:H_2:N_2$ (30:10:20:40): continuous feeding (10 mL/min)	6.2	0.7	–	–	[37]
C. beijerinckii TISTR 1461	STB	Sugarcane molasses	–	–	–	10.1	[20]
	GLB		–	–	–	10.6	
C. beijerinckii TISTR 1461	GLB	Sweet sorghum stem juice				11.0	[25]
C. beijerinckii DSM 6422	Serum bottles	Carrot discard	–	–	–	7.4	[39]
C. beijerinckii DSM 6423	Serum bottles	Sugarcane bagasse	3.5	3.5	0.5	8.0	[43]
C. beijerinckii DSM 6422	Serum bottles	Brewer's spent grain	–	–	–	8.0	[44]
C. beijerinckii DSM 6422	Serum bottles	Spent coffee grounds	–	–	–	6.7	[19]
C. beijerinckii TISTR 1461	Serum bottles	Sugarcane bagasse	–	–	–	12.0	[45]
Arthrobacter sp.: *C. beijerinckii* TISTR 1461	STB	Sweet sorghum stem juice	–	–	–	11.4	[46]
C. *beijerinckii* D9/pykA	Serum bottles	Puerariae slag	–	–	–	11.2	[47]
C. beijerinckii ATCC 10132	Serum bottles	Green coconut husk	–	–	–	3.4	[48]
C. carboxidivorans DSM 15243: *C. beijerinckii* DSM 6422	STB	$CO:CO_2:N_2$ (20:20:60) continuous feeding (50 mL/min) + 30 g/L fructose	1.7	3.7	0.9	6.7	This study
	GLB	$CO:CO_2:N_2$ (20:20:60) continuous feeding (200 mL/min) + 30 g/L fructose	1.2	4.4	0.5	6.6	
	STB	$CO:CO_2:N_2$ (20:20:60) continuous feeding (10 mL/min) + carrot discard (hydrolysate)	3.2	5.3	5.5	1.9	
	GLB	$CO:CO_2:N_2$ (20:20:60) continuous feeding (50 mL/min) + carrot discard	2.7	7.4	0.7	11.9	

STB: stirred tank biorreactor
GLB: gas-lift bioreactor.

butanol production was achieved by Naveira-Pazos et al. [37] from the fermentation of syngas by *C. carboxidivorans* P7 DSM 15243 in 2 L STB with continuous gas-feed, using CO_2 and CO as carbon sources from the syngas mixture and H_2 as energy source. However, high acetic acid levels (<6.2 g/L) were detected in this case. Therefore, as can be concluded by this comparison from the literature, the use of a co-culture (*C. carboxidivorans* and *C. beijerinckii*) and mixotrophic substrate (C1-gas and fructose), improves the production of butanol and organic acids. This could be attributed to the co-culture taking advantage of the metabolic ability of both microorganisms, as well as the mixotrophic strategy supplying two different carbon sources, thus stimulating different metabolic pathways and improving overall metabolic activity, while also preventing substrate limitations and maintaining a steady supply of carbon for both microorganisms [16,38].

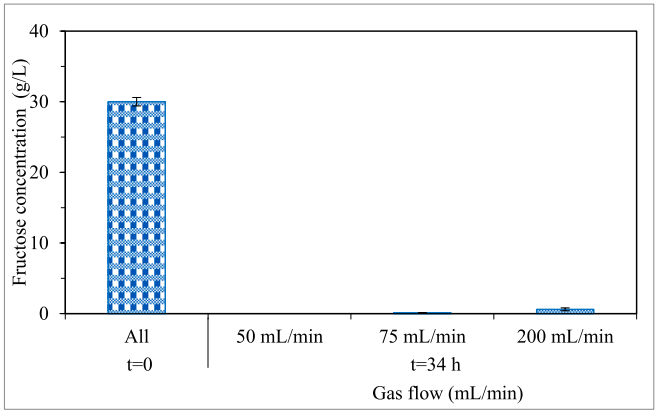
The observed fermentation performance in the co-culture system can be understood through the intricate metabolic interactions between *C. carboxidivorans* and *C. beijerinckii*. *C. carboxidivorans*, an acetogenic bacterium, is primarily responsible for the uptake and conversion of C1-gases (CO and CO_2) into acetate and other organic acids via the Wood–Ljungdahl pathway [16]. This acidogenic activity not only remediates gaseous substrates but also contributes to the initial pool of organic acids within the bioreactor. Simultaneously, *C. beijerinckii* is renowned for its biphasic metabolism, characterized by an initial acidogenic phase followed by a solventogenic phase where it converts sugars and certain organic acids into higher-value alcohols like butanol and ethanol [39]. In this co-culture, a synergistic relationship is hypothesized: the acetate produced by *C. carboxidivorans* from C1-gas uptake can potentially be assimilated by *C. beijerinckii* as a substrate for enhanced solventogenesis, especially during its solvent-producing phase. This metabolic cross-feeding mitigates product inhibition from acetate accumulation and channels carbon flow towards desired alcohol products. The efficient utilization of both C1-gases and the carrot discard enzymatic hydrolysate thus highlights a promising strategy for integrating waste streams into a robust bio-production platform, with *C. carboxidivorans* potentially serving as an in-situ supplier of key metabolic intermediates for *C. beijerinckii*.

3.2. Mixotrophic *C. carboxidivorans* and *C. beijerinckii* co-cultures in gas-lift bioreactor (GLB)

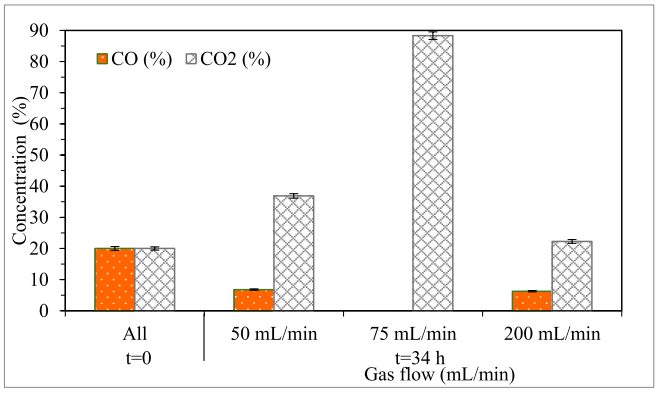
The mixotrophic (fructose (30 g/L)/C1-gases ($CO:CO_2:N_2$ (20:20:60)) co-culture fermentation by *C. carboxidivorans* and *C. beijerinckii* was also carried out in a gas-lift bioreactor (GLB), in this case using 50, 75 and 200 mL/min gas flow of $CO:CO_2:N_2$ (20:20:60) mixture. It is worth mentioning that distinct gas flow rates were used for GLB (50, 75 and 200 mL/min vs 10, 25, 35 and 50 mL/min for STB) to ensure adequate mixing and to achieve comparable gas-liquid mass transfer characteristics relevant to each bioreactor type. Operating the STB with very high gas flow rates led to significant liquid carryover and foam formation, making such conditions impractical. Conversely, lower gas flow rates in the GLB proved insufficient to generate the necessary mixing for effective mass transfer. While efforts were made to achieve similar superficial gas velocities (vvm - volumes of gas per volume of liquid per minute), the physical constraints and operational challenges specific to each bioreactor design precluded direct volumetric equivalence. Despite these volumetric differences, the comparative study remains valid because the larger working volume in the GLB (5.5 L) represents a more industrially relevant scale, allowing for a robust assessment of its potential for commercial application. Our selection of conditions aimed to optimize performance within the practical operational windows of each bioreactor, thereby providing meaningful insights into their respective capabilities for this bioprocess.

As mentioned previously by Özcan et al. [24], GLBs present a compelling alternative to STBs due to their inherent advantages, including lower energy consumption, reduced shear stress, diminished operational costs, and simpler design and operation [20]. This is primarily because agitation in GLBs is achieved pneumatically through gas circulation. These characteristics make GLBs particularly well-suited for sensitive co-cultures like *C. carboxidivorans* and *C. beijerinckii* and for processes involving C1-gases, where the gentle mixing and efficient gas-liquid mass transfer are critical. Our findings demonstrate the successful integration of these advantages within a novel co-substrate fermentation system.

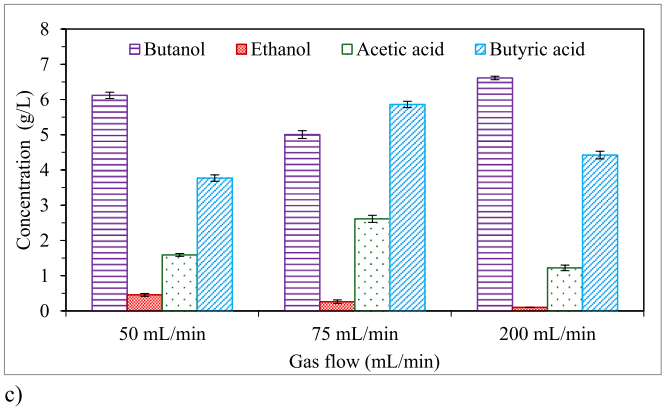
Fig. 2 shows the results obtained in GLB assays at the time of maximum butanol production (t = 34 h). Fructose was totally consumed at this fermentation time for the three gas flows studied (Fig. 2a), with a kinetic rate of −0.882 g/L·h (Table S4). The most fructose (79.2 %) was



a)



b)



c)

Fig. 2. Mixotrophic *C. carboxidivorans* (t = 0) and *C. beijerinckii* (t = 10 h) co-cultures (Fe^0 = 12.5 g/L) in gas-lift bioreactor (GLB) for different gas flows (50, 75 and 200 mL/min). (a) Fructose concentration (g/L), (b) Gas concentrations (CO and CO_2) (%), (c) Product concentrations (Butanol, Ethanol, Acetic Acid, and Butyric Acid) (g/L). The results are shown at the time of maximum butanol production obtained (t = 34 h).

metabolized after 10 h of fermentation (Fig. S2a), which was the time where *C. beijerinckii* was added to the GLB co-cultures. Then, the most fructose consumption was probably due to *C. beijerinckii*, as *C. carboxidivorans* metabolizes mainly C1-gases [26].

Regarding CO and CO_2 consumptions at the time of maximum butanol production (t = 34 h) (Fig. 2b), as can be observed, very high CO consumptions (66–100 %) were achieved for all gas flows at this fermentation time (specific speed of consumption from $-1.374{\cdot}10^{-3}$ to $-5.716{\cdot}10^{-3}$ g/L·h (Table S4)), with a total CO consumption when 75 mL/min gas flow was used. Nevertheless, observing the CO uptake kinetics, this gas was totally consumed at shorter fermentation times in some cases. For instance, for 50 mL/min gas flow, the CO had already been consumed at 23 h, starting to increase after 27 h of fermentation, and reaching a maximum value of 11.4 % at the end of process (Fig. S2b). This could be attributed to the partial loss of activity of *C. carboxidivorans* at this fermentation time [28].

Considering CO_2 at the time of maximum butanol production (t = 34 h), as can be seen in Fig. 2b, considerable levels (22.3–88.3 %) of this gas were achieved at this process time in all cases (with specific speed of production from $1.483{\cdot}10^{-3}$ to $1.678{\cdot}10^{-2}$ g/L·h (Table S4)), the highest concentration being obtained when 75 mL/min gas flow was used. As described before, for this same gas flow, the CO had been totally consumed at this fermentation time (t = 34 h). On the other hand, observing the CO_2 kinetics, considerable CO_2 consumption took place at shorter fermentation times. So, for instance, for 50 mL/min gas flow, 35.8 % of CO_2 had been consumed at t = 2.5 h (Fig. S2c), probably due to the presence of Fe^0, which performs the role of an electron donor for CO_2 conversion [18]. After short fermentation times, CO_2 continued being consumed by *C. carboxidivorans*, which has been reported previously in other works. So, for example, Fernández-Delgado et al. [26] analyzed the profiles of CO and CO_2 concentrations (%) during autotrophic fermentation (t = 7days) by *C. carboxidivorans*, at pH 6 and in presence of 50 g/L of Fe^0 (CO/CO_2: 20 %/20 %), observing that a considerable consumption of CO_2 (about 60 %) was achieved during the first days of fermentation by *C. carboxidivorans*. Anyway, although CO_2 continued being consumed by *C. carboxidivorans* after short fermentation times, the production of this gas in co-cultures (probably due to *C. beijerinckii*, which generates CO_2 within its metabolic pathway [30]) was too high, being produced at a rate higher than was being metabolized by *C. carboxidivorans*, thus reaching concentrations as high as 65.7 % (50 mL/min gas flow, t = 23 h) or 88.3 % (75 mL/min gas flow, t = 34 h) (Fig. S2c). However, CO_2 levels later decreased again after these fermentation times (Fig. S2c). So this could explain why the use of Fe^0 has a positive influence. As was described in Section 3.1., the advantageous of the presence of Fe_0 (12.5, 25 and 50 g/L) was previously studied in mixotrophic co-culture fermentation by *C. carboxidivorans* and *C. beijerinckii* [26], resulting in complete fructose and CO consumptions, much higher butanol concentrations (from 0.6 g/L – absence of Fe_0-up to 8 g/L –in presence of Fe_0) and lightly (about 1 g/L) higher levels of acetic and butyric acids.

In the gas-lift bioreactor (GLB), the product profile was also significantly dependent (p-value <0.05) on the gas flow rate, reaching peak butanol concentrations at a notably shorter time of 34 h (Fig. 2c). While high butanol concentrations (5.0–6.6 g/L) were achieved across all conditions, the maximum concentration (6.6 g/L) was obtained at the highest flow rate of 200 mL/min (kinetic rates = 0.194 g L/h (Table S4)), with a clear significant difference (p-value <0.05). Ethanol generation, however, remained minimal (0.1–0.5 g/L) and was largely independent of the gas flow rate (Fig. 2c), no being observed significant difference (p-value >0.05) in the case of this by-product.

A clear correlation emerged between gas flow, acid accumulation, and solvent production. The intermediate flow rate of 75 mL/min resulted in the lowest butanol concentration (5.0 g/L) and a significant (p-value <0.05) accumulation of precursor acids, with acetic and butyric acid concentrations reaching their peaks of 2.6 g/L and 5.9 g/L, respectively (kinetic rates of 0.077 and 0.172 g L/h, respectively (Table S4)). Conversely, the superior performance at 200 mL/min, which yielded the highest butanol concentration, corresponded to the lowest residual acetic acid (1.2 g/L). In a GLB, where liquid circulation is driven solely by gas injection, these results can be explained by mass transfer limitations. The accumulation of acids at 75 mL/min suggests that while the gas flow was sufficient to support robust acidogenesis by *C. carboxidivorans*, the resulting liquid circulation was inadequate for promoting efficient contact between *C. beijerinckii* and its acid substrates for conversion into butanol. This created a metabolic bottleneck. In contrast, the 200 mL/min flow rate generated more vigorous mixing and turbulence, enhancing the overall mass transfer of both gaseous substrates and dissolved acids, thus facilitating a more complete conversion to the final solvent product.

Furthermore, and consistent with the STB experiments, acidogenesis continued past the 34 h mark in all GLB cultures (Fig. S2e and S2f). This late-stage acid production, particularly prominent under the suboptimal mixing conditions of lower flow rates, further lends support to the hypothesis of a metabolic decoupling. Under this proposed scenario, acid production by *C. carboxidivorans* would persist even after the solventogenic phase of *C. beijerinckii* has ceased. However, it is important to note that a definitive mechanistic confirmation of this decoupling would necessitate targeted molecular analyses.

Moreover, in addition to the main products described above, some other by-products were produced in low concentrations in GLB co-cultures for all the gas flows studied, as described for the STB co-cultures (Section 3.1). In this way, maximum values of 0.6–0.8 g/L formic acid, 0.6–1.2 g/L acetoin and <0.1 g/L 2,3-butanediol were determined (Table S2).

On the other hand, STB and GLB mixotrophic co-culture fermentation by *C. carboxidivorans* and *C. beijerinckii* (at gas flow = 50 mL/min) were compared (Fig. 3 and S3). As can be seen, although fructose was consumed in both STB and GLB co-cultures, it was consumed much more quickly in the GLB co-culture, being completely metabolized in only 23 h (vs 45 h necessary in STB), with a significantly higher fructose consumption rate of 8.48 g/h (vs 0.8 g/h for STB) (Fig. 3a). With regards to CO consumption, as can be appreciated in Fig. 3b, the behavior of both STB and GLB co-cultures were also significantly different (p-value <0.05), reaching a CO consumption percentage of 100 % in the GLB co-culture at 20 h of process (versus a maximum of 30.7 % CO consumption reached at 45 h in the STB co-culture), which showed the excellent behavior of *C. carboxidivorans* in GLB co-cultures. Considering CO_2 generation (Fig. 3c), the maximum CO_2 concentrations achieved in both STB and GLB co-cultures were also enormously different (p-value <0.05) (65.7 % GLB vs 25.4 % STB), probably mainly due to *C. beijerinckii*, which also revealed the good behavior of this microorganism in GLB co-cultures. As for butanol production (Fig. 3d), although not much difference was observed in the maximum values recorded for both STB and GLB co-cultures (6.7 vs 6.1 g/L) (p-value >0.05), the maximum butanol concentration was reached more quickly using the GLB co-culture (34 vs 45 h) with a clear significant difference (p-value <0.05), again showing the good behavior of the gas-lift mechanism, where agitation was carried out through gas circulation [24].

The consistently superior performance of the gas-lift bioreactor, evidenced by faster substrate consumption rates and more rapid product accumulation, can be mechanistically attributed to its inherent hydrodynamic and mass transfer advantages over traditional stirred-tank bioreactors. Firstly, the pneumatic agitation within the GLB generates significantly lower shear stress compared to mechanical agitation in STBs. This gentle mixing regime is particularly beneficial for delicate, shear-sensitive microorganisms like *Clostridium* species, likely minimizing cellular damage and promoting sustained metabolic activity for both *C. carboxidivorans* and *C. beijerinckii* within the co-culture. Secondly, and critically for a gas-based fermentation system, GLBs facilitate highly efficient gas-liquid mass transfer. The circulatory flow pattern induced by gas sparging creates an extensive interfacial area between the gas bubbles and the liquid phase. This enhanced interfacial area,

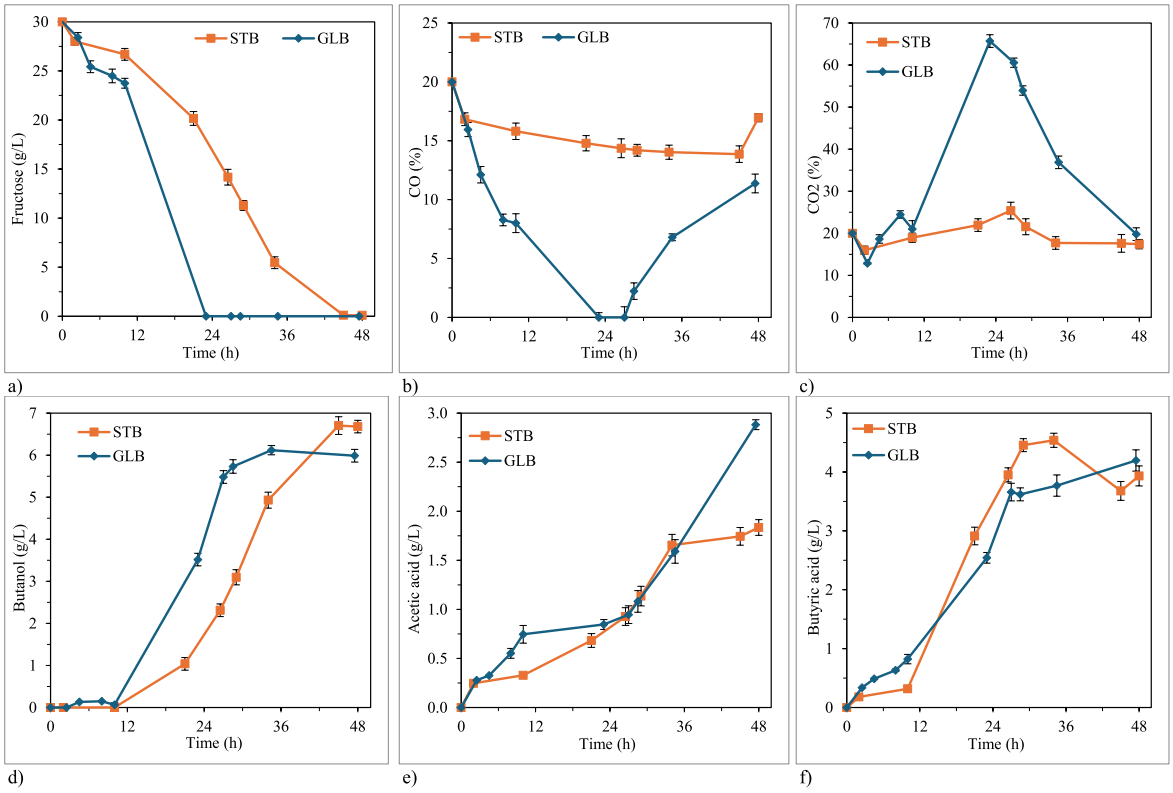


Fig. 3. Kinetics of mixotrophic *C. carboxidivorans* (t = 0) and *C. beijerinckii* (t = 24 h, STB; t = 10 h, GLB) co-cultures (Fe^0 = 12.5 g/L) in stirred tank bioreactor (STB) and gas-lift bioreactor (GLB) for 50 mL/min gas flow. (a) Fructose (g/L), (b) CO (%), (c) CO_2 (%), (d) Butanol (g/L), (e) Acetic acid (g/L), (f) Butyric acid (g/L).

coupled with effective liquid recirculation, significantly improves the volumetric mass transfer coefficient (k_La), which is paramount for the continuous supply of sparingly soluble C1-gases (CO and CO_2) to the cells. By effectively overcoming gas solubility limitations, the GLB ensures that the microbial consortium receives adequate gaseous substrates, thus enabling the observed rapid CO consumption and overall improved bioconversion efficiency [20–22,25].

In addition, higher concentrations of acetic acid (2.9 vs 1.8 g/L) (Fig. 3e) and butyric acid (4.2 vs 3.9 g/L) (Fig. 3f) were also obtained, at the end of process (t = 48 h), using the GLB co-culture, although in this case with no significant differences (*p*-value >0.05). Therefore, considering all these results, it can be concluded that the gas-lift mechanism is an interesting strategy for mixotrophic co-culture fermentation by *C. carboxidivorans* and *C. beijerinckii*. The gas-lift technology has also been used successfully to produce butanol and other main by-products by other authors from the literature [20–22,25]. As can be seen in Table 1, for instance, Thanapornsin et al. [20] compared fermentations in stirred-tank and gas-lift bioreactors, determining that gas-lift column bioreactors could be successfully used as low-cost bioreactors for butanol fermentation from sugarcane molasses by *C. beijerinckii* TISTR 1461; obtaining 10.1 and 10.6 g/L butanol for both STB and GLB fermentations, respectively. However, in this case, only a heterotrophic carbon source was used in monocultures; unlike in the current study, where mixotrophic fermentation in co-cultures was studied. Another study reported 11 g/L butanol from sweet sorghum stem juice by immobilized *C. beijerinckii* TISTR 1461 (on bamboo chopstick pieces), using an internal loop gas-lift bioreactor [25].

3.3. Carrot discard enzymatic hydrolysate/C1-gas co-cultures in stirred tank and gas-lift bioreactors by C. carboxidivorans and C. beijerinckii

Mixotrophic *C. carboxidivorans and C. beijerinckii* co-cultures, formed by carrot discard enzymatic hydrolysate and C1-gases, were carried out in STB and GLB (using 10 and 50 mL/min gas flows, respectively). These gas flows were selected from Sections 3.1. And 3.2., as were those flows where higher CO consumptions (Figs. S1b and 3) in co-cultures were achieved (considering the whole fermentation process) (Table S5). The composition of the carrot discard enzymatic hydrolysate was (g/L): glucose, 39.4; fructose + galactose, 25.4; arabinose, 1.8; formic acid, 0.1; acetic acid, 0.7; and total phenols, 0.8. So the total sugar content of the hydrolysate was 66.6 g/L.

Fig. 4 shows the results obtained in carrot discard enzymatic hydrolysate/C1-gas co-cultures by *C. carboxidivorans and C. beijerinckii*, comparing both STB and GLB. As can be seen, the glucose was consumed using both bioreactors (Fig. 4a); while galactose + fructose was completely metabolized only in the case of GLB (Fig. 4b and S4) (kinetic rat = −0.478 g L/h, Table S4), its consumption in STB being only 28.3 % (kinetic rate = −0.078 g L/h, Table S4). In addition, all the sugars were consumed much more quickly for the GLB co-culture, with glucose being completely consumed in a time as short as 43 h with a kinetic rate of −0.814 g L/h (Table S4) (vs 116 h in STB) and the galactose + fructose in only 47 h (vs no total consumption in 116 h for STB). This was different to those previously found with mixotrophic (fructose/C1-gases) co-cultures in STB (Section 3.1.), where the entire fructose was consumed in all cases.

Regarding CO consumption (Fig. 4c and S4), *C. carboxidivorans* was able to metabolize almost all (95 %) of this C1-gas (at t = 23–33 h) when the co-culture was carried out in GLB with a specific speed of $-7.993{\cdot}10^{-4}$ g/L·h (Table S4) (vs 54.9 % (t = 20 h) for STB) (*p*-value <0.05). These consumptions are similar to those described previously in Sections 3.1. And 3.2. For mixotrophic (fructose/C1-gases) *C. carboxidivorans and C. beijerinckii* co-cultures (using 10 and 50 mL/min gas flow for STB and GLB, respectively). CO_2 also behaved better in GLB, reaching a maximum consumption by *C. carboxidivorans* of up to 82.4 % (t = 33 h) (*p*-value <0.05), probably due to the presence of Fe^0 [26]. This was followed by a large production (with a specific speed of $1.908{\cdot}10^{-5}$ g/L·h, Table S4), reaching levels of about 27 % (t = 43 h), probably due to the action of *C. beijerinckii*, which produces CO_2 within

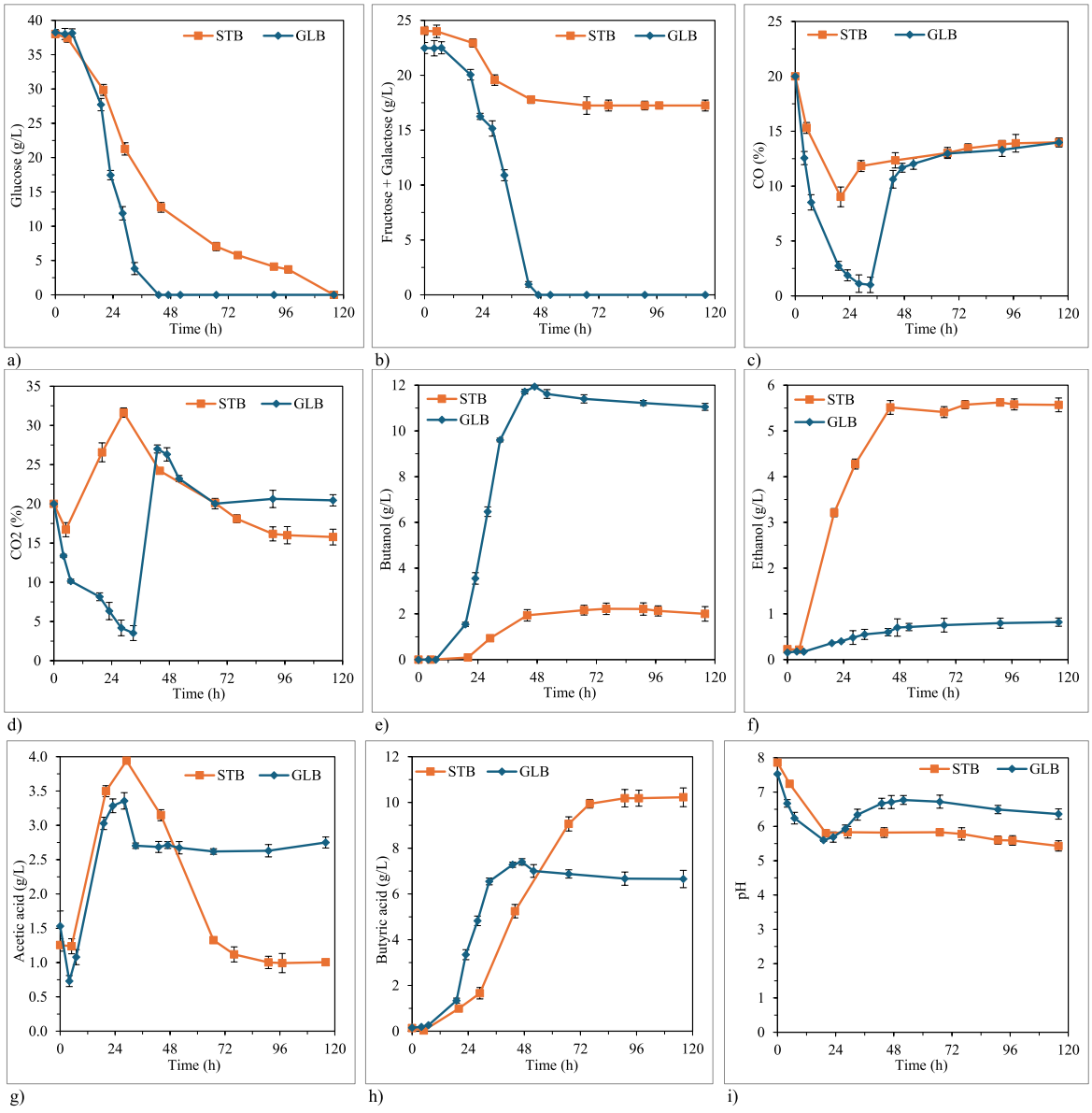


Fig. 4. Kinetics of carrot discard enzymatic hydrolysate/C1-gas co-cultures (Fe^0 = 12.5 g/L) in stirred tank bioreactor (STB) (10 mL/min gas flow) and gas-lift bioreactor (GLB) (50 mL/min gas flow) by *C. carboxidivorans* (t = 0) and *C. beijerinckii* (t = 24 h, STB; t = 10 h, GLB). (a) Glucose (g/L), (b) Fructose + Galactose (g/L), (c) CO (%), (d) CO_2 (%), (e) Butanol (g/L), (f) Ethanol (g/L), (g) Acetic acid (g/L), (h) Butyric acid (g/L), (i) pH.

its metabolic pathway [30] (Fig. 4d and S4). However, in STB, only a slight uptake of 16.5 % was observed over the first hours of the process (t = 5 h), followed by a sizeable production (reaching a concentration of 31.6 % at 29 h). Therefore, a significant difference (*p*-value <0.05) was observed in CO_2 uptake for both STB and BLB configurations.

Concerning butanol, as can be seen in Fig. 4e, the carrot discard enzymatic hydrolysate/C1-gas co-culture in GLB by *C. carboxidivorans and C. beijerinckii* was able to produce concentrations as high as 12.0 g/L (kinetic rate = 0.249 g L/h, Table S4), while only 2.2 g/L butanol were achieved using STB; then, a clear significant difference (*p*-value <0.05) was observed in butanol production for both bioreactors using the carrot discard enzymatic hydrolysate/C1-gas co-substrate. Moreover, this maximum value of butanol was reached at 47 h of fermentation, which is crucial for the economic profitability of the process [40]. Butanol production using carrot discard enzymatic hydrolysate/C1-gas co-culture in GLB (Fig. 4e) was twice that achieved when employing synthetic fructose/C1-gas GLB co-culture (Fig. 3d and S4) (12.0 vs 6.1 g/L), then also with a clear significant difference (*p*-value <0.05). This finding is particularly noteworthy as it highlights the synergistic value of valorizing two distinct waste streams - carrot discard (typically rejected due to physical imperfections and lacking commercial application) and C1-gases - to produce high levels of butanol. This approach addresses both waste management and sustainable biochemical production challenges.

Besides butanol, other main products, such as ethanol, acetic acid and butyric acid, were also generated in both STB and GLB co-cultures using carrot discard enzymatic hydrolysate/C1-gases as co-substrates. As can be observed in Fig. 4f and S4, up to 5.4 g/L ethanol (with a specific speed of 0.081 g L/h, Table S4) was obtained in the STB co-culture (at the time of maximum butanol production, t = 67 h); while, in the case of GLB, only 0.7 g/L ethanol were produced (t = 47 h), then being observed a clear significant difference (*p*-value <0.05) in this case. STB was able to generate mainly ethanol rather than butanol (5.4 vs 2.2 g/L (t = 67 h)), unlike those appreciated for GLB. This finding was also reported by Fernández-Naveira et al. [28], who achieved higher ethanol concentrations than for butanol (5.6 vs 2.7 g/L), also using 2 L STB with

continuous gas feed (10 mL/min), but employing only CO (100 %) as the sole gaseous substrate and only *C. carboxidivorans P7* DSM 15243. Vees et al. [16] also reported higher ethanol concentrations than butanol (5.7 vs 2.6 g/L) when employing a mixotrophic substrate (20 % CO, 10 g/L glucose) in 2 L STB with continuous gas-feed and only *C. carboxidivorans P7* DSM 15243. Regarding acetic acid (Fig. 4g and S4), 1.3 and 2.7 g/L acetic acid were achieved for STB and GLB, respectively, at the time of maximum butanol production (STB, t = 67 h; GLB, t = 47 h) (kinetic rates = 0.020 and 0.057 g L/h, respectively (Table S4)). However, much higher concentrations of butyric acid were achieved for both bioreactors (STB, 9.1 g/L; GLB, 7.4 g/L) (also with a much higher specific speed of production, 0.135 and 0.155 g L/h, respectively (Table S4))., also at the time of maximum butanol production (Fig. 4h and S4). Then, a clear significant difference (p-value <0.05) in acids accumulation was also appreciated between both bioreactors. After this fermentation time, for STB, while the acetic acid slightly decreased (Fig. 4g), the butyric acid considerably increased, reaching concentrations of up to 10.2 g/L (Fig. 4h). However, for GLB, no considerable increases were observed for any of these acids.

In addition, some other by-products (formic acid, acetoin and 2,3-butanediol) were also generated, but in low concentrations (Table S3). As can be seen, maximum values of 1.1 g/L formic acid, 1.9 g/L acetoin and 0.2 g/L 2,3-butanediol were determined for STB co-cultures; while up to 2.2 g/L formic acid, 1.6 g/L acetoin and 0.4 g/L 2,3-butanediol were obtained when GLB co-cultures were tested.

On the other hand, a different pH behavior was observed for both bioreactors (Fig. 4i). As can be observed, in the first hours of fermentation, the pH decreased to 5.6 for both bioreactors (t = 19 h). However, after 19 h of fermentation, while the pH slightly increased in STB (up to 5.8–5.9), a considerable increase was recorded for GLB, reaching values of up to 6.8, probably due to the high levels of butanol (12.0 g/L) [26,41].

To further evaluate the efficiency of the bioconversion process and elucidate substrate utilization, a detailed carbon balance was performed for both the STB and GLB configurations. The results are summarized in Table S6 and Table S7, respectively. These balances enabled a comprehensive assessment of carbon flow from initial substrates (sugars, CO, CO_2) to final products (butanol, ethanol, acetic acid, butyric acid).

In the STB configuration (Table S6), initial carbon mass of glucose and fructose were 15.20 and 9.62 g, respectively. At the end of fermentation (t = 116 h), complete consumption of glucose was observed, remaining 6.90 g fructose carbon without consuming. C1-gases (CO and CO2) were mainly consumed at the beginning of fermentation (t = 0–20 h), remaining 1.70 and 2.94 g carbon of CO and CO_2, respectively, at the end of fermentation (t = 116 h). The primary carbon products were butanol (1.40 g), ethanol (2.82 g), acetic acid (0.53 g), and butyric acid (4.94 g), at the time of maximum butanol production (t = 67 h). Notably, 19.41 g of carbon remained in the liquid phase at the time of maximum butanol production, alongside 2.73 g of carbon lost as gas outlet.

Conversely, the GLB configuration (Table S7) commenced with a higher initial substrate load of 99.47 g of glucose carbon and 58.45 g of fructose carbon. In this case, complete sugar consumption was achieved within 47 h. Carbon product formation was significantly enhanced in the GLB, yielding 50.31 g of butanol, 2.37 g of ethanol, 7.05 g of acetic acid, and 26.23 g of butyric acid carbon, at the time of maximum butanol production (t = 47 h). The liquid carbon content at this fermentation time was 85.96 g, and the carbon lost via gas outlet was 16.00 g.

These carbon balance results corroborate prior findings concerning the GLB's superior performance. The increased production of butanol in the GLB directly correlates with enhanced substrate utilization, particularly the more efficient uptake of CO and CO_2. This detailed carbon tracking provides quantitative evidence for the heightened metabolic activity and product synthesis capacity within the gas-lift bioreactor setup, thereby indicating a more effective conversion of both C1 gases and carrot discard hydrolysates into desired products.

In summary, carrot discard enzymatic hydrolysate/C1-gas co-culture by *C. carboxidivorans* and *C. beijerinckii* successfully proved to be more effective when gas-lift technology was applied, being able to produce butanol concentrations as high as 12.0 g/L, as well as 2.7 g/L acetic acid, 7.4 g/L butyric acid, 2.2 g/L formic acid, 1.6 g/L acetoin and 0.4 g/L 2,3-butanediol, at a fermentation time of 47 h, and with a complete consumption of sugars (glucose, fructose and galactose) and CO, as well as a considerable maximum CO_2 uptake (82.4 %, t = 33 h), showing the efficiency of the presence of Fe^0 [26]. This holistic approach represents a synergistic integration of C1-gas fermentation and agricultural waste valorization, offering significant industrial and environmental potential. The comprehensive valorization of these waste streams, yielding not only significant levels of butanol but also a diverse array of other valuable co-products, strongly indicates the high economic profitability of carrot discard enzymatic hydrolysate/C1-gas fermentation in GLB systems using these microorganisms, thereby presenting a sustainable pathway for industrial bioproduction [40,42].

As can be seen in Table 1, these results are much better than those reported in the literature for the same raw material (carrot discard), or other lignocellulosic residues, using conventional technologies. For instance, using the same raw material as in the current study (carrot discard), López-Linares et al. [39] obtained much lower concentrations of butanol (7.4 g/L) and ABE (acetone-butanol-ethanol) (11 g/L) from enzymatic hydrolysate by *C. beijerinckii* DSM 6422, using 100 mL serum bottles. 8 g/L of butanol, 3.5 g/L of butyric acid, 0.5 g/L of ethanol, and 3.5 g/L of acetic acid were achieved by *C. beijerinckii* DSM 6423 from sugarcane bagasse hydrolysates, using serum bottles [43]. 8 g/L butanol and 12 g/L ABE were also reported from enzymatic hydrolysates of microwave pretreated brewer's spent grain (147 °C, 2 min and 1.26 % H_2SO_4) by *C. beijerinckii* DSM 6422, using 100 mL serum bottles [44]. Enzymatic hydrolysate from microwave pretreated spent coffee grounds (160.47 °C and 1.5 % H_2SO_4) by *C. beijerinckii* DSM 6422, using 100 mL serum bottles, also resulted in only 6.7 g/L butanol and 10.4 g/L ABE [19]. Thanapornsin et al. [20] achieved 10.1–10.6 g/L butanol from sugarcane molasses by *C. beijerinckii* TISTR 1461, using stirred-tank and gas-lift bioreactors. Similar butanol production (12.0 g/L) to those obtained in this study were reported from sugarcane bagasse hydrolysate by C. *beijerinckii* TISTR 1461 [45], using 100-mL rubber-seal bottles. Daengbussadee et al. [46] also attained 11.4 g/L butanol from sweet sorghum stem juice (SSJ) by *Arthrobacter* sp. And *C. beijerinckii* TISTR 1461 co-culture, using a 2-L stirred-tank bioreactor. 11.2 g/L butanol and 13.7 g/L ABE were also achieved by C. *beijerinckii* D9/pykA from puerariae slag hydrolysate, employing anaerobic culture flasks (125 mL) [47]. However, a substantially lower yield of only 3.4 g/L butanol was reported by de Brito Bezerra et al. [48] from the green coconut husk hydrolysate using *C. beijerinckii* ATCC 10132 in 20 mL penicillin-type flasks. Therefore, this comparative analysis robustly demonstrates that the synergistic integration of co-substrates (carrot discard enzymatic hydrolysate and C1-gases), a dual-species co-culture (*C. carboxidivorans* and *C. beijerinckii*), and a specialized fermentation technology (gas-lift bioreactor) significantly enhances the production of butanol and other valuable by-products. From an industrial perspective, this maximizes resource efficiency by simultaneously transforming two distinct, low-cost waste streams—industrial C1-gases and agricultural carrot discard—into valuable biochemicals. Environmentally, it presents a compelling solution for waste valorization and carbon emission mitigation, contributing to a circular carbon economy. This innovative multi-pronged approach offers a distinct advantage in terms of resource utilization and product yield compared to conventional methods.

Nevertheless, the industrial application and scale-up of this promising bioprocess present inherent challenges that warrant careful consideration to fully realize its environmental and industrial potential. A primary concern revolves around potential gas-liquid mass transfer limitations, which can become significant at larger volumes and for highly metabolically active cultures requiring high rates of gas exchange. Optimizing these parameters for efficient C1-gas utilization in

our *C. carboxidivorans* and *C. beijerinckii* co-culture would be critical during scale-up, influenced by factors such as gas superficial velocity, column height-to-diameter ratio, and rheological properties of the fermentation broth. Furthermore, maintaining homogeneous mixing and preventing localized nutrient gradients, particularly with a complex substrate like carrot discard hydrolysate, can become more difficult in larger GLB systems, potentially impacting overall yield and productivity.

Future research should therefore focus on addressing these scale-up complexities, possibly through advanced computational fluid dynamics modeling or novel bioreactor designs, to fully harness the potential of GLBs for this unique bioconversion process. Additionally, subsequent investigations could involve comprehensive compositional characterization of various hydrolysate batches, coupled with sensitivity analyses, to fully understand the robustness of the bioconversion process to substrate heterogeneity. Such studies are crucial for optimizing industrial applications and ensuring process stability across different feedstock supplies. Moreover, future research efforts should include a thorough lifecycle assessment of the Fe^0 usage within this system, evaluating strategies for iron residue management and exploring methods to minimize any potential environmental impact. This holistic approach is essential for ensuring the overall sustainability and ecological viability of the process, thereby maximizing its industrial and environmental benefits.

4. Conclusions

This study successfully demonstrated the simultaneous bioconversion of C1-gases and carrot discard hydrolysate for the production of butanol and other valuable by-products, utilizing a Fe^0-supplemented co-culture of *Clostridium carboxidivorans* and *Clostridium beijerinckii*. Among the tested configurations, the gas-lift bioreactor was the most effective. Operating at a 50 mL/min gas flow, it achieved a high butanol concentration (12.0 g/L) and significant co-product titers within a short timeframe (47 h). The complete consumption of all substrates (sugars, CO) and high CO_2 uptake (82.4 %) underscore the efficacy of this integrated bioprocess. Furthermore, the inherent advantages of the gas-lift system, including its simple design and lower energy consumption, enhance the economic potential of this valorization pathway. Future research should prioritize the optimization of fermentation conditions, particularly the gas delivery strategy. Implementing advanced process controls, such as in-situ product removal to mitigate inhibition and continuous substrate feeding, could further elevate the process productivity and efficiency.

CRediT authorship contribution statement

Juan Carlos López-Linares: Writing – original draft, Project administration, Methodology, Investigation, Conceptualization. **Marina Fernández-Delgado:** Methodology, Investigation. **Cristina Betanzos-Salguero:** Methodology, Investigation. **María Ángeles Herrero-Vieira:** Methodology, Investigation. **Susana Lucas:** Supervision, Formal analysis, Conceptualization. **Mónica Coca:** Supervision, Formal analysis, Conceptualization. **M. Teresa García-Cubero:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2025.108473.

Data availability

Data will be made available on request.

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