Research Article

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Examining mixotrophic fermentation in fed-batch mode for C1-gas valorization

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Abstract

BACKGROUND: C1-gases like CO and CO_2, significant contributors to climate change, offer the potential for sustainable bioconversion into valuable products. The study explored mixotrophic fermentation using C1-gases in fed-batch mode to improve the production of target compounds, focusing on *Clostridium aceticum* and *Clostridium carboxidivorans*. It aimed to overcome the limitations of conventional gas fermentation (autotrophic fermentation and without fed-batch mode) and assess the potential of mixotrophic substrates for enhancing yields.

RESULTS: Results showed that mixotrophic fermentation with fructose as a co-substrate led to higher microbial growth in *C. aceticum*, increasing acetic acid (1200 *versus* 600 mg L^{-1}) and ethanol (600 *versus* 0 mg L^{-1}) production, compared to autotrophic fermentation. For *C. carboxidivorans*, constant CO consumption occurred in autotrophic and mixotrophic fermentation. Mixotrophic fermentation with fructose and C1-gases by *C. carboxidivorans* significantly boosted microbial growth and metabolic activity, increasing butanol (1600 *versus* 0 mg L^{-1}) and butyric acid (2400 *versus* 1800 mg L^{-1}) production, compared to autotrophic fermentation.

CONCLUSIONS: The study highlights mixotrophic fermentation's potential to enhance C1-gas valorization. It provides insights into microbial behavior under varied substrate conditions, contributing to sustainable biomanufacturing practices for biofuel and high-value bioproducts.

Keywords: autotrophic fermentation; C1-gases; *Clostridia* spp.; fructose; mixotrophic fermentation

INTRODUCTION

The global transition towards sustainability and a low-carbon economy has prompted innovative exploration for biomanufacturing.[1] Among these alternatives are carbon monoxide (CO) and carbon dioxide (CO_2), known as one-carbon (C1) gases, which are recognized as significant contributors to environmental degradation and climate change.[2] These gases predominantly originate from the combustion of fossil fuels, deforestation and other human activities.[3] For example, a waste gas from an industrial combustion process could be a mixture of CO, CO_2, H_2 and mainly N_2.[4] The valorization of CO and CO_2 has thus been converted into a strategic imperative in climate change mitigation and sustainable development agendas.[5] Biorefineries emerge as pivotal hubs in harnessing these unconventional raw materials to generate high-value products.[6] Through fermentation processes, C1-gases serve as substrates for producing different biofuels and have versatility in yielding a diverse array of precursor compounds of high-value chemicals.[7] By converting these gases into industrially relevant products, their atmospheric emissions are curtailed, thereby combating the adverse impacts of global warming.[8]

Acetogenic microorganisms, such as *Clostridium* spp. strains, possess the remarkable capability of fermenting C1-gases to synthesize organic acids and alcohols via the Wood–Ljungdahl pathway (WLP) under anaerobic conditions.[9] The WLP stands out as the most energy-efficient route for fixing C1-gases, allowing acetogenic bacteria to utilize these gases as sole sources of both energy and carbon.[10]

Clostridium aceticum and *Clostridium carboxidivorans* have garnered significant attention, and extensive research has delved into elucidating their behavior during gas fermentation, as well as how various process variables influence their metabolic activities.[10-16] However, despite their metabolic versatility, using C1-gases as substrates in fermentation processes often falls short of achieving the theoretical product yields predicted by the WLP.[17] In recent years, novel fermentation strategies have emerged as promising avenues to enhance the production of alcohols and organic acids from C1-gases. One such strategy involves the exploration of mixotrophic substrates, comprising a

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blend of heterotrophic and autotrophic substrates.[1,18] This mixotrophic approach has the potential to address the limitations of both sugar fermentations (carbon loss due to CO_2 production) and gas fermentations (low productivity and feedstock solubility in liquids).[19,20] Consequently, mixotrophic fermentation could lead to the development of adaptable and highly efficient production platforms.

The research reported here aimed to investigate mixotrophic fermentation with C1-gases in fed-batch mode to enhance the production of target compounds. Specifically, the study explored the behavior of two microorganisms, *C. aceticum* and *C. carboxidivorans*, under this fermentation mode. An industrial residual gas comprising 20% CO, 20% CO_2 and 60% N_2 is advantageous for C1-gas fermentation. This approach is cost-effective due to the lower cost of waste gases, promotes sustainability by recycling industrial emissions, provides optimal CO and CO_2 levels for microbial metabolism, maintains the necessary anaerobic conditions and enhances overall fermentation performance.

The successful implementation of mixotrophic fermentation using fructose as a heterotrophic source would pave the way for the comprehensive valorization of biomass waste, such as rejected fruits and vegetables, which could be used in this type of gas fermentation. This holistic approach to residue valorization represents a significant step forward in seeking sustainable alternatives in biofuel production and other industrially relevant products.

MATERIALS AND METHODS

Microorganisms and culture media

The microorganisms *C. aceticum* DSM 1496 and *C. carboxidivorans* DSM 15243, from the German collection of microorganisms (DSMZ, Leibniz, Germany), were employed. The strains were reactivated by inoculating the lyophilized cells into DSMZ liquid medium and grown for 24 h at 30 °C for *C. aceticum* and at 35 °C for *C. carboxidivorans* in an orbital shaker (Optic Ivymen Systems, Comecta, Spain) following the recommended procedure of DSMZ. Then, each strain was stored as glycerol stock (40% (v/v) sterile glycerol) at –80 °C until further use.

Both strain growths were carried out in septum bottles, with a rubber septum, with 50 mL of working volume and a mixture of CO, CO_2 and N_2 (20:20:60) as headspace. The cells were grown in a rotary shaker for 24 h and 200 rpm at 30 °C for *C. aceticum* and at 35 °C for *C. carboxidivorans*.

Table 1. Operation conditions for the different fermentations studied using two *Clostridium* bacteria

Initial medium pH	9	
Time (d)	0–7	
Microorganism	*Clostridium carboxidivorans* DSM 15243	*Clostridium aceticum* DSM 1496
Temperature (°C)	35	30
Agitation (rpm)	200	
Main products	Butanol; butyric acid	Ethanol; acetic acid
Type of fermentation	Autotrophic (C1-gases)	
	Mixotrophic (C1-gases and fructose)	
Fed-batch	C1-gases	

The composition of the liquid culture medium used for *C. aceticum* was modified DSMZ medium. It was as follows (per liter of distilled water): 0.5 g of yeast extract, 0.408 g of KH_2PO_4, 0.534 g of $Na_2HPO_4{\cdot}2H_2O$, 1 mL of resazurin (from a stock solution of 0.5 g L^{-1}), 0.3 g of NH_4Cl, 0.3 g of NaCl, 0.1 g of $MgCl_2{\cdot}6H_2O$, 1.8 mg of HCl 37%, 61.8 µg of H_3BO_3, 61.25 µg of $MnCl_2$, 943.5 µg of $FeCl_2$, 64.5 µg of $CoCl_2$, 12.86 µg of $NiCl_2$, 67.7 µg of $ZnCl_2$, 13.35 µg of $CuCl_2$, 5.5 mg of $CaCl_2{\cdot}2H_2O$, 400 µg of NaOH, 17.3 µg of Na_2SeO_3, 29.4 µg of Na_2WO_4, 20.5 µg of Na_2MoO_4, 0.5 mL of vitamin solution (containing (per liter of distilled water): 20 mg of D-biotin, 200 mg of nicotinamide, 100 mg of

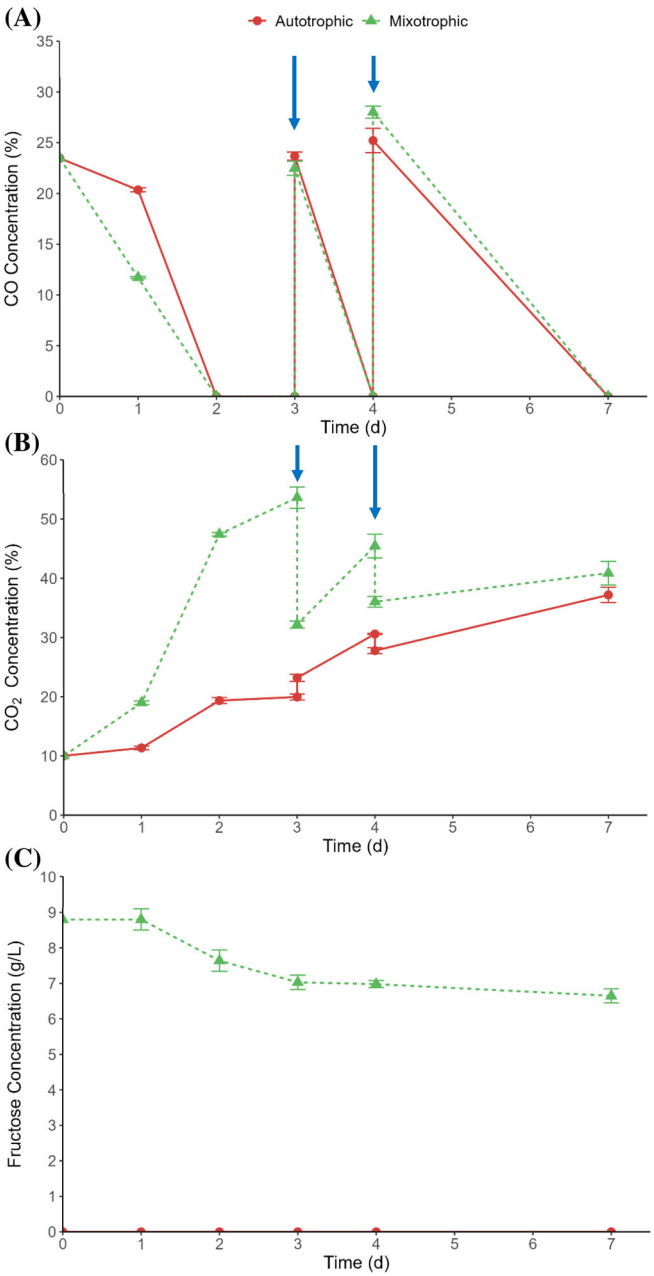

Figure 1. *Clostridium aceticum*. Comparison of substrate evolution for mixotrophic and autotrophic fermentation: (A) CO, (B) CO_2 and (C) fructose. Average values and error bars, representing plus and minus standard deviation from average experimental results, for samples in triplicate are shown. Arrows indicate those days on which a pulse of gas mixture was added to maintain the overpressure.

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p-aminobenzoic acid, 200 mg of thiamin (vitamin B1), 100 mg of pantothenic acid, 500 mg of pyridoxamine, 100 mg of cyanocobalamin (vitamin B12) and 100 mg of riboflavin), and 2.5 mL of reducing solution (containing (per liter of distilled water): 0.5 g of cysteine, 50 mL of $NaHCO_3$ (from a stock solution of 80 g L^{-1}) and 1 mL of $Na_2S{\cdot}9H_2O$ (from a stock solution of 240.2 g L^{-1})).

The composition of the liquid culture medium used for *C. carboxidivorans* was modified DSMZ medium. It was as follows (per liter of distilled water): 10 g of yeast extract, 5 g of trypticase peptone (BD BBL), 5 g of meat peptone (pepsin-digested), 0.5 mL of resazurin (from a stock solution of 0.5 g L^{-1}), 40 mL of salt solution, 1 g of Na_2CO_3 and 0.5 g of cysteine $HCl{\cdot}H_2O$. The salt solution contained the following (per liter of distilled water): 0.25 g of $CaCl_2{\cdot}2H_2O$, 0.5 g of $MgSO_4{\cdot}7H_2O$, 1 g of K_2HPO_4, 1 g of KH_2PO_4, 2 g of NaCl and 10 g of $NaHCO_3$.

In both cases, all medium components (except for vitamins and the reducing solutions for *C. aceticum*, and salt and secondary solutions for *C. carboxidivorans*) were sterilized at 121 °C for 15 min in septum bottles (previously flushed with nitrogen into the liquid). In contrast, the other solutions were prepared separately and sterilized by filtration using 0.2 μm cellulose nitrate filters (Sartorius 254 stedim Biotech, Göttingen, Germany).

Fermentation

Fed-batch fermentations were performed for both strains at initial pH of 9 to stimulate acid production during their acidogenic phase, and no pH control was employed during the fermentation. *C. carboxidivorans* enters this phase starting at pH 6, while *C. aceticum* does so at pH 8.[21] Using an initial pH of 9 ensures optimal conditions for both strains, enhancing the production of desired acids and increasing the fermentation process efficiency and yield. The fermentation studies were carried out in 100 mL sealed bottles equipped with rubber septa, each having a working volume of 50 mL (liquid culture medium described in the previous subsection) under anaerobic conditions. Headspace volume employed was also of 50 mL. The operation temperature and agitation were optimal for each strain, according to DSMZ recommendations. The operation conditions and leading products of strains are summarized in Table 1.

The bottles were sterilized at 121 °C for 15 min with the liquid culture medium without calcium/vitamin and reducing solutions (for *C. aceticum*), without salt and secondary solutions (for *C. carboxidivorans*) and without fructose (in the case of mixotrophic fed-batch fermentation). Once sterilized, the calcium/vitamin and reducing solutions, salt and secondary solutions, and fructose (about 10 g L^{-1}, considering DSMZ medium) (in the case of mixotrophic fed-batch fermentation) were added, and the liquid was flushed with nitrogen. In all cases, the headspace was replaced with a mixture of C1-gases ($CO{:}CO_2{:}N_2$, 20:20:60) after adding all solutions to the fermentation medium with an overpressure of 0.2 bar. Periodically, a pulse of gas mixture was added to maintain the overpressure. The inoculum

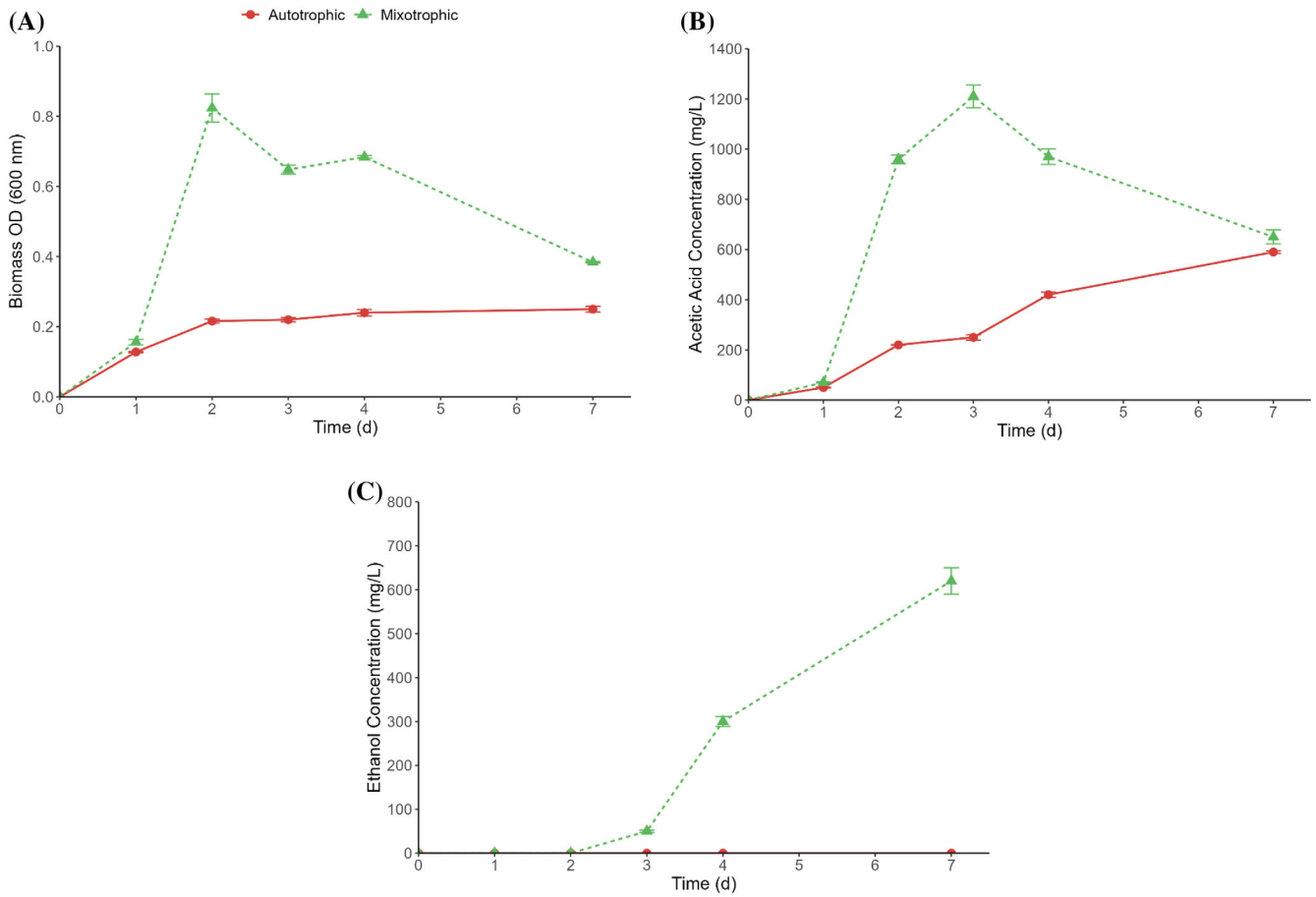

Figure 2. *Clostridium aceticum*. Comparison of optical density (A) and product evolution for mixotrophic and autotrophic fermentation: (B) ethanol and (C) acetic acid. Average values and error bars, representing plus and minus standard deviation from average experimental results, for samples in triplicate are shown.

loading was 10% (v/v), and no pH control was employed during the fermentation.

Liquid samples were taken every 24 h, centrifuged (at 13 500 rpm for 10 min) and analyzed for their content of fructose and fermentation products (ethanol, butanol and acetic and butyric acids). On the other hand, to quantify the behavior of C1-gases, 1 mL of gaseous sample was taken every 24 h and its composition, in terms of concentration of CO, CO_2 and N_2, was analyzed.

All fermentation tests were performed in duplicate.

Analytical methods

High-performance liquid chromatography was used to determine the content of fructose and fermentation products (ethanol, butanol and acetic and butyric acids) in the liquid phase, using a refractive index detector (Waters 2414), an Aminex HPX-87H column (at 60 °C) and 0.01 N H_2SO_4 (0.6 mL min^{-1}) as the mobile phase.

The possible presence of other fermentation products was checked as well.

The gas composition in gaseous samples was determined using an 8860GC gas chromatograph (Agilent Technologies, Spain) equipped with a thermal conductivity detector. The gas chromatograph was fitted with a 15 m HP-PLOT Molecular Sieve 5A column (inner diameter, 0.53 mm; film thickness, 50 µm), the oven temperature was maintained constant at 45 °C and in the injection port the temperature was kept constant at 250 °C in the detector. Helium was used as the carrier gas.

The optical density (OD) at 600 nm was measured using a spectrophotometer (Uvmini-1240, Shimazu Suzhou Wfg., Kyoto, Japan) to determine the concentration of microorganisms in the liquid samples.

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

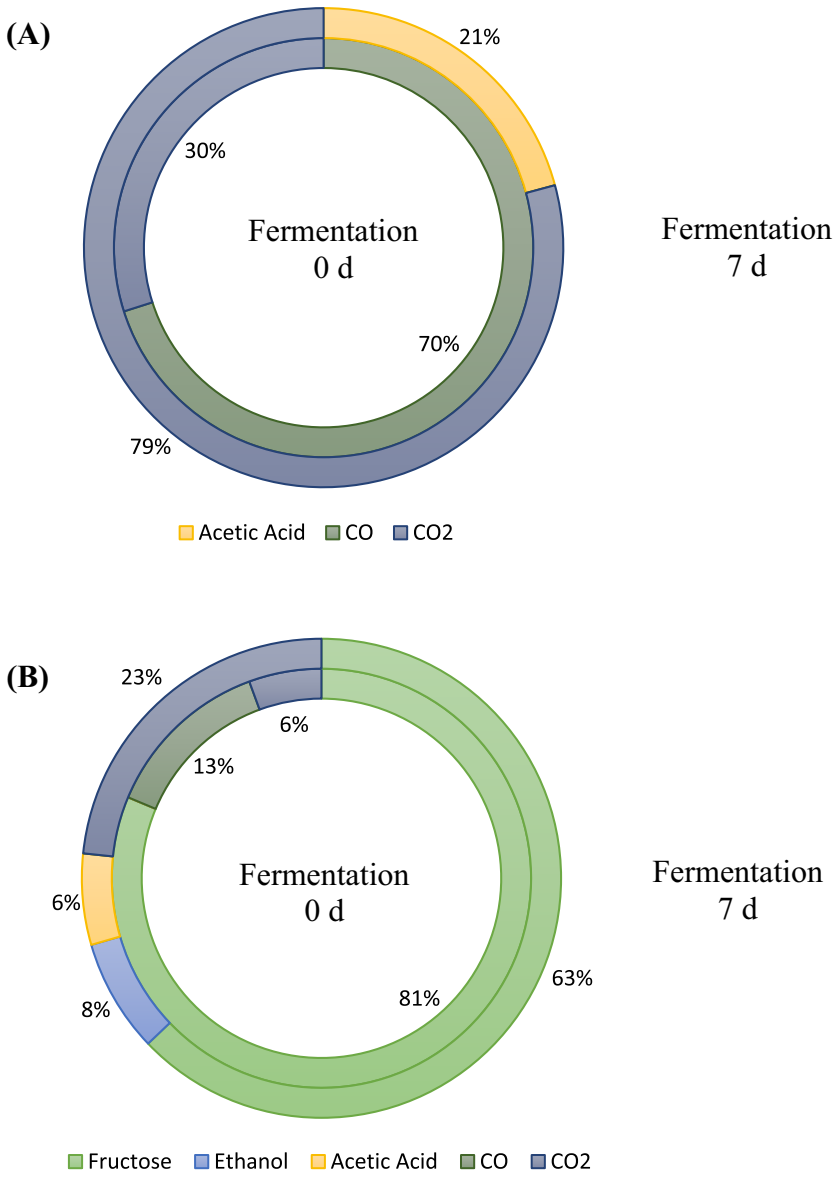

Figure 3. *Clostridium aceticum*. Carbon balance of autotrophic (A) and mixotrophic (B) fermentation. The inner circle represents day 0 and the outer circle represents day 7.

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Data analysis

Statistical software R (version 4.2.2 – Innocent and Trusting – 2022) was employed to investigate the influence of time on the fermentation process and explore correlations between fermentation variables and products. This analysis included carbon balances and heatmaps visualizing correlations between fermentation variables and their resulting products, alongside temporal visualizations to assess how these variables changed throughout the experiments.

RESULTS AND DISCUSSION

Clostridium aceticum

Firstly, regarding the previous results reported for *C. aceticum* under heterotrophic (fructose) and autotrophic (C1-gases) conditions,[22] in this work, *C. aceticum* was studied using gases as a fed-batch substrate to understand its behavior, with a semi-continuous gas feeding and, if this improved, with a heterotrophic substrate such as the additional use of fructose (mixotrophic fermentation). The first tests showed that the headspace could not be changed every day due to the stress of the microorganism, which could not produce either ethanol or acetic acid (data not shown). Therefore, it was decided not to change the headspace gases until it had been checked that the carbon monoxide (CO) was wholly consumed (Fig. 1(A)). This fed-batch configuration allowed the realization of CO consumptions of 100% by *C. aceticum* for the whole fermentation process (t = 1–7 d) in both autotrophic and mixotrophic conditions. This change reduced the amount of carbon dioxide (CO_2) generated during both fermentations in the headspace, decreasing the gas pressure (Fig. 1(B)).

On the other hand, it should be noted that mixotrophic fermentation generated a more significant amount of CO_2 in comparison to autotrophic fermentation, especially in the first days, reaching 50% of the headspace on the third day of fermentation (Fig. 1(B)). This superior CO_2 generation also coincides with the majority consumption of fructose present in the fermentation broth (Fig. 1(C)), which consumed only 2 g L^{-1} (fructose consumption = 24.3%). On the other hand, it is necessary to consider that fructose in the fermentation broth can repress the autotrophic metabolism of *C. aceticum*. This fact can result in the formation of acid inhibitors, causing the fructose not to be consumed, and the principal products will not be produced.[9] For instance, in this study case, formic acid production reached up to 1 g L^{-1} (data not shown), potentially contributing to this inhibitory effect.

One advantage of the presence of fructose in the fermentation broth is a higher OD than the OD of autotrophic fermentation, above all, in the first days of fermentation. The maximum OD reached was 0.82 for mixotrophic (day 2), almost four times higher than the maximum OD of autotrophic (0.22, day 7) (Fig. 2(A)). Even so, when comparing these results to the existing literature, it can be observed that OD was not exceptionally high, although the tendency is similar. For example,[9] a maximum OD of 2.5 was reached on the fourth day of heterotrophic fermentation with fructose and 1.8 on the ninth day of autotrophic fermentation with pure CO using *C. aceticum* as bacteria in both cases. Overall, the bacterial growth was slow in autotrophic fermentation due to the gas availability in the broth.[21] In autotrophic fermentation, the C1-gases must be dissolved in the liquid to be consumed by the bacteria, so it takes longer. However, in the case of mixotrophic fermentation, the bacteria can grow larger and faster because there is a heterotrophic carbon source (in this case, fructose) in the fermentation broth, employing a mixture of heterotrophic and autotrophic substrates, joining WLP and glycolysis pathways.[23] The growth of the bacteria also depends on the amount and type of the substrate. In the case of fructose, a sufficient quantity was added to ensure consumption. However, the addition of fresh gas on days 3 and 4 is necessary to maintain a constant supply of CO/CO_2 for dissolution in the liquid.

In autotrophic fermentation, pH was set at 9 to promote acetic acid production, inhibiting solventogenesis. This strategic adjustment was based on prior research findings.[21,24] Acetic acid was produced increasingly over time, reaching almost 600 mg L^{-1}

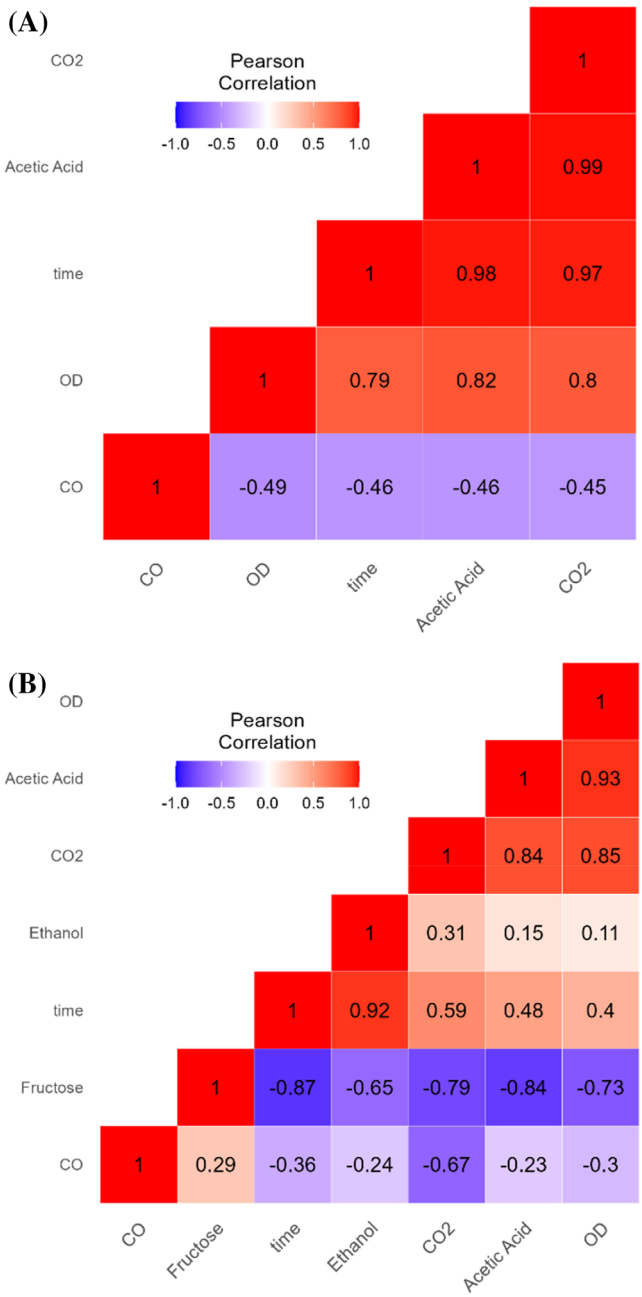

Figure 4. *Clostridium aceticum*. Heatmap of correlations between variables of autotrophic (A) and mixotrophic (B) fermentation.

on the seventh day of fermentation (Fig. 2(B)), which corresponds to a yield of 0.095 g of acetic acid per gram of carbon. On the other hand, no ethanol was produced in this study case (Fig. 2(C)). This may seem like a low concentration of acetic acid if compared with the literature because, for example, in the study of Arslan *et al.*,[9] pure CO resulted in a concentration of 3000 mg L^{-1} in the liquid phase. On the contrary, in this study case, utilizing a gas mixture containing 20% CO yields a concentration of almost 600 mg L^{-1} (Fig. 2(C)). This comparison highlights that, despite the difference in CO concentration, the proportional ratios of CO introduced into each system remain consistent. This demonstrates that the achieved concentrations align with the respective CO content in the gas supply. However, the previous explanation does not apply in the case of mixotrophic fermentation. In this type of fermentation, two metabolic pathways must be considered: the WLP and glycolysis pathways. In the first days of fermentation, the bacteria jointly consumed gases and fructose (Fig. 1). The consumption of fructose favored the production of pyruvate, which can then be converted into acetyl-CoA, an essential precursor in the WLP.[18,21] Because of this, acetic acid production was favored, and it reached its maximum production on the third day of fermentation with 1200 mg L^{-1} (0.1681 g of acetic acid per gram carbon) (Fig. 2(C)). However, from that day on, a decrease in the concentration of acetic acid is seen until it reaches 600 mg L^{-1} on the seventh day of fermentation (Fig. 2(C)). This reduction may be due to the continuation of the metabolic pathway, converting acetate into acetaldehyde and then into ethanol. This would make sense since ethanol appeared in the fermentation broth from the third day of fermentation, reaching 600 mg L^{-1} (0.0862 g of ethanol per gram of carbon) on the seventh day of fermentation (Fig. 2(B)), coinciding, in turn, with the amount of acetic acid that had been reduced.

Figure 3 illustrates the evolution of carbon from the initial compounds (CO/CO_2/fructose) over 7 days of fermentation for the two types studied. In autotrophic fermentation (Fig. 3(A)), it is evident that, initially, the headspace is predominantly occupied by CO carbons (70%). However, by the end of the fermentation, these CO carbons have entirely disappeared, transforming into acetic acid carbons (21%) and CO_2 (79%). In mixotrophic fermentation (Fig. 3(B)), fructose carbons are the majority at the beginning (81%), with CO and CO_2 carbons making up a smaller proportion (13% and 6%, respectively). At the end of the fermentation, the fructose carbons have significantly decreased to 63%. Notably, all CO carbons have been entirely consumed. This shift results in the production of ethanol carbons (8%) and acetic acid carbons (6%), along with an increase in CO_2 carbons (23%). These results suggest that while autotrophic fermentation excels in the conversion of CO, mixotrophic fermentation is less efficient in fully utilizing fructose but produces a wider variety of byproducts.

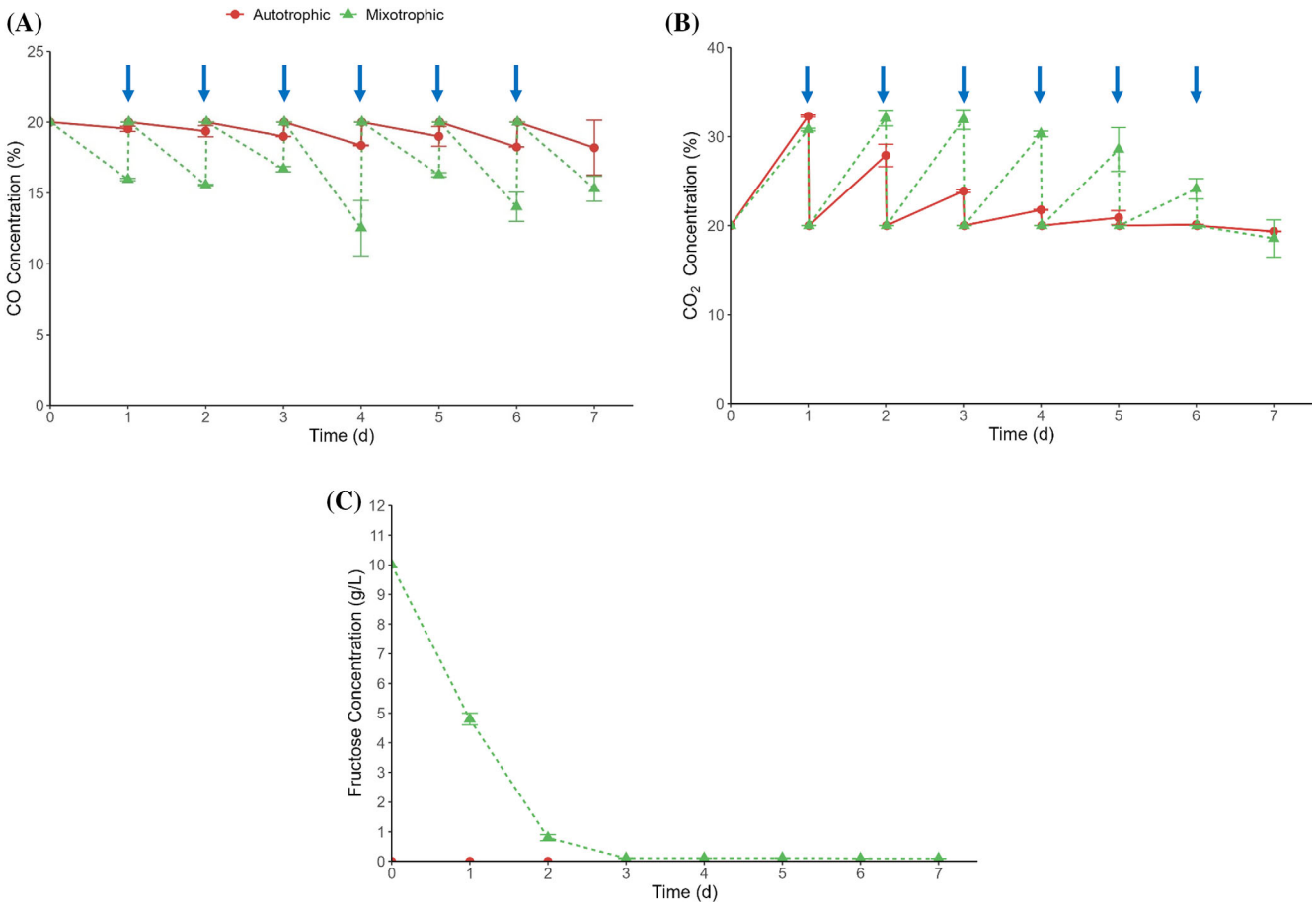

Figure 5. *Clostridium carboxidivorans*. Comparison of substrate evolution for mixotrophic and autotrophic fermentation: (A) CO, (B) CO_2 and (C) fructose. Average values and error bars, representing plus and minus standard deviation from average experimental results, for samples in triplicate are shown. Arrows indicate those days on which a pulse of gas mixture was added to maintain the overpressure.

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Finally, Fig. 4 shows heatmaps to represent the correlations of the variables with each other for both fermentations. The strength of the correlation is indicated by colors, with red indicating a positive correlation and blue indicating a negative correlation. For autotrophic fermentation (Fig. 4(A)), a strong positive correlation (>75%) is observed between CO_2 and acetic acid, time and OD. This means that these variables tend to increase together. This fact makes sense because, as observed previously, the amount of CO_2 generated increases alongside more significant cell growth and acetic acid production as time progresses. Conversely, all these variables show a weak negative correlation with CO. However, as CO serves as the substrate, its concentration decreased as the other variables increased, hence the negative correlation. On the other hand, for the mixotrophic fermentation (Fig. 4(B)), a robust positive correlation is evident among acetic acid, CO_2 and OD, as they exhibit similar trends consistent with our previous observations. Conversely, as previously noted, ethanol production displays a strong positive correlation with time, reflecting an increase in ethanol production over time. Regarding substrates, a notable negative correlation between fructose and all products is evident, as fructose is the primary carbon source stimulating fermentation development. In contrast, the correlation of CO with the variables is weaker, mainly due to its intermittent replacement as needed in the fed-batch fermentation mode. Consequently, it can be inferred that fructose promotes cell growth and enhances acetic acid and ethanol production when utilizing *C. aceticum* as the microorganism.

Clostridium carboxidivorans

Following the investigation of *C. aceticum*, a subsequent examination of *C. carboxidivorans* was undertaken to scrutinize autotrophic and mixotrophic fermentation processes employing a fed-batch regime with C1-gases. Daily changes to the headspace gases were found to exert no detrimental impact on the fermentation process. In Fig. 5(A), the variation in CO levels within the headspace is depicted. It is discernible that an overall decline in CO concentration occurs in both types of fermentation. However, a markedly higher average rate of CO consumption is observed in mixotrophic fermentation (16.6–29.9%) compared to autotrophic fermentation (5.1–9%), being calculated as the ratio between the CO consumption (difference between the initial and final CO concentration) and the initial CO concentration. This observed dissimilarity may be ascribed to the facilitative effect of fructose on CO consumption. As illustrated in Fig. 5(C), fructose (10 g L^{-1}) is rapidly depleted within the initial 3 days of fermentation (fructose consumption = 100%). This leads to a substantial proliferation of microorganisms in the mixotrophic fermentation medium, up to fivefold higher than in autotrophic fermentation (Fig. 6(A)). Consequently, this surge in microbial population engenders a significant daily production of CO_2, peaking at 50% in mixotrophic fermentation (Fig. 5(B)). So, routine gas replacement within the headspace became imperative to mitigate excessive pressure accumulation. Such pressure fluctuations can

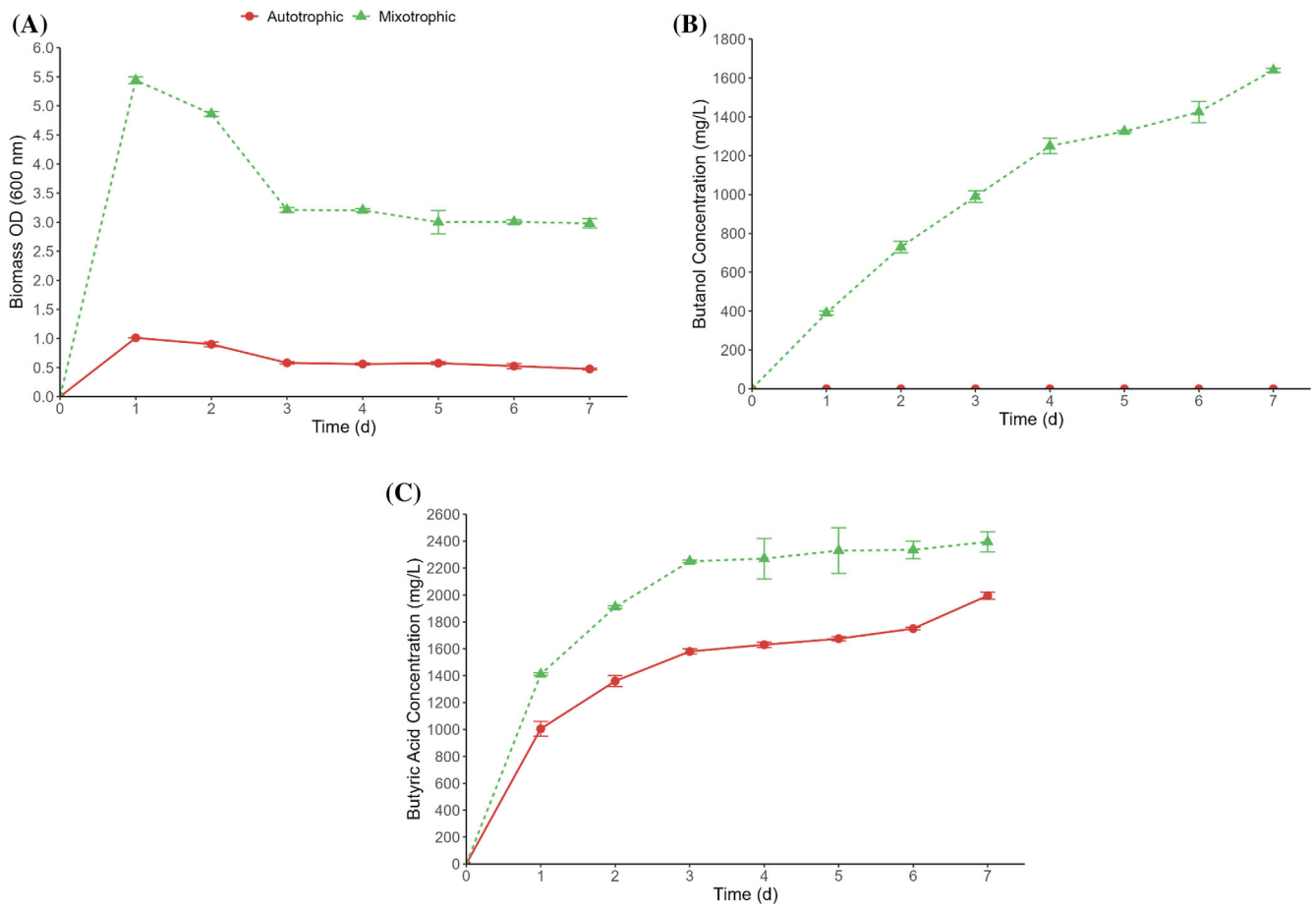

Figure 6. *Clostridium carboxidivorans*. Comparison of optical density (A) and product evolution for mixotrophic and autotrophic fermentation: (B) butanol and (C) butyric acid. Average values and error bars, representing plus and minus standard deviation from average experimental results, for samples in triplicate are shown.

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induce stress on the microorganisms, potentially impeding the fermentation process.[25] Notably, these consumption ratios were not replicated in autotrophic fermentation. As the fermentation progressed, a reduction in CO_2 levels was observed (Fig. 5(B)), primarily due to a lesser accumulation of microorganisms within the fermentation medium, resulting in diminished gas production. A comparison of substrate consumption patterns between *C. aceticum* and C. *carboxidivorans* reveals distinct differences regarding CO and fructose. In the case of *C. aceticum*, it displayed an inability to consume CO or thrive under conditions where the headspace was altered daily. Mixotrophic fermentation also exhibited a consumption rate of approximately 22% (about 2 g L^{-1} of fructose), being determined as the ratio between the fructose consumption (difference between the initial and final fructose concentration) and the initial fructose concentration. Conversely, *C. carboxidivorans* demonstrated a daily consumption of CO, maintaining a consistent rate in both autotrophic and mixotrophic fermentations despite daily changes to the headspace. Furthermore, it exhibited substantial enhancement in mixotrophic fermentation development due to its ability to metabolize all available fructose fully.

Regarding the main products obtained using *C. carboxidivorans*, pH 9 favored butyric acid production, unlike butanol. This phenomenon is observed in autotrophic fermentation, where butanol is not generated (Fig. 6(B)), yet the concentration of butyric acid reaches 1990 mg L^{-1} by the end of the fermentation process (Fig. 6(C)), which corresponds to a yield of 2.935 g of butyric acid per gram of carbon. When contrasting these products with those of mixotrophic fermentation, it becomes apparent that, by the seventh day of fermentation, the production levels rise to 1640 mg L^{-1} of butanol (0.2465 g of butanol per gram of carbon) (Fig. 6(B)) and nearly 2400 mg L^{-1} of butyric acid (0.3592 g of butyric acid per gram of carbon) (Fig. 6(C)). Comparing both mixotrophic and autotrophic fermentations, a plausible explanation

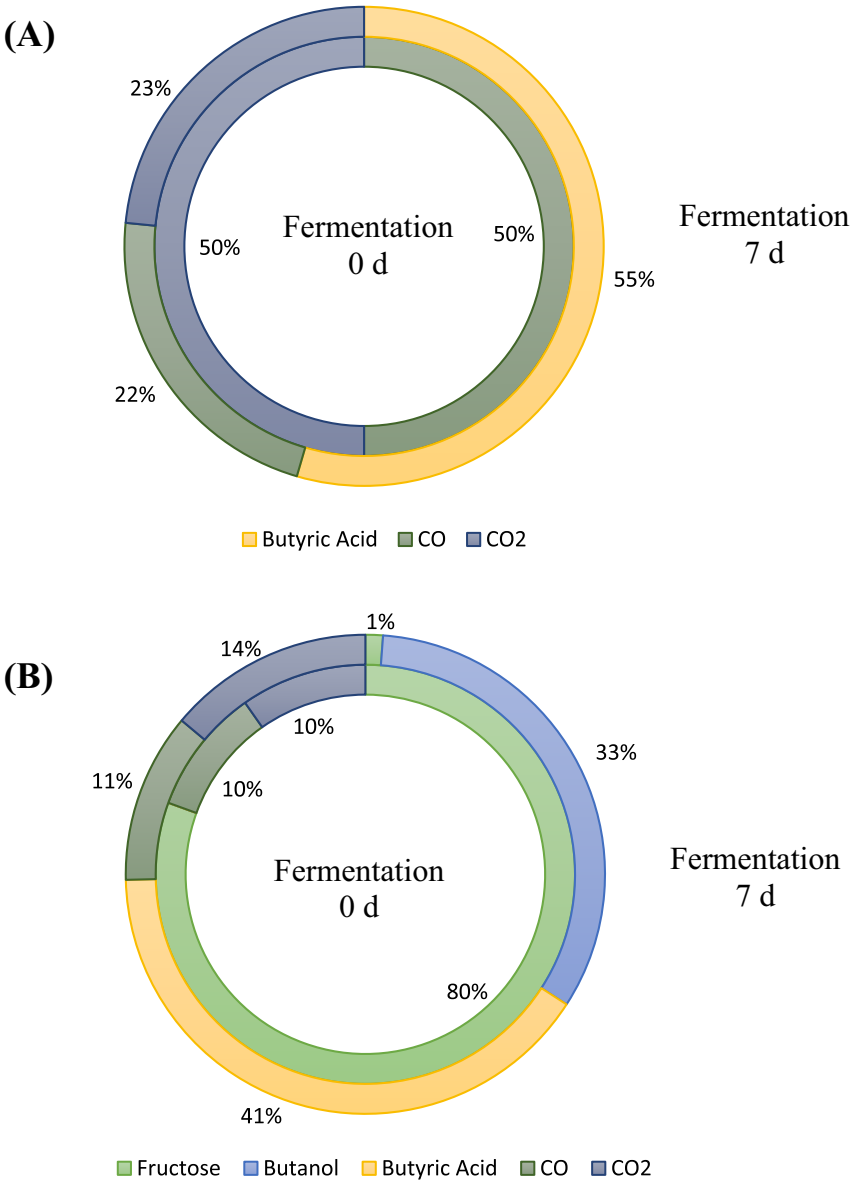

Figure 7. *Clostridium carboxidivorans*. Carbon balance of autotrophic (A) and mixotrophic (B) fermentation. The inner circle represents day 0 and the outer circle represents day 7.

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for this enhancement in butyric acid production, along with the additional butanol output, observed in mixotrophic fermentation, can be linked to the capability of fructose to offer an extra carbon and energy source to microorganisms during mixotrophic fermentation.[10] This mechanism is reminiscent of what is observed in the case of *C. aceticum*. According to the results obtained by Fernández-Naveira *et al.*[26] using glucose as a substrate and without pH control, levels of 1 g L^{-1} of butyric acid and 0.25 g L^{-1} of butanol were reached. In the study by Fernández-Naveira *et al.*,[27] using pure CO in a continuous system without pH control, the production of 0.3 g L^{-1} of butyric acid and 2.3 g L^{-1} of butanol was achieved at a pH of 5.75. On the other hand, Roell *et al.*[28] used a gas composition similar to ours, although without pH control, and obtained 1.1 g L^{-1} of butyric acid and 0.8 g L^{-1} of butanol. In contrast, Vees *et al.*[18] used a mixotrophic substrate with glucose and 20% CO in the gas, with a pH of 6, achieving higher production levels, reaching 2.6 g L^{-1} of butanol and 0.7 g L^{-1} of butyric acid. Comparing these results with ours, which were obtained out at a pH of 9, a similar trend is observed in butanol production, which is higher in mixotrophic conditions. However, butyric acid production is notably favored by an alkaline pH, as demonstrated by our research, where levels of 2400 mg L^{-1} of butyric acid were reached in the mixotrophic fermentation.

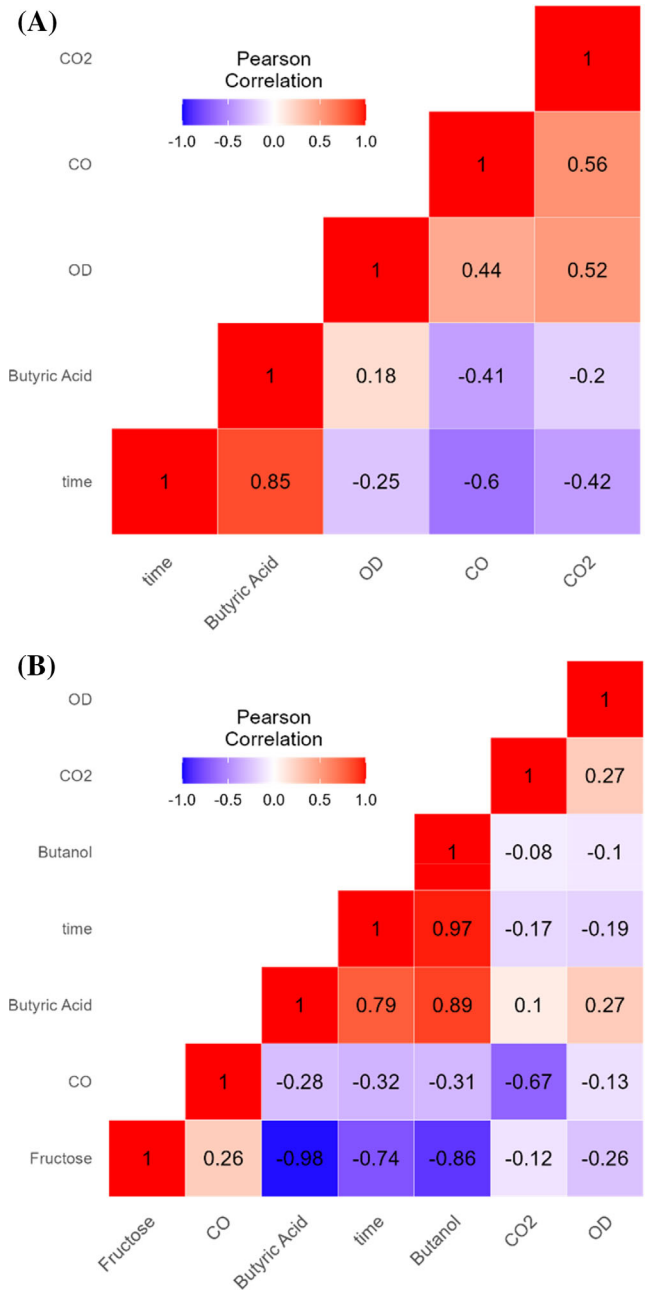

Figure 8. *Clostridium carboxidivorans*. Heatmap of correlations between variables of autotrophic (A) and mixotrophic (B) fermentation.

Figure 7 displays the transformation of carbon from the initial compounds (CO/CO_2/fructose) over a seven-day fermentation period for the two fermentation types studied. In autotrophic fermentation (Fig. 7(A)), it is clear that the headspace contains an equal distribution of CO and CO_2 carbons (50% each) at the start. By the end of the fermentation, these carbons have mostly been converted into butyric acid carbons (55%), with the CO_2 carbons constituting 22% and the remaining 23% still as CO. In mixotrophic fermentation (Fig. 7(B)), fructose carbons dominate at the beginning (80%), while CO and CO_2 carbons account for smaller portions (10% each). At the end of the fermentation, the fructose carbons had almost wholly been consumed, decreasing to 1%. The CO and CO_2 levels remain relatively constant due to the fed-batch process, which replenishes the headspace daily and halts consumption towards the end. This change produced butyric acid carbons (41%) and butanol carbons (33%). These findings indicate that autotrophic fermentation is highly effective at converting CO into butyric acid. In contrast, mixotrophic fermentation, although more efficient at entirely consuming fructose, generates various byproducts, such as butyric acid and butanol.

Figure 8 shows heat maps of *C. carboxidivorans* fermentations. The intensity of the correlation is depicted through colors, where red signifies a positive correlation, and blue denotes a negative correlation. Figure 8(A) illustrates the heat map of autotrophic fermentation. Notably, a robust positive correlation (85%) is observed between butyric acid and time, which is logical, as butyric acid displays a continuous increase throughout the fermentation period under study. Conversely, a slight positive correlation (<70%) is apparent between CO and CO_2, most likely attributable to the daily alteration of gas within the headspace. Additionally, weak negative correlations between CO and butyric acid are observed, which again may be influenced by the daily gas exchange. It should be noted that the biomass OD is not affected by time or by the increase in the concentration of butyric acid. Alternatively, Fig. 8(B) shows the heat map of mixotrophic fermentation. Three positive and three negative correlations are highlighted here. Regarding the positive correlations, it is evident, according to what has been observed in the previous figures, that both butanol and butyric acid increase over time and are strongly correlated as both products increase simultaneously with fermentation time. On the other hand, fructose negatively correlates with both fermentation products and time since this substrate is wholly consumed, favoring the appearance of fermentation products. It is worth noting in this case that C1-gases do not correlate with any of the study variables because the fermentation was working in a fed-batch system, and they are replaced daily so that no relationship can exist between them.

CONCLUSIONS

This study investigated the combined use of C1-gases and fructose in fed-batch fermentation to enhance the production of

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target compounds, focusing on *C. aceticum* and *C. carboxidivorans*. It found that combining gas fermentation with fructose increased microbial growth and ethanol/acetic acid in *C. aceticum*, while *C. carboxidivorans* showed higher butanol/butyric acid production. These productions highlight the potential of co-substrates to enhance C1-gas utilization, providing insights into microbial behavior under varied substrate conditions. Future work may concentrate on reactor optimization, continuous gas fermentation, improving fructose consumption, enabling CO_2 utilization without external H_2 and advancing sustainable biofuel and chemical production practices.

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DATA AVAILABILITY STATEMENT

Data are available upon request from the corresponding author.

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