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Clostridia as Promising Biofactory for Heterotrophic (Fructose) and Autotrophic (C1-Gas) Fermentation

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Abstract: This study compared the performance of *Clostridium ljungdahlii* and *Clostridium aceticum* in the fermentation of fructose and C1-gasses (CO, CO_2, N_2) to produce valuable products such as ethanol and acetic acid. In heterotrophic fermentation (fructose), *C. ljungdahlii* yielded high ethanol concentrations (350 mg/L) and acetic acid (500 mg/L), with optimal production at pH 8 on the first day of fermentation. Although autotrophic fermentation (C1-gasses) resulted in lower ethanol levels (200 mg/L), it remained a viable option. Conversely, *C. aceticum* predominantly produced acetic acid in both fermentation modes, with higher concentrations in the heterotrophic fermentation (1600 mg/L) than the autotrophic fermentation (380 mg/L). These findings demonstrate the versatility of both microorganisms for producing valuable metabolites. *C. ljungdahlii* shows promise for bioethanol production, while *C. aceticum* excels at generating acetic acid, a crucial component in bioplastics and various industrial processes.

Keywords: C1-gasses; Wood–Ljungdahl pathway; ethanol; acetic acid; *Clostridia* spp.

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1. Introduction

Carbon dioxide (CO_2) and carbon monoxide (CO), collectively known as one-carbon (C1) gasses, are two of the primary greenhouse gasses that significantly contribute to climate change. The escalating concentration of these gasses has emerged as a pressing concern for contemporary society [1]. These gasses predominantly originate from the combustion of fossil fuels, deforestation, and other human activities [2]. For example, a waste gas from an industrial combustion process is a mixture of CO (20–35%), CO_2 (20–30%), and mainly nitrogen (N_2, 50–60%) [3]. Global CO_2 emissions are alarmingly high, having recently reached a staggering 40.6 billion tons in 2022 [4]. And there is no indication that emissions will decline soon [5]. Consequently, an urgent demand is to develop effective and sustainable energy technologies that offer clean, affordable, safe, and customer-friendly fuel options to combat the escalating global warming crisis [6]. In parallel, innovative technologies are being explored to combat greenhouse gas emissions by converting them into valuable byproducts. These transformative processes encompass chemical catalysis [7,8] and gas fermentation [9,10].

Some acetogens, such as *Clostridium* spp., utilize the Wood–Ljungdahl metabolic pathway to transform CO_2 and CO into acetyl-CoA that serves as a key intermediate for the production of valuable compounds [11,12], including acetic acid and ethanol. The species commonly used in research include *Clostridium carboxidivorans* [13], *Clostridium aceticum* [14], and *Clostridium ljungdahlii* [15], which are considered non-pathogenic (class 1) strains. They could, therefore, be suitable for industrial-scale fermentation, as there is no biological safety level to consider. Of these microorganisms, *C. ljungdahlii* is a versatile producer, primarily

generating ethanol, but capable of synthesizing other valuable compounds, including acetic acid, formic acid, and 2,3-butanediol [16]. It is considered the model mesophilic acetogen, capable of growing autotrophically on H_2/CO_2 or CO and heterotrophically on fructose and xylose [17], as well as mixotrophically [18] and simultaneously consuming organic and inorganic carbon. Conversely, *C. aceticum* is highlighted for its high potential for acetic acid production from C1-gasses because it is reported as one of the first autotrophic acetogens capable of converting carbon dioxide and hydrogen to acetic acid and water in pure culture studies [19]. Moreover, it has been widely studied for its ability to grow autotrophically on CO and/or CO_2 + H_2 in fully automated bioreactor systems [20–22]. Although *C. aceticum* mainly produces acetic acid [14], recent studies explored the possibility of inducing ethanol production by manipulating fermentation conditions [22]. The ability of these microorganisms to ferment C1-gasses opens up new possibilities for the production of value-added products. Recent studies demonstrated that the operating conditions of the fermentation process can influence the production of specific compounds in *Clostridium* spp. For instance, solventogenesis, the production of such solvents as acetone and butanol, is triggered by metabolic stress conditions such as elevated C1-gas partial pressures or low pH levels [23]. In contrast, acidogenesis, producing organic acids like acetic acid and butyric acid, typically occurs at higher pH values [24].

The main objective of this study is to assess the feasibility of using C1-gasses (autotrophic fermentation) as a carbon source for producing industrially valuable metabolites (such as ethanol and acetic acid), being compared to different microorganisms (*C. ljungdahlii* and *C. aceticum*) and pH. These results are compared to the traditional method that uses fructose as a substrate (heterotrophic fermentation), which can be considered a reliable substrate that aligns with this study's focus on valuable metabolites [14,25]. Although existing studies address C1-gas compositions similar to those used in this research, a key distinction lies in the proposed process's absence of additional hydrogen sources. This unique aspect offers potential cost and process simplicity advantages, promoting a more sustainable approach to using C1-gasses in industrial fermentation, which could lead to new methods for integrating industrial processes and achieving a more efficient and sustainable utilization of available resources.

2. Materials and Methods

2.1. Microorganisms and Culture Media

The microorganisms *C. ljungdahlii* DSM 13528 and *C. aceticum* DSM 1496 from the German collection of microorganisms (DSMZ, Leibniz, Germany) were employed. The strain was reactivated by inoculating the lyophilized cells into a DSMZ liquid medium and grown for 24 h at 37 °C for *C. ljungdahlii* and 30 °C for *C. aceticum* in an orbital shaker (Optic Ivymen Systems, Comecta, Barcelona, Spain) following the recommended procedure of the DSMZ. Then, the strain was stored as glycerol stock (40% (*v*/*v*) sterile glycerol) at −80 °C until further use.

Autotrophic growth of the strain was carried out in septum bottles with a rubber septum, a 50 mL working volume, and a mixture of $CO:CO_2:N_2$ (20:20:60) as substrate. In contrast, fructose (about 25 g/L) was used for heterotrophic growth under anaerobic conditions, flushing nitrogen into the liquid. The composition of the liquid culture medium used for both strains was as follows (per liter 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), 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, and 0.5 mL of vitamin solution (containing (per liter distilled water): 20 mg of D-biotin, 200 mg of nicotinamide, 100 mg of *para-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 distilled water): 0.5 g of cysteine, 50 mL of $NaHCO_3$ (from a stock solution of 80 g/L), and 1 mL of $Na_2S{\cdot}9H_2O$ (from a stock solution of 240.2 g/L)).

All medium components (except for vitamins and the reducing solutions) were sterilized at 121 °C for 15 min in septum bottles (previously flushed with nitrogen into the liquid). In contrast, the vitamins and the reducing solutions were prepared separately and sterilized by filtration using 0.2 µm cellulose nitrate filters (Sartorius 254 stedim Biotech, Göttingen, Germany) to avoid possible compound degradation.

The cells were grown in a rotary shaker at 200 rpm for 24 h at 37 °C for *C. ljungdahlii* and 30 °C for *C. aceticum*.

2.2. Fermentation

Autotrophic and heterotrophic fermentations were performed for both strains at different initial pHs (5–9), and no pH control was employed during the fermentation. The operation conditions are summarized in Table 1. The fermentation studies were carried out in sealed bottles equipped with a rubber septum, each having a working volume of 50 mL (liquid culture medium described in Section 2.1. with the corresponding value of pH adjusted in each case) under rigorous anaerobic conditions. The operation temperature and agitation were optimal for each strain (Optic Ivymen Systems, Comecta, Barcelona, Spain).

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

Operational Condition	
Initial medium pH	5–6–7–8–9
Time (d)	0–7
Microorganism	*Clostridium ljungdahlii* DSM 13528 *Clostridium aceticum* DSM 1496
Main products	Ethanol Acetic Acid
Type of fermentation	Autotrophic (C1-Gasses) Heterotrophic (Fructose)

The bottles were sterilized at 121 °C for 15 min with the liquid culture medium without a calcium/vitamin solution, reducing solution, and fructose (in the case of heterotrophic fermentation). Once sterilized, the calcium/vitamin solution, the reducing solution, and fructose (in the case of heterotrophic fermentation) were added, flushing after nitrogen into the liquid. In the autotrophic studies, fructose was replaced with a mixture of C1-gasses ($CO:CO_2:N_2$) flushed after adding all solutions in the fermentation medium, reaching an overpressure of 0.2 bar. The inoculum loading was 10% *v*/*v*.

Liquid samples were taken every 24 h, centrifuged (at 13,500 rpm for 10 min) and analyzed for their content in fructose and fermentation products (ethanol, acetic acid, formic acid, acetoin, and 2,3-butanediol). On the other hand, to quantify the behavior of C1-gasses, 1 mL of a gaseous sample was taken every 24 h, and their composition, in terms of concentration of CO, CO_2, and N_2, was analyzed.

All fermentation tests were performed in duplicate.

2.3. Analytical Methods

High-Performance Liquid Chromatography (HPLC) was used to determine the content of fructose and fermentation products (ethanol, acetic acid, formic acid, acetoin, and 2,3-butanediol) in the liquid phase, using a refractive index detector (Waters 2414, Milford, MA, USA), an Aminex HPX-87H column (Bio-Rad, Alcobendas, Madrid) (at 60 °C), and 0.01 N of H_2SO_4 (0.6 mL/min) as mobile phase. The possible presence of other fermentation products was checked as well.

The gas composition in gaseous samples was determined using an 8860 GC gas chromatograph (GC, Agilent Technologies, Madrid, Spain) equipped with a thermal conductivity detector (TCD). The GC was fitted with a 15 m HP-PLOT Molecular Sieve 5A

column (ID, 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 liquid samples.

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

2.4. Data Analysis

The statistical software R (version 4.2.2.—Innocent and Trusting—2022) was employed to analyze the effects of time and pH on the fermentation experiments. Tukey's multiple-range tests analyzed the data to determine the statistically significant differences at a 95% confidence level ($p < 0.05$).

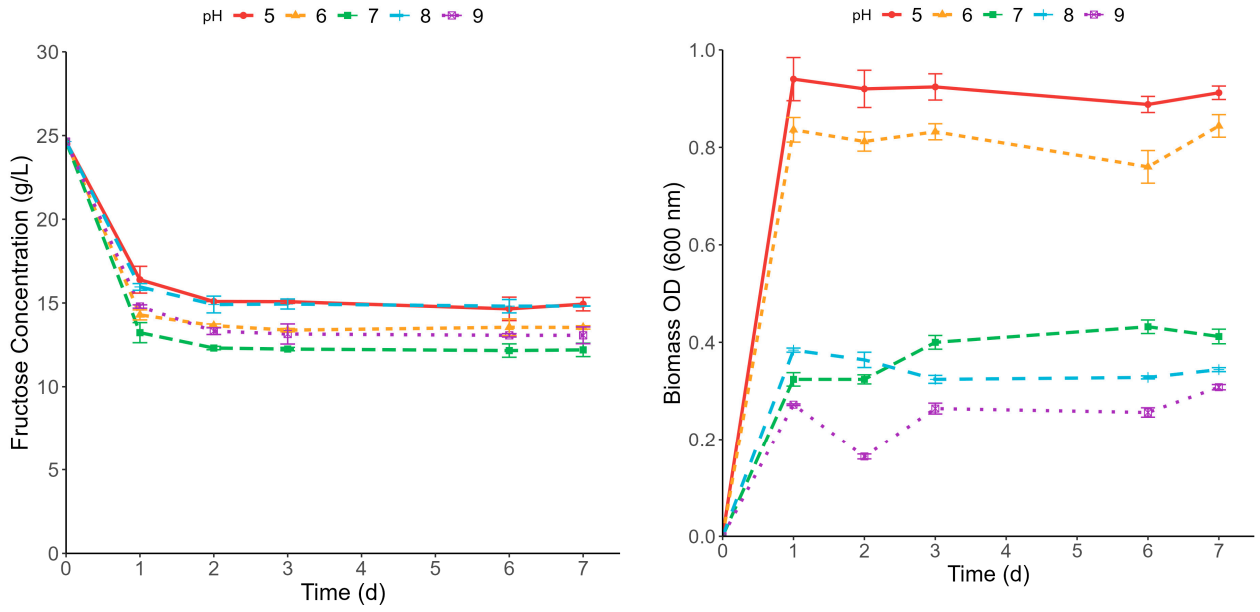
3. Results and Discussion

C. ljungdahlii and *C. aceticum* were studied comparatively in both heterotrophic and autotrophic fermentations, in order to assess the differences between both microorganisms and investigate the impact of the initial medium pH on fructose uptake, ethanol and acetic acid production, and biomass growth.

3.1. Heterotrophic Fermentation

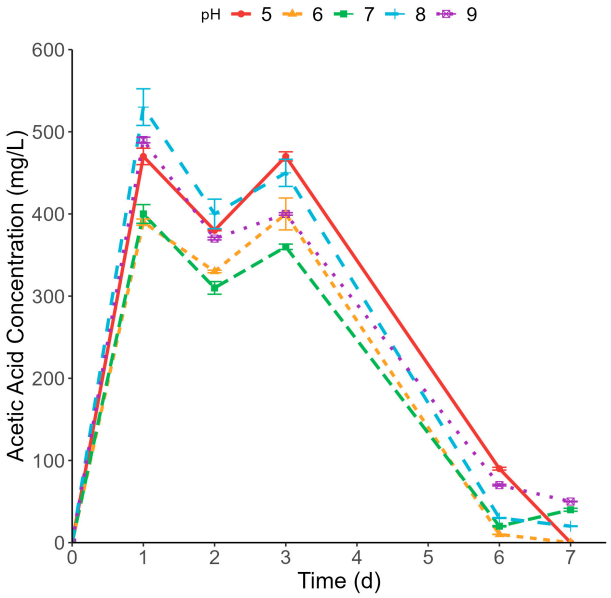
C. ljungdahlii and *C. aceticum* were studied using fructose as a substrate to understand its behavior at different pH levels (5, 6, 7, 8 and 9), which were not controlled during the experimental run.

Regarding the fructose uptake, in the case of *C. ljungdahlii*, it was around 10 g/L (Figure 1A). However, *C. aceticum* only consumed around 2 g/L of available fructose (Figure 2A). That is five times less fructose than *C. ljungdahlii* under the same conditions.



(**A**) (**B**)

Figure 1. *Cont.*



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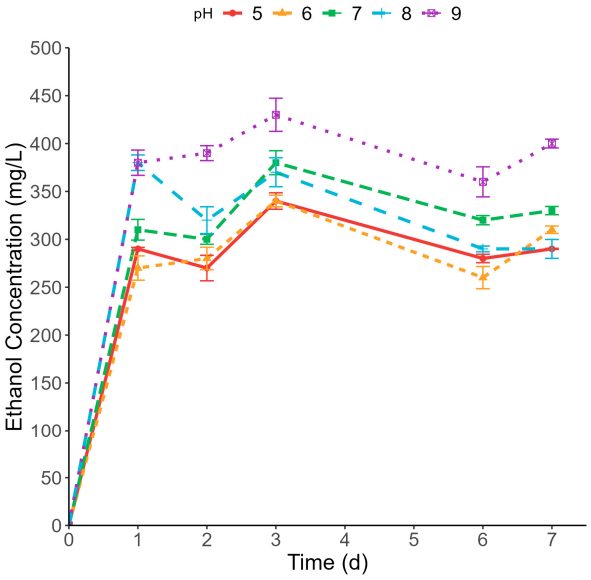
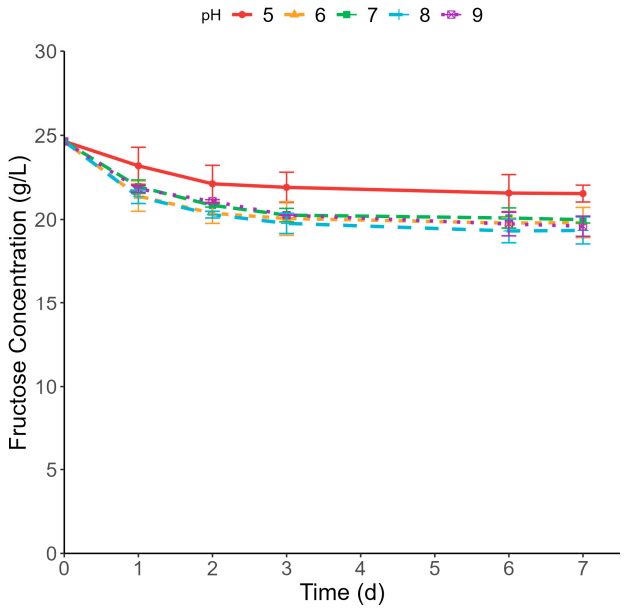
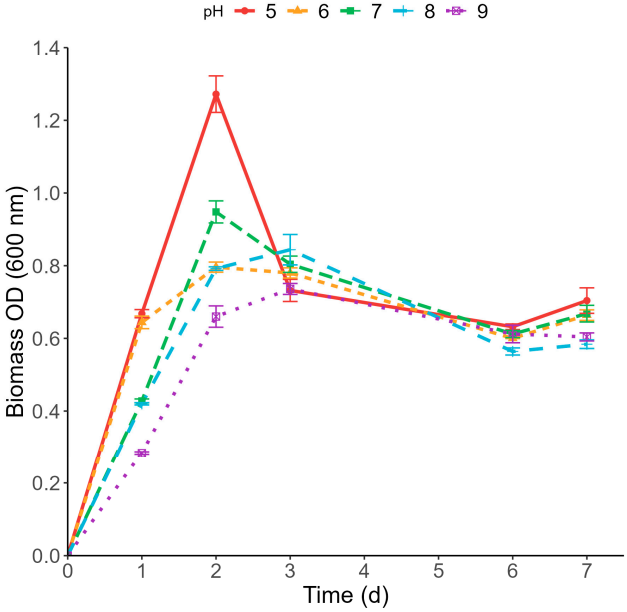


(**D**)

Figure 1. Fermentation kinetics by *Clostridium ljungdhalii:* Consumption of fructose (g/L) (**A**), evolution of biomass OD (**B**), and production of acetic acid (**C**) and ethanol (**D**) of heterotrophic fermentation at pH from 5 to 9. Error bars represent plus and minus standard deviation from average experimental results.



(**A**)



(**B**)

Figure 2. *Cont.*

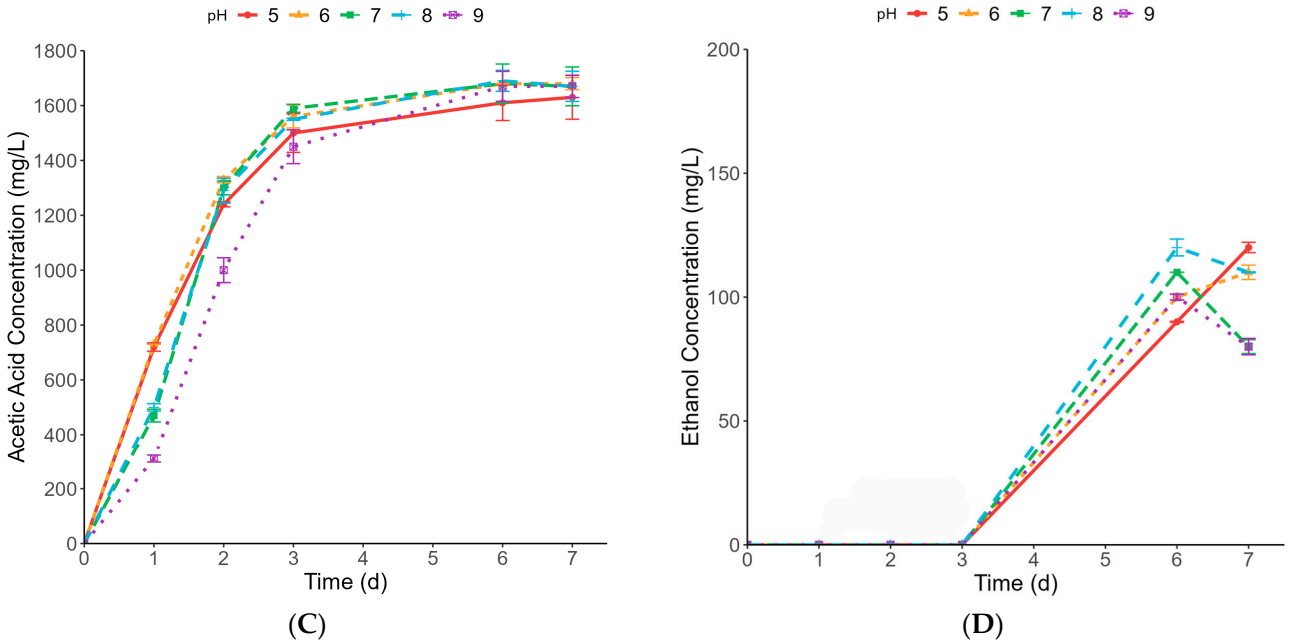


Figure 2. Fermentation kinetics by *Clostridium aceticum:* Consumption of fructose (g/L) (**A**), evolution of biomass OD (**B**), and production of acetic acid (**C**) and ethanol (**D**) of heterotrophic fermentation at pH from 5 to 9. Error bars represent plus and minus standard deviation from average experimental results.

Figures 1B and 2B show biomass OD for both *C. ljungdahlii* and *C. aceticum*, respectively. In general, a more acidic pH promotes strain growth [26,27]. In the case of *C. ljungdahlii* (Figure 1B), this action is observed at pH 5, where biomass OD reaches a value of around 0.95 after 1 d. In the same way, for *C. aceticum* (Figure 2B), pH 5 consistently exhibited superior growth performance for up to 2 days (~1.3). Beyond that point, all pH conditions showed a similar biomass OD of approximately 0.6, with no significant differences observed. A comparison of the biomass OD in *C. aceticum* with that of *C. ljungdahlii* reveals that *C. ljungdahlii* exhibits more excellent stability over time and achieves enhanced results at acidic pH ranges (5–6), attaining a biomass OD exceeding 0.8. However, their biomass OD performance at alkaline pH levels is not as remarkable, with values not exceeding 0.4. This observation might suggest that *C. aceticum* has a wider pH adaptability than *C. ljungdahlii*. Nevertheless, this adaptability also depends on the final products obtained.

Regarding acetic acid (Figures 1C and 2C), its formation by *C. ljungdahlii* peaked and then began to decline, eventually disappearing entirely by day six (Figure 1C), without increasing the ethanol concentration in the medium. To the metabolic pathway of *C. ljungdahlii*, acetic acid could have been reduced to obtain other intermediate products, like acetates or acetaldehydes [28], which could explain its disappearance in the fermentation broth [26,27]. However, it may also be due to the metabolic stress generated by the lack of CO or CO_2 in the gas phase of fermentation [16,23] since this microorganism is mainly autotrophic [15]. In contrast, when examining the behavior of *C. aceticum* in this type of fermentation, it becomes evident that *C. aceticum* primarily produces acetic acid (Figure 2C), reaching concentrations surpassing 1600 mg/L across all pH conditions at 7 days. This finding represents a threefold increase in the acetic acid yield obtained with the results obtained by *C. ljungdahlii* under identical conditions. It is worth noting that approximately 5 g/L of fructose was consumed during fermentation without producing any secondary metabolites. This observation might be attributed to the uncontrolled generation of acetic acid, leading to a decrease in pH that ultimately causes the demise of the bacteria involved [14]. Additionally, *C. aceticum* exhibits a distinct metabolic preference over *C. ljungdahlii* during heterotrophic fermentation with acidic pH, favoring acetic acid production over alcohol [14].

Considering the ethanol production for both *C. ljungdahlii* (Figure 1D) and *C. aceticum* (Figure 2D), *C. ljungdahlii* demonstrated a slight preference for basic pH environments. At pH 9, ethanol concentrations peaked at about 400 mg/L; at pH 5, ethanol levels only reached 300 mg/L (Figure 1D). It is worth noting that ethanol peaked and held steady after day one of fermentation. On the other hand, *C. aceticum* only starts producing ethanol on the third day, reaching a maximum concentration of 120 mg/L on the 7th day of fermentation (Figure 2D). This ethanol production, however, might be considered negligible due to its low concentration.

The ANOVA tables for ethanol and acetic acid production for both *C. ljungdahlii* and *C. aceticum* microorganisms are shown in Tables 2 and 3, respectively. In the case of *C. ljungdahlii* (Table 2), the main effects of time (p-value < 0.05) and pH (p-value < 0.1) significantly affected ethanol production by *C. ljungdahlii* on fructose. A low initial pH had a negative effect on ethanol production. However, Cotter et al. [29] noted that transferring dense cultures to lower pH media without waiting for significant growth has improved solvent production. In the case of this study, a large amount of ethanol was not produced, but *C. ljungdahlii* has produced other compounds such as formic acid, acetoin, and 2,3-butanediol (Table S1). The main effect of time significantly affected acetic acid production by *C. ljungdahlii* on fructose (p-values < 0.05). Although there are no significant differences between the acetic acid concentrations obtained at the different pH levels studied, the highest levels of acetic acid production were seen at pH 8 on the first day of fermentation (around 530 mg/L).

Table 2. *Clostridium ljungdahlii*: ANOVA tables for product concentrations obtained.

Fermentation	Product		DF	Mean Square	F-Value	Pr (>F)
Heterotrophic	Ethanol	pH	1	45,630	3.556	0.065
		time	1	239,966	18.702	0.000
		pH:time	1	511	0.040	0.843
	Acetic Acid	pH	1	1920	0.054	0.816
		time	1	412,727	11.708	0.001
		pH:time	1	5	0.000	0.991
Autotrophic	Ethanol	pH	1	213	0.058	0.81
		time	1	128,959	35.304	0.000
		pH:time	1	288	0.079	0.78
	Acetic Acid	pH	4	177	1.202	0.322
		time	1	12,313	83.752	0.000
		pH:time	4	21	0.142	0.966

Table 3. *Clostridium aceticum:* ANOVA tables for product concentrations obtained.

Fermentation	Product		DF	Mean Square	F-Value	Pr (>F)
Heterotrophic	Ethanol	pH	1	53	0.123	0.727
		time	1	118,374	272.800	0.000
		pH:time	1	193	0.444	0.508
	Acetic Acid	pH	4	34,940	0.270	0.896
		time	1	17,415,114	134.427	0.000
		pH:time	4	17,317	0.134	0.969
Autotrophic	Acetic Acid	pH	4	4198	1.196	0.327
		time	1	806,962	232.685	0.000
		pH:time	4	2897	0.835	0.511

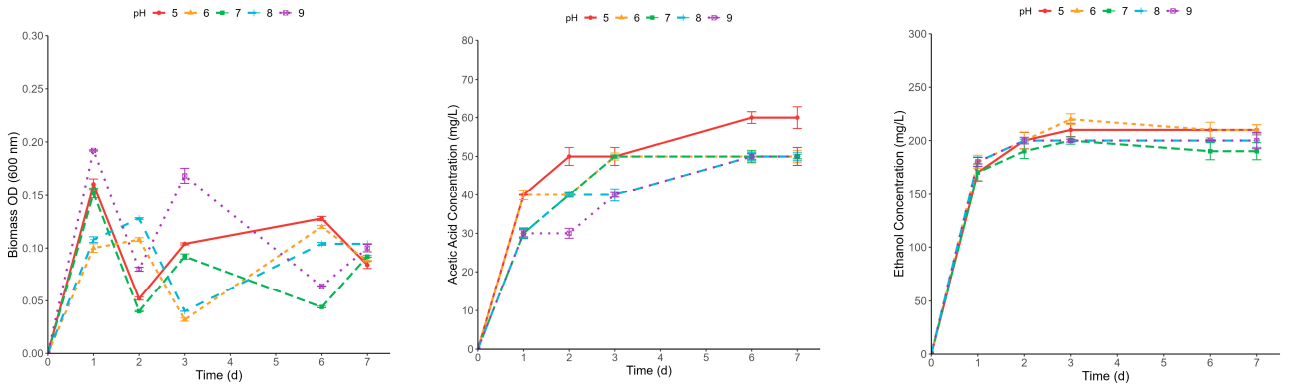
On the other hand, the ANOVA results for ethanol and acetic acid production by *C. aceticum* revealed that the fermentation time was the only significant parameter (p-value < 0.05) across both metabolites, as indicated in Table 3. Notably, a fermentation duration of at least 6 days is necessary to achieve a sufficiently high concentration of acetic acid (1600 mg/L). The ANOVA results also showed no significant main or interaction effects for the pH. This observation is consistent with the trends observed in Figure 2C,D, where the fermentation outcomes for each pH condition overlap, indicating the minimal impact of pH on producing these metabolites.

In summary, comparing both *C. ljungdahlii* and *C. aceticum* microorganisms in heterotrophic fermentations, *C. ljungdahlii* proved to be a very interesting microorganism that can succeed under heterotrophic conditions, exhibiting exceptional metabolic flexibility. It can utilize fructose as a substrate to generate diverse products, including ethanol and acetic acid. The acidity of the fermentation medium significantly affects product formation, with an acidic pH (5) favoring acetic acid production and a basic pH (9) favoring ethanol production. Moreover, *C. ljungdahlii* demonstrates superior ethanol production efficiency, reaching ethanol concentrations of up to 350 mg/L and acetic acid concentrations of up to 500 mg/L at pH 8 on the first day of fermentation, translating an ethanol yield and productivity of 44 mg/g and 127 mg L^{-1} d^{-1}, respectively, and acetic acid yield and productivity of 61 mg/g and 1559 mg L^{-1} d^{-1}, respectively (Table S2). On the other hand, *C. aceticum* displayed an evident superior acetic acid production capacity. *C. aceticum* achieved concentrations exceeding 1600 mg/L across varying fermentation pH conditions, achieving considerable yields between 310 and 530 mg/g and productivities between 230 and 280 mg L^{-1} d^{-1} (Table S2). Additionally, a modest amount of ethanol was produced following the third day of fermentation, reaching concentrations of 120 mg/L. In addition, the microorganism used in our study consumes a similar amount of fructose (~5 g/L, Table S3) as that reported by Arslan et al. [14]. However, the acetic acid concentration is lower, as these authors obtained 5000 mg/L, while 1600 mg/L was achieved here. Some authors indicated that the acetic acid production in heterotrophic fermentation could be harmed due to the formation of inhibitory byproducts, such as formic acid [20], and a change in the metabolic behavior of the microorganism in the presence of fructose [30].

3.2. Autotrophic Fermentation

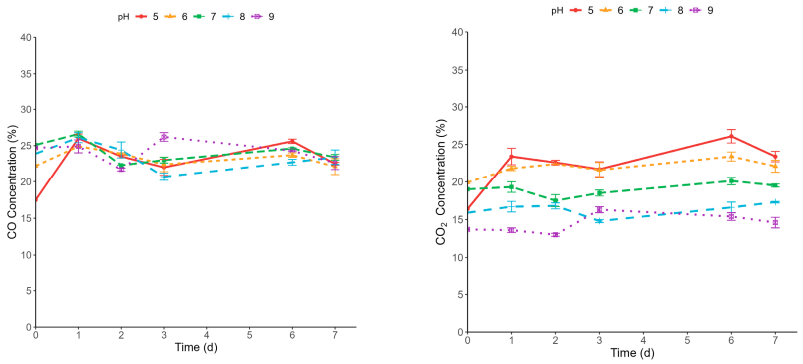
After the heterotrophic study, both *C. ljungdahlii* and *C. aceticum* were studied using a C1-gas mixture as a substrate to compare its behavior with the fructose using a different initial medium pH.

The first significant difference is the growth of the microorganisms (Figures 3A and 4A). In the case of *C. ljungdahlii*, the biomass OD was around 0.2 in all cases during the autotrophic fermentation. The difference between growing the microorganism with one substrate or another depends on its mechanisms for carbon fixation, which are not very efficient when gasses are used as a substrate [29]. For *C. aceticum*, in general, a basic pH under these conditions promotes acetogenesis, and solventogenesis remains inactive until there is a substantial acid accumulation in the fermentation broth, owing to the accompanying pH drop [22,31,32]. This action aligns with the case study's findings, demonstrating that basic pH conditions favor biomass growth (Figure 4A) and acetic acid production (Figure 4B).



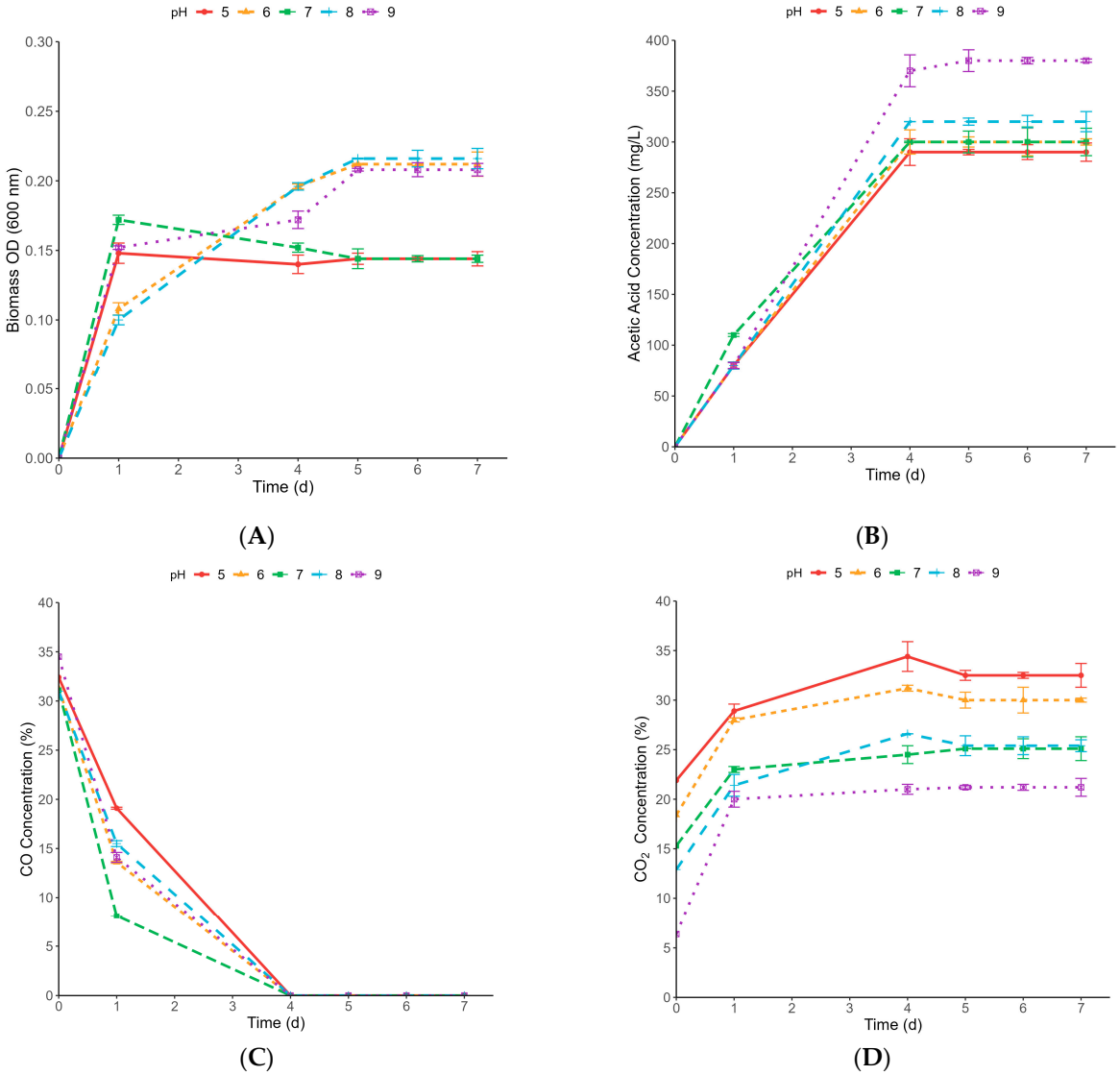
(**A**) (**B**) (**C**)

Figure 3. *Cont.*



(**D**) (**E**)

Figure 3. Fermentation kinetics by *Clostridium ljungdahlii:* Evolution of biomass OD (**A**), production of acetic acid (**B**) and ethanol (**C**), and evolution of CO (%) (**D**) and CO_2 (%) (**E**) of autotrophic fermentation at pH from 5 to 9. Error bars represent plus and minus standard deviation from average experimental results.



(**A**) (**B**)

(**C**) (**D**)

Figure 4. Fermentation kinetics by *Clostridium aceticum:* Evolution of biomass OD (**A**), production of acetic acid (**B**), and evolution of CO (%) (**C**) and CO_2 (%) (**D**) of autotrophic fermentation at pH from 5 to 9. Error bars represent plus and minus standard deviation from average experimental results.

Regarding acetic acid (Figures 3B and 4B) and ethanol production (Figures 3C and 4C) for both *C. ljungdahlii* and *C. aceticum* microorganims, the ethanol production by *C. ljungdahlii* reached a concentration of up to 200 mg/L with lower pHs (Figure 3C), but acetic acid did not exceed 60 mg/L (Figure 3B). This result was expected, since, during the solventogenic phase, acidic pH and low substrate levels favor ethanol production without producing a relevant concentration of acetic acid [16,23,29]. Comparing the products obtained with C1-gasses and fructose, C1-gasses cannot reach the same or higher concentrations of ethanol and acetic acid, but at the same time, only formic acid was produced as a byproduct (Table S3), and, even so, its concentration is less than the obtained in the case of heterotrophic fermentation. Possibly, this result is due to the carbon fixation and biodisponibility of the substrate. Considering the Wood–Ljungdhal metabolic pathway, CO can be converted into ethanol and CO_2 when CO is dissolved in water [23]. On the other hand, in the case of *C. aceticum*, acetic acid concentrations peak at pH 9, surpassing 380 mg/L, while other pH ranges (5–8) also yield respectable concentrations (300 mg/L) (Figure 4B). Furthermore, the fermentation remained predominantly in the acetogenic phase, as evidenced by the absence of ethanol production. This result can be attributed to the limited availability of substrate.

Concerning the CO (Figures 3D and 4C) and CO_2 (Figures 3E and 4D) evolution by both *C. ljungdahlii* and *C. aceticum* fermentations, in the case of *C. ljungdahlii*, as shown in Figure 3E, the CO_2 remains constant because H_2 is needed to use CO_2 as a substrate [29]. Still, the CO decreases slightly in the headspace (Figure 3D) since, as expected, it dissolves in the fermentation broth and transforms into the products of interest. However, the solubility of CO depends on such diverse factors as temperature, working volume, pressure, liquid–gas equilibrium, and even the number of cells in the fermentation broth [33]. On the other hand, when *C. aceticum* was used, as shown in Figure 4C, the amount of CO available in the medium diminishes substantially, and, by the fourth day of fermentation, all the CO has been consumed. This result coincides with the day of maximum biomass OD (Figure 4A) and the peak of acetic acid production (Figure 4B). Additionally, the accumulation of CO_2 in the headspace (Figure 4D) further supports the notion that fermentation proceeds efficiently, as CO_2 is a byproduct of fermentation. On the contrary, a comparison of both heterotrophic and autotrophic fermentations conducted with *C. aceticum* reveals that fructose yields up to 4.5 times more acetic acid than the C1-gas mixture, highlighting the inherent substrate advantage of fructose. However, it is essential to acknowledge that, with an increased availability of gasses, possibly through continuous fermentation instead of batch mode, the acetic acid production in autotrophic fermentation could also be enhanced.

Tables 2 and 3 summarize the ANOVA results for ethanol and acetic acid production by both *C. ljungdahlii* and *C. aceticum* microorganisms on C1-gasses. Firstly, with regard to *C. ljungdahlii*, both products (ethanol and acetic acid) were only significantly affected by the operation time (p-value < 0.05) between 0 and 24 h. After 24 h, no significant differences existed between the results obtained at all the pHs studied. This finding means that this microorganism can work at any pH without penalizing the final concentrations (mainly ethanol), and that, with the C1-gasses used, fermentation takes 24 h. On the other hand, in fermentations by *C. aceticum*, the ANOVA results for acetic acid production showed that the only significant factor (p-value < 0.05) was the fermentation time, as shown in Table 3, and not the pH, as one might expect. However, as discussed above for *C. aceticum*, if there had been a more significant amount of CO during fermentation, the pH would perhaps be a more determining factor, as it is when autotrophic fermentation was conducted with pure CO [14,22,34].

In brief, comparing both *C. ljungdahlii* and *C. aceticum* microorganisms in autotrophic fermentations, *C. ljungdahlii* showed to be also a very interesting microorganism that can succeed under autotrophic conditions. With no significant consumption ($p > 0.05$) of CO and CO_2 observed throughout fermentation, the peak ethanol production by *C. ljungdahlii* is attained after 3 days at pH 6, reaching 200 mg/L, equivalent to a productivity of 73 mg L^{-1} d^{-1} (Table S2). On the other hand, acetic acid production gradually increases

with the fermentation time, albeit insignificantly, as it never surpasses 100 mg/L, with productivities lower than 20 mg L^{-1} d^{-1}. Our research findings on ethanol production in autotrophic fermentation are comparable to those reported by Cotter et al. [29]. These authors achieved an ethanol concentration of 230 mg/L after 7 days of continuous fermentation (at pH 5) with syngas containing 20% CO. This research study, however, obtained a concentration of 200 mg/L after 2 days of batch fermentation using a gas containing 20% CO. This difference in approach offers a significant advantage: comparable results can be achieved without continuous operation, leading to reduced gas consumption and, consequently, lower resource utilization. Additionally, unlike in Cotter's study, the gas mixture used in this study does not contain hydrogen, highlighting that it does not rely on syngas, which typically requires more complex sourcing and handling. This novel method could pave the way for more efficient and sustainable ethanol production in autotrophic fermentation.

In contrast, autotrophic fermentation by *C. aceticum* yielded significantly higher acetic acid production, with the maximum concentration being 380 mg/L after 5 days of fermentation at pH 9, corresponding to productivity of 76 mg L^{-1} d^{-1} (Table S2). The results show a reasonable agreement with those reported by Arslan et al. [14] despite differences in fermentation methods and gas composition. Arslan et al. [14] attained an acetic acid concentration of 3000 mg/L after 7 days of continuous fermentation with CO. In this study, the acetic acid concentration was 380 mg/L after 7 days of batch fermentation using a gas mixture containing 20% CO. This finding suggests that the efficiency of CO conversion to acetic acid remained consistent despite some fluctuations in concentration levels. The observed variations could be due to the depletion of the gas supply by the fourth day in this study, impacting the continued production of acetic acid. However, despite this limitation, the CO consumption-to-acetic acid production ratios maintained a steady relationship throughout the fermentation period. This consistency implies that, while the overall gas supply might have influenced production, the underlying process efficiency remained stable, reflecting a reliable correlation between CO usage and acetic acid yield.

4. Conclusions

The results of this study demonstrate that *C. ljungdahlii* and *C. aceticum* are versatile microorganisms that can be used to produce industrially valuable products from autotrophic and heterotrophic fermentation. *C. ljungdahlii* can utilize fructose to produce ethanol and acetic acid. Ethanol production is more efficient at alkaline pH (9), while acetic acid production is more efficient at acidic pH (5). *C. ljungdahlii* can also utilize C1-gasses to produce ethanol, with a peak ethanol production of 200 mg/L after 2 days of fermentation. *C. aceticum* produces significantly higher acetic acid concentrations from fructose (up to 1600 mg/L) than C1-gasses (up to 380 mg/L). *C. aceticum* also exhibits superior biomass growth at acidic pH (5). Fermentation conditions should be further optimized in future work to improve metabolite production and C1-gas consumption. Co-substrates or co-cultures should also be investigated to improve metabolite yields. Additionally, continuous fermentation processes should be developed for cost-effective production.

Supplementary Materials: The following supporting information can be downloaded at the following: https://www.mdpi.com/article/10.3390/fermentation10110572/s1, Table S1: *C. ljungdahlii*: Fructose consumed in the heterotrophic fermentation and other produced compounds; Table S2: Yield (mg product/g fructose consumed) and productivity (mg product/(L·d)) of maximum concentration of ethanol and acetic acid obtained in heterotrophic and autotrophic fermentation using *C. ljungdahlii* and *C. aceticum*; Table S3: *C. ljungdahlii*: Other produced compounds in the autotrophic fermentation.

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