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Improving Carbon Fixation and Acetate Production from Syngas Fermentation: On-Demand Versus Continuous Feeding

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Abstract

Syngas fermentation is a promising carbon capture and utilization (CCU) technology for producing carboxylic acids while transforming low-cost waste gas into high-value products. This study evaluates the two bioreactor feeding strategies for synthesis gas (syngas) fermentation by *Eubacterium callanderi* (formerly *Butyribacterium methylotrophicum*) strain Marburg—on-demand feeding (ODF) and continuous feeding (CF)—with a synthetic syngas mixture of 23 vol% CO_2, 29 vol% CO, 32 vol% H_2, and 16 vol% CH_4, mimicking the syngas from lignocellulosic gasification. The ODF assay achieved a maximum syngas consumption rate of 112 mL/h, yielding 24.1 g/L acids, namely 22.9 g/L acetate and 1.3 g/L butyrate. CF of syngas at 223 mL/h required more gas (62.9 L) to produce 22.7 g/L total acids, from which 19.0 g/L acetate and 3.7 g/L butyrate were achieved. The CF-specific production rate ($g_{product}/g_{dry\_cell\_weight}$/hour) reached 0.5 g/g_{DCW}/h (acetate) and 0.17 g/g_{DCW}/h (butyrate), outperforming ODF with 0.3 and 0.02 g/g_{DCW}/h, respectively. ODF minimized gas wastage and enabled CH_4 accumulation inside the bioreactor up to approximately 78 vol%, while CF led to CO_2 accumulation, indicating a need for more efficient CO_2 utilization strategies, such as sequential fermentations. This work highlights the critical impact of the two feeding options studied with regard to scaling up the carbon-efficient production of carboxylic acids, and indicates that both strategies can have potential applications. ODF is ideal for increasing carbon fixation and achieving, simultaneously, gas cleaning, while CF fermentations are better suited to maximizing the acid production rate.

Keywords: acetogen; *Eubacterium callanderi* Marburg; *Butyribacterium methylotrophicum*; carboxylic acid; methane; carbon capture and utilization

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1. Introduction

The path towards decarbonization requires a multiangled approach, where several strategies must be followed simultaneously to guarantee that energy and societal transitions do not translate into deindustrialization and the loss of competitiveness, ensuring, instead, a quick and just response to climate change. In that sense, anaerobic C1 gas fermentation is a promising approach that can contribute to the decarbonization of the economy by reducing both direct emissions and fossil dependency [1–3]. In this process, carboxydotrophic acetogens use carbon monoxide (CO), and carbon dioxide and hydrogen (CO_2 + H_2) from a gas stream, as carbon and energy sources, through the Wood–Ljungdahl pathway, while producing biomass and a diversity of products with industrial application, such as short-chain volatile fatty acids (i.e., acetic acid and butyric acid) or alcohols (i.e., ethanol and

butanol) [4–6]. This process is a form of carbon capture and utilization (CCU) that results in the reduction in direct emissions, as well as in the production of molecules that can replace fossil-based compounds, turning what would otherwise be a discarded residue gas stream into an important source of raw materials [7]. Furthermore, compared to other more widespread technologies of carbon utilization, such as chemical synthesis, gas fermentation presents several advantages. It allows for the conversion of CO and CO_2 into platform chemicals at low temperatures and pressures, does not require organic solvents, metal catalysts, or complex gas concentration procedures, tolerates variable gas compositions, and, with the help of molecular engineering tools or manipulation of culture conditions, it can be tuned to produce different compounds to fit market needs [8–12]. While not as widespread as other carbon capture technologies, C1 gas fermentation has already seen implementation at pilot and demonstration scales in several installations, and even at commercial scale at Lanzatech's syngas fermentation plants in both China and Belgium [5,12]. However, it is still a process that has mostly been explored in niche applications.

In recent years, most studies on C1 fermentation have been focused on the strains of *Neomoorella thermoacetica* (formerly *Moorella thermoacetica*), *Clostridium carboxydivorans*, and *Clostridium ljungdahlii*, as the characteristics of these strains, coupled with the early availability of genetic manipulation tools, have enabled the study and manipulation of the metabolic pathways [12–16]. However, several microorganisms that use the Wood–Ljungdahl pathway have been described, many of which have shown potential for future application in industrial processes [5]. One such microorganism is *Eubacterium callanderi* strain Marburg, previously classified as *Butyribacterium methylotrophicum* strain Marburg [17]. Originally described by Zeikus et al. in 1980, this obligatory anaerobe is a mesophilic acetogenic strain that uses CO and CO_2 + H_2 as carbon and energy sources, producing mainly acetic and butyric acids as a fermentation product [17–20]. This strain has been used in different studies, and most works have been centered on its use for synthesis gas, or syngas, fermentation [19,21–28]. Syngas is the main product from the gasification process and is mostly composed of CO, H_2, and CO_2 at variable concentrations that depend on the gasification conditions and feedstock. This composition makes it an ideal substrate for acetogenic bacteria, especially if resulting from the gasification of sustainable or residual biomass [20,29–31]. However, gasification syngas can also contain CH_4 and low concentrations of small-chain hydrocarbons (C_nH_n), NH_3, H_2S, and other impurities (e.g., cyanides). Some of the latter are known inhibitors of microbial activity, which lead to the need for complex and exhaustive gas cleaning procedures before the fermentation stage [20]. In this area, the Marburg strain has demonstrated significant potential, as it could grow unaffected on "real syngas" from the gasification of technical lignin (a residue that is generated from second generation bioethanol production) with little purification and could simultaneously produce both butyric and acetic acids [19]. In fact, strain Marburg was shown to be not only highly resistant to many the syngas impurities, but to even thrive in their presence [19,20]. This is a clear advantage for future industrial applications, as it reduces the number of headspace cleaning stages, making the process cheaper and facilitating direct integration.

One important limitation in syngas fermentation is the low solubility of the gaseous substrate in the liquid fermentation media. This restricts mass transfer and becomes one of the bottlenecks that reduces the overall conversion rates [31–34]. One of the most common strategies to mitigate this issue is to increase the gas feeding rates, thereby compensating for the low solubility through increased agitation and replacement rates. While this results in greater biomass production and product formation, it also leads to lower carbon sequestration because large increases in dilution rate outpace the cells' capacity for carbon metabolism. This reduces the efficacy of syngas fermentation as a carbon sequestration technology [35].

A new syngas strategy was tested in a previous study [19], which did not rely on continuous gas inflow. Instead, it leveraged the pressure differential generated inside the bioreactor—caused by cellular gas consumption—to enable a form of sequential, on-demand syngas feeding. In this approach, the gas feeding rate was directly linked to the rate of carbon metabolization by the cells. Using the on-demand syngas feeding strategy, *Eubacterium callanderi* strain Marburg was able to achieve up to 88 mol% of carbon fixation from lignin-derived syngas, with the total depletion of CO and 71 mol% of CO_2 fixation. These values were comparable to those obtained with synthetic syngas, despite the presence of inhibitory impurities. The syngas used in these assays had a significant percentage of methane (CH_4) and it became apparent that strain Marburg was unable to metabolize it, leading to its accumulation in the headspace of the reactor. This revealed an opportunity to develop a syngas cleaning method that strips the syngas stream of other gas components, increasing the relative concentration of methane, and simultaneously producing platform chemicals from the CO and CO_2 + H_2 components without the need for intensive cleaning steps.

This study aims to compare the application of the on-demand feeding (ODF) mode to syngas fermentation with the more commonly used strategy of continuous gas feeding (CF) at constant rate, in order to better understand their respective advantages and limitations. In particular, it aims to compare the effects on syngas consumption profiles, carbon fixation, and acid production yields, and to assess the potential need for complementary strategies such as syngas recirculation or sequential batch operations.

2. Materials and Methods

2.1. Microorganism, Culture Media, and Syngas Composition

The bacterium used for this study was *Eubacterium callanderi* strain Marburg, previously classified as *Butyribacterium methylotrophicum* strain Marburg (DSM 3468, DSMZ, Braunschweig, Germany) [5,17,19,20]. The culture medium used for culture maintenance and all experimental assays was the Syn1 culture medium, as described in Pacheco et al. (2021), using 50 mM phosphate buffer at pH 7.0 as a pH stabilizer [19]. For the maintenance of syngas-adapted cells, these were transferred into a new flask with fresh culture medium every four days. These flasks contained the "four-component" commercial syngas from Air Liquide (Paris, France) with the following composition: 18 vol% H_2, 26 vol% CO, 19 vol% CO_2, and 37 vol% N_2, and the culture medium was inoculated at 2% (*v*/*v*), being incubated at 37 °C, 150 rpm in an orbital shaker. This syngas formulation was selected since it had already proven effective for long-term strain preservation in previous works [19,20].

For the bioreactor assays, the "four-component" commercial syngas, also from Air Liquide (Paris, France), with the same composition obtained from the gasification of technical lignin was used, as follows: 32 vol% H_2, 29 vol% CO, 23 vol% CO_2, and 16 vol% CH_4. The bioreactor was inoculated with 8%(*v*/*v*) of *E. callanderi*, which was pre-cultured at 37 °C, 150 rpm for 48 h, and a 2 M NaOH solution was used to maintain the pH at 7.0, when needed.

2.2. Experimental Set-Up

Strain Marburg was grown in a 2 L airtight glass bioreactor equipped with a pH sensor (405-DPAS–SC–K8S/250, Mettler Toledo, Columbus, OH, USA) and controller (Black Stone BL 931700 pH controller, Hanna Instruments, Germiston, South Africa), and all the necessary inlets/outlets for pH control, pressure measurement (LabQuest2 with gas pressure sensor, Vernier, Beaverton, OR, USA), syngas feed, liquid sampling/inoculum addition, and syngas outlet (where post-fermentation gas was collected for analysis). The initial operating volume used was 800 mL of Syn1 culture medium [19,20], and the cells

grew at pH 7.0 and 37 °C, until the syngas consumption rate lowered to values close to 0 mL/h.

For this work, two distinct syngas feeding modes were selected: on-demand syngas feeding (ODF) and continuous syngas feeding (CF). ODF was performed as previously described in Pacheco et al. (2021) [19]. This syngas feeding mode involved connecting an H_2 tight gas sampling bag (SKC 263-03, Standard FlexFoil® series with stainless steel fittings, SKC Inc., Eighty Four, PA, USA) filled with syngas to the bioreactor. All inlet and outlet ports were clamped to ensure the vessel was airtight. The only unclamped tube was the syngas inlet, allowing fresh syngas to flow from the bag into the bioreactor when the internal pressure of the glass vessel dropped sufficiently. The syngas inflow was monitored using an inline flow meter (μFlow, Bioprocess Control, Stockholm, Sweden) connected to the gas sampling bag. However, an improvement to the described method was the addition of headspace cleansing, which was performed approximately every 24 to 48 h, to minimize the effect of CH_4 and CO_2 accumulation in the reactor headspace, and no culture medium replacement was performed. Liquid and gas samples from the culture medium and reactor headspace, respectively, were taken before the headspace cleansing procedure.

As for the CF, the bio-reactor set-up is shown in Figure 1.

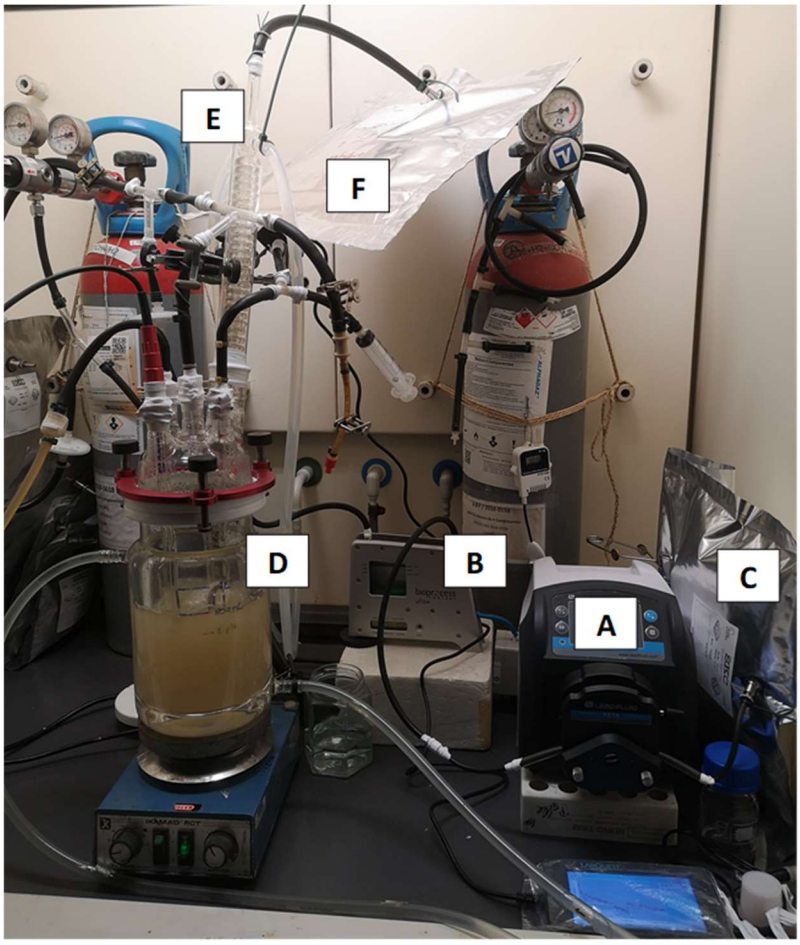

Figure 1. Representation of the bioreactor set-up used in the continuous syngas feeding assays (CF). Variable flow peristaltic pump (A), inline flow meter (B), H_2 tight gas sampling bag—syngas-in (C), glass vessel (D), water condenser (E), and H_2 tight gas sampling bag—post-fermentation gas (F).

A variable flow peristaltic pump (A) with Viton® tubing (Watson-Marlow, Falmouth, UK) was placed before the inline flow meter (μflow, Bioprocess Control, Sweden) (B) and connected to an H_2 tight gas sampling bag (SKC 263-03 Standard FlexFoil® series with stainless steel fittings, USA) filled with syngas A (C). This pump allowed a continuous flow

of syngas into the bioreactor for conversion by strain Marburg (Behringwerke, Marburg, Germany), while unconverted syngas flowed from the vessel (D), through a water condenser at 10 °C (E) to reduce volume fluctuations, to another H_2 tight gas sampling bag (F). The inlet and outlet gas sampling bags were replaced every 24 h. The outlet gas sampling bag was processed for gas composition and volume measurement. Liquid samples were also taken at the same time.

To compare the CF with ODF, two flow rates were selected to be tested during the CF mode, based on the maximum syngas consumption rate obtained on the ODF assay (112 mL/h). A rate equal to the maximum syngas consumption rate observed in the ODF assay was selected to evaluate the influence of the feeding method (CF and ODF) on key fermentation parameters. In addition, a higher rate, approximately double the maximum ODF consumption rate, was chosen to test the cells' ability to sustain faster growth. Due to limitations in tubing diameter and the pump used in this experiment, the lowest rate achieved during CF was 141 mL/h instead of the intended 112 mL/h, and the highest was 223 mL/h instead of 224 mL/h.

2.3. Analytical Methods

Microbial growth was monitored through optical density at 600 nm (Thermo Fisher Scientific spectrophotometer, Genesys 20, Thermo Fisher Scientific, Waltham, MA, USA), while the dry cell weight (DCW) was quantified according to official methods of analysis [36]. Acetic and butyric acids were quantified through high-performance liquid chromatography (HPLC) with a Biorad Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) at 35 °C, in an Agilent 1260 Infinity II (Agilent Technologies, Santa Clara, CA, USA) chromatographer equipped with refractive index and diode array detectors. To elute the samples, 0.5 mM H_2SO_4 was used at a flow rate of 0.6 mL/min. Solutions of the carboxylic acids with different concentrations were used as external standards. Gas samples were analyzed through gas chromatography (GC) in an Agilent/HP 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a gas sampling valve, two column packings (Molecular Sieve 5A and Porapak Q both from Agilent Technologies, Santa Clara, CA, USA), and two detectors (thermal conductivity detector and flame ionization detector) mounted in sequence. After injection, the oven temperature was kept at 40 °C for 17 min and then increased to 185 °C for 43 min, with a heating rate of 15 °C/min. Argon was the carrier gas, at a constant flow of 18.5 mL/min. Readings were performed under the following standard conditions of temperature and pressure: a temperature of 25 °C (293.15 K) and absolute pressure of 1.0×10^5 Pa.

2.4. Calculations

The molar concentrations of CO, H_2, and CO_2 were calculated from the GC analysis using the Peng–Robinson equation [37]. The concentrations of acetate and butyrate were calculated from a linear regression of standard peak area vs. standard concentration. The percentage of carbon fixation was calculated as described in Pacheco et al. (2021), and is the amount of CO and CO_2, in mol, that was used by the cells to form products and incorporated as biomass per mol of the CO and CO_2 fed, being estimated as follows in Equation (1) [19]:

$$\text{Carbon fixation}(\%) = \frac{\text{n}(\text{CO} + \text{CO}_2)_\text{i} - \text{n}(\text{CO} + \text{CO}_2)_\text{f}}{\text{n}(\text{CO} + \text{CO}_2)_\text{i}} \times 100 \quad (1)$$

where n(CO + CO_2) corresponds to the sum of the number of moles of CO and CO_2 in the headspace of the bioreactor, and i and f correspond to the initial and final fermentation cycle.

3. Results

3.1. On-Demand Syngas Feeding (ODF)

In this work, the ODF fermentation strategy was established as the baseline condition for comparison with the CF mode. This study optimized the procedure used in Pacheco et al. (2021), which employed a single gas bag and required the renewing of the culture media several times before a pH change from seven to six (acid shock) [19]. Key improvements included exchanging the gas bags every 24 h, which was the time when liquid and headspace gas samples were collected. At this time the headspace was also cleansed with fresh syngas. This procedural change allowed for headspace and liquid sampling with minimal destabilization of the system (increasing result reliability), avoided the premature end of the fermentation due to the accumulation of CH_4 and CO_2 inside the bioreactor, enabled the regular assessment of the effect of increasing concentrations of cells and fermentation products inside the system, and limited the influence of cell overexposure to low pressures.

Figure 2 is a representation of the syngas consumption rate, cell growth, and acid production under ODF, measured at each sampling point, over the course of 408 h of fermentation.

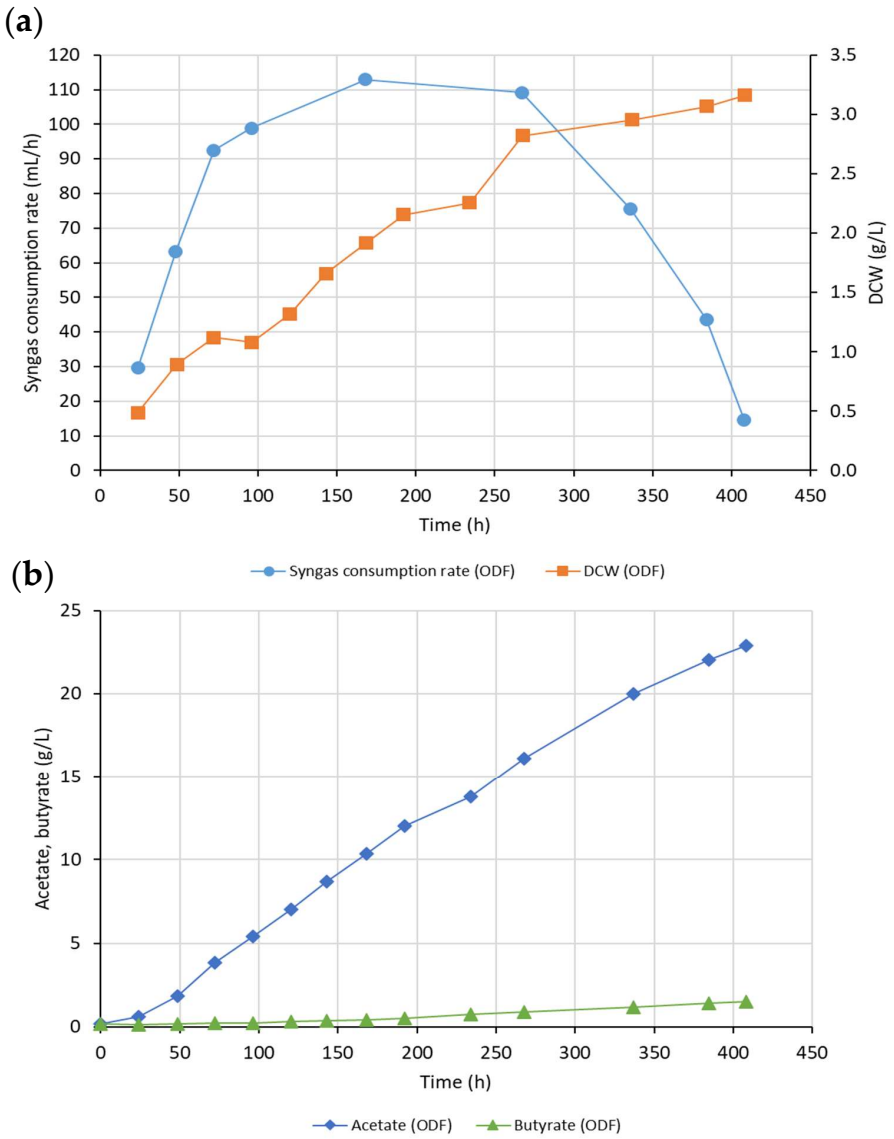

Figure 2. Graphical representation of (**a**) syngas consumption rate and dry cell weight (DCW), and (**b**) acetate and butyrate produced during the ODF fermentation of syngas by *E. callanderi* strain Marburg. Liquid and headspace sampling were performed every 24 to 48 h, after which the headspace was purged with fresh syngas.

Figure 2a shows the maximum rate at which syngas was consumed between each headspace cleaning step, every 24 h. The ODF fermentation was maintained for 408 h, the time when the syngas consumption rate declined to 15 mL/h. During the 408 h, a total of 23.4 L of syngas were consumed by the cells. The reactor headspace was cleansed a total of nine times with 1.2 L of fresh syngas during the fermentation, resulting in the total syngas being fed to the bioreactor being 34.2 L. Under these conditions, the highest maximum syngas consumption rate achieved was 112 mL/h, between 148 and 162 h. A maximum DCW of 3.2 g/L at 408 h and a corresponding maximum carbon consumed per gram of DCW of 0.4 mol/g was also achieved. Figure 2b shows the maximum acetate and butyrate concentrations reached at the end of the assay: 22.9 g/L and 1.3 g/L, respectively. The maximum specific production rates were 0.3 $g/g_{DCW}/h$ for acetate and 0.02 $g/g_{DCW}/h$ for butyrate. An increase in butyrate production was observed after approximately 200 h. This shift coincided with a decrease in the acetate production rate from 0.07 g/L/h to 0.04 g/L/h; both rates remained stable thereafter, until the end of the experiment. The total acid titter achieved during the ODF fermentation was 24.4 g/L, with an average volumetric production rate of 0.06 g/L/h.

To better represent the variations in the syngas consumption rate over the experiment, Figure 3 shows the volume of syngas consumed over time, resetting the volume at each headspace cleansing cycle.

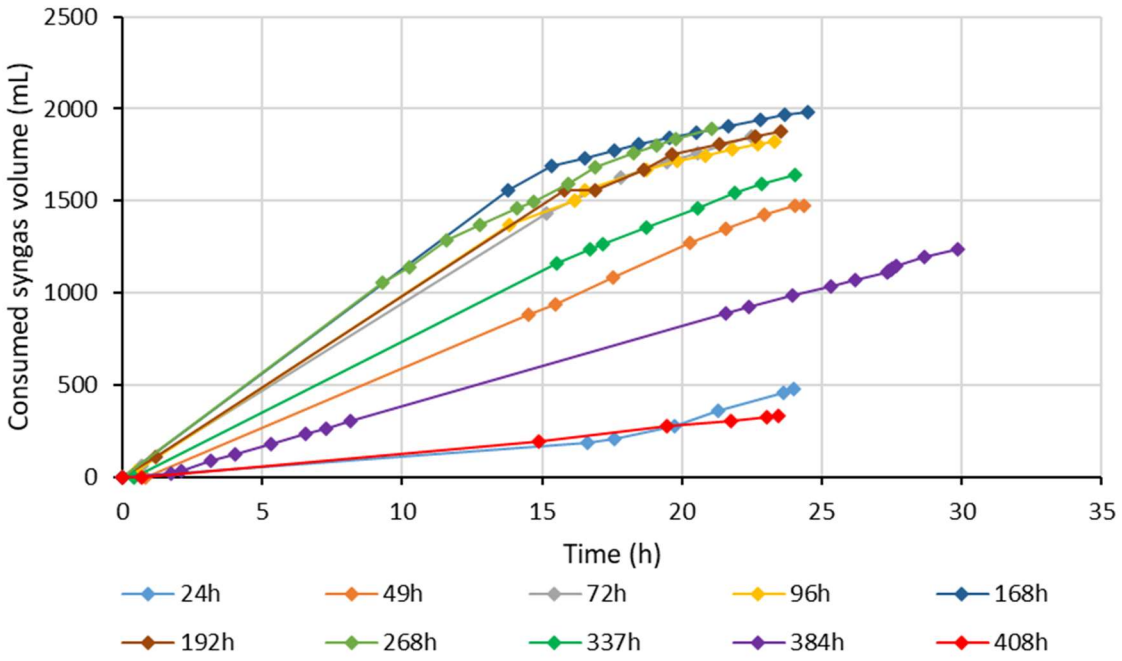

Figure 3. Time course profiles portraying the volume of syngas consumed between headspace cleansing procedures (ODF fermentation).

It is possible to observe that the syngas consumption rate was low in the first 24 h (light blue line) but increased rapidly over time. The rate more than doubled after 48 h (Figure 3, light orange line), achieving its maximum in the period between 143 and 168 h (Figure 3, dark blue line), as can also be observed in Figure 2a. The syngas consumption rate was almost stable from 168 to 267 h, with little variation (Figure 3, dark orange and light green lines). After this point, the syngas consumption started to decrease until the end of the assay (Figure 3, green, purple, and red lines).

Figure 4 shows the gas composition of the reactor headspace during the fermentation. It is important to note that the headspace was fully restored to the 0 h composition with every cleansing, being the results herein presented of the final composition immediately before the subsequent flush with new syngas.

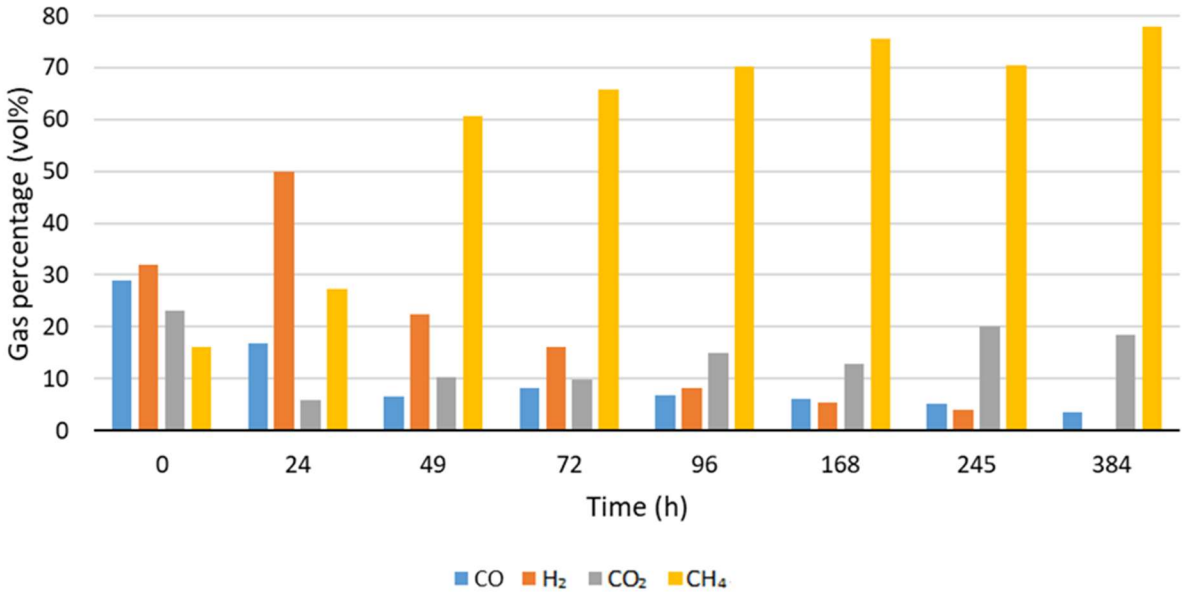

Figure 4. Reactor headspace composition during the ODF fermentation of syngas by *E. callanderi* strain Marburg.

From Figure 4, it is possible to observe that CO and H_2 rapidly decreased to values below 10 vol% in the first 49 and 96 h, respectively, while the CO_2 percentage varied along the course of the fermentation, fluctuating between 5 and 20 vol%, and started to accumulate after 168 h until the end of the fermentation. As for CH_4, it accumulated in the reactor headspace steadily during the course of the fermentation, from 16 vol% at 0 h to 77 vol% at 384 h. Furthermore, also at the end of fermentation, it was possible to achieve complete H_2 consumption, while the remaining CO amounted to only 3 vol%. In terms of carbon fixation, *E. callanderi* strain Marburg was able to convert a maximum of 67% of the fed CO and CO_2 into biomass and products, between 143 and 168 h.

3.2. Continuous Feeding of Syngas (CF) at 141 mL/h

Having determined a maximum syngas consumption rate of 112 mL/h during the ODF assay, this value was used to establish an approximate constant feeding rate for the first CF assay. The objective was to balance substrate delivery with consumption, thereby minimizing carbon waste while preventing substrate starvation and the consequent decline in metabolic activity. The temporal profiles of syngas consumption, cell growth, and acid production under CF at 141 mL/h are presented in Figure 5.

The fine-tuning the peristaltic pump's flow rate to closely match that of the ODF experiment resulted in an average continuous flow of syngas of 141 mL/h. Under this syngas feeding rate, the fermentation by strain Marburg lasted 559 h, at which point the bacteria halted syngas consumption. In total, 79.2 L of syngas was fed to the bioreactor during the fermentation, of which 29.2 L was consumed by strain Marburg (37% of the fed syngas was consumed by the bacteria), at a maximum consumption rate of 87 mL/h. A maximum DCW of 2.8 g/L was achieved at 485 h. A maximum carbon consumption per DCW of 1.2 mol/g was achieved. As for the fermentation products, the peak acetate and butyrate concentrations reached 21.2 g/L and 1.8 g/L, respectively, at the end of the assay, with a specific production rate of 0.5 $g_{acetate}/g_{DCW}/h$ and 0.05 $g_{butyrate}/g_{DCW}/h$. In

Figure 5b it is also possible to observe an increase in the butyrate production rate after 291 h, similarly to what was observed during the ODF fermentation. A total acid titter of 23.1 g/L was achieved during the CF fermentation at 141 mL/h, with an average volumetric production rate of 0.04 g/L/h.

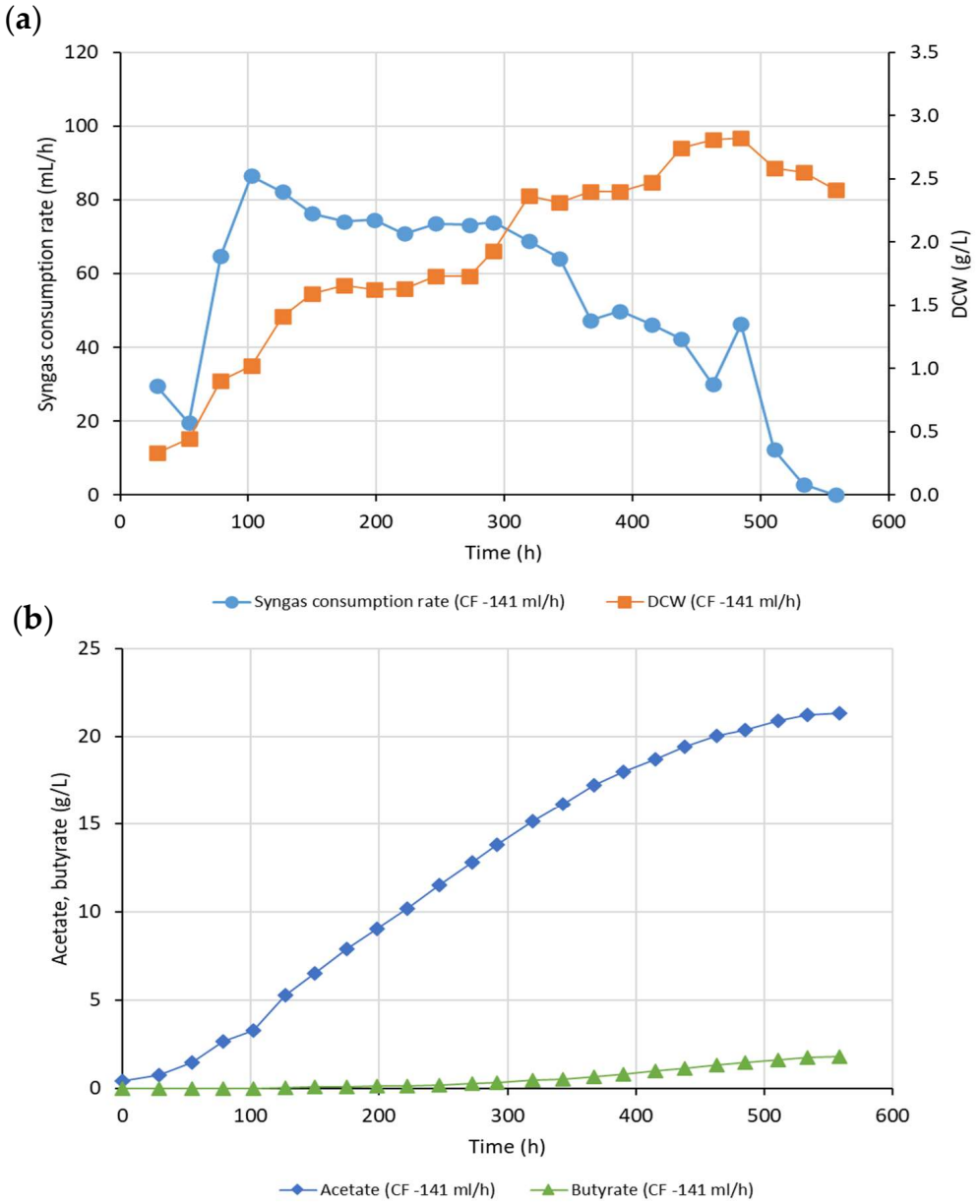

Figure 5. Graphical representation of (**a**) syngas consumption rate and dry cell weight (DCW), and (**b**) acetate and butyrate produced during the CF fermentation of syngas, at a rate of 141 mL/h, by *E. callanderi* strain Marburg.

To assess the syngas consumption by *E. callanderi* during the assay, the outflow gas sampling bags were removed approximately every 24 h and processed for analysis. Figure 6 is the graphical representation of the composition of the outflow syngas during the course of the experiment.

Contrary to the syngas profile obtained in ODF fermentation, CO_2 was the main component that accumulated in CF, approximately doubling its initial volumetric percentage, followed by CH_4, which achieved a maximum of 40 vol% at 127 h (Figure 6). In terms of CO and H_2, complete consumption was never achieved under CF fermentation at 141 mL/h,

since their vol% in the reactor headspace was never lower than 7 vol%. In terms of carbon fixation, the cells fixed 58% of the carbon supplied.

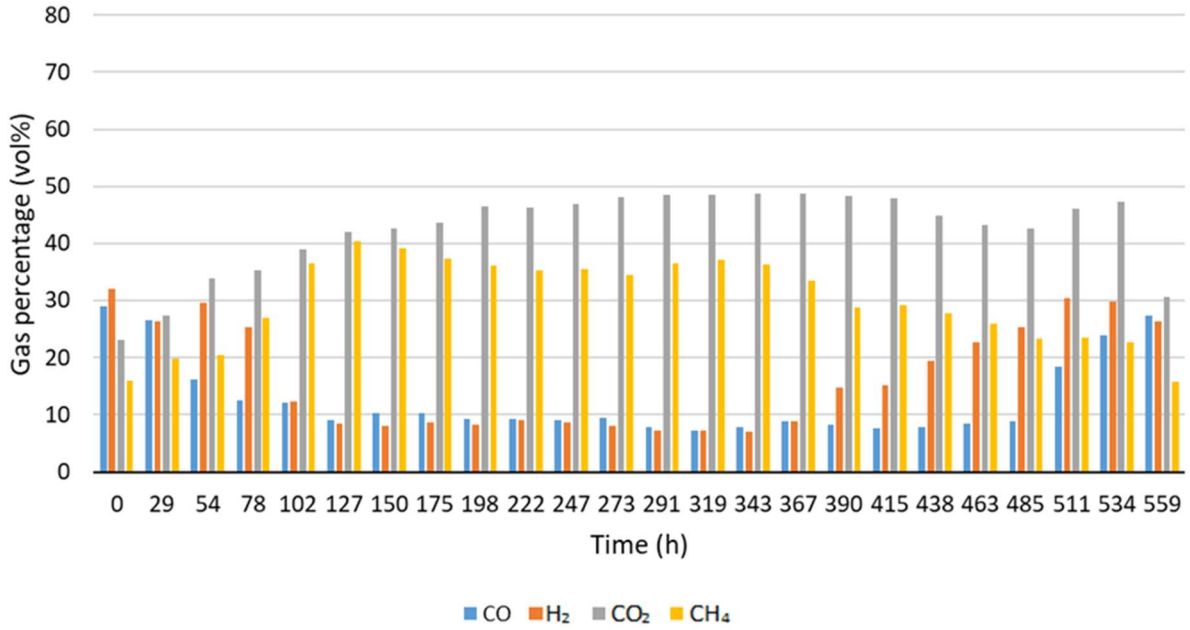

Figure 6. Syngas composition in the post-fermentation gas sampling bag during the CF fermentation of syngas, at a rate of 141 mL/h, by *E. callanderi* strain Marburg.

3.3. Continuous Feeding of Syngas (CF) at 223 mL/h

An advantage of the CF method versus the ODF method is that it is possible to increase or decrease the syngas feeding rates in order to test its influence on the cells, and the efficiency of carbon fixation and acid titters. Given that other works describe mass transfer as the major limitation of this technology and suggest increased flow rates to mitigate this limitation [10,11,33,34], the next step was to increase the syngas flow rate. The value was set to 223 mL/h to approximately double the syngas consumption rate of the first ODF experiment. Figure 7 is a graphical representation of the syngas consumption rate, cell growth (a), and acetate and butyrate production (b) during the fermentation under CF at 223 mL/h by strain Marburg.

The fermentation of syngas with a CF rate of 223 mL/h ended in just 284 h, when the syngas consumption rate was reduced to 30 mL/h. A total of 62 L of syngas was fed to the bioreactor, from which only 19.5 L was consumed by *E. callanderi* (31% of the fed syngas was consumed by the bacteria). The maximum consumption rate achieved was 91 mL/h at 168 h, while the maximum DCW was 2.1 g/L at 260 h. In this assay, the maximum carbon consumed per DCW was 2.7 mol/g, the highest obtained in these assays.

In terms of fermentation products, a maximum of 19.0 g/L of acetate and 3.4 g/L of butyrate was produced, at a specific production rate of 0.5 $g/g_{DCW}/h$ (acetate) and 0.08 $g/g_{DCW}/h$ (butyrate). Much like the previous assays, butyrate production increased in the second phase of the fermentation; in this case, it increased fourfold after 150 h. The acid titters were not unlike those of the previous assays, with the production of 22.4 g/L of total acids, at an average rate of 0.08 g/L/h.

As for the composition of the outflow syngas, Figure 8 illustrates the profile during the course of the fermentation.

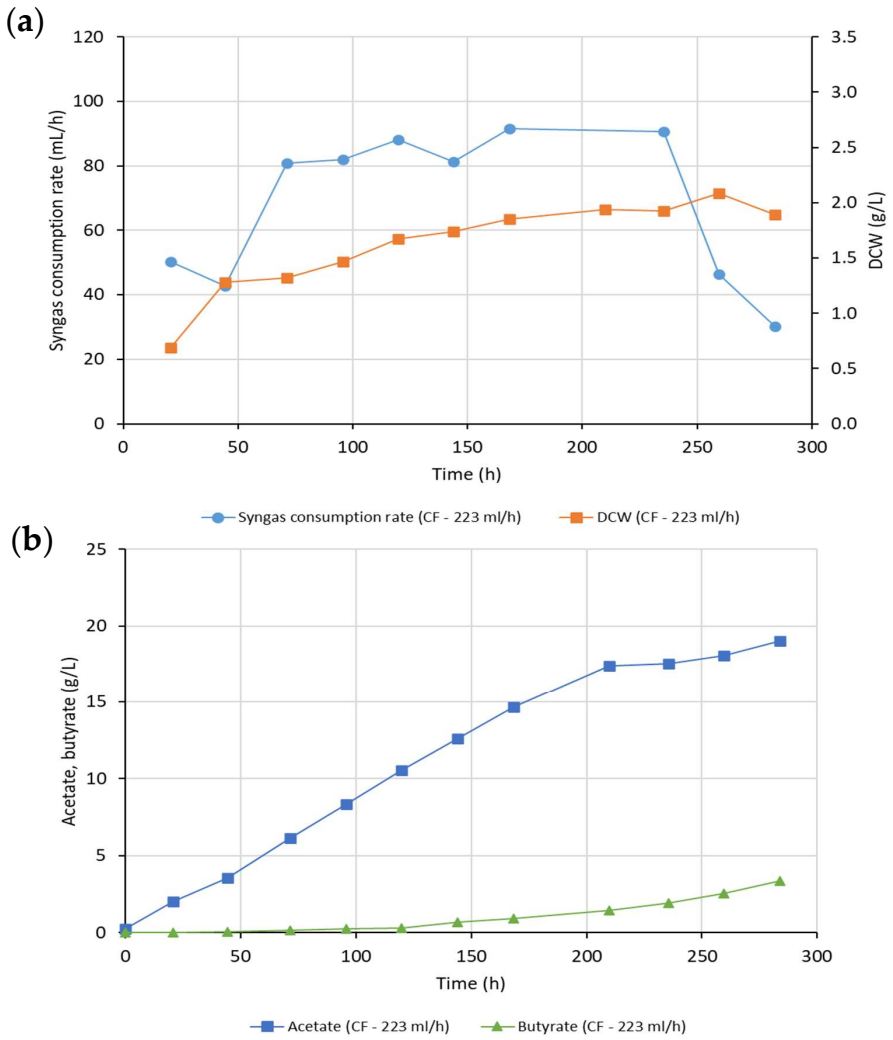

Figure 7. Graphical representation of (**a**) syngas consumption rate and dry cell weight (DCW), and (**b**) acetate and butyrate produced during the CF fermentation of syngas, at a rate of 223 mL/h, by *E. callanderi* strain Marburg.

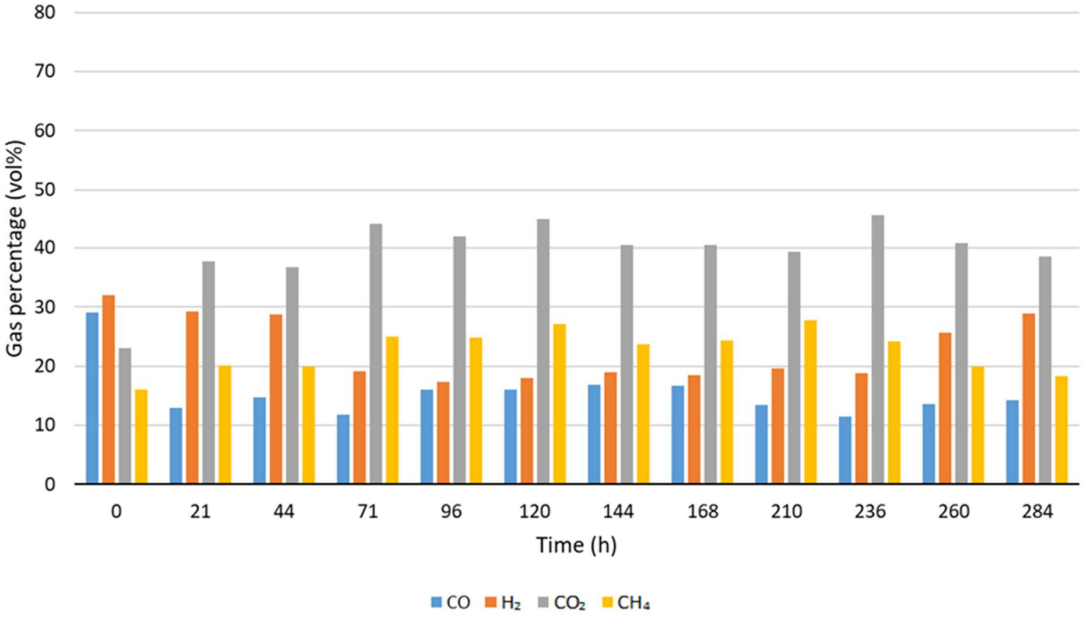

Figure 8. Syngas composition in the post-fermentation gas sampling bag during the CF fermentation of syngas, at a rate of 223 mL/h, by *E. callanderi* strain Marburg.

The accumulation trend of syngas components in this assay was similar to that obtained in the CF 141 mL/h assay, with CO_2 being the main component accumulated over time, achieving a maximum of 45 vol%, while CH_4 achieved only a maximum of 27.8 vol%. Furthermore, it was possible to observe a decrease in CO and H_2 conversion relative to the previous assay, from 29.1 to a minimum of 11.5 vol% for CO, and from 31.9 to a minimum of 17.3 vol% for H_2. This translates into a carbon fixation of 51%, the lowest of the three assays.

4. Discussion

Comparison Between ODF and CF Modes

From the analysis of Figures 2, 5 and 7a, it is possible to discern three stages that are common in all the fermentations. The first stage, that occurred between 0 and 50–100 h, involved the syngas consumption rate increasing rapidly along with cell growth, as is normal in conditions were the culture medium components and carbon and energy sources are abundant. The second stage occurred between 100 and approximately 250 h. In this stage, the syngas consumption rate was practically stable at its maximum; however, biomass kept increasing. At this point, the syngas consumption rate decoupled from cell growth, being probably limited by the mass transference of the syngas components into the liquid culture medium [9,32,38,39]. During the third and final stage, that occurred between 250 h (ODF and CF at 223 mL/h) or 300 h (CF at 141 mL/h) and the end of the experiments, the syngas consumption rate started decreasing rapidly, reaching values near 0 mL/h. At this stage, the cells began to lose the ability to consume syngas at the maximum rate even though the biomass concentration was still increasing and new cells were being formed, even if at a lower rate. To understand if this decrease in syngas consumption was related to the depletion of culture media components, namely to essential micronutrients or vitamins, both solutions were added aseptically to the culture medium at the end of the ODF fermentation (408 h); however, no variation in the syngas consumption rate was observed, which reached 0 mL/h 19 h later. This abrupt decrease in the syngas consumption rate has been similarly observed in batch fermentation experiments with other acetogenic microorganisms [30,35,40]. This phenomenon is frequently attributed to limited gas–liquid mass transfer, specifically a decrease in the solubility of the syngas components in the culture medium as the fermentation progresses [35,40,41]. However, in this case, the accumulation of acidic metabolites that have possibly caused an increase in the ionic strength of the medium may have also played a critical role. High ionic strength is known to reduce gas dissolution rates and impair cellular activity by disrupting proton motive force and osmoregulatory mechanisms [42,43]. The combined effects of diminished substrate solubility and compromised cellular osmoregulation likely led to the observed gradual and irreversible decline in the consumption rates, affecting both nascent and mature cells, and preventing their recovery even with subsequent medium supplementation. To mitigate the accumulation of inhibitory metabolites and elevated ionic strength, operational strategies, such as metabolite removal via membrane diffusion, cell concentration, and recirculation with fresh medium, or a continuous fermentation system with integrated product removal, should be considered [10,34,44–47].

An important observation is that the ODF fermentation functioned as a sequential batch system of ten cycles due to the periodic headspace resets. As shown in Figure 3, between 72 and 268 h, when syngas consumption rates achieved their maximum per cycle, gas consumption was faster during the initial 15 h of each cycle but slowed considerably thereafter. This pattern suggests that the 24 h cleansing intervals may have been suboptimal. The headspace composition data in Figure 4 provides a snapshot that correlates with the syngas consumption profiles of each cycle. For example, in Figure 3, between 24 and 49 h, approximately 1500 mL of syngas was consumed. Now looking at Figure 4, after 49 h, CH_4

already accumulated to 60 vol% in the reactor headspace. A correlation can then be made, explaining the sudden decrease in syngas consumed, which is observed via the dark blue line relative to "168 h" in Figure 3. At the point of inflection (after approx. 15 h in the 143–168 h cycle), the headspace maintained more than 60 vol%. CH_4 and the concentrations of CO and CO_2 were between 10 and 15 vol%, respectively. If the headspace cleaning step had been performed at this inflection point, the syngas consumption rates might have been higher than the ones observed during this experiment.

To facilitate the comparison between the ODF and CF fermentations, Table 1 shows a comparative summary of the main fermentation results of syngas consumption, and biomass and acid production. As can be observed in Table 1, even with headspace cleansing, less syngas was fed to the bioreactor in the ODF fermentation. However, this feeding mode still achieved the highest syngas consumption rate, along with the highest carbon fixation, which was 1.2 and 1.3 times higher than that obtained in the CF fermentation at 114 mL/h and 223 mL/h, respectively. The ODF fermentation achieved the near-complete consumption of CO and H_2, with accumulation of 78 vol% CH_4 and a minimum of 5 vol% of CO_2, whereas both CF fermentations accumulated more CO_2 than CH_4 due to lower H_2 consumption. The accumulation of CH_4 in the headspace during the ODF fermentation can be advantageous since CO, CO_2, and H_2 are metabolized by the acetogen into liquid acid compounds, while CH_4 remains up to a high-purity level, allowing it to be used for other purposes, such as energy generation.

Table 1. Comparative resume of the main syngas and metabolic parameters obtained during the ODF and CF fermentations.

		ODF	**CF**	
Syngas	Syngas feeding rate (mL/h)	-	141	223
	Max. syngas consumption rate (mL/h)	112 (162 h)	87 (102 h)	91 (168 h)
	Total fermentation time (h)	408	559	284
	Total syngas volume fed (L)	34.2	79.2	62.0
	Total syngas volume consumed (L)	23.4	29.2	19.5
	Carbon fixation (%) (see Equation (1))	67	58	51
Biomass	Max. DCW (g/L)	3.2	2.8	2.1
	Max. carbon consumed per DCW (mol/g)	0.4	1.2	2.7
Fermentation products	Total acids (g/L)	24.4	23.1	22.4
	Acetate titter (g/L)	22.9	21.2	19.0
	Butyrate titter (g/L)	1.5	1.8	3.4
	Specific production rate (Acetate) ($g/g_{DCW}/h$)	0.3	0.5	0.5
	Specific production rate (Butyrate) ($g/g_{DCW}/h$)	0.02	0.05	0.17

While carbon fixation was higher in the ODF fermentation, the maximum carbon consumed per gram of DCW was visibly higher under CF mode. Whereas 1.2 and 2.7 mol/g_{DCW} were achieved in the 141 and 223 mL/h fermentations, respectively, the cells grown under ODF only fixed 0.4 mol/g_{DCW}. This might be due to higher carbon availability in CF than ODF, allowing the cells to metabolize more CO, even at lower cell densities. This trend was also reflected in the specific production rates: for acetic and butyric acids, respectively, 0.3 and 0.02 $g/g_{DCW}/h$ (ODF); 0.5 and 0.05 $g/g_{DCW}/h$ (CF at 141 mL/h); and 0.5 and 0.17 $g/g_{DCW}/h$ (CF at 223 mL/h).

The carbon consumed during the ODF fermentation was primarily distributed between acetate production (22.9 g/L) and biomass formation (3.2 g/L), with lower butyrate production (1.5 g/L). At the end of the assay, 9 mmol of CO_2 remained, as it could not be metabolized due to the depletion of H_2. This was due to CO_2 being a byproduct of the bacterial metabolism and insufficient H_2 in the syngas composition to allow its complete consump-

tion. With the near absence of readily available carbon, the cells shifted their metabolism towards the production of acetate, the most energetically favorable product, which does not require an increase in the carbon chain, unlike butyrate. This is a well-known energy saving behavior that is present in acetogenic bacteria and has been extensively described in the bibliography [19,48,49]. However, in terms of carbon consumption per gram of DCW, the cells appeared less active, converting a maximum of only 0.4 mol of C/g_{DCW}.

During the CF fermentation at 141 mL/h, acid production was approximately equivalent to that of the ODF fermentation, with similar specific production rates and a difference in less than 0.4 g/L in biomass. However, a maximum of 17 mmol of CO_2 accumulated in this experiment, even in the presence of sufficient levels of H_2 to facilitate its fixation (Figure 5). This CO_2 accumulation is most likely an effect of unconverted CO in the headspace, due to the continuous feeding of fresh syngas. CO is a known hydrogenase inhibitor, hindering CO_2 conversion through the carbonyl branch of the Wood–Ljungdahl pathway [50]. This effect was not observed during the ODF fermentation, as the microbially induced demand pulled only low concentrations of syngas components into the reactor incrementally. This gradual feeding strategy supported sustained activity of the carbonyl branch, preventing metabolic inhibition (Figure 4).

The effects of the presence of increased CO concentrations in the headspace were even more visible during the CF fermentation at 223 mL/h (Figure 8), with more than 10 vol%. of CO or H_2 remaining unconverted, and a maximum of 29.8 mmol of CO_2 accumulated. Despite this, these cells demonstrated the highest carbon consumption per gram of DCW (2.7 mol of C/g_{DCW}), achieving not only a similar acetate titer but also a high butyrate titter of 3.4 g/L.

Regarding the yields obtained in the three experiments, especially the total acid titters achieved, these represent, to the authors' knowledge, the highest reported to date for *E. callanderi* strain Marburg through syngas fermentation. A comparison of the acetate titters achieved by strain Marburg in this study with those of other well-known acetogenic strains grown on syngas or C1 gases in a bioreactor is summarized in Table 2. The results are comparable to those of high-productivity strains such as *Neomoorella thermoacetica* (formerly *Moorella thermoacetica*) and *Acetobacterium woodii*, which recently achieved 22.3 and 21.1 g/L of acetate, respectively, when grown solely on syngas [31,51].

A further comparison can be made with the acetate production by *E. callanderi* (formerly *Eubacterium limosum*) KIST612, as reported by Lee et al. (2025), where dual bubble column reactors (with a cell retention system) were used to access cell viability under different operational conditions. The authors observed an increase from approximately 20 g/L to a maximum of 34.4 g/L of acetate when a sludge filtrate medium (SFM) from anaerobic digestion was added to the vessel and the gas feed composition was changed from 80 vol% CO/20 vol% CO_2 to 90 vol% CO/10 vol% CO_2 [47]. The initial acetate titter (~20 g/L) prior to SFM supplementation aligns with the results of the current study. However, the subsequent increase after SFM addition is not directly comparable, as the sludge filtrate introduces not only nutrients and vitamins but also variable volatile fatty acids, soluble organics, and other residual compounds. These components provide an alternative, and readily metabolizable the carbon source, bypassing the need for de novo carbon fixation from the supplied C1 gases. These results highlight the potential of the Marburg strain for producing acids from syngas, since this wild-type strain can achieve similar acid production values, even under suboptimal conditions, to those obtained in highly optimized systems or using genetically modified strains.

Table 2. Acetate production through syngas fermentation by different acetogenic strains, as reported in the literature, and the results obtained in the present study.

Acetogen	Syngas Composition (vol%)	Fermentation Type	Acetate Titter (g/L)	
E. callanderi (*B. methylotrophicum*) Marburg	Commercial gas CO/CO_2 (80/20)	Batch fermentation	4.9	[22]
Neomoorella thermoacetica (*Moorella thermoacetica*) DSM 6867	Commercial syngas $CO/H_2/CO_2$ (40/30/30)	Bubble column reactor	26	[52]
Acetobacterium woodii DSM 1030	Commercial gas $H_2/CO_2/N_2$ (35/25/40)	Batch fermentation	21.1	[51]
E. callanderi (*B. methylotrophicum*) Marburg	Commercial syngas $CO/H_2/CO_2/N_2$ (30/30/20/20)	Batch fermentation (sequential syngas batches + on-demand syngas feeding)	2.3	[19]
E. callanderi (*B. methylotrophicum*) Marburg	Syngas from the gasification of lignin $CO/H_2/CO_2/CH_4/N_2$ (24.2/16.5/23.9/13.6/18.1)	Batch fermentation (on-demand syngas feeding)	3.1	[19]
N. thermoacetica (*M. thermoacetica*) DSM 2955	Ultra-cleaned syngas from the gasification of crushed bark $CO/H_2/CO_2/N_2$ (34.3/15.1/17.5/33.1)	Batch fermentation (pilot scale)	22.3	[31]
Clostridium aceticum	Commercial syngas $CO/H_2/CO_2/N_2$ (30/20/10/40)	Batch fermentation	5.2	[53]
E. callanderi (*Eubacterium limosum*) KIST612	Commercial gas CO/CO_2 (80/20 and 90/10)	Dual bubble column reactor with cell retention (addition of anaerobic digestion sludge filtrate medium—SFM)	20 (pre-SFM addition) 34.4 (post-SFM addition)	[47]
E. callanderi (*B. methylotrophicum*) Marburg	Commercial syngas $CO/H_2/CO_2/CH_4$ (23.02/29.01/31.9/16.2)	Batch fermentation (on-demand syngas feeding-ODF)	22.9	This study

Overall, the results demonstrate that *E. callanderi* strain Marburg is a robust microorganism for producing value-added products from syngas. The fermentation experiments under different syngas feeding modes indicated that the optimal fermentation strategy depends on the final objective. The ODF strategy leverages the gas-cleansing capabilities of strain Marburg, making it suitable for accumulating high purity biomethane when coupled to a biomass gasification system, with simultaneously high CO and CO_2 conversion to liquid products. Conversely, the CF strategy enables higher and faster conversion rates, which is advantageous for continuous production systems using continuous gas streams. This syngas feeding mode also diminishes the effects of the poor mass transfer of syngas components to the liquid medium, increasing carbon availability and, therefore, cellular productivity. However, each system still has some limitations. ODF fermentations are time-consuming and require headspace purging to remove unconverted gases. Furthermore, to maximize ODF carbon conversion, depending on syngas composition, additional H_2 supplementation might be needed. In contrast, CF fermentations result in significant

amounts of unconverted carbon, which could be mitigated by implementing sequential bioreactors to maximize carbon conversion. This opens possibilities such as integrated product removal strategies, cell concentration steps, or the integration of chain elongation processes, resulting in a more varied portfolio of products.

5. Conclusions

The syngas fermentation performance of *Eubacterium callanderi* (formerly *Butyribacterium methylotrophicum*) strain Marburg was evaluated under two different syngas feeding regimes, namely on-demand syngas feeding (ODF) and continuous syngas feeding (CF), at two different rates (141 mL/h and 223 mL/h). The results obtained demonstrate the robustness and high carbon conversion efficiency of the strain for carboxylic acid production from syngas. The ODF and CF fermentation at 141 mL/h (a feeding rate similar to the maximum consumption rate obtained during the ODF fermentation) yielded approximately the same acetate and butyrate titters, 22.9 and 21.2 g/L, and 1.5 and 1.8 g/L, respectively. On the other hand, the CF fermentation shifted towards butyrate production, doubling the concentration achieved on the other two assays (3.4 g/L), with a still relevant production of acetate (19.0 g/L). The concentration of carboxylic acids obtained in this study were, to the author's knowledge, the best obtained to date by strain Marburg from syngas fermentation. Moreover, both the acetate and butyrate titters obtained herein were highly competitive with the established wild-type and genetically modified acetogens, such as some *Neomoorella thermoacetica* (formerly *Moorella thermoacetica*) strains.

A key finding was the trade-off between the two syngas feeding strategies. The ODF strategy proved effective for maximizing carbon conversion into liquid products and was ideal for harnessing the organism's gas-cleansing capabilities, e.g., for high-purity biomethane with the concomitant production of platform chemicals. In contrast, the CF strategy enabled higher and faster conversion rates, suitable for continuous production systems. However, each feeding method presented distinct limitations. ODF was time-demanding, required headspace cleansing, and might need additional H_2 supplementation to achieve its full potential. Conversely, CF resulted in significant amounts of unconverted carbon, namely CO, due to the continuous feeding of syngas components and the consequent probable inhibition of hydrogenases, which hindered CO_2 fixation through the Wood–Ljungdahl pathway. To overcome the limitation of unconverted carbon in CF systems, the implementation of sequential bioreactors could be proposed to achieve maximum carbon conversion.

Future work should focus on optimizing sequential feeding strategies to balance high productivity with high conversion yields, ensuring the scalability and economic viability of strain Marburg as a biocatalyst for sustainable chemical production. Furthermore, the potential to diversify gaseous carbon streams (e.g., incorporating industrial off-gases and syngas from alternative feedstocks, or focusing on the emissions of biogenic CO and CO_2) should be investigated to enhance the process's adaptability and cross-industrial applicability. Examples could cover the cement industry, municipal solid waste gasification, and/or a combination of waste streams. The appropriate life cycle assessment and techno-economic assessment of alternatives should also be tackled.

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