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**Chain Elongation by *Clostridium kluyveri*: Effect of Substrates**

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The microbial conversion of C1 gas produced by the gasification of C-based streams (e.g., biomass and plastic) is a promising strategy for the production of bioalcohols and biocommodities. Acetogenic bacteria can produce value-added metabolites, such as acetate and butyrate, but their recovery is costly and energy intensive. The success of exploiting these acids may be their conversion into medium-chain fatty acids (MCFAs), such as n-caproic and n-caprylic acids, in a second fermentation step. Interest in MCFAs is related to their high hydrophobicity, high values, and high energy densities. These MCAF features make them better suited for chemical manufacturing and as biofuel precursors. *Clostridium kluyveri* is known for its ability to produce MCFAs under anaerobic fermentation via the reverse β-oxidation pathway.

The present study reports the recent results of the characterization of *C. kluyveri* fermentation under batch conditions. In particular, attention was paid to assessing the effects of substrate composition on the production of MCFAs. Experiments were carried out in 0.25 L MiniBio bioreactors (Applikon), working volume of 0.12 L, operated at 37 °C and Ph = 6.8. The investigated substrates were acetic acid, ethanol, and butyric acid. The conversion process was characterized in terms of substrate conversion, metabolite and cell production, specific rates of cells/metabolite production, and yields.

The results indicated that *C. kluyveri* preferred acetic acid and ethanol as carbon sources for growth. Supplementation of butyric acid in the fermentation medium was associated with a high production of hexanoic acid.

* 1. Introduction

In the context of growing challenges regarding climate change, CO2 emissions, the country’s independence from fossil resources, and the production of renewable and carbon-neutral chemicals are key strategies for reducing emissions and supporting a circular economy.

Over the past century, microbial processes have been proposed to produce biochemicals and biofuels from renewable resources paving the way for a bio-based economy (Sivalingam and Dinamarca, 2021). These processes rely on sugars from crops or hydrolyzed lignocellulosic biomass. However, crop-based feedstocks raise ethical concerns due to competition with food production, whereas lignocellulosic biomass may require expensive – from economic and energy points of view–pretreatments owing to the presence of recalcitrant lignin. To overcome these challenges, there is growing interest in using alternative feedstocks such as CO2, glycerol, and organic waste (Blank et al., 2020). In recent years, significant progress has been made in developing biorefineries based on the C1-stream produced by the transformation of C-based streams (Qiao et al., 2022).

Syngas, a gas mixture of CO, H2, and CO2, can be produced via gasification of hydrocarbon resources or organic waste. It may also be produced during industrial processes such as steel mills (Liew et al., 2016). Acetogens, anaerobic bacteria can metabolize syngas to produce ethanol, butanol, and short-chain carboxylates (Wang et al., 2022). However, expanding the product spectrum, yields, and economic feasibility of the process is essential for the future of the process as a sustainable production platform.

Short-chain carboxylates and alcohols produced during anaerobic fermentation have several limitations for direct exploitation. Indeed, the main issues include: the bioproducts are fully miscible with the fermentation broth, making their extraction complex and expensive (Wu et al., 2019); the relatively low market value of these bioproducts raises concerns regarding the economic feasibility of the process, limiting its widespread industrial development; and the presence of residue of these by-products, such as short-chain carboxylates, alcohols, and lactates, in waste streams contributing to environmental pollution (Kucek et al., 2016).

Recent studies have explored chain elongation technology as a solution to the following limiting issues: selected microorganisms convert short-chain products into medium-chain carboxylates (MCCs) (Dellomonaco et al., 2011). Medium-chain carboxylates with 6-12 carbon atoms–caproate (C6), heptylate (C7), and caprylate (C8)–are target products owing to industrial interest and their low solubility in water-based solutions. The latter feature reduces the energy required for extraction and increases market value (Angenent et al., 2016). Consequently, the overall expenses decrease, enhancing the economic feasibility of the anaerobic fermentation process (Angenent et al., 2016).

*Clostridium kluyveri* is a known strain capable of chain elongation (Angenent et al., 2016). It isa mesophilic anaerobic bacterium that is found in aquatic environments. It can utilize ethanol and acetic acid as substrates to produce butyric and caproic acid (de Leeuw et al., 2021). This microorganism has gained significant interest from microbiologists because of its unique metabolic pathway, which enables it to add two carbon units to the acyl chain of a monocarboxylic acid during each reverse beta-oxidation cycle (Seedorf et al., 2008).

* 1. Materials and Methods
		1. Microorganism and media

The German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) provided an active culture of *Clostridium kluyveri,* DSM555. Active cultures were stored at -80 °C with glycerol as a cryoprotective agent. The thawed cells were inoculated into 0.010 L of DSMZ52 medium in 0.015 L Hungate tubes (precultures). The cells were grown under anaerobic conditions for 24 h at 37 °C and then transferred to the bioreactors. The composition of the fermentation medium used in this study was in agreement with the suggestion of Fernández-Blanco et al. (2024), with the exception of the carbon substrate. Carbon sources were acetic acid (AA) at 8.8 g/L and a mixture of butyric acid (6.6 g/L) and acetic acid (7.6 g/L). Throughout the fermentation tests, the ethanol concentration was set at 13 g/L.

The chemicals were purchased from Sigma-Aldrich (Milan, Italy).

* + 1. Apparatus and operative conditions

Batch fermentations were carried out in MiniBio bioreactors (Applikon) equipped with 0.25 L vessels. The operating volume (VL) was set at 0.12 L, and the operating conditions of the bioreactor were as follows: temperature of 37 °C, agitation speed of 250 rpm, and pH of 6.8 and kept constant by means of a controller unit that dispensed 1 M NaOH and 0.5 M HCl solutions.

The bioreactor was filled with 0.12 L (VL) of medium and autoclaved for 20 min at 121 °C. The sterilized bioreactor medium was N2-flushed to remove oxygen from the headspace and liquid medium. As the temperature dropped below 40 °C and anaerobic conditions were achieved, the N2 gas stream was stopped and cysteine-HCl, carbonate, sulfide, and vitamins from sterile-degassed stock solutions were added to the medium. Temperature and pH were continuously measured and recorded using a MiniBio acquisition unit. The bioreactors were inoculated with 0.010 L of a preculture of *C. kluyveri* grown aseptically for two days. At least once per day, fermentation status was characterized in terms of optical density (OD) and the concentration of acids and alcohols.

Each test was performed in triplicates (biological replicates). The data reported in the tables and figures represent mean values. The standard error was always lower than 5 %.

* + 1. Analytic methods

The pH of the culture was continuously monitored using a pH probe. Samples of the liquid culture (0.001 L) were periodically sampled to measure biomass and metabolite concentrations according to Lanzillo et al. (2020). The optical density (ODλ) of the culture at 600 nm was measured using a UV–visible spectrophotometer (SPECORD 50 UV-VIS, Analytik Jena, Jena, Germany). The cell concentration (gDM/L) was assessed according to a calibration curve (1 OD = 0.4 gDM/L).

The sampled culture was centrifuged at 13000 rpm for 10 min using a MiniSpin ® centrifuge (Eppendorf Italia, Milan, Italy), and the liquid composition was characterized. The concentration of soluble compounds (acids and alcohols, including acetic acid, butyric acid, hexanoic acid, ethanol) was measured by using an HPLC system (HP1100, Agilent Co., Santa Clara, CA, USA) equipped with a Rezex™ ROA-Organic Acid H+ column (8 %, 150 × 7.8 mm) and a RID detector (Column Temperature Controller, Thermasphere™ TS-130). The mobile phase was 3 mM H2SO4 solution delivered at a flow rate of 0.8 L/min at room temperature.

* 1. Results

The production of medium-chain fatty acids (MCFAs) by fermentation of some 2C-species – acting as electron acceptors - is discussed in detail in this section. The investigation was carried out using a pure *C. kluyveri* culture in fermentation batch experiments. The ethanol concentration in the medium (electron donor) was set to 13 g/L at the beginning of the fermentation tests. Fermentation test were grouped into: group I) acetic acid at 8.8 g/L was supplemented as substrate; group II) a mixture of butyric acid and acetic acid at 6.6 g/L and 7.6 g/L, respectively, was supplemented. These experiments aimed to assess the effects of the acidic substrate on the production of hexanoic acid and biomass in bioreactors under controlled operating conditions. Table 1 summarizes the initial substrate concentration, maximum product concentration, yield in terms of hexanoic acid production (YHA/X, YHA/AA, YHA/E, and YHA/BA), and specific cell growth rate (μ).

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| --- | --- | --- | --- | --- |
| Group | Initial substrate concentrationg/L | Maximum product concentrationg/L | Yieldsg/g | μ(h-1) |
|  | Ethanol | Acetic ac. | Butyric ac. |  | Butyric ac. | Hexanoic ac. | YHA/X | YHA/AA | YHA/E | YHA/BA |  |
| I | 13 | 8.8 | - |  | 2.9 | 9 | 13.6 | 1.4 | 0.7 | - | 0.074 |
| II | 13 | 6.6 | 7.6 |  | - | 15.8 | 27.7 | 4.0 | 1.2 | 4.6 | 0.038 |

Table 1: Concentration of initial substrate, metabolites**,** yields, and specific cell growth rate

Figure 1 shows the results of batch fermentation tests (group I) carried out at pH 6.8 with acetic acid and ethanol as carbon sources at initial concentrations of 8 g/L and 13.5 g/L, respectively.

Figure 1. Data measured during batch fermentation tests: A) concentration of cells and pH; B) concentration of ethanol, acetic acid, butyric acid, and hexanoic acid. Carbon sources: acetic acid and ethanol.

Bioreactor inoculation led to immediate cell proliferation, with no lag phase. The microorganisms began metabolizing ethanol and acetic acid since the beginning of the tests and the increase in the cell concentration was observed. After about 24 hours of fermentation, the cell exponential growth starts and a maximum of cell concentration was measured (0.69 gDM/L) after about 45 hours. The substrate concentration decreased with the increase of the cell concentration: ethanol and acetic acid concentrations approached a constant value, about 1.6 g/L and 0.1 g/L for acetic acid and ethanol, respectively. The specific cell growth rate was determined to be 0.074 h-1. This value is in agreement with the results of Zou et al. (2018) that reported 0.068 h-1 in an experiment to test the growth of *C. kluyveri* in the presence of acetate and ethanol as carbon sources. The main MCFAs produced during the fermentation tests were butyric acid and hexanoic acid, the latter being produced at remarkably higher concentrations than the former. Butyric acid production began as soon as ethanol and acetic acid were converted. The butyrate concentration vs. time was characterized by a maximum (2.9 g/L) at approximately time = 27 h and a plateau at a low value (2 g/L) after approximately 50 h. The concentration of hexanoic acid started to increase rapidly as butyric acid reached the maximum and approached 8.8 g/L at 45 h of fermentation. The dynamics of the butyric acid concentration are probably due to its two roles: product of the metabolism for the conversion of 2C species and conversion into hexanoic acid. The process was stopped at 142 h because the biomass concentration decreased to nearly zero, even though acetic acid was not completely consumed. It is possible that biomass growth was limited by the depletion of ethanol or other medium components. The final concentration of the MFCAs was in agreement with the data reported by Fernandez-Blanco et al. (2024). They carried out fermentation tests in bioreactors (working volume 1.2 L) operated at pH set at 6.8, initial acetate and ethanol concentration of respectively 6.77 g/L and 14.4 g/L. Indeed, they obtained for butyric and hexanoic acid a concentration of 3.10 g/L and 9.50 g/L, respectively, after 280 h of fermentation. The difference between butyric acid concentration and fermentation time could be due to the lower concentration of acetate (6.77 g/L) and the use of a titanium citrate solution as a reducing agent.

Figure 2 shows the results of the batch fermentation tests (group II) carried out at pH 6.8 with acetic acid, butyric acid, and ethanol supplied as carbon sources at initial concentration of 6.6 g/L, 7.6 g/L and 13.3 g/L, respectively. When the bioreactor was inoculated, the cells started to grow immediately. Ethanol was consumed since the beginning of the fermentation. The maximum of cell concentration (0.59 gDM/L) was measured at the ethanol depletion. Thereafter, there was a decrease in the biomass concentration, which was consistent with the substrate consumption, reaching a constant value of approximately 2.5 g/L for acetic acid and 4 g/L for butyric acid after approximately 66 h of fermentation. After the first 18 h of fermentation, both acetic and butyric acids started to be consumed.

Figure 2. Data measured during the batch fermentation test: A) cell concentration and pH; B) ethanol, acetic acid, butyric acid, and hexanoic acid concentrations. Carbon sources: acetic acid, butyric acid, and ethanol.

*C. kluyveri* was characterized by specific growth rate of 0.038 h-1. The rate is in agreement with the results reported by San Valero et al. (2019): they reported a specific growth rate of 0.039 h-1 for tests carried out under operational conditions close to those set in the present investigation. The production of hexanoic acid in the experiment presented in this work was in agreement with the substrate consumption, reaching a maximum value of 15.8 g/L at the end of the experiment. San Valero et al. (2019) observed that ethanol and acetic acid concentrations did not change over the first 4-5 days, coinciding with the bacterial growth lag phase. They noted that substrate utilization occurred with hexanoic acid formation, the concentration of which increased with time up to approximately 21 g/L. The difference between the results of San Valero et al. (2019) and those from the present investigation was likely affected by the small differences in substrate concentration, the use of Na-acetate instead of K-acetate, and the supplement of 2.5 g/L NaHCO3 to the fermentation media. Unfortunately, San Valero et al. (2019) stopped the fermentation test prematurely, despite the incomplete consumption of substrates, for several reasons. Issues included a significantly low cell concentration in the bioreactor as a consequence of cell mortality as well as the stop of C4 acid production and its conversion into hexanoic acid.

Acetic acid and butyric acid were simultaneously consumed. *C. kluyveri* did not show a clear preference for either of the two acids (San Valero et al., 2019). As a matter of fact, the microorganism is able to efficiently utilize both substrates at the same time. This feature highlights the flexibility of its metabolic pathways, which allows it to adapt to different available carbon sources.

A comparison between the results from the two fermentation tests indicated that: i) the maximum biomass concentration was slightly higher when only acetic acid was used as the electron acceptor, and ii) the cell specific growth rate was higher when only acetic acid was used. The highlighted results suggest that acetic acid potentially has two roles: it may contribute to more favorable biomass accumulation while improving metabolic efficiency, thus enabling microorganisms to exhibit accelerated growth rates.

Two issues deserve particular attention:

I) It is worth noting that undissociated fatty acids can be highly inhibiting/toxic for cells despite the acidic operating conditions. Even under neutral pH conditions, dissociated fatty acids may exert an inhibitory or toxic effect on bacteria involved in chain elongation, with inhibition/toxicity increasing with the length of the carbon chain. Consequently, the chemical form of fatty acids plays a significant role in determining their potential impact on microbial growth even under neutral pH conditions (Lonkar et al., 2016). Therefore, microbial growth may be slowed down and less efficient when acetic and butyric acids are used as substrates.

II) The use of a mixture of acetic and butyric acids as electron acceptors resulted in enhanced hexanoic acid production when compared to the use of only acetic acid. Indeed, the final concentration of hexanoic acid increased by more than 60 % upon butyric acid supplementation. The yield of hexanoic acid with respect to ethanol and acetic acid remarkably increased in group II fermentation compared to that in group I.

This enhanced behavior may be attributed to the metabolic pathways involved in the reverse beta-oxidation process, which play a key role in chain elongation. The process includes five acetyl-CoA molecules obtained from the oxidation of ethanol, combined with five additional acetyl-CoA molecules to produce acetoacetyl-CoA, which in turn is converted to butyryl-CoA. The merging of acetyl-CoA - from ethanol oxidation with butyryl-CoA may produce caproyl-CoA, which is in turn converted into (caproic) hexanoic acid (Angenent et al., 2016). The analysis of this metabolic pathway suggests that supplementation of butyric acid to the growth medium provides a key building block (butyryl-CoA) for the production of caproyl-CoA. As a result, the overall efficiency of hexanoic acid production was enhanced because butyric acid acts as a critical building block in the chain elongation process, facilitating the conversion of shorter-chain intermediates into longer-chain fatty acids. The synergistic action of acetic and butyric acids not only supports the production of butyryl-CoA but also enhances the overall metabolic flux, leading to a more efficient and higher yield of hexanoic acid.

* 1. Conclusions

Fermentation tests for a pure culture of *Clostridium kluyveri* were carried out in batch stirred tank reactors for preliminary investigation of the effects of substrate composition and concentration on the fermentation process. Fermentation was characterized in terms of metabolites, cell production, and yield. The results clearly demonstrated highly effective production of hexanoic acid, highlighting the ability of microorganisms to convert available substrates into the desired product under controlled fermentation conditions.

Tests with acetic acid and butyric acid as electron donors and ethanol as the electron acceptor were characterized by the maximum concentration of hexanoic acid, suggesting that the specific combination of substrates facilitated optimal metabolic conditions for the production of the target compound. *C. kluyveri* did not show a preference for either acetic acid or butyric acid because they were consumed simultaneously. The synergistic effect of acetic acid and butyric acid enhanced the production of hexanoic acid. The simultaneous utilization of both acids could potentially streamline carbon flow through the metabolic network, leading to higher yields of the desired product.

The experiments were concluded after approximately 144 hours of fermentation. The dynamics of the cell and metabolite concentrations suggest that the fermentation process stopped even though the substrate-acid conversion was not complete. It may be inferred that essential nutrients or the accumulation of inhibitory metabolites are responsible for the end of fermentation.

Nomenclature

AA – acetic acid concentration (g/L)

BA – butyric acid concentration (g/L)

E – ethanol concentration (g/L)

HA – hexanoic acid concentration (g/L)

ODλ – optical density

T – fermentation temperature (°C)

X – cell concentration (gDM/L)

μ – specific cell growth rate (h-1)

QG – gas flow rate (L/h)

VL – volume of culture (L)

VG – volume of gas inside the bioreactor (L)

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