**Medium chain fatty acids production via biological chain elongation**

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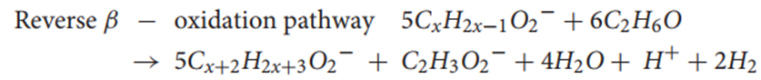
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**1.Introduction**

Medium-chain fatty acids (MCFAs) are saturated or unsaturated fatty acids having from 6 to 12 carbon atoms, which are mainly synthetized from fossil-base compounds or from vegetable oils such as palm oil, coconut oil or castor oil. Even in this last case, the MCFAs production cannot be considered sustainable as their extraction involves the adoption of no green solvents. Moreover, the MCFA content in the vegetable oils represents only the 5-15% w/w of the total compounds [1]. MCFAs are very attracting compounds as they have different applications in food, pharma, chemical and biofuel industries. The investigation of sustainable processes for MCFAs production has been receiving great attention from scientific community in last decade. In particular, anaerobic fermentation is a well-known technology for the organic wastes conversion into valuable bioproducts, such as Volatile Fatty Acids (VFAs), and biogas, a gaseous blend rich in methane. But the higher energy densities and economic value than the traditional methane and VFAs, make MCFAs more interesting in a biorefinery scope [2].

The biological process that appears as the most promising for MCFAs synthesis is represented by the “chain elongation”, where short chain VFAs, derived from the acidogenic fermentation of organic substrates, are converted into MCFAs through the addition in the reaction medium of electron donor-compounds, such as ethanol, lactate, methanol, n-propanol of biological origin. VFAs and ethanol conversion into MCFA is carried out by microorganisms able to elongate the VFAs’ carbon chain by reverse β-oxidation pathway, as reported in Figure 1.



**Figure 1**. Reverse β-oxidation pathway for the VFAs conversion into MCFAs.

Considering the caproic acid production from ethanol and acetate, ethanol is first converted into acetyl-CoA before entering into two cycles of reverse β-oxidation. The acetyl-CoA is stretched into butyrate along the first cycle of reverse β-oxidation reacting with acetate. The remaining acetyl-CoA is then used in the second cycle of reverse β-oxidation, reacting with the butyrate to generate the caproate.

The challenge of this work is to select caproic acid producing microorganisms by reverse β-oxidation from Microbial Mixed Culture (MMC). Firstly, batch tests were performed using synthetic acetate and ethanol at different molar ratios in order to verify the possible MCFAs production yields. In order to assure the presence of MMC, agricultural digestate was adopted as inoculum. With the optimization of the acetate-ethanol ratio, the chain elongation for the caproic acid production was also tested in a 1 liter CSTR reactor.

**2. Methods**

One of the main parameters governing the chain elongation process is represented by the acetate-ethanol molar ratio. The theoretical acetate-ethanol molar ratio for a complete conversion of the two substrates into caproate is 1:2. Previous studies demonstrated that this ratio must be lower than 1:2 because only 5/6 of the acetyl-CoA derived from ethanol enters in the reverse β-oxidation cycle for the caproate production, while the remaining 1/6 of the acetyl-CoA is used to provide ATP. But, acetate:ethanol ratios lower than 1:10 stop the process as consequence of the lack of enough acetate to support the reactions of the first cycle of reverse β-oxidation [3].

**2.1 Batch tests for the selection of the best acetate: ethanol molar ratio**

Different batch tests were carried out to individuate the optimal acetate: ethanol molar ratio and the best pH. Tests were performed in 0.5L reactors with a working volume of about 250 mL under anaerobic condition and at mesophilic temperature (37°C). The MMC was assured by the addition of agricultural digestate (inoculum), having a Total Solids (TS) and Volatile Solids contents of 5.29 and 3.31% w/w, respectively. The inoculum:substrate ratio was of 2:1 in terms of VS content, as suggested by Liu et al. (2017) [4]. Two rounds of batch tests were performed according to the acetate: ethanol molar ratios of Table 1, at pH of 7 and 9, respectively.

Table 1. Batch tests’ configuration

|  |  |  |  |
| --- | --- | --- | --- |
| Acetate: Ethanol ratio | Inoculum (g) | Acetate (g) | Ethanol (g) |
| 1:2 | 240 | 1.56 | 2.4 |
| 1:3 | 240 | 0.48 | 0.92 |
| 1:5 | 240 | 0.84 | 3.16 |
| 1:10 | 240 | 0.84 | 6.24 |

Batch tests had a duration of 10 days, considered the required time to allow the starting of the chain elongation for the acetate and ethanol conversion into caproic acid [4].

VFAs and MCFAs (C6, C8 and C10) concentrations were monitored at the end of the tests. The process performances were evaluated considering both the MCFAs and VFAs production yields, expressed as:

MCFAs yield (YMCFAs, % w/w) = \*100

VFAs Yield (YVFAs, % w/w) = \*100

In order to close the mass balance of the process, biogas production was also monitored following the Holliger et al. (2016)’s protocol [5].

**2.2 Continuous test**

The best acetate: ethanol molar ratio in terms of MCFAs yield from the batch tests was tested in continuous mode. A 1L CSTR reactor, with a working volume of about 0.6L, was initially fed with an inoculum amount of 580 g. Then, the CSTR were daily discharged and fed with about 5g of acetate and 20 of ethanol in order to have an acetate: ethanol molar ratio of about 1:5, the best one in term of MCFAs yield from the batch test. The Hydraulic Retention Time was set up at 25 days. Continuous test is still running and will be stopped at the end of 3 HRT. The corresponding results will not be included in the present abstract.

**3. Results and discussion**

The first main result from batch tests was that pH 9 inhibited completely any biological processes: acidogenic fermentation, reverse β-oxidation and biogas production, demonstrating that the neutral pH values are the ideal for all these processes.

Figure 2 summarized the MCFAs production yields for the batch tests carried at different acetate:ethanol molar ratios and pH 7.

**Figure 2.** MCFAs production yield for the different acetate: ethanol molar ratio

The tests confirmed that acetate:ethanol molar ratio is a fundamental parameter for the beginning of reverse β-oxidation reactions. In particular, the theoretical molar ratio of 1:2 was not adequate, as ethanol is not employed exclusively for the chain elongation but also for proving energy by ATP production, as commented above [2]. Instead, a too low acetate: ethanol molar ratio of 1:10 did not assure the minimal acetate amount to provide its elongation into MCFAs. The best acetate: ethanol molar ratio was of 1:5 with a MCFAs production yield of 6% w/w of the initial substrates amount (ethanol and acetate) fed in the reactor. Caproic acids accounted for the 90-95% w/w of the total MCFAs produced along the batch tests, while octanoic and decanoic acids represented the remaining part, suggesting that probably more days are required for their production.

Considering only batch test with the best acetate: ethanol ratio of 1:5, the VFAs production yield was of about the 27% w/w of the total amount of VS fed in the reactor, with butyric acid as the main product (around the 85-90% w/w). It demonstrated that reverse β-oxidation reactions were still running at the end batch tests, which had a duration of 10 days. For this reason, the following continuous test was set up at higher HRT of 25 days, that is also consistent with some previous research works [3, 4].

It is important to note that most of the VS derived from acetate and ethanol was converted into biogas, whose produced amount was about 2.3L. It corresponded to about 580 mLbiogas/gVS, for the batch test of an acetate: ethanol molar ratio of 1:5. Consequently, VS were converted for the 60% into biogas, for the 27% into VFAs and for the 6% into MCFAs. It demonstrated that batch configuration can assure the conversion of the substrates into MCFAs just for a minimal fraction, favoring the anaerobic digestion reactions for biogas production. The selection of MCFAs producing microorganisms could be made just with a continuous feeding of the acetate and ethanol, and consequently with a CSTR configuration.

**4. Conclusions**

A preliminary research work for MCFAs production at batch tests allowed the selection of the best operational conditions to increase the yield’s process. It emerged that a neutral pH and an acetate: ethanol molar ratio of 1:5 optimized the MCFAs production yield, achieving a final conversion of the 6% w/w of the substrates (VS based). Considering the high VS conversion into VFAs (27% w/w) and of butyric acid (representing the 90% w/w of the total VFAs), it emerged that 10 days were not enough to assure the completing of reverse β-oxidation reactions of the VFAs into MCFAs. Finally, it was demonstrated that batch test favored the anaerobic digestion reactions for biogas production rather than the selection of MCFAs producing microorganism.

A continuous test operated at neutral pH, an acetate: ethanol molar ratio of 1:5 and at high HRT (25 days) is still running and its results will be available in the next future.

**References**

[1] Venkateswar Reddy, M., Kumar, G., Mohanakrishna, G., Shobana, S., & Al-Raoush, R. I. (2020). Review on the production of medium and small chain fatty acids through waste valorization and CO2 fixation. *Bioresource Technology*, *309*(April), 123400. <https://doi.org/10.1016/j.biortech.2020.123400>

[2] Strazzera, G., Battista, F., Andreolli, M., ...Bolzonella, D., Lampis, S. (2021). Influence of different household Food Wastes Fractions on Volatile Fatty Acids production by anaerobic fermentation. Bioresource technology, 335, 125289. https:// 10.1016/j.biortech.2021.125289

[3] Zhang, C., Yang, L., Huo, S., Su, Y., & Zhang, Y. (2021). Optimization of the Cell Immobilization-Based Chain-Elongation Process for Efficient n-Caproate Production. *ACS Sustainable Chemistry and Engineering*, *9*(11), 4014–4023. https://doi.org/10.1021/acssuschemeng.0c07281

[4] Liu, Y., He, P., Shao, L., Zhang, H., & Lü, F. (2017). Significant enhancement by biochar of caproate production via chain elongation. *Water Research*, *119*, 150–159. https://doi.org/10.1016/j.watres.2017.04.050

[5] Holliger, C., Alves, M., Andrade, D., Angelidaki, I., Astals, S., Baier, U., Bougrier, Clair et al. (2016). Towards a standardization of biomethane potential tests. Water science and technology, 74(11), 2515-2522. https://doi.org/10.2166/wst.2016.336