



Aerobic and facultative bacteria: working horses at the service of Anaerobic Digestion

M. S. Duarte^{a1}, J. V. Oliveira^{a2}, C. P. Magalhães^{a3}, A. F. Salvador^{a4}, A. R. Castro^{a5}, A. J. M. Stams^{a,b,6}, A.J. Cavaleiro^{a7}, M. A. Pereira^{a8} and M. M. Alves^{a9}

^aCentre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal;

^bWageningen University & Research, Laboratory of Microbiology, Stippeneng 4, 6708 WE Wageningen, Netherlands

¹salomeduarte@ceb.uminho.pt, ²jvoliveira@ceb.uminho.pt, ³c.pereira.mg@ceb.uminho.pt, ⁴asalvador@ceb.uminho.pt,
⁵ritacastro@deb.uminho.pt, ⁶acavaleiro@deb.uminho.pt, ⁷fons.stams@wur.nl, ⁸alcina@deb.uminho.pt,
⁹madalena.alves@deb.uminho.pt

Abstract: It is clear that aerobic and facultative anaerobic bacteria have an important role in the first steps of the anaerobic digestion (AD) process, especially when complex organic compounds are degraded. However, their diversity, abundance and function, related to the fine control of process variables such as pH and ORP, and the potential establishment of networks with methanogens and acetogens are far from being fully understood. Here we show some examples demonstrating that microaerophilic and aerobic conditions are critical for accelerating the methane production rates from hydrophobic compounds such as lipids and hydrocarbons. In the later case, a combination of aerobic with methanogenic conditions allow to convert hydrocarbons to methane at accelerated rates, with bacterial lipids as main intermediates. We believe that hybrid fine-controlled microaerophilic AD processes will emerge as the next generation of applications of AD technology to boost the methane production rate from a myriad of anaerobically slow degraded complex substrates.

Keywords: Anaerobic digestion; Microaeration; Aerobic and facultative bacteria

Session – Microbiology of anaerobic digestion

Introduction

Aerobic and facultative anaerobic bacteria are usually found in methanogenic bioreactors and have a role in the first steps of the AD process, including the uptake of residual oxygen, thus protecting strict anaerobes from O₂ toxicity. However, their diversity, abundance and function, related to the fine control of process variables such as pH and ORP, and the potential establishment of networks with methanogens and acetogens are far from being fully understood. Some of these microorganisms are able to degrade hydrophobic compounds such as lipids or alkanes. Lipids are usually hydrolysed to LCFA, which are rather similar, in terms of chemical structure, to n-alkanes. Biodegradation of both LCFA and n-alkanes is thought to undergo beta-oxidation, but the initial activation of hydrocarbons under anaerobic conditions remains the critical step for the subsequent metabolism of these apolar substrates. After activation, it is proposed that these compounds are converted to LCFA, followed by beta-oxidation, forming ultimately acetate and hydrogen, the main substrates for methanogenesis.

We found recently that facultative anaerobic bacteria were directly involved in the transformation of unsaturated-LCFA (Duarte et al, 2018). In bioreactors where methanogenesis was inhibited, oleate (C18:1) was converted to palmitate (C16:0) and facultative bacteria (mainly *Pseudomonas* and *Rheinheimera* genera) represented approximately 50 % of the total microbial community (Cavaleiro et al., 2016). On the other hand facultative and aerobic bacteria, namely *Marinobacter*, *Alcanivorax* and *Rhodococcus*, can convert hydrocarbons into bacterial lipids under aerobic conditions, which can be further converted to methane. Thus, aerobic and/or facultative anaerobic bacteria seem to be key players in oils-to-methane bioconversion, probably by thriving in specific oxidation-reduction potential (ORP) conditions. We have been studying the effect of microaeration in methanogenic communities converting LCFA to methane, and a two-step aerobic-anaerobic process to transform n-alkanes to methane at accelerated rates. These two innovative strategies are herein presented and the role of facultative and aerobic bacteria in those conditions is demonstrated.

Degradation of LCFA Stimulated by Microaeration in Methanogenic Bioreactors

Very high loads of oleate (up to $14 \text{ g L}^{-1} \text{ d}^{-1}$, in COD) were converted to palmitate in a continuous plug flow reactor where the abundance of facultative anaerobic bacteria, mainly *Pseudomonas* spp., was strongly correlated ($p < 0.05$) with palmitate-to-total LCFA percentage (Duarte et al., 2018). Two *Pseudomonas* strains, isolated from this bioreactor, were incubated in batch under anaerobic and microaerophilic conditions, together with a syntrophic oleate degrading co-culture (*Syntrophomonas zehnderi* and *Methanobacterium formicicum*). The microbial consortia converted oleate to acetate and methane 1.5 times faster than the syntrophic culture alone (under strict anaerobic conditions) (Duarte, 2018), showing that facultative anaerobic bacteria (i.e., *Pseudomonas* isolates) significantly accelerate the methanogenic conversion of oleate.

In another study, bioreactors treating oleate-based wastewater were operated under different redox conditions (strictly anaerobic – AnR, -350 mV ; microaerophilic – MaR, -250 mV), at increasing organic loading rates (OLR) of $1 \text{ kg m}^{-3} \text{ d}^{-1}$ and $3 \text{ kg m}^{-3} \text{ d}^{-1}$ in COD. Palmitate accumulated 7 times more and facultative anaerobes were more abundant in the MaR than in the AnR, for the higher OLR. Methanogens became inhibited in both reactors, but MaR was able to recover the methanogenic activity after a batch period, while AnR did not recover. These results show the importance of microaeration and ORP on shaping robust microbial communities in continuous bioreactors converting LCFA to methane (Duarte et al., 2018).

In another preliminary study, by controlling the input of limited oxygen amounts, it was possible to fine-tune the ORP conditions. Four parallel CSTR (DASGIP® Parallel Bioreactor Systems from Eppendorf), inoculated with the same methanogenic sludge, were operated under different redox conditions for 18 days (ORP-R1= -200 mV , ORP-R2= -300 mV , ORP-R3= -400 mV and ORP-R4= -450 mV) and fed with an oleate-based effluent (10 g L^{-1} in COD; HRT=10 d). Due to the different ORP imposed, sludges behaved differently and the bioreactors presented distinct oleate degradation profiles (Fig.1).

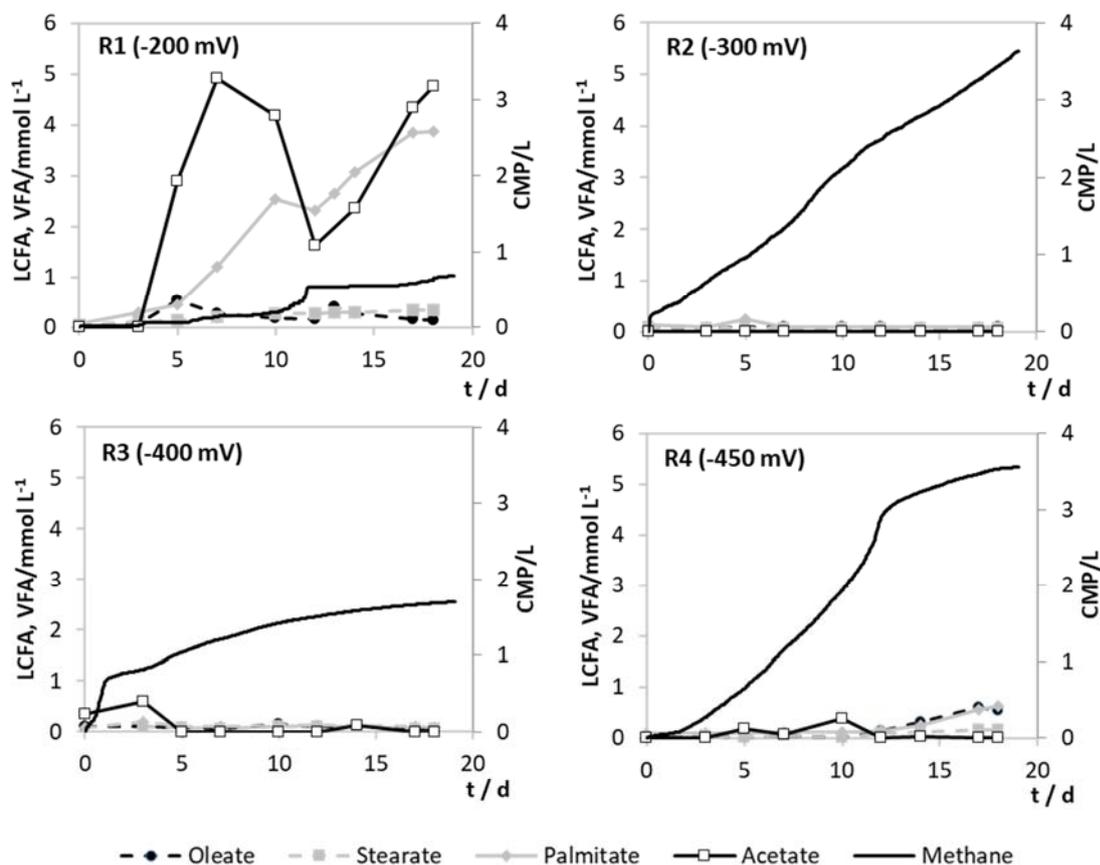


Figure 1 – LCFA and VFA (acetate) concentrations (primary axis) and cumulative methane production (CMP) (secondary axis) during the operation of reactors R1, R2, R3 and R4.

R1 can be distinguished from all the other reactors since, at the highest ORP tested, palmitate and acetate accumulated up to 4 and 5 mmol L^{-1} , respectively. This degradation profile resembles

previous bioreactor operations, where palmitate and acetate transiently accumulate during oleate conversion (Pereira et al., 2002; Cavaleiro et al., 2009). This does not mean that these intermediate products would not be further converted to methane in an extended bioreactor operation, even because the HRT used herein was only 10 days in a chemostat-type of reactor, and methanogens tended to be washed-out along the operating time. At lower ORP (reactors R2 and R3), accumulation of intermediate products was not observed, only in R4 palmitate and oleate started to accumulate mainly at the end of the reactor operation and at lower concentrations (Fig.1). The methane production rate was constant in R2 and reached the highest cumulative methane production (CMP) (3.63 L), showing that this bioreactor was the most efficient in the conversion of oleate to methane. These results suggest that residual amounts of oxygen, and consequently the variation of the ORP affects biological conversion rates. Further studies are needed to narrow the range of the ORP that maximizes the oleate conversion to methane and to better understand the process microbiology.

Aerobic conversion of hydrocarbons to lipids, followed by anaerobic degradation: a two-step strategy to speed up hydrocarbon biotransformation to methane

Anaerobic degradation of hydrocarbons is a very slow process, taking months to occur (Berdugo-Clavijo and Gieg, 2014), turning the AD applied directly to these compounds unsuccessful. Castro et al. (2017) have recently shown that a pre-incubation with the hydrocarbonoclastic bacterium *Rhodococcus opacus* B4, under aerobic conditions, of hydrocarbon contaminated cork used as oil-spill sorbent allowed a fast and effective conversion of the hydrocarbons into bacterial lipids, which was then converted to methane, overcoming the slow hydrocarbon AD drawback. *R. opacus* B4 consumed up to 96 % of hexadecane (1 g L⁻¹) impregnated in the cork sorbents after 48 hour incubation, resulting in a triacylglycerol (TAG) content of 0.77 ± 0.04 g g⁻¹ of cell dry weight (CDW). In a second anaerobic step, the resulting lipid-rich biomass (lipids containing cells + cork) was efficiently converted to methane (82 ± 4 % of conversion), reaching a maximum methane production of 0.40 L CH₄ g⁻¹ CDW, after 7 days of incubation. By applying this two-step process, a much faster and efficient conversion of hydrocarbons to methane was achieved.

Conclusions

The work presented shows that redox potential controlled by microaeration stimulates the growth of anaerobic facultative bacteria and enhances LCFA conversion to methane. The composition and function of the microbial communities, is currently under study, targeting the optimization of LCFA conversion to methane. We have also shown that the two-step aerobic/anaerobic process accelerates hydrocarbons transformation to methane, which represents an innovative strategy for the ex-situ restoration of petroleum contaminated sites or for recycling oil-biosorbents through methane production. We believe that hybrid fine-controlled microaerophilic AD processes will emerge as the next generation of applications of AD technology to boost the methane production rate from a myriad of anaerobically slow degraded complex substrates.

References

- Berdugo-Clavijo, C. and Gieg, L. M. (2014) Conversion of crude oil to methane by a microbial consortium enriched from oil reservoir production waters. *Frontiers in Microbiology*, **5**, 1–10.
- Castro, A. R., Guimarães, M., Oliveira, J. V., and Pereira, M. A. (2017) Production of added value bacterial lipids through valorisation of hydrocarbon-contaminated cork waste. *Science of the Total Environment*, **605–606**, 677–682.
- Cavaleiro, A. J., Pereira, M. A., Guedes, A. P., Stams, A. J. M., Alves, M. M., and Sousa, D. Z. (2016) Conversion of C_n-Unsaturated into C_n-2-Saturated LCFA Can Occur Uncoupled from Methanogenesis in Anaerobic Bioreactors. *Environmental Science & Technology*, **50**(6), 3082–3090.
- Cavaleiro, A. J., Salvador, A. F., Alves, J. I., and Alves, M. (2009) Continuous high rate anaerobic treatment of oleic acid based wastewater is possible after a step feeding start-up. *Environmental science & technology*, **43**(8), 2931–2936.
- Duarte, M. S. (2018) Microbial Interactions in the Transformation of Long-Chain Fatty Acids in Anaerobic Reactors.
- Duarte, M. S., Silva, S. A., Salvador, A. F., Cavaleiro, A. J., Stams, A. J. M., Alves, M. M., and Pereira, M. A. (2018) Insight into the Role of Facultative Bacteria Stimulated by Microaeration in Continuous Bioreactors Converting LCFA to Methane. *Environmental Science & Technology*, **52**, 6497–6507.
- Pereira, M. A., Pires, O. C., Mota, M., and Alves, M. M. (2002) Anaerobic degradation of oleic acid by suspended and granular sludge: identification of palmitic acid as a key intermediate. *Water science and technology*, **45**(10), 139–144.