Microbial Interactions in the Transformation of Long-Chain Fatty Acids in Anaerobic Reactors
Maria Salomé Lira Duarte
UMinho | 2018

Universidade do Minho
Escola de Engenharia

Maria Salomé Lira Duarte

Microbial Interactions in the Transformation of Long-Chain Fatty Acids in Anaerobic Reactors

dezembro de 2018

Universidade do Minho
Escola de Engenharia

Maria Salomé Lira Duarte

Microbial Interactions in the Transformation of Long-Chain Fatty Acids in Anaerobic Reactors

Tese de Doutoramento
Doutoramento em Bioengenharia

Trabalho elaborado sob a orientação da
Prof. Doutora Maria Madalena dos Santos Alves
da
Doutora Maria Alcina Alpoim de Sousa Pereira
e do
Prof. Doutor Alfons Johannes Maria Stams

dezembro de 2018

Nome

Maria Salomé Lira Duarte

Endereço eletrónico

salomeduarte@ceb.uminho.pt

Telefone

+351 253 601 963

Número do Cartão de Cidadão

13810448

Título

Microbial Interactions in the Transformation of Long-Chain Fatty Acids in Anaerobic Reactors

Interações Microbianas na Transformação de Ácidos Gordos de Cadeia Longa em Reatores Anaeróbios

Orientador(es)

Prof. Doutora Maria Madalena dos Santos Alves

Doutora Maria Alcina Alpoim de Sousa Pereira

Prof. Doutor Alfons Johannes Maria Stams

Ano de conclusão

2018

Doutoramento em Bioengenharia

Universidade do Minho, ____ / ____ / _____

Assinatura: _____

DECLARAÇÃO DE INTEGRIDADE

Declaro ter atuado com integridade na elaboração da presente tese. Confirmo que em todo o trabalho conducente à sua elaboração não recorri à prática de plágio ou a qualquer forma de falsificação de resultados. Mais declaro que tomei conhecimento integral do Código de Conduta Ética da Universidade do Minho.

Universidade do Minho, ___ / ___ / _____

_____

Maria Salomé Lira Duarte

AGRADECIMENTOS

Ao fim destes 4 anos, chegou finalmente a altura de agradecer e mostrar o apreço por todas as pessoas que, de uma forma ou de outra, me ajudaram a chegar até aqui.

Em primeiro ludar gostaria de agradecer aos meus orientadores: Madalena, Alcina e Fons.

Muito obrigada Madalena por sempre teres acreditado em mim, mesmo ainda antes de ter terminado o mestrado. Estou-te mesmo muito grata pela oportunidade de fazer o doutoramento neste tema, que sei ser importante para ti e para o grupo. Madalena e Alcina, agradeço-vos pelo apoio, partilha de conhecimentos e toda a ajuda e paciência demonstrada ao longo destes anos. Quando comecei o doutoramento era "pequenina", mas com a vossa ajuda sinto que cresci e que sou muito "maior" agora. Obrigada por me terem ajudado a crescer.

Thank you Fons, for sharing your knowledge and experience. It was very important to me all the meetings that we had, trying to link microbiology to biotechnology (reactors operation, etc.). I believe that this double concept has turned this work richer. Thank you for all your support.

Ana Júlia, agradeço-te mesmo muito por toda a ajuda. Foste sempre incansável e ensinaste-me muito. Muito obrigada por tudo!

Andreia, Joana e Rita Catita obrigada por toda a partilha e ensinamentos nas várias (muitas) dúvidas/pedidos de ajuda que foram surgindo ao longo desta caminhada. Tenho a certeza que vos vou continuar a chatear.

À Engenheira Madalena e ao Sr. Santos muito obrigada por me terem socorrido inúmeras vezes ao longo destes anos. Agradeço também à restante equipa técnica, às meninas da secretaria e ao Doutor Paulino por me terem ajudado sempre com muito agrado.

Obrigada Sérgio por todo o babyssiting, ensinamentos e socorrismo com os meus meninos (reatores). Sem ti e sem o teu apoio muitas inundações (ou outras catástrofes) poderiam ter acontecido.

Obrigada a todo o pessoal do BRIDGE, especialmente ao do LBA, a vossa boa disposição e companheirismo trouxeram uma leveza adicional ao trabalho ao longo desta etapa. João, Sérgio, Sónia, Jorge, Dani, Ana Lu, Carla(s), Cátia, Rita(s), Ricardo, Sofia Ferreira, Quim e todos os outros, obrigada pela vossa amizade e paciência ao longo destes anos.

Às minhas biológicas preferidas muito obrigada por tudo. Bé e Boguinhas o vosso apoio, compreensão e carinho significam mesmo muito para mim. Fico feliz por termos crescido juntas ao longo destes anos.

À minha família, especialmente aos meus pais, à Jó, à minha avó e à minha madrinha muito obrigada por tudo. O vosso apoio foi essencial, não só nesta etapa como em todas as outras que a precederam. Sem vocês eu não teria conseguido.

Ao João, obrigada por todo o apoio e por acreditares em mim (muito mais do que eu). Esta tese não é só minha, é nossa.

This PhD was supported by the European Research Council under the European Union's Seventh Framework Programme (FP/2007-2013)/ERC Grant Agreement No 323009. Additionally, this study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469 unit and COMPETE 2020 (POCI-01-0145-FEDER-006684) and BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 - Programa Operacional Regional do Norte.

SUMMARY

Conversion of long-chain fatty acids (LCFA) to methane in continuous bioreactors is not fully understood, in particular for unsaturated LCFA. For example, palmitate (C16:0, saturated LCFA) often accumulates during oleate (C18:1, unsaturated LCFA) biodegradation in anaerobic bioreactors, and the reason why this happens and which microorganisms catalyze this reaction remained unknown. Facultative anaerobic bacteria are frequently found in reactors operated at high loads of LCFA, but their function is also unclear.

In this thesis, oleate degradation in continuous bioreactors was studied to get further insights on the microbial interactions occurring in the transformation of LCFA. For that propose we compared bioreactors treating oleate-based wastewater (organic loading rates of 1 and 3 kg COD m^{-3} d^{-1}), operated under different redox conditions (strictly anaerobic-AnR, -350 mV; microaerophilic-MaR, -250 mV) (Chapter 3). For the higher load tested, palmitate accumulated 7 times more in the MaR, where facultative anaerobes were more abundant, and only the biomass from this reactor could recover the methanogenic activity, after a transient inhibition. In other experiment, two bioreactors were operated separately and the abundance of facultative anaerobic bacteria, namely *Pseudomonas* spp. (from which two strains were isolated), was strongly correlated ($p<0.05$) with palmitate-to-total LCFA percentage in the biofilm formed in a continuous plug flow reactor fed with very high loads of oleate (Chapter 4). The *Pseudomonas* isolates were further tested in batch and continuous reactors together with a syntrophic LCFA-degrading co-culture of *Syntrophomonas zehnderi* and *Methanobacterium formicicum* (Chapter 5). The results obtained show that facultative bacteria play an important role in anaerobic reactors treating oleate-based wastewaters, protecting the strict anaerobes from oxygen toxicity and decreasing the oleate toxicity towards the methanogens helping to improve oleate degradation. An additional study was performed to get insight into the production of biosurfactants and biopolymers by the *Pseudomonas* isolates and the anaerobic granular sludge during oleate degradation in continuous bioreactors (Chapter 6). The results indicate that possibly glycolipids and hydroxyalkanoates may have been produced, however it was not possible to identify these compounds. In conclusion, this thesis showed that the oxidation-reduction potential (ORP) and therefore the presence of facultative bacteria, regulate oleate to palmitate conversion, decreasing the LCFA toxicity toward methanogens, ultimately accelerating the methane production from lipid-rich wastewaters.

SUMÁRIO

A conversão de ácidos gordos de cadeia longa (AGCL) a metano em reatores operados em contínuo, não é totalmente compreendida, em particular para AGCL insaturados. A acumulação de palmitato (C16:0, AGCL saturado) ocorre frequentemente durante a biodegradação de oleato (C18:1, AGCL insaturado) em reatores em contínuo, no entanto a razão pela qual isso acontece e quais os microrganismos que catalisam essa reação continuam desconhecidos. Bactérias anaeróbias facultativas são frequentemente encontradas nesses reatores operados com elevadas cargas de AGCL, no entanto a sua função também não é clara. Neste trabalho comparámos bioreatores que trataram um efluente rico em oleato (cargas orgânicas de 1 e 3 kg COD m^{-3} d^{-1}), operados em diferentes condições de redox (estritamente anaeróbio – AnR, -350 mV; microaerofílico – MaR, -250 mV) (Capítulo 3). Para a carga mais elevada, a acumulação de palmitato foi 7 vezes mais elevada no reator MaR, no qual os microrganismos anaeróbios facultativos eram mais abundantes e apenas a biomassa desse reator conseguiu recuperar a atividade metanogénica depois de uma inibição transiente. Noutra experiência, dois reatores foram operados separadamente e a abundância de bactérias anaeróbias facultativas, nomeadamente de *Pseudomonas* spp (do qual duas estirpes foram isoladas), esteve fortemente relacionada com a percentagem de palmitato em relação ao total de AGCL presente no biofilme, o qual se formou num reator pistão alimentado com elevadas cargas de oleato (Capítulo 4). As *Pseudomonas* isoladas foram testadas em reatores batch e contínuos, juntamente com a co-cultura sintrófica composto por *Methanobacterium formicicum* e *Syntrophomonas zehnderi* (Capítulo 5). Os resultados obtidos mostram que as bactérias facultativas têm um papel importante nos reatores anaeróbios que tratam efluentes ricos em oleato, uma vez que protegem os microrganismos estritamente anaeróbios da toxicidade do oxigénio e diminuem também o efeito tóxico do oleato nos microrganismos metanogénicos, melhorando assim a degradação de oleato. Foi realizado ainda um estudo adicional de forma a se obter mais informação sobre a produção de biosurfactantes e biopolímeros pelos isolados de *Pseudomonas* e por biomassa granular anaeróbia durante a degradação de oleato em bioreatores operados em contínuo (Capítulo 6). Os resultados indicam que possivelmente glicolípidos e hidroxialcanoatos podem estar a ser produzidos, no entanto não foi possível identificar estes compostos. Em suma, esta tese mostrou que o potencial redox e consequentemente a presença da bactérias facultativas, regulam a conversão do oleato a palmitato, diminuindo a toxidade dos AGCL para os microrganismos metanogénicos, e por fim acelerando a produção de metano a partir de águas residuais ricas em lípidos.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

AD	anaerobic digestion
AnR	anaerobic reactor
An-MS	anaerobic test with *Methanobacterium formicicum* and *Syntrophomonas zehnderi*
An-MSP	anaerobic test with *Methanobacterium formicicum*, *Syntrophomonas zehnderi* and *Pseudomonas* isolates
BLASTN	standard nucleotide basic local alignment search tool
BrES	bromoethanesulfonate
COD	chemical oxygen demand
CSTR	continuous stirred tank reactor
CTR_ MSP	control test with *Methanobacterium formicicum*, *Syntrophomonas zehnderi* and *Pseudomonas* isolates.
CTR_MS	control test with *Methanobacterium formicicum* and *Syntrophomonas zehnderi*
CTR_P	control test with *Pseudomonas* isolates
DAPI	4',6-diamidino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
ENA	European Nucleotide Archive
FAB	facultative anaerobic bacteria
FID	flame ionization detector
FOG	fat, oil and grease
GC	gas chromatography
HPLC	high-performance liquid chromatography
HRT	hydraulic retention time
I1	*Pseudomonas* isolate 1
I2	*Pseudomonas* isolate 2
LCFA	long-chain fatty acids;
MaR	microaerophilic reactor
Mic-MSP	Microaerophilic test with *Methanobacterium formicium*, *Syntrophomonas zehnderi* and *Pseudomonas* isolates
MP	*Methanobacterium formicium* and *Pseudomonas* isolates
MS	*Methanobacterium formicium* and *Syntrophomonas zehnderi*
MSP	*Methanobacterium formicium*, *Syntrophomonas zehnderi* and *Pseudomonas* isolates
OCT	optimum cutting temperature
OLR	organic loading rate
ORP	oxidation-reduction potential
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

PFR	plug flow reactor
POME	palm oil mill effluent
R-GS	reactor with granular sludge
R-I1	reactor with isolate 1
R-I2	reactor with isolate 2
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
STP	standard temperature and pressure
VFA	volatile fatty acids
VS	volatile solids

Chapter 1.

Context, Aim and Thesis Outline

1.1. CONTEXT AND MAIN RESEARCH QUESTIONS

Among other substrates, lipids are ideal sources for methane production, since theoretically their biological degradation generate more biogas (1.425 L per g of substrate) richer in methane (69.5 %), when compared with other carbon sources such as carbohydrates (0.830 L g^{-1}, 50.0 %) and proteins (0.921 L g^{-1}, 68.8 %) (Alves et al., 2009).

The initial step in lipids degradation is the hydrolysis that generates glycerol and Long-Chain Fatty Acids (LCFA). The most abundant LCFA present in industrial and domestic wastewaters is oleic acid (C18:1 LCFA) (Baserba et al., 2012). When an oleate based effluent is continuously fed to an anaerobic reactor, a significant accumulation of palmitate (C16:0) occurs, and the sludge becomes encapsulated and loses methanogenic activity (Pereira et al., 2002b). But once LCFA-loaded sludge is incubated in batch, there is production of large amounts of methane which shows that efficient treatment of LCFA-rich wastewater can be achieved, if a correct equilibrium between accumulation and degradation is assured. The operational strategy for achieving an efficient oleate conversion in continuous bioreactors should involve the acclimation of the sludge based on successive continuous/batch cycles for the development of an anaerobic microbial community able to efficiently mineralize LCFA in continuous (Cavaleiro et al., 2009).

Although methanogenic LCFA degradation is known as an obligatory syntrophic process where hydrogen partial pressure has to be kept low enough (by methanogens) to allow continuous LCFA degradation, the conversion of oleate to palmitate is not dependent on the relationship between oleate degrading bacteria and methanogens (Cavaleiro et al., 2016). When methanogens were inhibited in continuous bioreactors, anaerobic sludge was still able to convert oleate to palmitate, although further palmitate degradation did not occur. That sludge was highly enriched in facultative anaerobic bacteria, namely *Rheinheimera* and *Pseudomonas*, among others, suggesting that they may have a role on oleate to palmitate conversion. Therefore, several questions on this topic remain and will be the focus of this thesis:

1) Why is oleate directly converted to methane in batch whereas in continuous mode transient palmitate accumulation occurs? Is the presence of small amounts of oxygen important for triggering the conversion of oleate to palmitate in continuous bioreactors?

2) Might one or more than one microorganism be involved in oleate conversion to palmitate and in palmitate degradation to the methanogenic substrates? Are facultative anaerobic bacteria involved in oleate to palmitate conversion?

3) What are the differences between the oleate biotransformation by pure cultures or by complex microbial communities? How different can be the role of a microorganism when grown alone or as part of a community?

1.2. AIM

The aim of this work is to get insight of the ecology and physiology of LCFA and lipids biodegradation, attempting to answer the research questions summarized above. Culture-dependent and culture-independent approaches were used to characterize LCFA-degrading microbial communities. Potential interactions between different microbial groups were also investigated in complex mixed or defined cultures.

1.3. THESIS OUTLINE

In **Chapter 1** the context and motivation of the thesis are presented. A state of the art regarding the anaerobic digestion of lipids/LCFA-rich wastewaters is presented in **Chapter 2**, with focus on the biochemistry and microbiology. In the **Chapter 3** two bioreactors were operated under different redox conditions to understand the influence of vestigial amounts of oxygen on oleate conversion to methane and on the microbial community developed. In **Chapter 4**, enrichment and isolation of facultative anaerobic bacteria was performed in a system composed by a continuous stirred tank reactor (CSTR) and a plug flow reactor (PFR), containing a biofilm rich in palmitate. In **Chapter 5**, the facultative anaerobic bacteria isolated in Chapter 4, were further incubated together with a strictly anaerobic LCFA degrading co-culture, composed by *Syntrophomonas zehnderi* and *Methanobacterium formicicum*, to study the interactions between strict anaerobes and facultative anaerobes during LCFA degradation to methane, in anaerobic reactors. In **Chapter 6** the screening for biosurfactants and biopolymers (PHA) produced during oleate degradation in continuous bioreactors was performed. In **Chapter 7** the main conclusions are summarized and futures perspectives for further research are suggested.

Chapter 2.

General Introduction to Anaerobic Microbial Transformation of LCFA

In circular economy's concept, anaerobic digestion plays an important role in transforming waste to renewable energy and nutrients. The anaerobic digestion of lipids/LCFA-rich waste(water) emerges as an interesting solution to deal with the fat, oil and grease (FOG) contaminated waters and to produce biogas. However, in high-rate anaerobic reactors lipids, and particularly LCFA, are not always effectively degraded. It is known that syntrophic bacteria together with methanogens are essential to convert LCFA to methane, nevertheless these bacteria are usually detected in low numbers, raising the question of which microorganisms play a central role in those reactors. Also, methanogenic communities are sensitive to high LCFA loads. This chapter reviews current knowledge on the anaerobic digestion of lipids, particularly LCFA, focusing on the biochemistry and microbiology involved in the anaerobic transformation of LCFA, with special attention on the role of syntrophic and anaerobic facultative bacteria. The toxicity of LCFA towards the microbial community and the possible influence of oxygen on LCFA degradation by methanogenic communities is also addressed.

2.1. Introduction to Anaerobic Digestion of Lipids

Over the past three decades, knowledge on anaerobic technology and microbiology has evolved significantly, allowing the development of anaerobic digestion (AD) as a sustainable treatment solution for a diverse range of wastes.

In AD processes waste lipids are ideal substrates for methane production, since theoretically their degradation produces more biogas and with higher methane content (70 %), yielding 1.4 L of biogas per gram of substrate ($L \cdot g^{-1}$), compared to 0.9 $L \cdot g^{-1}$ (69 %) and 0.8 $L \cdot g^{-1}$ (50 %), respectively for proteins and carbohydrates.

High-rate anaerobic wastewater technologies are well established for effluents from distilleries, pulp and paper, breweries among others. However, when dealing with industries producing high amounts of lipid-rich wastewaters, (e.g. dairy industry) several process problems still compromise a stable and efficient process operation (Alves et al., 2009). In general, economically feasible utilisation of lipids as a resource for AD is not yet widely applied (Dereli et al., 2014).

2.1.1. Nature and occurrence of waste lipids

Lipids usually designated as fat, oils and grease – FOG can be divided into three main groups: neutral lipids, glycolipids and phospholipids (Abraham, 2010).

The major components of oils are fatty acids and triacylglycerols, while minor components include phospholipids and sterols, among others (Gunstone and Harwood, 2007). Lipid-rich effluents and wastes present a similar composition. Over a thousand fatty acids are known with different chain lengths, positions, configurations and level of unsaturation, and also a wide range of additional alternatives along the aliphatic chain. Though, only approximately 20 fatty acids appear widely distributed in nature; of these, palmitic, oleic, and linoleic acids make up around 80 % of commodity oils and fats (Lalman and Bagley, 2001; Novak and Carlson, 1970; Scrimgeour and Harwood, 2007). Unsaturated fatty acids are components of oils while fats are normally composed of saturated fatty acids (Alves et al., 2009).

Lipids are present in most food and beverage production facilities' wastewaters, however their form can vary significantly depending on the industry. Food processing industries such as slaughterhouses, dairies, edible oils production, fish canning factories and wool scouring have wastewaters streams characterized by a high lipid content. The lipids present in the wastewater from industries, that use vegetable or animal oils and fats as raw material, are simple esters of straight

chain, even-numbered long-chain fatty acids and linear polyols (triglycerides, phospholipids). The hydrolysis products of these compounds are also present.

The composition of slaughterhouse wastewater is characterized by a complex mixture of fats, proteins and fibres (Ruiz and Veiga, 1997), being the main constituents in the suspended fraction depending considerably on the production process and the type of animals slaughtered. The dairy industry wastewaters have high concentrations of carbohydrates, proteins and fats, which come from the milk. Due to the fact that the dairy industry produces several products, the characteristics of this effluent vary significantly according to the industry and the process itself (Demirel et al., 2005; Rico Gutierrez, 1991). Another case is the palm oil mill effluent (POME) that is generated during the extraction and purification of palm oil (Igwe and Onyegbado, 2007). The separator sludge and sterilizer effluent are the two most important sources of POME wastewater, which contributes to the highly polluting characteristics of this wastewater (Borja and Banks, 1995; Ismail et al., 2010; Poh et al., 2010). The olive oils wastewaters produced by the traditional mill and press processes have an high organic fraction made up of sugars, polyphenols, polyalcohols, proteins and lipids (Boari et al., 1984) The bioethanol production from corn generates a lipid rich stream called thin stillage, which is a complex wastewater containing high concentrations of carbohydrates, proteins, lipids, glycerol and lactic acid (Kim et al., 2008). According to Becker (1999), the major constituents of wool scouring are fat and oils and the effluent characteristics vary largely between processes and materials (Bisschops and Spanjers, 2008). Regarding LCFA, palmitic and oleic acids are, respectively, the most abundant saturated and unsaturated LCFA present in raw products rich in lipids/fats (Table 2.1) and therefore in the respective waste/wastewater resulted from their process production.

2.2. THE BIOCHEMISTRY AND MICROBIOLOGY OF THE ANAEROBIC DIGESTION OF LIPIDS/LCFA

During anaerobic digestion, the triacylglycerols are hydrolyzed to glycerol and long-chain fatty acids (LCFA) (Figure 2.1). This step is catalyzed by extracellular lipases produced by acidogenic bacteria (Hanaki et al., 1981; Heukelekian and Mueller, 1958; Masse et al., 2002). The released LCFA are further degraded to acetate and hydrogen via β-oxidation (Novak and Carlson, 1970). Acetate and hydrogen are then finally converted to methane and carbon dioxide by methanogens (Bryant, 1979).

Table 2.1 – LCFA composition (%) in the principal products containing fat and/or lipids (adapted from Pereira, 2003)

LCFA (structure)	Palm oil [a]	Olive oil[a]	Soya bean oil[a]	Cotton seed oil[a]	Cocoa butter[a]	Chicken fat[a]	Beef tallow[a]	Whole milk[b]
Lauric (C12:9)	-	-	-	-	-	-	1.0	7.0
Myristic (C14:0)	1.4	-	1.0	1.4	-	1.4	2.6	6.0
Palmitic (C16:0)	42.9	14.3	11.0	25.7	26.7	21.0	28.1	21.0
Palmitoleic (C16:1)	0.7	1.4	-	1.0	0.5	6.7	3.8	2.0
Stearic (C18:0)	4.8	2.4	4.8	2.9	32.9	4.3	20.0	6.0
Oleic (C18:1)	39.0	71.4	21.9	15.2	33.8	42.4	37.6	39.0
Linoleic (C18:2)	10.0	5.5	49.0	51.9	4.3	20.0	2.9	13.0

[a]Taylor, 1965; [b] Hanaki et al., 1981

The hydrolysis of esters of fatty acids is known as a rapid step, leading in some situations to accumulations of free LCFA, indicating that LCFA oxidation is the rate-limiting step during the AD of lipids (Heukelekian and Mueller, 1958; Masse et al., 2002). LCFA are carboxylic acids with a wide range of chain lengths longer than 12 carbon atoms and a variable number of single and/or double bounds. Those with no double-bounds are called saturated, the ones with one or more double bounds are called unsaturated. LCFA have an amphiphilic structure, composed by a hydrophobic aliphatic tail and a hydrophilic carboxylic head. The main LCFA identified in domestic wastewaters and sludge are myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic (C18:1) and linoleic acids (C18:2) (Novak and Carlson, 1970).

The fermentation/acidogenesis step is the following step, in which the products resulting from hydrolysis are converted to fatty acids and alcohols. Frequently, in AD processes the primary fermenting bacteria responsible for hydrolysis also ferment the resulting monomers (glycerol and LCFA) further to fatty acids, succinate, lactate and alcohols (Schink, 1997). Some of these fermentation products, namely one-carbon compounds such as CO_2, and acetate and H_2, can be converted directly by methanogens into methane and carbon dioxide. The remaining intermediary products need the action of obligate proton reducers that will convert their substrates to acetate, hydrogen and carbon dioxide (Schink, 1997).

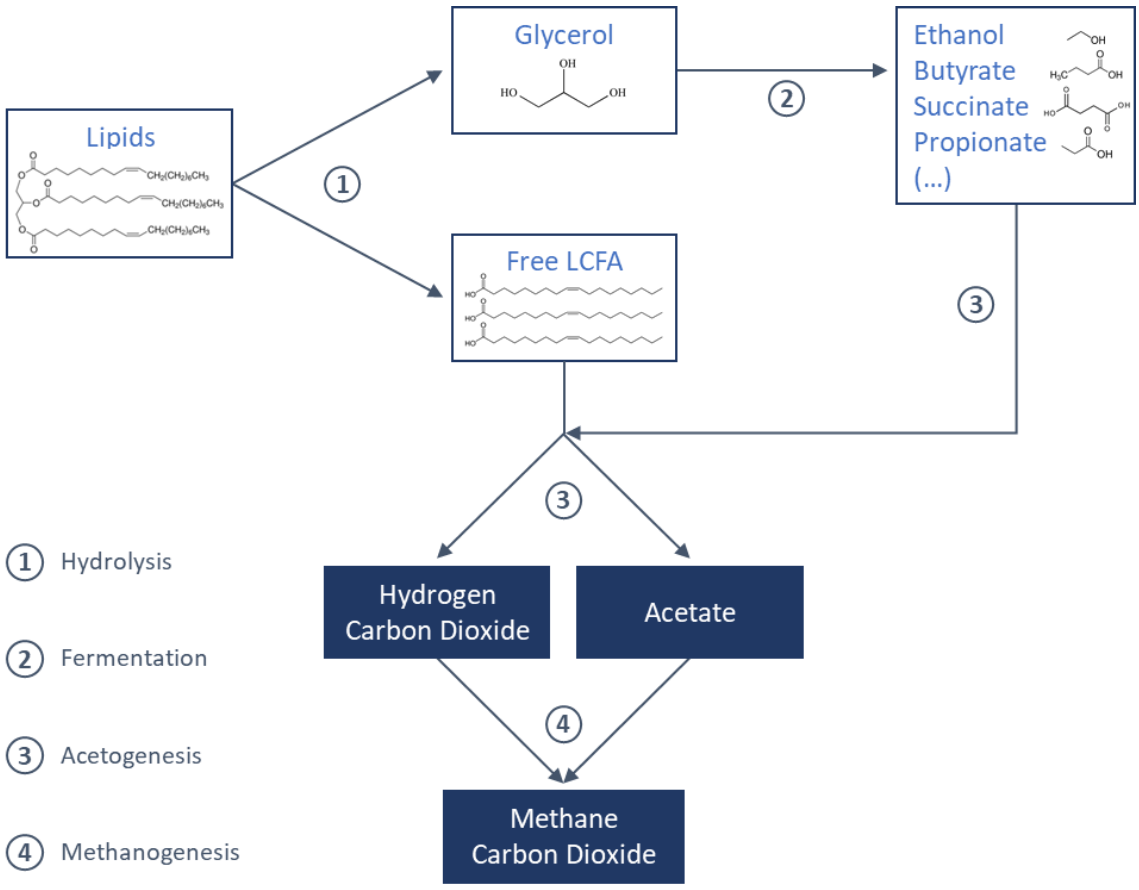


Figure 2.1 – Simplified scheme of the steps in the AD of lipids.

Particularly in the anaerobic digestion of lipids, after the hydrolysis of triacylglycerols, glycerol is fermented to fatty acids (acetate, lactate, succinate, etc.) and ethanol. LCFA degradation is assumed to occur via classical β-oxidation (Heukelekian and Mueller, 1958; Jeris and McCarthy, 1965; Weng and Jeris, 1976). This mechanism results in the sequential removal of two carbon atoms from the LCFA by forming acetate, hydrogen and a shorter LCFA, according to equation 2.1:

$$CH_3(CH_2)_nCOOH + 2H_2O \rightarrow CH_3(CH_2)_{n-2}COOH + CH_3COOH + 2H_2 \qquad \text{eq. 2.1}$$

Initially, the LCFA are taken up by active transport into the bacterial cell (Mackie et al., 1991) and activated to acyl-CoA thioesters by acyl-CoA synthetase. After this step, the fatty acyl-CoA undergoes β-oxidation. This oxidation pathway acts in a cyclic way, resulting, in each cycle, the shortening of the acyl-CoA by two carbon atoms to give ultimately acetyl-CoA and hydrogen (DiRusso et al., 1999). According to Weng and Jeris (1976), the most probable pathway for unsaturated LCFA degradation involves the preliminary hydrogenation of double bond, before going through the β-oxidation. As a result of a preliminary hydrogenation of the unsaturated-LCFA oleate (C18:1), stearate (C18:0) would be formed and further degraded via β-oxidation until acetate. However, several authors observed the

accumulation of palmitate during oleate degradation in bioreactors (Lalman and Bagley, 2001; Pereira et al., 2002b), suggesting that after the hydrogenation step, β-oxidation pathway would be interrupted after 1 cycle, generating palmitate according to the following reaction 4 (Table 2.2).

Moreover, Cavaleiro et al. (2016) have also shown that saturated C_{n-2}-LCFA (palmitate) accumulated both in the presence and absence of methanogens during the degradation of unsaturated C_n-LCFA (oleate), representing more than 50 % of total LCFA, which suggests that this pathway would be valid not only for oleate degradation but also for the remaining C_{n-2}-LCFA. Considering a hydrogen partial pressure of 1 Pa, the combination of both sequential step (hydrogenation + β-oxidation), is energetically favourable ΔG'= -73 kJ (Table 2.2).

Based on the assumption that unsaturated LCFA are degraded following these two sequential steps (hydrogenation and β-oxidation), it is still not clear if these steps are performed by one or more microorganisms (Sousa et al., 2009b). It has been suggested that in anaerobic degradation of oleate, the first step of saturation of oleate to stearate would be the limiting step, whereas the first step of β-oxidation of stearate to palmitate would proceed quickly (Pereira et al., 2005). The palmitate (C16:0) accumulation outside the cells in anaerobic bioreactors treating oleate based effluents (Pereira et al., 2002b) suggests that after one cycle of β-oxidation, this compound would be expelled

Table 2.2 – Gibbs free energy changes for some of the acetogenic and methanogenic reactions (presumably) involved in conversion of fatty acids. Adapted from Sousa et al. (2009b).

Reaction	$\Delta G^{0'}$/kJ	$\Delta G'$/kJ
Hydrogenation		
(1) $oleate^- + H_2 \rightarrow stearate^-$	-66	-38
β-Oxidation		
(2) $oleate^- + 2\ H_2O \rightarrow palmitoleate^- + acetate^- + 2\ H_2 + H^+$	+51	-23
(3) $stearate^- + 2\ H_2O \rightarrow palmitate^- + acetate^- + 2\ H_2 + H^+$	+51	-23
Hydrogenation + β-Oxidation		
(4) $oleate^- + 2\ H_2O \rightarrow palmitate^- + acetate^- + H_2 + H^+$	-15	-73
Complete β-Oxidation		
(5) $oleate^- + 16\ H_2O \rightarrow 9\ acetate^- + 15\ H_2 + 8\ H^+$	+338	-177
Methanogenic reactions		
(6) $acetate^- + H_2O \rightarrow HCO_3^- + CH_4$	-31	
(7) $4\ H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O$	-136	

ΔG' – Gibbs free energies (at 25 °C) for fatty acids concentrations of 1 mmol L^{-1}, considering acetate stoichiometric accumulation (9 mmol L^{-1}) and H_2 depletion to a partial pressure of 1 Pa.

$\Delta G^{0'}$ – Gibbs free energies (at 25 °C) calculated at standard conditions (solute concentrations of 1 mol L^{-1} and gas partial pressure of 10^5 Pa).

from the inside to outside the cell. Palmitate can be further degraded by a different or by the same microorganism, since bacteria that degrade unsaturated fatty acids are also able to degrade saturated fatty acids, while the opposite may not be true (Sousa et al., 2007b). Additionally, 16S rRNA gene sequences retrieved from unsaturated-LCFA degrading communities, cluster within the *Syntrophomonadaceae* (*Firmicutes*), while 16 rRNA gene sequences retrieved from saturated-LCFA degrading communities, cluster within *Syntrophaceae* (*Deltaproteobacteria*) (Sousa et al., 2009b).

Other alternative pathways have also been suggested, namely direct β-oxidation without previous fatty acid chain saturation (reaction 2, Table 2.2) (Roy et al., 1986), since unsaturated LCFA intermediates, i.e. palmitoleate (C16:1) and oleate, were also detected during oleate (C18:1) and linoleate (C18:2) degradation in anaerobic bioreactors, respectively (Lalman and Bagley, 2001, 2000). Previously, Weng and Jeris (1976) have also suggested that oleic acid (C18:1) could be converted initially to pelargonic acid (C9:1), prior to further degradation by β-oxidation to acetic and propionic acids. Thus far, experimental data are lacking to prove which pathway prevails during the degradation of unsaturated LCFA in continuous anaerobic reactors.

After β-oxidation, the products obtained during this acetogenic step – acetate and H_2/formate – are further used by methanogens to produce methane.

Lipids, and consequently LCFA, are usually separated from the wastewater before anaerobic digestion, due to operational problems caused mainly by accumulation of LCFA onto the sludge, resulting in biomass flotation and washout. However, LCFA account for more than 90 % of the degradable grease fraction and have a high potential for biogas production (Sousa et al., 2009b). Therefore, understanding the kinetics of LCFA degradation and keeping a high activity of microorganisms degrading LCFA is essential to achieve lipids degradation in anaerobic waste treatment systems.

2.2.1. Syntrophic bacteria

Under methanogenic conditions, LCFA degradation occurs through the cooperation between LCFA-consuming syntrophic bacteria and hydrogenotrophic methanogens, which consumes the hydrogen formed by bacteria to produce methane. None of these groups are able to degrade LCFA alone, so this relationship between syntrophic bacteria and methanogens is very important to achieve LCFA degradation anaerobically (Sousa et al., 2009b, 2007b).

In the absence of a hydrogen scavenger, hydrogen molecules tend to accumulate, increasing the hydrogen partial pressure. Due this fact, LCFA conversion turn out to be thermodynamically

unfeasible (McInerney et al., 2008), since the partial pressure of hydrogen should be maintained lower than 10 Pa (Schink, 1997).

It was only in 1986 that the first LCFA-degrading syntrophic bacteria *Syntrophomonas sapovorans* was isolated in co-culture with *Methanospirillum hungatei* (Roy et al., 1986). Up to now, only few species of syntrophic bacteria are described as able to use LCFA: *Syntrophomonas sapovorans* (Roy et al., 1986), *Syntrophomonas wolfei saponavida* (Lorowitz et al., 1989), *Syntrophomonas curvata* (Zhang et al., 2004), *Syntrophomonas zehnderi* (Sousa et al., 2007c), *Syntrophomonas palmitatica* (Hatamoto et al., 2007a), *Syntrophus aciditrophicus* (Jackson et al., 1999), *Thermosyntropha lipolytica* (Svetlitshny et al., 1996) and *Thermosyntropha tengcongensis* (Zhang et al., 2012). Among these microorganisms, the capability of degrading unsaturated LCFA is restricted to *S. sapovorans*, *S. curvata*, *S. zehnderi*, *T. lipolytica* and *T. tengcongensis* (Sousa et al., 2009b; Zhang et al., 2012). From the previous species described, *T. lipolytica* is the only one able to also hydrolyze lipids (Svetlitshny et al., 1996).

Besides hydrogenotrophic methanogens, acetoclastic methanogens have also an important role in the overall efficient conversion of LCFA to methane, since about 70% of the total theoretical methane obtained from LCFA degradation, derived from the acetate formed during the β-oxidation (table 2.1, reaction 5). Hydrogen scavengers other than methanogens, can also be partners for LCFA degrading syntrophic bacteria. Hydrogen- and acetate-consuming sulfate reducing bacteria (SRB) are commonly found in anaerobic bioreactors and can play the role of syntrophic partners in LCFA degradation, using sulfate as electron acceptor (Sousa et al., 2009a). *Desulfovibrio*, *Desulfomicrobium*- and *Desulforhabdus*-related species overgrew methanogens in LCFA-degrading enrichment cultures in the presence of 10 mmol L^{-1} of sulfate (Sousa et al., 2009a). It was not clear if SRB outcompete hydrogenotrophic methanogens because they may have higher affinity to hydrogen (since they are able to keep the hydrogen partial pressure lower than methanogens (Stams et al., 2003)) or because they could be more resistant to LCFA toxicity (Sousa et al., 2009a).

One important feature to take into account is that the flux of formate and hydrogen in syntrophic communities is highly dependent on the distance between the two organisms – syntrophic bacteria and hydrogen/formate consuming partner (Stams, 1994). Short distances between the syntrophic bacteria and methanogens are advantageous for the transfer of metabolites between each other, resulting in high conversion rates (Stams et al., 2012). For example, in granular sludge, microorganisms are organized in micro-colonies promoting a close proximity between hydrogen-producing and consuming microorganisms, shortening the distance that hydrogen/formate has to

diffuse (Stams et al., 2012). Regarding LCFA-degrading environments, the fatty matrix may be beneficial for hydrogen transfer since the hydrogen solubility is higher in the fatty matrix than in water (Alves et al., 2009). Due to the importance of the diffusion rates, it has also been suggested that formate transfer might be an additional or even a more important mechanism of electron transfer than hydrogen transfer, despite of the diffusion constant of formate (0.0015 mm^2 s^{-1}) in water being 30 times lower than that of hydrogen (0.045 mm^2 s^{-1}), the lower solubility of hydrogen in water originates concentration difference between the acetogens and the methanogens that can be more than 1000 times higher with formate than with hydrogen (Stams, 1994). Therefore, the flux of formate can be much higher than the flux of hydrogen (Stams, 1994). Indeed, the genome of the syntrophic fatty acid degrading bacteria *Syntrophomonas wolfei* encodes five gene clusters for formate dehydrogenases and only three for hydrogenases (Sieber et al., 2010), which suggests that formate may be more important than hydrogen in syntrophic co-cultures. (Schmidt et al., 2013). It was verified that the abundance of hydrogenases and formate dehydrogenases, and therefore the formation of hydrogen or formate depended on the growth conditions, i.e., in *S. wolfei* formate dehydrogenase activity was higher during syntrophic degradation of butyrate and hydrogenases were expressed at high levels specifically during axenic growth with crotonate (Schmidt et al., 2013). Nevertheless, these authors also suggested that H_2 or formate, or both simultaneously, can be used during syntrophic growth with butyrate.

Some SRB can perform the interconversion between formate and hydrogen. For example, in habitats with high hydrogen partial pressure and without sulfate as electron acceptor, SRB convert H_2 to formate for establishing syntrophic associations with methanogenic microorganisms that have higher affinity to formate than to hydrogen (Silva et al., 2013). This ability is not exclusive from SRB, since *S. wolfei* can also uptake formate and exchange formate into H_2, and use it as electron carrier depending on H_2/formate concentrations outside the cell (Schmidt et al., 2013).

Despite the fact that fatty acid-degrading syntrophic bacteria are highly associated to LCFA degradation, in bioreactors treating LCFA-based effluents however, these bacteria were detected in low numbers (maximum 3 %) (Stams et al., 2012). However, under certain operational conditions such as using long solids retention times (approximately 20 days) (Ziels et al., 2016) or during codigestion of dairy cattle manure with pulses of oleate (Ziels et al., 2017), *Syntrophomonas* genus were highly enriched, with up to 43 % of bacteria amplicon sequences assigned to *Syntrophomonas*.

2.2.2. Facultative anaerobic bacteria (FAB)

Diversity of microbial communities degrading LCFA goes beyond the typical LCFA degrading bacteria (e.g., *Syntrophomonas*) and usually include members from phyla *Firmicutes*, *Bacteroidetes*, *Synergistetes*, *Spirochaetes, Chloroflexi* and *Proteobacteria* among others, but their role in LCFA degradation is not clear (Hatamoto et al., 2007c; Kougias et al., 2016; Pereira et al., 2002; Shigematsu et al., 2006; Treu et al., 2016; Ziels et al., 2016) (Table 2.3).

Frequently, non-syntrophic bacteria are detected in LCFA-degrading communities in higher abundance than syntrophic bacteria. Their presence is usually associated to the conversion of side compounds formed during LCFA degradation (e.g., volatile fatty acids), which may enable these bacteria to outnumber the slowly growing syntrophic LCFA degraders (Sousa et al., 2009b). But FAB are also known to play an important role in the fermentation/acidogenesis step (Botheju and Bakke, 2011; Joubert and Britz, 1987), increasing the substrate available, like acetate, to be used by the methanogens, indirectly enhancing the methanogenesis step (Botheju and Bakke, 2011; Kato et al., 1997).

FAB have also been detected in methanogenic bioreactors treating lipid/LCFA-rich wastewaters (Table 2.3). In 2002, Pereira et al.(b) detected the presence of *Pseudomonas* in both suspended and granular sludge bed reactors, mainly in the floating top layer formed in result of LCFA accumulation. Other FAB were also identified, namely *Buchnera* and *Erysipelothrix* genera. In addition, Baserba et al. (2012) identified *Pseudomonas* spp. in reactors continuously fed with oleate and accumulating palmitate. Several *Pseudomonas* spp. are known to degrade fatty acids, including oleate when electron acceptors such as oxygen or nitrate are available (Sabina and Pivnick, 1956), but their role in anaerobic microbial communities degrading LCFA is unclear.

Despite the massive formation of palmitate from oleate in continuous reactor, one of the best known LCFA degrader - *Syntrophomonas zehnderi* - was never described to produce palmitate from oleate, though it is able to degrade both oleate and palmitate (Sousa et al., 2007c). Moreover, in bioreactors where methanogenesis was inhibited, oleate was converted to palmitate but the palmitate formed was no further degraded, showing that the initial steps of unsaturated LCFA degradation is independent from interactions between syntrophic bacteria and methanogens (Cavaleiro et al., 2016). In these reactors, facultative anaerobic bacteria (mainly *Pseudomonas* and *Rheinheimera* genera) were highly enriched and represented approximately 50 % of the total microbial community. Therefore, taking all this information together, microorganisms other than syntrophic bacteria and eventually FAB could be involved in palmitate formation from oleate.

Table 2.3 – Phylogenetic composition of microbial communities present in LCFA/lipids/Fat-oil and grease (FOG) degrading bioreactors or enrichment cultures.

Substrate and culture conditions	**Techniques applied**	**Bacterial community**	**Archaeal community**	**Reference**
Thermophilic oleate degrading enrichment cultures	ARDRA; sequencing	*Firmicutes* (*Clostridia*) *Synergistetes* (*Sinergistia*)	*Methanobacterium thermoautotrophicum* (added to the enrichment culture)	Menes et al. (2001)
Bioreactors with granular and suspended sludge fed with oleate	PCR-DGGE, sequencing, FISH	*Firmicutes* (*Syntrophomonas* and others) *Proteobacteria (Pseudomonas and others)* *Spirochaetales*	*Methanobacterium* sp. *Methanobacterium formicium* *Methanosaeta concilii*	Pereira et al., (2002)
Bioreactors with granular sludge fed with increasing loads of oleic acid	PCR-DGGE, cloning, sequencing	*Pseudomonas* *Desulfovibrio*	*Methanobacterium* sp. *Methanobacterium formicium* *Methanosaeta concilii*	Pereira et al. (2003)
Stearate degrading enrichment cultures	Culture dependent; RFLP; FISH; sequencing	*Deltaproteobacteria* (*Syntrophus gentianae*) *Bacteroidetes* (*Cytophaga* sp. BHI60-95B)	*Methanocalculus taiwanensis* *Methanosaeta concilii*	Grabowski et al. (2005)
Synthetic LCFA wastewater containing oleate and palmitate (chemostat cultivation)	Real-time PCR; FISH; DGGE; cloning; sequencing	*Firmicutes* (*Syntrophomonadaceae* and others) *Proteobacteria* *Bacteroidetes* *Spirochaetes*	*Methanosarcina* *Methanosaeta* *Methanospirillum*	Shigematsu et al. (2006)
Batch degradation of oleate or palmitate accumulated during continuous feeding in bioreactors	DGGE; real-time PCR; cloning; sequencing; FISH	*Firmicutes* (*Clostridiaceae*; *Syntrophomonadaceae*; uncultured) *Proteobacteria* *Bacteroidetes*		Sousa et al. (2007a)
Oleate or palmitate enrichment cultures	Culture dependent; PCR-DGGE; cloning; sequencing	*Syntrophomonas* – in both enrichments *Bacteroidetes/ Chlorobi* group (*Chlorobium*) – in oleate enrichment *Proteobacteria* (*Desulfovibrio*) – in oleate enrichment *Proteobacteria* (*Syntrophobacter*, *Halothiobacillus*) – in palmitate enrichment	Archaeal community was not studied	Sousa et al. (2007b)
Thermophilic or mesophilic palmitate, stearate, oleate or linoleate enrichment cultures	RNA-SIP; cloning; FISH; RFLP; Culture dependent;	*Firmicutes* (*Syntrophomonas*; *Syntrophothermus*; others) *Proteobacteria* (*Deltaproteobacteria*)	Archaeal community was not studied but *Methanosaeta* was detected by microscopic observation	Hatamoto et al. (2007b)

Table 2.3 (continued)

Substrate and culture conditions	Techniques applied	Bacterial community	Archaeal community	Reference
Incubations with palmitate under mesophilic or thermophilic conditions	RNA-SIP; RFLP sequencing	*Bacteroidetes* *Firmicutes (Clostridium; Syntrophomonas; Syntrophothermus; Tepidanaerobacter; Desulfotomaculum; Coprothermobacter)* *Deltaproteobacteria (Syntrophaceae; Geobacteraceae)* *Synergistetes; Deferribacteres; Bacteroidetes/Chlorobi; Thermotogae;* *Acidobacteria; Spirochaetes;* Others	Archaeal community was not studied	Hatamoto et al. (2007c)
Thermophilic bioreactor fed with manure with successive pulses of a LCFA mixture (oleate, stearate, palmitate)	PCR-DGGE; sequencing	*Firmicutes (Clostridium; Syntrophomonadaceae)* *Synergistetes*	*Methanosarcina*	Palatsi et al. (2010)
Thermophilic co-digestion of organic fraction of municipal solid wastes with FOG wastes	PCR-DGGE; cloning; sequencing	*Firmicutes (Clostridiales; Thermoanaerobacterales)* *Bacteroidetes* *Thermotogales* *Synergistetes* *Thermotogae*	*Methanobacterium* *Methanoculleus* *Methanosarcina* *Methanothermobacter wolfeii*	Martín-González et al. (2011)
Co-digestion of dairy and poultry wastes	Cloning; sequencing	Bacterial community was not studied	*Methanocorpusculum sp.* *Methanosarcina barkeri* *Methanosaeta concilii* *Methanoculleus palmolei* *Methanomethylovorans sp.*	Zhang et al. (2011)
Biodegradability batch tests of fresh pig/cattle slaughterhouse waste mixtures	PCR-DGGE; sequencing	*Firmicutes (Thermodesulfobiaceae; Syntrophomonadaceae)* *Synergistetes (Anaerobaculum sp)* *Bacteroidetes (Porphyromonadaceae)* *Chloroflexi (Anaerolineaceae)*	*Methanosaeta concilii* *Methanosarcina siciliae*	Palatsi et al. (2011)

Table 2.3 (continued)

Substrate and culture conditions	Techniques applied	Bacterial community	Archaeal community	Reference
Thermophilic bioreactor fed with manure with continuous addition of oleate	PCR-DGGE; sequencing	*Firmicutes* (*Clostidiaceae*; *Bacillaceae*; *Syntrophomonas*) *Bacteroidetes* *Proteobacteria (Pseudomonas)* *Thermotogae*	*Methanococcus* *Methanosarcina* *Methanobacterium* *Methanosaeta*	Baserba et al. (2012)
Oleate-rich wastewater treated in a bioreactor based on a sequence of step feeding and reaction cycles	PCR-DGGE; sequencing	Bacterial community was not studied	*Methanobacterium* *Methanosaeta*	Salvador et al. (2013)
Low-Temperature (10 °C) Anaerobic Digestion of Dilute Dairy Wastewater in an EGSB Bioreactor	Real-time PCR; PCR-DGGE	*Firmicutes* *Proteobacteria* *Spirochaetes* *Bacteroidetes* *Trichococcus* *Proteobacteria*	*Methanocorpusculum* *Methanospirillum hungatei* *Methanosaeta concilii*	Bialek et al. (2013)
CSTR co-digesting fish waste and cow manure	Pyrosequencing	*Firmicutes (Clostridium; Syntrophomonas;* others*)* *Proteobacteria* *Actinobacteria* *Synergistetes* *Tenericutes* *Cloacimonetes (Candidatus Cloacimonas acidaminovorans)*	*Methanobrevibacter,* *Methanoculleus* *Methanosarcina* *Methanosaeta*	Solli et al. (2014)
Batch-fed methanogenic bioreactors degrading oleic acid	Quantitative PCR; PCR; sequencing	*Clostridiales (Syntrophomonas)* *Anaerolineales (Levilinea)* *Synergistales (Synesgistes)* *Enterobacteriales (Escherichia;Shigella)*	*Methanomicrobiales* *Methanosaetaceae*	Ziels et al. (2015)
Biogas reactors disturbed with pulses of lipids	Sequencing	*Megamonas* *Flectobacillus* *Clostridium* *Syntrophomonas sapovorans*	*Methanoculleus* *Methanocorpusculum* *Methanocella*	Francisci et al. (2015)

Table 2.3 (continued)

Substrate and culture conditions	Techniques applied	Bacterial community	Archaeal community	Reference
Sequencing batch reactors treating dairy wastewater and cattle manure	PCR-DGGE; sequencing	*Bacteroidales* *Syntrophomonas* *Thermovirga*	*Methanospirillum* *Methanosarcinales* *Methanobacteriales*	Toumi et al. (2015)
Bioreactors subjected to inhibitory shock load induced by single pulses of unsaturated LCFA	Illumina HiSeq sequencing	*Clostridia (Syntrophomonas; Desulfotomaculum; Syntrophothermus)* *Gammaproteobacteria*	*Methanosarcina* *Methanoculleus*	Kougias et al. (2016)
Reactors co-digesting three agro-industrial wastes underwent abrupt and gradual changes of LCFAs concentrations	PCR-DGGE; FISH; Illumina MiSeq sequencing	*Clostridiales* *Syntrophomonadaceae (Syntrophomonas)* *Synergistetes* *Anaerobaculaceae* *Tissierellaceae* *Peptococcaceae*	*Methanosarcina-related methanogens*	Regueiro et al. (2016)
Comparison of bioreactors fed with cattle manure after oleate addition to the feeding	RNA Illumina MiSeq sequencing; shotgun reads	*Alcaligenaceae sp* *Eubacteriaceae sp* *Rikenellaceae sp.* *Clostridiales sp.* *Porphyromonadaceae sp.* *Halothermothrix* *Anaerobaculum*	*Methanoculleus sp.* *Methanosarcina sp.* *Methanothermobacter sp.*	Treu et al. (2016)
Anaerobic co-digestion of fats, oils, and grease with municipal sludge	Quantitative PCR; rRNA Illumina MiSeq sequencing	*Syntrophomonas* *Petrimonas* *Mahella* *Levilinea* *Sedimentibacter* *Ornithobacterium* Others	*Methanosaeta* *Methanospirillum*	Ziels et al. (2016)
Digesters were exposed to a subsequent OLR increase with FOG and glycerol	Pyrosequencing; Phospholipids and ether-linked isoprenoids analysis	*Firmicutes (Cloacibacillus)* *Sulphur reducing bacteria* *Bacteroidetes*	Archaeal community was not studied	Ferguson et al., (2016)

2.3. TOXICITY OF LCFA

Long-chain fatty acids were found to inhibit essential reactions in the anaerobic digestion process due to their toxic effect towards anaerobic microorganisms (Hanaki et al., 1981). In the early 90's, LCFA were considered to be bactericidal, exerting a permanent and irreversible toxic effect, particularly towards methanogens (Angelidaki and Ahring, 1992; Rinzema et al., 1994). Both acetoclastic and hydrogenotrophic methanogens are inhibited in the presence of LCFA (Hanaki et al., 1981) and pioneer studies suggested that acetoclastic methanogens are more sensitive than hydrogenotrophic methanogens (Hanaki et al., 1981; Hwu and Lettinga, 1997; Koster and Cramer, 1987; Lalman and Bagley, 2001; Rinzema et al., 1994). In previous studies acetoclastic archaea were unable to adapt to LCFA, neither after repeated exposure to toxic concentrations, nor after prolonged exposure to non-toxic LCFA concentrations (Rinzema et al., 1994). More recently, Silva et al. (2016) showed that pure cultures of *Methanosaeta concilii* and *Methanosarcina mazei* tolerate LCFA concentrations similar to those previously described for some hydrogenotrophic methanogens (Sousa et al., 2013), showing that these acetoclastic methanogens are more robust than considered previously.

LCFA are more toxic than neutral fats (Hanaki et al., 1981) and unsaturated LCFA, containing one or more double bonds, are considered even more toxic to microbial cells than saturated fatty acids (Demeyer and Henderickx, 1967; Lalman and Bagley, 2002, 2001). The increase of chain length and number of double bonds also increases LCFA toxicity (Lalman and Bagley, 2002, 2001).

LCFA adsorption onto the cell walls was reported as being the main cause of cell damage and toxicity by limiting membrane transport and decreasing its protective function (Demeyer and Henderickx, 1967; Galbraith and Miller, 1973; Heukelekian and Mueller, 1958). Indeed, the cell membranes integrity of pure cultures of methanogens was severely compromised after exposure to saturated and unsaturated LCFA (Sousa et al., 2013). Pereira et al. (2005) proved that the potential toxic effect of LCFA is mainly due to its accumulation onto the sludge, creating a physical barrier and delaying the transfer of metabolic substrates and products, which induces a delay on the initial methane production. This conclusion was based on the fact that, after the mineralization of the biomass-associated LCFA, the methanogenic activity can be recovered.

Koster and Cramer (1987) agreed that the adsorption of LCFA on the cell wall plays an important role in the mechanism of inhibition, but they said that the microbial inhibition was more correlated with the fatty acid concentration rather than with the amount of LCFA per unit of biomass. The biomass sensitivity to LCFA depends on the type of anaerobic sludge. Flocculent and suspended

sludge are more sensitive to LCFA toxicity (Hwu et al., 1996), but is more suitable for LCFA treatment than granular sludge due to their higher surface area, and adsorption capacity , when the toxicity can be overcome (Pereira et al., 2002b).

The adsorption of LCFA to the biomass causes other process complications, namely sludge flotation and washout. Due to these operational problems, not all anaerobic bioreactors configurations are suitable to treat lipid-rich effluents. High-rate anaerobic digestion of LCFA needs efficient mixing of the liquid in the reactor and sufficient contact between biomass and substrate, which can be obtained with an Expanded Granular Sludge Bed (EGSB), but not with an Upflow Anaerobic Sludge Blanket (UASB) reactors. When the performance of both reactor types was compared, the UASB systems failed at lipid loading rates exceeding 2 kg m^{-3} d^{-1} to 3 kg m^{-3} d^{-1} (in COD), while the EGSB reactor was able to handle organic loading rates (OLR) up to 30 kg m^{-3} d^{-1} (in COD) during anaerobic digestion of caproic or lauric acids as sole substrate (Rinzema et al., 1993a). However, when a triglyceride emulsion (simulating a lipid-rich wastewater) was tested, the performance of the EGSB reactor was clearly inferior to its performance during treatment of LCFA solutions, since this emulsion causes a very severe flotation and washout of sludge, even at very low OLR (1 kg m^{-3} d^{-1} to 2 kg m^{-3} d^{-1} in COD) (Rinzema et al., 1993b). Actually, the washout of the sludge is likely to occur before inhibition of methanogenesis, during the treatment of LCFA-rich effluents in UASB reactors (Hwu et al., 1998). Also, at short hydraulic retention times (HRT), the conceptual EGSB reactor was inadequate for an efficient treatment of oleate (Hwu et al., 1997a). However, the treatment performance is highly improved when the washed-out biomass is recirculated (Hwu et al., 1997b; Pereira et al., 2001a).

Throughout the years, several works have been done regarding the anaerobic digestion of LCFA under thermophilic conditions (Angelidaki et al., 1990; Angelidaki and Ahring, 1992; Hwu et al., 1997b, 1997a; Hwu and Lettinga, 1997). Since the composition of cell membranes of thermophilic and mesophilic microorganisms are different, distinct responses to LCFA toxicity are observed, due to the adsorption of LCFA onto the cell membrane (Hwu and Lettinga, 1997). These authors concluded that an increase in temperature aggravates the oleate toxicity, despite the faster recovery after LCFA inhibition resulting from thermophiles' faster growth rates. In Figure 2.2 is presented a timeline of most relevant research in methane production from LCFA.

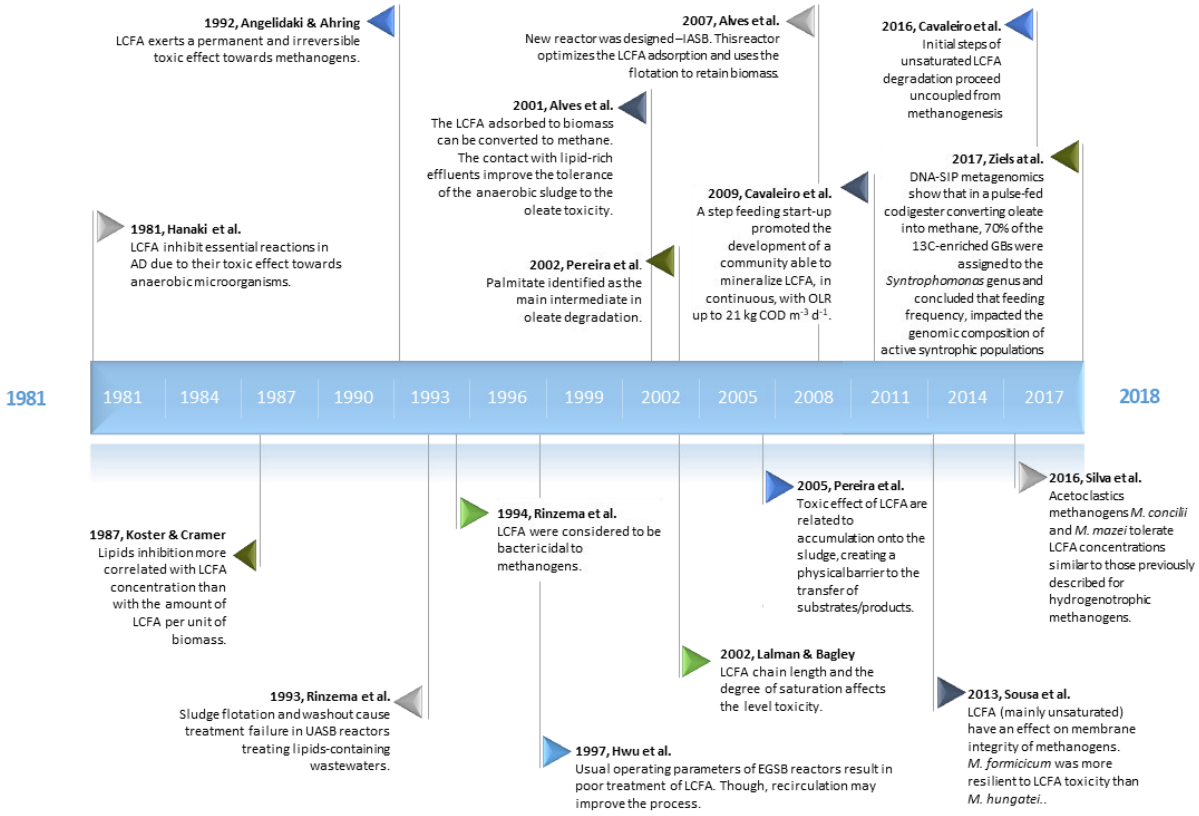


Figure 2.2 – Summary timeline of the most pioneering findings in the process of lipids AD, from 1981 to 2018.

2.3.1. How to overcome toxicity

Over the years, several strategies have been suggested to overcome the LCFA/lipids toxicity, namely bioaugmentation with LCFA degrading bacteria (Cavaleiro et al., 2010), sludge acclimatization to LCFA (Silva et al., 2014; Ziels et al., 2016), feeding strategies (Cavaleiro et al., 2009; Ziels et al., 2017), addition of adsorbents like bentonite (Angelidaki et al., 1990) and calcium salts (Hanaki et al., 1981).

One of the approaches to overcome the inhibitory effect of LCFA is by adding divalent cations, such as Ca^{2+} and Mg^{2+}, since it promotes LCFA precipitation and increases the interfacial tension (Galbraith et al., 1971; Hanaki et al., 1981). This strategy was also tested by Rinzema et al. (1989), in order to prevent inhibiton and avoid sludge flotation, but this approach just delayed slightly the sludge washout.

More recently, conductive materials (e. g. ferric hydroxide, magnetite and granular activated carbon) have improved the methane production rates from dairy wastewaters (containing lipids and consequently LCFA) (Baek et al., 2017; Martins et al., 2018). Some authors justify the enhancement

of methane production by the promotion of direct electron transfer in methanogenic communities however, the reasons by which methane production is improved are still not yet well understood.

It was shown that the inhibition caused by LCFA in the microbial community is not permanent since adaptation to lipids/LCFA can occur leading to methane production (Alves et al., 2001a; Kim et al., 2004; Pereira et al., 2004). For instance, the contact with lipid-rich effluents improve the tolerance of the anaerobic sludge to oleate (Alves et al., 2001a) and the tolerance level of acetoclastic methanogens increases after subjected to oleate pulses (Nielsen and Ahring, 2006). Recently Ziels et al., (2016) showed that microbial adaptation is important for the degradation of FOG to methane, i.e. more efficient LCFA conversion into methane can be achieved if the microbial community has enough time to enrich for LCFA-degrading bacteria. In the same line of research, another study suggested that an inoculum previously exposed to LCFA degrades LCFA more efficiently, due to the specialization of the microbial consortium (Kougias et al., 2016).

The degradation of biomass-associated LCFA was delayed by the addition of extra oleic acid (Pereira et al., 2001b). Further studies showed that palmitate was the main LCFA-associated to biomass, by mechanisms of adsorption, precipitation and entrapment when oleate was fed in a continuous bioreactor (Pereira et al., 2002b). Based on the observations that the biomass-associated LCFA, can be further degraded in batch mode to methane, a strategy based on intermittent feeding was suggested as adequate to treat effluents with high lipid content (Pereira et al., 2001a). To further test this hypothesis, an anaerobic bioreactor was operated based in cycles of continuous feeding phases, followed by batch reaction phases. This step-wise feeding start-up promoted sludge acclimation and the development of an anaerobic microbial community able to efficiently mineralize LCFA in continuous with OLR up to 21 kg m^{-3} day^{-1} (in COD) (Cavaleiro et al., 2009). Another work studied the impact of LCFA feed-frequencies on the microbial community and on the bioconversion kinetics and concluded that pulse feeding LCFA, rather than continuous feeding, resulted in higher conversion kinetics and functional stability, since the microbial community was predominately composed by syntrophic LCFA-degraders (Ziels et al., 2017).

Some studies have used alternative or additional electron acceptors in LCFA degradation (Guedes et al., 2013; He et al., 2015). Nitrate and sulfate could enhance the methane production from oleate when compared with the control (without external inorganic electron acceptor)(Guedes et al. (2013). Calcium palmitate degradation was also studied under different redox conditions by He at al.(2015). As expected, oxygen was the most favourable electron acceptor, and nitrate used as electron acceptor presented higher biodegradation efficiencies when compared to sulfate-reducing and

methanogenic conditions (He et al., 2015). If facultative bacteria are present these results may be explained by the redox tower (Figure 2.3). Preferentially, oxygen will be used by these microorganisms as electron acceptor since the energy released from the oxidation of the electron donor will be greater than if the same compound is oxidized with an alternative electron acceptor (Madigan et al., 2012). Following this principle, oxygen will be the preferred electron acceptor, followed by nitrate, sulfate and for last carbon dioxide.

2.3.2. Influence of oxygen in anaerobic transformation of lipids /LCFA

Although AD is an anaerobic process, the presence of vestigial amounts of oxygen or pulses of oxygen/air seems to improve the performance of the treatment (Lim and Wang, 2013) and to promote the growth of facultative anaerobic bacteria. Microaeration is defined as a strategy based

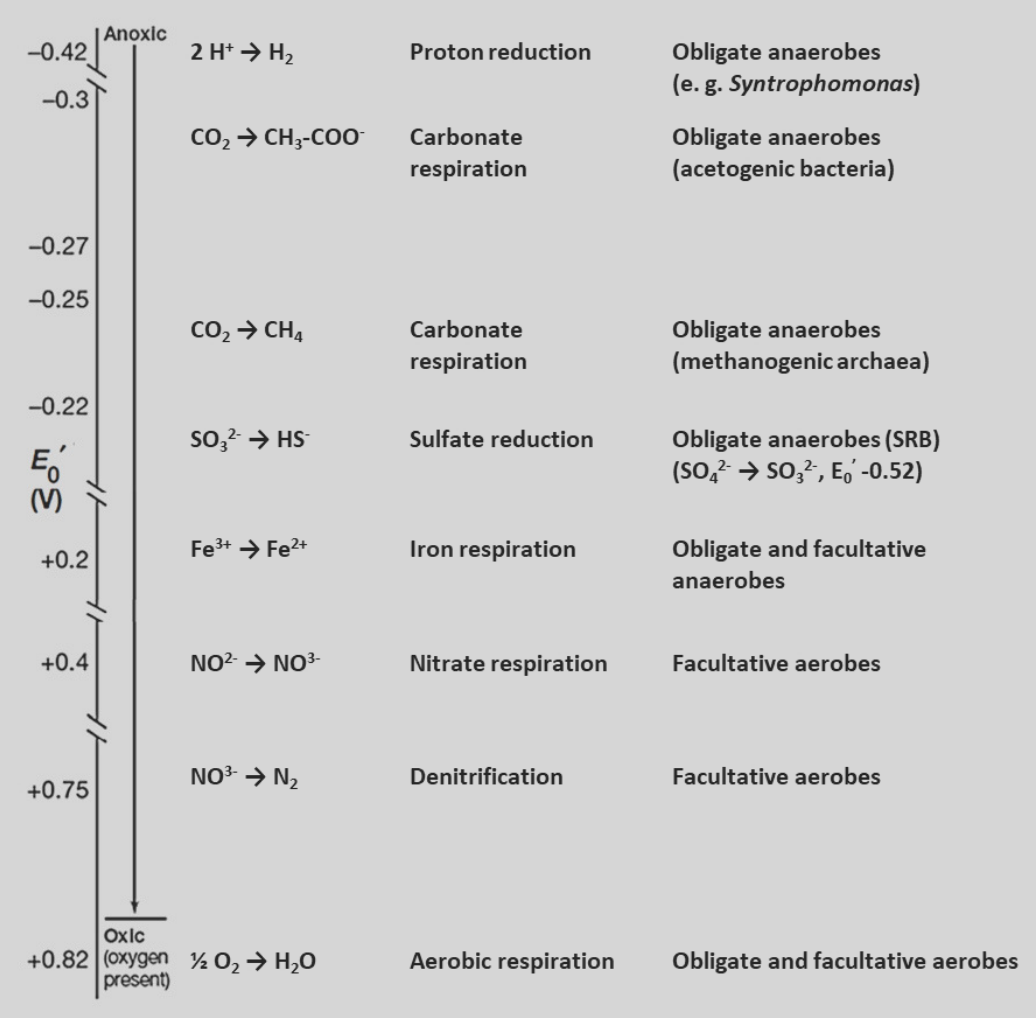


Figure 2.3 – Redox tower. The redox couples are organized from the most electronegative (top) to the most electropositive (bottom). Adapted from (Madigan et al., 2012).

on the introduction of small amounts of oxygen into an anaerobic biochemical process, to enable both anaerobic and aerobic biological activities to occur, within a single bioreactor (Botheju and Bakke, 2011). This approach was considered as an inexpensive and easily applicable pretreatment to efficiently improve the anaerobic biodegradability of recalcitrant compounds, since it promoted an increase in the biogas production (Tsapekos et al., 2017). The added oxygen is consumed fully by FAB, maintaining a reduced environment for the anaerobic degradation of organic matter (Lim and Wang, 2013).

The benefit of adding limited amounts of oxygen to accelerate and extend methanogenic processes has been reported for some organic substrates (Botheju and Bakke, 2011; Montalvo et al., 2016; Pedizzi et al., 2016; Tsapekos et al., 2017), including lipid-rich wastewaters (Nurliyana et al., 2015; Zouari and Jabiri, 2015). Nevertheless, the methodologies used so far are usually based on black box approaches, where oxygen is added in defined pulses or continuously, at variable concentrations and methane production and/or volatile fatty acids are recorded. Specificities related to substrate type, possible syntrophic relationships or in depth functional analysis of facultative bacteria need to be further studied.

The presence of facultative bacteria in reactors where oleate to palmitate conversion occurred, raised several questions related with the role of oxygen and/or facultative bacteria in anaerobic reactors treating LCFA-based wastewaters. Although the involvement of FAB in oleate conversion to palmitate is not confirmed, FAB were enriched in bioreactors accumulating palmitate from oleate (Cavaleiro et al 2016). Therefore, addition of small amounts of oxygen might be advantageous by stimulating the growth of FAB and promoting oleate to palmitate conversion, which is an important step during continuous treatment of oleate containing wastewater.

Based on what was mentioned above about syntrophic and facultative bacteria, as well as the role of oxygen on the anaerobic digestion processes, several hypotheses can be considered.

When sufficient oxygen is supplied facultative anaerobic bacteria degrade LCFA to carbon dioxide, nevertheless under microaerophilic conditions other reactions may occur, leading to the production of intermediary products. For example, in methanogenic reactors treating oleate-based effluents, palmitate may be accumulating due to a partial conversion of oleate by facultative microorganisms. Then, when the feeding is stopped in those reactors, there is an opportunity for the community to adapt and maybe enriched for syntrophic bacteria together with methanogens, which can at that point efficiently convert palmitate to methane under anaerobic conditions.

Additionally, several genera of facultative bacteria are able to produce extracellular polymeric substance (EPS), forming a biofilm as an auto-defense strategy against hostile conditions. LCFA, namely oleate, are often considered toxic to microorganisms which may trigger biofilm formation by these bacteria. This biofilm formation would contribute to the floating layer of LCFA-entrapped sludge that is often formed in reactors fed with oleate. Besides EPS, facultative bacteria, namely *Pseudomonas* spp. are also able to produce biosurfactants as a strategy to access hydrophobic compounds as hydrocarbons and lipids. Pseudofactin II is a lipopeptide surfactant with a palmitic acid connected to the terminal amino group produced by *Pseudomonas fluorescens* (Janek et al., 2012). In reactors degrading oleate, if a similar compound to Pseudofactin II is produced in large quantities, it could be hydrolyzed by hydrolytic bacteria releasing palmitic acid, which could explain palmitate accumulation on those systems.

There is a window of opportunities to explain the mechanisms involved in the anaerobic digestion of LCFA in methanogenic reactors that can be explored. Some of these features are addressed in the upcoming chapters, namely the influence of vestigial amounts of oxygen on oleate conversion to methane, the role of facultative bacteria in oleate to palmitate conversion and the interactions of these bacteria with syntrophic communities during oleate degradation in bioreactors.

Chapter 3.

Influence of Microaeration in Oleate Biodegradation to Methane in Continuous Anaerobic Reactors

Conversion of long-chain fatty acids (LCFA) to methane under anaerobic conditions, involves the syntrophic cooperation between bacteria performing β-oxidation and methanogens. In continuous reactors with high loads of oleate, this conversion involves the transient accumulation of high amounts of palmitate but the underlying mechanism is not clear. In reactors accumulating palmitate, facultative anaerobic bacteria are frequently found in high numbers. In this Chapter the effect of microaeration on LCFA conversion to methane is addressed. The performance of bioreactors treating oleate-based wastewater (organic loading rates of 1 kg m^{-3} d^{-1} and 3 kg m^{-3} d^{-1} in COD), operated under different redox conditions (strictly anaerobic – AnR, -350 mV; microaerophilic – MaR, -250 mV) was compared. Methane was produced in both reactors but in MaR, for the higher load, palmitate accumulated 7 times more and facultative anaerobes were more abundant. At the end of the bioreactors operation the methanogenic activity was inhibited but the sludge from MaR could recover while the AnR could not. This study shows that having a strict anaerobic feed or a feeding tank open to air resulted in different redox conditions, different microbial community composition and different oleate degradation profiles. Slightly higher redox potential promoted the transformation of oleate to palmitate, which is less toxic, avoiding a long term methanogenic inhibition in continuous reactors.

3.1. INTRODUCTION

Under methanogenic conditions, long-chain fatty acids (LCFA) are degraded by syntrophic communities of acetogenic bacteria that perform a β-oxidation pathway and hydrogenotrophic methanogens that maintain hydrogen concentrations at very low levels (Sousa et al., 2009b). For unsaturated LCFA, although the possibility of direct β-oxidation is not excluded (Canovas-Diaz et al., 1992; Lalman and Bagley, 2000), a mechanism starting with chain saturation followed by β-oxidation has been suggested by Weng and Jeris (1976). Nonetheless, stearate (C18:0) formation from oleate (C18:1) was only occasionally observed at low concentrations (Jeganathan et al., 2006; Salminen et al., 2001). The accumulation of stearate and palmitate (50:50 % total LCFA) was reported by Broughton et al., (1998) during the anaerobic digestion of sheep tallow and by Cavaleiro *et al.* (2009) during the stepwise feeding start-up of a reactor treating an oleate-based wastewater. Frequently, a significant accumulation of palmitate (C16:0) occurs associated to the sludge when anaerobic bioreactors are fed with oleate (Jeganathan et al., 2006; Lalman and Bagley, 2001; Novak and Carlson, 1970; Pereira et al., 2002b). The main mechanisms of palmitate accumulation are well described and include adsorption, entrapment in the filamentous structure of the sludge or precipitation (Pereira et al., 2005). Under those conditions, oleate conversion to palmitate is usually a fast process, while palmitate degradation proceeds slowly, raising the hypothesis that two different metabolic routes may be involved in the complete oleate degradation to methane (Cavaleiro et al., 2016). Accumulation of C_{n-2} saturated LCFA (palmitate) from C_n unsaturated LCFA (oleate) suggests an interruption of the β-oxidation after just one cycle. The mechanism of that process is not understood.

Recently, Cavaleiro et al. (2016) showed that the conversion of oleate to palmitate does not depend on the presence of methanogens, and that FAB are abundant in those reactors. The presence of these bacterial groups was also reported in several methanogenic bioreactors treating oleate-based wastewater (Baserba et al., 2012; Kougias et al., 2016; Palatsi et al., 2011; Pereira et al., 2002), but their role in unsaturated LCFA degradation is still not elucidated.

The occurrence and role of facultative anaerobes in the anaerobic digestion (AD) process is long recognized (Botheju and Bakke, 2011; Joubert and Britz, 1987). They are involved in hydrolysis and acidogenesis, that culminate in the production of substrates for the methanogens, and thus indirectly enhance methanogenic activity (Kato et al., 1997; Scott et al., 1983). Besides, the rapid consumption

of oxygen by facultative anaerobic bacteria, protects strict anaerobic microorganisms like methanogens from oxygen toxicity (Gerritse and Gottschal, 1993; Kato et al., 1997).

The benefit of microaeration to accelerate and extend methanogenic processes has been reported for some organic substrates (Botheju and Bakke, 2011; Montalvo et al., 2016; Pedizzi et al., 2016; Tsapekos et al., 2017), including lipid-rich wastewaters (Nurliyana et al., 2015; Zouari and Jabiri, 2015). Nevertheless, the methodologies used so far are usually based on black box approaches where oxygen is added in defined pulses or continuously at variable concentrations and methane production and /or volatile fatty acids are recorded.

In this work, the influence of different redox potentials on the activity and abundance of facultative anaerobes, and subsequent effect on oleate degradation profiles is studied. Redox potential differences were induced by the continuous addition of vestigial oxygen concentrations.

3.2. Materials and Methods

3.2.1. Experimental setup

Two 1 L working volume upflow bioreactors were operated in continuous mode at mesophilic temperature (37 °C). One of the reactors was operated under strict anaerobic conditions (Anaerobic Reactor, AnR in Figure 3.1), guaranteed by adding sodium sulfide to the feed and by refilling the headspace of the feeding tank from a bag of nitrogen. The other reactor was fed from a tank that was open to the air (Microaerophilic Reactor, MaR in Figure 3.1). Both systems were gently stirred twice a day during 5 min.

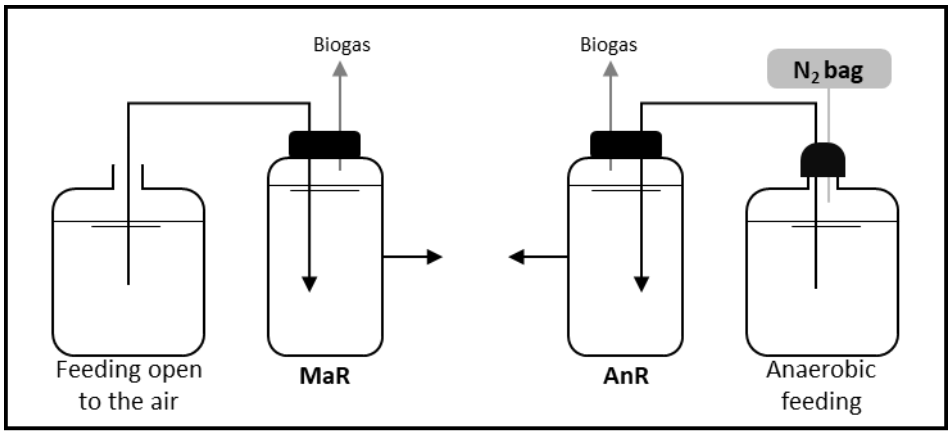


Figure 3.1 – Schematic diagram of the experimental setup of the experiment.

Disrupted anaerobic granular sludge from a brewery wastewater treatment plant was used as inoculum, at a final concentration of 10 g L^{-1} in volatile solids (VS). Feeding was prepared by mixing sodium oleate (≥ 82 % as oleic acid, and the remaining composed of small percentages of other fatty acids, Riedel-de-Häen, Seelze, Gremany) with tap water, and was supplemented with sodium bicarbonate (3 g L^{-1}), micronutrients (1 mL L^{-1}) and macronutrients (0.6 mL per g of Chemical Oxygen Demand – COD fed) according to Alves et al. (2001b). The AnR feed was first boiled and cooled down under N_2 flushing and then supplemented with 1 mmol L^{-1} of $Na_2S.9H_2O$.

The bioreactors operation was divided in two different periods according to the organic loading rate (OLR) applied. Oleate concentration in the feed (in COD) was 1 g L^{-1} during period I (52 days), and 4 g L^{-1} in COD in period II (11 days). The hydraulic retention time (HRT) was kept constant at 1 day in both periods. The reactors performance was monitored by measuring biogas production and methane content, pH, oxidation-reduction potential (ORP), total COD in the influent, soluble+colloidal COD in the effluent, volatile fatty acids (VFA) and LCFA inside the reactor. Duplicate samples of well homogenized sludge were collected for assessing microbial community composition in both reactors after 27 and 62 days of operation.

At the end of period II, the reactors were kept in batch mode under mild agitation and after 23 days the methanogenic activity was assessed

3.2.2. Methanogenic community activity assays

A volume of 12.5 mL of a well homogenized sample was incubated in 25 mL batch vials, flushed with N_2/CO_2 (80:20 % v/v). Ethanol (30 mmol L^{-1}) was chosen as substrate because it is easy to biodegrade and helps to overcome mass transfer limitations of the LCFA accumulation in the sludge (Pereira et al., 2005). The vials were incubated in the dark, at 37 °C, and stirred at 120 rpm. Pressure increase in the batch vials was measured using a hand-held pressure transducer, capable of measuring a pressure variation of 2 bar (0 kPa to 202.6 kPa) as described in Colleran et al., (1992). The methane content was periodically measured and the values of methane production were corrected for standard temperature and pressure (STP) conditions. The ethanol concentration was measured at the end of the assay by HPLC.

3.2.3. Analytical methods

COD and organic nitrogen were determined spectrophotometrically using cuvette test kits (Hach-Lange GmbH, Düsseldorf, Germany) and a DR 2800 spectrophotometer (Hach-Lange GmbH). Total and volatile solids were determined according to the Standard Methods (APHA et al., 1999). For

soluble+colloidal COD and organic nitrogen, samples were previously centrifuged for 15 min at 15000 rpm. ORP was measured using a multi-parameter analyzer C533 (Consort, Turnhout, Belgium) equipped with an ORP electrode D 223 (VWR, Radnor, PA). pH was measured with a Hanna (Woonsocket, RI) pH-meter. VFA were analyzed by high performance liquid chromatography HPLC (Jasco, Tokyo, Japan), using an Agilent Hi-Plex H column (300 mm x 7.7 mm), maintained at 60 °C and with UV detection at 210 nm. The mobile phase was sulfuric acid (5 mmol L^{-1}) at 0.6 mL min^{-1} and crotonic acid was used as internal standard. Biogas production was measured with a Ritter MilliGas counter (Dr. Ing. Ritter Apparatebau GmbH, Bochum, Germany). Methane content of the biogas was analyzed by GC (Chrompack 9000) with a flame ionization detector (FID) and Carbowax 20 M (2 m x 2 mm, 80–120 mesh) column and using nitrogen (30 mL min^{-1}) as the carrier gas. The detector, injector, and oven temperatures were 35 °C, 110 °C and 220 °C, respectively. Free saturated and unsaturated LCFA from C12 to C18 were esterified with HCl:1-propanol at 100 °C for 3.5 h, extracted with dichloromethane and quantified by GC-FID, as described by Neves et al. (2009). LCFA were separated using a Teknokroma TRB-WAX column (30 m x 0.25 mm x 0.25 μm) with helium as the carrier gas, at 1 mL min^{-1}. Temperatures of the injector and detector were 220 °C and 250 °C, respectively. The initial oven temperature was 50 °C, maintained for 2 min, followed by a 10 °Cmin^{-1} ramp up to 225 °C, and finally isothermal conditions were maintained for 10 min.

3.2.4. DNA extraction and 16S rRNA sequencing

Samples of well-homogenized sludge were centrifuged (4000 rpm, 10 min at 4 °C), the pellet was re-suspended in 0.5 mL of PBS (phosphate-buffered saline) and preserved at -20 °C until DNA isolation. Total genomic DNA was extracted using a FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH) and purified by ethanol precipitation. DNA amplification, Illumina libraries preparation, amplicon sequencing (Illumina MiSeq, Inc. SanDiego, CA) and bioinformatics analysis of the data were performed at Research and Testing Laboratory (RTL), Texas. Samples were amplified for sequencing using the universal primer pair, 515f and 806r (Caporaso et al., 2011), targeting the prokaryotic 16S rRNA gene. Details on the sequencing and bioinformatics data analysis can be found elsewhere (Paulo et al., 2017). The FASTq files were submitted in the ENA (European Nucleotide Archive), being available in the following link http://www.ebi.ac.uk/ena/data/view/PRJEB11469. Nucleotide sequences from this experiment are identified with accession numbers ERS937154 to ERS937157

3.2.5. Statistical analysis

To investigate if bioreactors performance were significantly different, student's t-test were applied. The set of COD values obtained for each reactor and in each period were compared. In period I n=14 and in period II n=4 (n represents the number of samples analyzed). The level of significance was defined at $p \leq 0.05$.

3.3. RESULTS

Oxygen concentrations in bioreactors AnR and MaR were below the detection limit (0.01 mg L^{-1}), but the ORP differed and was a clear indicator of the different operating conditions applied in both reactors. ORP values were approximately 100 mV higher in MaR than in AnR (Table 3.1). The ORP was also measured in the feeding tanks of both reactors, which were about -80 mV and -300 mV in MaR and AnR, respectively (Table S3.1), in accordance with the different oxygen exposure of the feeding tanks. COD removal efficiency during period I was higher ($p<0.001$) in MaR than in AnR, but in period II no significant differences were observed ($p>0.05$) (Table 3.1). With the increase of the OLR (period II), the reactors content became whitish, due to the accumulation of LCFA, and the biogas production decreased (Figure 3.2c). In the MaR a floating layer of LCFA-entrapped sludge was formed but the same did not occur in the AnR (Figure S3.1).

Palmitate (C16:0) and oleate (C18:1) represented more than 85 % of the total LCFA detected during the reactors operation. During period I, palmitate concentrations reached maximum values of 850 mg L^{-1} and 1100 mg L^{-1} in COD in the MaR and AnR, respectively, while lower oleate-COD concentrations, below 400 mg L^{-1}, were detected in both reactors (Figure 3.2a). Throughout period II, with the increase of the OLR, palmitate concentration inside the MaR accumulated up to 15500 mg L^{-1} in COD. Oleate (474 mg L^{-1} in COD), stearate (915 mg L^{-1} in COD) and myristate (686 mg L^{-1} in COD) were also detected. In the AnR, palmitate concentration also increased but only up to 2351 mg L^{-1} and oleate reached 1382 mg L^{-1}, in COD (Figure 3.2a). pH varied in both reactor from 6.7 to 8.5.

Table 3.1 – Operating conditions and performance data in MaR and AnR: organic loading rate applied in COD to both reactors (OLR_{COD}); oxidation-reduction potential (ORP) and COD removal efficiency ($COD_{removal}$).

Period	t/d	Mode	OLR_{COD}/ kg m^{-3} d^{-1}		ORP/mV		$COD_{removal}$/%	
			MaR	AnR	MaR	AnR	MaR	AnR
I	0-52	C	1.4 ± 0.6	1.0 ± 0.3	-289 ± 43	-374 ± 16	96 ± 1	89 ± 3
II	52-62	C	2.9 ± 0.5	3.3 ± 0.5	-255 ± 47	-366 ± 20	84 ± 4	70 ± 13

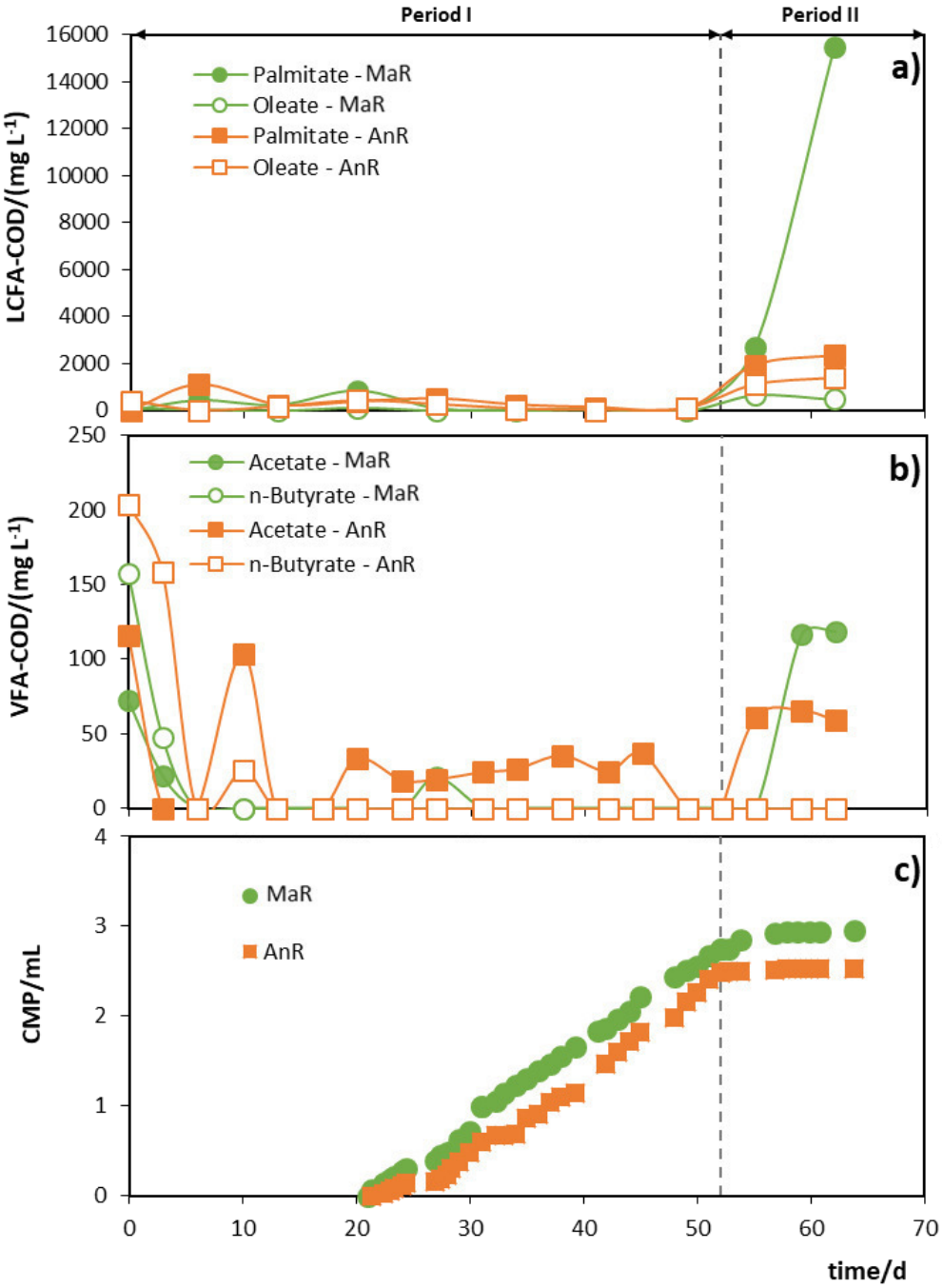


Figure 3.2 – LCFA inside the reactor (a), VFA (b) and cumulative methane production (CMP) (c) during periods I and II.

Acetate and n-butyrate were the only VFA detected in both reactors during the experiment (Figure 3.2b). In the MaR, acetate accumulated mainly during period II and reached a maximum value of 120 mg L^{-1} in COD. In the same period, acetate concentration (in COD) increased up to 60 mg L^{-1} in AnR.

Methane production ceased completely in both reactors in period II (Figure 3.2c). Subsequently, the feeding was stopped to allow the degradation of the accumulated fatty acids. After 23 days of batch

operation, palmitate concentration in MaR decreased by 91 %, whereas in AnR no measurable difference was detected. At this point, sludge was collected from both reactors to assess the specific methanogenic activity in the presence of ethanol, which gives information on the activity of different trophic microbial groups, the bacteria that convert ethanol to acetate and hydrogen, and the methanogens that produce methane from those intermediary products.

The sludge from MaR produced 37 mmol L^{-1} of methane after 11 days of incubation, which corresponds approximately to the theoretical value expected if all the ethanol was converted to methane (Figure 3.3). The complete degradation of ethanol was also confirmed by its absence at the end of the assay. On the other hand, no methane was produced by the sludge collected from AnR, though 80 % of the ethanol was converted to acetate (6.5 mmol L^{-1} of ethanol and 28 mmol L^{-1} of acetate were detected at the end of the activity test), showing that the methanogenic activity of the sludge was inhibited but not the acetogenic activity.

The microbial community composition was assessed in both reactors (Table 3.2). In period I, methanogenic communities were composed of *Methanobacterium* and *Methanosaeta* in both reactors, with the predominance of *Methanobacterium* (36 % of abundance) in the AnR. In the MaR these genera were present in similar relative percentages (approx. 10 %). The bacterial community in the AnR was composed of *Syntrophomonas*, *Syntrophobacter* and bacteria from the orders *Synergistales*, *Anaerolineales*, *Cytophagales* and *Syntrophobacterales*. In MaR facultative anaerobic bacteria accounted for 23 % of the total sequences retrieved (17 % *Stenotrophomonas* and 6 % *Deftia*). In period II, with the increase of the OLR, the relative abundance of Archaea decreased in both reactors. In addition, *Syntrophomonas* and *Syntrophobacter* became less abundant in the AnR and the relative abundance of facultative anaerobic bacteria (e.g. *Pseudomonas*, *Stenotrophomonas* and *Aeromonas*) increased to 21 %.

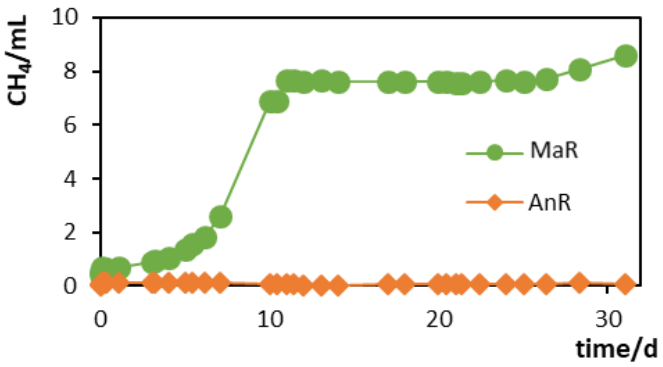


Figure 3.3 – Methane production during the activity assays performed with ethanol as substrate.

Table 3.2 – Taxonomic identification of the microbial reads identified at the genus level (at > 1 % relative abundance), in reactors MaR and AnR at days 27 (period I) and 62 (period II) of operation.

Phylum	**Order**	**Genus**	**MaR**		**AnR**	
			27 d	**62 d**	**27 d**	**62 d**
Proteobacteria	*Xanthomonadales*	*Stenotrophomonas*	**17**	4	<1	7
Unclassified *Bacteria*			**16**	2	**20**	3
Euryarchaeota	*Methanosarcinales*	*Methanosaeta*	**10**	3	**11**	5
Euryarchaeota	*Methanobacteriales*	*Methanobacterium*	**9**	**9**	**36**	**14**
Proteobacteria	*Burkholderiales*	*Delftia*	6	2	<1	2
Proteobacteria	*Syntrophobacterales*		4	<1	2	<1
Chloroflexi	*Anaerolineales*		4	<1	3	<1
Synergistetes	*Synergistales*		3	1	5	1
Thermotogae			3	<1	<1	<1
Planctomycetes			2	<1	<1	<1
Bacteroidetes	*Cytophagales*	*Cytophaga*	2	2	2	2
Proteobacteria	*Syntrophobacterales*	*Syntrophobacter*	2	<1	3	<1
Chloroflexi			2	<1	3	<1
Firmicutes			2	3	1	4
Spirochaetes			1	<1	2	1
Proteobacteria	*Rhodocyclales*	*Dechloromonas*	1	<1	<1	<1
Thermotogae	*Thermotogales*	*Mesotoga*	1	<1	<1	n.d.
Firmicutes	*Clostridiales*	*Syntrophomonas*	1	<1	2	<1
Bacteroidetes	*Bacteroidales*	*Bacteroides*	1	3	<1	<1
Proteobacteria	*Burkholderiales*	*Leptothrix*	<1	**25**	n.d.	n.d.
Proteobacteria	*Burkholderiales*	*Comamonas*	<1	**10**	<1	<1
Proteobacteria	*Rhodocyclales*	*Azospira*	<1	8	n.d.	**17**
Proteobacteria	*Pseudomonadales*	*Pseudomonas*	<1	3	<1	9
Proteobacteria	*Caulobacterales*	*Caulobacter*	<1	3	<1	<1
Synergistetes	*Synergistales*	*Aminiphilus*	<1	2	<1	7
Proteobacteria	*Burkholderiales*	*Acidovorax*	<1	2	<1	<1
Synergistetes	*Synergistales*	*Aminivibrio*	<1	1	<1	<1
Proteobacteria	*Xanthomonadales*	*Luteimonas*	n.d.	1	n.d.	n.d.
Bacteroidetes	*Sphingobacteriales*		<1	2	<1	n.d.
Proteobacteria	*Aeromonadales*	*Aeromonas*	n.d.	n.d.	n.d.	5
Proteobacteria	*Neisseriales*	*Microvirgula*	n.d.	<1	<1	3
Proteobacteria	*Desulfovibrionales*	*Desulfovibrio*	<1	<1	<1	2
Bacteroidetes	*Flavobacteriales*	*Chryseobacterium*	<1	<1	<1	2
Proteobacteria	*Caulobacterales*	*Brevundimonas*	<1	<1	<1	2

n.d. – not detected

3.3. Discussion

Microbial community analysis of sludge degrading LCFA showed the presence of facultative anaerobic bacteria in continuously fed methanogenic reactors (Cavaleiro et al., 2016; Pereira et al.,

2002). Currently, it is not known if these bacteria are involved in LCFA degradation, and if they are dependent on the presence of oxygen, or not.

Here, we investigated the possible effect of microaeration to trigger palmitate formation from oleate-based wastewater. This strategy led to an increase in the ORP from -350 mV (control, under strict anaerobic conditions – AnR) to -250 mV (MaR) although oxygen was not detected in the bulk medium. Facultative anaerobic bacteria were abundant in MaR (Table 3.2), possibly helping to create and maintain local oxygen-depleted conditions. In AnR, the community was mainly composed of methanogens and other strict anaerobes, including known syntrophic bacteria. In reactor MaR, LCFA removal (assessed indirectly by COD measurements) may have occurred anaerobically with conversion to methane, or aerobically to CO_2. However, from the cumulative methane production (Figure 3.2c), it is clear that similar amounts were obtained and even a slightly higher accumulation was observed in the microaerophilic reactor. This shows that COD was removed for the production of methane and not by an aerobic pathway, suggesting a cooperation rather than competition between aerobic and anaerobic metabolism.

When the OLR was increased, the methane production ceased in both reactors and LCFA accumulated. Low methane yields associated to LCFA accumulation in continuous bioreactors was described previously (Pereira et al., 2002a; Pereira et al., 2005), and subsequent degradation of biomass-associated LCFA to methane was demonstrated by suppressing the feeding and operating the reactor in batch mode for a certain period of time.(Cavaleiro et al., 2009) In this study, palmitate accumulated inside the reactor up to 7 times more in MaR than in AnR, for the higher organic load. Conversely, oleate concentration inside AnR was about three times higher than in MaR. After suppressing the feed to both reactors, the microbial community from MaR was able to degrade the LCFA accumulated in the reactor, but this was not the case in AnR, where the accumulation of LCFA was persistent. In addition, only the MaR sludge exhibited specific methanogenic activity when supplemented with ethanol. Oleate is reported to be more toxic than palmitate towards methanogens, in pure or in mixed cultures (Kim et al., 2004; Pereira et al., 2005; Silva et al., 2016; Sousa et al., 2013) and maybe this is the cause of the decrease in the methanogens' relative abundance and activity in AnR.

Information on LCFA toxicity and inhibition is controversial. In some studies, LCFA exerted a permanent and irreversible toxic effect towards methanogenic communities (Angelidaki and Ahring, 1992; Rinzema et al., 1994), while in other studies this was not the case (Alves et al., 2001b; Pereira et al., 2004). Differences in the operational methodologies, specifically the feeding procedures in

lab-scale reactors are usually not described in detail, and this may be the reason for the different conclusions previously reported. Here we show that, indeed, having a strict anaerobic feed or a feeding tank open to air, resulted in a different ORP, different microbial community profiles and a different behavior relative to oleate conversion. Interestingly, open to air feeding promoted the transformation of oleate to palmitate, avoiding a long term methanogenic inhibition as observed in AnR. From an applied point of view this information is important, since at industrial scale the feeding tanks/pipelines are not kept under strict anaerobic conditions and small amounts of oxygen can be introduced to the system.

This work encourages further systematic studies on the influence of ORP in the anaerobic digestion process, opening new perspectives on metabolic interactions where complex microbial networks are central. From an applied point of view, ORP is herein proposed as a potentially relevant control variable, whose fine-tuning can regulate oleate to palmitate conversion, decreasing the LCFA toxicity towards methanogens, ultimately accelerating the methane production from lipid-rich wastewaters.

Chapter 4.

Role of Facultative Anaerobic Bacteria in Oleate Biotransformation

Excessive accumulation of palmitate in methanogenic bioreactors fed with oleate is associated with a severe decrease in methane production. Bacteria known as aerobes or facultative anaerobes have been detected in those bioreactors. It was shown that oleate conversion to palmitate in continuous bioreactors is accompanied by the enrichment of facultative anaerobic bacteria (FAB) (Cavaleiro et al., 2016), suggesting that they may be involved in this step. In this Chapter, high organic loading rates and low hydraulic retention times were applied to selectively washout the microorganisms with slower growth rates (including methanogens) and simultaneously enrich for microorganisms capable of performing oleate to palmitate conversion. In two independent experiments, the abundance of FAB, namely *Pseudomonas* spp. (from which two strains were isolated), was strongly correlated ($p<0.05$) with palmitate-to-total LCFA percentage in the biofilm formed in a continuous plug flow reactor fed with very high loads of oleate. These results strongly suggest that facultative bacteria have a role in oleate conversion in continuous methanogenic bioreactors. Microbial networks and interactions of facultative and strict anaerobes in microbial communities should be considered in future studies.

4.1. Introduction

Under anaerobic conditions syntrophic bacteria are known to perform oleate conversion to methane together with methanogenic archaea (Sousa et al., 2009b). Nevertheless, palmitate accumulation in oleate-fed systems suggests the interruption of the degradation just after one β-oxidation cycle. Oleate conversion to palmitate is a fast process, while palmitate degradation proceeds slowly, raising the hypothesis that more than one microorganism may be involved in these reactions. Regarding this phenomenon, two major findings have been reported recently: i) the conversion of oleate to palmitate in anaerobic bioreactors can be independent of the activity of methanogens, since palmitate still accumulates in reactors in which methanogenesis was inhibited (Cavaleiro et al., 2016); ii) the relative abundance of several groups of facultative anaerobic bacteria increased in non-methanogenic bioreactors where accumulation of palmitate from oleate took place (Cavaleiro et al., 2016). Facultative anaerobic bacteria are also frequently found in methanogenic bioreactors treating oleate-based wastewater (Baserba et al., 2012; Kougias et al., 2016; Palatsi et al., 2011; Pereira et al., 2002). These observations strongly suggest that facultative anaerobic bacteria may play a major role in oleate degradation in anaerobic reactors.

The involvement of facultative anaerobes in hydrolysis and acidogenesis steps of anaerobic digestion (AD), that culminate in the production of substrates for the methanogens, is long recognized (Botheju and Bakke, 2011; Joubert and Britz, 1987). Facultative bacteria also protect strict anaerobes like methanogens by rapidly consuming traces of oxygen entering the system (Gerritse and Gottschal, 1993; Kato et al., 1997). However, information on the role and activity of facultative anaerobes in oleate to palmitate conversion and their possible metabolic interactions with strict anaerobes was never studied.

It is hypothesized that facultative anaerobic bacteria have a central role in the conversion of LCFA to methane in continuous high-rate bioreactors. In this Chapter, facultative anaerobic microorganisms able to degrade oleate were isolated and further studied in batch and continuous assays with diverse oxygen supply strategies.

4.2. Materials and Methods

This study was divided in two main experiments: experiment 1 (Figure 4.1a) which describes the enrichment and isolation of facultative anaerobic bacteria from continuous bioreactors fed with oleic

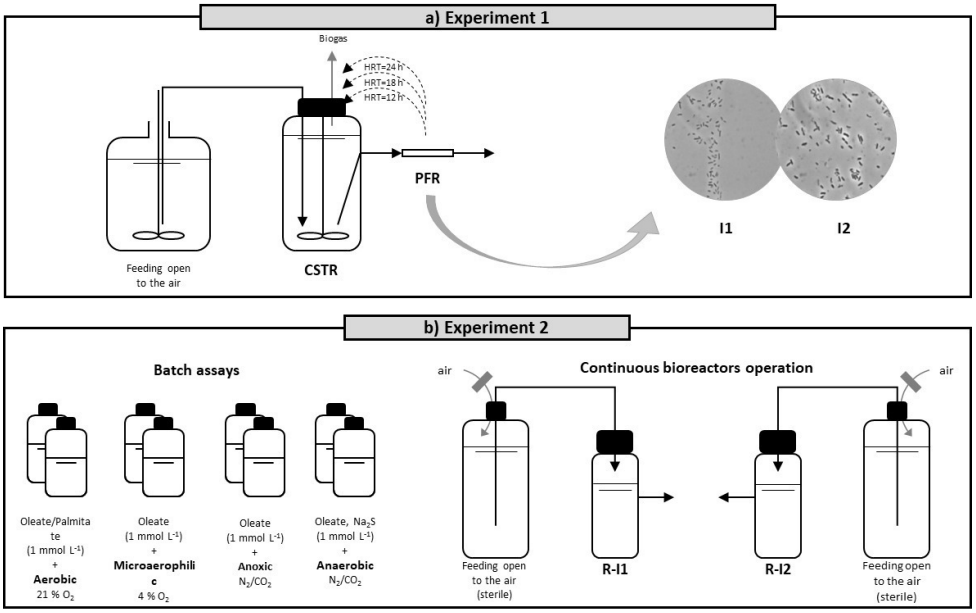


Figure 4.1 – Schematic diagram of the experimental setup of the enrichment strategy (a), and characterization of the PFR microbial isolates (b).

acid (section 4.2.1); experiment 2 (Figure 4.1b) in which the degradation of oleate by the isolated strains was studied under different ORP, promoted by diverse oxygen supply strategies (section 4.2.2).

4.2.1. Experiment 1 – Enrichment and isolation of facultative anaerobic bacteria from continuous bioreactors fed with oleic acid

Enrichment strategy

A continuous stirred tank reactor (CSTR) of 4.5 L was inoculated with an anaerobic sludge collected from the same source as the one utilized in Chapter 3 (section 3.2.1). The feeding was maintained in a tank open to air and prepared with a sodium oleate concentration of 4 g L^{-1} in COD, and supplemented with sodium bicarbonate (3 g L^{-1}), micronutrients (1 mL L^{-1}) and macronutrients (0.6 mL per g of Chemical Oxygen Demand – COD fed) according to Alves et al. (2001b). The CSTR was operated with an initial HRT of 6 h to promote the washout of methanogens and other slow growing microorganisms and therefore enriching for the faster growing facultative anaerobic bacteria which could possibly be involved in oleate to palmitate conversion.

A thick biofilm was observed developing in the effluent discharging tube, hereafter designated as plug flow reactor (PFR, volume of 7.5 mL) (Figure 4.1a). Samples from the PFR biofilm were collected periodically for LCFA quantification. Because significant accumulation of LCFA, other than oleate, was detected in the PFR, the operating strategy consisted in removing the biofilm from the PFR and

use it to re-inoculate the CSTR. The HRT was successively increased to 12, 18 and 24 h during the bioreactor operation (Figure 4.1). For each HRT, after reaching the steady state, the procedure was repeated, i.e., the biofilm from the PFR was re-inoculated in the CSTR, LCFA content was analyzed and the bioreactor performance was monitored. Samples were collected from the CSTR and the PFR biofilm at days 22, 78, 107 and 119 of operation for DNA isolation and further microbial community characterization.

In order to verify the reproducibility of the results obtained, the described bioreactor operation was repeated in an independent experiment (trial 2) in which the CSTR+PFR system was inoculated with fresh sludge and operated exactly under the same conditions of trial 1, but only for the HRT of 6 h and 12 h. Samples from the PFR biofilm were collected for LCFA quantification and DNA extraction in order to study the microbial community composition.

The performance of the system (CSTR + PFR) was monitored by measuring total COD in the influent; methane, pH, LCFA and VFA in the CSTR; soluble+colloidal COD, LCFA and VFA in the effluent of the PFR.

Isolation of facultative anaerobic bacteria

A biofilm sample (100 µL) taken from the PFR, when the CSTR was operated at a HRT of 12 h, was spread on 2 % agar plates containing basal bicarbonate-buffered medium (without reducing agent) (Stams et al., 1993) and 1 mmol L^{-1} of sodium oleate (≥ 99 %, Sigma-Aldrich, St. Louis, MO) as carbon source. The plates were incubated under air at 37 °C for 3 days. Single colonies were picked-up and transferred three times to new agar plates. Two different isolates with different colony morphologies (I1 and I2) could be obtained. Purity of the isolates was confirmed by phase contrast microscopic observations and the taxonomic identification was obtained by 16S rRNA gene sequencing (Sanger sequencing).

4.2.2. Experiment 2 – Degradation of oleate by the isolated strains under different ORP, promoted by diverse oxygen supply strategies

Batch assays

The ability of the isolates to metabolize oleate was studied in batch assays performed in closed serum bottles under four different redox conditions: strict anaerobic (supplemented with 1 mmol L^{-1} of Na_2S, as reducing agent), anoxic (without the addition of reducing agent), microaerophilic and aerobic (headspace with 4 % and 21 % (v/v) oxygen, respectively) (Figure 4.1b). All batch bottles contained bicarbonate-buffered mineral salt medium (Stams et al., 1993), 1 mmol L^{-1} of sodium oleate, and a

headspace initially composed of N_2/CO_2 (80:20 % v/v, 170 kPa), except for the aerobic assays. Air was added to the microaerophilic and aerobic assays with a pressure lock syringe (SGE, Ringwood, Australia) before autoclaving to obtain the required oxygen concentrations. Prior inoculation, isolate I1 and I2 were first grown on liquid media supplemented with 0.5 g L^{-1} of glucose, under aerobic conditions, at 37 °C and at 100 rpm. Aliquots of the microbial cultures were collected during the exponential growth phase and used to inoculate the bottles (10 % (v/v)). All tests were performed in duplicate and were incubated in the dark, at 37 °C, with stirring at 100 rpm. For the aerobic and microaerophilic conditions, the test ended after 15 days, and for anoxic and anaerobic conditions took 1 month. In the end, the whole content of the bottles was lyophilized for further LCFA quantification. The capacity to degrade palmitate (1 mmol L^{-1}) was also tested.

Continuous bioreactors

Two CSTR of 0.25 L were inoculated with the isolates I1 and I2 and were designated by R-I1 and R-I2, respectively. The inocula were prepared as described for the batch assays but using oleate (1 mmol L^{-1}) as carbon source instead of glucose. At the beginning of the experiment, the biomass concentration inside the bioreactors was approximately 2 mg L^{-1} in organic nitrogen. The reactors were operated in continuous mode at mesophilic conditions (37 °C) for 22 days, with HRT of 1 day and with 1 g L^{-1} in COD of sodium oleate (≥ 82 % as oleic acid, Riedel-de-Häen, Seelze, Germany). Feeding was prepared as described in Experiment 1, but in this case, it was autoclaved and the feeding tank was kept sterile by using a syringe filter in the air entrance (Figure 4.1b). The performance of the reactors was monitored by measuring pH, ORP, COD in the feed, and LCFA in the reactor. The morphology of the cells was examined by phase-contrast microscopy (Olympus-CX41, Olympus Corporation, Tokyo, Japan) and the viability of the isolates was tested periodically in agar plates, under the same conditions described for the isolation of the strains. The analytical methods were performed as described in Chapter 3 (section 3.2.3).

4.2.3. DNA extraction and 16S rRNA sequencing

Samples of well-homogenized sludge were centrifuged (4000 rpm, 10 min at 4 °C), the pellet was re-suspended in 0.5 mL of PBS (phosphate-buffered saline) and preserved at -20 °C until DNA isolation. Total genomic DNA was extracted using a FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH) and purified by ethanol precipitation. DNA amplification, Illumina libraries preparation, amplicon sequencing (Illumina MiSeq, Inc. SanDiego, CA) and bioinformatics analysis of the data were performed at Research and Testing Laboratory (RTL), Texas. Samples were amplified for

sequencing using the universal primer pair, 515f and 806r (Caporaso et al., 2011), targeting the prokaryotic 16S rRNA gene. Details on the sequencing and bioinformatics data analysis can be found elsewhere.(Paulo et al., 2017) The FASTq files were submitted in the ENA (European Nucleotide Archive), being available in the following link http://www.ebi.ac.uk/ena/data/view/PRJEB11469. Nucleotide sequences from this experiment are identified with accession numbers ERS1487522 to ERS1487524 and ERS1505407 for trial 1 and ERS2319672 to ERS2319677 for trial 2.

For the taxonomic identification of the isolates, a single colony was suspended in 50 µL of TE buffer. DNA was isolated after exposing the colonies at 95 °C during 10 min to promote cell lysis. The bacterial 16S rRNA genes were amplified by PCR (Polymerase Chain Reaction) with a Taq DNA polymerase (EP0402, ThermoScientific, San Diego, CA) and universal primers 27f and 1492r targeting the bacterial 16S rRNA gene, as detailed elsewhere.(Lane, 1991; Sousa et al., 2007a) Sanger sequencing was performed by Macrogen (Amsterdam, The Netherlands). The sequences were checked and cleaned using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Identification of 16S rRNA gene sequences was obtained by local alignment with NCBI nucleotide database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Isolates sequences are identified with accession numbers LT903717-LT903718 in ENA (www.ebi.ac.uk/ena/data/view/LT903717-LT903718) and its DNA sequences are detailed in the supplementary material section.

For DGGE analysis, PCR products were generated using bacterial 16S rRNA gene primers 968f and 1401r (Nübel et al., 1996). A description of PCR programs and primers sequences was described by Sousa et al. (2007) All primers used were synthesized commercially by Invitrogen (Life Technologies, Porto, Portugal). Size and yield of PCR products were estimated using a 1 kb extended DNA ladder (ThermoScientific, San Diego, CA) and a green safe staining via 1 % (w/v) agarose gel electrophoresis. DGGE analysis of the amplicons was performed as previously described by Zoetendal et al. (2001), using the Dcode system (Bio-Rad, Hercules, CA) with 8 % (v/v) polyacrylamide gels and a denaturant gradient of 30 – 60 %. A 100 % denaturing solution was defined as 7 mol L^{-1} urea and 40 % formamide. Electrophoresis was performed for 16 h at 85 V in a 0.5 x TAE buffer at 60 °C. DGGE gels were stained with $AgNO_3$ as described Sanguinetti et al. (1994) and scanned at 400 dpi.

4.2.4. Microscopy

PFR segments were cut with a scalpel, placed in OCT compound and immediately frozen with liquid nitrogen. Preserved samples were kept at -20 °C until being sliced in 50 µm thick sections with a

cryomicrotome (Leica Microtome 1900, Wetzlar, German). Before the staining, all the samples were fixed directly in the slides with 4 % formaldehyde solution (freshly prepared in PBS), in order to maintain the morphological integrity of the cells. The slides were washed with PBS and then double stained with DAPI (0.1 g L^{-1}) and Nile Red (10 mg L^{-1}). Both stains were incubated during 10 min at room temperature and the slides were washed with PBS after incubation with each one of the staining compounds. Biofilm images were acquired in a Confocal Scanning Laser Microscope (Olympus BX61, Model FluoView 1000, Tokyo, Japan). DAPI (Sigma, Darmstadt, Germany) was used to the detection of cells, as it links to DNA (laser excitation line 405 nm and emissions filters BA 430–470, blue channel) and Nile Red (Sigma, Darmstadt, Germany) to stain hydrophobic compounds (laser excitation line 559nm and emissions filters BA 575–675, red channel). Images were acquired with the program FV10-Ver4.1.1.5 (Olympus, Tokyo, Japan).

4.2.5. Statistical analysis

To investigate if bioreactors performance were significantly different, the student's t-test was applied. Palmitate-to-total LCFA percentage in the samples collected in trial 1 and trial 2 were initially compared to determine if the two trials were significantly different or not, with the objective of determine the reproducibility of the trials. After that, this set of values from trial 1 and trial 2 were used to compare palmitate-to-total percentage between periods. Canonical correspondence analysis (CCA) was performed to relate operational parameters (HRT and palmitate % in the PFR-biofilm) to microbial community composition, using the operational taxonomic units (OTU) with more than 1 % of relative abundance. CCA was done in Excel using the XLSTAT statistical software. All levels of significance were defined at $p \leq 0.05$.

4.3. Results

4.3.1. Experiment 1 – Enrichment and isolation of facultative anaerobic bacteria from continuous bioreactors fed with oleic acid

High organic loading rates and low hydraulic retention times were applied (Table 4.1) to selectively washout the microorganisms with slower growth rates (including methanogens) and simultaneously to enrich for microorganisms capable of performing oleate to palmitate conversion. Interestingly it was observed that the effluent washed out from the CSTR was partially retained in a thick biofilm formed in the discharge tube that acted as a PFR-biofilm reactor having a very low HRT in the order

Table 4.1 – Operating conditions and performance date in the CSTR and PFR: organic loading rate applied in COD to the CSTR (OLR-CSTR); hydraulic retention time in CSTR (HRT-CSTR) and in the PFR (HRT-PFR); LCFA content in the PFR biofilm.

Period	Trial	OLR-CSTR	HRT-CSTR	HRT-PFR	time	Oleate	Palmitate	Others
		kg m^{-3} d^{-1}	h	h	d	%	%	%
I	1	13.3 ± 0.6	6.3 ± 0.2	0.01 ± 0.00	22	31	51	18
					35	4	82	14
	2	12.8 ± 2.6	6.4 ± 0.8	0.01 ± 0.00	38	24	67	9
					45	22	69	9
					53	22	69	9
II	1	6.4 ± 0.3	12.7 ± 0.2	0.02 ± 0.00	43	47	33	20
					53	25	58	17
					78	15	72	13
	2	5.8 ± 1.2	12.7 ± 0.3	0.02 ± 0.00	60	49	44	7
					68	52	41	7
					76	28	64	8
III	1	4.2 ± 0.8	17.5 ± 0.5	0.03 ± 0.00	87	59	18	23
IV	1	3.0 ± 0.9	23.5 ± 0.6	0.04 ± 0.00	119	56	23	21
					125	84	8	8

of few minutes. Thus, during this bioreactor operation two different systems were distinguished and studied (CSTR and PFR, Figure 4.1a), presenting different reaction profiles and different microbial community composition.

The LCFA profiles in the bulk liquid washed out from the CSTR and from the PFR were similar, with oleate as the main fatty acid quantified (Figure 4.2).

VFA and methane were not detected during the experiment and pH varied from 7 to 9 in the CSTR. Biofilm formation was observed in the PFR in all the operational periods and contained both hydrophobic compounds and cells, as visualized by confocal microscopy (Figure 4.3). In periods I

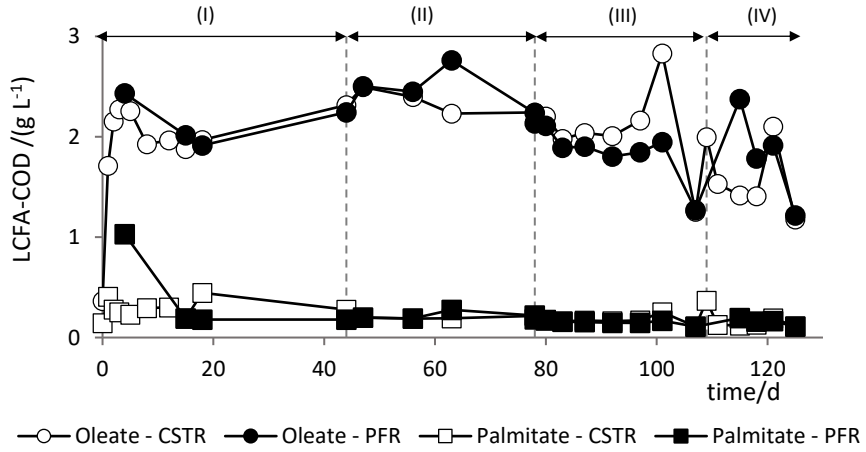


Figure 4.2 – LCFA concentration during periods I, II, III and IV in the effluent from the CSTR and from the PFR.

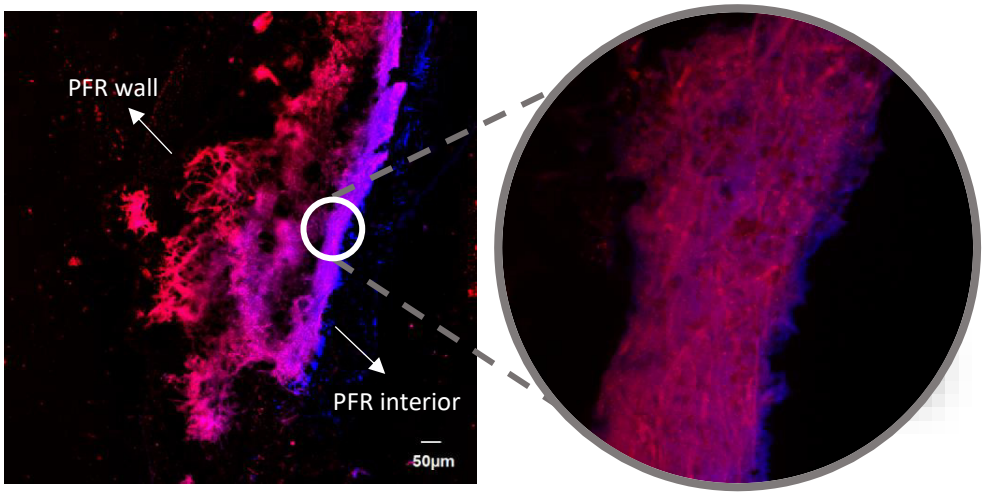


Figure 4.3 – Confocal Scanning Laser Microscope image of biofilm formed in trial 2 under an HRT of 6 h. Microbial cells are stained blue (DAPI staining) and hydrophobic compounds in red (Nile red staining).

and II, palmitate was the main LCFA detected in the biofilm, varying between 50 % and 80 % in trial 1 (except at day 43, immediately after increasing the HRT).

In a new independent experiment (trial 2 in Periods I and II) palmitate percentage in the biofilm was statistically equal ($p>0.05$) to the observed in trial 1 in the same periods, showing the consistency and robustness of the results. In periods III and IV (trial 1), a shift ($p<0.05$) was observed and oleate became the dominant LCFA detected in the biofilm, reaching a value of 84 %, which corresponds to the percentage of oleate in the feeding (Table 4.1).

The high amount of palmitate present in the biofilm during period I and II suggests the presence of bacteria that convert oleate to palmitate. These microbial communities were mainly composed by facultative anaerobic bacteria (Table 4.2). In trial 1, *Pseudomonas* was the predominant genus, representing 42 % and 58 % of the microorganisms detected in periods I and II, respectively (table 4.2). It was also one of the predominant genera in trial 2, representing up to 16 % of the total microbial community, together with other facultative anaerobic bacteria assigned to genera *Acinetobacter* (up to 36 %), *Delftia* (up to 29 %) and *Stenotrophomonas* (up to 13 %) (Table 4.2). The difference between trials is not surprising, as a different inoculum was used (obtained at different times), although from the same source (a brewery wastewater treatment plant). In periods III and IV, where longer HRTs were applied, the relative abundance of facultative anaerobes decreased and aerobic genus such as *Ochrobactrum* and *Microvirgula* became predominant. Interestingly, in these periods oleate was the main LCFA detected in the biofilm (Table 4.1).

Table 4.2 – Taxonomic identification of the microbial reads identified at the genus level (at > 1 % abundance), during the periods I to IV of the PFR operation.

Period	I	I	I	I	II	II	II	II	III	IV
Trial	1	2	2	2	1	2	2	2	1	1
Time / d	22	38	45	53	78	60	68	76	107	119
Genus / %										
Pseudomonas	**42**	7	**14**	**16**	**58**	**11**	6	**16**	2	n.d.
Acinetobacter	**20**	**36**	**34**	**19**	n.d.	**15**	**25**	**24**	<1	1
Unclassified *Bacteria*	**21**	3	4	6	3	4	1	8	<1	<1
Azoarcus	**10**	<1	<1	n.d.	**10**	n.d.	n.d.	n.d.	8	<1
Aeromonas	5	<1	<1	<1	3	<1	<1	<1	<1	<1
Delftia	<1	16	3	**18**	5	**19**	**29**	**12**	3	<1
Staphylococcus	n.d.	n.d.	n.d.	n.d.	4	n.d.	n.d.	n.d.	<1	<1
Comamonas	<1	<1	<1	<1	2	<1	<1	<1	<1	<1
Parabacteroides	n.d.	<1	<1	<1	2	<2	n.d.	<2	n.d.	<1
Ochrobactrum	n.d.	1	<1	<1	1	1	<1	<1	**46**	4
Aquamicrobium	n.d.	<1	<1	<1	<1	<1	n.d.	<1	**17**	3
Microvirgula	n.d.	**11**	3	4	n.d.	**16**	5	4	5	**65**
Bosea	n.d.	3	6	3	n.d.	2	1	<1	5	5
Escherichia	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1	<1
Stenotrophomonas	<1	**13**	**12**	**13**	<1	**12**	**10**	**12**	**10**	**10**
Proteocatella	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<1	4
Agrobacterium	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<1	2
Chryseobacterium	n.d.	3	5	3	n.d.	5	6	1	<1	<1
Dysgonomonas	<1	2	3	6	<1	4	4	6	<1	<1
Variovorax	n.d.	1	2	1	n.d.	<1	<1	<1	n.d.	n.d.
Sphingopyxis	n.d.	<1	2	<1	n.d.	<1	<1	<1	n.d.	n.d.
Camelimonas	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3	1	n.d.	n.d.
Parachlamydia	n.d.	n.d.	n.d.	n.d.	n.d.	<1	2	1	n.d.	n.d.
Arcobacter	n.d.	<1	<1	2	n.d.	<1	<1	<1	n.d.	n.d.
Azospira	n.d.	<1	<1	1	n.d.	<1	<1	<1	n.d.	<1
Pleomorphomonas	n.d.	<1	<1	<1	n.d.	1	<1	2	n.d.	<1

n.d. – not detected

CCA analysis using all data obtained in trials 1 and 2 demonstrated that the microbial genera are correlated to the tested variables, i.e., palmitate percentage and HRT ($p<0.05$). The abundance of facultative anaerobes, in particular of *Pseudomonas* spp, is highly correlated with the accumulation of palmitate (Figure 4.4).

Two *Pseudomonas* strains (I1 and I2) were isolated from the biofilm collected from PFR in period II and exhibit two distinct colony types. The colony formed by I1 was transparent and had a dispersive like shape; the colony formed by I2 has a circular raised shape and smooth appearance (Figure 4.5a). Both isolates were rod-shaped when observed by phase contrast microscopy (Figure 4.5b, c).

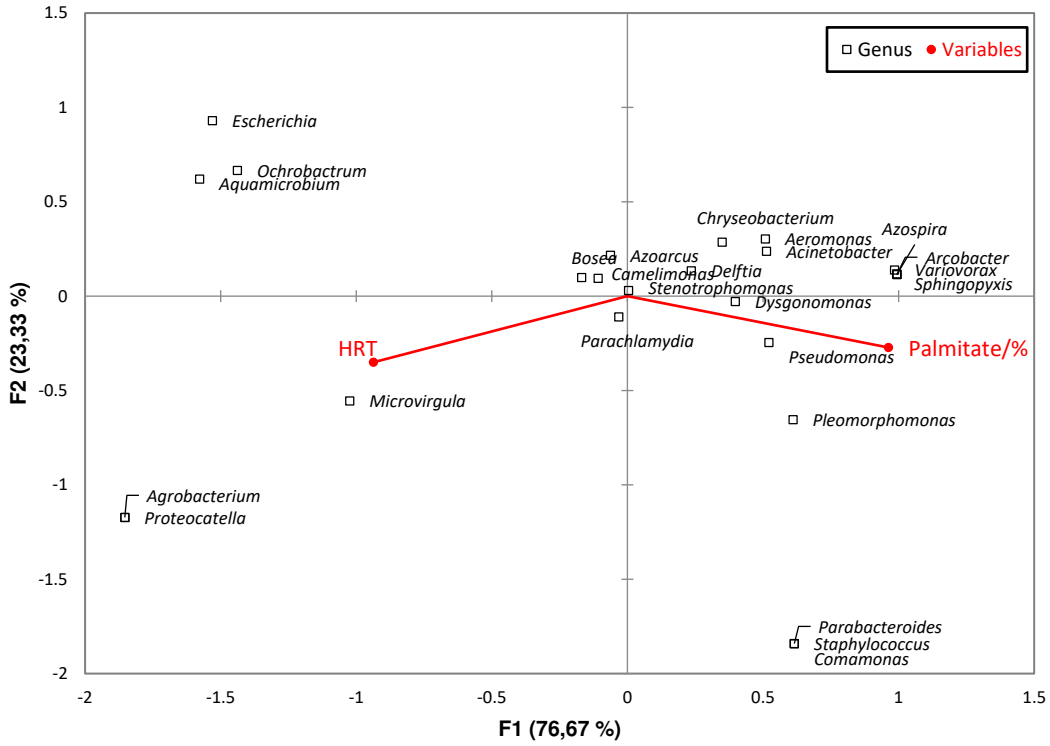


Figure 4.4 – Canonical correspondence analysis (CCA) between microbial community composition and environmental variables (palmitate % and HRT).

DGGE analysis confirmed the presence of I1 and I2 in CSTR and in PFR (Figure 4.6). Bands corresponding to I1 and I2 were rather intense, when compared with remaining bands, confirming their relative high abundance in the bioreactors.

Isolates I1 and I2 shared 98.2 % similarity of 16S rRNA gene and presented 99 % of identity with several species of *Pseudomonas* (data not shown). In general, closely related species of bacteria, like the ones belonging to *Pseudomonas* genus, cannot be differentiated based on the 16S rRNA gene, but can be grouped as suggested by Gomila et al. (2015). Such an analysis indicates that strain I1 and I2 are affiliated within the *P. oleovorans* and *P. stutzeri* groups, respectively.

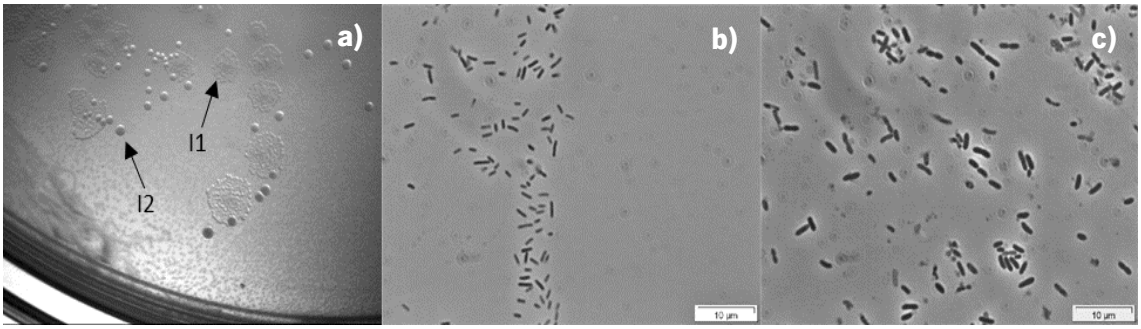


Figure 4.5 – Colonies formed by isolates 1 and 2 obtained in an Olympus stereomicroscope (SZ40) (a). Phase-contrast micrographs of cultures of isolate 1 (b) and isolate 2 (c) growing on oleate.

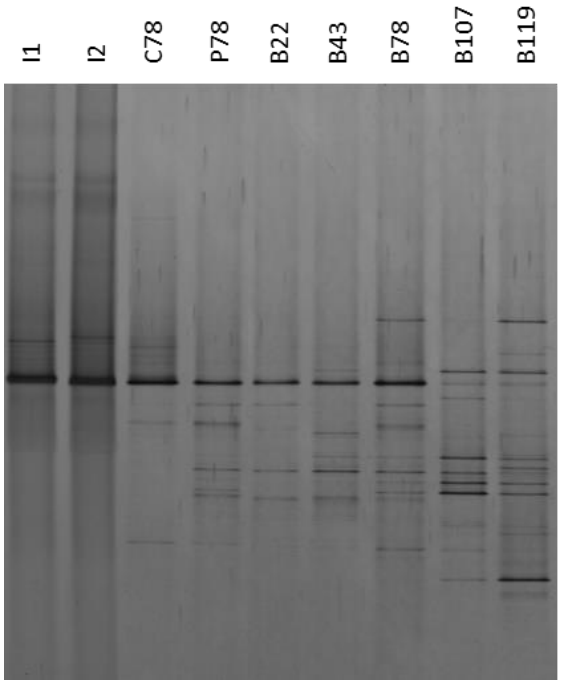


Figure 4.6 – DGGE profiles of the isolates growing in pure culture (I1 – Isolate 1, I2 – Isolate 2) and of the microbial communities present in the effluent from the CSTR (C), in the effluent from the PFR (P) and in the PFR biofilm (B). The numbers 22, 43, 78, 107 and 119 are related to the operation days.

4.3.2. Experiment 2 – Degradation of oleate by the isolated strains under different ORP, promoted by diverse oxygen supply strategies.

Under aerobic conditions and during an incubation of 15 days in batch, strain I1 was able to degrade around 84 % of the initial oleate added, while strain I2 was only able to degrade 36 %. Initial concentration of oleate was (0.76 ± 0.04) mmol L^{-1}. Palmitate was also completely degraded by both isolates. Under microaerophilic conditions (in the presence of approximately 4 % O_2) both isolates degraded up to 24 % in 15 days. Oleate degradation was below 10 % in the tests where anoxic and anaerobic conditions were applied, at least during 30 days of incubation (Table 4.3). No intermediary compounds, such as other LCFA, were detected in the assays.

Table 4.3 – Percentage of oleate degraded by the *Pseudomonas* spp. isolates at different redox conditions.

Incubation conditions	Duration of incubation/days	Percentage of oleate degraded/%	
		Isolate 1	Isolate 2
Aerobic	15	84 ± 4	36 ± 4
Microaerophilic	15	24 ± 6	24 ± 7
Anoxic	31	9 ± 3	5 ± 0
Anaerobic	31	10 ± 9	0 ± 2

In continuous bioreactors inoculated with strains I1 and I2 and operated at OLR of about 1 kg m^{-3} d^{-1} in COD (influent COD=1 kg m^{-3}), oleate concentration decreased till a minimum value of around 100 mg L^{-1} in COD (Figure 4.7). Palmitate concentration was also similar in both reactors and was always lower than 40 mg L^{-1} in COD. Other LCFA were detected in negligible amounts (< 20 mg L^{-1} COD). Both reactors presented a similar ORP with an average value of (118 ± 30) mV and (127 ± 18 mV), for R-I1 and R-I2 respectively.

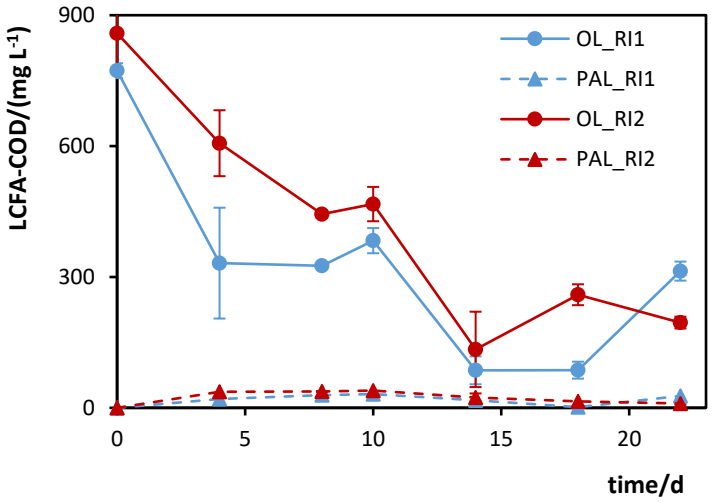


Figure 4.7 – LCFA concentrations in the reactors R-I1 and R-I2.

4.4. Discussion

It is well known that bacteria belonging to the genus *Syntrophomonas* perform LCFA degradation in syntrophy with methanogens (Sousa et al., 2009b). However, in high-rate continuous bioreactors inoculated with complex microbial communities, syntrophic fatty acid degrading bacteria are generally present in relative low numbers, *i.e.* 0.2 % – 3 % (Sousa et al., 2009b; Stams et al., 2012). In anaerobic bioreactors, accumulation of palmitate from oleate has been reported at organic loads higher than 3 g L^{-1} d^{-1} as oleate (in COD) (Jeganathan et al., 2006). However, palmitate was not detected in defined syntrophic co-cultures, for example of *Syntrophomonas zehnderi* and *Methanobacterium formicicum,* growing on oleate (Cavaleiro et al., 2016).

The involvement of facultative anaerobes in oleate conversion to methane was further investigated. By applying high hydraulic loading rates (Experiment 1) it was possible to enrich for facultative anaerobic microorganisms, which formed a thick palmitate-rich biofilm in a plug flow reactor fed with oleate. The abundance of facultative anaerobes, in particular of *Pseudomonas* sp., was highly

correlated with the accumulation of palmitate, strongly suggesting that these microorganisms are involved in palmitate formation from oleate. This is in accordance with the predominance of these bacteria in bioreactors where oleate to palmitate conversion occurred (Cavaleiro et al., 2016; Pereira et al., 2002).

The two *Pseudomonas* spp. strains isolated, were able to biodegrade oleate in batch assays under aerobic and microaerophilic conditions, but not in the absence of oxygen. Oleate was consumed according to the amount of oxygen supplied and no intermediates were detected. The isolates were further tested in continuous bioreactors, to simulate the continuous input of very low levels of oxygen where oleate degradation occurred again without palmitate formation. Since oxygen input was not quantifiable, the mass balance could not be determined in this case, but oleate was converted, and the viability of cells was confirmed. The tested conditions showed that *Pseudomonas* spp. were able to consume oleate, but we could not show that they formed palmitate. Nevertheless, it is noteworthy to highlight that our isolates were grown in planktonic form, while palmitate formation was associated with the development of a biofilm. Also in the CSTR, *Pseudomonas* were present in planktonic form and palmitate did not accumulate as it did in the biofilm. It is known that bacterial cells growing in biofilms may establish a different physiology than planktonic cells (Costerton et al., 1987). For example, when Heffernan et al. (2009) compared fluoroacetate degradation by *P. fluorescens* grown in biofilm, and in planktonic forms, differences were observed in the accumulation of an intermediate metabolite (glycolate) in the biofilm.

On the other hand, we cannot exclude that bacteria other than *Pseudomonas* are involved in oleate conversion to palmitate. In addition, possible interactions of facultative bacteria with syntrophic bacteria and methanogens when growing in complex microbial communities may govern the global metabolic pathway of oleate degradation in high rate continuous methanogenic bioreactors, which are difficult to mimic. Studies in batch assays with defined cultures or co-cultures are important to add evidence about possible mechanisms and metabolic traits taking place in microbial communities, but do not inform about interactions and relevance of potential multiple pathways occurring simultaneously in these complex systems. Further studies with defined cultures in continuous reactors are needed to understand the kind of metabolic cooperation or competition occurs between syntrophic and facultative anaerobic bacteria in methanogenic reactors treating LCFA-based wastewaters.

Chapter 5.

Syntrophic and Facultative Anaerobic Bacteria in Oleate Conversion to Methane: Cooperation or Competition?

To understand the microbial interactions between anaerobic and facultative anaerobic bacteria (FAB) during oleate conversion, a syntrophic co-culture of *Syntrophomonas zehnderi* and *Methanobacterium formicicum* was established and the effect of adding the two *Pseudomonas spp.* isolates (Chapter 4) was assessed in a continuous bioreactor fed with oleate.

The syntrophic co-culture converted oleate to acetate and methane, in a continuous bioreactor under strict anaerobic conditions. After reaching the steady-state, *Pseudomonas* isolates were inoculated in the bioreactor and the feeding tank was opened to the air, providing microaerophilic conditions (under sterile conditions). The bioreactor performance was maintained with methane and acetate as the main products detected. All different cell morphotypes could be observed inside the bioreactor during the entire operation (more than 240 days), showing that none of the microorganisms was washed-out and that all microorganisms were kept active. To get more insights into the possible microbial interactions, additional batch tests were performed. Under anaerobic and microaerophilic conditions, the consortia formed by *S. zehnderi*, *M. formicicum* and the two *Pseudomonas* isolates converted oleate to acetate and methane 1.5 times faster than the syntrophic culture alone (under strict anaerobic conditions). This work shows that facultative bacteria, and particularly *Pseudomonas*, play an important role in the anaerobic reactors treating oleate-based wastewaters, by improving oleate conversion to methane. Probably, FAB by rapidly consuming oxygen and oleate, protect strict anaerobes from oxygen and oleate toxicity.

5.1. INTRODUCTION

Few species are reported to degrade LCFA under methanogenic conditions. They belong to *Syntrophomonas*, *Thermosyntropha* and *Syntrophus* genera (Sousa et al., 2009b) and are dependent on the activity of hydrogenotrophic methanogens to keep the H_2 partial pressure and formate concentration low, in order to thermodynamically allow LCFA degradation.

Although *Syntrophomonas* are directly related to LCFA degradation, in anaerobic bioreactors they are usually detected in low percentages (Stams et al., 2012). Despite of being detected in low percentages, the importance of these bacteria is undoubtable and their enrichment was obtained (Sousa et al., 2008; Ziels et al., 2017, 2016). On the other hand, facultative anaerobic bacteria, as for example *Pseudomonas* species, are frequently abundant (Baserba et al., 2012; Cavaleiro et al., 2016; Pereira et al., 2002; Chapter 4). Their involvement in methanogenic LCFA degradation was never explored until recently, when Cavaleiro et al. (2016) showed that during the conversion of oleate to palmitate FAB became abundant. Further insight on this subject was obtained in Chapter 4 where the presence of *Pseudomonas* spp. was strongly correlated with palmitate-to-total LCFA percentage in bioreactors converting oleate to palmitate.

This Chapter aims to study the microbial relationships between syntrophic LCFA degrading bacteria, FAB and methanogens during anaerobic and microaerophilic oleate degradation. For this propose, a microbial consortium composed by *Syntrophomonas zehnderi* (a syntrophic LCFA degrading bacterium), two *Pseudomonas* spp. (isolated from an oleate degrading bioreactor: isolates I1 and I2, Chapter 4) and *Methanobacterium formicicum* (a hydrogen/formate utilizing methanogen) was studied in continuous and batch bioreactors fed with oleate.

5.2. MATERIAL AND METHODS

5.2.1. Continuous bioreactor operation

A continuous reactor of 0.5 L (working volume) and a headspace of 0.14 L was inoculated (10 % v/v) with a co-culture of *S. zehnderi* and *M. formicium* previously grown in 0.5 mmol L^{-1} of oleate (Figure 5.1). Ten small pieces (appx. 0.5 cm x 0.5 cm) of marprene tube were added to the reactor as a support for microbial cells attachment. The reactor was operated at mesophilic conditions at approximately 37 °C. The biogas produced was kept in the headspace of the reactor and the cumulative methane production was followed.

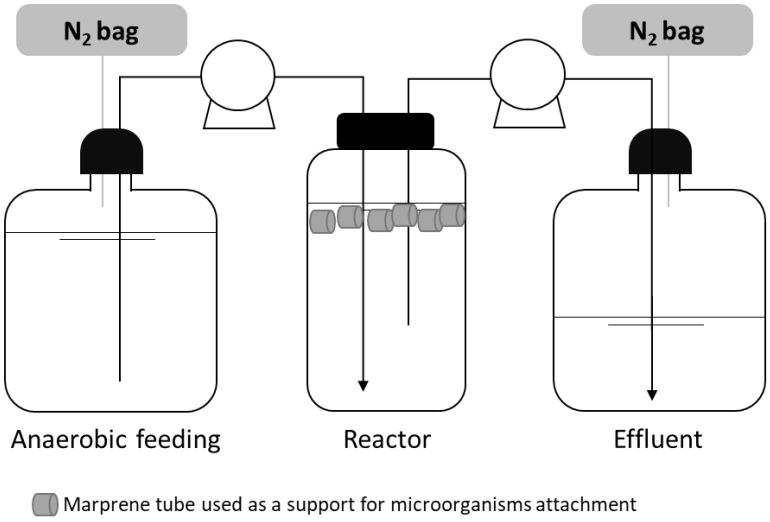


Figure 5.1 – Schematic diagram of the experimental setup for continuous culture of *S. zehnderi* and *M. formicicum*.

The bioreactor operation was divided in 10 different periods with different oleate concentrations, HRT and percentage of O_2 concentration in the feeding tank (Table 5.1). During periods I to IV the bioreactor was operated only with the syntrophic co-culture, under strict anaerobic conditions, until reaching a steady point where the culture was kept active inside the reactor during continuous operation. For that, oleate concentration and HRT were varied over periods I to IV (Table 5.1).

The feed consisted of anaerobic mineral salt medium (Stams et al., 1993) buffered with bicarbonate (4 g L^{-1}) and reduced with sodium sulfide (2 mmol L^{-1}). Sodium oleate (99 %, Sigma, Darmstadt, Germany) was used as carbon and energy source at an initial concentration of 0.25 mmol L^{-1} (Period I). The feeding was sterilized by autoclaving and the feeding tank was kept sterile and anaerobic by using a syringe filter in the gas entrance from a bag containing nitrogen (Figure 5.1). The headspace

Table 5.1 – Parameters set in the different operational periods: oleate concentration in the feed; HRT applied; mode: continuous or batch; percentage of oxygen in the feeding tank.

Microbial consortium	Period	Time/d	Oleate in the feed /mmol L^{-1}	HRT/d	Mode
M. formicicium *S. zehnderi*	I	0-13	0.25	20	continuous
	II	13-22	2.28 ± 0.09	20	continuous
	III	22-70	1.32 ± 0.76	10	continuous
	IV	70-112	2.63 ± 0.87	10	continuous
M. formicicium *S. zehnderi* *Pseudomonas* isolates	V	112-133	3.21 ± 0.47	10	continuous – 21 % O_2
	VI	133-160			batch
	VII	160-165	4.67 ± 0.70	10	continuous – 10 % O_2
	VIII	165-176			batch
	IX	176-216	1.36 ± 0.23	10	continuous – 5 % O_2
	X	216-245	2.13 ± 0.45	10	continuous – 5 % O_2

of the bioreactor was initially pressurized with a mixture of N_2/CO_2 (80:20 % v/v, 170 kPa).

In period V, the *Pseudomonas* isolates (Chapter 4) were inoculated to the bioreactor containing the syntrophic co-culture. The main difference in the experimental set up was that the headspace composition of the feeding tank changed from nitrogen to air (21 % of oxygen) during period V (Table 5.1). Bicarbonate concentration was set at 2 g L^{-1} in periods III to X. Although the conditions changed from anaerobic to aerobic or microaerophilic, sodium sulfide was still added (0.5 mmol L^{-1}) to provide a sulfur source for *M. formicicum*. Sporadically, when the ORP increased and the reactor become pink, and in the beginning of the batch periods, 0.5 mmol L^{-1} of sodium sulfide were added to the reactor.

The operational conditions were different between periods V to X because, when methane production dropped, showing a decrease in methanogenic activity, the operation mode was changed from continuous to batch. Also, oleate and oxygen concentrations were adjusted during the operation to guarantee the microbial activity of the oleate degrading consortium (Table 5.1).

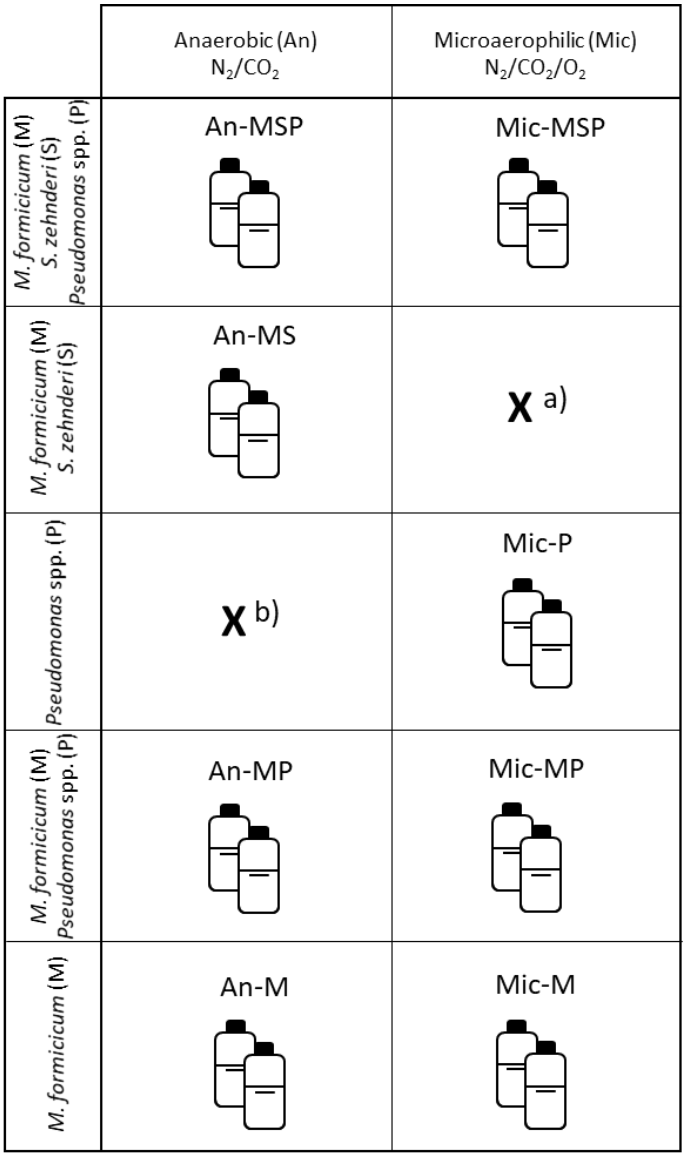
The feeding tank was kept at 60 °C in order to maintain the sodium oleate dissolved in the feed. Since the reactor was overpressured, the outlet was controlled using a peristaltic pump. Samples were collected for methane, LCFA and VFA quantification, pH and ORP monitorization, and for regular microscopic observations by phase-contrast microscopy (Olympus-CX41, Olympus Corporation, Tokyo, Japan). Detailed description of these procedures can be found in Chapter 3 (section 3.2.3). Methane content of the biogas was analyzed by GC-2014 Shimadzu ATF, with a Porapak Q (80-100 mesh) (2 m × 3.75 mm) column and flame ionization detector (FID). Nitrogen was used as the carrier gas (30 mL min^{-1}). The detector, injector, and oven temperatures were 35, 110 and 220 °C, respectively. The gas sample had a volume of 0.5 mL.

5.2.2. Batch assays

5.2.2.1. Syntrophic co-culture and Pseudomonas

The anaerobic consortium formed by *S. zehnderi*, *M. formicicum* and the *Pseudomonas* isolates was tested in batch assays, under strictly anaerobic and under microaerophilic conditions, containing oleate as the sole carbon and energy source (Figure 5.2).

The assays were performed in duplicate by using 120 ml closed serum bottles with a working volume of 50 mL. The anaerobic saline medium was prepared as described in Chapter 4 (section 4.2.2). In the microaerophilic assays, oxygen was added to the bottles headspace to a final concentration of 2 % (v/v).



a) Not tested
b) No growth (Chapter 4)

Figure 5.2 – Schematic representation of the conditions applied in the batch tests performed (section 5.2.2.1).

Control assays were performed as following: co-culture of *M. formicicum* (M) and *S. zehnderi* (S) under anaerobic conditions (An-MS); *Pseudomonas* (P) isolates I1 and I2 under microaerophilic conditions (Mic-P); *M. formicicum* (M) and *Pseudomonas* (P) isolates I1 and I2 under anaerobic and microaerophilic conditions (An-MP and Mic-MP); *M. formicicum* (M) under anaerobic and microaerophilic conditions (An-M and Mic-M).

The cultures were cultivated as following: (1) *M. formicicum* cultures were pre-grown in H_2/CO_2 (80:20 % v/v, 170 kPa) until reaching the exponential phase; (2) the headspace of the bottles was flushed four times with N_2/CO_2 (80:20 % v/v, 170 kPa) to remove the remaining hydrogen and the methane produced; (3) *S. zehnderi* was inoculated (10 % v/v) and oleate added to a final concentration of 0.5 mmol L^{-1}; (4) after reaching the exponential phase, the headspace of the bottles

was flushed four times with N_2/CO_2 to remove the methane produced prior *Pseudomonas* inoculation; (5) at the moment of isolates inoculation, oleate was added to a final concentration of approximately 1 mmol L^{-1} and air was added in the microaerophilic assays (Mic) to obtain a concentration in the headspace of 2 % (v/v) O_2, whereas in anaerobic assays (An) the headspace was composed only of N_2/CO_2. *Pseudomonas* isolates inoculum was grown on SOC medium (Super Optimal Broth with added glucose), under aerobic conditions, at 37 °C and at 100 rpm.

The tests to the culture composed by *M. formicicum* and *Pseudomonas* (MP) followed the previously described steps 1, 2 and 5. The control with only the *M. formicicum* (M) was pre-grown in H_2/CO_2 (80:20 % v/v, 170 kPa) until reaching the exponential phase and then flushed four times with H_2/CO_2. In Mic-M tests, air was added to the bottles to a final concentration of 2 % (v/v) of O_2.

5.2.2.2. Pseudomonas isolates

The two *Pseudomonas* isolates (I1 and I2) were characterized separately for their capacity to degrade oleate and acetate, under aerobic conditions. For this propose each isolate (I1 and I2) was incubated in Erlenmeyer flasks containing basal medium (composition described in Chapter 4 - section 4.2.2). Oleate was added in a concentration of 1 mmol $L^{-1}$and acetate in a concentration of 16 mmol L^{-1}. The utilization of hydrogen (60 mmol L^{-1}) and formate (20 mmol L^{-1}) by the co-culture composed by isolates I1 and I2 was tested under microaerophilic conditions (5 % O_2 in the hydrogen test and 4 % in the formate test) as described in the previous section 5.2.2.1. LCFA and VFA were analyzed as described in Chapter 3 (section 3.2.3). Hydrogen was quantified using a GC BRUKER SCION 456 (Billerica, MA) equipped with a thermal conductivity detector and a Molsieve packed column (13X, 80/100 mesh, 2 m of length, 2.1 mm of internal diameter). Argon was the carrier gas (30 mL min^{-1}) and the temperatures of the injector, column and detector were 100 °C, 35 °C and 130 °C, respectively.

5.3. RESULTS

5.3.1. Oleate degradation by the syntrophic co-culture and in association with facultative anaerobic bacteria, i.e. *Pseudomonas* spp., in a continuous bioreactor

The syntrophic co-culture of *S. zehnderi* and *M. formicicum* was efficiently cultivated in a continuous bioreactor. The products of oleate degradation were acetate, methane and small amounts of formate (Figure 5.3a). Oleate conversion to acetate and to methane (resulted from hydrogen) were calculated according with the stoichiometrically expected based on eq. 5.1:

$$1\ oleate \rightarrow 9\ acetate + 3.75\ methane \qquad \text{eq. 5.1}$$

The bioreactor was operated with the syntrophic co-culture for 112 days before inoculation of the *Pseudomonas* isolates. Periods I and II corresponded to the co-culture adaptation to the continuous bioreactor. During these periods, acetate concentration decreased and methane production stopped, which was accompanied by an increase in the pH to values reaching 8.6 (Figure 5.3), showing a decrease of the activity of the co-culture. In Period III, bicarbonate concentration was reduced to 2 g L^{-1}, the headspace of the reactor was flushed with N_2/CO_2 and the HRT was decreased to 10 days. These alterations in the bioreactor operation resulted in the decrease of pH to 7.8 and recovery

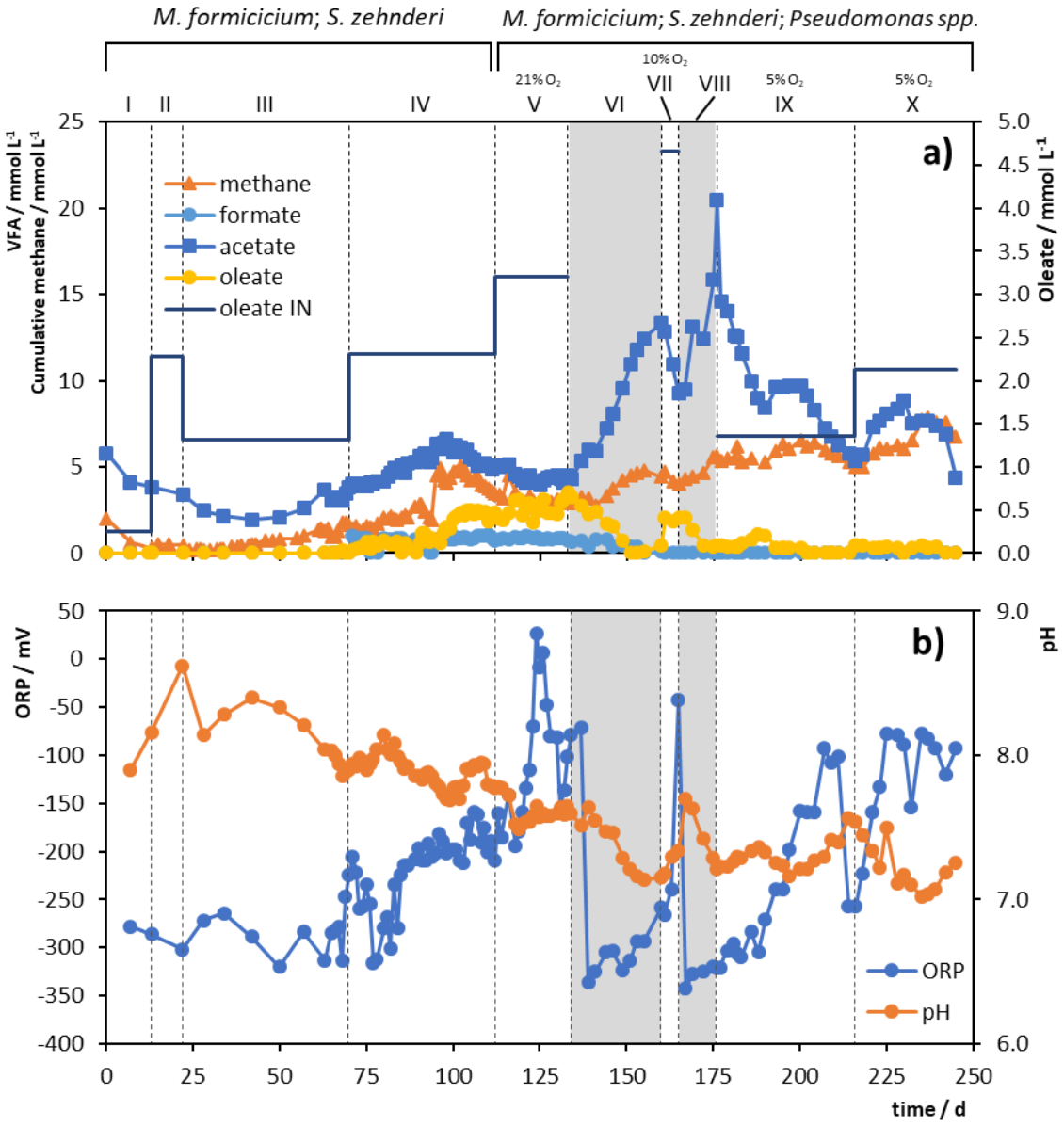


Figure 5.3 – Cumulative methane, VFA (acetate and formate) and oleate concentrations profile in the reactor and average oleate concentration in the feed (oleate IN) (a). ORP and pH inside the reactor (b)

of the microbial activity, with acetate and methane accumulating in the reactor (Figure 5.3). In this period, oleate conversion to acetate was (23 ± 5) % and to methane was (8 ± 4) % of the expected from complete oleate degradation according to eq 5.1. Oleate concentration in the reactor was however below the detection limit (25 mg L^{-1}), probably due to oleate adsorption to the feeding tank, to the feeding tube and to the reactor. After increasing oleate concentration in the feed to (2.63 ± 0.87) mmol L^{-1}, period IV (Table 5.1), oleate concentration inside the reactor increased up to a maximum of 0.5 mmol L^{-1}, formate to 1 mmol L^{-1}, acetate 6.6 mmol L^{-1} and methane 5.1 mmol L^{-1}. After detection of these maximum values (at day 98), both methane and acetate concentrations decreased (Figure 5.3). In this period (IV), oleate conversion to acetate ((21 ± 5) %) was similar to the former period but the conversion to methane increased to (20 ± 15) %.

At day 112 (period V) *Pseudomonas* isolates were inoculated in the reactor and the feeding tank was opened to the air (Figure 5.1). Oleate concentration inside the reactor increased up to a maximum of 0.7 mmol L^{-1}, acetate concentration decreased to 4 mmol L^{-1} and no methane was produced (Figure 5.3), showing that the methanogenic activity was compromised. To overcome this problem, an active co-culture of *M. formicicum* and *S. zehnderi* was supplemented to the reactor (5 % v/v, on days 118 and 126) and later on (on day 133) the reactor was operated in batch. This resulted in complete oleate consumption, acetate and methane concentrations were gradually increased (until a maximum of 13.4 and 4.8 mmol L^{-1}, respectively) and formate was no longer detected. This corresponds to an oleate conversion to methane of 62 % and to an oleate conversion to acetate around 184 %, suggesting that the oleate concentration in the reactor was 2 times higher than the 0.65 mmol L^{-1} quantified in the beginning of period VI. Once the system was recovering well (period VI), the reactor was again operated in continuous mode and with a percentage of oxygen in the feeding tank of 10 % (v/v) (period VII). However, the methane production decreased and oleate started accumulating inside the reactor (period VII). Therefore, the reactor was again closed (period VIII) to allow the recovery of the community, which was achieved after 11 days in batch. When the continuous operation was restarted (period IX), the oxygen concentration in the feeding was reduced to 5 % (v/v) to lower the impact of oxygen toxicity towards the anaerobes, and oleate concentration was decreased to (1.36 ± 0.23) mmol L^{-1} (Table 5.1). Although the conversion of oleate to methane was relatively low, methane production was quite constant for approximately 40 days and oleate conversion to acetate was circa 80 % (Table 5.2, period IX). In period X all the operational parameters

Table 5.2 – The oleate conversion to acetate and methane and yields oleate/acetate and oleate/methane calculated for periods III to X.

Period	Time/d	oleate conversion to acetate[1]/%	$Y_{acetate/oleate}$ mmol mmol^{-1}	oleate conversion to methane[2] /%	$Y_{methane/oleate}$ mmol mmol^{-1}
III	22-70	23 ± 5	2.2 ± 0.5	8 ± 4	0.3 ± 0.2
IV	70-112	21 ± 5	2.0 ± 0.3	20 ± 15	0.8 ± 0.6
V	112-130	18 ± 2	1.7 ± 0.1	24 ± 14	0.9 ± 0.5
VI	133-160	184	16.6	62	2.8
VII	160-165	25 ± 3	2.5 ± 0.3	0 ± 0	0.0 ± 0.0
VIII	165-176	240	21.6	119	4.5
IX	176-216	84 ± 24	8.0 ± 2.6	13 ± 14	0.5 ± 0.5
X	216-245	42 ± 8	3.2 ± 1.1	14 ± 7	0.5 ± 0.3

[1]Oleate conversion to acetate regarding the maximum acetate production expected (9 mmol L^{-1}) from 1 mmol L^{-1} of oleate.

[2]Oleate conversion to methane regarding the maximum methane production expected (3.75 mmol L^{-1}) from 1 mmol L^{-1} of oleate.

were maintained with exception of oleate concentration that was increased to (2.13 ± 0.45) mmol L^{-1} because oleate was being completely removed in the previous period, although oleate conversion to methane was only around 14 %.

During the bioreactor operation the pH was maintained between 7 and 8.6. Generally, pH decreased with the increase in acetate concentration (Figure 5.3). For example, when acetate concentration reached 13.4 mmol L^{-1} in period VI, the pH was 7.15, and when the pH reached the maximum value (pH = 8.6), the acetate concentration was 3.4 mmol L^{-1} (Figure 5.3b). The changes on the operation mode could be reflected in the ORP measured inside the reactor, with the ORP increasing during continuous operation (reaching a maximum value of 27 mV, in period V) and decreasing during batch periods (reaching a minimum value of -343 mV, in period VIII) (Figure 5.3b). This is justified by the fact that during continuous operation the feeding tank contained oxygen that consequently will promote the increase of the ORP. During period V, when the percentage of O_2 in the feeding was the highest (21 %, v/v), the ORP reached its higher value (27 mV), as well as in period VII when the oxygen in the feeding tank was kept at 10 % (v/v) the ORP sharply increased to -42 mV.

Over the experiment, samples were weekly collected for microscopic observations. Several microbial aggregates were observed (Figure 5.4). The curved rods were recognized as *S. zehnderi* (Sousa et al., 2007c) and the long rods corresponded to *M. formicicum* which is known to appear as single cells with some chains and filaments (Bryant and Boone, 1987). The straight rods were the *Pseudomonas* isolates described in Chapter 4.

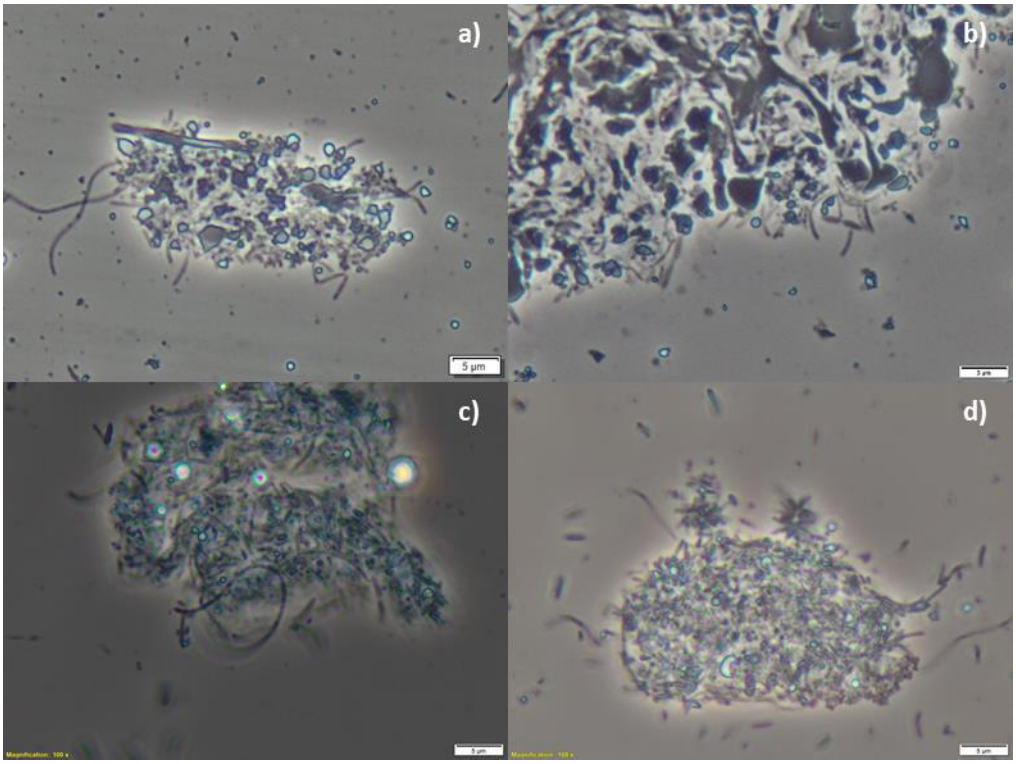


Figure 5.4 – Aggregates observed in samples from the bioreactor with only the syntrophic co-culture (a – day 98, period IV) and with the consortium composed by *M. formicicum*, *S. zehnderi* and the *Pseudomonas* isolates (b – day 132, period VI; c - day 195, period IX; d - day 225, period X).

5.3.2. Effect of the association of *Pseudomonas* spp. to the syntrophic co-culture on oleate conversion to methane.

The microbial consortium composed by the syntrophic co-culture and the *Pseudomonas* isolates, under batch anaerobic (culture An-MSP) and microaerophilic (culture Mic-MSP) conditions, converted oleate to methane 1.5 times faster than the control culture composed by the syntrophic co-culture only (An-MS) (Figure 5.5a). After 67 days, oleate was fully degraded in An-MSP and in Mic-MSP but took around 100 days to be completely degraded by the syntrophic co-culture alone (An-MS) (Figure 5.5c, Table 5.3). At the end of the incubations, methane concentration in An-MSP and Mic-MSP was (3.12 ± 0.13) mmol L^{-1} and (2.73 ± 0.08) mmol L^{-1}, and acetate concentration was (12.23 ± 0.38) mmol L^{-1} and (10.80 ± 0.21) mmol L^{-1}, respectively (Figure 5.5a,b, Table 5.3). The final values obtained for methane and acetate in An-MSP corresponded to 119 % of the expected from the initial oleate concentration. Regarding to Mic-MSP, the oleate conversion to methane and acetate were 107 % and 97 %, respectively. Part of the oleate could also have been degraded aerobically in the microaerophilic batch tests.

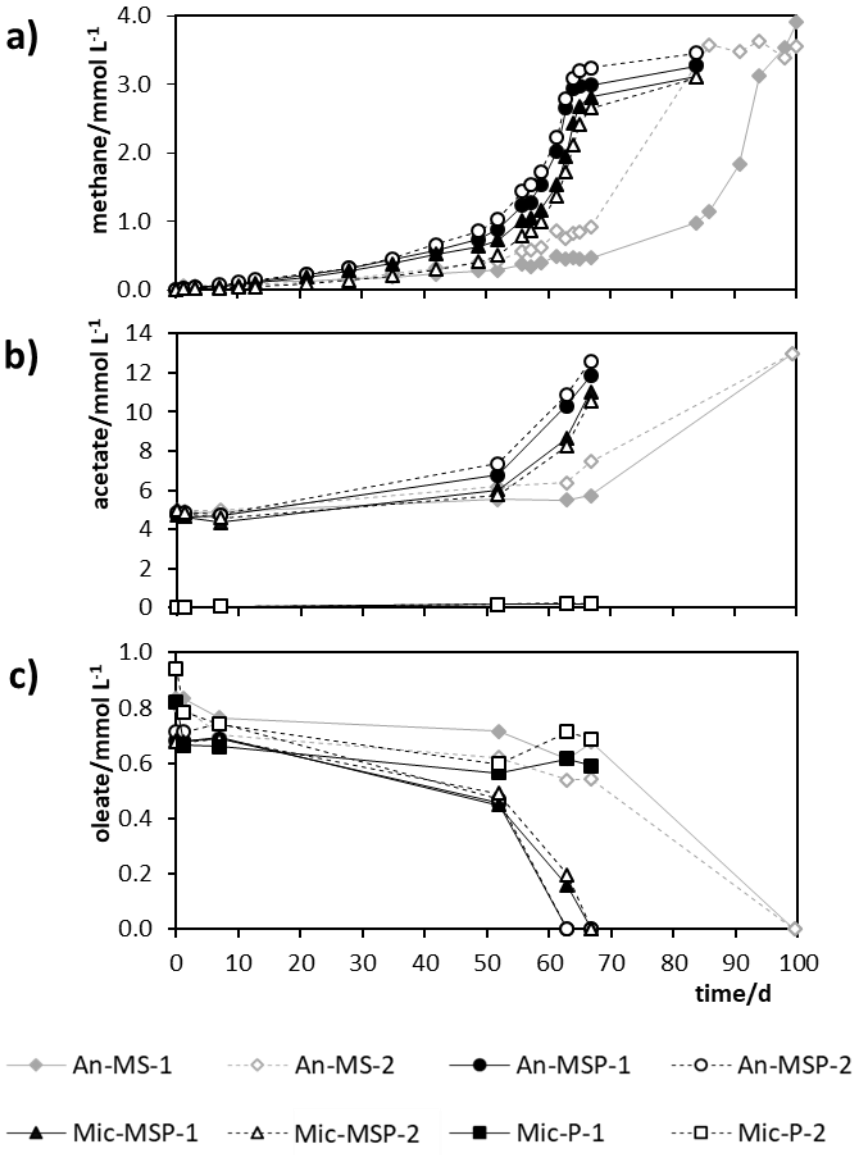


Figure 5.5 – Methane (a) and acetate (b) formation and oleate degradation (c) on the batch assays An-MS, An-MSP, Mic-MSP and Mic-P.

When the *Pseudomonas* isolates were incubated with oleate under microaerophilic conditions (Mic-P), circa 30 % of the initial oleate was fast degraded at the beginning of the incubation, but the remaining oleate was not further degraded (Figure 5.5c). The only product of oleate degradation in Mic-P was acetate, which was detected in residual concentrations (0.20 ± 0.00) mmol L^{-1} (Figure 5.5c).

The consortium composed by the *M. formicicum* and the *Pseudomonas* isolates (MP) did not present methane production under both microaerophilic and anaerobic conditions. Also, *M. formicicum* did not produced methane under microaerophilic conditions (Table 5.3).

Table 5.3 – Initial substrate concentration, final methane concentration and duration of the batch assays.

Assay	Substrate	$[Substrate]_{initial}$/ mmol L^{-1}	$[CH_4]_{final}$/ mmol L^{-1}	Duration/d
An-MSP	Oleate	0.70 ± 0.01	3.12 ± 0.13	67
Mic-MSP	Oleate	0.68 ± 0.00	2.73 ± 0.08	67
An-MS	Oleate	0.82 ± 0.01	3.72 ± 0.18	100
Mic-P	Oleate	0.88 ± 0.06	n. a.	67
An-MP	Oleate	0.74± 0.01	0.13± 0.05	52
Mic-MP	Oleate	0.75± 0.00	0.16± 0.03	52
An-M	Hydrogen	76	10.52 ± 0	32
Mic-M	Hydrogen	76	0.44± 0.26	52

n.a. – not applied

Additional tests showed that the co-culture composed by isolates I1 and I2 was able to use both hydrogen and formate as substrate under microaerophilic conditions (Table 5.4). The formate consumed was equivalent to the expected according to the initial oxygen concentration. Regarding hydrogen consumption, a depletion of 6 mmol L^{-1} could be detected which represents only 60 % of the theoretical expected (10 mmol L^{-1}) considering the amount of oxygen available (equations 5.4 and 5.5; Table 5.4).

In this work, the ability of *Pseudomonas* isolates I1 and I2 to degrade acetate was further tested in batch assays performed under aerobic conditions. Additionally, the profile of aerobic oleate degradation by the isolates was obtained by incubating *Pseudomonas* isolates I1 and I2 with 0.9 mmol L^{-1} of oleate.

Both isolates were able to degrade 16 mmol L^{-1} of acetate after approximately 24 h of incubation. Regarding oleate degradation, acetate was detected as the sole degradation product, after 3 days of incubation (Figure 5.6b). Isolate I1 converted approximately (0.5 ± 0.1) mmol L^{-1} of oleate to (1.1 ± 0.3) mmol L^{-1} of acetate. Similar results were obtained with isolate I2 which produced (1.0 ± 0.1) mmol L^{-1} of acetate from (0.5 ± 0.0) mmol L^{-1} of oleate. The results showed that not all oleate available

Table 5.4 – Initial oxygen and substrate (hydrogen and formate) concentrations (mmol per L of liquid media), final substrate concentrations and substrate expected to be consumed according with the initial oxygen concentration (eq. 5.4 and eq. 5.5).

Substrate	Time/d	$[Oxygen]_{initial}$/ % (v/v)	$[Substrate]_{initial}$/ mmol L^{-1}	$[Substrate]_{consumed}$/ mmol L^{-1}	$[Substrate]_{expected\ to\ be\ consumed}$/ mmol L^{-1}
Formate	15	4	18.93 ± 0.90	8.01 ± 1.24	8.62
Hydrogen	6	5	60.37	6.00 ± 0.03	10.14

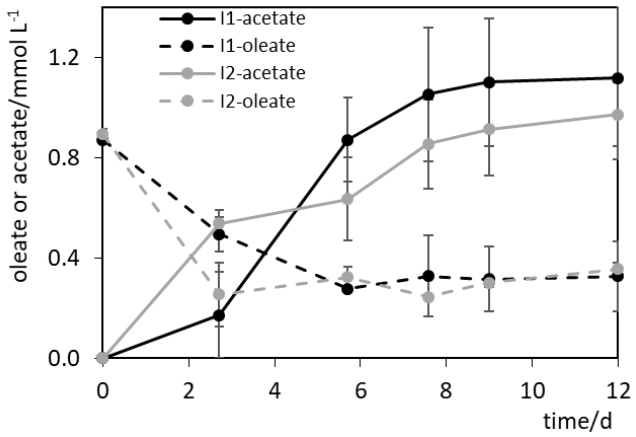


Figure 5.6 – Degradation of oleate by *Pseudomonas* isolates under aerobic conditions.

(0.9 mmol L^{-1}) was utilized by the *Pseudomonas* isolates, since (0.3 ± 0.1) mmol L^{-1} and (0.4 ± 0.0) mmol L^{-1} of oleate could still be quantified in at the end of the assays of I1 and I2, respectively (Figure 5.6b).

5.4. Discussion

In this work a continuous defined culture comprising a syntrophic co-culture of *S. zehnderi and M. formicium* and two *Pseudomonas* isolates was tentatively established. It was also demonstrated that oleate can be efficiently converted to acetate and methane in continuous bioreactors by the defined microbial consortium composed by *Syntrophomonas zehnderi*, *Methanobacterium formicicum* and the two *Pseudomonas* isolates. These microorganisms were maintained inside the reactor during the continuous operation, showing that all have a role in oleate degradation. The results suggest that in the continuous bioreactor, operated under microaerophilic conditions, *Pseudomonas* are probably utilizing the residual oxygen for oleate oxidation. This way *Pseudomonas* would protect the anaerobic syntrophic culture from oxygen toxicity and also contribute to decrease the oleate concentration and thus the potential toxicity.

The production of acetate from oleate in the continuous bioreactor may occur most likely by the activity of *S. zehnderi* but also by *Pseudomonas* isolates I1 and I2, once all these bacteria produce acetate from oleate when cultivated in pure culture under aerobic and microaerophilic conditions (Figures 5.5 and 5.6b). Nevertheless, the amount of acetate produced by the *Pseudomonas* isolates (in pure culture) is much less (approximately 1 mol of acetate per 0.5 mol of oleate) than the amount of acetate that accumulates during the syntrophic degradation of oleate by *S. zehnderi* (9 mol acetate per mol of oleate), and in the bioreactor (e.g., in the end of period V) circa 0.24 mmol d^{-1} of acetate

were produced from 0.15 mmol d^{-1} of oleate. These results highlight the importance of *S. zehnderi* as oleate degrading bacteria in the microbial consortia.

However, conversion of oleate to methane in continuous was not always constant and in some periods (IV, V), the methane production dropped and the oleate degradation slowed down (Figure 5.3a). The decrease in the methane production was accompanied by the detection of small amounts of formate in periods IV, V and VI (Figure 5.3a). Hydrogen was not quantified in the bioreactors, nonetheless, assuming that a hydrogen partial pressure of 0.1 to 1 Pa is equivalent to formate concentrations of around 1 to 10 μmol L^{-1} (Schink, 1994), then, 0.5 mmol L^{-1} of formate would correspond to a hydrogen partial pressure of 50 Pa. This value is 50 times higher than the 1 Pa of H_2 that allow the syntrophic degradation of LCFA (Sousa et al., 2009b, 2007b). These accumulation of electron carriers reflected the transient inhibition of *M. formicicum* by oleate, and by oxygen during the microaerophilic periods. In fact, formate accumulation has been associated before to the presence of inhibitors (Schink, 1997). Moreover, exposure to 0.5 mmol L^{-1} oleate was shown to cause damage in around 50 % of the cell membranes of *M. formicicum* cultures (Sousa et al., 2013), and oleate concentration in the bioreactor ranged from 0.4 to 0.7 mmol L^{-1}. Despite this transient inhibition, generally when the reactor was set in batch mode (periods VI and VIII) (Figure 5.3a), the methanogens were able to recover and formate was no longer detected. Nevertheless, it is important to note that oleate conversion to methane under continuous operation from period V on was always low (maximum of (14 ± 7) % of the methane expected from oleate conversion), but oleate degradation and acetate formation were quite stable (except in period VII). These results show that oleate degradation, by both *Pseudomonas* and *Syntrophomonas,* proceeded even when the methanogenic activity was low.

Some species of *Pseudomonas* were reported to grow on formate, hydrogen and CO_2. Autotrophic growth of *Pseudomonas oxalaticus* in the presence of formate has been reported (Blackmore and Quayle, 1968). Under microaerophilic conditions, the *Pseudomonas* isolates (I1 and I2) were able to degrade formate and hydrogen in batch assays according to eq. 5.2 and eq. 5.3 (Schink and Schlegel, 1978) (Table 5.4). This ability of *Pseudomonas* isolates may explain the decrease in formate concentration in the continuous reactor during period V (Figure 5.3a), that occurred when the *Pseudomonas* isolates were inoculated. In the bioreactor is possible that *Pseudomonas* were competing with *M. formicicum* for the electron carriers (H_2 and formate) produced by *S. zehnderi* from oleate oxidation. This agrees well with the fact that methanogenic activity decreases in the bioreactors even though oleate degradation proceeds (Figure 5.3a). Different syntrophic relationships

may occur simultaneously in this microbial consortium with *Pseudomonas* contributing as electron scavengers.

$$2\,HCOOH + O_2 \rightarrow 2\,CO_2 + 2\,H_2O \quad \text{eq. 5.2}$$

$$2\,H_2 + O_2 \rightarrow 2\,H_2O \quad \text{eq. 5.3}$$

Additional batch assays showed that oleate was degraded 1.5 times faster by a defined consortium of *S. zehnderi*, *M. formicicum* and the *Pseudomonas* isolates, than by the syntrophic co-culture alone (Figure 5.5c). Interestingly no significant differences were observed under microaerophilic and strict anaerobic conditions with the microbial consortia MSP (Figure 5.5). However, the exact role of *Pseudomonas* in this consortium is not unequivocally proved. Apart from degrading oleate, *Pseudomonas* spp. are also able to consume intermediates of oleate degradation (hydrogen, acetate and formate). *Pseudomonas* spp. degrade oleate via β-oxidation and comparing with *Escherichia coli,* this genus has a greater metabolic capacity for fatty acid degradation, since *Pseudomonas* can grow on short, medium and long chain fatty acids as sole carbon and energy sources (Zarzycki-Siek et al., 2013). In the presence of oxygen, the acetyl-coA formed by β-oxidation goes through citrate cycle producing CO_2.

The fact that the An-MSP and Mic-MSP behaved similarly is surprising and raises some questions that remain to be answered. The main question is which is the electron acceptor used by *Pseudomonas* in the absence of oxygen.

Under anaerobic conditions alternative electron acceptors may be used by *Pseudomonas* species, such as nitrate and nitrite (Lalucat et al., 2006). Sulfur and polysulfides has also been described as electron acceptor for *Pseudomonas* spp (Sun et al., 2018). Sulfur can be formed in the anaerobic medium because sodium sulfide, which is added to the medium as reducing agent, reacts with oxygen forming sulfur ($H_2S + \frac{1}{2}O_2 \rightarrow S^0 + H_2O$., eq. 5.4).

According to eq 5 from table 2.2 (Chapter 2), the complete β-oxidation of 1 mol of oleate results in 15 mol of H_2. Therefore 1 mmol of sulfur could be used to oxidize 0.06 mmol of oleate via hydrogen.

$$15\,H_2 + 15\,S^0 \rightarrow 15\,H_2S \quad \text{eq. 5.5}$$

Therefore, the ability of these isolates to use this alternative electron acceptor is worth to be investigated.

To sum up, facultative anaerobic bacteria, particularly *Pseudomonas* spp., play an important role in the anaerobic reactors treating oleate-based wastewaters, probably by protecting the strict anaerobic community from oxygen toxicity, eventually contributing to the decrease of oleate toxicity towards the anaerobic community and may represent an alternative hydrogen/formate scavenger for syntrophic LCFA degrading bacteria.

Chapter 6.

Screening for Biosurfactants and Biopolymers Formed During Oleate Biodegradation

In reactors treating oleate-based effluents (RI-1 and RI-2, Chapter 4) was possible to detect the presence of vesicles shapes by microscopic observations. These vesicles suggested the presence of some kind of surface-active compounds like biosurfactants. *Pseudomonas* spp. are known as biosurfactant producers. To better study these compounds, samples were collected from two bioreactors inoculated with the *Pseudomonas* isolates I1 (RI-1) and I2 (RI-2), and from a third bioreactor inoculated with granular sludge (R-GS) and subjected to biosurfactants screening and polyhydroxyalkanoates (PHA) analysis.

Palmitate was formed from oleate in R-GS but not in RI-1 and RI-2. Nevertheless, similar results were obtained regarding biosurfactants analysis, i.e., glycolipids were detected in the three bioreactors but lipopeptides were not produced. On the other hand, an unidentified compound, presumably a PHA, could be detected in R-GS samples but not in the RI-1 and RI-2.

6.1. INTRODUCTION

Several biofilm-forming bacteria, such as *Pseudomonas* spp. are able to produce biosurfactants that allow biofilm formation, migration within the biofilm and to form channels in the biofilm matrix in order to access nutrients (Masák et al., 2014; Raaijmakers et al., 2010). Moreover, they can also produce biosurfactants to access hydrophobic substrates (for example oils). Biosurfactants increase the oil/water surface area, accelerating the microbial degradation of various oils, improving the bioremediation of water and soil (Rosenberg and Ron, 1999). Biosurfactants are classified as glycolipids, lipopeptides, lipopolysaccharides, polysaccharide-protein complexes, fatty acids and lipids (Rosenberg and Ron, 1999). *Pseudomonas* are able to produce several kinds of surfactants, namely lipopeptides and glycolipids (Smyth et al., 2010b). The lipopeptides have a hydrophilic part containing polar amino acids, and a hydrophobic tail containing fatty acids and non-polar amino acids (Janek et al., 2010). Glycolipids contains a carbohydrate joined to fatty acids or hydroxy-fatty acids chains (Smyth et al., 2010a).

In a previous experiment (Chapter 4), two *Pseudomonas* strains were isolated from a biofilm formed in a reactor processing an oleate-based effluent. The *Pseudomonas* isolates were able to degrade oleate in continuous reactors. In those reactors and also in another inoculated with granular sludge, vesicles-like shapes were found during microscopic observations. These vesicles suggested that during oleate degradation a surface-active compound is formed. Besides the vesicles, inclusion bodies were also detected during microscopic observations of the *Pseudomonas* isolate I1 when incubated with oleate. The three most dominant types of lipid bodies inclusions in bacteria are polyhydroxyalkanoates (PHAs), triacylglycerols and wax esters (Thomson and Sivaniah, 2010). *Pseudomonas putida*, *Pseudomonas oleovorans*, *Pseudomonas aeruginosa* and many other microorganisms, can produce a wide range of PHAs when grown in the presence of various carbon sources (Huisman, 1989; Schmack and Mu, 1998). PHA is a polyester of hydroxyalkanoates that accumulates as carbon/energy storage material in microbial cells, which are synthesized and accumulated as intracellular granules when there is excess of carbon source and limitation of an essential growth-limiting component i.e. nitrogen, phosphate or oxygen (Salehizadeh and Loosdrecht, 2004).

The present chapter was designed to evaluate the potential of PHA accumulation by the *Pseudomonas* isolates as well as its capability to produce surface-active compounds.

6.2. MATERIALS AND METHODS

6.2.1. Culture conditions

Two *Pseudomonas* strains were isolated from a biofilm converting oleate to palmitate (Chapter 4). These isolates were inoculated separately in continuous reactors (Isolate 1 – RI1, Isolate 2 – RI2) fed with sodium oleate with an initial organic loading rate (OLR) of 1 g L^{-1} d^{-1} in COD for 33 days (Period I) according to section 4.2.2 (Chapter 4), and then the OLR was increased to 4 g L^{-1} d^{-1} in COD (Period II). The reactors content was examined by phase-contrast microscopy (Olympus-CX41, Olympus Corporation, Tokyo, Japan). In period II, samples from both reactors were collected to microscopic observations and for biosurfactants screening.

Besides the reactors inoculated with isolates, another bioreactor inoculated with smashed anaerobic granular sludge (Reactor Granular Sludge, R-GS) that was converting oleate to palmitate was also studied. The OLR applied to this reactor was 4 g L^{-1} d^{-1} in COD, and a sample was collected at day 41 of operation for microscopic observations and for biosurfactants and biopolymers screening.

6.2.2. Biosurfactants extraction

Samples RI-1(day 39), RI-2 (day 39) and RSG (day 41) were submitted to three different extraction procedures specific for lipopeptides, neutral lipids and glycolipids extraction. Lipopeptides were extracted three times with an equal volume of ethyl acetate and separated in separating funnels (Smyth et al., 2010b).

Neutral lipids were extracted with chloroform/methanol (2:1, v/v) in a proportion of 20 mg of lyophilized sample to 4 mL of chloroform/methanol mixture (Folch et al., 1957). In the end sodium thiosulfate was used to remove the traces of water and the extracts were dried in a TurboVap LV system (Biotage, Uppsala, Sweden).

For the extraction of glycolipids fraction, first the cells were removed by centrifugation and the supernatant was acidified to pH 3 and the glycolipid three times with an equal volume of ethyl acetate and separated in separating funnels (Smyth et al., 2010a).

6.2.3. Biosurfactants composition

The crude extracts were dissolved in 0.5 mL of chloroform and spotted on a TLC silica gel plates (Silica gel 60, Merck, Darmstadt, Germany). The lipopeptides were separated using a solvent system of chloroform/methanol/water (65:25:4, v/v/v) (Smyth et al., 2010b). For the detection of peptides, the plates were sprayed with a solution of 0.25 % (w/v) of ninhydrin in acetone and revealed at 105

°C for 5 min (Janek et al., 2010). For the separation of glycolipids was used a solvent system composed by chloroform/methanol/acetic acid (6.5:1.5:0.2, v/v/v) (Smyth et al., 2010a). The detection of glycolipids was performed by spraying the plates with anthrone reagent (0.125 g of anthrone dissolved in 63 mL of sulfuric acid and 25 mL of water) (Smyth et al., 2010a). Additionally, another plate was sprayed with orcinol-sulfuric agent for glycolipids detection. The different neutral lipids classes were separated using the solvent system of hexane/diethyl ether/acetic acid (80:20:1, v/v/v) and detected using iodine vapor as staining reagent (Alvarez et al., 1996). Rhamnolipids (90 % purity, AGAE Technologies, OR, USA) were used as glycolipids standard and surfactin produced by *Bacillus subtilis* (Pereira et al., 2013) as lipopeptides standard. Olive oil, oleic acid and palmitic acid were used as neutral lipids standards, for triacylglycerols and long chain fatty acids, respectively.

6.2.4. Glycolipids purification

The glycolipids extracted from the sample of reactor R-GS were further purified in a SPE silica column (SiOH) Chromabond 1g/6 cc, (Macherey-Nagel, Düren, Germany). The compounds were separated with increasing polarity, by use of the following solvent systems: chloroform/methanol (95:5, v/v) for fatty acids; chloroform/methanol/water (85:15:2, v/v/v) for glycolipids (Peng et al., 2007). The eluate was collected separately, dried in TurboVap LV and dissolved in chloroform.

6.2.5. Analysis of fatty acids composition

After glycolipids purification (section 5.2.4, sample from reactor R-GS day 41), these compounds were characterized regarding its content in LCFA. The fatty acids chain from the carbohydrate moiety of the purified glycolipid was hydrolyze under alkaline conditions performed with 1 mol L^{-1} NaOH in ethanol at 90 °C for 1 h. The reaction solution was then adjusted to pH 7.0 with 1 mol L^{-1} HCl and then extracted with ether three times (Peng et al., 2007). The solvent and the aqueous phase, each of which contained fatty acids and a carbohydrate, were collected separately. The solvent was evaporated, and the fatty acids suspended in chloroform.

Fatty acid composition was performed by analyzing the free fatty acids and the total fatty acids (free and non-free). Saturated and unsaturated free LCFA from C12 to C18 were extracted and quantified according to the method described by Neves et al. (2009) described in section 3.2.3 (Chapter 3). For the total LCFA (free and non-free) quantification, samples were methylated using a mixture of methanol/sulfuric acid (85:15, v/v) during 3.5 h at 100 °C (Brandl et al., 1988). Further extraction was performed with chloroform. The methyl esters were analyzed in a GC-FID (Varian 3800) under the same conditions as previously described for free fatty acids (section 3.2.3, Chapter 3).

6.2.6. Nuclear magnetic resonance spectroscopy (NMR)

The content of the lyophilized samples – RI-1 (day 46), RI-2 (day 46) and R-GS (day 41) – was extracted with chloroform/methanol (2:1, v/v) in a proportion of 20 mg of lyophilized sample to 4 mL of chloroform/methanol solution (Folch et al., 1957). After evaporation, the compounds were re-dissolved in deuterated chloroform ($CDCl_3$) (Acros, New Jersey, USA) and the respective ^{1}H-NMR spectra were recorded using Bruker Avance II 400 (400 MHz). The peak solvent was used as internal reference.

6.2.7. Quantification of polyhydroxyalkanoates

Samples collected from reactors RI-1 (day 8), RI-2 (day 8) and R-GS (day 41) were initially lyophilized. The analysis method for PHA quantification was adapted from Mesquita et al., 2015. Briefly, the polymers present in the samples were esterified with 1.5 mL of HCl:1-Propanol (1:4, v/v) and extracted with 1.5 mL of dichloromethane (including the internal standard – 1g L^{-1} of pentadecanoic acid dissolved in dichloromethane). Then the mixture was digested at 100 °C for 6 h, and after cooldown the content of the vial was transferred with 2 mL of ultra-pure water to another vial and closed with a rubber seal. The contact between the different phases was promoted and the vials were inverted to let the phases to separate. After 30 min, 1 mL of the organic phase was collected and injected in a GC with FID. The hydroxyalkanoates were separated using a Teknokroma TRB-WAX column (30 m x 0.25 mm x 0.25 μm) with helium as the carrier gas, at 1 mL min^{-1}. Temperatures of the injector and detector were 220 °C and 250 °C, respectively. The initial oven temperature was 50 °C, maintained for 2 min, followed by a 10 °C min^{-1} ramp up to 225 °C, and finally isothermal conditions were maintained for 25 min.

6.3. Results

Figure 6.1 shows the vesicular shapes observed in the microaerophilic reactors degrading oleate by pure cultures of *Pseudomonas* spp. (reactors R-I1 and R-I2) and granular sludge (reactor R-GS).
The vesicles formed raised the question of whether some kind of surface-active compound was being produced in the reactors RI-1, RI-2 and R-GS. Analysis of the samples collected from reactors RI-1 and RI-2 showed the absence of lipopeptides (Figure 6.2a). Nonetheless when tested for the presence of glycolipids, some spots were detected in the TLC (Figure 6.2 b). These spots do not match with the rhamnolipid standard run in lane 2. Long chain fatty acids were the predominant class of neutral lipids detected in all the samples from R-I1 and R-I2 (Figure 6.2c).

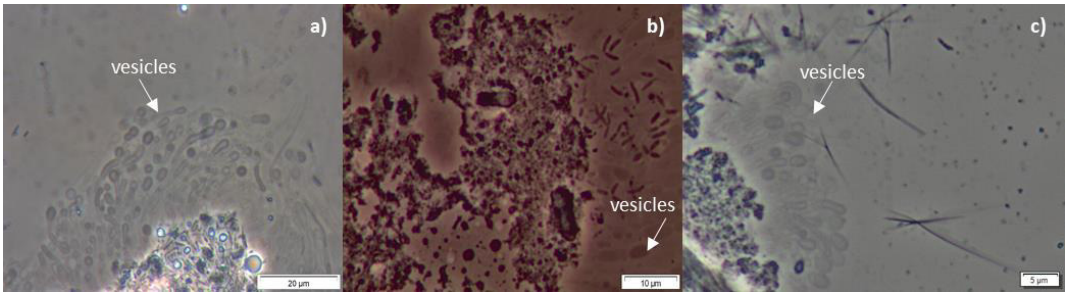


Figure 6.1 – Micrographs from reactors RI-1 (a) and RI-2 (b) at day 123, and reactor R-GS (c) at day 41.

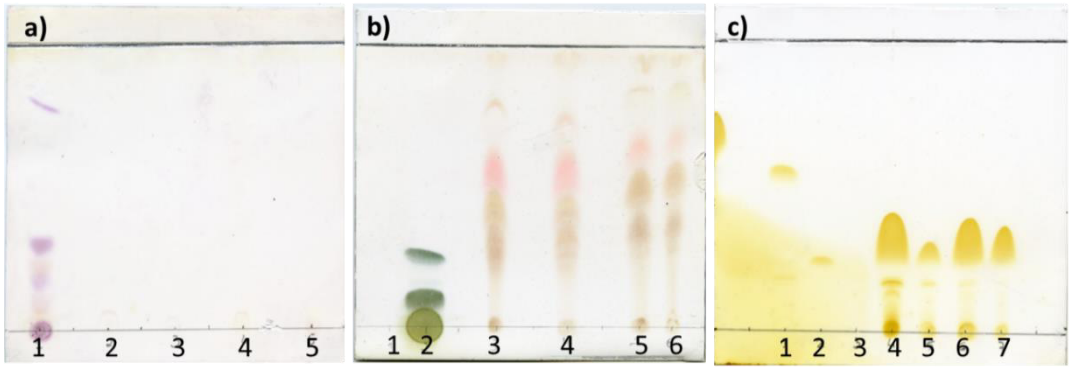


Figure 6.2 – TLC analysis of lipopeptides (a), glycolipids (b) and neutral lipid compounds (c). a) 1 – surfactin; 2 – R-I1 floating biofilm; 3 – R-I2 floating biofilm; 4 – R-I1 reactor sample; 5 – R-I2 reactor sample. b) 1 – oleic acid; 2 – rhamnolipid; 3 – R-I1 floating biofilm; 4 – R-I2 floating biofilm; 5 – R-I1 reactor sample; 6 – R-I2 reactor sample. c) 1 – olive oil; 2 – oleic acid; 3 – palmitic acid; 4 – R-I1 floating biofilm; 5 – R-I2 floating biofilm; 6 – R-I1 reactor sample; 7 – R-I2 reactor sample.

Since the color developed with anthrone reagent in Figure 5.2b was not the expected (green) according to the literature (Smyth et al., 2010a), orcinol was then used as an alternative dye (Figure 6.3). Using this reagent, the spots obtained were in accordance with the blue-green color expected (Smyth et al., 2010a).

The sample from reactor R-GS was also extracted and developed in TLC for detection of lipopeptides and glycolipids (Figure 6.4). The results were very similar to the ones obtained for reactors RI1 and

Figure 6.3 – TLC analysis of glycolipids using orcinol as detection agent. 1 – oleic acid; 2 – rhamnolipid; 3 – R-I1 floating biofilm; 4 – R-I2 floating biofilm; 5 – R-I1 reactor sample; 6 – R-I2 reactor sample.

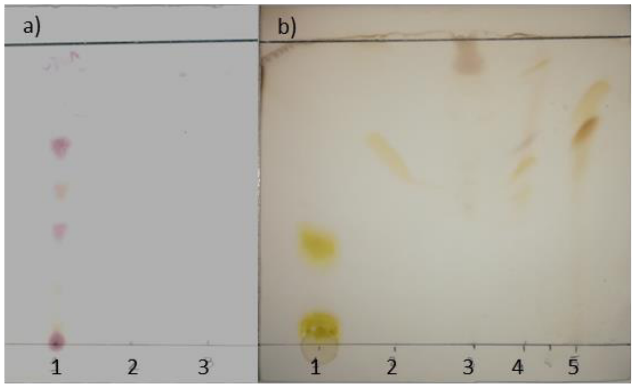


Figure 6.4 – a) 1 – TLC analysis of lipopeptides (a), glycolipids dyed with anthrone (b). a) 1 – surfactin; 2 – oleic acid; 3 – sample R-GS. b) 1 – rhamnolipid; 2 – oleic acid; 3 – sample R-GS (purified for glycolipids); 4 – sample R-GS (extraction glycolipids); 5 – feed of the reactors containing oleate.

RI2. No lipopeptides were detected (Figure 6.4a, lane 3) and some compound related to glycolipids was detected in the sample (Figure 6.4b, lane 4). Moreover, when the amount of oleic acid loaded was increased, a tenuous spot appeared (Figure 6.4, lane 2) as well as the spots present in the feeding sample (Figure 6.4, lane 5). When comparing the oleic standard and the feed profiles with the sample (Figure 6.4, lane 4), it seems that the R-GS samples have more bands.

The sample collected from reactor R-GS was submitted to a deeper characterization after glycolipids extraction. The extract was purified and 70 % (w/w) was recovered in the free fatty acids faction and 30 % (w/w) in the glycolipids faction. Then, the glycolipids faction was submitted to an alkaline hydrolyses and the fatty acids composition was determined (Table 6.1). Despite of palmitate being the predominant LCFA in the raw sample, in the purified glycolipids fraction stearate was the most abundant. Nevertheless, the concentrations were very low and below the limit of the calibration curve. The method used for total fatty acid quantification (section 6.2.5) was tested using the model compounds surfactin (lipopeptide) and rhamnolipid (glycolipid). No peaks were detected in the surfactin chromatograms, however the rhamnolipids analysis presented a large peak right before the pentadecanoic acid standard which may be the hydroxydecanoic acid that was described as the major component of the rhamnolipids standard R90 (AGAE technologies, OR, USA)

Table 6.1 – LCFA composition of the raw sample of reactor R-GS and the equivalent sample in which glycolipids were extracted and hydrolyzed.

	LCFA/ mg L^{-1}		
	Oleate (C18:1)	**Stearate (C18:0)**	**Palmitate (C16:0)**
Raw sample (free LCFA)	718	139	1672
Purified glycolipids (total LCFA)	0	11	7

(Aleksic et al., 2017). The R-I1, R-I2 and R-GS samples analyzed using this method showed similar results to the obtained with the free LCFA quantification method, suggesting that total (free plus non-free) LCFA corresponded to the free LCFA analyzed.

The characterization of biosurfactants, namely glycolipids, using NMR spectroscopy has been described (Smyth et al., 2010a). To obtain more information regarding the samples compositions, a ^{1}H-NMR spectroscopy analysis was carried on. The spectra obtained from RI-1 (Figure 6.5a) and

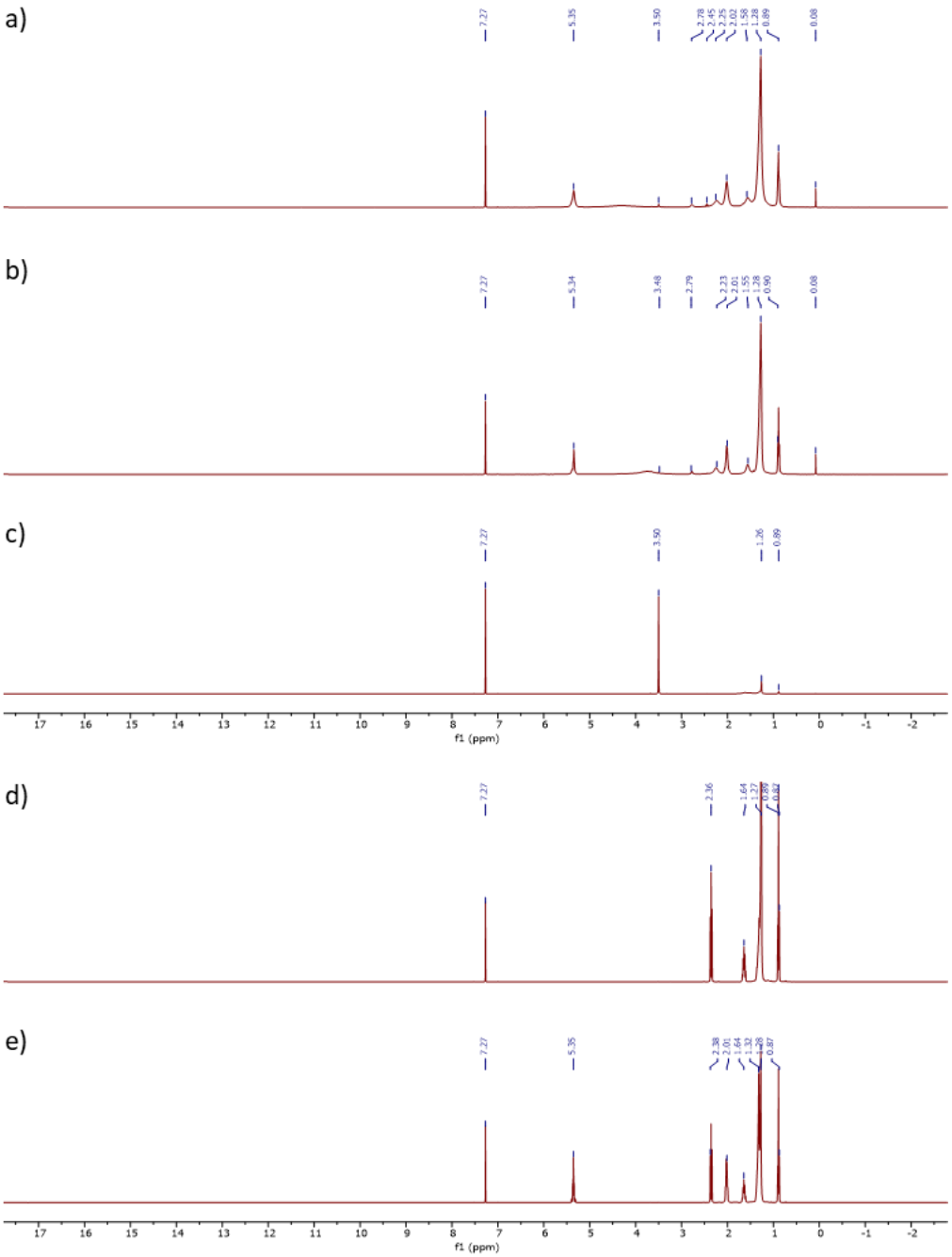


Figure 6.5 – ^{1}H-NMR spectra for samples of R-I1 (a), R-I2 (b) and R-GS (c). (d) and (e) are respectively the spectra from the standards palmitic and oleic acids.

RI-2 (Figure 6.5b) samples were very similar between them and also when compared with the standards of palmitic (Figure 6.5d) and oleic (Figure 6.5e) acids. Regarding the sample collected from reactor R-GS (Figure 6.5c), there is a shift around 3.50 ppm which is not present in the other samples neither in the standards. This suggests that some compound different from oleic and palmitic acids was being produced in reactor RSG.

Microscopic observations of samples collected from reactor RI-1 suggest the presence of inclusion bodies in *Pseudomonas* isolate I1 (Figure 6.6). Therefore, samples collected from reactors RI-1, RI-2 and R-GS were submitted to PHA analysis. Analysis of the samples collected from day 3, 4 and 41 from reactor R-GS showed the same LCFA profile as previously detected (total e free LCFA quantification method) but also an unidentified peak at the retention time of 35.6 minutes, as it can be seen in Figure 6.7. Samples from RI-1 and RI-2 were also analyzed and showed that palmitate and oleate were the major compounds, as previously identified (Chapter 4, section 4.3.2, Figure 4.7), but no peak was observed at a retention time of 35.6 minutes.

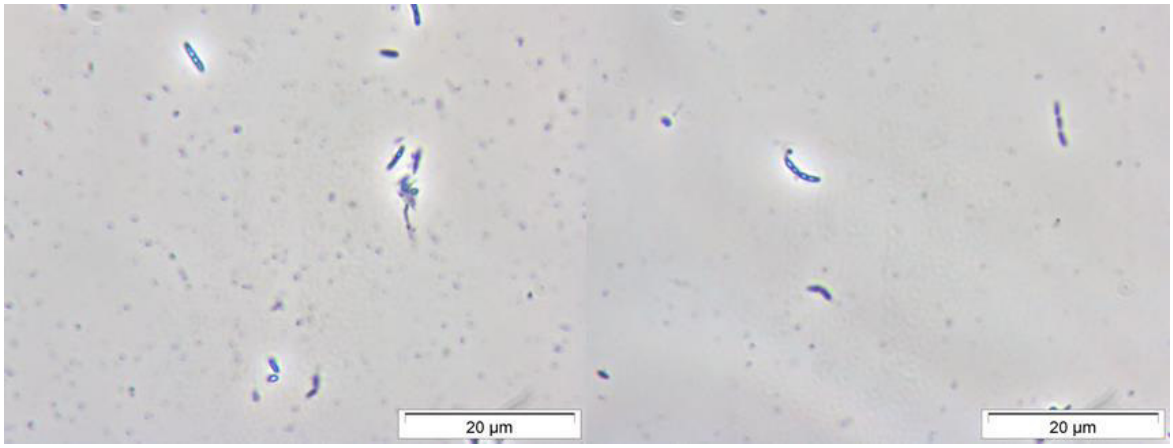


Figure 6.6 - Micrographs from reactors R-I1 at day 8 of operation.

6.4. Discussion

Vesicle-like shapes detected by microscopic observations in reactors where oleate degradation occurred presented fluidic movement and did not seem miscible with the medium, suggesting the presence of a surface-active compound.

TLC screening for the presence of biosurfactants in samples collected from reactors operated with the *Pseudomonas* isolates (RI-1 and RI-2) showed absence of lipopeptides and the possible presence

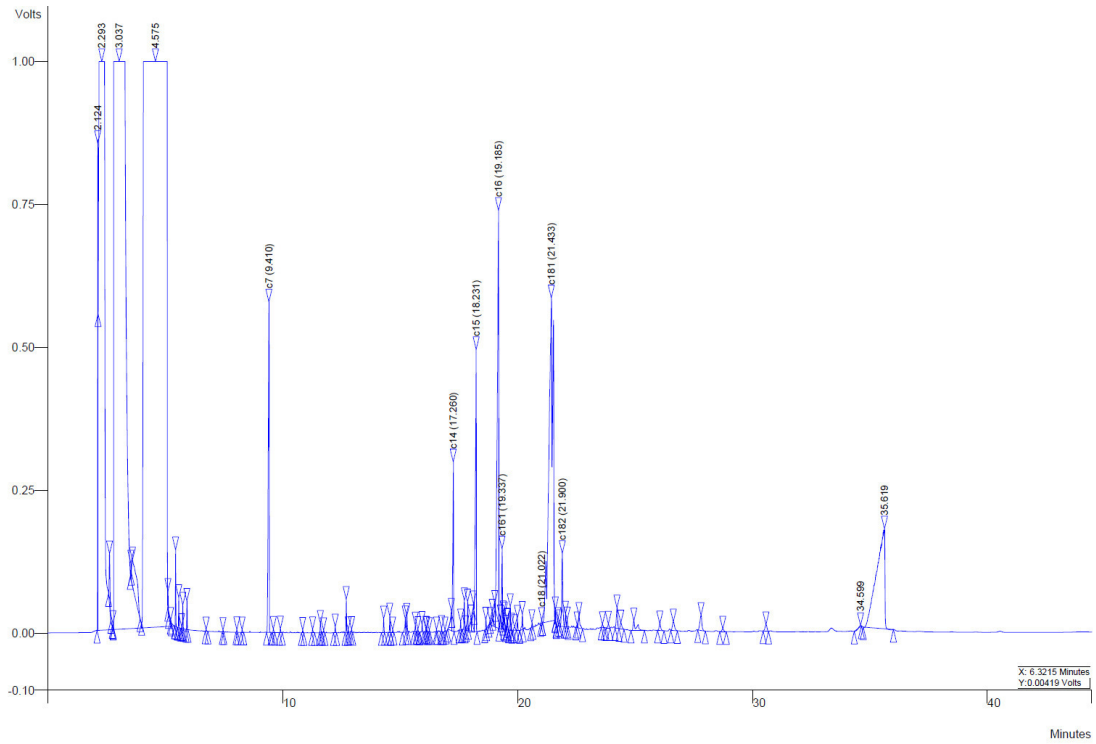


Figure 6.7 – Chromatogram of the analysis of hydroxyalkanoates fatty acid content present in the sample collected from reactor R-GS at 4th day of operation.

of glycolipid type compounds (Figure 6.2b and Figure 6.3). The sample from the reactor operated with anaerobic sludge (R-GS) presented a similar result... However, TLC analysis of the media containing oleate (Figure 6.4b, lane 5) and a standard of oleic acid (Figure 6.4b, lane 2) revealed some spots, suggesting that anthrone, the specific dye for carbohydrates, may also dye lipidic compounds, and therefore raises questions regarding the reliability of the TLC results that suggested the presence of glycolipids in the reactors. Nevertheless, the extract (with potential glycolipids) from the reactor R-GS was further analyzed, suggesting the presence of a glycolipid compound that has a stearate chain and/or palmitate in its composition. Nonetheless the extraction and purification methods were further tested using Rhamnolipids 90 % purity (AGAE Technologies, Corvallis, OR, USA) as model compound and the methods were not validated for the applied conditions since a great fraction of free fatty acids (70 %, w/w) was recovered from the standard solution containing 90 % of rhamnolipids. Solvent extraction and purification procedures are very specific methods that vary with the volume of solvents applied, type of solvent, time of extraction, among other variables. Therefore, the methods described in the literature, and used in this work, should be further optimized and validated with a standard with a higher level of purity.

In 1996, Burd and Ward have described an extracellular surface-active agent called PM-factor that formed ball-shaped particles when examined by electron microscopy, and was identified as a

lipopolysaccharide containing fatty acids, predominating palmitic, palmitoleic and oleic acids. There is a chance that some compound similar to PM-factor has been produced in the reactors studied. Further studies need to be performed to identify this compound.

Other hypothesis is based on the observation of inclusion bodies in the bacteria, suggesting the presence of PHA. Analyses to these compounds (Figure 6.6), resulted in the detection of a hydroxyalkanoate or LCFA longer that linoleate (C18:2) in samples collected from reactor R-GS. NMR results (Figure 6.5) also support the hypothesis that some compound like a biopolymer or a biosurfactant may be present in the reactor RSG. However, based on the spectra libraries, it was not possible to identify the chemical shift around 3.50 ppm observed in the obtained ^{1}H-NMR spectra.

In sum, the results here presented support the hypothesis that some biosurfactant and/or biopolymer is being produced when oleate is feed to methanogenic bioreactors and eventually might be further hydrolyzed/transformed into palmitic acid. Based on these results, further and deeper characterization should be carried on to clarify which compounds are being produced.

Chapter 7.

General Conclusions and Suggestions for Future Work

7.1. General conclusion

In anaerobic bioreactors, LCFA are converted to methane due to several microbial steps performed by a complex community. Under higher organic loads, in those reactors, oleate is mainly converted to palmitate and methanogens become transiently inhibited. Then, under batch mode the community present in the bioreactor is able to recover and to convert the accumulated palmitate into methane. The microbial interactions occurring during these main steps are still not well understood. The work presented in this thesis contributed to a further understanding of the microbial interactions occurring during the transformation of long-chain fatty acids in anaerobic reactors. The main conclusions reached were:

i. Open to air feeding promoted the transformation of oleate to palmitate, avoiding a long term methanogenic inhibition. The fast conversion of oleate to palmitate is here reported as an advantageous step, since palmitate is less toxic to methanogens than oleate. Having a strict anaerobic feed or a feeding tank open to air, resulted in a different ORP, different microbial community profiles and a different behavior relative to oleate conversion. From a practical point of view, ORP is proposed as a potentially relevant control variable, whose fine-tuning can regulate oleate to palmitate conversion, decreasing the LCFA toxicity towards methanogens, ultimately accelerating the methane production from lipid-rich wastewaters.

ii. The abundance of facultative anaerobic bacteria, namely *Pseudomonas* spp. (from which two strains were isolated), was strongly correlated ($p<0.05$) with palmitate-to-total LCFA in continuous reactors fed with very high loads of oleate. The work presented in Chapter 4 strongly suggests that facultative bacteria have a critical role in LCFA conversion in continuous methanogenic bioreactors.

iii. A constructed microbial community composed by facultative anaerobic bacteria (*Pseudomonas*) and a syntrophic LCFA degrader co-culture (*M. formicicum and S. zehnderi*) was able to grow and to degrade oleate in a continuous bioreactor and to faster convert oleate to methane in batch tests when compared to the syntrophic co-culture alone. These results suggest that facultative anaerobic bacteria, particularly *Pseudomonas* spp., play an important role in the anaerobic reactors treating oleate-based wastewaters, probably by protecting the strict anaerobic community from oxygen toxicity, eventually contributing for

decreasing oleate toxicity towards the anaerobic community and may represent an alternative hydrogen/formate scavenger for syntrophic bacteria LCFA degrading.

iv. A biopolymer and/or a biosurfactant seems to be produced in continuous bioreactors treating oleate. Whether these compounds may be intermediaries formed during the oleate transformation to methane in continuous bioreactors should be further addressed.

7.2. SUGGESTIONS FOR FUTURE WORK

This thesis presents some examples demonstrating that microaerophilic and aerobic conditions are critical for accelerating the methane production rates from LCFA. I suggest 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. Therefore, I expect that this work will encourage further systematic studies on the influence of ORP in the anaerobic digestion process, opening new perspectives on metabolic interactions of complex microbial networks.

Further studies are still needed to elucidate the mechanisms by which palmitate accumulates in continuous bioreactors fed with oleate and the role that facultative anaerobes have in the process. Two main hypotheses are proposed:

I. In oxygen limited zones occurring in biofilms, aggregates or when oxygen supply is controlled at very low levels, palmitate accumulates due to a direct partial conversion of oleate or by hydrolysis of an intermediate formed during oleate degradation by facultative anaerobes, followed by a subsequent rate-limiting step of palmitate conversion to methane, performed by slower growing strict anaerobes. This hypothesis is supported by the fact that the accumulated palmitate is efficiently converted to methane when the oleate feeding is stopped and so the oxygen supply, increasing the available time for that reaction and the anaerobic conditions needed to the strict anaerobic microorganisms. Why, in that case, oleate is a preferred substrate for facultative bacteria than the formed palmitate should be further investigated.

II. Palmitate is originated from an extracellular compound produced as a strategy to access oleate, and not a direct intermediary of oleate degradation. There are some surfactants

produced by *Pseudomonas* spp. that contain palmitate in its composition. For example Pseudofactin II produced by *Pseudomonas fluorescens* is a cyclic lipopeptide with a palmitic acid connected to the terminal amino group (Janek et al., 2012). The involvement of other microorganisms with similar strategies to facilitate the access to hydrophobic compounds is also possible. Additionally, the production of intracellular compounds, such as PHA, may also be occurring. A deeper characterization of these compounds should be addressed. For that, further optimization of the analytical methods should be performed.

III. *Pseudomonas* isolates may possess some alternative metabolic routes that are not described yet and somehow cooperating with the syntrophic co-culture during oleate conversion to methane. These microbial interactions should be further studied using novel high-throughput molecular methods, such as metaproteomics, for a deeper understanding about who is doing what.

These main hypotheses should be further studied to allow a better understanding of the role of facultative anaerobic bacteria in lipids/LCFA conversion to methane. In this way novel strategies can be further employed in anaerobic bioreactors to turn the available technologies to treat lipids more efficient. A systematic study of the ORP may allow a maximization of the oleate conversion to methane. Since other valuable compounds such biosurfactants may being produced, the AD of lipids/LCFA could be seen as a promising technology to generate also other value-added products besides methane. A metaproteomics analysis or the use of labeled substrates may help to understand better the dynamics of who is doing what in the consortium composed by *Methanobacterium formicicum*, *Syntrophomonas zehnderi* and *Pseudomonas.*

SCIENTIFIC OUTPUTS

The overall work presented in this PhD thesis gave origin to the following publications:

PAPERS IN JOURNALS WITH PEER REVIEW:

M. S. Duarte, S. A. Silva, A. F. Salvador, A. J. Cavaleiro, A. J. M. Stams, M. M. Alves and M. A. Pereira (2018) Insight into the Role of Facultative Bacteria Stimulated by Microaeration in Continuous Bioreactors Converting LCFA to Methane. *Environmental Science & Technology*, 52, 6497−6507.

PAPERS IN PREPARATION FOR SUBMISSION TO PEER REVIEWED JOURNALS:

B. C. Holohan, **M. S. Duarte**, M. A. Szabo Corbacho, A. J. Cavaleiro, A. F. Salvador, C. Frijters, S. Pacheco-Ruiz, M. Carballa, M. A. Pereira, D. Z. Sousa, A. J. M. Stams, V. O'Flaherty, J. B. van Lier and M. M. Alves. Anaerobic digestion of lipids: challenges and opportunities (in preparation for submission in *Environmental Science & Technology*)

M. S. Duarte, A. F. Salvador, A. J. Cavaleiro, A. J. M. Stams, M. A. Pereira and M. M. Alves. Syntrophic and facultative anaerobic bacteria in oleate conversion to methane: cooperation or competition?

ABSTRACTS AND POSTERS IN CONFERENCES:

M. S. Duarte, S. A. Silva, A. J. Cavaleiro, M. A. Pereira, A. J. M. Stams and M. M. Alves. Comparison of oleate conversion under microaerophilic and anaerobic conditions. 2015 MIT Portugal Annual Conference -Light: Designed by Nature, Transformed by Science, Lisbon, Portugal, June 19, 32, 2015.

M. S. Duarte, S. A. Silva, A. J. Cavaleiro, M. A. Pereira, A. J. M. Stams and M. M. Alves. Comparison of oleate conversion under microaerophilic and anaerobic conditions. AD14 -World Congress on Anaerobic Digestion, Viña del Mar, Chile, November 15-18, 2015.

A.J. Cavaleiro, **M.S. Duarte**, S.A. Silva, A.F. Salvador, A.P. Guedes, J.A. Ribeiro, D.Z. Sousa, M.A. Pereira, A.J.M. Stams, M.M. Alves. Energy production from lipids by novel anaerobes. Microbiotec2015, Évora, Portugal, December 10-12, 2015

M. S. Duarte, S. A. Silva, A. J. Cavaleiro, D. Z. Sousa, M. A. Pereira, A. J. M. Stams and M. M. Alves. What is the role of Pseudomonas sp. in the anaerobic digestion of long-chain fatty acids? MIT Portugal Annual Conference: 10 years engineering a better future, Braga, Portugal, June 30, 2016.

M. S. Duarte, S. A. Silva, A. F. Salvador, D.Z. Sousa, A.J. Cavaleiro, M.A. Pereira, A. J. M. Stams, M.M. Alves. 2016. Breakthroughs in Anaerobic Digestion of fat, oil, and grease (FOG). 2016 International Workshop on Environment and Alternative Energy, NASA's Jet Propulsion Laboratory (JPL) - Pasadena, CA October 18 - 21, 2016.

M. S. Duarte, B. C. Holohan, M. A. Szabo Corbacho, A. J. Cavaleiro, A. F. Salvador, C. Frijters,S. Pacheco-Ruiz, M. Carballa, M. A. Pereira, D. Z. Sousa, A. J. M. Stams, V. O'Flaherty, J. B. van Lier and M. M. Alves. Perspectives in anaerobic digestion of lipid-rich wastewater. AD15 -World Congress on Anaerobic Digestion, Beijing, China, October 17-20, 2017.

REFERENCES

Abraham, W.R., 2010. Biosynthetic Oils, Fats, Terpenes, Sterols, Waxes: Analytical Methods, Diversity, Characteristics, in: Timmis, K.N. (Ed.), Handbook of Hydrocarbon and Lipid Microbiology. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 80–93.

Aleksic, I., Petkovic, M., Jovanovic, M., Milivojevic, D., Vasiljevic, B., Nikodinovic-Runic, J., Senerovic, L., 2017. Anti-biofilm properties of bacterial di-rhamnolipids and their semi-synthetic amide derivatives. Front. Microbiol. 8, 1–16.

Alvarez, H.M., Mayer, F., Fabritius, D., Steinbüchel, A., 1996. Formation of intracytoplasmic lipid inclusions by *Rhodococcus opacus* strain PD630. Arch. Microbiol. 165, 377–386.

Alves, M.M., Mota Vieira, J.A., Álvares Pereira, R.M., Pereira, M.A., Mota, M., 2001a. Effects of lipids and oleic acid on biomass development in anaerobic fixed-bed reactors. Part II: Oleic acid toxicity and biodegradability. Water Res. 35, 264–270.

Alves, M.M., Pereira, M.A., Sousa, D.Z., Cavaleiro, A.J., Picavet, M., Smidt, H., Stams, A.J.M., 2009. Waste lipids to energy: how to optimize methane production from long-chain fatty acids (LCFA). Microb. Biotechnol. 2, 538–550.

Alves, M.M., Vieira, J.A., Pereira, R.M., Pereira, M.A., Mota, M., 2001b. Effect of lipids and oleic acid on biomass development in anaerobic fixed-bed reactors. Part I: Biofilm growth and activity. Water Res. 35, 255–263.

Angelidaki, I., Ahring, B.K., 1992. Effects of free long-chain fatty acids on thermophilic anaerobic digestion. Appl. Micriobiology Biotechnol. 37, 808–812.

Angelidaki, I., Petersen, S.P., Ahring, B.K., 1990. Effects of lipids on thermophilic anaerobic digestion and reduction of lipid inhibition upon addition of bentonite. Appl. Microbiol. Biotechnol. 33, 469–472.

APHA, AWWA, WPCF, 1999. Standard Methods for the Examination of Water and Wastewater, American P. ed. Washington D.C.

Baek, G., Jung, H., Kim, J., Lee, C., 2017. A long-term study on the effect of magnetite supplementation in continuous anaerobic digestion of dairy effluent – Magnetic separation and recycling of magnetite. Bioresour. Technol. 241, 830–840.

Baserba, M.G., Angelidaki, I., Karakashev, D., 2012. Effect of continuous oleate addition on microbial communities involved in anaerobic digestion process. Bioresour. Technol. 106, 74–81.

Becker, P., Köster, D., Popov, M.N., Markossian, S., Antranikian, G., Märkl, H., 1999. The biodegradation of olive oil and the treatment of lipid-rich wool scouring wastewater under aerobic thermophilic conditions. Water Res. 33, 653–660.

Bialek, K., Cysneiros, D., O'Flaherty, V., 2013. Low-ttemperature (10∘C) anaerobic digestion of dilute dairy wastewater in an EGSB bioreactor: microbial community structure, population dynamics, and kinetics of methanogenic populations. Archaea 1–10.

Bisschops, I., Spanjers, H., 2008. Literature review on textile wastewater characterisation. Environ. Technol. 37–41.

Blackmore, M.A., Quayle, J.R., 1968. Choice between autotrophy and heterotrophy in *Pseudomonas oxalaticus*. Growth in mixed substrates. Biochem. J. 107, 705–13.

Boari, G., Brunetti, A., Passinot, R., Rozzi, A., Blasio, V. De, 1984. Anaerobic Digestion of Olive Oil Mill Wastewaters Pgas. Agric. Wastes 10, 161–175.

Borja, R., Banks, C.J., 1995. Response of an anaerobic fluidized bed reactor treating ice-cream wastewater to organic , hydraulic , temperature and pH shocks. J. Biotechnol. 39, 251–259.

Botheju, D., Bakke, R., 2011. Oxygen Effects in Anaerobic Digestion – A Review. Open Waste Manag. J. 1–19.

Brandl, H., Gross, R.A., Lenz, R.W., Clinton, R., 1988. Pseudomonas oleovorans as a Source of Poly (b-Hydroxyalkanoates) for Potential Applications as Biodegradable Polyesters. Appl. Environ. Microbiol. 54, 1977–1982.

Broughton, M.J., Thiele, J.H., Birch, E.J., Cohen, A., 1998. Anaerobic batch digestion of sheep tallow. Water Res. 32, 1423–1428.

Bryant, M.P., 1979. Microbial Methane Production - Theoretical Aspects. J. Anim. Sci. 48, 193–201.

Bryant, M.P., Boone, D.R., 1987. Isolation and Characterization of *Methanobacterium formicicum* MF. Int. J. Syst. Bacteriol. 37, 171–171.

Burd, G., Ward, O.P., 1996. Physicochemical properties of PM-factor, a surface-active agent produced by *Pseudomonas marginalis*. Can. J. Microbiol. 42, 243–251.

Campbell, J.W., Morgan-kiss, R.M., Cronan, J.E., 2003. A new *Escherichia coli* metabolic competency : growth on fatty acids by a novel anaerobic b -oxidation pathway 47, 793–805.

Canovas-Diaz, M., Sanchez-Roig, M.J., Iborra, J.L., 1992. Myristic and oleic acid degradation by an acclimated anaerobic consortia: synergistic behavior, in: 6th E.C. Conference on Biomass for Energy, Industry, and Environment. Athens, Greece, pp. 580–584.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. PNAS 108, 4516–4522.

Cavaleiro, A.J., Pereira, M.A., Guedes, A.P., Stams, A.J.M., Alves, M.M., Sousa, D.Z., 2016.

Conversion of Cn-Unsaturated into Cn-2-Saturated LCFA Can Occur Uncoupled from Methanogenesis in Anaerobic Bioreactors. Environ. Sci. Technol. 50, 3082–3090.

Cavaleiro, A.J., Salvador, A.F., Alves, J.I., Alves, M., 2009. Continuous high rate anaerobic treatment of oleic acid based wastewater is possible after a step feeding start-up. Environ. Sci. Technol. 43, 2931–2936.

Cavaleiro, A.J., Sousa, D.Z., Alves, M.M., 2010. Methane production from oleate: assessing the bioaugmentation potential of *Syntrophomonas zehnderi*. Water Res. 44, 4940–4947.

Colleran, E., Concannon, F., Golden, T., Geoghegan, F., Crumlish, B., Killilea, E., Henry, M., Coates, J., 1992. Use of methanogenic activity tests to characterize anaerobic sludges, screen for anaerobic biodegradability and determine toxicity thresholds against individual anaerobic trophic. Water Sci. Technol. 25, 31–40.

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., Marrie, T.J., 1987. Bacterial Biofilms in Nature and Disease. Annu. Rev. Microbiol. 41, 435–464.

Demeyer, D.I., Henderickx, H.K., 1967. The Effect of C18 Unsaturated Fatty Acids on Methane Production in vitro by Mixed Rumen Bacteria. Biochem. Biophys. Acta 137, 484–497.

Demirel, B., Yenigun, O., Onay, T.T., 2005. Anaerobic treatment of dairy wastewaters: a review. Process Biochem. 40, 2583–2595.

Dereli, R.K., van der Zee, F., Heffernan, B., Grelot, A., van Lier, J.B., 2014. Effect of sludge retention time on the biological performance of anaerobic membrane bioreactors treating corn-to-ethanol thin stillage with high lipid content. Water Res. 49, 453–464.

DiRusso, C.C., Black, P.N., Weimar, J.D., 1999. Molecular inroads into the regulation and metabolism of fatty acids, lessons from bacteria. Prog. Lipid Res. 38, 129–197.

Ferguson, R., Coulon, F., Villa, R., 2016. Organic loading rate: A promising microbial management tool in anaerobic digestion. Water Res. 100, 348–356.

Filiatrault, M.J., Picardo, K.F., Ngai, H., Passador, L., Iglewski, B.H., 2006. Identification of *Pseudomonas aeruginosa* genes involved in virulence and anaerobic growth. Infect. Immun. 74, 4237–4245.

Folch, J., Lees, M., Stanley, G.H.S., 1957. A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues. J. Biol. Chem. 226, 497–509.

Francisci, D. De, Kougias, P.G., Treu, L., Campanaro, S., Angelidaki, I., 2015. Microbial diversity and dynamicity of biogas reactors dueto radical changes of feedstock composition. Bioresour. Technol. 176, 56–64.

Galbraith, H., Miller, T.B., 1973. Effect of Metal Cations and pH on the Antibacterial Activity and Uptake of Long Chain Fatty Acids. J. Appl. Microbiol. 36, 635–646.

Galbraith, H., Miller, T.B., Paton, A.M., Thompson, J.K., 1971. Antibacterial Activity of Long Chain Fatty Acids and the Reversal with Calcium, Magnesium, Ergocalciferol and Cholesterol. J. Appl. Microbiol. 34, 803–813.

Gerritse, J., Gottschal, J.C., 1993. Two-membered mixed cultures of methanogenic and aerobic bacteria in O2-limited chemostats. J. Gen. Microbiol. 139, 1853–1860.

Gomila, M., Peña, A., Mulet, M., Lalucat, J., 2015. Phylogenomics and systematics in *Pseudomonas*. Front. Microbiol. 6, 1–13.

Grabowski, A., Blanchet, D., Jeanthon, C., 2005. Characterization of long-chain fatty-acid-degrading syntrophic associations from a biodegraded oil reservoir. Res. Microbiol. 156, 814–821.

Guedes, A.P., Cavaleiro, A.J., Silva, S.A., Alves, M.M., Stams, A.J.M., Sousa, D.Z., 2013. Addition of electron acceptors stimulates methanogenesis from lipids by anaerobic sludge, in: 13th World Congress on Anaerobic Digestion. Santiago de Compostela.

Gunstone, F.D., Harwood, J.L., 2007. Occurence and characterisation of oils and fats, in: Gunstone, F.D., Harwood, J.L., Dijkstra, A.J. (Eds.), The Lipid Handbook. CRC Press - Taylor & Francis Group, pp. 37–143.

Hanaki, K., Matsuo, T., Nagase, M., 1981. Mechanism of Inhibition Caused by Long-Chain Fatty Acids in Anaerobic Digestion Process. Biotechnol. Bioeng. 23, 1591–1610.

Hatamoto, M., Imachi, H., Fukayo, S., Ohashi, A., Harada, H., 2007a. *Syntrophomonas palmitatica* sp. nov., an anaerobic, syntrophic, long-chain fatty-acid-oxidizing bacterium isolated from methanogenic sludge. Int. J. Syst. Evol. Microbiol. 57, 2137–42.

Hatamoto, M., Imachi, H., Ohashi, A., Harada, H., 2007b. Identification and cultivation of anaerobic, syntrophic long-chain fatty acid-degrading microbes from mesophilic and thermophilic methanogenic sludges. Appl. Environ. Microbiol. 73, 1332–1340.

Hatamoto, M., Imachi, H., Yashiro, Y., Ohashi, A., Harada, H., 2007c. Diversity of anaerobic microorganisms involved in long-chain fatty acid degradation in methanogenic sludges as revealed by RNA-based stable isotope probing. Appl. Environ. Microbiol. 73, 4119–4127.

He, X., Zhang, Q., Cooney, M.J., Yan, T., 2015. Biodegradation of fat, oil and grease (FOG) deposits under various redox conditions relevant to sewer environment. Appl. Microbiol. Biotechnol. 99, 6059–6068.

Heffernan, B., Murphy, C.D., Casey, E., 2009. Comparison of planktonic and biofilm cultures of

Pseudomonas fluorescens dsm 8341 cells grown on Fluoroacetate. Appl. Environ. Microbiol. 75, 2899–2907.

Heukelekian, A.H., Mueller, P., 1958. Transformation of Some Lipids in Anaerobic Sludge Digestion. Sewage Ind. Waste. 30, 1108–1120.

Huisman, G.W., 1989. Synthesis of Poly-3-Hydroxyalkanoates Is a Common Feature of Fluorescent Pseudomonads 55, 1949–1954.

Hwu, C.-S., Donlon, B., Lettinga, G., 1996. Comparative toxicity of long-chain fatty acid to anaerobic sludges from various origins. Water Sci. Technol. 34, 351–358.

Hwu, C.-S., Tseng, S.-K., Yuan, C.-Y., Kulik, Z., Lettinga, G., 1998. Biosorption of long-chain fatty acids in UASB treatment process. Water Res. 32, 1571–1579.

Hwu, C., Lettinga, G., 1997. Acute toxicity of oleate to acetate-utilizing methanogens in mesophilic and thermophilic anaerobic sludges. Enzyme Microb. Technol. 21, 297–301.

Hwu, C., Molenaar, G., Garthoff, J., van Lier, J.B., Lettinga, G., 1997a. Thermophilic high-rate anaerobic treatment of wastewater containing long-chain fatty acids : impact of reactor hydrodynamics. Biotechnol. Lett. 19, 447–451.

Hwu, C., van Beek, B., van Lier, J.B., Lettinga, G., 1997b. Thermophilic high-rate anaerobic treatment of wastewater containing long-chain fatty acids: effect of washed out biomass recirculation. Biotechnol. Lett. 19, 453–456.

Igwe, J.C., Onyegbado, C.C., 2007. A Review of Palm Oil Mill Effluent (POME) Water Treatment. Glob. J. Environ. Res. 1, 54–62.

Ismail, I., Ali, M., Aini, N., Rahman, A., Sau, C., 2010. Thermophilic biohydrogen production from palm oil mill effluent (POME) using suspended mixed culture. Biomass and Bioenergy 34, 42–47.

Jackson, B.E., Bhupathiraju, V.K., Tanner, R.S., Woese, C.R., Mcinerney, M.J., 1999. a new anaerobic bacterium that degrades fatty acids and benzoate in syntrophic association with hydrogen-using microorganisms. Arch. Microbiol. 107–114.

Janek, T., Łukaszewicz, M., Rezanka, T., Krasowska, A., 2010. Isolation and characterization of two new lipopeptide biosurfactants produced by *Pseudomonas fluorescens* BD5 isolated from water from the Arctic Archipelago of Svalbard. Bioresour. Technol. 101, 6118–6123.

Janek, T., Marcin, Ł., Krasowska, A., 2012. Antiadhesive activity of the biosurfactant pseudofactin II secreted by the Arctic bacterium *Pseudomonas fluorescens* BD5. BMC Microbiol. 12, 1–9.

Jeganathan, J., Nakhla, G., Bassi, A., 2006. Long-Term Performance of High-Rate Anaerobic

Reactors for the Treatment of Oily Wastewater. Environ. Sci. Technol. 40, 6466–6472.

Jeris, J.S., McCarthy, P.L., 1965. The Biochemistry of Methane Fermentation Using C14 Tracers. Water Pollut. Control Fed. 37, 178–192.

Joubert, W.A., Britz, T.J., 1987. Characterization of Aerobic , Facultative Anaerobic , and Anaerobic Bacteria in an Acidogenic Phase Reactor and Their Metabolite Formation. Microb. Ecol. 13, 159–168.

Kato, M.T., Field, J.A., Lettinga, G., 1997. Anaerobe Tolerance to Oxygen and the Potentials of Anaerobic and Aerobic Cocultures for wastewater treatment. Brazilian J. Chem. Eng. 14, 1–12.

Kim, S.-H., Han, S.-K., Shin, H.-S., 2004. Kinetics of LCFA Inhibition on Acetoclastic Methanogenesis, Propionate Degradation and β-Oxidation. J. Environ. Sci. Heal. Part A 39, 1025–1037.

Kim, Y., Mosier, N.S., Hendrickson, R., Ezeji, T., Blaschek, H., Dien, B., Cotta, M., Dale, B., Ladisch, M.R., 2008. Composition of corn dry-grind ethanol by-products : DDGS , wet cake , and thin stillage. Bioresour. Technol. 99, 5165–5176.

Koster, I.W., Cramer, A., 1987. Inhibition of Methanogenesis from Acetate in Granular Sludge by Long-Chain Fatty Acids. Appl. Environ. Microbiol. 53, 403–409.

Kougias, P.G., Treu, L., Campanaro, S., Zhu, X., Angelidaki, I., 2016. Dynamic functional characterization and phylogenetic changes due to Long Chain Fatty Acids pulses in biogas reactors. Sci. Rep. 6, 1–10.

Lalman, J., Bagley, D.M., 2002. Effects of C18 long chain fatty acids on glucose, butyrate and hydrogen degradation. Water Res. 36, 3307–3313.

Lalman, J.A., Bagley, D.M., 2001. Anaerobic Degradation and Methanogenic Inhibitory Effects of Oleic and Stearic Acids. Water Res. 35, 2975–2983.

Lalman, J.A., Bagley, D.M., 2000. Anaerobic Degradation and Inhibitory Effects of Linoleic Acid. Water Res. 34, 4220–4228.

Lalucat, J., Bennasar, A., Bosch, R., Garcı, E., 2006. Biology of *Pseudomonas stutzeri*. Microbiol. Mol. Biol. Rev. 70, 510–547.

Lane, D.J., 1991. 16S/23S rRNA sequencing, in: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. Wiley, pp. 115–175.

Lim, J.W., Wang, J.Y., 2013. Enhanced hydrolysis and methane yield by applying microaeration pretreatment to the anaerobic co-digestion of brown water and food waste. Waste Manag. 33, 813–819.

Lorowitz, W.H., Zhao, H., Bryant, M.P., 1989. *Syntrophomonas wolfei* subsp. *saponavida* subsp. nov. , a Long- Chain -Fatty- Acid-Degrading, Anaerobic, Syntrophic Bacterium; *Syntrophomonas wolfei* subsp. *wolfei* subsp. nov. ; and Emended Descriptions of the Genus and Species. Int. J. Syst. Bacteriol. 39, 122–126.

Mackie, R.I., White, B.A., Bryant, M.P., 1991. Lipid Metabolism in Anaerobic Ecosystems. Crit. Rev. Microbiol. 17, 449–479.

Madigan, M.T., Martinko, J.M., Stahl, D.A., Clark, D.P., 2012. Brock - Biology of Microorganisms, 13th ed. Pearson Education, Inc.

Martín-González, L., Castro, R., Pereira, M.A., Alves, M.M., Font, X., Vicent, T., 2011. Thermophilic co-digestion of organic fraction of municipal solid wastes with FOG wastes from a sewage treatment plant: reactor performance and microbial community monitoring. Bioresour. Technol. 102, 4734–41.

Martins, G.M., Salvador, A.F., Pereira, L., Alves, M.M., 2018. Methane production and conductive materials: a critical review. Environ. Sci. Technol. (Published ahead of print).

Masse, L., Masse, D.I., Kennedy, K.J., Chou, S.P., 2002. Neutral Fat Hydrolysis and Long-Chain Fatty Acid Oxidation During Anaerobic Digestion of Slaughterhouse Wastewater. Biotechnol. Bioeng. 79, 43–52.

McInerney, M.J., Struchtemeyer, C.G., Sieber, J., Mouttaki, H., Stams, A.J.M., Schink, B., Rohlin, L., Gunsalus, R.P., 2008. Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. Ann. N. Y. Acad. Sci. 1125, 58–72.

Menes, R.J., Fernández, A., Muxí, L., 2001. Physiological and Molecular Characterisation of an Anaerobic Thermophilic Oleate-degrading Enrichment Culture. Anaerobe 7, 17–24.

Mesquita, D.P., Amaral, A.L., Leal, C., Oehmen, A., Reis, M.A.M., Ferreira, E.C., 2015. Polyhydroxyalkanoate granules quantification in mixed microbial cultures using image analysis : Sudan Black B versus Nile Blue A staining. Anal. Chim. Acta 865, 8–15.

Montalvo, S., Huiliñir, C., Ojeda, F., Castillo, A., Lillo, L., Guerrero, L., 2016. Microaerobic pretreatment of sewage sludge : Effect of air fl ow rate , pretreatment time and temperature on the aerobic process and methane generation. Int. Biodeterior. Biodegradation 110, 1–7.

Neves, L., Pereira, M. a, Mota, M., Alves, M.M., 2009. Detection and quantification of long chain fatty acids in liquid and solid samples and its relevance to understand anaerobic digestion of lipids. Bioresour. Technol. 100, 91–6.

Nielsen, H.B., Ahring, B.K., 2006. Responses of the Biogas Process to Pulses of Oleate in Reactors

Treating Mixtures of Cattle and Pig Manure. Biotechnol. Bioeng. 95, 96–105.

Novak, J.T., Carlson, D. a, 1970. Kinetics of Anaerobic Long Chain Fatty Acid Degradation. Water Pollut. Control Fed. 42, 1932–1943.

Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W., Backhaus, H., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis . Sequence Heterogeneities of Genes Encoding 16S rRNAs in Paenibacillus polymyxa Detected by Temperature Gradient Gel Elect. J. Bacteriol. 178, 5636–5643.

Nurliyana, M.Y., H'ng, P.S., Rasmina, H., Kalsom, M.S.U., Chin, K.L., Lee, S.H., Lum, W.C., Khoo, G.D., 2015. Effect of C/N ratio in methane productivity and biodegradability during facultative co-digestion of palm oil mill effluent and empty fruit bunch. Ind. Crops Prod. 76, 409–415.

Palatsi, J., Illa, J., Prenafeta-Boldú, F.X., Laureni, M., Fernandez, B., Angelidaki, I., Flotats, X., 2010. Long-chain fatty acids inhibition and adaptation process in anaerobic thermophilic digestion: batch tests, microbial community structure and mathematical modelling. Bioresour. Technol. 101, 2243–2251.

Palatsi, J., Viñas, M., Guivernau, M., Fernandez, B., Flotats, X., 2011. Anaerobic digestion of slaughterhouse waste: main process limitations and microbial community interactions. Bioresour. Technol. 102, 2219–2227.

Paulo, A.M.S., Salvador, A.F., Alves, J.I., Castro, R., Langenhoff, A.A.M., Stams, A.J.M., Cavaleiro, A.J., 2017. Enhancement of methane production from 1-hexadecene by additional electron donors. Microb. Biotechnol. 1–10.

Pedizzi, C., Regueiro, L., Rodriguez-Verde, I., Lema, J.M., Carballa, M., 2016. Effect of oxygen on the microbial activities of thermophilic anaerobic biomass. Bioresour. Technol. 211, 765–768.

Peng, F., Liu, Z., Wang, L., Shao, Z., 2007. An oil-degrading bacterium : *Rhodococcus erythropolis* strain 3C-9 and its biosurfactants 102, 1603–1611.

Pereira, J.F.B., Gudiña, E.J., Costa, R., Vitorino, R., Teixeira, J.A., Coutinho, J.A.P., Rodrigues, L.R., 2013. Optimization and characterization of biosurfactant production by *Bacillus subtilis* isolates towards microbial enhanced oil recovery applications. Fuel 111, 259–268.

Pereira, M.A., Mota, M., Alves, M.M., 2002a. Operation of an anaerobic filter and an EGSB reactor for the treatment of an oleic acid-based effluent: influence of inoculum quality. Process Biochem. 37, 1025–1031.

Pereira, M.A., Mota, M., Alves, M.M., 2001a. Degradation of oleic acid in anaerobic filters: the effect

of inoculum acclimatization and biomass recirculation. Water Environ. Res. 73, 1–10.

Pereira, M.A., Pires, O.C., Mota, M., Alves, M.M., 2005. Anaerobic biodegradation of oleic and palmitic acids: evidence of mass transfer limitations caused by long chain fatty acid accumulation onto the anaerobic sludge. Biotechnol. Bioeng. 92, 15–23.

Pereira, M.A., Pires, O.C., Mota, M., Alves, M.M., 2002b. Anaerobic degradation of oleic acid by suspended and granular sludge: identification of palmitic acid as a key intermediate. Water Sci. Technol. 45, 139–144.

Pereira, M.A., Pires, O.C., Mota, M., Alves, M.M., 2001b. Sequencing adsorption and degradation cycles towards the methanization of long chain fatty acids : comparison between granular and suspended sludge 4, 605–610.

Pereira, M.A., Roest, K., Stams, A.J.M., Akkermans, A.D.L., Amaral, A.L., Pons, M.N., Ferreira, E.C., Mota, M., Alves, M.M., 2003. Image analysis, methanogenic activity measurements, and molecular biological techniques to monitor granular sludge from an EGSB reactor fed with oleic acid. Water Sci. Technol. 47, 181–188.

Pereira, M.A., Roest, K., Stams, A.J.M., Mota, M., Alves, M., Akkermans, A.D.L., 2002. Molecular monitoring of microbial diversity in expanded granular sludge bed (EGSB) reactors treating oleic acid. FEMS Microbiol. Ecol. 41, 95–103.

Pereira, M.A., Roest, K., Stams, A.J.M., Mota, M., Alves, M., Akkermans, A.D.L., 2002c. Molecular monitoring of microbial diversity in expanded granular sludge bed (EGSB) reactors treating oleic acid. FEMS Microbiol. Ecol. 41, 95–103.

Pereira, M.A., Sousa, D.Z., Cavaleiro, A.J., Mota, M., Alves, M.M., 2003. PhD thesis - Anaerobic Biodegradation of Long Chain Fatty Acids. Time. Universidade do Minho.

Pereira, M.A., Sousa, D.Z., Mota, M., Alves, M.M., 2004. Mineralization of LCFA associated with anaerobic sludge: Kinetics, enhancement of methanogenic activity, and effect of VFA. Biotechnol. Bioeng. 88, 502–511.

Poh, P.E., Yong, W., Chong, M.F., 2010. Palm Oil Mill Effluent (POME) Characteristic in High Crop Season and the Applicability of High-Rate Anaerobic Bioreactors for the Treatment of POME. Ind. Eng. Chem. Res. 49, 11732–11740.

Regueiro, L., Carballa, M., Lema, J.M., 2016. Microbiome response to controlled shifts in ammonium and LCFA leveis in co-digestion systems. J. Biotechnol. 220, 35–44.

Rico Gutierrez, J.L., 1991. Anaerobic Treatment of Cheese-Production Wastewater using a UASB Reactor. Bioresour Technol. 37, 271–276.

Rinzema, A., Alphenaar, A., Lettinga, G., 1993a. Anaerobic Digestion of Long-Chain Fatty Acids in UASB and Expanded Granular Sludge Bed Reactors. Process Biochem. 28, 527–537.

Rinzema, A., Alphenaar, A., Lettinga, G., 1989. The Effect of Lauric Acid Shock Loads on the Biological and Physical Performance of Granular Sludge in UASB Reactors Digesting Acetate. J. Chem. Technol. Biotechnol. 46, 257–266.

Rinzema, A., Boone, M., Knippenberg, K. Van, Lettinga, G., 1994. Bactericidal effect of long chain fatty acids in anaerobic digestion. Water Environ. Res. 66, 40–49.

Rinzema, A., van Veen, H., Lettinga, G., 1993b. Anaerobic Digestion of Triglyceride Emulsions in Expanded Granular Sludge Bed Reactors with Modified Sludge Separators. Environ. Technol. 14, 423–432.

Rosenberg, E., E. Z. Ron, 1999. High- and low-molecular-mass microbial surfactants. Appl. Micriobiology Biotechnol. 52, 154–162.

Roy, F., Samain, E., Dubourguier, H.C., Albagnac, G., 1986. *Synthrophomonas sapovorans* sp. nov., a new obligately proton reducing anaerobe oxidizing saturated and unsaturated long chain fatty acids. Arch. Microbiol. 145, 142–147.

Ruiz, I., Veiga, M.C., 1997. Treatment of slaughterhouse wastewater in a UASB reactor and an anaerobic filter. Bioresour Technol. 60, 251–258.

Sabina, L.R., Pivnick, H., 1956. Oxidation of Soluble Oil Emulsions and Emulsifiers by Pseudomonas oleovorans and Pseudomonas formicans1. Appl. Microbiol. 171–175.

Salehizadeh, H., Loosdrecht, M.C.M. Van, 2004. Production of polyhydroxyalkanoates by mixed culture : recent trends and biotechnological importance. Biotechnol. Adv. 22, 261–279.

Salminen, E., Einola, J., Rintala, J., 2001. Characterisation and Anaerobic Batch Degradation of Materials Accumulating in Anaerobic Digesters Treating Poultry Slaughterhouse Waste. Environ. Sci. Technol. 22, 577–585.

Salvador, A.F., Cavaleiro, A.J., Sousa, D.Z., Alves, M.M., Pereira, M.A., 2013. Endurance of methanogenic archaea in anaerobic bioreactors treating oleate-based wastewater. Appl. Microbiol. Biotechnol. 97, 2211–8.

Sanguinetti, C., Dias, N.E., Simpson, A., 1994. Rapid Silver Staining and Recovery of PCR Products Separated on Polyacrylamide Gels. Biotechniques 17, 914–921.

Schink, B., 1997. Energetics of Syntrophic Cooperation in Methanogenic Degradation. Microbiol. Mol. Biol. Rev. 61, 262–280.

Schink, B., 1994. Diversity, Ecology, and Isolation of Acetogenic Bacteria, in: Drake, H.L. (Ed.),

Acetogenesis. Springer US, Boston, MA, pp. 197–235.

Schink, B., Schlegel, H.-G., 1978. Hydrogen metabolism in aerobic hydrogen-oxidizing bacteria. Biochimie 60, 297–305.

Schmack, G., Mu, D.-, 1998. Biotechnological Production and Characterization of Polyesters Containing 4-Hydroxyvaleric Acid and Medium-Chain-length Hydroxyalkanoic Acids † 9297, 644–649.

Schmidt, A., Müller, N., Schink, B., Schleheck, D., 2013. A Proteomic View at the Biochemistry of Syntrophic Butyrate Oxidation in *Syntrophomonas wolfei*. PLoS One 8, 1–17.

Scott, R.I., Williams, T.N., Whitmore, T.N., Lloyd, D., 1983. Direct Measurement of Methanogenesis in Anaerobic Digestors by Membrane Inlet Mass Spectrometry. Eur. J. Appl. Microbiol. Biotechnol. 18, 236–241.

Scrimgeour, C.M., Harwood, J.L., 2007. Fatty acid and lipid structure, in: Gunstone, F.D., Harwood, J.L., Dijkstra, A.J. (Eds.), The Lipid Handbook. CRC Press - Taylor & Francis Group, pp. 1–36.

Shigematsu, T., Tang, Y., Mizuno, Y., Kawaguchi, H., Morimura, S., Kida, K., 2006. Microbial diversity of mesophilic methanogenic consortium that can degrade long-chain fatty acids in chemostat cultivation. J. Biosci. Bioeng. 102, 535–44.

Sieber, J.R., Sims, D.R., Han, C., Kim, E., Lykidis, A., Lapidus, A.L., McDonnald, E., Rohlin, L., Culley, D.E., Gunsalus, R., McInerney, M.J., 2010. The genome of *Syntrophomonas wolfei*: New insights into syntrophic metabolism and biohydrogen production. Environ. Microbiol. 12, 2289–2301.

Silva, S.A., Cavaleiro, A.J., Pereira, M.A., Stams, A.J.M., Alves, M.M., Sousa, D.Z., 2014. Long-term acclimation of anaerobic sludges for high-rate methanogenesis from LCFA. Biomass and Bioenergy 67, 297–303.

Silva, S.A., Salvador, A.F., Cavaleiro, A.J., Pereira, M.A., Stams, A.J.M., Alves, M.M., Sousa, D.Z., 2016. Toxicity of long chain fatty acids towards acetate conversion by *Methanosaeta concilii* and *Methanosarcina mazei*. Microb. Biotechnol. 9, 514–518.

Silva, S.M., Voordouw, J., Leitão, C., Martins, M., Voordouw, G., Pereira, I.A.C., 2013. Function of formate dehydrogenases in *Desulfovibrio vulgaris* Hildenborough energy metabolism. Microbiology 159, 1760–1769.

Smyth, T.J.P., Perfumo, A., Marchant, R., Banat, I.M., 2010a. Isolation and Analysis of Low Molecular Weight Microbial Glycolipids, in: Timmis, K.N. (Ed.), Handbook of Hydrocarbon and Lipid Microbiology. pp. 3705–3723.

Smyth, T.J.P., Perfumo, A., McClean, S., Marchant, R., Banat, I.M., 2010b. Isolation and Analysis of Lipopeptides and High Molecular Weight Biosurfactants, in: Timmis, K.N. (Ed.), Handbook of Hydrocarbon and Lipid Microbiology. pp. 3687–3704.

Solli, L., Håvelsrud, O.E., Horn, S.J., Rike, A.G., 2014. A metagenomic study of the microbial communities in four parallel biogas reactors. Biotechnol. Biofuels 7, 1–15.

Sousa, D.Z., Alves, J.I., Alves, M.M., Smidt, H., Stams, A.J.M., 2009a. Effect of sulfate on methanogenic communities that degrade unsaturated and saturated long-chain fatty acids (LCFA). Environ. Microbiol. 11, 68–80.

Sousa, D.Z., Pereira, M. a, Alves, J.I., Smidt, H., Stams, a J.M., Alves, M.M., 2008. Anaerobic microbial LCFA degradation in bioreactors. Water Sci. Technol. 57, 439–444.

Sousa, D.Z., Pereira, M.A., Smidt, H., Stams, A.J.M., Alves, M.M., 2007a. Molecular assessment of complex microbial communities degrading long chain fatty acids in methanogenic bioreactors. FEMS Microbiol. Ecol. 60, 252–65.

Sousa, D.Z., Pereira, M.A., Stams, A.J.M., Alves, M.M., Smidt, H., 2007b. Microbial communities involved in anaerobic degradation of unsaturated or saturated long-chain fatty acids. Appl. Environ. Microbiol. 73, 1054–64.

Sousa, D.Z., Salvador, A.F., Ramos, J., Guedes, A.P., Barbosa, S., Stams, A.J.M., Alves, M.M., Pereira, M.A., 2013. Activity and viability of methanogens in anaerobic digestion of unsaturated and saturated long-chain fatty acids. Appl. Environ. Microbiol. 79, 4239–45.

Sousa, D.Z., Smidt, H., Alves, M.M., Stams, A.J.M., 2009b. Ecophysiology of syntrophic communities that degrade saturated and unsaturated long-chain fatty acids. FEMS Microbiol. Ecol. 68, 257–272.

Sousa, D.Z., Smidt, H., Alves, M.M., Stams, A.J.M., 2007c. *Syntrophomonas zehnderi* sp. nov., an anaerobe that degrades long-chain fatty acids in co-culture with *Methanobacterium formicicum*. Int. J. Syst. Evol. Microbiol. 57, 609–615.

Stams, A.J.M., 1994. Metabolic Interactions Between Anaerobic Bacteria in Methanogenic Environments. Antonie Van Leeuwenhoek 66, 271–294.

Stams, A.J.M., Dijk, J.B. Van, Dijkema, C., Plugge, M., Dijk, J.B.V.A.N., Dijkema, C.O.R., Plugge, C.M., 1993. Growth of Syntrophic Propionate-Oxidizing Bacteria with Fumarate in the Absence of Methanogenic Bacteria. Appl. Environ. Microbiol. 59, 1114–1119.

Stams, A.J.M., Elferink, S.J.W.H.O., Westermann, P., 2003. Metabolic Interactions Between Methanogenic Consortia and Anaerobic Respiring Bacteria. Adv. Biochem. Eng. Biotechnol. 81,

31–56.

Stams, A.J.M., Sousa, D.Z., Kleerebezem, R., Plugge, C., 2012. Role of syntrophic microbial communities in high-rate methanogenic bioreactors. Water Sci. Technol. 66, 352–362.

Sun, R., Zhang, L., Zhang, Z., Chen, G.H., Jiang, F., 2018. Realizing high-rate sulfur reduction under sulfate-rich conditions in a biological sulfide production system to treat metal-laden wastewater deficient in organic matter. Water Res. 131, 239–245.

Svetlitshny, V., Rainey, F., Wiegel, J., 1996. *Themosyntropha lipolytica* gen. nov., sp. nov., a Lipolytic, Anaerobic, Alkalitolerant , Thermophilic Bacterium Utilizing Short- and Long-Chain Fatty Acids in Syntrophic Coculture with a Methanogenic Archaeum. Int. J. Syst. Evol. Bacteriol. 46, 1131–1137.

Taylor, R.J., 1965. The Chemistry of Glycerides. Unilever Ltd., London, UK.

Thomson, N., Sivaniah, E., 2010. Synthesis , properties and uses of bacterial storage lipid granules as naturally occurring nanoparticles 4045–4057.

Toumi, J., Miladi, B., Farhat, A., Nouira, S., Hamdi, M., Gtari, M., Bouallagui, H., 2015. Microbial ecology overview during anaerobic codigestion of dairy wastewater and cattle manure and use in agriculture of obtained bio-fertilisers. Bioresour. Technol. Technol. 198, 141–149.

Treu, L., Campanaro, S., Kougias, P.G., Zhu, X., Angelidaki, I., 2016. Untangling the Effect of Fatty Acid Addition at Species Level Revealed Different Transcriptional Responses of the Biogas Microbial Community Members. Environ. Sci. Technol. 50, 6079–6090.

Tsapekos, P., Kougias, P.G., Vasileiou, S.A., Lyberatos, G., Angelidaki, I., 2017. Effect of micro-aeration and inoculum type on the biodegradation of lignocellulosic substrate. Bioresour. Technol. 225, 246–253.

Weng, C., Jeris, J.S., 1976. Biochemical mechanisms in the methane fermentation of glutamic and oleic acids. Water Res. 10, 9–18.

Zarzycki-Siek, J., Norris, M.H., Kang, Y., Sun, Z., Bluhm, A.P., McMillan, I.A., Hoang, T.T., 2013. Elucidating the *Pseudomonas aeruginosa* Fatty Acid Degradation Pathway: Identification of Additional Fatty Acyl-CoA Synthetase Homologues. PLoS One 8, 1–14.

Zhang, C., Liu, X., Dong, X., 2004. *Syntrophomonas curvata* sp. nov., an anaerobe that degrades fatty acids in co-culture with methanogens. Int. J. Syst. Evol. Microbiol. 54, 969–973.

Zhang, F., Liu, X., Dong, X., 2012. *Thermosyntropha tengcongensis* sp. nov., a thermophilic bacterium that degrades long-chain fatty acids syntrophically. Int. J. Syst. Evol. Microbiol. 62, 759–763.

Zhang, Y., Zamudio Cañas, E.M., Zhu, Z., Linville, J.L., Chen, S., He, Q., 2011. Robustness of archaeal populations in anaerobic co-digestion of dairy and poultry wastes. Bioresour. Technol. 102, 779–785.

Ziels, R.M., Beck, D.A.C., Martí, M., Gough, H.L., Stensel, H.D., Svensson, B.H., 2015. Monitoring the dynamics of syntrophic β-oxidizing bacteria during anaerobic degradation of oleic acidby quantitative PCR. FEMS Microbiol. Ecol. 91, 1–13.

Ziels, R.M., Beck, D.A.C., Stensel, H.D., 2017. Long-chain fatty acid feeding frequency in anaerobic codigestion impacts syntrophic community structure and biokinetics. Water Res. 117, 218–229.

Ziels, R.M., Karlsson, A., Beck, D.A.C., Ejlertsson, J., Yekta, S.S., Bjorn, A., Stensel, H.D., Svensson, B.H., 2016. Microbial community adaptation influences long-chain fatty acid conversion during anaerobic codigestion of fats, oils, and grease with municipal sludge. Water Res. 103, 372–382.

Zoetendal, E.G., Akkermans, A.D.L., Vliet, W.M.A., Visser, A.G.M. De, Vos, W.M. De, 2001. The Host Genotype Affects the Bacterial Community in the Human Gastrointestinal Tract. Microb. Ecol. Health Dis. 13, 129–134.

Zouari, N., Jabiri, H. Al, 2015. Improvement by Micro-Aeration of Anaerobic Digestion of Slaughterhouse Wastewater At 38°C. Int. J. Innov. Res. Sci. Eng. Technol. 04, 807–816.

SUPPLEMENTARY MATERIAL

Table S3.1 – Oxidation-reduction potential (mV) measured in the feeding tanks of reactors MaR and AnR.

Period	MaR	AnR
I	-61 ± 79	-322 ± 49
II	-104 ± 29	-274 ± 19

Figure S3.1 - Photographs of reactors MaR (a) and AnR (b) in period II.

>Pseudomonas sp. isolate PFR-I1

AGCTACTTCTGGAGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCA
CCGTGACATTCTGATTCACGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCGG
ACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTACCGACCATTGTAGCA
CGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACC
GGCAGTCTCCTTAGAGTGCCCACCATAACGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGAC
TTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCA
CCAATCCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAATTAAAC
CACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCA
GGCGGTCAACTTAATGCGTTAGCTGCGCCACTAAGTTCTCAAGGAACCCAACGGCTAGTAGACATCGTTT
ACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAG
TCCAGGTGGTCGCATTCGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTC
CACCACCCTCTACCGTACTCTAGCTCGCCAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGCTTTCAC
ATCCAACTTAACGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTA
TTACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTGTCGGTAACGTCAAAACACTAACGTATTA
GGTTAATGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCACACACGCGGCATGGC
TGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTC
AGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTGAGCCATTACCTCACC
AACTAGCTAATCCGACCTAGGCTCATCTGATAGCGCAAGGCCCGAAGGTCCCCTGCTTTCTCCCGTAGGA
CGTATGCGGTATTAGCGTTCCTTTCGGAACGTTATCCCCCACTACCAGGCAGATTCCTAGGCATTACTCA
CCCGTCCGCCGCTAA

>Pseudomonas sp. isolate PFR-I2

AGTCGAGCGGATGAGAGAGCTTGCTCTCAGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGC
CTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGG
ACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAA
GGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTA
CGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG
AAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGCTAATACCTTGCTGTTTTGACG
TTACCGACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAA
TCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACC
TGGGAACTGCATCCAAAACTGGCGAGCTAGAGTATGGCAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGA
AATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCCACCTGGGCTAATACTGACACTGAGGTG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGT
TGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCATTAAGTCGACCGCCTGGGGAGTACGGCCGCAAG
GTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGC
GAAGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAGCTCTGA
CACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAAC
CCTTGTCCTTAGTTACCAGCACGTTAAGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAA
AGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAAC
TCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCT
TGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCT