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EDUARDO AUGUSTO FELIPE DE VASCONCELOS

ESTUDOS ECOLÓGICOS DE COMUNIDADES MICROBIANAS TRATANDO GLICEROL EM CONDIÇÕES ANAERÓBIAS PELA APLICAÇÃO DE BIOLOGIA MOLECULAR

FORTALEZA

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Orientadora: Prof[a]. Dr[a] Sandra Tédde Santaella
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Aprovado em 07/02/2017.

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RESUMO

Técnicas de biologia molecular aplicadas em digestão anaeróbia permitem estudar a ecologia microbiana e estrutura de comunidades em biorreatores. O glicerol é um subproduto da transesterificação e pode ser usado para produzir hidrogênio por digestão anaeróbia, portanto, conhecer as interações microbianas permite compreender a relação entre os fatores biológicos, operacionais e funcionais, aperfeiçoando os processos envolvidos e o desempenho do sistema. Esta pesquisa teve como objetivo estudar as variações de diversidade, riqueza e organização funcional da comunidade microbiana, relacionando os parâmetros ecológicos, operacionais, a funcionalidade e a produtividade de reatores anaeróbios. Reatores UASB hidrogênicos foram operados e monitorados, sob aumentos de COV. Os afluentes utilizados foram: glicerol residual e glicerol puro (duas fases), com solução de nutrientes em todos os afluentes. Analisou-se: DQO, metabólitos, pH, vazão, volume e composição de gás, organização funcional, riqueza, diversidade de Shannon, e espécies encontradas por sequenciamento de Sanger. Amostras do lodo foram coletadas e armazenadas a cada mudança de COV e o DNA foi extraído e amplificado com primers contendo grampos GC para os domínios *Bacteria* e *Archaea*, seguido por DGGE em gel de poliacrilamida (8%) para os dois domínios. As bandas resultantes foram excisadas, amplificadas, purificadas e sequenciadas. Os resultados mostraram que em cargas abaixo de 30 kg DQO/m^3.d houve elevação da capacidade de suporte e estabilidade do meio, com baixa organização funcional e aumento da riqueza e diversidade em ambos os estudos, para ambos os domínios e substratos. Os parâmetros populacionais de Bacteria nos estágios seguintes reduziu e a organização funcional aumentou, indicando aumento gradual no nível de especialização da comunidade. Os aumentos populacionais foram maiores com glicerol residual do que com puro devido à especificidade do segundo substrato quando comparado ao residual, mais generalista. Os parâmetros ecológicos de Archaea nas etapas posteriores decaíram por inibição pelo substrato, o que promoveu aumento da produtividade do domínio bacteria. O glicerol puro se mostrou um substrato pior para o desenvolvimento de metanogênicas do que o residual.

Palavras-Chave: Ecologia microbiana. Digestão anaeróbia. Biorreatores. Hidrogênio. PCR-DGGE.

ABSTRACT

Molecular biology techniques applied in anaerobic digestion allow the study of microbial ecology and community structure in bioreactors. Glycerol is a byproduct of transesterification and can be used to produce hydrogen by anaerobic digestion, so understanding the microbial interactions is necessary to assess the relation among biological, operational and functional factors, improving the processes and the performance of the system. This research aimed to study the variations of diversity, richness and functional organization of the microbial community, analyzing ecological and operational parameters, as well as the functionality and productivity of anaerobic reactors. Hydrogenic UASB reactors were operated and monitored under OLR increases. The feeding used were: crude glycerol and pure glycerol (two phases), with nutrient solution in all the affluents. The parameters analyzed were: COD, metabolites, pH, flow, volume and gas composition, functional organization, range-weighted richness, Shannon diversity, and species found by Sanger sequencing. Sludge samples were collected and stored at each OLR change and the DNA was extracted and amplified with primers containing GC clamps for Bacteria and Archaea domains, followed by DGGE on polyacrylamide gel (8%) for both domains. The resulting bands were excised, amplified, purified and sequenced. The results showed that in OLR below 30 kg COD/$m^3.d^{-1}$ there was an increase in the carrying capacity and stability of the environment, with low functional organization and increased richness and diversity in both studies, for both domains and substrates. Population parameters of Bacteria in the following stages decreased and functional organization increased, indicating a gradual increase in the level of community specialization. Populational increases were greater with residual glycerol than with pure due to the specificity of the second substrate compared to the residual, a more generalist substrate. The ecological parameters of Archaea in the later stages decreased due to inhibition by substrate excess, which promoted an increase in the productivity of the bacterial domain. Pure glycerol proved to be a worse substrate for the development of methanogens than the residual.

Key words: Microbial ecology. Anaerobic digestion. Bioreactors. Hydrogen. PCR-DGGE.

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1 INTRODUÇÃO

1.1 A origem do glicerol

Atualmente, a maior parte da energia utilizada no mundo, aproximadamente 80%, provém de fontes não renováveis de energia, como petróleo e carvão, com contribuição pouco significativa de fontes renováveis. A dificuldade cada vez maior de escavar novos poços de petróleo em camadas mais profundas e a custos mais elevados aumenta o preço deste recurso. A impossibilidade de renovação torna inviável o uso de combustíveis fósseis a longo prazo, principalmente o petróleo, sendo o seu uso limitado pelo tempo de exploração das reservas disponíveis. Durante o processamento e queima destes combustíveis ocorre liberação de poluentes, como monóxido e dióxido de carbono que, além de nocivos à saúde humana, contribuem para o aumento da concentração dos gases de efeito estufa na atmosfera (GONÇALVES; PEREZ; ÂNGELO, 2009).

O aumento da poluição pela produção de resíduos bem como a redução significativa das reservas de combustíveis fósseis tem aumentado a preocupação global com o uso de fontes de energia mais duradouras e menos degradantes, estimulando o desenvolvimento de novos combustíveis, limpos e renováveis, chamados de biocombustíveis (NORDHAUS, 2006). O biodiesel vem sendo amplamente estudado por suas vantagens como combustível, dentre as quais pode-se destacar o uso em motores a diesel convencionais sem necessidade de adaptações; não ocorrem alterações de performance e durabilidade do motor; e a redução de emissões (CHAND, 2002). Devido a estas vantagens, este biocombustível tem sido produzido em larga escala por representar uma alternativa sustentável e ambientalmente correta em relação aos combustíveis fósseis, o que promoveu o aumento na geração de resíduos do processo de transesterificação, que origina biodiesel e glicerol (BALAT, 2005).

Calcula-se que para cada 100 kg de biodiesel, 10 kg de glicerol são produzidos (YAZDANI; GONZALEZ, 2007; PAPANIKOLAOU *et al.*, 2008). Atualmente a indústria química é a principal consumidora de glicerol, porém, com os incentivos para a produção de biodiesel, a oferta em breve será bem mais elevada que a demanda, tornando necessário desenvolver usos alternativos para este glicerol residual (VIANA *et al.*, 2012). Uma alternativa viável é o uso de glicerol como substrato em reatores anaeróbios, trazendo uma nova fonte de energia renovável e reduzindo o problema causado por sua acumulação no meio.

1.2 Digestão anaeróbia de glicerol

Dentre as tecnologias de bioconversão, a digestão anaeróbia é uma opção interessante, pois agrega valor a rejeitos através de processos de baixo consumo de energia; baixos custos de implantação, operação e manutenção, baixa produção de sólidos e tolerância a elevadas cargas orgânicas (STERLING *et al.*, 2001; CANTRELL *et al.*, 2008). Este processo, bem consolidado no tratamento de resíduos sólidos e de águas residuárias, pode ser descrito como a conversão microbiológica de substratos em biogás, em um meio ausente de oxigênio molecular. Neste meio ocorrem interações ecológicas entre diferentes espécies de bactérias e Archaea promovendo a fermentação da matéria orgânica presente, resultando na produção de hidrogênio e metano (LETTINGA *et al.*, 1980; MCKENDRY, 2002).

A digestão anaeróbia é uma tecnologia bastante empregada na conversão de rejeitos em produtos de valor agregado, promovendo retorno financeiro. O glicerol, através de digestão anaeróbia, pode ser utilizado para a produção de diversos produtos, como 1,3-propanediol (PAPANIKOLAOU *et al.*, 2008), etanol (JARVIS; MOORE; THIELE, 1997), ácido propiônico (ZHANG, A. YANG, 2009), ácido butírico e acético (FORREST; SIERRA; HOLTZAPPLE, 2010), butanol (Biebl, 2001), dihidroxiacetona (GÄTGENS *et al.*, 2007), ácido succínico (LEE, *et al.*, 2001), hidrogênio (ROSSI *et al.*, 2011) e metano (SILES LÓPEZ *et al.*, 2009). O número de pesquisas que utilizaram glicerol na digestão anaeróbia de efluentes visando geração de energia vem crescendo, em especial para produção de metano (YANG *et al.*, 2008; HUTŇAN *et al.*, 2009; SILES LÓPEZ *et al.*, 2009; SELMA *et al.*, 2010) e hidrogênio (ITO *et al.*, 2005; WICHER *et al.*, 2010).

Dentre as diversas tecnologias utilizadas para digestão anaeróbia, o reator anaeróbio de fluxo ascendente e manta de lodo (Upflow Anaerobic Sludge Blanket - UASB) é o mais comum. O UASB, desenvolvido por Lettinga *et al.* (1980), pode ser operado com carga orgânica volumétrica elevada, baixo tempo de detenção hidráulica e baixa demanda de energia (CINTOLI *et al.*, 1995; NAJAFPOUR *et al.*, 2006). O UASB consiste em um tanque de fluxo vertical com ou sem recirculação, apresentando um leito de lodo na porção inferior, uma região de sedimentação, e um separador de fases (sólida, líquida e gasosa), que permite a passagem e coleta do biogás produzido (NARKOLI; MENROTRA, 1997).

No reator UASB, pode ocorrer a formação de lodo floculento ou granular, com concentração elevada de micro-organismos, a característica mais marcante deste tipo de reator. Os grânulos ou flóculos, contendo diversas espécies de bactérias em interação, são

agregados microbiológicos formados pela permanência e adaptação destes micro-organismos a ambientes com fluxos de líquidos sem um suporte de fixação. Devido à ausência de pontos de fixação, o fluxo de líquidos promove a seleção de indivíduos que sejam capazes de aderir uns aos outros, de modo a sobreviver e proliferar. Os agregados tornam-se gradativamente maiores e mais densos, tornando-se flóculos ou até grânulos compactos, com alta concentração de bactérias, com grande atividade microbiana e resistência ao fluxo hidráulico (LETTINGA *et al*., 1983).

Alguns estudos dedicados a entender a estrutura e diversidade microbiana em digestão anaeróbia, mostraram que as condições do substrato e do ambiente no reator apresentam grande impacto na estrutura das comunidades microbianas (TANG *et al*., 2005; LEE *et al*., 2008). Li *et al*. (2010) estudaram as mudanças na comunidade microbiana em um reator UASB alimentado por resíduos de criação de suínos, mostrando que as variações nos padrões de população bacteriana produziram efeitos diretos na eficiência da digestão dos efluentes das criações dos animais e no desempenho do reator. O desempenho do UASB depende também do processo de estruturação das comunidades nos flóculos ou grânulos, que apresentam um longo período de formação devido ao lento desenvolvimento destas estruturas (SHOW *et al*., 2006).

Estudar a divertsidade microbiana em reatores anaeróbios é fundamental para atingir maior produtividade, portanto é importante saber de que forma esta comunidade se desenvolve, se modifica e como utiliza a matéria orgânica ao longo das etapas operacionais. Compreender estes processos ecológicos pode melhorar o funcionamento e desempenho dos sistemas, pois o desenvolvimento e a estabilidade das comunidades microbianas estão ligados à eficiência dos reatores (HAWKES *et al*., 2002). Biorreatores anaeróbios atuam como um micro-ecossistema controlado, o que permite observar de forma mais aprofundada a diversidade microbiana, a estrutura da comunidade e os processos ecológicos que modificam estas comunidades ao longo do tempo. Este conhecimento pode promover maior eficiência e/ou estabilidade na produção sob determinados parâmetros operacionais e ecológicos, melhorando o desempenho de sistemas de biorreatores para produção de biogás.

1.3 Estudos ecológicos microbianos por meio de técnicas moleculares

Nos processos biotecnológicos para conversão da matéria orgânica em hidrogênio, as bactérias são os organismos mais abundantes e as responsáveis pela digestão anaeróbia (WAGNER *et al*., 2002; MOURA *et al*, 2009). Desta forma, caracterizar a dinâmica e a

estrutura das comunidades microbianas é essencial para compreender as características ecológicas destes organismos (DAIMS; STOECKER; WAGNER, 2005; SIYAMBALAPITIYA; BLACKALL, 2005). Ainda se conhece pouco das rotas biológicas de degradação de matérias orgânicas complexas, o que dificulta a melhoria dos processos de digestão anaeróbia (SANTOS *et al.*, 2009). O conhecimento da ecologia e da função das comunidades microbianas e seus processos de degradação de fontes de carbono é necessário para aperfeiçoar os processos biológicos envolvidos (NARIHIRO; SEKIGUCHI, 2007).

A caracterização e a identificação das comunidades microbianas possibilitam conhecer os processos ecológicos da microbiota. Quando diferentes populações de microrganismos são introduzidas em um meio, degradando um mesmo substrato, pode ocorrer mudanças na função, devido as mudanças na estrutura das comunidades provocadas pela competição, inibição ou facilitação microbianas. Este meio e suas características determinam o status de um organismo na comunidade em função de suas adaptações, fisiologia e comportamento (HUTCHINSON, 1957).

Em ambientes controlados podem ocorrer mudanças nas condições do meio ao longo do tempo, tornando necessária a observação das mudanças das comunidades microbianas, pois a medida que as condições se alteram, há aumento ou redução da complexidade estrutural e funcional do ecossistema (KREBS, 2009). As variações na diversidade e na riqueza de espécies ao longo do tempo, promovidas por alterações no meio, podem aumentar ou reduzir a capacidade de suporte de um ecossistema, reduzindo ou aumentando a estabilidade do meio. Em condições ambientais adequadas, a estabilidade pode atingir seu ponto máximo, e os organismos deste meio poderiam sobreviver por tempo indeterminado, em virtude da estabilidade ecológica atingida. Neste estágio estável, a comunidade atinge o máximo de desenvolvimento e equilíbrio (CLEMENTS, 1916).

Em processos de digestão anaeróbia as mudanças na estrutura e diversidade das comunidades, na forma de sucessão ecológica, podem influenciar os processos de digestão, impactando na produção de biogás (BOON *et al.*, 2002; FOSTER *et al.*, 2003). Xing *et al.* (2005) afirmam que a produção de H_2 é influenciada não somente por populações produtoras de hidrogênio, mas também por grupos microbianos que atuam no meio, equilibrando a comunidade, portanto consórcios microbianos apresentam estruturas e interações ecológicas que podem ser alteradas a cada mudança operacional dos reatores.

1.4 Objetivos e organização da tese

Esta tese de doutorado teve como objetivos estudar as variações de diversidade, riqueza e organização funcional da comunidade microbiana de reatores anaeróbios, relacionando os parâmetros ecológicos com os resultados operacionais obtidos para avaliar a influência biológica na funcionalidade e produtividade do reator ao longo das etapas do projeto.

A tese foi dividida em quatro capítulos, descritos abaixo:

Capítulo I: *Fermentative hydrogen production from residual glycerol: a review* (Publicado na revista *Biotechnology Letters*). Este manuscrito apresenta um levantamento de dados operacionais e ambientais que atuam na produção de hidrogênio a partir de glicerol, servindo como uma importante base para a compreensão e discussão dos resultados operacionais e metabólicos.

Capítulo II: *Factors that affect bacterial ecology in hydrogen-producing anaerobic reactors* (Publicado na revista *Bioenergy Research*). Este manuscrito apresenta um levantamento bibliográfico, relatando como os parâmetros ecológicos da comunidade microbiana em biorreatores podem ser direta ou indiretamente influenciadas por fatores operacionais, bem como apresenta uma discussão sobre a relação entre produtividade e diversidade nos reatores. Este manuscrito serve como uma importante base para a compreensão e discussão dos ecológicos e sua discussão com as variações operacionais e metabólicas ao longo do tempo.

Capítulo III: *Microbial ecology and community structure in an uasb reactor fed with residual glycerol* (A ser submetido para revista a ser definida). Este manuscrito apresenta os resultados da operação de um biorreator anaeróbio tratando glicerol residual, de modo a estudar a ecologia molecular microbiana e investigar como os parâmetros ecológicos e operacionais são influenciados pelas variações de diversidade, riqueza e organização funcional.

Capítulo IV: *Microbial community analisys of acidogenic uasb reactors treating glycerol under increasing organic loading rates* (A ser submetido para revista a ser definida). Este manuscrito apresenta os resultados da operação de um sistema consistindo de biorreatores anaeróbios, operados em duas fases distintas, tratando glicerol puro. O objetivo deste trabalho foi estudar a ecologia molecular microbiana sob efeito de um substrato restrito e sob efeito de inibição de metanogênese por clorofórmio, e investigar como os parâmetros e

operacionais e ecológicos dos domínios Bactéria e Archaea são influenciados pelas variações de diversidade, riqueza e organização funcional, bem como variações no substrato.

2 FERMENTATIVE HYDROGEN PRODUCTION FROM RESIDUAL GLYCEROL: A REVIEW

2.1 Introduction

Hydrogen, a renewable energy source obtained from organic waste (KOTAY; DAS 2008), can be used as a fuel for turbines, internal combustion engines, industrial processes and syntheses, and in fuel cells for power generation (ITO *et al*. 2005). Its production can be carried out using biological (DINAMARCA; BAKKE 2011) and physicochemical (KOTHARI *et al*. 2008) processes. The disadvantage of the latter is the negative energy balance since it is necessary to use large amounts of energy to generate high temperatures and pressures required for these processes (KOTHARI *et al*. 2008).

Using a biological method, H_2 may be generated from algae by bio-photolysis of water, or from bacteria through anaerobic digestion, known as the ‘dark fermentation’ (HALLENBECK; BENEMANN, 2002). Here, the organic matter is degraded in four basic steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. H_2 is produced in the second and third steps; it is consumed in the third step where homoacetogenic bacteria use it to produce acetate and in the fourth step where hydrogenotrophic methanogenic archaea use CO_2 and H_2 to produce methane (MCCARTY, 1964). Therefore, if the goal is to produce H_2, methanogenesis should be inhibited.

Glycerol is a by-product of biodiesel production, derived from the transesterification of oils from oilseeds and animal fats (LO *et al*., 2013). According to Yazdani and Gonzalez (2008), a large amount of glycerol is generated during the transesterification reaction (1 kg glycerol per 10 kg biodiesel produced), resulting in a total worldwide production of approx. 2,200,000 tons in 2013 and a projected 4,200,000 tons in 2020 (OECD/FAO 2012). This residual glycerol is currently being bought by the food, pharmaceutical, and cosmetics industries but an increasing demand for biodiesel and the consequent overproduction of this waste product will shortly make it an environmental liability (VIANA *et al*., 2012a). Therefore, to add value, this residue can be converted into a renewable energy source by using it as an organic substrate form the production of H_2 (ITO *et al*., 2005).The objective of this paper is to present a review of H_2 production from crude glycerol by anaerobic fermentations.

2.2 Biochemistry of the anaerobic digestion of glycerol

Anaerobic digestion of glycerol can occur via reductive or oxidative metabolic pathways. H_2 is produced only by the oxidative route where glycerol is converted to dihydroxyacetone by glycerol dehydrogenase (GDH) and then phosphorylated by dihydroxyacetone kinase. In the next step, the dihydroxyacetone is converted to pyruvate by the enzyme pyruvate-ferredoxin oxidoreductase (only by strict anaerobes) and to acetyl-CoA or formate by the enzyme hydrogenase (KUBIAK *et al.*, 2012). Formate can be converted to H_2 and CO_2 by certain species of enteric bacteria such as Escherichia coli and Enterobacter aerogenes. H_2 production is due to the action of formate-H_2 lyase, the activity of which occurs through ionisable groups in its active site that promote the ability to bind the substrate, catalysing the reaction (FABIANO; PEREGO, 2002).

After the formation of acetyl-CoA, enzymes act in the production of final metabolites, which depend on the specific nature of the microorganism and on the environment. Overall, H_2 production from pyruvate is relatively low because part of the energy generated from the fermentation is used for biomass growth. Each molecule of pyruvate yields only one or two H_2 molecules. Moreover, part of the pyruvate is used to generate ATP and is then excreted as a final product, acetate (HALLENBECK; BENEMANN, 2002).

In the reductive route, glycerol is first dehydrated to 3-hydroxypropionaldehyde by GDH and then to 1,3-propanediol by 1,3-propanediol oxidoreductase. Increased glycerol concentration causes an increase in the concentration of 1,3-propanediol and a decrease in H_2 production. This is because, during the conversion of pyruvate to acetyl-CoA, H_2 production is relatively high when glycerol is at a limiting concentration. However, excess glycerol causes an increase in the production of 1,3-propanediol as more $NADH_2$ is used for production of this metabolite (WU *et al.*, 2011). Only one-third of the substrate is used for the production of H_2 and the remaining for the production of acetate, butyrate, and alcohols (HALLENBECK, 2009).

If anaerobic fermentation continues, methane is formed from acetate by acetotrophic methanogens, and from H_2 and CO_2 by hydrogenotrophic methanogens.

Stoichiometrically, it is possible to produce up to 3 mol H_2 per mol glycerol. Although glycerol can be easily metabolized via pyruvate to form H2 and CO_2 (YAZDANI; GONZALEZ, 2008), the impurities present in crude glycerol, such as a high concentration of NaCl (59 g/l), may hinder anaerobic digestion (VIANA *et al.*, 2012b). Moreover, the

accumulation of some metabolites can also reduce the H_2 yield (MANGAYIL *et al.*, 2012). This has been observed in ethanol that is produced through a thermodynamically favourable reaction between acetic acid and H_2 (LEVIN *et al.*, 2004).

Table 1 Reactors used for H_2 production and the respective results

Ref.	Reactor	Inoculum	Temp. (°C)	Initial pH	[COD] (g/l)	S_0/X_0 ratio[a] (gCOD/gTVS)	Yield (molH_2/mol Glycerol)
1	Batch	*Thermotoga neapolitana*	75	6.8	5.0	–	2.73
2	Batch	Sludge from fixed-bed anaerobic reactor in lab-scale used for H_2 from sucrose-based synthetic wastewater	25	5.5	0.3	1	1.62
3	Fixed film (continuous)	*Enterobacter aerogenes*	37	6.8	0.72[b]	–	1.12
4	Batch	Activated sludge from waste water treatment plant	40	6.5	1	–	1.10
5	Batch	*Clostridium pasteurianum*	35	7.5	20	–	0.93
6	Batch	Activated sludge	37	8	15[c]	–	0.91
6	CSTR (continuous)	*Clostridium pasteurianum*	35	7.5	10	–	0.77
7	Batch	Mixed culture- wheat soil	30	7.0	3	–	0.31
8	Batch	Sludge from brewery thermically pre-treated	35	5.5	22.2	5.2	0.3
9	Batch	Mixed culture from hot spring sediments	55	5.5	20.3	5.4	0.3
10	Batch	*Klebsiella pneumonia*	40	8.0	20	–	0.25
11	Batch	Anaerobic sludge wastewater treatment plant thermically pre-treated	35	6.5	8.3	11.3	0.07

[1] Ngo *et al.* (2011); [2] Fernandes *et al.* (2010); [3] Ito *et al.* (2005); [4] Mangayil *et al.* (2012); [5] Lo *et al.* (2013); [6] Varrone *et al.* (2013); [7] Selembo *et al.* (2009); [8] Sittijunda and Reungsang (2012a); [9] Sittijunda and Reungsang (2012b); [10] Chookaew *et al.* (2012); [11] Vlassis *et al.* (2012)
[a] Substrate to microorganism concentrations ratio
[b] In terms of total organic carbon (TOC)
[c] Considering chemical oxygen demand (COD) concentration of crude glycerol of 1260 g/l (Viana *et al.* 2012a)

In anaerobic fermentations for H_2 production, hydrogenase catalyses the transfer of electron to H^+ generating H_2 (CHEN *et al.*, 2006a, b). These enzymes are classified into three types: [FeFe] hydrogenases, [FeNi] hydrogenases, and [Fe] hydrogenases (MATHEWS; WANG, 2009). Nitrogenase is responsible for the reduction of N_2 to NH_3. In the absence of N_2, the nitrogenase from Klebsiella pneumoniae, a facultative N2-fixing bacteria, can reduceH? toH_2 in the absence of available N_2. Since this enzyme preferably reduces N_2, the presence of NH_4^+ or N_2 inhibits the production of H_2.Other nitrogenases are also capable of catalysingH_2, but, because of their low activities and higher requirements for ATP, they are not considered efficient for H_2 production (HALLENBECK; BENEMANN, 2002). The production of one molecule of H_2 requires $NADH_2$ and at least four mol ATP. In contrast, Clostridium butyricum requires only $NADH_2$, which facilitates the production of H_2 (CHEN *et al.*, 2006a, b).

2.3 Factors affecting hydrogen production from glycerol

2.3.1 Type of reactor

The configuration of the reactor is crucial for the development of microbial consortia as it directly influences the microenvironment, hydrodynamic behaviour and the contact surface between the microorganisms and the substrate (MOHAN, 2009). Table 2.1 shows that batch reactors are frequently used to evaluate the production of H_2 fromresidual glycerol because this type of reactor is simple to operate and can produce fast results, achieving a yield of up to 2.73 mol H_2/mol glycerol (NGO *et al.*, 2011). However, in experiments carried out in flasks, the pH control (a crucial factor during H_2 production) can be difficult. It is recommended to perform tests of initial pH (KHANAL *et al.*, 2004), buffer type and concentration (DAVILA-VAZQUEZ *et al.*, 2011) and initial concentration of organic matter and sludge (CHEN *et al.*, 2006a, b) to determine optimal conditions. Another issue related with this type of reactor is that H_2 accumulates in the headspace, which could inhibit H_2 production by increasing the partial pressure of H_2 (HALLENBECK, 2009) and/or by promoting its consumption by homoacetogenic bacteria and hydrogenotrophic methanogens (DINAMARCA; BAKKE, 2011). Inert gas sparging is themain alternative to solve this kind of problem (NATH; DAS, 2004).

2.3.2 Inocula and pre-treatments

Microorganisms of the bacterial domain are predominant during anaerobic fermentations for H_2 production. H_2-producing bacteria can be found in several sources, such as domestic and industrial wastewater treatment systems (VLASSIS *et al.*, 2012; SITTIJUNDA; REUNGSANG, 2012a; MANGAYIL *et al.*, 2012; VARRONE *et al.*, 2013); hot spring sediments (SITTIJUNDA; REUNGSANG, 2012b); and rumen fluid (RATTI *et al.*, 2013). However, pre-treatment of the anaerobic sludge prior to inoculation is necessary to eliminate or inhibit the H_2 consumers, especially hydrogenotrophic methanogens (SELEMBO *et al.*, 2009; ROSSI *et al.*, 2011) that are also present in these environments.

Various types of sludge pre-treatments, such as thermal shock (WANG; WAN, 2008), addition of acid or base, freezing and thawing (ROSSI *et al.*, 2011), aeration and

addition of chemicals such as chloroform (NING *et al*., 2012a, b) or 2-bromoethane sulfonic acid (BESA) (KOSKINEN *et al*., 2007), can decrease or eliminate the activity of methane-producing microorganisms. The efficiency of the pre-treatment varies depending on the diversity of methanogen Archaea in the inoculum.

In addition to inhibiting the methanogenic activity, the pre-treatment induces the formation of spores, a typical phenomenon of some H_2-producing bacteria, e.g. *Clostridium* sp. (LEE *et al*., 2009; ROSSI *et al*., 2011). When medium containing a mixed culture of anaerobic microorganisms is exposed to adverse conditions, only the spore-forming bacteria, which are the H_2 producers, survive (ROSSI *et al*., 2011). The spores germinate when favourable conditions are available (ROSSI *et al*., 2011). Sludge from domestic wastewater treatment plant seems to be an adequate inoculum after pre-treatment due to the high concentration of spore-forming Clostridia (HU; CHEN, 2007).

Tests conducted by Rossi *et al*. (2011) using sludge from an upflow sludge blanket (UASB) reactor fed with soy industry effluent have shown that heat shock increases the H_2 yield from glycerol. However, while the efficiency of acid pre-treatment is controversial because of the high variability of the results from different investigations have shown a large variation, this can range from a complete absence of H_2 production due to death of the H_2-producing bacteria (ROSSI *et al*., 2011), to a large increase in the H_2 yield.

The use of a consortium of microorganisms eliminates the need for isolation and purification of a particular strain, minimizing costs and facilitating the application of these microorganisms in full-scale reactors (MOHAN, 2009). Despite the advantage of using a mixed culture, several researchers have investigated the use of pure cultures to produce H_2 from crude glycerol (ITO *et al*., 2005; NGO *et al*., 2011; CHOOKAEW *et al*., 2012; LO *et al*., 2013). Ngo *et al*. (2011) obtained up to 2.73 mol H_2/mol glycerol by utilizing *Thermotoga neapolitana* at 75º C. However, at mesophilic temperatures, the H_2 yield varies between 0.25 mol H_2/mol glycerol by using *K. pneumoniae* (CHOOKAEW *et al*., 2012), and approx. 1 mol H_2/mol glycerol by using *Clostridium pasteurianum* (LO *et al*., 2013) or *E. aerogenes* (ITO *et al*., 2005).

The results observed by Ngo *et al*. (2011) differ significantly from the ones seen by Eriksen *et al*. (2011), who used residual glycerol as substrate in three strains of *Thermotoga* and observed no H_2 production. Ngo *et al*. (2011) used pH control, HEPES buffer, N_2 sparging and pre-treatment of glycerol with removal of ethanol and methanol by rotary evaporation. The differences are probably due to the different methodologies used and the specific [FeFe] hydrogenase in *Thermotoga* that oxidizes reduced ferredoxin and NADH

simultaneously (SCHUT; ADAMS, 2009). In glycerol fermentation, the stoichiometric ratio NADH:reduced ferredoxin is 1:1. Ngo *et al*. (2011) possibly obtained this result through reduction of acetic acid to ethanol as a potential route for reoxidation of excessive NADH (ERIKSEN *et al*., 2011). The production of acetic acid under these conditions was high (22 mmol/l) (NGO *et al*., 2011), thus it is possible that there has been a reduction of acetic acid to ethanol. This data, however, was not mentioned by these authors.

2.3.3 Initial substrate to microorganisms ratio (S_0/X_0)

In general, for batch cultures, the initial substrate concentration (S0) represents a carbon and energy source for biosynthesis requirements and other energy purposes, while the initial biomass concentration (X0) is a source of substrate utilization (Liu 1996). According to Liu (1996), the initial S0/X0 ratio (g chemical oxygen demand [COD]/g total volatile solids [TVS]) can be chosen by changing S0 at a constant X0, or by varying X0 at a constant S0. For pure cultures, the calculation of the S0/X0 ratio is rather complicated as the inoculum concentration is usually expressed in optical density (NGO *et al*., 2011), and the methodology uses specific calibration curves to calculate the concentration of microorganisms.

The critical value of this ratio should be known to avoid decreasing H_2 production because, according to Monod's kinetic model, at high substrate concentrations the specific growth rate becomes independent of the substrate concentration (i.e., zero-order growth), decreasing the H_2 production (ANDREWS, 1968; BEKINS *et al*., 1998).

Some researchers have evaluated the influence of S0/X0 ratio on the H_2 yield. Chen *et al*. (2006a, b) changed the initial ratio S0/X0 from 0 to 30 gCOD/ gTVS and found the highest yield of H_2 (4 mol H_2/mol sucrose) at an initial S0/X0 ratio of 7.3 gCOD/gTVS. Hafez *et al*. (2010) reached a maximum yield of 2.8 mol H2/mol glucose by applying a S0/X0 ratio ranging between 4.4 and 6.4 gCOD/gTVS. Above this range, the yield decreased to 1.2 mol H2/mol glucose. Liu (1996) explains that this ratio reflects the initial energy level of cultivation, and may cause serious uncoupling between anabolism and catabolism leading to energy spilling in S0/X0 ratios that are higher than the critical value. Other authors report that the excess of organic matter causes resporulation of the H_2-producing bacteria and decreases the activity of H_2-producing bacteria (HAFEZ *et al*., 2010).

By analysing the H_2 yields obtained from crude glycerol, it can be concluded that a high initial S0/X0 tends to decrease the H_2 yield. Vlassis *et al*. (2012), Sittijunda and Reungsang (2012a,b), and Fernandes *et al*. (2010) used initial S0/X0 ratios of 11.3, 5.2, 5.4,

and 1 gCOD/gTVS, respectively, and reached H_2 yields of 0.07, 0.3, 0.3 and 1.62 mol H_2/mol crude glycerol, respectively. The lowest yields, found by Vlassis *et al*. (2012), can be explained by the high conversion rate of glycerol to 1,3-propanediol, which typically can be observed in environments with high S0/X0 ratios (SEIFERT *et al*., 2009).

To avoid inhibition by substrate excess, it is advised to perform tests to know the critical values of this ratio before inoculating a reactor, because for each inoculum there is an optimum initial substrate concentration.

2.3.4 Temperature

H_2 can be produced under mesophilic and thermophilic conditions (SITTIJUNDA; REUNGSANG, 2012b). Extreme conditions, such working at 75º and 90º C (CHOU *et al*., 2008; NGO *et al*. 2011), have also been investigated. We found no research on the production of H_2 under psychrophilic conditions from any energy source, including crude glycerol. This is possibly because the H_2 synthesis is thermodynamically unfeasible below 20º C since its optimal activity range is between 50º and 70º C (KOESNANDAR *et al*., 1991).

As the temperature increases, up to a certain limit, the rate of hydrolysis of complex organic matter can increase (LIN *et al*., 2008) and, correspondingly, the cellular activity of H_2-producing bacteria increases with the temperature (LEE *et al*., 2008). High temperatures favour the biochemical reactions for H_2 production, but not the H_2-consumption reactions (CHONG *et al*. 2009). Some bacteria, such as *Bacillus coagulans* and *Clostridium acetobutylicum*, have higher activities under mesophilic conditions, achieving 2.28 and 1.26 mol H_2/mol glucose, respectively (ZHANG *et al*., 2006; KOTAY; DAS, 2008). *Thermophilic* bacteria also have high hydrogenogenic activity, the most common genera being *Thermoanaerobacterium*, *Thermotoga*, *Thermoanaerobacter*, and *Caldoanaerobacter* (CHONG *et al*. 2009).

In studies where crude glycerol was used as the substrate, a wide range of temperatures have been tested (between 25º and 75º C) as shown in Table 2.1. As expected, the highest H_2 yield (2.73 mol H_2/mol glycerol) was under hyperthermophilic conditions (75º C) (NGO *et al*., 2011). According to Table 2.1, in experiments using lower temperatures, there was no evidence of a direct correlation between temperature and H_2 yield, showing high variability among the results of experiments using temperatures ranging from 30º to 55 º C (from 0.07 to 1.12 mol H_2/mol glycerol).

2.3.5 pH

Any biological H_2 production process is dependent on hydrogenases that are directly affected by temperature and pH; e.g., the activity of the enzymes [FeFe] hydrogenase and [NiFe] hydrogenase, essential for the growth of H_2-producing bacteria, dramatically decreases with a decrease in the medium pH to 4.5 (KHANAL *et al*., 2004). The catalytic activity of the enzymes and membrane proteins can be regulated by changes in extracellular pH (KODUKULA *et al*., 1988). The extracellular H^+ affects metabolic pathways leading to H_2 production in three ways: (1) by altering the activity of the catalytic sites; (2) by altering the substrate hydrolysis; (3) by altering the nutrient influx through proton motive force.

Hydrogenases differ in their location: from membrane-bound to periplasmic enzymes (DAS *et al*., 2006). Based on their structure and location in the cell, they can be classified into groups and, amongst them, the [FeFe] hydrogenase are periplasmic enzymes from strict anaerobic bacteria (DAS *et al*., 2006). This type is affected directly by the passive influx of protons through the membrane. Accordingly, the activity of hydrogenases can be regulated by the concentration of protons through the reduction potentials of the amino acids of the active sites, which are pH-dependent (CAMMACK *et al*., 1987). In these cases, the enzyme remains structurally intact but functionally altered. Furthermore, membrane-bound proton pumps, present in most bacteria, extrude protons from the cytoplasm, generating an electrochemical gradient of protons, as proton motive force allowing solute translocation (MITCHELL, 1973). Thus, the hydrolysis of carbon sources and the movement of nutrients, which occurs by a pH gradient across the membrane, can be altered by a change in pH (KODUKULA *et al*., 1988). The concentration of H^+ ions in the extracellular environment controls the direction of metabolic pathways and the ionization state of the enzyme functional groups involved in catalysis, thus affecting the reaction rate of H_2 production (FABIANO; PEREGO, 2002).

Several studies show that the optimum pH for H_2 production is in the range of 4.5–5.7 (considering the genus Clostridium as the main H_2 producer), because the synthesis or the activation of hydrogenase, which is necessary for solvent production, is negatively affected (GOTTWALD; GOTTSCHALK, 1985). In addition, in this pH range, the methanogenic archaea have their metabolic activity inhibited (ROSSI *et al*., 2011). Although the pH of crude glycerol is typically within this range (VIANA *et al*., 2012b), it is necessary to continuously control pH because, depending on the substrate concentration, the production of acids by acidogenic bacteria causes the pH to drop to an inadequate range for H_2

production (below 5). However, H_2 production can occur efficiently outside this range; e.g., between pH 6.5 and 7.5 (NGO *et al*., 2011; MANGAYIL *et al*., 2012).

2.3.6 Buffer

Carbonate (CO_3^{2-}) and phosphate (PO_4^{3-}) salts are most commonly used as buffers to maintain the pH within a range suitable for H_2 production (ITO *et al*. 2005; DAVILA-VAZQUEZ *et al*. 2011). However, when the pH range is between 5.5 and 6.5, the bicarbonate dissociates, diminishing its buffering capacity (DAVILA-VAZQUEZ *et al*., 2011) and decreasing the fraction of H_2 in biogas (LIN; LAY, 2004). Adding carbonate, in the form of NH_4HCO_3, leads to increased NH_4^+ concentration that can be toxic to the H_2-producing bacteria if this concentration exceeds 4–6 g/l (CHEN *et al*., 2008).When phosphate salts were used as buffer in concentrations different from the optimal of (600 mg PO4 3-/l), there was a decrease in H_2 production from sucrose at 20 g COD/l (LIN; LAY, 2004).

In addition to the aforementioned compounds, other buffers that can be used effectively in the case of H_2 production from crude glycerol include MES (SELEMBO *et al*., 2009) and HEPES (NGO *et al*., 2011). However, the latter has been tested only with *Thermotoga neapolitana* as the inoculum.

2.3.7 Microbial diversity in anaerobic hydrogenogenic reactors

In anaerobic reactors, changes in the operational variables (substrate type and concentration, organic loading rate [OLR], hydraulic retention time [HRT], pH, and temperature) can affect microbial diversity and dominance among species. Mariakakis *et al*. (2011) observed that an increase in OLR from 11 to 34 kg COD/m^3 per day promoted changes in the microbial community in the reactor. This was evident from the results of denaturing gradient gel electrophoresis (DGGE). These changes were probably due to accumulation of metabolites produced by the acidogenic bacteria. The highest H_2 production was achieved at 22 kg COD/m^3 per day, with *Clostridium* being the predominant species.

Temudo *et al*. (2008) performed DGGE analysis to investigate H_2 production using three substrates (glucose, glycerol, and xylose), and found that the presence of residual glycerol resulted in increased microbial diversity, with predominance of Clostridium intestinale and *Klebsiella oxytoca*. Sittijunda and Reungsang (2012b) also performed a DGGE analysis to investigate anaerobic reactors fed with glycerol and observed that at lower

concentrations of the substrate (up to 15 g glycerol/l), species from the genera *Thermoanaerobacterium* and *Alicyclobacillus* were dominant, with a H_2 yield of 0.3 mol H_2/mol crude glycerol. When the reactors were operated with higher concentrations of glycerol (19–28 g/l), there was a predominance of other genera such as *Bacillus*, *Geobacillus*, *Eubacteriaceae* and *Alicyclobacillus*. *Lactobacillus hilgardii* was observed during all operational periods, indicating its resistance to organic load variations.

A gradual decrease in HRT decreased microbial diversity through biomass loss (O-THONG *et al*., 2011). Demirel and Yenigun (2006) found that high upward speeds in an anaerobic reactor, caused by the reduction of HRT (from 20 to 12 h), hindered the sedimentation of the filamentous bacteria, such as *Methanosaeta* and filamentous archaea that are dominant in anaerobic reactors (HULSHOFF Pol *et al*., 2004). If the HRT imposed on the reactor is lower than the average time required for biomass growth, the H_2-producing bacteria will be negatively influenced by the decrease in HRT, also decreasing the H_2 yield (O-THONG *et al*., 2011).

The pH directly affects microbial diversity. Xing *et al*. (2005) evaluated the effects of pH on microbial dynamics within a hydrogenogenic CSTR. Until the 14th day, the microbial diversity reached the highest level (11 bands on DGGE analysis) and gradually decreased after the 21st day, probably due to the increase in OLR from 12 to 17 kg COD/m^3 per day, increasing the acidogenic population, mainly bacteria from the *Clostridium*, *Acidovorax*, and *Kluyvera* genera. Populations that are most fitted to the imposed pH prevail over the other species with dominance of the most adapted organisms, so these shifts can be explained by competition between populations. After the increase in the OLR, the pH changed, changing hydrolysis and nutrient availability, changing ecological niches and ultimately promoting ecological succession, followed by a climax state with dominance of the organisms most adapted to this new environmental condition. During the period of highest H_2 production, the pH was between 4 and 4.5 and *Ethanologenbacterium sp*. was predominant in the reactor. *Clostridium* and *Ethanologenbacterium sp*. are H_2-producing bacteria. Furthermore, a lower pH suppress the activity of methanogens since archaea grow optimally at pH near to neutral, thus reducing hydrogenotrophic methanogenic archaea in the substrate and increasing H_2 yields (ROSSI *et al*., 2011).

Temperature affects microbial diversity and H_2 production due to alterations in the community, since dominant H_2-producing bacteria might vary at different temperature ranges. Lin *et al*. (2008) evaluated shifts in the bacterial community caused by increases in temperature from 35 to 55° C and observed that H_2 production of enriched sewage sludge

microflora (dominated by *Clostridia sp*) was temperature-dependent. *K. pneumoniae* and *C. intestinale* dominated at 35º C and *Bacillus sp.* at 40º C, along with butyrateproducing bacteria. When operated in thermophilic conditions (50–55º C) there was another change in the community, with the emergence of thermophilic species (*Clostridium spp.*). When the reactor reached steady-state conditions, the highest H_2 yield (1.4 mol H_2/mol xylose) was at 50º C. The differences in H_2 yields between temperatures might be related to shifts in microbial community since *Clostridium*, a well-known H_2-producing genera, was predominant. Bacterial communities in extreme environmental conditions, such as high temperatures, usually exhibit low diversity. This influence of temperature on the diversity is directly linked to the specificity of the enzymes present in different bacterial types, each one working better within a certain temperature range, since enzymes work optimally at a specific temperature range. Ultimately, increasing the temperature can increase H_2 yields, since the hydrolysis rate is increased (DINAMARCA; BAKKE, 2011).

Microbial diversity is also related to the type of pretreatment applied to the inoculum (NING *et al.*, 2012a, b; PENDYALA *et al.*, 2012). Heat shock and pH pretreatments are performed to eliminate non-sporeforming microorganisms (Rossi *et al.* 2011), whereas chemical pre-treatments using chloroform aim to prevent methanogenic activity by inhibiting coenzyme M reductase (OREMLAND; CAPONE, 1988). Ning *et al.* (2012a, b) evaluated the effect of chloroform on H_2 production from glucose using the genera *Clostridium*, *Megasphaera* and *Janthinobacterium*. The authors studied different concentrations of chloroform (from 0.025 to 0.2 %) and observed that chloroform higher than 0.125 % inhibited to both methanogenic archaea and hydrogenogenic bacteria. The highest H_2 yield was 2.02 mol H_2/mol glucose with chloroform at 0.05 % with the predominance of uncultured *Olsenella*, an uncultured bacterium clone nbt176c01, *Janthinobacterium* sp.7, *M. paucivorans* strain VTTE 03234, *C. cellulosi* strain D3 and *Clostridium sp*. HPB-4.

Pendyala *et al.* (2012) observed that heat pretreatment is the most effective because it stimulates spore production and promotes greater diversity levels among hydrogenogenic bacteria. Their study established that the dominant populations belonged to the *Clostridium* and *Enterococcus* genera, showing the efficacy of pre-treatments in increasing the diversity index of H_2-producing bacteria. Eliminating methanogen archaea, however, might not be enough to avoid H_2 consumption, since homoacetogenic bacteria can also consume H_2, which could partially explain possible variations in H_2 yields. Heat or acid pre-treatments also may not be enough to avoid H_2 consumption because of some homoacetogenic bacteria are spore-forming, that could resporulate and consume H_2.

2.4 Final discussion

The results of several investigations dealing with the production of H_2 from glycerol have shown that it is possible to achieve yields near the maximum theoretical value (3 mol H_2/mol glycerol), especially using the thermophilic culture of *Thermotoga neapolitana* (NGO *et al*., 2011). However, this process has a negative energy balance as more energy is necessary to heat up the reactor to 75º C than the energy obtained by the increased H_2 production. Considering that each mol of H_2 can generate 68 kcal (JAIN, 2009), and that Ngo *et al*. (2011) found a specific production of up to 148 mol H_2/m^3 reactor, their system can generate 8,486 kcal H_2/m^3 reactor. However, to heat up 1 m3 of reactor with aqueous solution from 25º to 75º C, it is necessary to use 50,000 kcal.

There are no studies that deal with sludge bioaugmentation using glycerol as a substrate, but this seems to be a promising alternative, since the combination of metabolic pathways of the microbial consortium can improve the efficiency of decomposition and hydrogenation of the complex compounds (HUNG *et al*., 2011). This review shows that almost all investigations were carried out in batch mode, except for two that used pure culture (ITO *et al*., 2005; LO *et al*., 2013). A continuous flow reactor can be operated at constant high OLR so that the production of organic acids would be sufficient to inhibit the H_2-consuming bacteria.

Pre-treatments of the inoculum improve H_2 production and are widely used. There is no consensus on the pre-treatment that best selects microbiota with potential for H_2 production. Nevertheless, in practice, the pre-treatment of an amount of inoculum necessary for an industrial-scale reactor is costly and timeconsuming. An alternative would be to decrease or eliminate the methanogenic activity by operating the reactors at high OLR (HAFEZ *et al*., 2010) and control the medium pH to values between 5.0 and 5.5 (DINAMARCA; BAKKE, 2011).

Based on the current and projected (2020) production of crude glycerol (OECD/FAO2012), it is possible to calculate the impact of the use of such by-products as energy sources for biological H_2 production. Since each mol glycerol can be converted to a maximum of three mol H_2, the annual molar productions of H_2 are 7.2×10^{10} and 13.7×10^{10} mol for the years 2013 and 2020, respectively. Using the data predicting worldwide H2 consumption for 2020 (BOND *et al*., 2011), which is estimated to be 4.5×10^{13} mol (excluding oil refining), crude glycerol has a potential for providing merely 0.3 % of the

world demand. However, the future strategy for building the energy matrix seems to be seeking renewable sources wherever available, e.g., the glycerol produced in countries where oilseed is used for biodiesel production. Moreover, the production of H_2 can be associated to the production of methane in a two-phase reactor (LUO *et al.*, 2011), improving the energetic potential and environmental impact of the biodiesel production chain.

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3 FACTORS THAT AFFECT BACTERIAL ECOLOGY IN HYDROGEN-PRODUCING ANAEROBIC REACTORS

3.1 Introduction

Majority of the energy that is currently produced and consumed worldwide comes from non-renewable sources such as oil, gas, or coal with increasing contributions from renewable sources (DA ROSA, 2012; HÖÖK *et al.*, 2012; WEC, 2010). The intensification of industrial and technological development has encouraged the expansion of renewable energy sources to gradually replace fossil fuels along with increasing discussions on climate change due to concerns regarding global emissions, reduction of oil and gas reserves around the world, and the difficulty of finding and accessing new oil sources in deeper layers (BP, 2012; DA ROSA, 2012).

Hydrogen, a clean and renewable fuel, has been studied as a possible alternative to traditional energy sources because on combustion it only generates water as a by-product and presents more energetic capabilities than fossil fuels (CHENG; LIU, 2011). It is 50 % more efficient than gasoline, and its abundance is 2.75-fold greater when compared with hydrocarbon fuel sources (RAMACHANDRAN; MENON, 1998). Hydrogen has a high-energy content per unit of weight (142 kJ/g) and no greenhouse gases are produced as a result of combustion, making it an environmentally friendly alternative to fossil fuels (KOTAY; DAS, 2008).

Hydrogen can be produced through biological and physicochemical methods (DINAMARCA; BAKKE, 2011; SHOW *et al.*, 2012). Biological production of hydrogen is a low-cost technology that requires low energy for the process of gas generation and occurs mainly via three processes (SHOW *et al.*, 2012): photosynthesis, photofermentation, and dark fermentation. Both photofermentation and dark fermentation are technically simpler processes that can convert substrates like organic matter present in wastewater into a renewable energy source. However, to achieve this goal, it is necessary to understand the ecology and bacterial community functions to refine the biological processes and improve the biotechnological applications such as the treatment of wastewater, anaerobic digestion of organic co-products, and the production of biogas (MAINTINGUER *et al.*, 2015; SANTOS; PEIXOTO; ROSADO, 2009).

The study of ecological interactions in anaerobic reactors can provide information regarding how the bacterial community develops, changes, and degrades the substrate along

the stages of reactor operation. Changes in the reactor functioning can be associated with shifts in the genetic pool of bacterial communities. The development and stability of bacterial activity are linked to the efficiency of anaerobic hydrogen reactors (HAWKES *et al.*, 2002). Therefore, a better understanding of the factors that affect the diversity and bacterial ecology in anaerobic hydrogen reactors could lead to increased efficiency of anaerobic treatments and biogas production.

3.2 Interactions between hydrogen- and non-hydrogen-producing microorganisms

The structure of bacterial communities can be manipulated to achieve specific goals such as hydrogen production, which requires the appropriate design and operation of bioreactors. Anaerobic digestion is a four-stage process divided into hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Hydrogen is produced in both the second and third steps and is consumed at the fourth step when methanogenic archaea use carbon dioxide and hydrogen to produce methane (MCCARTY, 1964).

Methanogens are the main consumers of hydrogen in anaerobic environments (WEIJMA; LETTINGA; GUBBELS, 2002). To achieve hydrogen production through the final step of this method, methanogenesis must be avoided in order to prevent hydrogen consumption. To inhibit methanogenic activity, it is necessary to control certain operational parameters such as pH (WANG; WAN, 2011), organic loading rate (OLR) (MARIAKAKIS *et al.*, 2011), and pre-treatment of the inoculum (MAINTINGUER *et al.*, 2015; ROSSI *et al.*, 2011). Another option to inhibit methanogenesis is by using chemicals like 2-bromoethanesulfonic acid (BESA) or chloroform (ROSSI *et al.*, 2011). A few studies indicate that BESA is not able to eliminate hydrogen consumers (REN *et al.*, 2006; WANG *et al.*, 2003); additionally, BESA can reduce *Clostridia* diversity (WU *et al*, 2002) and inhibit hydrogen production (REN *et al.*, 2006). Furthermore, these treatments are not environmentally friendly and are too costly for large-scale operations (LI; FANG, 2007).

Other groups of non-hydrogen-producing microorganisms that play a strong role in anaerobic production of hydrogen are homoacetogenic bacteria (DIEKERT; WOHLFARTH, 1994), sulfate-reducing bacteria (SRB) (CORD-RUWISCH; SEITZ; CONRAD, 1988), and lactic acid bacteria (LAB) (NOIKE *et al.*, 2002).

Homoacetogenic bacteria are strictly anaerobic microorganisms that harbor special enzymes with the ability to catalyze the formation of acetyl-CoA, which subsequently converts acetate from hydrogen and carbon dioxide (DIEKERT; WOHLFARTH, 1994),

consuming hydrogen in the process. Siriwongrungson *et al*. (2007) operated a Continuous Stirred Tank Reactor CSTR under thermophilic temperatures (between 45 and 60 °C), using digested dairy manure as the inoculum, and reported that almost no hydrogen was produced from the oxidation of butyrate, indicating that the hydrogen produced from butyrate was consumed in a subsequent step. They found that the hydrogen produced from butyrate degradation promptly reacted with carbon dioxide to form acetate by homoacetogenesis.

The SRB consume hydrogen as they use sulfate as a terminal electron acceptor. This group of bacteria consumes hydrogen at a fast rate in the presence of sulfate or nitrate, even under low hydrogen concentrations (CORD-RUWISCH; SEITZ; CONRAD, 1988). The SRB are known competitors of acetogens and methanogens in anaerobic digestion for a variety of substrates such as propionate, butyrate, ethanol, and acetate (OUDE-ELFERINK *et al*., 1994).

The LAB are known to inhibit hydrogen production through the secretion of bacteriocins, antibiotic polypeptides (NOIKE *et al*., 2002) that inhibit *Clostridia*, thus affecting hydrogen production. Noike *et al*. (2002) studied the inhibition of hydrogen production by LAB and observed that hydrogen fermentation was replaced by lactic acid fermentation when two LAB strains were cultivated together with two hydrogen-producing strains. Under mesophilic conditions, LAB growth increased and the accumulation of lactic acid led to instability in the fermentation process. Wang and Zhao (2009) operated a continuous system using food waste as substrate and observed that LAB promoted a decrease in hydrogen yields, from 71 to 49 mL H_2 g^{-1} VS, while lactic acid increased from 2.3 to 4.4 g L^{-1}. Furthermore, an increase in OLR favored LAB indigenous to the inoculum, which increased lactic acid concentrations and led to instability of the system.

Some authors have observed cooperation between species, such as facilitation (CHENG *et al*., 2011), an ecological interaction in which at least one species benefits, causing no harm to any other participant of the relationship (STACHOWICZ, 2001). In anaerobic digestion, facilitation can cause a positive impact on hydrogen production. For example, *Klebsiella sp*. can consume low levels of oxygen in the environment thus, favoring the growth of strict or facultative anaerobes such as *Clostridium* species that produce hydrogen (CHENG *et al*., 2011). The same interaction was noted by Huang *et al*. (2010); during the lag phase, the dominant genus was *Bacillus sp*., a facultative anaerobe. Such dominance can be attributed to the fact that the authors did not sparge oxygen with nitrogen in the beginning of the experiment. As the community became established, the species of this genus consumed the remaining oxygen within the reactor. This allowed some strict anaerobes, such *Clostridium*

beijerinckii and *Clostridum perfringens*, to become the newly dominant species during the exponential phase and after the steady-state was reached. According to the authors, this change in the microbiota ultimately resulted in increased hydrogen production.

3.3 Bacterial diversity and stability in anaerobic hydrogenogenic reactors

Changes to the operational conditions of the reactor can promote changes in the bacterial community structure because it affects the anaerobic process and dominance between the established species (KIM *et al*., 2010; WON; LAU, 2011). After a disturbance, such as significant changes in one or more operational parameters, there will be a period where the microorganisms will readapt until a new community with a different organization from the previous stage is fully established. At that point, the reactor reaches the steady-state stage. This system's steady state is much simpler than what is described in Ecology as the "climax" community, a point of maximum biomass and development. However, for this set of conditions, including the functional stability promoted by a stable community, the steady state can be considered analog to the ecological climax state in a system (WHITTAKER, 1953; ZHAO; WANG; REN, 2010).

Some species that are inoculated into the reactor may disappear and previously undetected species can arise (REN *et al*., 2010). Because different species have different metabolic responses, each particular adaptation to the environment promotes different ecological interactions such as competition (REN *et al*., 2010) and/or facilitation (REN *et al*., 2010). Therefore, the start-up period in a reactor, together with the operating conditions, will establish a new climax community based on the genetic pool of the various microbial species found in the different types of inocula. Some of these species can be used for the production of hydrogen by anaerobic fermentation (HILIGSMANN *et al*., 2011; PENDYALA *et al*., 2012).

The ecological interactions can directly affect stability and/or function. Koskinen *et al*. (2007) monitored bacterial community dynamics inside a dark fermentation fluidized-bed bioreactor to identify the cause of the instability in hydrogen production. The authors concluded that the instability in the production was due to changes in the microbial community structure, which were caused by rapid enrichment. This led to a change in the bacterial community structure and its metabolism from acetate–butyrate to acetate–propionate production, consequently resulting in a decrease in hydrogen production.

In dark fermentation, pyruvate can be converted to formate (KUBIAK *et al*., 2012), which in turn can be converted to hydrogen and carbon dioxide by some hydrogen-producing bacteria such as *Escherichia coli* and *Enterobacter aerogenes*. Although hydrogen was neither produced nor consumed by *Desulfovibrio desulfuricans* in this study, it is known that this species can ferment pyruvate in the absence of sulfate or nitrate (SUH; AKAGI, 1966), thus becoming a competitor in hydrogen evolution.

The results of Koskinen *et al*. (2007) detail a community with increasing diversity, along with environmental changes. These findings bring an important question to light; what is the relationship between bacterial diversity and ecosystem stability? This subject has very divergent approaches in bacterial ecology, because the stability in a system can limit the capacity of a diversity change by minimizing the possible alterations in the established community through resistance or resilience and functional redundancy (MCCANN, 2000).

Resistance is defined as the ability to withstand perturbation, expressed as the degree to which the system (structure or characteristics) remains unchanged when affected by a disturbance (ALLISON; MARTINY, 2008). Resilience is defined as the ability to recover after the perturbation, expressed as the rate at which the system returns to its original state after a disturbance (ALLISON; MARTINY, 2008). In hydrogen reactors, resilience plays a major role in productivity, especially in mixed cultures. The nature of the microbial communities to function synergistically increases the resilience, when compared with pure cultures, recovering hydrogen producers after significant changes in environmental conditions (KLEEREBEZEM; VAN LOOSDRECHT, 2007). Functional redundancy implies that some members can act as “substitutes” for other members’ functions in the community; this is expressed as the ability to carry out a biological process at the same rate as another taxon, if the same environmental conditions are applied. Thus, the ecosystem functionality and process rates are not altered despite the changes to the population structure (ALLISON; MARTINY, 2008). The *Clostridia* genus, which contains many hydrogen-producing species, relies on redundancy to maintain the overall community function in anaerobic reactors (WERNER *et al*., 2010). Furthermore, resilience plays an important role in maintaining stable *Clostridium* populations in these conditions, as observed by Werner *et al*. (2010), who monitored digester performance coupled to microbial community composition.

Resistance and resilience play a role in diversity and stability, the greater range of species that are able to respond differently to diverse environmental perturbations (either by resisting the disturbance or being able to recover from it), the more likely the ecosystem will

stabilize in response to the applied disturbance (WALKER *et al*., 2004). However, even if the community can be recovered, the system function can be highly affected, thus altering the original function. The hydrogen reactor operated by Koskinen *et al*. (2007) did not recover hydrogen production, while the bacterial community diversity increased after the disturbance, resulting in significant changes in the bacterial community. The community showed low resilience, it recovered slowly and not to the previous structure. It also had an average resistance, since the disturbances did not have to be intense in order to disrupt the community structure, and no detectable functional redundancy, the function was altered despite the recovered community. The community showed a very unspecific recovery, and after the reactor configuration changed, the production was momentarily reestablished, decreasing afterwards. Both the structure changes and the function decrease must be considered together with the ecological aspects: no functional redundancy and low resilience of the community promoted high instability, despite recovery.

Several studies agree that a large number of species can sustain functioning ecosystems (HECTOR *et al*., 1999; TILMA; LEHMAN; THOMSON, 1997), which are based on two main components (TILMA; LEHMAN; THOMSON, 1997): selection, based in individual differences (e.g., metabolism, morphology) between species; and complementarity, which states that species discriminate between resources (niche diversification occurs). Based on probability, the richer a community is, the more likely this community contains one or more species that represents a significant effect on ecosystem functioning; thus, it can become more productive, because the range of resources they are able to use is larger and the system resilience tends to be higher (HECTOR *et al*., 1999; TILMA; LEHMAN; THOMSON, 1997). Some studies observed that thermophilic communities presented higher resilience and productivity when diversity was higher. Furthermore, the communities were able to recover growth and hydrogen production faster when compared with other temperature ranges, because of its higher resilience (GADOW *et al*., 2013; GAO; SHE; JIN, 2007; KUNDU; SHARMA; SREEKRISHNAN, 2013).

Xing *et al*. (2005) operated a hydrogenogenic reactor and observed that the diversity quickly increased and then gradually decreased. It can be inferred that the community shifts gradually selecting the most productive and stable communities, and that the hydrogen production increased while diversity decreased. Koskinen *et al*. (2007) demonstrated that a community increased in diversity during the fermentation process; however, they also demonstrated increased instability and decreased hydrogen production. The inoculum used by Koskinen *et al*. (2007) was enriched in a series of batch incubations, in

order to select the desirable species of hydrogen producers. The changes in diversity were due to new microorganisms that were gradually enriched, others that were likely already present in the inoculum, and some that may have entered with the unsterilized feed. Despite the selection of the most productive communities in the inoculum, the increasing presence of competitors changed the diversity and the function of the system. Thus, the higher the diversity, the more likely the system will be stable; however, if the initial diversity of the inoculum is lower than the final diversity, it indicates the growth of previously undetected species, or allochthonous microorganisms that further competed with or inhibited hydrogen producers, which possibly lead to system instability.

3.4 Molecular techniques for the characterization of mixed cultures in hydrogen reactors

Cultivation techniques, despite their value in microbiology, are very limited, especially considering that only a small fraction of the bacterial diversity can be cultivated. Although these techniques can be successfully applied in certain situations to study the microbial diversity and ecosystem functioning (XING *et al*., 2005), it remains a poor solution because the richness can only be manipulated at low levels. Therefore, identification and assessment tools that do not require cultivation have received attention as a possible strategy to acknowledge the microbiological diversity in an uncultured environment (BELL *et al*., 2005).

Major fingerprinting techniques that have been used to characterize bacterial communities in hydrogen production fermentation processes include denaturing gradient gel electrophoresis (DGGE) (PACE, 1997) and terminal restriction fragment length polymorphism analysis (TRFLP) (TORSVIK; ØVREÅS, 2002). These techniques are used to investigate structure and characteristics of microbial communities such as differences or changes in diversity and temporal changes in structure (KÖCHLING, 2007; LEE *et al*., 2008; PEREIRA *et al*., 2002; SANZ; NAKATSU, 2004; TORSVIK; ØVREÅS, 2002). A major quantitative technique is fluorescent in situ hybridization (FISH), a polymerase chain reaction (PCR)-independent technique that allows fast identification and quantification of bacterial cells by hybridizing target 16S rRNA molecules with fluorescently labeled oligonucleotide probes (CHAGANTI; LALMAN; HEATH, 2012; LIU *et al*., 1997; ZWIRGLMAIER, 2005).

In hydrogen production, these techniques play a significant role because they can be used to analyze the inoculum, to evaluate the effects of the applied pre-treatment to

determine the best pre-treatment used for each inoculum and carbon source, and to monitor the community structure along the reactor operation (BELL *et al*., 2005; CHAGANTI; LALMAN; HEATH, 2012; HUANG *et al*., 2009; LEE *et al*., 2008; LIU *et al*., 1997; PACE, 1997; ZWIRGLMAIER, 2005). Thus, during a hydrogen reactor operation, it is possible to rapidly determine variations in different samples by investigating the effects of the operational parameters on the hydrogen producing community, providing a reliable strategy to analyze and predict system disturbances (BELL *et al*., 2005; CHAGANTI; LALMAN; HEATH, 2012; HUANG *et al*., 2009; LEE *et al*., 2008; LIU *et al*., 1997; PACE, 1997; ZWIRGLMAIER, 2005).

3.5 Operational parameters that affect the community structure in hydrogenogenic reactors

Anaerobic digestion occurs in four distinct stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Hydrogen consumption occurs in the third stage by homoacetogenic bacteria that produce acetate and in the fourth stage by hydrogenotrophic methanogenic archaea that produce methane (MCCARTY, 1964). Some operational parameters are designed to avoid hydrogen consumption, mainly by inhibition of methanogenesis, thus affecting the original bacterial community structure.

Several factors can affect bacterial diversity in an anaerobic hydrogenogenic reactor (Table 3.1), including pH (XING *et al*., 2005), OLR (MARIAKAKIS *et al*., 2011; TEMUDO *et al*., 2008), carbon source (FERRAZ JÚNIOR; ETCHEBEHERE; ZAIAT, 2015), inoculum source (PENDYALA *et al*., 2012), and pretreatment of the inoculum (MAINTINGUER *et al*., 2015). Furthermore, to understand the causes of unstable operations, the relationship between stability and diversity in anaerobic reactors was investigated. The stability of a bacterial community depends on its structure, which can change due to environmental disturbances such as changes in operational conditions or ecological interactions (ALLISON; MARTINY, 2008) such as competition.

Table 3.1 Main factors affecting microbial diversity in anaerobic hydrogen reactors

Reference	Factors affecting dynamic	Carbon Source	Inoculum	Inoculum Source	Dominant Genera	Pre-Treatment	pH
Temudo *et al*. (2008)	Carbon Source, pH	Glucose, Glycerol, Xylose	*Clostridium, Klebsiella, Pectinatus, Enterobacter, Bacteroides, Uncultured bacteria*	Sludge from distillery wastewater treatment plant and sludge from a potato starch processing acidification tank	*Clostridium, Klebsiella*	N/A	4.0-8.5
Xing *et al*. (2005)	OLR, pH	Molasses from a beet sugar refinery	*Clostridium, Acidovorax, Kluyvera*	Silt of domestic sewage drainage	*Clostridium, Ethanologenbacterium, Acidovorax, Kluyvera, Bacteroides, Spirochaetes*	N/A	4.0-4.5
Mariakakis *et al*. (2011)	OLR	Sucrose	*Bacteriodetes, Firmicutes, Tetrasphaera, Olsenella, Clostridium*	Anaerobic digester of a sewage treatment plant	*Ethanoligenes, Prevotella, Selonomonas*	N/A	5.5
Ferraz Júnior *et al*. (2015)	OLR	Raw sugarcane vinasse	*Lactobacillus, Megasphaera*	Fermentation of vinasse (autochthonous microorganism growth)	*Megasphaera, Sutterella, Lactobacillus, Thermoanaerobacterium, Clostridium*	N/A	6.5 - 5.5
Pendyala *et al*. (2012)	Pre-treatment, Inocula source	Glucose	Granular sludge*: Clostridium, Bacillus, Enterococcus, Bacteroides, Eubacterium, methanogens (Methylophilus)* Flocculent sludge*: Clostridium, Bacillus, Enterococcus, Propionobacterium, Brevibacillus, Bacteroides, Lactobacillus*	Granular and flocculent sludge from wastewater facilities treating industrial and municipal effluents	Granular*: Clostridium, Enterococcus, Bacteroides* (heat, loading shock, LA, BESA, acid) Flocculent*: Clostridium, Enterococcus* (heat, loading shock, LA, BESA, acid) Granular/Flocculent: *Clostridum, Enterococcus, Bacillus, Clostridium, Fusobacterium* (alkali)	Heat, shock loading, acid, alkali, linoleic acid, 2-bromoethane sulphonic acid (BESA)	6.0
Maintinguer *et al*. (2015)	Pre-treatment, Inocula source	Xylose	(Phyla) *Proteobacteria, Firmicutes, Chloroflexi, Actinobacteria, Cyanobacteria, Fusobacteria, Deferribacteres, uncultured bacteria*	Sediment taken from Reservoir	*Firmicutes*	Heat shock	5.5
Koskinen *et al*. (2007)	Stability	Glucose	*Clostridium, Desulfovibrio, Escherichia, Schwartzia, Acidaminococcus, Anaerofilum*	Anaerobic digester treating municipal wastewater sludge	*Clostridium, Escherichia, Desulfovibrio, Megasphaera, Bacteroidetes, Lachnospiraceae*	N/A	6.0-4.6

N/A - Not applicable

3.5.1 OLR, pH and temperature

Some operational parameters, such as pH and OLR, can be changed to increase hydrogen yields and/or production. Mariakakis *et al*. (2011) noticed shifts in the bacterial community structure after increasing the OLR to up to 34 kg COD m^{-3} day^{-1}. They observed that the dominant population during the start-up period consisted of homoacetogenic bacteria, which were subsequently replaced by acidogenic species belonging to the *Selenomonas* genus at the steady state, with minor presence of *Ethanoligenes* and *Prevotella* being detected. The dominance of the acidogenic over the homoacetogenic species likely occurred due to an accumulation of by-products generated by the former group, which inhibited the latter. Additionally, an increase in OLR increases the amount of organic matter, favoring the development of acidogenesis, which provides an energetic advantage when compared with that of the homoacetogens. Moreover, species from the *Clostridium* genus were predominant when the OLR reached 22 kg COD m^{-3} day^{-1}, but they were no longer observed when the OLR was further increased. The species used in this study were probably strains sensitive to substrate concentration, where an OLR increase resulted in inhibition.

Mariakakis *et al*. (2011) also observed that along the reactor operation, the number of detected *Clostridium spp*. dramatically decreased during phases 5 and 6, the most productive stages in terms of hydrogen production. Subsequently, the number of detected *Clostridium spp*. increased again in phase 8, defined by poor reactor performance. When the number of species further increased in phase 9, hydrogen production ceased. These results imply that the amount of bacterial species adversely influenced reactor performance, an effect that was also suggested by Koskinen *et al*. (2007), Hafez *et al*. (2010), and Kim *et al*. (2006). These results showed higher microbial diversities with increasing OLR, due to higher substrate availability.

The pH is an environmental factor that is crucial to anaerobic microorganisms due to its effects on hydrogenase enzymes; it is essential for the growth of hydrogen-producing bacteria and, consequently, on metabolic pathways and bacterial community structure (FANG; LIU, 2002). Hydrogenase enzymes can be regulated by changes in extracellular pH (KODUKULA; PRAKASAM; ANTONISEN, 1988); this affects hydrogen production by altering the activity of the enzyme through a reduction in the amino acid potential at the active sites (CAMMACK *et al*., 1987). Substrate hydrolysis is also affected by changes in the external pH. Membrane-bound pumps extrude protons from the cell producing a gradient that allows for solute translocation (MITCHELL, 1973). This affects the hydrolysis of carbon

sources and nutrient influx, which occurs by a pH gradient across the membrane (KODUKULA; PRAKASAM; ANTONISEN, 1988). Thus, pH can directly affect the reaction rate of hydrogen production, the resource management of the bacterial community, and the survival of the most adapted microorganisms. The pH is also a factor that prevents methanogenic activity since the range in which most methanogens can grow is very limited (pH 6–8) (CHEN *et al*., 2002).

Xing *et al*. (2005) assessed the effects of both OLR and pH changes on the bacterial structure within a hydrogenogenic reactor and noted that the diversity increased on the first weeks of the experiment, reached its highest level, and then gradually decreased. This may have occurred due to increases in the OLR, which consequently increased the amount of organic matter, changing nutrient availability and ecological niches. These changes favor the development of acidogenesis, favoring the acidogenic population. The adapted species belonging to the *Clostridium*, *Acidovorax*, and *Kluyvera* genera were dominant and prevailed over other species; therefore, these community shifts could be explained by competition between the species. Both studies (MARIAKAKIS *et al*., 2011; XING *et al*., 2005) showthat diversity inside a reactor can constantly shift due to population changes through competition for resources; this is also indicated by changes in their metabolic by-products.

Liu, Chan, and Fang (2002) studied the start-up period of two acidogenic reactors. They monitored the microbial community dynamics and found that when the pH decreased, the communities of both Bacteria and Archaea domains changed, followed by a decrease in methane formation and an increase in hydrogen and volatile acid production. Further analysis showed that the bacterial population in the acidogenic reactor increased from 63.1 to 90.3 %, while the archaeal population decreased from 34.1 to 4.3 %, within the first 13 days. The study revealed that it is possible to establish a suitable microbial population in the acidogenic reactors in less than 2 weeks, but in order to obtain stable metabolic activity, a longer period (up to 71 days) is necessary.

The operation temperature of the reactor also affects the microbiota and, therefore, hydrogen production. Hydrogen can be produced at two major temperature ranges: in mesophilic conditions, between 20 °C and 45 °C (DAVILA-VAZQUEZ *et al*., 2008; ZHAO *et al*., 2008), and in thermophilic conditions, between 45 °C and 60 °C (KARGI; EREN; OZMIHCI, 2012; O-THONG. MAMIMIN; PRASERTSAN, 2011). Some bacteria have high activity under mesophilic conditions, such as *Bacillus coagulans* and *Clostridium acetobutylicum* (KOTAY; DAS, 2007; ZHANG; BRUNS; LOGAN, 2006). However, some thermophilic bacteria have even higher hydrogenogenic activity, as can be seen on the most

common thermophilic hydrogen-producing species, which belong to the *Thermoanaerobacterium*, *Thermotoga*, *Thermoanaerobacter* and *Caldoanaerobacter* genera (CHONG *et al*., 2009). This characteristic is due to the [Fe]hydrogenase enzyme (CHEN *et al*., 2006); the hydrogen production process is dependent on this enzyme, which is directly affected by temperature. Several studies have tested a wide range of temperatures for hydrogen production, between 25 °C and 75 °C (FERNANDES *et al*., 2010. NGO; KIM; SIM, 2011; SITTIJUNDA; REUNGSANG, 2012a, 2012b; VARRONE *et al*., 2012). The highest hydrogen yield (2.73 mol hydrogen/mol substrate) was found under thermophilic conditions (75 °C) (NGO; KIM; SIM, 2011), showing that this enzyme is more efficient at thermophilic, rather than mesophilic conditions.

3.5.2 Inocula

In hydrogen bioreactors, it is possible to use both pure (CHOOKAEW; O-THONG; PRASERTSAN, 2012; LO *et al*., 2013; NGO; KIM; SIM, 2011) and mixed cultures (HAN; SHIN, 2004; OH; VAN GINKEL; LOGAN, 2003). Viable hydrogen yields can be obtained using pure cultures. Ngo *et al*. (2011) achieved up to 2.73 mol hydrogen/mol substrate by utilizing glycerol as the carbon source and *Thermotoga neapolitana* as the selected thermophilic hydrogen-producing species. On the other hand, the use of a community eliminates the need for isolation or purification of any particular strain, which reduces the costs and complexity in full-scale reactors (MOHAN, 2009).

Many of the hydrogen-producing microorganisms belong to the *Clostridium* genus, which are strict anaerobes and spore-forming bacteria (COLLET *et al*., 2004; LIN; LAY, 2004; LIU; SHEN, 2004), *Enterobacter* (FABIANO; PEREGO, 2002; LIU; GROT; LOGAN, 2005; NATH; DAS, 2004), and other phylogenetically related microorganisms. Some of these microorganisms can be found in different sources including wastewater treatment systems (MANGAYIL; KARP; SANTALA, 2012; VARRONE *et al*., 2013; VLASSIS *et al*., 2012), rumen fluid (RATTI *et al*., 2013), and sediment (MAINTINGUER *et al*., 2015).

Maintinguer *et al*. (2015) studied the diversity of anaerobic bacteria in the sediment of a reservoir to evaluate the application of this inoculum in biohydrogen production. They observed a highly diverse source of microorganisms belonging to many phyla such as *Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Actinobacteria*, *Cyanobacteria*, *Fusobacteria*, *Deferribacteres*, and uncultured bacteria. The dominant phylum in the final

stage was *Firmicutes* and the production of hydrogen increased, confirming the efficiency of this community in hydrogen production. In this study, the community likely involves members with probable functional redundancy and facilitation.

3.5.3 Pre-treatments

Some species need to be eliminated or inhibited in order to induce the community to select the desirable genera. A pretreatment may be required to eliminate certain potential hydrogen consumers like methanogens (ROSSI *et al*., 2011; SELEMBO *et al*., 2009), which are also present in this environment. However, if inappropriately applied, pre-treatment of the seed sludge can also suppress the activity of hydrogen-producing bacteria (ZHU; BÉLAND, 2006).

Some of the hydrogen-producing bacteria, except methanogens and some homoacetogens (LI; FANG, 2007), are able to sporulate, a natural process that occurs when these microorganisms are in adverse conditions (DUANGMANEE *et al*., 2007; REN *et al*., 2008). Bacteria that can produce hydrogen during fermentation of glucose are mainly *Clostridium* and *Enterobacter*. As previously stated, *Clostridium* can form protective spores when they are under harsh conditions like heat shock and pH pre-treatments, which are the most commonly used conditions to eliminate nonspore-forming microorganisms that do not survive these processes (DUANGMANEE *et al*., 2007; REN *et al*., 2008). However, *Enterobacter* are not sporeforming, despite being hydrogen producers, which implies that many of these non-spore hydrogen producers will likely be destroyed after the pre-treatment, possibly affecting hydrogen production (REN *et al*., 2008).

Clostridium and *Enterobacter* comprehend strict and facultative bacteria. The latter can also survive in the presence of low oxygen levels (LI; FANG, 2007; ZHU; BÉLAND, 2006), while the homoacetogens are strict anaerobes, the presence of oxygen causes them to die. The aeration parameters vary (REN *et al*., 2008; WANG; WAN, 2008; ZHU; BÉLAND, 2006), resulting in different hydrogen production yields due to different aeration times. Therefore, an appropriate aeration pre-treatment could ensure hydrogen-producing diversity while avoiding homoacetogens by raising the oxidation–reduction potential (REN *et al*., 2008).

Another common type is a chemical pre-treatment that uses specific inhibitors such as chloroform (OREMLAND; CAPONE, 1988), nitrapyrin (SALVAS; TAYLOR, 1980), or BESA (LIU *et al*., 2008), to prevent the proliferation of methanogens. These

chemicals are competitive inhibitors of the coenzyme M-reductase, causing inhibition of the enzymatic activity that catalyzes the final step in the formation of methane, thus blocking the methanogenesis that is essential to their metabolism (GRAHAM; WHITE, 2002; GUNSALUS; WOLFE, 1978; NOLLET; DEMEYER; VERSTRAETE, 1997).

There are other methods of sludge pre-treatment such as hydraulic or organic shock loading and heating (WANG; WAN, 2008), acid/alkali, and freezing/thawing (ROSSI *et al*., 2011). Pendyala *et al*. (2012) studied mixed anaerobic cultures under the influence of various pretreatments (heat, shock loading, acid, alkali, linoleic acid, and BESA). The authors claimed that thermal pre-treatment was the most efficient and that it also increased the diversity of hydrogen-producing bacteria, with dominance of the *Clostridium* genus. The thermal pre-treatment stimulated spore production and, therefore, promoted an increase in the diversity index, specifically for hydrogen-producing bacteria. However, even by eliminating methanogens, hydrogen consumption can persist because homoacetogenic bacteria can consume hydrogen. Heat or acid pre-treatments induce the formation of spores by some hydrogen-producing bacteria (ROSSI *et al*., 2011); thus, these treatments may not be enough to improve hydrogen production because some of the homoacetogenic bacteria are also spore-forming and some hydrogenproducers are not spore-forming. Furthermore, sulfatereducing bacteria are hydrogen consuming and can tolerate high temperatures; therefore, this treatment is not effective.

Using a different approach, Ning *et al*. (2012) was able to inhibit methanogenic activity and obtain a stable hydrogen production using inocula treated with chloroform at different concentrations. The authors observed that the species changed as the chloroform concentration increased, promoting the selective inhibition of methanogens. Additionally, the appropriate concentration of chloroform was determined to enhance anaerobic hydrogen by 0.050 %.

3.5.4 Carbon source

The carbon source directly affects the bacterial dynamics in a community. Temudo *et al*. (2008) investigated how different carbon sources (glucose, glycerol, and xylose) affected the bacterial community structure. The authors found that the use of glycerol resulted in increased bacterial diversity when compared with the inoculum, which was withdrawn from a distillery wastewater treatment plant. Furthermore, after reaching a steady-state/climax condition, the observed dominant species were *Clostridium intestinale* and

Klebsiella oxytoca, both of which are able to convert glycerol into hydrogen (LIU; FANG, 2007).

The carbon source can also be changed during the operation, as determined by Jo *et al*. (JO *et al*., 2007). These authors observed changes to the bacterial community in a hydrogenogenic reactor where the *Clostridium* genus predominated, which was caused by a change to the initial substrate, food waste. Afterwards, the carbon source was changed to fermented vegetable waste, causing rapid growth of lactic acid bacteria. Additionally, a decrease in hydrogen-production due to an accumulation of lactic acid was observed. The dominance shifted, with the *Lactobacillus* genus dominating in this new stage. These were probably allochthonous species that entered the system along with the unsterilized influent. Incoming microorganisms from the carbon source can influence the community composition in a reactor if it has not been sterilized or pre-treated to avoid allochthonous microorganisms.

3.5 Bacterial ecology and system functioning of hydrogen reactors

Bacterial communities are vital for the adequate functioning of all ecosystems, including those of artificial origin, which emphasizes the need to understand bacterial processes and interactions (PEDROS-ALIO, 2006). A lab-scale anaerobic reactor is a controlled system; thus, it is a more manageable system for studying processes than a full-scale ecosystem. This system allows for an in-depth study of bacterial diversity, population structure, and the processes that modify these communities.

To improve hydrogen production and overcome the possibility of instability, the ecological processes must be investigated using methods capable of detecting and identifying microorganisms that exist in the community of a dark fermentation reactor. Some of these microorganisms have unclear ecological or productive roles, because they do not directly interact with the substrate nor produce hydrogen, but interact with the producing microorganisms (SIRIWONGRUNGSON *et al*., 2007; WEIJMA; LETTINGA; GUBBELS, 2002).

The stability and productivity of a diverse bacterial community depends on other species and on operational parameters, which contribute to community promoting interactions and functional characteristics that are important at every stage of the reactor operation, even if these secondary species are not directly related to the production. Ecological interactions such as competition and/or facilitation between bacterial populations can favor or hinder certain bacterial groups so that hydrogen-producing bacteria can act cooperatively with non

hydrogen-producing bacteria in the final stable community. Allochthonous or indigenous microorganisms could compete for the available resources with the hydrogen producers and other co-existing genera that provide beneficial interactions with the hydrogenogenic microorganisms. The operational parameters or the addition of specific inhibitors can be used to prevent the possible proliferation of undesired microorganism genera (WEIJMA; LETTINGA; GUBBELS, 2002).

As previously stated, the relationship between community diversity and ecosystem stability is still a matter of debate. Stability directly refers to the ability of the ecosystem to minimize fluctuations through resistance or resilience, defying or avoiding changes after disturbances (SIRIWONGRUNGSON *et al*., 2007). Resistance and resilience can be specially noted on environments with high functional redundancy states, which by definition are highly diverse communities with different microorganisms capable of maintaining some of the system's function (ALLISON; MARTINY, 2008).

Diversity alone cannot explain function stability since system stability is the outcome of functional redundancy, resistance, and resilience but is a strong indicative of an environment with higher probability of developing a successful community, depending on the disturbances applied to it (ALLISON; MARTINY, 2008; SIRIWONGRUNGSON *et al*., 2007; XING *et al*., 2005). A highly diverse bacterial community is more likely to possess higher functional redundancy, in which case, it could confer functional resilience to the community, even in major disturbances, maintaining a stable and functional community (ALLISON; MARTINY, 2008).

The point at which the community structure changes is also unclear. The community diversity must be at least partially sensitive (not highly resistant) to disturbances, not highly resilient, and the microorganisms have to be functionally dissimilar in order for changes to occur in the community, thus allowing the community to change (FANG; LIU, 2002).

The operating parameters in an anaerobic reactor can act as selective pressure on the community affecting population structure, diversity, and heterogeneity, as seen by Koskinen *et al*. (KOSKINEN; KAKSONEN; PUHAKKA, 2007). The performance of bioreactors depends on the bacterial activity in the system. Thus, understanding bacterial community structures could lead to higher hydrogen yields through selection of the most adequate genera by manipulation of the environmental conditions imposed to the community. Combining operational parameters with ecological factors could lead to maximizing the

development of effective bioprocesses by assessing the differences and synergies of bacterial ecology (VOLMER; SCHMID; BÜHLER, 2015; XING *et al*., 2005).

3.6 Conclusion

A successful operation in a hydrogen bioreactor can be achieved through the correct control of the operational parameters. The community's structure in mixed cultures is influenced by incoming microorganisms, by operating conditions, and by interactions among microorganisms. If the hydrogen production is based on an unsterilized carbon source, the inoculum has to be properly analyzed so the diversity shifts during reactor operation, from start-up to steady-state, may become more comprehensible. Utilizing the interplay between ecological factors and operational parameters to induce hydrogen production might result in a stable community with partial selection to hydrogen-producing bacteria to increase hydrogen yields.

Pre-treatments of the inoculum are widely used in improving hydrogen production; however, there is no consensus on the treatment that best selects for hydrogen-producing microorganisms. Negative and positive interactions between hydrogen-producing and other microorganisms must be considered when choosing the inoculum and the form of pretreatment that will be applied because it will have great impact on the community structure. In order to avoid hydrogen consuming microorganisms such as methanogens, homoacetogenic bacteria, SRB, and LAB, many pretreatment such as heat- and shock-loading are used. However, every pre-treatment based on the induction of spore formation can negatively affect hydrogen production by reducing non-spore-forming hydrogen producers. Treatment by aeration can inhibit these hydrogen consumers; however, this treatment can also inhibit some strict anaerobic hydrogen producers like *Clostridium butyricum*. One of the alternatives to select only the desired hydrogen-producing species would be chemical treatment with specific inhibitors. On the other hand, these solutions are not environmentally friendly. Furthermore, the addition of chloroform to the reactor influent, which is an efficient approach to avoid methanogens in lab-scale reactors, is an expensive and ecologically inadequate option when applied to real-scale anaerobic bioreactors.

Controlling the operational parameters presents the best approach to inhibit methanogenic and homoacetogenic activity, while maintaining a community with potential hydrogenproducing bacteria. Therefore, correct manipulation and selection of the community could be achieved through the control of the OLR and pH. The operating temperature also

plays a major role in hydrogen production, since hydrogen is more efficiently produced in thermophilic conditions, thus increasing hydrogen yields. However, if the temperature increases too much, enzymes will become inactivated, decreasing hydrogen production. The main disadvantage of thermophilic conditions is that more energy is used for heating the reactors, making mesophilic reactors the reasonable choice.

Mesophilic reactors, operating at high OLR and an acid pH range (4.0 to 6.0) could strongly favor hydrogen-producing bacteria, depending on the substrate and inoculum. Controlling these parameters is a preferable option as they are inexpensive and safe approaches to avoid methanogens. This leads to a natural induction of the sludge to shift its community into adapted hydrogenproducing bacteria.

The functional characteristics and interactions among species strongly influence ecosystem properties. Additionally, species loss or changes in composition can produce different results, depending on the functional redundancy. Furthermore, some species may not contribute significantly, or not contribute at all to ecosystem properties; however, the higher the diversity, the more likely it will be for a system to maintain its stability.

3.7 Acknowledgements

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4 MICROBIAL ECOLOGY AND COMMUNITY STRUCTURE IN AN UASB REACTOR FED WITH RESIDUAL GLYCEROL

4.1 Introduction

Glycerol is a by-product from the transesterification reaction of oils for biodiesel production (LO *et al*., 2013). During this process, 1 kg glycerol is produced for every 10 kg of biodiesel (YAZDANI; GONZALEZ, 2008). This by-product can be used as a substrate for anaerobic digestion in bioreactors, resulting in various products, such as 1.3-propanediol (PAPANIKOLAOU *et al*., 2008), ethanol (JARVIS *et al*., 1997), propionic acid (ZHANG; YANG, 2009), butyric and acetic acid (Forrest *et al*., 2010), butanol (BIEBL, 2001), dihydroxyacetone (GÄTGENS *et al*., 2007), succinic acid (LEE *et al*., 2001), caproic acid (DAMS *et al*., 2016), hydrogen (ROSSI *et al*., 2011), and methane (SILES-LÓPEZ *et al*., 2009).

During anaerobic digestion of biodegradable material, hydrogen and methane can be produced by microorganisms of the domains Bacteria and Archaea (VERHAART *et al*., 2010) in concurrent pathways. Therefore, to select the optimal operational parameters and improve the H_2 or CH_4 yields in anaerobic reactors it is necessary first to understand the microbial ecology due to the biological processes involved (NARIHIRO; SEKIGUCHI, 2007). Mixed bacteria have complex ecological interactions, such as cooperation and competition, and temporal effects in the different stages of a reactor. Thus, in order to obtain an optimally productive community, it is necessary to know the production efficiency, population characteristics and ecological function of different populations from both domains (LIN; CHANG, 2004).

Anaerobic bioreactors act as controlled ecosystems, allowing accurate evaluation of the community structure and diversity and the succession processes that modify these communities over time. The use of molecular biological techniques is essential to assess the microbial diversity, ecology and dynamics at different levels. Therefore, this research aimed to assess: (i) the ecological relationships like Shannon diversity index, range-weighted richness and species specialization and/or dominance; (ii) the community structure through populational shifts along the operation, and (iii) the relationship between the community structure and the ecological processes in the production yield and the VFAs produced along the operating stages of the reactors. This knowledge could promote the selection of a

microbial consortium and operational conditions with greater efficiency, improving the performance of anaerobic reactors.

4.2 Materials and Methods

4.2.1 Substrate and inoculum

The reactor was fed with residual glycerol from transesterification process for biodiesel production. Bovine tallow (44%) and soy (56%) were the feedstock for biodiesel production. The characteristics of the glycerol were: 1% non-glycerol organic matter; pH 5.5; 81.5% purity; 4.8% ash; 12.71% moisture; 0.03% methanol; 5.3% NaCl; density 1255.9 kg.m-3. The residual glycerol presented 1374 mg COD/L of organic matter concentration and was diluted to reach the desired chemical oxygen demand (COD) for each stage of operation. The dilution of the residual glycerol in the influent was gradually reduced, until it reached the least amount of water possible for the operation.

In every operational stage, a nutrient solution was added, adapted from Lin and Lay (2004), in the concentrations (mg.L-1): $MgCl_2 \cdot 6H_2O$ (40.0); $CoCl_2 \cdot 6H_2O$ (5.0); $CaCl_2 \cdot 2H_2O$ (0.1); $NiCl_2 \cdot 6H_2O$ (2.5); $MnCl_2 \cdot 4H_2O$ (10.6); KCl (1.1); $NH_4H_2PO_4$ (107.5); $ZnCl_2$ (0.1); $FeSO_4 \cdot 7H_2O$ (5.0); $MnSO_4 \cdot H_2O$ (0.3); $CuSO_4 \cdot 5H_2O$ (5.0); $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (51.1). The inoculum consisted of a mixed sludge obtained from a full-scale upflow anaerobic sludge blanket (UASB) reactor used for treatment of municipal wastewater. The inoculum did not suffer any form of pre-treatment, therefore the operational conditions were the only factors to induce or to prevent shifts in microbial diversity.

4.2.2 Bioreactor set-up

The bioreactor was a lab-scale UASB reactor, with working volume of 14.65 L. Eight taps were installed vertically along the reactor to allow sludge sampling. The pH was controlled automatically using a dosing pump (LMI Milton Roy, model P133-398TI) with NaOH solution 0,5% (v/v). The values of COD and total volatile solids (TVS) were determined according Eaton *et al*. (2005), methods titulometric and gravimetric, respectively. The biogas flow rate was monitored by a Ritter gasometer and the gas composition was determined by gas chromatography (C2V-200 micro GC; Thermo Fisher Scientific, The Netherlands).

The concentrations of volatile fatty acids (VFA) were determined by high-performance liquid chromatography (HPLC), under the conditions: Agilent Zorbax C18 column (150 x 4.6 mm) maintained at 25 °C; Ultraviolet/Visible detector Varian UV 325 Polaris 215 nm, having as mobile phase acetonitrile/water (3:7) with 0.01% sulfuric acid at 0.4 mL/min flow. The injected sample volume was 20 µL, the samples were previously filtered on cellulose acetate membrane ME25 with pores of 0.45 µm.

4.2.3 Operation strategy

The reactor was operated in five stages with increasing OLRs (from 14.4 to 54.5 kg $COD.m^{-3}.d^{-1}$), which were increased when the reactor was considered to be adapted to the substrate, observed when: the theoretical OLR for each stage was achieved and the pH, biogas volume and COD were within the range of planned values. In order to assess the acidogenic conditions and to verify the decrease in methanogenic production, the CH_4 and H_2 levels were analyzed.

4.2.4 DNA extraction and PCR amplification of the 16S rRNA

The DNA was extracted using the set for Fast extraction ® DNA Spin Kit for Soil (MP Biomedicals, LLC), following the manufacturer's protocol, with modifications: rotation speed (13900 rpm) and duration (20 min) in the centrifuge; speed and time of agitation equipment Mini-Bead-beater for cell disruption (30 s of disruption, followed by one minute on ice, followed by 20 s of disruption); turn-around time of samples to grip the DNA to the silica matrix (one hour). The extracted DNA was identified and stored at -18 °C. Samples of extracted DNA were quantified using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). After quantification, the samples were diluted to the correct concentration for PCR reaction.

DNA amplification occurred through the polymerase chain reaction (PCR) technique, done in both reactors, for both domains Bacteria and Archaea with primers containing 40bp GC-clamps for further analysis by DGGE. The 16S rRNA gene hypervariable regions V2-V3 of Archaea were amplified with the specific primers 0515R-GC: 5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAT CGT ATT ACC GCG GCT GCT GGC AC-3'; and 0109F-T: 5'-ACT GCT CAG TAA CAC GT-3' (Sigma Aldrich, St. Louis, MO, USA) (GROSSKOPF *et al.*, 1998; MUYZER *et al.*, 1993). In

the domain Bacteria, regions V6-V8 were amplified, using bacterial universal primers 1401R: 5'-CGG TGT GTA CAA GAC CC-3'; and 0968F-GC: 5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3' (Sigma Aldrich, St. Louis, MO, USA; NÜBEL *et al*., 1996).

The PCR mix (50 µL) contained: 10 µL of reaction buffer (5x); 5 µL of MgCl2 (25 mM); 0.25 µL of Taq polymerase (5 u/µL) (Promega, USA); 1 µL of deoxynucleotide triphosphates (10 mM); 1 µL of the extracted DNA; 1 µL of PCR primers (10 µM); and nuclease free water (Promega, USA) up to a final volume of 50 µL. The PCR was carried out in a T100 Thermal Cycler iCycler (Bio Rad Laboratories, Inc.). The amplification for Bacteria followed the protocol: initial denaturation at 95 °C for 2 min; 31 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 45 s, and elongation at 72 °C for 1 min; followed by a final elongation at 72 °C for 6 min. DNA amplification of Archaea followed the protocol: initial denaturation at 95 °C for 2 min; 31 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 40 s, and elongation at 72 °C for 90 s; followed by a final elongation at 72 °C for 6 min.

The sizes of PCR products were estimated with a DNA marker of 1000 base pairs Hypperladder II (Bioline, USA Inc.) in agarose gel electrophoresis (1.8%) and stained with SYBR Green I. The PCR products were verified in 1.7% agarose (w/v) gel electrophoresis, using the 1 Kb DNA Ladder (Promega, USA) as a molecular weight marker. The gels were stained with SYBR Green I nucleic acid gel stain (Sigma-Aldrich, USA) for 40 min and the result was analyzed in a Hood II universal transilluminator (Bio Rad Laboratories, USA).

4.2.5 Denaturant gradient gel electrophoresis (DGGE)

Denaturant gradient gel electrophoresis was performed in polyacrylamide gel (8%) with a urea/formamide denaturing gradient of 42-67% and 25-50% respectively for the domains Bacteria and Archaea (BEN-AMOR *et al*., 2005). The denaturing gradient for Bacteria followed the standard range observed in literature. The denaturing gradient for Archaea was adapted from the standard range observed in literature to better express the bands in the gel. The equipment used was a D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Inc.), with 0.5x TAE buffer at 60 °C and 85 V for 16 h for PCR products of Bacteria; and at 60 °C and 65 V for 18 h for Archaea PCR products. After the DGGE, the gels were stained with SYBR Green I dye (1:10.000 dilution; Sigma-Aldrich, St.

Louis, MO, USA) during 1 h and subsequently displayed in a UV transilluminator (Bio Rad Laboratories, Inc.).

Individual bands were excised from DGGE gels with a sterile blade, resuspended in 50 µl ultrapure water and kept at 60 °C for 1 h to allow the extraction of the PCR products from the gel. Excised PCR products were purified with PCR purification kit GenElute DNA Purification Kit (Sigma-Aldrich, St. Louis, MO, USA). The excised bands were chosen by presence, intensity and frequency in the gel.

4.2.6 Statistical analysis

The DGGE patterns obtained were processed (normalization and analysis) with Bionumerics software v.6.1 (Applied Maths BVBA, Sint-Martens-Latem, Belgium) to score the band pattern. Bands with more than 1% intensity, after background analysis, were considered in this process. Jaccard coefficient was used in order to obtain profile similarities. Cluster analyses were constructed using UPGMA algorithm (Bionumerics software).

Three ecological parameters were calculated, as described by Marzorati *et al.* (2008): the range-weighted richness (Rr) was calculated as the total number of bands; the Shannon diversity index (H) was calculated by the distribution of bands; and the functional organization (Fo) was calculated by measuring the normalized area between Pareto–Lorenz curve and the perfect evenness line.

The range-weighted richness (Rr) reflects the carrying capacity of a system; the Shannon diversity index (H) reflects the distribution of species in a given environment; and the functional organization (Fo) represents the relation between functional distribution (structure vs. functionality) of the microbial community (LEBRERO *et al*., 2013; MARZORATI *et al*., 2008).

4.2.7 Sequencing and phylogenetic analysis

The sequencing was performed by Sanger sequencing method (SANGER *et al*., 1977) at the Molecular Biology Laboratory, at the Institute of Marine Science. The taxonomic relationships of the sequences were determined using the RDP classification tool (WANG *et al*., 2007), with 90% confidence level. The obtained sequences (accession numbers upon acceptance) were also compared with GenBank gene bank through the search tool BLAST,

the National Center for Biotechnology Information (National Centre for Biotechnology Information NCBI) (MCGINNIS; MADDEN, 2004).

4.3 Results and discussion

4.3.1 Hydrogen production and yield

In order to induce a higher acidogenic production the methanogenic organisms must be inhibited, which gradually occurred due to the increase in OLR, which also increased biogas production, hydrogen production and yield and acetic and valeric acid concentration. Furthermore, methane production gradually decreased along the operation. The biogas detected in the reactors contained hydrogen, methane and carbon dioxide.

The operational stages, time, OLR, pH, hydrogen partial pressure, biogas production, H_2 and CH_4 percentages on biogas, H_2 production and yield of the reactor during stages I to V are presented in Table 4.1.

Table 4.1. Operational parameters of the UASB reactor fed with residual glycerol at each stage.

Stage	Time[a]	OLR[b]	pH	pH_2[c]	Biogas[d]	CH_4 (%)[e]	H_2 (%)[e]	H_2 Production[f]	H_2 yield[g]
I	24	14.4±3.9	5.7±0.5	0.04	2.43±1.6	12.49	17.62	0.30±0.4	0.005±0.01
II	45	26.5±5.3	5.3±0.2	0.11	6.81±2.1	25.03	20.13	1.39±0.4	0.017±0.01
III	59	39.2±5.5	5.4±0.2	0.19	8.82±1.5	29.74	19.24	1.70±0.3	0.015±0.00
IV	73	48.6±1.6	5.2±0.1	0.63	13.12±1.9	13.45	67.92	6.84±2.4	0.049±0.01
V	84	54.5±14.7	5.2±0.1	0.73	17.11±2.1	0.0	85.17	13.30±2.6	0.076±0.02

[a] Accumulated operating time (days); [b] Organic loading rate ($KgCOD.m^{-3}.d^{-1}$); [c] H_2 partial pressure (atm); [d] Biogas production ($L.d^{-1}$); [e] Gas concentration in biogas; [f] H_2 Production ($L.d^{-1}$); [g] H_2 yield (mol $H_2.mol^{-1}$ glycerol). Values after ± stand for standard deviation.

The biogas production started at an average of 2.43 $L.d^{-1}$, containing 17.62% of H_2 and 12.49% of CH_4 in its composition, for an OLR equal to 14.4 kg COD m^{-3} d^{-1} of residual glycerol and a yield of 0,005 mol $H_2.mol^{-1}$ glycerol. Hydrogen yields and gas production were very low until the 24th day (Stage I), and increased slowly at 45th (stage II) and 59th (stage III). At the 73rd day (stage IV) the gas production increased over 50%, the H_2 concentration and production increased over 200%, and the H_2 yield increased over 220%. At the 84th day (stage V) the H_2 production and yield increased 64% and the H_2 proportion increased over 20%, achieving a biogas production of 17.11 $L.d^{-1}$ with a S_0/X_0 ratio of 2.50. The CH_4 concentration increased between the 24th and the 59th day (stages I to III), gradually decreasing afterwards at the 73rd (stage IV), until no CH_4 was detected at the stage V, indicating that complete inhibition of the methanogenic activity could only be achieved at the final stage of operation.

When the OLR reached 54.5 kg $DQO.m^{-3}.d^{-1}$ (Stage V), the reactor reached its highest average yield (0.076 up to 0.096 mol $H_2.mol^{-1}$ glycerol). The highest biogas and hydrogen production also occurred at this stage (20.00 L $biogas.d^{-1}$ and 13.30 L $H_2.d^{-1}$), indicating that this stage had the most specialized microbiota. The yield was 27% higher than that observed by Vlassis *et al.* (2012), since these authors produced only 0.070 mol $H_2.mol^{-1}$ glycerol, with the same type of inoculum, despite the fact the this was considered to be below average (ITO *et al.*, 2005; NGO *et al.*, 2011; SELEMBO *et al.*, 2009). Furthermore, at stage V (54.5kg $DQO.m^{-3}.d^{-1}$) the reactor presented its highest instability period, including sludge flotation.

Even though the H_2 yield was higher at Stage V, it corresponds to only 8% of the maximum theoretical yield for this substrate (VIANA *et al.*, 2014), which could be caused by the deviation of the metabolic path due to operational aspects, like the one that produces 1,3-propanodiol. According to Seifert *et al.* (2009), this can be caused by an increase in glycerol concentration over 10 g $glycerol.L^{-1}$. Furthermore, this route not only reduces the H_2 yield by being a concurrent route, but it also consumes 1 mol of hydrogen in its metabolic path (SELEMBO *et al.*, 2009). In order to produce H_2 the metabolic route must be a pyruvate-formate, which produces acetic acid, butyric acid and ethanol Biebl (2001). If glycerol is used as substrate to produce hydrogen through butyric fermentation the main fermentation products are 1,3-propanodiol, butyric acid, lactic acid and ethanol (SEIFERT *et al.*, 2009).

Another factor that causes reduction in H_2 yield is the inhibition by high hydrogen partial pressure, which in fact occurred, since pH_2 was higher than 0.3 atm at stages IV and V. This increase in partial pressure changes NADH and ferredoxin mechanisms, causing

decrease in H_2 production (HAWKES *et al*., 2007). Due to these changes, the microbiota is induced to change its metabolic path from butyric acid to propionic acid, due to the NADH/NAD^+ ratio, since the propionic acid route produces more NAD^+ than the butyric route (SIVAGURUNATHAN *et al*., 2014).

The fermentation products can have a negative impact in H_2 yield, like propionic acid, one of the main products in this experiment, ranging from 32-46%, the higher proportion between the acids produced. The higher concentration of propionic acid also can inhibit acidogenic and H_2-producing bacteria, reducing the yield even further. The fermentation products produced along the operation are described in Table 4.2, according their concentration and stage in which they were produced.

Table 4.2. Average concentration of the main acids produced in each stage of operation of the UASB reactor fed with residual glycerol.

Stage	C2	C3	C4	C5	C6	C8	C9
I	0.7±0.5	2.9±1.8	0.3±0.3	1.4±1.3	0.1±0.1	0.0±0.1	0.3±0.3
II	1.0±0.3	3.1±1.4	0.7±0.3	1.8±1.4	0.1±0.1	0.1±0.1	0.4±0.2
III	0.4±0.3	1.8±1.7	0.3±0.2	1.9±1.3	0.1±0.1	0.1±0.2	0.2±0.2
IV	1.6±0.4	4.3±0.7	0.5±0.2	1.9±0.4	0.2±0.2	0.1±0.2	0.3±0.4
V	1.8±0.9	6.0±1.4	0.6±0.2	2.9±0.9	0.1±0.0	0.0±0.0	0.5±0.4

C2, C3, C4, C5, C6, C8, and C9 stand for concentration ($g.L^{-1}$) of acetic acid, propionic acid, butyric acid, valeric acid, caproic acid, caprylic acid, and pelargonic acid, respectively. Values after ± stand for standard deviation.

4.3.2 Microbial community characterization

Ecological interactions between microorganisms inside a reactor can directly affect stability and/or function of the system, either increasing or decreasing it. It is well known that the diversity inside a reactor can constantly shift due to population changes through competition for resources. Furthermore, allochthonous and indigenous microorganisms could compete for the available resources with the acidogenic population, directly affecting production and yield (MARIAKAKIS *et al.*, 2011; VASCONCELOS; LEITÃO; SANTAELLA, 2016; XING *et al.*, 2005). The increase in OLR produced different effects on the microbial community during the operation, according to the ecological parameters observed in Table 4.3.

The production and yields were related to influent COD and microbial ecology interactions. The rapid increase in bacterial community's richness (Rr) and diversity (H) from the seed to stages I, II and III indicate that these loading rates are inducing the community to grow more diverse and stable. Despite the fact that these stages do not correspond to the higher productivity, they do correspond to the highest diversity and richness. Elevated richness and diversity promote increased stability in an ecosystem (MILLS *et al.*, 2003), thus these stages correspond to the most stable stages of the reactor operation. Mariakakis *et al.* (2011) found similar results, decreasing the diversity when the OLR increased above the range of 20-30 $kgCOD.m^{-3}.d^{-1}$. The following stages (IV and V) presented a decrease in richness and diversity, which indicates that these species were probably sensitive to substrate concentration, where an increased OLR resulted in inhibition. Furthermore, some authors found that a significant increase in bacterial richness could adversely influence reactor performance (HAFEZ *et al.*, 2010; KIM *et al.*, 2010; KOSKINEN *et al.*, 2007), probably due to competition. A similar result was found in this study, since the stages with lowest richness presented the highest levels of productivity (Table 4.3).

Table 4.3. Ecological parameters of the reactor. richness (Rr), diversity (H) and functional organization (Fo) of domains Bacteriaand Archaea.

Stage	Time (days)[a]	OLR[b]	Bacteria			Archaea		
			Richness	Diversity	Functional Organization	Richness	Diversity	Functional Organization
Seed	N/A	N/A	38.72	2.32	48.35	119.24	3.35	31.93
I	24	14.4	136.23	2.98	53.69	169.44	3.65	31.90
II	45	26.5	194.06	3.32	54.73	135.36	3.53	30.35
III	59	39.2	59.63	2.52	59.36	92.40	3.43	26.47
IV	73	48.6	25.87	2.45	61.24	59.33	3.15	24.67
V	84	54.5	11.53	2.04	59.56	29.01	2.83	24.25

[a] Accumulated operating time (days); [b] Organic loading rate (KgCOD.m^{-3}.d^{-1})

The bacterial functional organization (Fo) gradually increased from the seed to stages I, II, III and IV, decreasing slightly at stage V. The stage IV presented the highest values of H_2 production, yield and concentration as well as the highest functional organization (Fo) for bacteria. According to Marzorati *et al*. (2008), the higher the Fo, the most specialized a community becomes, in which a small amount of the species is dominant. This is considered a highly functionally organized community, however lacks resistance to external changes because the low diversity and equitability promote lower functional redundancy and less available species to recover after the disturbance (MARZORATI *et al*., 2008). The decrease in Rr and H, while the increase in Fo indicates that the community is becoming more specialized towards the use of the substrate, which can be confirmed by the increased production of biogas, increased production and yield of H_2, increased production of acetic and valeric acid. Furthermore, the archaeal species were progressively inhibited, until no CH_4 was detected at the stage V, thus methanogenic activity could only be completely inhibited at the final stage of operation.

The archaeal community increased it richness (Rr) and diversity (H) significantly from the seed to stage I (OLR 14.4), decreasing onwards. The archaeal functional organization (Fo) gradually decreased from the seed to stages I to V. Methane production increased at stages II and III, achieving the highest levels of CH_4 production during the operation, despite the fact that the archaeal species richness and diversity were both significantly reduced at stage III. That occurs due to the average functional organization, which implies that these species are probably resistant and stable enough to endure the constantly increasing OLR. This resistance match what has been reported about the species *Methanosarcina sp*. This species has high growth rates and is resistant to changes caused by overloading when compared to other methanogens (CONKLIN *et al*., 2006; LIU *et al*., 2011; SHIN *et al*., 2010), being able to grow at low retention times (i.e. as low as 4 d) (Conklin *et al*., 2006) and high OLR (SCHÜRER; NORDBERG, 2008).

Even though methanogenic production was reduced down to not being further detected, archaeal organisms were still present. That might have occurred due to the inoculum being rich in *Methanosarcina sp*., which could start to grow faster than other archaeal species, and cause the methanogenic community to be dominated by *Methanosarcina sp*., promoting enhanced stability (CONKLIN *et al*., 2006; YU *et al*., 2006). Some authors suggest that certain substances present in the crude glycerol may increase the methane potential production, such as palmitic acid found in crude glycerol (between 1.5 and 3.0 $g.L^{-1}$), which

could produce many times more CH_4 than does the pure glycerol (HAZIMAH *et al*., 2003; VIANA *et al*., 2012).

4.3.3 Microbial community identification

A sequencing analysis was done in order to identify the main species in the reactor. The taxonomic relationships of the sequences were determined with 80% confidence level. The obtained sequences were also compared with other GenBank gene bank through the search tool BLAST (National Centre for Biotechnology Information NCBI – MCGINNIS; MADDEN, 2004). Table 4.4 presents the main species, with over 90% similarity. Figure 4.1 presents the DGGE patterns from both domains Archaea and Bacteria. The excised bands were numerated based on their sequence displayed on Table 4.4, from 1 to 4 for Archaea and 1 to 8 for Bacteria. Figure 4.2 presents the dendrograms of both domains.

The clones of the Bacteria domain contain sequences mainly related to *Firmicutes* and *Proteobacteria* and the Archaea domain contain sequences mainly related to Euryarchaeota, specially related to *Methanosarcina*, *Methanosarcinales* and *Methanobrevibacter*. *Methanosarcina*, and *Methanosarcinales* contain methane producing prokaryotes that utilize carbon dioxide (DEMIREL; SCHERER, 2008). Other study found these organisms in granular sludge of UASB reactors used to treat brewery wastes, along *Methanosaeta* and *Methanosarcina* (KEYSER *et al*., 2006).

Table **4.4**. Main species found in the UASB reactor fed with residual glycerol. Over 90% similarity.

Bacteria					Archaea				
Band	Closest Relative	% G+C	% identity	Phylum	Band	Closest Relative	% G+C	% identity	Phylum
B1	*Desulfovibrio sp.*	66.5	100%	Proteobacteria	A1	Uncultured *Methanosarcina sp.*	42.7	99%	Methanosarcinales
B2	*Klebsiella pneumoniae*	57.5	99%’	Proteobacteria	A2	Uncultured *Methanobrevibacter sp.*	31.3	99%	Euryarchaeota
B3	Uncultured *Firmicutes* bacterium	55	99%	Firmicutes	A3	Uncultured *euryarchaeote*	-	92%	Euryarchaeota
B4	*Acidaminococcus sp.*	50.2	99%	Firmicutes	A4	Uncultured *Methanosarcinales*	-	91%	Methanosarcinales
B5	Uncultured *Ruminococcus sp.*	41.3	98%	Firmicutes					
B6	Uncultured *Atopobium sp.*	35	99%	Actinobacteria					
B7	*Clostridium sticklandii*	33	98%	Firmicutes					
B8	*Pelomonas saccharophila*	-	100%	Proteobacteria					

Fig. 4.1. DGGE patterns of anaerobic communities from both domains Archaea (a) and Bacteria (b).

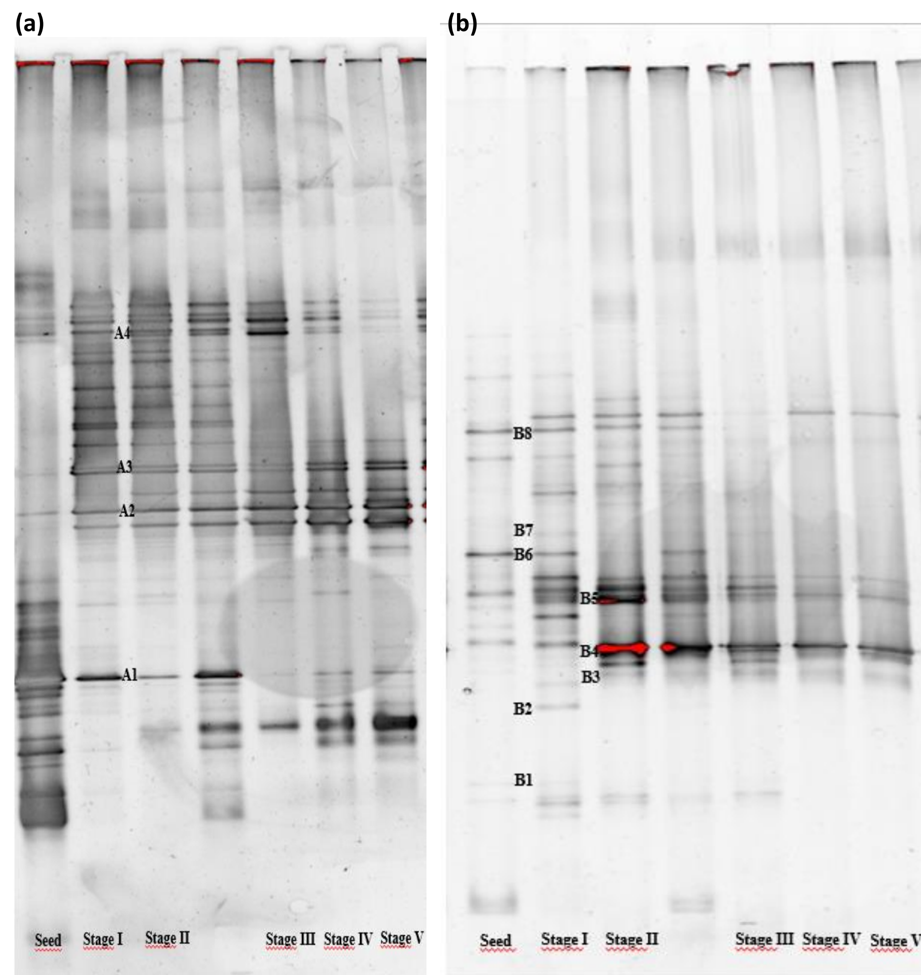
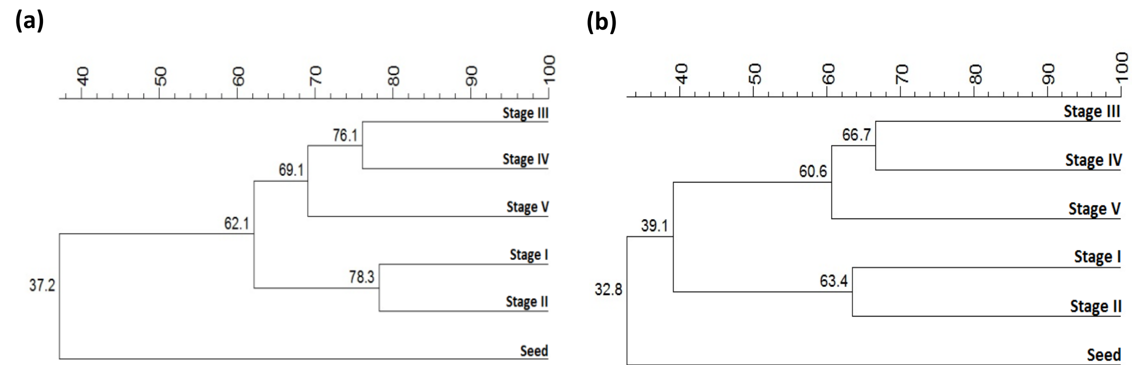


Fig. 4.2. Archaeal (a) and Bacterial (b) dendrograms.



The Firmicutes is a spore-forming taxon, widely studied and known as dominant bacterial group, capable of tolerating harsh conditions due to their spore-forming ability and able to colonize many types of environments (FILIPPIDOU *et al*., 2015). One of the identified species belonging to the phylum Firmicutes is Clostridium sticklandii, which is known as a producer of organic acids such as acetate, butyrate and propionate via the Stickland reaction (SNEATH *et al*., 1986). Kim *et al*. (2011) suggest that this species can be related to protein digestion, especially from the substrate or even cellular remains.

The similarity between stages for Archaea was:

- Seed and Stage I: 37,2%;
- Stages I and II: 78,3%;
- Stages II and III: 62,1%;
- Stages III and IV: 76,1%;
- Stages IV and V: 69,1%.

The similarity between stages for Bacteria was:

- Seed and Stage I: 32,8%;
- Stages I and II: 63,4%;
- Stages II and III: 39,1%;
- Stages III and IV: 66,7%;
- Stages IV and V: 60,6%.

The low similarity between seed and stage I in both domains Archaea (37,2%) and Bacteria (32,8%) occurred due to the development of a community populated by groups of generalist organisms, with no specific dominance, which can be explained by the OLR applied and impurities of the residual glycerol, which favor diversification, with increase in population Rr and H.

The dendrograms and patterns for Bacteria and Archaea between the five stages indicates that diversity and richness was most similar at the stages I to III than at the final stages IV and V, for both domains. However, the reason for each increase and decrease in parameters is different for each domain: for Bacteria, the similarity in the initial stages is probably due to the generalist species, since residual glycerol at low OLRs present a diverse community with low productivity. Subsequently, diversity and richness decrease, thus the similarity between stages also decreases.

The similarity of Archaea between stages I and II (78,3%) and stages II and III (62,1%) indicate the maintenance of general community diversification, promoted by the OLR and impurities of the residual glycerol observed on stage . However, despite the high

initial similarity, it gradually decreases over time, since the similarity index reached 62.5%, which indicates selection of resistant organisms, which resisted until the last stages (IV - 76,1% and V - 69,1%).

The similarity of Bacteria between stages I and II (63,4%) and stages II and III (39,1%) indicate that the initial community diversification and high similarity decreased abruptly, promoted by the increase in OLR and consequent initial dominance of most adapted and productive species, which is corroborated by the increase (10%) in Fo and decrease in population parameters (Rr and H) observed between stages II and III.

These similarities decreased on stages IV (66,7%) and V (60,6%), due to selection. At the final stage (V), the similarity decreases significantly, due to the level of specialization of the bacterial domain. For Archaea, the similarity in the initial stages is very similar to the one observed in Bacteria, also due to the presence of generalist species, however the decline in both diversity and richness occurs earlier, after stage II, since to most of the methanogens are not resistant to higher concentrations (OLR), thus only a few restricted species were capable of surviving.

Three main bacterial phyla were detected after sequencing: Actinobacteria, Firmicutes and Proteobacteria. In Archaea, two phyla were detected after sequencing: Euryarchaeota and Methanosarcinales.

4.4 Conclusions

The bacterial Rr/H at stages I/II/III indicates favoring of generalist species due to increased carrying capacity and niche diversification. Thus, higher Rr/H promotes higher stability and higher Fo promotes less stability/resilience, due to decreased genetic pool.

Increases in Fo and decreases in Rr/H indicate community specialization, therefore, the substrate degradation is more efficient. Archaeal, parameters decreased (stages III/IV) due to inhibition. Methane was undetected at stage V, indicating that the generalist species were subsequently inhibited.

H_2yield was limited by pH_2, Fo and propionic acid. OLR increases negatively impact methanogens, however, even in high loads could not eliminate the remaining archaea.

4.5 Acknowledgements

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5 MICROBIAL COMMUNITY ANALISYS OF ACIDOGENIC UASB REACTORS TREATING GLYCEROL UNDER INCREASING ORGANIC LOADING RATES

5.1 Introduction

Biodiesel is a biofuel with high potential due to its advantages when compared to other traditional fuels, including its renewable nature, high biodegradability, low emissions, no need for adaptations in the engines and absence of carbon dioxide emission (CHAND, 2002). Biodiesel is obtained through transesterification, which produces biodiesel and glycerol (BALAT, 2005); it is estimated that for every 100 kg of biodiesel, 10 kg of glycerol are produced (PAPANIKOLAOU *et al.*, 2008).

The biofuel production has increased worldwide: between 2000 and 2005 the world production has tripled, rising from less than 1 million tons in 2000, to 2.92 million tons in 2005 (MOUSDALE, 2008). According to Licht (2010), in 2009 the world production reached 16 million tons of biodiesel, which would result in an approximate production of 1.6 million tons of glycerol.

Currently, the glycerol produced is bought by food, pharmaceutical, and cosmetics industries, however an increasing demand for biodiesel as a fuel will result in overproduction of glycerol, turning it into an environmental liability (VIANA *et al.*, 2012). This residue can be converted through anaerobic digestion to volatile fatty acids (VFA) and biogas, including H_2 and CH_4 (ITO *et al.*, 2005).

The anaerobic digestion, also called dark fermentation", consists in four steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. H_2 and VFA are produced in the second and third steps, respectively, and are consumed in the fourth step, that consists in methane production by hydrogenotrophic methanogenic archaea to produce CH_4 (MCCARTY, 1964). Therefore, to increase the production and yield of a acidogenic reactor, the methanogenesis step must be inhibited.

Residual glicerol from biodiesel industry can be converted to methane (SILES-LÓPEZ *et al.*, 2009), as well as hydrogen and VFAs (ROSSI *et al.*, 2011) Studies show that a two-phase system comprised of an acidogenic reactor associated to a methanogenic reactor in series, increase the total energy efficiency, since the VFAs can be converted to CH_4 in the second reactor, in addition to the H_2 generated in the first (VIANA *et al.*, 2012).

This research is part of a macroproject, aiming to produce H_2 and CH_4 from residual and pure glycerol. Although the objectives were fulfilled, the lack of comprehension of the microbial community structure and ecology did not allow for a proper assessment of how the biological and ecological factors interfere with operational processes. It is known that the operational aspects affect the ecological parameters of the microbial community in a acidogenic reactor, thus, they are directly linked to the productivity.

Therefore, information on the microbial development through the use of molecular techniques are essential to understand the reactor performance because they reveal the correlation among biogas production, VFAs produced or consumed and the shifts in bacterial community at different stages (BUZZINI *et al.*, 2006; CASSERLY; ERIJMAN, 2003). Thus, it is necessary to study these relations in order to understand how they impact on performance.

The goals of this paper are to assess: (i) ecological parameters (Shannon diversity index, range-weighted richness and functional organization) in each stage of both operational phases of the reactor; (ii) changes in microbial community structure through diversity and richness shifts along the operation; and (iii) the relation between ecological and operational parameters and the impact on the reactor performance (biogas production, yield and VFAs produced) along the stages of both phases. This knowledge could promote the selection of a microbial consortium and/or operational conditions with greater efficiency in biogas production, improving the performance of the reactor.

5.2 Materials and Methods

5.2.1 Bioreactors set up

The system consisted of two upflow anaerobic sludge bed (UASB) reactors in lab-scale, made of a vertical cylinder of borosilicate glass. Eight taps were installed vertically along the reactor to allow sampling of the sludge. The operating volume of the reactors were different, due to the two phases:

- Phase I: the reactor was operated as UASB reactor with working of 14.85 L.
- Phase II: the reactor was filled with support medium comprised of polyvinyl chloride (PVC) cylinders (specific area: 907 $m^{-2}.m^{-3}$), thus being modified to a fixed bed reactor with a working volume of 13.08 L.

A dosing pump (LMI Milton Roy, model P133-398TI) was set to control automatically the pH, with a NaOH solution 0.5% (v/v). . DNA sampling were performed manually.

The chemical oxygen demand (COD) and total volatile solids (TVS) were determined according to Eaton *et al*., 2005. The biogas volume was verified by a Ritter gasometer and the composition was evaluated by a micro gas chromatography with nitrogen as carrier gas.

The concentrations of volatile fatty acids (VFA) were determined by high-performance liquid chromatography (HPLC), with adapted methodology of Fuentes *et al.* (2009), under the following conditions: Agilent Zorbax C18 column (150 x 4.6 mm) maintained at 25° C; Ultraviolet/Visible detector Varian UV 325 Polaris 215 nm, having as mobile phase acetonitrile/water (3:7) with 0.01% sulfuric acid at 0.4 $mL.min^{-1}$ flow. The injected sample volume was 20 uL, the samples were previously filtered on cellulose acetate membrane ME25 with pores of 0.45 um.

5.2.2 Substrate and inoculation

The H_2 reactor was fed with pure glycerol (99.5% purity) in both phases. The COD of the glycerol in both phases was 1418 g $COD.L^{-1}$ of organic matter concentration. In order to achieve the desired COD for each stage of operation, the influx was adapted at each stage (I to V), in both phases (I and II). The influent was fed upflow from the bottom to the upper part of the reactor by a dosing pump, and the wastewater was discharged through an effluent port.

The affluent used in phase 1 was composed of pure glycerol with addition of a nutrient solution. The affluent used in phase 2 was composed of pure glycerol, nutrient solution and of chloroform 0.05%. The nutrient solution used in every operational stage was adapted from Lin and Lay (2005), in the following concentrations (mg/L): $MgCl_2 \cdot 6H_2O$ (40.0); $CoCl_2 \cdot 6H_2O$ (5.0); $CaCl_2 \cdot 2H_2O$ (0.1); $NiCl_2 \cdot 6H_2O$ (2.5); $MnCl_2 \cdot 4H_2O$ (10.6); KCl (1.1); $NH_4H_2PO_4$ (107.5); $ZnCl_2$ (0.1); $FeSO_4 \cdot 7H_2O$ (5.0); $MnSO_4 \cdot H_2O$ (0.3); $CuSO_4 \cdot 5H_2O$ (5.0); $(NH_4)6Mo_7O_{24} \cdot 4H_2O$ (51.1). The inoculum consisted of a mixed sludge obtained from a UASB reactor for anaerobic digestion in a domestic sewage treatment plant. There was no pre-treatment of the inoculum therefore the operational conditions were the only factors to induce or to prevent shifts in microbial diversity.

5.2.3 *Operation strategy*

The reactor was operated in two phases:

- Phase I (UASB reactor): The reactor was operated in five stages with increasing OLRs (from 18.8 kg COD.m^{-3}.d^{-1} to 61 kg COD.m^{-3}.d^{-1});
- Phase II (UASB turned into a fixed bed reactor): The reactor was operated in five stages with increasing OLRs (from 20.0 kg COD.m^{-3}.d^{-1} to 42.9 kg COD.m^{-3}.d^{-1}) with pure glycerol as substrate and chloroform added. At Stage IV (41.2 kg COD.m^{-3}.d^{-1}) the substrate was changed to residual glycerol. Furthermore, at Stage V (50.0 kg COD.m^{-3}.d^{-1}), chloroform was not added anymore, in order to observe if the methanogens were completely inhibited.

The OLR was increased whenever the reactors were adapted, observed when: the theoretical OLR was achieved at each stage, when the pH, biogas production and COD were within the range of planned values.

5.2.4 *DNA extraction and amplification of the 16S rRNA*

The DNA extraction was performed using Fast extraction ® DNA Spin Kit for Soil (MP Biomedicals, LLC), following the manufacturer's protocol, with the following modifications: rotation speed (13900 rpm) and duration (20 minutes) in the centrifuge; speed and time of agitation equipment Mini-Bead-beater for cell disruption (30 seconds of disruption, followed by one minute on ice, followed by 20 more seconds of disruption); turn-around time of samples to grip the DNA to the silica matrix (one hour). The extracted DNA was identified and stored at -18° C and, subsequently, quantified using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific).

DNA was amplified by the polymerase chain reaction (PCR) technique for both domains Bacteria and Archaea using primers with GC clamps for further analysis by DGGE. In the domain Archaea, regions V2-V3 of 16S rRNA gene were amplified with the specific primers 0515R-GC: 5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAT CGT ATT ACC GCG GCT GCT GGC AC-3'; and 0109F-T: 5'-ACT GCT CAG TAA CAC GT-3' (Sigma Aldrich, St. Louis, MO, USA) (Grosskopf *et al.*, 1998; Muyzer *et al.*, 1993). In the domain Bacteria, regions V6-V8 of 16S rRNA gene were amplified, using bacterial universal primers 1401R: 5'-CGG TGT GTA CAA GAC CC-3'; and 0968F-GC: 5'-

CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3' (Sigma Aldrich, St. Louis, MO, USA; Nübel *et al*., 1996).

The PCR mix (50 µL) contained: 10 µL of reaction buffer (5x); 5 µL of $MgCl_2$ (25 mM); 0.25 µL of Taq polymerase (5u µL) (Promega, USA); 1 µL of deoxynucleotide triphosphates (10 mM); 1 µL of the extracted DNA; 1µL of PCR primers (10 µM); and nuclease free water (Promega, USA) up to a final volume of 50 µL. The PCR was carried out in a Thermal Cycler iCycler (Bio Rad Laboratories, Inc.). The amplification for Bacteria followed the protocol: initial denaturation at 95° C for two minutes; 31 cycles of denaturation at 95° C for 30 seconds, annealing at 58° C for 45 seconds, and elongation at 72° C for one minute; followed by a final elongation at 72° C for 6 minutes. DNA amplification of Archaea followed the protocol: initial denaturation at 95° C for two minutes; 31 cycles of denaturation at 95° C for 30 seconds, annealing at 52° C for 40 seconds, and elongation at 72° C for 90 seconds; followed by a final elongation at 72° C for 6 minutes. The sizes of PCR products were estimated with a DNA marker of 1000 base pairs Hypperladder II (Bioline, USA Inc.) in agarose gel electrophoresis (1.8%) and stained with SYBR Green I.

5.2.5 Denaturant gradient gel electrophoresis (DGGE)

Denaturant gradient gel electrophoresis analisys were performed in polyacrylamide gel (8%) containing denaturants urea/formamide forming grandients ranging from 42% to 67% and 30 to 60% respectively for the domains Bacteria and Archaea (BEN-AMOR *et al*., 2005). The equipment used was a D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Inc.), with TAE buffer 1x at 60° C and 85 V during 16h for PCR products of Bacteria; and at 60° C and 65 V during 18h for Archaea PCR products. After the DGGE, the gels were stained with SYBR Green I dye (1:10.000 dilution; Sigma-Aldrich, St. Louis, MO, USA) during 1h and subsequently displayed in a UV transilluminator (Bio Rad Laboratories, Inc.). Individual bands were excised with a sterile blade, ressuspended in 50 µl ultrapure water and kept at 60° C for 1h to allow the extraction of the PCR products from the gel. Excised PCR products were purified with PCR purification kit GenElute DNA Purification Kit (Sigma-Aldrich, St. Louis, MO, USA).

5.2.6 Statistical analysis

Normalization and analysis of the DGGE patterns were done with Bionumerics software v.6.1 (Applied Maths BVBA, Sint-Martens-Latem, Belgium) in order to determine band patterns. Only bands with more than 1% intensity, after background analysis, were considered in this procees. The profile similarities were calculated with Jaccard coefficient. Cluster analyses were constructed using UPGMA algorithm (Bionumerics software). After the analysis, three ecological parameters were obtained, according to Marzorati *et al*. (2008): the range-weighted richness (Rr); the Shannon diversity index (H); and the functional organization (Fo). The range-weighted richness (Rr) reflects the carrying capacity of a system. The Shannon diversity index (H) reflects the distribution of species in a given environment. The functional organization (Fo) represents the relation between functional distribution (structure vs. functionality) of the microbial community (LEBRERO *et al*., 2013; MARZORATI *et al*., 2008).

5.2.7 Sequencing and phylogenetic analysis

The Sanger sequencing method (SANGER *et al*., 1977) was performed at the Molecular Biology Laboratory, at the Institute of Marine Science, in order to identify the taxa. The taxonomic relationships of the sequences were determined using the RDP classification tool (WANG *et al*., 2007) with 80% confidence level. The obtained sequences were also compared with other GenBank gene bank through the search tool BLAST, the National Center for Biotechnology Information (National Centre for Biotechnology Information NCBI) (McGinnis and Madden, 2004).

5.3 Results and discussion

5.3.1 Operational results

In order to assess the relation between community structure and ecology with reactor functionality, some operational parameters were used: phases, time (days), OLR, pH, H_2 partial pressure, biogas production, H_2 and CH_4 percentages on biogas, H_2 production, and H_2 yield for both phases (I and II) of the reactor during stages I to V are presented in Table 5.1.

Table 5.1. Operational parameters of the reactor for Phases I (a) and II (b): Stages, time, OLR, pH, hydrogen partial pressure, biogas production, H_2 and CH_4 percentages, H_2 production and H_2 yield.

(a) Phase I

Stage	Time[a]	OLR[b]	pH	pH_2[c]	Biogas[d]	H_2 (%)[e]	CH_4 (%)[e]	H_2 Production[f]	H_2 yield[g]
I	9	18.8±2.7	5.5±0.4	0.00±0.0	1.0±0.3	-	-	0.0	0.000±0.00
II	23	28.9±4.9	5.3±0.2	0.03±0.0	0.9±0.5	4	89	0.1	0.000±0.00
III	37	37.4±3.8	6.0±1.2	0.30±0.0	1.9±1.2	5	56	0.1	0.002±0.00
IV	50	54.0±4.6	5.4±0.3	0.38±0.0	2.6±1.9	39	9	0.9	0.011±0.001
V	64	61.5±5.8	5.4±0.1	0.38±0.0	2.1±1.5	36	6	0.8	0.010±0.001

(b) Phase II

Stage	Time[a]	OLR[b]	pH	pH_2[c]	Biogas[d]	H_2(%)[e]	CH_4 (%)[e]	H_2 Production[f]	H_2 yield[g]
I	45	20.0±3.4	5.3±0.1	0.37±0.0	6.8±2.1	37	0	2.6	0.055±0.020
II	103	28.9±4.6	5.3±0.1	0.28±0.1	9.6±3.9	39	0	3.7	0.051±0.020
III	150	42.9±6.9	5.2±0.1	0.46±0.0	34.6±2.6	52	0	15.2	0.134±0.025
IV	184	41.2±4.0	5.6±1.2	0.41±0.1	22.3±5.1	45	0	10.6	0.113±0.030
V	234	50.0±3.4	5.1±0.6	0.41±0.0	24.0±7.1	41	0	10.3	0.105±0.020

[a] Accumulated operating time (days); [b] Organic loading rate ($kgCOD.m^{-3}.d^{-1}$); [c] Hydrogen partial pressure (atm); [d] Biogas production ($L.d^{-1}$); [e] Gas concentration in biogas; [f] Average H_2 -roduction ($L.d^{-1}$); [g] H_2 yield (mol $H_2.mol^{-1}$ glycerol). Values after ± stand for standard deviation.

5.3.1.1 Phase I - Hydrogen production

The average biogas production at the first stage (OLR 18.8 kg $COD.m^{-3}.d^{-1}$) was 1.0 L/d, containing 4% of H_2 and 89% of CH_4, of pure glycerol. Hydrogen yields and gas production were low until stage II (23th day), and gradually increased at stages III, IV and V. Gas production at these stages increased over 80%. During phase I, H_2 yields were low for the entire operation, however, despite the low values obtained, the yield increased over 500% between stages III and IV. Despite that increase, it corresponded to less than 2% of the maximum theoretical yield for this substrate (VIANA *et al.*, 2014).

The increase in H_2 yield and decrease in CH_4 production and concentration along Stages III, IV and V, indicate that this occurred due to inhibition of methanogenesis. However, the inhibition was not complete, since CH_4 production was observed during later stages (IV and V), in which the OLR was high. The highest concentration of H_2 on biogas (39%) occurred at Stage IV. At Stage V the concentration decreased, which indicates inhibition of the hydrogen producing bacteria by excess substrate (ANDREWS, 1968).

When the OLR reached 54,0 kg $COD.m^{-3}.d^{-1}$ (Stage IV), the reactor reached its highest average yield (0.011 mol $H_2.mol^{-1}$ glycerol). The highest biogas and hydrogen production also occurred at this stage (2.6 L $biogas.d^{-1}$ and 0.9 L $H_2.d^{-1}$), indicating that the microbiota was more adapted to the substrate, when comparing to previous or latter stages. The yield was 84% lower than that observed by Vlassis *et al.* (2012), which obtained 0.070 mol $H_2.mol^{-1}$ glycerol. Both yields are considered to be below average (ITO *et al.*, 2005; NGO *et al.*, 2011; SELEMBO *et al.*, 2009).

5.3.1.2 Phase II - Hydrogen production

In phase II a support medium was added and the reactor was inoculated again, in order to assess if the medium could retain the sludge, avoiding, or, at least, reducing the flotation and washouts. The biogas production started at an average 6,8 $L.d^{-1}$ (OLR 20.0 kg $COD.m^{-3}.d^{-1}$), containing 37% of H_2 and 0% of CH_4, of pure glycerol and a yield of 0.055 mol $H_2.mol^{-1}$ glycerol. At stage II (103rd day) the biogas production increased 29% and the yield decreased 7%. At the 150th day (stage III) the gas production increased 360% and the yield increased 262%. The H_2 production also increased 410%, from 3.7 $L.d^{-1}$ to 15.2 $L.d^{-1}$. This stage represents the most productive stage in the reactor.

The influx of chloroform was effective, since it properly inhibited the CH_4 production during the entire operation, even in OLR ranges that were adequate for methanogens to produce CH_4, as observed in phase I. On the other hand, the H_2 production increased further. This inhibition is only possible because chloroform prevents the activation of the coenzyme M reductase, thus preventing the CH_4 to be synthesized (NING *et al.*, 2012). Despite the positive effect on H_2 production by inhibiting the methanogens, chloroform can also inhibit fermentative bacteria, including H_2 producers if the concentration is higher than 0,10% (HU; CHEN, 2007).

During the entire operation, CH_4 was not detected on the biogas, proving the effectiveness of chloroform addition. The absence of CH_4 indicates that the methanogens were inhibited, however, they were not completely eliminated, since a few species could be found inside the reactor. Furthermore, the increase in OLR also decreases CH_4 production, partially inhibiting it, as observed in phase I (above 37 kg COD.m^{-3}.d^{-1}) and phase II, even when the chloroform influx was stopped, which corroborates other studies (VIANA *et al.*, 2014; VASCONCELOS; LEITÃO; SANTAELLA, 2016). During the entire operation, no flotation was observed at phase II, thus proving the effectiveness of the support medium in avoiding washouts.

5.3.1.3 Hydrogen yields

The low yields in phases I and II ocurred due to one or more of the following factors:

1) the toxicity caused by high concentration of Na^+;

2) the hydrogen partial pressure (pH2);

3) accumulation of 1,3-PPD and propionic acid; and

4) eventual sludge purges, caused by flotation, indicating instability.

The Na^+ along the operation ranged from 3.8 to 5.0 g.L^{-1}, reaching the inhibitory concentration of 4,5 g/L (MCCARTY, 1964) during part of the operation. The excess Na^+ promotes celullar plamolysis (ZHANG *et al.*, 2014), thus, decreasing the productivity due to reduction in microbial population. The accumulation of H_2 in the headspace can inhibit its production due to increase in hydrogen partial pressure (HALLENBECK, 2009), which was high (above 0,30 atm) during the most of the operation. One alternative to increase the yield would be purging H_2 with N_2 in future experiments.

The flotation in the reactor started at stage III (37.4kg COD.m^{-3}.d^{-1}), when the reactor presented high instability, with high frequency of sludge flotation. At stage IV, the flotation decreased slightly and the production, yield and H_2 percentage increased. At stage V (61.5 kg COD.m^{-3}.d^{-1}), the flotation increased significantly in volume and frequency and after 10 days, 88% of the original inoculated sludge mass had been purged off the reactor. When this large portion of the biomass was purged, the daily biogas production, H_2 production, concentration and yield decreased, when compared to the previous stages.

It has been reported that residual glycerol causes flotation and washouts due to impurities (HUTŇAN *et al.*, 2013; VIANA *et al.*, 2014) however, despite the purity of substrate, flotation occurred. Therefore, it occurred not due to impurities, but due to the sludge being dragged by the upflow of biogas bubbles, produced by the microbiota itself. In order to reduce the flotation and to properly retain the sludge, a support medium was added in phase II.

Despite the significant increase in H_2 yields of phase II when compared to phase I, these values are still below the maximum theoretical yield and the maximum observed yield found in other studies for this substrate (VIANA *et al.*, 2014).

5.3.1.3 Fermentation products

The fermentation products also have influence on the productivity, as shown in Table 5.2, that presents the concentrations of the main acids produced in each stage of the phase I and phase II, respectively.

Table 5.2. Average concentrations of the main acids produced in each stage for phases I (a) and II (b).

(a) Phase I

Stage	Concentration (g.L^{-1})				
	C2	C3	C4	C5	C6
I	0.1±0.0	0.9±0.0	0.3±0.0	0.1±0.0	0.2±0.0
II	0.9±0.4	1.4±0.3	0.5±0.1	0.2±0.1	0.4±0.2
III	0.4±0.0	1.2±0.3	0.9±0.4	0.9±0.7	1.0±1.7
IV	0.8±0.4	0.8±0.5	0.6±0.3	0.4±0.2	0.0±0.0
V	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.0

(b) Phase II

Stage	Concentration (g.L^{-1})							
	C2	C3	C4	C5	C6	1,3-PPD	EtOH	Glycerol
I	0.3±0.1	0.2±0.1	0.7±0.1	N.D	0.0±0.1	3.7±0.3	0.8±0.3	9.8±1.8
II	0.3±0.1	0.5±0.3	1.1±0.4	0.5±0.1	0.4±0.3	2.5±1.7	0.4±0.4	5.2±4.5
III	0.5±0.2	0.5±0.2	1.3±0.3	0.4±0.2	0.6±0.4	3.0±1.1	0.2±0.1	6.3±3.6
IV	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
V	0.3±0.1	1.2±1.7	0.9±0.2	0.3±0.1	0.0±0.0	3.3±1.1	0.2±0.1	11.8±2.0

The letters C2, C3, C4, C5 and C6 correspond to acetic acid, propionic acid, butyric acid, valeric acid and caproic acid, respectively. 1,3-PPD and EtOH correspond to 1,3-propanodiol and ethanol, respectively.

As it can be seen in the table, in stages I, II and III of phase I, the propionic acid was predominant over butyric and acetic acids, which is known to cause instability and also inhibits hydrogen-producing bacteria (KIM *et al*., 2006; SIVAGURUNATHAN *et al*., 2014). At stage II the highest concentration of propionic acid (1.4 $g.L^{-1}$) was achieved, which, considering the inhibitory concentration ranges from 0.8 to 0.9 $g.L^{-1}$, could be listed as one of the causes for the instability and low yield (WEIMER *et al*., 2015). From stage III to V the propionic acid production gradually decreased, since this process is affected by the increase of OLR.

The increase in 1,3-PPD and propionic acid are a direct consequence of the increase in pH_2 (MIZUNO *et al*., 2000; SEIFERT *et al*., 2009), due to shifts in the metabolic route. This is a concurrent metabolic route, thus negatively affecting the H_2 synthesis (BIEBL *et al*., 2001; SEIFERT *et al*., 2009). Since the beginning of the phase II 1,3-propanodiol was detected in concentrations above 2.0 $g.L^{-1}$.

5.3.2 Microbial ecology

The pH in both phases was controlled (between 5.3 and 5.5), therefore, favoring microbial selection and, consequently, improving H_2 production. The pH along the gradual increase in OLR in both phases I and II promoted operational and ecological responses, in both Bacteria and Archaea domains, during the entire operation, which were affected by three main ecological parameters: Range-weighted richness (Rr), Functional organization (Fo), and Shannon diversity index (H). These parameters can be observed in Table 5.3.

Table 5.3. Ecological parameters of the reactor for both phases I (a) and II (b), for domains Bacteria and Archaea.

(a) Phase I

Stage	Time (days)	OLR	Rr	H	Fo
Bacteria					
Seed	N/A	N/A	43.3	2.41	44.7
I	9	18.8	85.5	2.64	51.4
II	23	28.9	122.9	2.88	60.9
III	37	37.4	41.0	2.21	63.0
IV	50	54.0	30.7	1.93	72.4
V	64	61.5	19.4	1.84	62.3
Archaea					
Seed	N/A	N/A	134.3	2.47	37.2
I	9	18.8	75.1	2.24	33.7
II	23	28.9	34.5	2.05	31.2
III	37	37.4	25.8	1.63	28.2
IV	50	54.0	16.7	1.41	21.5
V	64	61.5	8.7	1.28	18.6

(b) Phase II

Stage	Time (days)	OLR	Rr	H	Fo
Bacteria					
Seed	N/A	N/A	40.0	2.42	4.1
I	45	20	67.7	2.61	59.8
II	103	28.9	117.0	2.79	67.6
III	150	42.9	27.8	2.20	79.1
IV	184	41.2	58.9	2.29	51.5
V	234	50	34.0	2.08	54.1
Archaea					
Seed	N/A	N/A	126,6	2,48	31.9
I	45	20	5,4	1,66	14.9
II	103	28,9	3,7	1,42	11.0
III	150	42,9	3,0	1,23	7.5
IV	184	41,2	4,3	1,21	8.8
V	234	50	10,9	1,32	14.6

The increase in richness and diversity of the bacterial community from seed to stages I and II in both phases indicates that the loading rates, especially in stage II (below 30 kg $COD.m^{-3}.d^{-1}$) can induce the community to a more diverse and stable state, with a higher carrying capacity, thus reflecting in the increases in Rr. However, when the OLR was increased above these values, the stability, richness and diversity decreased, which is in accordance with other study that observed a significant reduce in diversity when the OLR of glycerol increased over this same range of 20-30 kg $COD.m^{-3}.d^{-1}$ (MARIAKAKIS *et al*., 2011).

Despite the increase in richness, diversity and stability, these initial stages are not very productive, on the opposite; they represent the lowest productivity of the entire operation, in both phases. These findings corroborate with other study that shows that the relation between diversity and productivity in bioreactors is not linear (VASCONCELOS; LEITÃO; SANTAELLA, 2016). Notwithstanding, some authors show the possibility of reduced productivity in higher diversities and other adverse influences in reactor performance (HAFEZ *et al*., 2010; KIM *et al*., 2010; KOSKINEN *et al*., 2007; MARIAKAKIS *et al* 2011; VASCONCELOS; LEITÃO; SANTAELLA, 2016; XING *et al*., 2005).

The increase in functional organization (Fo) of bacteria in phase I indicates the successive increase of specialized species and the gradual reduction of generalist species, especially after the stage II, where diversity was maximum, but declined gradually from this point, reflection of the expertise of the community.

The successive increase of Fo on phase I had direct relationship with the amount of biogas produced ($L.d^{-1}$), the percentage of H_2 in the biogas, as well as the molar production of H_2 (mol $H_2.mol^{-1}$ of glycerol), which increased gradually, especially at stage IV, where the Fo for the bacterial community reached approximately 72.4%, highlighting the adaptation of more specialized and productive bacteria.

The functional organization (Fo) of bacteria in phase II increased from the inoculum, stages I, II and III and decreased on stage IV. This reduction of Fo in stage IV is directly related to the decrease in biogas production ($L.d^{-1}$) and yield (mol $H_2.mol^{-1}$ of glycerol), reflecting also the recovery of diversity and richness, due to the introduction of a substrate containing impurities (including long chain fatty acids), which increased the number of generalist species that took advantage of the less-specific substrate (residual) rather than the previous (VASCONCELOS; LEITÃO; SANTAELLA, 2016). In phase II, the highest Fo observed was at stage III, and it was directly related to biogas production ($L.d^{-1}$), H_2

percentage, as well as the yield (mol $H_2.mol^{-1}$ of glycerol), which reached 79.1%, highlighting the highest specialization and adaptation of more productive species.

The archaeal Rr, H and Fo in phase I decreased and affected the methane production, which probably occurred due to two factors: the substrate is more suitable for acidogenic bacteria than methanogens; and due to archaea being more sensitive to high concentrations of glycerol, promoting inhibition by increasing the OLR. In phase II, the same tendency can be observed up until stage III, however archaeal Rr, H and Fo decreased significantly faster than the previous phase, since the addition of chloroform inhibited the methanogens.

Notwithstanding, in stages IV and V the three ecological parameters (Rr, Fo and H) increased slightly, which can be seen by the decrease in H_2 production and yield, and the slight increase in methane percentage on biogas in the last stage. This occurred due to the change of substrate (in stage IV), which promoted a small recovery of methanogens (in stage V), after the chloroform influx was stopped. Despite that, the archaeal community did not recovery further. The absence of chloroform and the various substances present in residual glycerol induced this small recovery of parameters. A few studies suggest that certain substances in the crude glycerol could increase the methanogenic potential (HAZIMAH *et al.*, 2003; VIANA *et al.*, 2012). One of the possible substances would be palmitic acid (C16), found in crude glycerol (between 1.5 and 3.0 $g.L^{-1}$), since the carbons of this fatty acid could induce a greater production of CH_4 when compared to pure glycerol (VIANA *et al.*, 2012). This study corroborates the increase of richness, diversity and functional organization occurred only at stage V of phase II and did not occurred in phase I, despite the fact that this phase had no chloroform added.

Despite the fact the archaeal species richness and diversity were both significantly reduce, the presence of these species implies that they are probably resistant enough to endure the increasing OLR, which matches the endurance level that has been reported about *Methanosarcina* sp (CONKLIN *et al.*, 2006; SCHÜRER and NORDBERG, 2008; LIU *et al.*, 2011; SHIN *et al.*, 2010). However, no CH4 was detect further, even when methanogens were observed. This is probably due to the inoculum being rich in *Methanosarcina* sp., which could outcompete other species and rapidly grow, becoming dominant.

5.3.3 Microbial community structure and identification

The Table 5.4 presents the main species found in the reactor, in both phases, respectively. The figures 5.1 and 5.2 represent the patterns and dendrograms, respectively, for phases I and II, for both domains Bacteria and Archaea.

Table 5.4. Main species found in Phases I (a) and II (b) for both domains. Over 90% similarity.

		(a) Phase I		
		Bacteria		
Band	**Closest Relative**	**% G+C**	**% identity**	**Phylum**
B1	*Desulfovibrio sp.*	66.5	100%	Proteobacteria
B2	*Klebsiella pneumonia*	57.5	99%	Proteobacteria
B3	*Eubacterium sp.*	56.8	99%	Firmicutes
B4	Uncultured *Firmicutes* bacterium	55	99%	Firmicutes
B5	*Acidaminococcus sp.*	50.2	99%	Firmicutes
B6	Uncultured *Ruminococcus sp.*	41.3	98%	Firmicutes
B7	*Clostridium sticklandii*	33	98%	Firmicutes
		Archaea		
Band	**Closest Relative**	**% G+C**	**% identity**	**Phylum**
A1	Uncultured *Methanosarcina sp.*	42.7	99%	Methanosarcinales
A2	Uncultured *Methanobrevibacter sp.*	31.3	99%	Euryarchaeota
A3	Uncultured *euryarchaeote*	-	92%	Euryarchaeota
A4	Uncultured *Methanosarcinales*	-	91%	Methanosarcinales
		(b) Phase II		
		Bacteria		
Band	**Closest Relative**	**% G+C**	**% identity**	**Phylum**
B8	*Klebsiella pneumoniae*	57.5	99%	Proteobacteria
B9	*Eubacterium sp.*	56.8	99%	Firmicutes
B10	Uncultured *Firmicutes* bacterium	55	99%	Firmicutes
B11	*Acidaminococcus sp*	50.2	99%	Firmicutes
B12	*Clostridium sticklandii*	33	98%	Firmicutes
		Archaea		
Band	**Closest Relative**	**% G+C**	**% identity**	**Phylum**
A5	Uncultured *Methanosarcina sp.*	42.7	99%	Methanosarcinales
A6	Uncultured *Methanobrevibacter sp.*	31.3	99%	Euryarchaeota
A7	Uncultured *euryarchaeote*	-	92%	Euryarchaeota

Fig. 5.1. DGGE patterns of anaerobic communities from both domains Archaea (a) and Bacteria (b), for phases 1 and 2.

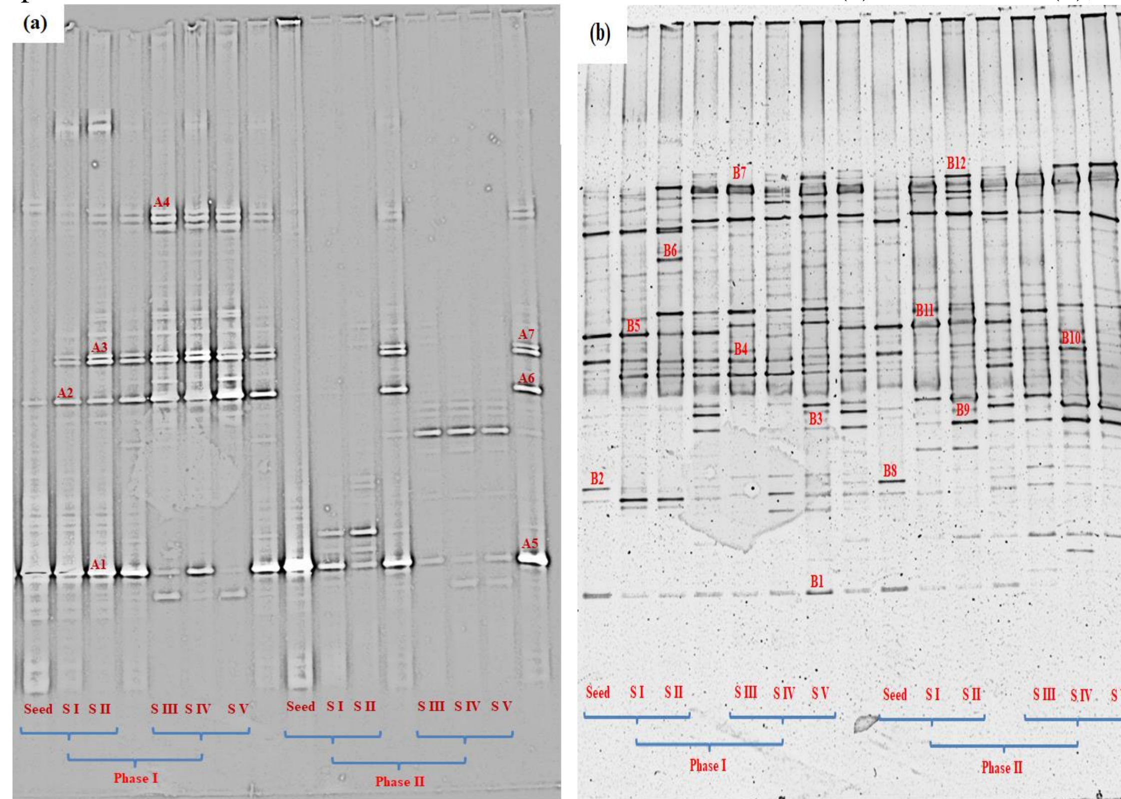
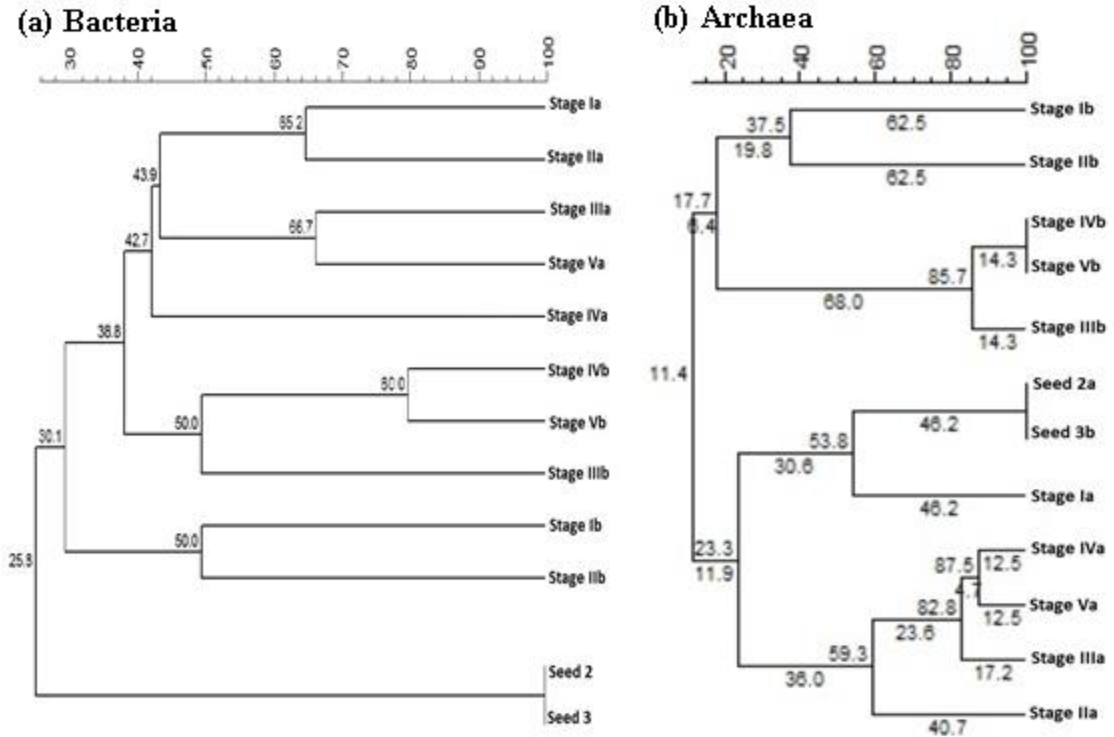


Fig 5.2. Bacterial (a) and Archaeal (b) dendrograms for both phases.



The stages I to V for Bacteria and Archaea are represented as letters, for both Phases I (Ia to Va) and II (Ib to Vb), respectively.

After sequencing, four phyla were detected, despite the richness and diversity of both Bacteria and Archaea domains: Proteobacteria, Firmicutes, Euryarchaeota and Methanosarcinales. The sequences obtained for the Bacteria domain were related to phyla Firmicutes and Proteobacteria for both phases I and II. For the Archaea domain, the sequences were related to phyla Euryarchaeota and Methanosarcinales at phase I, and related to only Methanosarcinales at phase II.

The Bacteria domain sequences found were mainly related to Firmicutes, a spore-forming taxon that is commonly known as a dominant bacterial group in anarerobic conditions. One of the main characteristics of this taxon is the ability to tolerate incredibly harsh conditions, due to their spores, which makes them suitable for colonizing many types of environments (FILIPPIDOU *et al.*, 2015). The phylum Firmicutes contains many species, one of them is Clostridium sticklandii, which was found in this study and is known to be an acidogenic bacteria capable of producing acetate, butyrate and propionate via the Stickland reaction, therefore, its name (SNEATH *et al.*, 1986). *C. sticklandii* is also capable to ferment protein, either from the substrate or from cellular remains (KIM *et al.*, 2011).

There were significant differences in ecological parameters for Bacteria between seed and stage I for both phases, due to the introduction of pure glycerol, a specific and limited substrate, which promoted selection of most adapted organisms and, furthermore, reduced the population in quantity and quality, hence, the high dissimilarity between seed and stages I (a and b). These findings indicate ecological succession, with quantitative differences, but qualitative similarity, due to changes in the populational species, maintaining the ecological niches.

Despite the high dissimilarities observed in most stages in both phases, a high similarity was observed between stages IV and V in phase II. This occurred due to the substrate change, which favored variability over productivity, starting at stage IV, due to the different substrate. This change increased niche diversification, since the substrate impurities favored generalist species, thus maintaining the high diversity on the next stage. Despite the qualitative similarity, the distribution and number of species were different between stages IV and V, which occurred due to selection of resistant species in stage V, when the OLR was further increased. The genetic similarity was maintained, hence the high similarity, due to the previously selected genetic pool, but with reduced overall populational status when compared to the previous stage.

In phase I this change in substrate did not occur on stages IV and V, favoring the selection of resistant/resilient species, therefore, the dissimilarity was higher, due to the specificity of the substrate, when compared to the same stages on phase I.

The divergence of the patterns and dendrograms observed for Bacteria indicates that the stages less productive (higher diversity and richness) were more homogeneous than the most productive stages, for both phases, due to adaptation and specialization of the most productive species, despite the significant changes observed in populational parameters (Rr and H). These changes in community structure were also observed by Xing *et al*. (2005). In the initial stages the low OLR promoted higher diversity, since at these substrate concentrations, there was no inhibition of sensitive organisms. The highest the substrate concentration, the less similar the stages, due to the reduced community, in accordance to the functional organization level.

The patterns and dendrograms observed for Archaea indicate that the most intense bands corresponded to two archaeal groups in phase I (Euryarchaeota and Methanosarcinales) and only one in phase II (Methanosarcinales). The *Methanosarcina* genus contains methane producing species, capable of utilizing CO_2 to produce CH_4 (DEMIREL; SCHERER, 2008). These species were also found in granular sludge of UASB reactors treating brewery wastes (KEYSER *et al*., 2006). Methanosaeta and Methanosarcina are known for being able to withstand harsh conditions (DEMIREL; SCHERER, 2008; KEYSER *et al*., 2006). The Uncultured *Methanosarcina* sp. (KM606590.1) was present until the last stage of phase II, with high OLR and after four stages with chloroform being added to the influent, which corroborates the resistance observed in other studies.

Is it can be observed in both phases for Archaea, the increase in Rr and H and reduction in Fo, mantaining a high similarity indicate a quantitative increase but without dominance, therefore, the archaeal community was dominated by generalists with a selected gene pool. This was corroborated by taxa identification, since Methanosaeta and Methanosarcina were the only sequenced taxa, evidencing the resistance and resilience of both groups.

5.4 Conclusions

The stages I/II in both phases were the most stable, however, were the least productive, since productivity was related to Fo and specialization, therefore, higher

functionality occurred at stages with lower stability. Production/yield were higher with pure glycerol and substrate change decreased Fo, since residual glycerol favors generalist species.

OLR and chloroform inhibited archaeal species, thus increasing H_2 yield/production by inhibiting H_2 consumers. Furthermore, residual glycerol increased archaeal Rr, due to increase in MPP, which did not occur on the other stages.

Despite productive stages, purges with N_2 could improve H_2 production/yield in future experiments, due to partial pressure limitations.

5.5 Acknowledgements

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6 CONSIDERAÇÕES FINAIS

O aumento da riqueza (Rr) e diversidade (H) da comunidade bacteriana a partir do inóculo até os estágios I e II nos reatores 1, 2 e 3 indica que essas cargas orgânicas aplicadas ao glicerol residual, especialmente no estágio II, promoveram maior diversificação e crescimento da comunidade, induzindo também aumento da capacidade de suporte e estabilidade no reator. Nos reatores 2 e 3, entretanto, o aumento da riqueza e diversidade foi menor do que nas etapas equivalentes no reator 1, devido à especificidade do substrato (glicerol puro) nestes dois reatores, em detrimento do substrato mais generalista (glicerol residual, contendo diversas impurezas e ácidos graxos) do reator 1. Estes estágios (I e II) correspondem às etapas mais estáveis da operação dos três reatores. Acima destes valores, a estabilidade decresceu. Mariakakis *et al.* (2011) obtiveram resultados similares, onde houve redução da diversidade com o aumento da COV acima da faixa de 20-30 kgCOD/m^3.d.

Sabe-se que a estabilidade da função não implica necessariamente em estabilidade da estrutura de uma comunidade, como pode ser observado nos resultados anteriores, em que o aumento da produtividade teve relação direta com a redução da estabilidade da comunidade microbiana. Apesar da maior estabilidade, riqueza e diversidade observadas nas etapas iniciais do presente estudo, estes estágios não correspondem às maiores produtividades encontradas nos reatores, corroborando com outros estudos que mostram a relação entre diversidade e produtividade em biorreatores não são interdependentes (VASCONCELOS; LEITÃO; SANTAELLA, 2016), podendo até mesmo apresentar efeitos contrários: maiores diversidades podem gerar menos estabilidade e produtividade no reator (XING *et al.*, 2005; MARIAKAKIS *et al.*, 2011; VASCONCELOS; LEITÃO; SANTAELLA, 2016).

O esperado seria o aumento de diversidade aumentar a produtividade por meio de redundância funcional e especialização de nicho (MILLS, 2003; MARIAKAKIS *et al.*, 2011; VASCONCELOS; LEITÃO; SANTAELLA, 2016). Porém, neste estudo mostrou-se que ocorreu o contrário, a partir de um determinado limiar. Para entender porque estas mudanças ocorrem é necessário entender a relação entre os parâmetros: riqueza ponderada (Rr) e organização funcional (Fo). A riqueza ponderada (Rr) pode ser interpretada da seguinte forma: se $Rr < 10$: baixa riqueza, típica de ambientes adversos, ou com restrição de qualquer tipo para sua colonização. Este tipo de ambiente tem uma alta seletividade, ou seja, somente poucas espécies bem adaptadas podem colonizá-lo. Se $10 < Rr < 30$: riqueza média, o que significa que o ambiente tem um bom potencial para colonizar, sem restrições graves. Este tipo de ambiente tem seletividade média, significando que muitos tipos de organismos podem

colonizar, com pouca ou nenhuma adaptação. Se Rr > 30: elevada riqueza, com alta probabilidade de colonização e capacidade de suporte notável. Típico de um ambiente altamente diversificado, com pouca ou nenhuma seletividade, significando que qualquer organismo pode colonizá-lo, não precisando de adaptações. O que também explica estas alterações é a organização funcional, que é a capacidade de uma comunidade de se organizar em uma distribuição adequada de organismos dominantes e resilientes, de forma a contrapor os efeitos de um distúrbio súbito a qual a comunidade seja exposta (FERNANDEZ *et al*., 2000; MARZORATI *et al*., 2008). Desta forma, quanto mais um meio for seletivo, menor a riqueza e a diversidade, porém, maior é a organização funcional, pois a comunidade se torna dominada por espécies mais adaptadas ou especializadas (MARZORATI *et al*., 2008; VASCONCELOS; LEITÃO; SANTAELLA, 2016).

Portanto, de forma a aumentar a produtividade de um meio, especialmente os de recursos restritos ou meios de produção de engenharia ecológica (como biorreatores), deve-se induzir o aumento da organização funcional e queda controlada de diversidade e riqueza, mantendo o nível populacional minimamente aceitável (mínimo de Rr e H) para que haja um pool mínimo de espécies para manter a funcionalidade.

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