UNIVERSIDADE ESTADUAL DE CAMPINAS
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Maria Paula Cardeal Volpi

Produção de biogás integrada ao conceito de biorrefinaria para biomassa lignocelulósica: aspectos operacionais e uso de nanopartículas

Biogas production integrated to the concept of biorefinery for lignocellulosic biomass: operational aspects and use of nanoparticles

Campinas
2022

MARIA PAULA CARDEAL VOLPI

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Tese apresentada à Faculdade de Engenharia de alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Ciências.

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RESUMO

A produção de biogás ocorre por meio da digestão anaeróbia (DA), que permite a recuperação energética da matéria orgânica através da produção de metano (CH_4). Substratos da atividade sucroalcooleira são considerados como potenciais facilitadores do desenvolvimento de biorrefinarias, tornando o sistema mais resiliente e versátil. Nesse contexto, a co-digestão de substratos de diferentes biodegradabilidades (mais degradáveis com menos degradáveis) surge como uma alternativa, sendo capaz de amenizar os efeitos inibitórios desses resíduos à DA, além de poder melhorar o processo de monodigestão de vinhaça que é "convencionalmente" realizado nas usinas de etanol. Portanto, o objetivo deste trabalho foi co-digerir resíduos da produção de etanol 1G (vinhaça e torta de filtro) e resíduos da produção de etanol 2G (licor de desacetilação) para obter biogás e propor a integração da biorrefinaria 1G2G. A etapa 1 do projeto consistiu em realizar a análise do Potencial Bioquímico de Metano (PBM) de cada resíduo, na etapa 2 foi realizada a operação em reator contínuo para a co-digestão de três resíduos e a etapa 3 foi uma otimização da produção de biogás com adição de nanopartículas de Fe_3O_4 (NP) na operação do reator de co-digestão (o mesmo operado na etapa 2). Para as etapas 2 e 3 foi feita a identificação do consórcio microbiano juntamente com proteínas extracelulares (análise proteômica), para traçar a rota metabólica em ambas as operações do reator. Os resultados de PBM mostraram que a co-digestão da vinhaça 1G com a torta de filtro e o licor de desacetilação melhorou o rendimento de CH_4 de substratos isolados, atingindo 605 $NmLCH_4\ gSV^{-1}$. A vinhaça e o licor de desacetilação como únicos co-substratos aumentaram o PBM em 38% em relação a vinhaça, indicando sinergismo nutricional. Na operação contínua do reator de co-digestão dos três resíduos o maior rendimento de CH_4 foi de 230 NmLCH4 gSV^{-1} com eficiência média de remoção de matéria orgânica de 83% ± 13 alcançados na Carga Orgânica Volumétrica (COV) de 4,16 $gSV\ L^{-1}dia^{-1}$. Além disso, o uso de Fe_3O_4 NP mostrou-se eficiente no processo de otimização da produção de CH_4, uma vez que o valor máximo foi 2,8 ± 0,1 $NLCH_4\ gSV^{-1}$ sendo 90% superior ao obtido na co-digestão sem a presença de NP. A principal *Archaea* metanogênica encontrada em ambos os reatores (estágio 2 e estágio 3) foi *Methanoculleus,* indicando que a possível rota metabólica predominante foi a oxidação do acetato sintrófico (SAO) acoplada à metanogênese hidrogenotrófica. Por meio desses resultados, foi possível realizar uma análise energética e obter a capacidade instalada para uma biorrefinaria integrada de etanol 1G2G de mais de 50 MW (considerando somente a capacidade energética do biogás) durante o período de safra. Convertendo o biogás em biometano foi provido a necessidade de biocombustível da frota da maior usina de etanol do Brasil e ainda

obtido um excedente que pode ser injetado na rede de gás e gerar eletricidade. De maneira geral, os resultados mostraram que a co-digestão dos resíduos propostos é uma alternativa viável para a produção de biogás e integração da biorrefinaria de etanol 1G2G.

Palavras-chave: Biorrefinaria, Co-digestão, Vinhaça, Metaproteomica, Torta de filtro

ABSTRACT

Biogas production occurs through anaerobic digestion (AD), which allows the energetic recovery of the organic source through the use of methane (CH_4). Substrates from the sugar-alcohol activity are considered as potential facilitators of the development of biorefineries, making the system more resilient and versatile. In this context, the co-digestion of substrates of different biodegradability (more degradable with less degradable) appears as an interesting alternative, being able to soften the inhibitory effects of those residues to AD, in addition to being able to improve the process of monodigestion of vinasse that is "conventionally " carried out in ethanol plants. Therefore, the objective of this work was to co-digest residues from the 1G ethanol-producing (vinasse and filter cake) and residues from the 2G ethanol-producing (deacetylation liquor) to obtain biogas and propose the integration of the 1G2G biorefinery. Stage 1 of the project consisted of performing the Biochemical Methane Potential (BMP) analysis of each residue, stage 2 was the operation in a continuous reactor for the co-digestion of three residues, and stage 3 was an optimization of biogas production with adding Fe_3O_4 nanoparticles (NP) in the co-digestion reactor operation (the same was operated in stage 2). For stages 2 and 3 was done identification of the microbial consortium together with extracellular proteins (proteome analysis), to trace the metabolic route in both reactor operations. BMP results showed that co-digestion of vinasse 1G with filter cake and deacetylation liquor improved the CH_4 yield of isolated substrates, reaching 605 $NmLCH_4\ gVS^{-1}$. Vinasse and deacetylation liquor as the only co-substrates increased PBM by 38% over vinasse, indicating nutritional synergism. In the continuous operation of the co-digestion reactor of the three residues, the highest CH_4 yield was 230 NmLCH4 gSV^{-1} with average organic matter removal efficiency of 83% ± 13 achieved at Organic Load Rate (OLR) of 4.16 gVS $L^{-1}day^{-1}$. Furthermore, the use of Fe_3O_4 NP proved to be efficient in the process of optimizing the production of CH_4, since the maximum value was were 2.8 ± 0.1 $NLCH_4\ gVS^{-1}$ being 90% higher than that obtained in the co-digestion without the presence of NP. The main methanogenic *Archaea* found in both reactors (stage 2 and stage 3) was *Methanoculleus*, indicating that the predominant metabolic route possible was syntrophic acetate oxidation (SAO) coupled with hydrogenotrophic methanogenesis. Through these results, was possible to perform an energy analysis and obtained the installed capacity for an integrated 1G2G ethanol biorefinery of more than 50 MW (considering only the energy capacity of biogas) during the season period. By converting biogas into biomethane, the need for biofuel in the fleet of the largest ethanol plant in Brazil was provided and still obtained a surplus that can be injected into

the gas grid and generate electricity. In general, the results obtained showed that the co-digestion of the proposed residues is a viable alternative for the production of biogas and integration of the 1G2G ethanol biorefinery.

Keywords: Biorefinery, Co-digestion, Vinasse, Metaproteomic, Filter cake.

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1 INTRODUCTION

Adopted in Paris at the United Nations Conference on Climate Change (COP21), the Paris agreement officially entered into force in November 2016, with governments' notorious commitment to key areas related to climate change, adaptation and enhancement in terms of capacity and energy technologies (GHEZLOUN; SAIDANE; MERABET, 2017). The importance of using biomass for power, heat and fuel generation is increasing on a global scale. International and national policies, such as the European Action Plan for biomass, support the expansion of bioenergy, as it is considered climate-friendly compared to fossil fuels (DRESSLER; LOEWEN; NELLES, 2012). In this promising and challenging context, the production of biogas is returning to prominence and, consequently, has received numerous initiatives. Anaerobic digestion (AD), an attractive process for the management of liquid and solid waste that allows energy recovery through methane (CH_4) and generation of added-value by-products for agriculture, develops in a finely balanced ecosystem. Different populations of microorganisms with specialized functions act together to promote the degradation of organic matter, in a process described, in synthesis, in four steps. In the first stage, facultative and anaerobic bacteria convert the complex organic compounds (carbohydrates, proteins and lipids) into simple organic compounds (glucose, aminoacids, fatty acids). In the second stage this organic sample are converted into volatile organic acids (e.g. lactate, butyrate, propionate) by acidogens microorganisms. In third stage this volatile acids are converted into CO_2, H_2 and acetate by acetogens bacterias. And in methanogenisis stage, organic acids and H_2 are converted to CH_4 and CO_2 by methanogenic *Archaea* (DEUBLIN; STEINHAUSER, 2008; WEILAND, 2010).

Biogas (60–70% CH_4, 30–40% CO_2 and rest being the impurities) is considered a versatile energy carrier, which can be used to replace fossil fuels in the production of both electricity and heat, as well as used as a gaseous fuel for vehicles. In addition, methane-rich biogas (90% methane) is considered biomethane, and can replace natural gas as a raw material for the production of chemicals (WEILAND, 2010). Recently, a study by the Brazilian Association of Biogas and Biomethane (Abiogás) indicated that Brazil has the potential to generate 23 billion cubic meters of CH_4 per year - the final product of a biogas plant. In this scenario, the residues and by-products from the sugar and alcohol activity are considered as raw materials for the generation of value-added products, such as biomethane (i.e. biogas containing 90% CH_4 v/v with characteristics similar to natural gas). Biogas can be a facilitator of the development of biorefineries, as well as improving the value of the product portfolio (HAGMAN et al., 2018).

The residues of the sugarcane agroindustry are already considered raw materials for recovery and generation of value-added products. Among the residues generated, vinasse is the one obtained in greater quantity, around 12-15 liter each 1 liter of ethanol produced. This residual liquid comes from fermented sugarcane juice distillation, with a dark color. It consists of water (93%), solids and organic minerals (7%). The main compound of vinasse is organic matter in the form of glycerol, some organic acids (as lactic acid), sulfate (anions) and some cations (GIANCHINI; FERRAZ, 2009; CHRISTOFOLETTI et al., 2013). The filter cake is a solid residue generated during the clarification (physical-chemical process) of sugar cane juice before being used in the production of sugar and first-generation (1G) bioethanol. Its composition is water, organic soil particles, sugars residuals and small pieces of sugar cane that are added to improve sucrose recovery in the rotary vacuum filter (ELSAYED et al., 2008). Sugarcane straw, a lignocellulosic residue, is another waste from ethanol production from sugarcane, and it is obtained from the sugarcane field. It highlight as an energy source with great potential for generating heat, electricity and producing cellulosic ethanol. This lignocellulosic residue is characterized by being composed of 40-44% cellulose, 30-31% hemicellulose and 22-25% lignin (ELSAYED et al., 2008).

The intensity of the expectations regarding the use of several biomasses and the production of biogas for energy purposes is outstanding. Despite all the scientific growth in this area, it is necessary to deepen the knowledge based on innovative issues and variations that investigate, in a comprehensive way, the interactions between the technological limitations prevailing in the bioprocess for the generation of CH_4, mainly in relation to residues that have low biodegradability, such as the presence of lignocellulosic materials, the presence of silica, soluble lignin, crystallized cellulose, high density and larger particle sizes, which can be limiting factors to be digested by microorganisms (SOEST, 1981).

In this context, the co-digestion could be a good option to use poorly biodegradable substrates in addition to providing and balancing macro and micronutrients for the AD process. This appears to be the case of residues from ethanol production from lignocellulosic biomass, usually recognized as complex substrates for AD, such as, the filter cake. However, there are gaps in the literature regarding the anaerobic co-digestion of waste from 2G ethanol production, especially for the recent and innovative pre-treatment of biomass and hydrolysis, e.g., deacetylation process, pre-treatment with ionic liquids, hydrolysis using genetically modified yeast, among others. Deacetylation liquor is a residue obtained from the alkaline pretreatment of sugarcane straw for the production of second-gerneration (2G) ethanol (BRENELLI et al., 2020), which has not yet been reported in the literature on its final deposition, or possible use

in biodigestion. The complexity of such substrates for AD may be one of the factors driving the integration of the 1G2G ethanol process by co-digestion of its residues, for example, with 1G vinasse that is already recognized as a substrate for biogas production (FERRAZ JÚNIOR et al., 2016a). It is worth mentioning that the integration of the 1G2G ethanol biorefinery can also be done through the use of 2G vinasse, which is already a waste that shows potential for biodigestion and CH_4 recovery (MORAES et al., 2014), but the use of waste from lignocellulosic pre-treatments are still in an initial scenario.

Scientific research was conducted to evaluate the potential of CH_4 production through Biochemical Methane Potential (BPM) of residues (TRIOLO et al., 2012). BPM monitors the volume of the biogas generated from a fraction of waste by assessing its biodegradability through the determination of the total cumulative CH_4 production (SILVA; MORAIS; ROCHA, 2016). By this approach, it is possible to reach the maximum experimental potential of the substrate organic fraction conversion in CH_4 and also assess the effect of specific conditions on AD: substrates sources (exclusive or in proportions of mixtures), temperature, nutrients, buffering, source of inoculum (anaerobic consortium), among other factors.

It is known that the microorganisms that are part of anaerobic digestion are diverse and distinct. They are considered highly diversified and high redundancy, meaning that several microorganisms are metabolically flexible and capable of doing the same job, being one of the reasons for the robustness of the anaerobic digestion process (ZUMSTEIN; MOLETTA; GODON, 2000). Identify the microbial community present in the reactor is also extremely important for the control of the AD process, because the biochemical reactions depend on the microbiota (BALAGURUSAMY, 2007). Recent advances in molecular microbial techniques (LAM et al., 2021; PING et al., 2020; PINHEIRO et al., 2020) are making it possible to determine the composition and dynamics of the microbiota in different biological systems by identifying the main groups of microorganisms, which extends in particular to the anaerobic processes.

In addition, identification of microorganism in the process is already a big step, suggesting its metabolic potential, but it may not be enough to attribute the function of these microorganisms, because is possible that a single microorganism has different functions at different stages of the metabolic pathways (CABEZAS et al., 2015). Therefore the analysis of the regulation and molecular function of proteins excreted by the microbial consortium is another fundamental approach for the understanding of biological systems (MUELLER et al., 2008). Proteins are molecules that play a number of essential functions and with wide functional diversity, for example, they can promote the fixation and aggregation of flakes into granules

and maintain their stability in the AD process (ZHANG et al., 2007). Proteomics analysis is used to characterize proteins within an environmental microbial consortium. This contributes to the knowledge of the functions of the main active and important metabolic pathways for AD (SU et al., 2012). In this context, the proteomic characterization of the microbial consortium may represent the key for understanding the mechanisms governing cell growth, metabolism and biosynthesis of products (such as CH_4) in order to improve the efficiency of production, quality and yield systems of final products (CABEZAS et al., 2015).

An important factor for the AD is the presence of micronutrients, a fundamental condition for biogas production, being the effect of the addition of certain compounds, such as iron, molybdenum and selenium, related to improvements in the CH_4 yield (ABDEL AZIM et al., 2017; CAI et al., 2017). Metals represent essential constituents of cofactors for enzymes and their addition to anaerobic digesters can stimulate and stabilize the performance of the biogas production process, emphasizing that the ideal combination of several elements supplementation can have a greater positive impact depending on the substrate (CHOONG et al., 2016; WINTSCHE et al., 2016). Scherer; Lippert; Wolff, (1983) classified that for methanogenic organisms the importance of micronutrients is given in the following order: Fe >> Zn> Ni> Cu = Co = Mo> Mn, indicating that such elements have essential roles as in the construction of methanogenic cells. In this sense, according to Abdelsalam et al., (2017a), some attempts have been made to increase biogas production by stimulating microbial activity using various biological and chemical additives under different operating conditions. According to the authors, nanotechnology, recognized by the European Commission as one of the most promising key technologies that can contribute to competitiveness and sustainable growth in various industrial sectors, is in line with the application of nanoparticles in biological CH_4 production. Nanotechnology can be described as the science of designing and constructing machinery where every atom and chemical bond is precisely specified (MUKHOPADHYAY, 2014). Opening prospects for AD field, some studies have recently reported better efficiencies of CH_4 production from the use of nanoparticles (ABDELSALAM et al., 2016, 2017b, 2017a; WANG et al., 2016b).

In this context, this research project aimed to fill gaps in the literature regarding the integration of biogas production in the concept of 1G2G sugarcane biorefineries, in order to exploit the potential of co-digestion of by-products from 1G2G ethanol production. The project was developed in three stages: (1) determination of BMP of the substrates (vinasse, filter cake and 2G ethanol pre-treatment residues) to analyze their CH_4 production potential for co-digestion; (2) parameters elucidation of the bioprocess in semi-continuous stirred reactor, by

monitoring its operating conditions, determining the extracellular proteins and analyzing the molecular biology of the microbial consortium; (3) optimization evaluation of the anaerobic biological process through the application of nanoparticles during the reactor operation to demonstrate possible effects on AD performance from substrates of the sugarcane agroindustry.

2 HYPOTHESIS

The project started from the hypothesis that would be possible to increase biogas/biomethane yield in integrated 1G2G sugarcane biorefineries by co-digesting their main residues/byproducts, when compared to the AD of 1G vinasse. Specific suppositions were raised, then, from that main hypothesis:

- The co-digestion process of 1G vinasse with filter cake and deacetylation liquor would be able to increase the biogas/methane yield compared to the mono-digestion;
- The molecular biology and metaproteomic tools and analysis would be useful to provide data on the AD metabolic pathways to support the reactor operational control;
- The addition of Fe_3O_4 nanoparticles would be able to increased biogas/methane yield of the co-digestion process.

3 BIBLIOGRAPHY REVIEW

3.1 Bioenergy and Biogas

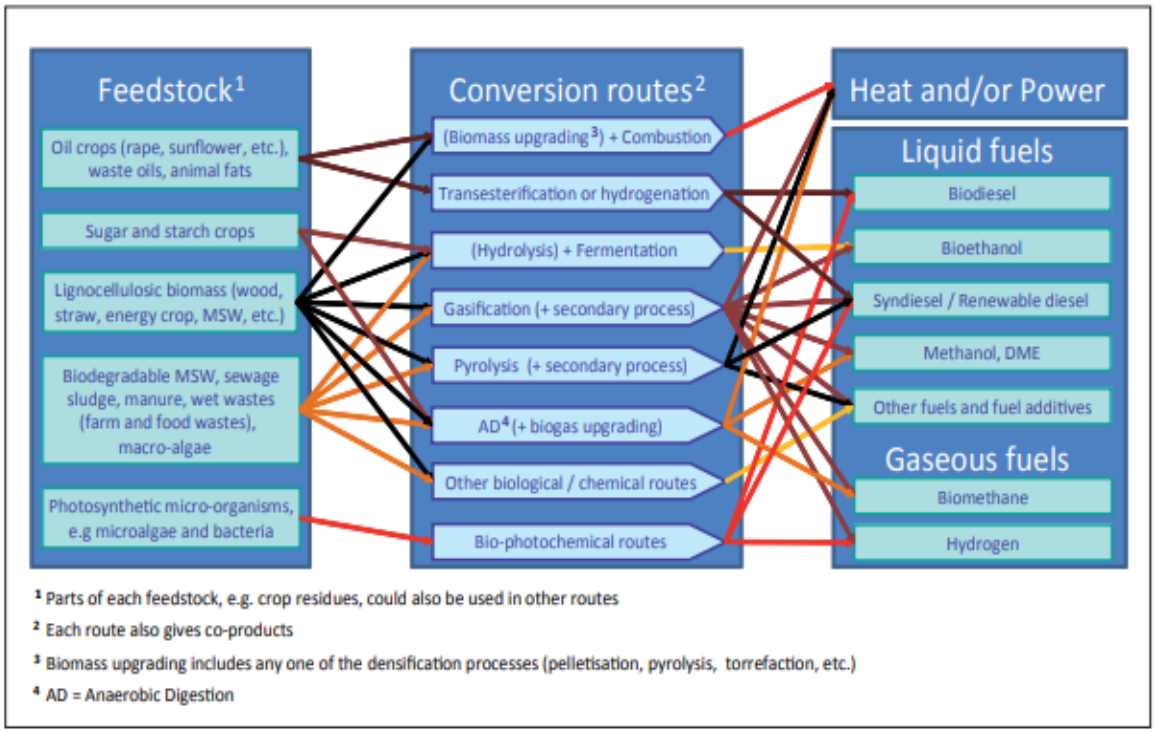
The debates on issues of global warming and the reduction of greenhouse gases are common knowledge, and in this scenario, bioenergy gains strength and stands out as an efficient alternative. With the industrial development of the countries, an exponential increase in energy consumption will occur, and at the same time, energy demand will increase by an annual average of 1.6% by 2030 (IEA, 2006). Despite its recognized sustainable character, obtaining the bioenergy is still a challenge for humanity. In this context, biomass can make a contribution to the supply of sustainable energy.

The importance of using biomass for power, heat and fuel generation is increasing on a global scale. International and national policies, such as the European Action Plan for biomass, support the expansion of bioenergy, as they are considered climate-friendly compared to fossil fuels (DRESSLER; LOEWEN; NELLES, 2012)

Forest, agricultural and municipal waste are the main raw materials for generating electricity and heat through biomass. Biomass provides about 50 EJ globally, representing 10% of global annual primary energy consumption (BAUEN et al., 2009).

There are some reactions that can be used to convert raw material from crude biomass into a final energy product. The conversion technologies are adapted to the physical and chemical nature of the biomass. Figure 1 shows the possible biomasses and the processes necessary to obtain bioenergy (BAUEN et al., 2009).

Figure 1. Schematic view of the wide variety of bioenergy routes



*Source:*copied from (BAUEN et al., 2009)

Biogas (60–70% CH_4, 30–40% CO_2 and rest being the impurities) is considered a versatile energy carrier, which can be used to replace fossil fuels in the production of both electricity and heat, as well as used as a gaseous fuel for vehicles. In addition, methane-rich biogas (> 90% methane, v/v) is considered biomethane, and can replace natural gas as a raw material for the production of chemicals (WEILAND, 2010). The production of biogas occurs through anaerobic digestion (AD) and this process was considered one of the most energy efficient and environmentally beneficial technologies for the production of bioenergy. Among the advantages that AD provides is that it reduces greenhouse gas emissions compared to fossil fuels by using resources that are available locally, such as solid urban waste, waste from forests, animal waste. In addition, the remaining digested AD is used as a fertilizer for crops and can replace mineral fertilizers (FEHRENBACH et al., 2008).

In Europe, biogas production reached 6 million tonnes of oil equivalent in 2007. Germany has become one of the largest biogas producers in the world thanks to the development of biogas plants on farms. In the European Union 1500 million tonnes of biomass can be anaerobically digested each year, and half of that is through of energy crops use (WEILAND, 2010).

All types of biomass can be used as substrate for the production of biogas. The composition of the biomass must be mainly of carbohydrates, proteins, fats, cellulose and hemicellulose. The composition of the biogas and the methane yield depends on the type of raw material used and the retention time. Strong lignified substances are not considered suitable for AD due to the slowness they can cause in the process, due to the difficulty of degradation by microorganisms (BRAUN, 2007).

One of the oldest substrates used in AD is animal manure and wastewater, as the focus was on the treatment of this waste and not the production of biogas. Currently, other residues are already used, such as crop residues, organic residues from the food industry, municipal biological residues and energy crops. Animal manure is used as co-substrate to increase the content of organic material and achieve a higher gas yield. Fats provide higher biogas yield, but require longer retention times due to their low bioavailability. Carbohydrates and proteins show faster conversion rates, but with lower gas yield. In AD the C / N ratio must be in the range of 15 and 30 and flaws in the process due to the accumulation of ammonia must be avoided (BRAUN, 2007; WEILAND, 2010).

Among the reactors that are commonly used for biodigestion, what stands out is the fermenter with vertical agitation that is applied in almost 90% of biogas plants in Germany (WEILAND, 2010), normally used for allowing the co-digestion of a variety of substrates with higher solids total. The agitators can vary, with the possibility of having slow or not rotating blades, larger or smaller blades and the shaft can also vary depending on the substrate. There is also pneumatic agitation with the biogas produced and hydraulic agitation by pumps. In addition to these reactors, other types are used as horizontal reactors, UASB reactor and fixed bed reactor, this being preferably with liquid only waste (KAPARAJU; SERRANO; ANGELIDAKI, 2010; ZHANG et al., 2012).

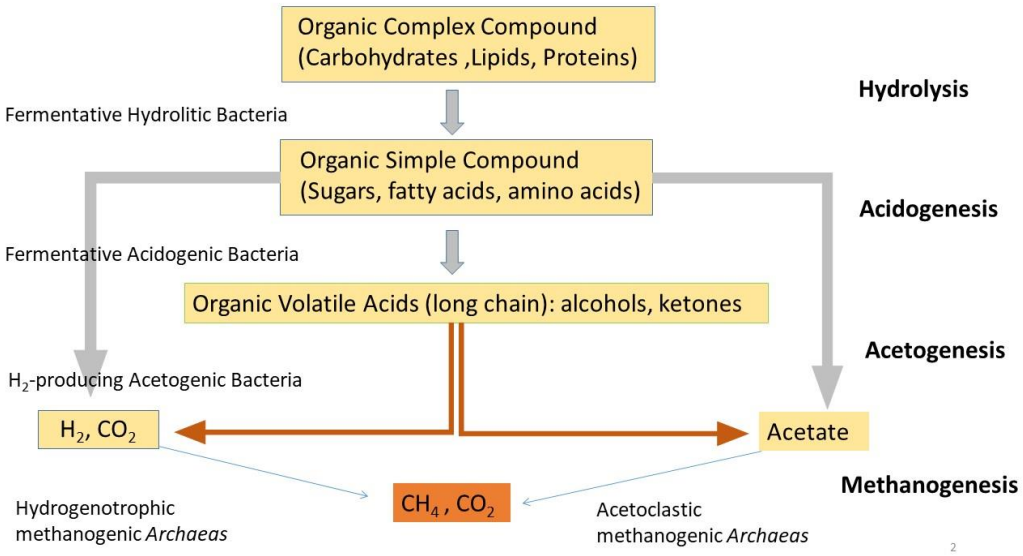
Biogas plays an important role in the context of the introduction of bioenergy in the current world, proving to be a strong candidate to supply the conditions for reducing greenhouse gases and generating renewable energy. In addition, its production can be carried out with different substrates, in different conditions and reactors, being very versatile. The AD is the process that allows the energy recovery of substrates through the actions of a microorganism's community.

3.2 **Anaerobic Digestion (AD) and Biochemical Process**

AD is a treatment process of waste in which chemicals or methane are obtained as products. The process occurs in two stages: acidogenic phase and methanogenic phase. The

process is quite complex, involves a lot of comunity of microorganisms and goes through four phases: hydrolysis, acidogenesis-acidification phase, acetogenesis and methanogenesis (Figure 2). There are biochemical involvement of various enzymes and prosthetic groups in the conversion of H_2 and CO_2 into methane and acetate into methane and CO_2, among which highlight Deazariboflavin derivative F420, methanopterin, methanofuran, nickel-tetrapirol factor F430 and coenzyme M (mercaptane sulfonate) (KRZYSZTOF ZIEMIŃSKI, 2012).

Figure 2. Metabolic pathway of methane production



Source: adapted from (KRZYSZTOF ZIEMIŃSKI, 2012)

In the hydrolysis phase, insoluble organic compounds such as carbohydrates, proteins, fats are broken down into mono sugars, amino acids and fatty acids. In this phase, it is the hydrolases group extracellular enzymes (amylase, protease, lipase) that act. Compounds that are more difficult to be degraded, such as cellulose, are the ones that limit the rate of digestion of the waste. Only 50% of organic compounds are degraded, the rest is not modified due to the lack of enzymes. The main genera of bacteria involved are: *Streptococcus* and *Enterobacterium* (KRZYSZTOF ZIEMIŃSKI, 2012; PARAWIRA et al., 2008).

In the acidogenesis phase, fermentative bacteria convert water-soluble chemicals (including products formed in the hydrolysis step) into short-chain organic acids (formic, acetic, propionic, butyric, pentanoic acid), alcohols (methanol, ethanol), aldehydes. These generated products cannot be used directly by methanogenic *Archaea* due to the accumulation of electrons. Therefore, it is necessary that obligatory bacteria from the acetogenesis process convert these compounds into hydrogen, acetate and CO_2, so that the methanogens can take

action. In the decomposition of proteins, peptides and amino acids emerge that can be used as a source of energy for anaerobic microorganisms. Acidogenesis can present two paths due to the various populations of microorganisms present in the anaerobic consortium. The process can be divided into hydrogenation and dehydrogenation. The other path in acidogenesis would be that the compounds from hydrolysis are converted directly into acetate, CO_2 and H_2. Of these products obtained, methanogens can directly use as substrate and energy source. It is worth mentioning that other products obtained in the acidogenesis phase is ammonia and together with activity of sulfate reducing bacteria (SRB) give an unpleasant smell to this phase of the process. The acid phase bacteria, belonging to facultative anaerobes, use the oxygen introduced in the process, favoring conditions for the growth of mandatory anaerobes such as: *Bacillus*, *Micrococus*, *Flavobacterium* (CONRAD, 1999).

As previously mentioned, in the acetogenic phase, bacteria of the genera *Syntrophomonas* and *Sytrophobacter* convert the products (alcohols, acids, aldehydes) obtained in the acidogenic phase into acetates and hydrogen that can be used by methanogenic *Archaea.* In this phase, 25% of acetates and 11% of hydrogen are formed. As a result of acetogenesis, hydrogen is released, which exhibits toxic effects on the microorganisms which carry out this process. Therefore, a symbiosis is necessary for acetogenic bacteria with autotrophic methane bacteria using hydrogen, hereinafter referred to as syntrophy. In addition, acetogenesis is characterized to describe the efficiency of biogas production, because 70% of methane comes from the reduction of acetates (KRZYSZTOF ZIEMIŃSKI, 2012).

The methanogenic phase consists of the production of methane by methanogenic *Archaea.* Methane is obtained through substrates from the previous phases, such as acetic acid, H_2, CO_2, methanol. Only 30% of the methane produced in this process comes from CO_2, a reduction made by autotrophic methane *Archaea*. During this process H_2 is used, creating good conditions for the development of acidic bacteria that originate short chain organic acids (acidogenesis) and consequently generates a low production of H_2 in the acetogenic phase (KARAKASHEV; BATSTONE; ANGELIDAKI, 2005). Methanogenic microorganisms are strict anaerobes, that is, the presence of oxygen is lethal to them. They lack the enzyme catalase and neither superoxide dismutase and due to their extreme sensitivity to oxygen, their biochemistry, physiology and ecology are less well known. They are also sensitive to changes in temperature and pH and their development can be inhibited by high levels of volatile fatty acids, hydrogen, ammonia. Methanogens are classified according to the temperature that exert the greatest activity, which are: mesophilic between 28°C and 42°C and thermophilic between

55°C to 72°C (KARAKASHEV; BATSTONE; ANGELIDAKI, 2005; KRZYSZTOF ZIEMIŃSKI, 2012).

The group of methanogens can be further subdivided into hydrogenotrophics or acetotrophics. Hydrogenotrophics are those that use only H_2 and CO_2 as substrates for conversion to CH_4, and acetotrophics use methyl groups such as acetate and CO_2 for the production of CH_4. Within these two groups of microorganisms, there are large quantities of species of methanogenic *Archaea*, in which pH, morphology, combination of substrates that they can use vary among themselves (DEMIREL; SCHERER, 2008).

As presented, AD is composed of different stages, with different microorganisms and different metabolic routes, which makes the process quite complex. What can vary the routes of AD is mainly the substrate to be degraded and the microorganism community that will be predominant. Different phyla, with different genera make up the microbial community of the anaerobic consortium, and the knowledge about them contributes to the improvement of the process. In addition, the co-digestion process can optimize the production of biogas, allowing the use of different substrates, balancing the nutrients necessary for the process.

3.3 Anaerobic Co-Digestion

The production of biogas from organic material depends on the content of substrates that will be converted into biogas, related to its biodegradability and chemical composition. Determining the degree of biodegradability, composition of substrates, amount of alkalinizer, help to improve methane production (SAHITO; MAHAR; BROHI, 2014). Hagos et al. (2017) reports that some studies were carried out on the production of biogas with mono-substrates but found that the direct use of substrates is difficult due to its nutritional imbalance, lack of diversified organisms and operational factors.

Within this context, anaerobic co-digestion emerged to improve biogas production in some cases. Co-digestion is characterized by the AD of two or more substrates which is an option to overcome disadvantages of mono-digestion, mainly in relation to the balance of nutrients and to improve the economic viability of AD plants (HAGOS et al., 2017a). Some studies have been done investigating the co-digestion of manure with other biomasses or food waste, showing that they are a very viable option, optimizing the production of methane and at the same time treating a greater volume of waste (ASTALS; NOLLA-ARDÈVOL; MATA-ALVAREZ, 2013; EL-MASHAD; ZHANG, 2010).

One of the main advantages of co-digestion is the improvement of biogas production and optimization of methane production, in addition to improving the stabilization of the

process, providing a better balance of nutrients, reducing the emission of greenhouse gases into the atmosphere, providing effects synergistic within the reactor, increases the load of biodegradable organic compounds, and generates economic advantages by sharing equipment and cost (HAGOS et al., 2017a).

Some works have been using the residues of the sugar cane industry as co-substrates in anaerobic digestion. Pinto et al. (2018) showed that using parchment in co-digestion with vinasse a production of 0.21 mLbiogas .g/VS_{added} is obtained. Janke et al. (2016) performed a co-digestion of filter cake with sugarcane bagasse, in which they conclude that co-digestion can produce 58% more biogas compared to mono-digestion of filter cake. In another study, Janke et al. (2015) point out that the co-digestion of sugarcane straw and bagasse would make economic sense, since it could partially replace the addition of high-cost chemicals, such as urea, which would be used to balance the C: N ratio, making the complementarity of these residues meet this need. However, they call the idea that the co-digestion of vinasse with other lignocellulosic residues could provide undesirable effects, as they could increase the production of H_2S and lead to the need for biogas desulfurization.

In this scenario, co-digestion presents as a promising and challenging concept, mainly related to sugarcane residues. Further investigation is still needed regarding the co-digestion of these residues, especially the lignocellulosic ones.

3.4 **Sugarcane Residues**

In Brazil, ethanol is produced from sugarcane (24.8 billion liters in the season 2021/22), and the state of São Paulo being the largest producing region (11 million liters in the season 2021/22) (CONAB, 2021). From the production of ethanol, large amounts of residual biomass (bagasse, filter cake, straw) are available at the plant. The sugarcane industry brings some problems from the planting of the cane to the harvest, for example, reduction of biodiversity caused by deforestation, contamination of the soil and water, generation of waste in large quantities (AGUIAR, 2011).

Among the residues generated, vinasse is the one obtained in greater quantity, around 12-15 liter each 1 liter of ethanol produced. This residual liquid comes from fermented sugarcane juice distillation, with a dark color. It consists of water (93%), solids and organic minerals (7%). The main compound of vinasse is organic matter in the form of organic acids, glycerol and cations (GIANCHINI; FERRAZ, 2009; CHRISTOFOLETTI et al., 2013).

The chemical composition of vinasse is generally 20-30 g L^{-1} of Chemical Oxigen Demand (COD), pH around 3.9-5.5, 4-250 mg.L^{-1} of phosphorus, (CHRISTOFOLETTI et al.,

2013), 24-58 g L^{-1} total solids (TS), 1.8-60 g L^{-1} volatile solids (VS) (MORAES; ZAIAT; BONOMI, 2015a). Among the alternatives to use of vinasse make a statement the practices of recycling in fermentation, fertigation (practices quite currently used in Brazil), combustion, production of yeast, animal feed production and the AD process (CHRISTOFOLETTI et al., 2013). It is worth mentioning that the use of vinasse when applied to the soil brings some negative points, such as the large amount of K and Na that can be added to the soil, and the production of gases such as CH_4 that can aggravate the greenhouse effect (DE OLIVEIRA et al., 2013).

The use of vinasse in biodigestion is already a technique considered an efficient alternative for the production of biogas. After AD, biodigested vinasse can later be used as a fertilizer, although it has a reduced organic load. (DJALMA NUNES FERRAZ JÚNIOR et al., 2016a; FUESS et al., 2017a; MORAES; ZAIAT; BONOMI, 2015a).

Other residue generate from sugarcane industry is filter cake. It is a solid residue generated during the clarification (physical-chemical process) of sugar cane juice before being used in the production of sugar and first generation bioethanol. Its composition is water, organic soil particles, sugars residuals and small pieces of sugar cane that are added to improve sucrose recovery in the rotary vacuum filter (ELSAYED et al., 2008).

Filter cake is generated around 3.4% of the sugarcane consumption annually. The best known options for using filter cake are to use it as an organic soil amendment, to act as fertilizers or in landfills, in addition to composting (MEUNCHANG; PANICHSAKPATANA; WEAVER, 2005). Some studies have already shown the use of filter cake in anaerobic digestion, mainly as a co-substrate, to balance the process nutrients (JANKE et al., 2015, 2016a, 2017a, 2017b). In the work of González; Reyes; Romero, (2017) for example, a yield of methane production of 365 LCH_4 $kg^{-1}VS^{-1}$ and biogas yields of 1.6 LL^{-1} were obtained in the co-digestion of filter cake with vinasse, which was 64 % higher compared to vinasse mono-digestion.

As the filter cake is derived from sugar cane with chemicals used to clarify cane juice, it contains nitrogen and phosphorus in addition to nutrients such as Na, Fe, Mn, Ca, Cu, Si, Mg, S and Zn, which are all essential for plant growth. In addition, the filter cake features 28.9% TS, 74.2% VS (% total solids), 47% carbon, 1.76% nitrogen, 0.6% phosphorus and 0.27% sulfur (% total solids) (JANKE et al., 2015).

Sugarcane straw, a lignocellulosic residue, is other waste from ethanol production from sugarcane, and obtained from the sugarcane field. It make a statement as an energy source with great potential for generating heat, electricity and producing cellulosic ethanol. This

lignocellulosic residue is characterized by being composed of 40-44% cellulose, 30-31% hemicellulose and 22-25% lignin, (SANTOS et al., 2012). In addition, it has 76.7% TS, 86.3% VS, 43.4% carbon, 0.52% nitrogen, 0.03% phosphorus and 0.06 sulfur (JANKE et al., 2015). Sugarcane straw was normally burned in the pre-harvest to reduce the cost of the harvest, mainly in mechanized operations, or remains in the field like fertilizer for soil (LEAL et al., 2013). Due to the great energetic potential of sugarcane straw, pre-treatments are being carried out to release sugars from the lignocellulosic material of cane straw, for the production of second generation ethanol (DA SILVA et al., 2010). Some works have also shown the use of sugar cane straw as a residue for anaerobic digestion and biogas production (JANKE et al., 2017a, 2017b).

In order to diversify the production in sugarcane industry and the possibility of ethanol production throughout the year, the second generation ethanol biofuels is being considered. Efforts are currently being made to produce ethanol by hydrolysis of sugarcane bagasse or straw. In addition, the production of liquid fuels through synthesis gas (biomass gasification) has been an alternative. There is a tendency to increase electricity production using residual sugarcane biomass, making it a product as important as ethanol and sugar (WALTER; ENSINAS, 2010).

3.5 Pre-treatment of lignocellulosic biomass to obtain 2G ethanol

Research to increase the yield of ethanol from sugarcane was focused on the production of second generation ethanol. The idea is to use technology that allows the recovery of sugars from the lignocellulosic material of sugarcane. In this way it is possible to use all lignocellulosic mass in an integrated way, optimizing the production of alcohol (MORAES; ZAIAT; BONOMI, 2015a).

The technology for converting lignocellulosic biomass into fermentable sugars for ethanol production has been considered as a promising alternative to meet the global demand for fuels. Although there are already technologies available for the processing of cellulose, most face technical or economic difficulties (SANTOS et al., 2012).

The sugars present in sugar cane straw are found in the form of polymers (cellulose and hemicellulose) and are covered by a macromolecule (lignin), forming the cellulosic microfibril. Due to its intermolecular interaction and complete absence of water in the microfibril structure, cellulose has a very recalcitrant structure that is difficult to break down and convert into fermentable monosaccharides (SANTOS et al., 2012). Due to these situations, different pretreatments are needed to release the sugars that are involved. The pre-treatments can be

classified as physical, chemical, physical–chemical, and biological, as well as them combinations (SILVA et al., 2010).

Alkaline pretreatments are common for the delignification of biomass, with additional effects on the removal of silica (ash insoluble component) or the partial removal of hemicelluloses (including acetyl and uronic acid groups) and the swelling of cellulose, resulting in a substantial increase in fiber surface (CARVALHO; QUEIROZ; COLODETTE, 2016). One of the reagents used in the alkaline pretreatment is sodium hydroxide (NaOH). Pre-treatment is usually carried out at room temperature or at higher temperatures (20-121°C) and may be for a short or long time. Alkaline pretreatment is effective for preparing biomass for use in the production of ethanol in bioconversion processes (MIRAHMADI et al., 2010).

Ionic liquids are another type of pre-treatment used for lignocellulosic biomass. They are characterized as molten salts below the melting point and have the ability to dissolve lignocellulosic biomass. The pre-treatment with ionic liquids showed some advantages in relation to the other pre-treatments, such as: changing the physico-chemical properties of the biomass, such as reducing the lignin content, extracting a specific macromolecular component, fractioning after the dissolution of the biomass (DA COSTA LOPES et al., 2013).

Pre-treatments release sugars from lignocellulosic biomass, making them available for fermentation and obtaining ethanol. However, little is found in the literature about what is done with the pre-treatment residue, what is left of this pre-treatment. Lima et al. (2018) performed anaerobic digestion of coffee husk hydrolyzate (CH), from a pre-treatment with ozone, in which the cellulose of the liquid phase of the CH would be used for the production of 2G ethanol. They obtained production of approximately 30 $NmLCH_4\ gCH^{-1}$ with the biodigestion of this hydrolyzate, however still presenting some inhibitions in the digestion due to some toxic products in the hydrolyzate. The effluent generated must be recovered to avoid environmental impacts in the presence of acids (MORAES; ZAIAT; BONOMI, 2015a), reinforcing the relevance of using these residues in biodigestion, and the need for more in-depth studies with the residues from the different pre-treatments that exist.

Brenelli et al. (2020) performed an alkaline and hydrothermal pretreatment of sugarcane straw to obtain xylo-oligosaccharides (XOS) and this was used to obtain 2G ethanol. As the hemicellulose of straw is highly acetylated and the acetate released has several harmful effects, deacetylation before the hydrothermal pretreatment was adopted as a strategy to increase the recovery of XOS in the resulting hydrolyzate and reduce its toxicity. Thus, this pre-treatment generates the deacetylation liquor, a residue that has not yet been explored. The

deacetylation liquor is rich in acetic acid, formic acid, carbohydrates (xylose, pentose) and lignin compounds, and its pH is next to 12.

Since many of these effluents generated from this pretreatments are rich in acids, it can be assumed that they may have potential for AD and biogas production. In view of this scenario, co-digestion/anaerobic digestion may contribute to the use of effluents generated from pre-treatments for the production of 2G ethanol. Based on the concept of biorefinery which is: "process of converting biomass into energy and chemicals" (DA COSTA LOPES et al., 2013), it is possible to integrate the first and second generation ethanol biorefineries, using residues from both processes for co-digestion and obtaining biogas. It is worth mentioning that the integration of the 1G2G ethanol biorefinery can also be done through the use of 2G vinasse, which is already a waste that shows potential for biodigestion and CH4 recovery (MORAES et al., 2014), but the use of waste from lignocellulosic pre - treatments are still in an initial scenario.

3.6 Biochemical Methane Potential Experimental and Theoretical (BMP and TBMP)

The biochemical methane potential (BMP) is a technique that was developed to determine the production of methane from an organic substrate during its anaerobic decomposition. This test is a simple and reliable method to obtain conversion rate of organic matter to methane (TRIOLO et al., 2012). The BMP technique is considered quite important, especially when working with unknown residues, or considered new residues, in which it provides results that predict the behavior of the residue in relation to its degradation and conversion to methane.

Studies in the literature already show the use of this methodology in a widespread way for different types of substrates. Gunaseelan (2004) performed BMP on fruits and vegetable solid. Owens and Chynoweth, (1993) perform BMP of municipal solid waste components. Janke et al. (2015) performed the BMP with residues from the sugar cane industry.

There is the experimental BMP and the theoretical BMP. In the experimental BMP an organic substrate is mixed with an anaerobic inoculum under defined operating conditions and the gas produced is measured. This operation takes place until the production of the gas is practically interrupted, that is, it ends, and in this way all the organic matter present in the substrate has been converted into methane. To perform the calculation of the experimental BMP, it can be done by dividing the net methane production of the residue under STP conditions by the weight of the added sample (base of VS or COD) (RAPOSO et al., 2011).

The theoretical potential of methane is used to predict methane production from a specific organic substrate. It can be expressed in mL of CH4 under standard temperature and pressure conditions (STP) or by the amount of organic material added or removed (volatile solids base or COD). The calculation of the biochemical potential of theoretical methane is generally calculated using the empirical formula: $C_aH_bO_cN_dS_e$ and using the Buswell equation (Equation 1) (RAPOSO et al., 2011).

$$TBMP_s = \frac{\left\{\left[\left(\frac{n}{2}\right)+\left(\frac{a}{8}\right)+\left(\frac{b}{4}\right)+\left(\frac{3c}{8}\right)\right]*22400\right\}}{12n+a+16b+14c} \quad \text{(Equation 1)}$$

TBMPs is the theoretical biological methane potential for solid substrate ($NmLCH_4\ gVS^{-1}$), and 22400 mL mol^{-1} represents the molar gas volume at standard temperature and pressure (STP, 273 K, and 1 bar). The molar content of hydrogen, oxygen, nitrogen, and carbon in the substrate is represented by a, b, c, and n, respectively.

Regression models were performed in which the methane yield of organic matter can be predicted from its chemical composition, so the calculation of theoretical potential can be done based on the COD. Theoretically 0.350 L of methane or 0.395 L at 35°C and 1 atm 1 g of removed COD can be obtained (GUNASEELAN, 2007), and is possible to calculate follow the Equation 2:

$$TBMP_L = VS_{added} \cdot \left(\frac{gCOD}{gVS}\right) * 350 \quad \text{(Equation 2)}$$

Where $TBMP_L$ is the theoretical biochemical methane potential for liquid substrate ($NmLCH_4\ gVS^{-1}$), VS_{added} is volatile solids added ($g\ mL^{-1}$), $_gCOD$ is chemical oxygen demand ($_gO_2\ mL^{-1}$) and 350 NmL is the theoretical CH_4 yield of 1 g COD at STP.

A disadvantage of performing the experimental BMP test is the duration of the tests and the fact that it does not provide a short-term result. The tests usually end when the variation in net methane production from one day to the next is 1%. It would be possible to limit the time required to perform a BMP test if one of the methods could predict the methane yield, but experimental tests are necessary to accurately verify the organic methane potential of the materials (RAPOSO et al., 2011).

The experimental methane yield can be used to calculate the level of anaerobic biodegradability compared to the calculated theoretical value, as shown in equation 3:

$$BD_{CH4}(\%) = \left(\frac{BMP}{TBMP}\right) * 100 \quad \text{(Equation 3)}$$

Where BD_{CH4} is the biodegradability, BMP is the Biochemical Methane Potential ($NmLCH_4\ gVS^{-1}$), and TBMP is the Theoretical Biochemical Methane Potential ($NmLCH_4\ gVS^{-1}$).

When biodegradability is calculated, the removed organic matter can be considered to be converted into methane, but some of that organic matter was used for the growth of microorganisms and the generation of new microbial mass. This value cannot be measured directly, but it can be estimated, but the literature show that this value to be less than 3% (RAPOSO et al., 2011).

BMP assays are generally performed in batch flasks, and are considered a starting point when starting an AD study. After BMP, other tests can be carried out, such as continuous reactor operation, with better targeting, since the behavior of the waste in relation to conversion to methane is known.

3.7 Factors affecting biodigestion to biogas production in Reactor Operation

The process of biodigestion is complex, involving several metabolic routes and a community of microorganisms that needs to be in balance, since the activity of one depends on the activity of the other, and there are some parameters that are extremely important for the good development of biodigestion in reactor like: pH, temperature, solids content, organic load, carbon nitrogen ratio, retention time.

The pH of an AD process varies over time. In the beginning, acid formation occurs in the acidogenesis process and the pH is in the range of 6 and CO_2 is released. After this stage, when the acetogenesis phase begins and the volatile acid is digested, the pH increases. It is necessary that the pH range in the digester is kept between 6.5 and 7.5 to always favor all stages of the process, so that the microorganisms are all active and biodigestion is efficient. If the pH happens to be less than 6.5 or greater than 7.5, the conditions may be harmful to methanogenic microorganisms. And a factor that must always be controlled is that the addition of any material does not cause a sudden change in pH, which can cause imbalance in the microbial population (SUTARYO; WARD; MØLLER, 2012; ZONTA et al., 2013).

There are two significant temperatures for AD microorganisms, either mesophilic (22-42°C) or thermophilic (55-72°C). The temperature maintained is extremely important as it represents the optimal activity of microorganisms (KRZYSZTOF ZIEMIŃSKI, 2012). In the works carried out by Janke et al. (2015, 2016b, 2017a) with AD from sugarcane residues,

temperature was used in the mesophilic range (38°C). Hartmann and Ahring, (2005) in their study with biodigetion of solid urban waste used thermophilic temperatures (55°C), as it can accelerate the AD process, in addition to reducing the number of pathogens during the anaerobic phase.

The solids content of a substrate used in AD in association with the organic load that is applied in the reactor is fundamental both for the performance and stability of the digesters, as well as for the cost of the operation. As the organic load increases, the CH_4 yield increases, and when the load decreases, CH_4 conversions decrease. However, with very high organic loads, methanogenic activities can be inhibited by high concentrations of long-chain fatty acids, volatile fatty acids and free ammonia (WU; HEALY; ZHAN, 2009). So it is always important to control the content of solids entering the reactor to achieve optimal methane production. In the work by Fernández; Pérez; Romero, (2008) in which fraction of municipal solid waste treatment was carried out, they reported that when the concentration of solids increased by 20 to 30%, the removal of chemical oxygen demand (COD) decreased from 80.69 % to 69.05% and the methane yield also decreased by 17%.

In addition to carbon and organic load, the nitrogen that is present in the waste is also very important for the production of biogas. Organisms need nitrogen to form cellular proteins. Carbon and nitrogen are considered the food for anaerobic bacteria. Carbon is where they get their energy from and nitrogen is used for cellular uptake (JAIN et al., 2015). The literature recommends an ideal C: N to thermophilic AD, ratio close to 25: 1 (WANG et al., 2012). If the C: N ratio is inadequate, it can generate results with a high release of total nitrogen or ammonia nitrogen, and an accumulation of volatile acids in the reactor. These substances (ammonia and volatile acids) are important intermediates and possible inhibitors of methanogenic activity (JAIN et al., 2015).

The period that the material stays inside the digester and the biogas is generated is known as the retention time. This time depends on the material used as a substrate and the process temperature. Methanogenic microorganisms take time to duplicate in 2 to 4 days, so the retention time cannot be less than that, as bacteria can escape with leachate and affect the entire biogas production process (KWIETNIEWSKA; TYS, 2014). In reactor continuous feed systems, the flow rate with which the reactor is fed is what will determine the retention time (JAIN et al., 2015).

The Oxidation Reduction Potential (ORP) is a parameter to control anaerobic digesters because measures the net value of all complex oxidation reduction reactions within an aqueous environment. In AD occur many complex reactions and it is difficult to identify each one of

them separately. A lot of biological reactions occur along of AD and some products from one reaction can be used as substrate for subsequent reactions (SUNG JAE LEE, 2008) and the ORP is important to understand the microbiological and operational interactions along the production of biogas from different raw materials. Some authors have already been using ORP as a control parameter for AD (NGHIEM et al., 2014; SUNG JAE LEE, 2008), but still nothing related to co-digestion with solid waste, and lignocellulosic residues.

Studies indicate that very high levels of ORP may indicate an inhibition of reactor activity. Under normal conditions of anaerobic digestion, the ideal operating range would be between -220 to -400 mV (BLANC; MOLOF, 1973). And that the ORP indicates different oxygen concentration conditions in a reactor (aerobic, anoxic and anaerobic). The ORP profile and sensitivity made it a parameter for monitoring process control (PEDDIE; MAVINIC; JENKINS, 1990). Studies were carried out relating ORP values to the production of volatile fatty acids. Wang; Zhou; Li, (2006) showed in their study that with the increase in ORP from -350 mV to -280 mV there was a greater production of propionic acid, and that high ORPs favor the production of propionic acid. As a result, the accumulation of proponic acid in an acidogenic phase reactor is not a good scenario for methane production, in a one-phase system, since an acetogenic rate of ethanol and butyrate by hydrogen-producing acetogensis is relatively higher than that of propionic acid.

According to Sung Jae Lee, (2008) results, a range of approximately -310 to -390 mV is ideal for the production of volatile fatty acids, and that this range suppresses the activity of methane-forming bacteria. And this way, ORP is considered as a successful parameter to control the chemical reactions of AD, since the organic material under anaerobic conditions is subjected to degradation by enzymes catalyzed by redox reaction.

All the factors mentioned above are important for the functioning of the biochemical processes that occur inside the reactors in the AD. As there are many microbial communities with different metabolic routes involved, it is necessary that the operational parameters of the reactor are aligned, so that there are no imbalances between the microorganisms.

3.8 Metabolic routes and microorganisms involved in Anaerobic Digestion

Among the most common phyla of bacteria found in AD reactors are Bacterioidetes, Firmicutes, Thermotogae, Euryarchaeota, Synergistes, Tenericutes, Proteocbateria (KIM et al., 2018). Thermotogae has often been reported in thermophilic digesters that treat organic waste like swine manure, organic market waste, and wastewater from food (JANG et al., 2016). Firmicutes are key cellulolytic bacteria in the thermophilic AD and contain acetogenic bacteria

as well, which degrade VFAs to produce acetic acid (WU et al., 2020). Within each of these phyla there are some genera that commonly appear in AD as can be seen in Table 1.

Table.1. Relationship between phylum and genus involved in the microbial community of AD

Phylum	Genus
Actinobacteria	*Actinomyces* *Atopobium* *Bacteriodes* *Prevotella* *Paludibacter*
Bacterioidetes	*Petrimonas* *Proteiniphilum* *Rekinellaceae*
Chloreflexi	*Blautia* *Coprothermobacter* *Enterococcus* *Gelria* *Erysipelotrichaceae* *Lanchnospiraceae*
Fibrobacters	*Cladicoprobacter* *Fastidiosipila* *Gelria* *Halocella* *Hydrogenispora*
Firmicutes	*Clostridium* *Lutispora* *Ruminiclostridium* *Ruminococcus* *Suntrophomonas* *Tepidanaerobacter*
Protobacteria	*Petrobacter* *Candidatus*
Spirochaetes	*Spirochaeta*
Synergistes	*Anaerobaculum* *Synergistaceae uncultured*
Ternicutes	*Haloplasma* *Fervidobacterium*
Thermotogae	*Kosmotoga* *Defluviitoga*

Source: Adapted (GUO et al., 2014)

The Euryarchaeota was the *Archaea* phylum and is composed for all methanogens such as genus: *Methanosaeta, Methanosarcina, Methanobacterium, Methanospirillum, Methanoculleus, Methanococcus, Methanobrevibacter Methanothrix*. They differ by their morphological and also metabolic characteristics (KRZYSZTOF ZIEMIŃSKI, 2012). As mentioned in section 2.7, methanogens are classified as hydrogenotrophic or acetotrophic, depending on the substrate they use. In the acetotrophic pathway, they can be caracterized by the groups *Methanosaeta* and *Methanosarcina*, for example. *Methanosarcina*, can utilize multiple substrates to produce methane and can be most abundant aceticlastic methanogens in unstable codigesters with high acetate concentrations (DEMIREL; SCHERER, 2008; WU et al., 2020). In the hydrogenotrophic pathway, the groups *Methanobacterium, Methanospirillum, Methanoculleus* and *Methanothermobacteria* that are part of it (DEMIREL; SCHERER, 2008; KIM et al., 2018). *Methanobacterium* plays key roles through interacting with other genera (e.g. *Syntrophomonas, Clostridium*), and tolarate high concentrations of ammonia (WU et al., 2020)

Each genus is characterized by producing certain enzymes and catalyzing specific substrates. Cai et al. (2018) detected the presence of this phylum in the AD with rice straw as a substrate. The genera *Fastidiosipila* and *Sedimentibacter* hydrolyze fermentation proteins and produce volatile fatty acids (VFA). Species of the genus *Propionispira* are characterized by fermenting carbohydrates and producing propionate acetate and CO_2 as final products. Generally, when high concentrations of propionate occur in the fermentative phases, it is due to the presence of this microorganism (AN et al., 2020). The *Mesotoga* genus, which belongs to the phylum Thermotogae, has characteristics of metabolizing sugar in the presence of sulfur or hydrogenotrophic sulfate reducers such as *Desulfovibrio vulgaris* and producing acetate, sulfide, CO_2 and traces of hydrogen. The phylum Proteobacteria is correlated with lignocellulose degradation at the hydrolysis phase (WU et al., 2020). Bacteria such as *Syntrophomonas c*an degrade VFAs such as butyrate and propionate (WU et al., 2020)

Within the AD stages, different chemical reactions occur depending on the substrate that is being degraded and different bacteria work cooperatively, one depending on the activity of the other within these metabolic routes. Bacteria of the genus *Syntrophomonas, Syntrophospora* and *Syntrophobacter* are syntrophic bacteria, which oxidize compounds such as propionate and butyrate and, thus, obligatorily use hydrogen as an electron acceptor. The butyrate oxidation reaction, carried out by these microorganisms, can occur only when the low pressure of H_2 is maintained (DEMIREL; SCHERER, 2008). Syntrophic bacteria cannot grow in the form of

pure cultures, but only when accompanied by microorganisms using hydrogen. This cooperative relationship between microorganisms, in which one synthesizes a product to be used by another is called syntrophy, and in particular the relationship of hydrogen consumption is called interspecific hydrogen transfer (KRZYSZTOF ZIEMIŃSKI, 2012).

Another relation of syntrophy that happens in AD is the syntrophic acetate oxidation (SAO) coupled with hydrogenotrophic methanogenesis. At SAO, both methyl and acetyl acetate groups are oxidized to CO_2 with hydrogen production. Because this reaction is energetically not favorable, hydrogenotrophic methanogenic *Archaea* consume H_2 and eliminate it from the reaction. In this situation, one microorganism also depends on the other since one needs to donate H^+ and the other receives it. Generally bacteria of the genus *Syntrophaceticus* and *Tepidanaerobacte*r participate in this type of reaction (HATTORI, 2008; KIM et al., 2018). Table 2 shows the chemical reactions involved in the metabolism of acetate and hydrogen.

Table 2. Relationship between phylum and genus involved in the microbial community of AD

Process	Reaction	ΔG (KJ/mol)
(1) Acetoclastic Methanogenesis	$*CH_3COO^- + H_2O \rightarrow *CH_4 + HCO_3^-$	-31.0
(2) Syntrophic Acetate Oxidation	$*CH_3COO^- + 4H_2O \rightarrow H*CO_3^- + 4H_2 + HCO_3^- + H^+$	+104.6
(3) H2-consuming methanogenesis	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-135.6
(4) Sum (2) + (3)	$*CH_3COO^- + H_2O \rightarrow H*CO_3^- + CH_4$	-31.0
(5) H2-consuming acetogenesis	$4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O$	-104.6

(*) represent the fate of the methyl group carbon acetate

Source: (HATTORI, 2008)

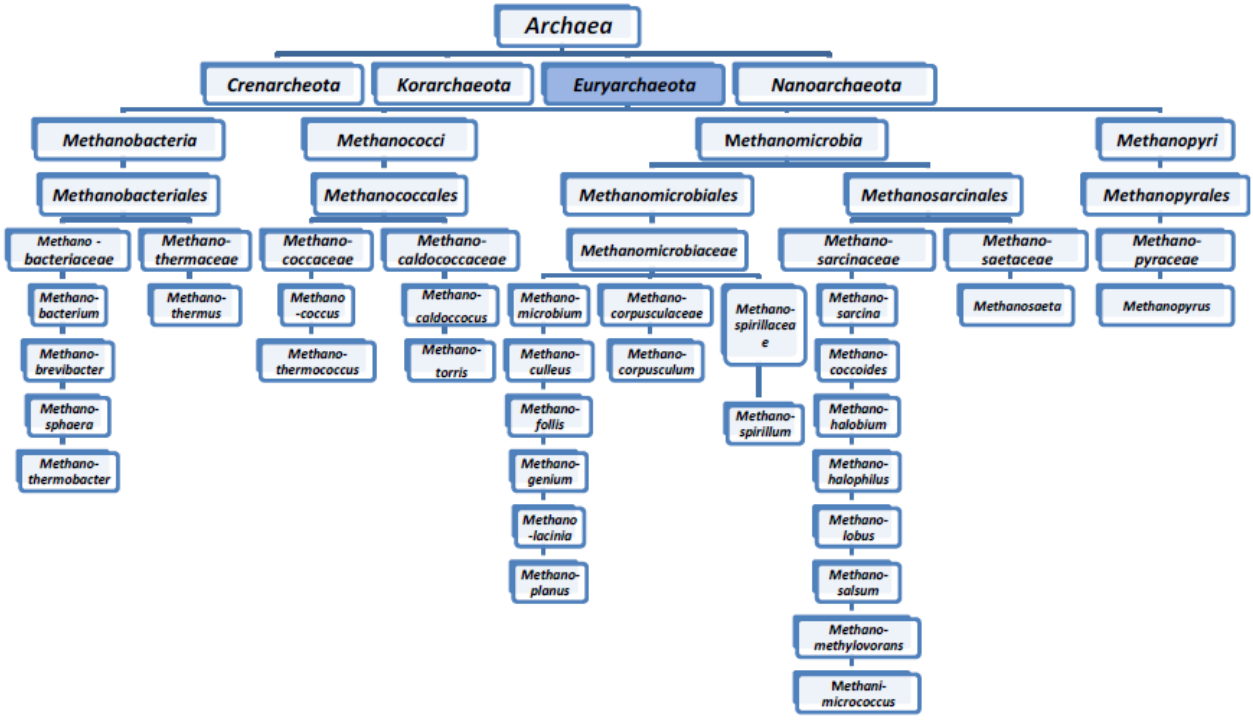
Most propionate oxidizing syntrophic bacteria belong to the *Syntrophobacter* genus of the δ-proteobacteria group. *Desulfotomaculum thermobenzoicum subsp. thermosyntrophicum* are examples of SRB and can act as syntrophic bacteria that oxidize thermophilic propionate (KRZYSZTOF ZIEMIŃSKI, 2012).

In view of the complexity of the microbial community involved in the AD process, molecular biology techniques were developed to make it possible to identify microorganisms, and to understand the steps involved in AD, as well as the consumption of substrates and generation of products, contributing to the AD optimization biogas production.

3.9 Biology Molecular Analysis

It is known that the microorganisms from the AD process are diverse and distinct. They are considered highly diversified and high redundant, meaning that several microorganisms are metabolically flexible and capable of doing the same job, being one of the reasons for the robustness of the anaerobic digestion process (ZUMSTEIN; MOLETTA; GODON, 2000). The figure 3 shows the different microorganisms that are involved in group of methanogenic *Archaea*.

Figure 3. Philogenetic hierarchy of methanogens



Source: copied (DEMIREL; SCHERER, 2008)

Cabezas et al. (2015) showed that molecular biology techniques serve to answer questions from microbial communities such as: "1-Who is there ?, 2-How the community change over time?, 3-How many microorganisms of the different groups are present?, 4-What are the specific functions of microorganisms in the community and its relationship with each other?"

The 16S rRNA gene analysis technique is one of the most frequently used techniques to identify microorganisms involved in a microbial community. This gene is considered a genetic

marker to study bacteria and phylogeny and *Archaea* taxonomy because it is present in almost all bacteria and *Archaea,* its function over time has not changed and that this gene is large enough for computing purposes (1500bp) (JANDA; ABBOTT, 2007). It is currently possible to determine the genus and species of a bacterium or *Archaea* by sequencing 16S rRNA population and comparing it with the available database sequence. During the last decades cloning in a plasmid vector followed by Sanger sequencing has been widely used (CABEZAS et al., 2015). In the study by Zumstein; Moletta; Godon, (2000) the structures of the bacterial and *Archaea* communities in an anaerobic digester were monitored in order to characterize the population dynamics, using the technique of fluorescence-based polymerase chain reaction (PCR) conformation polymorphism (SSCP) analysis using an automated DNA sequencer of 16S gene. Bibby; Viau; Peccia, (2010) also performed the technique of amplification and sequencing of the 16S rRNA gene to understand the diversity and abundance of pathogens in sewage sludge. Wilkins et al. (2015) carried out a study of the communities of *Archaea* in three sludge digesters for the production of biogas using the high-throughput sequencing of the methyl coenzyme M reductase (mcrA) and 16S rRNA genes.

Nelson; Morrison; Yu, (2011) performed an analysis based on all available results of the 16S rRNA gene sequence generated by the sanger sequencing of anaerobic digesters and found that up to 2010 there was a total of 19,388 sequences, 16,519 bacteria and 2869 *Archaea*, representing 28 bacterial phyla such as Proteobacteria, Firmicutes, Bacteroidetes, Chloroflexi. In addition to sequencing using the Sanger method, technologies using Next Generation Sequecing (NGS) are also used to identify microbial communities with the largest number of reactors. Werner et al., (2011) performed sequencing using NGS for a trial of 9 large-scale bioreactors for the treatment of brewery wastewater.

When different reactor operating parameters are tested, such as organic loading rate, hydraulic retention time, operating temperature to analyze the ideal conditions for anaerobic digestion, it is necessary to monitor together the microbial performance during the operation, and a suitable technique for this. It would be the digital printing technique like DGGE, T-RFLP or SSCCP (CABEZAS et al., 2015). These methods are based on the analysis of amplifications from PCR of the 16S rRNA community (DGGE, SSCP and T-RFLP) or the ribosomal system between the 16S and 23S rRNA genes (RISA). Through these analyzes a fingerprint of the microbial community based on the polymorphism of the sequence is generated, then making an assessment of the community structure and fluctuation over time in ecological studies (CABEZAS et al., 2015). Zhang et al. (2005) used the DGGE technique to compare the structure of microbial communities in sludge from two types of sparging flow anaerobic

reactors (UASB): a full-scale reactor for treating wastewater from potato processing and other three laboratory-scale reactors being fed with raw municipal sewage water, with CEPS (chemically enhanced primary sedimentation) pretreated municipal wastewater, and with a synthetic municipal sewage, respectively. Carballa et al. (2011) preferred to use the two techniques of DGGE and T-RFLP to compare the characteristics of the microbial community in continuous anaerobic reactors on a laboratory scale, obtaining results in which both techniques indicate that bacterial and mesophilic communities were richer and more even than *Archaea* and thermophilic communities, respectively.

In the AD process, quantifying the density and proportion of methanogens is important to ensure an efficient methanogenesis process. For this, techniques such as in situ hybridization (FISH) or quantitative PCR (qPCR) are considered adequate because they quantify different groups of microorganisms present in a complex community (CABEZAS et al., 2015). The FISH technique consists of application of fluorescently labeled probes to ribosomal rRNA in permeabilized whole microbial cells. Díaz et al. (2006) evaluated the different types of methanogenic granules of an anaerobic bioreactor that treated wastewater in a brewery using different molecular biology techniques and among them the FISH technique, being this technique responsible for identifying groups of bacteria as Firmicutes and *Archaea* like the Methanosaeta.

Given what has been presented, the techniques of molecular biology are already widespread within anaerobic digestion and considered of great importance mainly for the study of microbial communities. In addition, these techniques can be combined with information from analysis of proteins expressed by these bacteria, through proteomics analysis, corroborating to results that better explain the metabolic routes of the process.

3.10 **Proteomic Analysis**

Currently, discovering the roles that microorganisms play in anaerobic communities is one of the challenging issues. Relating the microorganism to its metabolic pathway is often a challenge. Identifying the microorganism in the process is already a big step, suggesting its metabolic potential, but it may not be enough to attribute the function of these microorganisms, because is possible that a single microorganism has different functions at different stages of the metabolic pathways. Within this, more sophisticated techniques such as proteomics can solve this problem, since it links identity to function (CABEZAS et al., 2015).

Omic techniques (genomics, transcriptomics and proteomics) are bioinformatic tools that allow the study of more complex ecosystems. Understanding the interaction between

species and responses to environmental conditions are only identified if the ecosystem is assessed as a whole. Omic techniques have come to reveal a complete picture of microbial functionality in an ecosystem (CABEZAS et al., 2015).

Proteomics analysis is used to characterize proteins within an environmental microbial consortium. This contributes to the knowledge of the functions of the main active and important metabolic pathways for AD. One of the main limitations that proteomics has is to extract a sufficient amount of high quality protein sample that is representative. This becomes complicated due to the complexity of the microbial communities involved and the presence of interfering compounds and the heterogeneity of natural environments. However, proteomics has great potential in linking genetic diversity and activities of microbial communities (SU et al., 2012).

Some works have already been carried out with proteomics techniques within anaerobic digestion and highlighting the importance of the relationship between the expressed proteins and the identified microorganisms that are expressed them. Abram et al. (2011) conducted a study in a wastewater treatment bioreactor basead in glucose in which a metaproteomic study was carried out and they identified 18 distinct proteins, excluding redundant identification. Of the 18 proteins, 14 were classified in the functional category of metabolism, related mainly to glycolysis, and methanogenesis, the other 4 proteins were classified as membrane proteins, reduction, transcription and degradation of proteins. In addition, they traced the metabolic pathway involved in AD and related proteins to possible microorganisms. In another study carried out by Hanreich et al. (2013), metagenomics and metaproteomics techniques were used to analyze microbial communities in AD with straw and hay as substrates they used mass spectrometer to identify proteins. The authors were able to detect transporter and flagellin proteins, which were expressed mainly by members of the phyla Bacteroidetes and Firmicutes and that 20 to 30% of the identified proteins were of *Archaea* origin and the main enzymes of methanogenesis were expressed in large quantities, indicating high activity metabolic rate of methanogens, although they represent only a smaller group within the microbial community.

Jing et al. (2017) carried out a study with the objective of investigating the effects that the addition of conductive magnetite can have on the degradation of propionate for methane production, and performed quantitative proteomic analysis of iTRAQ to analyze the alterations of the metabolic pathways induced with the addition of magnetite, and observed through this analysis that magnetite induced changes in the levels of protein expression involved in various metabolic pathways. In this context, proteomic analysis opens the way for explanations of

substrate degradation and conversion to methane, and can be a great ally in understanding co-digestion.

Currently, advances in the area of Proteomic analysis provide tools that allow a very thorough and advanced study of the proteins involved in AD. Heyer et al. (2019) analyzed the functionality of the microbial community of 11 reactors from a biogas plant, using the high-resolution metaproteomics pipeline, in which they were able to specifically identify by the MetaProteomeAnalyzer software specifically all the proteins involved, the species of microorganism that secreted it and the function it has.

In general, proteomics analyzes within AD are being introduced, in order to contribute to the explanations of the different and innumerable metabolic routes that exist in the AD process and being step to optimize the application of this technology. Proteomics has been improving the tools and techniques that can be applied and is an aspect that should be better exploited for the anaerobic consortium of microorganisms.

3.11 **Proteins involved in Anaerobic Digestion**

Within the biochemical processes of AD, a large number of enzymes play important roles. In the conversion of H_2 and CO_2 to methane and acetate to methane and CO2, several enzymes and prosthetic groups are involved, the basic of which is composed of: eazariboflavin derivative F420, methanopterin, methanofuran (MFR), nickel-tetrapirol fator F430 and coenzyme M (mercaptan sulfonate). The synthesis of cellular material with CO_2 occurs for example through the aceto-CoA pathway with pyruvate (HEYER et al., 2019). In the metabolic pathway, MFR, methanopterin and coenzyme M are methanogen C1 carriers, while coenzymes F420 and coenzyme B are electron donos (MASHAPHU, 2005). Enzymes such as Glyceraldehyde-3-phosphate dehydrogenase, enolase are usually involved in the glycolysis phase. LamB porin is related to membrane proteins, Iron containing alcohol dehydrogenase is related to the oxidation-reduction reaction (ABRAM et al., 2011).

Methanogenic metabolism is unique, as it requires co-enzymes that do not occur in any other organism except methanogens. In the first part of methanogenesis, CO_2 is limited by MFR, which is reduced to methenyl, methylene, methyl and at the final stage - methane, which is bound by coenzymes: Tetrahydromethanopterin, 2-methylthioethanesulfonic acid and 2-mercaptoethanesulfonic acid. Methanopterin is responsible for the reduction stage of CO_2 for methyl pyruvate groups. Methyl groups in carbonylation process are converted into carbonyl groups with a part of the enzyme carbon monoxide dehydrogenase. Hydrogenase is the enzyme responsible for the assimilation of H2, and they react with the F420 factor which is responsible

for providing a reducing force for the reaction to happen. Hydrogenase enzymes are very present in methanogenic processes because microorganisms use H2 as an electron source for oxidation-reduction reactions (KRZYSZTOF ZIEMIŃSKI, 2012; SAXENA; ADHIKARI; GOYAL, 2009). (F420) is an electron-transferring coenzyme used by several enzymes such as hydrogenase, formate dehydrogenase, methylene tetrahydromethanopterin (H4MPT) dehydrogenase, methylene H4MPT reductase, and heterodisulfide reductase (MASHAPHU, 2005)

Enzymes such as phosphate acetyltransferase and acetyl-CoA-decarbonylase have been linked to the metabolic pathway of acetoclastic methanogenesis, and the enzyme tetrahydromethanopterin S-methyltransferase has been linked to the hydrogenotrophic route and both pathways converge to the methyl-coenzyme M (methyl-CoM) reductase (HANREICH et al., 2012). Abram et al. (2011) detected both types of enzyme in an AD reactor with wastewater. In addition, the GroEL protein may also be present in AD processes, which is related to potentially syntrophic organisms (HANREICH et al., 2012). Table 3 shows the relation of proteins and microorganisms that was found in Hanreich et al. (2012) study.

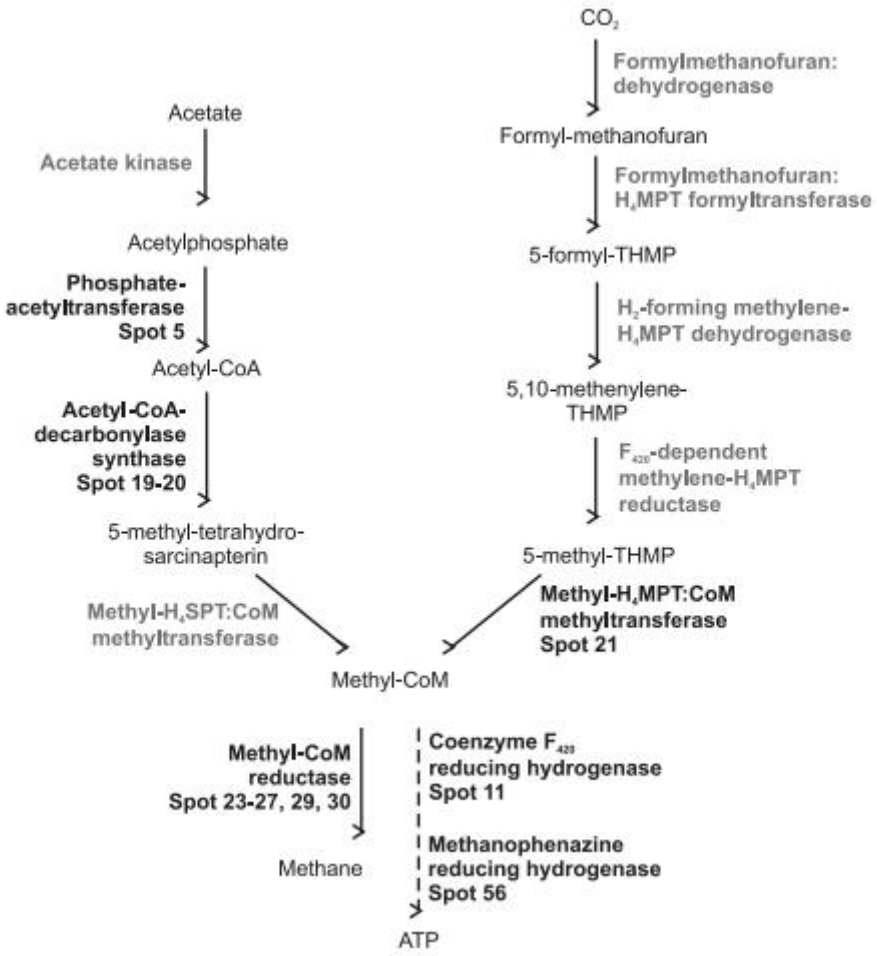
Table 3. Relation between protein and Microorganism from AD

Enzyme	Related Genus
Chaperonin GroEL	*Anaerobaculum*
Phosphate acetyltransferase	*Methanosacrina*
Coenzyme F420 reducing hydrogenase	*Methanocorpusculum*
Acetyl-CoA decarbonylase/synthase complex δ subunit	*Methanosarcina*
Tetradydromethaprotein S-methyltransferase, subunit H	*Methanosarcina*
Methanol corrinoid protein	*Methanosarcina*
Methyl-coenzyme M recutase, γ subunit	*Methanosarcina*
Methyl-coenzyme M recutase, β subunit	*Methanosarcina*
Methyl-coenzyme M recutase	*Methanoculleus*
Methanophenazine-reducing hydrogenase	*Methanosarcina*

Source: (HANREICH et al., 2012)

In the study by Hanreich et al. (2013) the map was drawn of the two pathways of methanogenesis: acetoclastic or hydrogenotrophic methanogenesis, specifying the related proteins in the thermophilic metabolic process (Figure 4). The substrates that are used was beet and rye silage. In this route, the presence of enzymes of the acetoclastic pathway expressed by members of the Methanosarcinaceae implies the activity of this metabolic route under thermophilic fermentation conditions. Enzymes expressed by members of Methanomicrobiales, which are hydrogenotrophic methanogens, have also been detected. In addition, the presence of proteins from the synergistic system, which can produce H_2, was also detected as a co-substrate for methane production. The enzyme methyl-CoM reductase was the most abundant protein, and they connected this enzyme with methane production which was constant. (HANREICH et al., 2012).

Figure 4. Draft of the acetotrophic and hydrogenotrophic pathway of methanogenesis



Source: copied (HANREICH et al., 2013)

In the study developed by Abram et al. (2011) in a reactor treating a synthetic, glucose-based wastewater at 15°C, a mapping of the metabolic route was made in which it presented different results from that found in the study by Hanreich et al. (2012) due to different temperature and substrates.

Microorganisms that contain Fe-Fe hydrogenases produce molecular hydrogen as a result of its energy metabolism and create trophic bonds in AD with the use of hydrogen methanogens. Proteins like glyceraldehyde-3-phosphatase, triosephosphate isomerase, fructose-biphosphate aldolase, glucose-6-phosphate isomerase, and lactate dehydrogenase are involved in the fermentation of sugars and carbohydrate metabolism (ZIGANSHIN et al., 2019).

Heyer et al. (2019) showed the metabolic routes involved in AD for the production of biogas, with the microorganisms involved and all the functionalities of the proteins, showing how abundant the number of proteins that are involved in the production of biogas, and how many different microorganisms can secrete them. In addition, they show the presence of phages

that can hinder the microbial consortium and decrease the rate of conversion of nutrients into biogas.

In addition, with the proteomics data, it is possible to relate how the microorganisms consumed the substrates for the production of CH_4, even to consider the possibility of optimizing the microorganisms necessary for the degradation of a specif substrate, due to their enzymatic production.

3.12 Oligoelements and application of Nanoparticles

It is already known that AD develops better in the presence of micronutrients. Ilangovan; Noyola, (1993) already reported that the availability of micronutrients such as Fe, Ni, Cu, Zn, Mo, Co, Se, Si, F, Mg, Na, are important for anaerobic microorganisms to obtain an efficient degradation of the residues. Some other older authors have also shown that such trace elements stimulate methanogenesis or are essential for cell growth (PERSKI; MOLL; THAUER, 1981). Nel; Britz; Lategan, (1985) used a solution of micronutrients and achieved a greater removal of COD, volatile acids and consequent greater production of methane. Other authors of more recent works, also continued to research and investigate the role of micronutrients in AD, and had positive results in the performance of the process, with different substrates such as corn silage (POBEHEIM et al., 2010) wheat vinasse (SCHMIDT et al., 2014) and rest of food (WEI et al., 2014). Cai et al. (2017) carried out a study comparing the effect of trace elements on the microbial community for methane production using rice straw as a substrate, and the results showed that the addition of Fe, Mo, Se and Mn reduces volatile fatty acids leaving dominant bacteria *Bacteriodetes* and *Methanoseata* and that the addition of trace elements increased the proportion of *Methanoseata* in relation to the control.

Scherer; Lippert; Wolff, (1983) classified that for methanogenic organisms the importance of micronutrients is given in the following order: Fe >> Zn> Ni> Cu = Co = Mo> Mn, indicating that such elements have essential roles as in the construction of methanogenic cells. Zhang et al. (2003) showed that if the content of the trace elements Co, Ni, Fe, Zn, Cu is less than 4.8, 1.32, 1.13, 0.12 $g.L^{-1}$ respectively, there is a limitation of the growth of the microoroganism culture methanogenic in terms of cell density.

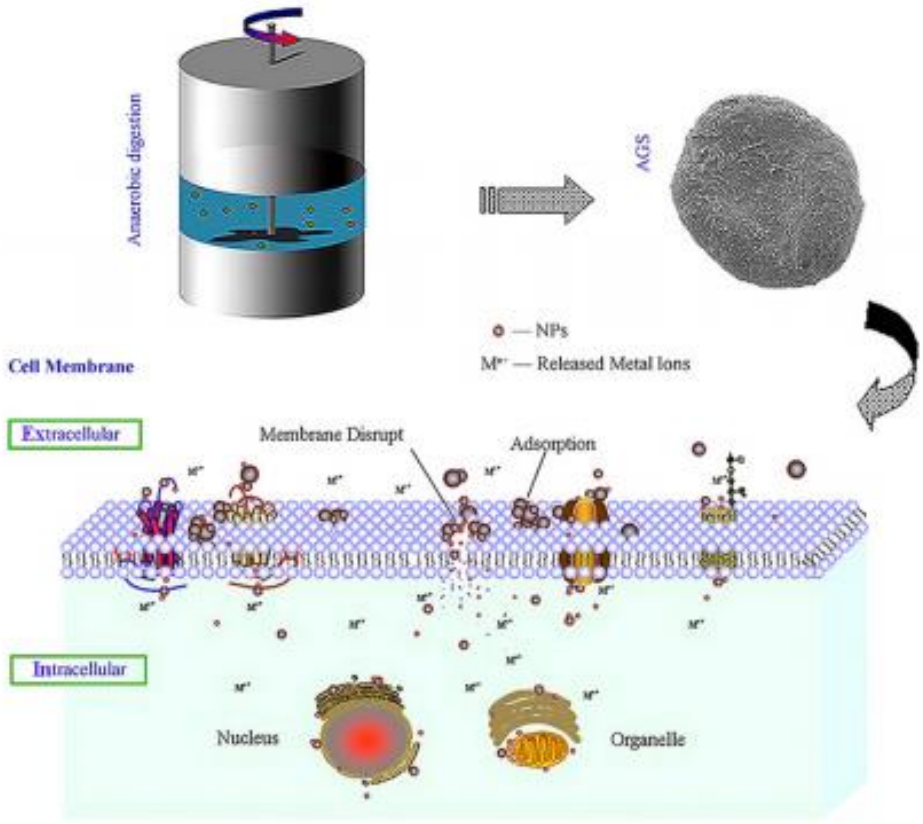
In addition to being a growth factor, Fe is also important in stimulating the formation of cytochromes and ferroxins that are vital for the cellular energy metabolism. With the increase in Fe, the rate of methane formation by *Methanosarcina barker* also increases with methanol as a substrate, so the AD process can only be successfully performed with correct concentrations of trace elements, so as not to become toxic (CHOONG et al., 2016; LIN et al.,

1990). In addition to methanogenesis, trace elements are also extremely important in the acetogenesis phase, as some metalloenzymes are involved in chemical reactions in this step, such as dehydrogenase formate, carbon monoxide dehydrogenase, and elements such as Fe, Se, Ni, Zn act in these processes as catalysts (CHOONG et al., 2016). Yu et al. (2015) showed that Fe supplementation accelerated the hydrolysis and acidification stage of AD, showing that hydrolytic and acidogenic bacteria also benefit from trace elements as a growth factor. In addition, Bini, (2010) reported that Ni is an essential cofactor for Ni-Fe hydrogenases, carbon monoxide hydrogenase and methyl reductase.

In view of so many studies about the importance of micronutrients in AD, other approaches are being made on how to add these trace elements in the AD process, in a way that their concentrations are ideal and do not cause toxic effects for de process and for the environment. Abdelsalam et al., (2016); Mu; Chen; Xiao, (2011) used nanoparticles as a means of adding trace elements in the AD process and were successful.

Nanoparticles are the particles found in nanometric size and nanotechnology is the engineering that allows the manipulation of matter on a nanoscale (1 to 100 nm). Nanoparticles have been offering potential for new functional materials, processes and devices allowing for a unique activity towards contaminants and greater mobility in the environment (LAROUI et al., 2011). The nano-size is important for interaction with the biological system, as it determines the ability to penetrate cell membranes, facilitating passage through biological barriers, absorption and distribution through metabolism. When compared to bulky atomic equivalents, nanometric materials have superior chemical and physical properties due to their mesoscopic effect, small object effect, quantum size effect and surface effect. In addition to having unique properties such as large surface area, dispersibility, high reactivity (ABDELSALAM et al., 2016, 2017b). Figure 5 shows how is the process of metal liberation by nanoparticle to microorganism cell in AD.

Figure 5. Effects of metal nanoparticles on an anaerobic digestion system



Source: copied (WANG et al., 2016b)

Abdelsalam et al. (2016) compared the effects on the production of biogas and methane from the anaerobic digestion of animal manure using nanoparticles (NPs) of trace metals such as Co, Ni, Fe and Fe_3O_4 and achieved an increase of 2, 2.17, 1.67 and 2.16 (respectively) times the volume of methane compared to the control used. In another study Abdelsalam et al. (2017b) carried out the digestion of slurry (raw manure) in order to accelerate the digestion process using Fe nanoparticles and Fe_3O_4 nanoparticles, obtaining increase results of 1.59 and 1.96 (respectively) times the volume of methane produced compared to the control, considering the Fe_3O_4 nanoparticle better for this substrate. Iron nanoparticles have been suggested as an important part of the bioavailable fraction of the metal (NI et al., 2013). Krongthamchat; Riffat; Dararat, (2006) showed that synthetic nano-iron was preferred over EDTA and Fe used in growth cultures for microalgae, suggesting that the nanoparticulate form of the metal is more bioavailable.

Mu; Chen; Xiao, (2011) found that among 4 types of nanoparticles such as TiO_2, Al_2O_3, SiO_2 and ZnO, only ZnO nanoparticles have an inhibitory effect on methane production

depending on its dose, if it is less than 6 mg.ST^{-1} there is no inbitory effect, using as substrate waste actived sludge.

Wang et al. (2016) studied the use of nanoparticles in the AD system in order to improve the efficiency of the process and avoid inhibitory effects. They work with the nanoparticles: nZVI (nano-zero-valent-iron), Ag, Fe_2O_3 and MgO for methane production with digestion of waste activated sludge. The presence of 10 mg.g^{-1} of nZVI of total suspended solids (TSS) and 100 mg.g^{-1} of TSS Fe_2O_3 increased methane production to 120% and 117% of the control, respectively, while 500 mg.g^{-1} of TSS Ag and 500 mg.g^{-1} TSS MgO generated lower levels of methane production (73.52% and 1.08% the control, respectively).

The European Commission recognizes nanotechnology as one of its six "main enabling technologies" that contribute to sustainable growth and competitiveness in various industrial sectors. The challenges of sustainability, food security and climate change are leading researchers to explore the field of nanotechnology as an improvement for the agricultural sector (PARISI; VIGANI; RODRÍGUEZ-CEREZO, 2015). Nanoparticles are considered a viable technology for introducing micronutrients in AD and consequent optimization of biogas production. It is an area that is still being applied in biodigestion, requiring some investigations regarding the use of different types of waste.

3.13 **Closure**

Given all the issues addressed, it is possible to notice that the literature already reports the use of 1G vinasse and filter cake through the co-digestion process to obtain CH_4, and even the use of 2G vinasse together with the residues from the production of 1G ethanol for the integration of the 1G2G ethanol biorefinery. However, there are still gaps regarding the use of pre-treatment residues of lignocellulosic materials, such as the pre-treatment of sugarcane straw, within co-digestion and CH_4 production, as a way of also proposing the integration of 1G2G ethanol biorefinery

Deacetylation liquor is still a little explored residue, mainly in relation to its use within biorefineries, therefore highlighting the importance of further studies on it in co-digestion with residues from the production of 1G ethanol (filter cake and vinasse).

One of the factors that can bring enriching results for the monitoring of co-digestion, is the control of the metabolic routes of the process, through proteomics analysis and identification of the microbial consortium. The literature shows that these molecular techniques are already widespread, and can be used within microbial consortiums. The use of these combined analyses

is challenging, but it manages to show the different interactions of the change of the microbial community with the different residues in the process steps.

In addition, co-digestion can be further optimized with the use of nanoparticles as a way of introducing micronutrients into AD, since the literature shows their effectiveness and better properties than the use of the "loose" micronutrient within the system.

4 OBJECTIVES

4.1 General

The purpose of this project was to integrate biogas production in an innovative concept of biorefinery by using organic substrates from the sugarcane processing to 1G2G ethanol in a anaerobic co-digestion, as relating metabolic microbial routes to operating parameters of AD process and the effects of nanoparticles application.

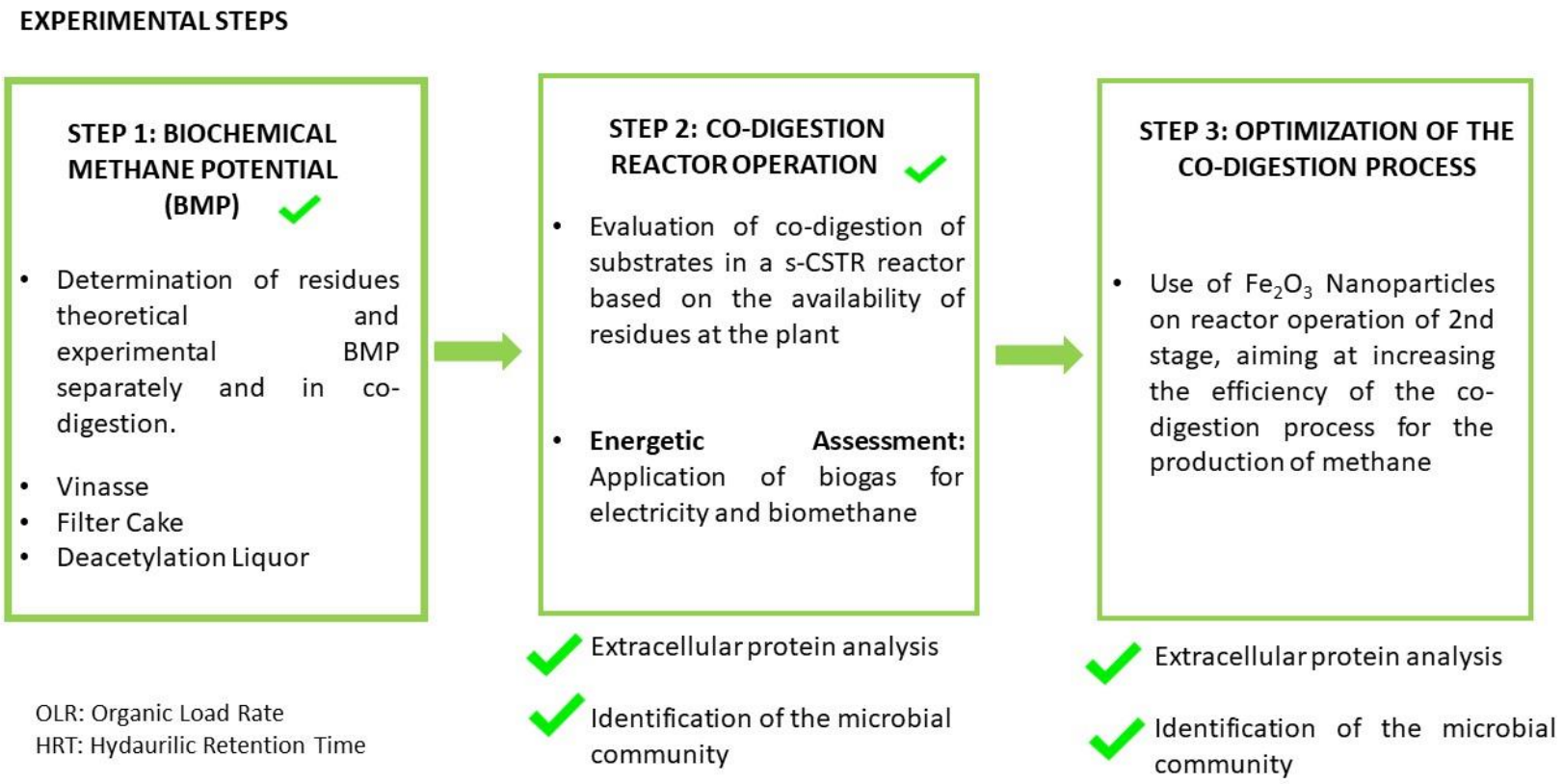
4.2 Specifics

The topics to follow are specific objectives:

- to evaluate the theoretical and experimental biochemical CH_4 potential and the digestibility of substrates from the production of 1G2G sugarcane ethanol (e.g. vinasse, filter cake and residue from straw/bagasse pre-treatment – deacetylation liquor);
- to investigate the biogas production from the co-digestion process of the aforementioned substrates through continuous reactor operation;
- to analyze the effect of nanoparticles used in the optimization of the continuous co-digestion process;
- to relate the operational aspects of the co-digestion process to the metabolic routes of the microbial community;
- to evaluate the energy potential of biogas in an integrated 1G2G ethanol biorefinery at the application level in electricity and biomethane
- to identify the microbial community involved in the co-digestion process.

Figure 6 shows represent the flowchart of all experimental steps performed.

Figure 6. Flowchart of experimental steps



5 RESULTS AND DISCUSSION

This session will be presented in the form of scientific articles that have been submitted or published to indexed journals.The first article entitled "Use of Lignocellulosic Residue from Second-Generation Ethanol Production to Enhance Methane Production Through Co-digestion" deals with the first stage of the project's development, which are the BMP and TBMP tests. This paper was published in the journal "*Bioenergy Research*". https://doi.org/10.1007/s12155-021-10293-1

The second article entitled "Operational and biochemical aspects of co-digestion (co-AD) from sugarcane vinasse, filter cake, and deacetylation liquor" deals with the second stage of the project's development, which was the operation of residues co-digestion reactor and the characterization of the microbial community. This paper was published in journal "*Applied Microbiology and Biotechnology*". https://doi.org/10.1007/s00253-021-11635-x

The third article entitled " Anaerobic co-digestion of residues in 1G2G sugarcane biorefineries for enhanced electricity and biomethane production " was carried out an energetic analysis of the results obtained in the second article, in order to propose a prospection of a 1G2G ethanol plant with biogas production both in the season and in the off-season. This article was published in the journal "*Bioresource Technology*". https://doi.org/10.1016/j.biortech.2021.124999

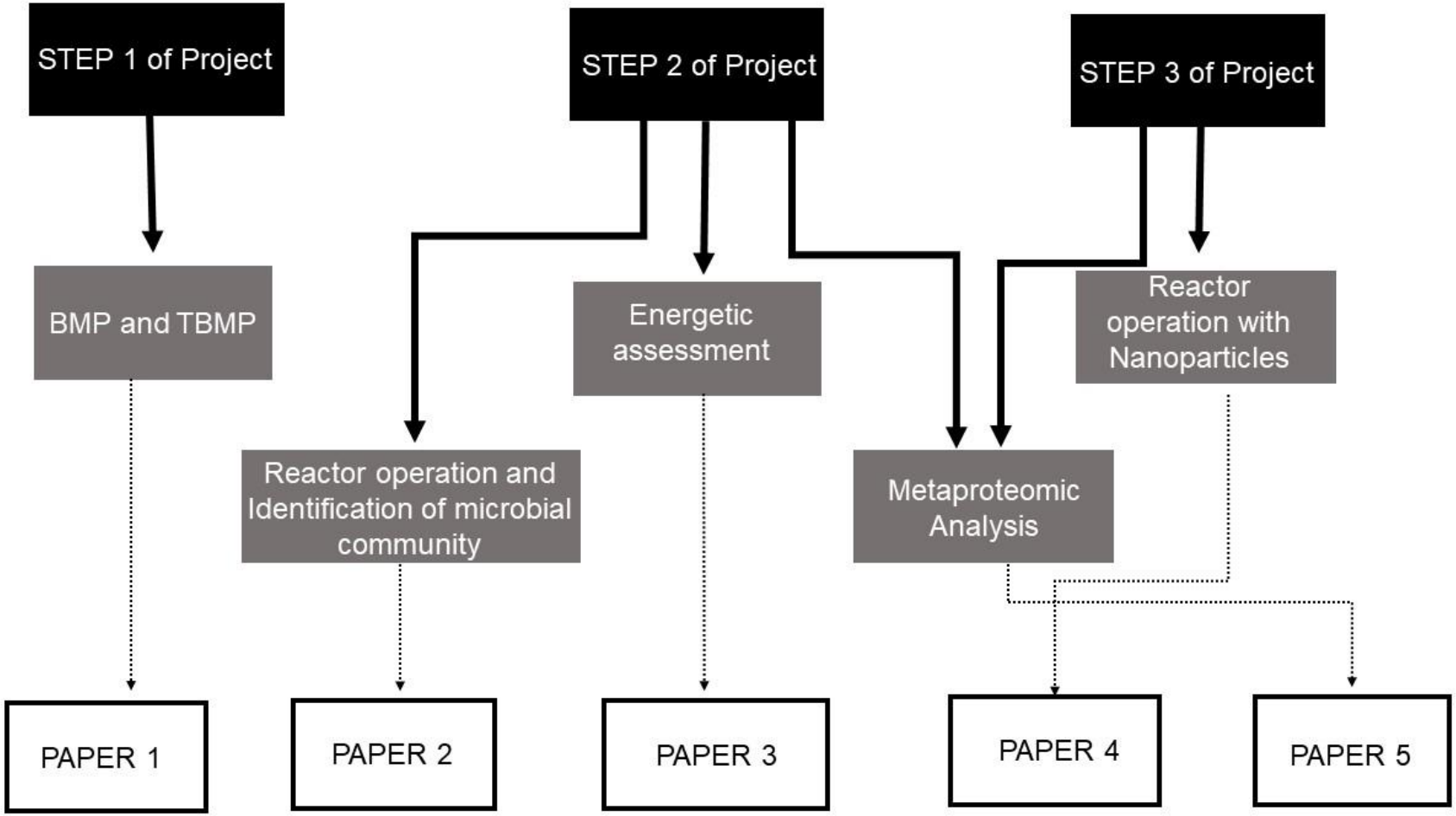
The fourth article entitled "Use of Fe_3O_4 nanoparticles in reactor co-digestion of residues from 1G2G ethanol biorefinery: microbiological routes and operational aspects" reports on the third stage of this project, in which the co-digestion of the residues was carried out, in the same way that it was carried out in stage 2, but now with the addition of Fe_3O_4 nanoparticles to evaluate the optimization of the process. In addition, the identification of microorganisms was also carried out through molecular biology analysis. This article has not yet been published, but it is in the final stages of production and was submitted as preprint in "*BioRxiv*" and the DOI to acess the paper is: **doi:** https://doi.org/10.1101/2022.03.21.484299.

The fifth article entitled "Metaproteomics of anaerobic co-digestion of residues from First and Second generation ethanol production with biogas generation" is related to the metaproteomic analysis stages of the second and third stages of this project, providing data on the possible metabolic routes followed by microorganisms in the production of CH_4 with the residues used. This paper was submitted in the journal "*Process Biochemistry*" and the proof of submission is just below the supplementary material of the article.

Additionally, in appendix sections are attached all the works presented in congresses and conferences during the development of this project.

The Figure 7 bellow show the flowchart about the thesis struscutre.

Figure 7. Flowchart of Thesis Structure



5.1 PAPER 1

This article was published in the journal BioEnergy Research-SPRINGER and the license granted by the journal for publication in this thesis can be found in Annex I

BioEnergy Research
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Use of Lignocellulosic Residue from Second-Generation Ethanol Production to Enhance Methane Production Through Co-digestion

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Abstract
This is a pioneer study evaluating the methane (CH_4) production potential from residues of integrated first (vinasse and filter cake) and second (deacetylation pretreatment liquor from straw) generation (1G2G) sugarcane biorefinery, providing a fully chemical characterization of them and their relation with the anaerobic digestion (AD) process. Small-scale assays provided fundamentals for basing the co-digestion optimization by assessing the optimal co-substrates synergistic conditions. Biochemical methane potential (BMP) tests showed co-digestion enhanced CH_4 yield of isolated substrates, reaching up to 605 $NmLCH_4\ gVS^{-1}$. The association of vinasse and deacetylation liquor as co-substrates increased the BMP by ~ 38% mostly by nutritionally benefiting the methanogenic activity. The kinetic analysis confirmed that the deacetylation liquor was the co-substrate responsible for improving the CH_4 production in the co-digestion systems due to the highest CH_4 conversion rate. The alkaline characteristic of the liquor (pH ~ 12) also prevented alkalizing from being added to the co-digestion, an input that normally makes the process economically unfeasible to implement on an industrial scale due to the large quantities required for buffering the reactor. The filter cake had the lowest BMP (262 $NmLCH_4\ gVS^{-1}$) and digestibility (≤ 40%), further limited by the required stirring to improve the mass transfer of biochemical reactions. The present study drives towards the more sustainable use of vinasse, the most voluminous waste from the sugarcane industry, and lignin-rich residues derived from pretreatment alkaline methods, aiming at an energy-efficient utilization, by at least 16% when compared to the traditional vinasse AD. The experimental and modeling elements from this work indicated the lignin-rich liquor is the main responsible for putting the co-digestion as a disruptive technological arrangement within the 1G2G sugarcane biorefineries, reinforcing the biogas production as the hub of the bioeconomy in the agroindustrial sector.

Keywords Biogas · Biorefinery · Micronutrients · Deacetylation liquor · Kinetic modeling · 1G2G ethanol

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Introduction

Anaerobic digestion (AD) is an attractive process for managing liquid and solid organic waste that allows energy recovery through biogas, rich in methane (CH_4). Organic matter conversion occurs by the activity of microbial consortia in a finely tuned balanced ecosystem. Digested material, i.e., digestate can also be exploited as a value-added by-product for agriculture [1, 2]. This biotechnological process is part of the current global context of searching for available residual substrates aligned to the diversification of product generation.

Despite all scientific growth in this area, gaining more knowledge based on innovative issues to comprehensibly investigate interactions between technological and fundamental bioprocess limitations entails optimizing CH_4 generation. For example, the availability of biodegradable fractions in the substrates from the sugar–energy industry (related to AD with consequent CH_4 production) still represents a bottleneck for this scientific field [3]. Insufficient knowledge on the principles and operation of AD bioreactors fed with such substrates often results in failed applications in Brazilian sugarcane mills. On the other hand, regarding pretreatment processes for lignocellulosic biomass to obtain hexose and pentose fractions for other bioprocesses, as in the case of 2G ethanol production, enormous advances in fundamental and technological aspects can be found in the literature [4, 5].

Some by-products from the sugarcane agroindustry are already considered raw materials for the recovery and generation of value-added products [6]. Vinasse generated from ethanol distillation is commonly directed to sugarcane culture as liquid-fertile. For each liter of alcohol produced, approximately 10 L of vinasse are generated, and its composition is 0.28–0.52 g. L^{-1} of nitrogen (N), 0.11–0.25 g L^{-1} of phosphorus (P), 1.0–1.4 g L^{-1} of potassium (K), and 20–30 g L^{-1} of chemical oxygen demand (COD) [7, 8]. Sugarcane bagasse, traditionally used in energy generation in combined heat and power (CHP) systems, can be used as a substrate to produce 2G ethanol and other added value by-products [9]. Sugarcane straw, also considered a potential organic source, has become available as lignocellulosic biomass since the progressive introduction of mechanical harvest without burning procedures in Brazil [10]. In addition to being left in the field for agricultural reasons, straw can be used as feedstock for thermochemical or biochemical conversion processes, which makes it feasible to incorporate it into a biorefinery. Sugarcane straw has a similar chemical composition to bagasse in terms of the major components of biomass: cellulose (30–40% w/w), hemicelluloses (20–30% w/w), and lignin (15–30% w/w) [11]. This biomass can be converted into value-added products as biofuels, after pretreatment methods and multi-enzyme complexes to liberate sugars. Among the diversity of methods that have been researching aiming at technological process improvements, Brenelli et al. [12] recently reported a promising alkaline pretreatment of sugarcane straw by deacetylation, in which acetic acid is removed as it is an inhibitor for microorganisms in fermentation processes, and thus, xylo-oligosaccharide (XOS) are recovered for being fermented to ethanol. Filter cake, another organic solid by-product, is generated from the filtration in rotary filters after cane juice clarification processes, presenting concentrations of 140–169 g kg^{-1} of lignin, 171–184.6 g kg^{-1} of cellulose, and 153–170 g kg^{-1} of hemicellulose [3, 13]. It has been used in intrinsic steps at the plant (improvements in permeability during sucrose recovery in the rotary filter) [14] and as a source of nutrients for the soil [15]. Non-controlled digestion of such waste in the fields may lead to the release of large amounts of CH_4, which may hinder the positive effect of bioenergy utilization on climate change mitigation [13].

The economic profitability of biorefineries can be supported by the integrated production of low-value biofuels [16]. In this context, co-digestion of residues can optimize CH_4 production by providing and balancing macro- and micronutrients for the AD process. It may also be the best option for substrates that are difficult to degrade. This appears to be the case for residues from ethanol production from the processing of lignocellulosic biomass, normally recognized as complex substrates for AD [7]. In addition to intrinsic improvements in the biological process (e.g., upgrading biogas production; better process stabilization by providing synergistic effects within the reactor; increased load of biodegradable organic compounds), the economic advantages of sharing equipment and costs are also successful [17]. Janke et al. [18] showed that co-digestion of filter cake with bagasse would produce 58% more biogas compared to large-scale filter cake mono-digestion. However, there are still gaps in the literature concerning the use of lignocellulosic residues from 2G ethanol production as co-substrates.

The biodegradation capacity of residues can be assessed by biochemical methane potential (BMP) assays. This approach shows the maximum experimental potential to convert the organic fraction of the substrates into CH_4. Specific conditions in AD can also be evaluated: substrate sources (exclusive or blend proportions), temperature, nutrients, buffering, and source of inoculum, among other factors. The BMP is the most used methodology by academic and technical practitioners to determine the maximum CH_4 production of a certain substrate [19, 20].

This work aimed to determine the BMP of the main residues from 1G2G sugarcane biorefinery—vinasse, filter cake, and deacetylation liquor (waste stream from

the pretreatment of sugarcane straw for the 2G ethanol production)—in assays as isolated substrates and as different blends of co-substrates for biogas production. The kinetic modeling from the experimental BMP data was further performed. The prior characterization of the substrates (in terms of nutrients) and its relation with the BMP results allowed to investigate the synergistic effects of the co-digestion on CH_4 production, which was further proved by the kinetic analysis. The BMP and kinetics evaluation accounted for the CH_4 production from the isolated substrates and from their co-digestion in different combinations.

Methodology

Substrates and Inoculum

Vinasse and filter cake from a 1G sugarcane ethanol production process were obtained from Iracema mill (São Martinho group), Iracemápolis municipality, São Paulo state, Brazil. The deacetylation liquor was obtained from a mild alkaline pretreatment of sugarcane straw under optimal conditions to remove acetate and lignin determined previously [12]. Briefly, 316L stainless steel batch reactor of 0.5 L capacity was filled with 20.0 g of raw sugarcane straw (dry basis) and NaOH aqueous solution at 8% w/w in 10% (w/w) of the final solid loading and incubated at 60 °C for 30 min. Afterward, the reactor was immediately cooled in an ice bath and the liquid fraction separated from the solid fraction through a muslin cloth stored at 4 °C until further use. The compositional analysis determined according to the NREL/TP-510–42,623 procedure [21] showed the deacetylation liquor was mainly composed of acetate, soluble lignin, and lignin-derived compounds and extractives.

This study compared two different inocula to perform the anaerobic co-digestion of sugarcane processing residues. In Experiment 1, the inoculum was obtained from the sludge of a mesophilic reactor (BIOPAQ®ICX, Paques) installed at Iracema mill from São Martinho group (22°35′17.6″S 47°31′51.5″W) treating sugarcane vinasse. Experiment 2 used an anaerobic consortium from the sludge of a mesophilic upflow anaerobic sludge blanket (UASB) reactor treating poultry slaughterhouse wastewater from Ideal slaughterhouse, at Pereiras municipality, São Paulo state, Brazil (23°05′10.5″S 47°58′58.9″W).

All microorganisms used in this study are registered in the Brazilian government's system SISGEN (access number A5E04AF), accordingly with Brazilian law for accessing genetic resources and associated traditional knowledge.

Biochemical Methane Potential (BMP) of Substrates

Theoretical biochemical methane potential (TBMP) of filter cake was based on the Buswell equation (Eq. 1) for solid substrates. TBMP of deacetylation liquor and vinasse was calculated from their organic matter concentration and VS content, as depicted by (Eq. 2) for liquid substrates [19]:

$$TBMP_S = \frac{\left[\left(\frac{n}{2} + \frac{a}{8} + \frac{b}{4} + \frac{3c}{8}\right) \bullet 22.4\right]}{12n + a + 16b + 14c} \quad (1)$$

where $TBMP_S$ is the theoretical biochemical methane potential for solid substrate ($NmLCH_4\ gVS^{-1}$) and 224,00 mL mol^{-1} is the molar gas volume in the standard temperature and pressure (STP, 273 K, and 1 bar). a, b, c, and *n* are the molar content of the substrate for hydrogen, oxygen, nitrogen, and carbon, respectively:

$$TBMP_L = \frac{0.35 \bullet OM}{VS} \quad (2)$$

where $TBMP_L$ is the theoretical biochemical methane potential for liquid substrate ($NmLCH_4\ gVS^{-1}$), 350 NmL is the theoretical CH_4 yield of 1 g COD at STP [22], VS is the volatile solids of the substrate (g mL^{-1}), and OM is the organic matter concentration, in terms of COD (gO_2 mL^{-1}).

BMP tests were performed to determine the biodegradability (BMP/TBMP) of crude substrates and their experimental potential for CH_4 production following the protocol of Triolo et al. [23] and the VDI 4630 methodology [24]. All experiments were conducted in triplicates of single batches using 250-mL Duran® flasks as bioreactors closed with a pierceable isobutylene isoprene rubber septum and stored in an Ethickthecnology (411-FPD) incubator at thermophilic condition (55 °C) as vinasse leaves the distillation columns at 90 °C and thus would have lower (or none) energy expenditure for cooling it to mesophilic conditions. As mesophilic sludges were used in thermophilic tests, the previous acclimatation of inocula was carried out for avoiding thermal shock to the microbial community. The temperature was gradually increased every 5 degrees until it reached 55 °C, which was kept for a week before the beginning of the tests, as already demonstrated in the literature [25]. The experiment was 2:1 inoculum to substrate ratio (w/w, in terms of Volatile Solids-VS) added to each flask, thus ensuring excess of inoculum to consume all the organic matter of the substrate and achieving its maximum experimental CH_4 production. The pH of solution flasks was corrected to neutrality by adding solutions of NaOH (0.5 M) or H_2SO_4 (1 M) when necessary. Nitrogen (N_2) gas was fluxed into the liquid medium for 10 min

and into the headspace for 5 min after closing the flasks. The headspace was kept at 40%. Biogas was collected from the headspace over the days by using a gastight Hamilton Super Syringe (1 L) through the flasks' rubber septum. The measured biogas was corrected for a dry gas base by excluding the water vapor content in the wet biogas. The pressure and temperature for one-liter gas were corrected to normal (NL) following the standard temperature and pressure (STP) conditions (273 K, 1013 hPa).

BMP was calculated through the average value of the replicates obtained at the end of each batch experiment, according to the traditional methodology [24] (Eq. 3) and also through the kinetic modeling as the approach suggested by Filer et al. [26], as this latter considers the trending of the values and the fundamental parameters of the process. Both BMP results were compared and used to calculate the biodegradability:

$$BMPexp = \frac{\left[\left(\left(NmLbg_{accumulated}^{residue} * \%CH_{4R}\right) - \left(\left(\frac{\left(NmLbg_{accumulated}^{inoculum} * \%CH_4 I\right)}{Inoculum_{CF}}\right) * V_{IF}\right)\right]}{VS_{residue} * Residue_{IF}} \quad (3)$$

where $BMPexp$ is the biochemical methane potential of each residue ($NmLCH_4\ gVS^{-1}$), $NmLbg_{accumulated}^{residue}$ is the accumulated production of biogas from the residue (NmL), $\%CH_{4\ R}$ is the CH_4 content from biogas of the residue, $NmLbg_{accumulated}^{inoculum}$ is the accumulated biogas production of inoculum (NmL), $\%CH_{4\ I}$ is the CH_4 content from biogas of the inoculum, $Inoculum_{CF}$ is the volume of inoculum in a flask of inoculum control (negative control) (mL), V_{IF} is the volume of inoculum added in each flask (mL), $VS_{residue}$ is the number of volatile solids from the residue ($gVS - g\ mL^{-1}$), and $Residue_{IF}$ is the amount of residue added in the flask (mL or g).

Analysis of variance (ANOVA) was used to identify the existence of significant differences between the treatments, and the Tukey test ($p < 0.05$) was performed to group BMP data. These analyses were performed by Microsoft Excel version 12.

Experimental arrangement

Two rounds of BMP tests were performed. Experiment 1 assessed the inoculum from vinasse treatment (section Substrates and inoculum) and equal percentages (in VS terms) of substrates for the co-digestion test. Experiment 2 assessed the inoculum from poultry slaughterhouse waste treatment (section Substrates and Inoculum), and the co-digestion conditions were expanded. The proportions of inoculum/substrate added in each flask were the same for both rounds of experiments (2:1 in terms of VS), as mentioned in section Biochemical Methane Potential (BMP) of Substrates. Two types of control were used in both experiments: positive and negative. Microcrystalline cellulose (Sigma-Aldrich Avicel® PH-101) was used as a positive control (+). Negative control (-) was conducted only with each inoculum. Digestion was terminated when the daily production of biogas per

Table 1 Experimental biochemical methane potential (BMP) design for this study

Assay		Bulk composition (%, w/w in terms of VS)				
		Vinasse	Filter cake	Deacetylation Liquor	Cellulose	Inoculum
Experiment 1	1	33	-	-	-	66
	2	-	33	-	-	66
	3	-	-	33	-	66
	4	11	11	11	-	66
	+	-	-	-	33	66
	-	-	-	-	-	100
Experiment 2	1	33	-	-	-	66
	2	-	33	-	-	66
	3	-	-	33	-	66
	4	17	17	-	-	66
	5	17	-	17	-	66
	6	-	17	17	-	66
	7	11	11	11	-	66
	+	-	-	-	33	66
	-	-	-	-	-	100

-, not added

batch was less than 1% of the accumulated gas production. The experimental design of both experiments is described in Table 1.

Kinetics

Co-digestion usually shows a multiple successive CH_4 production arrangement due to the difference between the biodegradability of each substrate. It is expected that the mixture of substrates leads to a better overall performance than co-digesting each substrate separately. A modified stacked sigmoidal function (Eq. 4), based on Boltzmann double sigmoid [27], was used for modeling CH_4 volumetric production in time. The mathematical adjustment was proposed in the present work to better predict the behavior of the residues about microbial community in the production of biogas since a co-digestion process was carried out and a residue that has not been reported in the literature is being used AD systems. Based on the parameters that the model provided, it was possible to obtain better conclusions regarding the BMP of the residues:

$$V_{CH_4}^{STP}(t) = V_{CH_4}^{max} \bullet \left(\frac{p}{1 + e^{\left(\frac{4r_1 \bullet (t_1 - t)}{V_{CH_4}^{max} \bullet p} \right)}} + \frac{1 - p}{1 + e^{\left(\frac{4r_2 \bullet (t_2 - t)}{V_{CH_4}^{max} \bullet (1 - p)} \right)}} \right) \quad (4)$$

where $V_{CH_4}^{STP}$ is the specific CH_4 production in time ($NmLCH_4\ g\ VS^{-1}$), $V_{CH_4}^{max}$ is the maximum specific volumetric production reached in the experiment ($NmLCH_4\ g\ VS^{-1}$), p is the proportion between ordinate values of the first and second stacked sigmoid, t_1 and t_2 are the time which the production of the first and second sigmoidal pattern reaches the maximum rate (d), and r_1 and r_2 are the maximum rate of CH_4 production for the first and second sigmoidal pattern, respectively ($NmLCH_4\ gVS^{-1}\ d^{-1}$).

The parameter $V_{CH_4}^{max}$ could be considered as a BMP of the assay since it represents the asymptotic maximum production of CH_4 (when $\lim_{t \to \infty} V_{CH_4}^{STP}(t)$). The main advantage of adopting $V_{CH_4}^{max}$ as the BMP is that it takes into consideration the trending of all experimental data, especially those in the step formed in the end phase of each batch. Thus, this approach to calculate the BMP is more precise than using the average of the last values of specific accumulated CH_4 production.

This model assesses the maximum rate for CH_4 production directly through both r_1 and r_2 parameters. Different from a classical Boltzmann sigmoidal function, all parameters in this model have a physical meaning. Thus, they could be useful to evaluate the studied process and for further scale-up work based on this present research. All data were processed and fitted using the software Microcal Origin© 2016.

Physicochemical Analysis

Biogas Composition

Gas chromatography (Construmaq MOD. U-13, São Carlos) analyses were performed to measure the concentration of CH_4. The carrier gas was hydrogen (H_2) gas (30 cm s^{-1}), and the injection volume was 3 mL. The stationary phase was a 3-m-long stainless steel packed column (Bio-Rad HPX-87H), a diameter of 1/8″ with a molecular tamper 5A for separation of O_2, N_2, and CH_4. Detection was performed through a thermal conductivity detector (TCD). Equipment was equipped with a specific injector for CH_4, with a temperature of 350 °C, an external stainless steel wall, and an internal refractory ceramic wall. The detection limit for CH_4 was 0.1 ppm.

Organic Matter

The organic matter content of samples was determined in triplicate according to the standard methods for the examination of water and wastewater [28] by the 5220B method for COD determination (digestion and spectrophotometry) and 2540 method for the solid series characterization. The solid series methodology accounted for the concentration of total (TS) and VS solids in the residue characterization.

Sugars and Acids

Concentrations of sugars and organic acids were determined in triplicate by high-performance liquid chromatography (HPLC, Shimadzu®), composed by pump equipped apparatus (LC-10ADVP), automatic sampler (SIL-20A HT), a CTO-20A column at 43 °C, (SDP-M10 AVP), Aminex HPX-87H column (300 mm, 7.8 mm, Bio-Rad), and a refractive index detector. The mobile phase was H_2SO_4 (0.01 N) at 0.5 ml min^{-1}.

Furfural and HMF were quantified using a Hewlett-Packard RP-18 column and acetonitrile–water (1:8 vv^{-1}) containing 1% (ww^{-1}) acetic acid as eluent in a flow rate of 0.8 mL min^{-1} and a UV detector at 274 nm.

Macro- and Micronutrient and Elementary Analysis

Elementary analysis and macro- and micronutrient analyses were performed at the Biomass Characterization and Analytical Calibration Resources Laboratory (LRAC), Unicamp. To determine the micronutrients, the substrate samples' ashes were analyzed using the X-ray fluorescence equipment (brand, Panalytical; model, Axios 1KW). The ashes were prepared as is described in standard methods for the examination of water and wastewater [28] for solid series analysis (2540 method). The elementary analysis

was possible only for solid samples, i.e., filter cake, by using an elementary carbon, nitrogen, hydrogen, and sulfur analyzer (brand, Elementar; model, Vario MACRO Cube; Hanau, Germany).

Total Lignin (Phenolic Compounds)

Total lignin (soluble + insoluble lignin) content in deacetylation liquor was determined according to [29]. Acid hydrolysis was performed in pressure glass tubes with H_2SO_4 at 4% (w/w) final concentration and autoclaved at 121 °C for 1 h. The resulting suspension was filtered and the filtrate was characterized by chromatography to determine concentrations of furan aldehydes (furfural and hydroxymethylfurfural (HMF) — as described in section Sugars and Acids).

Insoluble lignin was gravimetrically determined as the solid residue from hydrolysis. For the soluble lignin, an aliquot of the hydrolysate obtained in the acid hydrolysis step was transferred to a flask with distilled water, and the final pH was adjusted to 12 with a solution of 6.5 mol L^{-1} NaOH. Soluble lignin was determined from UV absorption at 280 nm using Eq. 5:

$$C_{lig} = \frac{\left(A_{280} \times DF\right) - \left(\varepsilon_{HMF} \times C_{HMF} + \varepsilon_{furfural} \times C_{furfural}\right) - B}{A} \quad (5)$$

where C_{lig} is the soluble lignin concentration in hydrolysate (g L^{-1}), A_{280} is the absorbance of hydrolysate at 280 nm, DF is the dilution factor, ε_{HMF} is the absorptivity of HMF (114.00 L g^{-1} cm^{-1} – experimental value), $\varepsilon_{furfural}$ is the absorptivity of furfural (146.85 L g^{-1} cm^{-1} – experimental value), C_{HMF} is the HMF concentration in hydrolysate (g L^{-1}), $C_{furfural}$ is the furfural concentration in hydrolysate (g L^{-1}), B is the linear coefficient (0.018 – experimental value), and A is the angular coefficient equal to absorptivity of lignin (23.7 L g^{-1} cm^{-1} – experimental value).

Results and Discussion

Characterization of Substrates

Table 2 shows the general characterization of substrates and inoculum. The COD value of vinasse was within the wide range generally found in the literature (15–35 g O_2 L^{-1}) [7, 8], as well as the VS content (0.015–0.020 g mL^{-1}) [9], while TS content was slightly higher than previously reported (0.020–0.024 g mL^{-1}) [8]. For the filter cake, the TS value was higher than normally reported (literature, 0.21–0.28 g mL^{-1}) [3], while VS content was much lower (literature, 0.70–0.74 g mL^{-1}) [13]. Such variations reflect the variability of ethanol production processes and the agricultural procedures affecting biomass characteristics, as well as the seasonality of sugarcane, already stated [8].

Elementary characterization of filter cake showed that it is mainly composed of 0.16% sulfur, 1.73% nitrogen, 31.56% carbon, and 3.11% hydrogen (in %TS). The values for S and N are close to those found in the literature (0.18% and 1.76%, respectively) [30]; however, the C value is below what is normally reported (40–42%) [18]. It resulted in the C:N ratio of the filter cake of 18:1, below what is recommended for AD, which is 20–40:1 [31].

Slaughterhouse inoculum presented higher values of COD, VS, and TS than the inoculum of the sugarcane mill (Table 2), already predicting that it may have a better development for biogas production as it probably contains high cellular mass, i.e., microbiological content. Additionally, the slaughterhouse inoculum visually presented a good-quality granular appearance from UASB reactors, while the mill inoculum had a liquid aspect. Both pHs were neutral, as expected for anaerobic inocula.

Table 2 Main parameter characterization for different substrates and inoculum

Residue	COD (gO_2 L^{-1})	Volatile solids (g mL^{-1})	Total solids (g mL^{-1})	Fixed total solids (g mL^{-1})	pH	Total lignin (phenolic compounds) (g L^{-1})
Vinasse [a]	28.81 ± 0.91	0.0184 ± 0.0002	0.0260 ± 0.0063	0.0077 ± 0.0005	4.50 ± 0.35	–
Filter cake [a]	–	0.2021 ± 0.0005	0.3173 ± 0.0009	0.1152 ± 0.0004	–	–
Deacetylation liquor [a]	32.90 ± 0.27	0.0163 ± 0.0006	0.0215 ± 0.0021	0.0112 ± 0.0001	12.40 ± 0.13	5.50
Iracema mill inoculum [a]	12.70 ± 0.42	0.0076 ± 0.0019	0.0154 ± 0.0003	0.0078 ± 0.0000	7.45 ± 0.58	–
Slaughterhouse inoculum [a]	20.01 ± 0.78	0.0466 ± 0.0076	0.0547 ± 0.0001	0.0081 ± 0.0000	7.32 ± 0.27	–

[a]Three replicates average ± standard deviation; –, not determined

The deacetylation liquor presented a strong alkali characteristic since it came from a mild alkaline pretreatment of sugarcane straw to remove acetyl groups and promote lignin solubilization [12]. Alkaline pretreatment is typically used in lignocellulosic materials such as wheat straw and sugarcane bagasse, thus decreasing its recalcitrance [4]. According to the deacetylation liquor composition (Table 2 and Table 3), a large amount of lignin fractions was detected (phenolic compounds) and high amounts of acids that can be transformed into CH_4, thus showing evidence of a potential high experimental CH_4 production. Several types of pretreatments are currently carried out with sugarcane lignocellulosic materials, such as chemical (acid, alkaline), biological, physical, and physicochemical, in which different types of residues are generated with different characteristics, pH, carbohydrate composition, and lignin content [32]. Thus, it is difficult to make comparisons with the literature. It is worth mentioning that the deacetylation liquor obtained from this work could be specially benefitted for the co-digestion with vinasse due to its basic character. The deacetylation liquor could neutralize the low pH of vinasse without adding large amounts of an alkalizing agent, proving some possible economic benefits of the AD system. The need to alkalize vinasse before AD is an economic disadvantage in terms of implementing this process in sugarcane mills [33]. The presence of C6 and C5 sugars, such as glucose, xylose, arabinose, and the presence of oligosaccharides, such as arabinoxylan and glucan (Table 3), is also highlighted which can be used by the anaerobic microbial community for conversion to CH_4, although constraints of AD from C5 sugars are commonly reported [34, 35].

High values of acetic acid were obtained for both vinasse deacetylation liquor (Table 3), in which this volatile fatty acid was reported as important and essential for the acetotrophic methanogenic metabolic route [36]. Also, Wang et al. [37] noted that concentrations of acetic acid and butyric acid of 2400 and 1800 mg L^{-1}, respectively, did not result in significant inhibition of methanogenic activity. Lactic acid was found in high concentrations in vinasse, and it is usually degraded to propionic acid, which is an undesirable terminal fermentation product; thus, high concentrations of propionic acid can result in methanogenesis failure [37]. Moreover, the high concentration of lactic acid in vinasse may result in the inhibitory effects for CH_4 production, highlighting the potential advantage of applying the co-digestion to balance the volatile fatty acid composition in the medium. Vinasse also presented malic acid which is generally from the sugarcane plant [37] and isobutyric acid, contributing to its acidic pH.

Table 3 Acid and sugar content of liquid substrates

	Vinasse[a] (mg L^{-1})	Deacetylation liquor[a] (mg L^{-1})
Acetate	2215.91 ± 0.80	3670.00 ± 0.89
Isobutyrate	2076.27 ± 1.50	0.00
Formate	0.00	63.00 ± 1.35
Malate	4944.00 ± 0.48	0.00
Lactate	2618.17 ± 0.98	0.00
Glucose	0.00	85.204 ± 2.45
Glucan	–	626.00 ± 1.12
Fructose	1045.25 ± 0.43	0.00
Arabinose	–	26.00 ± 0.44
Xylose	–	35.00 ± 0.95
Arabinoxylan	–	1747.00 ± 2.32

[a]Mean of three replicates ± standard deviation; –, not determined

Table 4 shows the macro- and micronutrient concentrations detected in the substrates. As no external micronutrient solution was added to the experiments, the effects of the nutrient content of the residues could be ascertained by comparing their BMP behavior with the positive control test (cellulose), which had an absence of nutrients. Menon et al. [38] showed optimal concentrations of 303 mg L^{-1} Ca, 777 mg L^{-1} Mg, 7 mg L^{-1} Co, and 3 mg L^{-1} Ni that increased biogas productivity by 50% and significantly reduced the processing time. Filter cake presented higher concentrations of the aforementioned micronutrients, except for Ni which was not detected. It is known that an excess of these compounds may cause inhibitory effects on AD, increasing the lag phase of the process [39] or reducing the specific CH_4 production [40]. A considerable amount of S was also detected in filter cake, which could decrease CH_4 formation from acetate due to the sulfate-reducing bacteria activity. Such bacteria compete by using acetate for sulfide production and can even inhibit methanogenesis activity, leading the process to failure [41]. Al and Fe were also present in inhibitory concentrations, which were reported in the literature with values greater than 2.5 g L^{-1} and 5.7 g L^{-1}, respectively [42]. Mg and Ca concentrations were also much above what is recommended for AD (ideally around 0.02 mg L^{-1} and 0.03 mg L^{-1}, respectively), which may also contribute to the inhibition of the process [43]. High concentrations of Mg ions stimulate the production of single cells of microorganisms with high sensitivity for lysis, leading to a loss of acetoclastic activity in anaerobic reactors, while high Ca concentrations can lead to an accumulation of biofilm, which impairs methanogenic activity and may also cause buffering capacity loss of the essential nutrients for AD [42]. On the other hand, cobalt (Co) was detected only in this substrate, within the stimulating concentration range for methanogenesis [44]. These findings reinforce the need of using co-substrates to dilute the potential inhibitory effects caused by excessive concentrations of nutrients in

Table 4 Macro and micronutrient concentration of substrates and inocula

Nutrients	Vinasse ($g\ L^{-1}TS^{-1}$)	Filter cake ($g\ L^{-1}TS^{-1}$)	Deacetylation liquor ($g\ L^{-1}TS^{-1}$)	Slaughterhouse inoculum ($g\ L^{-1}TS^{-1}$)	Iracema mill inoculum ($g\ L^{-1}TS^{-1}$)
Al	0.0137	16.1825	0.4164	0.3719	0.0402
Ba	0.0000	0.0000	0.0000	0.0014	0.0000
Br	0.0024	0.0000	0.0000	0.0003	0.0013
Ca	0.4682	6.0729	0.4055	0.2349	0.7875
Cl	1.2856	0.0657	0.2546	0.0572	0.7764
Co	0.0000	0.0330	0.0000	0.0023	0.0000
Cr	0.0000	0.0252	0.0000	0.0030	0.0000
Cu	0.0000	0.0230	0.0031	0.1097	0.0016
Fe	0.0163	10.6633	0.3227	1.1316	0.1062
Ga	0.0000	0.0051	0.0008	0.0003	0.0000
Ge	0.0000	0.0000	0.0021	0.0008	0.0000
K	2.6078	0.9487	1.1680	0.2709	1.7843
Mg	0.5372	1.4284	0.1286	0.1155	0.3595
Mn	0.0047	0.2864	0.0123	0.0073	0.0103
Mo	0.0000	0.0000	0.0000	0.0063	0.0004
Na	0.0849	0.0000	10.4902	0.7204	0.0000
Nb	0.0000	0.0040	0.0000	0.0000	0.0000
Ni	0.0000	0.0000	0.0000	0.0028	0.0000
P	0.0913	2.9929	0.1120	0.5496	0.2029
Pb	0.0000	0.0086	0.0000	0.0015	0.0000
Rb	0.0039	0.0042	0.0000	0.0006	0.0030
Si	0.5384	0.5304	1.1620	0.4663	0.0891
S	0.0739	18.1076	0.3345	0.5495	0.3779
Sr	0.0021	0.0419	0.0025	0.0025	0.0033
Ti	0.0013	1.9849	0.0374	0.0200	0.0025
V	0.0000	0.0529	0.0000	0.0000	0.0000
W	0.0000	0.0000	0.0000	0.0033	0.0000
Zn	0.0006	0.0491	0.0043	0.1292	0.0163
Zr	0.0000	0.0537	0.0000	0.0010	0.0000

the filter cake while taking advantage of beneficial effects that certain components of its composition may provide.

Deacetylation liquor presented in the main micronutrients in milder concentrations considered important for the development of methanogenic *Archaea*, such as Fe, Zn, Cu, and Mn, which stimulate reactions catalyzed by metalloenzymes, the formation of cytochromes, and ferroxins [45]. However, high concentrations of Si and especially Na were detected. The presence of large amounts of Si is intrinsic to lignocellulosic materials [46]. The use of Si as a trace element for AD is rarely reported, since it is often either volatilized in the biogas produced or else it remains in the digested material [47], not affecting the AD process. The Na can cause an inhibitory effect on the methanization of volatile fatty acids (mainly propionic acid) in concentrations between 3 to 16 $g\ L^{-1}$; however, for glucose-rich-substrates, this Na concentration does not significantly affect methanogenesis [48]. Methanogenic *Archaea* can also adapt to high Na concentration, leading to high CH_4 conversions [48]. Vinasse did not present known inhibitory concentrations for the assessed macro- and micronutrients [42].

Comparing the nutritional content of the inocula, the slaughterhouse inoculum presented a wider range of components in mild concentrations, indicating richer anaerobic microbial activity than the inoculum from the sugarcane mill, especially Co, Ni, and Fe content that together allows better development of methanogenic activity [49]. The mill's inoculum, on the other hand, had neither Co nor Ni trace metals and much lower Fe concentration. The nutritional poverty of the latter inoculum is accompanied by high K content, consistent with the vinasse treatment, a K-rich substrate.

Table 5 Values of experimental BMP, biodigestibility, pH (initial and final), and kinetic parameters of isolated and co-digested substrates of Experiment 1

Parameters	Cellulose	Vinasse [a]	Deacetylation liquor [b]	Filter cake [c]	(Vinasse + liquor + filter cake) [b]
Values of experimental BMP					
TBMP (NmLCH$_4$ gVS^{-1})	415	548	706	900	–
[1]BMP (NmLCH$_4$ gVS^{-1})	282 ± 13	476 ± 13	610 ± 50	353 ± 38	660 ± 49
Biodigestibily (%)					
[2]Average	68	87	46	40	–
[3]Model	71	89	95	45	–
pH initial	7.98 ± 0.47	7.13 ± 0.02	7.90 ± 0.85	7.77. ± 0.25	7.91 ± 0.01
pH final	7.69 ± 0.75	7.81 ± 0.15	7.80 ± 0.08	7.83 ± 0.01	7.78 ± 0.47
Kinetic model parameters					
$V_{CH_4}^{max}$(NmLCH$_4$ gVS^{-1})	295.83*	487.27*	673 ± 20	404.69*	688 ± 9
p_1	0.39 ± 0.02	0.48 ± 0.01	–	0.53 ± 0.02	–
r_1^{max}(NmLCH$_4$ gVS^{-1} d^{-1})	3.6 ± 0.06	7.4 ± 0.5	16 ± 0	5.5 ± 0.4	12 ± 0
t_1 (d)	25 ± 2	11 ± 1	57 ± 1	29 ± 2	32 ± 1
r_2^{max}(NmLCH$_4$ gVS^{-1} d^{-1})	15 ± 3	13 ± 1	–	11 ± 2	–
t_2 (d)	75 ± 1	72 ± 0	–	76 ± 1	–
R^2	0.89	0.97	0.99	0.96	0.99

[1]Average value of the replicates following the Eq. 3 ± standard deviation. [2]Calculated considering BMP; [3]calculated considering $V_{CH_4}^{max}$; –, not determined, *parameters values forced. Values within a row with the same letter are not significantly different at 5% probability by Tukey

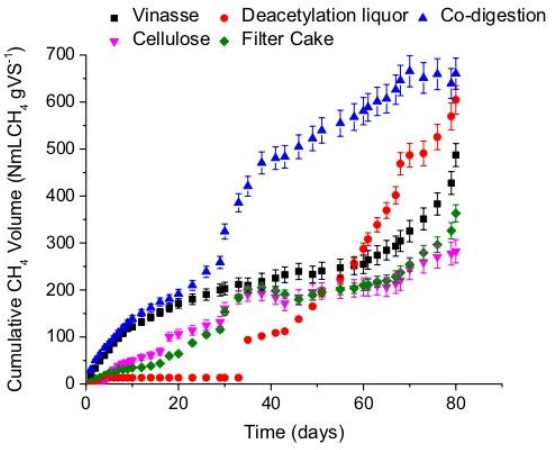

Fig. 1 Cumulative methane volume from BMP of Experiment 1

BMP: Experiment 1

The main results of the BMP tests of Experiment 1 are presented in Table 5, including the experimental values and the kinetic parameters obtained from the mathematical modeling and Tukey analysis. The respective curves of the cumulative volume of produced CH_4 are presented in Fig. 1. Co-digestion of substrates enhanced CH_4 production when compared to the AD of isolated substrates. However, the positive control (cellulose) did not reach the minimum recommendable BMP value (352 NLCH$_4$ kgVS^{-1}) to validate results as maximum potential values for specific CH_4 production [50]. That indicates that the maximum capacity for producing CH_4 from the assessed substrates may not have been reached. The most probable cause for this lack of performance observed in Experiment 1 might be occurred due to the inoculum. Although cellulose digestibility was low, high digestibilities were obtained for liquid substrates (vinasse and deacetylation liquor), which indicates that the presence of nutrients in the substrates (Table 4) has positively affected the inoculum activity as no nutritional supplementation was added in all assays. According to Menon et al. [38], the use of micronutrients remedies AD with a focus on CH_4 production in thermophilic process and increases biogas productivity. Also, a high concentration of acetate in vinasse and deacetylation liquor could be an important factor to boost CH_4 production, since acetate is the sole substrate used by acetoclastic methanogens *Archaea*. Despite the high organic content, the filter cake showed low biodigestibility compared to the other residues (53%). It is likely that the excess of micronutrients and S concentrations negatively affected methanogenesis (Table 4). Besides, the physical limitations on the biological process due to the higher TS content (at least 12-fold greater than the other co-substrates) (Table 2) were another major limiting factor. The absence of stirring may have hindered the mass

transfer between the substrate and the inoculum, reducing the microbiological reactions involved in the AD process and not allowing to achieve higher BMP values [51].

The pH of the assay was adjusted to between 7 and 8 at the beginning of the experiment, and throughout the experiment, it remained in this range, occurring neither acidification nor alkalinization.

Despite the high BMP value and high digestibility of deacetylation liquor, its lag phase was significantly long: CH_4 was produced only after 40 days. The long lag phase can be caused by the presence of pretreatment inhibitors for alcoholic microorganisms, which are commonly reported [52, 53]. However, the presence of furfural or HMF, commonly reported as inhibitors, was not identified. This fact raises two hypotheses: an excess of Na, which may have led to a longer time for methanogenic community adaptation (section Characterization of Substrates), and the presence of fractions of lignin and derived compounds, which may have caused the observed "delay" in the release of organic matter in the environment to access the microbiota. The degradation process of lignin to be used in AD is quite complex, in which some steps are involved before the acetogenesis process [54]. The lignin polymer is first depolymerized and then solubilized, in which different lignin monomers are formed, with varying chain sizes, such as phenylpropanoid derivatives with a carboxylic acid, alcohol, or amine groups. After this stage, these monomers undergo a wide variety of peripheral pathways to form other intermediates, which are the central monoaromatic intermediate, such as resorcinol (trihydroxybenzene). These elements proceed to the dearomatization and cleavage stage of the aromatic ring, forming aliphatic acids. This aliphatic acids enter in acidogenesis phase, and they are degraded into volatile fatty acids to continue in the following AD stages [55]. Thus, the long lag phase of deacetylation liquor AD observed in the BMP test may have happened due to the long process of degradation of lignin fractions and derived compounds, since lignin fractions (i.e., phenolic compounds) were detected in this substrate at significant levels (Table 3).

The biodigestibility predicted by the kinetic modeling showed higher values (from 2 to 10% higher) than the ones calculated from the experimental BMP, as a trend to produce CH_4 after ending the experiments was detected by the fitted model. This behavior indicates a trend towards bigger accumulated production of biogas in a longer time than the experiments were conducted. This fact was especially observed for the deacetylation liquor: the model predicted a larger lag phase (57 vs 34 days) and BMP (673 vs 610 $NmLCH_4\ gVS^{-1}$) values than the observed experimentally. These data confirm that the presence of phenolic compounds (lignin and derivatives) may have caused this long time in the lag phase.

The fitting of the kinetic model was not adequate for any of the studied substrates in Experiment 1. As depicted in Table 5, cellulose, vinasse, and filter cake must have the $V_{CH_4}^{max}$ value forced to the average of the CH_4 accumulated production at the end of each respective assay so the model could fit. The adjusted curves are presented in the Supplementary Materials (Fig. 1SM). It is noteworthy that although the experiments were completed according to VDI 4630 [24] (the process was finished when the production of biogas per batch was less than 1% of the accumulated gas production), the kinetic model showed a trend of increase in CH_4 production over a longer period. For this reason, the values of $V_{CH_4}^{max}$ can be considered the maximum BMP. Despite this fact, no distinguishable step was observed in Fig. 1, indicating that those assays might have reached the stationary phase. The absence of this last step in the experimental data leads to a lack of goodness of fitting, although reaching a good coefficient of correlation (R^2).

Experiment 1 showed that deacetylation liquor did not show a double sigmoid pattern, indicating that all the CH_4 production occurred in one single step. It confirms the peculiar behavior of this residue from producing CH_4: despite the large lag phase (t1 = 57 days) and the delay in the organic matter degradation, the overall rate for CH_4 conversion (observed by the r_1^{max} value) was the highest among the substrates, which occurred in one phase. This same pattern could be observed in the co-digestion of vinasse, liquor, and filter cake altogether, suggesting that the deacetylation liquor could have improved the process of CH_4 production. Another strong evidence that the deacetylation liquor is the substrate responsible for improving the co-digestion is the high value of the apparent kinetic parameter (r_1^{max}) in this experiment after the experiment with deacetylation liquor. Co-digestion increased the CH_4 production rate by 38% and 54% when compared to the isolated AD from vinasse and filter cake, respectively. This behavior is also confirmed by the analysis of ANOVA and the Tukey test that showed a significant difference between the treatments of the residues alone; however, concerning the deacetylation liquor and the co-digestion, there was no significant difference, indicating that the deacetylation liquor was responsible for the increase in CH_4 production in co-digestion.

BMP: Experiment 2

Table 6 shows the main results from the BMP tests of Experiment 2, including the experimental values, the kinetic parameters obtained from the mathematical modeling and Tukey analysis. The adjusted curves for this experiment can be consulted in the Supplementary Materials (Fig. 2SM). Unlike Experiment 1, high biodigestibility of cellulose (positive control) was reached (> 85%), thus

Table 6 Values of experimental BMP, biodigestibility, pH (initial and final), and kinetic parameters of isolated and co-digested substrates of Experiment 2

Parameters	Cellulose	Vinasse [a]	Deacetylation liquor [b]	Filter cake [c]	(Vinasse + liquor + filter cake) [a]	(Vinasse + filter cake) [a]	(Vinasse + deacetylation liquor) [b]	(Deacetylation liquor + filter cake) [b]
Values of experimental BMP								
TBMP ($NmLCH_4\ gVS^{-1}$)	415	548	706	900	–	–	–	–
[1]BMP ($NmLCH_4\ gVS^{-1}$)	380 ± 7	507 ± 6	853 ± 43	262 ± 2	605 ± 88	614 ± 8	971 ± 72	861 ± 24
Biodigestibily (%)								
[2]Average	92	93	121	29	–	–	–	–
[3]Model	89	94	122	30	–	–	–	–
pH initial	7.52 ± 0.45	7.47 ± 0.02	7.79 ± 0.15	7.59 ± 0.89	7.51 ± 2.85	7.45 ± 1.45	7.63 ± 0.39	7.71 ± 0.42
pH final	7.38 ± 0.08	7.12 ± 0.76	7.89 ± 0.61	7.49 ± 0.73	7.35 ± 0.92	7.32 ± 0.56	7.55 ± 0.03	7.87 ± 0.25
Kinetic model parameters								
$V_{CH_4}^{max}$ ($NmLCH_4\ gVS^{-1}$)	368 ± 7	513 ± 3	863 ± 9	274 ± 9	717 ± 119	797 ± 110	1298 ± 660	1021 ± 70
p_1	0.07 ± 0.01	0.44 ± 0.01	0.58 ± 0.03	0.32 ± 0.02	0.27 ± 0.08	0.28 ± 0.07	0.27 ± 0.32	0.31 ± 0.04
r_1^{max} ($NmLCH_4\ gVS^{-1}\ d^{-1}$)	5.6 ± 4.6	7.5 ± 0.4	20 ± 1	5.1 ± 0.4	7.2 ± 1.6	7.0 ± 0.4	8.3 ± 2.1	17 ± 1
t_1 (d)	7.4 ± 1.1	11 ± 1	12 ± 1	14 ± 1	13 ± 2	17 ± 1	18 ± 3	12 ± 0
r_2^{max} ($NmLCH_4\ gVS^{-1}\ d^{-1}$)	7.3 ± 0.3	11 ± 1	7.3 ± 0.6	3.7 ± 0.1	7.7 ± 0.7	6.4 ± 0.2	9.1 ± 0.8	7.9 ± 0.3
t_2 (d)	79 ± 1	75 ± 0	62 ± 2	93 ± 2	94 ± 8	100 ± 7	102 ± 25	89 ± 4
R^2	0.97	0.99	0.98	0.99	0.90	0.99	0.95	0.99

Notes: [1]Average value of the replicates following the Eq. 3 ± standard deviation; [2]calculated considering BMP; [3]calculated considering $V_{CH_4}^{max}$; –, not determined. Values within a row with the same letter are not significantly different at 5% probability by Tukey

validating the BMP tests as the maximum experimental CH_4 production from the assessed substrates [50]. The BMP values obtained in Experiment 2 are, thus, the representative ones for the assessed residues. This fact indicates better quality of anaerobic inoculum from the poultry slaughterhouse treatment when compared to the inoculum from sugarcane vinasse treatment. Biogas production constraints from vinasse on a scale (e.g., variation of vinasse composition throughout the season, AD reactor shutdown in the vinasse off-season) reflect the lack of robustness of the inoculum due to its continuous need for adaptation to the substrate, which weakens the microbial activity. It is noteworthy that the kinetic model prediction was close to the experimental values of BMP, reinforcing that the maximum BMP was experimentally reached: the biodigestibility (Table 6) calculated from both methods varied only by 3% on average. It confirms the robustness of the inoculum and proves the representativity of BMP results.

Lower filter cake BMP was obtained when compared to Experiment 1. The physical characteristics of inocula could have played a role in this case: the inoculum from poultry slaughterhouse treatment was composed of very well-formed granules (traditional upflow anaerobic sludge blanket, UASB sludge), while the inoculum from vinasse treatment was liquid without any granules. The mass transfer resistance in anaerobic granules might limit CH_4 production, since the larger the granule, the greater the resistance to mass transfer [56], which may have been attenuated with the liquid inoculum for the filter cake access. Additionally, in the co-digestion BMP tests, the highest value of BMP was obtained with only liquid substrates (deacetylation liquor + vinasse) while using filter cake as co-substrate caused a decrease in BMP values (Table 6). It reinforces that the mass transfer phenomena have an important influence on CH_4 production from filter cake, which must be considered for a reactor operation and inoculum sludge choice. The excess concentrations of

some macro- and micronutrients already discussed (section Characterization of Substrates) may also have contributed to the lower BMP.

Experimental BMP of deacetylation liquor showed an atypical result, as it was higher than its TBMP value. Deacetylation pretreatment liquor (with the alkaline character) has favorable characteristics for CH_4 production because it reduces the degree of inhibition on CH_4 fermentation [57], which may explain its high BMP value (Table 6). However, the lower TBMP than BMP implies the possibility that all organic matter in the deacetylation liquor was not accounted for in the COD value, underestimating the value of TBMP. Remnants of insoluble lignin may not have been quantified in the COD analysis [58], and during the BMP tests, they may have been hydrolyzed and made available as soluble lignin [55, 58]. CH_4 production from soluble lignin was already reported [59]. It is also worth mentioning that trace metals can act as catalysts, favoring the depolarization of the soluble lignin in the liquid medium, thus leaving more organic matter available [60]. The inoculum used in Experiment 1 had lower metal content when compared to the slaughterhouse inoculum of Experiment 2 (especially Al, Co, Fe, Cu), corroborating the hypothesis that the presence of metals may have contributed to the depolarization of soluble lignin in the deacetylation liquor. Thus, larger metal content in poultry inoculum may lead to larger amounts of available organic matter during the BMP test, which was not accounted for in the COD value of deacetylation liquor determined in the absence of inoculum. These assumptions highlight the need for deeper further studies on CH_4 production from liquid lignocellulosic substrates.

As in Experiment 1, the pH of Experiment 2 remained neutral throughout the operation, with no acidification or alkalinization of the medium, and no need for initial pH correction exclusively for the co-digestion test.

The co-digestion of substrates showed higher potential for CH_4 production than the AD of isolated residues, as in Experiment 1, except for the deacetylation liquor. However, considering the context of a sugarcane biorefinery, its most abundant residue (i.e., vinasse) must be properly managed, whereby AD is an advantageous alternative as already reported [7]. The enhancement of CH_4 production from vinasse can be achieved by adding other residues within the biorefinery boundary as co-substrates, as proved in the current work. By predicting a co-digestion reactor operation, in which the continuous stirred tank reactor (CSTR) is the traditional one [8], the disadvantage of the filter cake by having a higher ST content could be minimized due to stirring, avoiding its sedimentation and improving the substrate-inoculum contact and, therefore, resulting in increased CH_4 production.

Kinetic modeling performed in all assays of Experiment 2 (Table 6) showed a particularly good fitting. All assays showed a clear ending step at the end of each assay, and the model represented all data without any need of forcing parameters to a value.

As showed in Experiment 1, deacetylation liquor had the highest values for both $V_{CH_4}^{max}$ and r_1^{max}, demonstrating a higher and faster CH_4 production than any other assay. This value was 4 times greater than the r_1^{max} of the other isolated substrates. Co-digestion of deacetylation liquor and vinasse and deacetylation liquor and filter cake have been proved more capable of CH_4 production than other conditions. Kinetic parameters also confirmed that co-digestion was more effective than single substrate AD for CH_4 production, and the deacetylation liquor was probably the substrate that boosted methanogenesis in co-digestion assays. Besides, the r_1^{max} values of the deacetylation liquor were higher in the current experiment when compared to Experiment 1 (20 vs 16 $NmLCH_4\ d^{-1}$). This fact confirms that in Experiment 2, the phenolic compounds (lignin and derivatives) of deacetylation liquor may have been faster solubilized in shorter organic matter chains to be converted to CH_4 (section BMP: Experiment 2), making its r_1^{max} a value higher than in Experiment 1, where the lignin content took a longer period to be solubilized and thus resulting in a lower CH_4 production rate. This corroborates that TBMP was underestimated since lignin was not fully accounted for in the COD analysis in Experiment 1. The result of the Tukey test also confirms the hypotheses raised above, since there was no significant difference at 5% probability of the BMP of deacetylation liquor, vinasse and deacetylation liquor, and filter cake and deacetylation liquor tests. This situation shows that the AD assay of the deacetylation liquor alone or with the other residues will be no difference in final CH_4 production, indicating that the deacetylation liquor was the residue that leveraged methanogenesis when co-digesting with the other two residues.

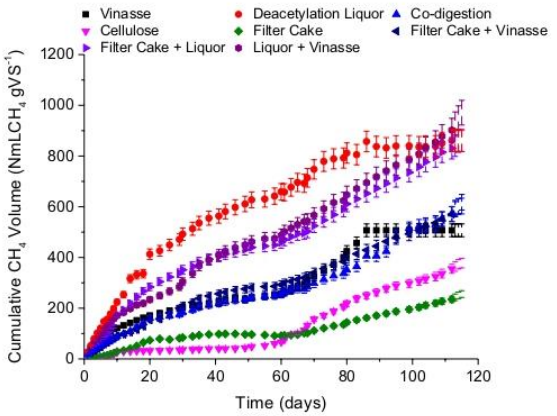

Fig. 2 Cumulative methane volume from BMP of Experiment 2

Although the Tukey test did not show a significant difference between the BMP test of the vinasse, of the co-digestion of the three residues, and the vinasse with the filter cake, it is notorious that the BMP values increased and a lot with the presence of the deacetylation liquor.

Figure 2 shows the curves of the cumulative volume of produced CH_4 in Experiment 2, presenting a more accentuated behavior of AD occurring in two phases when compared to Experiment 1: the acidogenic phase and the subsequent methanogenic phase [61]. This proves that the origin of the inoculum plays an important role in the production of CH_4, as the same substrates were used in the two rounds of experiments. The BMP of the substrates in Experiment 2 had a shorter lag phase when compared to Experiment 1, as confirmed by the obtained kinetic parameters (t_l), indicating that there was a better adaptation of the inoculum to the substrate. Gu et al. [62] observed distinct performance of biogas production using different inocula for the same substrate (rice straw), showing that some inocula were better adapted than others due to their specific enzymatic arsenal and to the degraded organic matter load capacity: the greater organic matter converted by the inoculum, the better it would be able to convert lignocellulosic residues.

The inoculum used in Experiment 2 came from a consolidated UASB reactor continuously treating poultry slaughterhouse waste, with higher organic loads fed to the reactor when compared to the inoculum used in Experiment 1 (from a reactor that has been in operation for only 4 years for the treatment of vinasse). This made the slaughterhouse inoculum more robust than mill inoculum, and, thus, more suitable and efficient to convert lignocellulosic materials, causing the smallest lag phase and making the digestion process more stable, which results in higher cumulative CH_4 volumes [63].

Conclusion

Anaerobic inoculum maturity improved the slow conversion of lignin-fraction monomers into CH_4 from deacetylation liquor. Its alkali characteristic may contribute to the AD operational costs reduction on an industrial scale as it avoided the reactor alkalizing demand. The highest filter cake TS content indicated operational adjustments are necessary, e.g., stirring to minimize the mass transfer resistance between substrate and microorganisms. The adjusted kinetic model confirmed the maximum experimental BMP values for the robust AD inoculum. This small-scale study shows how the co-digestion made use of residues' positive synergisms to increase CH_4 yield by at least 16%. This highlighted the advantage for the management of the voluminous residue of integrated 1G2G sugarcane biorefineries (vinasse) and new lignin-rich side streams derived from pretreatment technologies for sugarcane straw valorization, so far unexplored for CH_4 production.

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Author Contribution Maria Paula C. Volpi: Conceptualization, methodology, data curation, and writing — original draft preparation.

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Declarations

Conflict of Interest The authors declare no competing interests.

References

1. Parsaee M, Kiani Deh Kiani M, Karimi K (2019) A review of biogas production from sugarcane vinasse. Biomass Bioenerg 122:117–125. https://doi.org/10.1016/j.biombioe.2019.01.034
2. Moraes BS, Triolo JM, Lecona VP et al (2015) Biogas production within the bioethanol production chain: use of co-substrates for anaerobic digestion of sugar beet vinasse. Bioresour Technol 190:227–234. https://doi.org/10.1016/j.biortech.2015.04.089
3. Janke L, Leite A, Nikolausz M et al (2015) Biogas production from sugarcane waste: assessment on kinetic challenges for process designing. Int J Mol Sci 16:20685–20703. https://doi.org/10.3390/ijms160920685
4. Rabelo SC, Carrere H, Maciel Filho R, Costa AC (2011) Production of bioethanol, methane and heat from sugarcane bagasse in a biorefinery concept. Bioresour Technol 102:7887–7895. https://doi.org/10.1016/j.biortech.2011.05.081
5. Nakanishi SC, Soares LB, Biazi LE et al (2017) Fermentation strategy for second generation ethanol production from sugarcane bagasse hydrolyzate by Spathaspora passalidarum and Scheffersomyces stipitis. Biotechnol Bioeng 114:2211–2221. https://doi.org/10.1002/bit.26357

6. Adarme OFH, Baêta BEL, Filho JBG et al (2019) Use of anaerobic co-digestion as an alternative to add value to sugarcane biorefinery wastes. Bioresour Technol 287:121443. https://doi.org/10.1016/j.biortech.2019.121443
7. DjalmaNunesFerrazJúnior A, Koyama MH, de Araújo Júnior MM, Zaiat MM (2016) Thermophilic anaerobic digestion of raw sugarcane vinasse. Renew Energy 89:245–252. https://doi.org/10.1016/j.renene.2015.11.064
8. Moraes BS, Zaiat M, Bonomi A (2015) Anaerobic digestion of vinasse from sugarcane ethanol production in Brazil: challenges and perspectives. Renew Sustain Energy Rev 44:888–903. https://doi.org/10.1016/j.rser.2015.01.023
9. Fuess LT, Kiyuna LSM, Ferraz ADN et al (2017) Thermophilic two-phase anaerobic digestion using an innovative fixed-bed reactor for enhanced organic matter removal and bioenergy recovery from sugarcane vinasse. Appl Energy 189:480–491. https://doi.org/10.1016/j.apenergy.2016.12.071
10. Menandro LMS, Cantarella H, Franco HCJ et al (2017) Comprehensive assessment of sugarcane straw: implications for biomass and bioenergy production. Biofuels, Bioprod Biorefining 11:488–504. https://doi.org/10.1002/bbb.1760
11. Khaire KC, Moholkar VS, Goyal A (2021) Bioconversion of sugarcane tops to bioethanol and other value added products: an overview. Mater Sci Energy Technol 4:54–68. https://doi.org/10.1016/j.mset.2020.12.004
12. Brenelli LB, Figueiredo FL, Damasio A, et al (2020) An integrated approach to obtain xylo-oligosaccharides from sugarcane straw: from lab to pilot scale. Bioresour Technol 123637. https://doi.org/10.1016/j.biortech.2020.123637
13. Janke L, Leite A, Batista K et al (2016) Optimization of hydrolysis and volatile fatty acids production from sugarcane filter cake: effects of urea supplementation and sodium hydroxide pretreatment. Bioresour Technol 199:235–244. https://doi.org/10.1016/j.biortech.2015.07.117
14. Wongarmat W, Reungsang A, Sittijunda S, Chu CY (2021) Anaerobic co-digestion of biogas effluent and sugarcane filter cake for methane production. Biomass Convers Biorefinery. https://doi.org/10.1007/s13399-021-01305-3
15. Tellechea FRF, Martins MA, da Silva AA et al (2016) Use of sugarcane filter cake and nitrogen, phosphorus and potassium fertilization in the process of bioremediation of soil contaminated with diesel. Environ Sci Pollut Res 23:18027–18033. https://doi.org/10.1007/s11356-016-6965-x
16. Hernández-Pérez AF, de Arruda PV, Felipe M das G de A (2016) Sugarcane straw as a feedstock for xylitol production by *Candida guilliermondii* FTI 20037. Braz J Microbiol 47:489–496. https://doi.org/10.1016/j.bjm.2016.01.019
17. Hagos K, Zong J, Li D, et al (2017) Anaerobic co-digestion process for biogas production: progress, challenges and perspectives. Renew Sustain Energy Rev
18. Janke L, Leite AF, Nikolausz M et al (2016) Comparison of start-up strategies and process performance during semi-continuous anaerobic digestion of sugarcane filter cake co-digested with bagasse. Waste Manag 48:199–208. https://doi.org/10.1016/j.wasman.2015.11.007
19. Raposo F, Fernández-Cegrí V, de la Rubia MA et al (2011) Biochemical methane potential (BMP) of solid organic substrates: evaluation of anaerobic biodegradability using data from an international interlaboratory study. J Chem Technol Biotechnol 86:1088–1098. https://doi.org/10.1002/jctb.2622
20. Hobbs SR, Landis AE, Rittmann BE et al (2018) Enhancing anaerobic digestion of food waste through biochemical methane potential assays at different substrate: inoculum ratios. Waste Manag 71:612–617. https://doi.org/10.1016/j.wasman.2017.06.029
21. Sluiter A, Hames B, Ruiz R, et al (2008) Determination of sugars, byproducts, and degradation products in liquid fraction process samples, Technical Report NREL/TP-510–42623
22. Rodriguez-Chiang L, Llorca J, Dahl O (2016) Anaerobic co-digestion of acetate-rich with lignin-rich wastewater and the effect of hydrotalcite addition. Bioresour Technol 218:84–91. https://doi.org/10.1016/j.biortech.2016.06.074
23. Triolo JM, Pedersen L, Qu H, Sommer SG (2012) Biochemical methane potential and anaerobic biodegradability of non-herbaceous and herbaceous phytomass in biogas production. Bioresour Technol 125:226–232. https://doi.org/10.1016/j.biortech.2012.08.079
24. VDI 4630 (2006) Fermentation of organic materials. Characterization of the substrate, sampling, collection of material data, fermentation tests
25. Boušková A, Dohányos M, Schmidt JE, Angelidaki I (2005) Strategies for changing temperature from mesophilic to thermophilic conditions in anaerobic CSTR reactors treating sewage sludge. Water Res 39:1481–1488. https://doi.org/10.1016/j.watres.2004.12.042
26. Filer J, Ding HH, Chang S (2019) Biochemical methane potential (BMP) assay method for anaerobic digestion research. Water 11:29. https://doi.org/10.3390/w11050921
27. Mockaitis G, Bruant G, Guiot SR et al (2020) Acidic and thermal pre-treatments for anaerobic digestion inoculum to improve hydrogen and volatile fatty acid production using xylose as the substrate. Renew Energy 145:1388–1398. https://doi.org/10.1016/j.renene.2019.06.134
28. APHA, AWWA W (2012) Standard methods for the examination of water and wastewater, twenty-sec. Washington, DC
29. Sluiter JB, Chum H, Gomes AC et al (2016) Evaluation of Brazilian sugarcane bagasse characterization: an interlaboratory comparison study. J AOAC Int 99:579–585. https://doi.org/10.5740/jaoacint.15-0063
30. Janke L, Weinrich S, Leite AF et al (2017) Optimization of semi-continuous anaerobic digestion of sugarcane straw co-digested with filter cake: effects of macronutrients supplementation on conversion kinetics. Bioresour Technol 245:35–43. https://doi.org/10.1016/j.biortech.2017.08.084
31. Friehe J, Weiland P, Schattauer (2010) Fundamentals of anaerobic digestion In: FNR (Fachagentur Nachwachsende Rohstoffe e.V.) (Ed) Guide to Biogas—From Production to Use. Germany: FNR, pp 21–31
32. Tan L, Zhong J, Jin YL et al (2020) Production of bioethanol from unwashed-pretreated rapeseed straw at high solid loading. Bioresour Technol 303:122949. https://doi.org/10.1016/j.biortech.2020.122949
33. Junqueira TL, Moraes BS, Gouveia VLR, et al (2015) Use of VSB to Plan Research Programs and Public Policies. In: Bonomi A, Cavalett O, Pereira da Cunha M, Lima M (eds) Virtual biorefinery: an optimization strategy for renewable carbon valorization. Campinas: Springer, pp 257–282
34. Pérez-Pimienta JA, Icaza-Herrera JPA, Méndez-Acosta HO et al (2020) Bioderived ionic liquid-based pretreatment enhances methane production from: Agave tequilana bagasse. RSC Adv 10:14025–14032. https://doi.org/10.1039/d0ra01849j
35. Kamdem I, Hiligsmann S, Vanderghem C et al (2018) Enhanced biogas production during anaerobic digestion of steam-pretreated lignocellulosic biomass from Williams Cavendish banana plants. Waste and Biomass Valorization 9:175–185. https://doi.org/10.1007/s12649-016-9788-6
36. Demirel B, Scherer P (2008) The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: a review. Rev Environ Sci Biotechnol 7:173–190. https://doi.org/10.1007/s11157-008-9131-1

37. Wang Y, Zhang Y, Wang J, Meng L (2009) Effects of volatile fatty acid concentrations on methane yield and methanogenic bacteria. Biomass Bioenerg. https://doi.org/10.1016/j.biombioe.2009.01.007
38. Menon A, Wang JY, Giannis A (2017) Optimization of micronutrient supplement for enhancing biogas production from food waste in two-phase thermophilic anaerobic digestion. Waste Manag 59:465–475. https://doi.org/10.1016/j.wasman.2016.10.017
39. Ahn J-H, Do TH, Kim SD, Hwang S (2006) The effect of calcium on the anaerobic digestion treating swine wastewater. Biochem Eng J 30:33–38. https://doi.org/10.1016/j.bej.2006.01.014
40. Romero-Güiza MS, Mata-Alvarez J, Chimenos JM, Astals S (2016) The effect of magnesium as activator and inhibitor of anaerobic digestion. Waste Manag 56:137–142. https://doi.org/10.1016/j.wasman.2016.06.037
41. Koster IW, Rinzema A, de Vegt AL, Lettinga G (1986) Sulfide inhibition of the methanogenic activity of granular sludge at various pH-levels. Water Res 20:1561–1567. https://doi.org/10.1016/0043-1354(86)90121-1
42. Chen Y, Cheng JJ, Creamer KS (2008) Inhibition of anaerobic digestion process: a review. Bioresour Technol 99:4044–4064. https://doi.org/10.1016/j.biortech.2007.01.057
43. Issah A, Kabera T, Kemausuor F (2020) Biomass and bioenergy biogas optimisation processes and effluent quality : a review. Biomass Bioenerg 133:105449. https://doi.org/10.1016/j.biombioe.2019.105449
44. Bhattacharya SK, Uberoi V, Madura RL, Haghighi-Podeh MR (1995) Effect of cobalt on methanogenesis. Environ Technol 16:271–278. https://doi.org/10.1080/09593331608616269
45. Zieliński M, Kisielewska M, Dębowski M, Elbruda K (2019) Effects of nutrients supplementation on enhanced biogas production from maize silage and cattle slurry mixture. Water Air Soil Pollut 230. https://doi.org/10.1007/s11270-019-4162-5
46. Zhang J, Zou W, Li Y et al (2015) Plant science silica distinctively affects cell wall features and lignocellulosic saccharification with large enhancement on biomass production in. Plant Sci 239:84–91. https://doi.org/10.1016/j.plantsci.2015.07.014
47. Rasi S, Seppälä M, Rintala J (2013) Organic silicon compounds in biogases produced from grass silage, grass and maize in laboratory batch assays. Energy 52:137–142. https://doi.org/10.1016/j.energy.2013.01.015
48. Feijoo G, Soto M, Méndez R, Lema JM (1995) Sodium inhibition in the anaerobic digestion process: antagonism and adaptation phenomena. Enzyme Microb Technol 17:180–188. https://doi.org/10.1016/0141-0229(94)00011-F
49. Qiang H, Niu Q, Chi Y, Li Y (2013) Trace metals requirements for continuous thermophilic methane fermentation of high-solid food waste. Chem Eng J 222:330–336. https://doi.org/10.1016/j.cej.2013.02.076
50. Holliger C, Alves M, Andrade D et al (2016) Towards a standardization of biomethane potential tests. Water Sci Technol 74:2515–2522. https://doi.org/10.2166/wst.2016.336
51. Galaction AI, Cascaval D, Oniscu C, Turnea M (2004) Prediction of oxygen mass transfer coefficients in stirred bioreactors for bacteria, yeasts and fungus broths. Biochem Eng J 20:85–94. https://doi.org/10.1016/j.bej.2004.02.005
52. Rabelo SC, Andrade RR, Maciel Filho R, Costa AC (2014) Alkaline hydrogen peroxide pretreatment, enzymatic hydrolysis and fermentation of sugarcane bagasse to ethanol. Fuel 136:349–357. https://doi.org/10.1016/j.fuel.2014.07.033
53. Tramontina R, Brenelli LB, Sousa A et al (2020) Designing a cocktail containing redox enzymes to improve hemicellulosic hydrolysate fermentability by microorganisms. Enzyme Microb Technol 135:109490. https://doi.org/10.1016/j.enzmictec.2019.109490
54. Khan MU, Ahring BK (2019) Lignin degradation under anaerobic digestion: Influence of lignin modifications - a review. Biomass Bioenerg 128. https://doi.org/10.1016/j.biombioe.2019.105325
55. Mulat DG, Horn SJ (2018) Chapter 14: Biogas production from lignin via anaerobic digestion. RSC Energy Environ Ser 2018-Jan:391–412. https://doi.org/10.1039/9781788010351-00391
56. Gonzalez-Gil G, Seghezzo L, Lettinga G et al (2001) Kinetics and mass-transfer phenomena in anaerobic granular sludge. Biotechnol Bioeng 73:125–134. https://doi.org/10.1002/bit.1044
57. Krishania M, Vijay VK, Chandra R (2013) Methane fermentation and kinetics of wheat straw pretreated substrates co-digested with cattle manure in batch assay. Energy 57:359–367. https://doi.org/10.1016/j.energy.2013.05.028
58. Ganesh Kumar A, Sekaran G, Krishnamoorthy S (2006) Solid state fermentation of Achras zapota lignocellulose by Phanerochaete chrysosporium. Bioresour Technol 97:1521–1528. https://doi.org/10.1016/j.biortech.2005.06.015
59. Barakat A, Monlau F, Steyer JP, Carrere H (2012) Effect of lignin-derived and furan compounds found in lignocellulosic hydrolysates on biomethane production. Bioresour Technol 104:90–99. https://doi.org/10.1016/j.biortech.2011.10.060
60. Kim JY, Park J, Hwang H et al (2015) Catalytic depolymerization of lignin macromolecule to alkylated phenols over various metal catalysts in supercritical tert-butanol. J Anal Appl Pyrolysis 113:99–106. https://doi.org/10.1016/j.jaap.2014.11.011
61. Demirel B, Yenigün O (2002) Two-phase anaerobic digestion processes: a review. J Chem Technol Biotechnol 77:743–755. https://doi.org/10.1002/jctb.630
62. Gu Y, Chen X, Liu Z et al (2014) Effect of inoculum sources on the anaerobic digestion of rice straw. Bioresour Technol 158:149–155. https://doi.org/10.1016/j.biortech.2014.02.011
63. Quintero M, Castro L, Ortiz C et al (2012) Enhancement of starting up anaerobic digestion of lignocellulosic substrate: Fique's bagasse as an example. Bioresour Technol 108:8–13. https://doi.org/10.1016/j.biortech.2011.12.052

5.2 **PAPER 2**

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BIOENERGY AND BIOFUELS

Operational and biochemical aspects of co-digestion (co-AD) from sugarcane vinasse, filter cake, and deacetylation liquor

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Abstract

This work performed co-AD from the vinasse and filter cake (from 1G ethanol production) and deacetylation liquor (from the pretreatment of sugarcane straw for 2G ethanol production) in a semi-Continuous Stirred Tank Reactor (s-CSTR) aiming to provide optimum operational parameters for continuous CH_4 production. Using filter cake as co-substrate may allow the reactor to operate throughout the year, as it is available in the sugarcane off-season, unlike vinasse. A comparison was made from the microbial community of the seed sludge and the reactor sludge when CH_4 production stabilized. Lactate, butyrate, and propionate fermentation routes were denoted at the start-up of the s-CSTR, characterizing the acidogenic phase: the oxidation-reduction potential (ORP) values ranged from −800 to −100 mV. Once the methanogenesis was initiated, alkalizing addition was no longer needed as its demand by the microorganisms was supplied by the alkali characteristics of the deacetylation liquor. The gradual increase of the applied organic load rates (OLR) allowed stabilization of the methanogenesis from 3.20 $g_{VS}\ L^{-1}\ day^{-1}$: the highest CH_4 yield (230 $mL_N CH_4\ g^{-1}{}_{VS}$) and average organic matter removal efficiency (83% ± 13) was achieved at ORL of 4.16 $g_{VS}\ L^{-1}\ day^{-1}$. The microbial community changed along with the reactor operation, presenting different metabolic routes mainly due to the used lignocellulosic substrates. Bacteria from the syntrophic acetate oxidation (SAO) process coupled to hydrogenotrophic methanogenesis were predominant (~ 90% *Methanoculleus*) during the CH_4 production stability. The overall results are useful as preliminary drivers in terms of visualizing the co-AD process in a sugarcane biorefinery integrated to scale.

Key points

- *Integration of 1G2G sugarcane ethanol biorefinery from co-digestion of its residues.*
- *Biogas production from vinasse, filter cake, and deacetylation liquor in a semi-CSTR.*
- *Lignocellulosic substrates affected the biochemical routes and microbial community.*
- *Biomol confirmed the establishment of the thermophilic community from mesophilic sludge.*

Keywords Co-digestion · 1G2G Sugarcane biorefinery · Methane production · Continuous reactor operation

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Introduction

Anaerobic digestion (AD) of residues from sugarcane ethanol production has shown to be a promising strategy for waste management towards bioenergy enhancement (Moraes et al. 2015; Janke et al. 2016; Formann et al. 2020). Vinasse stands out among the residues of the sugarcane industry, consisting of a liquid with high potential pollution due to the high levels of organic matter, which can be harmful if improperly disposed (Moraes et al. 2015). This most voluminous waste from the ethanol distillation columns has already shown potential for methane (CH_4) production, even on a demonstration-scale, reaching up to 310 $mL_N CH_4$ per gram of removed chemical

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oxygen demand (COD) in a thermophilic Upflow Anaerobic Sludge Blanket (UASB) reactor from a Brazilian sugarcane mill in operation since the 1980s (Souza et al. 1992). The filter cake from the sugarcane juice filtration has also been considered a potential source for CH_4 production as co-substrate in the AD process, with only one Brazilian company currently announcing a co-AD technology, although scientific and widespread information is no longer provided (Zaparolli 2019). This residue has a positive environmental impact when left in the soil, since it promotes protection against erosion, increases biological activity, it is a temperature controller (Janke et al. 2015); however, the literature demonstrates its high capacity for energy conversion, through AD and CH_4 recuperation (Volpi et al. 2021a). It is known that such residue is rich in trace elements with a favorable balance of macronutrients that can contribute to co-AD besides having a suitable average C:N ratio (24:1) for the AD (Janke et al. 2015). Janke et al. (2018) showed that the filter cake after an alkaline pretreatment could be used as substrate to operate a continuous stirred-tank reactor (CSTR) for biogas production throughout the season without interruptions caused by the unavailability of vinasse during the off-season. In the same context, other liquid streams such as second-generation (2G) vinasse or the waste from the pre-treatment steps of 2G ethanol production could be used as co-substrates for keeping biogas production along the year, without the need of the filter cake pre-treatment (Volpi et al. 2021b).

The co-AD is an alternative process for AD of isolated substrates which may optimize the CH_4 yield. It allows the use of residues with low biodegradability and/or inhibitory substances content by providing its dilution, apart from balancing micro and macronutrients and supplying synergistic effects between microorganisms (Hagos et al. 2017). It seems to fit in the management of residues from 2nd generation (2G) ethanol production, which generates lignocellulosic waste usually recognized as complex substrates for AD. Within the co-AD concept, the integration of 1st and 2nd generation (1G2G) sugarcane biorefineries can be reinforced by blending their residues and maximizing their sustainable use to bioenergy generation.

Numerous types of pre-treatments of lignocellulosic biomass have been developed for the release of sugars (e.g., hexoses and pentoses) for the production of 2G ethanol (Moraes et al. 2015). Alkaline pretreatments are common for the delignification of biomass, having additional effects on the silica removal (ash insoluble component) or the partial removal of hemicelluloses (including acetyl and uronic acid groups) and the swelling of cellulose, resulting in a substantial increase in the fiber surface (Carvalho et al. 2016). The residues generated are potential sources for AD (Rabelo et al. 2011), although little has been studied about their co-AD, especially for the recent and innovative pre-treatment of biomass and hydrolysis, e.g., deacetylation process, pretreatment with ionic liquids, hydrolysis using genetically modified yeast, among others (Nakasu et al. 2020). The complexity of such substrates for AD may be one of the factors driving the integration of the 1G2G ethanol process by co-AD of its residues, e.g., with 1G vinasse that is already recognized as a substrate for biogas production (Ferraz Júnior et al. 2016).

Previous studies by our research group were carried out concerning Biochemical Methane Potential (BMP) tests of waste from alkaline pretreatment of sugarcane straw (straw deacetylation) in co-AD with other residues from the sugarcane 1G ethanol mill. The results confirmed beneficial effects from the synergisms of the co-substrates (Volpi et al. 2021a). Even the literature showing the use of energy cane for the production of biogas and 2G ethanol (Hoffstadt et al. 2020), to the best of our knowledge, the behavior of the aforementioned waste from 2G ethanol production in semi-continuous bench-scale reactors have not yet been studied, aiming to provide a preliminary basis for gradual scaling up of the process. Reactor operations should provide process parameters for continuous waste treatment and CH_4 production, enabling us to forecast the maximization of residue use within their specific availabilities in the 1G2G sugarcane biorefineries.

AD stability and efficiency depend on the synergistic activity of the microorganisms that belong to the anaerobic consortium, which performs hydrolysis, fermentation, acetogenesis, and methanogenesis activities (Li et al. 2009b). Relating the microorganism to its metabolic pathway is often a challenge. Identifying the microorganism in the process is already a big step, suggesting its metabolic potential, but it may not be enough to attribute the function of these microorganisms: a single microorganism may have different functions at different stages of the metabolic pathways (Cabezas et al. 2015). Furthermore, little has been found in the literature regarding the metabolic routes of microorganisms in AD from residues from the sugarcane industry with residues from 2G ethanol production in the co-AD system.

Given this context, the objective of the present work was to perform the anaerobic co-AD of residues from 2G ethanol production (i.e., lignocellulosic liquor from sugarcane straw deacetylation pre-treatment) and 1G ethanol production (i.e., vinasse and filter cake) in a stirred bench-scale reactor with semi-continuous feeding. Monitoring the operation aimed to reach the upper limit of the organic load applied to the reactor for maximizing stable CH_4 production, providing operational parameters for scale-up of the co-AD process. Fundamental aspects of AD during the operation were also investigated by the relation of reactor performance to monitoring analysis results. Microbial characterization was performed during stabilized CH_4 production to relate the microorganisms to potential metabolic routes, as well as to assess the modifications in the microbial community from the seed sludge.

Materials and methods

Residues and inoculum

The substrates were vinasse and filter cake from Iracema sugarcane mill (São Martinho group, Iracemápolis, São Paulo state, Brazil) and the liquor from the straw pre-treatment process, performed at the National Biorenovables Laboratory (LNBR) from the Brazilian Center for Research in Energy and Materials (CNPEM). Deacetylation pre-treatment was applied to sugarcane straw on a bench scale as described in Brenelli et al. (2020). Deacetylation liquor was used because it is rich in volatile fatty acids (mainly acetate) and it has a high Biochemical Methane Potential (BMP) (Volpi et al. 2021a) as co-substrate in reactor operation. The inoculum consisted of anaerobic consortium from the mesophilic reactor (BIOPAC®ICX - Paques) used for the treatment of vinasse from the Iracema sugarcane mill. This anaerobic consortium was used as inoculum in a previous study from our research group as describe in Volpi et al. (2021a).

Semi-continuous reactor: description and operation

The semi-Continuous Stirred Tank Reactor (s-CSTR) consisted of a 5L-Duran flask with 4L-working volume, closed with a pierceable isobutylene isoprene rubber septum kept, under agitation at 150 rpm by using an orbital shaking table Marconi MA 140. The operating temperature was 55°C, maintained by recirculating hot water through a serpentine. Thermophilic conditions were chosen because vinasse leaves the distillation columns at 90°C and thus would have lower (or none) energy expenditure to cool it to mesophilic conditions. Inoculum adaptation was performed because it was an anaerobic consortium from mesophilic conditions. The temperature of the inoculum was gradually increasing every 5 degrees per day until it reached 55°C, which was kept for a week before the beginning of the tests, as performed before by Boušková et al. (2005). The pH adjustment to neutrality was performed by adding NaOH (1M) solution when necessary. The reactor was fed once a day with the blend of co-substrates (in terms of volatile solids, VS): 70% of vinasse, 20% of filter cake, and 10% of deacetylation liquor, totaling 57.55 g_{VS} L^{-1}. These proportions were based on the residue's availability at the sugarcane mill, where the most abundant is vinasse (25 L vinasse per liter of ethanol total (1G+2G)) and the least would be the deacetylation liquor (7 L per liter of ethanol total (1G+2G)). The reactor was fully filled with inoculum during the start-up, in which aliquots of effluent were discharged and new feed was added in a fed-batch mode of 24h throughout the operation. The Organic Loading Rate (OLR) applied to the reactor was increased over time to maximize the volume of treated waste with concomitant reduction of Hydraulic Retention Time (HRT). Table 1 presents the values of operational parameters applied to the s-CSTR according to the respective operation phases. Biogas volume and CH_4 content were regularly monitored, as well as organic acids (OA), carbohydrates, alcohols, alkalinity, and organic matter (in terms of VS) content in the digestate. Oxidation-reduction potential (ORP) and pH were monitored both in the feed and digestate.

Analytical methods

Characterization of substrates

All the analyses followed the Standard Methods for the Examination of Water and Wastewater (APHA, Awwa W 2012). The substrates were characterized in terms of chemical oxygen demand (COD) (method 5220B), series of solids (method 2540), pH (pH meter PG 1800), OA, alcohol, carbohydrates, carbon, nitrogen, and phosphorus (method 4500P). COD measurement was performed for the characterization of liquid substrates (vinasse and deacetylation liquor), using the digestion method and reading in spectrophotometer RAC DR 6000. Analyses of carbon, nitrogen, and phosphorus were made using the TOC equipment Shimadzu-TOC-L-CNP. For the analysis of OA, carbohydrates, and alcohols, the samples were centrifuged for 10 minutes at 10,000 rpm, filtered in a porous membrane (0.2mm), and subjected to High-Performance Liquid Chromatography (HPLC, Shimadzu®). The HPLC consisted of a pump-equipped apparatus (LC-10ADVP), automatic sampler (SIL-20A HT), CTO-20A column at 43 °C, (SDP-M10 AVP), and Aminex HPX-87H column (300 mm, 7.8 mm, BioRad). The mobile phase was H_2SO_4 (0.01 N) at 0.5 ml min^{-1}. The series of

Table 1 Phases of reactor operation and the respective applied OLRs, feeding rate flows, and HRT

Phase in graph	OLR (g_{VS} L^{-1} day^{-1})	Feeding rate (L day^{-1})	HRT (days)
I	1.50	0.100	40
II	1.80	0.125	32
III	2.30	0.160	25
IV	2.75	0.190	21
V	3.20	0.222	18
VI	4.16	0.285	14
VII	4.80	0.333	12
VIII	5.23	0.363	11

solids included total solids (TS), volatile solids (VS), and fixed solids (FS), for all the substrates.

The elemental composition was performed for the characterization of filter cake in the Elementary Carbon, Nitrogen, Hydrogen and Sulfur Analyzer equipment (Brand: Elementar; Model: Vario MACRO Cube—Hanau, Germany).

Monitoring of semi-Continuous Stirred Tank Reactor (s-CSTR)

Daily biogas production was measured using a Ritter gas meter, Germany. The biogas was collected from the reactor by using a syringe through the rubber septum and CH_4 content was determined by gas chromatography (Construmaq São Carlos) five times a week. The carrier gas was hydrogen (30 cm s^{-1}) and the injection volume was 3 mL. The CG Column was made of 3-meter-long stainless steel, 1/8″ in diameter, and packaged with Molecular Tamper 5A for separation of O_2 and N_2 and CH_4 in the thermal conductivity detector (TCD). It had a specific injector for CH_4 with a temperature of 350 °C, an external stainless-steel wall, and an internal refractory ceramic wall. Detection (resolution) limits are 0.1 ppm for CH_4.

VS analyses were also carried out during the reactor operation. The determination was performed in the feeding and digestate to account for the organic matter (in terms of VS) removed during co-AD. The digestate was collected by using a syringe. The pH and the ORP of digestate were measured, immediately after sampling (before feeding) using a specific electrode for Digimed ORP. Alkalinity was performed using the titration method (APHA, Awwa W 2012). OA, carbohydrates, and alcohol analysis was performed for digestate three times a week.

Calculations

Principal component analysis (PCA) was performed using STATISTICA 10 through the correlation between the metabolites obtained (organic acids and alcohols) and the methane production, pH, partial and intermediate alkalinity, removal of organic matter, and ORP variables.

Gibbs free energies (ΔG°) of the conversion of propionate to acetate were calculated at room temperature in pH 7.0. The values were computed in accordance with (Alberty 1998; Dolfing 2015).

Biology molecular analysis

Microorganism identification analyses were carried out for the seed sludge samples (sample 1) before they were added to the reactor, and when the sludge was already stabilized in the s-CSTR with stable production of CH_4 under the OLR of 4.80 g_{VS} L^{-1} day^{-1} (sample 2). Genomic DNA was extracted in triplicate and the PowerSoil DNA Isolation Kit (Mobio) was used. For visual confirmation of the quality and integrity of the DNA extracted from the samples, a run on a 1% agarose gel stained with SYBR® Safe (Invitrogen) was performed. DNA quantification in the sample was performed with the Qubit® 3.0 equipment Fluorometer (Life Technologies) and the quality based on the 260/280 ratio, which was determined using the NanoDrop Lite equipment (Thermo Fisher). The large-scale sequencing of the V3-V4 region of the 16S ribosomal RNA gene from *Bacteria* and *Archaea* present in the samples was then determined with the forward primer (515) 5′ GTGYCAGCMGCCGCGGTAA and reverse primer (806) 5′ GGACTACNVGGGTWTCTAAT, in triplicate, using the Illumina MiSeq platform with paired-end sequencing (2 × 250 bp).

For the sequence analysis, the quality of readings was evaluated using the FastQC tool v.0.11.5 (Andrews, 2010), with quality strings lower than 30 (Phred score) and less than 100 base pairs were filtered with Trimmomatic 0.39 (Bolger et al. 2014). Bioinformatics analyses were performed using the Quantitative Insights into Microbial Ecology (QIIME2, version 2019.7, https://docs.qiime2.org/2019.7) (Bolyen et al. 2019) and its plugins. The taxonomic classification of Operational Taxonomic Units (OTUs) was performed with the q2-feature-classifier plug-in (Bokulich et al. 2018) in the classify-consensus-research program (Rognes et al. 2016), based on the SILVA Ribosomal RNA Gene version 132 database (Quast et al. 2013). The resulting Qiime output file containing the abundances of OTUs in the samples was analyzed using the Phyloseq package (McMurdie and Holmes 2013) from the R (Team, 2013) software for making graphs and tables.

The large-scale sequencing of amplicons from the ribosomal operon of the microbial community led to identifying the *Bacteria* and *Archaea* present in the samples in-depth to characterize the microbiota. The results of the genera found were expressed in percentage, reflecting the relative abundance of microorganisms in the samples. Raw sequences were deposited in BioSample NCBI under accession number PRJNA684620.

Results

Tables 2 and 3 show the characterization of the inoculum and the residues fed to the s-CSTR. Two different batches of vinasse and deacetylation liquor were used throughout the operation, called batch 1 and batch 2. Batch 1 was used in the first stages of the operation and batch 2 of vinasse and deacetylation liquor was fed from phases IV and VII, respectively. The differences in these substrate compositions directly affected the reactor supply, making it necessary to

Table 2 General characterization of residues

Characterization	Inoculum [a]	Vinasse lot 1 [a]	Vinasse lot 2 [a]	Liquor lot 1 [a]	Liquor lot 2 [a]	Filter cake [a]
COD (g L^{-1})	11.171 ± 0.901	28.660 ± 0.91	17.020 ± 0.45	32.920 ± 0.27	10.440 ± 0.01	-
Volatile solids (g mL^{-1})	0.015 ± 0.001	0.018 ± 0.00	0.099 ± 0.00	0.103 ± 0.00	0.008 ± 0.00	0.192 ± 0.01
Fixed solids (g mL^{-1})	0.010 ± 0.002	0.007 ± 0.01	0.005 ± 0.00	0.021 ± 0.00	0.005 ± 0.00	0.097 ± 0.00
Total solids (g mL^{-1})	0.025 ± 0.020	0.025 ± 0.00	0.015 ± 0.00	0.011 ± 0.00	0.014 ± 0.00	0.290 ± 0.02
pH	7.350 ± 0.210	4.030 ± 0.34	4.090 ± 0.48	12.450 ± 0.13	11.450 ± 0.21	-
Total carbon (mg L^{-1})	4892.00 ± 0.11	14110.94 ± 0.21	12658.00 ± 0.02	15919.00 ± 0.89	16652.00 ± 0.33	-
Organic total carbon (mg L^{-1})	3689.00 ± 0.58	14099.27 ± 0.12	12643.00 ± 0.45	15113.00 ± 0.56	15971.00 ± 0.56	-
Inorganic carbon (mg L^{-1})	1203.00 ± 0.69	11.69 ± 0.35	15.39 ± 0.63	805.60 ± 0.43	681.10 ± 0.33	-
Nitrogen (mg L^{-1})	596.30 ± 0.73	495.93 ± 0.95	302.50 ± 0.15	176.30 ± 0.96	121.20 ± 0.28	-
Phosphor (mg L^{-1})	13.72 ± 0.00	34.01 ± 0.00	9.96 ± 0.00	7.74 ± 0.00	2.98 ± 0.00	-
Soluble lignin (g L^{-1})	-	-	-	5.50	10.99	-

[a]Mean of three replicates ± standard deviation; - not carried out

adjust the feeding volume to maintain the applied OLR throughout the operation

Table 3 shows the main OA concentrations for the different batches of vinasse and deacetylation liquor, reinforcing the variability of such residues throughout the process and the season. Batch 1 of vinasse contained a larger variety of OA in higher concentrations, especially propionic acid, which can negatively affect the AD process (in concentrations as high as 900 mg L^{-1}) (Wang et al. 2009) by inhibiting the terminal process—the methanogenic *Archaea*—and resulting in the accumulation of hydrogen (H_2) and potentially raising the free energy (Marchaim and Krause 1993). Propionic acid was also detected in batch 2 of liquor, in non-inhibitory concentrations.

The results related to the monitoring of the reactor operation are presented from Figs. 1, 2, 3, 4 and 5. Figure 1 shows the removal efficiency of organic matter and CH_4 content in biogas produced in the reactor for each applied OLR.

In the initial OLR (phase I), large variations in the reactor efficiency occurred up to 55 days, when the organic matter removal stabilized at 71.27% ± 4.87% with the establishment of some metabolic routes for CH_4 production (55.91 ± 5.78 $mL_N CH_4\ g^{-1}v_S$). This behavior is in accordance with the results of digestate analysis (Figs. 2, 3 and 4). At each sequential increase of the applied OLRs, an initial disturbance on organic matter degradation was detected, representing firstly the adaptation of acidogenesis, at first 40 days (large variations on reactor efficiency) followed by the establishment of methanogenesis (little variations in the reactor efficiency). From about 90 days, CH_4 production as high as 90 $mL_N CH_4\ g^{-1}v_S$ was detected up to the penultimate applied OLR (phase VII), which

Table 3 Composition of acids, carbohydrates, and alcohols of residues

Compounds	Vinasse lot 1	Vinasse lot 2	Liquor lot 1	Liquor lot 2
Citric (mg L^{-1})	237.99	0.00	0.00	0.00
Succinic (mg L^{-1})	278.33	205.06	0.00	0.00
Propionic (mg L^{-1})	1695.30	0.00	0.00	822.74
Formic (mg L^{-1})	1019.35	586.98	0.00	22.01
Acetic (mg L^{-1})	657.20	0.00	3670.00	1063.37
Isobutiric (mg L^{-1})	1304.90	216.43	78.84	0.00
Butiric (mg L^{-1})	160.30	549.95	0.00	0.00
Malic (mg L^{-1})	259.18	160.80	0.00	34.11
Valeric (mg L^{-1})	39.01	0.00	0.00	0.00
Caproic (mg L^{-1})	791.05	0.00	0.00	0.00
Isovaleric (mg L^{-1})	0.00	0.00	0.00	1788.62
Latic (mg L^{-1})	3891.10	341.60	0.00	0.00
Glucose (mg L^{-1})	570.74	738.30	85.20	726.50
Fructose (mg L^{-1})	647.23	400.79	0.00	856.04
Ethanol (mg L^{-1})	114.30	0.00	0.00	0.00
Butanol (mg L^{-1})	556.62	0.00	0.00	0.00

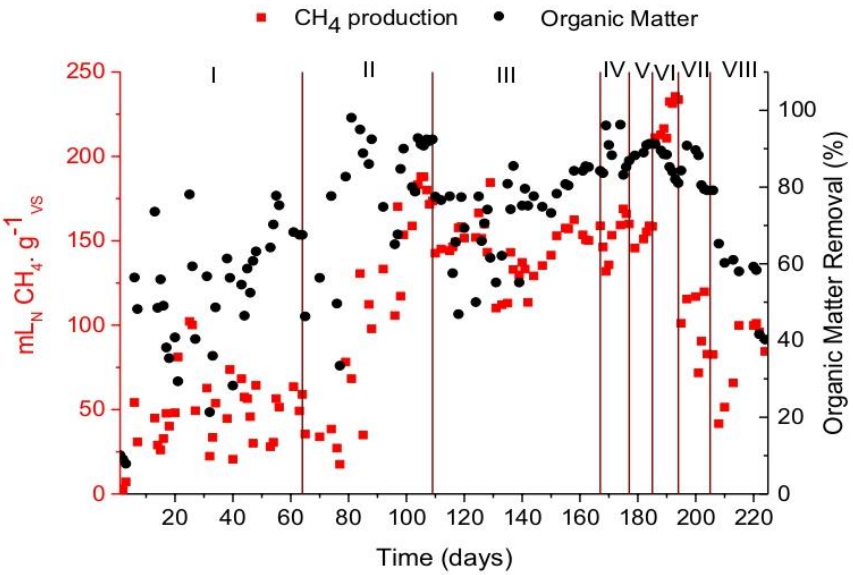

Fig. 1 Methane production and organic matter removal along the reactor operation according to the applied OLRs (g_{VS} L^{-1} day^{-1}): 1.50 (phase I); 1.80 (phase II); 2.30 (phase III); 2.75 (phase IV); 3.20 (phase V); 4.16 (phase VI); 4.80 (phase VII); 5.23 (phase VIII)

corresponded to the maximum applied OLR that the reactor was able to withstand with stability on CH_4 production (93.92 ± 17.62 $mL_N CH_4$ $g^{-1}v_S$ and 79.57 ± 4.54 % of organic matter removal), although this was not the maximum CH_4 yield. This fact indicates that methanogenesis activity started to become self-regulated from the end of phase II (change of applied OLR from 1.80 g_{VS} L^{-1} day^{-1} to 2.30 g_{VS} L^{-1} day^{-1}), which is reinforced by the results of digestate analysis presented in Figs. 2, 3 and 4.

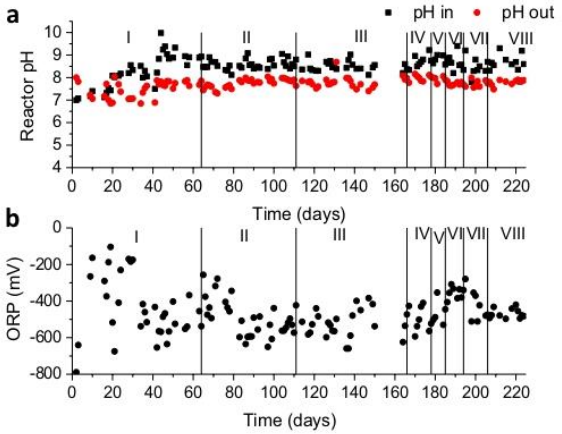

Fig. 2 Monitoring of pH (a) and oxidation reduction potential (ORP) (b) throughout the reactor operation according to the applied OLRs (g_{VS} L^{-1} day^{-1}): 1.50 (phase I); 1.80 (phase II); 2.30 (phase III); 2.75 (phase IV); 3.20 (phase V); 4.16 (phase VI); 4.80 (phase VII); 5.23 (phase VIII)

Figure 2 shows the monitoring of pH and ORP throughout the operation. Figure 2a is about pH and Figure 2b is about the ORP.

Figure 3 shows the results of the organic compounds monitored throughout the reactor operation according to the applied OLRs. In Fig. 3a are the acids, and in Fig. 3b are the carbohydrates and alcohols. In the start-up OLR (phase I), large amounts of OA were detected (Fig. 3a), mostly from the residue composition itself (Table 3). According to the AD fundamental phases and the establishment of methanogenesis, these concentrations were decreased throughout the operation due to the conversion by acetogens into acetate, H_2 and CO_2, and then to CH_4 by the methanogenic A*rchaea* to form biogas. The presence of the carbohydrates and their decrease in phases I and II indicate the establishment of acidogenesis (Fig. 3b).

Figure 4 shows the results of alkalinity indicators obtained during the reactor operation, with Fig. 4a representing total alkalinity and Fig. 4b showing the relationship between partial and intermediate alkalinity, for a better comparison with the data of the literature.

Figure 5 shows the results of the PCA analysis, which was carried out to better understand the relationship of the metabolic routes with the variables of CH_4 production, organic matter removal, alkalinity, ORP, and pH.

The results about molecular biology are presented in Figs. 6 and 7. Figure 6 shows the observed values of richness (number of species), the calculated values from diversity (Shannon index) (Fig. 6a), wealth estimate (Chao1 estimator) (Fig. 6b) of the samples, and the Shannon index (Fig. 6c). The number of species (Fig. 6a) and the richness (Fig. 6b) of sample 1 were greater than that of sample 2.

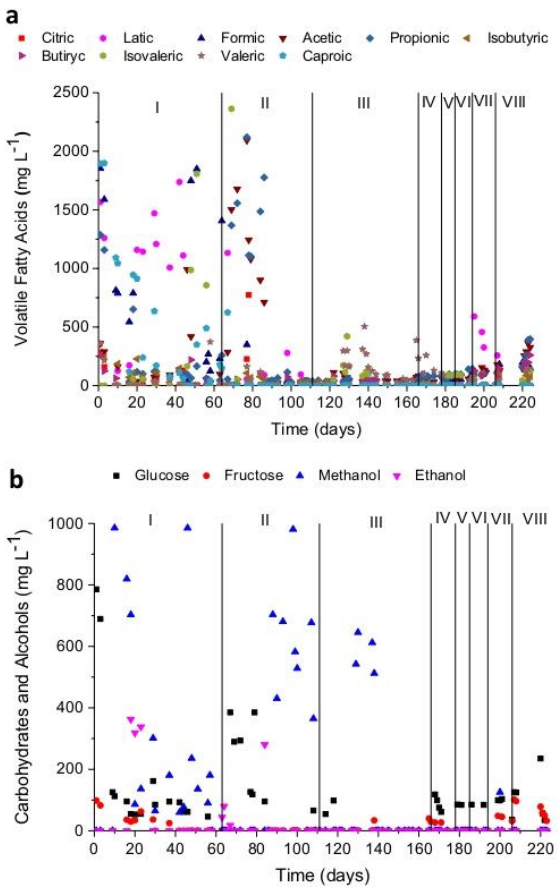

Fig. 3 Values of (a) organic acids and (b) carbohydrate and alcohol concentrations in the digestate monitored along the reactor operation according to the applied OLRs (g_{VS} L^{-1} day^{-1}): 1.50 (phase I); 1.80 (phase II); 2.30 (phase III); 2.75 (phase IV); 3.20 (phase V); 4.16 (phase VI); 4.80 (phase VII); 5.23 (phase VIII)

Figure 7a and b show the relative abundances of microorganisms found in the samples at the phylum and genus levels, respectively, in relation to *Bacteria* domain. Figure 8 shows the same information as Fig. 7 in respect to phylum and genus but in relation to *Archaea* domain.

Changes in the microbial community from one sample to the other, both for phylum (Fig. 7a) and genus (Fig. 7b), were observed in *Bacteria* domain. In sample 1 (Fig. 7a), there was a dominance of the microorganism phyla: (~3.5%) *Bacterioidetes*, (~7.5%) *Chloroflexi*, (~50%) *Firmicutes*, (~9%) *Synergistes*, (~7%) *Tenericutes*, (~11%) *Thermotogae*. For sample 2, the number of phyla was smaller, with a greater abundance of (~53%) *Firmicutes*, (~1%) *Tenericutes*, and (~26%) *Thermotogae*. Regarding the relative abundance of the genus of the *Bacteria* domain (Fig. 7b), the main genera found in sample 1 were: (~12%) *Defluviitoga*, (~6%) *Hydrogenispora,* (~2.5%) *Mesotoga*, (~2%) *Petrimonas*. In sample 2, the main genera were: (~35%) *Defluviitoga*, (~9%) *Hydrogenispora,* (~3%) *Ruminiclostridium*, (~0.75%) *Syntrophaceticus*, (~0.5%) *Tepidanaerobacter*.

In the *Archaea* domain there were also changes in relative abundance at the genus level from sample 1 to sample 2 (Fig. 8b). Regarding the phylum, the predominant phylum in both samples was the phylum *Euryarchaeota* (Fig. 8a), since most methanogenic organisms belong to it. The predominant genus of methanogenic *Archaea* in sample 1 was (~95%) *Methanobacterium* and the predominant genus in sample 2 was (~90%) *Methanoculleus* and also (~10%) *Methanothermobacter*.

Discussion

Characterization of residues

The C:N ratios of vinasse batch 1 (28:1) and batch 2 (40:1) were in the recommendable range for AD processes (20–40:1) (FNR, 2010), although the C:N ratio was slightly higher in the latter, mainly due to its N content being about 64% lower than in batch 1 (Table 2). The COD value of vinasse batch 1 was close to the values normally found in the literature (Moraes et al. 2015), whereas batch 2 had much lower COD values. Accordingly, the level of TS was also lower than the vinasse in batch 1, although the VS content was about 5 times higher. This fact shows the complexity of vinasse composition, which is significantly affected by factors such as the ethanol production processes and the sugarcane plant characteristics and cultivation. The choice of the suitable parameter for organic matter accounting and its maintenance for the reactor monitoring directly affects the successful operation and consequently CH_4 production. The COD of vinasse batch 1 takes into account non-organic materials, e.g., sulfide from yeasts after the fermentation cycle as a way to prevent flocculation. In this study, these differences resulted in different vinasse volumes from batch 1 and batch 2 to compose the feed keeping the same applied OLR. Based on VS contents, larger volumes of vinasse from batch 1 were used, which would be the opposite if only the COD was considered as a parameter for organic matter content. It highlights the importance of regular analysis for vinasse characterization throughout the season, especially related to the organic material, so that the applied OLR remains stable, thus avoiding organic load shocks, which can lead the reactor to failure (Fuess et al. 2017b). The "poor" operational control of vinasse AD normally adopted by Brazilian sugarcane plants results in an inefficient operation of the reactor, which has reflected in "negative marketing" for this process to scale in the sector. As the residue compositions vary throughout the sugarcane season

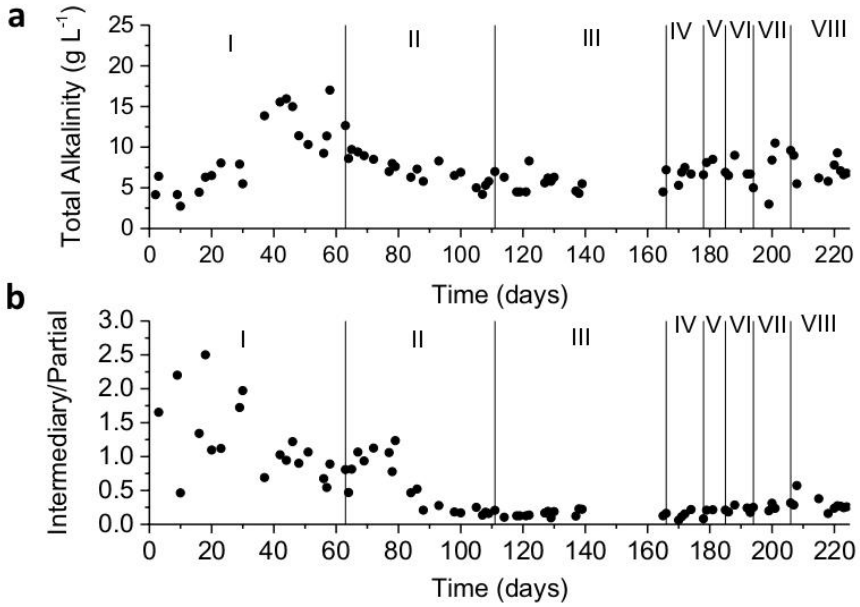

Fig. 4 Monitoring of reactor alkalinity: (a) total alkalinity; (b) alkalinity intermediary/alkalinity partial ratio according to the applied OLRs (g_{VS} L^{-1} day^{-1}): 1.50 (phase I); 1.80 (phase II); 2.30 (phase III); 2.75 (phase IV); 3.20 (phase V); 4.16 (phase VI); 4.80 (phase VII); 5.23 (phase VIII)

and ethanol processing, the strict operation and monitoring of AD reactor and substrates are essential for success. Uncertainties regarding the production of 2G ethanol and its residues make it even more difficult to insert AD, and bench-scale tests were extremely important to expand and deepen the knowledge of the main factors that affect this biological process.

Placing emphasis on the variability of the agro-industrial waste composition, phosphorus (P) content of vinasse in batch 2 was much lower than that detected in batch 1. The

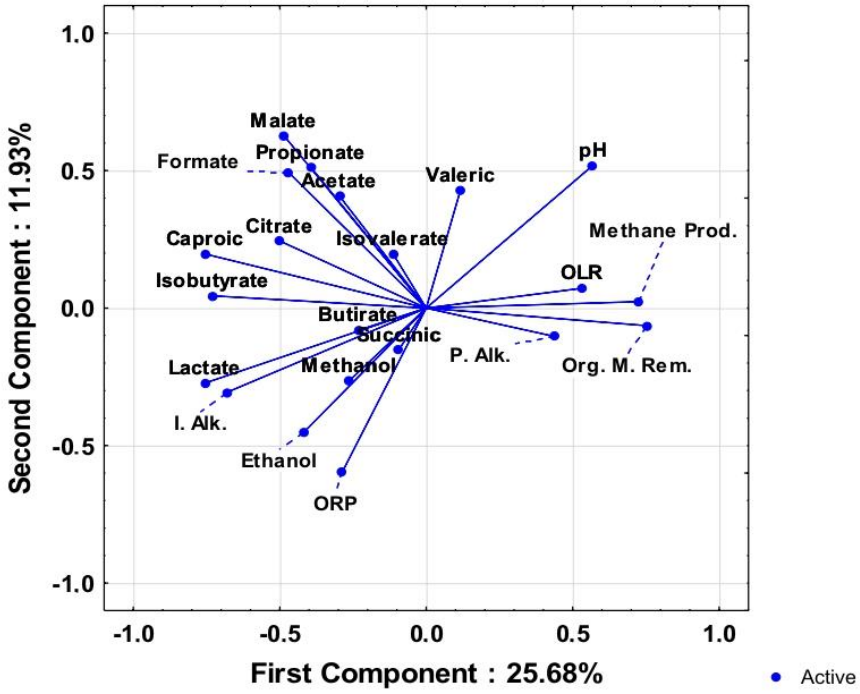

Fig. 5 Principal components analysis of CH_4-producing systems related with organic acids, pH, alkalinity, and organic matter removal

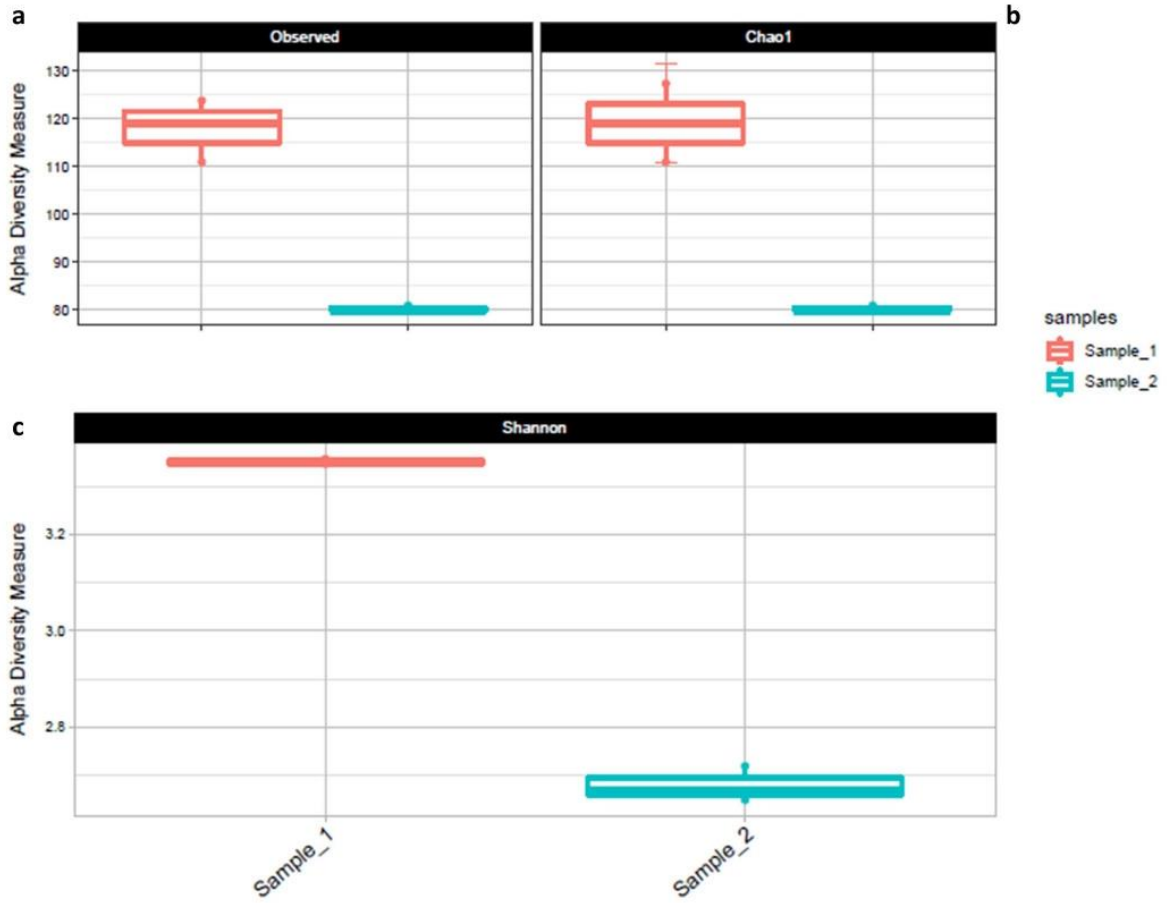

Fig. 6 Observed values of (a) richness (number of species), (b) richness estimate (Chao1 estimator), and (c) calculated values of diversity (Shannon index) of sample 1 (seed sludge) and sample 2 (sludge from the s-CSTR stable operation, phase VII)

values are within the wide range reported in the literature (4–250 mg L^{-1}) (Moraes et al. 2015). P can be accumulated in phosphorus-accumulating organisms (PAO) and these organisms can store VFA species as polyhydroxyalkanoates (PHA) materials, in the starvation period, that can be potentially degraded into varied fractions of individual VFA according to the PHA composition; however, when these concentrations are in excess they can form buffer solutions that precipitate important minerals from AD such as calcium, magnesium, aluminum, and iron (Wang et al. 2016), which was not observed in this work and, thus, P concentration was not inhibitory to the process.

Similar to vinasse, two batches of deacetylation liquor were used, in which batch 1 contained higher COD, VS, TS, P concentrations than the liquor of batch 2 (Table 2). On the other hand, soluble lignin content was twice as high in batch 2 as in batch 1. This compound was already reported to affect the AD process by its slow degradation, causing a "late" CH_4 production (Mulat and Horn 2018). Short HRT applied to reactors may not take full advantage of the deacetylation liquor's CH_4 potential. Thus, co-AD reactors (normally CSTRs), known for their long HRT may be suitable for fully making the most of this substrate. The deacetylation liquor has a C:N ratio of 90: 1 for batch 1 and 137: 1 for batch 2, showing insufficient N content against the C content. This reinforces the role of co-digestion for the balance of nutrients and dilution of components in excess, e.g., the liquor co-digested with vinasse and filter cake, both with higher levels of N.

High concentrations of lactic acid were detected in batch 1 of vinasse and were reported to generate possible inhibition of AD because it is a precursor of propionic acid in the hydrolysis-acidification process (Table 3) (Zhang et al. 2007). Formic acid was also detected in vinasse batch 1 (Table 3), which can be easily degraded by sulfate-reducing bacteria (SRB) (Dinsdale et al. 2000) and contributing to the

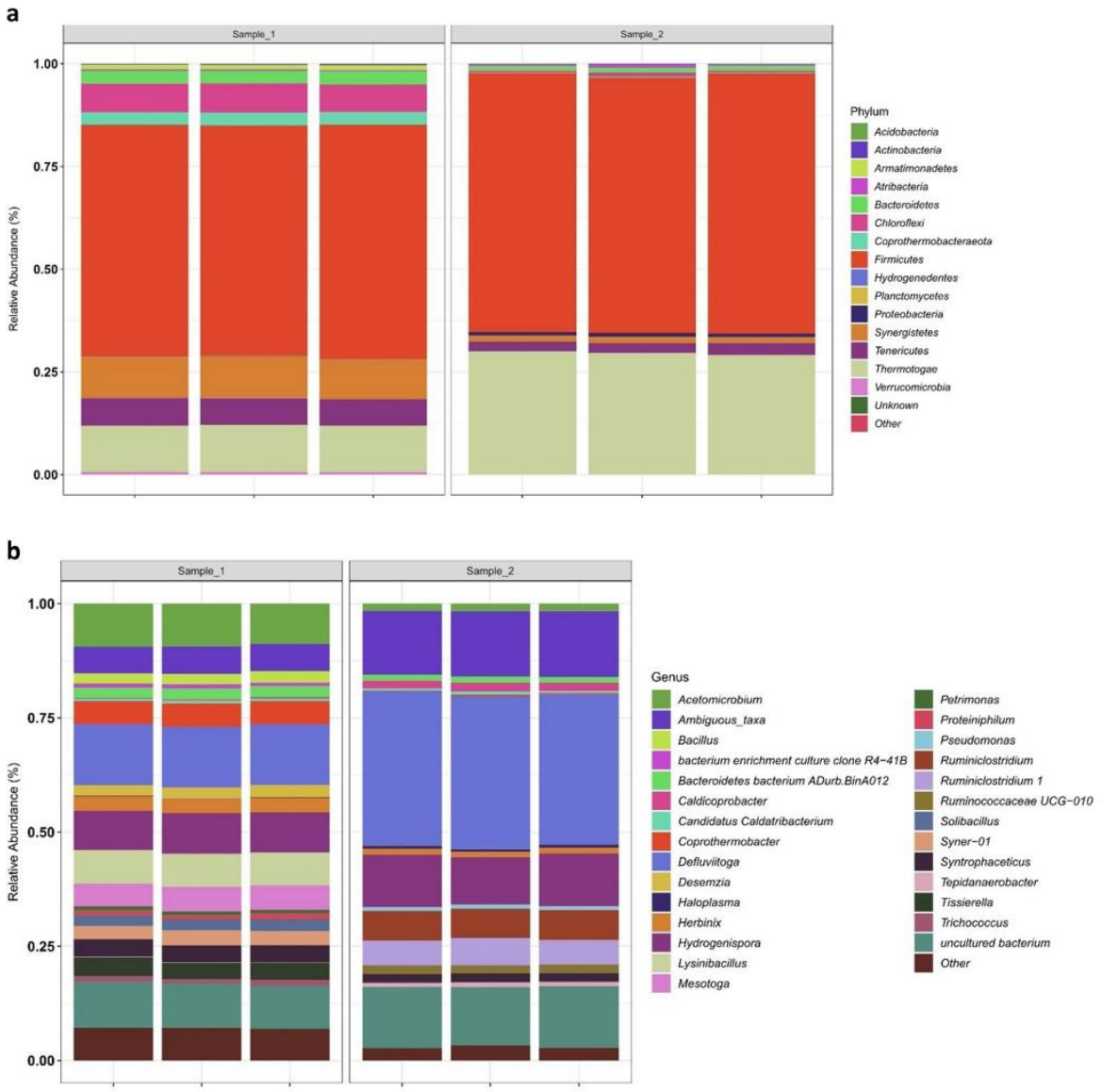

Fig. 7 Relative abundance of microorganisms at the phylum level (a) and genus level (b) in *Bacteria* domain from the seed sludge-sample 1 (1.1, 1.2, and 1.3) and from the s-CSTR sludge with stable CH_4 production-sample 2 (2.1, 2.2, 2.3)

sulfidric acid (H_2S) generation in biogas. In the presence of sulfate, SBR competes with methanogenic *Archaea* by the organic matter, leading part of the anaerobic metabolic pathways to sulfate reduction and lowering CH_4 formation. However, in addition to SRB, there is another metabolic route in which the formate can be used within the methanogenesis cascade. Through two groups of enzymes: iron-sulfur formate dehydrogenase (FDH) enzymes and NAD^+-dependent FDH enzymes it is possible that they can initially oxidize formate to H_2 and CO_2 and the CO_2 can then be reduced by methanogenic *Archaea* to form CH_4 and thus the format also contribute to CH_4 production (Crable et al. 2011). The acetic acid content in both batches of liquor (especially batch 1) indicates considerable potential for this residue to produce CH_4 as the acetotrophic pathway is the main one for CH_4 formation (Lata et al. 2002). The presence and concentration values of the different OAs may lead to the predominant metabolic routes of AD process, which can change due to

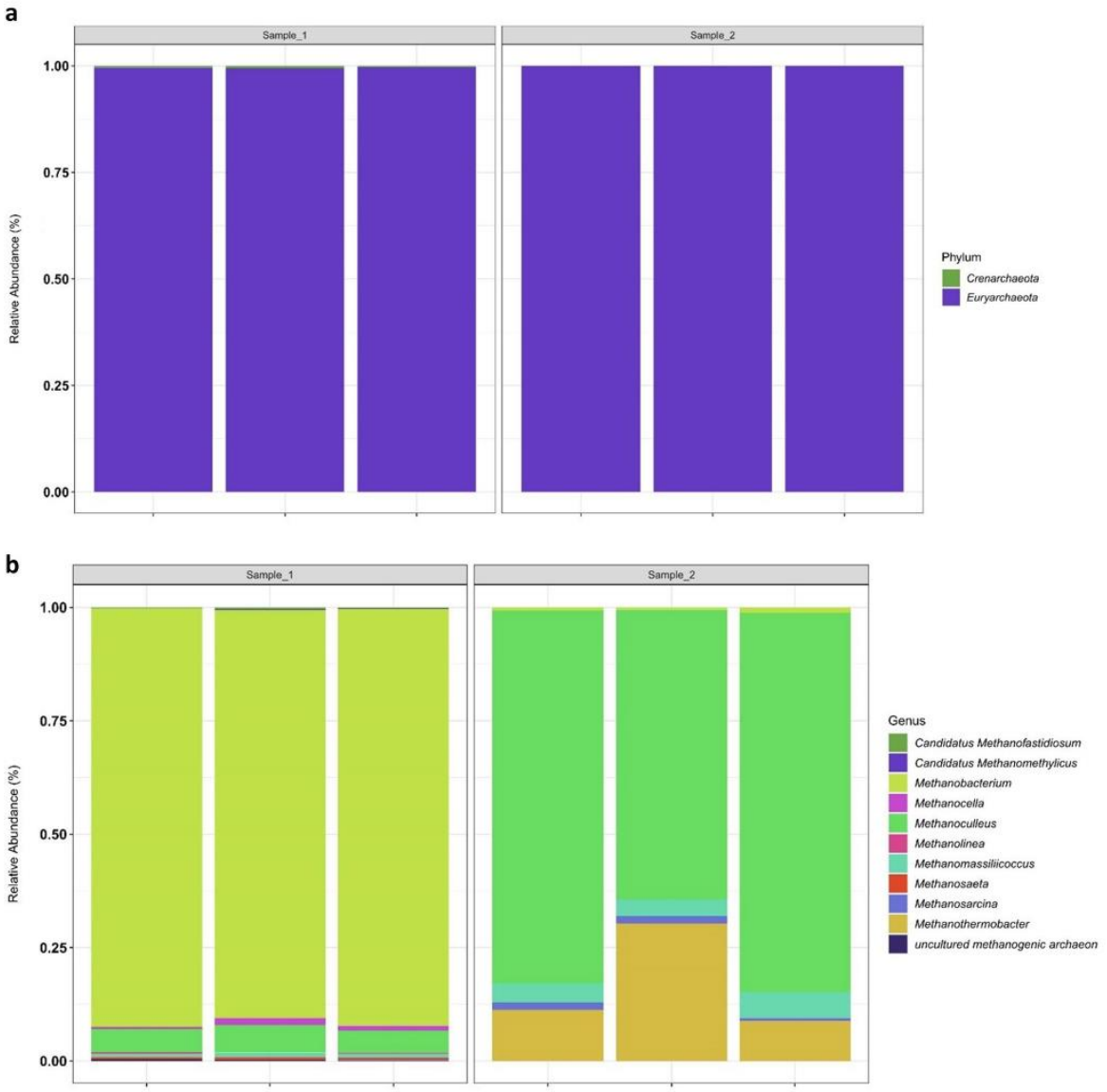

Fig. 8 Relative abundance of microorganisms at the phylum level (a) and genus level (b) in *Archaea* domain from the seed sludge-sample 1 (1.1, 1.2 and 1.3) and from the s-CSTR sludge with stable CH_4 production-sample 2 (2.1, 2.2, 2.3)

the variability of substrates composition and, thus, impairing the stable microbial consortia adaptation in AD reactors. Such complexity and specificities of these residues highlight the difficulty to introduce the co-AD process in continuous operation into the integrated 1G2G sugarcane biorefineries, despite their considerable CH_4 production potential.

The presence of isobutyric acid in vinasse batch 1 and isovaleric acid in deacetylated liquor batch 2 drew attention (Table 3). These iso-forms of such compounds have a worse rate of degradation in AD compared to their normal forms (butyric acid and valeric acid); however, the decomposition rate of the isoform of butyric acid is still higher than that of valeric and capric acid (Wang et al. 1999). Depending on the microbial consortia establishment, isobutyric acid can be degraded to acetic acid in the AD, which improves the CH_4 production, or it can undergo reciprocal isomerization and become butyric acid. Isovaleric acid, on the other hand, does not undergo this reciprocal isomerization in the AD process, encompassing different little elucidated metabolic routes from that of valeric acid (Wang et al. 1999).

Semi-Continuous Stirred Tank Reactor performance

Biogas production and reactor efficiency

The highest average reactor efficiency (83.08 ± 13.30 % organic matter removal and 233.20 ± 1.83 $mL_N CH_4\ g^{-1}v_S$) and CH_4 content (80.77% ± 0.28%) (Fig. 1) in biogas was achieved in phase VI, corresponding to the specific biogas production of 324.85 ± 2.76 mL g^{-1}_{VS}. These values are close to those obtained by Janke et al. (2016) in which 320 ± 0.48 mL biogas g VS^{-1} was achieved at the maximum ORL of 3.0 $g_{VS}\ L^{-1}\ day^{-1}$ in a s-CSTR treating sugarcane bagasse and filter cake (with tap water and cattle manure addition at mesophilic conditions, 38 °C). However, in their study, considerable OA accumulation in the digestate was observed (90% of OA, mainly propionic acid) and the average CH_4 content in biogas remained about 50%. Ferraz Junior et al. (2016) achieved the production of 250 mL_N -CH_4 $g^{-1}COD_{removed}$ and 72% removal of organic matter with the monodigestion of vinasse in an Upflow Anaerobic Sludge Blanket (UASB) reactor under thermophilic conditions. Santana Junior et al. (2019) reported the monodigestion of vinasse in a two-stage UASB reactor achieving biogas yield of 0.23 $m^3\ kg^{-1}\ COD_{removed}$, with 70% of CH_4. Both the removal of organic matter and the CH_4 content reported in the mentioned literature were lower than in the co-digestion process presented in our work, reinforcing the enhancement in the AD process applied to vinasse when co-digested with other residues from sugarcane ethanol process. Furthermore, the aforementioned authors reported the requirement of adding alkaline compounds to keep the pH neutrality, which was not necessary in the present study because the deacetylation liquor was able to contribute to the pH control when the methanogenesis was stabilized.

Although a considerable decrease in CH_4 content occurred at OLR 4.80 $g_{VS}\ L^{-1}\ day^{-1}$ in the present work (Fig. 1), CH_4 production and reactor efficiency remained stable as already described. In a scale operation, the choice of the OLR to be applied and maintained in the reactor will depend on the objective of the operation: maximum volume of treated waste or maximum energy production in the form of CH_4. The collapse of the studied s-CSTR occurred at the applied ORL of 5.23 $g_{VS}\ L^{-1}\ day^{-1}$, when the reactor efficiency significantly dropped with the accumulation of OA (Fig. 3a) and with the presence of carbohydrates in the digestate (Fig. 3b).

Degradation routes: pH and ORP indications

In the first 40 days, the pH output was around 6 to 6.5 (Fig. 2a), allowing the establishment of the acidogenesis process (Vongvichiankul et al., 2017), consistent with the starting behavior of the AD reactors, continuously adjusting the pH of the feed. After 90 days of operation, methanogenesis occurred and there was no need to adjust the pH, as the pH output was self-stabilized at around 7 until the end of the operation. ORP values followed the pH behavior, having larger variations (−800mV to −100mV) (Fig. 2b) and less stable CH_4 production (Fig. 1) during the first phase of operation. Some studies reported considerable drops in the ORP values (−350mV to −550mV) during the period of the highest H_2 production (Kataoka et al. 1997; Lin et al. 2008), corroborating the acidogenic step establishment at the beginning of the reactor operation. ORP variations during acidogenesis are related to the different metabolic pathways of acidogenic bacteria and the OLR applied to the reactor (Chen et al. 2015). The predominance of specific acidogenic routes may drive the methanogenic metabolic pathways, as well as the OA content of the fed substrates. After 90 days, the ORP variations decreased, although a considerable range still remained (−650mV to −450mV—Fig. 2b), which was further reduced (−300mV to −450mV) as CH_4 production became more stable at the end of the operation. These values during stability are within the range reported as ideal conditions for acidogenesis and methanogenesis (Golkowska and Greger 2013).

In phase II, when lactic acid was consumed, there were variations in ORP values, however, the predominance was −600mV (Fig. 2b). When the pH values of the outlet remained above 7 and ORP below −300mV, between 70 and 80 days (Fig. 2a and b—start of phase II), the biogas production increased by 200%, and was even better after 90 days (392%), when methanogenesis was consolidated (Fig. 1). Vongvichiankul et al. (2017) also reported a considerable increase in the biogas production (from 1.88 to 22.90 L day^{-1}) with the pH increase from 6.82 to 7.15 and the respective ORP increase from −359mV to −348mV.

Degradation routes: OA, carbohydrate, and alcohol indications

In accordance with Fig. 3, the consolidation of the methanogenic phase seemed to occur from about 90 days of operation, leading to a significant decrease in OA, alcohol and carbohydrates, although remaining methanol concentrations were detected. Methanol conversion in AD can occur by cultures of methanogenic *Archaea* or SRBs. The methanogens convert methanol into methyl-coenzyme M and in the presence of hydrogen methyl-coenzyme M is reduced to CH_4 (Weijma and Stams 1999). When methanol is the sole substrate, however, part of the methanol has to be oxidized to CO_2 to provide reducing equivalents for the reduction of methanol to CH_4. This oxidation of the methyl-group likely proceeds via a reversed pathway which methanogenic *Archaea* use to reduce CO_2 to CH_4 (Weijma and Stams 1999). In the presence of SRB, acetate is always necessary

as a carbon source. The biochemical pathway of methanol oxidation by SRBs is not known. It is likely that methanol is oxidized to formaldehyde by means of methanol dehydrogenase. Two other dehydrogenases oxidize formaldehyde to formate and then the formate is transformed into CO_2 (Weijma and Stams 1999). As methanogenic *Archaea* survive better in conditions of thermophilic temperature than SRBs, the methanogenic route from methanol should have been favored compared to sulfate reduction.

Lactic acid concentrations detected in phase I (Fig. 3a), mostly from batch 1 of vinasse composition, was probably converted into butyric acid (smaller proportions) or into propionic acid, both detected in phase II, when lactate content significantly dropped (average ORP values close to −500mV and pH around 7). Chen et al. (2015) reported that butyrate-type fermentation can happen between −300 and −250mV, which is a lower range than obtained in the present study. The differences in substrate compositions and inocula may explain this fact, indicating that the microorganisms can adapt differently to environmental conditions. Sugarcane vinasse is a highly acid substrate, and the butyrate-type fermentation is naturally favored by its composition (Fuess et al. 2020). On the other hand, Li et al. (2009a, b) reported that ethanol-type fermentation is favored when there are high concentrations of acetic acid and ethanol. Chen et al. (2015) also detected the ethanol-type fermentation in the ORP value of −120 mV and the pH lower than 5. In the present study, during phase II, acetic acid and small amounts of ethanol were also detected. It suggests the occurrence of butyric acid type fermentation (which may have been converted to acetate by SRBs) from the transition of phase I to phase II, with the ethanol type fermentation also taking place in the latter, even with the differences in the pH and ORP values reported in the few studies found in the literature, which reinforces the need for further research on this topic.

Although high content of malic and succinic acids was present in the fed substrates, no information regarding specific ORP values and their relationship with those acid-degradation metabolic pathways were found, but it is known they are propionate precursors in the AD process, as well as lactic acid (Scharer and Moo-young 1979). In phase II (Fig. 3a), with the stabilization of methanogenesis, the metabolic pathway of the lactate may have been shifted to form propionic acid, since this compound was detected and consumed in sequence, at the same time that CH_4 production increased. This situation can occur since the degradation of propionic acid is known to be the limiting factor in the CH_4 production phase under thermophilic conditions (Ferraz Júnior et al. 2016). The ORP was close to −280 mV (beginning of phase II) and pH of 7.5, in agreement with the ORP data proposed by Wang et al. (2006) for this metabolic route, except for the pH (values reported close to 5.5). The main acid precursor of CH_4, acetate, was detected up to 90 days of operation (ORP of −600mV), in parallel to the propionate appearance, when methanogenesis started to stabilize (Fig. 2a). The slight delay in propionate consumption after acetate uptake occurred as the former is the last OA to stabilize due to its slow degradation rate (Wiegant et al. 1986)

Lactate formation was also observed in phase VII (Fig. 2a), indicating that lactic acid bacteria (LAB) was established in the microbial consortia of the s-CSTR. Fuess et al. (2018) also reported that this bacteria group played a role in the microbial dynamics of vinasse-fed acidogenic systems by providing an alternative carbon source for both H_2-producing (butyric acid and H_2 production) and non-H_2-producing (propionic and acetic acids production) routes. With the organic overload of phase VIII, the lactate to propionate route may have prevailed and the methanogenic *Archaea* were not able to consume the latter acid, leading the reactor to collapse with a significant drop in the CH_4 production and removal organic matter efficiency (Fig. 1). It is worth mentioning that the increase of some carbohydrates (e.g., fructose and glucose) was also observed in phase VIII, which suggests that the acidogenic step was also affected by the organic overload.

In phase II, concentrations of propionic acid can be observed in the range of 1500 mg L^{-1} which is an inhibitory concentration for AD (Wang et al. 2009; Franke-Whittle et al. 2014). However, it did not inhibit the production of CH_4 (Fig. 1), which can be explained by the fact that different systems have their tolerance levels for OA due to the specific development and adaptation of the different microorganisms in the consortia to the different reactor conditions (Angelidaki et al. 1993). The decrease and stabilization of propionate concentrations along the reactor operation was a result of this self-regulation of the anaerobic microbial consortium, avoiding the inhibition process by such acid accumulation. The stabilization of ORP values lower than the favorable one for propionic type fermentation (−278 mV) (Ren et al. 2007) also confirmed the minimization of this route.

A simplified thermodynamic analysis of the identified Eq. 1 showed that there was no accumulation of propionic acid in the reactor, due to its conversion to acetic acid being favorable.

$$CH_3CH_2COO\_ + 3H_2O \rightarrow CH_3COO\_ + H^+ + HCO_3^- + 3H_2$$
$$\Delta G^\circ = -85.6 Kjmol^{-1}$$
$$Propionate conversion to acetate \quad (1)$$

Zhao et al. (2018) showed that the higher temperature (thermophilic process) had positive effects on propionate acetogenesis, favoring its conversion to acetate. In addition, the literature shows that propionic acid degradation is better in systems with low H_2 pressure and the concentration is kept low by H_2 consuming methanogens (Wiegant et al.

1986). Hydrogenotrophic methanogens have been identified in the microbial consortium (Fig. 7) that may have contributed to the consumption of H_2 and also favored the degradation of propionic acid when the methanogenesis stabilized (close to 90 days).

In phase V, the low concentration of OA remained practically stable, with no major increases in OA up to phase VI. In both phases, the reactor presented constant stabilization of CH_4 production and removal of organic matter (Fig. 1). It confirms that the AD biochemical routes were self-regulated with the synergisms between acidogenesis and methanogenesis established.

Degradation routes: alkalinity indications

The alkalinity of the reactor (Fig. 4) was in accordance with the behavior of pH, ORP, and OA variables (Figs. 2 and 3). Apart from the carbonate/bicarbonate system, the protonated forms of Volatile Fatty Acids (VFA) help to maintain the total alkalinity of anaerobic reactors, in which the intermediate alkalinity is caused by the ionized forms of VFA. The predominance of acidogenesis in the first 40 days of operation resulted in the low alkalinity values caused by the accumulation of VFA which is directly linked to the destruction of AD buffering capacity (Martín-González et al. 2013). In this period, the intermediate alkalinity/partial alkalinity (IA/PA) ratio remained between 1 and 2, much higher than the ideal value of 0.3, an indicator of the stability of AD process (Ripley et al. 1986). The gradual decrease in the IA/PA ratio close to 0.3 occurred over 90 days, coinciding with the consumption of the VFAs (Fig. 3a) and, thus, indicating the establishment of the self-controlled AD process. It is worth mentioning that alkalinizing were added only in the first days of phase I, and were no longer needed in the following phases, indicating that the deacetylation liquor as co-substrate provided the necessary alkalinity for the AD system. Fuess et al. (2017a) showed that $NaHCO_3$ is an alkalinizing used in AD processes, and is relatively expensive (USD 0.92 kg^{-1}) compared to the NaOH cost (USD 0.53 kg^{-1}); therefore, the use of deacetylation liquor can further reduce costs. The suppression of alkalinizing use may represent an economic advantage for reactor operations, which could be decisive for the implementation of the AD technology.

Relation of PCA and metabolic routes

According to Fig. 5, approximately 40% of the correlations can be explained by the PCA. The results showed that with the increase in OLR there was also an increase in CH_4 production and consequently greater organic matter removal. These variables form a group and have an inverse relationship to the metabolites of lactic acid, methanol, ethanol, succinic acid, which makes sense, since the butyric-type fermentation can happen together with ethanol-type fermentation, in the acidogenic phase (Li et al. (2009a, b)). In addition, lactic acid and butyric acid are precursors of propionic acid, also in the acidogenic phase (Krzysztof Ziemiński 2012).

The graph (Fig. 5) showed that the pH of the system, to favor CH_4 production, needs to be closer to neutrality, since CH_4 production is more related to partial alkalinity ($5.75 < pH < 8$) than to intermediate alkalinity ($4.3 < pH < 5.75$). In addition, intermediate alkalinity is indirectly related to pH, considering that low pH ($4.3 < pH < 5.75$) is associated with reduced end-products such as lactate and solvents (ethanol, butanol, and acetone) (Ferraz Júnior et al. 2020).

Through the analysis of PCA, it was also observed that as the concentration of OA (such as malic, acetic, propionic, formic acids) decreases, organic matter removal increases and consequently increases the production of CH_4. This behavior is consistent with the results since these acids are precursors of the phases of acidogenesis and acetoclastic methanogenesis (Vanwonterghem et al. 2015).

Microbial community characterization

Both samples came from anaerobic reactors for CH_4 production, being sample 1 (seed sludge) from a mesophilic process and sample 2 (s-CSTR sludge) from a thermophilic process. This temperature difference may have caused a selection of microorganisms, justifying these differences in the number of species and richness (Fig. 6). In addition, operational and substrate differences may have led to CH_4 production by different metabolic routes, selecting different microorganisms in both samples. Another reason that may explain this difference is that the microbial community in sample 2 comes from a reactor stabilized in the CH_4 operation, with the "selected" microorganisms.

Figure 6c shows the Shannon index, with values for both samples below 4.0, which potentially indicates a greater specificity of the microorganisms. Larger microbial diversity in anaerobic digesters is reported when this index is higher than 5.0 (Moraes et al. 2019). Even though both were below 5.0, sample 2 still presented a lower value, which indicates that the microbial community was even more specific and reinforces the idea that these microorganisms were acting on different metabolic routes.

In both samples, the relative abundance of the phylum *Firmicutes* was the highest, which is a common characteristic of the microbial community that makes up the anaerobic sludge (Fig. 7a) (Chen et al. 2016; Wu et al. 2020). Bacteria from the phylum *Firmicutes* are the main ones that produce cellulolytic enzymes within thermophilic AD. This phylum also contains acetogenic bacteria that degrade OA to produce acetic acid (Yu et al. 2018). The presence of the phylum *Firmicutes* may also be related to lignocellulosic

residues as substrate (deacetylation liquor and filter cake in this work), as reported by Yu et al. (2018) using rice straw in the thermophilic AD process. A decrease in the relative abundance of the phylum *Synergistes* and *Tenericutes* from sample 1 to sample 2 and an increase in the phylum *Thermotogae* in sample 2 were also observed (Fig. 7a). The phylum *Thermotogae* is the predominant one in thermophilic processes (Wang et al. 2018) and it has often been reported in thermophilic digesters treating organic wastes such as swine slurry, market biowaste, and food wastewater (Kim et al. 2018).

The Phylum *Proteobacteria* (~0.2%) is related to the degradation of lignocellulose in the hydrolysis phase (Yu et al. 2018; Wu et al. 2020), and this phylum was observed only in sample 2. The appearance of this phylum, even in small relative abundance (probably because the deacetylation liquor and filter cake were the co-substrate in minor proportions), indicates how the microbial population changed with the presence of different substrates for the CH_4 production.

The detected groups of methanogenic *Archaea* belong to the phylum *Euryacheota* (Wu et al. 2020) (Fig. 8a). The sample 2 presented different genera of methanogenic *Archaea* comparatively to sample 1: (~90%) *Methanoculleus* and (~10%) *Methanothermobacter* in sample 2, while in sample 1 the predominant was *Methanobacterium* (Fig. 8b). This difference in methanogenic *Archaea* between the samples confirms the change in the microbial community from one condition to another. Both microorganisms from the aforementioned genus from sample 2 are hydrogenotrophic methanogen, using mainly H_2 and CO_2 for conversion to CH_4 (Krzysztof Ziemiński 2012). Although *Methanobacterium* (predominant in sample 1) are also hydrogenotrophic methanogen, the literature shows that they appear mainly in mesophilic temperatures (30–35°C), which may explain the change in the hydrogenotrophic community through temperature change: in sample 1, the seed inoculum was mesophilic and, in sample 2, the microbial community worked at thermophilic temperature (Lin et al. 2018). Tian et al. (2015) carried out an analysis of the change from the mesophilic to thermophilic microbial community and the predominant methanogenic *Archaea* in the thermophilic process were *Methanoculleus* and *Methanothermobacter*, as in the present work. It is possible to infer that microorganisms preferred the metabolic route of CH_4 production from hydrogenotrophic methanogenesis in the anaerobic digestion of residues from the sugarcane industry, since hydrogenotrophic methanogens were detected in both samples.

The dominant methanogenic *Archaea* in sample 2 (*Methanoculleus* genus) have been reported to be predominant in the biogas production of mesophilic reactors with syntrophic acetate oxidation (SAO) coupled with hydrogenotrophic methanogenesis (Schnürer et al. 1999). On the other hand, Hattori (2008) reported that SAO coupled with hydrogenotrophic methanogenesis can also occur at elevated temperatures, and it was confirmed by analyses in thermophilic digesters. Thus, our results indicate that CH_4 was mainly produced from CO_2 reduction with H_2 in the s-CSTR, which implies the syntrophic oxidation of acetate coupled to hydrogenotrophic methanogenesis.

Syntrophaceticus and *Tepidanaerobacter* are known as SAO microorganisms which can also be coupled to hydrogenotrophic methanogenesis (Kim et al. 2018). Their presence in sample 2 (~0.75% and ~0.5%, respectively) confirmed the possibility of methanogenesis occurring from acetate via CO_2 reduction with H_2, and these microorganisms were involved in acetate catabolism. The SAO coupled to the hydrogenotrophic methanogenesis metabolic route was observed in AD processes from residues with high protein and ammonium contents such as chicken manure, but also in reactors with *Jatropha* press cake as substrate (Ziganshin et al. 2013), which have lignocellulosic characteristics such as the filter cake, i.e., a co-substrate used in the present work.

The predominance of the genus *Defluviitoga* (~35%), belonging to the phylum *Thermotogae*, was also observed in sample 2. *Defluvitoga* genus is reported to be dominant in the degradation of organic materials in CSTRs or thermophilic bioelectrochemical reactors (Guo et al. 2014). Some members of the aforementioned genus can also metabolize sugars to generate H_2 and OA in the hydrolysis phase of AD. This suggests that these microorganisms may also interact with hydrogenotrophic methanogenic *Archaea* (Kim et al. 2018).

A relative abundance of the genus *Ruminoclostridium* (~3%), belonging to *Firmicutes* phylum, was also observed in sample 2, while it was not detected in sample 1. Some species of the *Ruminoclostridium* genus are characterized by having acetate as their final product of sugar metabolism. They are known to metabolize materials with high concentrations of cellulose due to the high production of cellulolytic enzymes (Badalato et al. 2017). Peng et al. (2014) showed that bacteria of the *Ruminoclostridium* genus improved the efficiency of CH_4 production using lignocellulosic residues, such as wheat straw as substrate. The presence of this group of microorganisms in sample 2 indicates they were acting in the hydrolysis phase of cellulosic and lignocellulosic residues used in the co-digestion, thus explaining the reason for their absence in the seed sludge (sample 1).

It is worth mentioning that the molecular biological analysis was performed only at phase VII (OLR 4.80 $g_{VS}\ L^{-1}\ day^{-1}$) when the thermophilic methanogenesis was already established with stability, which is difficult to relate to the different transition metabolic routes mentioned in Section "Degradation routes: OA, Carbohydrate and Alcohol indications" about the fermentation of butyric, lactic and propionic acids. Detman et al. (2018) reported that lactic

acid is oxidized to acetate via acetotrophic methanogenesis, and the main methanogen was *Methanosarcina*. This genus was found in a small amount in sample 2 (~0.3%) (Fig. 8b). However, our results indicated the predominance of the SAO route, with hydrogenotrophic methanogenic organisms (sample 2), implying the possibility that in phases I and II of the reactor operation, there was a presence of acetotrophic microbial communities and SRB (such as *Desulfobulbus*) (Section "Degradation routes: OA, Carbohydrate and Alcohol indications"), which have lactate utilization genes (Detman et al. 2018), but in phase VII there has already been a change in the microbial community due to methanogenesis stability.

The co-AD in the s-CSTR proved to be a suitable alternative for energy recovery from the 2G ethanol production waste coupled to the residues from the 1G process. The reactor operation was effective for providing process parameters for continuous waste treatment and CH_4 production, enabling us to forecast the maximization of residue use within their specific availabilities in the 1G2G sugarcane biorefineries. The upper limit of the OLR applied without collapsing the reactor was 4.80 $g_{VS}\ L^{-1}\ day^{-1}$, maintaining the efficiency of the reactor and the stability of CH_4 production, although being 59% lower than the maximum CH_4 yield obtained at a lower OLR. These findings can guide practical operations in a biorefinery according to their current demand for energy production or maximizing waste treatment, changing the OLR applied to the reactor based on basic empirical science. The composition of substrates played a role in establishing the predominant metabolic routes into the s-CSTR: lactate and butyrate degradation pathways seemed to mostly occur due to the high content of such acids in vinasse and deacetylation liquor. The liquor composition also contributed to keeping the reactor buffer capacity as its alkaline characteristics favored the lower addition of alkalinizing along with the operation: it could result in cost reductions on an industrial scale as such co-substrate could partial or totally replace the alkalizer demand. The change in the microbial community from the seed sludge and from the reactor sludge when CH_4 production stabilized confirmed that the substrates composition and the operational conditions significantly affect the metabolic pathways for CH_4 production. The molecular biological analysis results proved the feasibility of the establishment of thermophilic methanogenic community (~26% *Thermotogae*) from the mesophilic sludge. The specific substrates and reactor conditions directed the co-AD process, selecting new community structures in a way that some members of the community increased while others decreased their abundance relatives.

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Data availability The datasets generated during and/r analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval "This article does not contain any studies with human participants or animals performed by any of the authors."

Conflict of interest The authors declare no competing interests.

References

Alberty RA (1998) Calculation of standard transformed gibbs energies and standard transformed enthalpies of biochemical reactants. Arch Biochem Biophys 353:116–130. https://doi.org/10.1006/abbi.1998.0638

Andrews S (2010) FastQC: a quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc. Accessed July 2020

Angelidaki I, Ellegraard L, Aharing BK (1993) A mathematical model for dynamic simulation of anaerobic digestion of complex substrates: focusing on ammonia inhibition. Biotechnol Bioeng 42:159–166

APHA, AWWA, WEF (2012) Standard methods for the examination of water and wastewater, 22nd edn. APHA, Washington, DC

Badalato N, Guillot A, Sabarly V, Dubois M, Pourette N, Pontoire B, Robert P, Bridier A, Monnet V, Sousa DZ, Durand S, Mazéas L, Buléon A, Bouchez T, Mortha G, Bize A (2017) Whole proteome analyses on *Ruminiclostridium cellulolyticum* show a modulation of the cellulolysis machinery in response to cellulosic materials with subtle differences in chemical and structural properties. PLoS One 12:1–22. https://doi.org/10.1371/journal.pone.0170524

Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, Huttley GA, Gregory Caporaso J (2018) Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6:1–17. https://doi.org/10.1186/s40168-018-0470-z

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, Da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J, Gauglitz

JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Bin Kang K, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciolek T, Kreps J, Langille MGI, Lee J, Ley R, Liu YX, Loftfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV, Metcalf JL, Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E, Rasmussen LB, Rivers A, Robeson MS, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, van der Hooft JJJ, Vargas F, Vázquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R, Caporaso JG (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 37:852–857. https://doi.org/10.1038/s41587-019-0209-9

Boušková A, Dohányos M, Schmidt JE, Angelidaki I (2005) Strategies for changing temperature from mesophilic to thermophilic conditions in anaerobic CSTR reactors treating sewage sludge. Water Res 39:1481–1488. https://doi.org/10.1016/j.watres.2004.12.042

Brenelli LB, Figueiredo FL, Damasio A, Franco TT, Rabelo SC (2020) An integrated approach to obtain xylo-oligosaccharides from sugarcane straw: from lab to pilot scale. Bioresour Technol 313:123637. https://doi.org/10.1016/j.biortech.2020.123637

Cabezas A, de Araujo JC, Callejas C, Galès A, Hamelin J, Marone A, Sousa DZ, Trably E, Etchebehere C (2015) How to use molecular biology tools for the study of the anaerobic digestion process? Rev Environ Sci Biotechnol 14:555–593. https://doi.org/10.1007/s11157-015-9380-8

Chen X, Yuan H, Zou D, Liu Y, Zhu B, Chufo A, Jaffar M, Li X (2015) Improving biomethane yield by controlling fermentation type of acidogenic phase in two-phase anaerobic co-digestion of food waste and rice straw. Chem Eng J 273:254–260. https://doi.org/10.1016/j.cej.2015.03.067

Chen S, Cheng H, Wyckoff KN, He Q (2016) Linkages of *Firmicutes* and *Bacteroidetes* populations to methanogenic process performance. J Ind Microbiol Biotechnol 43:771–781. https://doi.org/10.1007/s10295-016-1760-8

Crable BR, Plugge CM, McInerney MJ, Stams AJM (2011) Formate formation and formate conversion in biological fuels production. Enzyme Res 2011:1–8. https://doi.org/10.4061/2011/532536

de Carvalho DM, de Queiroz JH, Colodette JL (2016) Assessment of alkaline pretreatment for the production of bioethanol from eucalyptus, sugarcane bagasse and sugarcane straw. Ind Crops Prod 94:932–941. https://doi.org/10.1016/j.indcrop.2016.09.069

Detman A, Mielecki D, Pleśniak Ł, Bucha M, Janiga M, Matyasik I, Chojnacka A, Jędrysek MO, Błaszczyk MK, Sikora A (2018) Methane-yielding microbial communities processing lactate-rich substrates: a piece of the anaerobic digestion puzzle. Biotechnol Biofuels 11:1–18. https://doi.org/10.1186/s13068-018-1106-z

Dinsdale RM, Hawkes FR, Hawkes DL (2000) Anaerobic digestion of short chain organic acids in an expanded granular sludge bed reactor. Water Res 34:2433–2438. https://doi.org/10.1016/S0043-1354(99)00430-3

Dolfing J (2015) Protocols for calculating reaction kinetics and thermodynamics. 155–163 . https://doi.org/10.1007/8623_2015_109

Ferraz Júnior ADN, Koyama MH, de Araújo Júnior MM, Zaiat M (2016) Thermophilic anaerobic digestion of raw sugarcane vinasse. Renew Energy 89:245–252. https://doi.org/10.1016/j.renene.2015.11.064

Ferraz Júnior ADN, Pages C, Latrille E, Bernet N, Zaiat M, Trably E (2020) Biogas sequestration from the headspace of a fermentative system enhances hydrogen production rate and yield. Int J Hydrogen Energy 45:11011–11023. https://doi.org/10.1016/j.ijhydene.2020.02.064

FNR (2010) Guide to Biogas: From Production to Use. Fachagentur Nachwachsende Rohstoffe e.V (FNR), Gülzo

Formann S, Hahn A, Janke L, Stinner W, Sträuber H, Logroño W, Nikolausz M (2020) Beyond sugar and ethanol production: value generation opportunities through sugarcane residues. Front Energy Res 8:1–21. https://doi.org/10.3389/fenrg.2020.579577

Franke-Whittle IH, Walter A, Ebner C, Insam H (2014) Investigation into the effect of high concentrations of volatile fatty acids in anaerobic digestion on methanogenic communities. Waste Manag 34:2080–2089. https://doi.org/10.1016/j.wasman.2014.07.020

Fuess LT, de Araújo Júnior MM, Garcia ML, Zaiat M (2017) Designing full-scale biodigestion plants for the treatment of vinasse in sugarcane biorefineries: how phase separation and alkalinization impact biogas and electricity production costs? Chem Eng Res Des 119:209–220. https://doi.org/10.1016/j.cherd.2017.01.023

Fuess LT, Kiyuna LSM, Ferraz ADN, Persinoti GF, Squina FM, Garcia ML, Zaiat M (2017) Thermophilic two-phase anaerobic digestion using an innovative fixed-bed reactor for enhanced organic matter removal and bioenergy recovery from sugarcane vinasse. Appl Energy 189:480–491. https://doi.org/10.1016/j.apenergy.2016.12.071

Fuess LT, Ferraz ADN, Machado CB, Zaiat M (2018) Temporal dynamics and metabolic correlation between lactate-producing and hydrogen-producing bacteria in sugarcane vinasse dark fermentation: the key role of lactate. Bioresour Technol 247:426–433. https://doi.org/10.1016/j.biortech.2017.09.121

Fuess LT, dos Santos GM, Delforno TP, de Souza Moraes B, da Silva AJ (2020) Biochemical butyrate production via dark fermentation as an energetically efficient alternative management approach for vinasse in sugarcane biorefineries. Renew Energy 158:3–12. https://doi.org/10.1016/j.renene.2020.05.063

Golkowska K, Greger M (2013) Anaerobic digestion of maize and cellulose under thermophilic and mesophilic conditions–a comparative study. Biomass and Bioenerg 56:545–554. https://doi.org/10.1016/j.biombioe.2013.05.029

Guo X, Wang C, Sun F, Zhu W, Wu W (2014) A comparison of microbial characteristics between the thermophilic and mesophilic anaerobic digesters exposed to elevated food waste loadings. Bioresour Technol 152:420–428. https://doi.org/10.1016/j.biortech.2013.11.012

Hagos K, Zong J, Li D, Liu C, Lu X (2017) Anaerobic co-digestion process for biogas production: progress, challenges and perspectives. Renew Sustain Energy Rev

Hattori S (2008) Syntrophic acetate-oxidizing microbes in methanogenic environments. Microbes Environ 23:118–127. https://doi.org/10.1264/jsme2.23.118

Hoffstadt K, Pohen GD, Dicke MD, Paulsen S, Krafft S, Zang JW, Da Fonseca-Zang WA, Leite A, Kuperjans I (2020) Challenges and prospects of biogas from energy cane as supplement to bioethanol production. Agronomy 10:1–16. https://doi.org/10.3390/agronomy10060821

Janke L, Leite A, Nikolausz M, Schmidt T, Liebetrau J, Nelles M, Stinner W (2015) Biogas production from sugarcane waste: assessment on kinetic challenges for process designing. Int J Mol Sci 16:20685–20703. https://doi.org/10.3390/ijms160920685

Janke L, Leite AF, Nikolausz M, Radetski CM, Nelles M, Stinner W (2016) Comparison of start-up strategies and process performance during semi-continuous anaerobic digestion of sugarcane filter cake co-digested with bagasse. Waste Manag 48:199–208. https://doi.org/10.1016/j.wasman.2015.11.007

Janke L, Weinrich S, Leite AF, Sträuber H, Radetski CM, Nikolausz M, Nelles M, Stinner W (2018) Year-round biogas production in sugarcane biorefineries: process stability, optimization and

performance of a two-stage reactor system. Energy Convers Manag 168:188–199. https://doi.org/10.1016/j.enconman.2018.04.101

Kataoka N, Miya A, Kiriyama K (1997) Studies on hydrogen production by continuous culture system of hydrogen-producing anaerobic bacteria. Water Sci Technol 36:41–47. https://doi.org/10.1016/S0273-1223(97)00505-2

Kim E, Lee J, Han G, Hwang S (2018) Comprehensive analysis of microbial communities in full-scale mesophilic and thermophilic anaerobic digesters treating food waste-recycling wastewater. Bioresour Technol 259:442–450. https://doi.org/10.1016/j.biortech.2018.03.079

Krzysztof Ziemiński (2012) Methane fermentation process as anaerobic digestion of biomass: transformations, stages and microorganisms. African J Biotechnol 11:4127–4139. https://doi.org/10.5897/ajbx11.054

Lata K, Rajeshwari KV, Pant DC, Kishore VVN (2002) Volatile fatty acid production during anaerobic mesophilic digestion of tea and vegetable market wastes. World J Microbiol Biotechnol 18:589–592. https://doi.org/10.1023/A:1016314903817

Li J, Zheng G, He J, Chang S, Qin Z (2009) Hydrogen-producing capability of anaerobic activated sludge in three types of fermentations in a continuous stirred-tank reactor. Biotechnol Adv 27:573–577. https://doi.org/10.1016/j.biotechadv.2009.04.007

Li T, Mazéas L, Sghir A, Leblon G, Bouchez T (2009) Insights into networks of functional microbes catalysing methanization of cellulose under mesophilic conditions. Environ Microbiol 11:889–904. https://doi.org/10.1111/j.1462-2920.2008.01810.x

Lin C-Y, Wu C-C, Hung C-H (2008) Temperature effects on fermentative hydrogen production from xylose using mixed anaerobic cultures. Int J Hydrogen Energy 33:43–50. https://doi.org/10.1016/j.ijhydene.2007.09.001

Lin R, Cheng J, Ding L, Murphy JD (2018) Improved efficiency of anaerobic digestion through direct interspecies electron transfer at mesophilic and thermophilic temperature ranges. Chem Eng J 350:681–691. https://doi.org/10.1016/j.cej.2018.05.173

Marchaim U, Krause C (1993) Propionic to acetic acid ratios in overloaded anaerobic digestion. Bioresour Technol 43:195–203. https://doi.org/10.1016/0960-8524(93)90031-6

Martín-González L, Font X, Vicent T (2013) Alkalinity ratios to identify process imbalances in anaerobic digesters treating source-sorted organic fraction of municipal wastes. Biochem Eng J 76:1–5. https://doi.org/10.1016/j.bej.2013.03.016

McMurdie PJ, Holmes S (2013) Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8:1–11. https://doi.org/10.1371/journal.pone.0061217

Moraes BS, Zaiat M, Bonomi A (2015) Anaerobic digestion of vinasse from sugarcane ethanol production in Brazil: challenges and perspectives. Renew Sustain Energy Rev 44:888–903. https://doi.org/10.1016/j.rser.2015.01.023

Moraes BS, Mary dos Santos G, Palladino Delforno T, Tadeu Fuess L, José da Silva A (2019) Enriched microbial consortia for dark fermentation of sugarcane vinasse towards value-added short-chain organic acids and alcohol production. J Biosci Bioeng 127:594–601. https://doi.org/10.1016/j.jbiosc.2018.10.008

Mulat DG, Horn SJ (2018) Chapter 14: biogas production from lignin via anaerobic digestion. RSC Energy Environ Ser 2018-Janua:391–412 . https://doi.org/10.1039/9781788010351-00391

Nakasu PYS, Clarke CJ, Rabelo SC, Costa AC, Brandt-Talbot A, Hallett JP (2020) Interplay of acid-base ratio and recycling on the pretreatment performance of the protic ionic liquid monoethanolammonium acetate. ACS Sustain Chem Eng. https://doi.org/10.1021/acssuschemeng.0c01311

Peng X, Börner RA, Nges IA, Liu J (2014) Impact of bioaugmentation on biochemical methane potential for wheat straw with addition of *Clostridium cellulolyticum*. Bioresour Technol 152:567–571. https://doi.org/10.1016/j.biortech.2013.11.067

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41:590–596. https://doi.org/10.1093/nar/gks1219

Rabelo SC, Carrere H, Maciel Filho R, Costa AC (2011) Production of bioethanol, methane and heat from sugarcane bagasse in a biorefinery concept. Bioresour Technol 102:7887–7895. https://doi.org/10.1016/j.biortech.2011.05.081

Ren NQ, Chua H, Chan SY, Tsang YF, Wang YJ, Sin N (2007) Assessing optimal fermentation type for bio-hydrogen production in continuous-flow acidogenic reactors. Bioresour Technol 98:1774–1780. https://doi.org/10.1016/j.biortech.2006.07.026

Ripley LE, Boyle WC, Converse JC (1986) Improved alkalimetric monitoring for anaerobic digestion of high-strength waste. J Water Pollut Control Fed 58:1–6

Rognes T, Flouri T, Nichols B, Quince C, Mahé F (2016) VSEARCH: A versatile open source tool for metagenomics. PeerJ 2016:1–22. https://doi.org/10.7717/peerj.2584

Santana Junior AE, Duda RM, de Oliveira RA (2019) Improving the energy balance of ethanol industry with methane production from vinasse and molasses in two-stage anaerobic reactors. J Clean Prod 238:117577. https://doi.org/10.1016/j.jclepro.2019.07.052

Scharer JM, Moo-young M (1979) Methane generation by anaerobic digestion of cellulose-containing wastes. Advances in biochemical engineering, vol 11. Springer, Berlin Heidelberg, Berlin, Heidelberg, pp 85–101

Schnürer A, Zellner G, Svensson BH (1999) Mesophilic syntrophic acetate oxidation during methane formation in biogas reactors. FEMS Microbiol Ecol 29:249–261. https://doi.org/10.1016/S0168-6496(99)00016-1

Souza ME, Fuzaro G, Polegato AR (1992) Thermophilic anaerobic digestion of vinasse in pilot plant UASB reactor. Water Sci Technol 25:213–222. https://doi.org/10.2166/wst.1992.0153

Team RC (2013) "R: A language and environment for statistical computing."

Tian Z, Zhang Y, Li Y, Chi Y, Yang M (2015) Rapid establishment of thermophilic anaerobic microbial community during the one-step startup of thermophilic anaerobic digestion from a mesophilic digester. Water Res 69:9–19. https://doi.org/10.1016/j.watres.2014.11.001

Vanwonterghem I, Jensen PD, Rabaey K, Tyson GW (2015) Temperature and solids retention time control microbial population dynamics and volatile fatty acid production in replicated anaerobic digesters. Sci Rep 5:1–8. https://doi.org/10.1038/srep08496

Volpi MPC, Brenelli LB, Mockaitis G, Rabelo SC, Franco TT, Moraes BS (2021) Use of lignocellulosic residue from second-generation ethanol production to enhance methane production through co-digestion. Bioenergy Res. https://doi.org/10.1007/s12155-021-10293-1

Volpi MPC, Fuess LT, Moraes BS (2021) Anaerobic co-digestion of residues in 1G2G sugarcane biorefineries for enhanced electricity and biomethane production. Bioresour Technol 330:124999. https://doi.org/10.1016/j.biortech.2021.124999

Vongvichianku C, Jorawan D, Watsa K (2017) Relationship between pH Oxidation Reduction Potential (ORP) and Biogas Production in Mesophilic Screw Anaerobic Digester. Energy Procedia 138877-882. https://doi.org/10.1016/j.egypro.2017.10.113

Wang Q, Kuninobu M, Ogawa HI, Kato Y (1999) Degradation of volatile fatty acids in highly efficient anaerobic digestion. Biomass and Bioenerg 16:407–416. https://doi.org/10.1016/S0961-9534(99)00016-1

Wang L, Zhou Q, Li FT (2006) Avoiding propionic acid accumulation in the anaerobic process for biohydrogen production. Biomass and Bioenerg 30:177–182. https://doi.org/10.1016/j.biombioe.2005.11.010

Wang Y, Zhang Y, Wang J, Meng L (2009) Effects of volatile fatty acid concentrations on methane yield and methanogenic bacteria. Biomass Bioenerg. https://doi.org/10.1016/j.biombioe.2009.01.007

Wang R, Li Y, Chen W, Zou J, Chen Y (2016) Phosphate release involving PAOs activity during anaerobic fermentation of EBPR sludge and the extension of ADM1. Chem Eng J 287:436–447. https://doi.org/10.1016/j.cej.2015.10.110

Wang P, Wang H, Qiu Y, Ren L, Jiang B (2018) Microbial characteristics in anaerobic digestion process of food waste for methane production–a review. Bioresour Technol 248:29–36. https://doi.org/10.1016/j.biortech.2017.06.152

Weijma J, Stams AJM (1999) Methanol conversion in high-rate anaerobic reactors. Water Sci Technol 44(8):7–14. https://doi.org/10.2166/wst.2001.0452

Wiegant WM, Hennink M, Lettinga G (1986) Separation of the propionate degradation to improve the efficiency of thermophilic anaerobic treatment of acidified wastewaters. Water Res 20:517–524. https://doi.org/10.1016/0043-1354(86)90202-2

Wu X, Tian Z, Lv Z, Chen Z, Liu Y, Yong X, Zhou J, Xie X, Jia H, Wei P (2020) Effects of copper salts on performance, antibiotic resistance genes, and microbial community during thermophilic anaerobic digestion of swine manure. Bioresour Technol 300:122728. https://doi.org/10.1016/j.biortech.2019.122728

Yu Q, Tian Z, Liu J, Zhou J, Yan Z, Yong X, Jia H, Wu X, Wei P (2018) Biogas production and microbial community dynamics during the anaerobic digestion of rice straw at 39–50 °C: a pilot study. Energy Fuels 32:5157–5163. https://doi.org/10.1021/acs.energyfuels.7b04042

Zaparolli D (2019) Tapping more energy from sugarcane. In: Pesqui. FAPESP. Avaliable: https://revistapesquisa.fapesp.br/en/2020/02/19/tapping-more-energy-from-sugarcane/. Accessed December 2020

Zhang B, Cai W, He P (2007) Influence of lactic acid on the two-phase anaerobic digestion of kitchen wastes. J Environ Sci 19:244–249

Zhao J, Westerholm M, Qiao W, Yin D, Bi S, Jiang M, Dong R (2018) Impact of temperature and substrate concentration on degradation rates of acetate, propionate and hydrogen and their links to microbial community structure. Bioresour Technol 256:44–52. https://doi.org/10.1016/j.biortech.2018.01.150

Ziganshin AM, Liebetrau J, Pröter J, Kleinsteuber S (2013) Microbial community structure and dynamics during anaerobic digestion of various agricultural waste materials. Appl Microbiol Biotechnol 97:5161–5174. https://doi.org/10.1007/s00253-013-4867-0

5.3 **PAPER 3**

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Anaerobic co-digestion of residues in 1G2G sugarcane biorefineries for enhanced electricity and biomethane production

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HIGHLIGHTS

- Bioenergy production from sugarcane-derived residue co-digestion was assessed.
- Year-round operation of AD plants would offset limited 1G vinasse availability.
- Co-digestion provided over 400%-increase in bioenergy production (22.3 *vs.* 5.0 MW).
- Electricity (36 MW) and bioCH_4 (12.65×10^6 m^3) could be efficiently co-produced.
- Improved energy production from sugarcane depends on more efficient 2G technologies.

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ABSTRACT

The energy potential of residue-derived biogas via electricity and biomethane production was assessed in an integrated 1G2G sugarcane biorefinery concept. The mono-digestion of 1G-vinasse (1G-VN) was compared with different co-digestion systems, namely, 1G-VN + filter cake (FC) + deacetylation liquor (DL) in the season and FC + DL in the off-season. Gross energy output values and the resulting sugarcane use efficiency were also assessed in different biorefinery schemes. Electricity production from 1G to VN (5.0 MW) could be increased by over 400% through its co-digestion with FC and DL (22.3 MW). Alternatively, biomethane could fully supply the diesel-powered fleet (1.8×10^6 Nm^3 $month^{-1}$) of a sugarcane plant processing 10 million tons of sugarcane per harvest, and the surplus biogas could flexibly provide 36 MW of extra electricity. Biomethane could enhance the energy output of 1G2G sugarcane biorefineries by 15%. However, 2G processes still require marked improvements to maximize energy production from sugarcane.

1. Introduction

Biogas production through the anaerobic digestion (AD) of organic wastes, in particular the ones from agro-industrial activities, is very promising for the bioeconomy and bioenergy, because a wide range of applications, such as heat and power generation, may be exploited (Awosusi et al., 2020). Within this context, anaerobic co-digestion emerged to improve biogas production. Co-digestion is characterized by the AD of two or more substrates, which is an option to both overcome disadvantages of mono-digestion, mainly concerning the balance of nutrients, and improve the economic viability of AD plants by enhancing methane (CH_4) production (Hagos et al., 2017).

The application of AD within the sugarcane biorefinery context is a well-documented approach, based on the application of first generation vinasse (1G-VN)-derived CH_4 in combustion engines to generate electricity and thermal energy (Fuess et al, 2018; Moraes et al., 2014). Despite the great effort directed to the management of 1G-VN, additional residual streams resulting from sugarcane processing still require proper destination, which could be attained within the AD context targeting enhanced bioenergy production. Both the first (1G) and second (2G) generation approaches may be potentially exploited in this regard (Christofoletti et al., 2013; Ferraz Jr. et al., 2016; Moraes et al., 2015b),

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in order to address historical gaps regarding the energetic use of residues in sugarcane processing plants.

Considering 1G processing chains, filter cake (FC) is another relevant residue potentially used in AD. FC is generated in the steps of juice clarification and filtration (González et al., 2017), comprising a nutrient-rich residue, mainly in terms of calcium, nitrogen, phosphorus, magnesium, iron, manganese, and zinc (Janke et al., 2015, (Janke et al., 2016a)). Although the fibrous nature of FC limits its direct AD (González et al., 2017), it may be used to nutritionally supplement 1G-VN to obtain stable AD systems, as 1G-VN alone still presents some unregulated levels of micro and macronutrients that can impair methanogenesis (Moraes et al., 2015a). For instance, González et al. (2017) reported on beneficial impacts of the co-digestion of 1G-VN and FC in batch- and CSTR-based experiments. Blending 1G-VN with FC (50:50% on a chemical oxygen demand – COD – basis) increased CH_4 production by 13% relative to the mono-digestion of FC in batch systems. A 64%-increase in CH_4 evolution was further obtained in continuous experiments, considering a 1G-VN: FC proportion of 75:25 (%).

Regarding 2G processes, field experiences are still little, because specific aspects (e.g. the use of enzymes in pretreatment and hydrolysis steps) still require defining cost-effective approaches. This naturally limits studying the use of 2G residues within the AD context in comparison with 1G-based scenarios. Deacetylation liquor (DL), which results from the pretreatment of sugarcane trash, comprises an unexploited 2G residual stream recently used as a substrate in AD (Volpi et al., 2021a). The co-digestion of DL, FC, and 1G-VN has been pointed out as an efficient approach to improve CH_4 production from 1G to VN (Volpi et al., 2021a). Experiment-based studies are imperative to show the technical suitability of AD within the sugarcane biorefinery context, indicating the most efficient directions to achieve integrated management of residual streams in 1G2G plants. However, a broad assessment of AD requires more than lab-scale investigations, also demanding proper energetic investigations to guide the most efficient pathways to exploit such residual streams, which enables to quantify potential uses of the bioenergy recovered.

The energetic assessment of AD in sugarcane biorefineries has been massively assessed in recent years in the case of 1G-VN. Approaches for the energetic exploitation of 1G-VN-derived biogas are many, usually focusing on the cogeneration of electricity and heat (Fuess et al., 2018). Moraes et al. (2014) assessed the application of 1G-VN-derived biogas in boilers for the cogeneration of energy, indicating that 12% of the energy produced from bagasse burning could be replaced by that obtained from biogas. These authors highlighted the possibility to release bagasse from boilers to 2G ethanol production, integrating a 1G2G sugarcane biorefinery with increased ethanol yield and enhanced bioenergy recovery from residues and by-products.

Concurrently to electricity generation, biomethane ($bioCH_4$) production is a strategy to maximize the energetic exploitation of biogas in sugarcane processing plants (Junqueira et al., 2016; Fuess and Zaiat, 2018). $BioCH_4$ is the biofuel obtained from biogas purification (CH_4 fraction > 90%), which gives biogas heating value and composition equivalent to those of natural gas. A 16%-increase in natural gas consumption is expected by the European Union by 2030, which reinforces the importance of evaluating the contribution of $bioCH_4$ as a potential complement/ substitute to natural gas (Bordelanne et al., 2011). Because $bioCH_4$ directly replaces natural gas, no modifications in installations and equipment are required, which allows its prompt use in automobiles and trucks. The potential production of $bioCH_4$ within the sugarcane biorefinery context was also assessed exclusively for 1G-VN-fed AD plants (Junqueira et al., 2016; Fuess and Zaiat, 2018), with results highlighting economic advantages over electricity production. The replacement of diesel is a key factor to minimize economic and primarily environmental costs in sugarcane processing.

Practical experiences and scenarization-based results on the application of AD to exploit sugarcane-derived substrates reveal numerous energetic and environmental gains. However, research on the case of residue co-digestion is still required, mainly when considering the future implementation of 1G2G processing plants. While some experimental studies on the co-digestion of sugarcane-derived residues have already been presented (Adarme et al., 2019; González et al., 2017), preliminary energetic assessments of this approach are required to simultaneously indicate the potential full-scale energetic potential of AD, with emphasis on 2G residues. This study aimed to assess the energetic potential of the biogas produced in the co-digestion of residues from 1G2G sugarcane processing chains. The proposed scenarios innovate by including trash-derived DL as a potential substrate for biodigestion in integrated 1G2G schemes, providing details on the production of both electricity and $bioCH_4$ as strategies to exploit biogas streams in sugarcane biorefineries. A detailed study of the efficiency associated with the energy use of sugarcane was also presented, from which energetic bottle necks and prospects for the sucro-energetic industry were discussed.

2. Material and methods

2.1. 1G2G sugarcane biorefinery

Scenarization studies were based on a medium-to-large size integrated 1G2G ethanol-producing plant with a processing capacity of 4×10^6 tons of sugarcane (TC) per season (232 d) and the excess trash and bagasse in the off-season (133 d) (Junqueira et al., 2016). Hence, the considered biorefinery operates as a 1G-scheme in the season and a 2G-scheme in the off-season. The entire volume of 1G-VN produced in the season would be biodigested. The mass flow of FC directed to co-digestion was calculated based on a 20%-fraction of volatile solids (VS), following previous experimental results (Section 2.2). A similar approach was used to define the flow of DL, in which 10% of the VS content co-digested was considered. For the off-season, the entire volume of 2G-VN was considered to be biodigested in a separate scenario, i.e., mono-digestion. Compositional and AD-related performance data regarding 2G-VN were obtained from the literature (Rodriguez et al., 2019), i.e., no previous AD-based experiments were conducted. Fig. 1 presents the flowchart of the integrated 1G2G sugarcane biorefinery assessed, whilst Table 1 details input data used in calculations.

2.2. Performance data for co-digestion

Input data for the energy potential evaluation were obtained from experimental results in bench-scale semi-continuous CSTR system processing VN, FC (both from 1G ethanol production), and DL (from 2G ethanol production), following two co-digestion approaches (Volpi et al., 2021b). First, the three residues were co-digested in a VS-based proportion of 70:20:10 (VN:FC:DL; %), which was defined according to residue availability. In the second approach, only FC (50%) and DL (50%) were blended. Data regarding the mono-digestion of 1G-VN were also obtained elsewhere ()(Volpi et al., 2021a), considering experimental results. COD values in 1G-VN and DL were 28,660 and 32,920 mg L^{-1}, respectively, whilst VS concentrations were 0.018 g L^{-1} (1G-VN), 0.103 g L^{-1} (DL), and 0.192 g kg^{-1} (FC). Performance data regarding AD systems are summarized in Table 2.

2.3. Energetic assessment methodology

Three scenarios based on experimental results (Section 2.2) were considered for assessing the energy potential of biodigestion within the integrated 1G2G sugarcane biorefinery context: mono-digestion of 1G-VN in the season (base scenario); co-digestion of 1G-VN, FC, and DL in the season (scenario a); and, co-digestion of FC and DL in the off-season (scenario b). Because experiments with 2G-VN were not carried out due to substrate unavailability in the sugarcane mill, the mono-digestion of 2G-VN in the off-season (scenario c) was based on theoretical data. In all cases, calculations were based on single-stage AD, i.e., no phase-separation (which includes substrate hydrolysis/fermentation in a

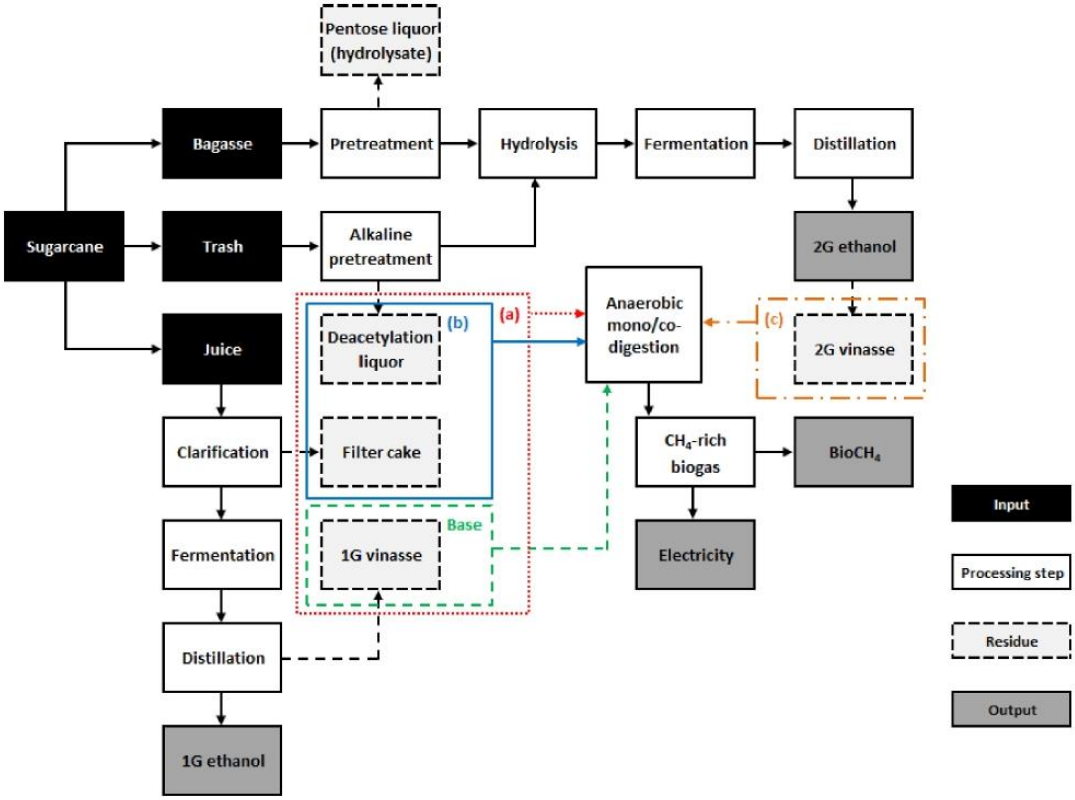

Fig. 1. Flowchart of the integrated 1G2G sugarcane biorefinery highlighting the residual streams and the proposed scenarios for the energetic assessment in mono/co-digestion plant.

Table 1
Main input data describing the integrated 1G2G biorefinery and material flows for the energetic assessment.

Input Data	Values	References
Integrated sugarcane biorefinery (1G2G)		
Sugarcane milling capacity-season (TC)	4×10^6	Junqueira et al. (2016)
Bagasse generation (kg TC^{-1})	276	Rodriguez et al. (2019)
Straw generation (kg TC^{-1})	140	Rodriguez et al. (2019)
Season period (d)	232	–
Off-season period (d)	133	–
1G-ethanol production (L TC^{-1})	85.4	Junqueira et al. (2016)
2G-ethanol production (L TC^{-1})	22.1	Junqueira et al. (2016)
1G-VN total production (season; m^3) [a]	341.6×10^4	Adapted from Rodriguez et al. (2019)
FC total production (season; kg) [b]	120×10^6	Adapted from Janke et al. (2016b)
DL total production (season; m^3) [c]	2.5×10^6	Adapted from Brenelli et al. (2020)
2G-VN total production (off-season; m^3) [d]	476.24×10^3	Adapted from Rodriguez et al. (2019)
Material flow rates for mono/co-digestion		
1G-VN volumetric flow rate (m^3 d^{-1}) [a]	9187.5	Fuess et al. (2017)
FC mass flow rate (kg d^{-1}) [e]	2625	Experimental data
DL volumetric flow rate (m^3 d^{-1}) [e]	1312.5	Experimental data
2G-VN volumetric flow rate (m^3 d^{-1}) [d]	3580.8	Adapted from Rodriguez et al. (2019)

Notes: [a]Considering the specific generation of 1G-VN of 10 L L^{-1} ethanol , [b]Considering the specific FC generation of 30 kg TC^{-1}, [c]Considering the specific DL generation of 4.47 L kg^{-1}straw, [d]Considering the specific generation of 2G-VN of 149.20 m^3 h^{-1}, [e]Considering 20% of VS of FC and 10% of VS of DL.

Table 2
Experimental (1G-VN, FC, DL) and estimated (2G-VN) performance data for the mono- and co-digestion processes considered in the energetic assessment.

Input Data	Values
VS removal efficiency (%)	80.0
Reactor volumetric flow rate of 1G-VN (m^3 d^{-1}) – base-scenario[a]	9187.5
Reactor mass flow rate (1G-VN + FC + DL) (kg d^{-1}) – scenario a[b]	13125
Reactor mass flow rate (FC + DL) (kg d^{-1}) – scenario b[a]	3937.5
CH_4 yield (NL-CH_4 gVS^{-1}) – base-scenario[a]	0.232
CH_4 yield (NL-CH_4 gVS^{-1}) – scenario a[b]	0.233
CH_4 yield (NL-CH_4 gVS^{-1}) – scenario b[a]	0.395
CH_4 yield (NL-CH_4 $gCOD^{-1}$) – scenario c[c]	0.133
CH_4 calorific value (MJ $Nm^{-3}CH_4$)	35.8
VS concentration (1G-VN) (g L^{-1}) – base-scenario[a]	18.2
VS concentration (1G-VN + FC + DL) (g L^{-1}) – scenario a[b]	57.55
VS concentration (FC + DL) (g L^{-1}) – scenario b[a]	18.4
COD of 2G-VN (kg m^{-3}) – scenario c[d]	92.30

Notes: [a]Data obtained experimentally from (Volpi et al., 2021a) [b]Data obtainded experimentally (Volpi et al., 2021b) considering that 70% of vinasse VS were added to the reactor, 20% of VS of FC and 10% of VS of DL, [c]Estimated considering the COD of 2G-VN equivalent to 92.30 kg m^{-3}, [d]Data from Rodriguez et al. (2019).

separate processing unit prior to methanogenesis) was considered. Hence, CH_4-rich biogas was the only product resulting from residue conversion. Fig. 1 also presents details of scenario description.

Power generation (MJ d^{-1}) from CH_4 production was calculated according to Eq. (1) adapted from Moraes et al. (2014), in which CP_{CH4} is the calorific power of CH_4 (MJ $Nm^{-3}CH_4$), $F_{co\text{-}AD}$ is the material flow rate

applied to mono/co-digestion systems (kg d^{-1} or m^3 d^{-1}), ER_{VS} is the removal efficiency of VS, $VS_{co\text{-}AD}$ is the amount of VS available for biodigestion (kgVS kg^{-1} or kgVS m^{-3}) and Y_{CH4} is the CH_4 yield (Nm^3CH_4 $kgVS^{-1}$).

$$P = CP_{CH4} \times F_{co-AD} \times ER_{VS} \times VS_{co-AD} \times Y_{CH4} \quad (1)$$

Power generation values calculated from Eq. (1) were further used to estimate the production of both electricity (in internal combustion engine – ICE – sets) and bioCH_4 (as an alternative vehicle fuel). Regarding electricity generation, a stationary ICE with an electric conversion efficiency of 38% (Caterpillar, Inc., model DM 5234, 50 Hz, 1500 rpm, 400 V; Peoria, IL, USA) was considered, as reported elsewhere (Moraes et al., 2014). In this case, a relatively conservative approach was selected to offset eventual uncertainties in full-scale biogas production/conversion.

BioCH_4 production was estimated considering a 2%-loss of CH_4 during biogas upgrading, reflecting the worst scenario within the range (1–2%) usually observed in upgrading technologies (Muñoz et al., 2015). BioCH_4 production and the corresponding energy production (MW) were calculated for all scenarios. The energetic reach of bioCH_4 production was compared with the capability to replace diesel in heavy trucks (rodotrens), representing the highest demand potentially observed for bioCH_4 in biorefineries. Assuming that bioCH_4 could achieve the minimum CH_4 content (96.5%) required by the National Agency for Petroleum, Natural Gas and Biofuels (ANP) to efficiently function as a fuel (Leme and Seabra, 2017), an autonomy of 1.6 km m^{-3} was assumed (Ornelas-Ferreira et al., 2020).

2.4. Conversion efficiency evaluation: Energy indicators

The energetic reach of the bioenergy potentially produced from biogas (considering both electricity and bioCH_4) was assessed through applying two indicators, namely, the gross energy output (GEO) and the sugarcane use efficiency (SUE). GEO (MJ TC^{-1}; Eq. (2)) is the sum of all energy outputs (ethanol and electricity/thermal energy or bioCH_4) extracted from sugarcane within a given processing chain, whilst SUE (dimensionless; Eq. (3)) measures the energy recovery efficiency associated with the conversion of sugarcane. This combined approach offsets limitations in the calculation of energy balances for 2G-based processes, because the availability of energy inputs largely vary according to process specificities, such as the type of pretreatment applied to obtain fermentable sugars and the level of integration used to manage thermal energy within the plant. In Eq. (2) and (3) the terms $P_{1G\text{-}EtOH}$, $P_{2G\text{-}EtOH}$, CP_{EtOH}, $El_{bagasse}$, P_{CH4}, CP_{CH4}, η and SEC are, respectively, the production of 1G (85.4 L TC^{-1}; Table 1) and 2G (22.1 L TC^{-1}; Table 1) ethanol, the calorific power of ethanol (21.4 MJ L^{-1}; Manochio et al., 2017), the thermoelectricity produced from bagasse (46.2 kWh TC^{-1} or 166.32 MJ TC^{-1}; Moraes et al., 2014), the production of CH_4 following the energetic scenarios described in Section 2.3 (Nm^3CH_4 TC^{-1}), the calorific power of CH_4 (35.8 MJ $Nm^{-3}CH_4$; Table 2), the energetic efficiency of the biogas application (dimensionless), and the sugarcane energy content (1.72×10^6 kcal TC^{-1} or 7188 MJ TC^{-1}; Moreira et al., 2019). Values for η were assumed as follows: [i] 0.38 considering a conservative electricity generation (as described in Section 2.3); [ii] 0.43 considering an optimistic electricity generation (ICE model J620 GS-F12, GE JenbacherGmbH & Co. OHG, Jenbach, Austria); and, [iii] 0.98 for bioCH_4 production, considering a 2%-loss in the upgrading step (Muñoz et al., 2015). In the particular case of optimistic electricity production, thermal energy recovery from exhaust gas was also included, using an efficiency factor of 0.515. Hence, the global conversion efficiency reached 0.945 in this case, which includes both electric and thermal energy.

$$GEO = P_{1G-EtOH} \times CP_{EtOH} + P_{2G-EtOH} \times CP_{EtOH} + El_{bagasse} + P_{CH4} \times CP_{CH4} \times \eta \quad (2)$$

$$SUE = \frac{GEO}{SEC} \quad (3)$$

The GEO and the associated SUE were assessed in four reference biorefinery schemes (BS1–BS4), as described in Table 3. For each biorefinery scheme the production of electricity (conservative and optimistic) or bioCH_4 from biogas was considered, totaling 12 calculation responses. The nomenclatures "el-cons", "el-opt" and "bioCH_4" further associated to each scheme (Section 3.3) refer to the production of electricity (conservative and optimistic approaches) and bioCH_4, respectively.

3. Results and discussion

3.1. Electricity production potential

Details of CH_4 production and the potential electric power in all four scenarios assessed are presented in Table 4. CH_4 production during the season for co-digestion (32.67×10^6 Nm^3CH_4; scenario a) was considerably higher than that obtained in the off-season (scenarios b and c, totaling 7.71×10^6 Nm^3CH_4), because of the large contribution of 1G-VN in association with the enhancement of the biological process provided by the co-substrates: the mono-digestion of 1G-VN (7.27×10^6 Nm^3CH_4; base scenario) resulted in lower CH_4 production compared with the total amount for the off-season. Consequently, the total production of electricity in scenario a was proportionally higher than that of scenarios b and c, i.e., 30.85 *vs.* 7.27 kWh TC^{-1} (Table 4). The association of proposed scenarios (a + b or a + b + c), compared to the base one, highlight the possibility to maintain both the anaerobic system and the biogas-derived energy production year-round, which may eliminate periods of re-startup by continuously maintaining active microbial communities. The maintenance of 1G-VN-fed reactors during the off-season has been pointed as one of the imperative factors to achieve efficient full-scale AD-derived energy generation in sugarcane biorefineries (Janke et al., 2016a; Santana Jr. et al., 2019). Because highly-efficient energy-generating systems depend on high biogas (CH_4) flowrates, re-startup periods will directly decrease the energetic exploitation of 1G-VN by requiring the application of lower organic loading rates as a strategy to prevent system acidification. Therefore, co-digestion systems may be the most immediate approach to eliminate the forced interruption of anaerobic systems due to substrate unavailability.

Although only FC and DL were used experimentally in the co-digestion reactor, considering the off-season, 2G-VN could also be used as co-substrate in that system. Scenario c revealed that the mono-digestion of 2G-VN could provide a 35% higher electricity production per ton of sugarcane in the off-season compared to scenario b, i.e., the

Table 3
Biorefinery schemes used to assess the GEO and the SUE in diversified sugarcane processing chains.

Biorefinery scheme	Description	Reference scenario (Section 2.3) for biogas generation
BS1[a]	1G-biorefinery with AD of 1G-VN (season)	Base scenario
BS2	1G2G-biorefinery with AD of 1G-VN (season) and 2G-VN (off-season)	Base scenario (1G-VN) and scenario c (2G-VN)
BS3	1G2G-biorefinery with co-digestion of 1G-VN, DL and FC (season) and co-digestion of DL and FC (off-season)	Scenarios a (1G-VN + DL + FC) and b (DL + FC)
BS4	1G2G-biorefinery with co-digestion of 1G-VN, DL and FC (season), co-digestion of DL and FC and AD of 2G-VN (off-season)	Scenarios a (1G-VN + DL + FC), b (DL + FC) and c (2G-VN)

Note: [a]Includes the production of (thermos)electricity from bagasse burning in boilers

Table 4
CH_4 production and potential electricity generation in each scenario.

Parameter	Base-scenario	Scenario a	Scenario b	Scenario c
Total CH_4 production (Nm^3CH_4)[a]	7.27×10^6	32.67×10^6	3.05×10^6	4.66×10^6
Potential power generated (MJ d^{-1})	112.32×10^4	504.05×10^4	81.91×10^4	125.48×10^4
Installed capacity (MW)	13.10	58.33	9.48	14.52
Electricity production (kWh)	27.50×10^6	123.43×10^6	11.50×10^6	17.62×10^6
Electricity production per ton of sugarcane (kWh TC^{-1})	6.87	30.85	2.87	4.40

Note: [a]Per period, i.e., season (232 d; base scenario and scenario a) or off-season (133 d; scenarios b and c).

co-digestion of FC and DL. The values of scenario c are very close to those experimentally obtained for 1G-VN mono-digestion (base scenario), which indicates that applying 2G-VN as a co-substrate along with DL and FC may also increase electricity production in a co-digestion system, because this approach normally enhances the CH_4 yield when compared to the mono-digestion of substrates (Adarme et al., 2019). However, experimentations with 2G-VN as co-substrate must be performed to corroborate this indication, because the efficiency of the biological process is strictly related to the substrate composition and its specific compounds interaction with the microorganisms: some inhibitors and nutrients may be accounted as organic material in the COD analysis but negatively affect the CH_4 yield (Moraes et al., 2015b).

Considering the season, the energetic performance of scenario a exceeded that of the base scenario by over 4-fold, i.e., installed capacities of 58.33 and 13.10 MW (Table 4). This particular result highlights the potential of co-digestion to markedly increase the energetic reach of AD in sugarcane biorefineries, in addition to maintaining the year-round operation through further processing residues (e.g. DL + FC as assessed in scenario b) during the off-season. Details of this sequential approach are discussed in Section 3.3. Proper consideration should only be directed to define adequate reactor configurations, as the higher solid content in the case of co-digestion (18.2 *vs*. 57.55 g L^{-1}; Table 2) would limit the use of some high-rate reactor configurations, such as the sludge blanket (Del Nery et al., 2018; Ferraz Jr. et al., 2016; Santana Junior et al., 2019) and fixed-film (de Aquino et al., 2017; Fuess et al., 2017) systems. Overall, high solid content hampers the granulation in sludge blanket reactors (van Lier et al., 2015), whilst it may trigger clogging-related operational problems in fixed-film systems (Fuess et al., 2017). CSTR systems could be suitable options to enable the implementation of the proposed co-digestion systems in full-scale plants, a technology that is widely applied in the European biogas plants for a range blend of residues (Janke et al., 2016a). On the other hand, both "waste to energy" technologies are not excluding and their complementarity should be addressed to envisage the integral exploitation of the aforementioned residues with enhanced energy yield.

In this sense, the entire volume of 1G-VN would be co-digested during the season in scenario a, whilst FC and DL would be used following the proportions mentioned in the experimental step of the co-digestion (Section 2.2). Considering the processing capacity of the plant (4×10^6 TC per harvest), 120×10^6 kg of FC would be produced in the entire season. The mass flow rate of FC supplying the co-digestion system (2625 kg day^{-1}; Table 1) would generate a surplus of 119×10^6 kg of FC to be used in the off-season and to guarantee co-digestion during this period. The same occurs with DL, with an annual production of 2.5×10^6 m^3 (Table 1) and a season-based consumption of only 304,500 m^3, resulting in a surplus of 2.2×10^6 m^3 to feed the reactor during the off-season (scenario b). The amount of waste that remains during the season, even with the co-digestion system, would be very high for both DL and FC. The storage of the FC for later use would not be a problem, because it is commonly carried out in sugarcane mills (similarly to bagasse storage).

In the case of DL, however, storage may result in a loss of opportunity for energy recovery and a potential source of environmental impacts due to its physicochemical characteristic, i.e., a liquid stream with high biodegradable organic content that could be managed by the AD technology. For treating only DL, high-rate reactors could be operated in parallel to the co-digestion system aiming at managing the surplus volume of that substrate in more compact systems, considering that DL has potential for CH_4 production from mono-digestion (Volpi et al., 2021a). The same could be applied for 2G-VN management, although no experimental data on CH_4 production potential was performed yet. In this configuration, the high-rate and the co-digestion technologies would complement each other to allow the integral use of DL for energy recovery by the AD process, which may enhance the energy balance of the biorefinery due to the increment in biogas production. Upflow anaerobic sludge blanket (UASB) reactors are already known for the management of vinasse in scale (Del Nery et al., 2018; Santana Jr. et al., 2019), and since DL is a liquid residue, this could be an alternative for its use, in addition to co-digestion. Alternatively, a larger proportion of DL could be added to the feeding of the co-digestion reactor (i.e., >10% in terms of VS), although investigations must be previously performed to confirm the technical viability of such hypothesis. 2G-VN could also play the role of 1G-VN in the off-season in a system co-processing three substrates, similarly to scenario a.

The electricity obtained through residue co-digestion in the season (scenario a) could supply a city with roughly 120×10^3 residences, considering the average consumption of 150 kWh per residence, according to the Brazilian National Electric Energy Agency (ANEEL) (Camilo et al., 2017). The number of residences supplied during the off-season (scenario b) would be lower (almost 13×10^3) because 1G-VN was not considered as a co-substrate in AD. When considering the mono-digestion of 2G-VN, the increment in electricity production during the off-season would result in a total supply of 20×10^3 residences. The energetic potential obtained for both scenarios with 1G-VN (mono or co-digestion) exceeded previous values reported in the correlate literature, considering similar scenarization conditions. Moraes et al. (2014) reported an installed capacity of 18 MW for the 1G-VN-derived biogas produced in an annexed distillery processing 2×10^6 TC per harvest. Although the reference biorefinery used herein has twice the processing capacity, i.e., 4×10^6 TC (Table 1), an almost 4-fold higher installed capacity (58.33 MW; Table 4) was obtained due to considering the co-digestion of FC and DL in association with 1G-VN. Accordingly, the monthly electricity production obtained herein (16.09×10^3 MWh) would exceed that reported by Moraes et al. (2014) (5×10^3 MWh). Installed capacity (9.48 MW; Table 4) and the monthly electricity production (2.62×10^3 MWh) of the co-digestion plant in the off-season (scenario b) corresponded to 50% of the performance data reported by Moraes et al. (2014). In particular, the same type of stationary engine was considered in the estimates compared herein, based on a conservative approach.

Considering sugarcane biorefineries with equivalent milling capacities (4×10^6 TC per harvest), comparative results may be found elsewhere (Fuess et al., 2018; (Junqueira et al., 2016).. Fuess et al. (2018) reported electric potential values in the range of 6.1–7.5 MW coupled to the single- and two-phase AD of 1G-VN, also using ICE as the prime mover. These authors reported electric potential values as high as 10.8 MW when considering combined cycle-based power plants, which combine gas and steam turbines (Fuess et al., 2018). These values are approximately 2–3.5-fold lower than the electric power achievable in scenario a (22.3 MW). The reported values (6.1–7.5 MW) are higher than the electric potential estimated for the base scenario (5.0 MW) mainly because of differences in the type of engine considered, i.e., conversion efficiency values of 43.0 *vs*. 38.0%. Fuess et al. (2018) also considered the operation of the AD plant during the off-season, based on the biodigestion of 1G-VN derived from energy cane processing. The electric potential values reported for the combustion engine (2.8–3.8

MW) were equivalent to that of scenario b (3.6 MW). However, when considering also the digestion of 2G-VN during the off-season (5.5 MW), higher electricity potential (9.1 MW) can be found for the present study.

A comparison in specific terms, which considers the amount of electricity obtained per amount of processed sugarcane, reveals another marked discrepancy of mono- and co-digestion systems. Moraes et al. (2014) and Fuess et al. (2018) reported values within the range 8.35–12.97 kWh TC^{-1}, whilst the value referring to scenario a reached 30.85 kWh TC^{-1} (Table 4), i.e., at least 2.5-fold higher than conventional 1G-VN-based systems.

According to the Brazilian National Supply Company (CONAB), the production of electricity from bagasse in sugarcane processing plants accounts for approximately 46.2 kWh TC^{-1}, from which 21.1 kWh TC^{-1} is consumed within the plant boundaries, generating a surplus of 25.1 kWh TC^{-1} (Moraes et al., 2014). Whereas energy consumption in AD plants is around 0.11–0.14 kWh TC^{-1} (Fuess et al., 2017), the co-digestion of 1G-VN, FC and DL could fully supply the plant's energy demand and still generate a surplus of 15 kWh TC^{-1} to be sold to the grid (scenario a). In scenario b, considering only the co-digestion of FC and DL in the off-season, it is possible to achieve 2.87 kWh TC^{-1}, whereas the mono-digestion of 2G-VN provided 4.40 kWh TC^{-1}, totaling a potential of 7.27 kWh TC^{-1} for the off-season, i.e., mono-digestion of 2G-VN provided 60% more energy relative to the co-digestion of FC and DL.

3.2. $BioCH_4$ production potential

A second potential application for the residue-derived biogas in the assessed scenarios included the production of $bioCH_4$, which can be used to replace diesel in vehicles and/or supply the gas grid, depending on the production level. The potential $bioCH_4$ production in each scenario and the corresponding energy production are depicted in Fig. 2a. Differently from electricity generation, in which heat losses usually account for over 50% of the available "raw energy", i.e., the installed capacity of the biogas plant, the energy potential of $bioCH_4$ production (Fig. 2a) could reach values as high as the ones estimated for the installed capacity (Table 4) due to the much lower loss levels (2% in this case). Despite the lower $bioCH_4$ production estimated for 2G-VN compared to 1G-VN (4.57 *vs.* 7.12×10^6 Nm^3;Fig. 2a), equivalent energetic potential levels would be reached (14.2 *vs.* 12.7 MW; Fig. 2a) because of the lower length of the off-season period, i.e., 133 *vs.* 232 d (Table 1). Apart from economic implications, $bioCH_4$ production largely outperforms the energetic exploitation of biogas compared to electricity generation on a

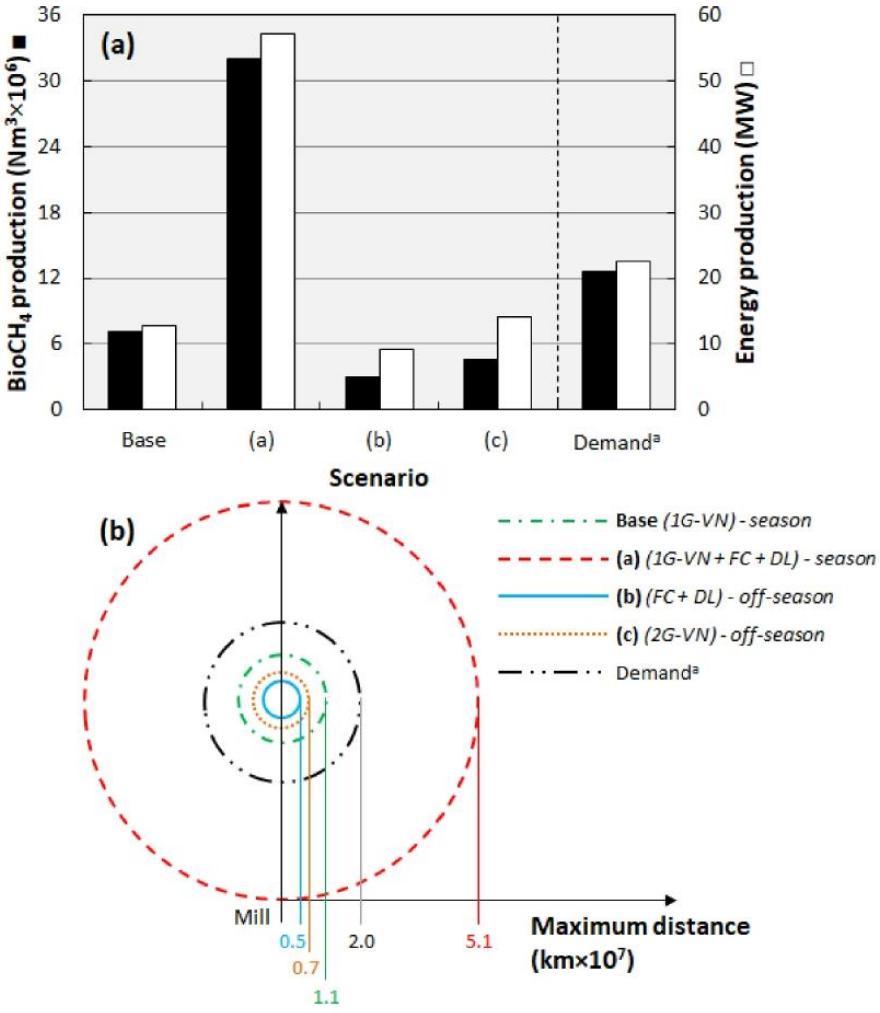

Fig. 2. Details of $bioCH_4$ production in the assessed scenarios: (a) total bioCH4 production per period (season or off-season) and corresponding energetic potential, and (b) maximum distance potentially traveled in a condition of high fuel consumption. Note: [a]Refers to the consumption of $bioCH_4$ required by an entire fleet of rodotrens (energetic efficiency of 1.6 km $Nm^{-3}CH_4$) to travel 2.02×10^7 km per season (average daily distance traveled – 87,200 km – equivalent to that of São Martinho mill's fleet).

conservative basis.

The magnitude of the $bioCH_4$-producing potential of the energetic scenarios may be assessed by using data from the São Martinho mill (Pradópolis, SP, Brazil), the world's largest sugarcane processing plant (milling capacity of approximately 10×10^6 TC per harvest). Considering the average distance traveled by its entire fleet of trucks, i.e., 87,200 km d^{-1}, and assuming the rodotrem as the reference vehicle (1.6 km $m^{-3}bioCH_4$; Ornelas-Ferreira et al., 2020), the consumption of $bioCH_4$ in the season would reach 12.65×10^6 m^3 (Fig. 2a). The potential $bioCH_4$ production (32.01×10^6 Nm^3bioCH_4; Fig. 2a) obtained in scenario a, i.e., the co-digestion of 1G-VN, FC, and DL, could easily supply the fleet, releasing 19.36×10^6 Nm^3bioCH_4 for possibly supplying the natural gas grid. The maximum distance traveled by the fleet of the reference plant during the harvesting period could be increased by

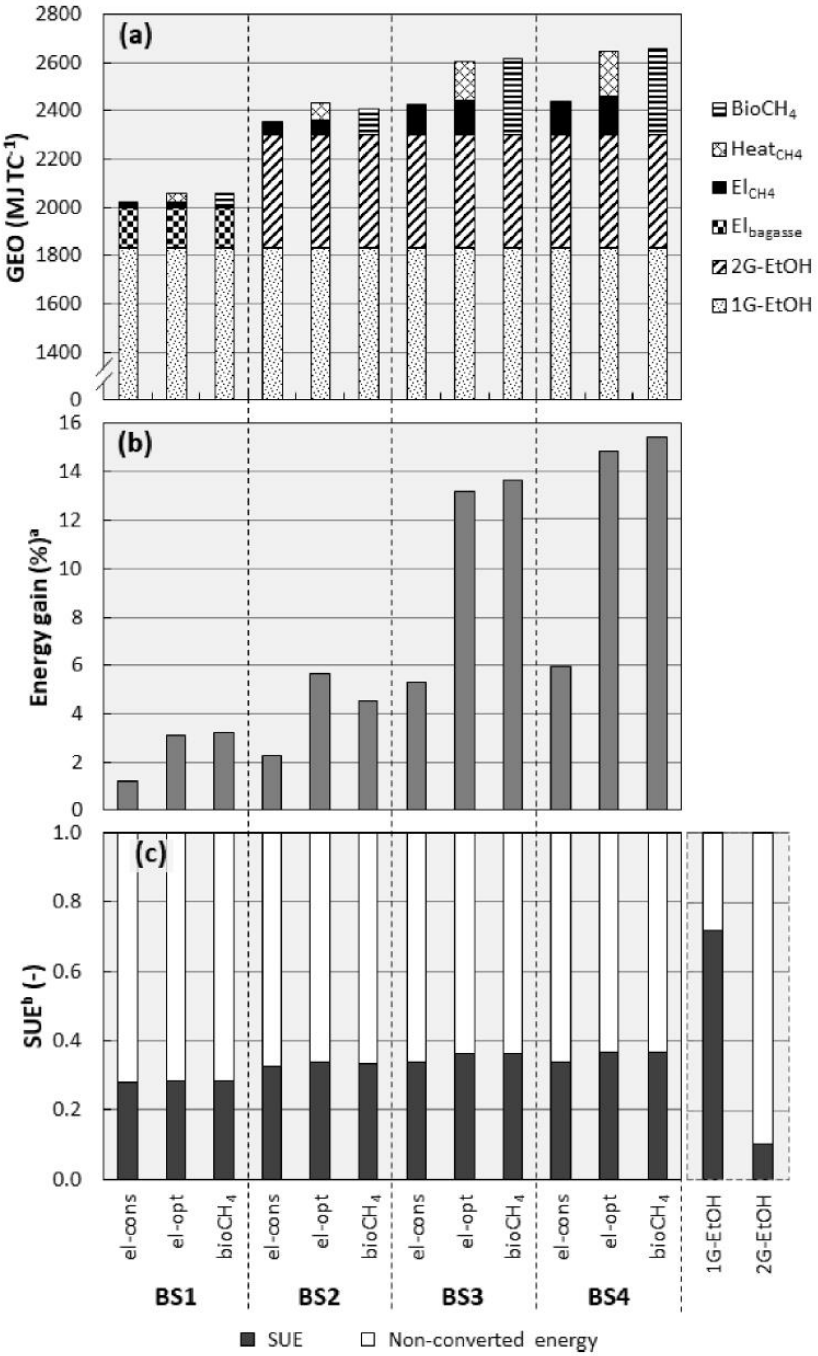

Fig. 3. Selected indicators used to assess the energy efficiency in different sugarcane biorefineries schemes (BS1-4): (a) gross energy output (GEO), (b) energy gain and (c) sugarcane use efficiency (SUE). Notes: [a]Relative to the processing chains without the anaerobic processing of wastewaters and/or residues, [b]SUE values for 1G- and 2G-ethanol production were calculated using the energy content of reducing sugars (2544 MJ TC^{-1}) and lignocellulosic materials (bagasse + trash = 4644 MJ TC^{-1}) as the reference, respectively. Descriptions for each scheme are found in Table 3.

over 150% (2.0 *vs.* 5.1 × 10^7 km; Fig. 2b). It is worth highlighting that estimates considered the worst scenarios for fuel demand, because the entire fleet was assumed to be composed of heavy duty trucks. Hence, higher efficiency levels would certainly be attained by replacing gasoline- and ethanol-fueled vehicles.

The considerable amount of surplus CH_4 could be also used to produce electricity, characterizing a highly flexible AD plant. Future techno-economic assessments should indicate the most feasible layouts for co-digestion plants, similar to the proposed for the mono-digestion of 1G-VN (Fuess and Zaiat, 2018; Junqueira et al., 2016; Moraes et al., 2014). BioCH_4 production via co-digestion in the off-season (scenario b) and through the mono-digestion of both 1G-VN (base-scenario) and 2G VN (scenario c) could reach, respectively, 2.98 × 10^6, 7.13 × 10^6, and 7.96 × 10^6 Nm^3bioCH_4 (Fig. 2a). These numbers indicate that base scenario and scenario c (both for mono-digestion processes of vinasse) would not meet the fleet's demand during the season (Fig. 2a-b), once again reinforcing the energetic relevance for co-digesting VN with other sugarcane-derived substrates. The installed capacity of the AD plant considering the production of both bioCH_4 and electricity would be 36 MW (scenario a), also considering the potential conservative power generation in stationary engines with 38% of conversion efficiency (Section 2.3). In this case, the electric power (9783 MWh $month^{-1}$) could supply over 65 × 10^3 residences.

Considering a scenario in which all the bioCH_4 produced would supply the entire fleet of treminhões or rodotrens (which travel the distance from the plant to the field), a fleet of almost 700 treminhões or 520 rodotrens could be fueled, which corresponds to a 2-fold higher fleet than that indicated by Moraes et al. (2014) for a sugarcane biorefinery processing 2 × 10^6 TC per season. Comparatively, the use of the bioCH_4 generated in the TMethar system to supply the COMLURB's own fleet of solid-urban waste collection trucks was assessed elsewhere (Ornelas-Ferreira et al., 2021). In this case, a potential production of 47,911 Nm^3bioCH_4 $month^{-1}$ was estimated through processing 23.5 ton d^{-1} of the organic fraction of municipal solid waste, which represents only 8% of the lowest monthly potential production estimated for the assessed scenarios in the 1G2G biorefinery (scenario b; 6.0 × 10^5 Nm^3bioCH_4 $month^{-1}$). Despite the higher length of the season period, co-digestion in scenario a could produce monthly 4.1 × 10^6 Nm^3bioCH_4, exceeding the production in scenario b by a factor of 6. Hence, the non-energetic exploitation of sugarcane-derived residues is an enormous strategic fault in the sucro-energetic industry, as evidenced by the energetic potential of biogas, irrespective of the final product.

3.3. Efficiency of bioenergy production from sugarcane

Results obtained for the indicators GEO and SUE in the different biorefinery schemes assessed are depicted in Fig. 3a-c. Prior to discussing impacts of AD/co-digestion, it is worthy highlighting that the energy output of integrated 1G2G biorefineries outperformed the one of 1G processes as a direct result of an approximately 3-fold higher production of usable energy (472.94 *vs.* 166.32 MJ TC^{-1}; Fig. 3a) when comparing the production of 2G-ethanol and electricity from bagasse (1G-plants). The contribution of CH_4-derived energy to the GEO directly depended on the biogas application. Overall, energy gains observed for the production of electricity/heat (optimistic approach) and bioCH_4 were over 2-fold higher than that of conservative electricity production within a given biorefinery scheme (Fig. 3b). Emphasis should be directed to the co-digestion of liquid and solid residues (schemes BS3 and BS4, as described in Table 3; Section 2.4), because the optimistic production of electricity (coupled to heat recovery) and the production of bioCH_4 could reach energy outputs (302.11–356.00 MJ TC^{-1}) as high as 64–75% of that associated with 2G-ethanol production (Fig. 3a), also exceeding the production of electricity from bagasse by approximately 200% depending on the scheme.

These results provide consistent figures to understand the impacts of selecting proper prime movers on the energetic efficiency of the AD-power plants. Optimized energy production (electricity + heat) could also be obtained by using gas turbines (GTB), considering electric and global (electricity + heat) conversion efficiency levels exceeding 40% and 80%, respectively (Fuess et al., 2018). An upgrade in electricity production would result from coupling a steam turbine in the GTB (combined cycle), in order to obtain over 55% of electric conversion efficiency to the detriment of complimentary heat recovery (Fuess et al., 2018). The thermal integration in 1G sugarcane biorefineries fully supply numerous processing steps with steam produced from bagasse combustion in boilers (Morais et al., 2016) and, therefore, targeting enhanced electricity production using the combined cycle may configure a more rational option for exploiting biogas. However, complimentary heat generation from biogas may be an imperative approach in 2G-based schemes, in an effort to partially replace the bagasse-derived thermal energy in periods of enhanced 2G-ethanol production within a flexible 1G2G biorefinery context (Dias et al., 2012). Apart from these applications, the thermal energy obtained from biogas could be used *in loco* to maintain desired temperature levels in AD plants, considering the recurrent indication of thermophilic conditions as the most appropriate ones for processing sugarcane-derived substrates, such as 1G-VN (Ferraz Jr. et al., 2016; Fuess et al., 2017).

Inserting residue co-digestion would naturally improve the energetic use of sugarcane, with SUE values peaking at 0.37 in scheme BS4 (considering both the optimistic production of electricity/heat and the bioCH_4 production; Fig. 3c). Regardless of this improvement, these results highlight a relatively inefficient use of sugarcane on an energy basis, indicating losses exceeding 60% of the global available energy content. Differences in the consolidation of 1G- and 2G-based technologies explain this scenario, because an individualized assessment of 1G-ethanol production reveals a SUE value of 0.72 (Fig. 3c), using exclusively the fraction of reducing sugars (2544 MJ TC^{-1}) as the source of convertible energy. Conversely, the SUE value in 2G-ethanol production represents only 10% of the available energy content found in the lignocellulosic fractions of sugarcane (Fig. 3c). Hence, this is not strictly a matter of wastewater/residue processing to obtain surplus energy, but it does reveal the need to optimize the energy extraction from bagasse and trash.

Opportunities for improving SUE values can include the replacement of the bio-based conversion route (fermentation) by the thermochemical one (syngas platform) in 2G ethanol production, which is based on the catalytic conversion of syngas (H_2 + CO) to ethanol (Morais et al., 2016). A 15%-increase in total (1G + 2G) ethanol production (517 *vs.* 596 × 10^6 L $year^{-1}$) was predicted to occur by simulating the replacement of biochemical 2G ethanol production by the one derived from the syngas platform (Dias et al., 2016). Using advanced technologies to efficiently extract fermentable substrates from lignocellulosic materials can also largely improve the energy yield in the biochemical route, as evidenced by a roughly 80%-increase in the 2G ethanol yield (22.1 *vs.* 39. 4 L TC^{-1}) simulated elsewhere (Dias et al., 2016). However, it is important highlighting that the values obtained with GEO/SUE indicators refer to a gross perspective, i.e., subtracting energy inputs to obtain the net values will result in lower efficiency use values. For instance, energy demands (renewable + fossil) in the industrial phase of 1G-ethanol production (19.4 MJ L^{-1}; Fuess and Garcia, 2014) are as high as the calorific power of ethanol (21.4 MJ L^{-1}; Manochio et al., 2017), which markedly decreases the effective output of usable energy.

Remarkable improvements in the energy use of sugarcane may be obtained only (or mainly) through modifying primary conversion steps in biorefineries. In other words, sugarcane could be used as the feedstock to obtain products other than ethanol targeting a more efficient energy conversion. In particular, an AD-based biorefinery could fit this demand, because of relatively low energy inputs required to produce biogas. Using the case of 1G-VN as the reference, previous research indicated maximum energy requirements within the range 1.67–1.95 kWh TC^{-1} (6.01–7.02 MJ TC^{-1}; Fuess et al., 2017), which correspond to less than 30% and 15% of the energy production as electricity (24.76 MJ TC^{-1};

conservative approach) and bioCH_4 (63.85 MJ TC^{-1}) in BS1 (Fig. 3a), respectively. Hence, in addition to providing an efficient management of residual streams, anaerobic processes may play a determining role in maximizing energy production from sugarcane, and the future implementation of AD-based sugarcane processing chains should not be considered illogical.

Consequently, a final question deserves attention: better producing electricity of bioCH_4? Regardless of the energetic pros of bioCH_4 production, there is no definitive answer to this question, because the best energetic and/or economic exploitation approach for biogas depends on numerous factors. Emphasis should be given to the location of the sugarcane processing plant, market demands and the amount of substrate available for anaerobic processing. On one hand, supplying the gas grid with bioCH_4 will directly depend on the distance of the biorefinery relative to the grid (Fuess and Zaiat, 2018; Junqueira et al., 2016), which may economically limit its use to *in loco* applications, such as supplying the fleet of biorefineries (Section 3.2). On the other hand, despite the saturated electric market in Brazil, increasing the local electricity supply through including biogas-derived electricity can be a key strategy to minimize energy losses in transmission lines, in addition to decreasing implementation and maintenance costs by eliminating long lines within a distributed energy generation context (Pereira et al., 2020). Alternatively, both biogas applications may be exploited under high substrate availability, similarly to the co-digestion scheme proposed in scenario a, because a kind of "economies of scale" could allow the simultaneous implementation of diversified biogas uses within a single industrial plant due to much higher revenues with the trade of bioenergy, regardless of its type. In particular, this should be a natural choice in the case of AD-based sugarcane biorefineries, considering the much higher availability of raw energy.

4. Conclusions

Energy-producing co-digestion plants could double the installed capacity of sugarcane biorefineries compared to VN-based mono-digestion. The co-digestion of VN, FC, and DL in the season followed by the co-digestion of FC and DL in the off-season could maintain continuous plant operation. Biogas could be also upgrade to BioCH_4, either as the sole product (up to 32×10^6 Nm^3 in the season) or as a co-product with electricity. Regardless of the enhanced energetic performance, an efficient exploitation of sugarcane still depends on the improvement of 2G technologies, because over 60% of the sugarcane's energy content is still wasted in unconverted lignocellulosic materials.

CRediT authorship contribution statement

Maria Paula C. Volpi: Conceptualization, Investigation, Methodology, Data curation, Writing - original draft. **Lucas T. Fuess:** Methodology, Resources, Data curation, Writing - original draft. **Bruna S. Moraes:** Conceptualization, Formal analysis, Writing - review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Adarme, O.F.H., Baêta, B.E.L., Filho, J.B.G., Gurgel, L.V.A., Aquino, S.F., 2019. Use of anaerobic co-digestion as an alternative to add value to sugarcane biorefinery wastes. Bioresour. Technol. 287, 121443.

de Aquino, S., Fuess, L.T., Pires, E.C., 2017. Media arrangement impacts cell growth in anaerobic fixed-bed reactors treating sugarcane vinasse: Structured vs. randomic biomass immobilization. Bioresour. Technol. 235, 219–228.

Awosusi, A., Sethunya, V., Matambo, T., 2020. Synergistic effect of anaerobic co-digestion of South African food waste with cow manure: Role of low density-polyethylene in process modulation. Mater. Today:. Proc. 38, 793–803.

Bordelanne, O., Montero, M., Bravin, F., Prieur-Vernat, A., Oliveti-Selmi, O., Pierre, H., Papadopoulo, M., Muller, T., 2011. Biomethane CNG hybrid: A reduction by more than 80% of the greenhouse gases emissions compared to gasoline. J. Nat. Gas Sci. Eng. 3 (5), 617–624.

Brenelli, L.B., Figueiredo, F.L., Damasio, A., Franco, T.T., Rabelo, S.C., 2020. An integrated approach to obtain xylo-oligosaccharides from sugarcane straw: From lab to pilot scale. Bioresour. Technol. 313, 123637.

Camilo, H.F., Udaeta, M.E.M., Veiga Gimenes, A.L., Grimoni, J.A.B., 2017. Assessment of photovoltaic distributed generation – Issues of grid connected systems through the consumer side applied to a case study of Brazil. Renew. Sustain. Energy Rev. 71, 712–719.

Christofoletti, C.A., Escher, J.P., Correia, J.E., Marinho, J.F.U., Fontanetti, C.S., 2013. Sugarcane vinasse: Environmental implications of its use. Waste Manage. 33 (12), 2752–2761.

Del Nery, V., Alves, I., Zamariolli Damianovic, M.H.R., Pires, E.C., 2018. Hydraulic and organic rates applied to pilot scale UASB reactor for sugar cane vinasse degradation and biogas generation. Biomass Bioenergy 119, 411–417.

Dias, M.O.S., Junqueira, T.L., Cavalett, O., Cunha, M.P., Jesus, C.D.F., Rossell, C.E.V., Maciel Filho, R., Bonomi, A., 2012. Integrated versus stand-alone second generation ethanol production from sugarcane bagasse and trash. Bioresour. Technol. 103 (1), 152–161.

Dias, M.O.S., Junqueira, T.L., Sampaio, I.L.M., Chagas, M.F., Watanabe, M.D.B., Morais, E.R., Gouveia, V.L.R., Klein, B.C., Rezende, M.C.A.F., Cardoso, T.F., Souza, A., Jesus, C.D.F., Pereira, L.G., Rivera, E.C., Maciel Filho, R., Bonomi, A., 2016. Use of the VSB to assess biorefinery strategies. In: Bonomi, A., Cavalett, O., Cunha, M.P., Lima, M.A.P. (Eds.), Virtual biorefinery: an optimization strategy for renewable carbon valorization. Springer, London, pp. 189–256.

Fuess, L.T., Garcia, M.L., 2014. Anaerobic digestion of stillage to produce bioenergy in the sugarcane-to-ethanol industry. Environ. Technol. 35 (3), 333–339.

Fuess, L.T., Zaiat, M., 2018. Economics of anaerobic digestion for processing sugarcane vinasse: Applying sensitivity analysis to increase process profitability in diversified biogas applications. Process Saf. Environ. Prot. 115, 27–37.

Ferraz Jr, A.D. N., Koyama, M.H., de Araújo Júnior, M.M., Zaiat, M., 2016. Thermophilic anaerobic digestion of raw sugarcane vinasse. Renewable Energy 89, 245–252.

Fuess, L.T., de Araújo Júnior, M.M., Garcia, M.L., Zaiat, M., 2017. Designing full-scale biodigestion plants for the treatment of vinasse in sugarcane biorefineries: How phase separation and alkalinization impact biogas and electricity production costs? Chem. Eng. Res. Des. 119, 209–220.

Fuess, L.T., Klein, B.C., Chagas, M.F., Alves Ferreira Rezende, M.C., Garcia, M.L., Bonomi, A., Zaiat, M., 2018. Diversifying the technological strategies for recovering bioenergy from the two-phase anaerobic digestion of sugarcane vinasse: An integrated techno-economic and environmental approach. Renewable Energy 122, 674–687.

López González, L.M., Pereda Reyes, I., Romero Romero, O., 2017. Anaerobic co-digestion of sugarcane press mud with vinasse on methane yield. Waste Manage. 68, 139–145.

Hagos, K., Zong, J., Li, D., Liu, C., Lu, X., 2017. Anaerobic co-digestion process for biogas production: Progress, challenges and perspectives. Renew. Sustain. Energy Rev. 76, 1485–1496.

Janke, L., Leite, A., Nikolausz, M., Schmidt, T., Liebetrau, J., Nelles, M., Stinner, W., 2015. Biogas production from sugarcane waste: Assessment on kinetic challenges for process designing. Int. J. Mol. Sci. 16, 20685–20703.

Janke, L., Leite, A., Batista, K., Weinrich, S., Sträuber, H., Nikolausz, M., Nelles, M., Stinner, W., 2016a. Optimization of hydrolysis and volatile fatty acids production from sugarcane filter cake: Effects of urea supplementation and sodium hydroxide pretreatment. Bioresour. Technol. 199, 235–244.

Janke, L., Leite, A.F., Nikolausz, M., Radetski, C.M., Nelles, M., Stinner, W., 2016b. Comparison of start-up strategies and process performance during semi-continuous anaerobic digestion of sugarcane filter cake co-digested with bagasse. Waste Manage. 48, 199–208.

Junqueira, T.L., Moraes, B., Gouveia, V.L.R., Mateus F. Chagas, E.R.M., Watanabe, M.D. B., Bonomi, Zaiat, M., Bonomi, A., 2016. Use of VSB to Plan Research Programs and Public Policies, in: Cavalett, A.B., Cavelet, O., Cunha, M.P., Lima, M. (Eds.), Virtual Biorefinery An Optimization Strategy for Renewable Carbon Valorization. Springer, London, pp. 257–282.

Leme, R.M., Seabra, J.E.A., 2017. Technical-economic assessment of different biogas upgrading routes from vinasse anaerobic digestion in the Brazilian bioethanol industry. Energy 119, 754–766.

Manochio, C., Andrade, B.R., Rodriguez, R.P., Moraes, B.S., 2017. Ethanol from biomass: A comparative overview. Renew. Sustain. Energy Rev. 80, 743–755.
Moraes, B.S., Junqueira, T.L., Pavanello, L.G., Cavalett, O., Mantelatto, P.E., Bonomi, A., Zaiat, M., 2014. Anaerobic digestion of vinasse from sugarcane biorefineries in Brazil from energy, environmental, and economic perspectives: Profit or expense? Appl. Energy 113, 825–835.
Moraes, B.S., Triolo, J.M., Lecona, V.P., Zaiat, M., Sommer, S.G., 2015a. Biogas production within the bioethanol production chain: Use of co-substrates for anaerobic digestion of sugar beet vinasse. Bioresour. Technol. 190, 227–234.
Moraes, B.S., Zaiat, M., Bonomi, A., 2015b. Anaerobic digestion of vinasse from sugarcane ethanol production in Brazil: Challenges and perspectives. Renew. Sustain. Energy Rev. 44, 888–903.
Morais, E.R., Junqueira, T.L., Sampaio, I.L.M., Dias, M.O.S., Rezende, M.C.A.F., Jesus, C. D.F., Klein, B.C., Gómez, E.O., Mantelatto, P.E., Maciel Filho, R., Bonomi, A., 2016. Biorefinery alternatives. In: Bonomi, A., Cavalett, O., Cunha, M.P., Lima, M.A.P. (Eds.), Virtual biorefinery: an optimization strategy for renewable carbon valorization. Springer, London, pp. 53–132.
Moreira, A., Moraes, L.A.C., de Aquino, G.S., Heinrichs, R., 2019. In: Sugarcane Biofuels: Status, Potential, and Prospects of the Sweet Crop to Fuel the World. Springer International Publishing, Cham, pp. 21–38.
Muñoz, R., Meier, L., Diaz, I., Jeison, D., 2015. A critical review on the state-of-the-art of physical/chemical and biological technologies for an integral biogas upgrading. Rev. Environ. Sci. Biotechnol. 14, 727–759.
Ornelas-Ferreira, B., Lobato, L.C.S., Colturato, L.F.D., Torres, E.O., Pombo, L.M., Pujatti, F.J.P., Araújo, J.C., Chernicharo, C.A.L., 2020. Strategies for energy recovery and gains associated with the implementation of a solid state batch methanization system for treating organic waste from the city of Rio de Janeiro - Brazil. Renewable Energy 146, 1976–1983.
Pereira, I.Z., Santos, I.F.S.dos., Barros, R.M., Castro e Silva, H.L.de., Tiago Filho, G.L., Moni e Silva, A.P., 2020. Vinasse biogas energy and economic analysis in the state of São Paulo, Brazil. J. Cleaner Prod. 260, 121018.
Rodriguez, R.P., Manochio, C., Moraes, B.de.S., 2019. Energy Integration of Biogas Production in an Integrated 1G2G Sugarcane Biorefinery: Modeling and Simulation. Bioenerg. Res. 12 (1), 158–167.
Santana Junior, A.E., Duda, R.M., Oliveira, R.A.de., 2019. Improving the energy balance of ethanol industry with methane production from vinasse and molasses in two-stage anaerobic reactors. J. Cleaner Prod. 238, 117577.
van Lier, J.B., van der Zee, F.P., Frijters, C.T.M.J., Ersahin, M.E., 2015. Celebrating 40 years anaerobic sludge bed reactors for industrial wastewater treatment. Rev. Environ. Sci. Biotechnol. 14, 681–702.
Volpi, M.P.C., Brenelli, L.B., Mockaitis, G., Rabelo, S. C., Franco, T.T., Moraes, B. S., 2021a. Biochemical methane potential (BMP) from sugarcane biorefinery residues: maximizing their use by co-digestion. bioRxiv, https://doi.org/10.1101/2021.02.19.432018.
Volpi, M.P.C, Ferraz Jr., A. D. J., Franco, T.T., Moraes, B.S., 2021b. Operational and biochemical aspects of co-digestion (co-AD) from sugarcane vinasse, filter cake and deacetylation liquor. bioRxiv, https://doi.org/10.1101/2021.02.24.432031.

5.4 **PAPER 4**

Use of Fe_3O_4 nanoparticles in reactor co-digestion of residues from 1G2G ethanol biorefinery: microbiological routes and operational aspects

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ABSTRACT

The co-digestion of residues from the sugarcane industry has already proven to be a highly attractive process for biogas production through anaerobic digestion (AD). The use of residues such as vinasse (1G) filter cake (1G) and deacetylation liquor (2G) in operation in a continuous CSTR reactor showed a possibility of integration of 1G and 2G ethanol biorefineries through AD in previous work by our research group. The use of nanoparticles (NP) is a favorable way to optimize AD processes, as these additives serve as a means of introducing nutrients into the process in a more assertive way from the point of view of distribution and interaction with microorganisms. In this context, the present work proposed the optimization of the co-digestion of vinasse, filter cake, and deacetylation liquor in a continuous reactor through the addition of Fe_3O_4 NP, by the purpose of comparison results with the operation of the same substrates and the same condition but without NP. Initially, tests were carried out in batches with different concentrations of NPs, to evaluate the best concentration to be added in the continuous reactor. A concentration of 5 mg L^{-1} was chosen, and it was added to each increase in organic rate load (ORL) used in the process. CH_4 production reached maximum values of 2.8 ± 0.1 $NLCH_4$ gVS^{-1} and organic matter removal 71 ± 0.9%, in phase VI, with ORL of 5.5 gVS L^{-1} day^{-1}. This production was 90% higher than the reactor co-digestion operation without the presence of NP. Furthermore, according to the results of pH, alkalinity, it can be concluded that the methanogenesis stabilized at 60 days of operation, being 30 days before when there was no NP added. The development of AD was stable, with low variations in the oxidation-reduction potential (ORP) and with stable organic acid (OA) concentrations, indicating the possibility of route propionic acid to produce CH_4. The main methanogenic *Archaea* found was *Methanoculleus*, indicating that the predominant metabolic route was that of syntrophic acetate oxidation (SAO) coupled with hydrogenotrophic methanogenesis. The use of Fe_3O_4 NP

managed to improve the AD operation of residues from the 1G2G ethanol production industry and did not modify the microbial community present, only stimulating their growth.

Keywords: Nanoparticles; Co-digestion; Methane optimization; 1G2G ethanol residues

1. INTRODUCTION

Anaerobic digestion (AD) is a process of managing liquid and solid waste that allows energy recovery through methane (CH_4) (Deublin and Steinhauser, 2008). This technique is used for different types of residues, and the literature has shown the great potential for CH_4 generation from sugarcane residues, especially vinasse (Djalma Nunes Ferraz Júnior et al., 2016; Fuess et al., 2017; Moraes et al., 2015).

In this setting, anaerobic co-digestion became popular as a way to boost biogas output. Co-digestion is defined as the AD of two or more substrates, and it is a method for overcoming the drawbacks of mono-digestion, particularly in terms of nutritional balance and improving the economic sustainability of AD plants (Hagos et al., 2017). Co-digestion has the advantages of optimizing CH_4 production, in addition to better stabilizing the process. With the presence of different substrates, it is possible to provide synergistic effects within the reactor, increasing a load of biodegradable compounds (Hagos et al., 2017).

Promoting the co-digestion of residues from the sugarcane industry can be an alternative to improve the management of the various residues obtained in this biorefinery, in addition to increasing CH_4 generation. Beyond vinasse, the filter cake is a lignocellulosic residue obtained from ethanol production that has a high potential for biogas production (Volpi et al., 2021a)(Janke et al., 2016) and can enhance the vinasse CH_4 production by co-digesting these two residues (Volpi et al., 2021a). However, the literature reports little about the use of residues from the production of 2G ethanol for AD, mainly emphasizing the use of only 2G vinasse (Moraes et al., 2014) but not reporting the use of liquors that can also be obtained from the 2G ethanol production process. In the work of Brenelli et al. (Brenelli et al., 2020), an alkaline pretreatment of sugarcane straw was performed to be used in the production of 2G ethanol. Within this process, straw deacetylation was carried out before the hydrothermal pretreatment,

since the straw hemicellulose is highly acetylated. The residue generated from this process, called deacetylation liquor, is rich in volatile fatty acids such as acetic acid, formic acid, being promising for CH_4 production through AD, or co-digestion (Volpi et al., 2021a).

In previous works by our research group, it was proposed to co-digest the residues of the sugarcane industry for the production of biogas, to promote the integration of the 1G2G ethanol biorefinery. The results showed that the co-digestion of vinasse, filter cake, and deacetylation liquor in semi-Continuous Stirred Tank Reactor (s-CSTR) reached production of 230 $NmLCH_4\ gSV^{-1}$ and organic matter removal efficiency 83% ± 13 , showing that the co-digestion of the proposed residues, increased the production of CH_4, about mono-digestion of vinasse (Volpi et al, 2021b).

To increase the production of CH_4 in operation in reactors, the literature reports that the use of additives can improve its performance, mainly related to the use of micronutrients (Demirel and Scherer, 2008). Scherer et al. (1983) classified that for methanogenic organisms the importance of micronutrients is given in the following order: Fe >> Zn> Ni> Cu = Co = Mo> Mn, indicating that such elements have essential roles as in the construction of methanogenic cells. Besides this, many of these micronutrients have concentrations that must be met, as cell growth may be limited or inhibited. Zhang et al. (2003) showed that for Co, Ni, Fe, Zn, Cu if the concentrations are less than 4.8, 1.32, 1.13, 0.12 $g\ L^{-1}$ respectively, there is a limitation of the growing culture of methanogenic microorganisms in terms of cell density.

Among the different trace elements, Fe is important to stimulate the formation of citrocomes and ferredoxins, important for cellular energy metabolism, mainly of methanogenic *Archaea* (Choong et al., 2016). In addition to methanogenesis, Fe is also important to catalyze chemical reactions of some metalloenzymes used in acetogenesis, such as dehydrogenase format, carbon monoxide dehydrogenase (Choong et al., 2016). The hydrolysis and

acidification phase of AD is also benefited by Fe as a growth factor since Fe supplementation can accelerate these steps (Yu et al., 2015).

In the work of Demirel and Scherer, (2011), the addition of Fe_3O_4 improved the production of biogas and the CH_4 content in biogas using cow dung and chicken litter. And Zhang et al. (2011) al showed that Zerovalent Iron (ZVI) helps to create an improved anaerobic environment for wastewater treatment and that promotes the growth of methanogens with greater removal of chemical oxygen demand (COD).

One of the ways to promote this addition of components to optimize AD is through the use of nanoparticles (NPs). Nanotechnology allows the manipulation of matter on a nanoscale (1 to 100 nm), and NPs are materials found in this size range (Abdelsalam et al., 2016). The nano-size is important because it allows greater mobility of the active compound in the environment, in addition to allowing interaction with the biological system, facilitating the passage of the compound in cell membranes, absorption, and distribution in the metabolism. This happens due to its mesoscopic effect, small object effect, quantum size effect, and surface effect and to have the greater surface area and dispersibility (Abdelsalam et al., 2017a, 2017b, 2016).

Some authors have already studied the use of different nanoparticles to optimize the production of biogas in different types of waste. Henssein et al. (2019) studied the use of NPs in AD of poultry litter. They observed that the production of CH_4 increased with the addition of NPs, being the NP concentrations (in mg L^{-1}) of 12 Ni (38.4% increase), 5.4 Co (29.7% increase), 100 Fe (29.1% increase), and 15 Fe_3O_4 (27.5% increase). Mu et al. (Mu et al., 2011a) studied the effect of metal oxide nanoparticles (nano-TiO2, nano-Al2O3, nano-SiO2, and nano-ZnO) on AD using activated sludge as a substrate, and the results showed that only Nano-ZnO had an inhibitory effect on CH_4 production in concentrations starting at 30 mg g^{-1}- total suspended solids (TSS). Abdeslam et al. (Abdelsalam et al., 2016) used the metallic NPs Co,

Ni, Fe, and Fe_3O_4 to compare the production of biogas and CH_4 from the anaerobic digestion of cattle manure and they obtained as a result that the methane yield increased significantly ($p < 0.05$) 2, 2.17, 1.67 and 2.16 times about the control, respectively. Wang et al. (2016) investigated the effects of representative NPs, (nZVI, Fe_2O_3 NPs) on CH_4 production during the anaerobic digestion of waste activated sludge, and the concentration of 10 mg g^{-1} TSS nZVI and 100 mg g^{-1} TSS Fe_2O_3 NPs increased methane production to 120% and 117% of the control, respectively. The literature has shown that experiments with Fe_3O_4, which are magnetic NPs, improved the AD process due to their characteristics of superparamagnetic, high coercivity, and low Curie temperature. In addition to these characteristics, Fe_3O_4 NPs are also non-toxic and biocompatible (Abdelsalam et al., 2017b; Mamani and Gamarra, 2014), which may favor AD processes.

To date, studies on the use of NPs to optimize the production of biogas in co-digestion with residues from the sugarcane industry have not been found in the literature. In our previous work (Volpi et al., 2021b) co-digestion of residues from the sugarcane industry and characterization of the microbial community was carried out. To promote optimization of the process, the objective of the present study was to co-digest vinasse, filter cake, and deacetylation liquor in an s-CSTR reactor, with the addition of Fe_3O_4 nanoparticles. First, batch assays were performed with different concentrations of Fe_3O_4 NPs, to assess what would be the best concentration to use in the reactor, and after that, the operation in the reactor was performed, with a characterization of the microbial community prior to inoculation and at the end of the operation, to compare the process with adding NPs in changing the microbial community.

2. METHODOLOGY

2.1 Residues and Inoculum

The substrates were vinasse and filter cake from Iracema sugarcane mill (São Martinho group, Iracemápolis, São Paulo state, Brazil) and the liquor from the straw pretreatment process, performed at the Brazilian Biorenewables National Laboratory (LNBR) from the Brazilian Center for Research in Energy and Materials (CNPEM). Deacetylation pre-treatment was applied to sugarcane straw on a bench-scale as described in Brenelli et al. (Brenelli et al., 2020). The anaerobic consortium of the mesophilic reactor (BIOPAC®ICX - Paques) from the aforementioned Iracema mill was used as inoculum. The substrates were characterized in terms of series of solids, volatile solids (VS), total solids (TS) Organic acids (OA), alcohol, carbohydrates, The inoculum was characterized in terms of VS and TS. The inoculum presented 0.0076 ± 0.00 g mL^{-1} in terms of VS and 0.0146 ± 0.00 in terms of TS. The vinasse presented 0.014 ± 0.00 g mL^{-1} of VS and 0.0176 ± 0.00 g mL^{-1} of TS, the deacetylation liquor 0.0123 ± 0.00 g mL^{-1} of VS and 0.0219 ± 0.00 g mL^{-1} of TS, and filter cake 0.5454 ± 0.53 g mL^{-1} of VS and 0.6197 ± 0.54 g mL^{-1} of TS. The pH of the inoculum was 8.57 ± 0.14, the pH of vinasse was 4.25 ± 0.17 and the deacetylation liquor the pH was 9.86 ± 0.15. The elemental composition was performed for the characterization of filter cake in the Elementary Carbon, Nitrogen, Hydrogen and Sulfur and was obtained 1.88% of N, 31.07% of C, 6.56% of H and 0.3% of S, all in terms of TS.

The characterization of OA, alcohol, and carbohydrates for liquid residues are presented in Table 5.4.1.

Table 5.4.1. Characterization of OA, carbohydrates, and alcohols of liquids residues

Compounds	Vinasse (mg L^{-1})	Deacetylation Liquor (mg L^{-1})
Acetate	1268.41	3250.00
Formate	--	650.00
Lactate	3706.94	423.18

Propionate	634.85	368.29
Butyrate	--	250.02
Isovalerate	931.63	269.03
Glucose	809.05	546.23

--: not carried out

2.2 Batch Tests

Batch tests were performed on the co-digestion of residues (vinasse + filter cake + deacetylation liquor in the proportion of 70:20:10 (in terms of VS) respectively following previous work (Volpi et al., 2021b) with different concentrations of Fe_3O_4 NPs to identify the best concentration to be used in the s-CSTR reactor. The tests were conducted in 250 ml Duran flasks, under 55 ° C, in which the inoculum was acclimated initially. On the first day, the temperature was increased to 40°C, then to 45°C and in 4 days it had reached 55°C. After reaching this temperature, the inoculum was kept for 1 week at 55°C, then from the beginning of the experiments. The experiments were in triplicate, with a 1:1 inoculum to substrate ratio (in terms of VS) added to each flask, following the s-CSTR operation. The pH of solution flasks was corrected to neutrality by adding solutions of NaOH (0.5 M) or H_2SO_4 (1 M) when necessary. N_2 has fluxed into the headspace of each vial. The biogas produced was collected from the headspace with the Gastight Hamilton Super Syringe (1L) syringe through the flasks' rubber septum. Gas chromatography analyses were also carried out to detect the concentration of CH_4 produced in the gas chromatograph (Construmaq MOD. U-13 São Carlos). The carrier gas was hydrogen (H_2) gas (30 cm s^{-1}) and the injection volume was 3 mL. The GC Column was made of 3-meter long stainless steel, 1/8 ”in diameter, and packaged with Molecular Tamper 5A for separation of O_2 and N_2 and CH_4 in the thermal conductivity detector (TCD). Digestion was terminated when the daily production of biogas per batch was less than 1% of

the accumulated gas production. After the assay, the values were corrected for standard temperature and pressure (STP) conditions (273 K, 1.013 hPa).

The different concentrations of Fe_3O_4 NP used in each bottle are described in Table 2. The choice of concentrations was made based on studies with NP and AD that the literature shows (Abdelsalam et al., 2016; Wang et al., 2016). It is worth mentioning that a control flask was made (Flaks 1-Table 5.4.2), adding only the inoculum and co-digestion, without NPs, to compare with the other bottles that contained NPs, and to evaluate the optimization of the process. Analysis of variance (ANOVA) was used to identify the existence of significant differences between the treatments ($p < 0.05$).

Table 5.4.2. Design of experiments of BMP

Flaks	Name in Graph	Fe_3O_4 NP Concentration (mg L^{-1})
1- Inoculum + Co-digestion	Control	0
2- Inoculum + Co-digestion + NP	NP 1	1
3- Inoculum + Co-digestion + NP	NP 2	5
4- Inoculum + Co-digestion + NP	NP 3	10
5- Inoculum + Co-digestion + NP	NP 4	20

2.3 Semi-continuous reactor: description and operation

The s-CSTR operation was followed according to previous work by our research group (Volpi et al., 2021b). 5L-Duran flask with 4L-working volume kept under agitation at 150 rpm by using an orbital shaking table Marconi MA 140. The operating temperature was 55°C, maintained by recirculating hot water through a serpentine. The reactor was fed once a day with the blend of co-substrates (in terms of volatile solids, VS): 70% of vinasse, 20% of filter cake, and 10% of deacetylation liquor, totaling 33.45 gVS L^{-1}. Throughout the operation, the

Organics Loads Rate (OLR) was increased to use the maximum OLR without collapsing the reactor. At the beginning of the operation, Fe_3O_4 NP was added and when the reactor stabilized the CH_4 production, the OLR was increased, expected to stabilize the CH_4 production again and added the same concentration of Fe_3O_4 NPs. This was done for all OLRs (excepted for the last one). Table 5.4.3 presents the values of operational parameters applied to the s-CSTR according to the respective operation phases and the days that were added Fe_3O_4 NPs.

Table 5.4.3. Phases of reactor operation and the respective applied ORLs, feeding rate flows, and HRT.

Phase in Graph	OLR (gVS L^{-1} day^{-1})	Feeding rate (L day^{-1})	HRT (days)	NP Addition Day
I	2	0.250	16	24
II	2.35	0.285	14	47
III	3	0.363	11	72
IV	4	0.500	8	95
V	4.70	0.571	7	109
VI	5.5	0.666	6	123
VII	6.6	0.800	5	136
VIII	8	1.000	4	150
IX	9	1.140	3.5	--

Note: --: not added

2.3.1 s-CSTR monitoring analyses

The volume of biogas produced was measured through the Ritter gas meter, Germany. The CH_4 content was determined by gas chromatography (Construmaq-MOD U-13, São Carlos) five times a week. OA, carbohydrates, alcohols, and organic matter content (in terms of VS) in the digestate were monitored following the same methodology described in the characterization of residues (section 2.1). The alkalinity from digestate also was determined using the titration method APHA, (APHA, AWWA, 2012). The pH and the Oxidation-

reduction potential (ORP) of digestate were measured, immediately after sampling (before feeding) using a specific electrode for Digimed ORP. The pH was monitored also in the feed. All reactor monitoring analyses followed as described in Volpi et al. (2021b).

2.4 Biology Molecular Analysis

Identification of the microbial community of the inoculum was carried out before being inserted in the reactor- Sample A1, and after the production of CH_4 was stabilized in the OLR of 4 gVS L^{-1} day^{-1} (Sample A2), to evaluate the change of the microbial community with the changes of the metabolic routes for the production of CH_4 and with the addition of Fe_3O_4 NP. The extraction and quantification and sequencing protocol were followed as described in Volpi et al. (2021b). Raw sequences were deposited in BioSample NCBI under accession number BioProject ID PRJNA781620.

2.5 NP preparations and characterization

Fe_3O_4 NP was used, due to the better performance of these NP in AD according to the literature (Abdelsalam et al., 2016; Ali et al., 2017; Zhang et al., 2020). The Fe_3O_4 NP used were IRON (II, III) OXIDE, NANOPOWDER, 50-100 N-SIGMA-ALDRICH. They were then diluted in distilled water at pH 7, in a glass bottle. Sodium dodecylbenzene sulfonate (SDS) at 0.1mM was used as a dispersing reagent to ensure NPs dispersion before use, as SDS has been shown to not significantly affect CH_4 production (Hassanein et al., 2019; Wang et al., 2016). To characterize the size of these NPs was performed analysis on the Laser Diffraction Particle Size Analyzer - MASTERSIZER-3000 (MALVERN INSTRUMENTS- MAZ3000- Worcestershire, U.K.). Measurement was made in Wet Mode - HIDRO EV. The mathematical model employed: Mie. It considers that the particles are spherical and that they are not opaque - thus taking into account the diffraction and diffusion of light in the particle and the medium. They were made for samples of pure NP.

3. RESULTS AND DISCUSSION

3.1 Characterization of Fe_3O_4 NP

Figure 5.4.1 shows the size and distribution of Fe_3O_4 NP diluted in water pH 7. Figure 5.4.1a shows two populations, one up to nano size (0.1 µm) and the other that starts from 0.3 µm and is no considered a nanoparticle.

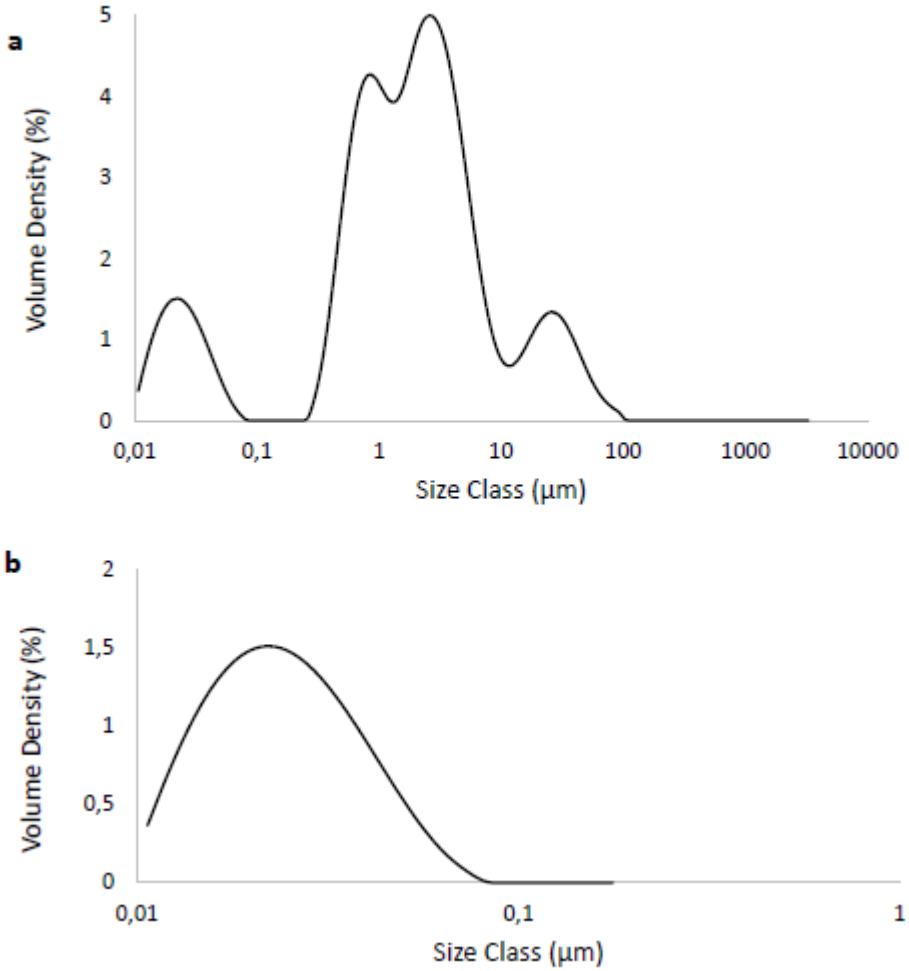

Figure 5.4.1. Size of Fe_3O_4 Nanoparticles (NP): (a): Involving all particles in the sample; (b) Nanosize cut of the particles

These results show that the sample used also contained particles larger than nanoparticles. The average size, including the two populations, was 180 ± 0.05 nm. This behavior of larger sizes found for Fe_3O_4 NP was also reported by Hansein et al. (2019) who found sizes between 96-400 nm. In the work by Abdsalam et al.(2017b), Fe_3O_4 NP sizes did not exceed 7 ± 0.2 nm. It is worth mentioning that the NPs used by Abdsalam et al. (2017b) were synthesized, and the NPs used by the present study and by Hansein et al. (2019) were obtained commercially. The size of NP is extremely important for the process since it can affect the binding and activation of membrane receptors and the expression of proteins (Jiang et al., 2008), thus acting to stimulate the growth of methanogenic *Archaea* (Mu et al., 2011b).

For better visualization, a cut in the graph was made of particles found only in nano size, which are shown in Figure 5.4.1b. The average size of these Fe_3O_4 NP was 23.56 ± 0.05 nm, which can be considered a greater size since some authors have reported a decrease in CH_4 production by using Fe NPs greater than 55 nm (Gonzalez-estrella et al., 2013; Hassanein et al., 2019). Another important factor is that these Fe_3O_4 NPs used in this work have a spherical shape (Figure 5.4.2), and this has improved the production of CH_4 in the work of Abdsalam et al. (2017b) which is explained by the greater membrane wrapping time required for the elongated particles.

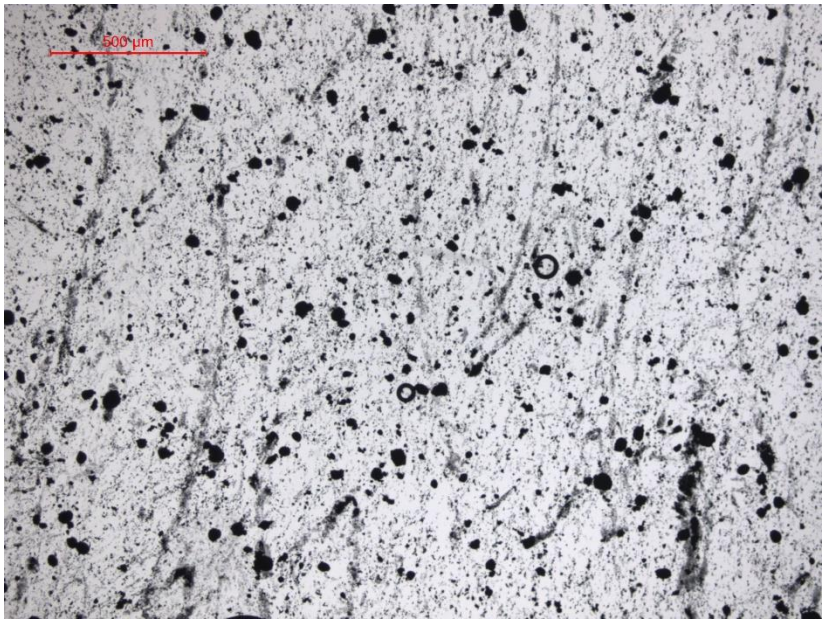

Figure 5.4.2. Mastersize images (50x) showing Fe_3O_4 NP

However, it is worth mentioning that besides size, another important factor to stimulate CH_4 production is the concentration in which the NP, in addition to the type of substrate being used and the interaction between them (Abdelsalam et al., 2016). For this reason, preliminary batch tests with different concentrations of NP Fe_3O_4 were carried out within the co-digestion of residues. It is worth mentioning that a zeta potential (ZP) analysis was carried out for the nanoparticles, to evaluate their dispersibility in the medium. However, it was not possible to obtain results, because they are magnetic particles, and have sizes larger than nano, the dispersion remained unstable, as has already been reported by Gonzalez et al. (2013).

3.2 Bacth preliminary assays

Table 5.4.4 shows the results of the accumulated CH_4 in triplicate of each of the tests with different concentrations of Fe_3O_4 NP, and Figure 5.4.3 shows the graph of CH_4 accumulated obtained over time.

Table 5.4.4. Final cumulative CH_4 production of co-digestion in different concentration of Fe_3O_4 NP

Assay	Cumulative CH_4 ($NmLgVS^{-1}$)[a]
Control	123.24 ± 9.60
NP 1	116.49 ± 17.45
NP 2	140.13 ± 95.60
NP 3	117.90 ± 10.68
NP 4	133.02 ± 106.29

[a:] mean of three replicates ± standard variation

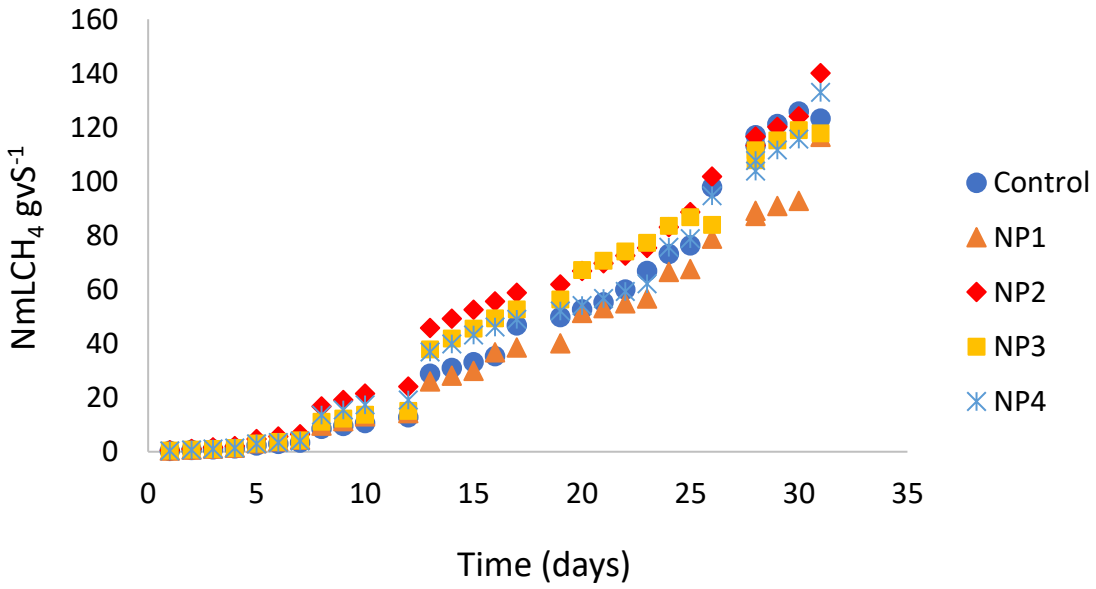

Figure 5.4.3 Cumulative CH_4 production testing four concentrations of Fe_3O_4 nanoparticle (NP) additions to co-digestion. NP1: 1 mg L^{-1}; NP2: 5 mg L^{-1}; NP3: 10 mg L^{-1}; NP4: 20 mg L^{-1}

Analysis of variance (ANOVA) was performed between the tests, but there was no significant difference between treatments with different concentrations of Fe_3O_4 NP (p-value = 0.1357 with $p < 0.05$). Perhaps what may have influenced this lack of difference between treatments was the use of filter cake, which can act as a nutritional supplement, since it is rich

in micronutrients (Volpi et al. 2021a). Although there is no significant difference, it can be seen that the NP 2 and NP 4 assays obtained CH_4 production greater than the control (Figure 5.4.3) and the NP 1 and NP 3 was below the control. Table 5.4.4 shows that the NP 2 test showed a 13% increase in CH_4 production compared to the control, while the NP 4 test showed a 7% increase in CH_4 production.

In the work of Hansein et al. (2019), they obtained a 25% increase in CH_4 production, using 15 mg L^{-1} of NP Fe_3O_4 in Biochemical Methane Potential (BMP) assays, with poultry litter residues, under mesophilic conditions. However, in this same work, a higher concentration of CH_4 (34%) was obtained using NP Ni with a concentration of 12 mg L^{-1}. The difference in substrates, experimental conditions, and the origin of the inoculum can influence these differences in production. It is worth mentioning the short lag phase found in the experiment could be because of the addition of Fe_3O_4 NPs, according to Krongthamchat et al. (2006).

Even in the preliminary test no significant difference in CH_4 production was observed, in the s-CSTR reactor, the concentration of 5 mg L^{-1} of NP Fe_3O_4 was used, the condition of the NP 2 experiment, which showed a greater increase in the CH_4 production concerning the control. It is also known that nanoparticles are not easy to be separated from biodegradable wastes, which may subsequently cause accumulation of inorganic pollutants (usually heavy metals) inside anaerobic digesters (Zhu et al., 2021). For this reason, it was decided to choose a lower concentration of NPs, to cause a less environmental impact on AD. With this, it is possible to observe differences in the operation of the continuous reactor with the addition of NP compared to the same reactor operation, but without the addition of NP (Volpi et al., 2021b).

3.3 Performance of a Semi-Continuous Stirred Tank Reactor

3.3.1 Biogas production

Figure 5.4.4 shows the results obtained from CH_4 production and removal of organic matter throughout the different OLRs used. In phases I and II, it is possible to observe an intense variation in the removal of organic matter, varying between approximately 30% and 70%. Along with this, there was also a small variation in CH_4 production, ranging from 0.1 to 0.5 $NLCH_4\ gVS^{-1}$. These variations are characteristic of the acidogenic phase, marking the start-up of the reactor. After approximately 60 days, that is, phase III, both the production of CH_4 and the removal of organic matter maintain stability, indicating the possibility that the reactor started the methanogenic phase. Between phase IV and phase V it is possible to observe that the production of CH_4 remains around 0.5 and 1 $NLCH_4\ gVS^{-1}$ showing a trend in the increase of CH_4 production. In phase VI, after the addition of Fe_3O_4NP, there was a 40% increase in CH_4 production (122 days), obtaining the highest CH_4 production throughout the entire operation, with $2.8 \pm 0.1\ NLCH_4\ gVS^{-1}$ and removal of $71 \pm 0.9\%$ of organic matter. In phase VII, the production of CH_4 begins to show a decrease, but the removal of the organic matter remains stable. In phase VII, the production of CH_4 remains low ($0.09 \pm 0.03\ NLCH_4\ gVS^{-1}$) and the removal of organic matter continues to decrease ($51 \pm 2.8\%$), reaching the collapse of the reactor in phase IX.

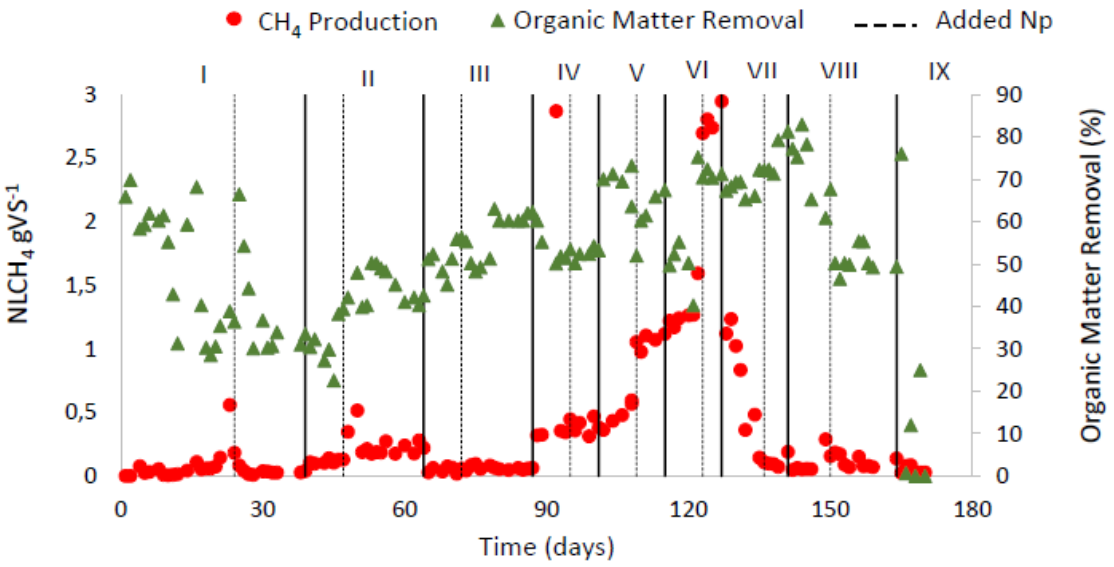

Figure 5.4.4. Methane production and organic matter removal along with the reactor operation according to the applied OLRs (g VS L^{-1} day^{-1}): 2.0 (Phase I); 2.35 (Phase II); 3.0 (Phase III); 4.0 (Phase IV); 4.7 (Phase V); 5.5 (Phase VI); 6.6 (Phase VII); 8.0 (Phase VIII); 9.0 (Phase IX)

In our previous study Volpi et al., 2021b (the operation was carried out in a reactor with co-digestion of the same residues as in this work and under the same experimental conditions) was obtained the maximum CH_4 production 0.233 ± 1.83 $NLCH_4$ gSV^{-1} and 83.08 ± 13.30% organic matter removal. The present work had an increase of 91% of the production of CH_4 about the previous work. This fact confirms that the presence of Fe_3O_4 NP contributed to better development and performance of the microbial community in the consumption of organic matter and conversion to CH_4 since Fe is a growth stimulant of methanogenic *Archaea* and they are dependent on this element to enzyme synthesis (Choong et al., 2016; Ni et al., 2013). In addition, the maximum production of CH_4 from the work of Volpi et al., 2021b was in the OLR of 4.16 gVS L^{-1} day^{-1}, with the reactor collapsed in the OLR of 5.23 gVS L^{-1} day^{-1}. In the present work it was possible to obtain the maximum performance of the reactor in the OLR of 5.5 gVS L^{-1} day^{-1}, and collapsing with OLR 9 gVS L^{-1} day^{-1}, showing that the presence of Fe_3O_4NP

made it possible to work with larger OLRs, resulting in greater volumes of feed in the reactor and consequent treatment higher volume of waste.

In the work of Hassanein et al. (2019) using poultry litter for BMP assays, with the addition of 15 mg L^{-1} Fe_3O_4 NP, maximum cumulative production of 339 $mLCH_4$ gVS^{-1} was obtained. In the work of Abdsalam et al. (2017b) in BMP tests with manure, 20 mg L^{-1} of Fe_3O_4 NP was added and 351 $mLCH_4$ gVS^{-1} was obtained. The literature has reported the use of NP in BMP assays, and smaller vials to assess NP activity. In the present study, it was possible to obtain 85% more CH_4 than those reported in the literature. It is worth mentioning that the use of the substrate with the type of NP interferes with the production of CH_4, in addition to the concentration of NP has been used also interfere. In the work by Abdsalam et al. (2016), it was confirmed that the use of Ni NP was the one that best impacted the increase in CH_4 production in the use of municipal solid waste. In the study by Ali et al. (2017) four concentrations of Fe_3O_4 NPs (50, 75, 100, and 125 mg L^{-1}) were tested in assays with municipal solid waste. The results showed that the addition of 75 mg L^{-1} Fe_3O_4NPs increases the CH_4 production by 53.3%. In contrast, less CH_4 production was observed by adding a high concentration of Fe_3O_4 NPs.

Absalam et al. (2016) showed that the addition of Fe_3O_4 magnetic NPs increased bacterial activity during onset up to 40 days of HRT. However, in the present study, an increase in bacterial activity was observed in the middle of the operation (phase IV, V, and VI, after 90 days), in agreement with Quing Ni et al (2013) who indicated that during the exposure of 50 mg L^{-1} of magnetic NPs the adverse effects were insignificant in bacteria and concluded that magnetic NPs appeared to be non-toxic during long-term contact. The best performance is due to the presence of $Fe2^{+}$ / $Fe3^{+}$ ions, introduced into the reactor in the form of nanoparticles that could be adsorbed as the growth element of anaerobic microorganisms (Abdelsalam et al., 2016). In addition, Fe_3O_4 magnetic NP ensures a distribution of the iron ions in the slurry through the corrosion of the NPs, thus maintaining the iron requirement of the reactor supplied

(Abdelsalam et al., 2016). The presence of NPs also shows a possible effect on the hydrolysis-acidification process, increasing the reduction of the substrate, since there were increasing amounts of organic matter removed in phases V, VI and VII, and a subsequent increase in the production of CH_4.

3.3.2 pH, ORP, and Alkalinity

Figure 5.4.5a shows the results obtained from the reactor inlet and outlet pH, as well as the results of oxide-reduction potential (ORP).

It is possible to observe that in the first days, the effluent pH is very acid, around 6 and the ORP values vary a lot. In addition, the feeding pH was daily adjusted to a neutral pH. These characteristics mark acidogenesis, and the intense oxidation-reduction reactions typical of the AD process (Vongvichiankul et al., 2017). After 60 days, it is possible to observe that the pH remains between 7.5 and 8 until the end of the operation, indicating that from this date on, the reactor started the methanogenesis phase, maintaining the pH stable and no more adjustment of the pH at the entrance. The same is true for ORP values, which after 60 days, remain around -460 and -490 mV.

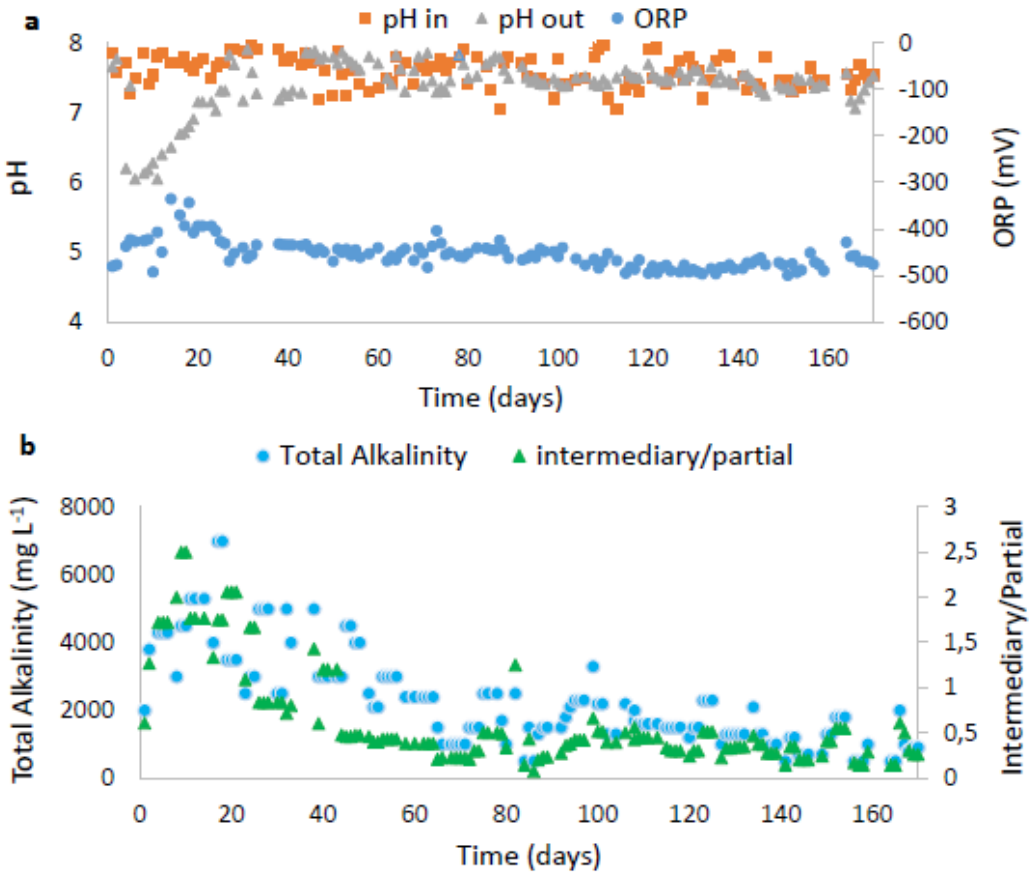

Figure 5.4.5. pH Oxidation Reduction Potential (a) and Alkalinity (b) along with the reactor operation according to the applied OLRs (g VS L^{-1} day^{-1}): 2.0 (Phase I); 2.35 (Phase II); 3.0 (Phase III); 4.0 (Phase IV); 4.7 (Phase V); 5.5 (Phase VI); 6.6 (Phase VII); 8.0 (Phase VIII); 9.0 (Phase IX)

In our previous work, methanogenesis was established only after 90 days, with stabilization of the pH and ORP values (Volpi et al., 2021b). In this present work, methanogenesis was established before, next 60 days, and everything indicates that it may have been due to the presence of NPs, since Abdsalam et al. (2017b) showed that the addition of Fe_3O_4 NP reduces the lag phase of AD. In addition, Feng et al. (2014) showed that the addition of Fe in the AD system can directly serve as an electron donor to reduce CO_2 in CH_4 through hydrogenotrophic methanogenesis causing improvement of CH_4 production, according to the reactions below (5.4.1), (5.4.2) and (5.4.3).

$$4H_2 + Fe_3O_4 = 4H_2O + 3Fe^0 \quad \text{(reaction 5.4.1)}$$

$$CO_2 + 4Fe^0 + 8H^+ = CH_4 + 4Fe^{2+} + 2H_2O$$ (reaction 5.4.2)

$$CO_2 + 4H_2 = CH_4 + 2H_2O$$ (reaction 5.4.3)

From this process the substrates would be deprived of hydrogen ions (H^+) which will increase the pH of the substrate and the capture of CO_2 also prevents the formation of carbonic acid, increasing the pH of the substrate (Abdelsalam et al., 2017b). This may explain the increase in pH after 24 days (Table 5.4.3) since it was the first addition of Fe_3O_4 NPs. The methanogenesis process stage was also stimulated, as this nano additive served as an electron donor that could reduce CO_2 to CH_4.

At the beginning of the operation, the ORP varied between -350 and -550 mV, and this variation is a characteristic of acidogenesis and reactor start-up (Volpi et al., 2021b). However, this variation in a start-up was much smaller than reported in Volpi et al. (2021b) (-800 and -300 mV) indicating greater stability of the operation. After approximately 40 days (Figure 5.4.5a), it is observed that the ORP remains practically constant until the end of the operation, varying between -480 and -400 mV, although the literature shows that the ORPs characteristic of the acidogenic and methanogenic phase is between -330 and -428 mV (Golkowska and Greger, 2013). This demonstrates the stability of the prevalence targeting of metabolic routes for the production of CH_4 and development of methanogenic A*rchaea* entire operation, which may have been optimized by the presence of Fe_3O_4 NP, since in the work of the same reactor operated and without the NPs the ORP values in methanogenic phase varied much more (-650 and -400 mV). These low ORP values in the system are characteristic of the presence of Fe NPs since they reduce the system's ORP to increase the conversion of complex compounds to volatile fatty acids and to be able to provide ferrous ions for the growth of fermentative and methanogenic *Archeae* (Lee and Lee, 2019).

It is important to demonstrate that the ORP values practically constant are in agreement with the OA values (section 3.3.3), which are in extremely low concentrations when the reactor

stabilizes in methanogenesis. Here it is worth emphasizing the differences in the ORP values found in the literature are varied due to the different raw materials applied, experimental conditions and the type of NP used.

Figure 5.4.5b shows the results of alkalinity obtained during the operation. It is possible to observe that the alkalinity is high up to 60 days, being following the pH and ORP and also with the presence of OA (Figure 5.4.6a section 3.3.3) characterizing the acidogenic step of the process. After 60 days, the intermediate/partial alkalinity (IA/PA) is below 0.3, which is considered ideal for AD, as it demonstrates stability (Ripley et al., 1986). As was the behavior of the ORP, the IA/PA also remained stable throughout the process, showing self-regulation of methanogenesis. In our previous study (Volpi et al., 2021b), this stability of alkalinity also only happened after 90 days, confirming the hypothesis that the presence of Fe_3O_4NP has reduced the lag phase. In addition, Fe_3O_4NP can absorb inhibitory compounds and act as a pH buffer, further improving the alkalinity of the process.

3.3.3 Indications of OA, Carbohydrate, and Alcohol indications

Figure 5.4.6 shows the results obtained from OA and carbohydrates and alcohols.

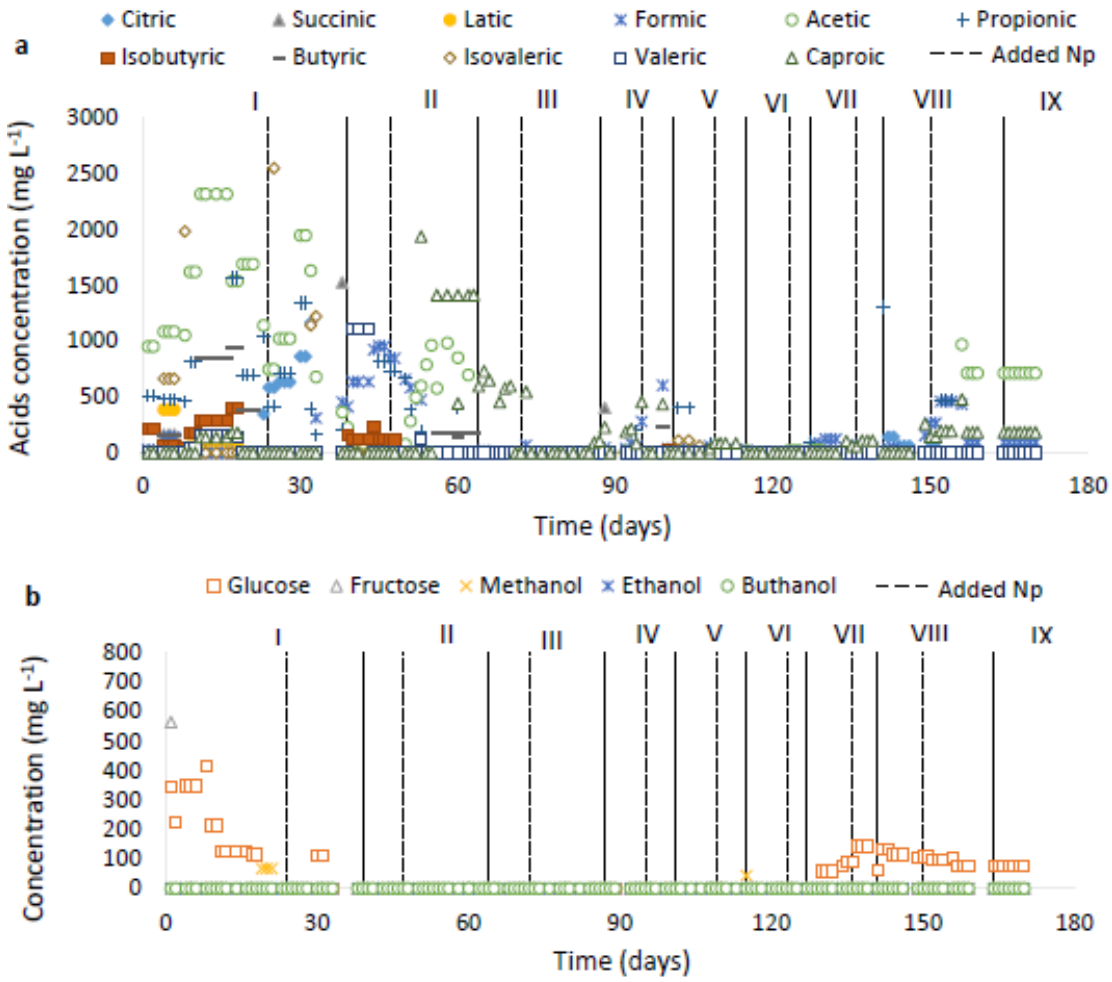

Figure 5.4.6 Values of Organic acids (a), Carbohydrates, and Alcohols (b) along with the reactor operation according to the applied OLRs (g VS L^{-1} day^{-1}): 2.0 (Phase I); 2.35 (Phase II); 3.0 (Phase III); 4.0 (Phase IV); 4.7 (Phase V); 5.5 (Phase VI); 6.6 (Phase VII); 8.0 (Phase VIII); 9.0 (Phase IX)

In phases, I and II (Figure 5.4.6a) the presence of high concentrations of OA confirms the start-up of the reactor, in the acidogenic phase. After 60 days, the concentrations of these OA decrease considerably, indicating the entrance of the reactor in the methanogenic phase and agreeing with what was discussed in the previous sections (section 3.3.1, 3.3.2).

At the beginning of the reactor operation (phase I), the concentration of acetic acid is relatively high, which is favorable for the CH_4 production process, since it is the main precursor of the CH_4 metabolic route (Wiegant et al., 1986). In addition to acetic acid, there is also the presence of propionic acid, which in concentrations above 1500 mg L^{-1} can be inhibitory to the

metabolic pathway of CH_4 production (Wang et al., 2009). But, this concentration decrease in phase I and phase II, and in acetic acid concentration increase at the end of phase II, indicating that the route of conversion of propionic acid to acetic acid may have prevailed at the beginning of the operation, as also occurred in our previous study (Volpi et al., 2021b). It is worth mentioning that in the presence of low H_2 pressure, propionic acid consumption is favored (Wiegant et al., 1986), and Fe is a trace element whose main substrate for oxidation-reduction reactions is H_2 (Choong et al., 2016). The presence of Fe_3O_4 NP may have favored the consumption of H_2, according to reaction 5.4.4 and reaction 5.4.1, and consequently helped in the consumption of propionic acid, favoring the formation of acetic acid and this having been converted to CH_4.

$$H_2 \leftrightarrow 2e^- + 2H^+ \quad \text{(reaction 5.4.4)}$$

In phases I and II it is also possible to observe the presence of formic acid, and its conversion to acetic acid is typical of acidogenesis (Choong et al., 2016). Therefore, in addition to the conversion of propionic acid to acetic acid, the conversion of formic acid to acetic acid may also have occurred at the end of acidogenesis, marking the beginning of methanogenesis (phase III-Figure 5.4.6a). In addition, the presence of Fe NP can increase the production of acetate and donate electrons for direct conversion of CO_2 into CH_4 by autotrophic via methanogenesis (Feng et al., 2014).

The Fe (III) reduction reaction is a favorable process to directly oxidize organics into simple compounds (Romero-Güiza et al., 2016), increasing the consumption of OA, and eliminate compounds that may be toxic to the process, by stimulating microbial growth, synthesis of necessary enzymes within the oxidation-reduction reactions and consequently greater efficiency in the digestion of organic matter (Choong et al., 2016; Lee and Lee, 2019). The positive effect of Fe (III) supplementation was attributed to the favorable redox conditions, which all evited the thermodynamic limitations on organic acid degradation. Furthermore, Fe

(III) can precipitate H_2S minimizing related inhibition phenomena (Romero-Güiza et al., 2016). The control of OA can allow a greater capacity of feed of the digester, without affecting the performance of digestion significantly (Zhang et al., 2015), this is what happened in the present study, since the used OLRs were higher than the experiment previous (Volpi et al., 2021b), with higher volumes of feed, and a stable operation, reaching high CH_4 production.

The presence of caproic acid draws attention at the end of phase II and the beginning of phase III (Figure 5.4.6a). Caproic acid is produced by elonging the chain of short-chain volatile fatty acids, such as acetic acid and butyric acid through an oxidation reaction, in which some species can gain energy by increasing the length of the volatile organic acids chain with reducing substrates such as ethanol and lactic acid (Owusu-Agyeman et al., 2020). However, in the operation, neither the presence of lactic acid nor ethanol was detected (Figure 5.4.6a and Figure 5.4.6b), but it seems that Fe_3O_4NP may have acted as this reducing substrate, donating electrons and allowing an increase in the chain of butyric and acetic acids. This fact may also have been caused by the continuous feeding process of the reactor, in which Fe_3O_4NP was added with a certain frequency, having a constant availability of the electron donor for the formation of caproic acid and in agreement with what was reported by Owusu-Agyeman et al. (2020). Even with the possible change of the route for the production of caproic acid, the production of CH_4 prevailed, indicating the self-regulation of the microbial consortium for the metabolic route of CH_4. Although not the focus of this work, the addition of Fe_3O_4 NP with the residues of the sugarcane industry can stimulate the production of caproic acid, and organic acid has high added value because it is used as antimicrobials for animal feed and precursors aviation fuel (Angenent et al., 2016).

Figure 5.4.6b shows that at the beginning of the operation there was greater availability of glucose, and when the reactor entered the phase of methanogenesis, the concentration of this glucose was very low, indicating the self-regulation of the process for CH_4 production. When

the reactor begins to decrease its production of CH_4, phases VII and VIII, the concentration of glucose increases again, indicating the start of the collapse of the operation.

2.6 Microbial community characterization

Figure 5.4.7 shows the observed values of richness (number of species-a), and the calculated values from diversity (Shannon index-b) and wealth estimate (Chao1 estimator-c) of the Samples. The results show that the number of species (Figure 5.4.7a) and richness (Figure 5.4.7b) of the A1 samples was higher than that of the A2 sample. This behavior is as expected for these results since the A1 samples are samples from the initial inoculum, that is, from the inoculum without having been inserted into the reactor. The A2 samples are from the inoculum when the CH_4 production was stabilized, that is to say, that the microbial community present is already "selected" for the specific metabolic route of CH_4 production according to the substrates used. In addition, the inoculum of Sample A1 comes from a mesophilic reactor, while Sample A2 comes from a thermophilic reactor and this temperature change may also have led to this difference between species of microorganisms. These results are consistent with what happened in our previous work (Volpi et al., 2021b) indicating that the presence of NP did not influence the diversity of microorganisms and the change in the microbial community from one sample to another.

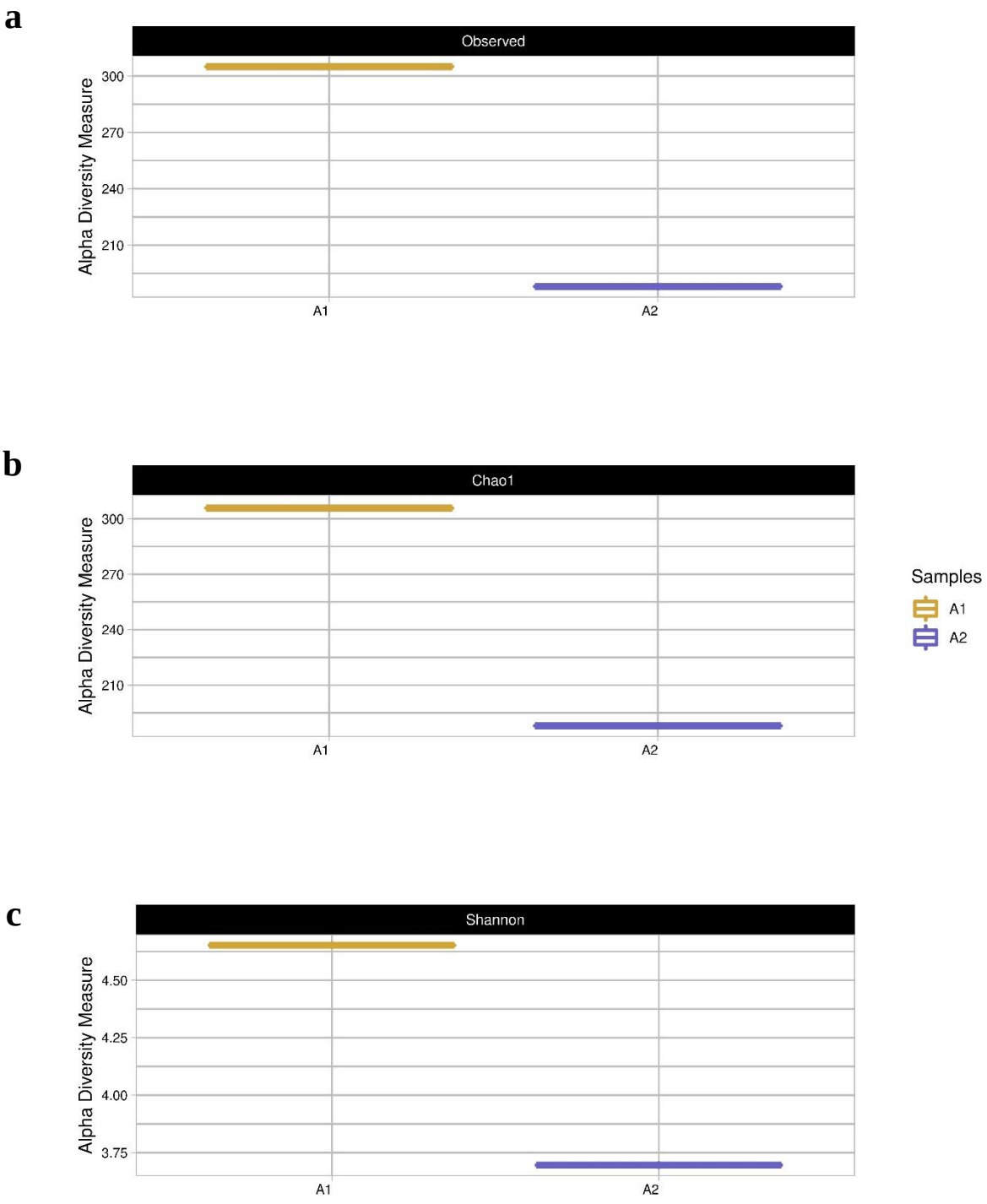

Figure 5.4.7. Observed values of (A) richness (number of species), (B) richness estimate (Chao1 estimator), and (C) calculated values of diversity (Shannon index) of Sample 1 (seed sludge) and Sample 2 (sludge from the s-CSTR stable operation, Phase IV)

The Shannon index obtained from sample A1 was close to 5.0, while that from sample A2 was less than 3.75. As discussed in Volpi et al., 2021b, when the value of the Shannon index

is greater than 5.0, it indicates greater microbial diversity in anaerobic digesters (Moraes et al., 2019). Thus, it can be seen that the A2 sample has a much lower microbial diversity than the A1, since these microorganisms are in stabilized metabolic routes for CH_4 production (due temperature change), indicating that this microbial community is even more specific.

Figure 5.4.8 shows the results obtained from phylum in relation to *Bacteria* order (a) and *Archaea* order (b) from samples A1 and A2.

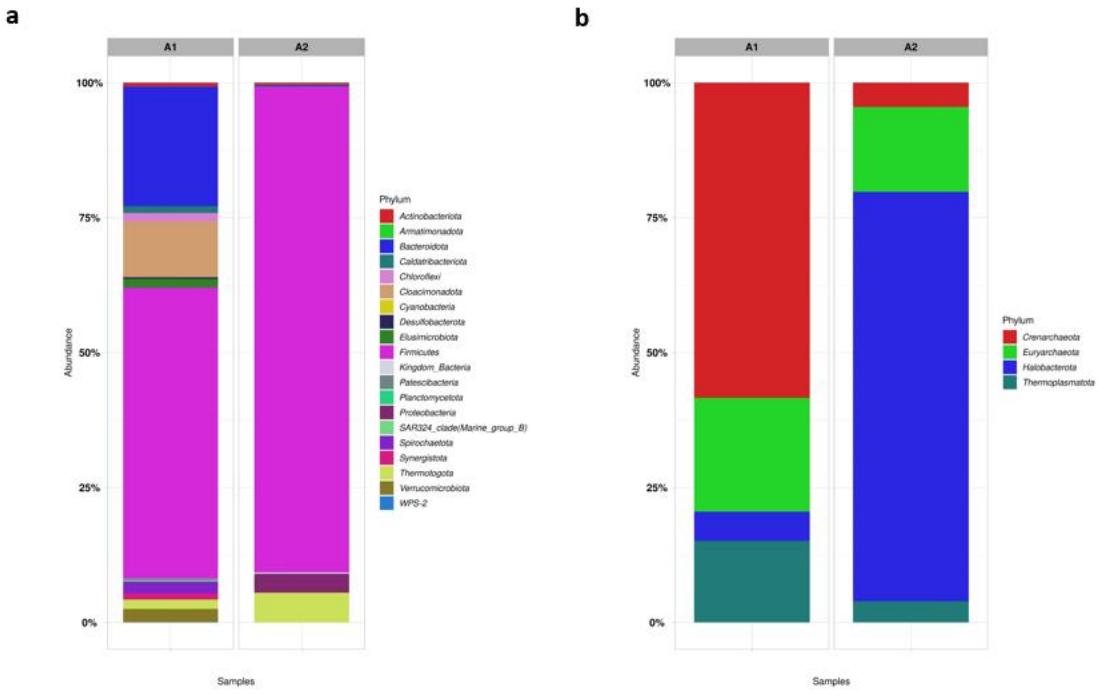

Figure 5.4.8. Relative abundance of microorganisms at the phylum level from Bacteria order (a) and *Archaea* order (b) from the seed sludge- Sample A1 and from the s-CSTR sludge with stable CH_4 production-Sample A2

Following what was discussed above, the phyla variety found in sample A1 (Figure 5.4.8a) is much larger than those found in A2. In sample A1 the main phyla found from *Bacteria* order was: (~25%) *Bacteroidota*, (~15%) *Cloacimonadota*, (~50%) *Firmicutes* and (~2%) *Spirochaetota*. Microorganisms of the phylum *Bacteroidota*, *Cloacimonadota*, and *Spirochaetota* are generally found in mesophilic processes and are bacteria responsible for the

fermentative and hydrolytic steps of AD (Xie et al., 2020; Zhang et al., 2021). The presence of these three phyla in the A1 sample and the absence of them in the A2 sample indicates how temperature influenced the change in the bacterial community since the A1 inoculum comes from a mesophilic process. The large presence of the *Firmicutes* phylum is to be expected since they are one of the main phyla of anaerobic processes, and most cellulolytic bacteria belong to them (Wu et al., 2020). In sample A2 the main phyla found are (~80%) *Firmicutes*, (~2%) *Protobacteria*, and (~5%) *Thermotogota*. The *Thermotogota* phylum is characteristic of thermophilic processes (Wang et al., 2018), and bacteria of the *Protobacteria* phylum are characteristic for degrading lignocellulosic material (Wu et al., 2020). It is important to mention that these two last phyla are present in smaller proportions in sample A1, indicating the possibility of a change in the microbial community due to experimental conditions and used substrates. Furthermore, in the previous co-digestion work (Volpi et al., 2021b) these same phyla were found in the sample when the reactor was stabilized for CH_4 production, indicating that the presence of Fe_3O_4 NP did not influence the change in the microbial community concerning order Bacteria. Zhang et al. (2021) showed that the presence of *Proteobacteria* followed by *Firmicutes* the bacteria were the central syntrophic acetogens for propionate oxidation via the methylmalonyl-CoA pathway, perhaps indicating the presence of this metabolic route when CH_4 production stabilized, as discussed in section 3.3.3.

About *Archaea* order phyla, in sample A1 it was observed (~25%) *Euryarchaeota* and in sample A2 (~20%) of the same phylum. This phylum is characteristic of methanogenic *Archaea*, responsible for the production of CH_4. In addition to this main phylum, other phyla of the *Archaea* order were also found, such as *Crenarchaeota, Halobacterota*, that also have methanogenic genera (Lyu et al. 2018).

Figure 9 shows the main genera found for samples A1 and A2 to the order *Bacteria* (a) and the order *Archaea* (b). As previously discussed, the A1 sample presented a very large microbial diversity, with no genus that was predominant in the process about the *Bacteria* order. Its genera of microorganisms come from the main phyla (*Bacteroidota, Cloacimonadota, Firmicutes*) and are characteristic of acidogenic and hydrolytic processes.

Sample A2 has some genera of the order *Bacteria* that emphasized such as (~5%) *Defluvitoga*, (~3%) *Hydrogenispora*, (~9%) *Ruminiclostridium*. These genera were also present in the reactor operation without the presence of Fe_3O_4 NP (Volpi et al., 2021b).

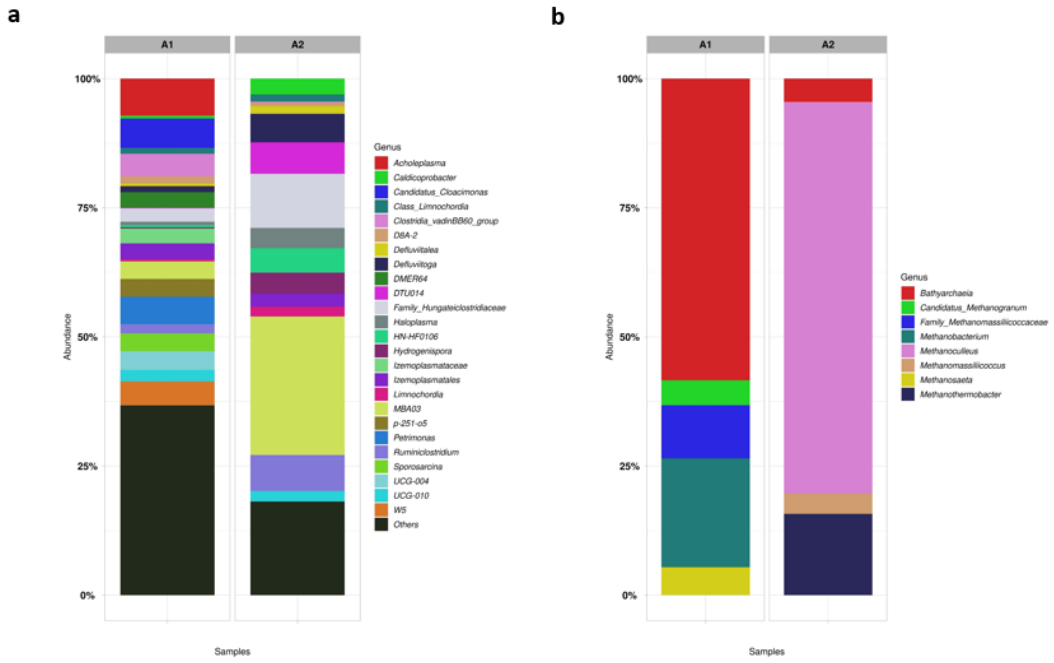

Figure 5.4.9. Relative abundance of microorganisms at the genus level from Bacteria order (a) and *Archaea* order (b) from the seed sludge- Sample A1 and from the s-CSTR sludge with stable CH4 production-Sample A2.

Defluvitoga genus, belonging to the phylum *Thermotogota*, is reported to be dominant in the degradation of organic materials in CSTRs or thermophilic bioelectrochemical reactors (Guo et al., 2014). *Ruminiclostridium*, belonging to the phylum *Firmicutes*, are hydrolytic bacteria characterized by metabolizing cellulosic materials, with a high concentration of lignocellulose (Peng et al., 2014), which is the case of residues used in reactor operation. In the

work by Kang et al. (2021) wheat straw was used for anaerobic digestion, and bacteria belonging to the genus *Ruminiclostrium* and *Hydrogenispora* were found as the main microorganisms. This fact leads to the association that such bacteria are present in the degradation of lignocellulose substrates since wheat straw and residues from the present work have a similar composition.

Hydrogenispora is acetogenic bacteria, which can ferment carbohydrates such as glucose, maltose, and fructose into acetate, ethanol, and H_2 (Kang et al., 2021). These bacteria can act in conjunction with hydrogenotrophic methanogens. In Figure 9b, the predominant methanogenic *Archaea* in sample A2 was (~70%) *Methanoculleus*. This methanogenic *Archaea* participates in the syntrophic oxidation of acetate (SAO) coupled with hydrogenotrophic methanogenesis pathway (Schnürer et al., 1999). Furthermore, it was also the main methanogenic found in the work by Volpi et al. (2021b). Therefore, it can be seen that despite the addition of NP in the reactor, the presence of the main *Archaea* comunity was not altered (*Methanoculleus*), but the presence of other methanogenic phyla that were not present in the first reactor was stimulated (*Crenarchaeota, Halobacterota)* .The genus (~15%) *Methanotermobacter* was also found in sample A2. This genus is characterized by being present in thermophilic anaerobic digestions and belongs to the obligate-hydrogenotrophic methanogens (Li et al., 2020). This fact corroborates the possibility that the predominant metabolic route in the co-digestion of vinasse, filter cake, and deacetylation liquor is syntrhophic acetate oxidation (SAO) coupled with hydrogenotrophic methanogenesis. Furthermore, it was discussed in section 3.3.3 that in the presence of low H_2 pressure, propionic acid consumption is favored, and Fe is a trace element whose main substrate for oxidation-reduction reactions is H_2. This confirms the fact that the presence of Fe_3O_4 NP may have reinforced that the main metabolic pathway for the co-digestion of these residues is through hydrogenotrophic methanogens.

In sample, A1 (Figure 5.4.9a) were found (~20%) *Methanobacterium* and (~7%) *Methanosaeta. Methanobacterium* is known as hydrogenotrophic methanogens while *Methanosaeta* is known as obligate-acetoclastic methanogen and has a strong affinity to acetate (Li et al., 2020). These two genera are not found in sample A2, indicating how there was a change in the microbial community from sample A1 to A2 due to different substrates and experimental conditions.

4. CONCLUSIONS

Through the present work, it was possible to conclude that the use of Fe_3O_4 NP is an additive that optimized the co-digestion of 1G2G ethanol industry residues, providing an increase of approximately 90% in CH_4 production. Despite not having significant differences between the different concentrations of NP in the batch process, the concentration of 5 mg L^{-1} of Fe_3O_4 NP was ideal for a stable continuous operation, with production stimulation, and without process inhibitions.

These nanoparticles proved to favor the reduction of the lag phase of the process, through a stabilized reactor operation. The reactor collapsed in OLRs of 9 gVS L^{-1} day^{-1}, being an OLR almost 2 times larger than that used in the operation without the presence of NP (9 *vs* 5 gVS L^{-1} day^{-1}). Furthermore, the methanogenesis was stabilized after 60 days of operation, being 30 days earlier than the operation without the addition of NP.

Fe_3O_4 NP did not influence the possible metabolic pathways of the process, on the contrary, they stimulated the growth of methanogenic *Archaea*, reinforcing that the main metabolic pathway of these residues in co-digestion is through SAO with hydrogenotrophic methanogenesis. *Methanoculleus* are the main methanogenic *Archaea* found in the process, and *Defluvitoga*, *Ruminiclostridium*, and *Hydrogenispora* are the main genus of *Bacteria* order in process, both with or without the addition of NP.

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5. REFERENCES

Abdelsalam, E., Samer, M., Attia, Y.A., Abdel-Hadi, M.A., Hassan, H.E., Badr, Y., 2017a. Effects of Co and Ni nanoparticles on biogas and methane production from anaerobic digestion of slurry. Energy Convers. Manag. 141, 108–119. https://doi.org/10.1016/j.enconman.2016.05.051

Abdelsalam, E., Samer, M., Attia, Y.A., Abdel-Hadi, M.A., Hassan, H.E., Badr, Y., 2017b. Influence of zero valent iron nanoparticles and magnetic iron oxide nanoparticles on biogas and methane production from anaerobic digestion of manure. Energy 120, 842–853. https://doi.org/10.1016/j.energy.2016.11.137

Abdelsalam, E., Samer, M., Attia, Y.A., Abdel-Hadi, M.A., Hassan, H.E., Badr, Y., 2016. Comparison of nanoparticles effects on biogas and methane production from anaerobic digestion of cattle dung slurry. Renew. Energy 87, 592–598. https://doi.org/10.1016/j.renene.2015.10.053

Ali, A., Mahar, R.B., Soomro, R.A., Sherazi, S.T.H., 2017. Fe3O4 nanoparticles facilitated anaerobic digestion of organic fraction of municipal solid waste for enhancement of methane production. Energy Sources, Part A Recover. Util. Environ. Eff. 39, 1815–1822. https://doi.org/10.1080/15567036.2017.1384866

Angenent, L.T., Richter, H., Buckel, W., Spirito, C.M., Steinbusch, K.J.J., Plugge, C.M., Strik, D.P.B.T.B., Grootscholten, T.I.M., Buisman, C.J.N., Hamelers, H.V.M., 2016. Chain Elongation with Reactor Microbiomes: Open-Culture Biotechnology to Produce Biochemicals. Environ. Sci. Technol. 50, 2796–2810. https://doi.org/10.1021/acs.est.5b04847

APHA, AWWA, W., 2012. Standard Methods for the Examination of Water and Wastewater, twenty-sec. ed. Washington, DC.

Brenelli, L.B., Figueiredo, F.L., Damasio, A., Franco, T.T., Rabelo, S.C., 2020. An integrated approach to obtain xylo-oligosaccharides from sugarcane straw: from lab to pilot scale. Bioresour. Technol. 123637. https://doi.org/10.1016/j.biortech.2020.123637

Choong, Y.Y., Norli, I., Abdullah, A.Z., Yhaya, M.F., 2016. Impacts of trace element supplementation on the performance of anaerobic digestion process: A critical review. Bioresour. Technol. 209, 369–379. https://doi.org/10.1016/j.biortech.2016.03.028

de Souza Moraes, B., Mary dos Santos, G., Palladino Delforno, T., Tadeu Fuess, L., José da Silva, A., 2019. Enriched microbial consortia for dark fermentation of sugarcane vinasse towards value-added short-chain organic acids and alcohol production. J. Biosci. Bioeng. 127, 594–601. https://doi.org/10.1016/j.jbiosc.2018.10.008

Demirel, B., Scherer, P., 2011. Trace element requirements of agricultural biogas digesters during biological conversion of renewable biomass to methane. Biomass and Bioenergy 35, 992–998. https://doi.org/10.1016/j.biombioe.2010.12.022

Demirel, B., Scherer, P., 2008. The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: A review. Rev. Environ. Sci. Biotechnol. 7, 173–190. https://doi.org/10.1007/s11157-008-9131-1

Deublin, D., Steinhauser, A., 2008. Biogas from Waste and Renewable Resources: An Introduction. Wily Online Library, Weinheim, Germany.

https://doi.org/1010029783527621705

Djalma Nunes Ferraz Júnior, A., Koyama, M.H., de Araújo Júnior, M.M., Zaiat, M., 2016. Thermophilic anaerobic digestion of raw sugarcane vinasse. Renew. Energy. https://doi.org/10.1016/j.renene.2015.11.064

Feng, Y., Zhang, Y., Quan, X., Chen, S., 2014. Enhanced anaerobic digestion of waste activated sludge digestion by the addition of zero valent iron. Water Res. 52, 242–250. https://doi.org/10.1016/j.watres.2013.10.072

Fuess, L.T., Kiyuna, L.S.M., Ferraz, A.D.N., Persinoti, G.F., Squina, F.M., Garcia, M.L., Zaiat, M., 2017. Thermophilic two-phase anaerobic digestion using an innovative fixed-bed reactor for enhanced organic matter removal and bioenergy recovery from sugarcane vinasse. Appl. Energy 189, 480–491. https://doi.org/10.1016/j.apenergy.2016.12.071

Golkowska, K., Greger, M., 2013. Anaerobic digestion of maize and cellulose under thermophilic and mesophilic conditions – A comparative study. Biomass and Bioenergy 56, 545–554. https://doi.org/https://doi.org/10.1016/j.biombioe.2013.05.029

Gonzalez-estrella, J., Sierra-alvarez, R., Field, J.A., 2013. Toxicity assessment of inorganic nanoparticles to acetoclastic and hydrogenotrophic methanogenic activity in anaerobic granular sludge. J. Hazard. Mater. 260, 278–285. https://doi.org/10.1016/j.jhazmat.2013.05.029

Guo, X., Wang, C., Sun, F., Zhu, W., Wu, W., 2014. A comparison of microbial characteristics between the thermophilic and mesophilic anaerobic digesters exposed to elevated food waste loadings. Bioresour. Technol. 152, 420–428. https://doi.org/10.1016/j.biortech.2013.11.012

Hagos, K., Zong, J., Li, D., Liu, C., Lu, X., 2017. Anaerobic co-digestion process for biogas production: Progress, challenges and perspectives. Renew. Sustain. Energy Rev. 76, 1485–1496. https://doi.org/10.1016/j.rser.2016.11.184

Hassanein, A., Lansing, S., Tikekar, R., 2019. Bioresource Technology Impact of metal nanoparticles on biogas production from poultry litter. Bioresour. Technol. 275, 200–206. https://doi.org/10.1016/j.biortech.2018.12.048

Janke, L., Leite, A., Batista, K., Weinrich, S., Sträuber, H., Nikolausz, M., Nelles, M., Stinner, W., 2016. Optimization of hydrolysis and volatile fatty acids production from sugarcane filter cake: Effects of urea supplementation and sodium hydroxide pretreatment. Bioresour. Technol. 199, 235–244. https://doi.org/10.1016/j.biortech.2015.07.117

Jiang, W.E.N., Kim, B.Y.S., Rutka, J.T., Chan, W.C.W., 2008. Nanoparticle-mediated cellular response is size-dependent 145–150. https://doi.org/10.1038/nnano.2008.30

Kang, Y.R., Su, Y., Wang, J., Chu, Y.X., Tian, G., He, R., 2021. Effects of different pretreatment methods on biogas production and microbial community in anaerobic digestion of wheat straw. Environ. Sci. Pollut. Res. https://doi.org/10.1007/s11356-021-14296-5

Krongthamchat, K., Riffat, R., Dararat, S., 2006. Effect of trace metals on halophilic and mixed cultures in anaerobic treatment. Int. J. Environ. Sci. Technol. 3, 103–112. https://doi.org/10.1007/BF03325913

Lee, Y.J., Lee, D.J., 2019. Impact of adding metal nanoparticles on anaerobic digestion performance – A review. Bioresour. Technol. 292, 121926. https://doi.org/10.1016/j.biortech.2019.121926

Li, Z., Wachemo, A.C., Yuan, H., Korai, R.M., Li, X., 2020. Improving methane content and yield from rice straw by adding extra hydrogen into a two-stage anaerobic digestion system. Int. J. Hydrogen Energy 45, 3739–3749. https://doi.org/10.1016/j.ijhydene.2019.07.235

Lyu Z., Shao N., Akinyemi T., Whitman W.B. 2018. Methanogenesis. Curr Biol 28:R727–

R732 . https://doi.org/10.1016/j.cub.2018.05.021

Mamani, J.B., Gamarra, L.F., 2014. Synthesis and Characterization of Fe3O 4 Nanoparticles with Perspectives in Biomedical Applications 17, 542–549.

Moraes, B.S., Junqueira, T.L., Pavanello, L.G., Cavalett, O., Mantelatto, P.E., Bonomi, A., Zaiat, M., 2014. Anaerobic digestion of vinasse from sugarcane biorefineries in Brazil from energy, environmental, and economic perspectives: Profit or expense? Appl. Energy 113, 825–835. https://doi.org/10.1016/j.apenergy.2013.07.018

Moraes, B.S., Zaiat, M., Bonomi, A., 2015. Anaerobic digestion of vinasse from sugarcane ethanol production in Brazil: Challenges and perspectives. Renew. Sustain. Energy Rev. 44, 888–903. https://doi.org/10.1016/j.rser.2015.01.023

Mu, H., Chen, Y., Xiao, N., 2011a. Bioresource Technology Effects of metal oxide nanoparticles (TiO 2 , Al 2 O 3 , SiO 2 and ZnO) on waste activated sludge anaerobic digestion. Bioresour. Technol. 102, 10305–10311. https://doi.org/10.1016/j.biortech.2011.08.100

Mu, H., Chen, Y., Xiao, N., 2011b. Effects of metal oxide nanoparticles (TiO 2, Al 2O 3, SiO 2 and ZnO) on waste activated sludge anaerobic digestion. Bioresour. Technol. 102, 10305–10311. https://doi.org/10.1016/j.biortech.2011.08.100

Ni, S.Q., Ni, J., Yang, N., Wang, J., 2013. Effect of magnetic nanoparticles on the performance of activated sludge treatment system. Bioresour. Technol. 143, 555–561. https://doi.org/10.1016/j.biortech.2013.06.041

Owusu-Agyeman, I., Plaza, E., Cetecioglu, Z., 2020. Production of volatile fatty acids through co-digestion of sewage sludge and external organic waste: Effect of substrate proportions and long-term operation. Waste Manag. 112, 30–39. https://doi.org/10.1016/j.wasman.2020.05.027

Peng, X., Börner, R.A., Nges, I.A., Liu, J., 2014. Impact of bioaugmentation on biochemical

methane potential for wheat straw with addition of Clostridium cellulolyticum. Bioresour. Technol. 152, 567–571. https://doi.org/10.1016/j.biortech.2013.11.067

Ripley, L.E., Boyle, W.C., Converse, J.C., 1986. Improved alkalimetric monitoring for anaerobic digestion of high-strength waste.

Romero-Güiza, M.S., Vila, J., Mata-Alvarez, J., Chimenos, J.M., Astals, S., 2016. The role of additives on anaerobic digestion: A review. Renew. Sustain. Energy Rev. 58, 1486–1499. https://doi.org/10.1016/j.rser.2015.12.094

Scherer, P., Lippert, H., Wolff, G., 1983. Composition of the major elements and trace elements of 10 methanogenic bacteria determined by inductively coupled plasma emission spectrometry. Biol. Trace Elem. Res. 5, 149–163. https://doi.org/10.1007/BF02916619

Schnürer, A., Zellner, G., Svensson, B.H., 1999. Mesophilic syntrophic acetate oxidation during methane formation in biogas reactors. FEMS Microbiol. Ecol. 29, 249–261. https://doi.org/10.1016/S0168-6496(99)00016-1

Sh, U., 2010. Mineral Requirements for Mesophilic and Thermophilic Archive of SID. Arch. SID 4, 33–40.

Volpi, M.P.C., Brenelli, L.B., Mockaitis, G., Rabelo, S.C., Franco, T.T., Moraes, B.S., 2021a. Use of lignocellulosic residue from second-generation ethanol production to enhance methane production through co-digestion. Bioenergy Res. https://doi.org/10.1007/s12155-021-10293-1

Volpi, M.P.C., Junior, A.D.N.F., Franco, T.T., Moraes, B.S., 2021b. Operational and biochemical aspects of co-digestion (co-AD) from sugarcane vinasse, filter cake, and deacetylation liquor. Appl. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-021-11635-x

Vongvichiankul, C., Deebao, J., Khongnakorn, W., 2017. Relationship between pH,

Oxidation Reduction Potential (ORP) and Biogas Production in Mesophilic Screw Anaerobic Digester. Energy Procedia 138, 877–882. https://doi.org/10.1016/j.egypro.2017.10.113

Wang, P., Wang, H., Qiu, Y., Ren, L., Jiang, B., 2018. Microbial characteristics in anaerobic digestion process of food waste for methane production–A review. Bioresour. Technol. 248, 29–36. https://doi.org/10.1016/j.biortech.2017.06.152

Wang, T., Zhang, D., Dai, L., Chen, Y., Dai, X., 2016. Effects of metal nanoparticles on methane production from waste-activated sludge and microorganism community shift in anaerobic granular sludge. Sci. Rep. 6, 1–10. https://doi.org/10.1038/srep25857

Wang, Y., Zhang, Y., Wang, J., Meng, L., 2009. Effects of volatile fatty acid concentrations on methane yield and methanogenic bacteria. Biomass and Bioenergy. https://doi.org/10.1016/j.biombioe.2009.01.007

Wiegant, W.M., Hennink, M., Lettinga, G., 1986. Separation of the propionate degradation to improve the efficiency of thermophilic anaerobic treatment of acidified wastewaters. Water Res. 20, 517–524. https://doi.org/https://doi.org/10.1016/0043-1354(86)90202-2

Wu, X., Tian, Z., Lv, Z., Chen, Z., Liu, Y., Yong, X., Zhou, J., Xie, X., Jia, H., Wei, P., 2020. Effects of copper salts on performance, antibiotic resistance genes, and microbial community during thermophilic anaerobic digestion of swine manure. Bioresour. Technol. 300, 122728. https://doi.org/10.1016/j.biortech.2019.122728

Xie, S., Li, X., Wang, C., Kulandaivelu, J., Jiang, G., 2020. Enhanced anaerobic digestion of primary sludge with additives: Performance and mechanisms. Bioresour. Technol. 316, 123970. https://doi.org/10.1016/j.biortech.2020.123970

Yu, B., Lou, Z., Zhang, D., Shan, A., Yuan, H., Zhu, N., Zhang, K., 2015. Variations of organic matters and microbial community in thermophilic anaerobic digestion of waste activated sludge with the addition of ferric salts. Bioresour. Technol. 179, 291–298.

https://doi.org/10.1016/j.biortech.2014.12.011

Zhang, L., Gong, X., Wang, L., Guo, K., Cao, S., Zhou, Y., 2021. Metagenomic insights into the effect of thermal hydrolysis pre-treatment on microbial community of an anaerobic digestion system. Sci. Total Environ. 791, 148096. https://doi.org/10.1016/j.scitotenv.2021.148096

Zhang, W., Wu, S., Guo, J., Zhou, J., Dong, R., 2015. Performance and kinetic evaluation of semi-continuously fed anaerobic digesters treating food waste: Role of trace elements. Bioresour. Technol. 178, 297–305. https://doi.org/10.1016/j.biortech.2014.08.046

Zhang, Y., Jing, Y., Zhang, J., Sun, L., Quan, X., 2011. Performance of a ZVI-UASB reactor for azo dye wastewater treatment. J. Chem. Technol. Biotechnol. 86, 199–204. https://doi.org/10.1002/jctb.2485

Zhang, Y., Zhang, Z., Suzuki, K., Maekawa, T., 2003. Uptake and mass balance of trace metals for methane producing bacteria. Biomass and Bioenergy 25, 427–433. https://doi.org/10.1016/S0961-9534(03)00012-6

Zhang, Z., Guo, L., Wang, Y., Zhao, Y., She, Z., Gao, M., Guo, Y., 2020. Application of iron oxide (Fe3O4) nanoparticles during the two-stage anaerobic digestion with waste sludge: Impact on the biogas production and the substrate metabolism. Renew. Energy 146, 2724–2735. https://doi.org/10.1016/j.renene.2019.08.078

Zhu, X., Blanco, E., Bhatti, M., Borrion, A., 2021. Impact of metallic nanoparticles on anaerobic digestion: A systematic review. Sci. Total Environ. 757. https://doi.org/10.1016/j.scitotenv.2020.143747

5.5 PAPER 5

Metaproteomics of anaerobic co-digestion of residues from First and Second generation ethanol production with biogas generation

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ABSTRACT

The proteomic analysis has been highlighted as a powerful tool for deeper investigation of the anaerobic digestion (AD), but less information was found about the co-digestion of ethanol production residues. In this context, this study aimed to analyze the repertoire of proteins from anaerobic co-digestion performed in reactors that contained residues from the production of First- generation (1G) and Second- generation (2G) ethanol for biogas production. Proteomics analysis was performed for three types of samples: anaerobic sludge before being inserted into the reactor (SI), semi-continuous stirred reactor (s-CSTR) with co-digestion of filter cake, vinasse, and deacetylation liquor (R-CoAD) and s-CSTR with co-digestion of these same residues with the addition of Fe_3O_4 nanoparticles (R-NP). Protein extracts were analyzed by shotgun high-resolution Mass Spectrometry for a Metaproteomics analysis. Most proteins identified were related to the carbohydrate metabolism and amino acid metabolism. The metabolic routes annotated for the three samples were very similar, with minor changes in the initial stages of the bioprocess. The main metabolic routes annotated for the generation of residues or metabolic products from the production of 1G2G ethanol in co-digestion was syntrophic acetate oxidation process coupled with hydrogenotrophic methanogenesis, with the production of CH_4 occurring preferentially via CO_2 reduction.

Keywords: Proteins, vinasse, filter cake, anaerobic inoculum, deacetylation liquor, metabolic pathway

1. INTRODUCTION

Biogas production through anaerobic digestion (AD) and the energy recovery from methane (CH_4) are some of the ways that have been studied for the generation of renewable energy and reduction of greenhouse gas (GHG) emissions (Longati et al. 2020). Literature shows as substrates for AD, a variety of wastes can be employed such as restaurant food waste, agro-industrial waste, animal manure, lignocellulosic waste, and domestic sewage (Meyer et al. 2018; Parralejo et al. 2019; Pramanik et al. 2019). Among several agro-industry residues, the use of by-products from ethanol production, such as vinasse, distincts itself in the production of biogas, because it is a residue with high amount of organic matter and provides a way for the integration of ethanol plants in biorefinery concepts since there is the conversion of waste into energy (Moraes et al. 2015; Fuess et al. 2017).

The distilleries of First generation (1G) ethanol production generate residues in addition to vinasse, such as filter cake, sugarcane straw, which also have huge potential to produce methane (CH_4) within anaerobic digestion (Volpi et al. 2021a), making sugarcane industrial plants to produce bioethanol, and another type of bioenergy. An additional strategy to produce biogas is from the residues of second-generation ethanol (2G) production from lignocellulosic residues, such as sugarcane straw, thus further increasing the productivity of bioethanol and biogas (Longati et al. 2020). For the production of 2G ethanol, several pretreatments are applied to lignocellulosic materials. Brenelli et al. (2020) demonstrated the efficiency of acetic groups removal through the alkaline pretreatment of sugarcane straw to produce 2G ethanol. From this process, a residue rich in acetate emerges, which is the deacetylation liquor that has a high biochemical methane potential (BMP) (Volpi et al. 2021a).

To promote the integration of 1G2G ethanol biorefineries, we have previously demonstrated that the anaerobic co-digestion of vinasse, filter cake, both from the production of 1G ethanol and the deacetylation liquor (2G ethanol) in a continuous reactor allows the

recovery of 234 $NmLCH_4\ gVS^{-1}$ (Volpi et al. 2021c). In addition, by converting the biogas produced into electricity, it is possible to obtain an installed capacity of 58 MW for a 1G2G ethanol plant, and if this biogas is purified using biomethane, it is possible to supply the entire truck fleet of the largest ethanol plant in Brazil during the season period (Volpi et al. 2021b).

Despite all the operational discussion of the co-digestion of residues from ethanol production for CH_4 recuperation, biochemical aspects of the process need to be better understood. The AD process is quite complex from a microbiological point of view. The consortium of bacteria responsible for the metabolism of waste to the final product is composed of several species of Bacteria and *Archaea*, and they may prefer different metabolic routes, depending on the experimental conditions, the substrates used, since there are numerous metabolic routes within the process (Abram et al. 2011). We have demonstrated that in the microbial consortium of co-digestion of vinasse, filter cake, and deacetylation liquor, the predominant *Archaea* group was *Methanoculleus*, that are part of the metabolic route syntrophic acetate oxidation (SAO) coupled with hydrogenotrophic methanogenesis (Volpi et al. 2021c), that indicates this metabolic route was possibly the main one for the production of CH_4 from that residues. However, just by identifying the existing microorganisms, it is difficult to distinguish which possible route is being preferred in the process, according to the experimental conditions. Little is known about the functional activities of the various abundant groups of anaerobic sludges from AD bioreactors (Abram et al. 2011). Therefore, it is important to gain insights into the biochemistry of the bioprocess to achieve better performance and optimization of sugarcane residues co-digestion.

The literature is quite scarce regarding proteomics analysis of anaerobic reactors that use sugarcane residues, mainly 2G ethanol residues. Even less information is found on the proteomics analysis of residues anaerobic co-digestion from the sugar-energy industry. Studies report that the metaproteomics approach was successful applied to analyze the expression of

key microbial functions in various environments, including improved biological phosphorus removal reactors, activated sludge, and local mine acid drainage, as well as under local soil and seascapes (Wu et al. 2013).

Therefore, the present work reports the metaproteomic analysis of samples from anaerobic reactors that contained residues from the production of 1G ethanol and the production of 2G ethanol in co-digestion for biogas production. Metaproteomic analyses were performed for three types of samples: the first type is the anaerobic sludge used as inoculum, that is, the seed sludge; second type is from a reactor with co-digestion of filter cake, vinasse, and deacetylation liquor, and the last sample type is from another reactor with the same previous co-digestion condition with the addition of Fe_3O_4 nanoparticles (NP). The goal was to analyze if there would be changes in the expressed proteins between the different sample types and if there could be a change or preference in the metabolic routes detected in these complex microorganism communities.

2. MATERIAL AND METHODS

2.1 Residues and Inoculum

The residues used in the reactor operation as substrate were vinasse, filter cake, and deacetylation liquor. The vinasse and filter cake, both from the production of 1G ethanol, were collected from Iracema sugarcane plant (São Martinho group, Iracemápolis, São Paulo state, Brazil) and the liquor from the straw pretreatment process, performed at the Brazilian Biorenewables National Laboratory (LNBR) from the Brazilian Center for Research in Energy and Materials (CNPEM).

The microbial consortium used as inoculum in the process was obtained from a mesophilic reactor (35°C) BIOPAC®ICX – Paques treating vinasse, also from the same Iracema sugarcane plant in Iracemápolis.

2.2 Semi-continuous stirred reactor (s-CSTR) operation

Proteomics analysis was performed on three different samples, one sample being the seed inoculum, that is, the inoculum before being inserted into the reactor that was called sample SI, and two samples from two different semi-continuous stirred reactors. The sample from the first reactor is called the R-CoAD sample, which was composed by microbial communities from the co-digestion of vinasse, filter cake, and deacetylation liquor. The sample from the second reactor is called the sample R-NP, which is composed of the same co-digestion as the first reactor, but with the addition of Fe_3O_4NP. The NP concentration in s-CSTR was 5 mg L^{-1}.

The sample R-CoAD was obtained from the reactor operation described in our previous work (Volpi et al. 2021c) and the sample R-NP was obtained from the reactor operation as described in our previous work (Volpi et al. 2022). The two s-CSTR were operated under 55°C with 4 liters-working volume and the co-digestion of the residues was added in the proportion of volatile solids (VS): 70% VS of vinasse, 20% VS of filter cake, and 10% VS of deacetylation liquor. Both samples were collected when the CH_4 operation was stabilized during reactor operation. The inoculum was characterized in terms of VS and total solids (TS). The inoculum presented 0.0076 ± 0.00 g mL^{-1} in terms of VS and 0.0146 ± 0.00 g mL^{-1} in terms of TS. Figure 5.5.1 exemplifies how the samples from the microbial consortium were obtained to perform the metaproteomics analyses. Table 5.5.1 summarizes some indicators of the process during the stable operation of the two reactors (Volpi et al. 2021c; Volpi et al., 2022).

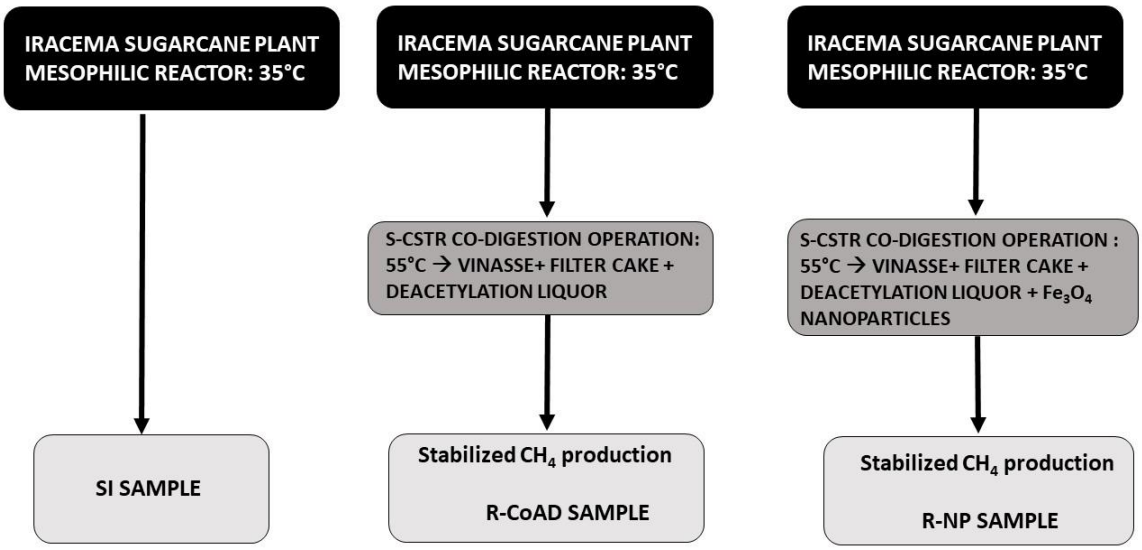


Figure 5.5.1 s-CSTR operation scheme and obtaining samples for proteomics analysis

Table 5.5.1. Summary of operating indicators results of the two reactors used for sample removal for proteomics analysis

	R-CoAD reactor	R-NP reactor
Organic Matter Removal (%)	83	71
CH_4 production ($NmLCH_4\ gVS^{-1}$)	234	2800
Organic Load Rate ($gVS\ L^{-1}\ day^{-1}$)	4.16	5.23

2.3 Metaproteomic Analysis

2.3.1 Protein Extraction

Proteins were recovered from the sludge following the protocol Hurkman and Tanaka (1986) in triplicates. All samples were lyophilized in the Modulyod FR-Drying Digital Unit lyophilizer (Thermo Fisher, USA). After lyophilization, PVPP (1%) and (2 %) B-mercaptoethanol extraction buffer was added to 1 mL of lyophilized sample. Washes were

carried out in a cold room at 4°C and in an orbital shaker, followed by washes with Phenol in a 10,000g centrifuge for 30 minutes at 4°C. After the washes, new washes of the sample with 100% methanol + 0.1 M Ammonium acetate were started, and then the sample was precipitated with acetone. This sample was dried, resuspended in Urea (7M) and Thiourea (2M) solubilization buffer and then the sample was desalted using a fresh 50mM NH_4HCO_3 solution, with the aid of an Amicon®Ultra Centrifugal Filters column from Millipore (cat # UFC 5003BK), selective for 3000-10000 NMWL.

After this process, digestion with Trypsin, through RapiGest SF (0.2%) and TFA solution. The samples were dried in a "speed vac". After drying, they passed through a C18 purification column ZipTip Reversed-Phase ZipTip C18, P10 (Millipore, cat# ZTC18M096, USA) before being inserted into the mass spectrometer.

2.3.2 Two-Dimensional LC-MS/MS Analysis

The LC-MS was performed on a NanoElute (Bruker Daltonik) system coupled online to a hybrid TIMS-quadrupole TOF mass spectrometer (timsTOF Pro) (Meyer et al. 2018) (Bruker Daltoniks, Germany) via a nano-electrospray ion source Captive Spray (Bruker Daltoniks, Germany). For the gradient run (22 min. total run), approximately 200 ng of peptides were separated on a Bruker TEN column 10 cm × 75 µm ID, 1.9 µm C18 reversed-phase column (Bruker) at a flow rate of 500 nL min^{-1} in an oven compartment heated to 50°C. To analyze samples from whole-proteome digests, we used a gradient starting with a linear increase from 2% B to 35% B over 18 min, followed by a further linear increase to 95% B in 2 min which was held constant for 2 min. The column was equilibrated using 4 volumes of solvent A. The mass spectrometer was operated in data-dependent PASEF (Meier et al. 2015) mode with 1 survey TIMS-MS and 4 PASEF MS/MS scans per acquisition cycle. We analyzed an ion mobility range from 1/K0 = 1.3 to 0.85 Vs cm-2 using equal ion accumulation and ramp time

in the dual TIMS analyzer of 100 ms each. Suitable precursor ions for MS/MS analysis were isolated in a window of 2 Th for m/z < 700 and 3 Th for m/z > 700 by rapidly switching the quadrupole position in sync with the elution of precursors from the TIMS device. The collision energy was lowered stepwise as a function of increasing ion mobility, starting from 27 eV for 1/K0 = 0.85 Vs cm-2 and 45 eV for 1/K0 = 1.3 Vs cm-2. We made use of the m/z and ion mobility information to exclude singly charged precursor ions with a polygon filter mask and further used 'dynamic exclusion' to avoid re-sequencing of precursors that reached a 'target value' of 20,000 a.u. The ion mobility dimension was calibrated linearly using three ions from the Agilent ESI LC/MS tuning mix (m/z, 1/K0: 622.0289, 0.9848 Vs cm-2; 922.0097, 1.1895 Vs cm-2; and 1221.9906, 1.3820 Vs cm-2). The Mass Spectrometry was performed at the Max Feffer Laboratory of Plant Genetics Department of Genetics Esalq/Usp.

2.3.3 Processing Parameters and Database Search

All MS/MS samples were processed using PEAKS Studio Version 10.6 (Bioinformatics Solutions Inc., Waterloo, ON, USA) Software. Mass spectra were searched against the UniProtKB/SwissProt database (January 16th of 2019), using the following search parameters. Carbamidomethylation of cysteine was used as fixed amino acid modification, and oxidation of methionine and acetylation (Protein N-term), as variable modifications. Trypsin was selected as the proteolytic enzyme, with a maximum of two potential missed cleavages. Peptide and fragment ion tolerance was set to 20 ppm and 0.05 Da, respectively. The maximum false-positive discovery rate (FDR) in Scaffold was set up to 1% at protein and peptide level, with one unique peptide criterion to report protein identification. All protein hits were identified with confidence of > 95%. Protein quantification was performed using signal intensity (area under the curve, AUC).

The predicted protein identifications were obtained with the embedded ion accounting algorithm of PEAKS Software searching into the database for *NCBI non-redundant database.* The mass spectrometry proteomics data were deposited in the ProteomeXchange consortium via the PRIDE partner repository with the dataset identifier PXD029938.

2.3.4 Data Analysis

For metaproteomics data analysis, a list of potential contaminant proteins that were not from microorganisms, including plant and animal proteins were removed from our main data set of identified proteins. One protein from each Protein Group were included in the data set. For quantitative analysis the intensities were transformed to log base two and filtered to have at least two valid values in each group. More than two non-valid values for the same protein identified were assigned as zero. We used InteractVenn (Herbele et al., 2015) to determine common and exclusive proteins in each biological condition. Differentially expressed proteins between biological samples were examined by two-tailed Student's t-test.

The annotation of the identified proteins was performed with the updated Gene Ontology analysis of frequency of GO terms in ID/mapping module from UniProt and database of Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.kegg.jp/kegg/). The version of KEGG used for annotation was the number 5 of KEGG Mapper [2] accessed on August 20th, 2021. The KEGG was mainly used to trace the metabolic routes of the samples. Protein sequences were submitted to BlastKOALA (https://www.kegg.jp/blastkoala/) in the KEGG for functional annotation.

To analyze the correlation between samples in the protein expression, a heatmap was created with R version 5.1.2 (http://www.r-project.org) using the heatmap function, a heat map.2 function in R's 'gplot' package (version 3.0.1). The proteins were classified based on the Pearson correlation coefficients as a similarity measure in protein expression and Euclidean

hierarchical clustering method. The figure 1SM in supplementary materials (SM) show the Pearson correlation graphic.

3. Results and Discussion

3.1 Identified Proteins

In general, a total of 439 proteins were found, being 319 proteins for the SI sample, 293 proteins for the R-CoAD sample, and 299 proteins for the R-NP sample. From this data, contaminating proteins and proteins from other organisms were excluded, but taking into account proteins of the same suggested function attributed to different microbial species. Table 5.5.1SM, Table 5.5.2SM and Table 5.5.3SM show the identified proteins that were differentially expressed between the samples. These tables also contain the NCBI-Uniprot identifier of each protein. The samples R-NP and R-CoAD were the ones with the highest numbers of differentially expressed proteins since this samples were from different reactors. However, this does not mean that they have followed different metabolic routes for CH_4 production, but that the addition of Fe_3O_4 NP in the reactor may have influenced a higher or lower expression of a certain protein, as NP acts as an *Archaea* growth stimulant and also acts as a protein cofactor (Abdelsalam et al. 2017; Hassanein et al. 2019) improving the AD process.

Figure 5.5.2 shows the results obtained from Venn diagram where shows how many proteins were exclusive to each sample and how many proteins the sample has in common. For Venn diagram and functional analysis, the Uniprot identifier of proteins that obtained the highest coverage within the same group of proteins was used since many proteins of the same group with the same function were identified. The SI sample got exclusive 48 proteins, while the R-CoAD sample got 10 and the R-NP sample got 17. The identification of each of these proteins can be found in Table 2SM. Sample R-CoAD and Sample R-NP have 24 proteins in

common, that is the more quantity of proteins in common, this can indicate that these two samples may have the same metabolic route as expected because they have the same substrates used in AD process.

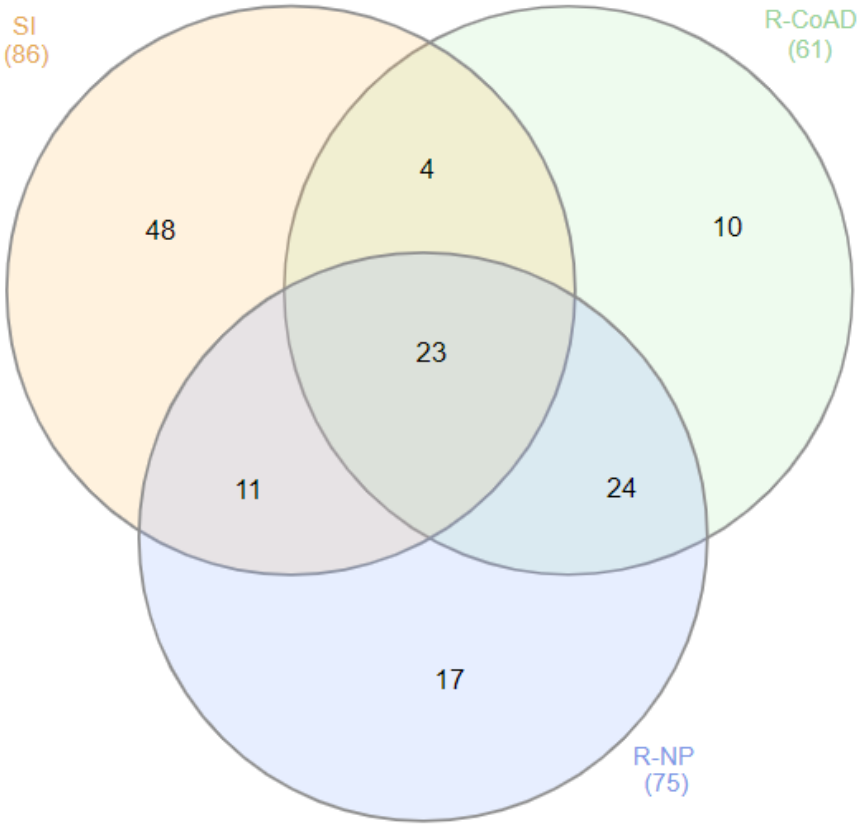


Figure 5.5.2. Venn Diagram about proteins of each sample. SI: seed inoculum, R-NP: Nanoparticles reactor, R-CoAD: Co-digestion reactor

Figure 5.5.3 shows the relative abundance of proteins found in each of the samples. It is possible to observe that sample SI has a greater diversification of proteins than the other samples, like was identified in Figure 5.5.2. This could have happened because the anaerobic sludge from sample SI was not analyzed at the time when the CH_4 production was stable, indicating that there may be more metabolic pathways in the process, compared to samples R-CoAD and R-NP. This situation was seen in the work by Volpi et al. (2021c) about the

identification of the microbial community in samples SI and R-CoAD. The sample of seed inoculum had a greater diversification of microorganisms concerning sample of reactor, making the substrates and reactor conditions directed the co-digestion process, selecting new community structures in a way that some members of the community have increased while others have decreased their abundance relatives.

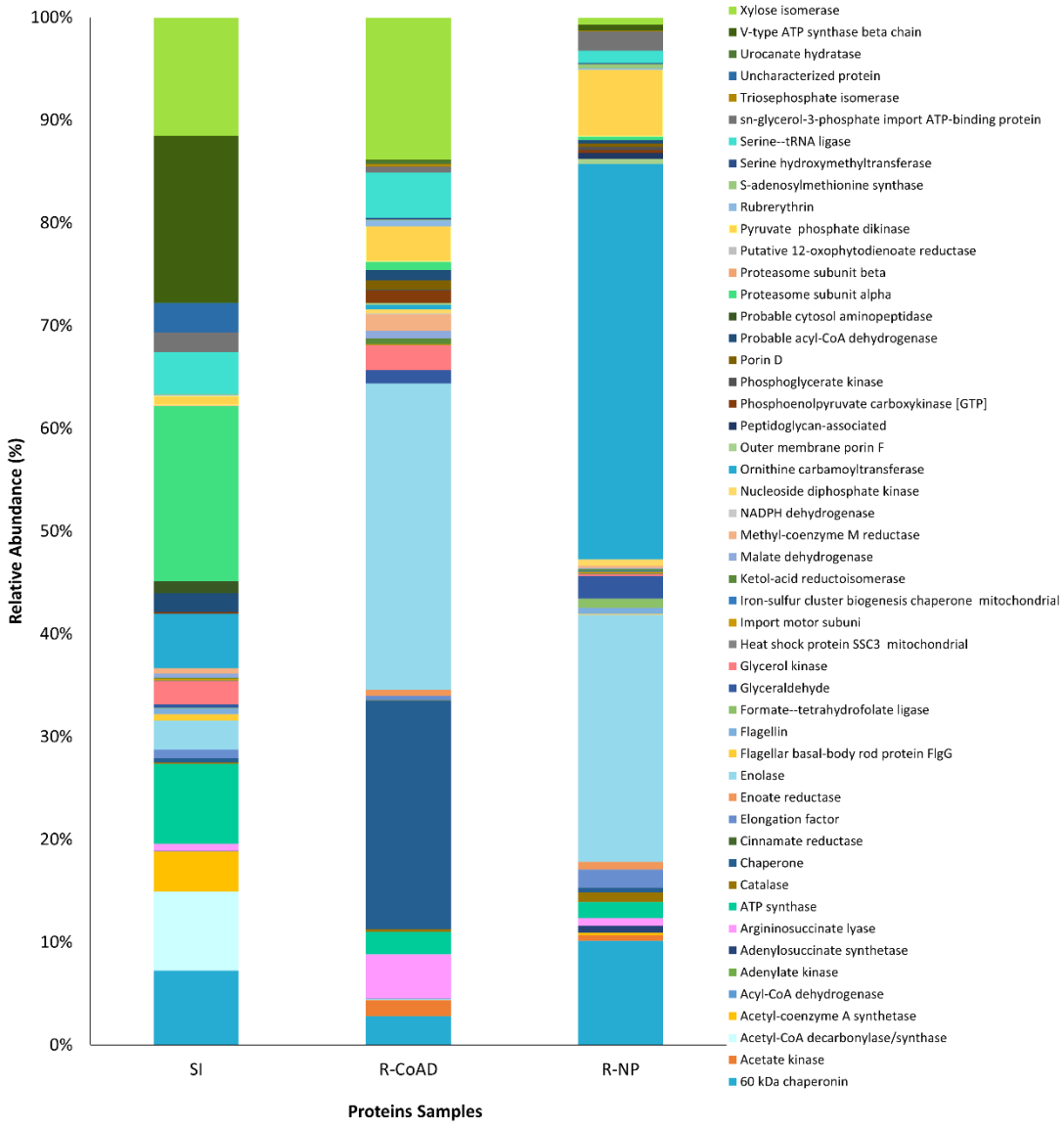


Figure 5.5.3. Relative abundance of proteins found in each of the samples. Average of triplicates. SI: seed inoculum, R-NP: Nanoparticles reactor, R-CoAD: Co-digestion reactor

The main proteins found in the sample R-NP are classified in the following functional classes: (~20%) Chaperone, (~30%) Enolase, (~13%) Xylose Isomerase, (~4%) Argininosuccinate lyase. For sample R-CoAD, the main proteins identified were belonging to the functional classes: (~10%) 60 kDa Chaperonin, (~24%) Enolase, (~38%) Ornithine carbamoyltransferase, and (~6%) Pyruvate phosphate dikinase.

In both samples, the presence of the Enolase enzyme is highly represented and carbohydrate appeared to be the main initial carbon source. This enzyme is associated with glucose metabolism, and reactors are treating samples with a high cellulose content that can be used in the glycolysis pathway or the pentose phosphate pathway once degraded (Abram et al. 2011). In addition to Enolase, another enzyme associated with cellulose metabolism is Xylose isomerase, indicating that the metabolic route of Xylulose also can be involved in the cellulose catabolism that takes place under AD. The Pyruvate phosphate dikinase enzyme could work at end of the glycolytic pathway metabolizing phosphoenolpyruvate into pyruvate (Abram et al. 2011).

The presence of proteins such as chaperones and chaperonins is usually related to stress responses due to environmental conditions and survival challenges in extreme or changing conditions and is not directly related to metabolic pathways involved in the AD of polysaccharides and biogas production. However, these proteins may be common in anaerobic reactor sample analyses as reported by Lam et al. (2021) and may be important to ensure the proper cellular response and protein folding under AD (Lam et al. 2021).

Among the main proteins related to the metabolic process of CH_4 production within anaerobic digestion, methyl coenzyme-M is a key enzyme at the end of CH_4 production (Abram et al. 2011). This enzyme was found in the three samples, (~0.5% in sample SI, ~1.6 % in sample R-NP, and 0.2% in sample R-CoAD) indicating that the CH_4 production route was probably active in the sludge.

The acetate kinase enzyme was also found (~1.5%) in sample R-NP and (~0.5%) in sample R-CoAD. This enzyme is responsible for the conversion of acetyl phosphate into acetate (and vice versa) within the metabolic pathway to produce CH_4. The acetate is the main precursor of CH_4 production (Pan et al. 2016) and the presence of this protein, which is widespread in bacteria fermentation consortiums, suggests that complex organic matter is degraded to acetate as well in our AD conditions, to produce CH_4.

Acetyl-coenzyme A synthetase, Acyl-CoA dehydrogenase, all related to the acetoclastic pathway in CH_4 production were also identified in the three samples. The SI sample showed the highest number of these proteins (e.g. ~7% Acetyl-CoA decarbonylase/synthase), being expressed by *Archaea methanosarcina* and *Methanothrix*, possibly indicating that the acetoclastic route was predominant in the inoculum before being inserted into the reactor. The fact that these enzymes were not found in greater amounts in the R-CoAD and R-NP samples do not indicate that the acetoclastic route was not present inside the reactor, but perhaps that as the CH_4 production was already stable, only proteins related to the final steps of methanogenesis, as it was the case with Methyl-CoM, were detected.

3.2 Relationship of proteins and the microbial community

Figure 5.5.4 shows the relative abundance of proteins expressed by each genus of microorganism. The R-NP and R-CoAD samples also had proteins that are produced by methanogenic *Archaea* and also by groups of *Bacteria* that are important for AD, such as *Thermoanaerobacter, Thermobifida, Thermomicrobium, Thermosipho, Thermotoga, Syntrophomonas, Ruminiclostridium, Pseudomonas.* Our proteomics results revealed a high number of proteins identified and annotated to the microorganisms of the *Thermotoga* genus, which is characteristic of thermophilic processes (Volpi et al. 2021c). The presence of these

proteins was more abundant in the samples from the two reactors which operated at 55°C than in the SI sample that comes from a mesophilic reactor.

These same samples showed proteins that are expressed from acetogenic and hydrolysis phases from the organisms from genus *Clostridium* and *Thermoanaerobacter*, which are part of the first stages of AD and they are important for drive in the entire metabolic process (Merlin Christy et al. 2014). Microorganisms of the genus *Lactobacillus* for example, whose proteins were identified in the sample R-NP and R-CoAD, are responsible for converting pyruvate into lactic acid, which can then be converted to acetate (Lam et al. 2021). Species of the genus *Clostridium* are involved in the degradation of pyruvate to butyrate (Lam et al. 2021) and possibly this butyrate was converted to acetate. All these microorganisms from the early stages of AD are important to prepare substrates that will be reduced to CH_4, such as acetate and CO_2, which will be used by methanogenic *Archaea* species.

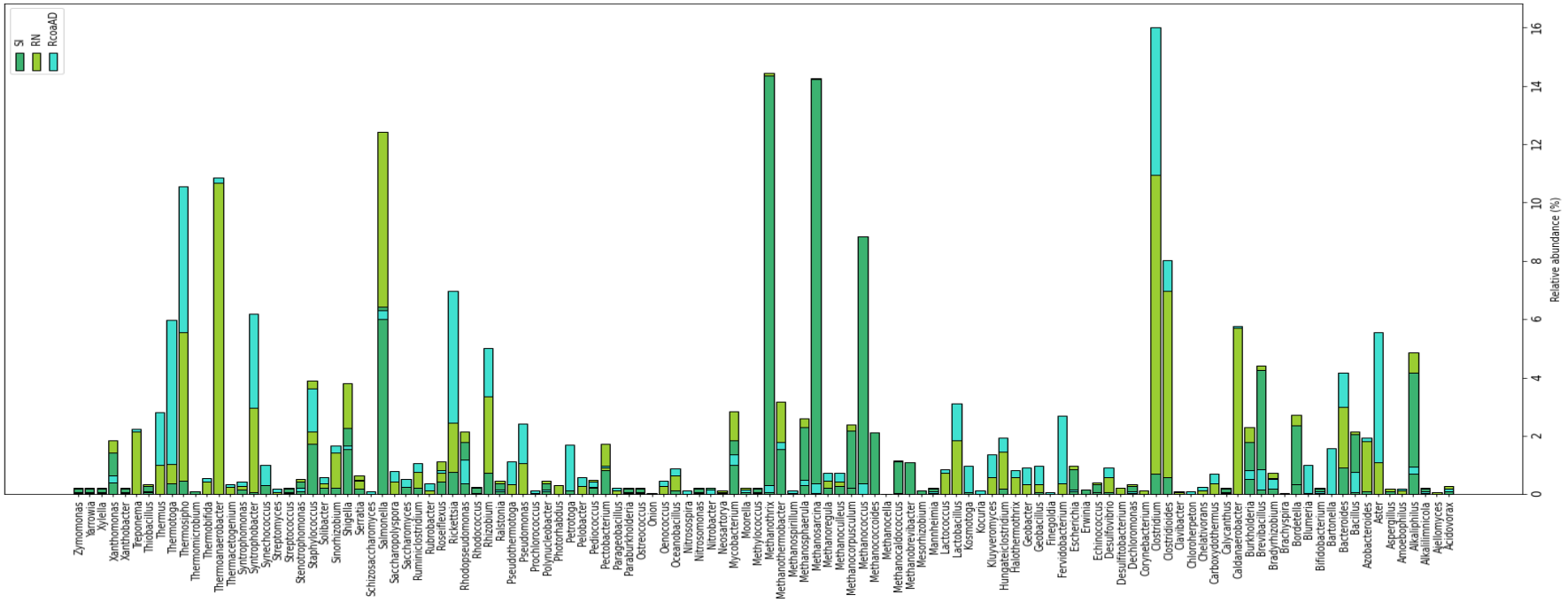


Figure 5.5.4. Relative abundance and distribution of identified proteins assigned to Bacterial and *Archaea* genera in each sample. Average of triplicates. SI: seed inoculum, R-NP: Nanoparticles reactor, R-CoAD: Co-digestion reactor

It is noteworthy that all samples have proteins from methanogenic *Archaea* annotated from the acetoclastic pathway such as *Methanosarcin,* as well as from the hydrogenotrophic pathway such as *Methanoculleus*. We have previously identified the main *Archaea* genus found in the samples of AD was *Methanoculleus* in the co-digestion of vinasse, filter cake, and deacetylation liquor, indicating that the probable metabolic route with these substrates would be syntrophic acetate oxidation (SAO) process coupled to hydrogenotrophic methanogenesis (Volpi et al., 2021c). The fact that the two reactors under AD have an expression of enzymes from both the acetoclastic metanogenisis route (e.g. ~3% and ~1% of acetate kinase, for R-CoAD and R-NP respectively) and the hydrogenotrophic metanogenisis route (e.g. ~5% and ~3% of acetyl CoA descarbonylase/synthase, for R-CoAD and R-NP respectively) confirms the possibility that SAO was coupled to the hydrogenotrophic route and may be the most likely within the waste co-digestion (vinasse, filter cake and deacetylaion liquor) from the ethanol production industry

3.3 Protein functional analysis

Figure 5.5.5a shows the heatmap of the results obtained from clustering the protein groups of the 3 samples, with the 3 repetitions performed. From this graphic it can be seen that samples were clustered in two groups, being the one in purple and the two group in red.

In the first cluster it can be seen in general that the R-CoAD samples and the R-NP sample have similar patterns of protein abundance, while in the second cluster most proteins that were expressed in the SI sample were not expressed in the other two samples. To assess the biological function of the set of expressed proteins of the two clusters identified, we performed analysis using Blastkoala, which is shown in Figure 5.5.5b.

The Cluster 1, which has the proteins from the R-CoAD and R-NP samples with high abundance (Figure 5.5.5b), had an intense carbohydrate metabolism activity, in addition to amino acid metabolism. The Cluster 2 had the highest expression of proteins in the SI sample, the greatest functions of the detected proteins were metabolism of other amino acids, energy metabolism, cellular process. In general, these protein functions, even though none are directly related to the CH_4 route, were already shown to have different metabolic pathways, and the R-CoAD and R-NP samples were classified under the same functions. It is worth remembering that these samples come from the anaerobic co-digestion operation of the same residues with stabilized CH_4 production.

According to the functions represented in the BlastKOALA (Figure 5.5.5b), there was a higher expression of proteins that act in the first AD phases, being the hydrolysis and acidogenic phase, for all samples, even samples R-NP and R-CoAD were removed from the reactor in the methanogenesis phase. This can be confirmed through Figure 5.5.6, which shows the frequency of each of the enzymes identified in the samples according to the analysis of the Gene ontology (GO) of UniProtKB in relation to the molecular function of these proteins. In all samples, active carbohydrate and protein metabolism enzymes were detected, such as hydrolase, lyase, peptidase and some other auxiliary enzymes (Bertucci et al. 2019) that are part of the hydrolysis steps. Still, these enzymes are more frequent in SI samples than in the other two coming from methanogenesis. These enzymes are important in the fatty acid biosynthesis process, which is an essential step for biogas production (Ping et al. 2020). What probably occurred is that smaller amounts of proteins related to methanogenesis were identified, which were not detected in the analysis carried out in the blastkoala because of low quantification. Even with this difficulty, it was possible to detect proteins related to the CH_4 metabolic route, as described in section 3.1.

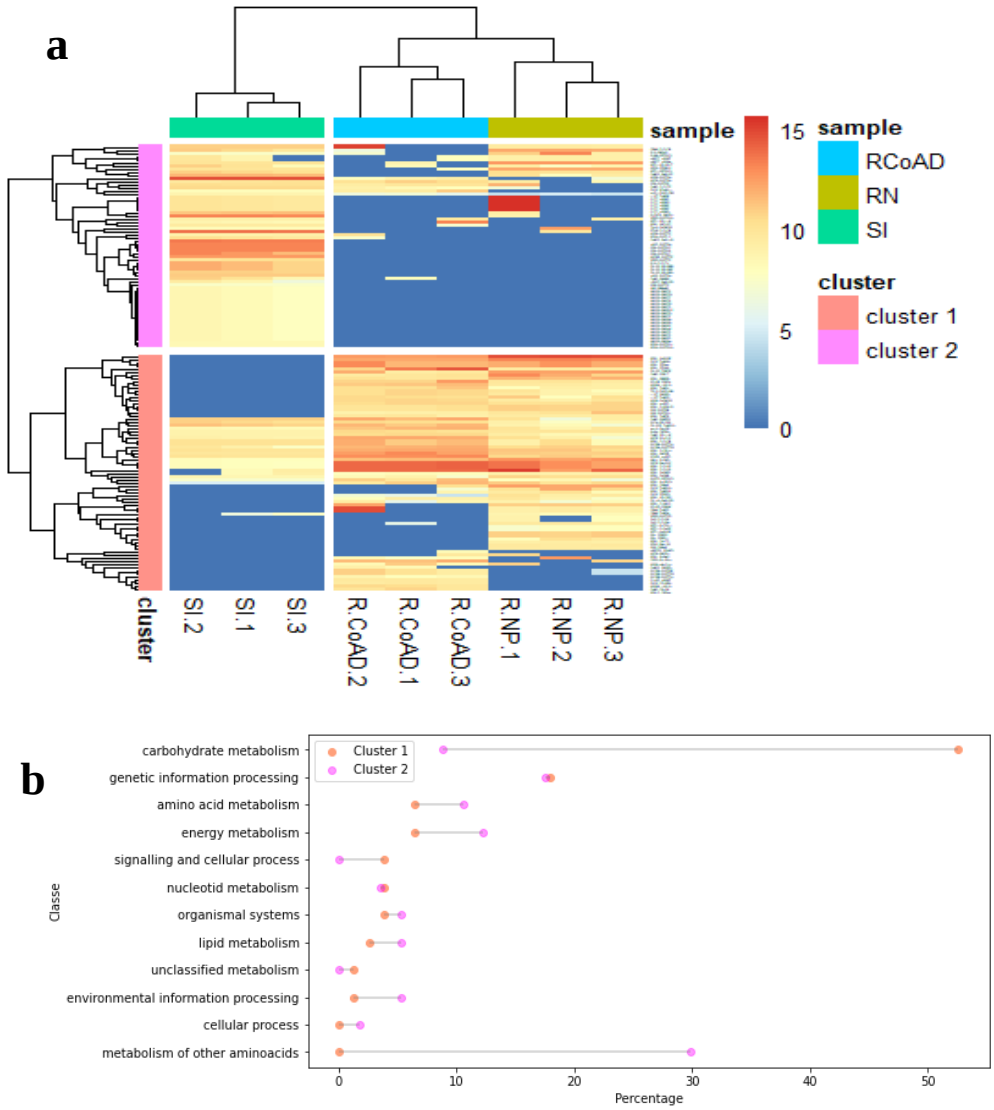


Figure 5.5.5. Hierarchical clustering analysis of the expression profiles of the identified 139 proteins. The nine columns represent triplicates of different treatments (SI, R-CoAD and R-NP). The rows represent individual proteins. The more and less abundant proteins are respectively indicated in orange and blue. The intensity of color increases with increased abundance differences, as shown in the bar. (b) Functional categories of clusters originated in figure 5.5.5a. The two different clusters were analyzed using BlastKOALA and comparative are shown as a bar chart.

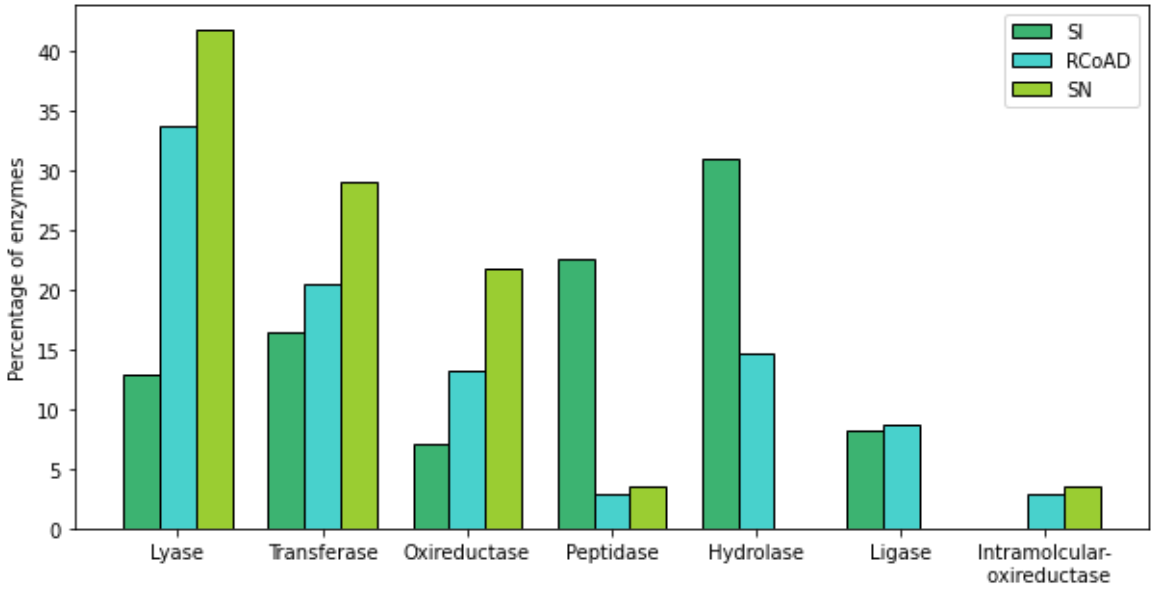


Figure 5.5.6. Molecular functions of samples according to UniProtKB ontology (GO) gene classification. SI (seed inoculum), R-CoAD (Co-digestion reactor), R-NP (Nanoparticles reactor)

3.4 Metabolic Pathway

Figure 5.5.7 shows the metabolic route that was proposed for the 3 samples together, according to the identification of proteins. To propose this route, KEGG's specific metabolic routes (http://www.kegg.jp/kegg/1) were followed. According to Figure 5.5.7, the main differences between the three samples occurred mainly in the early stages of AD, such as hydrolysis and acetogenesis, which are a process where macromolecules such as cellulose, lignin, xylose were broken down by different types of microorganisms, so that the volatile fatty acids, enter the phase of acetogenesis and methanogenesis.

Likely, methanogenesis was not different for the three samples, because the same residues were used as substrates, with the same compositional characteristics (Volpi et al. 2021a).

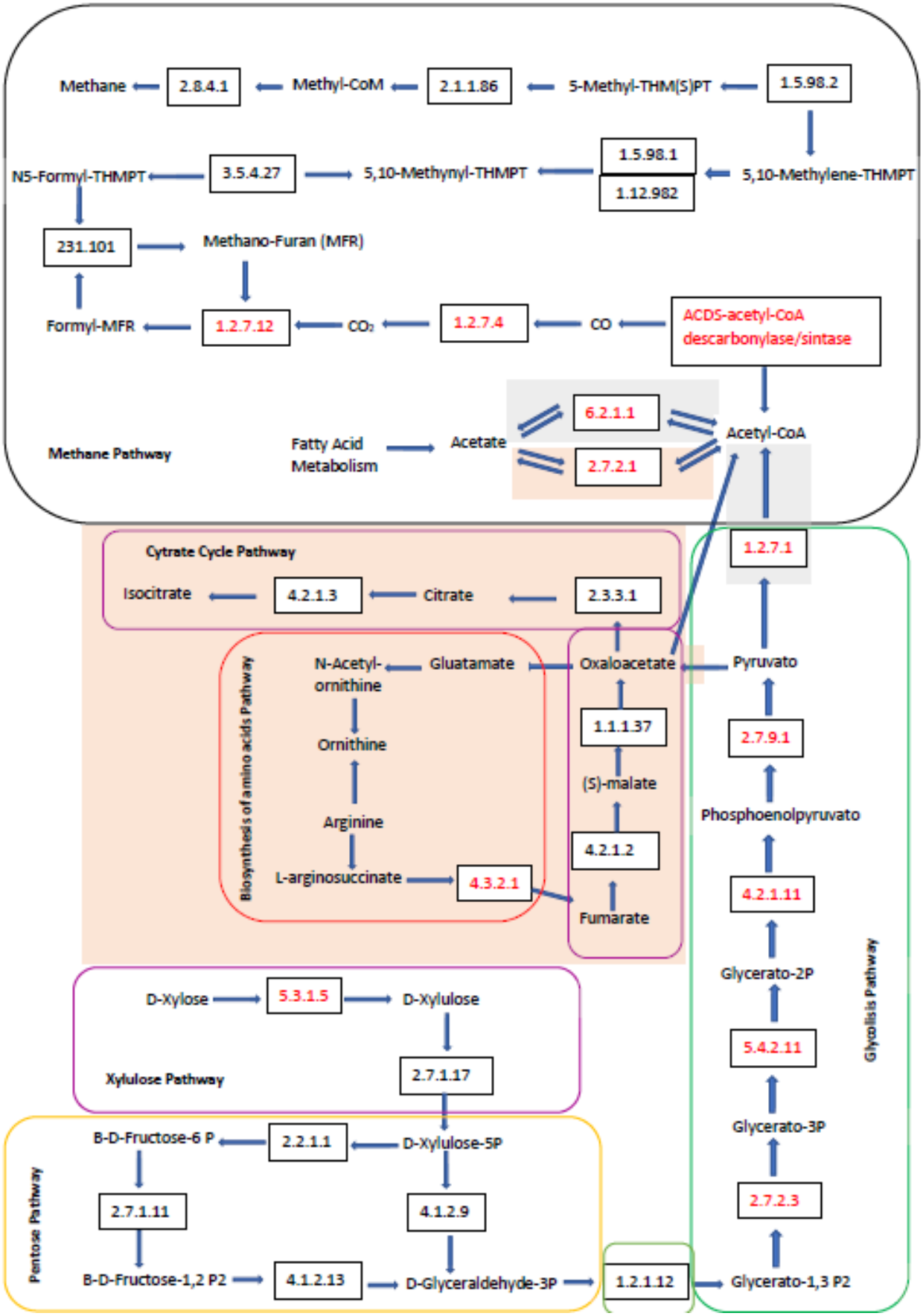

Methane
2.8.4.1
Methyl-CoM
2.1.1.86
5-Methyl-THM(S)PT
1.5.98.2
5,10-Methylene-THMPT
1.5.98.1
1.12.982
5,10-Methynyl-THMPT
3.5.4.27
N5-Formyl-THMPT
231.101
Methano-Furan (MFR)
Formyl-MFR
1.2.7.12
CO2
1.2.7.4
CO
ACDS-acetyl-CoA descarbonylase/sintase
Methane Pathway
Fatty Acid Metabolism
Acetate
6.2.1.1
2.7.2.1
Acetyl-CoA
Cytrate Cycle Pathway
Isocitrate
4.2.1.3
Citrate
2.3.3.1
Biosynthesis of amino acids Pathway
N-Acetyl-ornithine
Gluatamate
Ornithine
Arginine
L-arginosuccinate
4.3.2.1
Oxaloacetate
1.1.1.37
(S)-malate
4.2.1.2
Fumarate
1.2.7.1
Pyruvato
2.7.9.1
Phosphoenolpyruvato
4.2.1.11
Glycerato-2P
5.4.2.11
Glycerato-3P
2.7.2.3
Glycerato-1,3 P2
Glycolisis Pathway
D-Xylose
5.3.1.5
D-Xylulose
2.7.1.17
Xylulose Pathway
B-D-Fructose-6 P
2.2.1.1
D-Xylulose-5P
2.7.1.11
4.1.2.9
B-D-Fructose-1,2 P2
4.1.2.13
D-Glyceraldehyde-3P
1.2.1.12
Pentose Pathway

Figure 5.5.7 General proposed map of the metabolic pathways for 3 samples. SI: seed inoculum, R-NP: Nanoparticles reactor, R-CoAD: Co-digestion reactor. The red enzymes were detected in process. The white background is the metabolic maps for all samples, the pink background maps are found for the sample R-NP and R-CoAD, and the map with a gray background is found in the sample SI.

At the beginning of the metabolic route, the pathway of xylose, pentose, and glycolysis probably occurred in the 3 samples since the presence of the enzyme Xylose isomerase (EC: 5.3.1.5) was detected. Then, the proteins phosphoglycerate kinase (EC: 2.7.2.3) enolase (EC: 4.2.1.11), and Pyruvate phosphate dikinase (EC: 2.7.9.1) were detected, all involved in the glycolysis pathway, probably for the synthesis of pyruvate.

From this, there were some differences in the SI sample routes with the R-CoAD and R-NP samples. The R-CoAD and R-NP samples showed several proteins related to the metabolism of amino acids such as Arginine succinate lyase, Ornithine Carbometyltransferase. The presence of these proteins allowed the route Biosynthesis of amino acids, Arginine Biosynthesis, and Citrate Cycle (all pink in the map in Figure 4.5.7) to be explored. However, these routes are not extremely important for the production of CH_4, as they are part of the initial stages of the process.

In the SI sample, acetyl-CoA may have followed an acetoclastic methanogenic metabolic route, since the protein Acetyl-Coenzyme A synthase (EC: 6.2.1.1) was detected. In the sample R-CoA and R-NP the acetoclastic pathway also can be identified since protein Acetate kinase (EC: 2.7.2.1) was detected in greater proportions in both samples. In general, all samples follow this route of Acetyl-CoA generation, both from metabolites such as acetate, glycolise, citrate cycle, and fatty acid metabolism pathways.

In the end, it is likely that acetate could be converted to CO and later to CO_2 by the protein Acetyl-CoA decarbonylase/synthase complex subunit alpha (EC: 1.2.7.4) inside of Acetyl-CoA pathway (M00422-methane metabolic route-KEGG) (Lam et al. 2021). This

protein is part of the ACDS complex that catalyzes the reversible cleavage of acetyl-CoA, allowing autotrophic growth from CO_2. This CO_2 could then be used by the hydrogenotrophic methanogens, as reported by Lam et al. (2021), following the degradation processes until the formation of CH_4, since the Methyl-CoM enzyme was detected.

In this study, proteins that are part of the metabolic pathway of the acetoclastic methanogenic (M00357-Methane metabolic route KEGG) and also proteins that participate in the hydrogenotrophic pathway (M00567 Methane metabolic route-KEGG) were identified in all samples. This is a common situation for bioreactors that are fed with glucose as reported by Abram et al. (2011) methane production from both CO_2 and acetate correlates with the observation of a temporally increasing ratio (2.1–3.3 times) of hydrogenotrophic to acetoclastic methanogenic activity. Figure 5.5.8 was taken from the KEGG site and has highlighted the routes of the acetoclastic methanogenesis, the hydrogenotrophic methanogenesis, and also the Acetyl-CoA pathway.

The initial proposal from previous work by our research group (Volpi et al. 2021c) reported that the predominant metabolic route in the process would be SAO coupled with hydrogenotrophic methanogenesis (SAO-HM) was proved, since, by the identified proteins, acetate may be oxidized, being converted into CO_2 (Figure 5.5.7 and Figure 5.5.8). As this reaction generates H_2 and is thermodynamically unfavorable (reaction 5.5.1), hydrogenotrophic methanogenic *Archaea* consume the present H_2 and generate CH_4 (reaction 5.5.2) (Pan et al. 2016). Furthermore, this route is (reaction 5.5.1) favored at elevated temperatures. That is why it is common in thermophilic reactors. Therefore, as proteins from the two methanogenic routes and microorganisms classified as participants in the SAO-HM were identified (Volpi et al., 2021b) the two reactions may be coupled, and it was probably the predominant one in co-digestion with residues from ethanol production 1G2G.

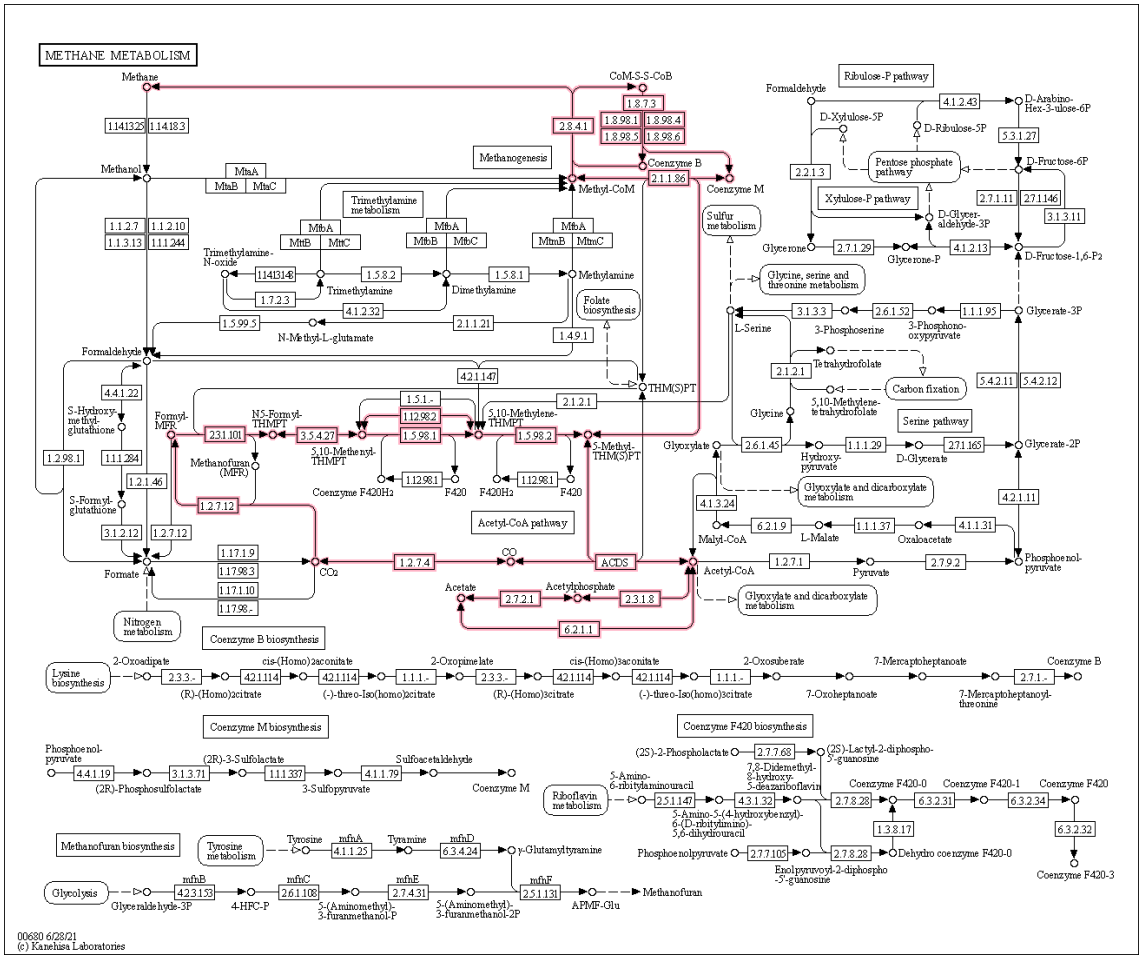


Figure 5.5.8 Acetoclastic methanogenesis, Hydrogenotropic methanogenesis, and Acetyl-CoA pathway inside of Methane metabolic route. (Source: KEGG: https://www.genome.jp/pathway/map00680)

Syntrophic acetate oxidizing reaction

$CH_3COOH + 2H_2O \rightarrow 2CO_2 + 4H_2$ $\Delta G° = +104.6$ KJ (Reaction 5.5.1)

Hydrogenotrophic methanogenesis

$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ $\Delta G° = -135.0$ KJ (Reaction 5.5.2)

4. CONCLUSIONS

A change in the metabolic route within the anaerobic co-digestion reactor with residues from the production of 1G2G ethanol was observed compared to the metabolic route of the microbial community before being inserted into the reactor. Many enzymes related to the hydrolysis and acidogenic phases of AD were detected, since the substrates used are rich in carbohydrates and composed of cellulosic and lignocellulosic material. The predominant metabolic route for co-AD from residues of ethanol production was the syntrophic acetate oxidation (SAO) process coupled to hydrogenotrophic methanogenesis, with the production of CH_4 occurring preferentially via CO_2 reduction. These results may contribute to a possible selection of microorganisms according to their metabolic pathway, in a biogas optimization process from sugarcane residues.

5. REFERENCES

Abdelsalam E, Samer M, Attia YA, Abdel-Hadi MA, Hassan HE, Badr Y (2017) Effects of Co and Ni nanoparticles on biogas and methane production from anaerobic digestion of slurry. Energy Convers Manag 141:108–119 . https://doi.org/10.1016/j.enconman.2016.05.051

Abram F, Enright AM, O'Reilly J, Botting CH, Collins G, O'Flaherty V (2011) A metaproteomic approach gives functional insights into anaerobic digestion. J Appl Microbiol 110:1550–1560 . https://doi.org/10.1111/j.1365-2672.2011.05011.x

Bertucci M, Calusinska M, Goux X, Rouland-lefèvre C, Untereiner B, Ferrer P (2019) Carbohydrate Hydrolytic Potential and Redundancy of an Anaerobic Digestion Microbiome Exposed to Acidosis, as Uncovered by Metagenomics. Appl Environ Micro 85:16

Brenelli LB, Figueiredo FL, Damasio A, Franco TT, Rabelo SC (2020) An integrated

approach to obtain xylo-oligosaccharides from sugarcane straw: from lab to pilot scale. Bioresour Technol 123637 . https://doi.org/10.1016/j.biortech.2020.123637

Fuess LT, Kiyuna LSM, Ferraz ADN, Persinoti GF, Squina FM, Garcia ML, Zaiat M (2017) Thermophilic two-phase anaerobic digestion using an innovative fixed-bed reactor for enhanced organic matter removal and bioenergy recovery from sugarcane vinasse. Appl Energy 189:480–491 . https://doi.org/10.1016/j.apenergy.2016.12.071

Hassanein A, Lansing S, Tikekar R (2019) Bioresource Technology Impact of metal nanoparticles on biogas production from poultry litter. Bioresour Technol 275:200–206 . https://doi.org/10.1016/j.biortech.2018.12.048

Hurkman WJ, Tanaka CK (1986) Solubilization of Plant Membrane Proteins for Analysis by Two-Dimensional Gel Electrophoresis. Plant Physiol 81:802–806 . https://doi.org/0032-0889/86/8 1/0802/05/$0 1.00/0

Kanehisa M, Sato Y, Morishima, K, (2016) BlastKOALA and GhostKOALA: KEGG Tools for Functional Characterization of Genome and Metagenome Sequences. J Mol Biol.;428:726–31. https://doi.org/10.1016/j.jmb.2015.11.006

Lam TK, Yang JT, Lai SJ, Liang SY, Wu SH (2021) Meta-proteomics analysis of microbial ecosystem during the anaerobic digestion of chicken manure in biogas production farm. Bioresour Technol Reports 13:100643 . https://doi.org/10.1016/j.biteb.2021.100643

Longati AA, Lino ARA, Giordano RC, Furlan FF, Cruz AJG (2020) Biogas Production from Anaerobic Digestion of Vinasse in Sugarcane Biorefinery: A Techno-economic and Environmental Analysis. Waste and Biomass Valorization 11:4573–4591 . https://doi.org/10.1007/s12649-019-00811-w

Meier F, Beck S, Grassl N, Lubeck M, Park MA, Raether O, Mann M (2015) Parallel accumulation-serial fragmentation (PASEF): Multiplying sequencing speed and sensitivity by synchronized scans in a trapped ion mobility device. J Proteome Res

14:5378–5387 . https://doi.org/10.1021/acs.jproteome.5b00932

Merlin Christy P, Gopinath LR, Divya D (2014) A review on anaerobic decomposition and enhancement of biogas production through enzymes and microorganisms. Renew Sustain Energy Rev 34:167–173 . https://doi.org/10.1016/j.rser.2014.03.010

Meyer AKP, Ehimen EA, Holm-Nielsen JB (2018) Future European biogas: Animal manure, straw and grass potentials for a sustainable European biogas production. Biomass and Bioenergy 111:154–164 . https://doi.org/10.1016/j.biombioe.2017.05.013

Moraes BS, Zaiat M, Bonomi A (2015) Anaerobic digestion of vinasse from sugarcane ethanol production in Brazil: Challenges and perspectives. Renew. Sustain. Energy Rev.

Pan X, Angelidaki I, Alvarado-Morales M, Liu H, Liu Y, Huang X, Zhu G (2016) Methane production from formate, acetate and H2/CO2; focusing on kinetics and microbial characterization. Bioresour Technol 218:796–806 . https://doi.org/10.1016/j.biortech.2016.07.032

Parralejo AI, Royano L, González J, González JF (2019) Small scale biogas production with animal excrement and agricultural residues. Ind Crops Prod 131:307–314 . https://doi.org/10.1016/j.indcrop.2019.01.059

Ping Q, Zheng M, Dai X, Li Y (2020) Metagenomic characterization of the enhanced performance of anaerobic fermentation of waste activated sludge with CaO 2 addition at ambient temperature : Fatty acid biosynthesis metabolic pathway and CAZymes. Water Res 170:115309 . https://doi.org/10.1016/j.watres.2019.115309

Pramanik SK, Suja FB, Zain SM, Pramanik BK (2019) The anaerobic digestion process of biogas production from food waste: Prospects and constraints. Bioresour Technol Reports 8:100310 . https://doi.org/10.1016/j.biteb.2019.100310

Volpi MPC, Brenelli LB, Mockaitis G, Rabelo SC, Franco TT, Moraes BS (2021a) Use of lignocellulosic residue from second-generation ethanol production to enhance methane

production through co-digestion. Bioenergy Res. https://doi.org/10.1007/s12155-021-10293-1

Volpi MPC, Fuess LT, Moraes BS (2021b) Anaerobic co-digestion of residues in 1G2G sugarcane biorefineries for enhanced electricity and biomethane production. Bioresour Technol 330:124999 . https://doi.org/10.1016/j.biortech.2021.124999

Volpi MPC, Junior ADNF, Franco TT, Moraes BS (2021c) Operational and biochemical aspects of co-digestion (co-AD) from sugarcane vinasse, filter cake, and deacetylation liquor. Appl Microbiol Biotechnol. https://doi.org/10.1007/s00253-021-11635-x

M.P.C. Volpi, G. Mockaitis, G. Bruant, J. Tremblay, B. Moraes, Use of Fe3O4 nanoparticles in reactor co-digestion of residues from 1G2G ethanol biorefinery: microbiological routes and operational aspects, BioRxiv Prepr. (2022). https://doi.org/10.1101/2022.03.21.484299.

Wu J horng, Wu F yau, Chuang H ping, Chen W yu, Huang H jen, Chen S hui, Liu W tso (2013) Community and proteomic analysis of methanogenic consortia degrading terephthalate. Appl Environ Microbiol 79:105–112 . https://doi.org/10.1128/AEM.02327-12

Heberle, H.; Meirelles, G. V.; da Silva, F. R.; Telles, G. P.; Minghim, R. (2015). ***InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams***. BMC Bioinformatics 16:169 (2015).10.1186/s12859-015-0611-3

SUPPLEMENTARY MATERIALS DATA

Table 5.5.1SM. Proteins that were differentially expressed between samples SI and R-NP

ID protein from UNIPROT	Protein Description	Expression significance value
ACDA_METSH		0.00
ENO_CLOK5	Enolase	0.00
ENO_CLOK1	Enolase	0.00
ENO_CLOD6	Enolase	0.04
ENO_RUMCH	Enolase	0.00
GLPK2_THEMA	Glycerol kinase 2	0.34
ENO_OCEIH	Enolase	0.38
CH60_CLOTE	60 kDa chaperonin	0.12
RUBY_DESVH	Rubrerythrin	0.01
PPDK_RICFE	Pyruvate phosphate dikinase	0.27
PPDK_RHIME		0.00
PPDK_RICPR		0.00
PPDK_RICCN	Pyruvate phosphate dikinase	0.01
PPDK_RICBR	Pyruvate phosphate dikinase	0.01

PPDK_RICTY	Pyruvate phosphate dikinase	0.01
ENO_THEAB	Enolase	0.17
PSA_METB6	Proteasome subunit alpha	0.06
PSA_METPE	Proteasome subunit alpha	0.12
PSA_METMJ	Proteasome subunit alpha	0.10
ARLY_SINFN	Argininosuccinate lyase	0.00
ARLY1_RHIME	Argininosuccinate lyase 1	0.00
ARLY_RHIE6	Argininosuccinate lyase	0.00

Table 5.5.2SM. Proteins that were differentially expressed between samples SI and R-CoAD

ID protein from UNIPROT	Protein Description	Expression significance value
FLA_BACHD		0.04
ENO_CLOK5	Enolase	0.03

ENO_CLOK1	Enolase	0.03
CH603_BRADU	60 kDa chaperonin 3	0.36
CH602_RHOPA	60 kDa chaperonin 2	0.36
CH602_RHOP5	60 kDa chaperonin 2	0.21
CH606_BRADU	60 kDa chaperonin 6	0.36
CH602_NITHX	60 kDa chaperonin 2	0.36
ENO_BACV8	Enolase	0.05
ENO_RUMCH	Enolase	0.05
ENO_BACTN	Enolase	0.05
ENO_BACFR	Enolase	0.05
ENO_BACFN	Enolase	0.05
UGPC_BARBK	sn-glycerol-3-phosphate import ATP-binding protein UgpC OS=Bartonella bacilliformis	0.00
RUBY_DESVH	Rubrerythrin	0.14
PPDK_CLOSY		0.00
PPDK_RICFE	Pyruvate phosphate dikinase OX=315456 GN=ppdK PE=3 SV=1	0.08

PPDK_RHIME		0.00
PPDK_RICPR		0.00
PPDK_RICCN	Pyruvate phosphate dikinase	0.00
PPDK_RICBR	Pyruvate phosphate dikinase	0.00
PPDK_RICTY	Pyruvate phosphate dikinase	0.00
VATB_METPE	V-type ATP synthase beta chain	0.00
ENO_SYNSC	Enolase	0.15
ENO_THEAB	Enolase	0.00
ATPA_DECAR	ATP synthase subunit alpha	0.06
ARLY_SINFN	Argininosuccinate lyase	0.02
ARLY1_RHIME	Argininosuccinate lyase 1	0.03
ARLY_RHIE6	Argininosuccinate lyase	0.03

Table 5.5.3SM Proteins that were differentially expressed between samples R-NP and R-CoAD

ID Protein from UNIPROT	Protein Description	Expression significance value
ENO_CLOK5	Enolase OS=Clostridium kluyveri	0.10

ENO_CLOK1	Enolase OS=Clostridium kluyveri	0.10
ENO_CLOD6	Enolase OS=Clostridioides difficile	0.07
ENO_HUNT2	Enolase	0.13
CH60_PSELT	60 kDa chaperonin	0.16
CH60_FERNB	60 kDa chaperonin	0.18
CH60_THEAB	60 kDa chaperonin	0.01
CH60_THENE	60 kDa chaperonin	0.01
CH60_THEM4	60 kDa chaperonin	0.01
CH60_THEMA	60 kDa chaperonin	0.01
CH60_THEP1	60 kDa chaperonin	0.01
CH60_THESQ	60 kDa chaperonin	0.01
ENO_THEFY	Enolase OS=Thermobifida fusca	0.19
ENO_BREBN	Enolase OS=Brevibacillus brevis	0.10
ENO_AYWBP	Enolase OS	0.00
ACKA1_LACLA		0.11
MCRA_METTH		0.04

MCRA_METTM		0.04
ACDP_MYCLE	Probable acyl-CoA dehydrogenase fadE25	0.14
ACDP_MYCBO	Probable acyl-CoA dehydrogenase fadE25	0.26
ACDP_MYCTU	Probable acyl-CoA dehydrogenase fadE25	0.26
ACDP_MYCTO	Probable acyl-CoA dehydrogenase fadE25	0.26
ENO_RUMCH	Enolase	0.04
ENO_OCEIH	Enolase	0.02
G3P_THEAQ	Glyceraldehyde-3-phosphate dehydrogenase	0.00
ENO_LACF3	Enolase OS=Lactobacillus fermentum	0.31
ENO_LACCB	Enolase OS=Lactobacillus casei	0.08
ENO_OENOB	Enolase	0.18
ENO_PEDPA	Enolase	0.05
ENO_LACP3	Enolase	0.05
ENO2_LACGA	Enolase 2	0.05
ENO_PELCD	Enolase	0.32
ENO1_LACJO	Enolase 1	0.05

ENO_LACS1	Enolase	0.05
ENO_LACSS	Enolase	0.05
ILVC_SACEN	Ketol-acid reductoisomerase (NADP(+))	0.08
ILVC_THEPS	Ketol-acid reductoisomerase (NAD(+))	0.02
RUBY_DESVH	Rubrerythrin	0.01
CH60_SOLUE	60 kDa chaperonin	0.06
PPDK_CLOSY		0.32
PPDK_RICFE	Pyruvate phosphate dikinase	0.01
PPDK_RHIME		0.01
PPDK_RICPR		0.08
PPDK_RICCN	Pyruvate phosphate dikinase	0.08
PPDK_RICBR	Pyruvate phosphate dikinase	0.00
PPDK_RICTY	Pyruvate phosphate dikinase	0.08
PORF_PSESY	Outer membrane porin F	0.03
ENO_THET8	Enolase	0.16
ENO_THET2	Enolase	0.16

GLYA_RHOP2	Serine hydroxymethyltransferase	0.14
ARLY_SINFN	Argininosuccinate lyase	0.01
ARLY1_RHIME	Argininosuccinate lyase 1	0.01
ARLY_RHIE6	Argininosuccinate lyase	0.01

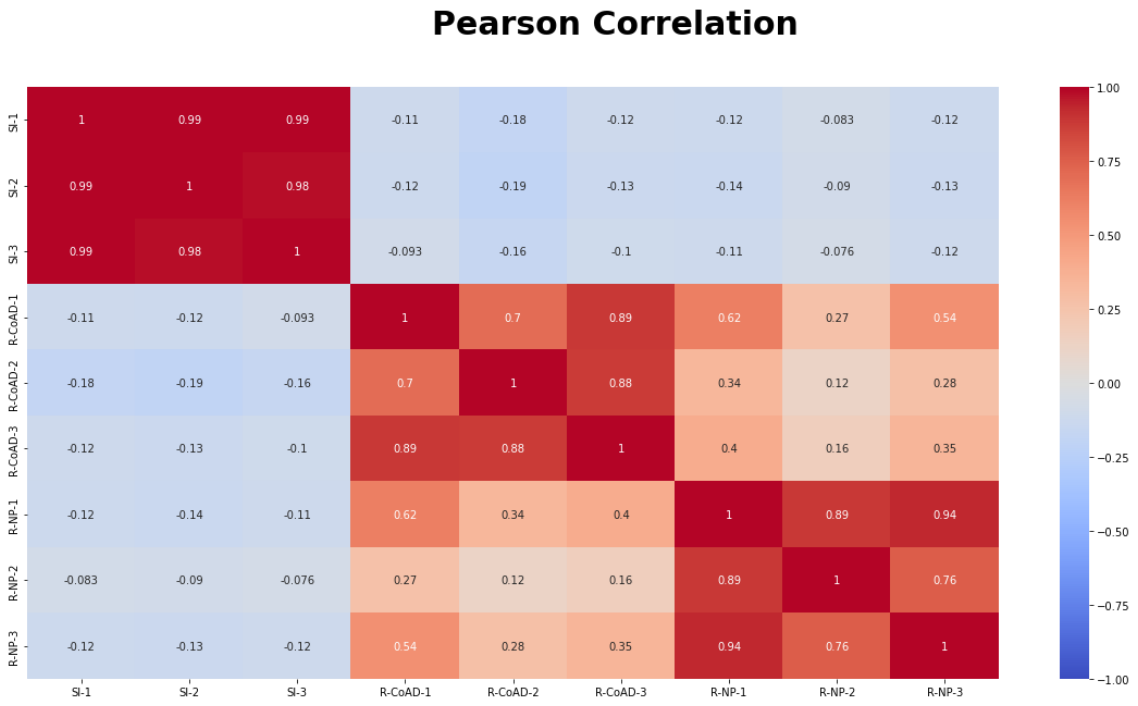
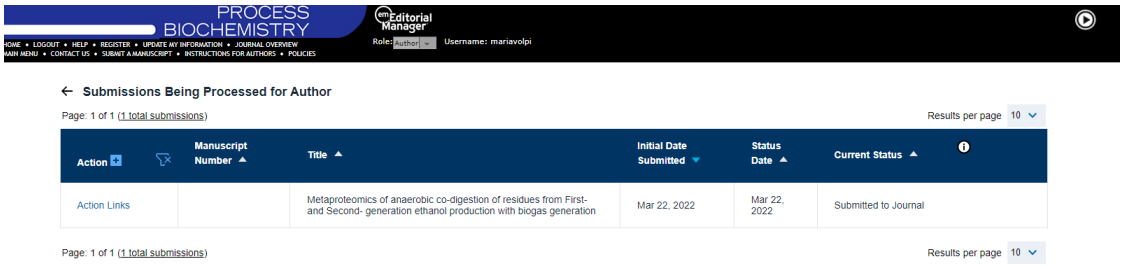


Figure 5.5.1SM. Heatmap with Pearson correlation coefficients between SI, R-CoAD and RN samples. All positive correlations are shown in red and negative correlations are shown in blue. The numbers inside the square represent the correlation values.

Comprovante de Subimissão Process Biochemistry Journal



Process Biochemistry

Metaproteomics of anaerobic co-digestion of residues from First- and Second-generation ethanol production with biogas generation

--Manuscript Draft--

Manuscript Number:	
Article Type:	Full Length Article
Section/Category:	Biomass & Bioenergy - Bioenergy production
Keywords:	proteins; vinasse; filter cake; anaerobic inoculum; deacetylation liquor; metabolic pathway
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	Flávia V. Winck
	Mônica T. V. Labate
	Bruna S. Moraes
Abstract:	The proteomic analysis has been highlighted as a powerful tool for deeper investigation of the anaerobic digestion (AD), but less information was found about the co-digestion of ethanol production residues. In this context, this study aimed to analyze the repertoire of proteins from anaerobic co-digestion performed in reactors that contained residues from the production of First- generation (1G) and Second-generation (2G) ethanol for biogas production. Proteomics analysis was performed for three types of samples: anaerobic sludge before being inserted into the reactor (SI), semi-continuous stirred reactor (s-CSTR) with co-digestion of filter cake, vinasse, and deacetylation liquor (R-CoAD) and s-CSTR with co-digestion of these same residues with the addition of Fe_3O_4 nanoparticles (R-NP). Protein extracts were analyzed by shotgun high-resolution Mass Spectrometry for a Metaproteomics analysis. Most proteins identified were related to the carbohydrate metabolism and amino acid metabolism. The metabolic routes annotated for the three samples were very similar, with minor changes in the initial stages of the bioprocess. The main metabolic routes annotated for the generation of residues or metabolic products from the production of 1G2G ethanol in co-digestion was syntrophic acetate oxidation process coupled with hydrogenotrophic methanogenesis, with the production of CH_4 occurring preferentially via CO_2 reduction.
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6 GENERAL CONCLUSIONS

With the results obtained it can be concluded that co-digestion is a viable alternative for biogas production inside sugarcane plants. With the co-digestion of these residues, it is possible to obtain biogas production throughout the year inside the 1G2G biorefinery, enabling the use of biogas for both electricity application and bioCH_4 generation and making the 1G2G plant self-sufficient.

The deacetylation liquor, being a still little explored residue, presented high BMP values, being very promising for AD. In addition, it appraised it as a co-substrate that contributed even more to the digestion of vinasse, since its pH close to 12 brings an alkalizing power to the reactor, it is not necessary to adjust the pH with external chemical substances.

The use of nanoparticles can be a technological advance in the area of biodigestion for the production of CH_4, due to its stimulating properties for bacteria growth.

With the residues used in the present study, it was possible to detect the preference of bacteria for the metabolic route syntrophic acetate oxidation (SAO) coupled with hydrogenotrophic methanogenesis, indicating the influence that substrate composition and operating conditions have on the metabolic pathway that bacteria follow.

Proteomics techniques have shown to be a promising advance for the biochemical understanding of AD, and consequently to optimize the process, even with the difficulties of this technique for application in anaerobic inoculum.

7 SUGGESTION FOR FUTURE WORK

- Simulation of biogas production for the sugarcane off-season by co-digestion filter cake, the deacetylation liquor and 2G vinasse
- Experimental assessment of the alternative technological arrangement of biogas production aiming at biogas production along the year as proposed in PAPER 3
- Economic assessment of different technological arrangement of biogas production in 1G2G integrated sugarcane biorefineries
- Reactor sludge sample metabolomics analysis for better understanding of metabolic pathways within AD

8 REFERENCES

AGUIAR, A.N. Vinhaça da cana aumenta emissão de gases do efeito estufa, 2011. Agência USP, 2009. Available at: https://www.inovacaotecnologica.com.br/. Acessado em 26.03.2020 as 13h10.

CONAB. Acompanhamento da Safra Brasileira - Cana de açúcar - V.8 - SAFRA 2021/22 - N.3 - Terceiro levantamento - Novembro 2021. v. 8, n. 3, p. 63, 2021.

INTERNATIONAL ENERGY AGENCY. World Outlook 2006. IEA. Paris, 2006. Available at: http://www.economicswebinstitute.org/essays/energy2006.pdf. Acess in 04.04.2020 09h35.

GIANCHINI, C. F., FERRAZ, M. V. Benefícios da utilização de vinhaça em terras de plantio de cana-de-açúcar-Revisão de Literatura. Revista Científica Eletrônica de Agronomia, 15, 2009.

ABDEL AZIM, A. et al. The physiology of trace elements in biological methane production. **Bioresource Technology**, v. 241, p. 775–786, 2017.

ABDELSALAM, E. et al. Comparison of nanoparticles effects on biogas and methane production from anaerobic digestion of cattle dung slurry. **Renewable Energy**, v. 87, p. 592–598, 2016.

ABDELSALAM, E. et al. Effects of Co and Ni nanoparticles on biogas and methane production from anaerobic digestion of slurry. **Energy Conversion and Management**, v. 141, p. 108–119, 2017a.

ABDELSALAM, E. et al. Influence of zero valent iron nanoparticles and magnetic iron oxide nanoparticles on biogas and methane production from anaerobic digestion of manure. **Energy**, v. 120, p. 842–853, 2017b.

ABRAM, F. et al. A metaproteomic approach gives functional insights into anaerobic digestion. **Journal of Applied Microbiology**, v. 110, n. 6, p. 1550–1560, 2011.

AHN, J.-H. et al. The effect of calcium on the anaerobic digestion treating swine wastewater. **Biochemical Engineering Journal**, v. 30, n. 1, p. 33–38, 2006.

AN, Q. et al. Performance and energy recovery of single and two stage biogas production from paper sludge: Clostridium thermocellum augmentation and microbial community analysis. **Renewable Energy**, v. 148, p. 214–222, 2020.

ANGELIDAKI, I. et al. Thermophilic anaerobic digestion of source-sorted organic fraction of household municipal solid waste: Start-up procedure for continuously stirred tank reactor. **Water Research**, v. 40, n. 14, p. 2621–2628, 2006.

ANGELIDAKI, I.; ELLEGRAARD, L.; AHARING, B. K. on Ammonia Inhibition. **Biotechnology and Bioengineering**, v. 42, p. 159–166, 1993.

ASTALS, S.; NOLLA-ARDÈVOL, V.; MATA-ALVAREZ, J. Thermophilic co-digestion of pig manure and crude glycerol: Process performance and digestate stability. **Journal of Biotechnology**, v. 166, n. 3, p. 97–104, 2013.

BADALATO, N. et al. Whole proteome analyses on Ruminiclostridium cellulolyticum show a modulation of the cellulolysis machinery in response to cellulosic materials with subtle differences in chemical and structural properties. **PLoS ONE**, v. 12, n. 1, p. 1–22, 2017.

BALAGURUSAMY, N. a Preliminary Study on Molecular Characterization of the. **Journal Of Applied Bacteriology**, v. 6, n. 3, p. 237–242, 2007.

BARAKAT, A. et al. Effect of lignin-derived and furan compounds found in lignocellulosic hydrolysates on biomethane production. **Bioresource Technology**, v. 104, p. 90–99, 2012.

BAUEN, A. et al. BIOMASS RESOURCES AND POTENTIALS. In: **BIOENERGY – A SUSTAINABLE AND RELIABLE ENERGY SOURCE A review of status and prospects**. Netherlands: IEA BIOENERGY, 2009. p. 108.

BHATTACHARYA, S. K. et al. Effect of Cobalt on Methanogenesis. **Environmental Technology**, v. 16, n. 3, p. 271–278, mar. 1995.

BIBBY, K.; VIAU, E.; PECCIA, J. Pyrosequencing of the 16S rRNA gene to reveal bacterial pathogen diversity in biosolids. **Water Research**, v. 44, n. 14, p. 4252–4260, 2010.

BINI, E. Archaeal transformation of metals in the environment. **FEMS Microbiology Ecology**, v. 73, n. 1, p. 1–16, 2010.

BLANC, F. C.; MOLOF, A. H. Electrode Potential Monitoring and Electrolytic Control in Anaerobic Digestion. **Journal of the Water Pollution Control Federation**, v. 45, n. 4, p. 655–667, 1973.

BOKULICH, N. A. et al. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. **Microbiome**, v. 6, n. 1, p. 1–17, 2018.

BOLADO-RODRÍGUEZ, S. et al. Effect of thermal, acid, alkaline and alkaline-peroxide pretreatments on the biochemical methane potential and kinetics of the anaerobic digestion of wheat straw and sugarcane bagasse. **Bioresource Technology**, v. 201, p. 182–190, 2016.

BOLGER, A. M.; LOHSE, M.; USADEL, B. Trimmomatic: A flexible trimmer for Illumina sequence data. **Bioinformatics**, v. 30, n. 15, p. 2114–2120, 2014.

BOLYEN, E. et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. **Nature Biotechnology**, v. 37, n. 8, p. 852–857, 2019.

BRAUN, R. Anaerobic digestion: A multi-faceted process for energy, environmental management and rural development. **Improvement of Crop Plants for Industrial End Uses**, p. 335–416, 2007.

BRENELLI, L. B. et al. An integrated approach to obtain xylo-oligosaccharides from sugarcane straw: from lab to pilot scale. **Bioresource Technology**, p. 123637, 2020.

CABEZAS, A. et al. How to use molecular biology tools for the study of the anaerobic digestion process? **Reviews in Environmental Science and Biotechnology**, v. 14, n. 4, p. 555–593, 2015.

CAI, Y. et al. Effects of adding trace elements on rice straw anaerobic mono-digestion: Focus on changes in microbial communities using high-throughput sequencing. **Bioresource Technology**, v. 239, p. 454–463, 2017.

CAI, Y. et al. Effects of molybdenum, selenium and manganese supplementation on the performance of anaerobic digestion and the characteristics of bacterial community in acidogenic stage. **Bioresource Technology**, v. 266, n. June, p. 166–175, 2018.

CARBALLA, M. et al. Correlations between molecular and operational parameters in continuous lab-scale anaerobic reactors. **Applied Microbiology and Biotechnology**, v. 89, n.

2, p. 303–314, 2011.

CARVALHO, D. M. DE; QUEIROZ, J. H. DE; COLODETTE, J. L. Assessment of alkaline pretreatment for the production of bioethanol from eucalyptus, sugarcane bagasse and sugarcane straw. **Industrial Crops and Products**, v. 94, p. 932–941, 2016.

CHEN, S. et al. Linkages of Firmicutes and Bacteroidetes populations to methanogenic process performance. **Journal of Industrial Microbiology and Biotechnology**, v. 43, n. 6, p. 771–781, 2016.

CHEN, X. et al. Improving biomethane yield by controlling fermentation type of acidogenic phase in two-phase anaerobic co-digestion of food waste and rice straw. **Chemical Engineering Journal**, v. 273, p. 254–260, 2015.

CHEN, Y.; CHENG, J. J.; CREAMER, K. S. Inhibition of anaerobic digestion process: a review. **Bioresource technology**, v. 99, n. 10, p. 4044–64, jul. 2008.

CHOONG, Y. Y. et al. Impacts of trace element supplementation on the performance of anaerobic digestion process: A critical review. **Bioresource Technology**, v. 209, p. 369–379, 2016.

CHRISTOFOLETTI, C. A. et al. Sugarcane vinasse: Environmental implications of its use. **Waste Management**, v. 33, n. 12, p. 2752–2761, 2013.

CONRAD, R. Contribution of hydrogen to methane production and control of hydrogen concentrations in methanogenic soils and sediments. **FEMS Microbiology Ecology**, v. 28, n. 3, p. 193–202, 1999.

DA COSTA LOPES, A. M. et al. Pre-treatment of lignocellulosic biomass using ionic liquids: Wheat straw fractionation. **Bioresource Technology**, v. 142, p. 198–208, 2013.

DA SILVA, A. S. A. et al. Milling pretreatment of sugarcane bagasse and straw for enzymatic hydrolysis and ethanol fermentation. **Bioresource Technology**, v. 101, n. 19, p. 7402–7409, 2010.

DE OLIVEIRA, B. G. et al. Soil greenhouse gas fluxes from vinasse application in Brazilian sugarcane areas. **Geoderma**, v. 200–201, p. 77–84, 2013.

DE PAOLI, F. et al. Utilization of by-products from ethanol production as substrate for biogas production. **Bioresource Technology**, v. 102, n. 11, p. 6621–6624, 2011.

DE SOUZA MORAES, B. et al. Enriched microbial consortia for dark fermentation of sugarcane vinasse towards value-added short-chain organic acids and alcohol production. **Journal of Bioscience and Bioengineering**, v. 127, n. 5, p. 594–601, 2019.

DEMIREL, B.; SCHERER, P. The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: A review. **Reviews in Environmental Science and Biotechnology**, v. 7, n. 2, p. 173–190, 2008.

DEMIREL, B.; YENIGÜN, O. Two-phase anaerobic digestion processes: A review. **Journal of Chemical Technology and Biotechnology**, v. 77, n. 7, p. 743–755, 2002.

DETMAN, A. et al. Methane-yielding microbial communities processing lactate-rich substrates: A piece of the anaerobic digestion puzzle. **Biotechnology for Biofuels**, v. 11, n. 1, p. 1–18, 2018.

DEUBLIN, D.; STEINHAUSER, A. **Biogas from Waste and Renewable Resources: An**

Introduction. Weinheim, Germany: Wily Online Library, 2008.

DÍAZ, E. E. et al. Phenotypic properties and microbial diversity of methanogenic granules from a full-scale upflow anaerobic sludge bed reactor treating brewery wastewater. **Applied and Environmental Microbiology**, v. 72, n. 7, p. 4942–4949, 2006.

DINSDALE, R. M.; HAWKES, F. R.; HAWKES, D. L. Anaerobic digestion of short chain organic acids in an expanded granular sludge bed reactor. **Water Research**, v. 34, n. 9, p. 2433–2438, 2000.

DJALMA NUNES FERRAZ JÚNIOR, A. et al. Thermophilic anaerobic digestion of raw sugarcane vinasse. **Renewable Energy**, 2016a.

DJALMA NUNES FERRAZ JÚNIOR, A. et al. Thermophilic anaerobic digestion of raw sugarcane vinasse. **Renewable Energy**, v. 89, p. 245–252, 2016b.

DRESSLER, D.; LOEWEN, A.; NELLES, M. Life cycle assessment of the supply and use of bioenergy: Impact of regional factors on biogas production. **International Journal of Life Cycle Assessment**, v. 17, n. 9, p. 1104–1115, 2012.

EL-MASHAD, H. M.; ZHANG, R. Biogas production from co-digestion of dairy manure and food waste. **Bioresource Technology**, v. 101, n. 11, p. 4021–4028, 2010.

ELSAYED, M. T. et al. Impact of filter mud applications on the germination of sugarcane and small-seeded plants and on soil and sugarcane nitrogen contents. **Bioresource Technology**, v. 99, n. 10, p. 4164–4168, 2008.

FEHRENBACH, H. et al. Kriterien einer nachhaltigen Bioenergienutzung im globalen Maßstab. **UBA-Forschungsbericht**, v. 206, n. 206, p. 41–112, 2008.

FERNÁNDEZ, J.; PÉREZ, M.; ROMERO, L. I. Effect of substrate concentration on dry mesophilic anaerobic digestion of organic fraction of municipal solid waste (OFMSW). **Bioresource Technology**, v. 99, n. 14, p. 6075–6080, 2008.

FRANKE-WHITTLE, I. H. et al. Investigation into the effect of high concentrations of volatile fatty acids in anaerobic digestion on methanogenic communities. **Waste Management**, v. 34, n. 11, p. 2080–2089, 2014.

FUESS, L. T. et al. Thermophilic two-phase anaerobic digestion using an innovative fixed-bed reactor for enhanced organic matter removal and bioenergy recovery from sugarcane vinasse. **Applied Energy**, v. 189, p. 480–491, 2017a.

FUESS, L. T. et al. Designing full-scale biodigestion plants for the treatment of vinasse in sugarcane biorefineries: How phase separation and alkalinization impact biogas and electricity production costs? **Chemical Engineering Research and Design**, v. 119, p. 209–220, 2017b.

FUESS, L. T. et al. Temporal dynamics and metabolic correlation between lactate-producing and hydrogen-producing bacteria in sugarcane vinasse dark fermentation: The key role of lactate. **Bioresource Technology**, v. 247, n. September 2017, p. 426–433, 2018.

FUESS, L. T. et al. Biochemical butyrate production via dark fermentation as an energetically efficient alternative management approach for vinasse in sugarcane biorefineries. **Renewable Energy**, v. 158, p. 3–12, 2020.

GALACTION, A. I. et al. Prediction of oxygen mass transfer coefficients in stirred bioreactors for bacteria, yeasts and fungus broths. **Biochemical Engineering Journal**, v. 20,

n. 1, p. 85–94, 2004.

GANESH KUMAR, A.; SEKARAN, G.; KRISHNAMOORTHY, S. Solid state fermentation of Achras zapota lignocellulose by Phanerochaete chrysosporium. **Bioresource Technology**, v. 97, n. 13, p. 1521–1528, 2006.

GHEZLOUN, A.; SAIDANE, A.; MERABET, H. The COP 22 New commitments in support of the Paris Agreement. **Energy Procedia**, v. 119, p. 10–16, 2017.

GOLKOWSKA, K.; GREGER, M. Anaerobic digestion of maize and cellulose under thermophilic and mesophilic conditions – A comparative study. **Biomass and Bioenergy**, v. 56, p. 545–554, 2013.

GONZALEZ-GIL, G. et al. Kinetics and mass-transfer phenomena in anaerobic granular sludge. **Biotechnology and Bioengineering**, v. 73, n. 2, p. 125–134, 2001.

GU, Y. et al. Effect of inoculum sources on the anaerobic digestion of rice straw. **Bioresource Technology**, v. 158, n. 2014, p. 149–155, 2014.

GUNASEELAN, V. N. Biochemical methane potential of fruits and vegetable solid waste feedstocks. **Biomass and Bioenergy**, v. 26, n. 4, p. 389–399, 2004.

GUNASEELAN, V. N. Regression models of ultimate methane yields of fruits and vegetable solid wastes, sorghum and napiergrass on chemical composition. **Bioresource Technology**, v. 98, n. 6, p. 1270–1277, 2007.

GUO, X. et al. A comparison of microbial characteristics between the thermophilic and mesophilic anaerobic digesters exposed to elevated food waste loadings. **Bioresource Technology**, v. 152, p. 420–428, 2014.

HAGMAN, L. et al. The role of biogas solutions in sustainable biorefineries. **Journal of Cleaner Production**, v. 172, p. 3982–3989, 2018.

HAGOS, K. et al. Anaerobic co-digestion process for biogas production: Progress, challenges and perspectives. **Renewable and Sustainable Energy Reviews**, v. 76, n. November 2016, p. 1485–1496, 2017a.

HAGOS, K. et al. **Anaerobic co-digestion process for biogas production: Progress, challenges and perspectivesRenewable and Sustainable Energy Reviews**, 2017b.

HANREICH, A. et al. Metaproteome analysis to determine the metabolically active part of a thermophilic microbial community producing biogas from agricultural biomass. **Canadian Journal of Microbiology**, v. 58, n. 7, p. 917–922, 2012.

HANREICH, A. et al. Metagenome and metaproteome analyses of microbial communities in mesophilic biogas-producing anaerobic batch fermentations indicate concerted plant carbohydrate degradation. **Systematic and Applied Microbiology**, v. 36, n. 5, p. 330–338, 2013.

HARTMANN, H.; AHRING, B. K. Anaerobic digestion of the organic fraction of municipal solid waste: Influence of co-digestion with manure. **Water Research**, v. 39, n. 8, p. 1543–1552, 2005.

HATTORI, S. Syntrophic acetate-oxidizing microbes in methanogenic environments. **Microbes and Environments**, v. 23, n. 2, p. 118–127, 2008.

HERNÁNDEZ-PÉREZ, A. F.; DE ARRUDA, P. V.; FELIPE, M. DAS G. DE A. Sugarcane

straw as a feedstock for xylitol production by Candida guilliermondii FTI 20037. **Brazilian Journal of Microbiology**, v. 47, n. 2, p. 489–496, 2016.

HEYER, R. et al. Metaproteome analysis reveals that syntrophy, competition, and phage-host interaction shape microbial communities in biogas plants. **Microbiome**, v. 7, n. 1, p. 1–17, 2019.

HOLLIGER, C. et al. Towards a standardization of biomethane potential tests. **Water Science and Technology**, v. 74, n. 11, p. 2515–2522, 2016.

ILANGOVAN, K.; NOYOLA, A. Availability of micronutrients during anaerobic digestion of molasses stillage using an upflow anaerobic sludge blanket (Uasb) reactor. **Environmental Technology (United Kingdom)**, v. 14, n. 8, p. 795–799, 1993.

ISSAH, A.; KABERA, T.; KEMAUSUOR, F. Biomass and Bioenergy Biogas optimisation processes and effluent quality : A review. **Biomass and Bioenergy**, v. 133, n. June 2019, p. 105449, 2020.

JACKSON-MOSS, C. A.; DUNCAN, J. R. The effect of iron on anaerobic digestion. **Biotechnology Letters**, v. 12, n. 2, p. 149–154, 1990.

JAIN, S. et al. A comprehensive review on operating parameters and different pretreatment methodologies for anaerobic digestion of municipal solid waste. **Renewable and Sustainable Energy Reviews**, v. 52, p. 142–154, 2015.

JANDA, J. M.; ABBOTT, S. L. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. **Journal of Clinical Microbiology**, v. 45, n. 9, p. 2761–2764, 2007.

JANG, H. M. et al. Effect of increased load of high-strength food wastewater in thermophilic and mesophilic anaerobic co-digestion of waste activated sludge on bacterial community structure. **Water Research**, v. 99, p. 140–148, 2016.

JANKE, L. et al. Biogas Production from Sugarcane Waste: Assessment on Kinetic Challenges for Process Designing. **International journal of molecular sciences**, v. 16, n. 9, p. 20685–20703, 2015.

JANKE, L. et al. Optimization of hydrolysis and volatile fatty acids production from sugarcane filter cake: Effects of urea supplementation and sodium hydroxide pretreatment. **Bioresource Technology**, v. 199, p. 235–244, 2016a.

JANKE, L. et al. Comparison of start-up strategies and process performance during semi-continuous anaerobic digestion of sugarcane filter cake co-digested with bagasse. **Waste Management**, v. 48, p. 199–208, 2016b.

JANKE, L. et al. Improving anaerobic digestion of sugarcane straw for methane production: Combined benefits of mechanical and sodium hydroxide pretreatment for process designing. **Energy Conversion and Management**, v. 141, p. 378–389, 2017a.

JANKE, L. et al. Optimization of semi-continuous anaerobic digestion of sugarcane straw co-digested with filter cake: Effects of macronutrients supplementation on conversion kinetics. **Bioresource Technology**, v. 245, p. 35–43, 2017b.

JING, Y. et al. iTRAQ quantitative proteomic analysis reveals the pathways for methanation of propionate facilitated by magnetite. **Water Research**, v. 108, p. 212–221, 2017.

JUNQUEIRA, T. L. et al. Use of VSB to Plan Research Programs and Public Policies. Green

Energy and Technology. In: BONOMI, A. et al. (Eds.). . **Virtual Biorefinery: An Optimization Strategy for Renewable Carbon Valorization**. Campinas: Springer, 2015.

KAMDEM, I. et al. Enhanced Biogas Production During Anaerobic Digestion of Steam-Pretreated Lignocellulosic Biomass from Williams Cavendish Banana Plants. **Waste and Biomass Valorization**, v. 9, n. 2, p. 175–185, 2018.

KAPARAJU, P.; SERRANO, M.; ANGELIDAKI, I. Optimization of biogas production from wheat straw stillage in UASB reactor. **Applied Energy**, v. 87, n. 12, p. 3779–3783, 2010.

KARAKASHEV, D.; BATSTONE, D. J.; ANGELIDAKI, I. Influence of Environmental Conditions on Methanogenic Compositions.pdf. **Applied and Environmental Microbiology**, v. 71, n. 1, p. 331–338, 2005.

KARTHIKEYAN, O. P.; VISVANATHAN, C. Bio-energy recovery from high-solid organic substrates by dry anaerobic bio-conversion processes: A review. **Reviews in Environmental Science and Biotechnology**, v. 12, n. 3, p. 257–284, 2013.

KATAOKA, N.; MIYA, A.; KIRIYAMA, K. Studies on hydrogen production by continuous culture system of hydrogen-producing anaerobic bacteria. **Water Science and Technology**, v. 36, n. 6, p. 41–47, 1997.

KHAN, M. U.; AHRING, B. K. Lignin degradation under anaerobic digestion: Influence of lignin modifications -A review. **Biomass and Bioenergy**, v. 128, n. August, 2019.

KIM, E. et al. Comprehensive analysis of microbial communities in full-scale mesophilic and thermophilic anaerobic digesters treating food waste-recycling wastewater. **Bioresource Technology**, v. 259, n. January, p. 442–450, 2018.

KIM, J. Y. et al. Catalytic depolymerization of lignin macromolecule to alkylated phenols over various metal catalysts in supercritical tert-butanol. **Journal of Analytical and Applied Pyrolysis**, v. 113, p. 99–106, 2015.

KOCH, F. A.; OLDHAM, W. K. Oxidation-reduction potential - A tool for monitoring, control and optimization of biological nutrient removal systems. **Water Science and Technology**, v. 17, n. 11–12, p. 259–281, 1985.

KOSTER, I. W. et al. Sulfide inhibition of the methanogenic activity of granular sludge at various pH-levels. **Water Research**, v. 20, n. 12, p. 1561–1567, 1986.

KRISHANIA, M.; VIJAY, V. K.; CHANDRA, R. Methane fermentation and kinetics of wheat straw pretreated substrates co-digested with cattle manure in batch assay. **Energy**, v. 57, p. 359–367, 2013.

KRONGTHAMCHAT, K.; RIFFAT, R.; DARARAT, S. Effect of trace metals on halophilic and mixed cultures in anaerobic treatment. **International Journal of Environmental Science and Technology**, v. 3, n. 2, p. 103–112, 2006.

KRUGER, J. S. et al. Lignin Depolymerization with Nitrate-Intercalated Hydrotalcite Catalysts. **ACS Catalysis**, v. 6, n. 2, p. 1316–1328, 2016.

KRZYSZTOF ZIEMIŃSKI. Methane fermentation process as anaerobic digestion of biomass: Transformations, stages and microorganisms. **African Journal of Biotechnology**, v. 11, n. 18, p. 4127–4139, 2012.

KWIETNIEWSKA, E.; TYS, J. Process characteristics, inhibition factors and methane yields of anaerobic digestion process, with particular focus on microalgal biomass fermentation.

Renewable and Sustainable Energy Reviews, v. 34, p. 491–500, 2014.

LAM, T. K. et al. Meta-proteomics analysis of microbial ecosystem during the anaerobic digestion of chicken manure in biogas production farm. **Bioresource Technology Reports**, v. 13, n. February, p. 100643, 2021.

LAROUI, H. et al. Nanomedicine in GI. **American Journal of Physiology - Gastrointestinal and Liver Physiology**, v. 300, n. 3, p. 371–383, 2011.

LATA, K. et al. Volatile fatty acid production during anaerobic mesophilic digestion of tea and vegetable market wastes. **World Journal of Microbiology and Biotechnology**, v. 18, n. 6, p. 589–592, 2002.

LEAL, M. R. L. V. et al. Sugarcane straw availability, quality, recovery and energy use: A literature review. **Biomass and Bioenergy**, v. 53, p. 11–19, 2013.

LI, J. et al. Hydrogen-producing capability of anaerobic activated sludge in three types of fermentations in a continuous stirred-tank reactor. **Biotechnology Advances**, v. 27, n. 5, p. 573–577, 2009a.

LI, T. et al. Insights into networks of functional microbes catalysing methanization of cellulose under mesophilic conditions. **Environmental Microbiology**, v. 11, n. 4, p. 889–904, 2009b.

LIMA, D. R. S. et al. Influence of different thermal pretreatments and inoculum selection on the biomethanation of sugarcane bagasse by solid-state anaerobic digestion: A kinetic analysis. **Industrial Crops and Products**, v. 111, n. May 2017, p. 684–693, 2018.

LIN, C.-Y.; WU, C.-C.; HUNG, C.-H. Temperature effects on fermentative hydrogen production from xylose using mixed anaerobic cultures. **International Journal of Hydrogen Energy**, v. 33, n. 1, p. 43–50, 2008.

LIN, D. et al. Enhanced cytochrome formation and stimulate methanogenesis rate by the increased ferrous concentrations in Methanosarcina barkeri culture. **FEMS Microbiology Letters**, v. 68, n. 1–2, p. 89–92, 1990.

LIN, R. et al. Boosting biomethane yield and production rate with graphene: The potential of direct interspecies electron transfer in anaerobic digestion. **Bioresource Technology**, v. 239, p. 345–352, 2017.

LÓPEZ GONZÁLEZ, L. M.; PEREDA REYES, I.; ROMERO ROMERO, O. Anaerobic co-digestion of sugarcane press mud with vinasse on methane yield. **Waste Management**, v. 68, n. January 2018, p. 139–145, 2017.

MARCHAIM, U.; KRAUSE, C. Propionic to acetic acid ratios in overloaded anaerobic digestion. **Bioresource Technology**, v. 43, n. 3, p. 195–203, 1993.

MARTÍN-GONZÁLEZ, L.; FONT, X.; VICENT, T. Alkalinity ratios to identify process imbalances in anaerobic digesters treating source-sorted organic fraction of municipal wastes. **Biochemical Engineering Journal**, v. 76, p. 1–5, 2013.

MASHAPHU, N. THE MICROBIAL COMPOSITION OF A NATURAL METHANOGENIC CONSORTIUM Nthabiseng Mashaphu. n. November, 2005.

MASPOLIM, Y. et al. The effect of pH on solubilization of organic matter and microbial community structures in sludge fermentation. **Bioresource Technology**, v. 190, p. 289–298, 2015.

MCMURDIE, P. J.; HOLMES, S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. **PLoS ONE**, v. 8, n. 4, 2013.

MENANDRO, L. M. S. et al. Comprehensive assessment of sugarcane straw: implications for biomass and bioenergy production. **Biofuels, Bioproducts and Biorefining**, v. 11, n. 3, p. 488–504, 15 maio 2017.

MENON, A.; WANG, J. Y.; GIANNIS, A. Optimization of micronutrient supplement for enhancing biogas production from food waste in two-phase thermophilic anaerobic digestion. **Waste Management**, v. 59, p. 465–475, 2017.

MEUNCHANG, S.; PANICHSAKPATANA, S.; WEAVER, R. W. Co-composting of filter cake and bagasse; by-products from a sugar mill. **Bioresource Technology**, v. 96, n. 4, p. 437–442, 2005.

MIRAHMADI, K. et al. Alkaline pretreatment of spruce and birch to improve bioethanol and biogas production. **BioResources**, v. 5, n. 2, p. 928–938, 2010.

MORAES, B. S. et al. Anaerobic digestion of vinasse from sugarcane biorefineries in Brazil from energy, environmental, and economic perspectives: Profit or expense? **Applied Energy**, v. 113, p. 825–835, 2014.

MORAES, B. S. et al. Biogas production within the bioethanol production chain: Use of co-substrates for anaerobic digestion of sugar beet vinasse. **Bioresource Technology**, v. 190, p. 227–234, 2015.

MORAES, B. S. et al. Reduction in greenhouse gas emissions from vinasse through anaerobic digestion. **Applied Energy**, v. 189, n. 2017, p. 21–30, 2017.

MORAES, B. S.; ZAIAT, M.; BONOMI, A. **Anaerobic digestion of vinasse from sugarcane ethanol production in Brazil: Challenges and perspectivesRenewable and Sustainable Energy Reviews**, 2015a.

MORAES, B. S.; ZAIAT, M.; BONOMI, A. Anaerobic digestion of vinasse from sugarcane ethanol production in Brazil: Challenges and perspectives. **Renewable and Sustainable Energy Reviews**, v. 44, p. 888–903, 2015b.

MU, H.; CHEN, Y.; XIAO, N. Effects of metal oxide nanoparticles (TiO 2, Al 2O 3, SiO 2 and ZnO) on waste activated sludge anaerobic digestion. **Bioresource Technology**, v. 102, n. 22, p. 10305–10311, 2011.

MUELLER, L. N. et al. An assessment of software solutions for the analysis of mass spectrometry based quantitative proteomics data. **Journal of Proteome Research**, v. 7, n. 1, p. 51–61, 2008.

MUKHOPADHYAY, S. S. Nanotechnology in agriculture: Prospects and constraints. **Nanotechnology, Science and Applications**, v. 7, n. 2, p. 63–71, 2014.

MULAT, D. G.; HORN, S. J. Chapter 14: Biogas Production from Lignin via Anaerobic Digestion. **RSC Energy and Environment Series**, v. 2018-Janua, n. 19, p. 391–412, 2018.

NAKANISHI, S. C. et al. Fermentation strategy for second generation ethanol production from sugarcane bagasse hydrolyzate by Spathaspora passalidarum and Scheffersomyces stipitis. **Biotechnology and Bioengineering**, v. 114, n. 10, p. 2211–2221, 2017.

NAKASU, P. Y. S. et al. Interplay of acid-base ratio and recycling on the pretreatment performance of the protic ionic liquid monoethanolammonium acetate. **ACS Sustainable**

Chemistry & Engineering, 2020.

NEL, L. H.; BRITZ, T. J.; LATEGAN, P. M. The effect of trace elements on the performance efficiency of an anaerobic fixed film reactor treating a petrochemical effluent. **Water SA**, v. 11, n. 3, p. 107–110, 1985.

NELSON, M. C.; MORRISON, M.; YU, Z. A meta-analysis of the microbial diversity observed in anaerobic digesters. **Bioresource Technology**, v. 102, n. 4, p. 3730–3739, 2011.

NGHIEM, L. D. et al. Oxidation reduction potential as a parameter to regulate micro-oxygen injection into anaerobic digester for reducing hydrogen sulphide concentration in biogas. **Bioresource Technology**, v. 173, p. 443–447, 2014.

NI, S. Q. et al. Effect of magnetic nanoparticles on the performance of activated sludge treatment system. **Bioresource Technology**, v. 143, p. 555–561, 2013.

OKADA, D. Y. et al. Evaluation of the microbial community of upflow anaerobic sludge blanket reactors used for the removal and degradation of linear alkylbenzene sulfonate by pyrosequencing. **International Biodeterioration and Biodegradation**, v. 96, p. 63–70, 2014.

OWENS, J. M.; CHYNOWETH, D. P. Biochemical methane potential of municipal solid waste (MSW) components. **Water Science and Technology**, v. 27, n. 2, p. 1–14, 1993.

PARAWIRA, W. et al. Energy production from agricultural residues: High methane yields in pilot-scale two-stage anaerobic digestion. **Biomass and Bioenergy**, v. 32, n. 1, p. 44–50, 2008.

PARISI, C.; VIGANI, M.; RODRÍGUEZ-CEREZO, E. Agricultural nanotechnologies: What are the current possibilities? **Nano Today**, v. 10, n. 2, p. 124–127, 2015.

PEDDIE, C. C.; MAVINIC, D. S.; JENKINS, C. J. USE OF ORP FOR MONITORING AND CONTROL OF AEROBIC SLUDGE DIGESTION. v. 116, n. 3, p. 461–471, 1990.

PENG, X. et al. Impact of bioaugmentation on biochemical methane potential for wheat straw with addition of Clostridium cellulolyticum. **Bioresource Technology**, v. 152, p. 567–571, 2014.

PÉREZ-PIMIENTA, J. A. et al. Bioderived ionic liquid-based pretreatment enhances methane production from: Agave tequilana bagasse. **RSC Advances**, v. 10, n. 24, p. 14025–14032, 2020.

PERSKI, H. J.; MOLL, J.; THAUER, R. K. Sodium dependence of growth and methane formation in Methanobacterium thermoautotrophicum. **Archives of Microbiology**, v. 130, n. 4, p. 319–321, 1981.

PING, Q. et al. Metagenomic characterization of the enhanced performance of anaerobic fermentation of waste activated sludge with CaO 2 addition at ambient temperature : Fatty acid biosynthesis metabolic pathway and CAZymes. **Water Research**, v. 170, p. 115309, 2020.

PINHEIRO, T. et al. Differential proteomic analysis by SWATH-MS unravels the most dominant mechanisms underlying yeast adaptation to non-optimal temperatures under anaerobic conditions. **Scientific Reports**, v. 10, n. 1, p. 1–17, 2020.

PINTO, M. P. M. et al. Co–digestion of coffee residues and sugarcane vinasse for biohythane generation. **Journal of Environmental Chemical Engineering**, v. 6, n. 1, p. 146–155, 2018.

POBEHEIM, H. et al. Influence of trace elements on methane formation from a synthetic model substrate for maize silage. **Bioresource Technology**, v. 101, n. 2, p. 836–839, 2010.

QUAST, C. et al. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. **Nucleic Acids Research**, v. 41, n. D1, p. 590–596, 2013.

QUINTERO, M. et al. Enhancement of starting up anaerobic digestion of lignocellulosic substrate: Fique's bagasse as an example. **Bioresource Technology**, v. 108, p. 8–13, 2012.

RABELO, S. C. et al. Production of bioethanol, methane and heat from sugarcane bagasse in a biorefinery concept. **Bioresource Technology**, v. 102, n. 17, p. 7887–7895, 2011.

RABELO, S. C. et al. Alkaline hydrogen peroxide pretreatment, enzymatic hydrolysis and fermentation of sugarcane bagasse to ethanol. **Fuel**, v. 136, p. 349–357, 2014.

RAPOSO, F. et al. Biochemical methane potential (BMP) of solid organic substrates: Evaluation of anaerobic biodegradability using data from an international interlaboratory study. **Journal of Chemical Technology and Biotechnology**, v. 86, n. 8, p. 1088–1098, 2011.

REN, N. Q. et al. Assessing optimal fermentation type for bio-hydrogen production in continuous-flow acidogenic reactors. **Bioresource Technology**, v. 98, n. 9, p. 1774–1780, 2007.

RIPLEY, L. E.; BOYLE, W. C.; CONVERSE, J. C. Improved alkalimetric monitoring for anaerobic digestion of high-strength waste. 1986.

RODRIGUEZ-CHIANG, L.; LLORCA, J.; DAHL, O. Anaerobic co-digestion of acetate-rich with lignin-rich wastewater and the effect of hydrotalcite addition. **Bioresource Technology**, v. 218, p. 84–91, 2016.

ROGNES, T. et al. VSEARCH: A versatile open source tool for metagenomics. **PeerJ**, v. 2016, n. 10, p. 1–22, 2016.

ROMERO-GÜIZA, M. S. et al. The effect of magnesium as activator and inhibitor of anaerobic digestion. **Waste Management**, v. 56, p. 137–142, 2016.

SAHITO, A. R.; MAHAR, R. B. U. X.; BROHI, K. M. Anaerobic Co-Digestion of Canola Straw and Buffalo Dung: Optimization of Methane Production in Batch Experiments. v. 33, n. 1, p. 49–60, 2014.

SANTOS, F. A. et al. Potential of sugarcane straw for ethanol production. **Quimica Nova**, v. 35, n. 5, p. 1004–1010, 2012.

SAXENA, R. C.; ADHIKARI, D. K.; GOYAL, H. B. Biomass-based energy fuel through biochemical routes: A review. **Renewable and Sustainable Energy Reviews**, v. 13, n. 1, p. 167–178, 2009.

SCHARER, J. M.; MOO-YOUNG, M. **Methane generation by anaerobic digestion of cellulose-containing wastes**. Advances in Biochemical Engineering, Volume 11. **Anais**...Berlin, Heidelberg: Springer Berlin Heidelberg, 1979

SCHERER, P.; LIPPERT, H.; WOLFF, G. Composition of the major elements and trace elements of 10 methanogenic bacteria determined by inductively coupled plasma emission spectrometry. **Biological Trace Element Research**, v. 5, n. 3, p. 149–163, 1983.

SCHMIDT, T. et al. Trace element supplementation in the biogas production from wheat

stillage - Optimization of metal dosing. **Bioresource Technology**, v. 168, p. 80–85, 2014.

SCHNÜRER, A.; ZELLNER, G.; SVENSSON, B. H. Mesophilic syntrophic acetate oxidation during methane formation in biogas reactors. **FEMS Microbiology Ecology**, v. 29, n. 3, p. 249–261, 1999.

SILVA, G. A.; MORAIS, J. A.; ROCHA, E. R. Proposta de procedimento operacional padrão para o teste do Potencial Bioquímico do Metano aplicado a resíduos sólidos urbanos. **Engenharia Sanitaria e Ambiental**, v. 21, n. 1, p. 11–16, 2016.

SLUITER, J. B. et al. Evaluation of Brazilian sugarcane bagasse characterization: An interlaboratory comparison study. **Journal of AOAC International**, v. 99, n. 3, p. 579–585, 2016.

SOEST, P. J. V. A. N. Limiting factors in plant residues of low biodegradability. **Agriculture and Environment**, v. 6, p. 135–143, 1981.

SOUZA, M. E.; FUZARO, G.; POLEGATO, A. R. Thermophilic anaerobic digestion of vinasse in pilot plant UASB reactor. **Water Science and Technology**, v. 25, n. 7, p. 213–222, 1992.

STAMS, A. J. M.; PLUGGE, C. M. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. **Nature Reviews Microbiology**, v. 7, n. 8, p. 568–577, 2009.

SU, C. et al. Culture-independent methods for studying environmental microorganisms: Methods, application, and perspective. **Applied Microbiology and Biotechnology**, v. 93, n. 3, p. 993–1003, 2012.

SUNG JAE LEE. Relationship between Oxidation Reduction Potential (ORP) and Volatile Fatty Acid (VFA) Production in the Acid-Phase Anaerobic Digestion Process. **Thesis**, n. January, 2008.

SUTARYO, S.; WARD, A. J.; MØLLER, H. B. Thermophilic anaerobic co-digestion of separated solids from acidified dairy cow manure. **Bioresource Technology**, v. 114, p. 195–200, 2012.

TAN, L. et al. Production of bioethanol from unwashed-pretreated rapeseed straw at high solid loading. **Bioresource Technology**, v. 303, n. December 2019, p. 122949, 2020.

TELLECHEA, F. R. F. et al. Use of sugarcane filter cake and nitrogen, phosphorus and potassium fertilization in the process of bioremediation of soil contaminated with diesel. **Environmental Science and Pollution Research**, v. 23, n. 18, p. 18027–18033, 2016.

TRAMONTINA, R. et al. Designing a cocktail containing redox enzymes to improve hemicellulosic hydrolysate fermentability by microorganisms. **Enzyme and Microbial Technology**, v. 135, n. August 2019, p. 109490, 2020.

TRIOLO, J. M. et al. Biochemical methane potential and anaerobic biodegradability of non-herbaceous and herbaceous phytomass in biogas production. **Bioresource Technology**, v. 125, p. 226–232, 2012.

VONGVICHIANKUL, C.; DEEBAO, J.; KHONGNAKORN, W. Relationship between pH, Oxidation Reduction Potential (ORP) and Biogas Production in Mesophilic Screw Anaerobic Digester. **Energy Procedia**, v. 138, p. 877–882, 2017.

WALTER, A.; ENSINAS, A. V. Combined production of second-generation biofuels and electricity from sugarcane residues. **Energy**, v. 35, n. 2, p. 874–879, 2010.

WANG, L.; ZHOU, Q.; LI, F. T. Avoiding propionic acid accumulation in the anaerobic process for biohydrogen production. **Biomass and Bioenergy**, v. 30, n. 2, p. 177–182, 2006.

WANG, P. et al. Microbial characteristics in anaerobic digestion process of food waste for methane production–A review. **Bioresource Technology**, v. 248, p. 29–36, 2018.

WANG, Q. et al. Degradation of volatile fatty acids in highly efficient anaerobic digestion. **Biomass and Bioenergy**, v. 16, n. 6, p. 407–416, 1999.

WANG, R. et al. Phosphate release involving PAOs activity during anaerobic fermentation of EBPR sludge and the extension of ADM1. **Chemical Engineering Journal**, v. 287, p. 436–447, 2016a.

WANG, T. et al. Effects of metal nanoparticles on methane production from waste-activated sludge and microorganism community shift in anaerobic granular sludge. **Scientific Reports**, v. 6, n. April, p. 1–10, 2016b.

WANG, X. et al. Optimizing feeding composition and carbon-nitrogen ratios for improved methane yield during anaerobic co-digestion of dairy, chicken manure and wheat straw. **Bioresource Technology**, v. 120, p. 78–83, 2012.

WANG, Y. et al. Effects of volatile fatty acid concentrations on methane yield and methanogenic bacteria. **Biomass and Bioenergy**, 2009.

WEI, Q. et al. Performance and kinetic evaluation of a semi-continuously fed anaerobic digester treating food waste: Effect of trace elements on the digester recovery and stability. **Chemosphere**, v. 117, n. 1, p. 477–485, 2014.

WEIJMA, J.; STAMS, A. J. M. Methanol conversion in high-rate anaerobic reactors. p. 7–14, 1999.

WEILAND, P. Biogas production: Current state and perspectives. **Applied Microbiology and Biotechnology**, v. 85, n. 4, p. 849–860, 2010.

WERNER, J. J. et al. Bacterial community structures are unique and resilient in full-scale bioenergy systems. **Proceedings of the National Academy of Sciences of the United States of America**, v. 108, n. 10, p. 4158–4163, 2011.

WIEGANT, W. M.; HENNINK, M.; LETTINGA, G. Separation of the propionate degradation to improve the efficiency of thermophilic anaerobic treatment of acidified wastewaters. **Water Research**, v. 20, n. 4, p. 517–524, 1986.

WILKINS, D. et al. Pyrosequencing of mcrA and archaeal 16s rRNA genes reveals diversity and substrate preferences of methanogen communities in anaerobic digesters. **Applied and Environmental Microbiology**, v. 81, n. 2, p. 604–613, 2015.

WINTSCHE, B. et al. Trace elements induce predominance among methanogenic activity in anaerobic digestion. **Frontiers in Microbiology**, v. 7, n. DEC, p. 1–12, 2016.

WU, G.; HEALY, M. G.; ZHAN, X. Effect of the solid content on anaerobic digestion of meat and bone meal. **Bioresource Technology**, v. 100, n. 19, p. 4326–4331, 2009.

WU, X. et al. Effects of copper salts on performance, antibiotic resistance genes, and microbial community during thermophilic anaerobic digestion of swine manure. **Bioresource Technology**, v. 300, n. 30, p. 122728, 2020.

YU, B. et al. Variations of organic matters and microbial community in thermophilic

anaerobic digestion of waste activated sludge with the addition of ferric salts. **Bioresource Technology**, v. 179, p. 291–298, 2015.

YU, Q. et al. Biogas Production and Microbial Community Dynamics during the Anaerobic Digestion of Rice Straw at 39-50 °c: A Pilot Study. **Energy and Fuels**, v. 32, n. 4, p. 5157–5163, 2018.

ZAPAROLLI, D. **Tapping more energy from sugarcane**. Disponível em: <https://revistapesquisa.fapesp.br/en/2020/02/19/tapping-more-energy-from-sugarcane/>.

ZHANG, B.; CAI, W.; HE, P. Influence of lactic acid on the two-phase anaerobic digestion of kitchen wastes. **Journal of Environmental Science**, v. 19, n. 1, p. 244–249, 2007.

ZHANG, D. et al. Bioreactor performance and methanogenic population dynamics in a low-temperature (5-18°C) anaerobic fixed-bed reactor. **Bioresource Technology**, v. 104, p. 136–143, 2012.

ZHANG, L. et al. Role of extracellular protein in the formation and stability of aerobic granules. **Enzyme and Microbial Technology**, v. 41, n. 5, p. 551–557, 2007.

ZHANG, Y. et al. Uptake and mass balance of trace metals for methane producing bacteria. **Biomass and Bioenergy**, v. 25, n. 4, p. 427–433, 2003.

ZHANG, Y. et al. Study of microbial community structures in UASB sludge treating municipal wastewater by denaturing gradient gel electrophoresis of 16S rDNA. **Science in China, Series C: Life Sciences**, v. 48, n. SUPPL.1, p. 128–135, 2005.

ZIELIŃSKI, M. et al. Effects of Nutrients Supplementation on Enhanced Biogas Production from Maize Silage and Cattle Slurry Mixture. **Water, Air, and Soil Pollution**, v. 230, n. 6, 2019.

ZIGANSHIN, A. M. et al. Microbial community structure and dynamics during anaerobic digestion of various agricultural waste materials. **Applied Microbiology and Biotechnology**, v. 97, n. 11, p. 5161–5174, 2013.

ZIGANSHIN, A. M. et al. Spatial separation of metabolic stages in a tube anaerobic baffled reactor: reactor performance and microbial community dynamics. **Applied Microbiology and Biotechnology**, p. 3915–3929, 2019.

ZONTA, Ž. et al. Modelling inhibitory effects of long chain fatty acids in the anaerobic digestion process. **Water Research**, v. 47, n. 3, p. 1369–1380, 2013.

ZUMSTEIN, E.; MOLETTA, R.; GODON, J. J. Examination of two years of community dynamics in an anaerobic bioreactor using fluorescence polymerase chain reaction (PCR) single-strand conformation polymorphism analysis. **Environmental Microbiology**, v. 2, n. 1, p. 69–78, 2000.

ZWIETERING, M. H. et al. Modeling of the bacterial growth curve. **Applied and Environmental Microbiology**, v. 56, n. 6, p. 1875–1881, 1990.

9 APPENDIX A

The work bellow was presented at IWA - 16th World Conference on Anaerobic Digestion, in Delft Netherland. 23-19 June 2019.

Biogas production integrated to the concept of biorefinery for lignocellulosic biomass

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Abstract: In the challenging context of the Paris Agreement (COP21-2015), biogas production is once again receiving global prominence and, consequently, governmental and industrial initiatives. In a scenario of high expectation, substrates from the sugar-alcohol activity are considered as potential facilitators of the development of biorefineries. However, there are gaps in the literature regarding anaerobic digestion (AD) of waste from the production of lignocellulosic (or second generation, 2G) ethanol, but it is known that its recalcitrance may be an obstacle to the biological process. The results of this work showed that sugarcane vinasse and filter cake have potential for biogas production and that co-digestion is an alternative to use residues that have low biodegradability, as is the case of pre-residue treatment of sugarcane straw

Keywords: Sugarcane biorefinery; Co-digestion; Lignocellulosic residue,

Introduction

Adopted in Paris at the United Nations Conference on Climate Change (COP21), the agreement officially entered into force in November 2016, with governments' notorious commitment to key areas related to climate change, adaptation and enhancement in terms of capacity and energy technologies (Ghezloun et al., 2017). Each country intends to continue with its own efforts to achieve objectives and targets of emission reduction. The RenovaBio Program, launched by the Brazilian Ministry of Mines and Energy (MME) in December 2016, was designed to address the new expansion of biofuel production and use in a more sustainable way (Addington, 2017). In this promising and challenging context, the production of biogas is returning to prominence and, consequently, has received numerous initiatives.

Recently, a study by the Brazilian Association of Biogas and Biomethane (Abiogás) indicated that Brazil has the potential to generate 23 billion cubic meters of CH_4 per year - the final

product of a biogas plant. In this scenario of high expectations, the substrates from the sugar and alcohol activity are considered as raw material for the generation of value-added products, such as biomethane. Biogas can be a facilitator of the development of biorefineries, as well as improving the value of the product portfolio (Hagman et al., 2017).

The by-products of the sugarcane industry are already considered raw materials for recovery and generation of value-added products. Vinasse, a by-product of the distillation process (10 L of vinasse produced per liter of ethanol) is commonly directed to the soil (sugarcane plantation) as liquid-fertile. The filter cake, another solid compound, is generated after the process of clarifying the cane juice prior to the production of first generation sugar and / or ethanol (1G) from the filtration in rotary filters. It has been used in intrinsic steps at the plant (improvements in permeability during sucrose recovery in the rotary filter) (Janke et al., 2016) and as a source of nutrients for the soil (Tellechea et al., 2016).

Currently the search for available residual substrates is in line with the diversification of product generation. Allied to this, the intensity of the expectations regarding the use of several biomasses and the production of biogas for energy purposes is outstanding. In spite of all the scientific growth in this area, it is necessary to deepen the knowledge based on innovative issues and variations, that investigate in a comprehensive way the interactions between the technological limitations prevailing in the bioprocess for the generation of CH_4 (Rabelo et al., 2014; Nakanishi et al., 2017).

Substrate co-digestion can optimize CH4 production by providing and balancing macro and micronutrients for the AD process, and may also be the best choice for poorly biodegradable substrates. This appears to be the case with residues from ethanol production from the processing of lignocellulosic biomass, usually recognized as complex substrates for AD. However, there are gaps in the literature regarding the anaerobic co-digestion of waste from the production of 2G ethanol, especially for the recent and innovative pretreatment of biomass and hydrolysis, e.g., deacetylation process, pre-treatment with ionic liquids, hydrolysis using genetically modified yeast, among others. The complexity of such substrates for AD may be one of the factors driving the integration of the 1G2G ethanol process by co-digestion of its residues, for example, recognition of the biogas production from 1G vinasse (Júnior et al., 2016).

In this context, this research project aims to fill gaps in the literature regarding the integration of biogas production in the concept of 1G2G sugarcane biorefineries, in order to explore the potential of co-digestion of by-products of production of 1G2G ethanol. The project was developed according the determination of Biochemical Mehtane Potential (BMP) in Duran

flasks of the substrates (vinasse, filter cake and 2G ethanol pretreatment residues) to analyze their CH_4 production potential for co-digestion.

Material and Methods

The substrates used was the vinasse and filter cake (from 1G ethanol production), obtained from the Iracema Plant (from the São Martinho group) and the pre-treatment residue from the deacetylation process of straw (from the production of 2G ethanol) from the National Laboratory of Bioethanol Technology Science (CTBE). As inoculum, an anaerobic consortium from the BIOPAQ®ICX reactor of the Iracema Plant (from the São Martinho group) was used. The experiments was conducted at 55 ° C. The experimental BMP will be performed according to the VDI4600 methodology in Duran flasks. BMP was performed for each substrate in the flaks separately and a co-digestion of the 3 substrates was done together in a flask.

Results and Conclusions

According to the results obtained, it is possible to observe that among the produced methane potencial, the filter cake (Figure A3) is the one with the best potential for methane production, followed by vinasse (Figure A1). The liquor coming from the deacetylation pretreatment of sugarcane straw (Figure A2) did not present good biogas production, evidencing that pentoses alone did not produce biogas.

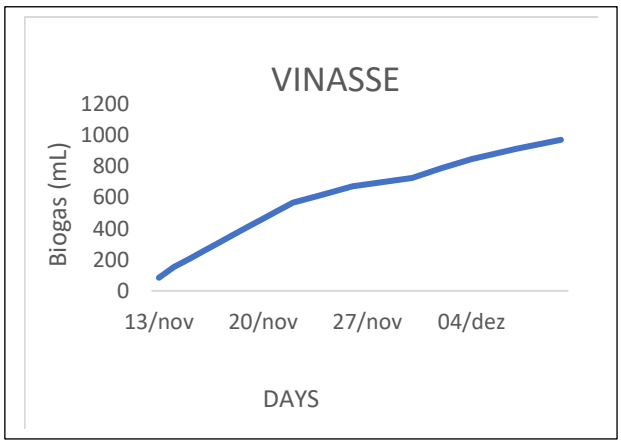

Figure A1. BMP of Vinasse

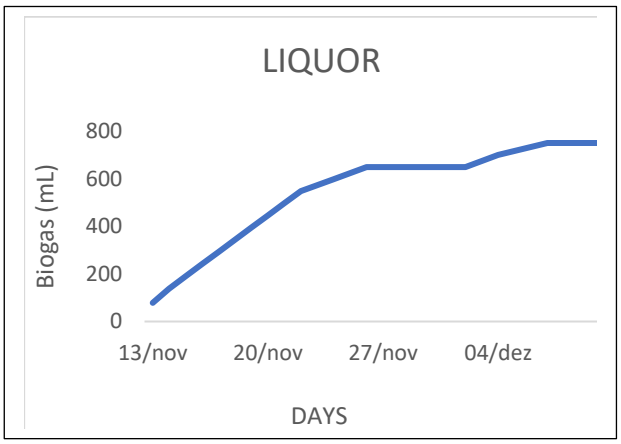

Figure A2. BMP of Liquor

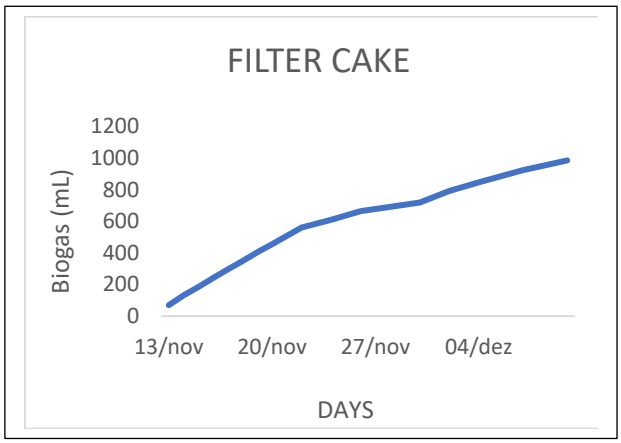

Figure A3. BMP of Filter Cake

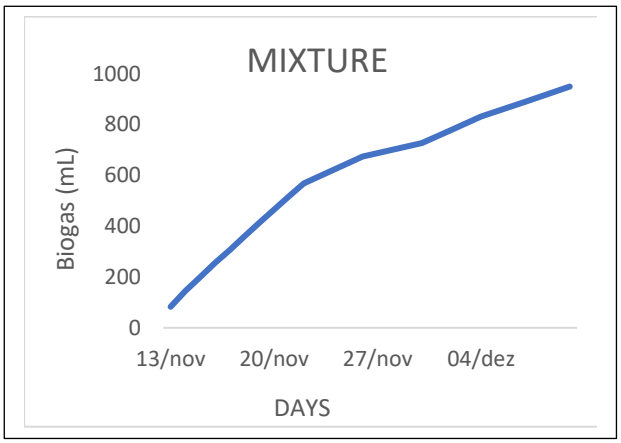

Figure A4. BMP of Mixture

However in the co-digestion (Figure A4) it was possible to obtain a better production, making it clear that it becomes a good alternative to use residues that have a low biodegradability, as is the case of the desacetilation residue.

Thus, the present work shows that co-digestion has become a good option for the process of anaerobic digestion, waste management and an optimization of the methane production, thus allowing the integration of 1G and 2G ethanol biorefinery.

References

Addington, B. (2017), RenovaBio: A Paradigm Shift for Biofuels in Brazil**.** Avaliable: <http://www.udop.com.br/download/noticias/2017/24_07_17_artigo_renovabio.pdf>.

Ghezloun, A., Saidane, A., Merabet, H. (2017), International Conference on Technologies and Materials for Renewable Energy, Environment and Sustainability, TMREES17, 21-24 April 2017, Beirut Lebanon THE COP 22. Ener. Proced**.,** 119,10-16,
Hagman, L., Alyssa, B., Mats, E., Niclas, S.,(2017), The role of biogas solutions in sustainable biorefineries. J. Clean. Produc**.,** 1-8.

Janke, L., Leite, A., Batista, K., Weinrich, S., Sträuber, H., Nikolausz, M., Nelles, M., Stinner, W, (2016), Optimization of hydrolysis and volatile fatty acids production from sugarcane filter cake: Effects of urea supplementation and sodium hydroxide pretreatment. Biores. Technol**.**, 199, 235-244.

Nakanishi, S.C., Soares, L. B., Biazi, L. E., Nascimento. V. M., Costa, A. C., Rocha, G. J. M., Ienczak, J. L., (2017), Fermentation strategy for second generation ethanol production from sugarcane bagasse hydrolyzate by Spathaspora passalidarum and Scheffersomyces stipitis. Biotechnol. Bioengineeri., 114, 2211-2221.

Sarita, C.R., Andrade, R. R., Filho, R. M., Costa, A. C, (2014), Alkaline hydrogen peroxide pretreatment, enzymatic hydrolysis and fermentation of sugarcane bagasse to ethanol. Fuel, 136, 349-357.

Tellechea, F. R., Martins, M. A., Silva, A. A., Gama-Rodrigues, E. F., Martins, M. L., (2016), Use of sugarcane filter cake and nitrogen, phosphorus and potassium fertilization in the process of bioremediation of soil contaminated with diesel. **Envir. Sci. Pollut. Researc.**, 23, 18027-18033

16TH IWA World Conference on Anaerobic Digestion
Accelerating natural cycles with anaerobic digestion

Biogas production integrated to the concept of biorefinery for lignocellulosic biomass

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INTRODUCTION

The by-products of the sugarcane industry (e.g vinasse, filter cake from 1G etanol production) are already considered raw materials for recovery and generation of value-added products, like biogas for energy production.

Substrate co-digestion can optimize CH4 production by providing and balancing macro and micronutrients for the anaerobic digestion (AD) process. However, there are gaps in the literature regarding the anaerobic co-digestion of waste from the production of 2G ethanol, especially for the recent and innovative pretreatment of biomass.

This research project aims to fill gaps in the literature regarding the integration of biogas production in the concept of 1G2G sugarcane biorefineries, in order to explore the potential of co-digestion of by-products of production of 1G2G ethanol.

MATERIALS AND METHODS

Sugarcane

Vinasse (1G)

Filter Cake (1G)

Liquor from straw deacetylation (2G)

Biochemical Methane Potential (BMP)

RESIDUE + INOCCULUM

Temperature: 55ºC

GAS VOLUM

GAS CROMATOGRAPHY ANALYSIS

RESULTS

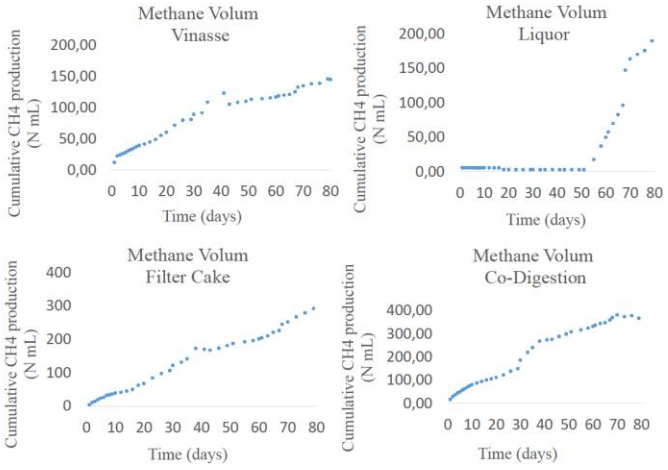

TABLE 1- Biochemical Methane Potential (BMP)

RESIDUE	BMP (N mL de CH4 / g SV)
VINASSE	488,38
LIQUOR	604,54
CO-DIGESTION	660,34
FILTER CAKE	362,06

CONCLUSIONS

Co-digestion proved to be a good option for waste management and enhanced methane generation from by-products of 1G and 2G ethanol production, contributing for structuring the concept of integrated sugarcane biorefinery.

AKNOWLEDGMENT

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16TH IWA World Conference on Anaerobic Digestion

Accelerating natural cycles with anaerobic digestion

From: **IWC International Water Conferences**
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Subject: **Certificate of attendance**

IWC - 16th World Conference on Anaerobic Digestion 23-27 June 2019, The Netherlands, Sunday June 23, 2019 - Thursday June 27, 2019

The Hague, 2019 July 2th

To whom it may concern:

On behalf of the Royal Netherlands Water Network (KNW) / IWC International Water Conferences, Delft University of Technology and the Ghent University, we hereby confirm you attend the 16th World Conference on Anaerobic Digestion in Delft, The Netherlands, 23 – 27 June 2019. The conference was a highly successful and exciting event and we werehonored with your presence at this event.

Title of your presentation during the conference: Biogas Production Integrated to the concept of Biorefinery for ligonocellulosic biomass

Authros: Maria Paula Cardeal Volpi, Danilo Herculano Silva, Brenno Vinicius de Medeiros Lima, Lucas Monteiro Galotti Souza, Gabriela Pereira de Freitas , Bruna de Souza Moraes

Type here the title of your presentation(s): Poster

With kind regards,

Drs M.M. Bekkenutte
Royal Netherlands Water Network

10 APPENDIX B

The expanded summary below was presented at the Online Latin Meetings on Anaerobic Digestion-DAAL 2020, in poster format, on November 12th, in an online congress. The certificate is below.

Oxidation Reduction Potential (ORP) for monitoring co-digestion reactor start-up fed with residues from sugarcane industry

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Highlights

- Co-digestion can improve the low biodegradability of lignocellulosic residues.
- ORP can be a useful control parameter of anaerobic digesters
- Variations in ORP values reflected CH_4 production instability during reactor start-up
- Optimal ORP value for co-digestion of sugarcane residues was close to -500 mV

Abstract: Co-digestion is characterized as the Anaerobic Digestion (AD) of two or more substrates, which is an option to overcome disadvantages of mono-digestion. The oxidation-reduction potential (ORP) is an important control parameter of AD from a biological point of view related to electron transfer between species of bacteria and archeas. It allows understanding the microbiological and operational interactions along the production of biogas from different raw materials. ORP have been used as a control parameter for AD, but there are gaps in literature related to ORP for monitoring co-digestion reactor start-up, especially in the sugarcane industry. The objective of the present work was to evaluate the content of methane in biogas integrated to the monitoring of ORP values in the co-digestion of residues from sugarcane industry. The results showed that the ORP values varied considerably at the beginning of the operation as well as the values of methane content, reflecting the reactor start-up phase and the microbial consortia adaptation. ORP values stabilized after 38 days of operation followed by the steady state methane content. The optimal ORP value for stabilized methane content was close to -500 mV. ORP proved to be an effective control parameter to monitor the co-digestion reactor start-up.

Keywords: Co-digestion; Sugarcane Residues; Oxidation Reduction Potential; Methane Content; Reactor Start-up

Introduction

Anaerobic digestion (AD) is a process used to treat organic waste such as agro-waste, animal manure or municipal waste. During this process, the organic material is transformed into biogas, which is composed mainly of methane and carbon dioxide, by a faithfully balanced ecosystem of microorganisms (KARTHIKEYAN; VISVANATHAN, 2013). In addition, co-digestion is characterized by the AD of two or more substrates which is an option to overcome disadvantages of mono-digestion, mainly in relation to the balance of nutrients and to improve the residues that have low biodegradability, as lignocellulosic residues (HAGOS et al., 2017a).

From a biological point of view, AD occurs with different microorganisms that are involved in different stages of the process: hydrolysis, acidogenesis, acetogenesis and methanogenesis (DEUBLIN; STEINHAUSER, 2008).

Within AD the efficiency of energy conversion and process stability can be easily disturbed by biological and environmental factors, such as temperature, pH, hydrodynamics, retention time (LIN et al., 2017). In addition to these parameters, there is the oxidation-reduction potential (ORP), which can also cause changes in AD mainly from a biological point of view, related to electron transfer between species of bacteria and archeas (STAMS; PLUGGE, 2009).

ORP is a useful parameter to control anaerobic digesters, because measures the net value of all complex oxidation reduction reactions within an aqueous environment. The ORP indicates different oxygen concentration conditions in a reactor (aerobic, anoxic and anaerobic) and it is a parameter for monitoring process control (PEDDIE; MAVINIC; JENKINS, 1990). Studies show that very high levels of ORP may indicate an inhibition of reactor activity. Under normal conditions of AD, the ideal operating range would be between -220 to -400 mV (BLANC; MOLOF, 1973).

Many complex reactions occur during AD process and it is difficult to identify each one separately. Some products from one biological reaction can be used as substrate for subsequent reactions (SUNG JAE LEE, 2008) and the ORP is important to understand the microbiological and operational interactions along the production of biogas from different raw materials. Some authors have already been using ORP as a control parameter for AD (NGHIEM et al., 2014; SUNG JAE LEE, 2008), but there is little information related to co-digestion of solid waste and lignocellulosic residues in the context of sugarcane biorefineries. In addition, ORP could be an effective parameter to monitor especially the start-up of reactor and the subsequent stabilization of methane production. Start-up is an important step for establishing an appropriate microbial community in anaerobic biological treatment processes, indicating the period of acclimatization of the inoculum. When methane production stabilizes, it indicates that the reactor's start-up period is over (ANGELIDAKI et al., 2006). Several oxidation reduction reactions occur in the first steps of AD (acidogenesis) and then these reactions end up being focused on methane production, achieving stability with negative ORP values.

Given this context, the objective of this work was to co-digest residues from the sugarcane industry, monitoring the reactor's ORP integrated to methane content in biogas to understand the behaviour of that parameter during the start-up of the co-digestion reactor.

Material and Methods

Residues and Inoculum

The substrates were vinasse, filter cake (from 1G ethanol production), obtained from Iracema Mill (Iracemápolis-SP-BR) and a lignocellulosic liquor obtained from sugarcane straw deacetylation pre-treatment process (in bench-scale) from 2G ethanol production performed at National Biorenovables Laboratory (LNBR)-Campinas-SP-BR. Inoculum was obtained from mesophilic anaerobic reactor (BIOPAQ-ICX) treating vinasse at the aforementioned sugarcane mill.

Reactor operation

The reactor consisted in a 5 L flask fed daily with an Organic Load Rate (OLR) of 2 $g.VS.L^{-1}.day^{-1}$, co-digesting 70% vinasse, 20% filter cake and 10% liquor in terms of volatile solids. The reactor was kept under agitation and 55°C. The methane content was measured by gas chromatography (Construmaq-São Carlos) and the ORP was measured by a DIGIMED probe, with both analyses being performed 3 times a week.

Results and Discussion

Figure B1 shows the methane content in biogas related to ORP values during the reactor start-up. Considerable variation in methane content in biogas was observed in the beginning of operation, with stabilization only after 45 days. This shows the first stages of the AD phase, hydrolysis and acidogenesis, so that only in the end does methanogenesis enter and thus stabilize methane content. Furthermore, it can be observed the period of adaptation of the microbial consortium, especially the methanogenic ones. This is intrinsic of the start-up of anaerobic reactors, which is a decisive phase for the success of the operation. Dissatisfaction in biological start-up treatment systems can lead to a prolonged period of acclimatization and ineffective removal of organic matter (Angelidak et al., 2006).

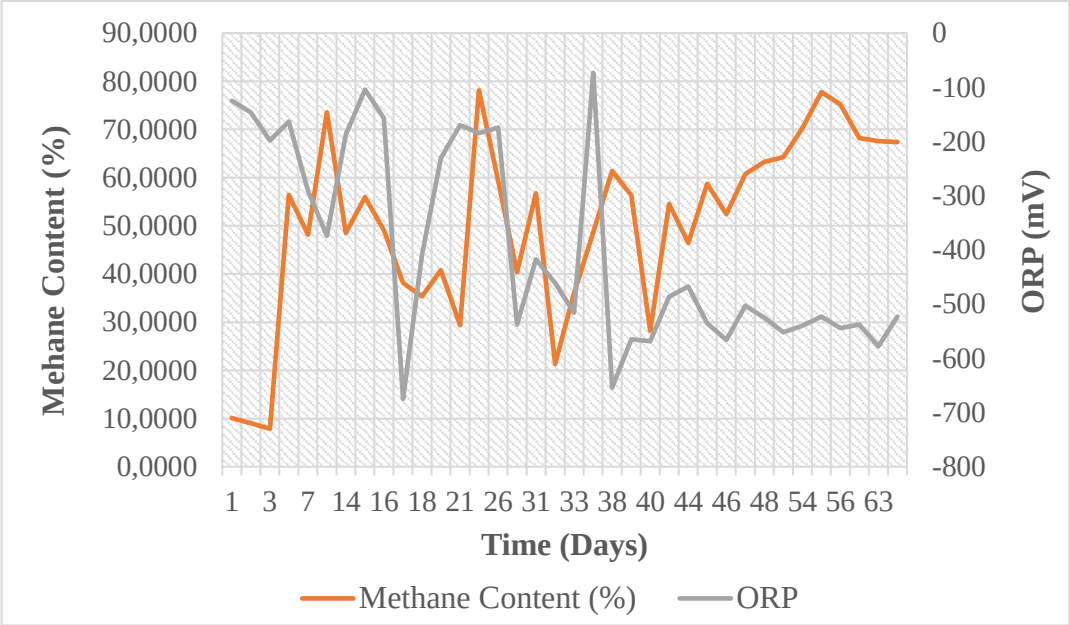

Figure B1. Methane Content in biogas and ORP values of Reactor Operation in co-digestion

Large variation in ORP values was observed in the first 39 days. Stabilization of ORP occurred few days before the methane content stabilizes, staying between -400 and -500 mV. Thus, OPR was effective in previously indicating the methanogenesis stabilization. It shows that the ORP values proceed the same behaviour as methane content. The large variation in ORP values at the beginning of AD represents the phase of hydrolysis, acidogenesis and acetogenesis, with the formation of acids and activities of different bacterial groups, showing the phase of adaptation of these bacteria in the reactor. In these three initial phase, organic compounds of complex chains such as lipids, carbohydrates and proteins are hydrolyzed through the formation of compounds with smaller carbon chains. These compounds are biologically oxidized and converted into organic acids, such as acetic acid, propionic by facultative bacteria (STAMS; PLUGGE, 2009). The different ORP values represent this intense exchange of electrons and reactions of reduction and oxidation of substrates, marking the reactor start-up phase. In the methanogenic phase, acids are converted into methane, carbon dioxide and chemicals substances or carbon dioxide is reduced to form methane by anaerobic microorganisms (STAMS; PLUGGE, 2009). When the reactor is already stabilized in the methane content, it favors the presence of methanogenic microorganisms, acting in chemical reactions for only the production of methane, making the ORP values not vary as much, also remaining stabilized.

Koch and Oldham, (1985) obtained optimized methane production when the ORP reached between -500 mV and -520 mV. In the work of Vongvichiankul et al., (2017) for the treatment of synthetic and leachate food waste, the optimal ORP value for methane production was -335 mV. In the present work, the optimal ORP value for stabilized methane content in biogas in the co-digestion of residues from the sugarcane industry was close to -500 mV.

Conclusions

Co-digestion of sugarcane vinasse, filter cake and lignocellulosic liquor from sugarcane straw pre-treatment was suitable for methane production. ORP stabilization preceded stable methane content, proving to be an effective control parameter to previously indicate the final step of start-up reactor.

Acknowledgements.- Thanks to FAPESP for financing this research project, to the Sanitation and Environment Laboratory of the Faculty of Agricultural Engineering at UNICAMP and to NIPE UNICAMP.

References

Blanc, F.C., Molof, A.H., 1973. Electrode Potential Monitoring and Electrolytic Control in Anaerobic Digestion. *J. Water Pollut*. Control Fed. 45, 655–667.

Deublin, D., Steinhauser, A., 2008. Biogas from Waste and Renewable Resources: *An Introduction. Wily Online Library, Weinheim, Germany*. https://doi.org/1010029783527621705

Hagos, K., Zong, J., Li, D., Liu, C., Lu, X., 2017. Anaerobic co-digestion process for biogas production: Progress, challenges and perspectives. *Renew. Sustain. Energy Rev*. 76, 1485–1496. https://doi.org/10.1016/j.rser.2016.11.184

Karthikeyan, O.P., Visvanathan, C., 2013. Bio-energy recovery from high-solid organic substrates by dry anaerobic bio-conversion processes: A review. *Rev. Environ. Sci*. Biotechnol. 12, 257–284. https://doi.org/10.1007/s11157-012-9304-9

Koch, F.A., Oldham, W.K., 1985. Oxidation-reduction potential - A tool for monitoring, control and optimization of biological nutrient removal systems. *Water Sci. Technol*. 17, 259–281. https://doi.org/10.2166/wst.1985.0237

Lin, R., Cheng, J., Zhang, J., Zhou, J., Cen, K., Murphy, J.D., 2017. Boosting biomethane yield and production rate with graphene: The potential of direct interspecies electron transfer in anaerobic digestion. *Bioresour. Technol*. 239, 345–352. https://doi.org/10.1016/j.biortech.2017.05.017

Nghiem, L.D., Manassa, P., Dawson, M., Fitzgerald, S.K., 2014. Oxidation reduction potential as a parameter to regulate micro-oxygen injection into anaerobic digester for reducing hydrogen sulphide concentration in biogas. *Bioresour. Technol*. 173, 443–447. https://doi.org/10.1016/j.biortech.2014.09.052

Peddie, C.C., Mavinic, D.S., Jenkins, C.J., 1990. Use Of Orp For Monitoring And Control Of Aerobic Sludge Digestion. *J. Environ. Eng*. 116, 461–471.

Stams, A.J.M., Plugge, C.M., 2009. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nat. Rev. Microbiol*. 7, 568–577. https://doi.org/10.1038/nrmicro2166

Sung Jae Lee, 2008. *Relationship between Oxidation Reduction Potential (ORP) and Volatile Fatty Acid (VFA) Production in the Acid-Phase Anaerobic Digestion Process*. Master Thesis, University of Canterbury, New Zeland.

Vongvichiankul, C., Deebao, J., Khongnakorn, W., 2017. Relationship between pH, Oxidation Reduction Potential (ORP) and Biogas Production in Mesophilic Screw Anaerobic Digester. *Energy Procedia* 138, 877–882. https://doi.org/10.1016/j.egypro.2017.10.113

Certificate of Participation

Hereby we state that the POSTER paper

Oxidation reduction potential (ORP) for monitoring co-digestion reactor start-up fed with residues from sugarcane industry

Volpi Maria Paula, Lima Brenno V. M, Moraes Bruna S.

Was presented at the Latin American Meetings on Anaerobic Digestion (Mexico Session)

Adalberto Noyola
Chair Organizing Committee

Germán Buitrón
Chair Scientific Committee

11 APPENDIX C

The expanded summary below was presented at the XLIX Congresso Brasileiro de Engenharia Agrícola-CONBEA 2020, in poster format, on November 23-25, in an online congress. The certificate is below.

CO-DIGESTÃO DE VINHAÇA COM TORTA DE FILTRO PARA A OTIMIZAÇÃO DA PRODUÇÃO DE METANO

MARIA PAULA CARDEAL VOLPI[1], AGATHA SANTOS[2], BRENNO VINICIUS MEDEIROS LIMA[3], BRUNA DE SOUZA MORAES[4]

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Apresentado no

XLIX Congresso Brasileiro de Engenharia Agrícola - CONBEA 2020

23 a 25 de novembro de 2020 - Congresso On-line

RESUMO: No cenário mundial a busca por fontes alternativas de energia vem sendo recorrente, fazendo com o que biogás se destaque. Sua produção ocorre através da digestão anaeróbia (DA), que permite a recuperação energética da fonte orgânica através do uso do metano (CH4), além da possível geração de subprodutos com valor para a agroindústria. Além disso a co-digestão tem se mostrado uma alternativa para o uso de resíduos que possuem uma baixa biodegradabilidade e também para melhorar o rendimento de CH_4. Em um contexto de elevada expectativa, substratos provenientes da atividade sucroalcooleira são considerados potenciais facilitadores para a DA. A vinhaça e a torta de filtro já são usadas como substratos para a produção de metano, porém ainda apresentam algumas lacunas a respeito das limitações tecnológicas dentro da DA, como a disponibilidade da fração biodegradável. Diante disto, o presente trabalho teve como objetivo realizar a co-digestão da vinhaça com a torta de filtro para obtenção de CH_4. Os resultados mostraram que a co-digestão dos dois resíduos teve maior produção de metano se comparada com a digestão isolada de cada um deles, alcançando 615,96 N mL CH_4/ gSV e corroborando para que a co-digestão, além de otimizar o processo, permita um gerenciamento de maior número de resíduos

PALAVRAS-CHAVE: **Co-digestão, Metano, Resíduos, Vinhaça, Torta de Filtro**

CO-DIGESTION OF VINASSE WITH FILTER CAKE FOR THE OPTIMIZATION OF METHANE PRODUCTION

ABSTRACT: In the world scenario, the search for alternative energy sources has been recurrent, making biogas spotlight. Its production occurs through anaerobic digestion (AD), which allows the energy recovery of the organic source through the use of methane (CH_4), in addition to the possible generation of by-products with value for the agribusiness. In addition, co-digestion has been shown to be an alternative for the use of residues that have a low biodegradability and also to improve CH_4 yield. In a context of high expectations, substrates from sugar and alcohol activity are considered potential facilitators for AD. Vinasse and filter cake are already used as substrates for the production of methane, but they still have some gaps regarding technological limitations within AD, such as the availability of the biodegradable fraction. In view of this, the present study aimed to co-digest vinasse with the filter cake to obtain CH_4. The results showed that the use of the two residues together had a higher methane production than if they were used separately, reaching 615.96 N mL CH_4 / g SV, corroborating that the co-digestion in addition to optimizing the process, allows a management greater number of waste

KEYWORDS: **Co-digestion, Methane, Residues, Vinasse, Filter Cake**

INTRODUÇÃO: A digestão anaeróbia (DA) é um processo atrativo para o gerenciamento de resíduos líquidos e sólidos que permite a recuperação energética através do biogás, que é rico em metano (CH_4), e geração de bioprodutos com valor agregado para agricultura, sendo desenvolvido sob um ecossistema fielmente equilibrado de microrganismos.

É evidente a busca por substratos residuais disponíveis que estejam alinhados com a diversificação de geração de produtos. Aliado a isto, é de destaque a intensidade das expectativas quanto ao uso de diversas biomassas e a produção de biogás para fins energéticos. Apesar de todo crescimento científico nesta área, faz-se necessário aprofundar o conhecimento com base em questões e variações inovadoras, que investiguem, de forma abrangente, as interações entre as limitações tecnológicas predominantes no bioprocesso para geração de CH_4. Por exemplo, a disponibilização da fração biodegradável presente nos substratos provenientes da indústria sucroenergética (relacionada à biodigestão anaeróbia com consequente produção de CH_4) ainda representa um gargalo para este campo científico (JANKE et al., 2015).

Neste contexto, o processo de co-digestão vem ganhando destaque exibindo melhor eficiência no processo de DA por oferecer benefícios complementares como melhor rendimento de produção, disponibilidade de nutrientes, menor volume de alimentação, variabilidade de substrato, diluição de toxicidade, sinergismo e microrganismos robustos (MEHARIYA et al., 2018).

Dentre os resíduos que são utilizados na DA, é de destaque os subprodutos da indústria sucroalcoleira como vinhaça, torta de filtro e bagaço, que já mostram seu potencial para a produção de metano (MORAES et al., 2015; JANKE et al., 2015).

Diante do cenário abordado, o objetivo deste trabalho foi realizar a DA da vinhaça e da torta de filtro separadamente e também a co-digestão de dos resíduos para comparar a produção de CH_4

MATERIAL E MÉTODOS: Substratos e Resíduos-Os substratos vinhaça e torta de filtro (da produção de etanol 1G) foram obtidos da Usina Iracema (do grupo São Martinho), assim como o inoculo anaeróbio proveniente de um reator mesofílico (BIOPA®CICX - Paques) para tratamento de vinhaça da mesma usina.

Ensaio de Potencial Bioquímico de Metano (PBM)-Para avaliar a produção do metano foram realizados ensaios de PBM segundo a metodologia VDI 4630 (2006), em frascos Duran em triplicata. Em um frasco foi adicionado a torta de filtro com o inoculo, em outro frasco a vinhaça e o inoculo, e em outro frasco os dois resíduos juntos com o inoculo. Ensaios apenas com inoculo foram utilizados como controle negativo. Todos os frascos foram incubados a 55°C e analisado o volume de biogás com o uso da seringa Hamilton e a concentração de metano através de cromatografia gasosa. O ensaio durou um total de 120 dias.

RESULTADOS E DISCUSSÃO: A Tabela C1 mostra os valores de PBM da vinhaça, da torta de filtro e dos dois resíduos juntos.

TABELA C1. Potencial Bioquímico de Metano (PBM) da vinhaça, torta de filtro e co-digestão

Resíduos	PBM (NmL CH_4/ g SV)
Vinhaça	506,23
Torta de Filtro	260,17
Torta de Filtro + Vinhaça	615,96

De acordo com os resultados obtidos, fica evidente que o processo de co-digestão potencializa a produção de metano para ambos os resíduos. A vinhaça sozinha atingiu 506,23 N mL CH_4/ g SV e a torta de filtro apenas 260,17 N mL CH_4/ g SV. A co-digestão aumentou em até 17% a produção de metano quando comparada à digestão isolada da vinhaça e 57% quando comparada com a digestão isolada da torta de filtro. A digestão de mais de um substrato no mesmo reator pode estabilizar positivamente o sinergismo e adicionar macro e micronutrientes que podem suportar o crescimento microbiano (MATA-ALVAREZ; MACÉ; LLABRÉS, 2000), além disso permite o gerenciamento de um maior número de resíduos, inclusive aqueles que ainda não têm um destino final adequado.

A Figura C1 mostra a produção de volume de metano que foi acumulado ao longo do tempo, ficando claro que a co-digestão dos resíduos foi a melhor condição. É possível observar que a vinhaça acaba estabilizando a produção de metano em um tempo muito menor do que a torta e a co-digestão e alcança uma produção de volume acumulado próximo á 400 N mL CH_4, enquanto que a co-digestão chega a mais de 1400 N mL CH_4. Este fato pode ser devido a maior biodegradabilidade da vinhaça, que pode estar relacionada ao menor teor de sólidos totais.

E vale ressaltar que os ensaios realizados em batelada não tiveram agitação, o que pode ter feito os sólidos da torta de filtro sedimentarem. Considerando a possibilidade de uma futura agitação, como por exemplo em um reator, a produção de CH_4 da co-digestão pode ser ainda maior.

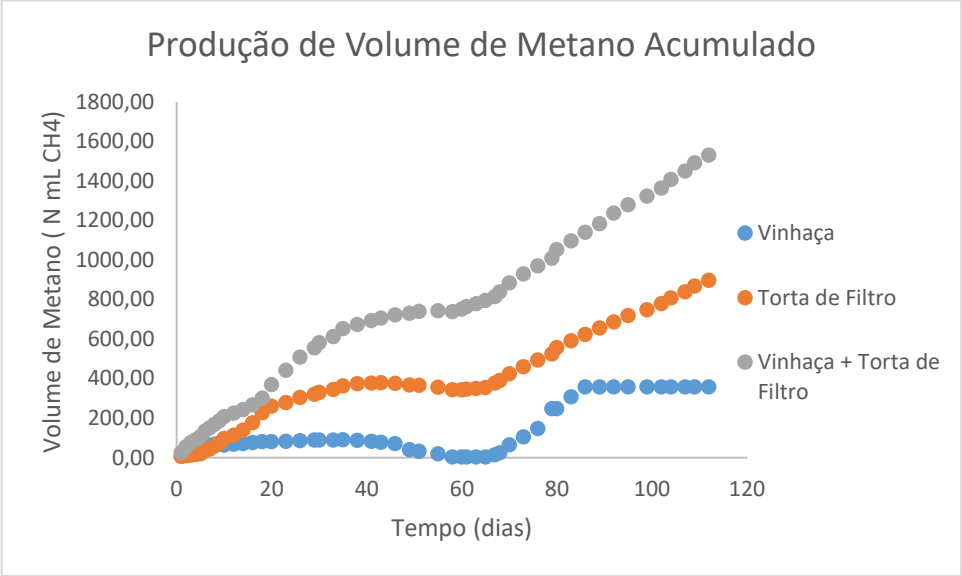

FIGURA C1. Volume acumulado de metano de Vinhaça, Torta de Filtro e Co-digestão

CONCLUSÕES: A co-digestão da vinhaça e da torta-filtro foi eficaz para a produção de CH_4, sendo considerada um processo de otimização da DA, além de ser uma boa alternativa para o uso de resíduos que possuem baixa biodegradabilidade e/ou baixo PBM.

REFERÊNCIAS: JANKE, L.; LEITE, A.; NIKOLAUSZ, M.; SCHMIDT, T.; LIEBETRAU, J.; NELLES, M.; STINNER, W. Biogas Production from Sugarcane Waste: Assessment on Kinetic Challenges for Process Designing. **International Journal Of Molecular Sciences,** v. 16, n. 9, p.20685-20703, 31 ago. 2015

MATA-ALVAREZ, J; MACÉ, S; LLABRÉS, P. Anaerobic digestion of organic solid wastes. An overview of research achievements and perspectives. **Bioresource Technology**, v. 74, n. 1, p.3-16, ago. 2000. Elsevier BV. http://dx.doi.org/10.1016/s0960-8524(00)00023-7

MEHARIYA, S.; PATEL, A. K.; OBULISAMY, P. K.; PUNNIYAKOTT, E.; WONG, J. W.C. Co-digestion of food waste and sewage sludge for methane production: Current status and perspective. **Bioresource Technology**, v. 265, p.519-531, out. 2018. Elsevier BV. http://dx.doi.org/10.1016/j.biortech.2018.04.030.

MORAES, B. S.; PETERSEN, S. O.; ZAIAT, M.; SOMMER, S. G.; TRIOLO, J. M. Reduction in greenhouse gas emissions from vinasse through anaerobic digestion. **Applied Energy**, v. 189, p.21-30, mar. 2017. Elsevier BV. http://dx.doi.org/10.1016/j.apenergy.2016.12.009.

CERTIFICADO

A Associação Brasileira de Engenharia Agrícola (SBEA) certifica que **MARIA PAULA CARDEAL VOLPI** apresentou o trabalho intitulado **CO-DIGESTÃO DE VINHAÇA COM TORTA DE FILTRO PARA A OTIMIZAÇÃO DA PRODUÇÃO DE METANO**, na modalidade **PÔSTER**, no XLIX Congresso Brasileiro de Engenharia Agrícola - CONBEA 2020, realizado no período de 23 a 25 de novembro de 2020, no formato on-line.

Jaboticabal - SP, 25 de novembro de 2020.

Prof. Dr. André Luís Teixeira Fernandes
Presidente da SBEA

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23 a 25 de novembro de 2020 - Congresso On-line
"A Engenharia Agrícola no desenvolvimento bioenergético"

12 APPENDIX D

The abstract below was presented at 8th International Conference on Energy, Sustainability and Climate Crisis (ESCC 2021), which took place in Volos, Greece during August 30 – September 3, 2021. The certificate is below.

Presentation Title: "The use of biogas as a source of bioenergy within 1G2G ethanol biorefineries through residues co-digestion "

Keywords: "Methane; sugarcane residues; anaerobic digestion; bioenergy

Abstract (min 300 words – max 500 words): The debates on issues of global warming and reduction of greenhouse gases are common knowledge, and in this scenario, bioenergy gains strength and stands out as an efficient alternative. With the industrial development of the countries, an exponential increase in energy consumption will occur, and at the same time, energy demand will increase by an annual average of 1.6% by 2030. The importance of using biomass for power, heat, and fuel generation is increasing on a global scale. In this promising and challenging context, the production of biogas is returning to prominence and, consequently, has received numerous initiatives. Biogas (60–70% CH_4, 30–40% CO_2, and the rest are the impurities) is considered a versatile energy carrier, which can be used to replace fossil fuels in the production of both electricity and heat, as well as used as a gaseous fuel for vehicles. Anaerobic digestion (AD), an attractive process for the management of liquid and solid waste that allows energy recovery through methane (CH_4). The co-digestion, process where is used two or more residues in AD, has been highlighted since is an option to use poorly biodegradable substrates and providing and balancing macro and micronutrients for the AD process. The AD of vinasse, residue from the production of 1G ethanol, is already successfully disseminated in the literature, reaching CH_4 productions that can be used as an energy source within the ethanol mills. In addition to vinasse, the production of 1G ethanol generates other residues with the potential for CH_4 production through AD, such as filter cake. However, the literature reports little about the use of residues from the production of 2G ethanol, mainly from the use of liquors originated from pretreatments of lignocellulosic residues such as sugarcane straw or bagasse. Among these residues, the deacetylation liquor is a residue obtained from the pretreatment of sugarcane straw and has a high biochemical potential. Therefore, the present study aimed to co-digest vinasse, filter cake, and deacetylation liquor to produce CH_4 in a continuous reactor, in a thermophilic process. It was possible to obtain a production of approximately 230 $NmLCH_4$ gVS^{-1} (VS-volatile solids), emphasizing that the co-digestion of these residues is effective for energetic recuperation in sugarcane biorefinery, in addition to allowing the integration of 1G2G ethanol biorefineries. Considering the entire volume of waste produced in the harvest (232 days ~ 7 months) it is possible to obtain a monthly electricity production of 17 x 10^6 kWh (considering an engine with 38% efficiency) for the sugarcane mill. Considering that the residential energy consumption per capita in Brazil is 38 kWh per month, this amount of electricity generated is capable of supplying a city with 4.6 x 10^6 inhabitants. These results also show an advance for the use of bioenergy within these biorefineries and show that biogas plays an important role in the context of the introduction of bioenergy in the current world, proving to be a strong candidate to supply the conditions for reducing greenhouse gases and generating renewable energy.

To: Maria Paula Volpi

Certificate of Participation in ESCC 2021

Dear Maria Paula Volpi,

I hereby certify that you participated and presented your research during the 8th International Conference on Energy, Sustainability and Climate Crisis (ESCC 2021), which took place in Volos, Greece during August 30 – September 3, 2021.

Thank you for your contribution to the conference.

UNIVERSITY OF THESSALY - SCHOOL OF ENGINEERING - DEPARTMENT OF MECHANICAL ENGINEERING

As. Professor, Dr. Georgios K.D. Saharidis,

Managing Director of ESCC 2021

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13 APPENDIX E

The paper below was presented at The 9th Microbial Ecology and Water Engineering (MEWE) specialist conference of the internation water association (IWA), online 18-20 october 2021. Delft. The certificate is bellow.

Microbial community change in methane production in co-digestion of residues from ethanol production

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Preferred topic #: Engineering and managing microbial communities

The biogas (rich in methane CH_4) production is carried out through the process of anaerobic digestion (AD), in which residues are substrates for a microbial consortium that by their metabolism stabilize organic matter and generate by-products. Through co-digestion of residues from 1G and 2G ethanol production is possible to obtain sufficient CH_4 to supply an integrated 1G2G plant ethanol. However, the AD process is highly complex from a microbiological point of view, since these microorganisms can follow different metabolic routes depending on the substrate or experimental conditions. The present work aimed to realize the characterization of the microbial community present in a co-digestion reactor of filter cake (1G), vinasse (1G), and deacetylation liquor (2G), under 55°C, to assess the change in this microbial community when the CH_4 production was stable. Genetic sequencing of the 16S ribosomal RNA gene of samples from the microbial consortium was performed before being inserted into the reactor (sample 1), and when the CH_4 production was stabilized (sample 2). Figure E1 shows the Family characterization made of the two samples. It is possible to notice that there is a difference between the two characterized samples. Among the main families found in Sample 2, the family stands out (~30%) *Petrotogaceae*, characteristic in thermophilic processes. Two other families that stand out in Sample 2 are (~3%)

Syntrophomonadaceae and (~20%) *Ruminococcaceae*, which are bacteria from the acetogenic group and characterized by degrading cellulosic materials, which are predominant in the reactor. *Methanomicrobiaceae* which was present just in sample 2, indicating that the metabolic route of syntrophic oxidation of acetate coupled with hydrogenotrophic methanogenesis, was possibly predominant. With these results, it is possible to better understand the change that the microbial consortium undergoes, and which *Bacteria* and *Archeae* are involved in the degradation of residues from ethanol production.

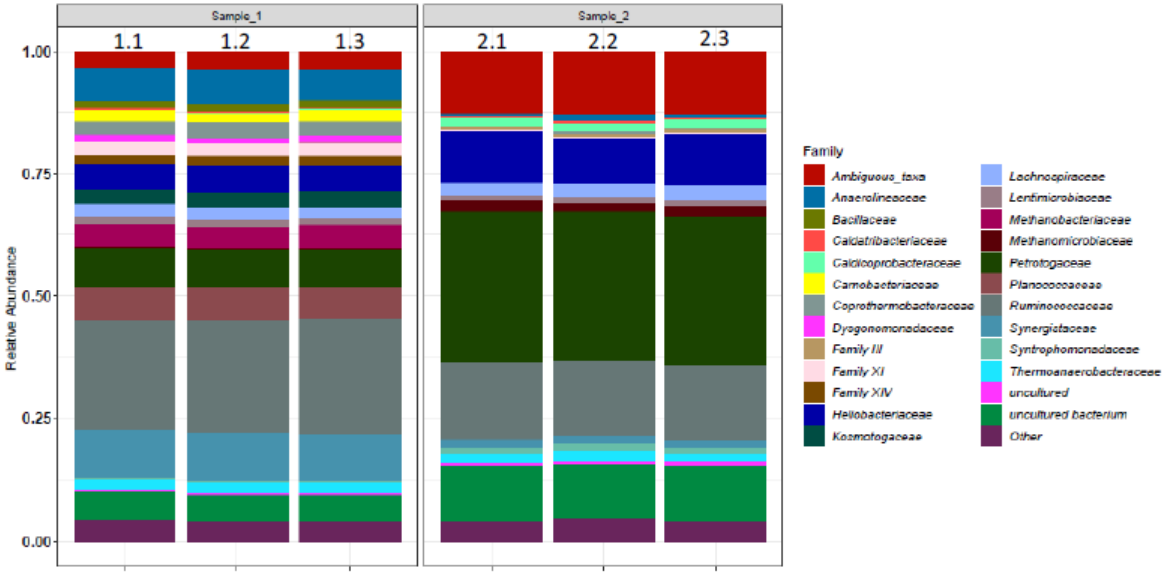

Figure E1. Relative abundance of microorganisms at the Family level from the seed sludge-Sample 1 (1.1, 1.2, and 1.3) and from the s-CSTR sludge with stable CH_4 production-Sample 2 (2.1, 2.2, 2.3)

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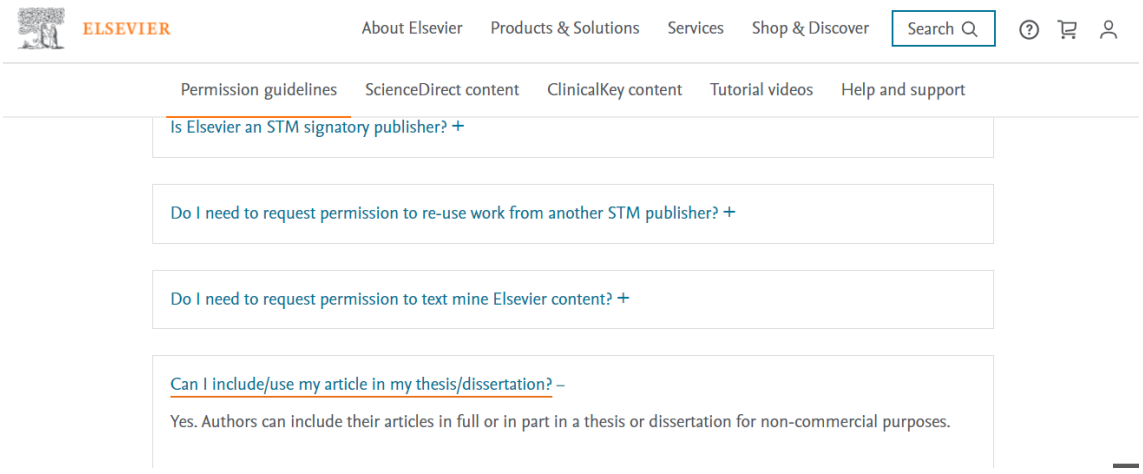

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