Molecular Identification of Microbial Communities in the Methane Production from Vinasse: A Review

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Sugars, starches, and cellulose materials are used for ethanol production. When producing a liter of alcohol, 10 to 15 liters of liquid waste are generated. This waste is called vinasse, and it generates negative impacts on the environment. The process of storing and disposing vinasse in soils generates emissions to the atmosphere, mainly methane. Anaerobic treatment allows for the capture and generation of more biogas, therefore allowing mitigation of the environmental impacts. The microbial diversity present in the anaerobic digestion (AD) of vinasse is strongly related to the efficiency and quality of methane production. The gene 16s rDNA-based molecular techniques have been the most commonly used techniques for monitoring microbial communities present in the digesters. However, the identification is not enough. Rather, it is necessary to know the metagenomic functionality in this type of habitat. This review provides a comprehensive overview of methods to identify the microorganisms in the anaerobic digestion of vinasse. In addition, microbial community identification in vinasse reactors and their relationship with methane production are reviewed.

Keywords: Biogas; Vinasse; Anaerobic biological treatment; Wastewater; Digester

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INTRODUCTION

There are three main types of raw materials used in the manufacture of ethanol *via* fermentation: sugars, starches, and cellulose materials (Lin and Tanaka 2006). The distillation stage generates up to 10 to 15 L liquid waste per liter of ethanol (García *et al.* 1997; Moraes *et al.* 2015). This acidic waste liquid is very aggressive to the environment due to its high content of toxic and recalcitrant organic matter (Robles-González *et al.* 2012). Vinasse has the following characteristics: a pH between 3.9 to 5.1, a chemical demand of oxygen (COD) in the range of 50,000 to 95,000 mgL^{-1}, a high concentration of total solids (TS) (79,000 to 37,500 mg L^{-1}), and a high level of volatile solids (VS) (79,000 to 82,222 mgL^{-1}) (Moran-Salazar *et al.* 2016).

Vinasses are effluents that are difficult to treat; soil fertilization has been a common technique for final disposal (Moran-Salazar *et al*. 2016). During such disposal, the waste is not actually treated. It has been reported that this disposition, storage, and fertilization generates CO_2 and methane emissions (do Carmo *et al.* 2012; de Oliveira *et al.* 2013; Moraes *et al.* 2017), as well as negative environmental impacts (Cruz *et al.* 1991). In addition, methane is the second most important greenhouse gas induced by man after carbon dioxide (Saunois *et al.* 2016).

The anaerobic digestion (AD) allows for the capture and generation of biogas. The

anaerobic treatment of the vinasse also generates a low production of sludge and can be used to reduce the contamination while producing biogas, which can be used as a source of renewable energy (Belhadj *et al.* 2013; Fuess *et al.* 2018; Volpini *et al.* 2018). In the anaerobic treatment of vinasse, the production of biogas is between 400 and 600 L per kg of COD, which is eliminated with a methane content of 60 to 70% (Moletta 2005). The advantage of methane is that it is odorless, colorless, and non-poisonous. Furthermore, it is easily separated from the liquid phase, which can contribute to the reduction of the process costs (Marty *et al.* 2001; Lu *et al.* 2009). Due to the presence of mezcal, vinasses have similar physiochemical characteristics with tequila, sugarcane, and beet vinasse (Moran-Salazar *et al.* 2016). These could also be used for the generation of renewable energy and reduce the polluting potential (Leme and Seabra 2017).

The efficiency and quality of the process depends on the composition and activity of the microbial community that is present (Christy *et al.* 2014; Bailón-Salas *et al.* 2017a), *i.e.*, temperature and pH (Basu 2010). So, the lack of knowledge of the microbial communities present in AD of vinasse limits the capacity to maximize the methane production. In AD of vinasse, changes in the structure of microbial communities have been rarely studied (Jiménez *et al.* 2018). In the next sections, molecular techniques for the microorganism identification in diverse vinasses are summarized. The National Center for Biotechnology Information (NCBI) database is an important resource relevant to biotechnology and has been used in this review. Besides, a search about microorganisms identified in several studies was made. The methane yield also depends on using varied inoculum sources in varied vinasse types.

Vinasse Types of and Methane Yield

The vinasses composition varies depending on the biomass used for the ethanol production (España-Gamboa *et al.* 2011). Many feedstocks have been used for alcohol production, and these confer unique characteristics to each vinasse generated. The feedstocks include sugar crops (sugarcane, sugar beet, molasses, and sweet sorghum), starch crops (corn, wheat, rice, cassava, and barley), cellulosic material (harvesting crop residues, bagasse, and wood), fruit sources and Agavaceae family plant. Tables 1 to 4 detail physicochemical properties and composition of vinasses.

The distillation of sugar crops for the production of alcohol generates an effluent with a high organic matter (COD 109,700 to 57,600 mg L^{-1}) (Table 1).

Sugarcane vinasse is a liquid produced in the unit of rectification and distillation in bioethanol production (Parsaee *et al.* 2019). Some of the main parameters of sugarcane vinasse characterization are given in Table 1. Low pH (3.34), COD greater than 58,000 mg L^{-1}, and a BOD of 23,182 mg L^{-1} were reported.

The waste from solid-state fermentation of sorghum, corn and/or wheat is called solid vinasse (Wang *et al*. 2010; Ao *et al.* 2019). The shown value of pH (4.36) (Ao *et al.* 2019) was higher compared to sugarcane vinasse.

The most important source of starch for bioethanol is cassava. This is due to its abundance and low cost (Zhang *et al.* 2016). The cassava vinasses characterization is shown in the Table 2. The pH near 4, COD, BOD, suspended solids, total nitrogen, and total phosphorus of up to 70,000, 35,000, 45000, 900 and 400 mg L^{-1}, respectively, was reported (Luo *et al.* 2009). Rice wine vinasse also have low pH (3.8) (El-Zaiat *et al.* 2019) and lower concentrations of organic material (Table 2).

Table 1. Characteristics of Vinasse from Sugar Crops

Vinasse type	Parameter	Value	Reference
Sugarcane	pH	3.3	Santos *et al*. 2019
	Chemical oxygen demand	58,533	
	Biochemical oxygen demand	23,182	
	Butyric acid	468	
	Lactic acid	4,200	
	Ethanol	15,848	
	Methanol	594	
	Phenols	1,706	
	Volatile suspended solid	5,553	Fuess *et al.* 2019
	Acetic acid	1,722	
	Propionic acid	127	
	Total carbohydrates	7,275	
	Glycerol	3,914	
	Sulfate	2,993	Correia *et al.* 2017
	pH	4	Moraes *et al.* 2015
	Chemical oxygen demand	109,700	
	Biochemical oxygen demand	87,700	
	Phenols	12.4	
	Total solids	5.8	
Sugar beet	pH	5.1	Robertiello 1982
	Biochemical oxygen demand	78,300	
	Chemical oxygen demand	81,200	
Cane molasses	Chemical oxygen demand	57,600	Bories *et al.* 1988
	Acetic acid	616	
	Propionic acid	90	
	Butyric acid	290	
	Sulfate	3,820	

All values, except pH are expressed in mg L^{-1}.

Many cellulosic materials have been used in ethanol production (Lu-Chau *et al.* 2019). These cellulosic materials include sugarcane bagasse (Liu *et al.* 2015; Joppert *et al.* 2017), agave bagasse (Aguilar *et al.* 2018), newspaper (Wu *et al.* 2014), and coffee husks (Gouvea *et al.* 2009), *etc*. However there are few studies on physicochemical characteristics of the cellulosic vinasses. A study about the production of 2G ethanol from sugarcane bagasse reported COD values of 38,800 mg L^{-1} in the vinasse (Tian *et al.* 2013). Chemical characterization of cotton vinasse gave the following results: pH 4.7, nitrate 350 mg L^{-1}, and ammonium 90 mg L^{-1} (Diaz *et al.* 2003). Wheat straw processing for ethanol resulted in pH 3.6, COD 150,000 mg L^{-1}, ammonium 160 mg L^{-1}, and phenols 61 mg L^{-1} (Kaparaju *et al.* 2010).

Table 2. Vinasse Characterization from Some Starch Crops

Vinasse type	Parameter	Value	Ref.
Rice vinasse	pH	4.8-5.9	Yu *et al*. 2002
	COD	29,500–35,400	
	BOD	15,600–18,700	
	Total N	70–140	
	Total P	20–30	
Casssava vinasses	pH	4-5	Yang and Li 2013
	COD	40,000-50,000	
	BOD	20,000-25,000	
	SS	25,000-30,000	
	pH	4-4.2	(Luo *et al.* 2009)
	COD	40,000–70,000	
	BOD	24,000–35,000	
	SS	30,000–45,000	
	Total N	800–900	
	Total P	200–400	

All values, except pH are expressed in mg L^{-1}.

In the fruit wine production, large amounts of water are used in the cleaning and distillation stages (Pap *et al*. 2004). The vinasses are complex effluents with variable physicochemical properties (Sousa *et al*. 2019). Table 3 shows high levels of organic compounds, principally polyphenols, as well as other parameters. It has been reported that the phenolic compounds are toxic and can inhibit the bacterial activity (Borja *et al*. 1993).

Table 3. Physicochemical Properties and Composition of Fruit Vinasses

Vinasse type	Parameter	Value	Ref.
Mixture of apples and pears	pH	3.4	Robertiello 1982
	BOD	22,000	
	COD	48,900	
Grape vinasse	pH	4.71	Diaz *et al.* 2002
	Nitrates	350	
	Ammonium	50	
	pH	4.03	Díaz-Reinoso *et al.* 2017
	COD	70,710	
	Sulfates	900	
	Total solids	61,500	
	Phosphates	1,740	
	pH	3.88	Sousa *et al.* 2019
	COD	29,150	
	Ammonium	218.2	
	Nitrate	0.01	
	Polyphenols	1,700	
	Potassium	2142.0	

All values, except pH are expressed in mg L^{-1}.

Some *Agave* species are used for liquor production (Ramírez-Malagón *et al.* 2008). Sotol is obtained from the genus *Dasylirion*, whereas tequila is produced exclusively from *Agave tequilana* and mezcal from several species of *Agave* (Gentry 1982; Pardo-Rueda *et al*. 2015; CRM 2018). The physicochemical characteristics of tequila and mezcal vinasses are shown in Table 4. Based on this review, mezcal vinasse have more sulfate content than sugarcane vinasse.

Table 4. Tequila and Mezcal Vinasse Characterization

Vinasse type	Parameter	Value	Ref.
Tequila	COD	38,000	García-Becerra *et al.* 2019
	pH	3.6	
	Acetic acid	1000	
	Butyric acid	100	
	BOD	29,900-30,500	Buitrón *et al.* 2014
	Phenols	44-81	
	Sulfates	915	
	Ammonium	110	
	pH	3.2-4.0	
Mezcal	pH	3.8	Cruz-Salomón *et al.* 2017
	COD	120,221	
	BOD	102,180	
	Total N	1,600	
	Total P	723	
	Acetic acid	15,140	
	Sulfates	3499.14	

All values, except pH are expressed in mg L^{-1}.

All types of biomass can be used as substrates for biogas production (Braun 2007). However the anaerobic digestion of wood is not suitable due to the slow decomposition (Weiland 2010).

The pH values of all vinasses are very low (Tables 1 to 4). So the pH must be adjusted before starting anaerobic digestion. Weiland (2010) recommended an initial pH in the digestion systems in the range 7.0 to 8.0.

To avoid process failure by ammonia accumulation, the C/N ratio should be between 15 and 30 (Zubr 1986; Weiland 2010), and the macronutrients phosphorus and sulfur are necessary in a ratio of 15:5:1 (Weiland 2010).

Moreover, the inoculum selection as well is used to increase the methane production from vinasse (Ordaz-Díaz and Bailón-Salas 2019). Table 5 shows the methane yield using varied inoculum sources and vinasse types. In methane production from vinasses, different types of inoculum have been used, such as brewery sludge, sludge from a wastewater plant, rumen waste, sludge from poultry slaughterhouse reactor, pulp and paper wastewater, swine wastewater, sludge from distillery waste, and sludge from anaerobic reactor. Based on Table 5, the maximum methane yield was obtained using brewery sludge as the inoculum.

Furthermore regarding the inoculum selection, a mesophilic and constant process is recommended. Fluctuations have been found to affect the biogas production negatively (Levén *et al.* 2007).

Table 5. Methane Yield Using Varied Inoculum Source

Vinasse type	Methane yield (L kg^{-1} of COD)	Inoculum source	Reference
Tequila	357	Brewery	Jáuregui-Jáuregui *et al.* 2014
Tequila	240-280	Brewery	Arreola-Vargas *et al.* 2018
Tequila	290	Brewery	Arreola-Vargas *et al.* 2017
Tequila	290	Brewery	Toledo-Cervantes *et al.* 2018
Tequila	257.9	Brewery	Buitrón *et al.* 2014
Mezcal	307.5	Wastewater plant	Cruz-Salomón *et al.* 2017
Sugarcane	139.17	Rumen	Syaichurrozi *et al.* 2013
Sugarcane	299	Granular sludge from Poultry slaughterhouse reactor	Del Nery *et al.* 2018
Sugarcane	246	Pulp and paper wastewater	Janke *et al.* 2015
Sugarcane	185	Swine wastewater	de Barros *et al.* 2016
Sugarcane	170-240	UASB reactor treating sugarcane vinasse	de Barros *et al.* 2017
Grape	340	-	Petta *et al.* 2017
Sorghum, corn and wheat Mixture	214 [a]	Anaerobic reactor fed with vegetable wastes	Ao *et al.* 2019
Cane molasses	6.5–8·4 [b]	Sludge from distillery waste	Bories *et al.* 1988
Cassava	220	Anaerobic granular sludge from a mesophilic UASB from cassava viasse	Luo *et al.* 2009
Corn whole stillage (synthetic)	15.8[b]	Secondary anaerobic digested sludge from the wastewater treatment plant	Andalib *et al.* 2012
Corn thin stillage	1.41[b]	Sludge from the mesophilic anaerobic digester (cattle waste)	Lee *et al.* 2011
Wheat straw	324[a]	Sludge from a potato-processing wastewater treatment plant	Kaparaju *et al.* 2010

[a]L kg^{-1} TS, [b]m^3 m^{-3} day^{-1}.

BACKGROUND ON ANAEROBIC DIGESTION

Anaerobic digestion is the fermentation of organic waste in the absence of oxygen (Abbasi *et al.* 2012). In the anaerobic wastewater treatment, microorganisms carry out the degradation of the organic matter to produce methane, carbon dioxide, and nutrient-rich sludge (Tabatabaei *et al.* 2010).

Stages and Microorganisms Involved in Methane Production

The stages of AD are hydrolysis, acidogenesis, acetogenesis, and methanogenesis, where the archaea and bacteria kingdoms participate in the process (Dugba and Zhang 1999).

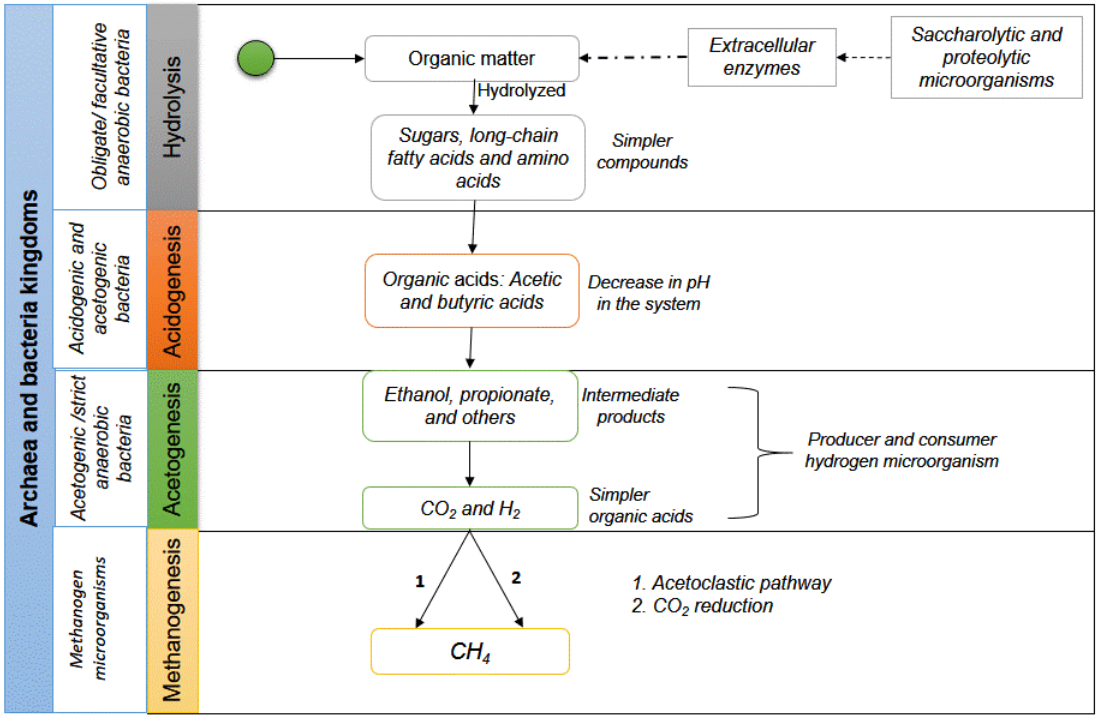

Fig. 1. Flowchart of AD process for the methane production

In the hydrolysis step (Fig. 1), the organic matter (large chains of organic polymers) is hydrolyzed to simpler compounds or monomers by the action of extracellular enzymes produced by hydrolytic bacteria. The saccharolytic and proteolytic microorganisms decompose the sugars and proteins, respectively (Demirel and Scherer 2008). The monomers can be used as a carbon source by other bacteria and by the same hydrolytic bacteria. At this stage, obligate or facultative anaerobic bacteria participate (Vavilin *et al.* 1996).

Acidogenesis (fermentation) is the quickest reaction, where the hydrolyzed products are further transformed into simpler organic compounds. The sugars, long-chain fatty acids, and amino acids from hydrolysis are used by fermentative microorganisms that produce organic acids (Kalyuzhnyi *et al.* 2000; Demirel and Scherer 2008). This stage is of great importance, because mainly acetic and butyric acids are precursors for the formation of methane (Hwang *et al.* 2001). The biotransformation of organic matter to organic acids causes a decrease in pH in the system. This environment favors acidogenic

and acetogenic bacteria (Demirel and Yenigün 2002).

In the acetogenesis, strict anaerobic bacteria participate, and these microorganisms grow slowly (Xing *et al.* 1997). Acetogenic bacteria produce intermediate products such as ethanol, propionate, and others. The intermediate products are converted to simpler organic acids such as CO_2 and H_2. Microorganisms that produce and consume hydrogen are possible under this condition. The monitoring and reduction of acetogenic microorganisms in addition to the constant elimination of hydrogen are essential to ensure that acetate production is not interrupted or drastically reduced (Demirel and Scherer 2008; Schuchmann and Müller 2016). At this stage, the methane production could be improved by injecting CO_2, which produces more acetic acid yield in this stage. This is due to the fact that acetic acid is the direct substrate for methanogenic microorganisms (Li *et al.* 2019).

Subsequently, methanogen microorganisms consume organic acids and generate biogas. The CH_4 is produced by two major pathways: the acetoclastic pathway where approximately two-thirds of the methane is produced, and by CO_2 reduction where CO_2 reducing methanogens produce the remaining amount of methane. The sulfate content in the vinasse can inhibit methanogenic archaea richness, since the sulfate-reducing bacteria are competing for the carbon sources (Moestedt *et al*. 2013; Buitrón *et al.* 2019). Acetotrophic methanogens convert acetate into biomethane and CO_2, where 70% of methane is formed from acetate (Demirbas *et al.* 2006; Demirel and Scherer 2008). At the end of the process, the biogas produced contains 60% methane, 40% carbon dioxide, water vapor, and minimum amounts of hydrogen sulfide (Christy *et al.* 2014).

As can be seen in Fig. 1, the microorganisms that participate in each stage of methane production are classified at the class level. Microbial consortia composition has been studied for the production of methane from sugarcane vinasse (dos Reis *et al.* 2015; Dias *et al.* 2016; de Barros *et al.* 2017; Iltchenco *et al.* 2019), brewery vinasse (Enitan *et al.* 2014), and synthetic vinasse (Rodríguez *et al.* 2012). These studies just focused on microbial composition identification at initial and/or final times. There has been a lack of available information about other stages that are crucial for methane production. Li *et al.* (2019) mentioned that the improvement of methane production requires the improvement of each step of anaerobic digestion. Moreover, this cannot be improved if the changes in the microbiota that are responsible for performing a specific function are not known.

Molecular Techniques for the Identification and Monitoring of Microorganisms in the Anaerobic Digestion of Vinasse

Metagenomics allows the study of microbial communities without the necessity of obtaining pure cultures (Ghosh *et al.* 2019). Instead, nucleic acids are isolated directly from the sample (Haynes 2008). The basic stages in the study of microbial communities using molecular techniques involves the metagenomic DNA extraction, amplification and sequencing. Other molecular tools are fingerprint methods, such as denaturing gradient gel electrophoresis (DGGE).

The molecular identification of microbial communities is mainly based on the sequence of 16s ribosomal DNA (rDNA) amplified by the polymerase chain reaction (PCR) (Takami 2019). The V3 and V4 region of the 16s gene has been studied to compare the structures of microbial communities due to the precision in taxonomic assignments (Liu *et al*. 2007). However, universal single-copy "marker" genes are also ideal candidates for taxonomic analysis of environmental samples (Segata *et al.* 2012). For example, the rpoB

gene can be used to calculate relative abundances and provide better bootstrap support for phylogenetic reconstruction (Walsh *et al.* 2004; Adékambi *et al.* 2009).

DNA extraction

Traditional techniques for DNA extraction are based on the use of hazardous chemicals including phenol and chloroform (Griffiths *et al.* 2000; Nwokeoji *et al.* 2016) and on the guanidine thiocyanate method (Godon *et al.* 1997). However, for complex samples of wastewater it has been recommended to use the QIAamp DNA Mini Kit and MO BIO Power Soil DNA Isolation Kit due to the high integrity in terms of diversity (Martínez *et al.* 2014; Dias *et al.* 2016; Walden *et al.* 2017).

Nucleic acid amplification methods

In studies based on specific genes, amplification is necessary. The PCR allows generating multiple copies of a specific fragment of DNA or RNA (Hoy 2013). The advantage of PCR-based methods is that they are fast and accurate (Tong 2014). However, there are modified methods such as Real-Time PCR (RT-qPCR) or alternative methods based on isothermal amplification.

RT-qPCR

In Real-Time PCR DNA, amplification is detected when the reaction is progressing through a fluorescent reporter, where the intensity of the signal is proportional to the number of amplified DNA molecules (Jia 2012). In the microbial community studies in the AD of tequila vinasse, the primers W49F / W104R and W274R / W275F were used to amplify the V3 region of the 16S rRNA gene (Jáuregui-Jáuregui *et al.* 2014; Toledo-Cervantes *et al.* 2018).

Isothermal amplification of specific sequences

In isothermal amplification, specialized equipment is not required, such as a thermocycler. Various proteins help DNA polymerase to replicate the DNA (Gill and Ghaemi 2008). There are several types of isothermal amplification methods such as loop-mediated isothermal amplification (LAMP). This amplification method allows for the amplification of six different regions. It is suitable for Sanger and pyrosequencing sequencing (Nagamine *et al.* 2002; Gill and Ghaemi 2008; Fakruddin and Chowdhury 2012). Other technologies such as strand displacement amplification (SDA), cross priming amplification (CPA), Nicking Enzyme Amplification Reaction (NEAR), and Nicking enzyme-mediated amplification (NEMA) require an additional enzyme, such as a restriction endonuclease or a nicking enzyme. A disadvantage of isothermal amplification of specific sequences is that the components of the reaction mixture and the primer design are more complicated compared to conventional PCR (Tong 2014).

Sequencing and Analysis

The microbial communities can be identified by high-throughput sequencing, which allows sequencing of the amplicon library for rDNA (Haynes 2008). Other technology includes the single molecule real time (SMRT) that allows for the generation of a full sequence data of 16s rRNA genes. The objective of SMRT is to identify bacterial diversity and community structure at the species level (Yang *et al*. 2018).

Once the sequence is obtained, they are submitted to a database such as BLAST

(Altschul *et al.* 1990), HBLAST (O'Driscoll *et al.* 2015), or to the Metabolic and Physiological Potential Evaluator (MAPLE) system using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Takami 2019). DNA extraction, 16s rDNA amplification, sequencing, and later analysis of the sequences by means of software are required. For example, the Quantitative Insights into Microbial Ecology (QIIME) package allows the analysis of the microbial community based on data from DNA sequences (Navas-Molina *et al.* 2013).

DNA Fingerprinting Techniques

Molecular fingerprinting techniques based on the amplification of the 16s rDNA are powerful tools for the study of microbial communities in environmental samples (Kuhn *et al.* 2017). The PCR-DGGE allows the separation of DNA fragments (same size) previously amplified and the analysis of resulting banding patterns. The double-stranded fragments are separated in polyacrylamide gel with urea-formamide depending on their nucleotide sequence (Myers *et al.* 1987; Muyzer *et al.* 1993). In PCR-DGGE, P338f-GC and P518r (Jiménez *et al.* 2018), 1055F / 1392R-GC (Dias *et al.* 2016), and 968FGC-1401R (Rodríguez *et al.* 2012; dos Reis *et al.* 2015) primers have been used for the study of bacterial communities in the AD of vinasse. For the Domain Archaea, Parch519fGC / Arch915r and A109 (T) -F / 515-GC-R primers have been used (Rodríguez *et al.* 2012; dos Reis *et al.* 2015). Also, denaturing gradients ranging from 45% to 60% (Jiménez *et al.* 2018) and 30% to 70% (Dias *et al.* 2016) have been used. Other studies have used a gradient of 42% to 67% and 30% to 60% for bacterial and archaeal communities. The DGGE profiles have been analyzed with the Bionumerics software 5.1 (Applied Maths, Kortrijk, Belgium) (Jiménez *et al.* 2018), BioNumerics 7.1 (Dias *et al.* 2016) and BioNumerics 2.5 (dos Reis *et al.* 2015). The bands obtained are excised, crushed, eluted in milliQ water, re-amplified, purified, and sequenced (Bailón-Salas *et al.* 2017b). In some studies, bands are not sequenced (Jiménez *et al.* 2018).

FUNCTIONAL DIVERSITY OF WHOLE MICROBIAL COMMUNITIES

The objective of metagenomic analysis is to know the function that microbial communities perform in different environments (Takami 2019). However, all the reports of microbial communities related to the production of methane from vinasse and other environments are based on the 16s rDNA gene.

In AD, there has been a limited understanding of the relationship between microbial community structure and function (Venkiteshwaran *et al.* 2015). To evaluate the metagenomic functionality of microbial communities, Takami *et al.* (2012, 2014) developed a method. Subsequently Takami *et al.* (2016) and Arai *et al.* (2018), respectively, developed and improved a system to evaluate metagenome functionality. The system was called MAPLE, which allows a homology to search much faster than the Basic Local Alignment Search Tool (BLAST) (Takami 2019). In the KEGG module the methane metabolism is available (Takami 2019).

MICROBIAL COMMUNITIES IDENTIFICATION IN VINASSE REACTORS AND THEIR RELATIONSHIP WITH METHANE PRODUCTION

Molecular techniques for the microbial identification in reactors for the production of methane from vinasse have been studied in samples of sugarcane vinasse (Martínez *et al.* 2014; dos Reis *et al.* 2015; Dias *et al.* 2016; de Barros *et al.* 2017; Jiménez *et al.* 2018), synthetic vinasse (Rodríguez *et al.* 2012), and tequila vinasse (Jáuregui-Jáuregui *et al.* 2014; Toledo-Cervantes *et al.* 2018).

Table 5. Microorganisms Identified in the Production of Methane from Vinasse

Microorganism	Accession number	Source	Reference
Uncultured *archaeon*	JF937237.1	Fluidized bed reactor treating **synthetic vinasse** at anaerobic conditions	Rodríguez *et al.* 2012
Sporomusa sp.	JF937208.1		
Acetobacterium sp.	JF937206.1		
Tissierella sp.	JF937209.1		
Anaerostipes sp.	JF937202		
Propionibacterium sp.	JF937214.1		
Coriobacterium sp.	JF937213.1, JF937215.1 and JF937216.1		
Wolinella sp.	JF937218.1		
Desulfovibrio sp.	JF937211.1 and JF937212.1		
Uncultured *Halothiobacillaceae*	JF937210.1		
Uncultured *Prevotella* sp.	KM820902.1	Methane production from **sugarcane vinasse**	dos Reis *et al.* 2015
Uncultured *Clostridia*	KM820906.1		
Uncultured *Megasphaera* sp.	KM820904.1		
Uncultured *archaeon*	KM820901.1		
Uncultured *Methanobacterium* sp.	KM820898.1		
Clostridium beijerinckii	KT626859.1	Vinasse biodigestor	Database NCBI, 2015
Uncultured *Chloroflexi*	CU917991.1	Production of biogas from **sugarcane vinasse**	Dias *et al.* 2016
Desulfomicrobium sp.	JN828421.1		
Thioalkalimicrobium sp.	GU735085.1		
Acinetobacter soli	KJ806407.1		
Thioalkalimicrobium sp.	GU735085.1		
Pseudomonas sp.	KJ950456.1		
O. ureolytica	CU927589.1		
Clostridium beijerinckii	MWMH01000028.1	Upflow Anaerobic Sludge Blanket biodigestor used to treat **sugarcane vinasse**	Database NCBI, 2017

Few studies have been conducted with a metagenomic analysis in methane production from vinasses (Rodríguez *et al.* 2012; Enitan *et al.* 2014; dos Reis *et al.* 2015; Dias *et al.* 2016; de Barros *et al.* 2017; Iltchenco *et al.* 2019). In general such approaches are not able to identify microorganisms at the species level, so the specific function in the process is uncertain. Besides, present attempts have not been made to understand the microbial community structure in AD of mezcal vinasses.

The microorganisms identified in the methane production from vinasse are shown in Table 6. Some references are available in the public database of the NCBI and others was made based in reports of journals.

Sporomusa sp. is strictly an anaerobic bacterium (Möller *et al.* 1984), isolated from the alcohol distillation industry (Ollivier *et al.* 1985), and synthetic vinasse (Rodríguez *et al.* 2012). In the acidogenesis, sugars and fatty acids are converted to organic acids such as acetic, propionic, and butyric acids. In the AD process, butyrate and propionate are important intermediate compounds (Schink and Stams 2006). It has been reported that some species of the genus *Anaerostipes* are butyrate producing bacteria (Eeckhaut *et al.* 2010). *Clostridium* sp. is a solvent producing bacterium that has the capacity to convert a range of carbohydrates to end products such acetone, butanol, and ethanol. Specifically, *Clostridium beijerinckii* produces butyric acid and acetic acid (Mitchell 1997; Nimcevic *et al.* 1998; Little *et al.* 2015). Some species of *Megasphaera* have the capability of producing various volatile fatty acids including lactic, formic, acetic, propionic, and butyric acids using sugarcane vinasse (Marx *et al.* 2011; Sydney *et al.* 2014). In the raw sugarcane vinasse, high concentrations of propionic acids have been reported as indicating a highly active *Propionibacteria* community (Júnior *et al.* 2016). *Coriobacterium glomerans* has been isolated from the intestinal tract of insects. Glucose, L-arabinose, D-xylose, D-ribose, mannose, sucrose, maltose, cellobiose, mannitol, and salicin are used as a carbon source that are fermented to acetic acid, L-lactic acid, ethanol, CO_2, and H_2 (Haas and König 1988).

Ethanol and propionate are mainly transformed into simpler organic acids, CO_2, and H_2 in the acetogenesis step. *Acetobacterium* sp. converts H_2/CO_2 into acetate through acetogenic fermentation (Bainotti and Nishio 2000). *Acetobacterium woodii* has been the most studied species (Bertsch and Müller 2015; Schuchmann and Müller 2016). At this stage, methylamine is also produced. *Tissierella* sp. is strictly anaerobic and can produce methylamine (Harms *et al.* 1998). They were also found to be greatly correlated with the recovered biogas (Chen *et al.* 2018).

In methanogenesis, the conversion of CO_2 and hydrogen to methane is carried out by hydrogenotrophic methanogens (Zabranska and Pokorna 2018). Some genera of *Methanobacterium* have been associated with this activity (Visser *et al.* 1991; Harada *et al.* 1996). The H_2 produced in the previous stage must be monitored and eliminated so that the acetate is not reduced. *Wolinella succinogenes* compete with methanogens microorganisms by consuming H_2 (Asanuma *et al*. 1999). *Prevotella* sp. utilizes saccharides such as xylan, xylose, pectin, and carboxymethylcellulose, and produces acetate and succinate (Ueki *et al.* 2007). The acetoclastic microorganism consumes acetate, methanol, and some amines. *Pseudomonas* sp. can facilitate the extracellular electron transfer and can oxidize various organic electron donors, such as acetate and ethanol (Maruthupandy *et al.* 2015; Barua *et al.* 2018).

It has been reported that *Thioalkalimicrobium* sp. oxizes the sulfur to sulfates (Sorokin *et al*. 2002). Vinasse obtained from the ethanol distillation has sulfate-rich, liquid

substrates (Barrera *et al*. 2013). Methane production can be affected by alternative hydrogen sinks such as sulfates (Johnson and Johnson 1995), where bacteria could remove sulfate in wastewater before anaerobic treatment for biogas production (Promnuan and Sompong 2017). *Desulfovibrio* sp. removes the dissolved sulfate and produces small amounts of H_2 (Martens and Berner 1974; Guyot and Brauman 1986). *Desulfomicrobium aspheronum* also removes sulfate (Rozanova *et al.* 1990), and *Halothiobacillaceae* sp. utilizes reduced sulfur for energy needs (Quek *et al.* 2017).

CONCLUSIONS

1. The studies reported in this review focused on microbial composition identification at initial and/or final times. Therefore there is little available information about other stages that are crucial for methane production. Furthermore, there is little information on microbial communities associated with the production of methane from tequila vinasse and null for the mezcal vinasse.
2. Research of microbial communities that participate in the production of biogas from mezcal vinasses is necessary because each microorganism performs a specific function at each stage of the methane production process. In addition, the quality and performance of methane's production process are related to the composition and activity of the microbial community. In each reactor subjected to different conditions, the bacterial diversity that develops in the reactor should be monitored. This information should correlate to maximize methane production, and increase knowledge in this field of research and industry.
3. The molecular tools allow rapid advancement in the knowledge of microbial communities in these habitats. Furthermore, it is time to enrich the functional knowledge of microbial communities, so that cellular metabolism and key functional genes of the microorganisms are better understood.

ACKNOWLEDGMENTS

The support of the Science and Technology National Council (CONACyT) is gratefully appreciated.

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Article submitted: October 4, 2019; Peer review completed: January 23, 2020; Revised version received and accepted: February 16, 2020; Published: February 25, 2020.
DOI: 10.15376/biores.15.2.Diaz