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Effect of the inoculum/substrate ratio on the biochemical methane potential (BMP) of grape marc

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ABSTRACT

The grape industrialization process produces large volumes of solid organic waste, with the grape bagasse being the main waste generated in the winemaking process. Anaerobic digestion can be used to treat and dispose of agro-industrial biomass waste. The objective of this study was to evaluate the effect of the inoculum/substrate ratio on the Biochemical Methane Potential (BMP) of grape marc. The experiment was performed in laboratory scale through a system of reactor bottles in batches, removing a set of triplicate flasks for sampling and analysis every 48 hours, with the test lasting 12 days. The reactors contained residue, inoculum and 20% of nutritive solution, maintaining 20% of headspace. The reactors were incubated in an incubator at a mesophilic temperature (35 ± 2°C) and shaken manually every 24 hours. Three different inoculum/substrate (I/S) ratios of 0.75, 1.5 and 3 were used to evaluate the methane yield, organic removals and at the end of degradation the morphology of the bacterial community was evaluated by means of scanning electron microscopy. The I/S 3 ratio provided the best results for loading anaerobic systems, indicating that grape marc presents potential for biological treatment through anaerobic digestion.

Keywords: agroindustrial waste, anaerobic digestion, biogas, biomethane.

Efeito da relação inóculo/substrato no potencial bioquímico de metano (PBM) no bagaço de uva

RESUMO

O processo de industrialização da uva produz grandes volumes de resíduos sólidos orgânicos, sendo o bagaço de uva o principal resíduo gerado no processo de vinificação. A digestão anaeróbica pode ser utilizada para o tratamento e disposição dos resíduos de biomassa

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agro-industrial. O objetivo deste estudo foi avaliar o efeito da relação inóculo/substrato no Potencial Bioquímico de Metano (PBM) do bagaço de uva. O experimento foi realizado em escala laboratorial através de um sistema de frascos reatores em lotes, retirando um conjunto de triplicatas dos frascos para amostragem e análise a cada 48 horas, com a duração do teste de 12 dias. Os reatores continham resíduo, inóculo e 20% de solução nutritiva, mantendo 20% de headspace. Os reatores foram incubados em uma incubadora à temperatura mesófila (35 ± 2°C) e agitados manualmente a cada 24 horas. Três diferentes razões inóculo/substrato (I/S) de 0,75, 1,5 e 3 foram utilizadas para avaliar a produção de metano, as remoções orgânicas e no final da degradação a morfologia da comunidade bacteriana por meio de microscopia eletrônica de varredura. A relação I/S 3 forneceu os melhores resultados para carregamento de sistemas anaeróbicos, indicando que o bagaço de uva apresenta potencial para tratamento biológico através da digestão anaeróbica.

Palavras-chave: biogás, biometano, digestão anaeróbica, resíduos agroindustriais.

1. INTRODUCTION

Wine production is an activity that stands out all over the world (Devesa-rey *et al.*, 2011), producing large volumes of organic solid waste. According to Mello (2018), the production of grapes in Brazil for processing (wine, juice and derivatives) was 818,783 million kg in 2017, 48.74% of the total production of grapes, with the rest (51.26%) for consumption in natura.

For Zhang *et al.* (2017), solid organic by-products of wine production include grape marc, stalk, wine lees and sludge. The main one is grape marc, composed mainly of seeds and peelings (Christ and Burrit, 2013; Duba *et al.*, 2015).

Although potentially polluting, for Avaci *et al.* (2013), biogas can be recovered through anaerobic digestion (AD), which is seen as an ideal form of biomass waste treatment. It is a promising technology in the management of organic materials and renewable energy production, nutrient recycling and reduction of waste streams (Li *et al.*, 2018).

The AD process is regulated by environmental factors, some of the main ones being temperature (mesophilic or thermophilic regime) and pH, waste composition, physical state and substrate structure and the inoculum/substrate ratio (I/S) (Angelidaki *et al.*, 2009; Zarkadas *et al.*, 2016; Córdoba *et al.*, 2018).

The ratio between substrate and inoculum is an important parameter in anaerobic digestion and is one of the most important factors for the initiation of a balanced microbial population in the anaerobic system (Haider *et al.*, 2015; Zhu *et al.*, 2014). In a study by Latifi *et al.* (2019), the results showed that the decrease in the I/S ratio led to the accumulation of fatty acids and high ammonia concentrations in the reactor, resulting in lower methane yields. In this sense, the relationship between inoculum/substrate, which is incubated in the reactors, influences the specific production of methane, taking into account that different residues require different proportions due to intrinsic characteristics of their generating processes.

Given the diversity of factors that affect anaerobic digestion, anaerobic biodegradation tests are used to establish the potential for biodegradation and determine the methane production potential of waste (Angelidaki *et al.*, 2009), and tests of Biochemical Methane Potential (BMP) can be used for such purposes (Holliger *et al.*, 2016). Also, the BMP of the I/S ratios can be used to design installation parameters of a full-scale reactor operation, and according to Filer *et al.* (2019), even the size of the digesters and possibilities of biogas exploitation. For example, Holliger *et al.* (2017) found that the weekly methane production rates of BMP were similar and followed the same pattern compared to the full-scale reactor installation.

Thus, faced with the generation of waste that is part of the grape industrialization process, anaerobic digestion becomes a viable alternative for treatment and disposal of such waste, and may also be reverted into benefits for the production system of the winemaking itself. Therefore, the objective of this study was to compare the influence of the inoculum/substrate ratio on the biochemical methane potential of grape marc, evaluating at the end of the degradation the morphology of the bacterial community by means of scanning electron microscopy.

2. MATERIALS AND METHODS

2.1. Substrate and Inoculum

For the BMP assays, the bagasse of the pressed grape (hybrid cultivars Isabel and BRS Violeta) was used as substrate, reminiscent of the winemaking process, composed of peelings, seeds and moisture, without stalks. The residue was collected at Vitivinícola Quintas São Braz, located in the rural area of the city of Petrolina-PE, Brazil.

The waste collection process was performed according to the procedures of the German Guideline VDI 4630 (VDI, 2016) and ABNT NBR 10007, where simple samples were collected, taking four sub-samples of three sections (top, middle and bottom of the waste pile) to integrate the composite sample, which was packed, identified and transported under refrigeration. Physical-chemical characterizations of the substrate were performed in triplicate, analysis of total solids and total volatile solids, pH, humidity, phosphorus, total nitrogen and chemical oxygen demand (COD), according to APHA *et al.* (2012), and organic matter (OM). The metals nickel, iron, potassium, sodium, calcium, copper, manganese, zinc and lead were determined by means of nitric acid digestion and subsequent analysis in atomic absorption spectrophotometer with atomization by air-acetylene flame in the specific wavelength for each chemical element analyzed according to APHA *et al.* (2012).

The origin, quality criteria and preparation of the inoculum met the recommendations of VDI 4630 (VDI, 2016), using anaerobic sludge from the discharge of a UASB (Upflow Anaerobic Sludge Blanket) reactor treating domestic sewage in a treatment station of the Companhia Pernambucana de Saneamento, located in Petrolina - PE, Brazil. The specific methanogenic activity (SMA) of the sludge for propionate, butyrate and acetate substrates is 0.125 L CH_4 kg STV $d^{-1,}$ considered a good activity for anaerobic sludge (Angelidaki *et al.*, 2009).

2.2. Experimental Configuration of the BMP Assay

By adapting VDI 4630 (2016) to the sacrifice bottle methodology adopted by Amorim *et al.* (2013), the experiment was conducted in a system of 66 reactor bottles in batches, each with a total volume of 115 mL (Holliger *et al.*, 2016). The reactors contained residue, inoculum and 20% nutrient solution, filling a working volume of 92 mL, to maintain 20% of headspace (Angelidaki *et al.*, 2009). The assay lasted 12 days, in order to make the identification of the potential faster, considering that normally most of the biogas forms in the first week of the trial. A set of triplicates was removed every 48 hours for sampling and analysis.

Prior to that, 24 hours before the installation of the experiment, the microbial biomass was acclimatized in the reactors, placing only the anaerobic sludge and the nutritive solution, enriching them to later receive the grape marc. After mixing and homogenization of the inoculum and bagasse, the pH of the contents of the bottles was checked using sodium bicarbonate, leaving them close to neutrality. Subsequently, the bottles were sealed with nitrile rubber caps and aluminum seals, with crimping pliers. The biogas was measured every 24 hours, a 10mL syringe was inverted into the lid of the reactor, through to the nitrile rubber cap and aluminum seal. The pressure inside the reactor pushes the piston of the syringe, allowing the measurement of biogas. The reactor bottles were incubated in an incubator at a mesophilic temperature (35 ± 2°C) and shaken manually every 24 hours. Methane production due to the possible presence of residual substrate in the inoculum was subtracted by performing blank controls.

2.3. Inoculum/substrate Ratios Used in BMP

Three inoculum/substrate ratios (I/S) of 0.75, 1.5 and 3.0 were tested and achieved by maintaining a fixed inoculum concentration (15 g VS L^{-1}) and varying the substrate concentration from 5 to 20 g VS L^{-1} (Table 1).

Table 1. Experimental conditions used for the different BMP tests performed.

Ratios (I/S)	Inoculum		Substrate	
	Volume (L)	**Concentration (g VS L^{-1})**	**Volume (L)**	**Concentration (g VS L^{-1})**
0.75	0.015	15	0.007	20
1.50	0.015	15	0.004	10
3.00	0.015	15	0.002	5

The evaluation of the effect of the I/S ratio on BMP was determined by the percentage of removal of volatile solids and COD, and by the methane yield per mass of volatile solids added (L CH_4 kg^{-1} $VS_{Aditioned}$). The methane yield (%R) was calculated in relation to the theoretical volume of methane (TV_{CH4}) obtained according to Chernicharo (2007) (Table 2). The characteristics of the substrate, the anaerobic inoculum and the I/S ratios are shown in Table 2.

Table 2. Physical-chemical characterization of inoculum, substrate and I/S ratios.

Parameters	Units	Inoculum	Grape marc	I/S		
				0.75	**1.5**	**3.0**
pH	-	8.15	3.72	7.23	7.40	7.53
Humidity	%	51.75	68.37	-	-	-
TS	%	9.07	47.33	217.25	125.78	172.29
VS	%	4.40	40.92	197.54	107.72	148.97
COD	g L^{-1}	-	36.30	4.88	3.39	2.71
OM	%	-	98			
NT	%	0.89	1.31	-	-	-
NH_3	g kg^{-1}	1.82	0.02	-	-	-
P	mg L^{-1}	90.14	542.98	-	-	-
K	g kg^{-1}	5.80	0.14	-	-	-
Na	g kg^{-1}	6.50	0.0022	-	-	-
Mg	g kg^{-1}	1.50	0.0167	-	-	-
Ca	g kg^{-1}	1.60	0.0024	-	-	-
Nitrate	g kg^{-1}	0.11	0.062	-	-	-
Zn	mg kg^{-1}	-	0.000	-	-	-
Cu	mg kg^{-1}	-	0.034	-	-	-
Mn	mg kg^{-1}	-	0.099	-	-	-
Fe	mg kg^{-1}	-	1.648	-	-	-
Ni	mg kg^{-1}	-	0.054	-	-	-
Pb	mg kg^{-1}	-	0.000	-	-	-
VFA	mg HAc L^{-1}	-	-	612.39	528.41	538.90
TA	mg HAc L^{-1}	-	-	22.67	9.33	12.00
Theoretical volume of CH_4 (TV_{CH4})	(NL CH_4 kg^{-1} VS_{Ad})	-	-	45.51	68.49	136.79
SMA	L CH_4 kg VS d^{-1}.	0.125	-	-	-	-

2.4. Collection and Characterization of Biogas

The collection of biogas for the characterization was performed within 24 and 48 hours of the experiment, time in which production peaks were observed. The characterization of the biogas proceeded regarding the composition of methane and carbonic gas. To this end, part of the biogas was transferred to 10 mL syringes with the aid of 3-way valves that allowed the connection between the syringes, and from these to gas collectors with nitrilic rubber.

The biogas was characterized in gas chromatograph Agilent Model 7890 A with detector type FID equipped with a methanator. The column used was agilentHayesep Q 80/100. N_2 with flow of 25 mL min^{-1} was used as carrier gas. The temperature of the detector was 300ºC, the temperature of the column oven was 60ºC and the running time was 11 min. To calculate the concentrations, two calibration curves were constructed, one for CO_2 (250, 500 and 1000 ppm) and CH_4 (0.5, 1 and 3 ppm).

The volume of methane produced (VCH_4) was obtained through the composition and volume of biogas, the rate of methane production was determined in relation to the VS added. The values were normalized to standard temperature and pressure (VDI, 2016).

2.5. Morphology of the Inoculum Microbial Community

At the end of the BMP assays, samples of the biomass contained in the reactors (inoculum) were characterized under VEGA3 TESCAN scanning electron microscope (SEM). Adapting to the methodology of Araújo *et al.* (2003), samples of 0.5 mL of sludge were fixed with phosphate buffer (pH 7.3) containing 25% glutaraldehyde, for 2 h at 4ºC. After fixation, dehydration with ethanol solutions was performed, washing the samples 6 times at 10-minute intervals at increasing concentrations (45, 70, 80, 95 and 100% v/v). These samples were fixed on aluminum supports (stubs) with double-sided carbon tape and dried at 30ºC in an incubator for 2 hours. The samples were then covered with a 20 nm thick gold layer in the Sputter Quorum Model Q 150R ES, with 15 mA current and 90 seconds metallization time.

2.6. Statistical Analysis

The results were statistically evaluated in the Sisvar® software (Version 5.6), through descriptive statistics and analysis of variance (ANOVA) applying the Tukey test at the 5% significance level.

3. RESULTS AND DISCUSSION

3.1. Organic Removals

Figure 1a shows the removal of volatile solids at the end of the digestion time as a function of the I/S ratios studied. There was no significant difference according to the Tukey test at 5% significance, between the relations of 0.75 and 3.0, which provided the greatest reductions, 59.22 and 59.24%, respectively. The ratio of 1.5 obtained the lowest percentage of reduction around 56.73%.

In studies carried out by Li *et al.* (2016; 2018) the largest VS reductions were obtained in reactors with the highest methane yields, different from the present study, in which it can be observed that the largest removals were obtained in the ratios with different load extremes. Showing an uncertain trend regarding the removal of organic matter and the inoculum/substrate ratios, as well as in Córdoba *et al.* (2018). Ros *et al.* (2016) obtained percentages of VS removal, also with grape marc residue of 12 - 35%.

Figure 1b shows that the I/S 3.0 ratio obtained the best performance in percentage of removal (77.5%), a ratio that also obtained the best specific production of methane in relation to volatile solids. While the ratios of 1.5 and 0.75 obtained 61.7% and 57.2%, respectively.

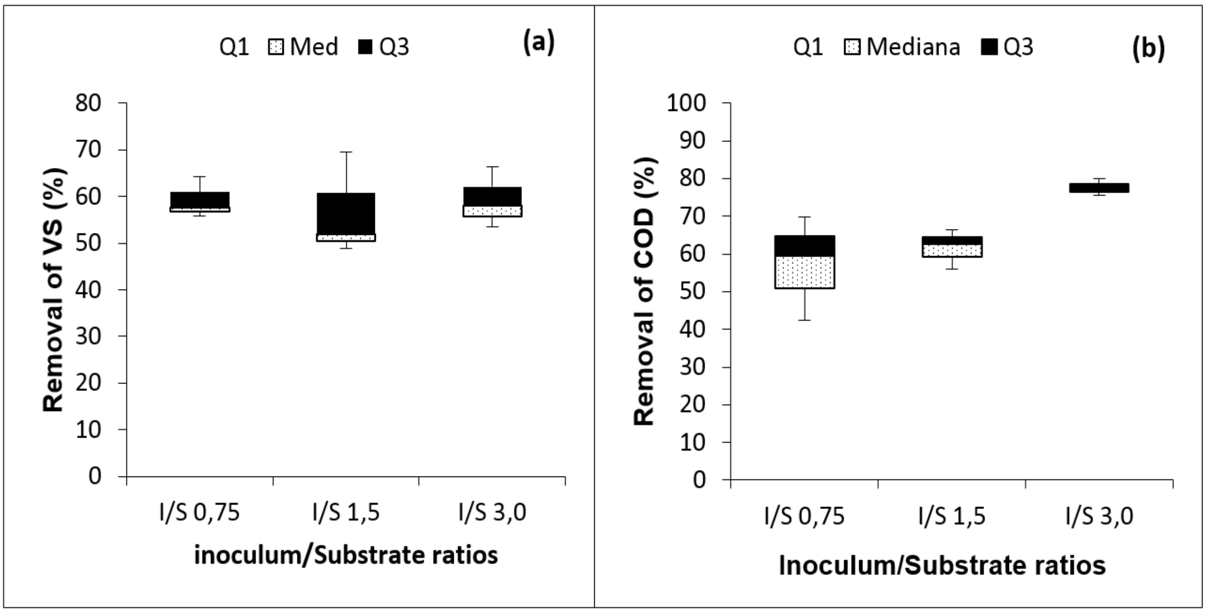

Figure 1. Organic removals in the different I/S relations. (a) Removal of VS. (b) Removal of COD.

The results were promising when compared to the study conducted by Leite *et al.* (2014) that when using typically vegetable waste, in addition to sewage sludge, a removal of 51% of COD was obtained. In contrast, Gueri *et al.* (2018) obtained a reduction of 80.98 - 81.63%, using food waste and anaerobic sludge in bench reactors. Talha *et al.* (2016) working with sugarcane bagasse, obtained a COD removal of 69.64%.

The organic removal of COD is more cohesive in relation to that of volatile solids, showing higher removals as the I/S ratios increase.

3.2. Methane Yield

For the calculation of the methane yields, the values obtained for the volume of biogas, percentage of methane and volume of methane, presented in Table 3, were used, in which there was a significant difference between the I/S ratios applied. It can be observed that the I/S ratio 3, despite having the lowest volume of biogas produced, obtained the best percentage of methane. On the other hand, the I/S ratio of 0.75 obtained the lowest percentage of methane, even generating the highest amount of biogas.

Table 3. Biogas volume, percentage, volume and methane yields for I/S ratios.

I/S Ratios	V_{biogas}	CH_4			
	(mL)	%	(mL)	%R	L CH_4 $Kg^{-1}VS_{Ad}$
0.75	136.76a	26.20b	35.93ab	42.80b	19.42c
1.5	133.60a	31.64a	42.27a	67.00a	45.96b
3.0	87.40b	33.80a	29.54b	47.00b	64.22a
%CV	8.30	6.74	12.41	7.89	9.63

*Averages followed by the same letter in the column do not differ according to Tukey's test at 5% significance; CV: Coefficient of variation.

The I/S 3 ratio was statistically higher according to the Tukey test at 5% significance (Table 3), providing the best results for the cumulative production of methane in relation to the amount of VS added as a function of time, with 64.22 L CH_4 Kg^{-1} $VS_{additioned}$, followed by I/S1.5 (45.96 L CH_4 $Kg^{-1}VS_{add}$) and I/S 0.75 (19.42 L CH_4 Kg^{-1} VS_{add}). Evidencing that the higher the concentration of added inoculum in relation to the substrate, the higher the production of methane per gram of volatile solids, with an increase of 70% when the I/S ratio varied from 0.75 to 3.

Ros et *al*. (2016) obtained for grape marc, during 40 days, 360 L CH4 $Kg^{-1}VS_{add}$. Fabbri *et al*. (2015) also using grape marc, obtained 145-254 L CH4 $Kg^{-1}VS_{add}$. This difference in yields may be associated with the fact that the above studies had an average duration of 60 days. However, in order to obtain uniformity between the duration times used in BMP tests, it is recommended that the experiment should be completed when the daily rate of biogas is equivalent to about 1% of the total volume of biogas produced up to that moment, according to the VDI 4630 (2016).

Moset *et al.* (2015), varying I/S ratio from 0.5 - 2, found that a higher ratio provides a higher BMP for maize (397.5 L CH_4 Kg^{-1} VS_{add}). Pellera and Gidarakos (2016) used I/S ratios of 0.5, 1, 2 and 4, obtaining the best yields in I/S ratio 4 for cotton ginning waste and in ratio 2 for grape waste (skin, seed and stalk). Rouches *et al.* (2019), evaluating the impact of I/S ratios (0.9, 0.5, 0.3, 0.1) on anaerobic digestion of wheat straw, observed a drastic drop in methane production in the lowest ratios.

Thus, the biochemical methane potential test is an essential technique to evaluate the implementation and optimization of anaerobic biotechnologies (Silva *et al.*, 2018). Although this technique is characterized by long periods of digestion, from 20 to more than 100 days, which is not appropriate to the need for rapid decision-making, as in the case of agroindustries (Raposo *et al.*, 2012; Silva *et al.*, 2018). Thus, taking into account that the experimental period of the present study, 12 days, compared to other studies with other organic waste mentioned above, whether they come from the winemaking process or not, reveals the potential of grape marc for specific production of methane.

The methane yield (%R) shows that the experimental methane production was above 40% for I/S ratios 0.75 and 3 of the theoretical volume of methane ($TVCH_4$), while the I/S ratio 1.5 obtained 67% yield from $TVCH_4$ (Table 3), indicating that the organic fraction removed anaerobically converted into methane, from the treatment of grape marc, and that this method is a viable alternative for the use of residues from grape processing.

3.3. Morphology of the Inoculum Microbial Community

In the SEM micrographs of the 0.75 inoculum I/S (Figure 2), it can be seen that even with this ratio, which provided the lowest percentage of methane among the studied ratios, there was a predominance of bacterial morphologies in the form of coconuts similar to *methanosarcine sp* (coconuts adhered to the granule indicated by the arrows) belonging to the domain of the arches, in addition to long (ellipses) and short (circle) bacilli. *Methanosarcines sp* are the only group together with *Methanosaeta sp* to metabolize the acetate for methane production. *Methanosarcin* is capable of growing on substrates other than acetate such as hydrogen and predominates in environments with high acetate concentration and pH between 5.9 and 6.4. The I/S ratio of 0.75 presented a value of 4872 mg H Ac L^{-1} and pH of 6.86.

It is also observed that short bacilli and rounded bacilli were also found (circles) indicating bacteria belonging to the order of *Methanobacteriales*, of which, according to Gerardi (2006), the two main genera are *Methanobacterium* and *Methanobrevibacter*. The presence of these groups of bacteria and arches demonstrates a diversified biomass suggesting a balance between the communities of acetotrophic (coconut and filamentous) and hydrogenotrophic (bacilli) methanogenic microorganisms.

A B

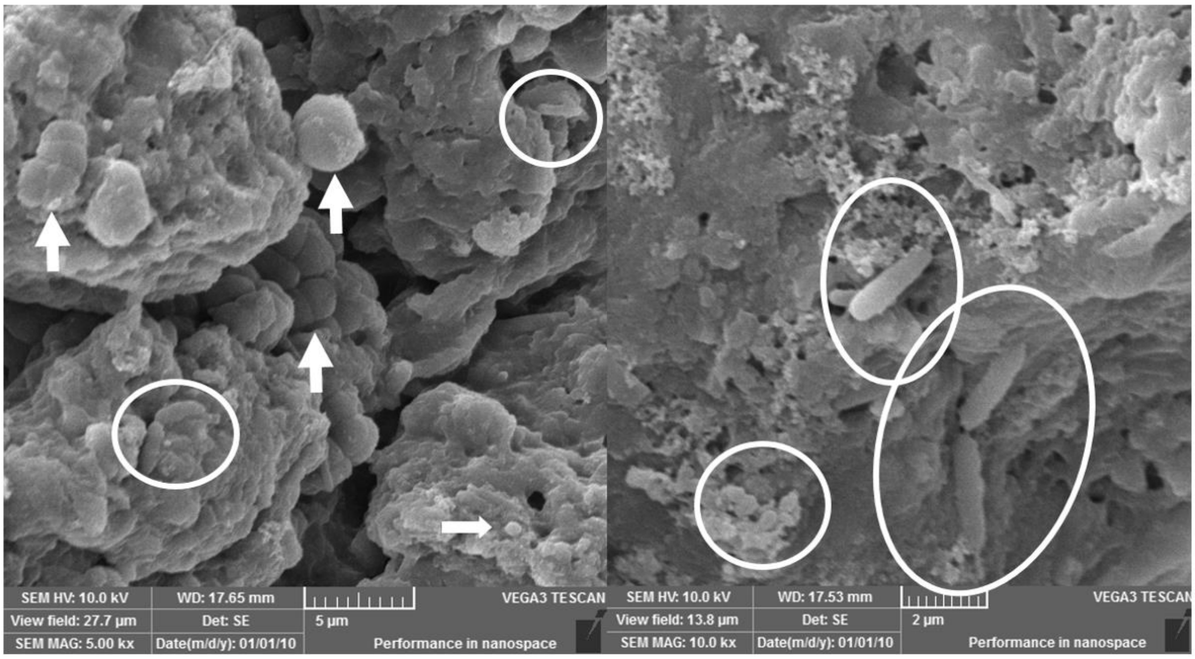

Figure 2. Sample micrograph of inoculum I/S 0,75. (a) predominance of clustered cocci (*Methanosarcines sp*) and isolated cocci (arrows) besides short bacilli and bacilli with rounded ends (circles). (b) Long bacilli (ellipses) and short bacilli (circle).

4. CONCLUSIONS

The experimental results indicated that grape marc presents potential for biological treatment by anaerobic digestion, offering volumetric production of methane in 12 days, and showing the functionality of the BMP test in identifying the potential of grape marc establishing bases for its use as a substrate in the implementation of anaerobic biotechnologies.

The I/S 3.0 ratio obtained the best performance, providing the best performance of methane in relation to the added VS, methane and efficiency in the removal of COD, thus standing out as the most promising ratio for the loading of anaerobic digestion systems to the full-scale grape marc residue.

Microscopic analysis of the morphology of inoculum microorganisms showed microbial groups such as *Methanosarcin sp.* and bacilli with morphology of *Methanobacteriales*, even though this is the treatment with the lowest methane yield.

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