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# *Methanotrophic activity and microbial community dynamics in a UASB sludge*

This study determined the methanotrophic activity in anaerobic sludge from a pilot-scale UASB reactor. Four batch experiments, with three replicates, were performed in 110 mL antibiotic flasks. The results showed that the maximum rate was 115 $\mu molCH_4.d^{-1}$ and the methanotrophic activity was 2.3 $mmolCH_4.gTVS^{-1}.d^{-1}$, indicating that the methanotrophic microorganisms play a key role within the UASB reactor since they are part of the sludge microbiota and may consume some of the methane produced inside the reactor. Therefore, these microorganisms may reduce possible methane losses, either atmospheric and/or dissolved in the treated effluent. The microbial community was investigated by molecular tools (PCR-DGGE) and two DNA sequences related to methanotrophic bacteria, *Methylocystis* sp. (similarity of 93%) and *Methylocaldum* sp. (similarity of 98%) to 16S rRNA gene sequences, were detected. The methanotrophic activity and the identification of the community of main microorganisms involved allow the reduction of methane into the atmosphere and contribute to the system's mass balance between production and consumption.

**Keywords:** Anaerobic Gas; Chromatograph; Methane Oxidation; Pilot-Scale; Sludge.

# *Atividade metanotrófica e dinâmica da comunidade microbiana em lodo de UASB*

Para determinação da atividade metanotrófica em lodo anaeróbio de um reator UASB operando em escala piloto, foram montados quatro experimentos em triplicata utilizando frascos de antibióticos de 110 mL. Os resultados mostraram que a taxa máxima produzida foi de 115 $\mu molCH_4.d^{-1}$ e a atividade metanotrófica 2,3 $mmolCH_4.gTVS^{-1}.d^{-1}$, indicando que os microrganismos metanotróficos desempenham um papel fundamental dentro do reator UASB, uma vez que fazem parte da microbiota do lodo e podem consumir parte do metano produzido dentro do reator. Portanto, esses microrganismos podem reduzir possíveis perdas de metano, seja atmosférico e/ou dissolvido no efluente tratado. A comunidade microbiana foi investigada com auxílio de ferramentas de biologia molecular (PCR-DGGE) e duas sequências de DNA relacionadas a bactérias metanotróficas, *Methylocystis* sp. (similaridade de 93%) e *Methylocaldum* sp. (similaridade de 98%) para sequências do gene 16S rRNA, foram detectados. A atividade metanotrófica e a contribuição predominante da comunidade de microrganismos envolvidos permitem a redução do metano na atmosfera e contribuem para o balanço de massa do sistema entre produção e consumo.

**Palavras-chave:** Gás Anaeróbio; Cromatógrafo; Oxidação de Metano; Escala Piloto; Lodo.

Topic: **Engenharia Sanitária**

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**INTRODUCTION**

Methane is formed through the methanogenesis process which is the organic matter fermentation final step. This process occurs in a variety of anoxic environments, e.g., rice fields, animals' intestines, soils, sediments, freshwater sediments, landfills, and in wastewater treatment plant (WWTP) processes such as anaerobic systems treating domestic sewage or industrial wastewater (KALYUZHNAYA *et al*., 2015). About 60% of the global $CH_4$ budget came from these processes and environments resulting in a methane global rate estimated at 500 to 600 $TgCH_4.yr^{-1}$ (CONRAD, 2009).

In anaerobic systems, such as upflow anaerobic sludge blanket (UASB) reactors, methane is produced within the system to form the biogas, which is captured by the three-phase separator (CHERNICHARO *et al*., 2015). However, it may also be detached from the final effluent as dissolved methane, which contributes to the greenhouse effect, representing environmental and economic losses (CAKIR *et al*., 2005; LOBATO *et al*., 2012).

It is known that methane oxidizer microorganisms are present in these systems. Methanotrophic groups use methane and other C1 compounds as a source of carbon and energy (MATSUURA *et al*., 2017). Aerobic oxidation is performed by bacteria that use methane monooxygenase (MMO) enzymes to oxidize methane to methanol (LUESKEN *et al*., 2011). Anaerobic oxidation may be performed by nitrite-dependent anaerobic methane oxidation (N-DAMO) bacteria and anaerobic methanotrophic archaea (ANME) (KAMPMAN *et al*., 2018).

Studies have investigated methane oxidation in WWTPs. Meulepas *et al*. (2010) obtained an oxidation rate of 11.4 $\mu molCH_4.gVSS^{-1}.d^{-1}$ evaluating methane oxidation of UASB sludge treating industrial wastewater, and also evaluated the role of methane oxidation in activated sludge system, sludge digesters, and an oxygen-limited autotrophic biological system (nitrification/denitrification) used to treat high nitrogen concentration wastewater. The highest oxidation potential could be verified in the activated sludge which presented an oxidation rate of 160.32 $mmolCH_4.gVSS^{-1}.d^{-1}$ (HO *et al*., 2014). In this sense, new possibilities for the dissolved methane removal in anaerobic effluent would be a post-treatment by microorganisms, which could biologically oxidize methane (KALYUZHNAYA *et al*., 2015). According to Brandt *et al*. (2018), it is possible to design biofilters for the removal of residual methane released from effluents. In this case, it is recommended to install two biofilters arranged in series: the first one will treat odorous gases, and the second one directed to treat $CH_4$, since the acidic conditions found in biofilters may affect the methanotrophic activity.

In the Brazilian context, basic sanitation needs investments in the overall sectors (e.g., water supply, solid waste management, and mainly in sewerage). About 45.9% of Brazilians still do not have access to sewage collection networks (SNIS, 2020). Optimizing sewage treatment technologies may greatly contribute to reducing this inequality, because like most developing countries, Brazil is limited to the secondary treatment level by aerobic or anaerobic processes (VON SPERLING, 2014). Furthermore, methane production in treatment systems would be relevant from environmental and socio-economic aspects with great

relevance in developing countries.

Therefore, we aimed to determine the maximum methane consumption performed by anaerobic batch tests from a UASB reactor sludge, as well as to determine the predominant microbial community selected over time using biological molecular tools (PCR-DGGE).

## MATERIALS AND METHODS

### Anaerobic methane oxidation test

The experiment was performed to evaluate anaerobic methanotrophic activity in UASB sludge samples. Four batch experiments (tests 1, 2, 3, and 4), with three replicates each, were performed in 110 mL antibiotic flasks. Before sludge sampling, the concentration of total volatile solids (TVS) was measured according to APHA (2005) in order to determine the volume of sludge to be added to 110 mL flasks, to obtain 5 $gTVS.L^{-1}$ (CHERNICHARO, 2017). After adding the inoculum, the sludge was supplemented with 20 mL of culture medium previously sterilized according to Luesken *et al*. (2011). The flasks were sealed with rubber bungs (butyl) and aluminum seals and the atmosphere was "washed" with inert gas ($N_2$ - 99.9%) to anaerobiosis and incubated at 30 °C in an orbital shaker at 250 rpm. 20 mL of $CH_4$ was added utilizing a glass ground syringe. Autoclaved sludge flasks were incubated as control.

### Gas analysis

Subsequently, 1 mL of headspace gas sample was taken twice a week to check methane decay. Gas was measured using a gas chromatograph (GC) (Perkin Elmer Auto System) equipped with a thermal conductivity detector (TCD). Chromatographic analyses occurred using He gas as a carrier gas and column temperature at 220 °C. Each run had a total duration of 4.5 min, and the methane retention time occurred around 2.75 min. Standard curves for the calculation of the gas concentrations were established using standard gas (47.1% $CH_4$, 25% $CO_2$, 2% $H_2$, 5% CO, and 20.9% $N_2$) and area of known peaks.

After 34 days, accumulated methane consumption data was processed in programming language R 3.6.3 (R Development Core Team) for methane consumption determination. For each test, linear and sigmoidal models (Logistic and Gompertz) were adjusted. For the model comparison, it was considered the coefficient of determination ($R^2$), the residual sum of squares (RSS), and the significance level of the coefficients (p-value).

Methane consumption rate ($mLCH_4.d^{-1}$) was obtained by dividing the maximum consumption value ($mLCH_4$) by the load of total volatile solids (gTVS) of the sludge added to each flask.

### DNA extraction and PCR-DGGE

Inoculated sludge samples, as well as the enriched biomass at the assay end (after 34 days of incubation), were collected for molecular analysis of Test 3. DNA was extracted according to Egli *et al*. (2003) and amplified by polymerase chain reaction (PCR). For this purpose, 30 $ng.\mu L^{-1}$ of DNA was added to 50 µL of

reaction. The primers used were 1055F (5'ATGGCTGTCGTCAGCT 3') and 1392R-GC (5'ACGGGCGTGTAC 3'), for the RNAr 16S V8 region of the Bacteria domain. To confirm the amplicons presence, 2 µL of each one was submitted to 1% agarose gel electrophoresis with GelRed solution (Biotium®), 10x buffer (Phoneutria®), and Low Mass DNA Ladder (Invitrogen®), as the reference standard for DNA quantification. The software ImageJ (Image Processing and Analysis in Java[1]) was used for gel photograph analysis and estimating the DNA present amount.

The amplicons containing 400 ng of DNA from the inoculum samples and oxidation test end were submitted to electrophoresis run at 60 °C in TAE (Tris-Acetate-EDTA) buffer 0.5 for 17 h at 80 V in a Bio-Rad DCode Universal Mutation Detection System (Hercules, CA, USA), using an 8% polyacrylamide gel and a denaturing gradient of 45 to 75%. The gels colored with Sybr-Gold (Life Technologies) solution for 30 minutes were visualized under ultraviolet light and the bands contained excised from the gels and eluted in 50 µL of TE (Tris-EDTA) buffer remaining 4 °C for 48 h.

**Analysis of DGGE band sequences**

Eluted DNA was re-amplified with primers 1055-1392 (without the GC clamp). The purified PCR products were sent for unidirectional sequencing (primer 1055F) at Macrogen Inc (South Korea) by a 3730XL sequencer. From the direct sequence obtained (forward), the reverse sequence (reverse complement) was also obtained through the software Sequence Manipulation Suite[2] (STOTHARD, 2000). The direct and reserve sequences (forward-reverse) were aligned through the software FASTA[3] (University of Virginia).

Aligned sequences were used for comparison by the Ribosomal Database Project databases through the software RDP Classifier[4] and the NCBI (National Center of Biotechnology Information[5]) through the BLAST (Basic Local Alignment Search Tool).

**RESULTS AND DISCUSSION**

**Methanotrophic activity**

Data were adjusted for the Logistic, Gompertz, and Linear models and showed the optimum $R^2$ (Test 1 = 0.96; Test 2 = 0.99; Test 3 = 0.98; Test 4 = 0.99), RSS, and p-value of coefficients (0 and 0.001) for Gompertz Model. Figure 1 presents the models adjustment for the tests.

It was verified that the UASB sludge methanotrophic activity should be taken into account, as it is part of the reactor methane balance. The maximum methane rate was 115.5 µmol$CH_4$.$d^{-1}$and the maximum methanotrophic activity average was 2.33 mmol$CH_4$.g$TVS^{-1}$.$d^{-1}$. Table 1 presents maximum specific methanotrophic activity values obtained for each test (1 to 4) and average values.

[1] http://imagej.nih.gov/ij/
[2] http://www.bioinformatics.org/sms2/rev_comp.html
[3] http://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi?rm=select&pgm=fad
[4] https://rdp.cme.msu.edu/classifier/classifier.jsp
[5] http://www.ncbi.nlm.nih.gov/

**Table 1:** Maximum methanotrophic activity consumption rate and methanotrophic activity of UASB sludge samples.

| Sample | $CH_4$ maximum rate ($mLCH_4.d^{-1}$) | $CH_4$ maximum rate ($\mu molCH_4.d^{-1}$) | Methanotrophic activity ($mLCH_4.gTVS^{-1}$) | Methanotrophic activity ($mmolCH_4.gTVS^{-1}.d^{-1}$) |
|---|---|---|---|---|
| **Test 1** | 1.6 | 65 | 33.8 | 1.4 |
| **Test 2** | 2.9 | 119 | 89.1 | 3.6 |
| **Test 3** | 2.7 | 112 | 35.0 | 1.4 |
| **Test 4** | 4.0 | 164 | 68.9 | 2.8 |
| **Average** | **2.8** | **115.5** | **56.7** | **2.33** |

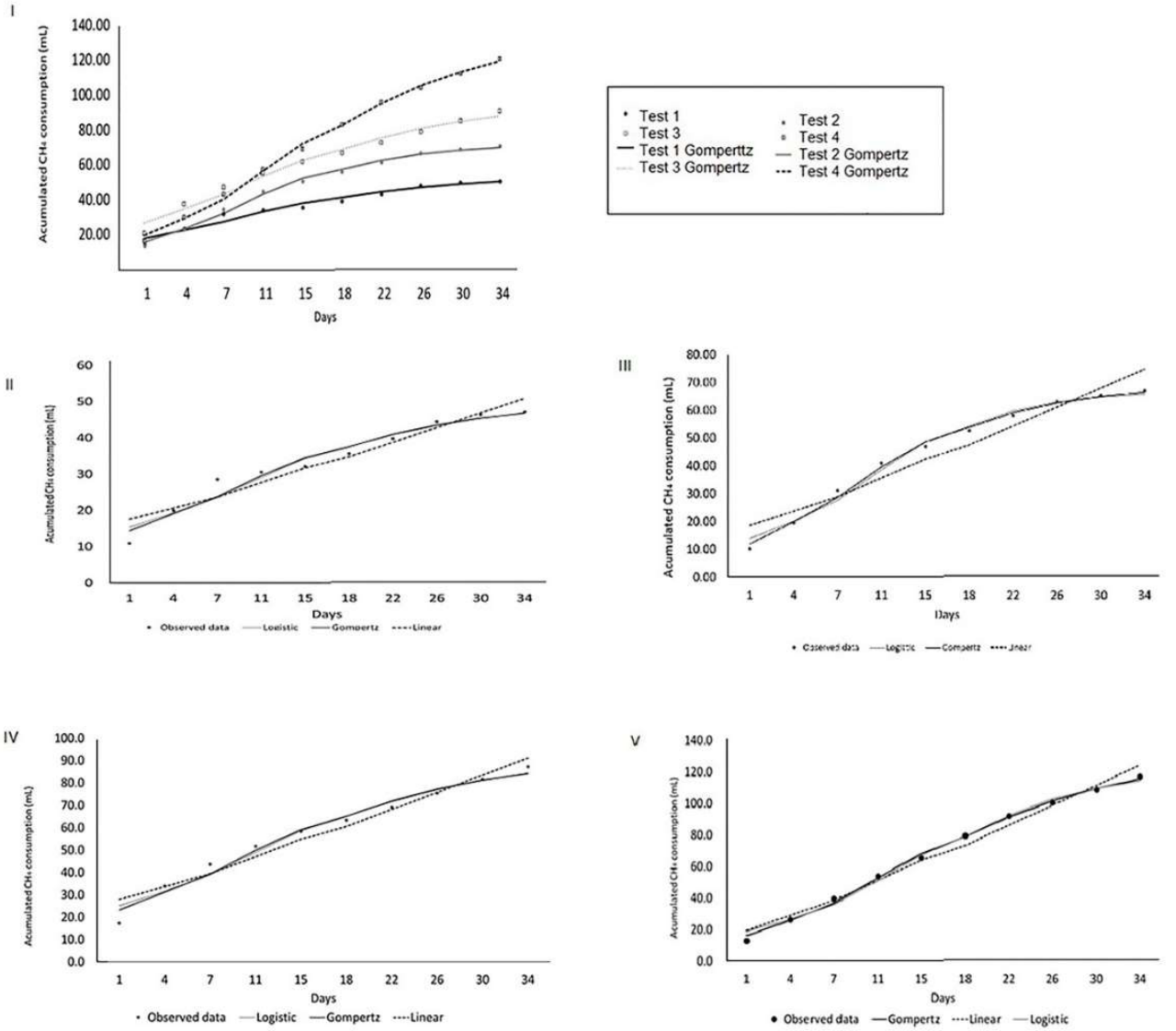


**Figure 1:** Accumulated $CH_4$ consumption scatter plot. I. All tests (1 to 4) and Gompertz adjust. II to V. Tests containing the curve adjusted to the data by the Logistic, Gompertz, and Linear models. II: Test 1. III: Test 2. IV: Test 3. V: Test 4.

Results were superior to those found by Knief *et al*. (2003), who estimated methane oxidation maximum rates ranging from 0.01 to 3.11 $nmolCH_4.g^{-1}.h^{-1}$. However, the study used samples of the soil superficial horizon (5 to 20 cm) from Germany, Italy, and Holland, and they were incubated at 25 °C-temperature below the present study. Some authors (ETTWIG *et al*., 2009) present a methane consumption activity rate of approximately 0.18 $nmolCH_4.min^{-1}$ per mg of protein with a culture enriched from sediments of agricultural ditches and freshwater in batch experiments using a culture enriched with methanotrophic microorganisms.

Moreover, methane uptake was carried out in 100 mL batch reactors incubated in a shaker (40 rpm)

at 30 °C by methanotrophs in methanotrophic denitrificant reactors (RDM) (CUBA *et al*., 2001). The RDM was fed with nitrate and nitrite 0.009 and 0.005 mol.$L^{-1}$, respectively, corresponding to uptake rates of 0.52 mol$CH_4$.$g^{-1}$$NO_3$-N and 0.17 mol$CH_4$.$g^{-1}$ $NO^{2-}$-N.

After analyzed samples of Canadian Arctic soil (permafrost) incubated in a medium containing mineral salts or added directly to glass flasks, the researchers (MARTINEAU *et al*., 2010) detected concentrations ranging from 2.5 to 7.5 nmol$CH_4$.$g^{-1}$.$d^{-1}$ (4 °C ) and 7.5 to 11.5 nmol$CH_4$.$g^{-1}$.$d^{-1}$ (at 25 °C). In contrast, when the mineral medium was added, the oxidation rates at 4 °C were approximately 100 nmol$CH_4$.$g^{-1}$.$d^{-1}$, whereas the rates for oxidation at 25 °C ranged from 300 nmol to 550 nmol$CH_4$.$g^{-1}$.$d^{-1}$. The nutrient deficiency may be an important factor that limits the activity of methanotrophic bacteria in Arctic soils (MARTINEAU *et al*., 2010) and explains low oxidation values.

Some authors investigated the labeled methane (13C) oxidation in samples of granular sludge obtained from three UASB reactors in full scale. The composite sample was incubated and the oxidation rate presented was 11.4 µmol$CH_4$.g$VSS^{-1}$$d^{-1}$ (MEULEPAS *et al*., 2010). In a study by Ho *et al*. (2013), the methane oxidation potential from sludge samples from different WWTPs was 160.32 (± 17.79) mmol$CH_4$.g$VSS^{-1}$ for anoxic sludge from the activated sludge system (AS) and 34.66 (± 0.94) mmol$CH_4$.g$VSS^{-1}$ for the AS return sludge. For sludge from anaerobic digesters, the total methane consumed was 22.09 (± 1.02) mmol$CH_4$.g$VSS^{-1}$; and finally, for the laboratory-scale OLAND system, the result was 85.16 (± 2.69) mmol$CH_4$.g$VSS^{-1}$ and 35.19 (± 5.71) mmol$CH_4$.g$VSS^{-1}$ for industrial-scale OLAND.

It was observed that values achieved in the present study were lower than those found for anaerobic digesters treating waste and AS (MEULEPAS *et al*., 2010; HO *et al*., 2013), however, higher than rates of UASB granular sludge oxidation treating industrial effluent. It is worth noting that different methodologies promote tests with different temperatures, pressure (BHATTARAI *et al*., 2018), compositional change of culture media (MARTINEAU *et al*., 2010), low concentrations of dissolved oxygen (KAMPMAN *et al*., 2018), and inoculum (SINISCALCHI *et al*., 2017). These experiments stimulate different biotic interactions which occur over time and can potentially alter the methane oxidation activity (HO *et al*., 2013).

Furthermore, this research aimed to determine the maximum accumulated production over the test period in order to demonstrate how important it would be to consider the presence of methanotrophic in the treatment systems. When considering smaller scale reactors, methane oxidation is interesting from an environmental and economic point of view (GÜR *et al*., 2016). However, it is worth mentioning that in the large-scale case, dissolved methane would represent energy loss, since methane could be used to generate electricity at the treatment plant itself or for local use and/or with the concessionaire (LOBATO *et al*., 2012). In anaerobic reactors, the methane composition in the biogas is estimated to be 60-85%. The methane calorific value is 35.9 MJ.$Nm^{-3}$ and for biogas with 60% methane is 21.5 MJ.$Nm^{-3}$, corresponding to 67% of the natural gas calorific value (LOBATO *et al*., 2012; ROSA *et al*., 2015). The electricity generation through biogas from anaerobic sewage treatment systems in Brazil indicates economic viability for cities with a population of over 300,000 inhabitants (SANTOS *et al*., 2016).

In Minas Gerais, state of the present study, Campello *et al*. (2020) evaluated the economic viability

of exploiting for electric power generation, biogas generated by anaerobic digestion of sewage and sewage sludge in treatment plants. They demonstrated potential for electricity generation of about 47,140 MWh per year and a potential reduction in emissions of greenhouse gas (GHG) close to 325,800 $tCO_2eq.yr^{-1}$.

The impact of dissolved methane from sewage treatment systems on the greenhouse effect could be as high as the impact of emissions from recovered methane on biogas, especially in the case of low chemical oxygen demand (COD) influent concentrations, in which there are no economic possibilities to implement energy recovery from biogas (CAKIR *et al*., 2005). In this sense, the possibility of using biological oxidation is a way to minimize the environmental impacts of these systems.

**Microbiological community dynamics**

A large number of bands was obtained and kept from the two samples analyzed, however only the sequences of the bands listed on the gel (Figure 2) were recovered, and this amount proved to be enough to assess the diversity of the samples (Table 2). Channel 1 corresponded to the test of the inoculum sample (UASB sludge), while channel 2 contained the biomass after 34 days of the test start-up. Most bands showed high similarity with non-cultivated bacteria clones from environmental samples.

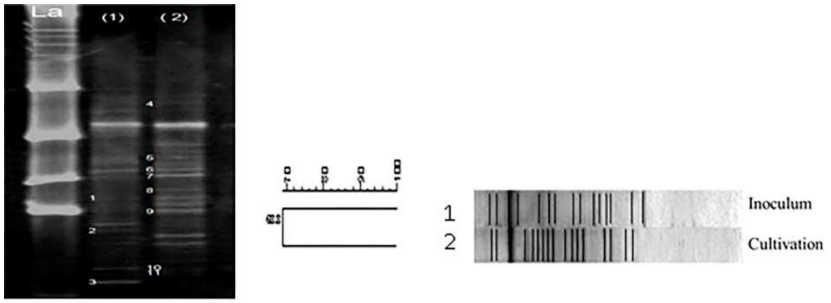


**Figure 2:** DGGE gel colored with SYBR and Dendrogram generated based on band profile. Gold containing a 16S ribosomal DNA fragment amplified with universal Bacteria primers (La) molecular weight marker (Ladder Express); (1) UASB sludge used as inoculum for the anaerobic methane oxidation test; (2) sludge sample at the end of the anaerobic methane oxidation test (34 days of incubation).

**Table 2:** Alignments of the sequences obtained from the bands with the software RDP Classifier and Blast.

| Band number | RDP Classifier | Blast | GenBank Access Number | Similarity (%) |
|---|---|---|---|---|
| 1 | Class *Alphaproteobacteria* | *Methylocystis* sp. | EU647258.1 | 93 |
| 2 | Domain Bacteria | Uncultivated *Mycobacterium* sp. clone | EU631845.1 | 87 |
| 3 | Family *Synergistaceae* | Uncultivated *Synergistetes* | AB908684.1 | 98 |
| 4 | Domain Bacteria | Uncultivated *Acidobacteriaceae* | KP717505.1 | 98 |
| 5 | Domain Bacteria | Uncultivated *Desulfuromonadaceae* | KC009900.1 | 77 |
| 6 | Domain Bacteria | *Acidovorax* sp. | KP126996.1 | 86 |
| 7 | Genus *Methylocaldum* | Uncultivated *Methylocaldum* sp | HF565149.1 | 98 |
| 8 | Domain Bacteria | Uncultivated *Planctomycetales* clone | FJ710625.1 | 95 |
| 9 | Genus *Solirubrobacter* | Uncultivated *Solirubrobacter* sp. | HQ213036.1 | 99 |
| 10 | Phylum *Chloroflexi* | Uncultivated *Chloroflexi* | AY921707.1 | 98 |
| 11 | Domain Bacteria | Uncultivated *Armatimonadetes* | KJ600041.1 | 95 |

Methanotrophic microorganisms were observed in bands 1 and 7. Band 1 showed 93% similarity with the genus *Methylocystis* sp. (class Alphaproteobacteria), and band 7 showed 98% similarity with the sequence of *Methylocaldum* sp. (class Gammaproteobacteria).

One of these sequences (band 1) showed high similarity with the *Methylocystis* genus.

Methanotrophs of the genus *Methylocystis* sp. are generally in the rod-shape and simple arrangements (BRENNER *et al*., 2005). They have been reported in environments such as rice fields, sewers, and freshwater sediments (BRENNER *et al*., 2005). The community of the acidic peatlands of southern Brazil was investigated and found methanotrophs related to *Methylocystaceae* and *Methylococcaceae* (ETTO *et al*., 2012), as well as in the Amazon region where detected the same families (*Methylocystaceae* and *Methylococcaceae*) (FINN *et al*., 2020; TESSARO, 2012).

Bands 2 and 6 were classified at the domain level by the RDP Classifier, showing 87 and 86% of the similarity with the genera *Mycobacterium* and *Acidovorax*, respectively. The genus *Mycobacterium* (phylum *Actinobacteria*) is composed of numerous pathogenic bacteria and species whit bioremediation ability (DAS *et al*., 2015).

*Acidovorax* has also been described in domestic sewage sludge samples and soils, including contaminated soils with polycyclic aromatic hydrocarbons (SINGLETON *et al*., 2009). Band 9 was classified at genre level with 99% similarity with *Solirubrobacter* (phylum *Actinobacteria*). Members of this genus are chemorganotrophic, growing at a temperature of 19 to 38 °C, and have already been isolated from soils containing crops (SINGLETON *et al*., 2009).

Bands 3, 10, and 11 were classified at the phylum level, with band 3 showing identity with *Synergistetes*, band 10 with the phylum *Chloroflexi*, and band 11 with the phylum *Armatimonadetes* (previously classified as OP10). The three phyla are composed of microorganisms that contain anaerobic representatives and have also been mentioned as present in sewage treatment systems (GANESAN *et al*., 2012).

Bands 4 and 8 were identified as *Acidobacteriaceae* and *Planctomycetales*, respectively. The *Acidobacteriaceae* family is composed of chemorganotrophic microorganisms, found in soils, sediments, wastewater, water distribution systems, acid mine drainage, hydrothermal vents, among others (ZEKKER *et al*., 2015).

The DGGE band profile was compared using the software BioNumerics (version 7.1), which indicated the similarity coefficient between the initial sample (inoculum) and after 34 days of incubation (anaerobic methane oxidation test) by analyzing the presence/absence of similar bands (Figure 2).

By using the Dice coefficient, it was observed 69% of similarity among the samples, indicating that there was a change in the microbial community inside the flasks, under the test conditions, such as the methane and culture medium addition. Therefore, the sample incubation for 34 days led to a methanotrophic selection and enrichment related to the genus *Methylocaldum* (i.e., aerobic methanotrophs), but have also been reported in anoxic environments, such as in UASB reactor and fermenter sludges (SINISCALCHI *et al*., 2017).

Diversity was also calculated using the Shannon index (H'). The H' index showed practically the same diversity for both samples (H' = 2.4 for the inoculum and 2.6 for 35 days of testing), suggesting that the incubation time was short to reveal a difference in diversity and possible community selection, however sufficient to perform the anaerobic methane oxidation test and, consequently, methanotrophic activity

calculation of the UASB reactor sludge.

## CONCLUSIONS

The most appropriate conditions to determine methanotrophic activity were established, and methanotrophic activity from a UASB sludge was determined through four repetitions. Methanotrophs bacteria were identified by DGGE in the batch flasks and show the coexistence of aerobic bacteria in anoxic environments. A large part of methane consumption occurs from biological oxidation by methanotrophic organisms, and therefore the contribution and importance of these microorganisms within these systems are highlighted.

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