# Biodegradation of Leather Solid Waste and Manipulation of Methanogens and Chromium-resistant Microorganisms

by

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## Abstract

The final solid wastes generated by tanneries are usually separately disposed of in hazardous industrial landfills, which are characterized as places of waste confinement where the residues undergo undesired and uncontrolled biological treatment. The storage and corresponding evolution of the sludge has not yet focalized the attention of the scientific community. Apart from developing knowledge of the ecology and function of the microbial community in anaerobic digestion processes, it is necessary to control this biological process. The manipulation of strict anaerobic microorganisms is the limiting factor in their isolation due to their high sensitivity to oxygen; therefore, cultivation under anaerobic conditions is essential. This work presents an evaluation of biogas generation and quality. In addition, microbial biodegradation is carried out in bench bioreactors containing two main solid wastes from tanneries: wet-blue shavings and sludge from a tannery wastewater treatment plant (WWTP). We analyzed two storage conditions of the sludge: storage under ambient conditions and under refrigeration. Sludge kept at ambient conditions showed a significantly higher methane production; methane was only detected in vials containing high concentrations of microorganisms. The manipulation of microorganisms did not interrupt biodegradation. The isolation technique with vials proved to be appropriate for the quantification of methanogenic microorganisms in biodegradation tests, although it was not fully elucidated for their isolation.

## Introduction

Globally, tanneries process an average of 15 million tons of hides and skins each year, but the economic importance of the tanning industry contrasts with the environmental issues of residues generated in the tanning process.[1] The solid wastes generated by tanneries are wet-blue shavings (chromed solid waste generated to regulate leather thickness), hide and leather trimmings, sawdust and sludge from wastewater treatment plants (WWTP). The residues generated after the chromium tanning process are usually separately disposed of in hazardous industrial landfills, which are characterized as places of waste confinement where the residues undergo undesired and uncontrolled biological treatment. The major residue of WWTP is sludge, and the amount of sludge has globally increased as a consequence of an increasing quantity of treated industrial wastewater.[2,3] In recent years, the organic fraction of the solid waste has been recognized as a valuable organic resource that can be converted into useful products through microbial transformation.[4–6]

Methane and carbon dioxide are the main products of microbial anaerobic digestion to degrade organic and inorganic compounds in the absence of molecular oxygen. Hereby, the types, availability, complexity of the substrate, temperature, moisture and the presence of inhibitory substances, such as ammonia, sulphides and heavy metals are factors that influence this process. Biogas produced by anaerobic digestion is composed of 48–65% of methane, 36–41% of carbon dioxide and have less significant quantities of hydrogen sulphide, other sulphur compounds and ammonia. However, the actual composition of biogas varies between different landfills as well as with the landfill itself, due to changes in the process and in raw material conditions.[7] The calorific value of biogas is assessed by its methane content, which has a typical calorific value of 21–24 MJ/$m^3$ or 6 kWh/$m^3$.[8,9] The methane-producing microorganisms belong to the Archaea domain, which comprises prokaryotic microorganisms that are evolutionarily distinct from members of the domain Bacteria in terms of their genomic organization, gene expression, cellular composition and phylogeny. The methanogens are strict anaerobes. Their metabolism is unique in a sense that obtained through methane production.[10] Due to the difficulties for microorganisms to degrade waste containing chromium, tannery WWTP pre-adapt the inoculum to chromium, resulting in a higher biogas production.[11] Isolating

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Manuscript received May 23, 2016, accepted for publication May 25, 2016.

microorganisms with the potential to produce methane from tannery WWTP sludge, hydrolyzed collagen and wet-blue shavings, only sporulating Gram-positive bacteria were identified.[12] Biodiversity in landfill areas is decreasing, and since the late 20th century, it has been recognized that reclamation of this areas is necessary to restore environmental services and also to achieve optimum economic values.[13] It is now encouraged to exploit the biomass of long-term landfill.

Sludge storage plays an important role in selector systems due to its influence on population dynamics and selection of microorganisms in mixed cultures.[14] The storage of sewage sludge has not yet focalised the attention of the scientific community since the available data only concerns the liming treatment or the fate of the soils receiving the sludge and not the evolution of the sludge during storage.[15] Knowledge of the ecology and function of the microbial community in anaerobic digestion processes is necessary to control this biological process. The manipulation of strict anaerobic microorganisms is the limiting factor in their isolation due to their high sensitivity to oxygen; therefore, they require cultivation under anaerobic conditions.[16,17]

This work aims to evaluate and verify the biogas and methane production using biodegradation tests for incubated mixed solid wastes from tanneries. This study is novel in several important aspects: it evaluates the controlled mixture of sludge from tannery WWTP and wet-blue leather shaving, it statistically analyses the influence of sludge storage conditions (in view of scaling-up of this process) and it reports the collection, manipulation and quantification of anaerobic microorganisms (methanogenic archaea).

## Material and Methods

**Bioreactor Assembling**

Tannery waste biodigestion was carried out in bench bioreactors. The bioreactors were made of transparent glass with volume of 350 mL and kept in a thermostatic bath at 35°C. They featured two valves: a superior one to measure the produced gas and a lateral one to collect samples for gas chromatography analysis. The total volume of gas generated in the bioreactors was measured daily based on the water volume shift through pressure equalization, shown in Figure 1.

The experiments were conducted with 1 g of wet-blue leather shaving and 25 ml of activated sludge collected from an aerobic bioreactor of a tannery WWTP (using chromium salts as tanning agents).[11] Nutrient solution (250 ml) was added to each bioreactor to ensure favorable conditions for the initial growth of microorganisms. The nutrient solution (Dettmer, 2012) was composed of 2 g $l^{-1}$ yeast extract, 1 g $l^{-1}$ peptone, 7 g $l^{-1}$ $K_2HPO_4$ and 3 g $l^{-1}$ $KH_2PO_4$. The characterization of the wet-blue leather shavings and the activated sludge were, respectively, 41.39% and 18.95 g $l^{-1}$ of volatile matter, 3.94% and 7.67% of chromium oxide

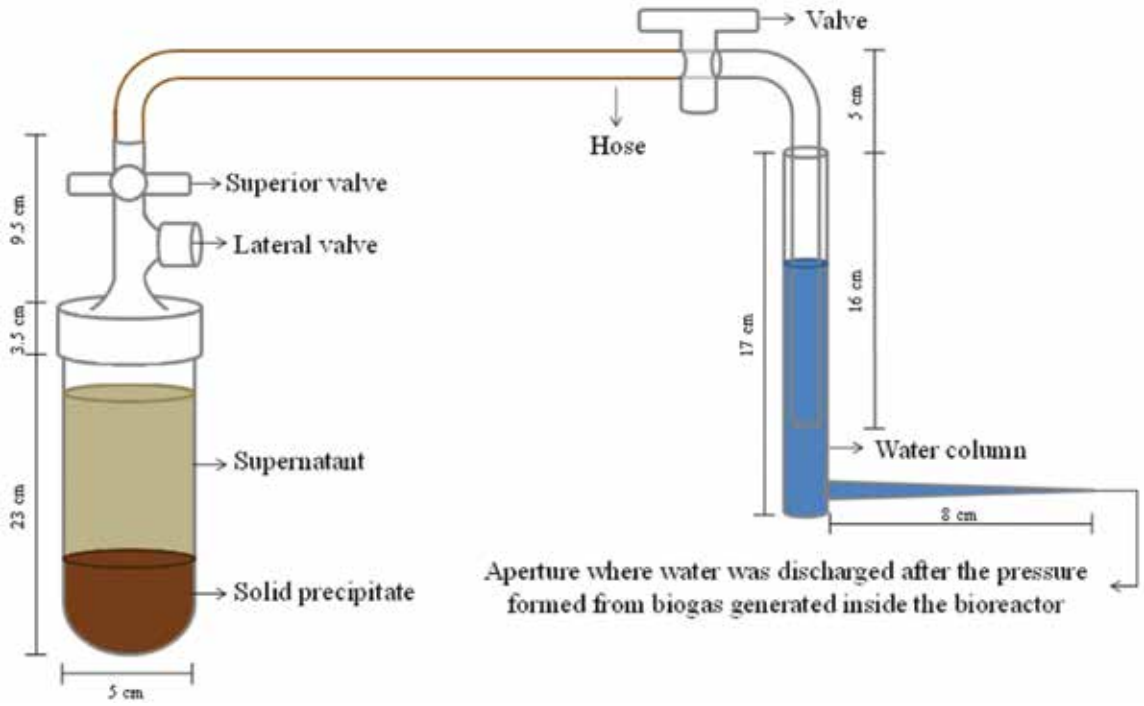


Figure 1. Bench bioreactor design and apparatus used to measure the total volume of gas generated in the bioreactors.

content and 8.90% and 245.56 mg $l^{-1}$ of Nitrogen TKN. The chromium content of the wet-blue shavings was in agreement with the specification of wet-blue leather. In the literature, chromium oxide levels ranging from 1.95 to 27.10%, regarding the management and segregation of the wastewater streams, are found for tannery sludge.[11]

Two experimental conditions were tested in duplicate for 118 days, with different sludge storage conditions prior to the experiment. In scenario 1 (bioreactors 1 and 2), the sludge was stored for six months at room temperature (ranging from about 10 to 35°C) in contact with atmospheric oxygen, simulating ambient storage conditions. In scenario 2 (bioreactors 3 and 4), sludge was stored for six months under refrigeration (4°C) and without contact with atmospheric oxygen, simulating approved conservation conditions. Bioreactor 4 was used for determination of the colony-forming units (CFU) per ml of total prokaryotic cells. Biodigestion and collection experiments are summarized in Table I.

For measurements, a hose was connected between the device and the bioreactor superior valve. First, the device valve was opened to relieve the remaining pressure and the left over water was discarded. Thereafter, the bioreactor valve was opened, which resulted in the release of a volume of water equivalent to the volume of biogas generated in the bioreactor. This amount of water was weighed and converted to volume by the density of water at room temperature.

**Biogas Composition Monitoring**

To determine the composition of the generated gases, a gas chromatograph, fitted with a thermal conductivity detector (TCD) and two packed columns, Porapak Q (80–100 mesh) and Molecular Sieve 13X (80–100 mesh) was used. The Porapak Q determined the fraction of air ($N_2$ + $O_2$), methane and carbon dioxide. The Molecular Sieve 13X determined the ratio between nitrogen and oxygen in the air. Helium was used as carrier gas in both columns. The equipment used was a gas chromatograph Auto System XL/GC, Perkin-Elmer, with interface command via Turbochrom 6.0 software.

**Table I**
**Biodigestion and collection experiments.**

| Bioreactor | Sludge storage condition for 6 months | Bioreactors with CFU/ml determined |
|---|---|---|
| 1 | Ambient aerobic conditions | |
| 2 | | |
| 3 | Refrigerated and anaerobic conditions | |
| 4 | | x (118 day of biodegradation) |

From the peak area values obtained in the chromatograms, the amount of methane generated during the process was estimated. Firstly, all areas obtained were divided by response factors, required to obtain the real response of the amounts present in the bioreactor.[11] With the new calculated areas, only the percentage among them was taken into consideration. The peak time was not considered due to its great sensibility to sample introduction in the equipment. To calculate the percentage of methane in biogas, only the area proportions obtained through the Porapak Q column analysis were considered, because the peak related to methane in Molecular Sieve 13X column cannot efficiently separate carbon dioxide from methane and would amplify the amount of methane. The results obtained from Molecular Sieve 13X analysis were used to monitor the amount of oxygen in the bioreactors.

Once the methane percentage was obtained on a weekly basis, a linear behavior was estimated for each of these days to estimate the percentage of methane for the days when chromatography was not carried out. With the percentage of methane obtained for all days of the experiment, these values were multiplied by the volume of biogas measured for each day, resulting in the volume of methane produced daily.

**Statistical Analysis**

To analyze the sludge storage condition, a two-way ANOVA was performed in Microsoft Excel 2010®, where the two factors considered were sludge storage condition (factor A; two levels: ambient and refrigerated conditions) and time (factor B; eleven levels: from 10 to 110 days, at 10 day intervals). The response variables analyzed were accumulated volume of biogas and of methane in each condition. The influence of gas production difference at different levels of time (factor B) has no scientific relevance. However, the variation of gas production with respect to the sludge storage over time is important and was therefore considered.

**Microbial Collection and Analysis**

For determination of CFU and microbial isolation, it was necessary to open the bioreactor top cover, exposing it to atmospheric oxygen. Samples from the precipitated solid and from the supernatant of bioreactor 4 were collected, both with a long needle (BD Spinal™, 15 cm, code: 405211), in duplicate. A series of fivefold centesimal dilutions was performed in mineral solution with both solid and supernatant samples before inoculation on melted culture media for UFC determination. Sterile insulin syringes were used for sample transfer. Each dilution was dissolved in archaea melted media in duplicate.[18] All dilution solutions or culture media were prepared in 50 ml

penicillin vials, sealed with a rubber stopper and an aluminum flip-off cap, where $N_2$ (Nitrogen 5.0 IBG) and $CO_2$ (Carbon Dioxide 4.0 IBG) in equal proportions were added up to the top of the vials before sterilization. After inoculation of all dilutions in the archaea media, all vials were kept in a slanted position still medium solidification. The inoculated vials were incubated at 35°C for 21 days. Growth and gas production, by GC analysis, were observed every seven days. To estimate CFU/ml, vials containing between 30 and 300 CFU were chosen. The schematization of the bioreactor collection procedure is shown in Figure 2.

After CFU determination and methane detection, isolation of morphologically different colonies from growth results in the vials was carried out on agar plates with the same culture media. Plates were incubated in an anaerobic jar system (Anaerobac Probac of Brazil) at 35°C for seven days in the dark. The transfer from the vials to the plates took place in less than eight minutes. Isolated colonies were tested by Gram staining.

## Results and Discussion

**Biogas and Methane Production**

Accumulated biogas volumes for each sludge storage condition are shown in Figures 3 and 4. Each bioreactor was daily measured for the 118 days of the experiment.

In all curves, there were three distinct phases of gas generation. The first, observed until the 20$^{th}$ day, shows a low gas generation because the microorganisms are adapting to new incubation conditions and the aerobic microbiota was probably growing and using the residual oxygen (Figures 3, 4a, 4b). The second, observed between the 20$^{th}$ and the 70$^{th}$ day, presents an increasing gas production, probably due to the large amounts of nutrients available in the medium and the establishment of an anaerobic atmosphere, resulting in methane production (Figures 3, 4c, 4d). The last phase, observed after the 70$^{th}$ day, shows arrested gas generation because of nutrient depletion in the closed vessel.

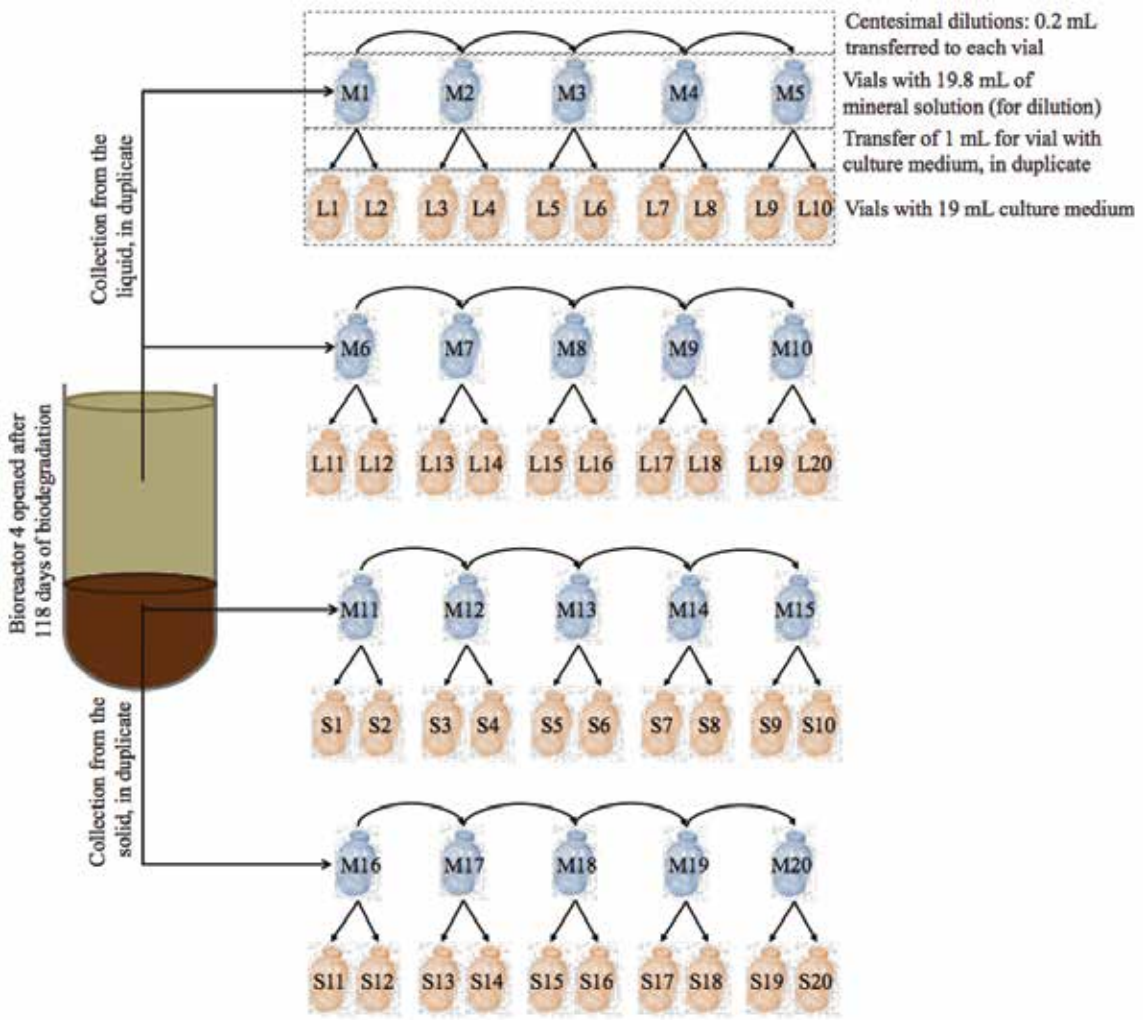


Figure 2. Schematization of the bioreactor 4 collection procedure.

Methane production showed a similar pattern. In scenario 1 (bioreactors 1 and 2 – sludge kept at ambient temperature and in contact with oxygen), higher amounts of biogas and methane were produced than in scenario 2 (bioreactors 3 and 4 – sludge kept refrigerated and without contact with oxygen). Biogas production in bioreactors 1 and 2 stabilized around the 70th day of the experiment, while in bioreactor 3 and 4, stabilization occurred around the 55th day. Also, the initial growth in scenario 1 was less pronounced than in scenario 2. The cumulative volume of methane followed the same tendency as the cumulative volume of biogas produced in both scenarios.

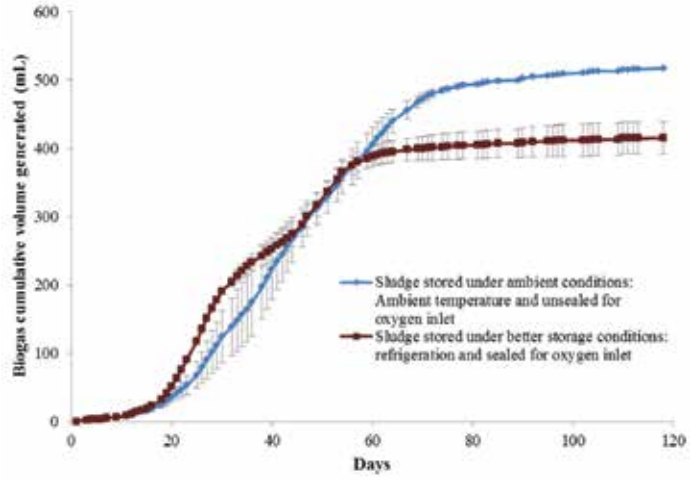


Figure 3. Biogas cumulative volume generated in each sludge storage condition tested, indicating the average standard deviation of the duplicate at each point.

**Statistical Results**

The ANOVA results show that there was no significant dependence between the volume of produced biogas and sludge storage conditions. Figure 3 shows that 450 mL of biogas were produced on average during the period observed, representing about 17 mL of biogas produced per mL of the mixed residue biodegraded. The two-way ANOVA with methane production as response variable showed a significant relationship between methane production and sludge storage conditions (p-value = 0.00027). Figure 5 shows that 265 mL of methane were produced on average during the period observed in scenario 1, where sludge was stored under ambient conditions, while 225 mL of methane were produced on average for the same period in scenario 2, where sludge was stored under better preservation conditions. This represents about 10 mL of methane produced

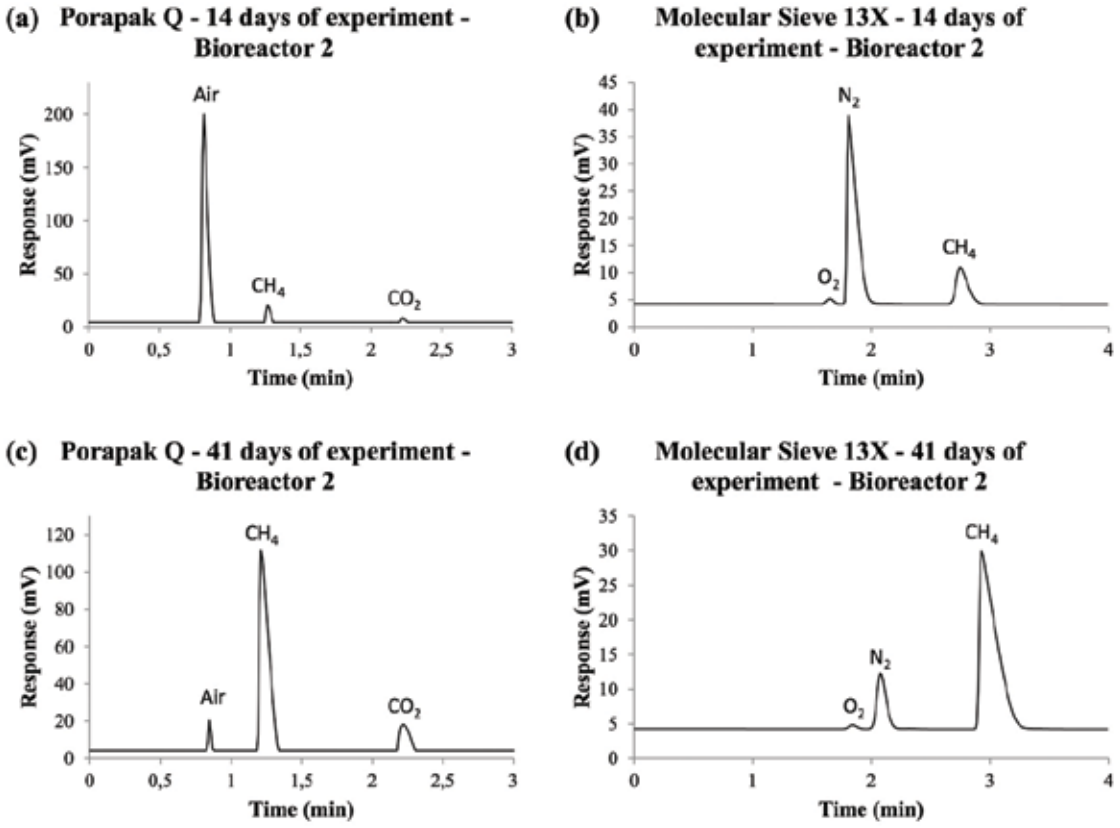


Figure 4. Examples of chromatograms generated in mV x s (a) and (b) with samples collected in the 2nd week of the experiment, and (c) and (d) with samples collected at the 6th week of the experiment. The cumulative volume of methane generated in each bioreactor is shown in Figure 5.

per mL of the mixed residue biodegraded in scenario 1 and about 8.5 mL in scenario 2. The results are summarized in Table II.

Authors who have studied the production of biogas and methane from the same mixed solid waste in similar conditions found about 400-515 ml of biogas and 290-320 ml of methane.[11]

**Microbial Results**

As expected, the CFU/mL, analyzed in bioreactor 4, was significantly greater in the solid phase than in the liquid phase due to the better availability of nutrients, shown in Table III.

We did not isolate any archaea, as all colonies isolated were sporulating Gram-positive cells. It was found[19] the following rod-shaped Gram-positive staining microorganisms in a system of biogas produced from organic waste of a natural gas pipeline system: *Clostridium perfringens*, *Bacillus cereus*, *Corynebacterium spp.* and *Leucobacter acridicollis.*

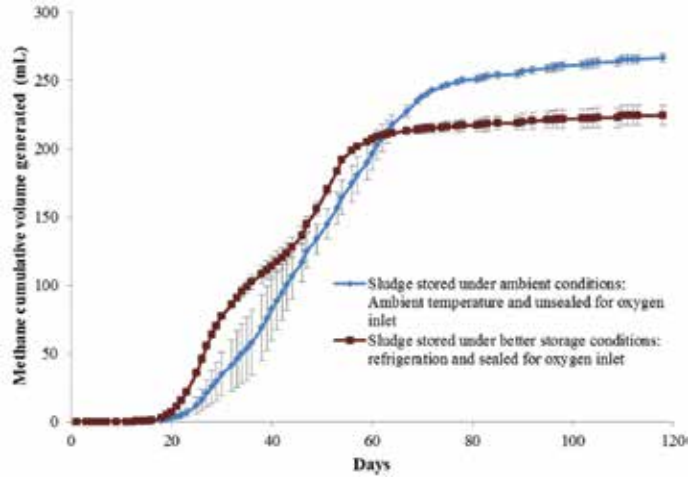


Figure 5. Cumulative methane volume generated in each sludge storage condition tested, indicating the average standard deviation of the duplicate at each point.

## Table II
**Sludge storage conditions and impacts on biogas production.**

| Sludge storage condition influence | | Ambient conditions with contact with oxygen | Refrigerated and without contact with oxygen |
|---|---|---|---|
| Biogas | Average standard deviation along 118 days of biodegradation (ml) | 29.39 | 25.57 |
| | p-value | 0.31 (> 0.05 – not significant) | |
| | F(p-value = 0.31 ) ($F_{tabled}$ (0.05) = 4.96) | 1.14 (< 4.96 – not significant) | |
| | Average volume produced (ml) | 450 | |
| | Average volume produced per ml of the mixed residue biodegraded (ml) | 17 | |
| | Average volume produced per initial VS of the mixed residue biodegraded (ml) | 230 | |
| Methane | Average standard deviation along 118 days of biodegradation (ml) | 14.06 | 6.95 |
| | p-value | 0,00027 (< 0.05 – significant) | |
| | F(p-value = 0.00027) ($F_{tabled}$ (0.05) = 4.96) | 29.80 (> 4.96 – significant) | |
| | Average volume produced (ml) | 265 | 225 |
| | Average volume produced per ml of the mixed residue biodegraded (ml) | 10 | 8.5 |
| | Average volume produced per initial VS of the mixed residue biodegraded (ml) | 135.5 | 115 |

As those colonies are not methanogenic archaea, chromatographic analyses of the remainder vials were performed to investigate the presence of methane. All vials used for CFU determination were monitored for the presence of methane by chromatography. From the total of 40 vials analyzed (previously identified in Figure 2), only four presented methane. These vials presented bubbles after three weeks of cultivation (Figure 6) and corresponded to the first dilution of culture media showing an elevated CFU; it was therefore impossible to recognize and isolate the colonies responsible for methane production. The percentages of methane obtained after three weeks of cultivation for each of the four vials are presented in Table IV.

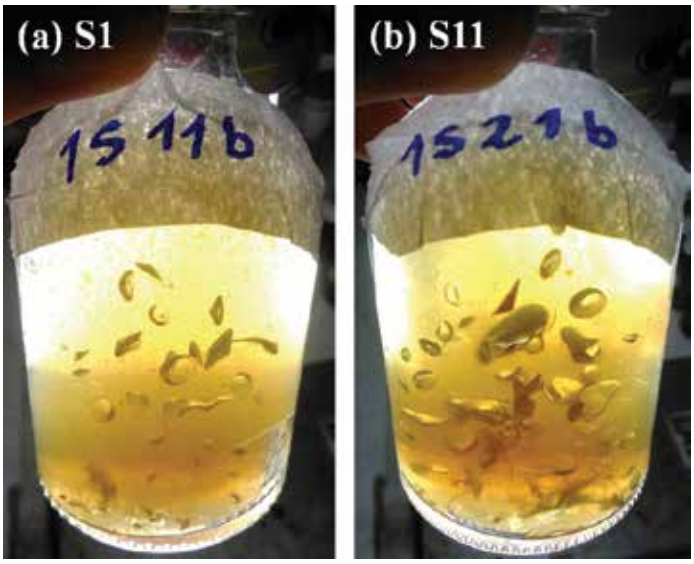


Figure 6. First dilution of the two samples from the precipitated solid phase presenting bubbles in the culture medium.

**Table III**
**CFU/mL in each phase of Bioreactor 4.**

| Phase | CFU/mL |
|---|---|
| Liquid | $4.45 \times 10^6$ |
| Solid | $1.90 \times 10^9$ |

**Table IV**
**Methane percentages of the four vials that presented methane in vials used for CFU determination of the solid phase from bioreactor 4.**

| Vials | Methane percentage after 3 weeks of cultivation |
|---|---|
| S1 | 53,66% |
| S2 | 35,60% |
| S11 | 42,95% |
| S12 | 25,75% |

## Conclusions

Our findings suggest that the sludge storage condition does not influence the amount of biogas produced. However, it significantly influences the quality of the produced biogas, i.e., the amount of methane produced. The sludge storage condition that showed higher methane production was the ambient condition, i.e., at room temperature and with contact to atmospheric oxygen. We therefore assume that oxygen diffusion into the sludge did not prevent the formation of anaerobic zones, thereby benefiting the anaerobes, regardless of the temperature variation. This storage condition imitates the actual procedure made in the landfill. The mixed residues (wet-blue shavings and sludge) produced about 17 ml of biogas, 10 ml of methane from ambient storage conditions and 8.5 ml of methane from better conservation conditions assays per mL of residue in the tested conditions. The culturing technique using penicillin-type vials proved to be suitable for the quantification of anaerobic microorganisms present in biodegradation tests and for the determination of methane production, because the manipulation of anaerobic microorganisms did not interrupted biodegradation. The absence of methane-producing archaea in subsequent vials after dilutions can be linked to the temporary exposure of samples to oxygen during the transfers between vials. Also, the initial presence of other microorganisms in higher numbers can facilitate the fast establishment of better atmospheric and/or nutritive conditions for archaea growth.

## Acknowledgements

This study was supported by CNPq (Edital UNIVERSAL – MCTI/CNPq Nº 14/2013), FAPERGS (Edital FAPERGS/CAPES 17/2012) and FINEP (Edital MCTI/FINEP CT-HIDRO 01/2013).

## References

1. Piccin, J. S., Gomes, C. S., Feris, L. A. & Gutterres, M.; Kinetics and isotherms of leather dye adsorption by tannery solid waste. *Chem. Eng. J.* **183,** 30–38, 2012.
2. Athanasoulia, E., Melidis, P. & Aivasidis, A.; Optimization of biogas production from waste activated sludge through serial digestion. *Renew. Energy* **47,** 147–151, 2012.

3. Akyol, Ç., Demirel, B. & Onay, T. T.; Recovery of methane from tannery sludge: the effect of inoculum to substrate ratio and solids content. *J. Mater. Cycles Waste Manag.* **17,** 808–815, 2015.
4. Narihiro, T. & Sekiguchi, Y.; Microbial communities in anaerobic digestion processes for waste and wastewater treatment: a microbiological update. *Curr. Opin. Biotechnol.* **18,** 273–278, 2007.
5. Khalid, A., Arshad, M., Anjum, M., Mahmood, T. & Dawson, L; The anaerobic digestion of solid organic waste. *Waste Manag.* **31,** 1737–1744, 2011.
6. De Meester, S. *et al.*; The environmental sustainability of anaerobic digestion as a biomass valorization technology. *Bioresour. Technol.* **121,** 396–403, 2012.
7. Rasi, S., J. & Rintala, J.; Trace compounds affecting biogas energy utilisation - A review. *Energy Convers. Manag.* **52,** 3369–3375, 2011.
8. Baciocchi, R. *et al.*; Innovative process for biogas upgrading: results from pilot plant operation. *Biomass Bioenergy* 128 – 137, 2012. doi:10.1016/j.biombioe. 2012.11.016
9. Bond, T. & Templeton, M. R.; History and future of domestic biogas plants in the developing world. *Energy Sustain. Dev.* **15,** 347–354, 2011.
10. Brochier-Armanet, C., Forterre, P. & Gribaldo, S.; Phylogeny and evolution of the Archaea: One hundred genomes later. *Curr. Opin. Microbiol.* **14,** 274–281,2011.
11. Priebe, G. P. S., Kipper, E., Gusmão, A. L., Marcilio, N. R. & Gutterres, M. ;Anaerobic digestion of chrome-tanned leather waste for biogas production. *J. Clean. Prod.* **129,** 410–416, 2016.
12. Schacker, P., Priebe, G., Corção, G. & Gutterres, M.; Isolation, Characterization and Effects of Inhibitors on Bacteria Producing Biogas from Tannery Sludge. *Congr. Int. union leather Technol. Chem. Soc. Istanbul* **32,** 1–12, 2013.
13. Adibee, N., Osanloo, M. & Rahmanpour, M.; Adverse effects of coal mine waste dumps on the environment and their management. *Environ. Earth Sci.* **70,** 1581–1592 (2013).
14. Martins, A. M. P., Heijnen, J. J. & Van Loosdrecht, M. C. M.; Effect of feeding pattern and storage on the sludge settleability under aerobic conditions. *Water Res.* **37,** 2555–2570, 2003.
15. Aarab, T., Smeyers, M., Remy, M., Godden, B. & Delhaye, J. P.;The storage of sewage sludge: Influence of liming on the evolution of copper chemical fractions. *Waste Manag.* **26,** 1024–1032, 2006.
16. Tarasov, A. L., Borzenkov, I. A., Chernykh, N. A. & Belyayev, S. S.; Isolation and investigation of anaerobic microorganisms involved in methanol transformation in an underground gas storage facility. *Microbiology* **80,** 172–179, 2011.
17. Stieglmeier, M., Wirth, R., Kminek, G. & Moissl-Eichinger, C.; Cultivation of anaerobic and facultatively anaerobic bacteria from spacecraft-associated clean rooms. *Appl. Environ. Microbiol.* **75,** 3484–3491, 2009.
18. Sarmiento, F., Mrázek, J. & Whitman, W. B.; Genome-scale analysis of gene function in the hydrogenotrophic methanogenic archaeon Methanococcus maripaludis. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 4726–31, 2013.
19. Vinneras, B., Schonning, C. & Nordin, A. ;Identification of the microbiological community in biogas systems and evaluation of microbial risks from gas usage. *Sci. Total Environ.* **367,** 606–615, 2006.