Isolation, Characterization and Effects of Inhibitors on Bacteria Producing Biogas from Tannery Sludge

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Abstract

The leather market trades millions of units of hides per year, producing a ton of residual sludge and leather waste at the end of the production chain. Such waste is not given a specific, useful destination, so it is eventually deposited in Hazardous Industrial Waste Landfills where it is degraded by micro-organisms, releasing gases that cause the greenhouse effect. When organic composites are oxidized by aerobic micro-organisms, carbon dioxide and water vapor are generated, and afterwards, without oxygen, which was previously consumed by aerobic organisms, methane is formed from the degradation of organic material by anaerobic organisms.
As methane typically has higher calorific power as well as energy generation potential, it is very important to study the biodegradation of different substrates through anaerobic digestion. Thus, this study aims to isolate and characterize micro-organisms present in the tannery sludge with potential for biogas generation by biodegradation of waste leather. They were isolated with culture media in Petri dishes and characterized by the Gram staining technique. In addition, a review of the literature describes the effect of specific inhibitors that can cause the reduction of specific methanogenic activity of these bacteria; such activity is defined as the maximum methane production by a consortium of anaerobic micro-organisms. The fraction of methane generated was obtained from gas chromatography.

Keywords: micro-organisms, biogas, isolation, characterization.

1. Introduction

Industrial growth, coupled with increasing global energy demand, is a cause of concern to experts over waste disposal and power generation. Because greenhouse gas emissions need to be reduced, studies are conducted to generate cleaner energy by using industrial waste.
Waste and sludge generated by the leather industry have little choice of destination, the main one being Hazardous Industrial Waste Landfills (HIWLs). In these landfills, a huge amount of gases is released from the biodegradation of discarded organic compounds. After tanned solid

waste is isolated in landfill cells, the organic components begin to be degraded by biochemical reactions using micro-organisms present in the media (Priebe, 2010). In the presence of atmospheric air, when the trenches or cells are sealed, organic compounds are aerobically oxidized by combustion-like reactions, but the major biotransformation which occurs in such landfills is anaerobic digestion (Priebe *et al.* 2011).
Anaerobic digestion occurs in the absence of oxygen through the performance of various microbial communities with distinct physiological characteristics that work syntrophically in successive degradation of organic matter to methane, carbon dioxide, water, hydrogen sulfide and ammonia, as well as new cells (Chernicharo, 1997). Biogas, formed by the gases aforementioned, is comprised of approximately 50% methane, 45% carbon dioxide, 5% nitrogen, less than 1% of hydrogen sulphide and 2700 ppmv volatile organic compounds (Themelis and Ulloa, 2007).

1.1. Biogas Generation

There are several studies on biogas production using various raw materials, including cattle manure and straw, for example. In a study by Poeschl (2011), manure produced a small amount of biogas (469 MJ/t) with 55% methane, while straw produced a larger amount of biogas (5367 MJ/t) with 51% methane. The amount of methane formed is important because this component has the highest calorific value for energy among the gases that make up biogas, with a generation capacity of 21-24 MJ/m³ (Dimpl, 2010) or about 6 kWh/m³ (Bond, 2011). Each raw material used produces a different property in the biogas generated. This generation occurs through two subsequent mechanisms:

$$Acetogenesis: C_6H_{12}O_6 \rightarrow 2C_2H_5OH + CO_2$$
$$Methanogenesis: CH_3COOH \rightarrow CH_4 + CO$$
$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

The difference in the amount of gases formed may be due to the ability of methanogenic micro-organisms present in the environment to form biogas.

1.2. Microbiology in biogas production

Studies conducted by various researchers show that biogas is produced anaerobically, i.e. in the absence of oxygen. These bacteria are known as anaerobic bacteria. There are bacteria which produce methane from acetate; therefore, they are called acetoclastic or acetotrofic. And there are those that produce methane from hydrogen and carbon dioxide; for this reason, they are called hydrogenotrophic methanogenic (Amaral, 2004).
Nearly all methanogenic species are able to produce methane from hydrogen and carbon dioxide. The genera of hydrogenotrophic bacteria more frequently isolated in anaerobic reactors are *Methanobacterium*, *Methanospirillum* and *Methanovibacter* (Chernicharo, 1997). Three types of acetoclastic methanogenic bacteria have been described: *Methanosarcina sp*. uses acetate, H_2, methanol and methylamine; *Methanosaeta soehngenii* uses acetate; and species belonging to the genus *Methanobacterium* use formate (Quaresma, 1992). Although there are few methanogenic species, these are prevalent in anaerobic digestion. They account

for 60% to 70% of methane production from acetate. The rest comes from H_2 and CO_2 (Chernicharo, 1997). Table 1 shows previously identified methanogenic bacteria from municipal solid waste and their respective morphology.

Table 1: Morphological aspects of methanogenic bacteria.

Genus	Morphology
Methanobacterium sp.	Rod-shaped
Methanobrevibacter sp.	Rod-shaped
Methanomicrobium sp.	Rod-shaped
Methanogenium sp.	Flagellum
Methanospirillum sp.	Irregular Cocci
Methanoplanus sp.	Filamentous
Methanothermus sp.	Plate-shaped
Methanococcus sp.	Rod-shaped
Methanosarcina sp.	Irregular Cocci
Methanosaeta sp.	Cocci cluster
Methanolobus sp.	Rod-shaped/filamentous, Cocci

Source: Foresti (s.d) apud Catelli (1996).

One of the important applications of microbiology is the production of enzymes. Enzymes are organic substances, usually proteins, known as biocatalysts of multiple chemical reactions. They are traded in industries of detergents, food, pharmaceutical drugs, diagnostics, fine chemicals, biodegradation of waste, among others. However, production of enzymes is only possible when reproducibility of results occurs in the isolation of microorganisms.
This study aims to isolate and characterize microorganisms responsible for producing biogas from the biodegradation of tannery sludge and waste from fur and leather.

2. Materials e Methods

To isolate and characterize the microorganisms responsible for the digestion of waste, four benchtop bioreactors were mounted (Figure 1) with sampling points of the gases generated inside them, which were also used for collecting the internal solution with a disposable syringe (3 ml) for cultivation of the microorganisms responsible for biodigestion on the plates.

Figure 1: Bioreactor system immersed in a thermostatic bath.

The experiments were conducted with two different carbon sources. Two bioreactors were filled with hydrolyzed collagen and two others with wet-blue leather shavings. They were all inoculated with aerobic biological sludge from a tannery effluent treatment plant (Priebe et al, 2012) and a nutrient solution composed of yeast extract, peptone, potassium phosphate monobasic and dibasic potassium phosphate (Dettmer *et al.* 2012) (Table 2) was added to obtain an optimum condition for growth and metabolism of the microorganisms. The bioreactors were kept in a thermostatic bath, with the temperature controlled to 35°C. Analyses of the biogas generated in the bioreactors were made thirty days after the experiment.

Table 2: Components found in each bioreactor.

Components	***Hydrolyzed Collagen***	***Wet-blue Shaving***
	Reactor	
	1 2	**3 4**
Substrate (g)	1.7	1.7
Biological Sludge (mL)	25	25
Yeast Extract (g/L)	2	2
Peptone (g/L)	1	1
K_2HPO_4 (g/L)	7	7
KH_2PO_4 (g/L)	3	3

To dilute the aforementioned internal solution, six test tubes with saline solution 0.9% were used. After cultivation and dilution, disposable Petri dishes and Drigalsky spatula were used

for spreading the solution and promote the growth of micro-organisms. Both a Kolle handle and a platinum loop end were used for isolation. Nitrogen gas was used to reduce the amount of oxygen in the surface of the plates. After growth and cultivation of the microorganisms on the plates, the colonies were isolated also in Petri dishes and characterized using microscope slides for microscopic analysis. The mole fractions of methane, carbon dioxide, oxygen and nitrogen were evaluated as the solution was spread on the plates.

2.1. Determination of Gas Composition by Chromatography

To investigate the production of biogas, the composition of the biogas generated in each bioreactor was determined by gas chromatography (GC) using a thermal conductivity detector (TCD). By using a Porapak Q packed column (80/100 mesh), it was possible to separate the fractions of air (N2 and O2), CH4 and CO2. Helium was used as a carrier gas with a flow rate of 30 mL min-1, and the temperatures of the injector, oven and detector were maintained at 100 ºC, 60 ºC and 100 ºC, respectively (Priebe *et al*., 2012).
The ratio O_2/N_2 present in the air fraction was determined using a molecular sieve packed column (Molecular Sieve 13X). Again, helium was used as a carrier gas at a flow rate of 20 mL min^{-1}. The temperatures of the injector and the oven were 80 ºC and 100 ºC, respectively (Priebe *et al.*, 2012).
Analyses were performed sequentially, and in both cases, analysis time was 5 minutes. The equipment used was a Perkin-Elmer Autosystem XL/GC with a command interface through software *Turbochrom* 6.0 developed by DEQUI/UFRGS. The chromatographic conditions were developed based on standards ASTM D 1945, ASTM D 1946, USEPA Methods 3A and 3C (Priebe *et al*. 2012).

2.2. Isolation

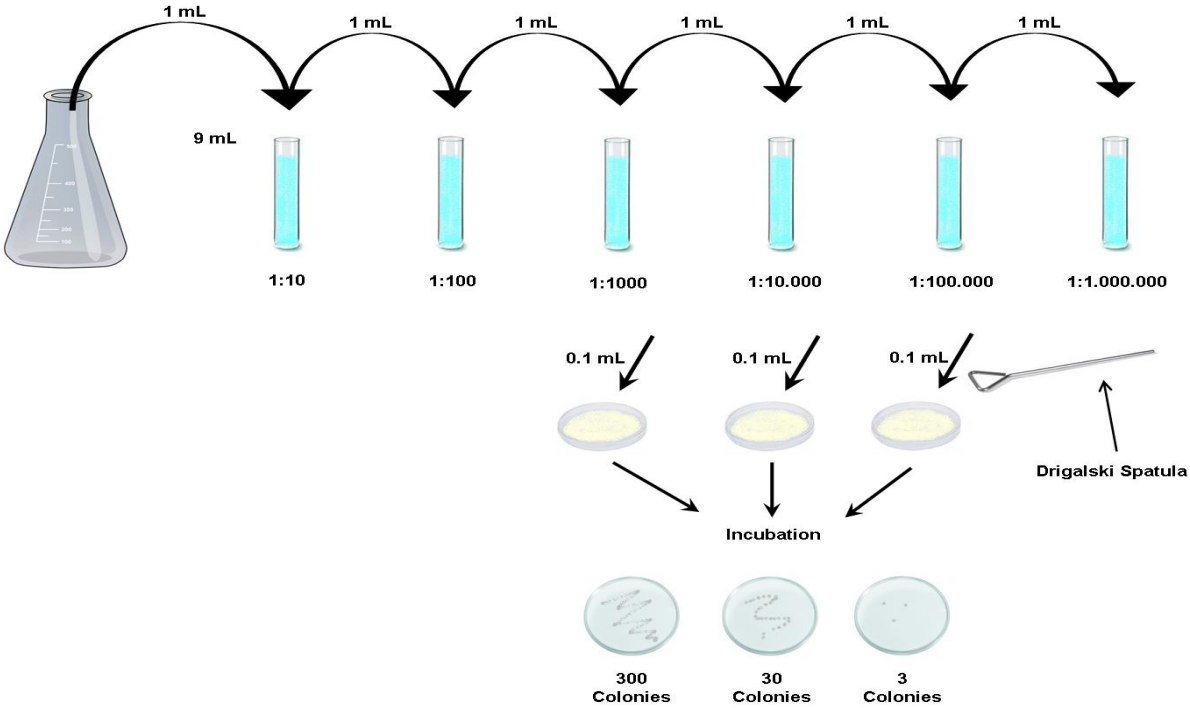
The plates were prepared with peptone, NaCl, yeast extract, agar, hydrolyzed collagen and skim milk in compositions as shown in Table 3.

Table 3: Composition of culture plates.

Components	***Concentrations (g/L)***
Agar	15
NaCl	10
Yeast Extract	7
Peptone	10
Collagen	4
Skim Milk	10

To foster growth of the microorganisms on the plates, the solutions were collected directly from the bioreactor and spread onto two plates, one contained nitrogen and one was closed under atmospheric air. Thus, cultivation was also done with dilute solutions of bioreactors at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}, as shown in Figure 2. The solutions were spread on the plates with a Drigalsky spatula and afterwards they were incubated in a kiln at 40°C for 24

hours. The higher the concentration of the solution, the greater the number of colonies growing.



Source: Dettmer *et al.*, 2013.

Figure 2: Methods for isolating microorganisms from biodigestion.

2.3. Characterization

After isolation of each strain generated on the plates from the solutions of bioreactors, smears were made on microscope slides to characterize the microorganisms by means of the Gram staining technique, as shown in Figure 3.

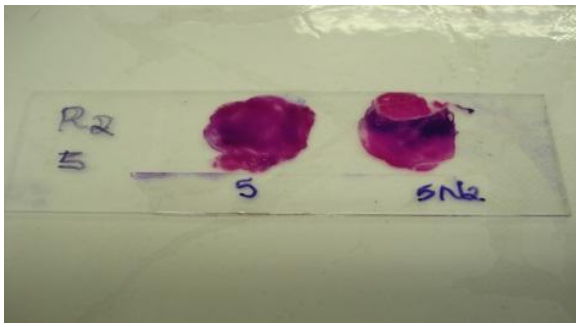


Figure 3: Gram staining technique

This technique consists in dividing bacteria into two groups: Gram-positive and Gram-negative bacteria according to their reaction to Gram staining. After Gram staining, the Gram-positive bacteria are stained in purple, while Gram-negative bacteria, in pink. Such a

difference in reaction to Gram staining is due to differences in the cell wall structure of Gram positive and Gram-negative cells, i.e., differences in the complexity of their structure.

3. Results and Discussion

The data obtained by gas chromatography prior to isolation on the plates showed that biogas was being produced in an acceptable amount for plating, as shown in Tables 4 and 5. The production of biogas in the reactors was analyzed for the duplicates where there was a greater amount of gas, and the differences among microorganisms were checked based on the substrate used.

Table 4: Biogas composition in reactor with hydrolyzed collagen.

Reactor with hydrolyzed collagen	
Component	**Percentage**
O_2	0.00%
N_2	0.00%
CH_4	93.18%
CO_2	6.82%

Table 5: Biogas composition in reactor with wet-blue leather shaving.

Reactor with wet-blue leather shaving	
Component	**Percentage**
O_2	0.31%
N_2	20.70%
CH_4	72.30%
CO_2	6.69%

No growth of strains was observed on the plates that had been inoculated with diluted solutions; therefore, this dilution did not favor the growth of the microorganisms. Plating was then conducted with the solution obtained directly from the bioreactor, without dilution. Growth was observed in a wider range of strains in the solution from the bioreactor where hydrolyzed collagen had been used as a substrate, as shown in Figures 4 and 5.

Figure 4: Growth of microorganisms on the plates.

Figure 5: Isolated colony of microorganisms.

Platings were performed in duplicate in the selected bioreactor. Because the literature reports that methane-producing microorganisms have strictly anaerobic characteristics, nitrogen was placed on one of the duplicate plates before they were closed in order to achieve a low oxygen concentration in the media. There were no differences in the growth of the microorganisms on the plates with nitrogen compared to those with air only. This result may have been due to the fact that the microorganisms perform as a consortium, i.e., some of them consume the oxygen present in the media and, afterwards, gases begin to be produced, favoring the growth of various species under different conditions.

Each strain was also isolated in duplicate and, as carried out with the solutions, nitrogen was placed on the surface of one of the duplicate plates in order to analyze the behavior of each individual colony of microorganisms under such conditions. Again, the morphological analyses with the Gram staining technique showed no significant differences. This result can lead to the conclusion that the micro-organisms in this study may have facultative anaerobic and aerobic characteristics.

The 12 species observed in the plating with the solution of the bioreactor with hydrolyzed collagen and the 8 species observed in the plates isolated with the solution of the bioreactor with wet-blue leather shavings were characterized morphologically, as shown in Tables 6 and 7.

Table 6: Characterization of microorganisms with the Gram staining technique in the reactor with hydrolyzed collagen.

Reactor with hydrolyzed collagen			
Plate	**Morphology**	**Gram Staining**	**Characteristic**
1	Rod-shaped	Positive	Sporulated
1 with N_2	Rod-shaped	Positive	Sporulated
2	Rod-shaped	Positive	Sporulated
2 with N_2	Rod-shaped	Positive	Sporulated
3	Rod-shaped	Positive	NOT Sporulated
3 with N_2	Rod-shaped	Positive	NOT Sporulated
4	Rod-shaped	Positive	Sporulated
4 with N_2	Rod-shaped	Positive	Sporulated
5	Rod-shaped	Negative	Sporulated
5 with N_2	Rod-shaped	Positive	Sporulated
6	Rod-shaped	Positive	Sporulated
6 with N_2	Rod-shaped	Positive	Sporulated
7	Rod-shaped	Positive	Sporulated
7 with N_2	Rod-shaped	Positive	Sporulated
3	Rod-shaped	Positive	Sporulated
8 with N_2	Rod-shaped	Positive	Sporulated
9	Rod-shaped	Positive	ACTINOMYCETES
9 with N_2	Rod-shaped	Positive	ACTINOMYCETES
10	Rod-shaped	Positive	Sporulated
10 with N_2	Rod-shaped	Positive	Sporulated
11	Rod-shaped	Positive	Sporulated
11 with N_2	Rod-shaped	Positive	Sporulated
12	Rod-shaped	Positive	Sporulated
12 with N_2	Rod-shaped	Positive	Sporulated

Table 7: Characterization of micro-organisms with the Gram staining technique in the reactor with wet-blue leather shavings.

Reactor with wet-blue leather shaving.

Plate	Morphology	Gram Staining	Characteristic
1	Rod-shaped	Positive	Sporulated
1 with N_2	Rod-shaped	Positive	Sporulated
2	Rod-shaped	Positive	Sporulated
2 with N_2	Rod-shaped	Positive	Sporulated
3	Rod-shaped	Positive	Sporulated
3 with N_2	Rod-shaped	Positive	Sporulated with endospores
4	Rod-shaped	Positive	Sporulated
4 with N_2	Rod-shaped	Positive	Sporulated
5	Rod-shaped	Positive	Sporulated
5 with N_2	Rod-shaped	Positive	Sporulated
6	Rod-shaped	Positive	Sporulated
6 with N_2	Rod-shaped	Positive	Sporulated
7	Rod-shaped	Positive	Sporulated
7 with N_2	Rod-shaped	Positive	Sporulated
8	Rod-shaped	Positive	Sporulated
8 with N_2	Rod-shaped	Positive	Sporulated

These results show that the bacteria producing biogas from tannery sludge are rod-shaped and have very thick cell walls consisting of several layers of peptidoglycan, which is a polysaccharide. The existence of spores indicates the ability of cells to protect themselves from harsh conditions and survive.

The actinomycetes identified on plate 9 of the reactor with hydrolyzed collagen shows the existence of filamentous bacteria, forming branched filaments, whose behavior may range from anaerobes to facultative aerobes.

Experimentally, it was observed that gas production is directly affected by the presence of oxygen, with no damage to the bacteria, i.e., biogas production is suspended until the residual oxygen is consumed by bacteria and then methanogenic bacteria continue the production of methane and carbon dioxide.

Studies were conducted on the influence of other factors that inhibit methanogenic activity, and they indicate that increased concentrations of sodium, chromium or phenol may increase the inhibition of biological sludge, while sulfates reduce such inhibition (Alves *et al.*, 2005). The formation of biogas can also be affected by the presence of ammonia in the media, which is toxic during methanogenesis (Shanmugam *et al.*, 2009). The susceptibility or inhibition of microorganisms depends on the tanning performed. When there are no strong links with the tanning agent, no degradation occurs and the reactivity of the agent continues to inhibit enzymatic activity, as is the case of vegetable tannins (Covington *et al.*, 2003).

4. Conclusion

It was noted that the microorganisms responsible for producing biogas are stable and easily reproducible; thus, enzymes can be produced from these microorganisms in order to generate gases from isolated colonies. Further research needs to be conducted to investigate the use of microorganisms individually or as a consortium for the production of enzymes.
More characterizations have to be made for more information on the microbial community structure. Analyses are required of gene RNAr 16s, which is found in all living cells. Based on genetic genetic sequencing, such analyses can provide information on the molecular phylogeny of microorganisms.

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