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Effect of enzymatic pretreatment on the anaerobic digestion of milk fat for biogas production

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ABSTRACT

The lipids present in dairy wastes, in addition to representing an important industrial loss, interfere negatively in wastewater systems. Nevertheless, if properly and separately considered, this material may be an interesting substrate for methane production. The objective of the present research was to evaluate the anaerobic degradation of milk fat *in natura* and when separately hydrolyzed by two lipases, one produced by *Geotrichum candidum* (GCL) and the other produced by *Candida rugosa* (CRL). The main purpose was to evaluate whether the enzymes' mechanisms of action would interfere with the anaerobic digestion of fats. The rates of biogas production and specific methane production both indicated CRL as the most advantageous. In addition to offering no benefit, pre-hydrolysis with GCL showed a higher degree of microbial inhibition.

1. Introduction

The food industry is extremely important worldwide, and the future food supply relies on this productivity. However, production is accompanied by a large amount of residues, and the stabilization of which implies costs both for the industry and for the environment, depending on the chosen technology. Thus, the proper selection of residue treatment technique is imperative.

Among the technologies used for residue stabilization, anaerobic digestion stands out because both pollution control and energy recovery can be achieved through such practice (Chen, Cheng, & Creamer, 2008). According to Kapid, Vijay, Rajesh, and Prasad (2004), the biogas obtained from anaerobic reactors is a friendly and environmentally clean source of energy that is also inexpensive and versatile.

The dairy industry is one of the largest sectors in the world and emits flow rates ranging from 3 to 6 L per liter of processed milk. According to Demirel, Yenigun, and Onay (2005), lipids are potentially inhibitory compounds consistently encountered in dairy wastewaters. According to Mata-Alvarez et al. (2014), due to its high methane potential, lipids are very interesting co-substrates for solid-state anaerobic codigestion, nonetheless, its dosing rate must be limited in order to avoid high inhibition.

In addition to representing an important industrial loss, high concentrations of lipids can cause microorganism inhibition, the clogging of pipes, and increases in the hydraulic detention times inside biological reactors, among other negative effects (Mendes, Castro, Pereira, & Furigo, 2005). Alves et al. (2009) asserted that to date, effluents containing high concentrations of lipids have not been effectively treated in anaerobic high-rate reactors, noting that the production of methane from such substrates or intermediates remains a challenge. These authors stressed however that the methanogenic production potential of lipids is higher than that of other complex substrates such as proteins and carbohydrates. Thus, if milk fat could be removed in a first step, and separately digested, a higher methane yield could be achieved. Nevertheless, there is little information available in the literature regarding the anaerobic digestibility of lipids (Demirel, Yenigun and Onay, 2005). Accordingly, milk fat anaerobic degradation needs to be better understood and improved, as both the environment and dairy industry can be benefit from this situation.

Because of its low solubility, the accessibility of lipids is also low, causing lower biological conversion rates and resulting in an increase in its permanence within treatment systems, which ultimately maximizes its negative effects. According to Hamawand (2015), removing lipids from the wastewater could be a good solution, nevertheless this would create another type of waste. Alternatively, enhancing the availability or solubility of these materials for digestion by any sustainable method would be preferable due to their high biogas potential.

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Several pre-treatments have been studied to improve lipid availability and degradability. Battimelli, Torrijos, Moletta, and Delgenes (2010) reported that by increasing the initial bio-availability of fatty wastes, without any modification to the long-chain structure, an enhancement of fatty carcass waste biodegradability was obtained. According to Cammarota and Freire (2006), the application of a pre-treatment to hydrolyze and dissolve lipids may also improve the biological degradation of fatty wastes, accelerating the process and improving time efficiency. The use of lipases for co-digestion of sewage sludge and grease trap may be feasible due to the saving in operational costs and the increase in the biogas production (Donoso-Bravo & Fdz-Polanco, 2013).

Indeed, several authors have reported good results using enzymes to hydrolyze fatty wastes or wastewaters. According to Donoso-Bravo and Fdz-Polanco (2013), although grease trap addition to anaerobic digestion of sewage sludge showed a negative effect on the waste biodegradability, the inhibition was completely overcome by the addition of lipase. Furthermore, enzyme addition remarkably improved the methane production for all grease trap fractions studied (2, 5 and 10%w). Mendes, Pereira, and Castro (2006) used pancreatin for the pre-hydrolysis of dairy effluents and analyzed their degradation in biomethane potential assays. When compared with the use of a non-hydrolyzed effluent, the flasks fed with the pancreatin-treated effluent showed the highest conversion rates and larger volumes of biogas production. Leal, Cammarota, Freire, and Sant'anna (2002) observed high efficiency in an anaerobic treatment fed with effluents pretreated with a highly active enzyme extract produced by *Penicillium restrictum*. With a fat concentration of 1200 mg L^{-1}, removal efficiencies of organic matter of 19 to 80% were obtained with and without prior enzymatic treatment, respectively. Gomes et al. (2011) however found severe inhibition when subjecting an upflow anaerobic sludge blanket reactor to continuous feeding with dairy effluent hydrolyzed with pancreatin. Siguemoto et al. (2009) studied an anaerobic sequencing batch reactor fed with dairy effluent hydrolyzed with an enzyme from *Candida rugosa* and also observed signs of severe inhibition.

Among the products of hydrolysis, long-chain fatty acids (LCFAs) are formed in addition to glycerol (easily converted), and LCFAs are toxic to important anaerobes. As another problem encountered, Hwu, Dolon, and Lettiga (1996) cited the difficulty in transporting nutrients into cells, which occurred in function of the adsorption of LCFAs. Pitk, Palatsi, Kaparaju, Fernández, and Vilu (2014) observed that lipid addition at over 2% of feed mixture (lipid rich solid slaughterhouse wastes and dairy manure) resulted in formation of floating granules and process efficiency decrease. In addition, the formed floating granules had low biodegradability and its organic part was composed of lipids and calcium salts of LCFAs.

According to Chen, Ortiz, Steele, and Stuckey (2014), LCFA inhibition of methanogenesis could cause the failure of the LCFA fermentation and, consequently, of the whole anaerobic digestion bioprocess. The limiting step was suggested to be closely related to the initial concentration of LCFAs. Nonetheless, according to Mendes, Castro, Pereira and Furigo (2005), once inside the cells, LCFA can be incorporated into lipid complexes such as the plasma membrane or converted into intermediates of anaerobic processes.

Lipases (glycerol ester hydrolases, EC 3.1.1.3) catalyze the hydrolysis of ester linkages in lipids, and these enzymes can differ considerably in their positional specificity in the hydrolysis of triacylglycerols (Schmid & Verger, 1998). Therefore, the present study evaluated the anaerobic degradation of fat milk by promoting two types of enzymatic hydrolysis using enzymes with different mechanisms of action, one that is ester specific and another that is ester unspecific. The main purpose was to verify whether the type of pre-hydrolysis process affects the anaerobic process and if biogas production can be related to the mechanism of action of the enzyme used. A comparison between the digestion of an untreated fatty substrate and pre-hydrolyzed substrates was also performed. In addition, the inhibitory effect of the hydrolyzed substrates was evaluated through specific methanogenic activity (SMA) assays.

2. Material and methods

2.1. Materials

Butter (MF) was used as a model substrate in this study. The enzymes used were two lipases, one that is ester unspecific and produced by *C. rugosa* and another that is ester specific and produced by *Geotrichum candidum*. The lipase produced by *C. rugosa* (CRL) was supplied by Sigma Aldrich, lot BCBC4593V, with a nominal activity of 5.95 U mg^{-1}. The lipase produced by *G. candidum* (GCL) was obtained through submerged fermentation according to Silva (2012) in Erlenmeyer flasks at 30 °C with agitation of 180 r.p.m. for 48 h. Enzyme precipitation was performed according to Secades and Guijarro (1999). The solid phase was resuspended in 0.05 M phosphate buffer, pH 7.0, and dialyzed against the same buffer at 4 °C. The dialyzed suspension was frozen at −25 °C and lyophilized. The powder resulting from the process presented a nominal enzymatic activity of 2.3 U mg^{-1}.

2.2. Pre-hydrolysis reaction

The reaction system was composed of 0.7808 g of MF and 16 mL of lipase suspension. The mass of MF was chosen according to the methodology used for enzymatic activity determination (described in item 2.3), in which the substrate (oleic acid) is present in an excess amount (intended situation for the hydrolysis reactions). Both LCR and LGC were suspended in phosphate buffer to obtain a final activity of 20 U mL^{-1}. Prior to its use, the suspension of LGC lipase was centrifuged at 8500 ×*g* for 6 min. The optimum pH of the enzymatic reactions was determined using buffer solutions prepared as follows: 0.1 M acetate buffer (at pH 3.5; 4.0; 4.5; 5.0; 5.5), 0.1 M phosphate buffer (at pH 6.0; 6.5; 7.0; 7.5) and 0.1 M borate buffer (at pH 8.0; 8.5; 9.0). The effect of temperature on enzymatic activity was determined using incubation temperatures ranging from 35 to 45 °C with the aid of a thermostatic agitated water bath. This temperature range was based on preliminary results achieved for the enzyme preparation of *G. candidum* (Silva, 2012) and on the results obtained by and Mendes, Pereira and Castro (2006) when using *C. rugosa* lipase. After the determination of the optimum pH and temperature values, the hydrolysis reactions were monitored for 24 h to determine the reaction time; the reaction progress was assessed through the determination of free fatty acids. The final optimum conditions were a pH of 6.6 and a temperature of 40 °C for LCR and a pH of 7.0 and a temperature of 40 °C for LGC. The hydrolysis reactions lasted for 16 and 8 h for LCR and LGC, respectively.

2.3. Analytical methods

The determination of lipase activity was based on the procedure described by Macedo, Pastore, and Park (1997), as reviewed in Kamimura, Mendieta, Sato, Pastore, and Maugeri (1999). An emulsion composed of 25% olive oil and 75% Arabic gum (7% p/v) was used as the substrate. The reaction was conducted in 125-mL Erlenmeyer flasks with 5 mL of emulsion, 2 mL of 0.1 M sodium phosphate buffer at pH 7, and 1 mL enzymatic suspension. The Erlenmeyer flasks were incubated at 45 °C in a Dubnoff bath with agitation for 30 min. The reaction was quenched with 10 mL of a solution of acetone and ethanol (1:1). The fatty acids released during the reaction were determined. The activity is expressed in lipase units (U), which correspond to 1 μmol of fatty acid released per minute under the specified conditions.

The determination of the concentration of free fatty acids was performed through titration with 0.05 M NaOH solution in the presence of phenolphthalein as an indicator. The volume of NaOH was converted to micromoles of oleic acid using a standard curve (Eq. (1)) constructed

by the titration of emulsions of oleic acid with concentrations ranging from 50 to 900 μmol:

$$\text{Oleic acid } (\mu\text{mol}) = 50.299 \cdot \text{vol NaOH } (\text{mL}) + 30.518. \quad (1)$$

The concentration of organic matter is expressed as the chemical oxygen demand (COD), and the total Kjeldahl nitrogen (TKN-N) concentration and solids were measured according to the methods described in Standard Methods for Examination of Water and Wastewater (APHA, AWWA & WEF, 1995).

2.4. Biomethane potential (BMP) assays

The inoculum consisted of cow manure and sludge from a 1-m^3 pilot anaerobic sequencing batch reactor fed with dairy effluents, with an average organic matter concentration of 5454 ± 1935 mg L^{-1} and a lipid concentration of 240 ± 37 mg L^{-1}. The inoculum was analyzed using common light and fluorescence microscopy. The exams evidenced an abundance of *Methanosaeta* sp.-like morphologies, cocci and non-fluorescent bacilli. The average solid concentration was 8.5 g L^{-1}, and the specific methanogenic activity (SMA) was 5.1 mL $CH_{4@STP}$ g^{-1}TVS h^{-1}, where TVS is total volatile solids in the analyzed sludge.

BMP assays were conducted in triplicate, as described in Cavaleiro, Ferreira, Pereira, Tommaso, and Alves (2013), in 100-mL flasks to which 20 mL anaerobic sludge, 20 mL of cow manure suspension (20 g L^{-1}) and 5 mL of fat suspension were added (48 g L^{-1}). Nutrients and trace metals were supplied according to Zhender, Huser, Brock, and Wuhrmann (1980). Flasks fed with fat hydrolysed using GCL (MF*Cr*), fat hydrolyzed using CRL (MF*Cr*) and non hydrolyzed fat (MF) were incubated at 35 °C. Table 1 presents the details of the assay. After the contents of the flasks were assembled, they were exposed to N_2 flow to assure anaerobic conditions. The bottles were sealed with butyl rubber septa and aluminum screw caps. Biogas production was analyzed by the transduction of pressure, and the concentration of methane was determined by gas chromatography. Methane production from the inoculum was analyzed in flasks containing only sludge and manure. Methane production at STP conditions (P_{CH_4}) was calculated by subtracting the production measured in blank flasks. To compare the BMP values obtained with the theoretical potential of the substrates, the methane recovery coefficient (CH_4%) was calculated for the different conditions tested according to Eq. (2):

$$CH_4\,\% = \frac{\text{BMP} \times 100}{\text{Theoretical } CH_4 \text{ potential}} \quad (2)$$

The theoretical methane potential was calculated according to Speece (1996), who stated that 0.350 L of $CH_{4@STP}$ is generated per gram of COD. Specific methane production was calculated according to Eq. (3), where COD_{add} is the mass of the total volatile solids from the inoculum:

$$SP_{CH_4} = \frac{P_{CH_4}}{COD_{add}} \quad (3)$$

The initial rates of biogas production were calculated by linear regression of the biogas production curve from 0 to 50 h.

2.5. Biogas analysis

A volume of 0.5 mL of biogas sample was injected into a gas chromatograph equipped with a thermal conductivity detector (DCT) and a Carboxen 1010 PLOT column, 30 m × 0.53 mm. The injector temperature was 220 °C. Helium was used as the carrier gas with a flow rate equal to 5.66 mL min^{-1}. The detector temperature was 230 °C. The current was 35 mA, with negative polarity. The oven temperature was 130 to 135 °C, with a 46 °C min^{-1} ramp.

2.6. Toxicity determination

This test was conducted using the methodology adapted from Hwu and Lettinga (1997) in which flasks fed with hydrolyzed fat were used for verifying the extent of possible inhibition on acetoclastic methanogens caused by the exposure to long-chain fatty acids. After verification at the end of biogas production, flasks MF*Gc* and MF*Cr* received 1 mL of 0.8 M sodium acetate solution, which corresponded to the same amount of carbon source previously used for the SMA assay performed for the inoculum characterization. Biogas production was analyzed by pressure transduction, and the concentration of methane was analyzed by gas chromatography. The SMA was estimated and compared to the value obtained for the inoculum.

3. Results and discussion

After hydrolysis, total fatty acid concentrations of 37 and 26 μmol mL^{-1} were registered using GCL and CRL, respectively. Thus, the addition of total fatty acids was 187 μM and 133 μM for MF*Gc* and MF*Cr*, respectively. It is important to observe that in the control flasks, 44 μM of LCFAs were added from the non-hydrolyzed MF. The mass of COD added to the flasks and the mass of fat are presented in Table 2. These amounts were similar for the control flasks and MF*Cr*; nevertheless, the reductions in COD and fat concentration were verified during the hydrolysis process when GCL was used. This reduction caused a 23.4% decrease in the mass of COD added to MF*Gc* compared to the control flasks.

The COD reduction was most likely caused by the presence of viable cells of *G. candidum* in the process, as the broth containing the enzymes was lyophilized but not sterilized after lipase production.

Table 1
Biomethane potential assay details.

Condition	Biomass (mL)	MS (mL)	MFS (mL)	COD_{add}	$O\&G_{add}$	Tap water (mL)	MaS (mL)	MiS (mL)
Blank	20.01	20	0	nd	nd	19.7	0.2	0.1
Control	20.03	20	5	0.416	0.243	14.7	0.2	0.1
MF*Gc*	20.02	20	5	0.264	0.186	14.7	0.2	0.1
MF*Cr*	20.14	20	5	0.403	0.235	14.7	0.2	0.1

Control — flask with non-hydrolyzed fat.
MF*Gc* — flask with fat hydrolyzed using *G. candidum* lipase.
MF*Cr* — flask with fat hydrolyzed using *C. rugosa* lipase.
MS — manure suspension.
MFS — milk fat suspension.
COD_{add} — COD added.
$O\&G_{add}$ — Oil and grease added.
MaS — macronutrient solution.
MiS — micronutrient solution.
nd — not determined.

Table 2
Methane production (P_{CH_4}), total LCFA added, methane recovery (CH_4%) and specific methane production (SP_{CH_4}) observed in BMP assays.

Condition	P_{CH_4} (mL $CH_{4@STP}$)	TLCFA added (mM)	CH_4%	SP_{CH_4} (mL $CH_{4@STP}$ $gCOD_{add}^{-1}$)
Control	91.3 ± 1.5	0.73	62.7	219.4
MF*Gc*	56.7 ± 2.1	3.1	61.4	214.9
MF*Cr*	168.4 ± 14.6	2.2	119.3	417.9

Control — flask with non-hydrolyzed fat.
MF*Gc* — flask with fat hydrolyzed using *G. candidum* lipase.
MF*Cr* — flask with fat hydrolyzed using *Cn rugosa* lipase.
TLCFA — total long-chain fatty acids.
COD_{add} — COD added.

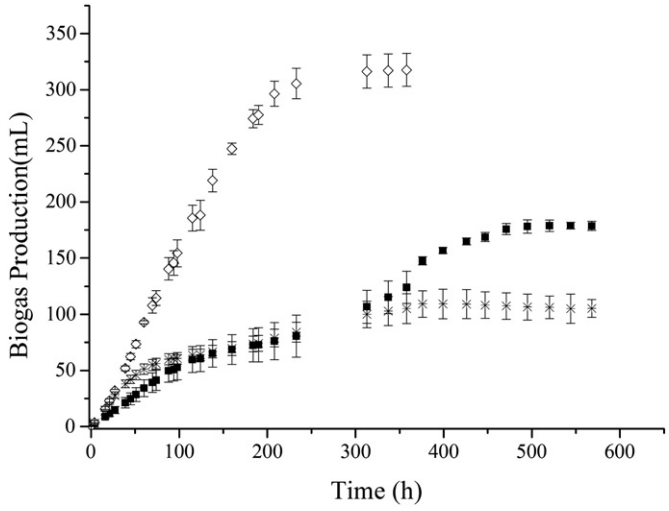

Fig. 1. Biogas production in flasks fed with non-hydrolyzed milk fat (■), milk fat hydrolyzed by *G. candidum* lipase (∗) and milk fat hydrolyzed by *C. rugosa* lipase (◊).

Fig. 1 shows the production of biogas obtained in the assays, and it is possible to observe the biogas production from the fatty substrates (P_{CH_4}). Two plateaus were observed for biogas production in the control flasks, indicating that the mixture had to overcome intermediate conditions that temporarily impaired biogas production. When working with hydrolyzed and non-hydrolyzed dairy effluents, Mendes, Pereira and Castro (2006) did not observe similar behavior, which could possibly be explained by differences in the concentration of the fat present in such assays (150 mg L^{-1}), which was lower than that used in this study.

Smoother plateaus were observed in the biogas production curve obtained from MF*Gc*. Such a result is consistent with the fact that GCL is an ester-specific hydrolase and promotes the partial hydrolysis of triacylglycerols, yielding rather unsaturated LCFAs. Although being fed with hydrolyzed MF, the accumulated volume of biogas produced in MF*Gc* was lower than that observed in the control flasks, indicating the occurrence of an inhibitory process. In fact, Baillargeon, Bistline, and Sonnet (1989) described the GCL produced by NRRL Y-552 (used in this study) as a moderate ester-specific enzyme, as it failed to discriminate between palmitic and oleic acids. Thus, the GCL used in the present study, being a moderate ester-specific enzyme, most likely promoted partial hydrolysis, without releasing glycerol, a rapidly usable substrate, but yielded both oleic and palmitic acid. Oleic acid is an unsaturated LCFA that represents from 26 to 30% of the LCFAs in MF (Solomons and Fryhle (2006); Damodaran, Parkin, and Fennema, 2007). According to Komatsu, Hanaki and Matsuo (1991), unsaturated LCFAs appear to have a greater inhibitory effect than saturated LCFAs and strongly inhibit methane production from acetate. However, according to Pereira, Sousa, Mota, and Alves (2004), palmitic acid is expected to accumulate in LCFA-inhibited anaerobic reactors. These observations provide an explanation for the lower biogas production obtained for MF*Gc*.

Different from the two trials presented above, the biogas production observed for MF*Cr* showed only a single plateau, which occurred at the end of the assay. In addition, the biogas production was the highest among the assays performed.

The highest initial rate of biogas production occurred with MF*Cr*, followed by the value obtained for MF*Gc*. The initial rates of biogas production were 1.3 mL h^{-1}, 0.82 mL h^{-1} and 0.47 mL h^{-1} in the assays fed with CRL-hydrolyzed MF, GCL-hydrolyzed MF and non-hydrolyzed MF, respectively.

As observed by Mendes, Pereira and Castro (2006) and Gomes et al. (2011) in BMPs fed with hydrolyzed dairy effluent, no adaptation phase (lag phase) of biomass was observed for biogas production.

Table 2 shows data on the maximum methanogenic production (P_{CH_4}) measured in the tests, the added mass of total long-chain fatty acids (TLCFAs), the specific methanogenic production (SP_{CH_4}) and the percentage of methane recovery (CH_4%). The P_{CH_4} values of the different fats were significantly different. The methane production from MF*Cr* was 84% higher than that observed in the control flasks. This fact allows the conclusion that pre-hydrolysis with CRL is very suitable when the production of biogas from milk fats is required.

The values of CH_4% and SP_{CH_4} for the control flasks and MF*Gc* did not present any difference, but both were significantly different from the values obtained from MF*Cr*. The CH_4% from the control flasks and MF*Gc* was 62.7 and 61.4%, respectively. The CH_4% calculated for MF*Cr* was 19% higher than the theoretical value, leading to the conclusion that CRL most likely hydrolyzed the fatty cellular material from the sludge.

The SMA values obtained for MF*Gc* and MF*Cr* were 1.51 and 1.73 mL $CH_{4@STP}$ $gTVS^{-1}$ h^{-1}, respectively. Compared to the values measured for the inoculum sludge, decreases of 71 and 66% were recorded for MF*Gc* and MF*Cr*, respectively. Considering the volumes of the reaction systems (60 mL), the mass of substrate provided, and the hydrolysis yields, the total LCFA concentrations of 0.73 mM, 3.1 and 2.2 mM were added to the control, MF*Gc* and MF*Cr*, respectively. Damodaran, Parkin, and Fennema (2007) reported that based on the fatty acid profile of milk fat (butter), 28.2% is related to oleic acid and 18:1Δ9 carbon. Hwu and Lettinga (1997) found 50% inhibition of acetoclastic methanogenesis with oleate concentrations ranging from 2.35 mM to 4.3 mM, values similar to those occurring when MF was hydrolyzed. It is important to note however that there is no biomass recirculation in conventional anaerobic reactors treating manure or sludge. In addition, as the microorganisms are part of a secondary product called biofertilizers, the decrease in SMA would not represent a problem when the goal is methane production. Nonetheless, it is indeed an issue to be considered in continuous reactors, fed with hydrolyzed fatty substrates.

4. Conclusions

The biogas initial velocities as well as the values of specific methanogenic production and the recovery methane coefficient indicated that pre-hydrolysis with the lipase produced by *C. rugosa* is the most suitable for processes aimed at methane production. The use of lipase produced by *G. candidum* (NRRL Y-552) did not offer a benefit when comparing its anaerobic degradation process with the anaerobic process fed with non-hydrolyzed milk fat. After exposure to long-chain fatty acids from the hydrolysis of milk fats, the biomass showed reductions in acetoclastic methanogenic activity.

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