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

R.F. Domingues, T. Sanches, G.S. Silva, B.E. Bueno, R. Ribeiro, E.S. Kamimura, R. Franzolin Neto, G. Tommaso *

Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo, R. Duque de Caxias Norte, 225, Campus da USP, 13635-900 São Paulo, Brazil

A B S T R A C T

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.

© 2015 Elsevier Ltd. All rights reserved.

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.

* Corresponding author at: Laboratório de Biotecnologia Ambiental, Departamento de Engenharia de Alimentos, Faculdade de Zootecnia e Engenharia de Alimentos. Av. Duque de Caxias Norte, 225 Pirassununga 13635-900, Brazil. Tel.: +55 19 35654304; fax: +55 19 35654284.

E-mail address: tommaso@usp.br (G. Tommaso).

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