

Enzymatic pre-hydrolysis applied to the anaerobic treatment of effluents from poultry slaughterhouses

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Abstract

A pool of hydrolases with 21.4 U g⁻¹ lipase activity was produced through solid-state fermentation of the fungus *Penicillium restrictum* in waste from the *Orbignya oleifera* (babassu) oil processing industry. Enzymatic hydrolysis and anaerobic biodegradability tests were conducted on poultry slaughterhouse effluents with varying oil and grease contents (150–1200 mg l⁻¹) and solid enzymatic pool concentrations (0.1–1.0% w/v). Enhanced anaerobic treatment efficiency relative to raw effluent was achieved when a 0.1% concentration of enzymatic pool was used in the pre-hydrolysis stage with 1200 mg oil and grease l⁻¹ (chemical oxygen demand (COD) removal efficiency of 85% vs. 53% and biogas production of 175 ml vs. 37 ml after 4 d).

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Scientific relevance of the paper

Slaughterhouse wastewater contains high levels of fats and proteins that present low biodegradability. A large number of pre-treatment systems are employed to remove oil and grease to prevent a host of problems that may otherwise arise in the biological process, and reduce the efficiency of the treatment station. Problems caused by excessive oil and grease include a reduction in cell-aqueous phase transfer rates, sedimentation hindrance due to the development of filamentous microorganisms, development and flotation of sludge with poor activity, clogging and the emergence of unpleasant odors. Thus, the application of a pre-treatment to hydrolyze and dissolve lipids may improve the biological degradation of fatty wastewaters, accelerating the process and improving time efficiency. However thus far, only a few studies describing the degradation of

fats and oils by alkaline/acid/enzymatic hydrolysis have been reported; the treatment of effluents from several origins is a new and promising application for lipases. Among the organisms that produce the hydrolytic enzymes studied, the fungus *Penicillium restrictum* is a particularly promising one. The use of low-cost enzymatic preparations represents a vital development in the treatment of effluents, since the use of high-cost commercial enzymatic preparations would make the pre-treatment procedure economically infeasible. In this context, it seems that the solid-state fermentation process will be a suitable technology once enzyme production can be performed *in situ* and industrial wastes can be employed as a source of nutrients for the fermentative process. When cultivated in low-cost solid medium composed of agro-industrial waste, *P. restrictum* produces a pool of hydrolases capable of degrading highly complex organic compounds. This degradation enables a considerable increase in organic matter removal efficiency to be realized, which produces a high-quality effluent for subsequent biological treatment. Accordingly, there is

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presently a wide variety of ongoing scientific investigation in the field of developing enzymatic hydrolysis processes to precede traditional biological treatment.

1. Introduction

Slaughterhouse effluents have high concentrations of biodegradable organic matter and are composed mostly of fats and proteins (Salminen and Rintala, 2002; Masse and Massé, 2005). Many studies that have focused on anaerobic treatment of these effluents have reported operational problems due to high oil and grease contents in the effluents. Oil and grease may solidify at lower temperatures and thus cause operational damage associated with clogging and unpleasant odors. The development and flotation of sludges with different physical characteristics or poor activity are known to cause biomass loss through the reactor's outflow, decreasing its concentration inside the reactor and the treatment efficiency. Oil and grease that is adsorbed on the surface of anaerobic sludge may limit the transport of soluble substrates to the biomass and consequently may reduce the substrate conversion rate. Thus, it is clear that the removal or pre-hydrolysis of fat present in these effluents is vital for efficient functioning of the commonly used biological treatment processes (Martínez et al., 1995; Ruiz et al., 1997; Massé and Masse, 2001; Caixeta et al., 2002; Fuchs et al., 2003; Wang and Banks, 2003).

The use of lipases in the treatment of highly fatty effluents is a promising alternative to traditional methods. However, there are few studies in the literature that have examined this application, especially with slaughterhouse effluents. A chief obstacle preventing more studies and more widespread use of enzymes in environmental technology is the high cost of commercial enzymes. Consequently, the employment of hybrid technology (enzymatic treatment combined with biological treatment) using enzymatic pools produced through solid-state fermentation from low-cost industrial wastes may represent an important advancement in the treatment of effluents with high oil and grease contents (Gombert et al., 1999; Palma et al., 2000; Cammarota et al., 2001; Jung et al., 2002; Cammarota and Freire, 2006; Leal et al., 2002, 2006).

Thus, the aim of this study was to evaluate the use of different concentrations of a hydrolases pool obtained through solid-state fermentation as an alternative enzymatic-biological hybrid treatment for poultry slaughterhouse effluents across a range of fat content levels.

2. Materials and methods

2.1. Solid-state fermentation

Enzyme pools were produced through solid-state fermentation of the fungus *P. restrictum* isolated from *Orbignya oleifera* (babassu) oil extraction industrial waste (Freire et al., 1997). The agro-industrial waste generated in the babassu seed oil extraction stage, known as babassu cake, was used as a culture medium (Gombert et al., 1999). This cake was

supplemented with 2.5% (w/w) molasses, a waste product from the sugar industry, and inoculated with a suspension of *P. restrictum* spores (10^8 spores g^{-1} cake). The fermentation was conducted in tray reactors incubated for 20 h in a fermentation chamber with controlled temperature ($35^\circ C$) and humidity (70%). Finally, the fermented babassu cake was dried for 4 h at $45^\circ C$, resulting in a solid enzymatic pool (SEP) powder containing hydrolases and microorganisms. The composition of the babassu cake (in w/w) was 6.6% water, 22.8% proteins, 61.8% overall carbohydrates, 4.5% lipids and 4.3% ash. After solid-state fermentation the SEP contained $21.4 U g^{-1}$ lipase, $2.7 U g^{-1}$ protease and $7.1 mg$ glucosamine g^{-1} (Gombert et al., 1999; Palma et al., 2000; Cammarota and Freire, 2006).

2.2. Collection and characterization of the effluent

The effluent from a poultry slaughterhouse was collected after the flotation stage and kept at $4^\circ C$. Three collections were performed during this study with an average pH 5.7 ± 0.4 , chemical oxygen demand (COD) = $1181 \pm 371 mg l^{-1}$, biochemical oxygen demand = $652 \pm 193 mg l^{-1}$, nitrogen Kjeldahl total = $194 \pm 44 mg l^{-1}$, total phosphorus = $59 \pm 75 mg l^{-1}$, oil and grease (O&G) = $230 \pm 320 mg l^{-1}$, and total solids = $2960 \pm 183 mg l^{-1}$. Only the effluent from the third collection was supplemented with $0.03 g KH_2PO_4 l^{-1}$ in order to correct for low phosphorus levels.

The fat added to the effluent to create different oil and grease concentrations (150, 300, 750 and $1200 mg O\&G l^{-1}$) was collected in the same slaughterhouse after separation in a flotation unit and was characterized in terms of moisture (75.5%) and oil and grease content (38.4% in dry base) and kept at $4^\circ C$. The anaerobic sludge used as inoculum in biodegradability tests was collected in the upflow anaerobic sludge blanket (UASB) reactor of the treatment station and the concentration of volatile suspended solids (VSS) was determined ($17,422 mg l^{-1}$).

2.3. Enzymatic hydrolysis of the effluent

Effluents containing different oil and grease contents were pre-treated with 0.1, 0.5 or 1.0% (w/v) SEP. The pre-hydrolysis stage of effluent treatment was conducted in stirred jacket vessels (0.51, containing 0.41 l of effluent) incubated for 22 h at $35^\circ C$ and 120 rpm after the specified oil and grease concentration was achieved and the pH was brought to 7.0 ± 0.2 . Follow-up analysis of the quantity free acids released was performed (every 2 h during the 22 h pre-hydrolysis stage) for both hydrolyzed and control (without the addition of SEP) effluents. All experiments were conducted in triplicate.

2.4. Anaerobic biodegradability tests

Anaerobic biodegradability tests were conducted with enzymatically pre-treated effluents containing different oil and grease and SEP contents. Control experiments were conducted with effluents without the addition of SEP. All tests were conducted in 100 ml penicillin-type flasks with 90 ml of raw or pre-hydrolyzed effluent mixed with anaerobic sludge and in order to maintain an initial COD:VSS ratio of 1:1. The flasks were sealed with a rubber lid and an aluminum seal and incubated at $35^\circ C$ for 6–7 d, during which the biogas production and composition and the COD removal efficiency were evaluated. In order to evaluate the effects of the accumulated oil and grease on the biomass as well as the adaptation of the biomass to the effluent and SEP constituents, sequential tests with biomass reutilization were conducted in three sequential batches. All experiments were conducted in triplicate.

2.5. Analytical methods

Determination of SEP activity levels was performed according to the methodology described by Gombert et al. (1999). One lipase activity unit

(U_L) was defined as the amount of enzyme that produces 1 μmol of fatty acid equivalents and one protease activity unit (U_p) was defined as the unitary difference in absorbance between the reaction blank and the sample per minute under the assay conditions. Enzymatic hydrolysis efficiency was monitored through the formation of free acids, measured by automatic titration with 40 mM NaOH.

The volume of biogas produced in the anaerobic biodegradability tests was measured through the dislocation of the embolus of calibrated plastic syringes connected to the flasks. The methane concentration in the biogas was determined by gas chromatography. The COD and other parameters employed in effluent characterization were determined according to Standard Methods (Greenberg et al., 1992). All quantitative data are presented as means \pm standard deviation.

3. Results

Table 1 summarizes the production of free acids from oil and grease present in poultry slaughterhouse effluent after 22 h of enzymatic hydrolysis. We observed a progressive decrease in the amount of released acids (ΔT) with increasing oil and grease content among the three lower oil and grease content levels (150–750 mg O&G l⁻¹) within SEP concentration conditions. Meanwhile, we observed increases in the final concentration of free acids with increasing SEP concentration for all oil and grease contents evaluated. Although effluent, SEP and added fat contain some initial free acid which increased with increasing fat concentration, the final free acid contents following

Table 1

Concentration of free acids in the enzymatic hydrolysis of poultry slaughterhouse effluent using 0.1, 0.5 and 1.0% (w/v) of solid enzymatic pool (SEP) with lipase activity of 21.4 U g⁻¹ at the beginning (T_0) and after 22 h of hydrolysis (T_{22}) and production after 22 h ($\Delta T = (T_{22} - T_{22\text{Control}}) - (T_0 - T_{0\text{Control}})$) at 35 °C for different oil and grease contents

mg O&G l ⁻¹	SEP % (w/v)	Free acids ($\mu\text{mol ml}^{-1}$)		
		T_0	T_{22}	ΔT
150	0.1	13.50	14.24	1.0
	0.5	14.53	17.62	3.4
	1.0	17.32	21.68	4.6
Control	0.0	13.07	12.80	—
300	0.1	14.40	15.23	0.6
	0.5	16.10	18.44	2.1
	1.0	18.45	22.31	3.6
Control	0.0	13.83	14.10	0.3
750	0.1	15.17	16.17	0.1
	0.5	17.36	20.16	1.9
	1.0	19.69	23.61	3.0
Control	0.0	14.85	15.75	0.9
1200	0.1	20.51	19.88	3.2
	0.5	21.45	21.82	4.2
	1.0	21.24	24.82	7.3
Control	0.0	18.81	15.02	—

The quantity free acids measured in control experiments (conducted with effluents without the addition of SEP) was discounted in the calculation of ΔT .

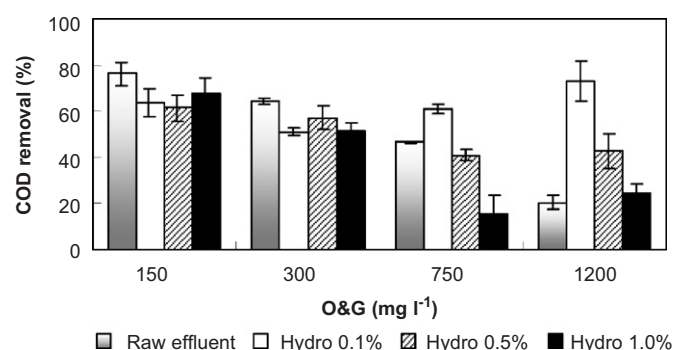


Fig. 1. Total COD removal (%) in the first batch (after 6–7 d) with raw and pre-hydrolyzed effluents containing different oil and grease (O&G) concentrations. Raw effluent (effluent without pre-hydrolysis); hydro 0.1% (effluent pre-hydrolyzed with 0.1% SEP); hydro 0.5% (effluent pre-hydrolyzed with 0.5% SEP) and hydro 1.0% (effluent pre-hydrolyzed with 1.0% SEP).

hydrolysis were higher in the experiments with SEP than in the control experiments (without SEP).

Fig. 1 summarizes the total COD removal in the first batch obtained after 6–7 d of treatment for all tested oil and grease and SEP concentrations in the effluent. The COD removal efficiencies did not differ substantially between SEP concentrations (61–76%) at the lowest oil and grease concentration (150 mg l⁻¹). However, when the oil and grease concentration was increased in the control experiments (without pre-hydrolysis), there was a reduction in the total COD removal efficiency. Indeed COD removal efficiency fell to as low as 21% in control tests with 1200 mg O&G l⁻¹. In contrast, in effluents previously hydrolyzed with 0.1% SEP, the same increase in oil and grease concentration did not affect the COD removal efficiency, which remained between 64% (for 150 mg O&G l⁻¹) and 73% (for 1200 mg O&G l⁻¹). However, at the highest oil and grease concentrations evaluated (750 and 1200 mg O&G l⁻¹), higher SEP concentration levels were associated with a decline in COD removal efficiency (40% with 0.5% SEP and 20% with 1.0% SEP).

Fig. 2 illustrates the quantification of accumulated biogas production over time. In the presence of 150 or 300 mg O&G l⁻¹ (Fig. 2a and b), the biogas produced from raw effluent initially exhibited a 1-d adaptation stage (lag phase) and then proceeded to stabilize by the fifth day. The biogas production kinetics profile of effluent hydrolyzed with 0.1% SEP approximated that obtained with the raw effluent. However, greater levels of biogas production were consistently observed in effluent samples hydrolyzed with higher SEP contents. A longer lag phase (3 d) was observed in the experiments evaluating raw effluent containing 750 or 1200 mg O&G l⁻¹ (Fig. 2c and d). Meanwhile in previously hydrolyzed effluents, the commencement of biogas production could be observed after a lag phase of only 1 d.

Fig. 3 summarizes the maximum methane production rates determined for the first batch of each effluent sample containing the specified oil and grease concentration. These

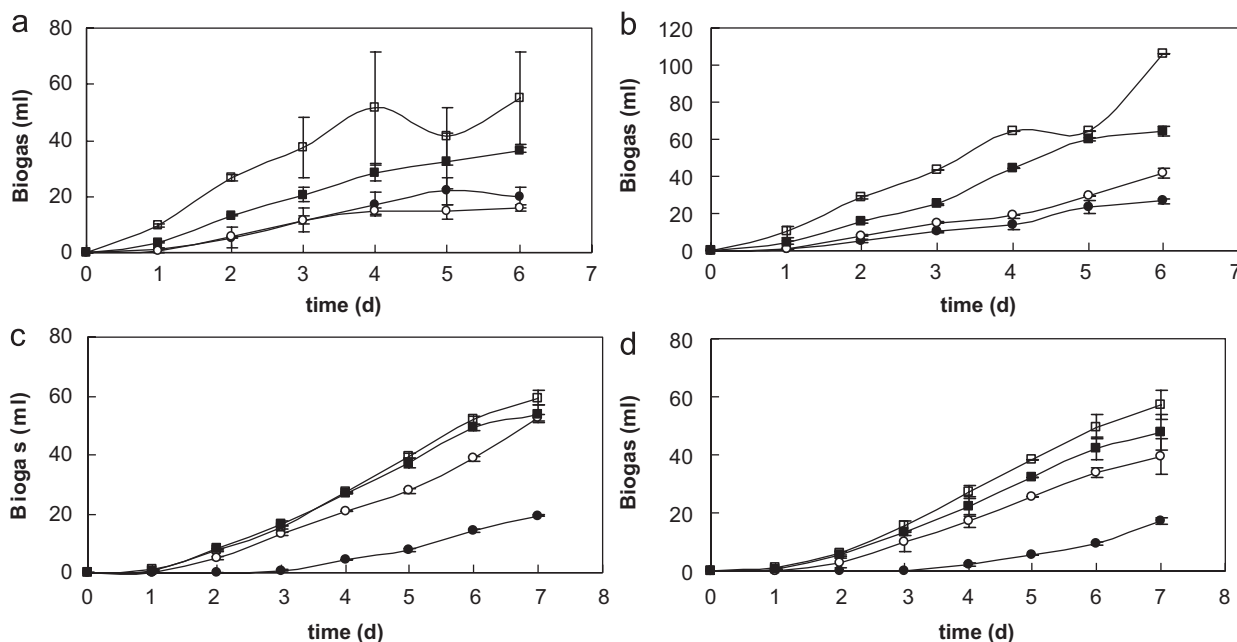


Fig. 2. Biogas production from raw and pre-hydrolyzed effluents in the first batch containing 150 mg O&G l⁻¹ (a), 300 mg O&G l⁻¹ (b), 750 mg O&G l⁻¹ (c) and 1200 mg O&G l⁻¹ (d). Raw effluent (●: raw effluent without pre-hydrolysis); hydro 0.1% (○: effluent pre-hydrolyzed with 0.1% SEP); hydro 0.5% (■: effluent pre-hydrolyzed with 0.5% SEP) and hydro 1.0% (□: effluent pre-hydrolyzed with 1.0% SEP).

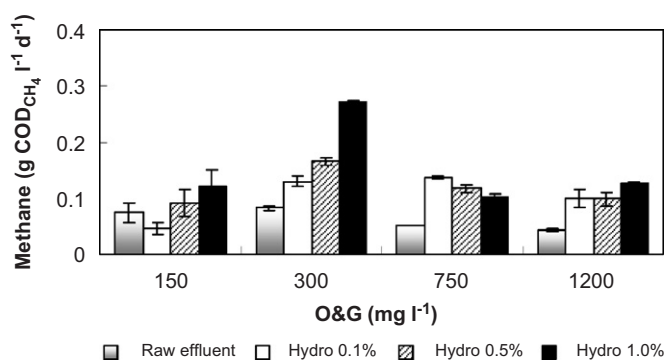


Fig. 3. Maximum methane production rates of raw and pre-hydrolyzed effluents in the presence of different oil and grease concentrations. Raw effluent (raw effluent without pre-hydrolysis); hydro 0.1% (effluent pre-hydrolyzed with 0.1% SEP); hydro 0.5% (effluent pre-hydrolyzed with 0.5% SEP) and hydro 1.0% (effluent pre-hydrolyzed with 1.0% SEP).

rates were calculated based on the methane concentrations obtained at the end of each experiment and are expressed in terms of converted COD. Previously hydrolyzed effluents generally had higher methane production rates than did raw effluent with the exception of those with the lowest oil and grease and SEP concentrations. In the raw effluent experiments, inhibition of methane production was observed when the oil and grease concentration exceeded 300 mg l⁻¹, while in experiments with 0.1% SEP, methane production was inhibited only when the oil and grease concentration exceeded 750 mg l⁻¹.

Fig. 4 illustrates the COD removal efficiencies (a) and the maximum methane production rates (b) after 6–7 d for three consecutive batches with effluent containing

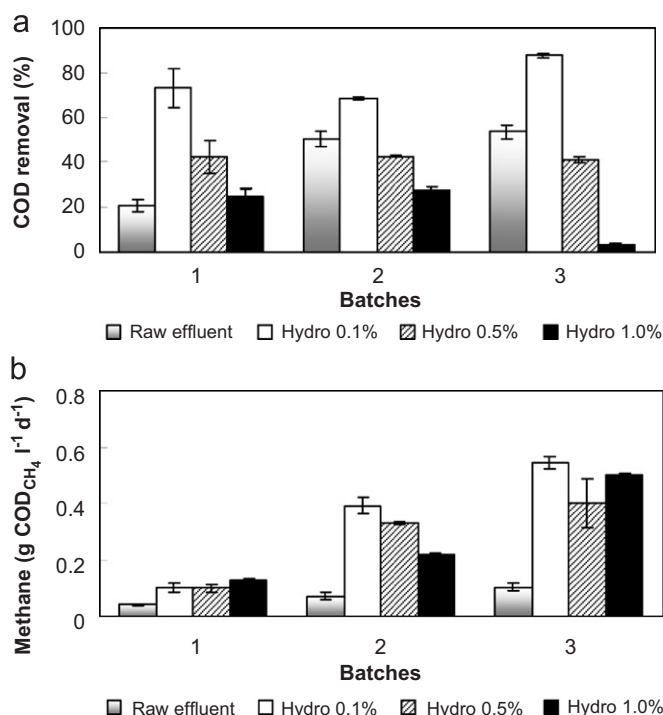


Fig. 4. Effects of adaptation time on COD removal efficiency (a) and maximum methane production rate (b) in raw and pre-hydrolyzed effluents with 1200 mg O&G l⁻¹. Raw effluent (raw effluent without pre-hydrolysis); hydro 0.1% (effluent pre-hydrolyzed with 0.1% SEP); hydro 0.5% (effluent pre-hydrolyzed with 0.5% SEP) and hydro 1.0% (effluent pre-hydrolyzed with 1.0% SEP).

1200 mg O&G l⁻¹. In tests with raw effluent and with effluent pre-treated with 0.1% (w/v) SEP, increases in COD removal efficiency were observed from the first to the third

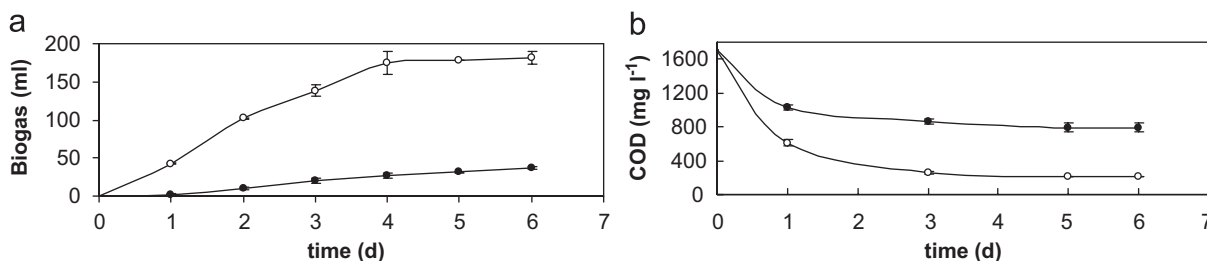


Fig. 5. Evolution of biogas production (a) and COD decline (b) in tests with raw effluent and with effluents hydrolyzed with 0.1% SEP containing 1200 mg O&G l⁻¹. (●) Raw effluent without pre-hydrolysis and (○) effluent pre-hydrolyzed with 0.1% SEP.

batch. In the experiments conducted with effluent pre-hydrolyzed with 0.5% (w/v) SEP, the COD removal efficiency remained stable across all batches. In contrast, the COD removal efficiency decreased from the second to the third batch in effluent hydrolyzed with 1.0% (w/v) SEP (Fig. 4a). Hydrolyzed effluent experiments yielded higher methane production rates than those obtained with raw effluent, and this tendency increased with adaptation (Fig. 4b).

Based on the results obtained in the third batch (adapted sludge), the anaerobic degradation kinetics of the effluent were monitored at the highest fat content (1200 mg O&G l⁻¹); these data are summarized in Fig. 5. After 4 d, the COD removal efficiency (85%) and biogas production (175 ml) of pre-hydrolyzed effluent were greater than that of raw effluent (53% and 37 ml, respectively) containing the same initial fat content.

4. Discussion

The decrease in the amount of released acids with increasing oil and grease contents (150–750 mg O&G l⁻¹) within SEP concentration conditions (Table 1) was not expected because higher oil and grease concentrations in the effluent should result in a higher production of free acids at a fixed SEP content. This decrease may be attributed to the consumption of free acids by microorganisms present in the effluent and in the fat added to the effluent. This interpretation is consistent with the work of Leal et al. (2002) who corroborated the hypothesis that produced acids can be consumed as substrate by aerobic microorganisms. In experiments carried out in the presence of the respiratory chain uncoupler sodium azide (1% w/v), acid contents continued to increase with time throughout the assay. Meanwhile in the experiments carried out in the absence of sodium azide, free fatty acids release decreased after 7 h of incubation as a consequence of microbial substrate consumption.

The substantial consumption of free acids in the raw effluent control experiment with 1200 mg O&G l⁻¹ demonstrated that effluent and fat contain microorganisms that consume these acids as substrate. At a concentration of 1200 mg O&G l⁻¹, the release of free acids in the presence of 0.5% or 1.0% SEP increased, indicating that at these oil

and grease and SEP concentrations, the free acid production exceeded the microorganisms' consumption capacity.

Enzymatic pool hydrolysis rates in the poultry slaughterhouse effluent samples were based on the results free acid data shown in Table 1. These values (maximum of 0.33 μmol free acids ml⁻¹ h⁻¹) were far below previously reported rates obtained with dairy effluents, which were approximately 2.0 μmol free acids ml⁻¹ h⁻¹ (Camarota et al., 2001; Jung et al., 2002; Leal et al., 2002). The markedly lower hydrolysis rates with poultry slaughterhouse effluent relative to those with dairy effluents indicates that the hydrolases present in this SEP likely have a higher affinity for the fat and protein constituents in dairy effluents.

The purpose of adding SEP to the effluent in the stage prior to anaerobic biodegradation is to hydrolyze triglycerides and proteins into simpler and more easily assimilated molecules (fatty acids, glycerol and amino acids). Since the exact composition of the fat in slaughterhouse effluent is not known, we evaluated the anaerobic system response with a range of SEP contents in the hydrolysis stage (Fig. 1). The decline in COD removal efficiency observed in the presence of higher SEP concentrations (0.5% and 1.0% SEP) may be related to the formation of large amounts of long-chain fatty acids (LCFA). LCFA are known to inhibit microbial activity, due to the slow growth rate of organisms that consume LCFA and to toxicity on anaerobic microorganisms (Salminen and Rintala, 2002). The use of 0.5% and 1.0% SEP during effluent hydrolysis resulted in free acid release that was markedly more than that obtained in the presence of 0.1% (w/v) SEP (Table 1). LCFA may be among these acids and may contribute to elevated inhibition of microbial activity.

The reduction of the biogas production lag phase period in the biodegradation experiments (Fig. 2) with addition of SEP illustrates that SEP can efficiently accelerate the substrate consumption by the microbial consortium. The observed inhibition of methane production rates at higher oil and grease concentrations (Fig. 3) was expected since low oil and grease biodegradability rates, associated with the accumulation of these materials on the cell surface, reduces permeability and the absorption of substrate and nutrients (Perle et al., 1995). In experiments with 0.5% and 1.0% SEP, methane production increased with increasing fat content up to 300 mg O&G l⁻¹, and then decreased with

higher fat content effluent. This pattern is consistent with the possibility that the presence of more LCFA results in an inhibition of microbial activity.

Increases in COD removal efficiency from the first to the third batch (Fig. 4a) may be due to the longer contact time of effluents with the sludge, which may allow a progressive adaptation of the microorganisms to the constituents of these effluents, and thereby improve the removal efficiency. In effluents hydrolyzed with 1.0% SEP, two opposing influences upon microorganism activity were observed. Long contact time of effluents with the sludge provides for better adaptation, thus contributing to an increase in COD removal rate. However, this also leads to a high initial concentration of LCFA and concomitant inhibition of the anaerobic microorganisms, which results in attenuation of the COD removal rate (Salminen and Rintala, 2002). Therefore, in the range of concentrations examined herein, COD removal efficiency decreased as enzymatic pool content increased. The results shown in Fig. 4b confirm the effect of sludge adaptation on the constituents of the effluent containing $1200 \text{ mg O\&G l}^{-1}$ on maximum methane production rate. Note that the methane production rates increased from the first to the third batch in all experiments conducted with pre-hydrolyzed effluents. However, in the second and third batches with the high enzymatic pool contents, the observed rates were lower than those for 0.1% SEP; this observation confirms that high LCFA concentrations inhibit microbial activity and methane production.

Our data clearly demonstrate a positive effect of a pre-hydrolysis stage on fat and protein content in the effluent as evidenced by enhanced COD removal efficiency and increased biogas production (Fig. 5). Caixeta et al. (2002) treated slaughterhouse effluents with oil and grease contents ranging from 40 to 600 mg l^{-1} in an UASB reactor, and observed COD removal efficiencies in the range 77–91%. However, the authors used oil and grease contents that were far below those used in the present study. Ruiz et al. (1997) working with slaughterhouse effluents containing 8000 mg l^{-1} of COD in an UASB reactor followed by anaerobic filter obtained COD removal efficiencies in the range 60–90%; however, when higher organic loads were applied, problems such as sludge flotation and loss of biomass were reported. The impact of the enzymatic hydrolysis of fat particles on the efficiency of a downstream anaerobic digestion process was evaluated by Masse et al. (2003). In that study, slaughterhouse wastewater containing fat particles was pre-treated with 250 mg l^{-1} of pancreatic lipase PL-250 and delivered to an anaerobic sequencing batch reactor operated at 25°C . Approximately 35% of the neutral fat was hydrolyzed during pre-treatment. However, the pre-treatment presented only a small overall effect on the fat particle digestion, manifested by a decrease of about 5% (3 h) in the digestion time to achieve 80% of reduction in the neutral fat and LCFA concentrations.

5. Conclusions

In anaerobic biodegradability tests, pre-treating effluent with 0.1% SEP resulted in high COD removal efficiencies and increased biogas production, even at the highest fat contents studied. Pre-hydrolysis with higher SEP concentrations (0.5% and 1.0%) did not result in high COD removal efficiencies; this failure at the higher enzymatic pool concentrations was probably due to the presence of high LCFA levels in the medium. The anaerobic sludge underwent adaptation both to the constituents of the effluent and to the enzymatic pool, such that increases in COD removal efficiency and methane production rate were observed even in the raw effluent in later batches. The enzymatic pre-hydrolysis effect was evident when the degradation kinetics of the raw and pre-treated (0.1% SEP) effluents were compared. While a 53% COD removal efficiency and 37 ml of biogas production were observed with raw effluent, a markedly enhanced 85% COD removal efficiency and 175 ml of biogas production were observed with previously hydrolyzed effluent. In summary, our results indicate that the use of 0.1% SEP in the hydrolysis stage of poultry slaughterhouse effluents provides an effective strategy for achieving higher methane production rates, shorter processing times and lower reactor volumes.

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