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HYDROLYTIC ENZYMES AS COADJUVANTS IN THE ANAEROBIC TREATMENT OF DAIRY WASTEWATERS

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Abstract. - An enzymatic extract produced by *Penicillium restrictum* having a high level of lipase activity (17.2 $U.g^{-1}$) was obtained by solid-state fermentation using babassu cake as substrate. The enzymatic extract was used in the hydrolysis of a dairy wastewater with high fat contents (180, 450, 900 and 1,200 $mg.L^{-1}$). Different hydrolysis conditions were tested, and it was determined that it should be carried out at a temperature of 35ºC, without agitation, with 10% v/v enzymatic extract and a hydrolysis time of 12 hours. Both crude and hydrolysed effluents were then submitted to an anaerobic biological treatment. It was observed that for the enzymatically pretreated effluent there was a significant improvement in the efficiency of the anaerobic treatment. For the highest fat content tested (1,200 $mg.L^{-1}$), removal efficiencies of 19 and 80% were attained for crude and hydrolysed effluents, respectively. In addition, a tenfold increase in the removal rate of COD from the hydrolysed effluent (1.87 kg $COD.m^{-3}.d^{-1}$) was observed in relation to the crude effluent (0.18 kg $COD.m^{-3}.d^{-1}$). The results obtained in this study illustrate the viability of using a hybrid treatment (enzymatic-biological) for wastewaters having high fat contents.
Keywords: dairy wastewater; enzymes; lipases; anaerobic treatment; enzymatic treatment.

INTRODUCTION

Due to its continental dimensions and both its climatic and territorial characteristics, Brazil has a unique biodiversity in the world and a great abundance of biomass and agroindustrial solid wastes, which constitute raw material for biotechnological processes. These factors show that it is imperative to develop bioprocesses in this country, which would provide new products and reduce production costs.

In this context, in this work a strain of the fungus *Penicillium restrictum*, previously isolated from the waste of a babassu-processing industry as a lipase producer (Freire et al., 1997a,b), was employed.

Lipases (glycerol ester hydrolases, E.C. 3.1.1.3) are hydrolases which catalyse the hydrolysis of carboxyl ester bonds present in acylglycerol with the consequent release of organic acids and glycerol. They are particularly important due to the fact that they specifically hydrolyse oils and greases, which is of great interest for different industrial applications, among them the treatment of industrial wastewaters having high fat contents, such as in the case of wastewaters generated in dairy production plants.

Basically, both solid-state and submerged fermentations can be employed for the production of lipases. In the present work, solid-state fermentation was studied due to several advantages, such as a lower initial investment, low energy consumption,

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and the possibility of employing agroindustrial wastes as raw materials, contributing to a reduction in production costs (Gombert et al., 1999).

The utilisation of a hybrid technology – enzymatic treatment associated with anaerobic biologic treatment – enables a reduction in hydraulic retention time and, consequently, in reactor volume, since it promotes hydrolysis of fats which cause problems of clogging of the sludge bed in anaerobic reactors of the UASB type (Chernicharo, 1997).

The purpose of this work was to study the production of hydrolytic enzymes (lipases and proteases) by solid-state fermentation, evaluating the enrichment of the babassu cake medium with different supplements under previously optimised fermentation conditions (Gombert et al., 1999). Additionally, the use of these enzymes for hydrolysing a wastewater from a dairy industry prior to the biological anaerobic treatment was studied.

MATERIALS AND METHODS

Microorganism

The strain of *Penicillium restrictum* used was isolated from babassu cake samples kindly supplied by Tocantins Babaçu S.A. (Freire, 1996).

Culture Media and Fermentation

Four cultivation media based on crushed and screened babassu cake (Tyler 35 – 60) were tested with different supplements. A spore suspension (10^8 spores/g of medium) and the nutrients were added to 10 g of cake in all the media prepared in order to obtain a final moisture of approximately 70%. The supplements tested were *Leitelho,* a waste from butter production; a liquid waste from the fat separation tank, here called *liquid RTSG*; a solid waste from the fat separation tank, here called *solid RTSG*; and 1% w/v *olive oil*. The amounts of each of the supplements used were determined according to the C and N contents of the substances and the C/N ratio recommended in the literature (Gombert et al., 1999). Table 1 shows the C/N ratio for each one of the supplements tested. Beakers containing the cultivation media were incubated at 30ºC for 63 hours with injection of humidified air. At selected time intervals (15, 20, 24, 38, 48 and 63 h), whole beakers were taken as samples.

Sample Preparation and Enzyme Extraction

From each beaker two aliquots of 0.5g were taken out for pH and moisture analysis. Then 45 mL of 50 mM phosphate buffer pH 7.0 was added to the remaining fermented solids. The mixture was incubated in a rotary shaker at 35°C and 200 rpm for 30 minutes. The liquid fraction was then extracted by manual pressing and centrifugation at 3000 rpm for 2 minutes. The supernatant (enzyme extract) was used for the other analytical assays.

Enzymatic Wastewater Hydrolysis

The wastewater from the dairy industry (Cooperativa de Cantagalo/RJ, Brazil) was treated with 10 and 20% v/v enzymatic extract of 2.1 $U.mL^{-1}$ lipase activity. Wastewater hydrolysis was carried out for 12 hours at a temperature of 35ºC, without agitation. Four different initial concentrations of oils and greases were tested: 180, 450, 900 and 1,200 $mg.L^{-1}$.

Adaptation of Anaerobic Sludge to the Wastewater

The anaerobic biodegradation experiments were carried out with anaerobic sludge obtained from a biological reactor from the same dairy industry. An adaptation of this sludge, both to the wastewater used in this study and to the enzymatic extract added in the hydrolysis step, was required. Therefore, 100 mL sludge was incubated in a sealed glass flask of 400 mL working volume, containing a mixture of wastewater and sludge. The whole assembly was installed in a climatized room at approximately 35ºC. The microbial flora was gradually adapted to the enzymatic extract-wastewater mixture. Adaptation of the sludge was evalueted by monitoring pH and COD (chemical oxygen demand).

Table 1: C/N ratio of each one of the supplements.

Supplements	C/N ratio
Leitelho	11.3
Liquid RTSG	12.1
Solid RTSG	12.4
Olive oil (1% w/v)	13.3

Anaerobic Wastewater Treatment

The anaerobic biodegradability of the industrial wastewater either with or without enzymatic pretreatment was evaluated in experiments carried out in sealed glass flasks (reactors) having a working volume of 120 mL, coupled to gasometers for collecting the gases produced. The adapted sludge (30 mL) and either crude or enzymatically pretreated wastewater (90 mL) were added to the reactors, which were operated in a batch mode and were fed every fourth day. During this period samples were taken for pH and COD assays. A comparison of COD removal kinetics allowed evaluation of the effect of wastewater enzymatic pretreatment on the efficiency of the anaerobic treatment.

Analytical Assays

i) Moisture of the cultivation medium was determined after drying 0.5 g of fermented material at 70°C for 24 h.
ii) pH both of cake and the effluent, was determined using a potentiometer (direct reading).
iii) Protease activity was determined according to Charney and Tomarelli (1947).
iv) Lipase activity was determined according to Freire et al. (1997 a, b).
v) Total Kjeldhal Nitrogen (TKN) in effluents was determined according to a procedure described in the Standard Methods (APHA, 1992).
vi) Total Organic Carbon (TOC) in effluents was determined according to a procedure described in the Standard Methods (APHA, 1992).
vii) Chemical Oxygen Demand (COD) of effluent was measured by closed reflux (Hach) according to a procedure described in the Standard Methods (APHA, 1992).
viii) Oils and greases were determined by Soxhlet extraction using hexane as a solvent, according to procedures described in the Standard Methods (APHA, 1992).

RESULTS AND DISCUSSION

Fermentation

Solid-state fermentations using the four different culture media described in Materials and Methods were carried out. Lipase and protease activities were determined throughout the fermentation. The purpose of these experiments was to use the waste generated by dairy production, which is available in great amounts and at no cost, as carbon and nitrogen sources for enriching the cultivation medium employed in enzyme production. Figure 1 shows the results obtained in terms of the ratio of lipase/protease activity. It can be concluded that none of the wastes used was a satisfactory supplement to the babassu cake. In the medium supplemented with liquid RTSG, a level of lipase activity (19.8 $U.g^{-1}$) similar to that in the medium supplemented with 1% w/v olive oil (17.2 $U.g^{-1}$) was obtained. However, the production of protease in this medium (liquid RTSG) was high (29.9 $U.g^{-1}$), which would cause hydrolysis of the lipase produced. Figure 2 shows the typical results obtained for both lipase and protease activities for the medium supplemented with 1% w/v olive oil. Thus, although supplementation with olive oil results in a higher cost of enzyme production, its higher lipase/protease ratio (Fig. 1) subsequently enabled more efficient hydrolysis. Additionally, using olive oil as supplement the lipase activity peak was reached within 24 hours of fermentation, while in the case of liquid RTSG this only occurred after 48 hours (results not shown).

Enzymatic Wastewater Treatment

Initially, the concentration of enzymatic extract (10 or 20% v/v) with and without agitation was tested. Additionally, an assay was also carried out where sodium azide was added (1% w/v) in order to avoid the growth of microorganisms and to discriminate between the action of enzymatic catalysis and that of microbial biodegradation.

As shown in Fig. 3, up to about 6 hours of hydrolysis, no significant difference in the evolution of free acids was observed for the three conditions tested with 10% v/v enzyme extract. After that, a decline in the concentration of free acids was only observed in the experiment carried out with agitation. This fact must be related to the use of the acids produced as a substrate by aerobic microorganisms which can grow more rapidly in this assay.

Corroborating this hypothesis, this behaviour was not observed in the experiments carried out in the presence of sodium azide (1% w/v), where acid content continues increasing throughout the assay. In the experiment carried out without agitation and with agitation with 20% v/v enzymatic extract, there was even an increase in the concentration of free acids up to 7 hours. After that, the free acids began to be consumed by the microorganisms present in the reaction medium, so a decrease in their concentration was observed.

Doubling the concentration of enzyme extract resulted in an increase in free acid contents in the medium, but the excessive use of crude enzyme extract would make this process economically unfeasible on larger scales.

Based on the results obtained in this preliminary phase, wastewater hydrolysis was carried out under the following conditions: 35ºC, 10% v/v enzyme extract (2.1 $U.mL^{-1}$), no agitation and a hydrolysis time of 12 hours.

Anaerobic Treatment of the Wastewater

The effect of the enzymatic pretreament on the COD removal kinetics may be observed in Figure 4, which shows the COD variation with time for an effluent having an initial content of oils and greases of 1,200 $mg.L^{-1}$. The initial COD removal rate was much higher for the hydrolysed wastewater than for the crude . During the first 24 hours, the rate was 0.18 kg $COD.m^{-3}.d^{-1}$ for crude wastewater, whereas for the hydrolysed wastewater it was 1.87 kg $COD.m^{-3}.d^{-1}$, i.e., the COD removal rate was ten times higher for the pretreated effluent.

Table 2 summarises the results obtained for effluents with different initial contents of oils and greases. In addition to the significant increase in the COD removal efficiency and the reduction in treatment time, the hydrolysis of the fat present in the wastewater using the crude enzymatic extract enables a remarkable improvement in the operating conditions of the anaerobic reactor as well as in the quality of the treated wastewater (lower levels of suspended solids and of oils and greases). Thus, the use of this hybrid technology (enzymatic-biological treatment) was shown to be a very promising alternative for treating wastewaters having high fat contents, such as those from the dairy industry.

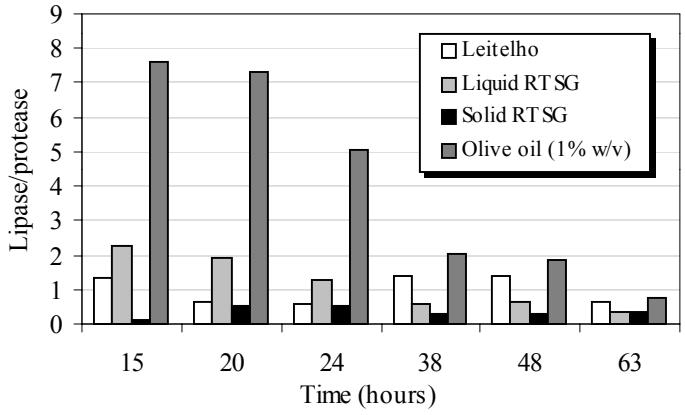

Figure 1: Variation in activity ratio (lipase/protease) throughout the fermentation time for different types of supplement.

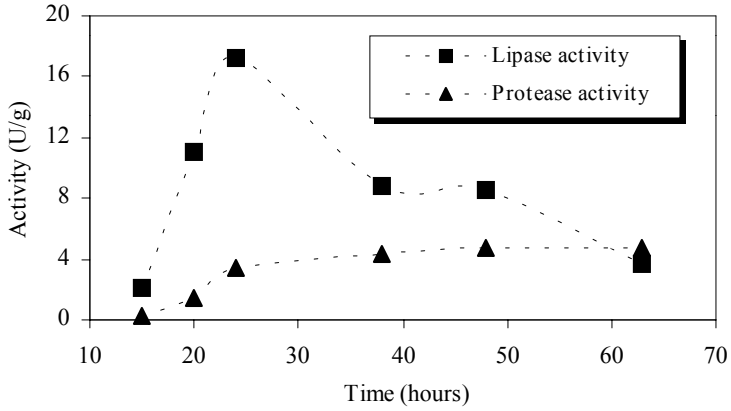

Figure 2: Variation in lipase and protease activities for the medium supplemented with olive oil (1% w/v) throughout the fermentation time.

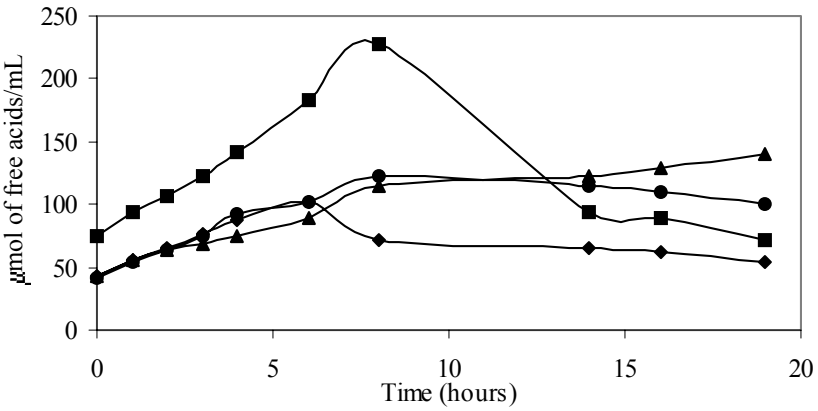

Figure 3: Evolution of the production of free acids throughout the hydrolysis time. Average values of measurements carried out in duplicate are shown. (■) agitation with 20% v/v enzymatic extract, (▲) sodium azide with 10% v/v enzymatic extract, (♦) agitation with 10% v/v enzymatic extract and (●) without agitation with 10% v/v enzymatic extract.

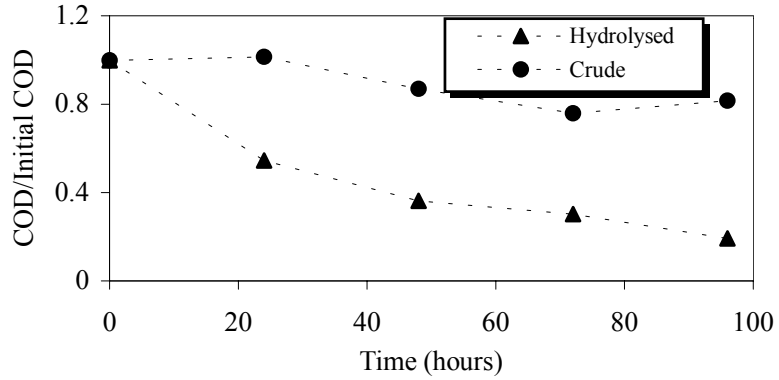

Figure 4: COD evolution with anaerobic treatment time for an effluent containing an initial oil and grease content of 1,200 mg.L^{-1}.

Table 2: Comparison of COD removal efficiency in crude and hydrolysed effluents after 48 h of anaerobic treatment.

Initial O&G Content (mg.L^{-1})	COD Removal Efficiency (%)	
	Crude	**Hydrolysed**
180	55	95
450	70	91
900	37	80
1,200	19	80

CONCLUSIONS

The enzymatic/biological treatment evaluated has shown very satisfactory results, such as

i) the possibility of producing *in situ* an enzymatic extract having a high lipolitic activity, at low cost, using a locally developed technology and a microorganism isolated and adapted to the conditions of the local environment;

ii) the possibility of treating wastewaters having fat contents up to 1,200 $mg.L^{-1}$ by anaerobic processes with no operating problems;

iii) obtaining high COD removal efficiencies with lower treatment times, contributing to a reduction in the reactor volume.

The results obtained in the present work indicate the feasibility of the alternative proposed in this research. Further studies will focus on optimisation of the hydrolysis conditions, aiming at a reduction of the amount of enzyme applied, and on evaluation of combined enzymatic and biological treatment in continuous anaerobic reactors of the UASB type on bench and pilot scales.

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