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Application of Home-Made Enzyme and Biosurfactant in the Anaerobic Treatment of Effluent with High Fat Content

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Abstract

The Solid State Fermentation (SSF) lipase production from *Penicillium simplicissimum* using an agro industrial residue and the biosurfactant from *Pseudomonas aeruginosa* were employed for a combined pre-treatment of wastewater from poultry processing industry. During the hydrolysis step of the wastewater fat better results were obtained at 34°C with biosurfactant 0.4% (v/v) and liquid enzyme preparation 6.2% (v/v) or at 46°C with biosurfactant 0.1% (v/v) and liquid enzyme preparation 3.8% (v/v). The pre-hydrolysis in two different conditions of enzyme and biosurfactant concentrations at 34°C and posterior anaerobic treatment allowed the COD removal and methane production, while the no hydrolyzed wastewater besides the COD removal, did not show methane production. Those results suggest the importance and efficiency of the pre-treatment with home-made lipases and biosurfactant during the high fat content wastewater pre-treatment.

Keywords: Anaerobic treatment; Biosurfactant; Fat; Lipase; Wastewater

Introduction

In Brazil, poultry slaughtering industry stands out as one of the most important industrial activities. Brazil is the third largest chicken's producer, reaching 11 million tons of chicken meat produced, with 3.6 million for export [1]. However, the poultry slaughtering generate high volumes of effluent containing biodegradable organic matter, lipids, proteins and cellulose. Lipids can represent over 67% of the COD particulate in slaughterhouses wastewater [2].

Nowadays, with demand for energy, the search for alternative energy sources is increasing. Thus, the anaerobic processes involving as effluents generated in the food industry become advantageous, since they allow the energy production in form of methane gas [3].

For an efficient anaerobic wastewater treatment containing high fat content, it becomes necessary a step of pre-hydrolysis in order to avoid operational problems. Enzymatic fats hydrolysis has been studied using commercial enzyme. However, the utilization of these enzymes involves the increase of treatment costs [4].

Several research groups evaluated the process of solid state fermentation (SSF) as a viable and economic alternative for the enzymes production, mainly due to the possibility of using agro industrial wastes as culture medium. Castilho et al. [5] demonstrated that lipase production SSF using the babassu cake as residue requires 78% less investment in comparison to the submerged fermentation. Lipases from *Penicillium simplicissimum* produced by SSF have high biotechnological potential, since they have higher production and yield [6].

In the environmental area, many studies have reported the use of biosurfactants to increase interaction water/oil, accelerate the degradation of several oils by microorganisms and promote the contaminated soils and waters bioremediation [7]. However, there are a few reports in the literature of the combined use of enzymes and biosurfactant to increase the treatment efficiency of wastewater from food industries [8].

Considering the need to promote the proper treatment of wastewater with high fat content, this paper aims the use of an extracellular lipase from *Penicillium simplicissimum* produced by SSF in babassu cake and a rhamnolipid produced by Pseudomonas *aeruginosa* PA1 and its application in wastewater pre-treatment from a poultry processing industry.

Material and Methods

Solid-state fermentation

The enzyme pool was produced by solid state fermentation of waste from babassu oil production by *Penicillium simplicissimum* microorganism in tray reactor containing 15 g of basal medium (babassu cake). After milling and supplementation with 6.25% (w/w) molasses, to a C:N ratio of 12.8:1, the cake was autoclaved at 121°C/15 min and inoculated with a spores suspension to an initial concentration of 107 spores/g [6]. After 48 h of growth conducted at 30°C and 95% humidity, a liquid enzyme preparation (LEP) was obtained by extraction with phosphate buffer pH 7.0 and 0.5% (w/v) Tween 80. The mixture was incubated in a rotary shaker at 25°C and 200 rpm for 40 minutes. The liquid fraction was then extracted by manual pressing and centrifugation. The supernatant (LEP) was used for the assays.

Biosurfarctant production

The biosurfactant was produced using *Pseudomonas aeruginosa*. After 7 days, the cell-free crude fermented broth was recovered from the fermented medium and characterized. The main characteristics of

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this cell-free fermented medium are: rhamnolipid concentration (4.7 g/L), surface tension (28mN/m), critical micelle concentration (CMC = 205 mg/L), emulsification index (61%) and chemical oxygen demand (COD = 20767 mg/L) [8]. It was stored at -20°C until use.

Collection and characterization of the effluent and sludge

The effluent was obtained from a poultry processing industry in the city of Rio de Janeiro (Rio de Janeiro - Brazil) at the treatment plant before the flotation step. After collection, properly preserved aliquots were taken for determining the characterization parameters and the remaining volume was stored in freezer until the moment of use (Table 1). The amounts of nitrogen and phosphorus in the effluent were enough to supply the necessities of the microorganisms in the anaerobic biodegradability tests. The sludge used in the anaerobic biodegradability tests was collected from a bioreactor UASB (upflow anaerobic sludge blanket) operating in this poultry processing industry, being characterized in terms of total volatile solids (14895 ± 1113mg/L) and oil and grease accumulated according standard procedures [9] (Table 1).

Pre-treatment with enzyme and biosurfactant

Firstly, a study was conducted in order to determine the time for enzymatic hydrolysis of fat present in the effluent (2034 mgO&F/L). Times of 4, 8 and 24h, using concentrations of 1 and 5% (v/v) LEP, at 400 rpm and 30°C, were evaluated. These experiments were conducted with 100 mL of effluent in 500mL flasks. Then, the best conditions were set for hydrolysis employing an experimental design 2^k type, having as variables the temperature, LEP and biosurfactant concentrations. The actual and coded values of these variables are shown in Table 2. The experiments were conducted with 100 mL of raw wastewater in glass reactors with stirring (400 rpm) and temperature control (34°C, 40°C or 46°C) for 8 h. The hydrolysis was monitored through free acid measurement performed by titration with 0.04M NaOH until pH 11 [8] (Table 2).

Variable	Average ± SD
pH	6.4 ± 0.1
T (°C)	33
COD (mg/L)	8692 ± 1262
mgO&F/L	2403 ± 521
Total Solids (mg/L)	6717 ± 3049
Fixed Total Solids (mg/L)	1807 ± 151
Volatile Total Solids (mg/L)	4910 ± 2898
Total Nitrogen (mg/L)	434 ± 2
Total Phosphorus (mg/L)	6.5 ± 0.7

Average and standard deviations of three samples of collected effluent

Table 1: Characterization of the raw effluent from the poultry processing industry. The effluent was characterized in terms of pH, COD, suspended solids (total, fixed and volatile), total nitrogen, total phosphorus and oils and greases, according to standard procedures (APHA 2005).

Variable	Levels		
	-1	0	1
Biosurfactant (% v/v)	0.10	0.25	0.40
Enzyme pool (% v/v)	3.8	5.0	6.2
T (°C)	34	40	46

Average and standard deviations of three samples of collected effluent

Table 2: Experimental design 2^3 type, having as variables the temperature, LEP and biosurfactant concentrations. Conditions used in the pretreatment with enzyme pool and biosurfactant.

Anaerobic biodegradability tests

Anaerobic biodegradability tests were conducted with the raw and treated effluents with LEP and biosurfactant. All the tests were conducted in batch using six penicillin flasks of 100mL. The flasks were incubated at 30°C for up to 8 days with 90mL of a mixture of anaerobic sludge and effluent with pH adjusted to 7.0 ± 0.1. The effluent and sludge volumes were calculated in order to obtain an initial COD: sludge VSS ratio of 1:1 in the flasks. The biodegradability was assessed by measuring the COD removal efficiency and biogas production, performed by the piston displacement of 20 mL plastic syringes connected to the flasks. Aliquots for the initial COD determination were taken after pre-treatment and before contact with the anaerobic sludge. The final soluble COD was determined on the last day of the degradation test, after collecting the biogas for analysis in gas chromatograph. Specific methane production (L CH_4/g removed COD) was also used to assess the biodegradation process.

Analytical methods

The lipase activity was determined by colorimetric and titrimetric methods using p-nitrophenil laureate and olive oil as substrates, respectively [6], where one lipase unit was defined as the enzyme amount that causes the release of 1µmole of fatty acids per minute, under the assay conditions (titrimetric) and one unit of lipase activity is defined as the amount of enzyme which releases 1 µmole of p-nitrophenol under the assay conditions (colorimetric). Enzyme activity was expressed as units per gram of dry babassu cake. The rhamnolipid concentration, reported as rhamnose, was determined according to the method of Pham et al. [10]. The emulsification index was determined according to the method of Cooper and Goldenberg [11]. The determination of the crude extract surface tension (mN/m) was performed on an Aqua-Pi tensiometer (Kibron Inc., Helsinki) at 25°C, using the Du Noüy [12] method. The critical micelle concentration (CMC) was determined according to methodology described by Cooper et al. [13]. The biogas composition was determined in gas chromatograph VARIAN MICRO GC 4900 employing 10 m x 0.32 mm PPQ column, column temperature of 50°C, thermal conductivity detector (TCD) with temperature of 250°C, injector temperature of 80°C and helium as carrier gas. The other parameters used in the effluent and sludge characterization and in the monitoring of the anaerobic biodegradability tests were determined according to procedures described in Standard methods [9].

Results and Discussion

Determination of enzymatic hydrolysis pre treatment time

A preliminary study was conducted in order to determine the optimum pre hydrolysis time. We investigated the kinetic of hydrolysis with two different amounts of liquid enzyme preparation (LEP) (1 and 5% v/v) at 30°C, 400 rpm. The concentration of free acids was monitored after 4, 8 and 24h hydrolysis, as shown in Table 3. For both concentrations of LEP, the maximum free acids production was

Hydrolysis time (h)	1% LEP	5% LEP	Δ (1%)	Δ (5%)
0	30.5	30.5	0	0
4	31.2	31.5	0.7	1.0
8	34.4	41.3	3.9	10.8
24	28.7	33.7	--	3.2

Δ Variation between 4, 8 and 24 h and the zero time

Table 3: Free acid production (µmol/ml) for different concentrations of LEP (with 4.7 U/mL of lipase activity) and hydrolysis times (Standard deviation less than 5%).

obtained with 8 h hydrolysis. So, this time was selected for the next assays and the LEP concentration of 5% (v/v) was the basis for the experimental design (Table 3).

Valladão et al. [14] found maximum values of hydrolysis at 22 h (7.3 mol/ml) using 1% w/v (0.21 U/mL) lipase from *P. restrictum* in treating poultry slaughterhouse wastewater containing 1200mgO&F/L. In this work it was used substantially the same lipase activity (0.23 U/mL or 5% v/v) to hydrolyze almost twice as fat (2034 mgO&F/L), yielding 8.10mol/mL of free acids in only 8 h. These results show that the enzyme preparation produced by the fungus *P. simplicissimum* is most effective for hydrolysis of fats present in the effluent from a poultry processing industry.

Determination of optimum conditions for the pre-treatment

In order to evaluate the synergistic effect on the pretreatment step of the enzyme pool with the biosurfactant, an experimental design was carried out with effluent containing 2034 mgO&F/L. The free acids concentrations obtained in each condition evaluated are presented in Table 4.

The standard variables effects (t) and the significance probability test (p) were used to evaluate the effects of biosurfactant concentration, LEP concentration and temperature on the fat hydrolysis present in the effluent. Using a 10% confidence level ($p<0.10$) was observed that the LEP concentration, the biosurfactant concentration and interaction between biosurfactant concentration and temperature showed higher significant effect on the fat hydrolysis in the effluent (Table 5). The positive effect of the interaction between the variables biosurfactant and enzyme concentration prove the success of use of these products on the pretreatment when combined (Table 5).

Based on the results it was possible to construct an empirical model for quantification of free fatty acids released due to the LEP concentration, biosurfactant concentration and temperature (Equation 1) that includes the statistically significant and marginally significant variables, considering $p<0.1$.

$$FA = 8.48 + 1.38\,LEP - 1.26\,B - 0.97\,T + 1.01\,LEP \times B - 1.75\,B \times T \quad \text{(Eq. 1)}$$

Condition	LEP (% v/v)	BS (% v/v)	T (°C)	Free acids (µmol/ml)*
1	3.8	0.10	34	8.73
2	6.2	0.10	34	10.07
3	3.8	0.40	34	6.81
4	6.2	0.40	34	13.97
5	3.8	0.10	46	10.94
6	6.2	0.10	46	13.29
7	3.8	0.40	46	6.00
8	6.2	0.40	46	8.41
9	5.0	0.25	40	7.2
10	5.0	0.25	40	6.97
11	5.0	0.25	40	6.92

*Difference between sample and control after 8h. Standard deviation less than 5%

Table 4: Conditions (experimental design) and results of the pretreatment with enzyme pool (LEP) and biosurfactant (BS).

Variable	Effect	Standard Error	*t*	*p*
LEP concentration	2.75	1.00	2.73	0.0525
Biosurfactant concentration	-2.52	1.00	-2.51	0.0663
Temperature	-1.93	1.00	-1.91	0.1280
LEP conc x Biosurfactant conc.	2.04	1.00	2.01	0.1135
Biosurfactant conc. x Temperature	-3.52	1.00	-3.49	0.0251

Table 5: Standard variables effects (*t*) and the significance probability test (*p*).

Where: FA = free acids concentration (µmol/ml), LEP = liquid enzyme preparation concentration (coded values), T = Temperature (coded values), B = Biosurfactant concentration (coded values).

The model generated was considered predictive by analysis of variance (ANOVA) and it showed satisfactory determination coefficient (R^2=0.84) and F test value (7.71) greater than the critical value (2.14). This model allowed the construction of outline curves, which show the predicted values of free acids produced for each condition between studied ranges (Figure 1).

At Figure 1a, it was observed that at 34°C it was possible to obtain high free acids concentrations in the hydrolysis applying high LEP concentrations and biosurfactant (within the studied range). However, keeping the LEP concentration and biosurfactant in their highest levels, the concentration of free acid liberated in hydrolysis decreases with increasing temperature (Figure 1b). This could be explained by deactivation of the enzyme at higher temperature, but it is known that the enzyme preparation used in this work presents optimal activity at 50°C [6]. It was also observed in Figures 1c and Figures 1d that high amounts of free acids are obtained in high temperature employing low concentrations of biosurfactant and LEP. Increasing the temperature can lead to a greater solubility in oils and greases from wastewater, reducing the concentration of biosurfactant necessary to maintain the emulsion quality and consequently the access of the enzyme to the substrate, combined with a good enzyme activity at higher temperatures.

However, when comparing the hydrolysis conditions only with the effluent from a poultry processing industry, the free acids production obtained in the present studied with *P. simplissicimum* LEP (7 a 14µmol/mL) demonstrates that optimization of the enzymatic hydrolysis combined with biosurfactant had positive effects, producing better results in enzymatic hydrolysis step of the wastewater fat. Results indicate two optimum conditions of pretreatment (with a maximum hydrolysis 8h): (1) hydrolyzing the effluent to 34°C, which, thereafter, follow to one biological treatment with mesophilic microflora and (2) hydrolyzing the effluent at 46°C for further treatment with thermophilic microorganisms.

Anaerobic biodegradability tests

The anaerobic biodegradability tests were done after enzymatic hydrolysis optimization step, using the hydrolyzed obtained under two distinct conditions: in the first one, the hydrolyzed shows a high acids content (condition 1); in the second one, the hydrolyzed shows a not so high (medium) acids content (condition 2). Such strategy was made to combine good hydrolysis efficiency with a high specific methane production (SMP), once the free acids released during the enzymatic hydrolysis can be inhibitors to anaerobic biodegradability step [15]. Those conditions were selected fixing the temperature in 34°C, once it was used mesophilic microorganisms during the anaerobic biodegradability tests. Condition 1 had hydrolysis conditions at 34°C, biosurfactant 0.4% (v/v) and enzyme (LEP) 6.2% (v/v), reaching 14 µmol/mL of free acids; for condition 2 was used the same temperature and biosurfactant quantity, however with enzyme 3.8% (v/v), reaching nearly 8 µmol/mL of free acids. Table 6 shows the results in terms of COD and methane specific production (Table 6).

It's possible to see that for the hydrolyzed wastewater (conditions 1 and 2) it was possible to obtain methane as principal product during this step. Besides that, the reduction of initial fat content provides a high COD removal, proving the efficiency of enzymatic treatment. However, the specific methane production was higher for the wastewater with

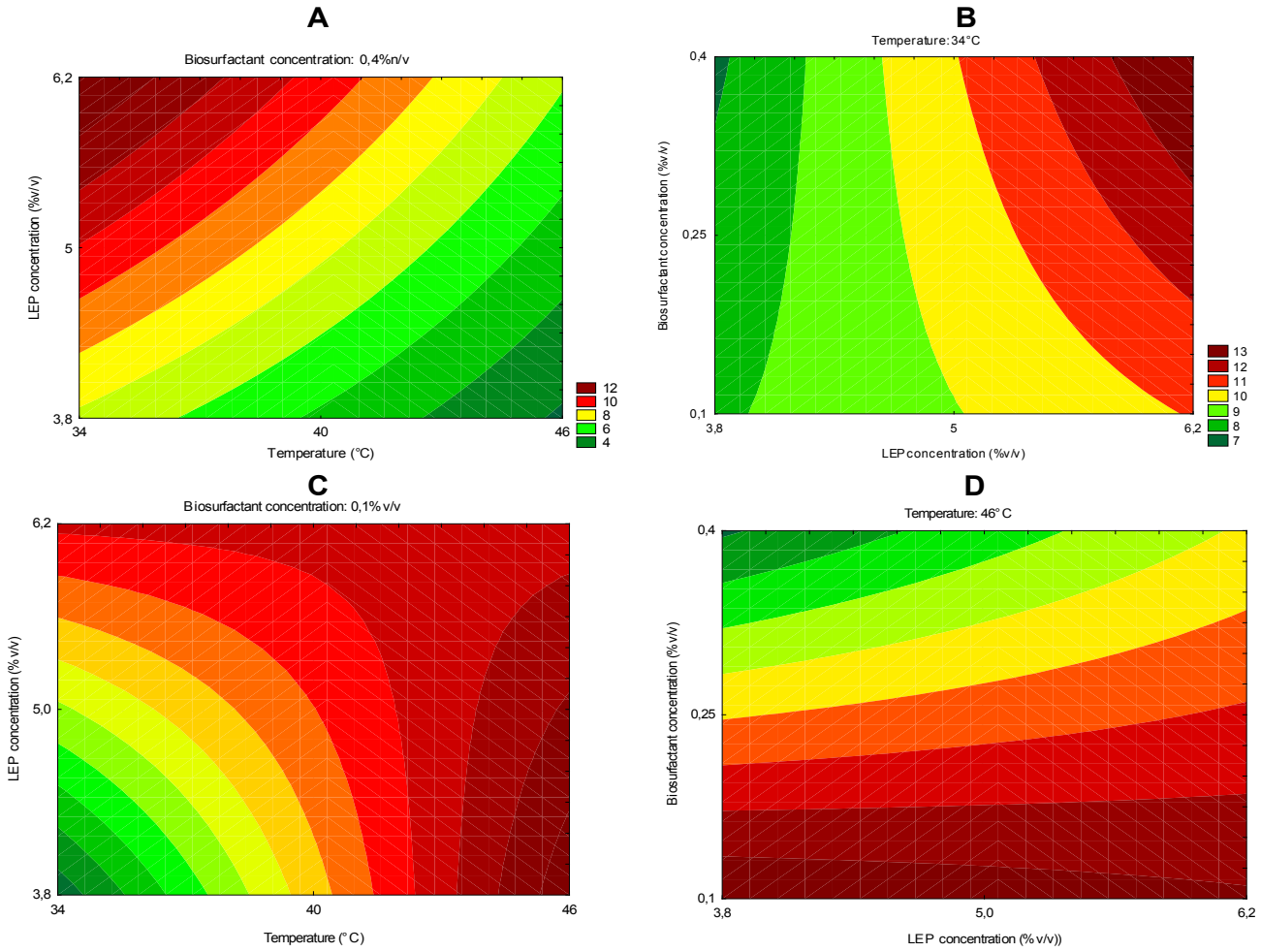

Figure 1: Contour curves for the hydrolysis of the effluent as a function of the variables biosurfactant concentration and LEP concentration to 34°C (a); LEP concentration and temperature for biosurfactant concentration of 0.4% v/v (b); concentration of LEP and temperature for the biosurfactant concentration of 0.1% v/v (c); and biosurfactant concentration and the concentration of LEP at 46°C (d).

Condition	Initial COD (mg/L)	Final\ COD (mg/L)	COD removal (%)	Biogas volume (mL)[a]	Methane (%)	Methane volume (mL)[a]	mL CH_4/g removed COD[a]
Control	3400	240	93	65	nd	nd	0
Condition 1	6147	412	93	79	80	63.2	122.4
Condition 2	4867	304	94	80	82	65.6	159.7

[a]30ºC, 1 atm, 8 days

Table 6: Anaerobic biodegradability tests with the addition of the enzyme pool and biosurfactant under different conditions (Standard deviation less than 5%).

lower acids content released (condition 2), once in this condition the wastewater may contain lower concentration of long chain fat acids, potential inhibitors of methanogen archaeas [15]. In condition control case (without enzymatic hydrolysis) there was COD reduction (93%) and gas productions (65 mL), however it was not possible to detect the methane production. The value of COD reduction in control is the same reached by hydrolyzed wastewater and the gas production was lower when compared with hydrolyzed wastewater. This indicate that part of the COD is removed by other metabolic pathways and/or by other microorganisms producing another by-products and gas as CO_2 (detected by chromatography), or H_2 and volatile acids (not determined). Other possibility is that part of COD removal was reached by adsorption of the fat content on the sludge or onto flasks wall used during the test.

While Valladão et al. [14] obtained 165 mL CH_4/g COD provided (at 34ºC) with effluent containing 1200 mgO&F/L hydrolyzed with 0.21 U/mL for 22 h, this work yielded 150 mLCH_4/g COD provided (at 34ºC) with wastewater containing 2034 mgO&F/L hydrolyzed with 0.19 U/mL and 0.4% biosurfactant for 8h. These results shows that higher concentrations of fat can be degrade, keeping the same enzymatic activity for shorter pre-hydrolysis time when adding a small quantity of biosurfactant. Probably the biosurfactant facilitates access of the enzyme to particles of fat.

Conclusions

The hydrolysis of wastewater from poultry processing industry with a home-made lipase showed high efficiency compared to other lipases.

The effect of biossurfactat alone was not evaluated in this study, but the use of it aimed to assist and minimize the use of the enzymatic preparation. After the optimization step, the use of the biosurfactant resulted in a reduction of 30% in the quantity of enzyme to release the same amount of fatty acids (8 μmol/mL). While an increase of about 35% in the quantity of enzyme preparation led to a 75% increase in the content of free fatty acids (14 μmol/mL) due the use of the biosurfactant. The enzymatic preparation and ramnolipid showed higher production of free fat acids in two different conditions of enzyme and biosurfactant concentration, and temperature due to variables interaction effect of biosurfactant and enzyme concentration, and temperature and biosurfactant concentration as showed in experimental design analysis. This result allows the pre-treatment to be conducted in 34 or 46ºC with efficiency and indicate a subsequent anaerobic treatment application with mesophilic or thermophilic microflora.

Is notable the favorable joint application between enzyme and biosurfactant also for methane production. The treatment shows high COD removal efficiency and methane production for a more concentrated effluent treatment with shorter time probable allowing operation of the system for long periods without problems caused by fat accumulation, reducing costs of the process.

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