ISSN 1330-9862
(FTB-2333)

original scientific paper

Production of a Fermented Solid Containing Lipases of *Rhizopus microsporus* and Its Application in the Pre-Hydrolysis of a High-Fat Dairy Wastewater

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Received: August 21, 2009
Accepted: November 10, 2009

Summary

The filamentous fungus *Rhizopus microsporus* CPQBA 312-07 DRM was grown in solid--state cultivation and the fermented solid produced was used to hydrolyze triacylglycerols in a high-fat dairy wastewater. For the solid-state cultivation, a mixture of sunflower seed meal and sugarcane bagasse (1:3 by mass on dry basis) was selected. After 18 h of culture, the fermented product had an activity, measured titrimetrically against triolein, of 26 U per gram of dry solids. This substrate mixture does not suffer from compaction and therefore can be used in large scale solid-state cultivation bioreactors. When used to pretreat a high-fat dairy wastewater, with an oil and grease level above 1300 mg/L, the fermented solid reduced the oil and grease level to below 300 mg/L after 72 h at 35 °C. Further work is required to improve the production of lipolytic activity in the solid-state cultivation step and to find the optimum pretreatment time in the wastewater pretreatment step.

Key words: lipolysis, enzymatic pretreatment, solid-state fermentation, solid-state cultivation, dairy wastewater, *Rhizopus microsporus*

Introduction

High concentrations of oil and grease (O&G) in effluents, such as those issuing from dairy processing factories and slaughterhouses, reduce the efficiency of conventional biological processes for wastewater treatment (*1*). In the activated sludge process, high O&G levels lead to the formation of films around the biological flocs, hindering the transfer of oxygen and substrate to the floc microorganisms and leading to the proliferation of filamentous microorganisms (*2*). This is undesirable, since these filamentous organisms cause the formation of loose flocs, which float in the sedimentation tank. In the case of anaerobic digestion, excessive amounts of O&G inhibit the action of acetogenic and methanogenic bacteria (*1*,*3*,*4*).

Oily effluents can be pretreated with lipases (triacylglycerol acylhydrolases, EC 3.1.1.3), but the viability of such pretreatment processes depends on the costs of the enzyme. Immobilized preparations of commercial lipases have been studied (*5*–*7*), but these are too expensive. The production of lipases by solid-state cultivation (SSC) has the potential to be more economic, with production

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costs in SSC being predicted to be only 30 % of those in submerged culture, if solid agro-industrial residues are used as substrates (*8*). Based on these considerations, a research group at the Federal University of Rio de Janeiro (UFRJ) in Rio de Janeiro, Brazil, has used a 'fermented solid', produced by SSC, to pretreat synthetic and real effluents that contained initial O&G levels above 800 mg/L (*9–15*). The pretreatment led to improved removal of COD (chemical oxygen demand), in subsequent conventional aerobic or anaerobic treatment processes, in comparison with the effluent that was not pretreated (*16*).

The aims of the present study are to produce a fermented solid containing the lipases of *Rhizopus microsporus* and to study the changes that occur during the pretreatment of a high fat dairy wastewater with this fermented solid. During the pretreatment step, we monitored not only the free acid profiles, as previous workers have done, but also the profiles for lipase activity and O&G levels. BOD_5 (biological oxygen demand, 5-day test) and COD tests were also done.

Materials and Methods

Fungal strain and media

Rhizopus microsporus, originally isolated from soil from Guadalajara, Mexico, and found in preliminary tests to produce good levels of lipolytic activity, was identified by CPQBA (Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas) of UNICAMP, Campinas, São Paulo, Brazil, where it is deposited as strain CPQBA 312-07 DRM. Spore suspension was prepared by growing the strain on potato dextrose agar at 30 °C for five days and harvesting the spores with a Tween solution (0.01 % *m/V*). The spore concentration in the suspension was determined using a Neubauer chamber.

Solid-state cultivation

Sugarcane bagasse (donated by Usina de Álcool Melhoramentos, Jussara, Paraná, Brazil), when pretreated, was sieved to obtain particles between 0.85 and 1.7 mm, washed three times with distilled water, followed by drying at 80 °C for 24 h. When not pretreated, it was simply dried, without sieving, and contained particles between 0.8 mm and 1.5 cm. Sunflower seeds (purchased at a local market) were always milled and then sieved to obtain particles between 0.85 and 1.4 mm. Nutrient medium (NM) contained (in g/L): $MgSO_4 \cdot 7H_2O$ 0.2, K_2HPO_4 0.7, KH_2PO_4 0.4, yeast extract 2.0, glucose 1, and 1 % (by volume) olive oil (*17*).

Various wetting solutions were tested, namely: 0.1 M phosphate buffer, pH=7.0; nutrient medium; nutrient medium plus 5 % (by volume) olive oil; nutrient medium plus 5 % (by volume) bovine fat; nutrient medium plus 5 % (by volume) dairy wastewater fat; and dairy wastewater. When fats were used, they were liquefied at 50 °C and then added to the culture medium under vigorous agitation.

Each 250-mL Erlenmeyer flask contained 10 g of dry substrate and wetting solution. Flasks were plugged with cotton wool and autoclaved at 121 °C for 15 min. After cooling, the initial moisture content (around 75–79 % by mass on wet basis) was measured in an infrared moisture balance (Gehaka IV 2000, São Paulo, SP, Brazil) and the flasks were inoculated with spore suspension (1 mL, containing $3.0 \cdot 10^8$ spores) and incubated at 40 °C.

Drying of the fermented solid and extraction of lipolytic activity

For enzyme extraction, each flask received 100 mL of 20 g/L NaCl solution and was then agitated on a rotary shaker for 1 h at 200 rpm and 30 °C. The suspension was filtered through cheesecloth, with the excess liquid being squeezed out manually. The extract was centrifuged for 10 min at 12 500×*g*. In the drying tests, two methods were compared: lyophilization (for 24 h at –40 °C in a Jouan LP3 lyophilizer, Allerød, Denmark) and drying in an oven at 55 °C.

Collection, characterization and preparation of the dairy wastewater

Both effluent samples were collected from the Parshall flume (localized after the equalization tank) in the effluent treatment station of a dairy industry located in the state of Paraná, Brazil and stored at –18 °C until use. The first sample had the following composition (in mg/L): COD 3680, BOD_5 3255, oil and grease 640, total Kjeldahl nitrogen 17.6, total phosphorus 1.76, sedimentable solids 0.30, total suspended solids 752, volatile suspended solids 735, total solids 3160, and pH=13. The second sample had pH=2 and was composed of (in mg/L): COD 2789, BOD_5 2431, oil and grease 427, total Kjeldahl nitrogen 67.9, total phosphorus 11.2, sedimentable solids 0.90, total suspended solids 179, volatile suspended solids 157, and total solids 4138. The analyses were performed according to standard procedures (*18*).

O&G levels above 1000 mg/L are an intermittent problem in the dairy industry; therefore not all samples contain them. Following a strategy previously used by other workers (*9,11*), we obtained such levels by collecting a low-fat wastewater and then supplementing it with extra dairy fat. This dairy fat was collected in the equalization tank of the effluent treatment station of the same dairy industry. It contained 87 % O&G by mass (dry basis) and was kept at –18 °C. It was incorporated into the wastewater using a mechanical impeller to produce a stable emulsion. Depending on the experiment, the volume of the wastewater and the mass of the dairy fat varied. In a typical experiment, 2 L of the effluent and 5 g of the dairy fat were mixed during 4 h at room temperature, at 5000 rpm. The mixture was then adjusted to pH=7.0 and, if sterilized, was autoclaved at 121 °C for 15 min. The wastewater with added dairy fat, prepared as described here, was denominated as 'high fat dairy wastewater' (HFDWW).

Hydrolysis of the high fat dairy wastewater (HFDWW)

Hydrolysis was tested in 250-mL Erlenmeyer flasks with 70 mL of HFDWW. A mass of 0.3 g of dried fermented solid, containing 33 U/gds (lipolytic units per gram of dry solid, measured against tricaprylin), was added per 100 mL of HFDWW (*i.e.* 0.3 % *m/V*). Flasks were incubated at 35 °C and 150 rpm on an orbital shaker. At each sampling time, the contents of three

flasks were filtered through a nylon mesh. As a coarse estimate of fungal growth, the contents retained in this mesh were transferred to a pre-weighed qualitative filter paper and then dried at 55 °C to constant mass. The biomass was calculated as the difference between the mass of these dry solids and the mass of dry solids from autoclaved fermented solid added to the HFDWW in control flasks. The filtrate was used for the analysis of free acids, lipolytic activity (titrimetric method) and O&G content. Another control experiment involved the incubation of HFDWW without the addition of fermented solid. All experiments were done in triplicate.

Determination of colony forming units (CFU)

Samples were diluted with saline solution (9 g/L NaCl), in a 1/10 dilution series until a dilution of 10^{-8}. Spread plates with a nutrient medium, containing (in g/L): peptone 5, meat extract 3, NaCl 1, agar 15, were inoculated with 0.1 mL of each dilution and then incubated at 30 °C for 24 h before counting of the colonies.

Lipase activity measurement

Lipolytic activity was measured directly in the fermented solid, in the aqueous extract prepared from the fermented solid and in the effluent during the enzymatic treatment. Two methods were used: the spectrophotometric method of hydrolysis of *p*-nitrophenyl palmitate (*p*NPP, Sigma-Aldrich, St. Louis, MO, USA) (*19*) and the titrimetric method (*20*), in which tributyrin (C4), tricaprylin (C8) and triolein (C18) were used as substrates (all obtained from Sigma-Aldrich, St. Louis, MO, USA).

For the *p*NPP method, 1 mL of solution A (3 mg of *p*NPP in 1 mL of 2-propanol) was added to 9.0 mL of solution B (50 mM, pH=8, Tris-HCl buffer, polyvinyl alcohol 0.25 % *m/V*), dropwise, with intense stirring. A volume of 0.9 mL of the mixture was transferred to a cuvette and 0.1 mL of aqueous extract containing the enzyme was mixed in. An absorbance coefficient of $1.4 \cdot 10^4$ L/(mol·cm) was used for *p*-nitrophenol. One unit of activity (U) was defined as the production of 1 μmol of *p*-nitrophenol per minute at 40 °C and pH=8.0.

For the titrimetric method, emulsions of the substrates were prepared and the free fatty acids were titrated using 0.05 M NaOH in a pH-Stat (Metrohm 718 Stat Titrino, Herisau, Switzerland) set at pH=7.0. Each substrate, tributyrin (54 mM), tricaprylin (63 mM) and triolein (62 mM), was pre-emulsified with gum arabic (3 % *m/V*), 2 mM $CaCl_2$, 2.5 mM Tris-HCl buffer and 150 mM NaCl (*21*). For each assay, 20 mL of the emulsion and 250 mg of dried solid were placed in a thermostated vessel (37 °C) at pH=7 and the hydrolysis reaction was accompanied for 10 min. One unit of activity (U) was defined as the liberation of 1 μmol of fatty acid per minute, under the assay conditions.

Analytical methods

The parameters COD and BOD_5 were determined according to standard methods (*18*). The O&G content was determined by Soxhlet extraction using hexane as the solvent (*18*). Free acids (FA) were determined by the titration of samples: 5 mL of the effluent (treated or non-treated) were added to 15 mL of ethanol and then titrated, using 0.005 M NaOH, with phenolphthalein as the indicator (*22*).

Results

Production of lipolytic activity in solid-state cultivation

We initially used sunflower seed meal, which was found to induce lipase production in SSC more effectively than several oily agro-industrial residues (*23*). This meal contains 25.9 % (by mass) lipids, 45.7 % carbohydrates and 18 % protein. With this substrate, *R. microsporus* produced a maximum *p*NPP-hydrolyzing activity of 4.0 U/gds at 48 h (Table 1). However, during this culture, the solid substrate compacted significantly, a problem that can be attributed to the high lipid content. If this were to happen in a large-scale production process, it would significantly reduce the ability to aerate the substrate bed. We therefore tested the addition of sugarcane bagasse as a bulking agent, as has been done for the production of biosurfactants by SSC (*24*).

Several different mixtures of sunflower seed meal with sieved, washed and dried sugarcane bagasse were tried (Table 1). With sunflower seed meal alone, the peak of *p*NPP-hydrolyzing activity occurred at 48 h, whereas

Table 1. Production of *p*NPP-hydrolyzing activity by *Rhizopus microsporus* CPQBA 312-07 DRM in solid-state cultivation using different ratios of sunflower seed meal and sugarcane bagasse

Substrates	SSM/SCB	Maximum activity/(U/gds)	Time at which maximum obtained/h	Activity per g dry sunflower seed meal/(U/g)
SSM+SCB	1:9	1.6±0.2	18	16.4
	1:3	3.6±0.1	18	14.3
	1:1	4.6±0.3	18	9.4
	3:1	2.8±0.4	18	3.7
SSM	1:0	4.5±0.4	48	4.5
SCB+NM+olive oil	0:1	0*	–	–
SCB+NM+effluent fat	0:1	0*	–	–
SCB+effluent	0:1	0*	–	–
SCB+NM+animal fat	0:1	0*	–	–

SSM sunflower seed meal, SCB sugarcane bagasse, NM nutrient medium (see Materials and Methods)
* there was no visual evidence of growth

for all mixtures it occurred at 18 h (Table 1). The 1:1 mixture gave a peak activity (4.6 U/gds) that was comparable to that obtained with sunflower seed meal alone (4.5 U/gds), while all the other mixtures gave lower yields. However, although the 1:1 mixture gave the highest activity based on the overall mass of solids, the 1:3 and 1:9 mixtures gave higher activities per mass of sunflower seed meal used. This calculation is important not only because sunflower seed meal is significantly more expensive than sugarcane bagasse, but also because it is desirable to minimize the introduction of oil into the waste treatment system within the fermented solid. For example, based on the initial lipid content of the sunflower seed meal of 25.9 % (by mass) and the addition of fermented solid to the effluent at 0.3 % (m/V), the 1:1, 1:3 and 1:9 mixtures have the potential to contribute up to 390, 194 and 78 mg/L O&G, respectively, to the effluent. The 1:9 mixture gave a slightly higher activity per mass of sunflower seed meal than the 1:3 mixture, but a greater reactor volume would be necessary to produce the same amount of enzyme. Therefore the 1:3 mixture was used for all further studies.

Diaz *et al*. (*25*) produced lipases using sugarcane bagasse as the only solid material, this bagasse being impregnated directly with a nutrient solution. We tried a similar strategy, in which sugarcane bagasse was impregnated with a nutrient medium and an inducing agent (in different treatments, olive oil, fat obtained from a dairy effluent, dairy effluent itself and fat obtained from an abattoir). However, this test was unsuccessful since there was no growth and, as a result, no *p*NPP-hydrolyzing activity was produced (Table 1).

The *p*NPP-hydrolyzing activity was used in the studies described above, due to the convenience of the method. However, since the objective of the process is to use the fermented solid to hydrolyze fats and oils in effluents, it was necessary to demonstrate that the *p*NPP--hydrolyzing activity was in fact indicative of real lipolytic activity. We therefore used the titrimetric method to determine the activity against tributyrin, tricaprylin and triolein for a sample of fermented solid removed at 18 h and added directly into the emulsion. Activities against tributyrin and tricaprylin were similar, with values of (50±5) U/gds and (51±4) U/gds, respectively. The activity against triolein was (26±2) U/gds, confirming the presence of true lipases. Tricaprylin was chosen for further assays since it is easier to prepare emulsions with it than with triolein.

The sugarcane bagasse used in the studies described above had been sieved, washed and dried. Since these processes would be expensive at large scale, we investigated whether they were necessary. A substrate mixture made with sieved, washed and dried bagasse gave a tricaprylin-hydrolyzing activity of 56 U/gds at 18 h, while a substrate mixture made with bagasse that was neither sieved nor washed before drying gave a slightly higher activity, of (61±6) U/gds at 18 h. In the remaining studies the bagasse was therefore neither sieved nor washed.

Up until this point the fermented solid was dried by lyophilization before its activity was determined. However, such a process would be prohibitively expensive at large scale. We therefore tested whether it was feasible to use mild heat drying (55 °C). Also, we incubated a crude aqueous solution of the enzyme extracted from the fermented solid at the same temperature. During the first two hours of drying of the fermented solid, the original tricaprylin-hydrolyzing activity fell by a third (Fig. 1). A similar loss was observed in the crude aqueous extract. However, after this point the behaviour was quite different. The activity in the aqueous extract continued to decrease, until it completely disappeared after 9 h. In the case of the fermented solid, further losses were quite small, with the value stabilizing at around 44 U/gds. When a sample of the fermented solid was dried by lyophilization, the final activity was 47 U/gds, so mild heat drying is a feasible alternative to lyophilization.

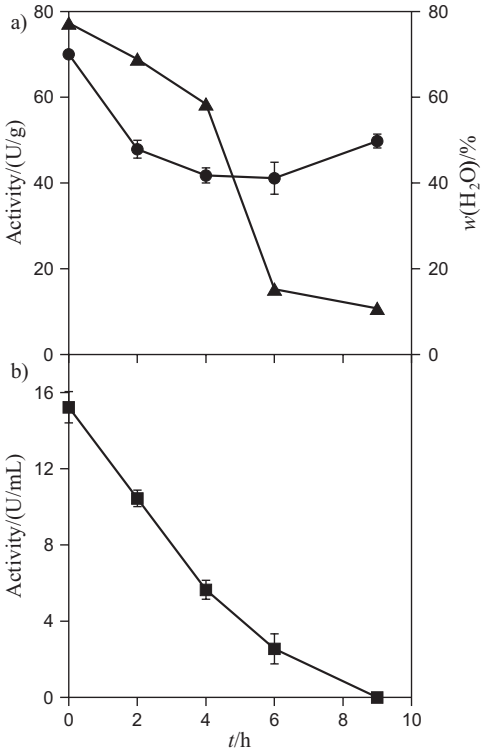

Fig. 1. Loss of lipolytic activity during the drying of the fermented solid (obtained by growing *Rhizopus microsporus* CPQBA 312-07 DRM on a 1:3 (by mass) mixture of sunflower seed meal and sugarcane bagasse): (a) drying of fermented solid at 55 °C, key: (●) residual lipolytic activity and (▲) moisture content; (b) incubation of a crude aqueous extract of the fermented solid at 55 °C, for comparative purposes. The symbols represent the means of triplicate determinations±the standard errors of the means

Experiments with the first batch of high fat dairy wastewater (HFDWW)

In our studies with HFDWW we used O&G contents above 1000 mg/L. Such high contents occur intermittently in dairy wastewaters, causing problems for the operation of biological treatment processes (*10*,*11*). The initial experiments were done with sterilized HFDWW in order to investigate the behaviour of *R. microsporus* in the wastewater, while avoiding interferences caused by the wastewater microflora.

Fermented solid was added at 0.3 % (*m*/*V*) and hydrolysis was followed over 120 h. The rate of O&G removal fell over time (Fig. 2a), which is not surprising since the lipolytic activity detected in the effluent also fell (Fig. 2b). The O&G content of the effluent was reduced to 250 mg/L by 72 h, whereas in control incubations (with sterilized effluent and with sterilized effluent plus autoclaved fermented solid), the O&G content remained at the original level (Fig. 2a). The maximum lipolytic activity in the effluent (3500 U/L) was detected at 12 h (Fig. 2b). The maximum free acid level (4.4 mM) also occurred at this time (Fig. 2c). In the control experiment the free acid contents remained around 3 mM. The fungus grew in the effluent (Fig. 2b). The maximum biomass value was around 1.5 g/L, obtained at 24 h.

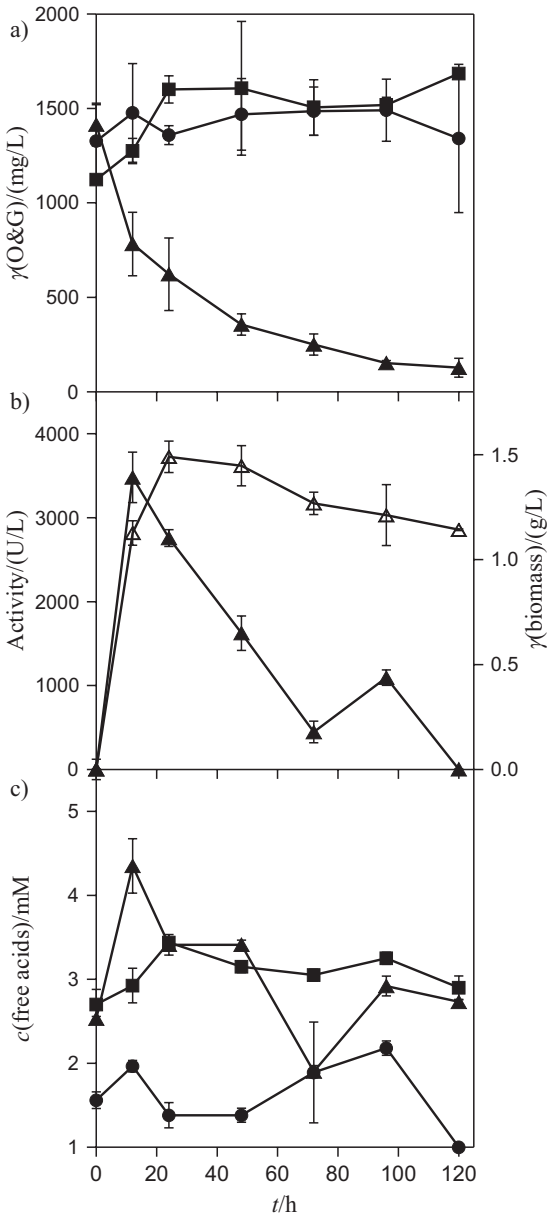

Fig. 2. Reaction profiles during the pretreatment of sterilized high-fat dairy wastewater (HFDWW) with 0.3 % (*m*/*V*) fermented solid: (a) oil and grease (O&G) levels; (b) lipolytic activity (▲) and biomass concentration (Δ); (c) free acid levels. Key to parts a and c: (●) sterilized effluent, (■) sterilized effluent with autoclaved fermented solid, (▲) sterilized effluent with fermented solid. Pretreatment was carried out at 35 °C and 150 rpm. The symbols represent the means of triplicate flasks±the standard errors of the means

We evaluated the COD and BOD_5 of HFDWW pretreated with fermented solid for 12 and 72 h (Table 2). The COD fell throughout the pretreatment process. This was not the case with the BOD_5 value. The addition of the extra fat to bring the original O&G level over 1000 mg/L did not increase the BOD_5 value, indicating that this fat was not used by the sludge microflora during the 5 days of the BOD test. After 12 h of pretreatment, the BOD_5 level had increased by about 1000 mg/L, indicating that the hydrolysis of the fat by the lipase had made the fat available to the sludge microflora. After 72 h of pretreatment the BOD_5 and COD values had both fallen significantly. The fall of the COD during the pretreatment indicates that the fungus had used up a significant amount of organic matter present in the HFDWW, given that the lipolytic activity itself does not remove COD.

Table 2. BOD_5 and COD levels in the first sample of high-fat dairy wastewater (HFDWW) during treatment with 0.3 % (*m*/*V*) fermented solid obtained using *Rhizopus microsporus* CPQBA 312-07 DRM

Time/h	Sample BOD_5/(mg/L)	COD/(mg/L)
0	3250	6908
12	4253	6747
72	1134	2570

Experiments with the second batch of high fat dairy wastewater (HFDWW)

The experiment with the second batch of HFDWW had two aims. Firstly, since a real enzymatic pretreatment process would involve non-sterile wastewater, we included a treatment in which the fermented solid was added to unsterilized HFDWW. Secondly, given that the physicochemical properties and the composition of effluents vary over time, we tested whether our enzymatic pretreatment process would be effective with HFDWW prepared from raw effluent collected at a different time.

Significant removal of O&G was obtained in both sterilized and unsterilized HFDWW treated with fermented solid, with a reduction of over 1000 mg/L (Table 3). When HFDWW was not sterilized and received no fermented solid, the endogenous microflora only removed around 500 mg/L O&G, with the level falling from the original value of 1358 to 848 mg/L at 72 h.

The BOD_5 level remained essentially constant in the sterilized HFDWW that did not receive fermented solid (Table 3). The other three treatments led to significant reduction in BOD_5. As in Table 2, the sterilized HFDWW with fermented solid first increased its BOD_5, from around 3000 mg/L in the original HFDWW to around 4000 mg/L

Table 3. Parameters determined in the second sample of high-fat dairy wastewater (HFDWW) during treatment with 0.3 % (*m*/*V*) fermented solid (FS) obtained using *Rhizopus microsporus* CPQBA 312-07 DR

Time/h	Treatments			
	Sterilized effluent	Unsterilized effluent	Sterilized effluent+FS	Unsterilized effluent+FS
	Colony forming units/(CFU/L)			
0	–	$2.42 \cdot 10^9$	–	$2.42 \cdot 10^9$
12	–	$6.76 \cdot 10^9$	–	$1.63 \cdot 10^{10}$
72	–	$1.07 \cdot 10^{12}$	–	$3.16 \cdot 10^{12}$
	γ(fungal biomass)/(g/L)			
0	–	–	0	0
12	–	–	0.85±0.03	–0.23±0.08
72	–	–	1.53±0.07	–0.39±0.15
	BOD_5/(mg/L)			
0	3130	4499	3130	4499
12	3352	2190	3977	3035
72	3551	808	1329	371
	COD/(mg/L)			
0	5378	5892	5378	5892
12	5657	7335	6235	6733
72	6813	4048	2988	3126
	γ(O&G)/(mg/L)			
0	1598±20	1358±20	1598±20	1358±20
12	1357±16	1210±4	1118±74	1088±51
72	1345±22	848±50	348±17	268±25

at 12 h, and then decreased to a value around 1000 mg/L at 72 h. In both unsterilized treatments the BOD_5 decreased throughout, reaching values below 1000 mg/L. The greatest removal was in the unsterilized HFDWW with fermented solid, indicating synergistic action between the enzyme released from the fermented solid and the endogenous microorganisms of the effluent.

The COD in the sterilized HFDWW without the addition of fermented solid did not decrease over the 72 h of the pretreatment process (Table 3). In the other three treatments, by 72 h the COD had been reduced by between 1800 and 2700 mg/L.

In the unsterilized HFDWW, both with and without the addition of fermented solid, the viable count of the endogenous microorganisms increased by several orders of magnitude, from an initial value of $2.4 \cdot 10^9$ to over $1 \cdot 10^{12}$ CFU/L at 72 h. Fungal biomass developed in the sterilized HFDWW to which fermented solid had been added, reaching 1.53 g/L at 72 h (Table 3). However, there was no production of fungal biomass when fermented solid was added to unsterilized HFDWW. Indeed, in this case the fermented solid lost mass during the incubation. Growth of the fungus is therefore inhibited by the microflora of the original effluent, so the contribution of the fermented solid in the unsterilized HFDWW is due to the lipolytic activity produced in the prior SSC process.

Discussion

The present work makes two contributions. Firstly, it provides an insight into the phenomena that occur during the enzymatic pretreatment of high-fat effluents. Secondly, although optimization of the production of the enzyme was not the major aim, our results allow us to undertake a preliminary evaluation of the potential of our system. These two items will be discussed separately below.

The insights obtained into the pretreatment process

Our work represents the first time that lipase activity, O&G, BOD_5 and COD have been followed during the treatment of the wastewater with fermented solid. Previous workers who have used fermented solid in enzymatic pretreatment processes have demonstrated that the pretreatment step leads to better COD removal in a subsequent anaerobic digestion, but they have focused their characterization of the pretreatment step on free acid levels (*9–12,14,26*). There is no guarantee that selecting pretreatment times solely on the basis of free acid levels will optimize the efficiency of the treatment in subsequent conventional effluent treatment processes.

In the first pretreatment experiment, lipase levels in the effluent were reasonably high (above 1500 U/L) until 48 h, suggesting that it may be appropriate to carry out the pretreatment for slightly longer than the times of

8 to 24 h that have been used by previous workers (*9–12*,*14*,*26*). However, the use of longer pretreatment times to increase O&G removal must be balanced against the greater capital costs associated with the construction of larger pretreatment tanks. In fact, it would be desirable to investigate the effect of time of pretreatment, and the resulting values for lipase activity and free acid, BOD_5, COD and O&G levels obtained in the pretreatment process, on the subsequent efficiency of the treatment of the effluent in conventional processes. Special attention should be given to the BOD_5 to COD ratio, since low values of this ratio can compromise the efficiency of conventional processes (*27*,*28*). Based on this consideration, pretreatment times as long as 72 h should be avoided: from the results shown in Table 3, the pretreatment of unsterilized effluent with 0.3 % (*m*/*V*) fermented solid during 72 h led to a low BOD_5 to COD ratio of 0.12.

The potential of the pretreatment process with Rhizopus microsporus

The only previous work that has been undertaken with the addition of fermented solid to pretreat high-fat effluents is that of the group at UFRJ (*16*). Their production process involves the growth of *Penicillium restrictum* on a solid substrate in which the major component is babassu cake, a waste material left over after the recovery of oil from seeds of the babassu palm. In their system, lipolytic activities of around 30 U/gds after 20 h of fermentation have been reported, obtained using the titrimetric method with olive oil (*14*). Our production process has a comparable yield, with 26 U/gds being obtained, using the titrimetric method with triolein, a not too different substrate.

The UFRJ process involves a substrate composed totally of waste materials, namely babassu cake supplemented with molasses (*14*), while our substrate contains a non-waste component, namely ground sunflower seeds. In laboratory-scale production studies done in tray-type bioreactors (*i.e.* without forced aeration), their substrate would be preferable because of its lower cost. However, if an enzymatic pretreatment process were to be implemented in a wastewater treatment plant, it would probably be necessary to produce the fermented solid in a large-scale forcefully-aerated bioreactor. In such a bioreactor, babassu cake, with the particle size of 0.21 to 0.42 mm used by Rosa *et al*. (*14*), would be likely to form compact masses, leading to large pressure drops and poor aeration of the bed. Our use of sugarcane bagasse as a bulking material has the potential to prevent such problems from occurring at large-scale, as we have demonstrated using 8 kg of substrate (dry basis) in an intermittently-agitated, forcefully-aerated bioreactor (results not shown). Of course, we need to substitute the ground sunflower meal with a waste material that will induce lipases. Note that this would not include babassu cake, which is not produced in southern regions of Brazil. In an SSC process for production of fermented solid it would be essential to minimize transport costs by using locally available wastes.

The work of Diaz *et al*. (*25*) suggests that it should be possible to increase lipase production levels significantly. When they grew a related strain, *Rhizopus homothallicus*, in SSC, on a medium consisting of sugarcane bagasse impregnated with a liquid broth containing olive oil, they obtained tricaprylin-hydrolyzing activities (determined by the titrimetric method) of up to 1500 U/gds at 12 h. The most probable explanation as to why their tricaprylin-hydrolyzing activities were around 30-fold higher than those obtained in the current work is that they used forcefully-aerated column bioreactors, whereas we used 5-cm high layers in Erlenmeyer flasks without forced aeration. As a result, oxygen limitation is likely to be more severe in our system. In fact, the absence of forced aeration, combined with poor bed porosity due to the presence of fat in the impregnating media, might have contributed to the lack of growth in the last four treatments shown in Table 1.

It would be highly desirable to achieve levels of lipolytic activity comparable to those obtained by Diaz *et al*. (*25*). In the current work we added 0.3 % (*m*/*V*) to the HFDWW. In other words, it would be necessary to add 3 kg of dry fermented solid per cubic meter of effluent. Given the large effluent volumes generated by dairy industries, this would imply the need for the production of large quantities of fermented solid. With a fermented solid containing 1500 U/gds, it would be possible not only to reduce the required production levels by an order of magnitude (*i.e.* adding solids at a rate of 0.3 kg per cubic meter of effluent) but also to reduce the effluent pretreatment time.

It is not possible to compare directly the effectiveness of our fermented solid in the pretreatment process with that used by the UFRJ group, since they evaluated the efficacy of their pretreatment on the basis of a subsequent anaerobic treatment, whereas we did our evaluation on the basis of BOD_5, COD and O&G analyses. In any case, our results are promising and suggest that we should work to optimize the system involving *Rhizopus microsporus* CPQBA 312-07 DRM, including both the production of the fermented solid and its application in the pretreatment of the high-fat effluent.

Conclusions

Rhizopus microsporus CPQBA 312-07 DRM produced a lipolytic activity against triolein of 26 U/gds when grown in solid-state cultivation on a 1:3 (by mass) mixture of sunflower seed meal and sugarcane bagasse. When this fermented solid was dried and added at 0.3 % (*m*/*V*) to a high fat dairy wastewater, the oil and grease level decreased from 1358 to 268 mg/L after 72-hour incubation at 35 °C. However, such long pretreatment times lead to a wastewater of relatively low biodegradability (BOD_5/COD ratios as low as 0.12). Further work is required to improve the production of lipolytic activity in the solid-state cultivation step and to find the optimum pretreatment time in the wastewater pretreatment step.

Acknowledgements

The work was supported financially by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brasil), a Brazilian government agency for the advancement of science and technology. Research scholarships were granted to David Mitchell, Nadia Krieger, Patrício Zamora and Dayane Alberton by CNPq.

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