SELECTION OF MICROORGANISMS PRODUCER OF LIPASE FOR FAT REMOVAL FROM BIODIESEL PURIFICATION WATER

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ABSTRACT: The objective this study has been the selection of lipase productor microorganism, for removal of oils and grease, in the pre-treatment of biodiesel wastewater washing. For this, analyses of the physicist-chemistries characteristics had been made with the wastewater of the biodiesel washing, and then it had been isolated and chosen, by means of determinations of the lipase activity. Following, it was made a test of fat biodegradation, in the conditions: pH (5.95), temperature (35 ºC), rotation (180 rpm) and ammonium sulfate as nitrogen source (3 g L^{-1}) and establishing as variable the two microorganism preselected and the time (24; 48; 72; 96 and 120 h). The biodiesel purification wastewater had presented high potential of environmental impact, presenting a concentration of O&G of 6.76 g L^{-1}. From the six isolated microbiological cultures, two microorganisms (A and B) had been selected, with enzymatic index of 0.56 and 0.57, respectively. The treatment of the wastewater using the isolated microorganism (*Klebsiella oxytoca)* had 80% of the fatty removal in 48 h.

KEYWORDS: enzymatic degradation, fatty effluent, lipolytical activity.

SELEÇÃO DE MICRORGANISMOS PRODUTORES DE LIPASE PARA REMOÇÃO DE GORDURA DE ÁGUA DE PURIFICAÇÃO DO BIODIESEL

RESUMO: Este trabalho teve por objetivo selecionar cepas de microrganismos com alto potencial de produção de lipase, a fim de remover óleos e graxas, no pré-tratamento da água residuária de purificação do biodiesel. Para tanto, foram feitas análises das características físico-químicas do efluente, e a partir desse resíduo foram isolados e selecionados microrganismos com potencial de produção de lipase. Em seguida, foi feito um ensaio de biodegradação de gordura, nas condições: pH (5,95), temperatura (35 ºC), rotação (180 rpm) e sulfato de amônio como fonte de nitrogênio (3 g L^{-1}), e tendo como variáveis os microrganismos pré-selecionados e o tempo (24; 48; 72; 96 e 120 h). A água residuária de purificação do biodiesel apresentou uma concentração de O&G de 6,76 g L^{-1}. Das seis culturas microbianas isoladas do tanque de decantação deste efluente, dois microrganismos (A e B) foram selecionados, com índice enzimático de 0,56 e 0,57, respectivamente. O tratamento da água residuária, utilizando o microrganismo isolado *Klebsilla oxytoca*, obteve uma remoção de O&G de 80% em 48 h.

PALAVRAS-CHAVE: biodegradação enzimática, efluentes gordurosos, atividade lipolítica.

INTRODUCTION

The high demand for energy in the industrialized world and in the domestic sector as well as the pollution problems caused due to the wide use of these fuels, have stimulated recent interests in finding alternative sources for petroleum-based fuels (GERIS et al., 2007).

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Therefore, vegetable oils and animal fat have been studied as potential renewable source for biofuel production. The transesterification process is the most frequently used for production of biodiesel. This process consists in the chemical reaction of vegetable oils or animal fat with alcohol, ethanol or methanol, in the presence of a basic catalyst, acidic or enzymatic (RAMOS et al., 2003). After this production process, during the stage of biodiesel purification, there is the formation of wastewater with high content of oils and greases, which have the potential to contaminate water bodies, damaging the balance necessary to maintain aquatic ecosystems.

The biodiesel wastewater purification features several parameters, such as oil and grease, DQO, DBO, color, turbidity, pH, not complying with resolution of CONAMA nº 430/2011 laying down detailed conditions for the discharge of effluents into water bodies observing a need of treatment of these effluents (DRANKA et al., 2008).

In order to try solving these problems, alternative methods have been used in order to reduce the concentration of lipids contained in effluents with high organic load. The enzymatic treatment of effluents is interesting because it helps to improve the efficiency of conventional treatments (VALLADÃO et al., 2007).

The use of enzymes in effluent treatment offers several potential advantages, such as simplicity and ease in process control; there is no need of acclimatization of biomass; there is no effects of shock by load of pollutants; may be applied in processes with high or low concentration of pollutants and operate in wide ranges of pH, temperature and salinity. Among different possibilities, the enzymes may be used in the treatment of effluents generated in the oil, textile, paper and cellulose derivatives, foods in general, among other (MENDES et al., 2005).

One proposal is to use enzymatic hydrolysis by lipases for removing oil and grease from these effluents, either by the use of microorganisms that produce lipase, or by the use of enzymes (isolated or not) (DURLI, 2007).

These enzymes feature particular importance, by the fact that they hydrolyze specifically fats and oils, which may be of great interest for the treatment of effluents with high fat content (MENDES et al., 2005). Lipase (triacylglycerol hydrolases, EC 3.1.1.3) is responsible for the catalysis of reactions involving hydrolysis and ester synthesis from glycerol and long-chain fatty acids, causing them to be considered as an important group of biocatalysts, occurring its performance in oil-water interface (CASTRO & ANDERSON, 1995).

The application of a pre-treatment to hydrolyze and dissolve fats can improve the biological degradation of effluents with a high content of oils and greases, accelerating the process and reducing the time of it. (ALESSI, 2005). Thus, this study aimed to select strains of microorganisms with high potential of lipase production, in order to remove oils and greases, in the pre-treatment of the wastewater of biodiesel purification.

MATERIAL AND METHODS

The experiment was conducted in the "Laboratório de Saneamento Ambiental e Enzimologia e Tecnologia das Fermentações da Universidade Estadual do Oeste do Paraná - UNIOESTE" (Laboratory of Environmental Sanitation and Enzymology, and Fermentation Technology of the State University of Western Paraná - UNIOESTE) - *campus* Cascavel, state of Paraná, Brazil. It was used the wastewater of biodiesel purification from an industry that produces biofuel in the northern Paraná, in which the process occurs by transesterification between soybean oil and methanol, with sodium methylate as catalyst.

The effluent was collected in the output, after the purification process, end of the box of fat retention, in sterilized glass vials, packed in thermic box for transportation to the laboratory.

For the characterization of this residue, parameters and methodologies (described in Table 1) were considered.

TABLE 1. Parameter evaluated for wastewater characterization.

Parameter	Unity	Method	Protocols APHA, AWWA, WEF (2005)	Different Method
ST	mg L^{-1}	gravimetric	2540-B	
SV	mg L^{-1}	gravimetric	2540-E	
O&G	mg L^{-1}	gravimetric by centrifuging	-	SUEHARA et al. (2005)
Turbidity	UNT	nephelometric	2130-B	
Color	UC	spectrophotometric	2120-C	
pH	-	potentiometric	4500-H^+ - B	
NTK	mgN L^{-1}	Semi-micro-Kjeldahl	4500-N_{org}-B	
Free acids	%	titrimetric	-	ROSA (2004)

The microorganisms were isolated in Petri plates previously prepared and sterilized, containing culture medium composed of (g L^{-1}): ammonium sulphate 1.0; yeast extract 1.0; potassium phosphate diacid 0.2; sulfate magnesium sulfate heptahydrate 0.2; and soybean oil 1.0. Through the streak-plate technique of the wastewater was inoculated on the plates, which were incubated for 24 hours at 30 °C for growth of microorganisms (SUEHARA et al., 2005). This procedure was repeated until each colony was found totally isolated from each plate.

Each microorganism isolated was tested regarding the potential of biodegradation of the waste under study, by determining the enzyme (lipase). To this end, a handle of the inoculum was inoculated isolated in Petri plates containing (g L^{-1}): peptone 10.0; sodium chloride 5.0; calcium chloride dihydrate 0.1; and agar 18.0. The plates were incubated at 30 °C for 96 hours and the Enzyme Index (EI) was determined after this time. The EI expresses the relationship between the diameter of the degradation halo of the substrate by the diameter of the growth of the microorganism colony (VIEIRA et al., 2006).

In order to define which microorganism, previously selected, had higher efficiency in the fat removing from the effluent under study, and find their respective times of maximum degradation, it was carried out a degradation test with the two strains with higher enzyme activity previously selected.

For this purpose, standardization of the inoculum was performed by optical density, adjusting the absorbance of the inoculum in a spectrophotometer at 580 nm wavelength, adding the cell suspension, at a dilution 10^{-1}, until reaching the absorbance of 0.2. This absorbance corresponded to 3.6×10^8 cells.mL^{-1} for the two microorganisms, measured by cell count after the construction of a dilution curve. The substrate used was prepared with 50 mL of biodiesel washing water, added a nitrogen source (3 g L^{-1} $(NH_4)_2SO_4$). The amount of microorganism seeded on the agar plate should be standardized across all samples to be tested to enable comparison of results (TORTORA et al., 2006).

The biodegradation test was conducted under the conditions of 35 °C and 180 rpm rotation , having the microorganisms preselected (A and B) and the time (24; 48; 72; 96 and 120 h) as variables, with three replications for each sample. After cultivation, the substrate was spun at 2000 rpm for 15 minutes, the precipitate was discarded and in the supernatant was analyzed the content of O&G (SUEHARA et al., 2005) and the lipase activity (VIEIRA et al., 2006).

The previously selected microorganisms, used in the initial test of degradation of lipids, were identified as to their morphology and Gram staining. After defining the microorganism with higher efficiency of fat removal, they were sent to the Institute André Tosello (in Campinas, state of São Paulo, Brazil), in order to identify genus and species, by means of biochemical methods.

The evaluation of the results was performed by analysis of variance (ANOVA parametric) supported by the software *Statistica 7.0*®. Differences between means were evaluated by Tukey's test *a posteriori*. For all analyzes it was adopted a significance level of 5% ($p < 0.05$).

RESULTS

Characterization of the residue

The obtained values in the characterization of the residue are shown in Table 2. Only the pH shows value within the parameters established by CONAMA Resolution 430/2011, for effluent release. The other parameters show high values, requiring treatment for their subsequent release into water bodies.

TABLE 2. Characterization of biodiesel wastewater purification.

Parameters	Unity	Mean value	Standard deviation	Variation coefficient (%)
ST	g L^{-1}	23.89	3.01	12.60
SV	g L^{-1}	18.48	2.65	14.34
O&G	g L^{-1}	6.76	0.32	4.73
TURBIDITY	UNT	290	-	-
COLOR	UC	5.450	-	-
pH	-	6.2	0.10	1.61
NTK	mgN L^{-1}	0	0.00	0.00
Free acids	%	17.21	0.25	1.45

According to DRANKA et al. (2008), the pH found in their study of wastewater of biodiesel purification was 6.3, similar to that presented in this study of 6.2. In the levels of oil and grease (O&G) it was obtained a value of 2678 ± 10 mg L^{-1}, value lower than that found in the residue of this research, underlining the need for treatment with the adoption of a tightening of discharge standards of residual water. About the amount of nitrogen present in the washing water from biodiesel was null, demonstrating the need for the addition of this nutrient in possible biological treatments.

To control such situation, the National Council of Environment (CONAMA), by resolution 430 of May 13, 2011, settled conditions for release effluents into water bodies: pH from 5.0 to 9.0; temperature less than 40 °C; concentration of mineral oil up to 20 mg L^{-1}, and for vegetable oils and animal fats up to 50 mg L^{-1} (CONAMA, 2011).

Microorganisms selection

Based on the diameters of halos and colonies found in the test of the activity of lipase production, it was determined the enzymatic index - EI (Table 3). The lower the EI, the greater the potential presented by the microorganism for enzymatic production; if the EI is equal to 1.0 means that the enzyme production is null; if it is between 1 and 0.64 the production is positive; and if it is less than 0.64 indicates the microorganism has a production strongly positive (RIBAS et al., 2009).

TABLE 3. Average enzymatic index (EI) found for each microorganism, based on the relationship between colony diameter and colony diameter + halo.

Microorganism	Colony Diameter (cm)	Colony Diameter +Halo (cm)	EI
A	2.67	4.37	0.56
B	2.20	3.77	0.57
C	3.17	4.53	0.69
D	3.90	5.00	0.74
E	3.07	4.13	0.74
F	1.33	1.33	1.00

Thus, it can be observed that, the isolated strain "F" showed no production of lipase and the "A" has the highest enzyme activity, but most strains with a positive production (Figure 1).

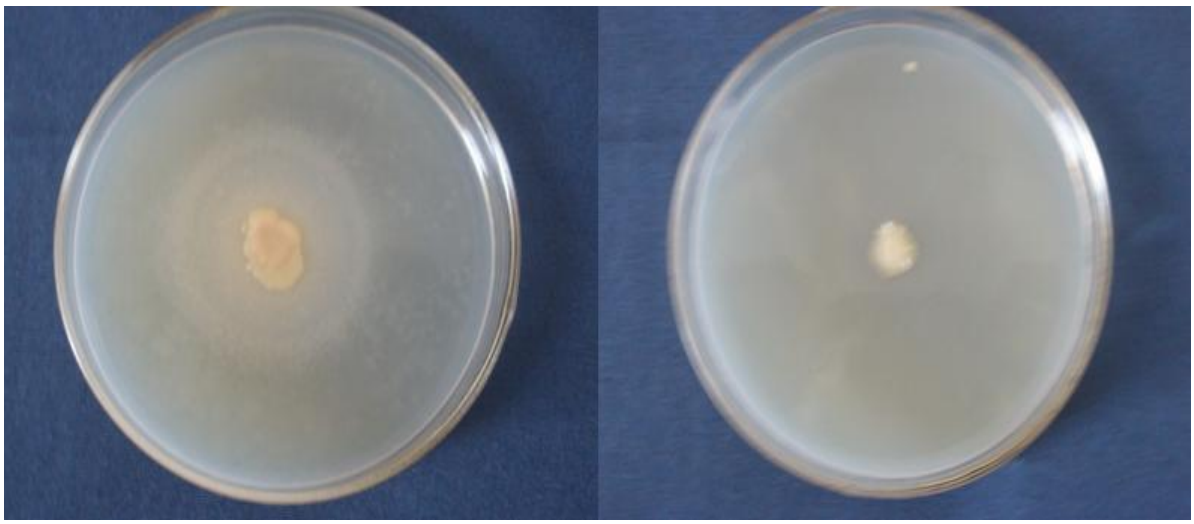
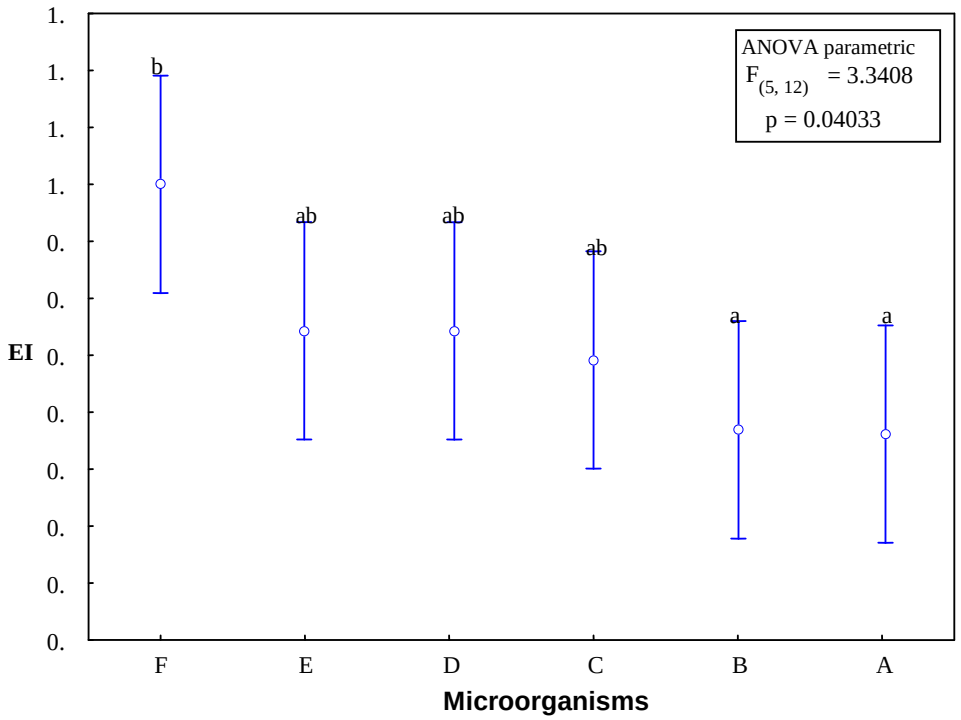


FIGURE 1. Pictures of the isolated strains; on the left (microorganism B) with degradation halo; on the right (microorganism F) without degradation halo.

Based on statistical analysis carried out, the significance of the variable in the microorganism producing the enzyme may be confirmed. The microorganisms identified with A and B were statistically similar, at 5% significance level, and the lowest EI, that is, the highest potential for fat degradation (Figure 2).



* Different letters indicate significant differences (p <0.05) by Tukey's test *a posteriori*.

FIGURE 2. Mean ± 95% EI for enzymatic index values obtained by different microorganisms.

LOPERENA et al. (2009), when isolating microorganisms natives of aerobic treatment of wastewater from dairy products, 41 cultures were obtained, from which 19 have produced lipase, 12 protease and two cultures have produced both enzymes, and 21 had high potential of lipolytic activity and / or proteolytic activity.

DARTORA et al. (2003) in order to select microorganisms that produce extracellular enzymes of the type lipases have isolated microorganisms using liquid culture medium based on cheese whey

under conditions of 30 °C for 48 hours. As a result, from 12 isolated strains (seven yeast strains, two filamentous fungi and three bacteria), demonstrated lipolytic activity through enzymatic index (EI) in three yeast strains, two strains of bacteria and two filamentous fungi are producers of lipases.

VIEIRA et al. (2006), studying the ability of degradation of biodiesel from palm oil by bacteria previously isolated from diesel storage tanks, with capacity of degrading diesel, ascertained that 16 out of 25 bacterial isolates, or 64%, had at least one enzyme activity (lipase and/or esterase). Moreover, they concluded that the test of enzyme activity using Tween 20 and Tween 80 proved to be effective for the selection of bacteria with the capacity of using biodiesel, because in the degradation tests of biodiesel, the selected bacteria were able to use this product.

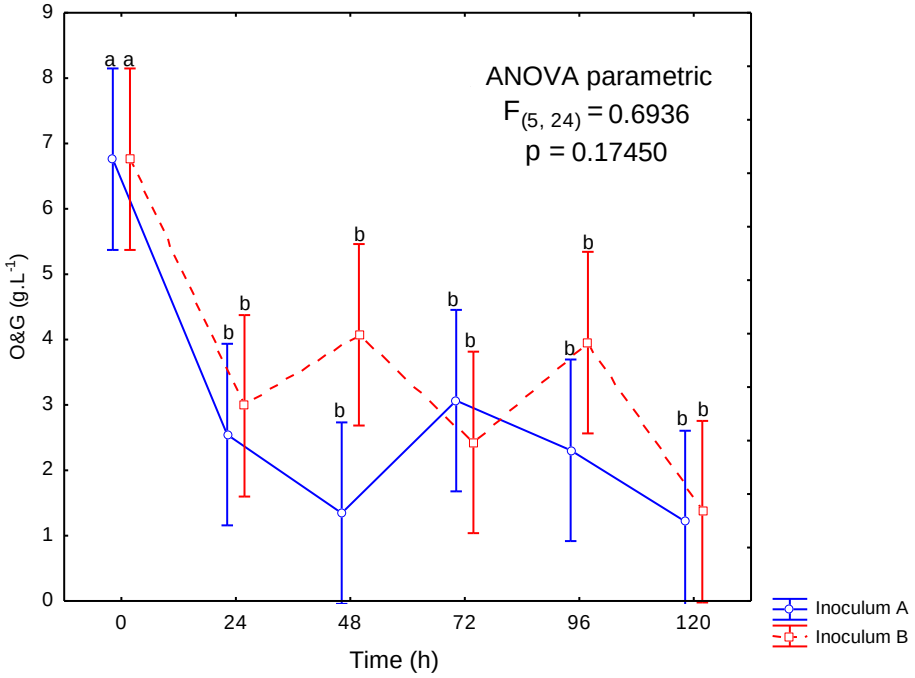
Test of degradation of lipids

Considering that the content of oil and grease (O&G) as a variable of response, and the type of inoculums used and the incubation period as predictors in the ANOVA test, it can be verified that only the variable "time" has been statistically significant at 5% probability (Table 4).

TABLE 4. Analysis of variance (ANOVA) for O&G content.

	Sum of the Squares	Liberty level	Sum of Means	F	P
Time	101.67	5	20.33	14.97	0.000
Inoculums	4.68	1	4.68	3.44	0.076
Time*Inoculums	11.50	5	2.30	1.69	0.175
Error	32.59	24	1.36		

Based on the curve of reducing the content of O & G, throughout the 120 hours of incubation for two selected microorganisms (Figure 3), it can be observed that despite not differ statistically, the microorganism A showed a removal of fat higher than the microorganism B. At time of 120 hours, the microorganism "A" provided a removal of 87% of the fat of the effluent under the conditions, producing a lipase activity of 0.30 U mL^{-1} min^{-1}, compared to 79% of removal of fat and lipase activity of 0.29 U mL^{-1} min^{-1} produced by microorganism “B”. The variable "time", as from 24 hours of incubation, variation was not made in the removal of fat in the Tukey test (0.05), at the time of 48 h the microorganism removed 80% of the O&G of effluents.



* Different letters indicate significant differences (p <0.05) by Tukey's test *a posteriori.*

FIGURE 3. Assay of reducing of O&G content.

DURLI (2007), in order to reduce the amount of fat contained in the effluent, used lipases of *Burkholderia cepacia* LTEB11, and the removal of O&G predicted by the model was 60%, after 48 hours, under a condition from 29 to 32 ºC, pH from 8.0 to 9.2.

RAJENDRAN & THANGAVELU (2009) evaluated the lipase production by *Rhizopus arrhizus*, in a mixture of components, and found a maximum value of lipase production equal to 3.98 U mL^{-1}. BROZZOLI et al. (2009) have produced lipase using wastewater of the industry producer of olive oil as the culture medium for *Candida cylindracea* and obtained values that reached 20.4 U mL^{-1}. These values are much greater than the lipase production obtained in this experiment, possibly, due to the characteristics of the waste that have not favored microbial growth.

Microorganism Identification

Morphological analysis showed that two strains with high potential for microbial production of lipase, known as A and B, are bacteria which have the form of bacilli with coloring negative Gram (Figure 4).

The strain called "A", which presented the best results for removal of fat of the wastewater from biodiesel washing, under the conditions studied it was identified as *Klebsiella oxytoca* (Flugge 1886) Lautrop 1956 (Figure 4). This is a microorganism that is found in the intestinal tract of living beings and animal, and it may be isolated from various pathological processes and from the aquatic and botanical environments.

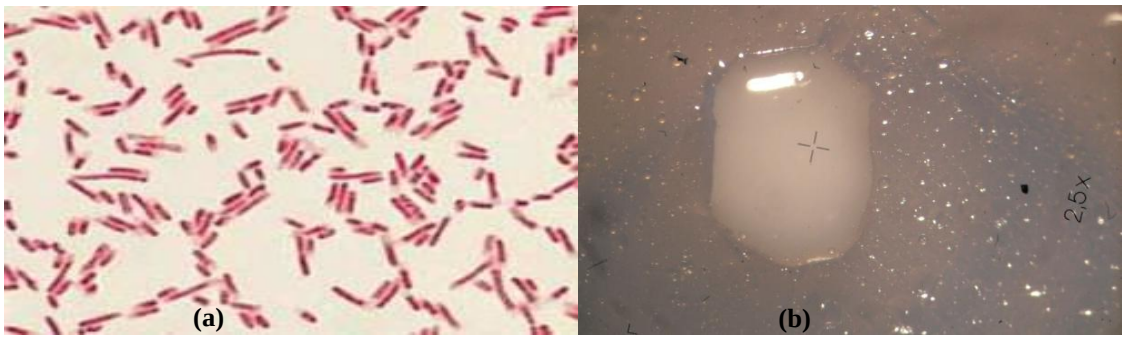


FIGURE 4. (a) Illustrative picture of Gram negative bacilli. (b) Photograph of the isolated colony of Klebsiella oxytoca (Flugge 1886) Lautrop 1956.

CONCLUSIONS

However, it is concluded that the biodiesel wastewater purification, has a high potential of environmental impact and needs an efficient treatment to fit the standards of releases required by CONAMA 430/11, the concentration of oil and grease (O&G) with a concentration of 6.76 g L^{-1} is highlighted.

From the six microbial cultures isolated from the settling tank, where the collect from this effluent took place, two microorganisms were selected presenting high potential for production of lipase, with enzymatic index of 0.56 and 0.57. These microorganisms are, therefore, indicated for further study with the aim of being used in the pretreatment of removal of fat from wastewater of biodiesel purification, once they have adapted to the residue with such specific characteristics. We highlight that the isolated strain *Klebsiella oxytoca* the one that achieved better treatment outcomes.

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