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Biodegradation of dairy wastes using crude enzymatic extract of *Yarrowia lipolytica ATCC 9773*

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Arnulfo Tarón Dunoyer[1*]; Rafael Emilio González Cuello[1]; Rosangela Perez Salinas[2]

[1]Faculty of Engineering. University of Cartagena, Campus Piedra de Bolivar. Consulate Avenue, 30th Street, n° 48-152, Cartagena, Colombia. E-mail: rgonzalezc1@unicartagena.edu.co
[2]Faculty of Health Sciences. University of Santander, Center neighborhood, 6th Race, n° 14-27, Valledupar, Colombia. E-mail: ros.perez@mail.udes.edu.co
*Corresponding author. E-mail: atarond@unicartagena.edu.co

ABSTRACT

Effluents generated by the food industry have become a serious environmental concern. Bioremediation is a biological process developed as an alternative for the treatment of contaminated areas. In current research, the biodegradation of fat, Biochemical Oxygen Demand (BOD_5), Chemical Oxygen Demand (COD) and total solids were evaluated in dairy waste employing enzymatic extract of *Yarrowia lipolytica* ATCC 9773 as biological agents. All the variables were determined following the specifications of the Standard Methods of the American Water Works Association. Enzymatic extract of *Y. lipolytica* at different concentrations (8, 12 and 16.0%) was used in a fermentative medium at two pHs (5.0 and 6.5) for 32 h. The highest percentages (%) of fat (82.88), BOD (43.32), COD (44.3) and total solids (13.58) removal were obtained using an inoculum concentration of 16% at pH 5.0 for 32 h of fermentation. These results may have industrial relevance for the reduction of contamination of industrial effluents with high levels of fat and other contaminants.

Keywords: biological treatment, fatty effluents, removal, *Y lipolytica.*

Biodegradação de um residuo leiteiro usando *Yarrowia lipolytica* ATCC 9773

RESUMO

Os efluentes gerados pela indústria de alimentos tornaram-se uma séria preocupação ambiental. A biorremediação é um processo biológico desenvolvido como alternativa para o tratamento de áreas contaminadas. Na pesquisa atual, a biodegradação de gordura, a demanda bioquímica de oxigênio (DBO_5), a demanda química de oxigênio (DQO) e os sólidos totais foram avaliados como rejeitos lácteos empregando extrato enzimático de *Yarrowia lipolytica* ATCC 9773 como agentes biológicos. Todas as variáveis foram determinadas seguindo as especificações dos Métodos Padrão da American Water Works Association. O extrato enzimático de Y. lipolytica em diferentes concentrações (8, 12 e 16,0%) foi utilizado em meio fermentativo a dois pH (5,0 e 6,5) durante 32 h. As maiores porcentagens (%) de remoções de gordura (82,88), DBO (43,32), DQO (44,3) e sólidos totais (13,58) foram obtidas utilizando

uma concentração de inóculo de 16% a pH 5,0 durante 32 h de fermentação. Esses resultados podem ter relevância industrial para a redução da contaminação de efluentes industriais com altos níveis de gordura e outros contaminantes.

Palavras-chave: efluentes gordurosos, remoção, tratamento biológico, *Y lipolytica*.

1. INTRODUCTION

The food industry is a sector with a high incidence of environmental pollution. Different industries such as dairy, meat and vegetable oil refining produce large amounts of wastewater. The high oil content of these increase the spoilage of some ecosystems (Porwal *et al.,* 2015).

Dairy wastes are pollutants that affect the environment when they are discarded without adequate treatment (Liu *et al.,* 2015b). In the last decades, fatty effluents have been released to the environment without previous treatment (Kumari *et al.,* 2017; Tarón-Dunoyer *et al.,* 2017), which affects public health and environmental sustainability. Considering the aforementioned, the treatment of fatty effluents is an economic and hygienic requirement.

Many of these effluents require pretreatment in order to remove incompatible substances before they are discharged into sewer systems (Kumari *et al.,* 2017; Pilusa *et al.,* 2013). The main components present in wastewater are: oils, fats and long-chain fatty acids, which represent a great problem in the pretreatment due to the fact that they are contaminants of aquatic ecosystems (Becerra-Gutiérrez *et al.,* 2015; Fachin *et al.,* 2013; Abass *et al.,* 2011)

Currently, there has been an emphasis on finding new biotechnological alternatives for the treatment of wastewater that also minimize the adverse effects previously mentioned. Biological treatment is one alternative used to decontaminate wastewater, where lipolytic properties of living organisms are employed to eliminate high-fat waste from the aquatic ecosystem (González *et al.,* 2012; Kushwaha *et al.,* 2011). Various microorganisms such as filamentous fungi, bacteria and yeast are well known as lipolytic microorganisms. Within this group of microorganisms, the yeast *Yarrowia lipolytica* is highlighted due to its extracellular and intracellular activity (Darvishi *et al.,* 2017). Its lipase production depends on the medium composition and environmental conditions (Deive *et al.,* 2010).

Yarrowia lipolytica is an adequate biological agent for biodegradation of substrates with a high fat content (Lopes *et al.,* 2018). *Y. lipolytica* has been approved as GRAS (Generally Recognized As Safe) in several industrial processes. *Yarrowia sp. have* been isolated from lipid-rich foods such as cheese and olive oil as well as from wastewater (Aloulou *et al.,* 2007). The preference of *Y. lipolytica* for these substrates has been attributed to the efficient production and secretion of proteolytic enzymes (Fickers *et al.,* 2005; Liu *et al.,* 2015a; Brigida *et al.,* 2014). In this sense, there are no references in the literature regarding the use of crude enzymatic extract for the biodegradation of dairy waste. Hence, this research focused on the utilization and evaluation of the biodegradation capacity of the crude enzymatic extract of *Yarrowia lipolytica* ATCC 9773 in dairy waste.

2. MATERIALS AND METHODS

2.1. Biological material

Yarrowia lipolytica strain (ATCC 9773) was purchased from Medimark © Europe, 38033 Grenoble Cedex 2. France.

2.2. Industrial dairy waste

The dairy waste sample was obtained from a dairy industry located in Valledupar (Colombia). The sample was collected in an 8 L plastic container. The container used for sample

collection was pre-treated by washing with alcohol and later rinsed four times with distilled water. The sample was stored at a temperature below 4°C to avoid any physico-chemical changes in the effluent. Finally, the sample volume was divided in order for the pH values to be adjusted to 5.0 and 6.5 by employing HCl solution 0.5 N.

2.3. Activation and conservation of *Yarrowia lipolytica* ATCC 9773

The strain was inoculated at 25°C for three days in petri dishes containing PDA agar (Figure 1a) and olive oil as a lipid source. A microscopic morphology (Figure 1b) was then carried out employing lactophenol blue as colorant. Lastly, 0.5 mL of the inoculum was adjusted by turbidimetry at MacFarlan scale (3) and stored at 4°C until use.

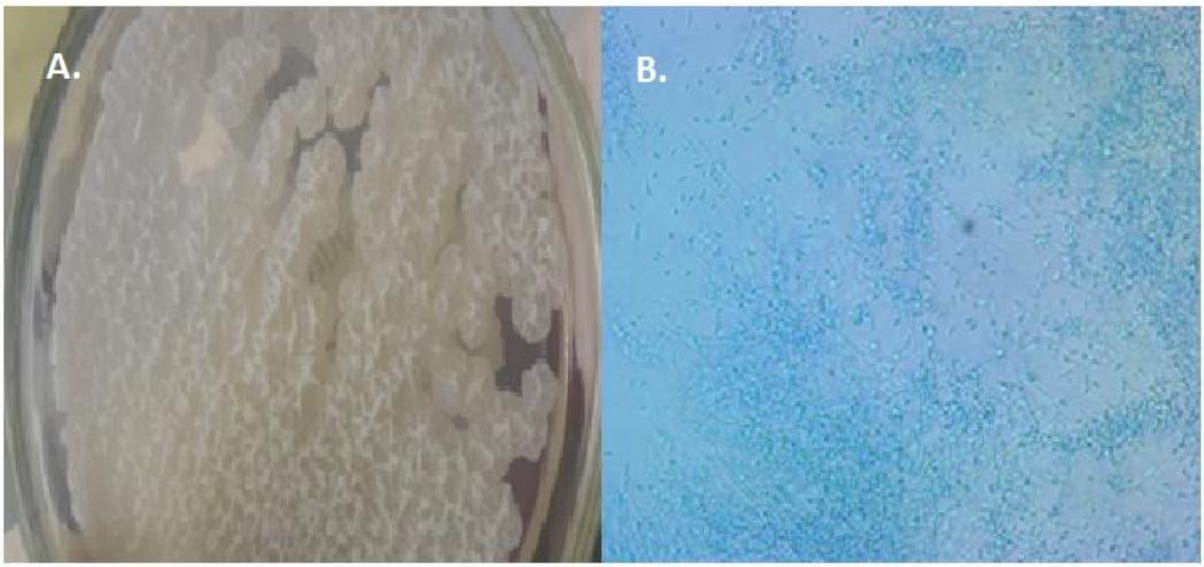

Figure 1. Morphology of *Yarrowia lipolytica* ATCC 9773: A) Macroscopic., B) Microscopic morphology.

2.4. Preparation of the inoculum and obtaining the crude enzymatic extract (CEE)

The inoculum was obtained from a suspension of mature spores of *Y. lipolytica* cultivated for five days at 25°C in PDA agar supplemented with olive oil. The biomass obtained was suspended in a solution of 0.9% (w/v) sodium chloride (NaCl). Subsequently, 200 mL of culture medium containing salt water (30% SW), sodium chloride (5.0%), yeast extract (0.5%), olive oil (1.0%) and Triton X-100 (0.1%) were inoculated with a suspension of *Y. lipolytica* for an incubation time of 8 hours. The fungal biomass was then separated from the supernatant by centrifugation at 5000 rpm for 10 min. Finally, the supernatant [enzymatic extract (EE)], was filtered through cellulose acetate membranes (0.22 to 0.45 mm) and the suspension cell viability was determined by spectrophotometry (Spectronic 20D) at 600 nm (Wu *et al.*, 2009).

2.5. Physicochemical effluent characterization

The physicochemical analyses were performed using the Standard Methods protocols established for raw water and wastewater. The fat and oil content was determined by the Soxhlet method according to the Standard Methods of the APHA *et al.* (2012). The hardness was measured by titration using EDTA solution as titrating agent, the results were expressed as mg of $CaCO_3$/L (Method 2340 C). Biological oxygen demand (BOD) was estimated by preparing the required volume of dilution water with the addition of nutrients and incubating for a period of five days at 20°C, while chemical oxygen demand (COD) was determined based on the rapid dichromate oxidation method (APHA *et al.*, 2012). The phosphorus content was determined by acid digestion, using the ascorbic acid method expressed in mg of P/L. Protein content was

determined by the Kjeldahl method. Hydrogen potential was determined potentiometrically using a digital potentiometer (Bench pH-Conductivity meter PC 510).

2.6. Evaluation of biodegradation

This test was performed during the effluent's discontinuous fermentation using different concentrations (8%, 12% and 16%) of CEE of *Y. lipolytica*, considering the volume of the effluent's residual fat (pH 5 and 6.5 at 25°C). Fat content was determined each 8 hours until reaching 32 hours of fermentation. It is noteworthy to mention that BOD_5 and COD were calculated only for that time where the best fat removal was reached (inoculum concentration: 16%, incubation time: 32h and pH: 5.0).

2.7. Statistical analysis

The percentage of fat removal was used as the response variable. These data were analyzed by means of analysis of variance (ANOVA one way) in order to determine statistically significant differences ($P < 0.05$) between the samples. SPSS software (Version 17.0 for Windows) and the multiple comparison test of Tukey were used. All tests were completed in triplicate.

3. RESULTS AND DISCUSSION

Growth of *Y. lipolytica*

The growth curve of *Y. lipolytica ATCC 9773* under experimental conditions is illustrated in Figure 2a y 2b. These conditions were chosen from previous analyses of the research group. The figures show that there were mainly three phases: (1) lag phase, which lasted from 0 to 8 h, and the absorbance was 0.332 (pH 5) and 0.044 (pH 6.5); (2) logarithmic phase, which lasted from 8 to 36 h, and the absorbance increased from 0.332 to 1.319 (pH 5) and 0.044 to 0.412 (pH 6.5); and (3) death phase, the absorbance decreased after 42 h. It is interesting to mention that no stationary phase was observed, since the fermentation process was stopped at 44 h, which was before the appearance of the stationary phase.

This experimental result showed that *Y. lipolytica* was capable of using oil salts as the sole source of carbon, nitrogen and energy, and *Y. lipolytica* presented remarkable growth in oil and salt wastewater.

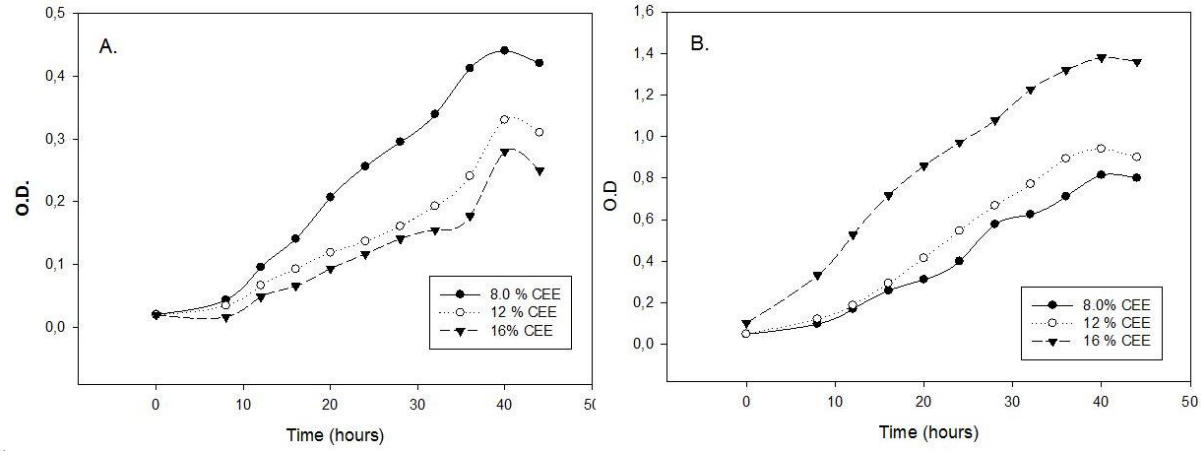

Figure 2. Growth curve of *Yarrowia lipolytica* ATCC 9773 at different pH values. A: pH 6.5; B: pH 5.0.

Table 1 shows the physical-chemical characterization of the effluent over the course of the experiment:

Table 1. Physical-chemical characterization of the residual fat effluent before and after the biodegradation process using Y. lipolytica ATCC 9773 under different conditions.

Parameters	C_i	C_f *(a)*	C_f *(b)*	*Unit*
BOD_5	17299±14.8	9805±21.2	10680±75.5	mg de 0_2/L
COD	53118±27.5	29576±15.2	32316±15.2	mg de 0_2/L
Fat	3260±20.80	558±5.290	1083±8.540	mg/L
pH	8.080±0.00	5.0±0.000	6.5±0.000	U de pH
Total solids	21308±14.4	18415±29.7	19676±92.9	mg/L
Phosphorus	<0.07	<0.075	<0.075	mg de P/L
Hardness	486.6±5.77	487.3±7.50	503.3±5.7	mg $CaCO_3$/L
Protein	2.26±0.057	1.94±0.051	1.81±0.028	%

C_i: Initial conditions of the effluent. C_f (a): Final conditions; pH 5,0 inoculum concentration of 16%, 32 hours of fermentation.
C_f (b): Final conditions; pH 6,5 inoculum concentration of 8%, 32 hours of fermentation.

According to the results of the BOD_5 and COD analyses, the dairy residue can be considered highly biodegradable. *Y lipolytica* ATCC 9773 is able to reduce BOD_5 43.32% and COD 44.30%. The best result in fat removal were obtained when a pH of 5 and an inoculum concentration of 16% were used. It seems that a pH increase in the effluent has effects on *Y. lipolytica* which influence its growth and therefore the lipases production.

One-way ANOVA of BOD_5 and COD values revealed significant differences ($p<0.05$) among the studied samples when the effluents' pH increased. Regarding to the inoculum concentration, also significant differences ($p<0.05$) were found at pH 5.0 when inoculum increased; however, the same was not observed at pH 6.5. Similar results have been reported by other researchers (Wu *et al.*, 2009; Wu and Wan, 2008) due to the fact that a high inoculum concentration can encourage microbial growth. Studies involving considerable reduction in COD and BOD in wastewater by using bacterial isolates have been published by Das and Santra (2010), Gaikwad *et al.* (2014). As with most other agro-industries, the dairy industry produces strong wastewaters characterized by COD and high BOD absorptions signifying their elevated organic content (Orhon *et al.*, 1993). The drop in COD values may be due to the concentration of nutrients, which microbial cultures could use for growing. Current results are in accordance with the reduction in COD reported by Guillen-Jimenez *et al.* (2000), where maximum COD fall was found up to 65–70%. Similar decrease in COD of the dairy wastewater (99.9%) was noted by Cosa and Okoh (2014) with a consortium of two marine species.

When the pH is 5.0 and the inoculum concentration 16%, the total solids removal reached values close to 13.58%. These results are opposite those reported by Stefańsk *et al.* (2018) who reported similar removal values employing a pH of 6.5 and an inoculum concentration of 8%. On the other hand, the hardness values are slightly higher than those initial values; this increase could be caused by the production of solid materials generated by the microbial growth.

Figure 3 shows the values of fat removal at different concentrations of inoculum (8%, 12% and 16%) in the effluent, adjusted at two pH values (5.0 and 6.5). To pH 5.0 the highest fat removal values (88. 82%) were achieved using an inoculum concentration of 16% and 32 h fermentation time. No significant differences were found ($p>0.05$) among different inoculum concentrations at the same fermentation times. It should be mentioned that significant differences ($p<0.05$) were found in fat biodegradation for all fermentation times.

The fermentative process at a pH of 6.5 using different inoculum concentrations is illustrated in Figure 3, where a removal percentage of 49.63% after 8 hours of fermentation may be observed. When the inoculum amount was increased, no significant differences ($P>0.05$) were appreciated in the fat removal percentage for different samples in the same

fermentation time. After 8 hours of fermentation, the percentage of fat removal remained constant, although Y. lipolytica ATCC 9773 continues to present lipase activity, which results in a decreasing of fat percentage until 32 hours of fermentation time is reached.

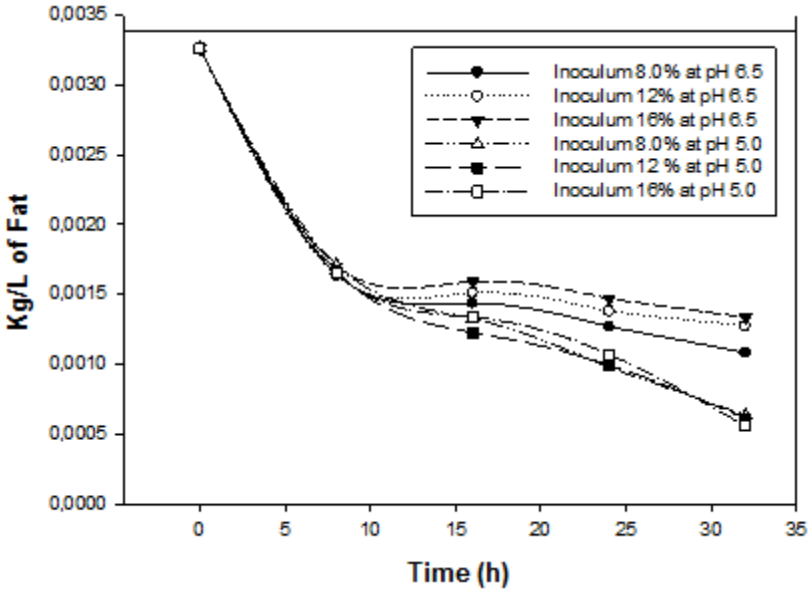

Figure 3. Fat removal from industrial effluent at different concentrations of CEE and a pH of 5.0 and 6.5.

It must be highlighted that significant differences are found ($p < 0.05$) in the percentages of biodegradation obtained at higher fermentation times. At 8% inoculum concentration, more than 16% fat biodegradation was obtained during the same fermentation time (32 hours). It seems that an increase in cell concentration causes cyanic changes in the microorganism, which result in a low metabolic activity, resulting in a reduction of lipase activity. On the other hand, no significant differences were found ($p > 0.05$) between inoculum concentrations of 12% and 16%.

The values of fat removal at different pH concentrations and fermentation times are shown in Table 2. The highest fat removal value (82.88) was obtained at pH 5 using an inoculum concentration of 16%, while the lowest value (47.50) was reached at pH 5 and an inoculum concentration of 8%. It should be highlighted that significant differences ($p < 0.05$) were found for all the fermentation times.

Table 2. Percentage of fat biodegradation using CEE at two pH values over 32 days.

Fat removal (%)						
Time (hours)	*pH 5*			*pH 6.5*		
	Concentration of CEE Y. lipolytica			*Concentration of CEE Y. lipolytica*		
	8%	12%	16%	8%	12%	16%
8	47.50[a]	48.89[a]	50.18[a]	49.63[a]	49.81[a]	47.79[a]
16	59.29[b]	62.39[b]	58.98[b]	55.88[b]	53.65[b]	51.16[b]
24	70.00[c]	69.60[c]	67.23[c]	61.07[c]	57.66[c]	54.93[c]
32	80.52[d]	81.22[d]	82.88[d]	66.77[d]	60.85[d]	59.01[d]

Rows with no common letter showed statistically significant difference (significance level\0.05).

4. CONCLUSIONS

The study showed that fatty effluent from the dairy industry contains high levels of BOD_5, COD, total solids and fat. The results show that CEE from strain culture (*Yarrowia lipolytica*) reduced the levels of BOD_5 and COD until reaching the values of 43.32 and 44.3%, respectively. Likewise, the *Y. lipolytica* ATCC 9773 strain is able to biodegrade the fat content of the effluent to values close to 82.88%. These results may have relevant implications in the industry for the reduction of the contamination of effluents with large amounts of contaminating material, specifically fat.

5. ACKNOWLEDGMENT

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