Revista
Torreón Universitario
www.faremcarazo.unan.edu.ni / ISSN 2410-5708 / e-ISSN 2313-7215
Year 10 | No. 27 | February - May 2021

Lipolytic activity of fungi isolated from used cooking oil and machine shop floors

Zulma Pérez
Specialist in laboratory analysis
Biotechnology Laboratory
Universidad Nacional Autónoma de Nicaragua, Managua
https://orcid.org/0000-0002-1957-902X
kzulma@gmail.com

Gabriela Gazo
Graduate of Industrial Chemistry, Faculty of Sciences
Universidad Nacional Autónoma de Nicaragua, Managua
https://orcid.org/0000-0002-9366-2454

Rommel Uriarte Ortiz
Specialist in laboratory analysis
Biotechnology Laboratory
Universidad Nacional Autónoma de Nicaragua, Managua
https://orcid.org/0000-0001-8930-5369

Submitted on May 7, 2020 / Accepted on October 9, 2020
https://doi.org/10.5377/torreon.v10i27.10845

Keywords: Lipolytic fungi, lipase, enzyme activity, used cooking oil

ABSTRACT

The objective of the research[1] was to select the genus with the highest enzymatic activity by using used cooking oil as the only source of the substrate. Fungi were isolated from used cooking oil and contaminated soils by mechanical car repair shops. The samples were inoculated in general fungal media (PDA agar), when obtaining axenic cultures they were inoculated in selective agar for lipolytic fungi (tributyrin agar), the genus was identified by bright field microscopy and phase contrast. In the selective medium, the

1. This article is part of the project "Insulation and identification of fungi from used cooking oil". It was financed by the Vice-Rectory of Research, Postgraduate and University Extension, of the UNAN-Managua through the Funds for Research Projects (FPI). It began in 2016 and ended in May 2020.

presence of halo around the colony or crystallization close to the colony was considered as an indicator of the lipolytic genus. The genus identified in soil were *Fusarium* and *Aspergillus*, the genus *Aspergillus* was identified in the oil. The tests were carried out with aeration, in the tests without aeration, the interaction between the genera isolated from the soil was evaluated, which was named consortium. The enzymatic activity in the first phase for the genus *Aspergillus* in tests without aeration, isolated from the oil was 0.0148 (U / ml). For *Aspergillus* and *Fusarium* isolated from the soil, the enzymatic activity was 0.0051 and 0.0055 (U / ml) respectively; in the consortium, it was 0.003 U / ml. The genus *Aspergillus* isolated from the oil showed higher enzymatic activity and was selected for the second phase of lipolytic activity measurement. The highest enzymatic activity 0.0777 (U / ml) was obtained at 144 hours when the culture was evaluated with aeration interrupted and continuous feeding. When the cultures were subjected to an aeration interrupted and discontinuous feeding, the enzymatic activity was similar to that achieved without aeration (0.026 and 0.034 (U / ml) respectively). These results suggest that the genus studied are capable of utilizing used cooking oil as the only carbon source for their metabolic processes.

INTRODUCTION

Lipases are ubiquitous proteins widely distributed in nature, present in animals, plants, and microorganisms, generally secreted to the extracellular environment, thus facilitating the processes of extraction and purification for the industry. (Rabbani, and others, 2013)

The variability, adaptability, and advantages in the production of microbial lipases, has allowed its promotion within the group of the most produced enzymes in the world, with biotechnological applications in the processing of dairy foods, oils, detergents, cosmetics, leather, pharmaceutical products, paper, production of surfactants, biodiesel and recently as a promising alternative for the treatment of wastewater contaminated with high lipid content. Microbial lipases are highly appreciated as biocatalysts due to their peculiar characteristics, such as the ability to use a wide range of substrates, high activity, and stability. However, in certain industrial segments, the use of lipases is still limited by their high cost. Therefore, there is a great interest in obtaining low cost, highly active, and stable lipases that can be applied in different industrial branches. (Anobom, and others, 2014)

Lipases (triacylglycerolacil hydrolases, EC. 3.1.1.3), are enzymes with biological activity on the ester bond of triacylglycerol molecules present in fats or oils. When they act in an aqueous medium they can produce glycerol, fatty acids, monoglycerides, and diglycerides, while in the absence of water, the reaction is reversible (esterification, interesterification, and transesterification), generating glycerides. (Pedroza Padilla, Romero Tabarez, & Orduz, 2017)

This research arises from the analysis of the environmental impact of the final deposition of used cooking oil in Nicaragua, coming from the dining rooms of institutions, soup kitchens, restaurants, and hotels. Used cooking oil is not collected adequately; there is no regulation to control these discharges. The only company that promotes the collection of this waste is the Green Products Company in the Biotechnology Laboratory at UNAN Managua and uses it as raw material for the production of cleaning soap. In the case of contaminated soil in the mechanical workshops, the oils are dumped directly into the soil without prior treatment. By isolating fungal genera capable of using hydrocarbons and used cooking oils as substrate, they could be used in industry to produce enzymes and bioremediate contaminated soils and water.

Lipase activity can be determined qualitatively and quantitatively, the main objective of the research was to select the genus with the highest enzymatic activity by using used cooking oil as the only source of a substrate.

The tests were done in modified solid substrate fermentation systems, without aeration, with interrupted aeration / batch feeding and interrupted aeration / continuous feeding. For a solid substrate fermentation process, a biological matrix, i.e. natural substrates, or inert substrates, can be used. These are impregnated synthetic substrates. The choice of one or another type of support will depend on the particularities of the process and the type of product to be obtained. Bearing this in mind, most of the processes developed in recent years have been valid for natural substrates that come from agro-industrial processes and that are mostly by-products with no economic value. In this way, this type of fermentation is increasingly proposed as an alternative to add value and take advantage of organic matter. In the trials, we worked with a medium of basic mineral salts, using sugar cane bagasse as a solid substrate.

METHODOLOGY

Used cooking oil sample collection

From the oil stored in barrels in the green products company, sub-samples were taken at random, then homogenized to obtain a final sample, which was left exposed to the environment for fungal spores to colonize the surface.

Contaminated soil sample collection in mechanical workshops

Random subsamples were collected in areas where oil was observed, then homogenized to obtain a final sample, and from this, inoculations were made.

Isolation

Soil and oil samples were inoculated directly into dextrose potato agar, this culture medium is non-selective, its use is especially indicated for the isolation of filamentous fungi

and yeasts. Two inoculation techniques were used, spill on plate and surface extension or by exhaustion. In both techniques, used cooking oil was used as a substrate, sterilized with 0.22 µm filters, and incubated for 7 days at 35 ºC.

Isolation in tributyrin agar

Once axenic cultures were obtained from both matrices, they were inoculated in tributyrin agar, which is used for the detection of lipolytic microorganisms. The peptic digestion of the animal tissue and the yeast extract provides nutrients to the microorganisms. The degradation of tributyrin by the microorganisms is recognized by clear areas surrounding the lipolytic colonies in the cloudy culture medium. They were incubated for 7 days at 35 °C.

Microscopic identification of isolated fungal genera

The colonies of axenic cultures isolated in tributyrin agar were observed in the microscope, the adhesive tape technique was used because the original juxtaposition of spores and hyphae segments is preserved; it consists of taking with the help of tweezers, a strip of tape of approximately 4 cm, with the adhesive side out, pressing firmly against the surface of the colony of the fungus, then, the tape was placed in a slide containing a drop of lactophenol blue. (Arias Cifuentes & Piñeros Espinoza, 2008). For the identification of the genera, dichotomous keys were used according to Agrios' phytopathology.

Lipase production with solid substrate fermentation systems without aeration, with genera isolated from used cooking oil and soil contaminated by mechanical workshops

The culture medium was composed of (g/L): NaH_2PO_4 1,2, KH_2PO_4 2, $MgSO_4.7H_2O$ 0.3 and $CaCl_2$ 0.25. (Gwen Falony, Armas, Dustet Mendoza, & Martínez Hernández, 2006). The tests were carried out in triplicate, 20 ml of culture medium plus 1 ml of spore suspension (10^7 CFU/ml) in saline solution were added to each 100 ml Erlenmeyer. The carbon source was the used cooking oil 2% (v/v), this was applied in the form of emulsion in phosphate buffer and as solid substrate bagasse of cane 2% (w/v). The trials lasted 8 days, at 35 ºC, without aeration. Besides making the assays with each one of the genera identified by each matrix, there were carried out assemblies in the fungal consortium (*Aspergillus sp.* and *Fusarium sp.* in soil), to evaluate its interaction among genres and consequently the enzymatic activity. It was considered an analytical interference, the coloration of sugarcane bagasse in the cultivation means, therefore, it was decided to mount a control, which contained cultivation means plus bagasse, in the same conditions that the assays with identified fungal genres. Once the concentration of lipases in the samples was quantified, it was proceeded to subtract the concentration identified in the control, it was applied the same in all the tests.

Lipase production with solid substrate fermentation systems, with interrupted aeration / discontinuous feeding, with isolated genera from used cooking oil.

The culture medium was composed by (g/L): NaH_2PO_4 1,2, KH_2PO_4 2, $MgSO_4.7H_2O$ 0.3 and $CaCl_2$ 0.25. (Gwen Falony, Armas, Dustet Mendoza, & Martínez Hernández, 2006). The tests were carried out in triplicate, 20 ml of culture medium plus 1 ml of spore suspension (10^7 CFU/ml) in saline solution were added to each 100 ml Erlenmeyer. The source of carbon was the used cooking oil 2% (v/v) this was applied in the form of emulsion in phosphate buffer and as solid substrate bagasse of cane 2% (w/v). The trials lasted 8 days, at 35 °C, with interrupted aeration.

Lipase production with solid substrate fermentation systems, with interrupted aeration /alimentación continuous

The test was done in percolators, composed of filtration units with a capacity of 500 ml, the upper chamber, and the receiver with graduation. Two lateral ways allow the connection to the vacuum line, the adapters with vacuum tubes from 1/4 to 5/16 of an inch (6 to 8 mm) of internal diameter. The filtration equipment was connected to a vacuum pump, which was controlled by a timer, whose operation was programmed every 5 minutes of work and 15 minutes of rest, the cultures were aerated in an interrupted way, guaranteeing 5 minutes of aeration during the 10 days of the test at 35 °C. The culture medium used was the same as in the production of lipase without aeration. The samples were collected at 0, 48, 96, 144, and 240 hours.

Every two days, 25 ml of culture medium (to avoid nutrient depletion), 1 ml of sterile used cooking oil (0.22 μm filter), and emulsified in sterile phosphate buffer (autoclave) were added. The number of spores used was the same as in the non-aerated trials.

In each test, a control assembly was made, which was subtracted when quantifying the enzymatic activity of lipase.

The bagasse used in the tests without aeration and with aeration was previously sieved (850 μm) and later sterilized in autoclave.

Quantitative analysis of enzymatic activity

The extracellular lipase of all the isolates was quantified by spectrophotometric method. Hydrolysis of p-NPP was measured to release p-nitrophenol (p-NP). To prepare the calibration curve, the method proposed by (Margesin & Shinner, 2005) was used with modifications. 1.25 mg of p-nitrophenol (p-NP) was dissolved in a buffer solution and measured at 25 ml, the concentration was 50 μg/ml. As a substrate p-NPP was used at a concentration of 20 mM, it was dissolved in 2-propanol. The phosphate buffer used NaH_2PO_4 /NaOH (100 mM was adjusted to pH 7.25 with a 1N sodium hydroxide solution.

Calibration curve

A standard curve was prepared (62.5, 50, 37.5, 25, 12, 6 µg/ml) to quantify enzymatic activity as a function of pNP concentration. Phosphate buffer solution with pH 7.25 was used to calibrate to 5 ml, they were prepared in conical polypropylene centrifuge tubes.

To read the blank, 5 ml of buffer solution was added to the conical tube, each of the tubes was vortexed for 5 seconds, and incubated for 10 min at 40 °C. Then, the tubes were read in a spectrophotometer at 410 nm, and the buffer solution was used as blank to calibrate the equipment.

Procedure for reading the samples

Before reading the samples, 25 ml of distilled water were added and filtered, 4 ml of buffer and 800 µl of the sample were added to each polypropylene tube and incubated at 40 °C for 10 minutes, then 200 µl of the substrate (pNPP) was added and incubated again for 10 minutes at 40 °C, the reaction was stopped with ice for 10 minutes, centrifuged for three minutes at 3000 rpm and read at 410 nm as well as the calibration curve. (Sadati, Barghi, & Larki, 2015).

Calculations and expression of results

The pNP concentration of the samples was calculated using the calibration curve, from which the calculated pNP concentration in the control sample was subtracted. One enzyme unit was defined as 1 µmol of 4-nitrophenol, enzymatically released from the substrate in milliliters per minute (ml/min).

RESULTS AND DISCUSSION

Isolation of lipolytic microorganisms in tributyrin agar

Some methods where the production of lipase is confirmed by the formation of turbid zones or white crystals around the colonies in the agar. It is possible to use agar media with substrates or indicator dyes added for the detection of lipolytic organisms. Qualitative identification includes gel diffusion tests, based on the incorporation of lipids, substrates in the media, and tests based on the addition of a color dye to the media. (Lanka & Latha, 2015)

From the oil sample, the genus *Aspergillus* was identified. From the contaminated soil sample, two genera, *Aspergillus* and *Fusarium*, were identified.

Although the genus *Aspergillus* was identified in both matrices, it cannot be assured that they are the same species.

In figure 1, image a), the formation of halos around the colonies can be seen, this colony was isolated from used cooking oil. Image b) is a colony isolated from the soil contaminated

by mechanical workshops, even though both matrices were inoculated into the same culture medium under the same conditions. The behavior of these genera was different. For *Fusarium* genus (Fig. 1 b), crystallization was observed around the colony, which is an indicator of lipolytic microorganisms.

In tests carried out by (Sierra , 1957), they added tween as a substrate in the culture media, it was observed around the colonies of lipolytic microorganisms the formation of a halo, this is due to the crystals of calcium salts, of the fatty acid released by the lipolysis.

In a study carried out by (Gopinath, Anbu, & Hilda, 2005) they isolated fungi from five samples from different stations, contaminated by oil residues, they previously inoculated them in PDA agar and then they carried out qualitative evaluation for lipolytic fungi, in different solid means (solid culture medium with tween 20, LTB agar and tributyrin agar), to determine which of the species presented the halo with greater diameter, this behavior pattern is an indicator of the production of extracellular lipase in the medium; it was observed the development of a clear crystallized zone in the solid medium, where tween 20 was added as substrate, which they took as criteria to consider *Aspergillus fumigatus* and *Aspergillus nidulans* as lipolytic microorganisms, the reaction in this medium by the extracellular lipase, In the first days of incubation, it was observed; however, in species such as *Aspergillus niger* and *Aspergillus versicolor*, the lipolytic activity was visualized after 72 hours of incubation. The peak of the highest enzyme activity (>7cm) occurred at 168 hours for *Aspergillus fumigatus* and *Aspergillus nidulans*. For species such as *Aspergillus clavatus*, *Aspergillus flavus*, *Aspergillus ochraceus*, *Aspergillus versicolor*, and *Fusarium sp.* weak lipolytic activity was observed with halos smaller than 4 cm. Regarding the evaluation of the fungal species analyzed on tributyrin agar, the behavior was different compared to the results using tween 20 as a substrate. Tributyrin is a conveniently used substrate, it does not need the addition of emulsifiers, the hydrolysis of tributyrin by fungal microorganisms can be qualitatively evaluated, measuring the increase of the diameter of the clear zone. *Aspergillus japonicus*, *Aspergillus* versicolor, and *Fusarium sp.* presented halo after 48 hours of incubation. *Aspergillus ochraceus* presented lipolytic activity after 72 hours of incubation. In this study, haloes in the identified genera (*Aspergillus* and *Fumigatus*) were observed after 5 days of incubation in tributyrin agar.

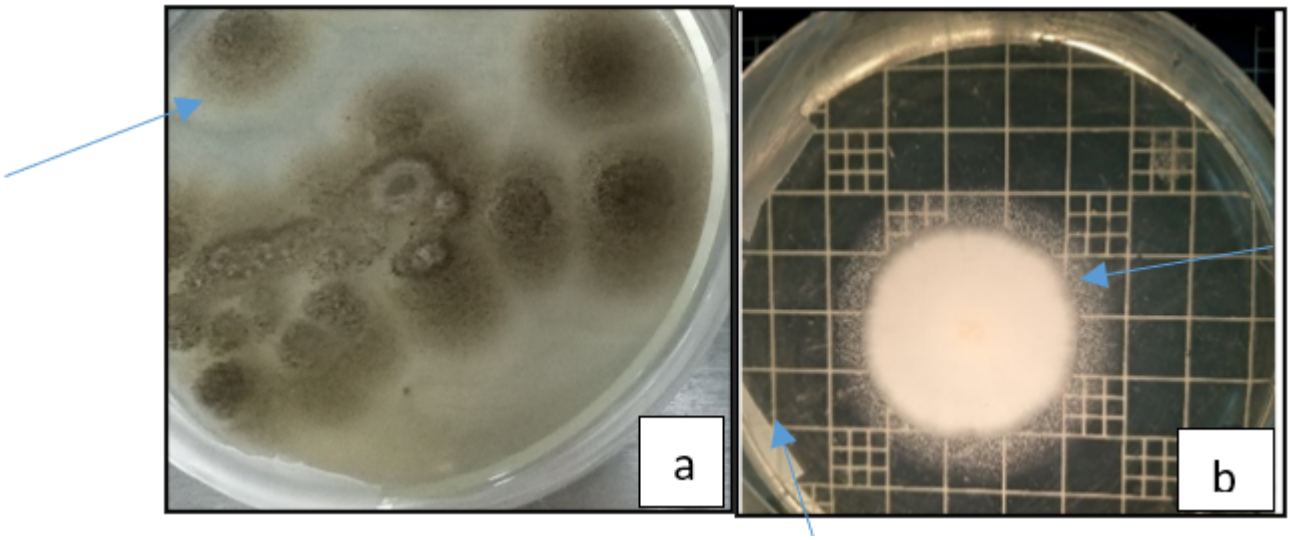

Figure 1. a) Aspergillus genus isolated from used cooking oil. b) Fusarium genus isolated from contaminated soil by mechanical workshops.

Enzymatic activity without aeration with genera isolated from used cooking oil and contaminated soil by mechanical workshops

Lipases are produced by many microorganisms, as are other enzymes of the hydrolase family, such as esterases. Among the microorganisms, fungi are widely recognized as preferable sources of lipase because they generally produce the enzyme in an extracellular manner, which facilitates the recovery of the enzyme from the fermentation broth. (Rai, Shrestha, Sharma, & Joshi, 2014)

Figure 2 shows the genera identified by matrices, in the soil matrix the genera *Aspergillus* and *Fusarium* were identified, and in oil, only *Aspergillus* was identified.

The highest enzymatic activity (0.0148 U/ml) was determined in the genus *Aspergillus*, isolated from used cooking oil. The technique of solid-state fermentation used, involves the development and metabolism of microorganisms with the smallest volume of water, and also offers greater advantages than submerged state fermentation, however, also has its limitations, for example, it is not possible to control and monitor some parameters such as pH, temperature, humidity, and air flow. (Gwen Falony, Armas, Dustet Mendoza, & Martínez Hernández, 2006)

Figure 2 shows the interaction between *Aspergillus* and *Fusarium* genera (Consortium(s)), the enzymatic activity determined in the consortium was 0.003 U/ml (considered low), this could be because one of the two genera is highly competitive for nutrients present in the medium.

The activity determined in the genera *Aspergillus* (s) and *Fusarium* (s) individually, was 0.0051 and 0.0055 respectively, there was no significant difference between them. The genus

Aspergillus isolated from oil, presented enzymatic activity 27 times higher approximately, than the enzymatic activity determined in the genera isolated from the soil.

These results may be because the genera identified are capable of using used cooking oil as their only source of carbon. The low enzymatic activity in genera such as *Fusarium*, could be since enzyme production is expressed in an intracellular way because these genera probably do not present mechanisms for extracellular expression.

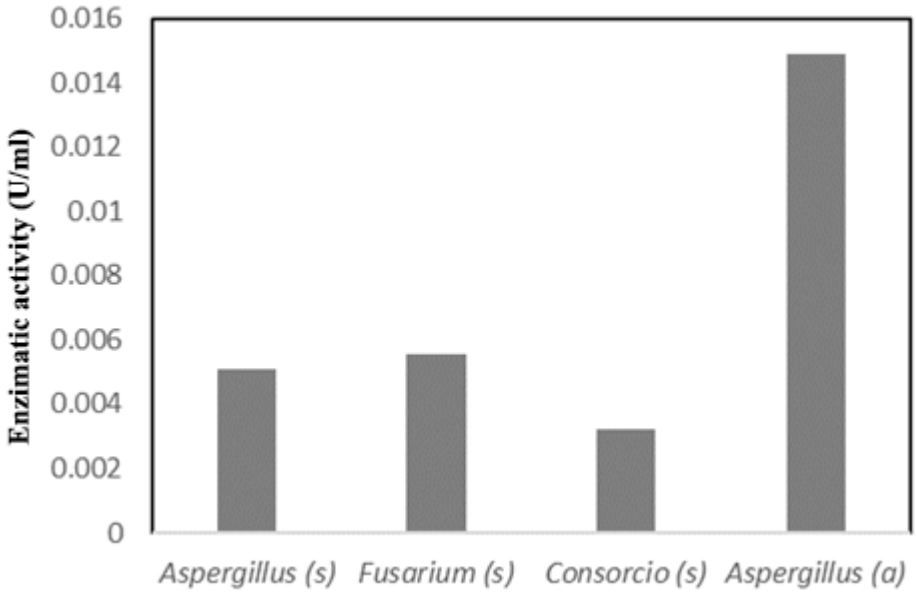

Figure 2. Enzymatic activity through identified genera isolated from the soil contaminated by oils from mechanical workshops and used cooking oil.

s: Identified genera in contaminated soil from mechanical workshops

a: Genres identified in oil-contaminated by mechanical workshops

Enzymatic activity with interrupted aeration /alimentación discontinuous

The aeration was through an innovative system, which consisted of the design of a timer adapted to a vacuum pump to oxygenate the cultures in an interrupted way. There was no significant difference in the results of enzymatic activity, between aerated cultures (0.0267 U/ml), and without aeration 0.03482 (U/ml); both trials had the same substrate concentration. This may be because the substrate was not sufficient and led to the stress of the microorganisms. (Fig.3)

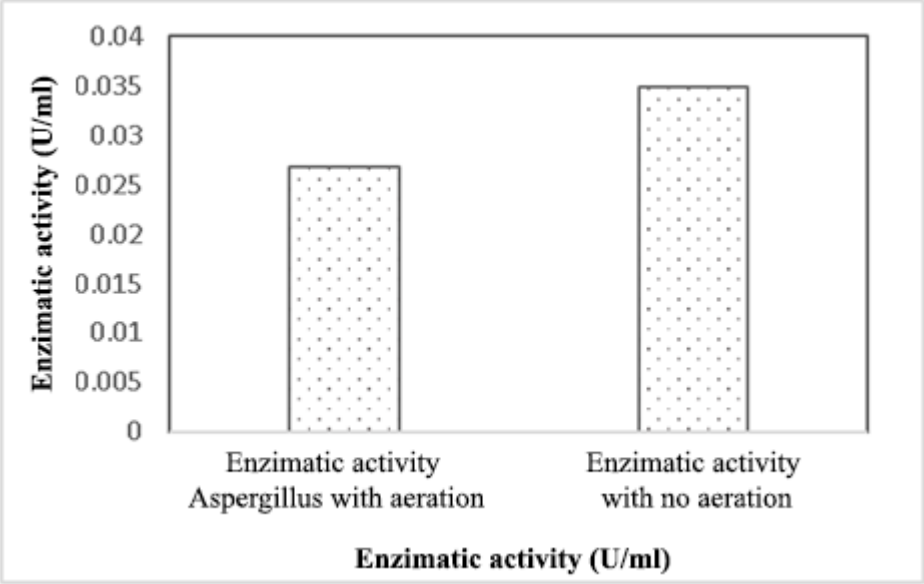

Figure 3. Aspergillus genus with higher enzyme activity (U/ml)

Enzymatic activity with interrupted aeration /alimentación continuous

Figure 4 shows the enzymatic activity of the genus *Aspergillus*. Based on previous trials where low enzymatic activity was recorded, assemblies were made providing continuous feeding and interrupted aeration, the highest calculated enzymatic activity (0.0777 U/ml) was reached at 144 hours, being higher than the previous fermentation system.

Ethanol production trials have been conducted with *Saccharomyces cerevisiae* (Laopaiboon, Thanonkeo, Jaisil, & Laopaiboon, 2007), comparing continuous and batch feed fermentation systems, and the results show that ethanol production improved in terms of product concentration and yield, using continuous fermentation systems.

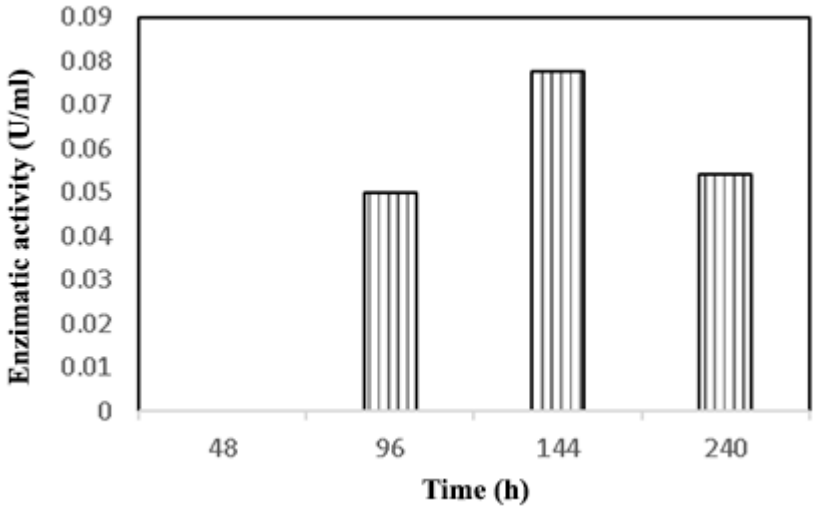

Figure 4. Enzymatic activity with interrupted aeration and continuous feeding

Figure 5 compares the values of enzyme activities under different conditions. The highest enzymatic activity (0.0777 U/ml) was reached when the culture was submitted to continuous feeding with aeration at 144 hours, however, when the culture was aerated and with discontinuous feeding, the enzymatic activity was similar to the one reached without aeration (0.026 and 0.034 (U/ml) respectively). This is probably, although the culture was aerated, the amount of substrate and nutrient medium was not sufficient.

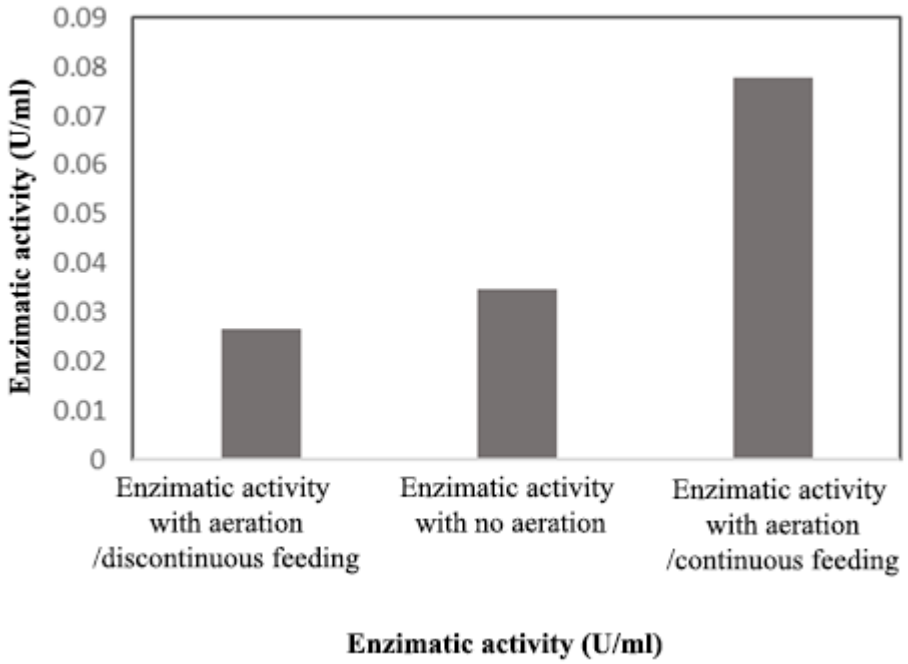

Figure 5. Comparison of the enzyme activity of Aspergillus genus with aeration/ discontinuous feeding, without aeration and aeration/continuous aeration

CONCLUSIONS

The results show that the genera identified, especially the *Aspergillus* genus, can use used cooking oil as their only source of carbon.

By providing continuous aeration and feeding to the crops, enzyme activity was doubled compared to crops with aeration and discontinuous feeding.

The trials were adapted to innovative and economical aeration systems.

This study is the precedent for further trials, where it is intended to improve the production of the enzyme lipase, to be purified and commercialized.

Another work to take into account that resulted from this study is the possibility of using these fungal genera in the bioremediation of sites contaminated by oils and hydrocarbons.

THANKS

To the Vice-Rector's Office for Research, Graduate Studies, and University Extension and the Research Directorate of UNAN-Managua, for funding through the Research Project Fund (FPI).

To Ph.D. Martha Lacayo, head of the Biotechnology Laboratory, for always supporting research.

To Pablo Vallecillo for designing the timer for crop aeration.

To Gabriela Garcia Gazo for her unconditional support in carrying out the trials.

WORKS CITED

Anobom, C. D., Pinheiro, A. S., De-Andrade, R. A., Aguieiras, E. C., C., A. G., Moura, M. V., . . . Freire, D. M. (2014). From structure to catalysis: Recent Developments in the Biotechnological application of Lipases. BioMed Research International, 2014, 1-11. http://dx.doi.org/10.1155/2014/684506

Arias Cifuentes, E. L., & Piñeros Espinoza, P. A. (2008). Aislamiento e identicación de hongos filamentosos de muestras de suelos de los páramos de Guasca y Cruz verde. (Tesis de grado) Pontificia Universidad Javeriana. Bogotá, Colombia.

Gopinath, S., Anbu, P., & Hilda, A. (2005). Extracellular enzymatic activity profiles in fungi isolated from oil-rich environments. Mycoscience, 46, 119-126. https://doi.org/ 10.1007/s10267-004-0221-9

Gwen Falony, G., Armas, J. C., Dustet Mendoza, J. C., & Martínez Hernández, J. L. (2006). Production of Extracellular

Lipase from Aspergillus niger by Solid-State Fermentation. Food Technol. Biotechnol., 44(2), 235-240.

Lanka, S., & Latha, N. L. (2015). A Short Review on Various Screening Methods to Isolate Potential Lipase Producers: Lipases-the Present and Future Enzymes of Biotech Industry. International Journal of Biological Chemistry, 9(5), 207-219. https://doi.org/10.3923/ijbc.2015.207.219

Laopaiboon, L., Thanonkeo, P., Jaisil, P., & Laopaiboon, P. (2007). Ethanol production from sweet sorghum juice in batch and fed-batch fermentations by Saccharomyces cerevisiae. World J Microbiol Biotechnol, 1497-1501. https://doi.org/10.1007/s11274-007-9383-x

Margesin, R., & Shinner, F. (2005). Manual of soil Analysis. Heidelberg, Germany: Springer.

Pedroza Padilla, C. J., Romero Tabarez, M., & Orduz, S. (2017). Actividad lipolitica de microorganismos aislados de aguas residuales contaminados con grasas. Biotecnología en el sector Agropecuario y Agroindustrial., 15(1), 36-44. http://dx.doi.org/10.18684/BSAA(15)36-44

Rabbani, M., Reza Bagherinejad, M., Sadeghi, H. M., Shariat, Z. S., Etemadifar, Z., Moazen, F., . . . Zaghian, S. (2013). Isolation and characterization of novel thermophilic lipase-secreting bacteria. Brazilian Journal of Microbiology, 44(4), 1113-1119.

Rai, B., Shrestha, A., Sharma, S., & Joshi, J. (2014). Screening, Optimization and Process Scale up for Pilot Scale Production of Lipase by Aspergillus niger. Biomedicine and Biotechnology, 2(3), 54-59. https://doi.org/10.12691/bb-2-3-3

Sadati, R., Barghi, A., & Larki, R. A. (2015). Isolation and Screening of Lipolytic Fungi From Coastal Waters of the Southern Caspian Sea (North of Iran). Jundishapur J Microbiol., 8(4), 1-7. https://doi.org/10.5812/jjm.8(4)2015.16426

Sierra, G. (1957). A simple method for the detection of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. J. Microbiol., 23, 15-22.