# Biotechnology Education

# All-Around Management of a Fungal Isolate Obtained from Cheese Spoilage as an Environmental Source: Direct Approach from an Undergrad Student to a Biotechnological Characterization

**Eugenia Guadalupe Ortiz Lechuga**
**Omar Eduardo Tovar Herrera**
**Katiushka Arévalo Niño***

*From the Biotechnology Institute, Biological Sciences Faculty, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, Mexico*

## Abstract

In this work, we present the results of an undergrad student from the perspective of its first approach as a principal researcher in a project. In order to gain practical experience, one of the options for students that have interest in pursuing a postgraduate program corresponds to a research stay in a laboratory of their selected field conducting a project for a period of 6 months. In this particular project, a fungal sample was isolated from Parmesan cheese spoilage and its enzymatic activity evaluated. Using simple and standardized protocols, the student was capable of identifying a possible biotechnological application for the isolate by detecting and categorizing the lipolytic activity. Through microculture characterization in potato dextrose agar (PDA) the genus of the sample was determined as *Penicillium* and confirmed by molecular analysis of the ITS1 and ITS4 regions. In order to examine comprehensively the potential of the new isolate, the extracellular and intracellular enzymatic activities were analyzed as well as four methods of cell rupture. From these results, sonication was determined as the best technique with 211 U/L as a maximum lipolytic value. To finalize the evaluation of the sample, the student determined the optimal pH and temperature as well as the thermotolerance of the crude extract obtaining a residual activity of 13% after 60 minutes of incubation at 45 °C. Upon conclusion of the research we could recognize that through a direct characterization of a fungal isolate using techniques that are widely known, the student was capable of determining and value one of the most interesting capabilities fungi has to offer; enzymatic activity, and that the knowledge obtained from established protocols enables and encourages the students to correlate the source from where they were obtained with potential biotechnological applications. © 2019 International Union of Biochemistry and Molecular Biology, 47(6):681–688, 2019.

***Keywords:*** *Fungi; lipase; parmesan;* Penicillium

## Introduction

*Parmigiano-Reggiano* or Parmesan cheese is produced from unpasteurized cow's milk and as products like *Tequila* or *Champagne*, its Protected Designation of Origin status limits its manufacturing areas to the provinces of Parma, Reggio Emilia, Bologna, Modena and Mantova, Italy [1]. Since rennet-coagulated cheeses like *Parmigiano-Reggiano* take as much as 24 months to ripen, the ecosystem in which it develops is of high importance and so are the un-inoculated microorganisms that can positively or negatively affect the organoleptic characteristics of the product itself. During the ripening, the biochemical changes during these primary events include actions directed to the metabolism of residual lactose, lactate and citrate followed by lipolysis and proteolysis [2]. As a part of the ripening process, there is a triglyceride hydrolysis by the action of lipases.

Bacterial ripened varieties such as Cheddar and Gouda have no strong lipolytic agents of their own, although lipolysis progresses as a result of enzymes from the starter and non-starter microflora. Following this strategy, levels of lipolysis in cheeses in which a secondary microflora grows are frequently linked to the lipolytic ability of the second starters, with examples from the genus *Penicillium* spp. [2].

*Volume 47, Number 6, November/December 2019, Pages 681–688*
**To whom correspondence should be addressed. Tel.: 52 81 8329 4000 ext. 6492 and 6440. E-mail: karevalo01@hotmail.com; katiushka.arevalonn@uanl.edu.mx.*
*Received 10 January 2019; Revised 19 June 2019; Accepted 18 July 2019*
*DOI 10.1002/bmb.21288*
*Published online 6 August 2019 in Wiley Online Library (wileyonlinelibrary.com)*

With reports that contamination can come from a number of different origins, over 40 non-starter yeasts and mold species have already been isolated from cheeses [3].

Commonly, mold isolated from cheese include *Penicillium*, *Aspergillus*, *Cladosporium*, *Mucor*, and *Trichorderma* being *Penicillium* the most commonly found contamination with a persistency of up to 7 years in coating and unpacking areas of cheese factories [4].

In the present work we have evaluated the skills acquired by the first approach of an undergrad student to a research environment through the study of an abundant fungal isolate from the surface of a Parmesan cheese sample; the assessment made from the traditional morphological description combined with the molecular identification and enzymatic activity allowed the student to learn in an integral way that biochemistry and molecular biology are key subjects when analyzing the characteristics of new isolates and the enormous potential that unconventional sources of isolation can budge for industrial applications.

## Materials and Methods

**Characteristics of the student:** The student that selected this program and was responsible for the development of this particular work was a 3rd year college student with a biotechnology major, his basic formation was centered in bioinformatics, industrial biotechnology and omics sciences, with an interest in pursuing a graduate program in Food Chemistry.

### Experimental Activities

The student was instructed to meet a minimum of 4 hours of work per day (Monday to Friday) at the laboratory facilities under the supervision of an assigned researcher. As a part of the training the student was required to keep a work log with the protocols and observations made during the experimental process. The work was divided into three main phases with a programmed schedule determined by the time the standardized protocols usually take. The first stage corresponded to the purification and detection of biotechnological potential for the isolate, in this case and pondering the origin of the sample (cheese) lipolytic activity was considered to be of relevance. The second phase was to identify the isolate through morphological analysis and to conduct a DNA extraction to confirm the identity of the strain. Finally, as a third phase, the lipolytic activity was confirmed and quantified to later be optimized following a step by step approach using basic parameters such as pH and temperature.

#### *Phase 1*

*Isolation and Evaluation*—The fungal sample used in the present work was isolated from commercial Parmesan cheese by taking spores from the spoilage surface and maintained using PDA plates. The sample was preserved in 10% glycerol and kept at −20 °C.

*1.2 Lipolytic activity screening*—To determine lipolytic activity a central inoculation point in 0.002% Rhodamine B Agar (RBA) supplemented with olive oil at 1% was used and its fluorescence analyzed under UV light (350 nm) after 96 hours of incubation at 25 ± 2 °C.

#### *Phase 2*

*Morphological characterization*—The fungal sample was streaked on all four panels of a PDA cube (1x1 cm) and incubated at 25 °C for 72 hour (Fig. 1), with observations every 24 hour stained with lactophenol blue and analyzed under optical microscope at 100×.

*Molecular Identification and sequencing analysis*—The DNA extraction was made from grounded fungal biomass using liquid nitrogen. The quality of the extraction was tested through 1% agarose gel followed by Nanodrop™ Lite. The PCR was prepared in a total volume of 50 μL, with a final concentration of 0.2 mM dNTPs and 10 μM of forward oligonucleotides "*ITS1*" (5'TCC GTA GGT GAA CCT TGC GG 3') and reverse "*ITS4*" (5'TCC TCC GCT TAT TGA TAT GC 3') 2 mM of $MgCl_2$, 0.5 U of Taq DNA polymerase and 150 ng of genomic DNA as template DNA.

The amplification was done on a TC9610 Multigene Optimax gradient thermocycler (Labnet®) with an initial cycle of 5 minutes at 95 °C, followed by 28 cycles of 30 seconds at 95 °C, 30 seconds at 58 °C, 45 seconds at 72 °C with a final extension of 10 minutes at 72 °C. Bio Basic Inc.'s EZ-10 Spin column PCR kit was used to purify the ITS amplicon. The PCR fragment of the purified ITS was sequenced using BioEdit® 7.2.5 software. To determine the degree of similarity BLASTn offered by GenBank was used. Once the consensus sequence of the samples was obtained the phylogeny was made with 70 ITS sequences of fungi of the same genus.

#### *Phase 3*

*Liquid fermentation*—A standardized concentration of spores ($5x10^6$/mL) was inoculated in 200 mL of basal medium: 0.5 g of $NaNO_3$, 0.5 g of KCl, 0.5 g of $MgSO_4.7H_2O$, 2 g of $KH_2PO_4$, 1 g of yeast extract, 5 g of bacto peptone and 10 mL of olive oil adjusted to pH 7.6, incubated at

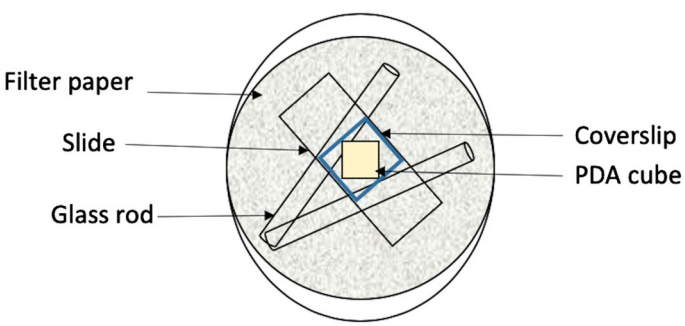


**FIG 1** *Microculture preparation. [Color figure can be viewed at wileyonlinelibrary.com]*

25 °C ± 2 °C and 150 rpm for seven days. A 25 mL sample was taken every 24 hours and centrifuged at 7,000 rpm for 30 minutes and 4 °C.

*Lipolytic activity determination*—The enzymatic crude extract (supernatant) was separated from the biomass and stored at 4 °C. A p-nitrophenol standard curve was prepared in Tris–HCl (pH 7.4) and the absorbance measured at 410 nm. The substrate solution (0.02 g of SDS, 0.02 g of 4-nitrophenyl palmitate and 1 g of triton X-100 in 100 mL of $H_2O$) was prepared with 1 mL of 50 mM phosphate buffer (pH 7.4) and 0.5 mL of the enzymatic extract with readings every minute for 15 minutes. The enzymatic unit (U) was defined as the amount of enzyme that releases 1 μmol of para-nitrophenol per minute.

$$U = \frac{[(\Delta abs)(total\ reaction\ volumen)]*1000}{[(slope)(standard\ cuvette\ lenght)(sample\ volume)]}$$

*Temperature*—The optimal temperature was determined in a range of 25 °C to 65 °C with increments of 10 °C, each maintained for 15 minutes. In order to test thermostability, the samples were pre-incubated in a water bath controlling the temperature at 35 °C, 45 °C and 55 °C for 60 minutes.

*pH*—The influence of pH on the enzymatic activity was examined by adjusting this parameter using four different buffers; citrate buffer (pH 3–6.2), acetate buffer (pH 3.6–5.6), phosphate buffer (pH 5.8–7.4) and carbonate buffer (pH 9.2–10.6).

*Cell rupture evaluation*—The fungal biomass was left to *air-dry* at 37 °C; samples of 100 mg were used to assess four different methods of extraction. To test the effectiveness of mechanical force, the sample was subjected to homogenization and resuspended in 1 mL of phosphate buffer to subsequently be placed in vortex for 5 minutes. As a second method, the use of detergents was evaluated with a mix of 5 μl of Triton X-100 and 1 mL of phosphate buffer with constant agitation for 30 minutes with ice as a temperature stabilizer. The sonication technique was evaluated by adding 1 mL of phosphate buffer to the biomass and placed in a Branson® 1200 sonicator for 6 minutes using distilled water at 4 °C as a conductor. The final method was carried out by grinding the biomass with liquid nitrogen to a fine powder, after which 1 mL of phosphate buffer was added and vortexed for 5 minutes. Samples from the four methods were centrifuged with two repetitions at 13,000 rpm for 20 minutes.

### Statistical Analysis

Once the enzymatic activity was obtained, we performed a multiple range test with a Fisher's Minimum Difference Procedure (LSD) with 5.0% risk to establish significant difference between the different methods of lysis analyzed using the Statgraphics® Centurion XVI software version 16.1.03.

## Results and Discussion

### Isolation and Screening for Enzymatic Activity

The sample was already isolated from commercial Parmesan cheese and cataloged as FOAN-1, any work that was done further with it corresponded to the project assigned to the student. The initial description of the fungus was observed to be olive green with white margins and abundant sporulation (Figs. 2A and 2B). Given that the species of fungi mainly found as the principal colonizers for Parmesan cheese are reported to be in the genus *Cladosporium*, *Epicoccum*, *Mucor* and *Penicillium* [5] it allowed us to determine a starting point for the identity of the isolate.

**Educational insights**—The screening for lipolytic activity was confirmed from the observation of orange fluorescence around the colony in rhodamine B agar (RBA) (Fig. 2B). With the formulation of a complex detection media like RBA, that requires two stages for preparation, the student had the opportunity to reaffirm his knowledge in solution preparation and relate the reaction of the dye with the detection of the enzyme. Regarding morphology, the appearance in this growth medium was less "dusty" observing white spots around the surface of the mycelium.

In order to allow the evaluation of the growth rate in different nutrient media, the student was instructed to monitor the radial growth of the colonies in both media (Fig. 3).

When examining the radial growth, we observed a linear tendency expressed in both PDA and RBA with a slightly faster growth time in PDA, which can be explained by Tremarin *et al.* (2015) [6] as they observed a typical radial growth behavior when incubating at temperatures close to the optimum for their fungi; by making this remark the student had the capability of defining stress related behavior from the microorganism growth rate and determine that when a stress factor (nutrient availability) is present it produced a slower growth rate and a clear modification on morphology.

### Microscopic Description

The morphological characteristics of the strain after 24 hours presented a segmented hyphae and conidiophore

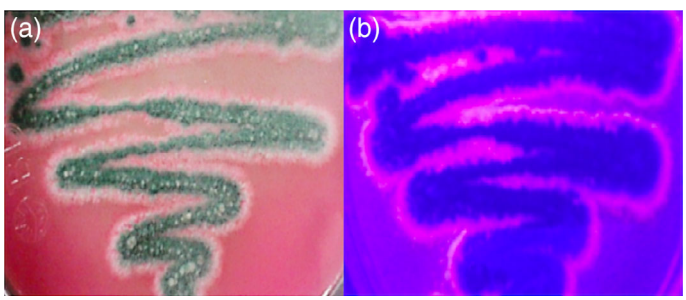


**FIG 2** *(A) Macroscopic morphology observed in Rhodamine b agar. (A) Orange fluorescence. [Color figure can be viewed at wileyonlinelibrary.com]*

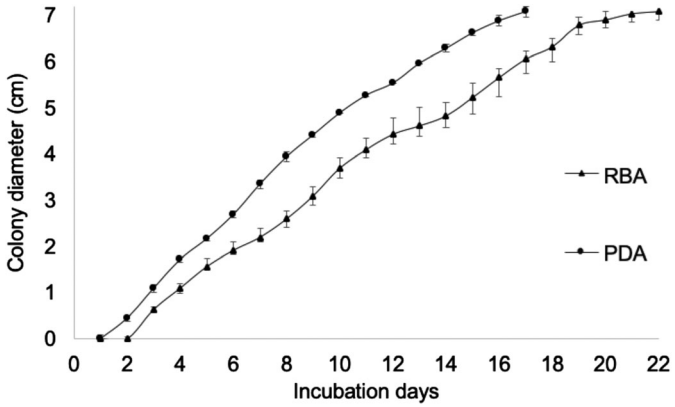


**FIG 3** *Colony diameter.*

(Fig. 4) with its late end closer to the metula. The phialides and conidia had a paintbrush shape; all characteristics of the genera *Penicillium* and shared among several species.

**Educational insights—**Considering that the student was a biotechnology major with little experience in microbiology, it was of great importance that the description was made to complete the registration of a new isolate in the collection of fungal strains in our laboratory and therefore know the information necessary to provide a classification according to the standards of the institution, including crucial data such as; isolation area, macroscopic and microscopic characteristics.

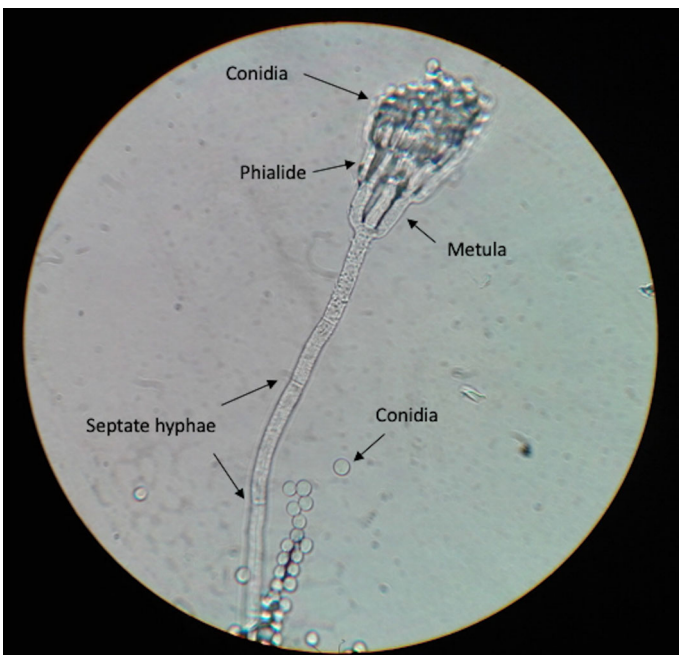


**FIG 4** *Microscopic observation of the sample at 100X. [Color figure can be viewed at wileyonlinelibrary.com]*

## Molecular Analysis and Identification

The DNA extraction was made at standard conditions with dried biomass and visualized in agarose gel with a concentration of 151.9 ng/μL and a A260/A280 ratio of 2.11. The control sample corresponded to a known size fungus (600 bp). The result of the alignment (Table I) using the ITS sequence was consequent on a phylogenetic tree where not all the branches and clades were completely resolved; with our sample of interest grouped on a clade among *Penicillium tricolor*, *Penicillium polonicum*, *Penicillium commune* and *Penicillium crustosum*.

**Educational insights—**With this information, the student concluded that the ITS sequence was not enough to determine the species among the *Penicillium* genera [12] given that they can be easily confused, providing a non-reliable determination of identity whereas these species are commonly mistaken phylogenetically [13]. When analyzing these results in context with the morphological characterization and the environmental description of the most common habitats where these species are found, the student was capable to differentiate information in a critical and focused manner according to the established work objectives and limit the information in order to concentrate his efforts on two possible species, *Penicillium commune* and *Penicillium crustosum*. Regarding *Penicillium commune*, this specie has spores that are often isolated in indoor air and can act as a deteriorator of hard cheeses and other dairy products [14], contrasting with *Penicillium crustosum*; commonly found in fruits in deterioration (apples, pears, strawberries *etc*.) with occasional reports in cheeses [15].

Relaying on the microscopic morphology of the strain, the student performed an analysis by comparison of the observations made (Fig. 4) with the morphology reported for *P. commune* and *P. crustosum*; as for the conidiophore, both are very similar, however, they have some differences. According to the branching patterns of the conidiophores for *P. crustosum*, they are usually tri-verticilated and strongly compacted [16], while for *P. commune*, they may also be tri-verticilated and on occasion can have a quadri-verticilated morphology and dissimilarly to *P. crustosum* not compact or even appear as separated [17]. Finally, the macroscopic growth of the sample showed a higher similarity with the characteristics of *Penicillium crustosum* [18] for which a homogeneous green color colony with white borders has been reported and was observed in the strain isolated in this study. Contrarily, the appearance of *Penicillium commune* presents a light green hue that turns yellow the closer it is to the center of the colony in PDA [19], diverging to the observations made in our work.

## Lipolytic Activity

There are scarce reports of screening for lipolytic activity for specific isolates of *Penicillium crustosum* using rhodamine B agar [20, 21], however this technique has been widely used for a variety of microorganisms showing its effectiveness [22, 23] reaffirming its use by the results obtained in our research.

**Educational insights—**Once the identity of the strain was bound to a closer range and the presence of lipolytic potential

**TABLE I** *Alignment results from BLAST analysis*

| *Description* | *Query cover (%)* | *Identity (%)* | *Accession number* | *Locality* | *Author* |
|---|---|---|---|---|---|
| *P. commune* NIJP03 | 100 | 97.35 | HQ710533.1 | Soil/India | [7] |
| *P. commune* ASM-07 | 100 | 97.12 | LC092113.1 | Soil/Saudi Arabia | [8] |
| *P. crustosum* HSf3 | 100 | 97.12 | KR425648.1 | Thermal water/Saudi Arabia | [9] |
| *P. crustosum* UFMGCB8594 | 100 | 97.12 | KP903322.1 | Lichen/Antarticta | [10] |
| *P. commune* | 100 | 97.12 | KR012904 | Common bean/Colombia | [11] |

was observed, the student was given the task to adequate the protocols established for quantification of lipolytic activity for his particular strain, taking into consideration the behavior already shown (growth rate and sporulation) and to determine the best option for intracellular quantification of lipolytic enzymes; with this approach we stimulate the exploration and examination component for a forefront theoretical framework by discriminating between the numerous scientific papers that present determination of lipolytic activity and our specific work protocols.

The initial intracellular activity evaluated (Fig. 5) from a standard fermentation at pH 7.6 and 25°C, had the highest value at 24 hours and presented a descending behavior during the course of the following seven days of incubation. As reported for the extracellular activity the highest peak was observed at 72 hours similar to the results showed by *Penicillium verrucosum* with 2.63 U/mL and *Penicillium restrictum* with 13 U/mL [24, 25] allowing the student to observe a very distinct adjustment in the manifestation of the enzymatic apparatus and in turn denote the adaptability process of the fungus to an exogenous carbon source in the form of olive oil (Fig. 5). Similar to the reports made by Takac *et al.* (2009) [26] in their work with submerged fermentations of *Candida rugosa*, when detecting the association with growth rate and presence of extracellular enzymes, leading to modulation as

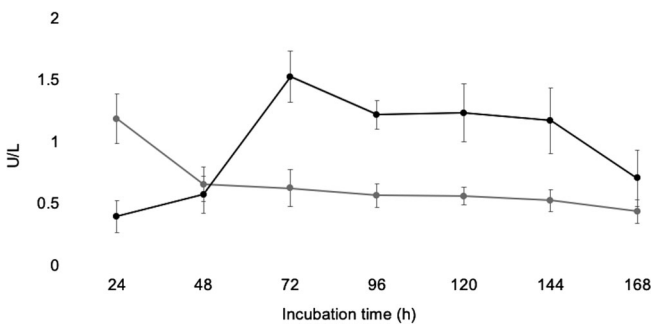


**FIG 5** *Lipolytic activity from extracellular (black lines) and intracellular samples (gray lines).*

well as a reduction of nutrients by the increased incubation time, as was the case for *Penicillium chrysogenum* reporting lipolytic activity at 120 hours [27, 28].

To further the analysis of the results obtained and considering that, numerous variables can have a direct effect of the production of enzymes we proceeded to examine some of the most important factors that can affect the production of lipases in a step by step strategy.

## Parameter Optimization

By this stage, the student was familiarized with the behavior shown by lipolytic enzymes and as a final step on the characterization, the analysis of significant variables was conducted.

Microbial lipases have their production greatly influenced by medium composition being the carbon source a major factor; since lipases are inducible and mostly extracellular, they are generally produced in the presence of a lipid source [29], therefore the use of olive oil as an inductor relays in that its major components are triacylglycerols; additionally, lipases are significantly influenced by physicochemical factors such as temperature and pH [30].

The pH had a critical influence on the crude extract defining the activity of the enzyme as neutral with an optimal value of pH at 7, observing significant loss of activity when tested at both the acid and the alkaline spectrum (Fig. 6).

Regarding temperature, the optimal value was registered at 45 °C corresponding to 20.9 U/L observing a decreasing result at 55°C with 12.68 U/L and finally at 65 °C with 7.6 U/L (Fig. 7). The optimum temperature and pH were within the normal range reported [31] and was comparable to the behavior of lipases from the same genus, like the enzymatic extract from *Penicillium verrucosum* reported by Pinheiro *et al.* (2008) [25]. Concerning the thermotolerance analysis, after 60 min of incubation at 45°C the enzymatic extract retained a 13% of residual activity, becoming inactive once challenged at 55 °C and 65 °C. When compared to the stability showed by several microorganisms like *Pseudomonas fluorescences*, *Pseudomonas cepacia* and *Aspergillus flavus* (optimal range of 40–50 °C) with inactivation up to 70 °C to 80 °C [32] we can discern that the temperature limit to maintain stability, is

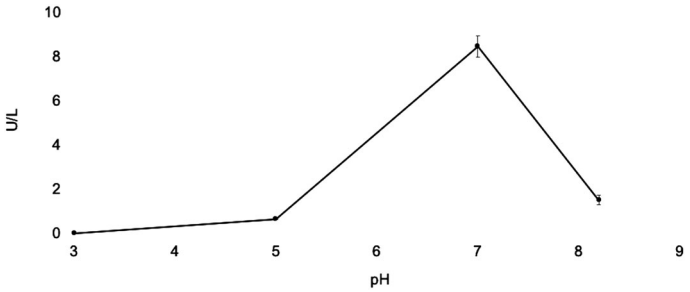


**FIG 6** *Evaluation of pH.*

significantly lower in our strain than those exhibited by the reported isolates, however, when considering the locality of isolation we believe that the particularity of the sample from which we obtain the strain (Parmesan cheese) which is regularly maintained at a storage temperature between 4–8 °C never exceeding 18 °C [33] could have played a key role in the adaptation process of the strain.

**Educational insights—**From the results obtained, the student could observe the improvement on the lipolytic activity with the adjustment of physical–chemical parameters such as pH and temperature and determine potential on the enzyme to be optimized to conclusively ponder that the source from which it was isolated had a direct effect on the ability developed from this enzymatic machinery. These findings are of great significance given that it allowed the student to correlate concepts that previously only knew from his regular theoretical courses giving him a clearer view on complex biological systems.

## Intracellular Lysis

**Educational insights—**To finalize the objectives presented, the student designed a protocol that consisted of four different methods for intracellular enzymatic determination that consider a range of possible mechanical, chemical and molecular interactions deciding on the use of homogenization, detergents, freezing with liquid nitrogen and sonication. Given that the strain that we work in this project was neither fully characterized nor had a definitive identification, we view this as a learning opportunity where we could assess different techniques for the same purpose, in this case intracellular lysis.

For the evaluation of the lysis methods aimed at attaining intracellular lipases, out of the four techniques tested (Fig. 8) the best results were obtained with sonication with 211 U/L. Centering the analysis on this method, diverse reports indicate that the use of sonication is an efficient method for cell lysis for organisms with resistant cell membranes and has been used by several authors, with some mentions that physical methods are the most common at industrial level [29, 34]. The discussion about sonication presented by Klimek *et al.* (2011) [35] refers that the use of it depends on factors such as the percentage of chitin in the cell walls which can lead to the variation in effectiveness from the same lysis method between organisms; however, said methods do not leave residues and by not having a direct interaction with the enzyme, like some detergents may have for the inherent presence of charges that could affect the integrity or enzymatic yield of the enzyme of interest, comprise in our case a preferable technique.

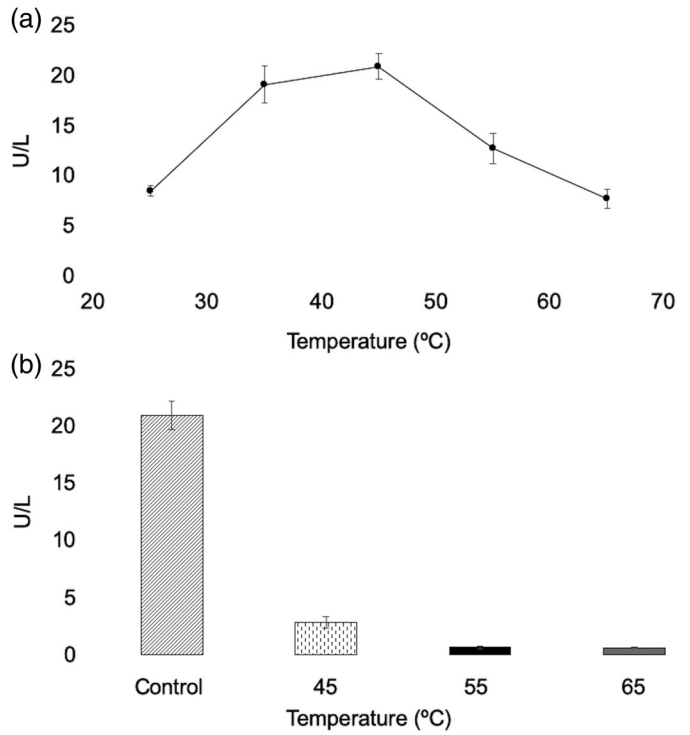


**FIG 7** *(A) Optimal temperature and (B) Thermotolerance.*

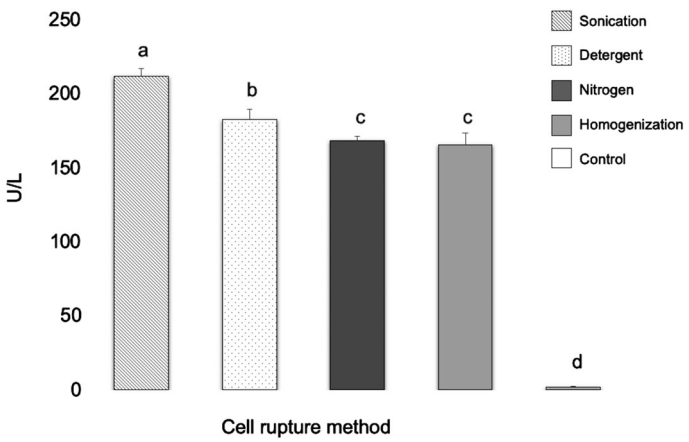


**FIG 8** *Lysis methods evaluated.*

## Conclusions

With the use of an integral approach to the characterization and identification of a novel fungal isolate we provided the student with valuable practical experience in areas such as microbiology, biochemistry, molecular biology, bioinformatics and biotechnology, reinforcing the knowledge obtained through the theoretical curses and mainly gathering that through a direct experience the student was able to work under pressure, meet deadlines and retain the curiosity and commitment that graduate school requires.

As for the final decision of the student that conducted the research, he completed the program successfully, however his original intentions shifted from the research environment towards a position on quality management within the food industry. With these results, we, as a research lab that welcomes students reinforce our main objective, an holistic formation of future human resources capable of development in either the scientific, industrial or the academic fields of work.

## Acknowledgments

We would like to thank the Biotechnology Institute of the Universidad Autónoma de Nuevo León for the support provided for the completion of this work.

### REFERENCES

[1] Dias, S., Oliveira, M., Semedo-Lemsaddek, T., Bernardo, F. (2014) Probiotic potential of autochthone microbiota from São Jorge and Parmigiano-Reggiano cheeses. Food Nutr. Sci. 5, 1793–1799. https://doi.org/10.4236/fns.2014.518193.

[2] McSweeney, P. L. H. (2004) Biochemistry of cheese ripening. Int. J. Dairy Technol. 57, 127–144. https://doi.org/10.1111/j.1471-0307.2004.00147.x.

[3] N. Banjara (2014) *Debaryomyces hansenii*: a foodborne yeast that produces anti-candida killer toxin. [Thesis] Master of Sciences. University of Nebraska-Lincoln, Food Science and Technology Department.

[4] Ledenbach, L. H., Marshall, R. T. (2009) Microbiological spoilage of dairy products. In: Sperber W. H., Doyle M. P., Eds. Compendium of the Microbiological Spoilage of Foods and Beverages. Food Microbiology and Food Safety, Springer, New York, NY, pp. 41–67. https://doi.org/10.1007/978-1-4419-0826-1_2.

[5] Lavoie, K., Touchette, M., St-Gelais, D., Labrie, S. (2012) Characterization of the fungal microflora in raw milk and specialty cheeses of the province of Quebec. Dairy Sci. Technol. 92, 455–468. https://doi.org/10.1007/s13594-011-0051-4.

[6] Tremarin, A., Longhi, D., Salomão, B., Aragão, G. (2015) Modeling the growth of *Byssochlamys fulva* and *Neosartorya fischeri* on solidified apple juice by measuring colony diameter and ergosterol content. Int. J. Food Microbiol. 193, 23–28.

[7] Jain, N., Bhargava, A., Tarafdar, J. C., Singh, S. K., Panwar, J. (2013) A biomimetic approach towards synthesis of zinc oxide nanoparticles. Appl. Microbiol. Biotechnol. 97, 859–869.

[8] Mohamed, S., Al-Yami, M., Sadik, A. (2016) Detection of Mycophages associated with fungal strains isolated from soil of KSA and identified via18S rRNA gene. Res. J. Pharm. Biol. Chem. Sci. 7, 1375–1380.

[9] Selim, S., Hassan, S., Hagagy, N. I., Krakova, L., Grivalsky, T., Pangallo, D. (2016) Assessment of microbial diversity in Saudi Springs by culture-dependent and culture-independent methods. Geomicrobiol. J. 34, 443–453. https://doi.org/10.1080/01490451.2016.1219430.

[10] Santiago, I. F., Soares, M. A., Rosa, C. A., Rosa, L. H. (2015) Lichensphere: a protected natural microhabitat of the non-lichenised fungal communities living in extreme environments of Antarctica. Extremophiles 19, 1087–1097. https://doi.org/10.1007/s00792-015-0781-y.

[11] Parsa, S., García-Lemos, A. M., Castillo, K., Ortiz, V., López-Lavalle, L. A., Braun, J., Vega, F. E. (2016) Fungal endophytes in germinated seeds of the common bean, *Phaseolus vulgaris*. Fungal Biol. 120, 783–790. https://doi.org/10.1016/j.funbio.2016.01.017.

[12] Scoch, C., Seifert, K., Huhndorf, S., Robert, V., Spouge, J., Levesque, A., Chen, W., Fungal Barcoding Consortium (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. PNAS 109, 6241–6246. http://www.pnas.org/content/109/16/6241.full.pdf.

[13] Peterson, S. (2000) Phylogenetic analysis of *Penicillium* species based on ITS and LSU-rDNA nucleotide sequences. In: Samson R. A., Pitt J. I., Eds. Integration of Modern Taxonomic Methods for Penicillium and Aspergillus Classification, Harwood Academic Publishers, Amsterdam, the Netherlands. pp. 163–178.

[14] Ostenfeld, L. T., Gareis, M., Frisvad, J. C. (2002) Cell cytotoxicity and Mycotoxin and secondary metabolite production by common Penicillia on cheese agar. J. Agric. Food Chem. 50, 6148–6152. https://doi.org/10.1021/jf020453i.

[15] Camardo Leggieri, M., Decontardi, S., Bertuzzi, T., Pietri, A., Battilani, P. (2016) Modeling growth and toxin production of toxigenic fungi signaled in cheese under different temperature and water activity regimes. Toxins 9, 4. https://doi.org/10.3390/toxins9010004.

[16] CBS-KNAW. (2003) *Penicillium crustosum*. Fungal Biodiversity Centre of The Royal Netherlands Academy of Arts and Sciences. http://www.westerdijkinstitute.nl/Penicillium/BioloMICS.aspx?Table=Species%20of%20subgenus%20Penicillium&Rec=19&Fields=All/ (Revised 25 May 2018).

[17] CBS-KNAW. (2004) *Penicillium commune*. Fungal Biodiversity Centre of The Royal Netherlands Academy of Arts and Sciences. http://www.cbs.knaw.nl/Penicillium/BioloMICS.aspx?Table=Species%20of%20subgenus%20Penicillium&Rec=14&Fields=All/ (Revised 25 May 2018).

[18] Eunjong, B., Hyojin, K., Hyejung, C., Sun, Y., Jeongho, K. (2012) Antifungal activity of *Leuconostoc citreum* and Weissella confuse in rice cakes. J. Microbiol. 50, 842–848.

[19] Dhakar, K., Sharma, A., Pandey, A. (2014) Cold, pH and salt tolerant *Penicillium* spp. inhabit the high-altitude soils in Himalaya, India. World J. Microbiol. Biotechnol. 30, 1315–1324. https://doi.org/10.1007/s11274-013-1545-4.

[20] Bavaro, S. L., Susca, A., Frisvad, J. C., Tufariello, M., Chytiri, A., Perrone, G., Mita, G., Logrieco, A. F., Bleve, G. (2017) Isolation, characterization, and selection of molds associated to fermented black table olives. Front Microbiol. 8, 1356. https://doi.org/10.3389/fmicb.2017.01356.

[21] Sibanda, T., Selvarajan, R., Tekere, M., Nyoni, H., Meddows-Taylor, S. (2017) Potential biotechnological capabilities of cultivable mycobiota from carwash effluents. MicrobiologyOpen 6(5), e00498. https://doi.org/10.1002/mbo3.498.

[22] Carissimi, M., Ottonelli Stopiglia, C. D., Furtado de Souza, T., Corbellini, V. A., Scroferneker, M. L. (2007) Comparison of lipolytic activity of *Sporothrix schenckii* strains utilizing olive oil-rhodamine b and tween 80. TECNO-LÓGICA 11, 33–36.

[23] Suseela, L., Anupama, M., Prudhvilal, B., Narasaiah, T. V., Naveena, J. L. L. (2014) Isolation and characterization of lipase producing fungi from palm oil mill effluent obtained from Pedavegi, A. P, India. Int. J. Biol. Pharm. Res. 5, 559–565.

[24] Freire, D., Teles, E., Bon, E., Sant, G. (1997) Lipase production by *Peniillium restrictum* in a bench-scale fermenter: effect of carbon and nitrogen nutrition, agitation, and aeration. Appl. Biochem. Biotechnol. 63, 63–65.

[25] Pinheiro, T., Menoncin, S., Domingues, N., Oliveira, D., Treichel, H., Diluccio, M., Freire, D. (2008) Production and partial characterization of lipase from *Penicillium verrucosum* obtained by submerged fermentation of conventional and industrial media. Food Sci. Technol. 28, 444–450. https://dx.doi.org/10.1590/S0101-20612008000200028.

[26] Takaç, S., Ünlü, A. E., Erdem, B. (2010) Oxygen transfer strategy modulates the productions of lipase and esterase enzymes by *Candida rugosa*. J. Mol. Catal. B: Enzym. 64, 150–154.

[27] Cho, H., Bancerz, R., Ginalska, G., Leonowicz, A., Cho, N., Ohga, S. (2007) Culture conditions of Psychrotrophic fungus, *Penicillium chrysogenum* and its lipase characteristics. J. Fac. Agr. Kyushu Univ. 52, 281–286.

[28] Iftikhar, T., Abdullah, R., Iqtedar, M., Kaleem, A., Aftab, M., Niaz, M., Tabassum, B., Majeed, H. (2015) Production of lipases by *Alternaria* sp. (mbl 2810) through optimization of environmental conditions using submerged fermentation technique. Int. J. Biosci. 7, 178–186.

[29] Ramnath, L., Sithole, B., Govinden, R. (2017) Identification of lipolytic enzymes isolated from bacteria indigenous to *Eucalyptus* wood species for application in the pulping industry. Biotechnol. Rep. 15, 114–124. http://doi.org/10.1016/j.btre.2017.07.004.

[30] Zarevúcka, M. (2012) Olive oil as inductor of microbial lipase. In: Boskou D., Ed. Olive Oil - Constituents, Quality, Health Properties and Bioconversions, InTech, Rijeka, Europe. https://www.intechopen.com/books/olive-oil-constituents-quality-health-properties-and-bioconversions/olive-oil-as-inductor-of-microbial-lipase (Revised 25 May 2018).

[31] Yuan, D., Lan, D., Xin, L. R., Yang, B., Wang, Y. (2016) Screening and characterization of thermostable lipase from marine *Streptomyces* sp. strain w007. Biotechnol. Appl. Biochem. 63, 16–22. https://doi.org/10.1002/bab.1338.

[32] Colla, L., Ficanha, A., Rizzardi, J., Bertolin, T., Reinehr, C., Vieira, J. (2015) Production and characterization of lipases by two new isolates of *Aspergillus* through solid-state and submerged fermentation. Biomed. Res. Int. 2015, 725959.

[33] Jaster, H., Pires de Campos, A. C. L., Auer, L. B., Los, F. G. B., Salem, R. D. S., Esmerino, L. A., Nogueira, A., Demiate, I. M. (2014) Quality evaluation of parmesan-type cheese: a chemometric approach. Food Sci. Technol. 34, 181–188.

[34] Kanmani, P., Kumaresan, K., Aravind, J. (2015) Gene cloning, expression, and characterization of the *Bacillus amyloliquefaciens* PS35 lipase. Braz. J. Microbiol. 46, 1235–1243. http://doi.org/10.1590/S1517-838246420141068.

[35] Klimek, M., Brzezińska, M., Żymańczyk, E., Lejczak, B., Kafarski, P. (2011) Comparative study of fungal cell disruption—scope and limitations of the methods. Folia Microbiol. 56, 469–475.