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# Biotechnological valorization of oils from agro-industrial wastes to produce lipase using *Aspergillus sp.* from Amazon

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ABSTRACT

Lipases are enzymes with a large number of applications at the industrial level. New producers and the development of bioprocess with high productivity are the major challenges of these enzymes industrially. Regarding the production process, oils from agro-industrial waste can emerge as efficient enzymes inductor reducing the production cost. Moreover, this process can minimize environmental problems related to agro-industrial disposal. Thus, this work proposes to evaluate a new *Aspergillus* specie from Amazon forest in Brazil as lipase producer using oils from agro-industrial waste (grape seed oil (GSO) and cotton seed oil (CSO)) as an inductor. It was used olive and soybean oil as standard. The microorganism metabolized all oils studied; however, the ones with a higher percentage of fatty acid esters (> 80%), namely, soybean, olive, and GSO, promoted the highest lipase production. By comparing the activity of the lipase produced, GSO allowed an enzyme activity 2 fold higher than CSO. The enzyme produced using GSO was characterized and pre-purified by precipitation, showing an optimum pH of 7.5 and stability for pH between 7 and 7.5 values; an optimum temperature of 45 °C and a thermal stability between 30 and 50 °C was achieved. In conclusion, a new isolated from Amazon, *Aspergillus sp.*, can produce lipase for industrial purpose using GSO. Moreover, other oils from agro-industrial waste with a high percentage of fatty acid esters can be used as an inductor for lipase production.

## 1. Introduction

Lipases (EC 3.1.1.3) are hydrolases enzymes, also called as glycerol esters hydrolases, with the natural function of triglycerides hydrolysis in an aqueous medium into glycerol and fatty acids. Because of their characteristics, these enzymes can be applied in the production of detergents, cosmetics, water treatment, bioremediation, oleochemical industry, production of structured lipids (Ferreira-Dias et al., 2013; Palomo et al., 2004; Sharma et al., 2001), among others, replacing synthetic compounds or exploring new areas.

In 2016 the value of the global market for industrial enzymes has reached USD 5 billion being lipases responsible for 10% of the global industrial enzyme (Lai et al., 2018). These enzymes correspond to the third biggest-selling group in the world (Messias et al., 2011). It is expected a compound annual growth rate (CAGR) of 4.7% each year, increasing from US $ 5.0 billion to US $ 6.3 billion during the period from 2016 to 2021 (Dewan, 2014). In this way, the market of enzymes remains interesting and as lipases correspond to an important percentage of this market, new sources and production procedure are of industrial interest. Lipases can be produced by animals, plants and microorganisms being the enzyme produced by the last one more stable, with substrate specificity and with lower production cost as compared to other sources (Contesini et al., 2010). Moreover, from the economic and industrial point of view, microbial lipases have advantages because of fermentative processes (Padilha et al., 2011), which can be in the solid state or submerged culture. In the submerged culture, the microorganism develops in the liquid medium and factors such as temperature, pH, carbon and nitrogen source, the presence of inductors, among others can interfere in the production process (Domínguez et al., 2003). Although in the literature there are several studies evaluating the lipase production in culture media using different oils as carbon source and/or inductor (soybean oil (Colla et al., 2015); olive, soybean, corn and

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sunflower oils (Lima et al., 2003); olive, soybean, lard, peanut and sunflower seed oil (Jia et al., 2015); sunflower, coconut, palm, sage, almond, mustard, ghee, castor, olive and sesame (Sethi et al., 2016)), there is still interest in studies using vegetable oils from alternative sources, mainly agro-industrial waste. Oils from agro-industrial waste are an environmental problem because the most part of them are disposal by burning, dumping or unplanned landfilling creating different problems (Sadh et al., 2018). Grape seed oil (GSO) is a residue from wine industry (Shinagawa et al., 2015) while cotton seed oil (CSO) is a waste from cotton industry (Panagiotopoulos et al., 2013). Both oils are composed of long-chain esters of fatty acids (Lakshmi et al., 1999) being oils rich in linoleic and oleic acid (Agostini et al., 2012; Carbonell-Verdu et al., 2015; Shinagawa et al., 2015). So, these oils can be used as an alternative to extensively studied soybean and olive oils and others aforementioned in order to produce lipases by bioprocesses.

Taking into consideration the above, this work aimed to study the production of lipases by submerged culture using a new strain of *Aspergillus sp.* isolated from Amazon forest using oils from agro-industrial waste, namely, GSO and CSO as a carbon source and enzyme inductor. The enzyme produced using GSO was characterized in relation to pH and temperature and studies of pre-purification employing precipitation methods were also carried out. The objectives proposed in the present study are of great interest in industrial terms because they aim to valorize an industrial residue producing lipase which is an enzyme with numerous industrial application.

## 2. Materials and methods

### 2.1. Materials

Bacteriological peptone and Potato Dextrose Agar were purchased from Acumedia®. Olive oil and soybean oil were purchased from Carbonell® and Soya® acquired at a local market (Araraquara, São Paulo, Brazil), respectively; grape seed oil and cotton seed oil were donated by Distriol® (São Paulo, Brazil). The substrate *p-nitrophenyl palmitate* (*pNPP*) was purchased from Sigma-Aldrich® (St. Louis, MO). All the other reagents were of analytical grade and used as received.

### 2.2. Microorganism maintenance

The fungus *Aspergillus sp.* DPUA 1727 was generously provided by the Culture Collection of the Federal University of Amazonas, DPUA, AM, Brazil. The cultures were preserved in distilled water, reactivated in Potato Dextrose Agar supplemented with yeast extract (PDA) and maintained at 30 °C for 7 days. After this period, the cultures were kept in the refrigerator at 4 °C. The inoculum was prepared in PDA plate, and the cultures were maintained at the same reactivation conditions. The PDA medium had the following composition (g/L, in deionized water): agar potato dextrose (39.0) and yeast extract (5.0).

### 2.3. Fungus identification

The total fungal genomic DNA was isolated using the BioPur Mini Spin Extraction Kit® (Biometrix, Brazil), according to manufacturer's recommendations. A portion of the calmodulin (*CaM*) gene was amplified using the primer-pair cmd5/cmd6 (Hong et al., 2006). PCR was performed in 25 µl reaction mixture containing a 1 × PCR buffer, 2.0 mM of MgCl2, 0.2 mM of dNTP mixture (Invitrogen, Life Technologies), 0.4 µM of each primer, 1 U of Taq DNA polymerase (Invitrogen, Life Technologies), and 10 ng of DNA template. Thermal cycling conditions were 95 °C for a 5 min denaturation step, followed by 34 PCR cycles (94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min) and a final extension at 72 °C for 5 min. The PCR amplicon was purified using ExoProStarTM 1-Step (GE Healthcare Life Sciences, UK) and submitted to direct sequencing in both directions using a BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA). The reaction product was processed in ABI 3500XL Genetic Analyzer (Applied Biosystems, USA). The sequence obtained was compared using the Basic Local Alignment Search Tool (Altschul et al., 1990) against the GenBank checking the "sequences from type material" (https://blast.ncbi.nlm.nih.gov/Blast.cgi). In addition, the sequence here obtained was aligned by a ClustalW algorithm with the type strains sequences from all *Aspergillus* section *Nigri* species available in the NCBI database. A Maximum Likelihood (mL) tree was reconstructed, with 1000 bootstrap replicate, using MEGA7 software (Kumar et al., 2016).

### 2.4. Production of lipase by submerged culture

For the submerged culture, Erlenmeyer flasks (250 mL) containing 50 mL of culture medium was inoculated with 5 mycelial agar discs (8 mm diameter) of *Aspergillus sp.* DPUA 1727 strain. After incubation for 72 h at 30 °C and 150 rpm in an orbital shaker, the fermented broth was filtered using a filter paper of 80 g $m^{-2}$ (Whatman, UK). The biomass was determined by dry weight using a moisture balance (Shimadzu® MOC-63U). The cell-free filtrate was used to measure the lipase activity, total proteins, and pH (pHmeter).

First, soybean, olive, grape seed, or cotton seed oils were evaluated as carbon source and inductor. To this purpose, the media had the following composition: 20.0 g/L bacteriological peptone, 0.6 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L $KH_2PO_4$, 1.0 g/L $NH_4NO_3$ and 8.0 mL/L oil. The experiments were performed in triplicate and the initial pH of the fermented medium was adjusted to 6.3. Subsequently, the lipase production over time was studied in the same conditions aforementioned but evaluating only grape seed oil. Samples were taken after 24, 48, 72, 96 and 120 h of fermentation process.

### 2.5. Lipase stability studies

Initially, it was determined the optimal pH and temperature of the lipase. The optimal pH was carried out in the range of pH from 3 to 10 diluting the fermented broth containing the enzyme in the respective buffer in the rate 1:1 at 37 °C. The buffers used were: Mcllvaine buffer (pH 3, 4, 5 and 6), 0.1M phosphate buffer (pH 7 and 8) 0.1 M, and 0.1M bicarbonate-carbonate buffer (pH 9 and 10) 0.1 M The optimal temperature was determined in the fermented broth containing the enzyme diluted in 0.1 M phosphate buffer pH 7.5 in the range of temperatures from 20 to 60 °C.

Following, the lipase stability under several values of pH (from 3 to 10 at 25 °C, buffer aforementioned) and temperature (from 20 to 60 °C at 0.1 M phosphate buffer pH 7.5) was studied. In both experiments the fermented broth was diluted in the buffer in the ratio of 1:1 and the solutions were incubated in a thermoregulated bath (model 521/2DE, New Ethics, SP, Brazil) during 48 h. Aliquots were taken after 0, 1, 3, 6, 24 and 48 h of incubation.

For all the stability studies, the lipase relative activity (RA%) was determined according to the Eq. (1):

$$RA = \frac{LA}{LA_0} \times 100 \quad (1)$$

where *LA* and *LA0* are the lipase activity (U $mL^{-1}$) after the incubation time and initial enzymatic activity, respectively.

### 2.6. Pre-PurificationLipase pre-purification using precipitation methods

#### 2.6.1. Precipitation with ammonium sulfate

Precipitation with ammonium sulfate was performed according to Bharti et al. (2013), with modifications. The cell-free fermented broth was precipitated using ammonium sulfate at 80% of saturation (w/v), left overnight under stirring at 4 °C and then, at 10,886 × *g* for 15 min at 4 °C, in the Thermo Scientific Sorvall Legend RT Centrifuge. Subsequently, the precipitated was resuspended with 6 mL of phosphate buffer (0.1 M, pH 7.5) and dialyzed using dialysis membrane (cut-off:

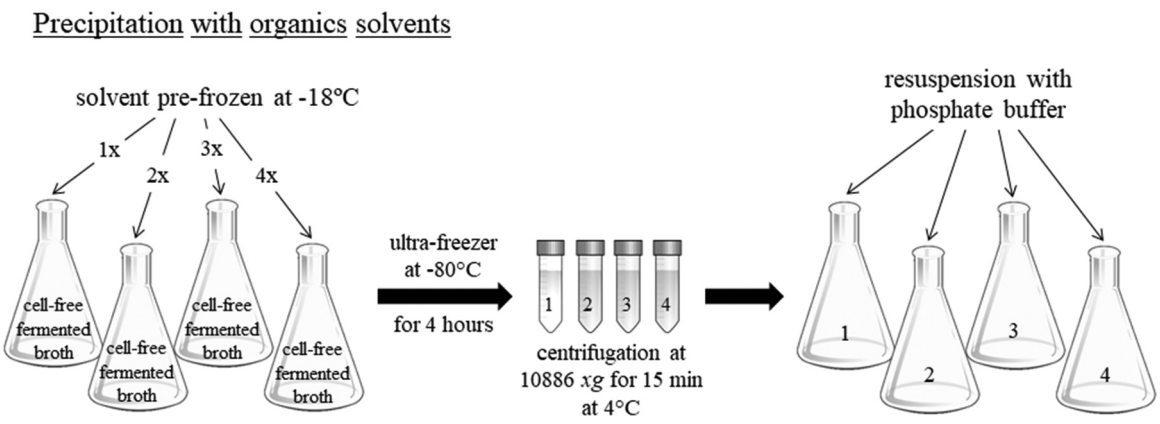


**Fig. 1.** Scheme elucidating the procedure of precipitation with organic solvents.

10,000–12,000 Da) for 48 h at 4 °C. The buffer solution was exchanged every 6 h. Afterward, the dialyzed precipitate was resuspended in phosphate buffer (0.1 M, pH 7.5), in the final concentration of 1 g/mL, for measurement of enzyme activity and total proteins.

#### 2.6.2. Precipitation with organic solvents

Acetone and ethanol were used to precipitate lipase. The protocol used for both solvents was based on the work of Yadav et al. (2015), with modifications. The solvents were pre-frozen at −18 °C and the tests were carried out by adding 1, 2, 3 and 4 times the volume of the cell-free fermented broth to each solvent and brought into ultra-freezer at −80 °C for 4 h. After incubation, the solution was centrifuged at 10,886 × *g*, for 15 min at 4 °C, in the centrifuge Hitachi CR22N Shimadzu®. The precipitate was resuspended in phosphate buffer (0.1 M, pH 7.5), to a final concentration of 2.5 g/mL and the measurements of enzyme activity and total proteins were performed. A scheme to illustrate the precipitation with organic solvents is shown in Fig. 1.

In the studied that evaluated the effect of different oils on lipase biosynthesis (Section 3.2) and in the purification tests employing precipitation (Section 4), it was calculated the specific activity (U $mg^{-1}$ of proteins). In the purification tests was also calculated the purification factor. The following equations were used to calculate the aforementioned parameters:

$$\textbf{Specific activity}(\textbf{U mg}^{-1}) = \frac{\text{enzymatic activity(U mL}^{-1})}{\text{total proteins (mg mL}^{-1})} \quad (2)$$

$$\textbf{Purification factor} = \frac{\text{specificactivity of the purified enzyme(U mg}^{-1})}{\text{specificactivity of the fermented medium (U mg}^{-1})} \quad (3)$$

### 2.7. Analytical methods

#### 2.7.1. Lipase activity

The lipase activity was measured according to the methodology described by Mayordomo et al. (2000), with modifications. The reaction was carried out by adding 250 µl of a solution containing 200 mg Triton X-100, 50 mg gum arabic and 0.1 M phosphate buffer (pH 7.5) to a total volume of 50 mL, 250 µl of enzyme solution (filtered broth and solution in the precipitated assays) and 45 µl of solution containing 15 mg of *p-nitrophenyl palmitate* (*pNPP*) diluted in 10 mL of isopropanol. The reaction took place in a 40 °C bath for 30 min. After the reaction, 0.5 mL of Trizma base 2% (w/v) solution was added. The quantification of the activity was performed from the formation of *pNP*, which produces yellow coloration, quantified by absorbance at 398 nm in a multimode plate reader (EnSpire® PerkinElmer). One unit of activity was defined as the amount of enzyme required to hydrolyze 1 µmol *pNP* per minute under the described conditions.

#### 2.7.2. Protein content analysis

The concentration of total proteins was determined by the method of Lowry et al. (1951). To this purpose, initially, the Biuret reaction involved the reduction of copper ($Cu^{2+}$ to $Cu^{+}$) by proteins in alkaline solutions, followed by the enhancement stage, the reduction of the Folin–Ciocalteu reagent producing a characteristic blue color. The absorbance was measured in the wavelength of 595 nm in multimode plate reader EnSpire PerkinElmer®

#### 2.7.3. SDS-PAGE analysis

The electrophoresis analyzes were made based on the Laemmli (1970) method. The gel was made with 10% polyacrylamide by applying 20 µl of the sample in each well and 2 µl of 10 kDa molecular weight marker.

#### 2.7.4. Scanning Electron Microscopy (SEM)

The samples were made from the inoculum of *Aspergillus sp.* in PDA plate, grown for 7 days. The mycelium discs were removed and fixed in a flat bottom plate, with glutaraldehyde 5%. After drying for 5 days at ambient temperature, the wells were washed with ethanol 70% and 80% to remove excess spores for better visualization of the structures. The samples were analyzed at the Chemistry Institute of Unesp Araraquara, under an scanning electron microscope of the JEOL (model JSM-7500F, PC-SEM operating software v. 2.1.0.3), equipped with secondary electron detectors, backscatter and chemical analysis (energy dispersive spectroscopy - EDS) of Thermo Scientific (Ultra Dry model, operation software NSS 2.3).

#### 2.7.5. Statistical analysis

Statistical analyzes were performed using the statistical software IBM SPSS® version 21.0. The experimental results were presented by the mean values of the triplicates and standard deviations of the means. To verify the equality between the means, the statistical test used was one-way ANOVA, with Welch's correction for variables with unequal variances, with a significance level of 5% and Tukey's post-test.

## 3. Results and discussion

### 3.1. Fungus identification

Although internal transcribed spacer rDNA region (ITS1–5.8S-ITS2) is the official DNA barcode for fungi (Schoch et al., 2012) it does not contain enough variation for distinguishing among all *Aspergillus* section *Nigri* species. As recommended by some authors (Raja et al., 2017; Samson et al., 2004) it was used DNA sequences of the gene encoding calmodulin (*CaM*) to identify the fungal isolate here studied. This kind of sequences is useful for species-level identification in certain lineages of fungi such as *Aspergillus*, one of the most prolific genera of fungi for

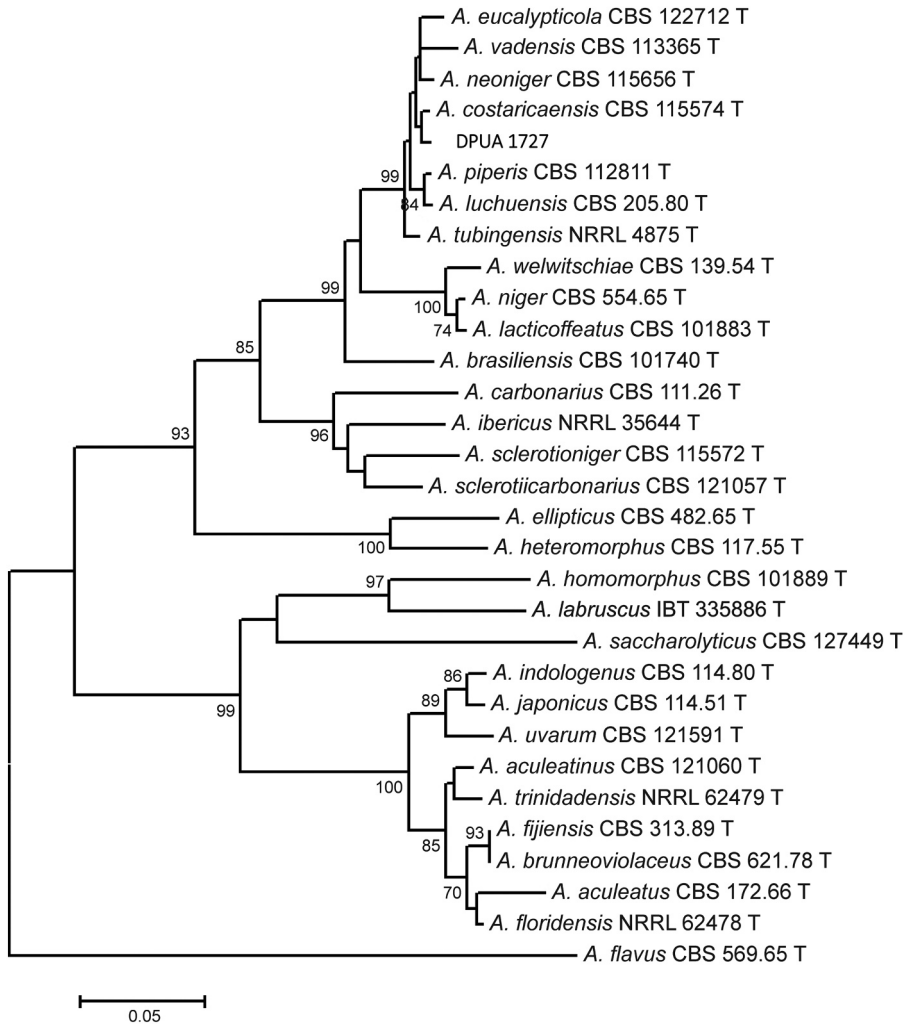


**Fig. 2.** *Calmodulin* based phylogenetic tree for *Aspergillus sp.* DPUA 1727 showing relatives fungus (Kimura two-parameter model; Maximum Likelihood algorithm). Bootstrap values (1000 replicate runs) greater than 70% are listed.

secondary metabolites and which include numerous medicinally and industrially important species (Raja et al., 2017). The *CaM* gene sequence obtained in the present study for the DPUA 1727 strain was most similar to that of *Aspergillus costaricaensis* type strain (I = 99%), based on BLASTn search checking the "sequences from type material". The *CaM* based phylogenetic tree reconstructed with the sequence obtained for DPUA 1727 strain and those retrieved from the GenBank database for each type species placed in *Aspergillus* section *Nigri* is shown in Fig. 2. The isolate DPUA 1727 was phylogenetically recognized as a member of *A. costaricaensis* species. *A. costaricaensis* is a biseriate species (Epithet: costaricaensis) that was firstly isolated from soil in Costa Rica (Samson et al., 2004). It is not an ochratoxin-producing species and may be especially interesting for biotechnological exploration (Samson et al., 2004). In CYA media the colony of *A. costaricaensis* presents black color and reverse colony straw-colored (Silva et al., 2011). The strain 1727 presents phylogenetic very similar to *A. costaricaensis,* however, probably it is a new strain that was isolated from Amazon soil. Therefore, in this work, we will call the microorganism as *Aspergillus sp.* DPUA 1727. Fig. 3 presents the microorganism morphology in BDA plate (A) and obtained by scanning electron microscopy (SEM).

### 3.2. Effect of different oils on lipase biosynthesis

Microorganisms require a carbon and nitrogen source to their grown and biosynthesis of bioproducts. In the case of lipase biosynthesis by a microorganism, it is necessary the presence of an inductor such as a fat or oil that has their ester bonds break down releasing free fatty acids (Gupta et al., 2015). The inductor can also be used as the main carbon source of the microorganism (Gupta et al., 2015). The study of an alternative lipase production process by submerged culture of *Aspergillus sp.* strain DPUA 1727 using oils from agro-industrial waste, GSO, and CSO, was carried out. The results of lipase hydrolytic activity with GSO and CSO were compared with conventional inductors soybean and olive oil (Colla et al., 2015; Jia et al., 2015), as can be observed in Table 1 which also shows the results of pH and biomass.

Among the inducers evaluated, the lipase activity decreased according to the following sequence: SO > OO = GSO > CSO. The result achieved can be related to the carbonic acid portion presented in the fatty acid of each oil studied (Dobrev et al., 2015; Sethi et al., 2016). Table 2 shows the main components in terms of fatty acids of each oil used.

As can be seen at Table 2, the percentage of fatty acid esters in the oils was 89.15%, 85.5%, 84.4%, and 77% (v/v) to grape seed, soybean, olive and cotton seed, respectively. These values were calculated considering the mean between the minimum and maximum values of each C18:n fatty acid shown in the table. In this way, the actual value may have variations between these extremes. The lipase biosynthesis was more pronounced in oils with a high amount (> 80%) of C18:n fatty acids present. These results are in agreement with the work of Lakshmi et al. (1999), which studied the production of lipase by *Candida rugosa*. In this work, the quantity of lipase produced also had a correlation with

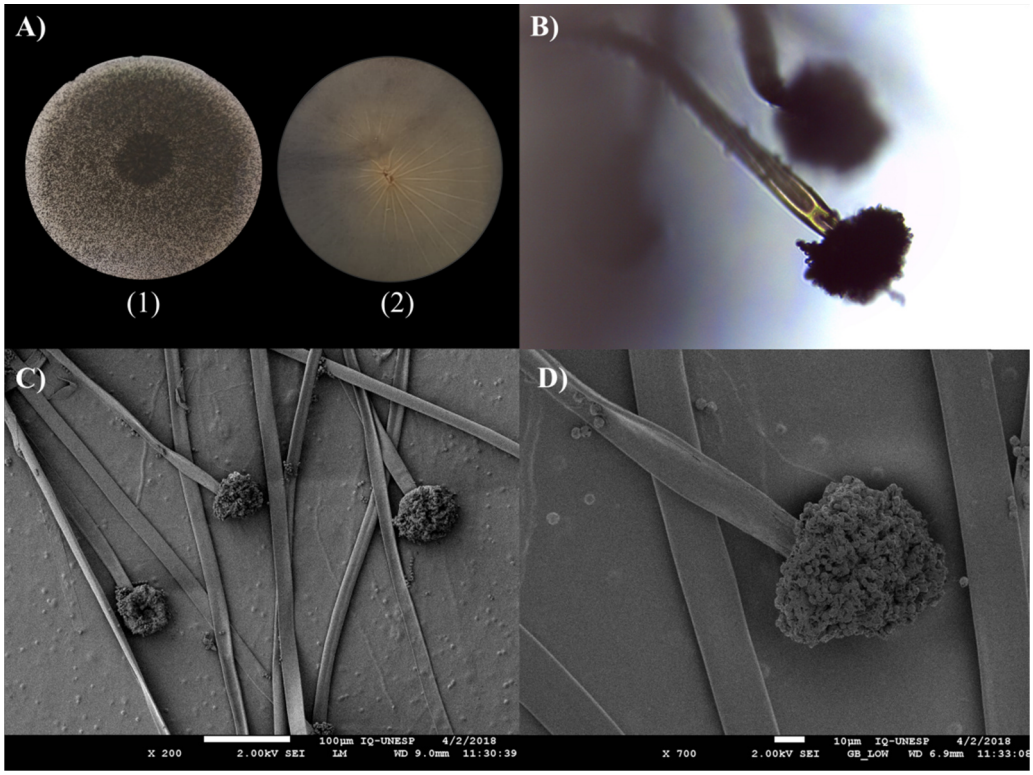


**Fig. 3.** Images of *Aspergillus sp.* DPUA 1727 A) growth for 7 days in BDA plate, B) conidiophore and conidia saw by optical microscopy and C and D) hyphae, conidiophores, dehydrated after treatment with 70% ethanol, and conidia seen by Scanning Electron Microscopy (SEM).

**Table 1**
Lipase hydrolytic activity, pH, and biomass obtained by submerged culture of *Aspergillus sp.* DPUA 1727 strain using the following oils: cotton seed (CSO), grape seed (GSO), olive (OO) and soybean (SO).

| Oil | Enzymatic activity (U mL$^{-1}$) | pH | Biomass (g L$^{-1}$) |
|---|---|---|---|
| **Cotton seed** | 7.7 ± 1.3$^{c}$ | 3.7 ± 0.1$^{a}$ | 8.8 ± 0.9$^{a}$ |
| **Grape seed** | 13.5 ± 1.1$^{b}$ | 3.9 ± 0.2$^{a}$ | 7.0 ± 0.1$^{b}$ |
| **Olive** | 14.4 ± 0.6$^{b}$ | 3.6 ± 0.1$^{a}$ | 7.2 ± 0.4$^{b}$ |
| **Soybean** | 17.9 ± 0.8$^{a}$ | 3.6 ± 0.4$^{a}$ | 7.7 ± 0.7$^{ab}$ |

Statistical test Anova one way (with Welch correction for unequal variances) and Tukey post-test. The results are presented by means and standard deviations of triplicates. Equal letters represent values equal to a significance level of 5%.

the relative percentage of C18:n fatty acids esters present in the oils used (sesame, groundnut, sunflower, palm, coconut and castor oils). Dobrev et al. (2015) studying different vegetable oils (sunflower, soybean, olive, rapeseed, corn and soap stock) in the production of lipase by *A. carbonarius* observed that as higher the content of free fatty acids in the oil more pronounced was the lipase production. The authors

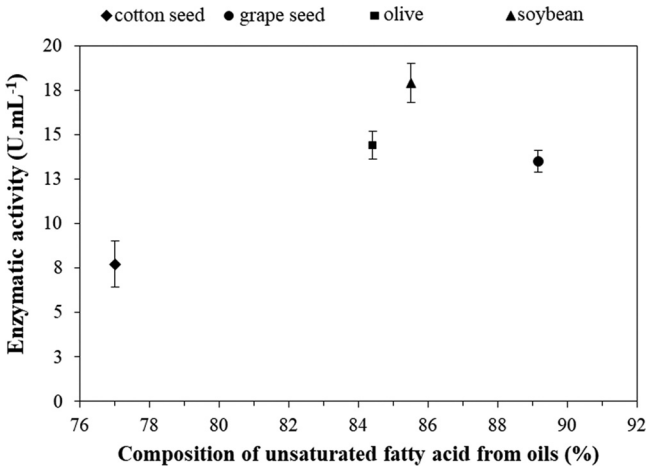


**Fig. 4.** Correlation between the composition of unsaturated long chain fatty acids (%) of the different oils used and the enzymatic activity (U mL$^{-1}$) of lipase produced.

**Table 2**
Percentage of the constituent in terms of long and unsaturated fatty acids in cotton seed oil, grape seed oil, soybean and olive oil, used in the lipase biosynthesis.

| Oil | Stearic C18:0 | Oleic C18:1 | Linoleic C18:2 | Linolenic C18:3 | Arachidonic C20:0 |
|---|---|---|---|---|---|
| **Cotton seed**[b] | 2.00 | 35.00 | 42.00 | – | – |
| **Grape seed**[a] | 3.50 | 14.30 | 74.70 | 0.15 | 0.16 |
| **Olive**[a] | 2.30 | 66.40 | 16.40 | 1.60 | 0.43 |
| **Soybean**[b] | 2.00–6.00 | 22.00–34.00 | 43.00–56.00 | 5.00–11.00 | – |

[a] Orsavova et al. (2015).
[b] Chempro (available in http://www.chempro.in/fattyacid.htm).

evaluated the influence of sunflower oil and soap stock, oils that showed the greatest activity in previous tests, in the concentration of 15 g/L. The lipase activity with soap stock as carbon source was 4 times higher than with sunflower oil. Soap stock presents in its composition 89.31% of unsaturated fatty acids (linoleic and oleic). According to the authors, the presence of fatty acids with a chain length higher than 16 carbons atoms in oils affect positively the lipase biosynthesis. The process of lipases biosynthesis during the microorganism metabolism is not well understood (Yadav et al., 2015). However, the lipase structure and consequently its specificity seems to be dependent on strain metabolism. It is known that from the fatty acid side the lipase may present affinity for short-chain fatty acids (until 10 carbons), others for unsaturated fatty acids and others are non-specific (Ghosh et al., 2016). Unsaturated fatty acids present double bends that after break generate ethylene chain structure. Saturated fatty acids are straighter with a compact structure (Saravanan et al., 2007). So, unsaturated long chain fatty acids are easier to be hydrolyzed. Fig. 4 presents a relation among the percentage of C18:n fatty acids present in each oil used and the enzymatic activity achieved under the experimental conditions. In general, the lipase activity was more pronounced with oils that presented a porcentage of unsaturated fatty acids higher than 80%, namely, SO, OO and GSO. Moreover, the lipases biosynthesis was more favored by unsaturated fatty acid than by the chain length of fatty acids. It is known that microorganisms adjust their metabolic activity according to the environment, consequently, the amount of enzyme produced may change depending on this variable.

Considering the lipase specificity, these enzymes can be grouped into sn-1,3-regioselective lipases (triacylglycerol yielding more 1 (3), 2-diacylglycerol that 1,3-diacylglycerol), sn-2-regioselective lipases (yield more 1,3-diacylglycerol than 1 (3), 2-diacylglycerol), and nonselective lipases (no selectivity toward either position) (Tong et al., 2016). The enzyme specificity cannot be determined with the results presented, however, preliminary studies (data not presented) showed that the fermented broth presents mostly 1,3-diacylglycerol, which is an indicator that the lipase studied is sn-2-regioselective.

Regarding the other fermentation parameters, CSO and SO promoted a growth 25% higher than the other sources. The pH values of the enzymatic filtrates with all oils presented statistically equal values ($p = 0.358$) at the end of fermentation, around 3.7. As the initial pH was 6.3, there was an acidification of the medium probably due to the hydrolysis of the oils triglycerides with consequent release of fatty acids in the medium and decrease of the pH.

Although soybean oil allowed a lipase enzymatic activity 1.33 times higher than GSO, this work was focused on evaluating mainly agro-industrial waste. As aforementioned, SO and OO was used as standard since their use as lipase inductor is well described in the literature (Andrade et al., 2014; Colla et al., 2015; Coradi et al., 2013; Hosseinpour et al., 2011). In this way, grape seed oil appears as an interesting carbon source aiming to produce lipase by submerged culture of *Aspergillus sp.* In the wine industry, it is estimated that from 20% to 25% of the biomass generated comes from the seeds, which are discarded (Coelho et al., 2018) and its reuse, besides reducing waste disposal an alternative because of the characteristics of the grape oil. The oil obtained from these seeds has a high content of polyunsaturated fatty acids (PUFA), representing about 85–90% of the total composition, among them linoleic acid (C18:2), abundantly present in vegetable oils, and linolenic acid (C18:3) (Shinagawa et al., 2015). As previously discussed, these characteristics are positive in the induction of lipase during a fermentative process. In addition, as it is industrial waste oil, its reuse would reduce environmental problems. To our knowledge, there are no reports in the literature using grape oil to produce microbial lipases until the present study.

In order to study the microorganism behavior and lipase production using GSO, further experiments of lipase production by *Aspergillus sp.* DPUA 1727 through time was performed. The data of hydrolytic activity (U $mL^{-1}$), specific activity (U $mg^{-1}$), pH of the enzymatic filtrate and biomass (g $L^{-1}$) in Fig. 5.

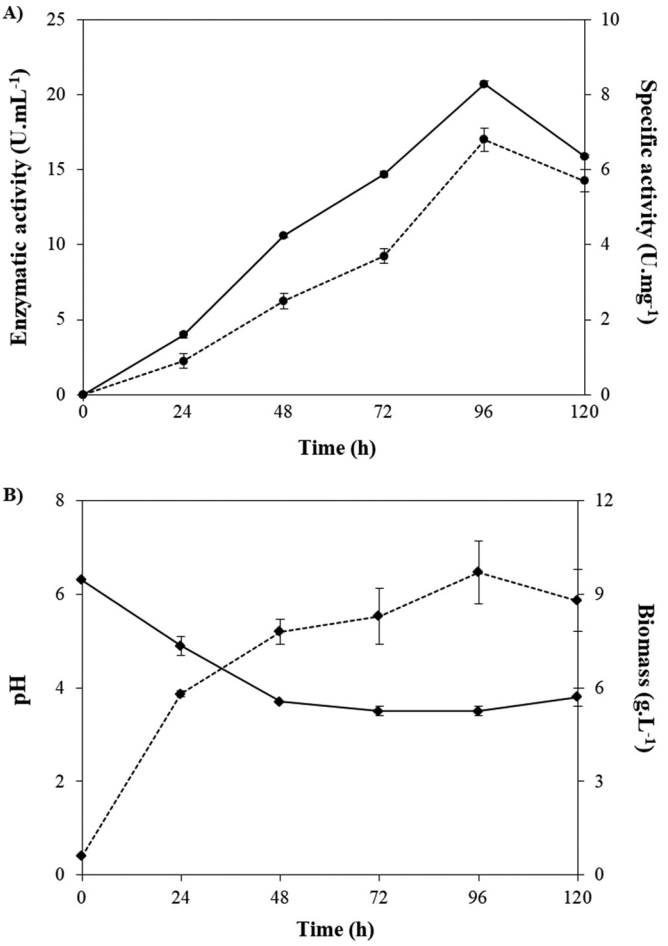


**Fig. 5.** Growth curve of *Aspergillus sp* DPUA 1727 in submerged culture during 120 h. **A)** Enzymatic activity (U $mL^{-1}$) (full line) and Specific activity (U $mg^{-1}$) (dashed line); **B)** pH of the enzymatic filtrate (full line) and Biomass (g.$L^{-1}$) (dashed line). The error bars represent 95% confidence limit for the measurements.

The average growth per day was 25% up achieving the maximum after 96 h of submerged culture. In 24 h of production, there was an increase of 9.7 times in the amount of biomass produced, and the quantity obtained with 96 days of production was 16.2 times greater in relation to time zero. According to Dalmau et al. (2000), usually at the end of the exponential phase of growth occurs the appearance of extracellular lipase in the culture medium. In the presence of cotton oil, longer production time is required to increase the enzyme production by the microorganism. However, this procedure will reduce the productivity being not an interesting alternative.

The initial pH of the culture medium was 6.3, it becomes acid with the development of fermentation probably because of oil hydrolysis which generates fatty acids and others organic acids. Regarding the lipolytic enzyme, the highest hydrolytic lipase activity (20.7 U $mL^{-1}$) and specific activity (6.8 U $mg^{-1}$) was also obtained after 96 h of fermentation. The lipase activity increases through time as a consequence of microorganism metabolism and enzyme accumulation in the fermented broth. Initially, the enzyme produced by the microorganism is consumed in the oils hydrolysis, afterward, the oil concentration is reduced and the enzyme produced accumulates in the fermented broth. From the results achieved, it is clear that 96 h of the fermentation process is the best for lipase production since it generates around 41% more enzyme (determined through enzyme activity). Moreover, considering the productivity at this time it was obtained the highest productivity (0.22 U/mL h). Aiming to increase the knowledge about the

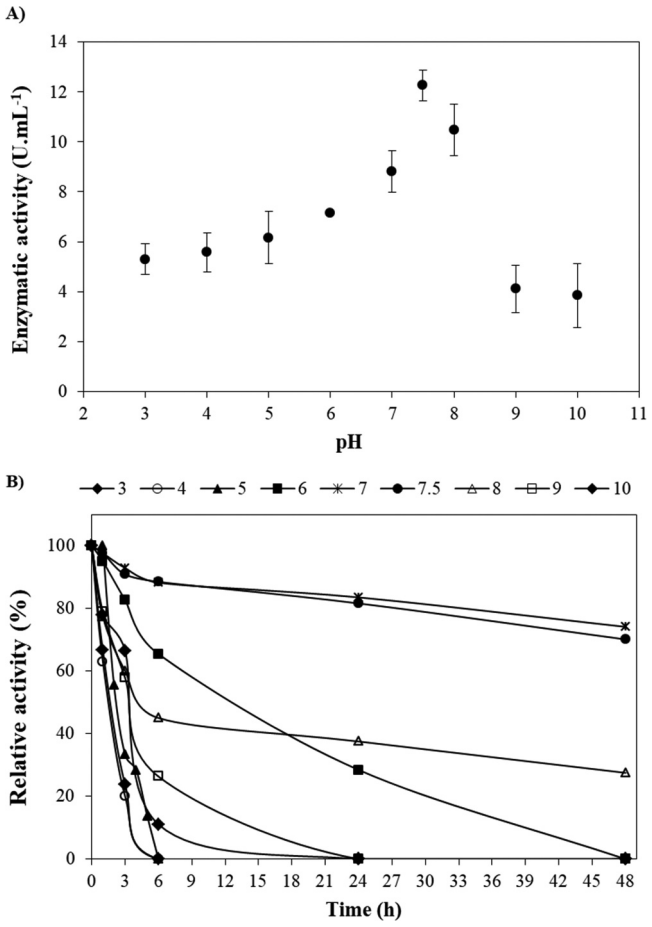


**Fig. 6.** Characterization of the lipase enzyme produced by submerged cultivation of *Aspergillus sp.* DPUA 1727 at 37 °C in relation to optimum pH (A) and pH stability (B) after 0, 1, 3, 6, 24 and 48 h of incubation. The error bars represent 95% confidence limit for the measurements. In the pH stability, 100% refers to the activity achieved at time 0 for each pH.

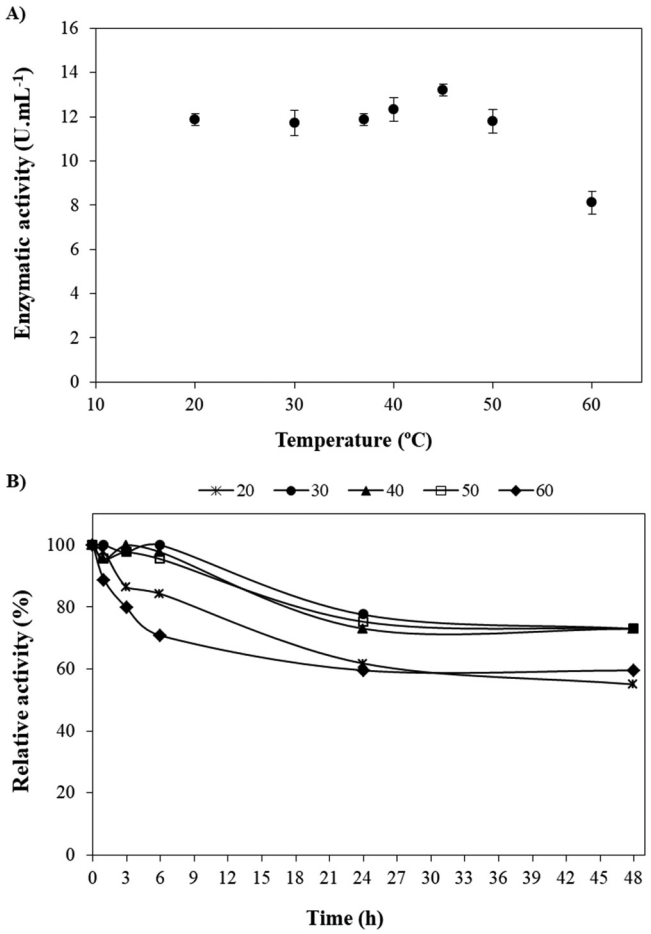


**Fig. 7.** Characterization of the lipase enzyme produced by submerged cultivation of *Aspergillus sp.* DPUA 1727 at pH 7.5: optimal temperature (A) and stability at a temperature (B) after 0, 1, 3, 6, 24 and 48 h of incubation. The error bars represent 95% confidence limit for the measurements. In the temperature stability results, 100% refers to the activity achieved at time 0 for each temperature.

enzyme produced by *Aspergillus sp.* DPUA 1727 strain using GSO as a carbon source, the lipase characterization was performed with the enzyme produced after 96 h.

### *3.3. Characterization of lipase produced by Aspergillus sp. DPUA 1727 strain using grape oil as a carbon source: effect of pH and temperature*

The main constitution of enzymes are amino acids and changes in the environment, such as pH and temperature, can affect the enzyme structure and, consequently, its activity. Therefore, it is essential to characterize the enzyme in terms of pH and temperature. Fig. 6 presents the results attained for optimum pH (A) and pH stability (B) at the following times: 0, 1, 3, 6, 24 and 48 h.

The lipase enzyme produced by DPUA 1727 strain had an optimum pH at 37 °C of 7.5 and remained stable at pHs 7 and 7.5 for 48 h, maintaining 74.13% and 70.12% of the initial activity, respectively. The enzyme exposition at acid or alkaline values of pH decreased significantly the enzyme activity and a hydrolytic activity loss of 100% was achieved after 24 h of incubation in the extreme conditions (pH values: 3.0, 4.0, 5.0, 9.0 and 10.0). The influence of pH on the activity of the enzyme is due to the presence of the ionizable groups present around the active site, making the substrate binding to the enzyme and consequently, the effectiveness of the catalysis is optimized (Marzzoco and Torres, 2007). In the literature, several lipolytic enzymes produced by *Aspergillus*species exhibited optimum pH between 5 and 6 (Liu et al., 2015; Mhetras et al., 2009; Namboodiri and Chattopadhyaya, 2000).

The results of lipase optimum temperature and temperature stability after 0, 1, 3, 6, 24 and 48 h of incubation at pH 7.5 in the range of temperatures from 20 to 60 °C, are presented in Fig. 7(A and B), respectively.

Considering the optimal temperature, the enzyme exhibits a temperature range of maximum activity since there was no statistical difference between the temperatures activated achieved with 37, 40 and 45 degrees (Statistical test Anova one way, $p = 0.0994$). However, at 60 °C, the lipase activity was considerably reduced and at lower temperatures (20 and 30 °C) the activity was close to optimum condition. The temperature influences the rate of enzymatic reaction, at low temperatures the kinetic energy in the system is smaller not favoring the formation of the enzyme-substrate complex. To the other hand, at high temperatures the reaction rate increases, as a consequence, the number of productive collisions (E→S) per unit of time, but with an increase of temperature and time of exposure, there may be a decrease in the rate of product formation or denaturation caused by the loss of the tertiary structure of the enzyme necessary for catalytic reactions to occur (Gomes et al., 2006). Although in the optimal temperature studies, 20 °C showed a lipase activity close to optimum conditions, in the stability studies the behavior of lipase was equaled incubating the enzyme at 20 and 60 °C. At these conditions, the loss of activity was around 40% after 48 h of incubation while in the others conditions around 25%. Depending on the time of incubation the enzyme behavior was different, the enzyme maintained an average of 98.49% of the

initial activity at temperatures of 30, 40 and 50 °C for 6 h, following the same profile and ending with 73.03% activity at the end of 48 h of testing.

Some studies with lipases produced by different genera of *Aspergillus* have shown that the temperature range where lipase is most commonly stable is around 37 °C: *A. flavus* (37 °C (Colla et al., 2015)), *A. niger* (35 °C (Colla et al., 2015)), *A. tamarii* JGIF06 (37 °C (Das et al., 2016)) which is in agreement with the stability produced by DPUA 1727 strain. The optimal conditions of pH and temperature achieved for the lipase produced by DPUA 1727 strain using GSO as a carbon source are quite similar to results of lipase from *A. niger* F044 which presented optimum pH and temperature of 7.0 and 45 °C, respectively. In this work, the authors demonstrated that lipolytic activity declined rapidly at a temperature over 65 °C (Shu et al., 2007). As in the present work a restrict range of temperature (20–60 °C) was evaluated no further evidence about denaturation can be assessed.

Following with the lipase characterization, a pre-purification step using precipitation was performed in order to remove some contaminants and make the SDS-electrophoresis to determine the enzyme molecular weight.

## 4. Pre-Purification Lipase pre-purification using precipitation methods

The downstream process is composed at least for ultrafiltration, precipitation, ion exchange chromatography and/or liquid-liquid extraction and depending on the biomolecule application a final polishing stage is carried out (Santos et al., 2018). Among these techniques, precipitation is one of techniques more used to recovery proteins and in the most part of published protein purification protocols include at least one precipitation step (Sharma et al., 2001; Souza et al., 2014; Ventura and Coutinho, 2016). In the present work, this traditional methodology was evaluated through the lipase precipitation with ammonium sulfate, acetone and ethanol (both with several cycles) and the results are presented in Table 3.

First, the lipase precipitation was performed using ammonium sulfate followed by a dialysis step to remove inorganic salts and other compounds with low molecular weight. However, as can be seen in Table 3 a considerable reduction in the lipase activity was achieved after the dialysis process. One reason to these findings can be the formation of aggregate during the precipitation process because of lipase dehydration (water affinity with ammonium sulfate), leaving the lipase active sites, which are hydrophobic, exposed and thus interact with each other, forming aggregates that precipitate with the other contaminants. It is known that precipitation with ammonium sulfate is very used in both laboratory and industrial levels to extract enzymes, however, the significant activity loss (> 50%) in the present work with the lipase studied encouraged that other precipitant agents were evaluated, namely, ethanol and acetone. The principal effect of the organic solvent is the reduction in water activity. A decrease in the dielectric constant with the addition of an organic solvent leads to the decrease in the solvating power of water for a charged, hydrophilic protein molecule, and thus protein solubility decreases and precipitation occurs (Hatti-Kaul and Mattiasson, 2007). It is important to highlight that lipases from different microbial sources can differ in their structure and the results achieved in the present work is specific to lipase from *Aspergillus sp.* DPUA 1727. Thus, in terms of industrial application, lipases from different microbial source has to be characterized before their application.

In the experiments with organic solvents, the aggregation phenomenon was not observed. In the experiments with addition of different volumes of ethanol there was an increase in lipase activity after precipitation by adding 1:1 (v/v) ethanol to the fermented broth, and it was maintained with the other volumes, reaching a purification factor of 10.72 after 3 cycles of precipitation. The application of more cycles did not promote higher purification factor. However, with acetone, a higher number of cycles increased the enzymatic activity and purification factor. After 4 cycles of acetone addition, an enzymatic activity of 70.50 U $mL^{-1}$ with a purification factor of 16.76 was obtained. Depending on lipase application both organic solvents can be used. The results with ethanol are quite interesting because besides ethanol be a good precipitant agent for lipases, it is a solvent frequently used in the process in which lipase is applied such as biodiesel production. So, if ethanol is used as a precipitant agent of lipases, its excess do not need to be removed before its application.

The SDS-electrophoresis was performed with the lipase precipitated using 3 cycles of ethanol(Fig. 8). The fermented broth presented only one representative band (~64 kDa) but this can be the result of the low concentration of other compounds. After the precipitation technique, the precipitated resuspended contained multiple bands proving that precipitation is a method that not promote high purification. Depending on lipase microbial source, the lipase molecular weight can be in the range of 20–75 kDa (De Castro et al., 2004) and further purification studies has to be performed in order to prove which band achieved in the electrophoresis is related with the target lipase.

## 5. Conclusions

In this work, a new strain of *Aspergillus, Aspergillus sp.* DPUA 1727 was studied in order to produce lipase using agro-industrial waste as an inductor. It was showed that agro-industrial waste can be used to this purpose mainly if it presents the higher percentage of fatty acid esters (> 80%). In this sense, grape seed oil showed to be an alternative to inductors already used to produce lipases because it is an industrial

**Table 3**
Results of enzymatic activity (U $mL^{-1}$), total proteins (mg $mL^{-1}$), specific activity (U $mg^{-1}$) and the purification factor of each lipase purification method tested.

| | Organic Solvent ratio | Enzymatic activity (U $mL^{-1}$) | Total proteins (mg $mL^{-1}$) | Specific activity (U $mg^{-1}$) | Purification factor |
|---|---|---|---|---|---|
| **Fermented broth** | n/a | 30.36 ± 1.28 | 3.68 | 8.25 ± 0.35 | n/a |
| **Precipitation with ammonium sulfate and dialysis** | n/a | 6.48 ± 0.66 | 0.21 | 30.86 ± 0.66 | 3.74 ± 0.38 |
| **Precipitation with acetone** | 1× | 22.46 ± 1.46$^{c}$ | 0.46 | 48.83 ± 3.16$^{c}$ | 5.92 ± 0.39$^{c}$ |
| | 2× | 21.76 ± 0.79 $^{c}$ | 0.26 | 83.69 ± 3.05$^{A,b}$ | 10.14 ± 0.37 $^{A,b}$ |
| | 3× | 40.87 ± 0.57 $^{A,b}$ | 0.47 | 86.96 ± 1.22$^{A,b}$ | 10.54 ± 0.15 $^{A,b}$ |
| | 4× | 70.50 ± 1.79 $^{a}$ | 0.51 | 138.24 ± 3.52$^{a}$ | 16.76 ± 0.43 $^{a}$ |
| **Precipitation with ethanol** | 1× | 35.26 ± 2.98 $^{B,b,c,d}$ | 0.94 | 37.51 ± 3.17$^{d}$ | 4.55 ± 0.38 $^{d}$ |
| | 2× | 33.37 ± 2.15 $^{d}$ | 0.47 | 71.00 ± 4.57$^{b}$ | 8.61 ± 0.55 $^{b}$ |
| | 3× | 39.80 ± 1.06 $^{A,a,c}$ | 0.45 | 88.44 ± 2.36 $^{A,a}$ | 10.72 ± 0.29 $^{A,a}$ |
| | 4× | 39.10 ± 0.44 $^{A,B,a,b}$ | 0.66 | 59.24 ± 0.66 $^{c}$ | 7.18 ± 0.08 $^{c}$ |

Statistical test Anova one way (with Welch correction for unequal variances) and Tukey post-test. The results are presented by means and standard deviations of triplicates. Equal letters represent values equal to a significance level of 5%, capital letters: between groups in the same column, and lowercase letters: intra groups in the same column.

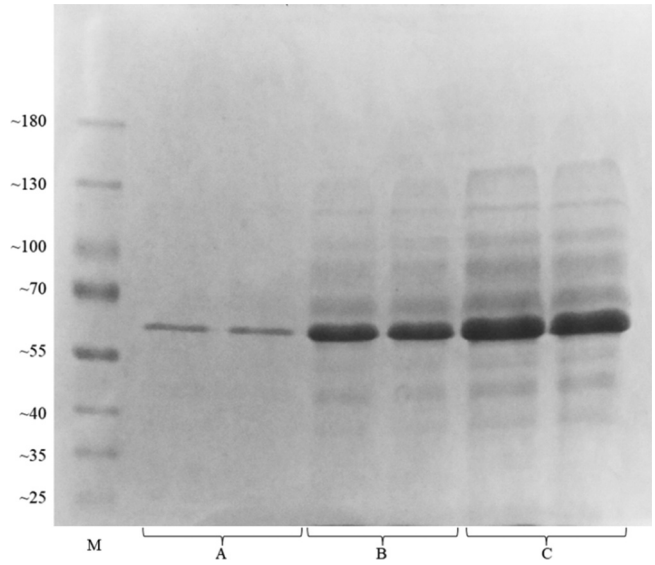


**Fig. 8.** SDS-PAGE analysis of the lipase, in duplicate: M) molecular weight marker (25–180 kDa), A) fermented broth, B) 90 mL of fermented broth precipitated and resuspended in 5 mL of 5 mM phosphate buffer pH7.5, and C) 90 mL of fermented broth precipitated and resuspended in 2.5 mL of 5 mM phosphate buffer pH 7.5.

residual with desired characteristics. The enzyme produced presents an optimum pH of 7.5 and stability between 7 and 7.5 and an optimum temperature of 45 °C and a thermal stability between 30 and 50 °C. As final remarks, the integration of new strains with suitable agro-industrial waste can emerge as an important aspect to improve the lipases bioprocess since these enzymes present a wide range of applications.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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