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Isolation of a *Aspergillus niger* lipase from a solid culture medium with aqueous two-phase systems

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ABSTRACT

The aim of this work is to find the best conditions to isolate lipase from a solid culture medium of *Aspergillus niger* NRRL3 strains using aqueous two-phase systems formed with polyethylene glycol and potassium phosphate or polyethylene glycol and sodium citrate. We studied the partitioning of a commercial lyophilizate from *A. niger*. Also, the lipase enzymatic activity was studied in all the phases of the systems and the results indicate that citrate anion increases lipase activity. An analysis by fluorescence spectroscopy of the interaction between lipase and the bottom and top phases of the systems shows that the protein tryptophan-environments are modified by the presence of PEG and salts. Separation of the enzyme from the rest of the proteins that make up the lyophilized was achieved with good yield and separation factor by ATPS formed by PEG 1000/Pi at pH 7, PEG 2000/Ci at pH 5.2 and PEG 4000/Ci at pH 5.2. The above mentioned systems were used in order to isolate extracellular lipase from a strain of *A. niger* in submerged culture and solid culture. The best system for solid culture, with high purification factor (30.50), is the PEG 4000/Ci at pH 5.2. The enzyme was produced in a solid culture medium whose production is simple and recovered in a phase poor in polymer, bottom phase. An additional advantage is that the citrate produces less pollution than the phosphate. This methodology could be used as a first step for the isolation of the extracellular lipase from *A. niger*.

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1. Introduction

Lipases (EC 3.1.1.3) are a group of enzymes which catalyze hydrolysis and synthesis of triglycerides in vivo, producing or consuming fatty acid esters and even the synthesis of the latter in low water content environments. This feature has increased the biotechnological interest in these enzymes for a number of industrially significant biotransformations. Potential applications include modification of sugars, synthesis of flavor esters for the food industry, the resolution of racemic mixtures and obtention of biofuel [1]. From an industrial standpoint, fungi are more interesting sources of these enzymes than animals or plants for their potential use in biotechnology due to their availability and high stability. In the case of the *Aspergillus niger* yeast, the produced lipase is mainly used in industry [2]. It has 298 amino acids, of which 4 are tryptophans [3]. It is of great interest from the theoretical and practical point of view to learn about the conditions of the medium that stabilize the enzyme for the design and development of a biotechnological process where the macromolecule participates as a catalyst.

Downstream process is defined as a sequence of procedures that results in a purified product [4]. Aqueous two-phase systems (ATPS) extraction is one of them. Protein partition involves adding one or more neutral polymers to a buffered salt solution, to separate the protein into two phases enriched with polymer or salt. Proteins tend to partition differentially between the phases depending on size, conformation, net electrical charge (pI) and specific interactions with phase-forming polymers. A single partition step involves adding the material to be partitioned to a two-phase system. Then, they are mixed gently until the system reaches the equilibrium. Partition has a variety of advantages such as low energy consumption, large scale and high cost-efficiency according to the biotechnological processes [5]. Enzymatic extraction from culture media should allow the partition with aqueous two-phase systems without loss of activity.

In this work, we studied the partitioning of a commercial lyophilized from *A. niger* using aqueous two-phase systems formed with polyethyleneglycol and potassium phosphate or polyethyleneglycol and sodium citrate. An analysis by fluorescence spectroscopy and enzymatic activity of the interaction between a commercial lipase and the bottom and top phases of the systems

Abbreviations: PEG 1000, PEG 2000, PEG 4000 and PEG 8000, polyethylene glycol of average molecular weight 1000, 2000, 4000, and 8000 g/mol; pNPL, 4-nitrophenyl laurate; ATPS, aqueous two-phase system; Pi, phosphate buffer; Ci, citrate buffer; SCM, solid culture medium; LCM, liquid culture medium.

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Table 1
Composition of the two-phase systems, partitioning coefficient of total proteins (K_p) and lipase (K_r) of the commercial lyophilized, recovery (Y%) in both phases, separation and purification factors (β, PF).

Systems	PEG MM	% PEG (w/w)	% Pi (Ci) (w/w)	K_r	K_p	Y (%) top	Y (%) bottom	β	PF
PEG/Pi pH 7	1000	17.00	16.20	17.23	2.23	95.23	4.77	7.73	2.92
	2000	15.00	15.00	4.86	2.25	84.93	15.07	2.16	0.53
	4000	15.08	12.03	0.27	1.16	22.09	77.91	0.23	0.26
	8000	14.30	10.90	0.11	1.33	9.01	90.99	0.08	0.65
PEG/Ci pH 5.2	1000	15.92	13.97	1.05	0.78	54.91	45.09	1.35	2.83
	2000	14.50	10.00	0.02	0.43	2.27	97.73	0.05	8.34
	4000	10.50	14.20	0.08	0.58	8.49	91.51	0.14	6.93
	8000	11.69	9.52	0.04	0.35	4.21	95.79	0.10	3.74
PEG/Ci pH 8.2	1000	10.50	13.13	0.34	0.64	28.28	71.72	0.53	0.48
	2000	9.75	10.87	0.08	0.51	7.06	92.94	0.16	0.37
	4000	9.70	8.82	0.12	0.43	13.31	86.69	0.28	0.15
	8000	6.24	8.48	n.d.					

n.d., not determined.

mentioned was also made. The best systems were chosen in order to purify a lipase from a solid culture medium of *A. niger* NRRL3 strains with the aim of applying it as a first step to isolate the enzyme from a culture broth.

2. Materials and methods

2.1. Chemicals

Lipase from a lyophilized culture medium of *A. niger* and lipase from *A. niger* were purchased from Sigma Chem. Co. (USA) and used without further purification. Its solutions were prepared for direct weighed into 50 mM, pH 7 potassium phosphate buffer or into 65 mM, pH 5.2 sodium citrate buffer. PEGs of average molecular weight 1000, 2000, 4000, and 8000 g/mol (PEG 1000, PEG 2000, PEG 4000 and PEG 8000) were purchased from Sigma Chem. Co. (USA). All other chemicals were of analytical grade. The specific substrate for the lipase determination, 4-nitrophenyl laurate (pNPL), was acquired from Sigma Chem. Co. (USA).

2.2. Lipase assay

Lipase activity was determined by the enzymatic capacity to hydrolyse the substrate p-nitrophenil lauric (pNPL), which liberates p-nitrophenol (pNP) that absorbs at 400 nm at pH 7. A solution 25 mM of pNPL in ethanol–water (1:1) was prepared. The reaction was carried out for 30 min at 37 °C, with 100 μL of an adequate dilution of the enzymatic sample in 800 μL of 50 mM phosphate buffer at pH 7. Then, 100 μL of substrate was added for saturation final concentration (0.4 mM). After that, 250 μL of 1 M Na_2CO_3 were added and kept at 0 °C for 15 min. The mixture was centrifuged at 16,000 × g for15 min and the supernatant was read at 400 nm. The pNP molar absorptivity coefficients were determined on each medium studied (data not shown). Blanks with all the reaction compounds and the corresponding phase without the enzyme were assayed [6]. One unit of activity lipase is the amount of enzyme that liberates 1 μmol of pNP/min from pNPL/mg of protein under the conditions mentioned above.

2.3. Preparation of the aqueous biphasic system

Table 1 shows the composition of the systems used in this work according to the binodial diagrams from the bibliography [7,8]. The systems were prepared with PEG of four different molecular masses. In order to prepare 50 mL two-phase aqueous systems, we mixed the corresponding amount of PEG and a 28% w/w pH 7 potassium phosphate buffer or 25% w/w pH 5.2 or pH 8.2 sodium citrate buffer stock solutions and water. Low-speed centrifugation was used after gentle mixing of the system components to speed up phase separation; then 2.0 mL of each phase were mixed. The prepared systems have a top phase volume/bottom phase volume ratio (R_V) equal to the unity. Then, the systems were stored at the corresponding work temperature (6 °C and 37 °C).

The systems used for the partitioning of the culture medium supernatant were prepared replacing a percentage of water on the previous systems and then R_V was measured in each case.

2.4. Determination of the partition coefficient (K_r) and the purification parameters

The partition coefficient of the lipase lyophilizate between both phases was determined by adding 100 μL of a 24.56 mg/mL solution in the two-phase system containing 2.0 mL of each equilibrated phase, obtained from a pre-formed system. After gently mixing by inversion for 1 h and letting it to settle for 30 min, the system was centrifuged at low speed for the two phase separation. Samples were withdrawn from separated phases; the lipase activity and the total protein concentration in each phase were determined in order to calculate the partition coefficients defined as K_r and K_p, respectively:

$$K_r = \frac{[\text{Act}]_{\text{TOP}}}{[\text{Act}]_{\text{BOTTOM}}}$$

$$K_p = \frac{[\text{TP}]_{\text{TOP}}}{[\text{TP}]_{\text{BOTTOM}}}$$

where $[\text{Act}]_{\text{TOP}}$ and $[\text{Act}]_{\text{BOTTOM}}$ are equilibrium enzymatic activities of the partitioned enzyme in the PEG and phosphate rich phases, respectively. $[\text{TP}]_{\text{TOP}}$ and $[\text{TP}]_{\text{BOTTOM}}$ are the total protein concentration in the same phases.

The β factor was calculated as the quotient between K_r and K_p and indicates the capacity of the systems to separate the target protein from the impurities.

In order to evaluate the purification process, the enzyme recovery percentage in the bottom and top phases ($Y_{\text{TOP}}(\%)$) were calculated according to the following equations:

$$Y_{\text{BOTTOM}}(\%) = \frac{100}{1 + R_V K_r}, \quad Y_{\text{TOP}}(\%) = \frac{100}{1 + 1/R_V K_r} \tag{4}$$

where $R_V = V_T/V_B$, V_B and V_T being the bottom and top phase volume, respectively, and K_r the lipase partition coefficient [9].

The purification factor (PF) was calculated as:

$$\text{PF} = \frac{\text{Act}_{\text{phase}}}{\text{Act}_{\text{LIOFILIZATE}}} \tag{5}$$

where $\text{Act}_{\text{PHASE}}$ and $\text{Act}_{\text{LIOFILIZATE}}$ are the lipase specific activities in the corresponding phase and in the original mixture.

2.5. Determination of the thermodynamical functions associated with protein partitioning

Lipase partitioning coefficients were determined at two different temperatures (T_1: 6 °C and T_2: 37 °C), applying van't Hoff equation and considering independent ΔH on the range of temperature [10–12]:

$$\ln \frac{K_2}{K_1} = \frac{\Delta H^\circ}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \tag{6}$$

where K_1 and K_2 are the coefficients determined at temperatures T_1 and T_2, R is the constant of the gases, the enthalpic change (ΔH°) was determined. The change of free energy (ΔG°) was obtained from the following equation:

$$\Delta G^\circ = -RT \ln K \tag{7}$$

and the entropy variation (ΔS°) from:

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} \tag{8}$$

2.6. Quenching of the intrinsic fluorescence of lipase by acrylamide

The measurement of the native fluorescence of the enzyme was analyzed and the fluorescence emission spectrum of the protein in a 50 mM phosphate buffer medium was obtained. The scanning rate was 60 nm/min and the data acquisition was obtained every 0.2 nm with a slit of 0.2 nm. The fluorescence spectra were obtained in an Aminco Browman spectrofluorometer Serie 2000 Model FA354 using a thermostatized cuvette of 1 cm pathlength and were corrected using a software provided by the instrument manufacturer. The fluorescence spectra were obtained for solutions of 1.27 mg/mL lipase in the presence and absence of the corresponding phases, in order to determine the fluorescence (F_0) on the wavelength of the maximum emission (λ_{max}) for tryptophan (Trp). The same procedure was carried out with aliquots of 4 M acrylamide and the fluorescence intensity was redetermined (F). Final quencher concentrations ranged from 0 to 0.14 M. The data were analyzed using the mathematical model for the sphere of action according to Lakowicz [13]:

$$\frac{F_0}{F} = (1 + K_D[Q])e^{([Q]\nu N/1000)} \tag{9}$$

where $[Q]$ is the quencher concentration, K_D is the Stern Volmer constant related to the lifetime of the fluorophore and the bimolecular quenching constant, ${K_D}^{-1}$ is the quencher concentration at which $F_0/F = 2$ or 50% of the intensity is extinguished [13], N is the Avogadro's number and ν is the volume of the "sphere of action", i.e., the sphere within which the probability of immediate quenching is unity, and whose radius is only slightly larger than the sum of the radii of the fluorophore and quencher.

2.7. A. niger production

Strains of *A. niger* NRRL3 were provided by the culture collection of Agricultural Research Service, USDA. The production of lipase from *A. niger* NRRL3 strain, was carried out with a solid culture medium (SCM) according to Adham and Ahmed [14] with some modifications. Liquid culture media (LCM) were used as control. The organism was reactivated on a potato-dextrose agar medium (PDA) at 30 °C for 5 days. The spores were removed from the PDA with sterile distilled water, with the help of a sterile magnetic stirrer. The numbers of spores were counted using a Thoma counter chamber type. A spore suspension was prepared in sterile distilled water to get 10^6 spores/mL concentration in each specific medium. This solution was used as a source of inoculum. The culture medium (pH 6) components were: 1% glucose, 1% olive oil, 3% casein peptone, 0.05% $MgSO_4 \cdot H_2O$, 0.05% KCl and 0.2% K_2HPO_4. This medium was autoclaved at 121 °C for 15 min and was inoculated when it reached room temperature. The SCM was carried out in 250 mL Erlenmeyer with 6 g of milled polyurethane (particle size: 1.68–3.36 mm, approximately); it was sterilized and 14 mL of culture medium was added. Then the SCM was homogenized and incubated for 5 days at 30 °C. The extract was obtained by addition of 14 mL of 50 mM phosphate buffer at pH 7 to the SCM, compressed with a syringe, filtered and centrifuged at 6000 rpm during 20 min al 8 °C. The LCM was carried out in 250 mL Erlenmeyer containing 50 mL of the culture medium and was incubated on a shaker at 150 rpm and 30 °C for 5 days. The extract was obtained by filtration through No. 40 filter paper.

The content of protein and nucleic acid was estimated by the Warburg and Christian method [15].

3. Results and discussion

3.1. Lipase partitioning in PEG/phosphate or citrate systems

Phosphate/PEG systems are well-known conventional systems. Citrate systems were selected since citrate is a salt that does not pollute the environment. For this latter type of systems, the binodial diagrams are available at pH 5.2 and 8.2. At that pH, the lipase is either near its isoelectric point (lipase IP: 4.4) or negatively charged.

Table 1 shows the results of partitioning coefficients of the commercial lyophilized, and the recovery, separation and purification factors for the systems assayed at 6 °C. Enzyme partition coefficients in the PEG 1000/Pi and PEG 2000/Pi systems were the largest and higher than the unity, which indicates that the protein is partitioning towards the polymer-rich phase. In the other systems, the partition coefficients are close to 1 or less, which indicates that the protein is partitioning towards the salt-rich phase.

The difference in the protein behavior between the systems with Pi pH 7 and Ci pH 5.2 led us to use another pH for the citrate systems (pH 8.2) where the protein have net negative charge, the same on the pH 7 PEG/Pi systems. The partitioning results are similar at pH 5.2 Ci or pH 8.2 Ci. This would suggest that the electric protein charge is not an important factor on its partitioning in this pH range. Lipase from *A. niger* has a molecular mass around 35,000 Da, with capacity to form dimmer, the structural characteristic gives to this enzyme high hydrophobic balance. Works previous [11] has reported a poor pH effect on its partitioning coefficient in ATPSs. This hydrophobic characteristic can explain a pH insensitive of K.

Some of the drivers for lipase partition are the excluded volume of the protein, given by the free solution available to the protein, and the medium–protein interactions. The K_r values decrease according to an increase in the PEG molecular mass for both PEG/Pi or PEG/Ci systems. Our finding suggests that, in systems with PEG 1000 or PEG 2000/Pi pH 7, the lipase partition is driven by the enzyme–polymer interaction, which accounts for K_r coefficient with values greater than one, while in PEG 8000 the excluded volume had an effect over the protein–polymer interaction, resulting in a decrease in the partition coefficient. There is a tendency to a decrease in the partition coefficients with increasing molecular mass of PEG, consistent with the increase of the PEG excluded volume, because this produces the transfer of protein to the salt-rich phase, this effect has been reported for others proteins [16].

The recovery percentage results in the top phase of PEG 1000 and PEG 2000/Pi systems and all the bottom phases of the citrate systems with molecular mass above 1000 are appropriate although the separation and purification factors can be determinants for the choice of the best ways to work on the culture media.

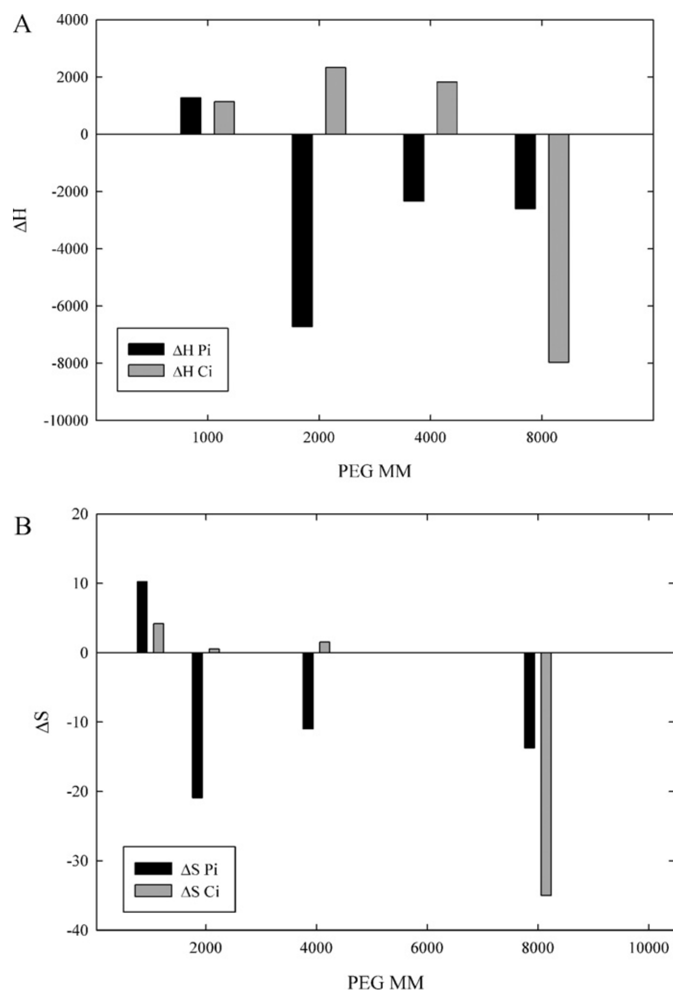

Fig. 1. Thermodynamics functions associated to the lipase partitioning process into two-phase systems: PEG/Pi pH 7 and PEG/Ci pH 5.2. (A) Enthalpy change as a function of the PEG molecular mass and (B) entropic change as a function of the PEG molecular mass.

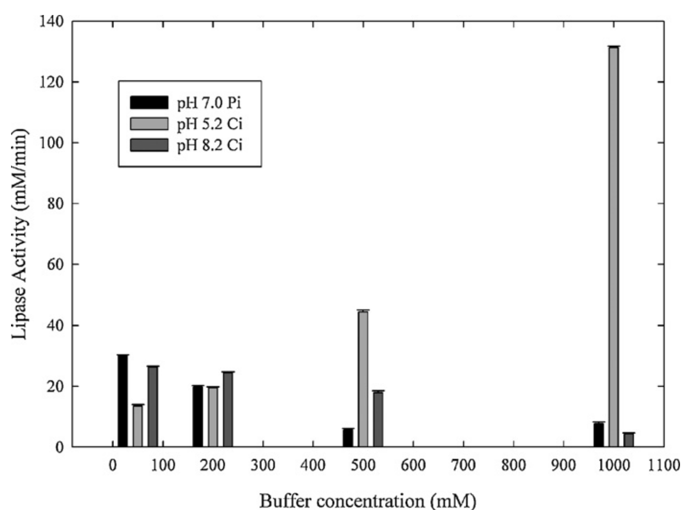

Fig. 2. Lipase activity in Pi buffer at pH 7.0, Ci buffer at pH 5.2 and Ci buffer at pH 8.2 of different concentrations.

3.2. Temperature effect on lipase partitioning

The partition coefficients of lipase were determined at different temperatures in order to calculate the enthalpy change and the entropic change associated with the protein partition by applying the Eqs. (6), (7) and (8). Fig. 1 shows the enthalpic (A) and entropic (B) changes for lipase partition equilibrium in both systems studied (PEG/Pi and pH 5.2 PEG/Ci). The enthalpy and entropic change values obtained for the PEG 1000 systems are positive; while for PEG 8000 both functions acquire negative values, both for systems PEG/Pi and PEG/Ci.

The interaction between PEG 1000 and lipase, in both systems (Pi or Ci), is driven by the entropic factor possibly due to the significant loss of structured water in the hydrophobic region of the lipase. Interaction between PEG of low molecular mass and a protein is carried out when a previous loss of the structured water around the hydrophobic region of both molecules occur [17]. In saline medium, the proteins are well hydrated due to the exclusion of the salt, which increases the protein hydration. When the macromolecule is transferred to a hydrophobic phase (like a PEG of low molecular mass) the polymer have the possibility to access to the protein domain, displacing the hydration water, as result there is an increase in the disorder of the system and positive entropic values. According to the equation: $\Delta G = \Delta H - T\Delta S$, positive ΔS values contribute to making negative the free energy of the protein partitioning. On the other hand, the loss of water molecules from the hydrophobic surfaces is associated to positive enthalpic changes, however, these values are not enough to exceed the term $(-T\Delta S)$. A similar explanation can be applied to the case of PEG 2000/Ci and PEG 4000/Ci systems.

On the contrary, the interaction between PEG 8000 and lipase, for both PEG/Pi and PEG/Ci systems, is driven by the enthalpic factor possibly due to the predominance of electrostatic interactions. For the partitioning in PEG/Pi systems (except for PEG 1000), the negative entropy changes observed suggest the formation of an ordered final state when the protein is transferred from the bottom to the top phase. In the PEG/Pi systems, there is enthalpic–entropic compensation (data not shown), which suggests that liquid water plays a role in the molecular mechanism of this process. This compensation is the thermodynamic manifestation of structure-making and structure-breaking effects of the cosolute on the solvent [18]. The pattern of compensation was not observed for the enthalpic–entropic changes of the PEG/Ci.

3.3. Biological activity of lipase in different buffers

The enzymatic activity can be modified by the medium composition where it is placed. The *A. niger* lipase activity was analyzed at different pHs and in the presence of some specific anions. Pinna et al. [19] found that the lipase has its maximum activity at pH 6 and that salts induced water structure modification, this affects the activity more than the pH, and it can be justified in terms of an interaction specific of the anions on the enzyme surface [19].

Fig. 2 shows the activity of lipase in Pi or Ci buffers at different concentrations. The pHs were chosen according to the pH of ATPS used in previous sections. The pNP molar absorptivity was determined in all the studied media (data not shown). Lipase activity increases with citrate molar concentration at 5.2 pH and decreases with Pi concentration or Ci at the other analyzed pH. It is not possible to state from the result which of the two factors is the important one.

In Table 2, the relative activity percentages to the lipase activity in the top and bottom phases used in the ATPSs are expressed. The lipase relative activity was calculated taking its activity in a medium of 50 mM, pH 7.0 potassium phosphate buffer or 65 mM, pH 5.2 sodium citrate buffer as a hundred per cent.

In the phases where the PEG is in high concentration (top phases), the relative enzymatic activity decreased as the PEG molecular mass increased. In the saline phases, it has higher

Table 2
Wavelength of maximum fluorescence emission spectrum (λ), Stern Volmer constant (k_Q), sphere action volume (υ) for the native fluorescence quenching with acrylamide and relative biological activity for the lipase in the absence and presence of the different phases.

Lipase in phase		% Act	λ (nm)	υ (Å^3)	k_Q
50 mM Pi pH 7		100	342	7604.3	8.2
Top phase PEG/Pi	PEG 1000	283 ± 5	337	9998.7	3.3
	PEG 2000	96 ± 4	336	7076.6	4.3
	PEG 4000	59 ± 4	324	12850.0	5.0
	PEG 8000	46 ± 4	337	11056.0	1.4
Bottom phase PEG/Pi	PEG 1000	106 ± 2	338	7207.9	6.2
	PEG 2000	107 ± 6	338	7030.7	5.9
	PEG 4000	115 ± 3	340	4843.6	7.8
	PEG 8000	126 ± 4	338	4727.9	7.2
65 mM Ci pH 5.2		100	342	6268.1	10.0
Top phase PEG/Ci	PEG 1000	1109 ± 4	338	5117.1	8.2
	PEG 2000	300 ± 5	336	7274.5	3.9
	PEG 4000	323 ± 5	325	14422.0	2.1
	PEG 8000	153 ± 2	338	10537.0	3.7
Bottom phase PEG/Ci	PEG 1000	289 ± 5	334	5094.8	4.5
	PEG 2000	1166 ± 4	336	3945.4	4.5
	PEG 4000	1456 ± 5	336	5430.3	3.1
	PEG 8000	1259 ± 5	334	5413.1	2.9

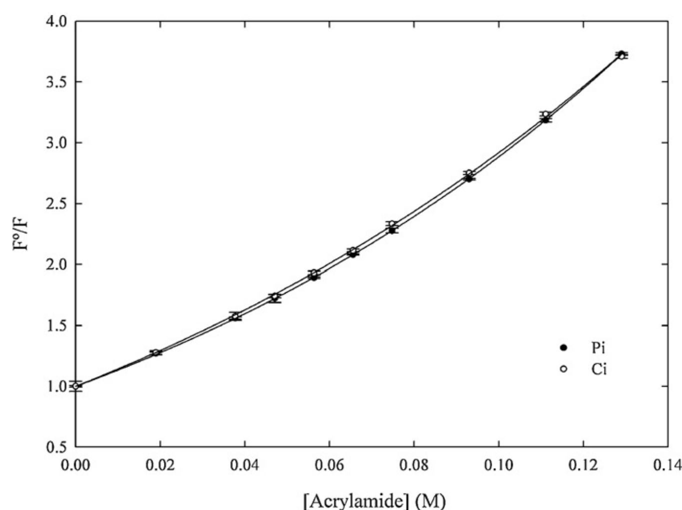

Fig. 3. Quenching of the native fluorescence of lipase by acrylamide in 50 mM Pi buffer at pH 7.0, and 65 mM Ci buffer at pH 5.2.

relative activities than in the corresponding Pi and much higher in Ci. An increase in the activity was observed for the top phase of PEG 1000 both for systems PEG/Pi and PEG/Ci.

The presence of the PEG molecules probably acts as a barrier around the protein domain in the proximity of the active site, introducing a steric hindrance in the interaction between the substrate and the lipase specific site in the case of the Pi buffer. When the anion is citrate, the presence of PEG molecules never decreased the activity of the enzyme compared with 65 mM citrate. From the results presented in Table 2, we can state that the most important factor in the activity is the kind of anion used.

The medium effect on the enzyme activity is an evidence of the interaction of the medium with the protein, which results in a modification of the tertiary structure of the active site. This fact is very important in the sense that these polymers are used in the bioseparation process of different proteins; therefore, a previous study about the polymer effect on the protein structure is important to determine if the method could be used.

3.4. Determination of the ATPS separation capacity for lipase

The experiments above were performed using a non-pure lyophilizate of lipase which contains other unknown proteins. The selection of the best systems for the recovery of lipase was made based on the analysis of the parameter β, Y (%) and PF. When the above experiments were carried out, the total protein concentrations in the top and bottom phases were determined and the partition coefficients of the total proteins were calculated. In Table 1 the mentioned parameters are presented. According to the results of the previous section, the purification factors (PF) were calculated as the ratio of the specific activity in the phase and the specific activity of the lyophilizate in the Pi or Ci buffer as required.

The systems chosen to work on the culture media were PEG 1000/Pi at pH 7, PEG 2000/Ci and PEG 4000/Ci at pH 5.2 and PEG 2000/Ci at pH 8.2 according to the best value of the percentage of recovery in combination with the values of the purification and separation factors.

3.5. Fluorescence measurements

This methodology was used to analyze the interaction between the phases of the chosen systems and lipase. The native fluorescence spectra of lipase in both top and bottom phases were obtained (data not shown). The peak position is a measure of the accessible tryptophan perturbation of the protein. The energy of the emitted photon is a direct measurement of the fluorophore–medium interaction [13]. In our case, the position of the peak allows us to have an idea of the strength of the PEG perturbation on the protein surface exposed to the solvent. Table 2 shows the values of the fluorescence emission peak of the protein in the assayed medium. In a medium of 50 mM, buffer phosphate pH 7.0 or 65 mM buffer citrate pH 5.2, lipase showed native fluorescence spectra centered at 342 nm, which suggests that the tryptophan residue environment is similar to many proteins which have a peak at about 340 nm. Except for the top phases of PEG 4000 systems where a blue shift is very important (18 and 17 nm in Pi and Ci, respectively), the protein showed a blue shift (2–8 nm) in the native fluorescence emission in the presence of other phases. The magnitude of the shift, in general, does not depend on the PEG molecular mass or its concentration. It suggests that the solvent reaches the protein tryptophan and that an increase in the hydrophobicity on the tryptophan microenvironment is produced in the presence of either the top or the bottom phases [13].

Fig. 3 shows the quenching of the native fluorescence of protein by acrylamide in the presence of 65 mM, pH 5.2 sodium citrate buffer and 50 mM, pH 7.0 potassium phosphate buffers at constant concentration of protein. The data were presented as a Stern Volmer plot. Positive deviations from the Stern–Volmer equation were observed for lipase when the extent of the quenching was large. This phenomenon is frequently interpreted as a "sphere of action", within which the probability of quenching is the unity [13]. From the fitting data and applying Eq. (9), the diffusion quenching constant (K_D) and the sphere radio were calculated, as shown in Table 2. According to Lackowitz [13], $K_D{}^{-1}$ is the quencher concentration at which $F_0/F=2$ or 50% of the intensity is quenched, therefore, an increase of K_D indicates greater affinity between the quencher and the fluorophoro. This technique gives information about the quencher-fluorophore accessibility and affinity. Moreover, this technique provides information about any modifications at the tryptophan microenvironment induced by the presence of cosolute [13].

It can be seen that the rich polymer phases induce decreased K_D values with increases of radii (except the top phases of PEG 2000/Pi and PEG 1000/Ci systems) for the PEG/Pi and PEG/Ci systems, which suggests a decrease in the quencher accessibility and affinity by the presence of the phases. Furthermore, in all cases, the area of the microenvironment of the tryptophan residues affected

Table 3
Partitioning coefficient of total proteins (K_p) and lipase (K_r) of the solid and liquid culture mediums, recovery (Y%) in both phases, separation and purification factors (β, PF).

Culture medium	ATPS	K_r	K_p	Y (%) top	Y (%) bottom	β	R_V	PF
SCM	PEG 1000/Pi pH 7.0	8.61	17.13	85.77	14.23	0.50	0.70	1.57
	PEG 2000/Ci pH 5.2	0.03	2.77	5.20	94.80	0.01	1.83	10.90
	PEG 2000/Ci pH 8.2	0.08	67.90	3.85	96.15	0.0012	0.50	26.00
	PEG 4000/Ci pH 5.2	0.07	47.76	4.86	95.14	0.0015	0.73	30.50
LCM	PEG 1000/Pi pH 7.0	1.11	3.91	43.01	56.99	0.28	0.68	0.72
	PEG 2000/Ci pH 5.2	0.04	1.32	6.82	93.12	0.03	1.83	7.90
	PEG 2000/Ci pH 8.2	0.29	3.14	12.66	87.34	0.09	0.50	0.75
	PEG 4000/Ci pH 5.2	0.36	1.73	15.25	84.75	0.21	0.50	0.32

by the phase is greater than when the protein is alone. When the enzyme is in contact with the bottom phase, there is a minor effect on the values of K_D or the radii for PEG/Pi systems. In contrast, in systems PEG/Ci, K_D decreased with similar radii in all cases. PEG rich phases of higher molecular mass produce major effects on the sphere volume. Therefore, it can be assumed that the PEG, which is in higher concentration in the top phase, induces the changes. This finding agrees with the minor capacity of the quencher to interact with the protein domain due probably to a steric hindrance.

The quenching with iodide was carried out (data not shown). The spectra of the protein native fluorescence at constant concentration of protein in the corresponding buffers (Pi or Ci) overlap with the spectra in presence of iodide. This fact indicates that the tryptophan residues are inside of lipase from *A. niger.*

3.6. Isolation of lipase from LCM and SCM culture media

To control the lipase production, activity measurements were carried out for both culture media extracts. The SCM culture medium extract reported higher activity than LCM (data not shown). These extracts were subjected to bioseparation studies. On 10 g of the chosen ATPS, a 23 ± 2% of total system was replaced by the filtering of culture media both the LCM and SCM. The K_r, K_p, Y%, β and PF were determined and presented in Table 3. The R_V was also determined and indicated in the table. These values are different from one in all cases. K_p values differ greatly from those with the commercial lyophilized, they are higher in all cases. K_r values are more similar to those obtained with the lyophilized. All these results indicate that the composition of the culture media differs greatly from to the two-phase systems and the results of the partitioning in them. With the Warburg method, used for total protein determination, the content of nucleic acids is also measured these are much higher in the culture media than in the commercial lyophilizate, this is at least an important difference between them. All differences are reflected in the PF results. Taking all this into account, the citrate systems are the best for the isolation of lipase from both culture media (LCM and SCM). Considering the SCM results and the simplicity in the preparation, we believe it to be ideal for lipase isolation.

4. Conclusions

In order to isolate lipase from a culture media using ATPSs, it is necessary to recover high enzymatic activity in the corresponding phase. The methodology should be simple, inexpensive, it should involve recyclable materials and produce no impact on the environment. Lipase was partitioned in PEG/Pi systems and in PEG/Ci systems with less pollution. The PEG 1000–protein interaction is basically hydrophobic and allows lipase, in the PEG 1000/Pi or Ci systems, to be partitioned towards the polymer-rich phase. The opposite effect occurs in PEG 8000/Pi or Ci systems and it can be seen that PEG 8000–lipase interaction is fundamentally electrostatic. Partitioning of lipase in PEG/Pi two-phase systems with high molecular mass (PEG 2000, PEG 4000 and PEG 8000) is an enthalpically driven process, which indicates that electrostatic PEG–protein interactions predominate. PEG 8000/Ci systems function in the same way.

It is important to know how the media employed affects the protein. The different phases of the systems affect the enzymatic activity in various ways. The top phases of the PEG/Pi systems modify the activity far more than the bottom phases where it is little affected. In bottom phases, there is low polymer concentration in relation to the top phase. The presence of polymer would be producing occlusion of the active site of the enzyme. The PEG/Ci systems considerably increase the activity in relation with the PEG/Pi systems in both top and bottom phases. All the systems studied turn the environment of protein tryptophan more hydrophobic; they modify the diffusion quenching constant (K_D) and increase the "sphere of action" radio, which would indicate a modification of the enzyme tertiary structure in the presence of the phases. These effects are more important in the top phases; therefore, they would probably be produced by the presence of PEG. The difference in the values of K_D and υ (Å^3) between PEG/Pi and PEG/Ci systems cannot explain the large variation in the enzymatic behavior of the protein in the presence of Pi or Ci. The tryptophan residues may not be involved in the active site of the protein.

Separation of the enzyme from the rest of the proteins that make up the lyophilized was achieved with good yield and separation factor with the ATPS formed by PEG 1000/Pi, PEG 2000/Ci at both pH studied and PEG 4000/Ci at pH 5.2. The above mentioned systems were used in order to isolate extracellular lipase from a strain of *A. niger* in submerged and solid culture. PEG 4000/Ci at pH 5.2 presented high purification factor for the solid culture. This has several advantages: the enzyme is recovered in a medium poor in polymer, from a solid culture medium which is simple to produce, and the citrate produces less pollution than the phosphate. This methodology could be used as a first step for the isolation of the extracellular lipase from *A. niger.*

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References

[1] Z.-Y. Shu, H. Jiang, R.-F. Lin, Y.-M. Jiang, L. Lin, J.-Z. Huang, J. Mol. Catal. B: Enzym. 62 (2010) 1.
[2] F. Bjorkling, S.E. Godtfredsen, O. Kirk, Trends Biotechnol. 9 (1) (1991) 360.
[3] Y. Jiangke, S. Jin, Y. Yunjun, Biotechnol. Lett. 32 (2010) 951.
[4] R. Scopes, Protein Purification, Springer Verlag, New York, 1988.
[5] R. Hatti-Kaul (Ed.), Methods in Biotechnology, vol. 11, Humana press, 2000.
[6] L. Castro-Ochoa, C. Rodríguez Gómez, G. Valerio-Alfaro, R. Oliart Ros, Enzym. Microb. Technol. 36 (6) (2005) 648.
[7] B. Zaslavsky, in: B.Y. Zavslavsky (Ed.), Physical Chemistry and Bioanalytical Applications, Marcel Decker, New York, 1994.
[8] G. Tubío, B. Nerli, G. Picó, J. Chromatogr. B 799 (2004) 293.
[9] Q.K. Shang, W. Li, Q. Jia, D.Q. Li, Fluid Phase Equilib. 219 (2004) 195.
[10] D. Spelzini, B. Farruggia, G. Picó, J. Chromatogr. B 821 (2005) 60.

[11] G. Bassani, B. Farruggia, B. Nerli, D. Romanini, G. Picó, J. Chromatogr. B 859 (2007) 222.
[12] D. Forciniti, C.K. Hall, M.R. Kula, Bioseparation 2 (1991) 115.
[13] J.R. Lakowicz, Principles of Fluorescense Spectroscopy, Plenum Press, New York, 1983.
[14] N. Adham, E. Ahmed, Indian J. Microbiol. 49 (2009) 77.
[15] O. Warburg, W. Christian, Biochemistry Z. 310 (1942) 384.
[16] T. Arakawa, S. Timasheff, Biochemistry 24 (1985) 6756.
[17] G. Picó, G. Bassani, B. Farruggia, B. Nerli, Int. J. Biol. Macromol. 40 (2007) 268.
[18] R. Lumry, L. Rajeender, Biopolymer 9 (1970) 1125.
[19] M.C. Pinna, A. Salis, M. Monduzzi, B. Ninham, J. Phys. Chem. B Lett. 109 (2005) 5406–5408.