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# Liquid-liquid extraction of protease from cold-adapted yeast *Rhodotorula mucilaginosa* L7 using biocompatible and biodegradable aqueous two-phase systems

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**ABSTRACT**

This work aimed to optimize the extraction of an extracellular protease produced by the cold-adapted yeast *Rhodotorula mucilaginosa* L7 using aqueous two-phase systems (ATPS) comprising polyethylene glycol (PEG) and sodium citrate or sodium tartrate. First, the biocompatibility of the phase forming agents was assessed. The results obtained with PEG-2000, PEG-4000, and PEG-6000 demonstrated that even at large PEG concentrations (32 wt%) the protease maintains its activity after 3 h of reaction, whereas an increase in salt concentration provokes a gradual decrease in protease stability. Subsequently, the partitioning of the protease in both types of ATPS was assessed, evaluating the effect of temperature, molecular weight, and concentration of PEG on protease purification, using two $2^3$-full factorial designs. The best partitioning conditions were obtained in PEG-6000/sodium tartrate-based ATPS, at 30°C (with a yield of 81.09 ± 0.66% and a purification factor of 2.51 ± 0.03). Thus, considering the biodegradable characteristics of the system, the PEG/sodium tartrate ATPS is a viable and economic low-resolution step in protease purification, with a strong potential for future industrial application.

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Protease; cold-adapted microorganisms; liquid-liquid extraction; aqueous two phase systems; purification



## Introduction

Currently, the use of enzymes at an industrial level has become a common practice, and the most utilized are the hydrolytic enzymes. Among them, carbohydrases, proteases, and lipases are the dominant enzymes on the market, accounting for more than 70% of all sales. Proteases refer to a group of proteins whose catalytic function is to hydrolyze peptide bonds, and have been used in a large array of applications, mostly in the chemical, food, cosmetic, and pharmaceutical industries. In addition to these main applications, proteolytic enzymes have been applied in leather treatment and in several bioremediation processes (1).

Although proteases are widespread in nature, microorganisms are the preferred source of these enzymes because of their rapid growth, the small space required for cultivation, and the ease with which they can be genetically manipulated to produce enzymes with customized properties (2). Among the several classes of industrially used microorganisms, the cold-adapted type have been the focus of interest, because the enzymes they produce catalyze reactions at low and moderate temperatures more efficiently than at high temperatures. Thus, the use of these enzymes decreases the energetic and processing costs that are typically associated with high-temperature processes and industrial heating steps (3). Moreover, due to their heat-labile activity, these enzymes can be quickly inactivated by a mild heat treatment, which is useful in many biotechnological applications that require a rapid heat-inactivation rate (3-6).

Traditionally, the purification of extracellular proteases involves an initial fractionation step with ammonium sulfate (a compound of some environmental concern), followed by several steps of chromatography (high cost), which is difficult in their large-scale bioproduction (7-9). Thus, as stated by Straathof (10), it is imperative to continue the search for more sustainable, economical, and suitable separation methods.

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Since the beginning of the 1970s, aqueous two-phase systems (ATPS) have been suggested as a good alternative for the purification of several biomolecules and other biological products (11). By definition, ATPS are systems that are spontaneously formed by the mixing of two different aqueous solutions above certain concentrations, such as the aqueous solutions of two polymers, one polymer and one salt, or two salts (11-13). Because of the water-rich content of both co-existing phases, ATPS provides a more biocompatible environment for the separation of a broad range of biological materials. Moreover, the majority of components used in the formation of these systems are not expensive, easily recycled, and, similar to other liquid–liquid extraction operations, ATPS can be readily scaled up (14). Although largely studied (15-18), conventional ATPS using polymer and inorganic salts presents several disadvantages, mainly observed when potassium phosphate is employed as the phase forming agent, because the high salt concentration required for ATPS formation leads to environmental concerns, due to the high eutrophication potential of phosphate (19). This phenomenon leads to the depletion of dissolved oxygen in water, resulting in a consequent loss of aquatic life (20-23). Therefore, ATPS composed of biodegradable anions (such as citrate or tartrate) and polyethylene glycol (PEG) has been suggested to be a more biocompatible alternative (14, 24-25). These two salts are particularly interesting because of their low environmental impact and high biodegradability, and because they can be discharged into common biological wastewater treatment plants.

Despite the large number of studies employing traditional polymer/salt ATPS (15-18), these biodegradable ATPS are less studied, mainly to extract proteases from fermentative broths. Thus, in this paper, the extraction and pre-purification of the protease secreted by the cold-adapted yeast *R. mucilaginosa* L7 using biodegradable polymer/salt ATPS is reported. First, the protease stability and activity in different concentrations of each phase forming compound was evaluated. To determine the significance of different parameters (sodium salt anion, PEG molecular weight, PEG concentration, and temperature) on protease purification, a $2^3$-full factorial design was implemented for each ATPS. In the latter part of the paper the effect of protease re-concentration changing the ATPS volume ratio ($V_r$) was studied. The goal of this paper was to find the best conditions to separate proteases from the cold-adapted yeast *R. mucilaginosa* L7.

## Experimental section

### Chemicals

Polyethylene glycols (PEG) 2000, 4000, and 6000 g/mol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Each PEG is identified in the manuscript as the following acronyms: PEG-2000, PEG-4000, and PEG-6000. Both salts used in the study, sodium citrate and sodium tartrate, were also acquired from Sigma-Aldrich (St. Louis, MO, USA). All of the reagents were of analytical grade with a minimum purity of 99% and were used as received. All of the stock solutions were prepared using water purified by filtration through a Millipore Milli-Q ion exchange system (Bedford, MA, USA).

### Yeast strain and culture conditions

The yeast studied herein (strain L7) was previously isolated from an Antarctic marine alga and taxonomically identified as *Rhodotorula mucilaginosa* through similarity to the partial 26S rDNA gene, according to the procedure described by Duarte et al. (26). The strain was deposited at the Brazilian Collection of Environmental and Industrial Microorganisms (Paulinia, Brazil) under the access number CBMAI 1528. The microorganism was maintained in potato-dextrose-agar (PDA) solid medium at 15°C in a dark room.

The *R. mucilaginosa* L7 strain was grown in Sabouraud-dextrose liquid medium (compose of 40 g/L dextrose and 10 g/L peptone) at 25°C, pH 5.6 with shaking at 150 rpm until the exponential phase (18 h). One mL aliquots from this culture were pipetted into 250 mL flasks containing 50 mL of fresh Sabouraud-dextrose medium and were grown at 25°C, 150 rpm, and an initial pH of 5.6 for 48 h. Then, the cells were removed by centrifugation at 3500 x*g* in a centrifuge model Jouan B4i (Thermo, Waltham, MA, USA). The supernatant, which exhibited an enzymatic activity equal to 200 U/mL, was stored in an ultrafreezer at -70°C for use as the protease source in the partitioning studies.

### Evaluation of proteolytic activity and stability

Protease stability was evaluated in the presence of the polymers, PEG-2000, PEG-4000, and PEG-6000, or the biodegradable salts sodium citrate and sodium tartrate. For this purpose, stock solutions of each polymer (48 wt%) and salt (22 wt% and 34 wt%, pH 5.0, for the sodium tartrate and sodium citrate,

respectively) were prepared. Then, different volumes of each stock solution were mixed with 75 µL of crude extract, and brought to a final volume of 150 µL with purified water. Each mixture was placed at 20°C and aliquots of the solutions were withdrawn at time intervals of 0, 1, 2, and 3 h to determine the residual proteolytic activity at 50°C, as described below.

The percent of residual proteolytic activity ($A_r$,%) of each solution was determined by calculating the ratio of the enzymatic activity after treatment ($A$, U/mL) to that of the initial extract ($A_0$, U/mL), according to the following equation:

$$A_r(\%) = \frac{A}{A_0} \times 100$$

### Protease partitioning studies using the biodegradable ATPS

According to our previous studies, the protease is stable at acidic pH, and has an optimum pH of 5.0 (9). Thus, the selected ATPS were systems of PEG/salt whose phase diagrams had been determined at an acidic pH. PEG/sodium tartrate and PEG/sodium citrate-based ATPS previously reported (26, 27), were chosen to develop the respective assays. The ATPS were prepared with sodium citrate or sodium tartrate and various PEG molecular weights (MW) and concentrations. All of the mixture points were prepared according to the previously determined phase diagrams (27-28). All of the components of the ternary systems were added to 15 mL glass tubes by weight, and mixed by turning the tubes up-side down several times. After complete homogenization, 4.6 g of the culture supernatant (free of cells) was added to the tubes and brought to 10.0 g with deionized water. Then, all of the systems were mixed at 8 rpm for 30 min at 25°C in an end-to-end mixer (Branstead/Thermolyne, Dubuque, IA, USA). After mixing, the ATPSs were stood at each temperature of interest (4, 17, and 30°C) in a thermo bath model 521/2DE (New Ethics, Vargem Grande Paulista, SP, Brazil) until they reached two-phase separation (at least 1 h). After the phase settling, the volumes of the upper and lower phases were measured and aliquots from both phases were carefully collected using Pasteur-pipettes for the subsequent quantification of proteolytic activity and total protein concentration. The partitioning behavior of the protease in each ATPS was evaluated according to the protease partition coefficient ($K_e$), protease yield ($Y$,%) and purification factor ($FP$). All of the partitioning parameters and their respective equations are detailed below.

The protease partition coefficient ($K_e$) was defined as the ratio of the enzymatic activity (U/mL) in the top phase ($A_{Top}$) to that in the bottom phase ($A_{Bot}$):

$$K_e = \frac{A_{Top}}{A_{Bot}}$$

The protease yield ($Y$,%) was determined by the ratio of the total activity (U) in the top phase to that added in the starting extract, and expressed as a percentage:

$$Y(\%) = \left( A_{Top} * V_{Top} / A_0 * V_0 \right) \times 100$$

where $V_{Top}$ and $V_0$ are the volumes of the top phase and of the crude extract added to the system, respectively.

The protein partition coefficient ($K_P$) was calculated as the ratio of the equilibrium concentration (mg/mL) of the total protein in the top phase $[P]_{Top}$ to that in the bottom phase $[P]_{Bot}$:

$$K_P = \frac{[P]_{Top}}{[P]_{Bot}}$$

The purification factor ($PF$) of the protease was calculated as the ratio of the specific activity (U/mg) in the phase where it was preferentially partitioned (top phase) to the specific activity in the culture supernatant before partitioning:

$$PF = \frac{A_{Top}}{C_{Top}} \Big/ \frac{A_0}{C_0}$$

where $C_{Top}$ and $C_0$ are the total protein concentrations (expressed as mg/mL) in the top phase and the crude extract, respectively.

### Determination of proteolytic activity

Proteolytic activity was measured by digestion of azocasein (Sigma, St. Louis, MO, USA), in accordance with Charney and Tomarelli (29). An appropriate dilution of culture supernatant (150 µL) was incubated with 150 µL of 0.5 wt% azocasein in 50 mM sodium acetate buffer (pH 5.0) during 40 min at 50°C. The reaction was then stopped by adding 150 µL of 10% (w/v) trichloroacetic acid. After centrifugation of the reaction mixture, 100 µL of the supernatant was mixed with 100 µL of 0.5 M KOH, and the absorbance at 430 nm was measured against an appropriate blank. All of the samples were measured in duplicate, and the respective proteolytic activity was expressed in units of enzyme activity (U). One U was defined as the amount of enzyme leading to a

0.001 increase in the absorbance under the assay conditions. The enzyme activity was determined using a microplate spectrophotometer (Spectra Max Plus 384, Molecular Device, Sunnyvale, CA, USA).

### Determination of total protein concentration

The total protein content in both phases of all of the systems was determined using the Bicinchoninic Acid (BCA) method according to previously reported procedures (30). To avoid interference from the phase components, samples were diluted at least tenfold with distilled water and 10 µL of each was pipetted in triplicate into a 96 well microplate. Bovine serum albumin (BSA) standards were made ranging from 1,500 µg/mL to 25 µg/mL, and 10 µL of each standard was pipetted in triplicate into the same plate. For blanks, 10 µL of distilled water was pipetted in triplicate into the plate. Next, the working BCA reagent was prepared by diluting 1 part of copper (II) sulfate pentahydrate 4% (w/v) solution (Sigma Aldrich, St. Louis, MO, USA) into 50 parts bicinchoninic acid solution (Sigma Aldrich, St. Louis, MO, USA), and 200 µL of the working reagent was placed in each well. The plate was briefly mixed on a plate shaker and incubated at 37°C for 30 min. The UV/Vis measurements were performed in a microplate spectrophotometer at 562 nm (Spectra Max Plus 384, Molecular Device, Sunnyvale, CA, USA). The protein concentration was determined by comparison with the absorbance values of the BSA standard curve.

### Statistical analysis

Taking into account the large number of variables in the study, two $2^3$-full factorial experimental designs were performed: one for PEG/sodium tartrate ATPS and another for PEG/sodium citrate ATPS. In both cases, the independent variables (*Xi*) were PEG molecular weight (PEG MW) ($X_1$), PEG concentration [PEG] (wt%) ($X_2$), and temperature (T) ($X_3$), whereas the responses (*Yi*) were protease partition coefficient ($K_e$) ($Y_1$), purification factor (*PF*) ($Y_2$), and protease yield in the top phase (*Y*,%) ($Y_3$).

As shown in Table 1, three different levels were adopted for each independent variable, namely PEG-2000, PEG-4000, and PEG-6000 for $X_1$; 13.00, 13.50, and 14.00 wt% for $X_2$ (in PEG/Sodium Tartrate ATPS); 15.01, 15.24, and 15.48 wt% for $X_2$ (in PEG/Sodium Citrate ATPS); and 4, 17, and 30°C for $X_3$, respectively. It is important to note that the ATPS compositions (27-28) were selected to avoid a significant loss of protease activity in the presence of high salt concentrations (see below in Section titled "Influence of Salts and Polymers on Protease Stability" and Fig. 1).

**Table 1.** Factor levels of the $2^3$ experimental designs used for the study of protease partitioning in PEG/sodium tartrate (a) and PEG/sodium citrate (b) ATPS.

| | Levels | | |
|---|---|---|---|
| Variables | Low (−1) | Central (0) | High (+1) |
| PEG MW (g/mol) | 2000 (a, b) | 4000 (a, b) | 6000 (a, b) |
| [PEG] (wt%)* | 13.00 (a) | 13.50 (a) | 14.00 (a) |
| | 15.01 (b) | 15.24 (b) | 15.48 (b) |
| Temperature (°C) | 4 | 17 | 30 |

*A maximum concentration of PEG was defined for all of the ATPS studied (16 wt%) because the protease loses activity at high salt concentrations and high polymeric concentrations induces the precipitation of azocasein not allowing the respective protease to be measured.

For statistical elaboration, the actual values of each independent variable were coded to give −1, 0, and +1 coded levels according to the equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i}$$

where $x_i$ represents the corresponding coded values, $X_0$ the actual values at the central point, and $\Delta X_i$ the step change value. Both experimental design approaches were performed with four repetitions at the central point.

Furthermore, due to several experimental limitations related to proteolytic quantification (at high polymeric concentrations the precipitation of azocasein occurs), a maximum PEG concentration was defined for all ATPS (lower than 16 wt%). It is important to note that in both experimental design approaches, the same amount of crude extract (4.6 g) (which correspond to 200 U/mL of total protease activity) was used. Similar concentrations of PEG-2000, PEG-4000, or PEG-6000 were combined with aqueous solutions of sodium tartrate (10 wt %) and sodium citrate (11.5 wt%) to perform the experimental design analysis.

After the experiments at pre-determined conditions, the statistical results were determined with the aid of the software *Statistica* version 10 (StatSoft, Tulsa, OK, USA).

## Results

### Influence of salts and polymers on protease stability

Before performing the protease partitioning, an analysis of its stability in the presence of each phase forming agent was carried out. The stability of the protease secreted by *R. mucilaginosa* L7 was investigated by incubating the enzyme without the substrate for 3 h, in the presence of PEG-2000, PEG-4000, and PEG-

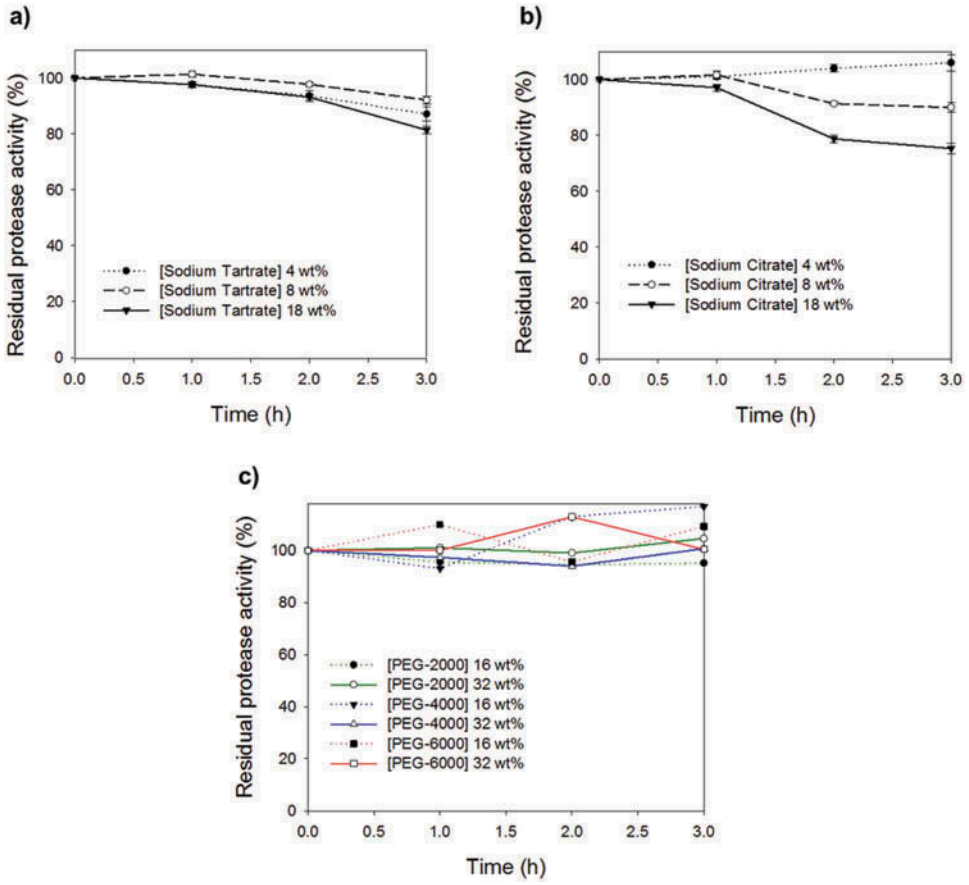


**Figure 1.** Residual protease activity (%) of *R. mucilaginosa* L7 protease at different concentrations of: a) sodium tartrate; b) sodium citrate; and c) PEG with different molecular weights (2000, 4000, and 6000 g/mol). The stability of the enzyme was determined at 1, 2 and 3 h at 20ºC. The residual enzyme activity was assessed with 0.5 % (w/v) azocasein at pH 5.00 and 50ºC.

6000, or with the sodium citrate and sodium tartrate aqueous solutions. Afterwards, the residual proteolytic activity in 0.5% (w/v) azocasein was evaluated. The concentrations of each PEG and salt aqueous solutions were defined according to the previously reported phase diagrams (27-28).

As shown in Figs. 1a and 1b, a gradual decrease in protease stability was observed with an increase in salt concentration; the highest reduction was observed after 3 h of incubation with 18 wt% of added salt (in the presence of sodium tartrate and sodium citrate the residual protease activities were 20% and 25% lower than the initial values, respectively). A detailed analysis of the protease activity of Fig. 1b clearly indicates that after a small addition of sodium citrate (4 wt%) no negative deviations were observed after 1, 2, or 3 h of incubation (a small positive deviation was observed after 3 h of incubation, which can be related to some interferences in the azocasein quantification). However, when a large amount of sodium citrate was added, a significant decrease in protease activity was observed after 2 h of incubation. From the analysis of Fig. 1a, small deviations in the protease activity were observed with different sodium tartrate aqueous solutions (4, 8, or 18 wt%), but in all cases, the protease only began to lose activity after 2 h of incubation. The mechanisms of proteins-ion interactions are poorly understood. However, previous evidence has indicated that several kosmotropic anions, such as tartrate and citrate, can reduce the hydration shell of macromolecules and consequently induce the process of protein aggregation (31). Accordingly, the decrease of proteolytic activity (observed in Figs. 1a and 1b) can be related to the low protein solubility as a consequence of its aggregation in the presence of a high concentration of sodium tartrate or sodium citrate.

In contrast, the presence of different PEGs did not affect protease stability, as exhibited in Fig. 1c. The results obtained with PEG-2000, PEG-4000, or PEG-6000 clearly demonstrated that even at large PEG concentrations (32 wt%), the protease maintains its activity after 3 h of reaction. These results were expected because PEG is frequently used as a polyhydroxy stabilizer to improve the biocompatibility of proteins for

both *in vivo* and *ex vivo* applications (32). Similar results have been reported for other enzyme-PEG pairs, in which it was demonstrated that in the presence of PEGs with molecular weight above 4000 g/mol, the enzymatic activity was not significantly affected due to an unspecific exclusion of the polymer from the protein surface and a structure-stabilizing effect, as predicted by Timasheff´s theory (33).

### Protease partitioning with PEG/sodium tartrate and PEG/sodium citrate-based ATPS

In light of the protease stability results, in which a decrease in enzymatic activity with an increase in the concentration of salts was demonstrated, we decided to work with the minimal salt concentration that permits the formation of each proposed ATPS (27-28). Thus, for all assays, the salt concentration was defined: 10.0 wt% for sodium tartrate and 11.5 wt% for sodium citrate-based ATPS, and the PEG MWs (g/mol), PEG concentrations and temperatures varied as presented in Table 1. The results of the protease partitioning in each PEG/sodium tartrate and PEG/sodium citrate-based ATPS are summarized in Tables 2 and 3, respectively.

A quick analysis of Tables 2 and 3 shows that in all of the ATPS studied, the $K_e$ values are higher than 1, indicating a preferential partitioning of the protease to the top phase (PEG-rich phase). This preferential migration of protease to the PEG-rich phase is extremely interesting when the aim is to extract enzymes from fermentation broths, because cellular debris and other particulate matter typically remain in the bottom phase (salt-rich phase) (34).

**Table 2.** Experimental values of the protease partitioning with PEG/sodium tartrate ATPS. *Ke* is the protease partition coefficient, *PF* is the protease purification factor of the top phase and *Y* (%) is the protease yield of the top phase. The ternary systems were chosen according to the $2^3$ factorial design with centred face and four repetitions at the central point.

| Ternary system | PEG MW (g/mol) | [PEG] (wt%) | T (°C) | *Ke* | *PF* | *Y* (%) |
|---|---|---|---|---|---|---|
| 1 | 2000 | 13.00 | 4 | 2.46 ± 0.05 | 1.68 ± 0.04 | 74.71 ± 0.86 |
| 2 | | 13.00 | 30 | 2.60 ± 0.02 | 1.56 ± 0.02 | 69.63 ± 0.53 |
| 3 | | 14.00 | 4 | 2.94 ± 0.02 | 1.49 ± 0.01 | 66.11 ± 0.43 |
| 4 | | 14.00 | 30 | 2.90 ± 0.06 | 1.53 ± 0.01 | 62.22 ± 0.36 |
| 5 | 6000 | 13.00 | 4 | 3.71 ± 0.01 | 1.40 ± 0.04 | 78.07 ± 0.76 |
| 6 | | 13.00 | 30 | 3.90 ± 0.02 | 2.51 ± 0.03 | 81.09 ± 0.66 |
| 7 | | 14.00 | 4 | 4.06 ± 0.07 | 2.42 ± 0.05 | 74.93 ± 0.76 |
| 8 | | 14.00 | 30 | 3.77 ± 0.03 | 2.12 ± 0.07 | 71.85 ± 0.63 |
| 9 | 4000 | 13.50 | 17 | 3.23 ± 0.06 | 1.93 ± 0.03 | 72.32 ± 0.84 |
| 10 | | 13.50 | 17 | 3.15 ± 0.02 | 2.01 ± 0.02 | 71.10 ± 0.46 |
| 11 | | 13.50 | 17 | 3.17 ± 0.06 | 2.00 ± 0.01 | 71.24 ± 0.35 |
| 12 | | 13.50 | 17 | 3.06 ± 0.02 | 2.02 ± 0.08 | 70.30 ± 0.90 |

**Table 3.** Experimental values of the protease partitioning with PEG/sodium citrate ATPS. *Ke* is the protease partition coefficient, *PF* is the protease purification factor of the top phase and *Y* (%) is the protease yield of the top phase. The ternary systems were chosen according to the $2^3$ factorial design with centred face and four repetitions at the central point.

| Ternary System | PEG MW (g/mol) | [PEG] (wt%) | T (°C) | *Ke* | *PF* | *Y* (%) |
|---|---|---|---|---|---|---|
| 1 | 2000 | 15.01 | 4 | 4.58 ± 0.01 | 1.53 ± 0.01 | 55.74 ± 0.14 |
| 2 | | 15.01 | 30 | 6.06 ± 0.18 | 1.51 ± 0.04 | 61.24 ± 0.82 |
| 3 | | 15.48 | 4 | 7.63 ± 0.10 | 1.62 ± 0.02 | 57.95 ± 0.76 |
| 4 | | 15.48 | 30 | 13.64 ± 0.06 | 1.68 ± 0.01 | 65.01 ± 0.30 |
| 5 | 6000 | 15.01 | 4 | 5.38 ± 0.11 | 2.13 ± 0.16 | 58.50 ± 0.46 |
| 6 | | 15.01 | 30 | 7.31 ± 0.05 | 1.80 ± 0.01 | 58.61 ± 0.42 |
| 7 | | 15.48 | 4 | 8.74 ± 0.11 | 2.01 ± 0.03 | 58.58 ± 0.71 |
| 8 | | 15.48 | 30 | 11.97 ± 0.03 | 2.18 ± 0.06 | 66.28 ± 0.68 |
| 9 | 4000 | 15.25 | 17 | 5.19 ± 0.05 | 2.00 ± 0.07 | 62.72 ± 0.45 |
| 10 | | 15.25 | 17 | 5.47 ± 0.06 | 1.85 ± 0.02 | 62.81 ± 0.65 |
| 11 | | 15.25 | 17 | 5.56 ± 0.03 | 2.00 ± 0.08 | 62.45 ± 0.41 |
| 12 | | 15.25 | 17 | 5.42 ± 0.02 | 2.07 ± 0.09 | 63.58 ± 0.83 |

In Table 2, there is a comparison of the different protease partitioning parameters for all of the PEG/sodium tartrate ATPS. The highest value observed for *Y (%)* (81.09 ± 0.66%) and for *PF* (2.51 ± 0.03) occurred in assay 6, which was carried out at 30°C, 10 wt% of sodium tartrate, and 13 wt% of PEG-6000. Contrarily, the worst purification values were obtained from assays 1 through 4; all of these experiments using PEG-2000 based ATPS.

All of the PEG/sodium citrate-based ATPS are depicted in Table 3. The highest values of *Y (%)* (66.28%) and *PF* (2.18 ± 0.03) were obtained in assay 8, which corresponds to a system composed of 11.5 wt % of sodium citrate and 15.48 wt% of PEG-6000 and prepared at 30°C.

However, taking into account the several variables under study, statistical analysis was used to evaluate the responses of *Y* (%) to the PEG/sodium tartrate and PEG/sodium citrate ATPS. The results are presented in Figs. 2 and 3, respectively. Analyzing the Pareto chart, the length of each bar is proportional to the standardized effect of the related variable or interaction, and the bars extending beyond the vertical

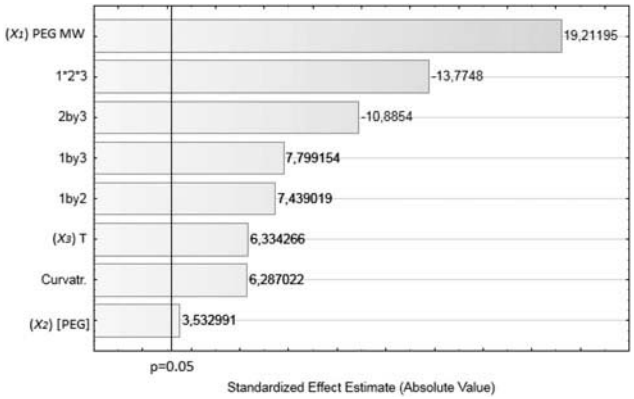


**Figure 2.** Pareto chart of ANOVA applied for the evaluation of the effects of different PEG molecular weights (PEG MWs, g/mol) ($X_1$), PEG concentrations ([PEG], wt%) ($X_2$), and Temperature (T, ºC) ($X_3$) in protease partitioning using the PEG/sodium tartrate ATPS obtained for *Y (%)* as response.

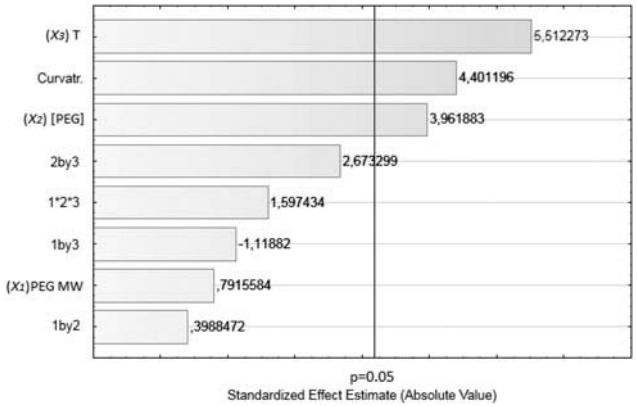


**Figure 3.** Pareto chart of ANOVA applied for the evaluation of the effects of different PEG molecular weights (PEG MWs, g/mol) ($X_1$), PEG concentrations ([PEG], wt%) ($X_2$), and temperature (T, ºC) ($X_3$) in protease partitioning using the PEG/sodium citrate ATPS obtained for *Y (%)* as response.

line correspond to the statistically significant effects at a confidence level of 95%. Thus, according to Fig. 2, it was observed that in the PEG/sodium tartrate system the strongest effects on *Y* (%) were those exerted by PEG MW ($X_1$) (positive sign) and PEG concentration ($X_2$) (negative sign). This statistical analysis means that such parameters would be improved by a simultaneous decrease in concentration and increase in the MW of PEG. Conversely, when the Pareto chart was analyzed for the PEG/sodium citrate ATPS (Fig. 3), the significant variables influencing *Y* (%) were PEG concentration ($X_2$) and temperature ($X_3$), both with positive signs. According to this analysis, an increase in the temperature and/or the PEG concentration causes an increase in *Y* (%).

In summary, according to the statistical analyses, it is clear that the protease partitioning in the PEG/sodium tartrate or PEG/sodium citrate is controlled by different factors for each set of ATPS. The MW and PEG concentration were the main limiting factors of the first set of ATPS, while the temperature and PEG concentration were the driving factors for the second set. A general comparison between both systems clearly indicates that the *Y (%)* and *PF* are significantly higher in the PEG/sodium tartrate ATPS. However, when it is compared to the $K_e$ obtained in both series of ATPS, the $K_e$ values are usually superior in PEG/sodium citrate ATPS. This opposite behavior may be a result of the protein aggregation capacity of citrate salts, as explained in the section titled "Influence of Salts and Polymers on Protease Stability" (35). It is important to note that these results were also confirmed by the total mass balance results which were always lower than 100% in the PEG/sodium citrate ATPS (data not shown).

Taking into account all of the results presented above, the protease concentration and partitioning optimization assays were performed using the ATPS composed of sodium tartrate as the salt and PEG-6000 (high MW leads to high *Y* (%) and *PF*) at the lowest possible concentration (a decrease in concentration increases the *Y* (%) and *PF*) at 30°C.

## Protease concentration and partitioning optimization

A complementary reduction of volume and an increase in the purification factor are fundamental when the aim of the liquid-liquid extraction is an industrial separation process (36). Thus, in the last part of our work we studied the effect of volumetric ratio ($V_r$) in protease partitioning using PEG/sodium tartrate ATPS, which exhibited the best purification performances (defined with the statistical analysis of the responses in terms of *Y* (%) and the *FP*). Five mixture points of ATPS were prepared by combining different compositions of PEG-6000 (with the lowest possible concentration defined according to the critical point of the phase diagram), sodium tartrate, and crude extract (because a small amount of each phase forming component was used, the amount of crude extract added was increased to 5.18 g). The protease concentration studies were prepared to obtain a $V_r$ between 0.3 and 12 (as depicted in Figs. 4a and 4b). After the preparation of the ATPS, the partitioning tests were performed at 30ºC, as previously described in the section titled "Protease Partitioning Studies using the Biodegradable ATPS." A detailed composition and $V_r$ of each PEG/sodium tartrate

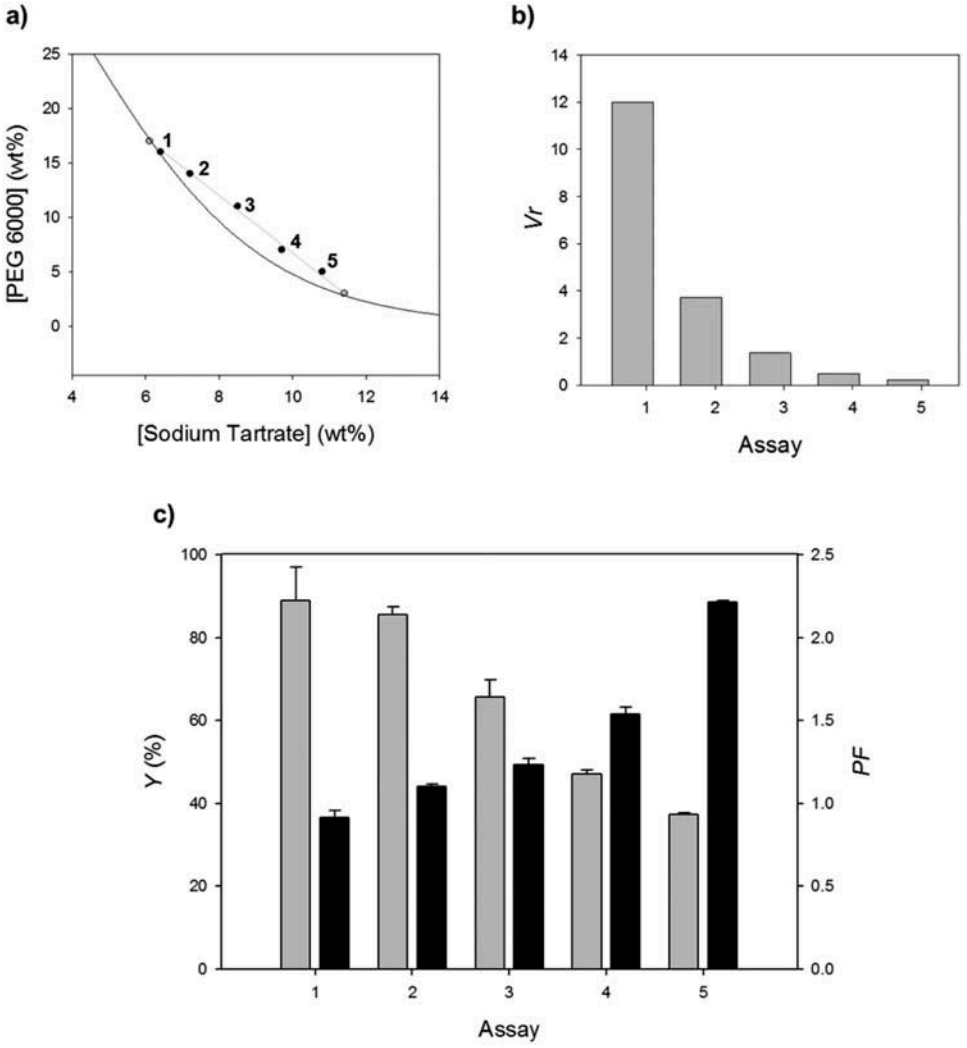


**Figure 4.** Effect of different volume ratios ($V_r$ is the volumetric ratio between top and bottom phase) in protease partitioning using the system PEG-6000/sodium artrate-based ATPS, in which: **a)** corresponds to the experimental ternary phase diagram with the mixture points studied (numbering 1 to 5), tie-line (grey line) and binodal curve (black line); b) shows the experimental values of volume ratio ($V_r$); and c) shows the experimental values of protease yield *Y* (%) and purification factor (*PF*).

ATPS is presented in Supplementary Information (Table S1).

As observed in Fig. 4c, a decrease in $V_r$ resulted in a decrease of *Y* (%), and a proportional increase in *PF*. The increase observed in the *PF* was expected, because at lower *Vr*, the volumetric activity (U/mL) was higher in the top phase (high values of $K_e$), while the concentration of total protein between the co-existing phases remained almost constant ($K_P \approx 0.8$) (Table S1). These results are in accord with those of previous studies, in which a strong influence of $V_r$ in the $K_e$ was demonstrated (37-39). The reduction observed in the *Y* (%) parameter with a decrease in $V_r$ can be attributed to the reduction of the top phase volume, and the subsequent reduction of the total protease activity (U) in this phase.

For a comparison, a summary of the results obtained in previous work in protease purification using different types of PEG/salt ATPS is presented in Table 4. With respect to the type of salt chosen for protease recovery, Bacha et al. (2012) evaluated the influence of potassium phosphate, sodium sulfate, ammonium sulfate, and sodium citrate solutions (50% w/v) in combination with PEG-4000 (50% w/v) on the purification of an extracellular protease from *Serratia marcescens P3* (40). These authors achieved maximum yields (approximately 68%) and purification factors (2.7-fold) with the systems containing sodium citrate. Similarly, for the PEG/citrate ATPS (composed of 11.5 wt% of sodium citrate and 15.48 wt% of PEG-6000), we obtained maximum yields of 66.28% and *PF* of 2.18 ± 0.03. It is important to highlight that we used lower concentrations of sodium citrate to diminish the effect of the higher concentrations of salts on protein stability and to achieve a more

**Table 4.** Comparison of proteases purification (*PF*) and yields (*Y*, %) obtained from different biological sources using aqueous two-phase systems (ATPSs).

| ATPS | Biological source | *Y* (%) | *PF* | Ref |
|---|---|---|---|---|
| PEG-6000/Sodium Tartrate | *Rhodotorula mucilaginosa* | 81 | 2.5 | current work |
| PEG-600/Sodium Tartrate | Bovine pancreas | 87 | 2.8 | Pellegrini et al. 2011 (38) |
| PEG) 4000-sodium citrate | *Serratia marcescens* | 68.7 | 2.77 | Bacha et al. 2012 (40) |
| PEG-1000/Sodium Citrate | *Clostridium perfringens* | 131 | 4.2 | Porto et al. 2008 (41) |
| PEG-4000/Potassium Phosphate | *Penicillium roqueforti* | 56 | 3.1 | Pericin et al. 2008 (42) |
| PEG-8000/Potassium Phosphate | *Azadirachta indica* leaves | 85 | 1.1 | Subatrha et al. 2012 (43) |
| PEG-1000/Potassium Phosphate | *Bacillus subtilis* | 62 | 6.1 | Chouyyok et al. 2005 (44) |
| PEG-6000/Potassium Phosphate | *Lentinus citrinus* | 151 | 1.1 | Kirsh et al. 2012 (45) |
| PEG-6000/Potassium Phosphate | *Escherichia coli* | 95 | 1.7 | Loc et al. 2013 (46) |

economic and ecological system. Porto et al. (2008) (41) also used an ATPS composed of PEG-10000 and citrate-salts to remove proteases from a *Clostridium perfringens* fermentation broth. Similarly to our results, these authors found an increase in protease recovery in the top phase with a decrease in citrate concentration (*Y* of 131% and *PF* of 4.2).

In the case of PEG/tartrate ATPS used for the purification of proteases, Pellegrini et al. (38) evaluated the capability of PEG/sodium tartrate-based ATPS to purify α-chymotrypsinogen from bovine pancreas at a pH of 5.0. The authors found that the systems based on PEG-600 (20.62 wt%) and sodium tartrate (12.85 wt%) with a $V_r$ equal to one have the highest enzyme purification performance (*PF* = 2.8; *Y* = 87%). However, in contrast to our results, which demonstrated that it is possible to concentrate and increase the purity of the protease in a PEG-rich phase using PEG-6000/sodium tartrate-based ATPS by decreasing the $V_r$, these authors suggested that volume ratio changes were not suitable variables to improve the protease purifying performance in PEG-600 and PEG-4000 ATPSs.

A large number of studies that analyzed the recovery of proteases in PEG/phosphate ATPS have been performed (42-46), in which the *PF* and *Y* values for the partitioning of the proteases were similar to the values reported by our group. However, the concentration of phosphate used in these systems was between 15-50 wt %, which leads to environmental concerns, avoiding a large industrial application. Phosphate pollution is recognized as a major pollution problem throughout the world and some countries have introduced control acts to limit the use of phosphate-based compounds. In fact Switzerland banned the use of phosphate in detergents (47). A report prepared by the Swedish Chemical Agency (2006) (48) indicated a series of substances that should be used instead of phosphates. According to that report, citrate salts, at concentrations between 2.5 wt% and 15 wt% were found to be easily biodegradable, not bio-accumulative and non-toxic to aquatic organisms. Likewise, tartrate was found to have a low toxicity and was not bio-accumulative. Thus, it is evident that the ATPS studied in this work (with citrate and tartrate sodium salts) are particularly interesting because of their low environmental impact and high biodegradability and, because they can be discharged into common biological wastewater treatment plants.

## Conclusions

The potential applicability of biodegradable ATPS to purify proteases directly from *Rhodotorula mucilaginosa* L7 culture was studied. First, it was demonstrated that the protease can remain fully stable in different aqueous solutions of PEG (different MWs and concentrations) and in the presence of low concentrations of sodium tartrate or sodium citrate aqueous solutions. The protease purification results revealed that the best conditions to purify the protease were obtained in the system composed of PEG-6000 and sodium tartrate at 30°C. Additionally, with an experimental design and subsequent statistical analysis, it was shown that the PEG MW or PEG concentration had a strong influence on protease purification parameters.

As discussed, our protease recovery results are similar to or even higher than those reported by other authors, where PEG/salt ATPS were also used to purify different enzymes (38, 40-46). Furthermore, although the *PF* obtained in our study were generally lower than other systems reported in the literature, it is important to note that this parameter is directly related with to the initial protease/total protein ratio. In our case, because *R. mucilaginosa L7* secretes a small amount of proteins into the extracellular medium (49), the protease sample purified with our biodegradable ATPS exhibited a high degree of purity.

Finally, in order to optimize the protease extraction and its concentration in small volume size, the influence of volume ratio on the enzyme recovery and purification was also evaluated. Our results demonstrated that it is possible to concentrate and to increase the purity of the protease in a PEG-rich phase using PEG-6000/Sodium Tartrate-based ATPS, since a decrease of $V_r$ resulted in the increment on the protease *PF*. Taking into account all of the interesting purification results and the biocompatible (high protease stability) and biodegradable characteristics of PEG and sodium tartrate, in addition to their low costs, it is

clear that PEG/sodium tartrate-based ATPS may be a potential and viable liquid-liquid technique to purify proteases for industrial purposes.

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