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Recovery of pectinases from *Aspergillus niger* using aqueous two-phase systems

Recuperação de pectinases de *Aspergillus niger* usando sistemas aquosos bifásicos

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ABSTRACT
This work aimed to study the purification of pectinases by aqueous two-phase systems (ATPS). The crude enzymatic extract was produced by *Aspergillus niger* ATCC 9642 and contained exo-polygalacturonase (exo-PG), pectinmethylesterase (PME) and pectin lyase (PMGL). The ATPS systems tested consisted in the combinations of polyethylene glycol (PEG) and potassium phosphate and sodium citrate, alcohol (ethanol, n-propanol and isopropanol) and salt (ammonium sulfate, potassium phosphate and sodium citrate). The experiments showed higher recoveries using ATPS system - PEG/phosphate for the exo-PG were using 16% PEG 4.0 kDa/4.8% NaCl and 16% PEG 1.5 kDa/without NaCl, obtaining purification factors (PF) of 1.37 and 1.21 times and recovery (R) of 49 and 59%, respectively. However, for the enzymes PME and PMGL were of 4.8 and 4.7 fold and 478 and 241%, respectively. When used ATPS system - PEG/sodium citrate the best PF were of 2.4, 7.85 and 5.7 and R of 100, 331 and 239% for exo-PG, PME and PMGL, respectively. The ATPS system is an alternative and efficient method for the recovery and/or purification of pectinases.

Key words*:* alcohol, enzymes, polyethylene glycol, purification, salt.

RESUMO
Este trabalho teve como objetivo estudar a purificação de pectinases por sistemas aquosos bifásico (SAB). O extrato enzimático bruto foi produzido por *Aspergillus niger* ATCC 9642, composto pelas enzimas exo-poligalacturonase (exo-PG), pectinmetilesterase (PME) e pectina-liase (PMGL). Os sistemas SABs testados consistiram nas combinações de polietilenoglicol (PEG) e fosfato de potássio e citrato de sódio, álcool (etanol, n-propanol e isopropanol) e sal (sulfato de amônio, fosfato de potássio e citrato de sódio). Os experimentos mostraram maiores recuperações utilizando o sistema SAB - PEG/fosfato para o exo-PG, utilizando 16% PEG 4,0 kDa/4,8% NaCl e 16% PEG 1,5 kDa/sem NaCl, obtendo fatores de purificação (PF) de 1,37 e 1,21 vezes e recuperação (R) de 49 e 59%, respectivamente. No entanto, para as enzimas PME e PMGL foram de 4,8 e 4,7 vezes e 478 e 241%, respectivamente. Quando utilizado o sistema SAB - PEG/ citrato de sódio, os melhores PF foram de 2,4, 7,85 e 5,7 e R de 100, 331 e 239% para exo-PG, PME e PMGL, respectivamente. O sistema SAB é um método alternativo e eficiente para a recuperação e/ou purificação de pectinases.

Palavras-chave: álcool, enzimas, polietileno glycol, purificação, sal.

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1 INTRODUCTION

The pectinolytic enzymes or pectinases are produced by most of higher plants, filamentous fungi, by bacteria and some yeasts [1]. Their commercial application was first observed in 1930, for the preparation of wines and fruit juices [2].

As an emerging class of purification technology, two-phase aqueous system (ATPS) prossesses attractive properties such as time saving, simplicity and easy scale-up that make its application economically feasible [3], technique that can be applied in the purification of enzymes. The ATPS is formed by adding two water soluble polymers or water-soluble polymer and a component of low molecular weight, such as inorganic salts that above certain concentrations can separate into two phases. For purification, the target product should be partitioned to a different phase from the contaminants [4].

Most studies carried purification by chromatographic processes [5-7], with the ultimate goal to obtain an enzyme with very high degree of purity. These studies seek only the complete characterization of the enzyme, without worrying about costs and the relationship of purity to the desired application. Studies on purification strategies using simple and low cost processes but that allow to achieve high recovery and purification factors of the enzyme are important from the industrial point of view [8,9].

Purification of enzymes by ATPS is highly effective, while maintaining a high level of enzyme activity [10-13]. According to Clonis [14] 50-80% of the cost of biological product is due to the purification strategy. The aqueous organic phase system (ATPS), which integrates the concentration, clarification and initial purification, has become a desirable method for the purification and recovery of many biological products [15,16]. The simplicity, easy scale-up, rapid operation and low material cost of this method and the minimal denaturation of bio-molecules makes an ATPS very attractive for obtaining industrial enzymes compared with conventional methods of purification [9,10,17,18].

However, only a few studies on partitioning and/or purification of pectinases (exo-PG, PME and PMGL) produced by microorganisms are addressed in literature. In this way, it is necessary to study one alternative method and of low cost for partitioning and/or purification of enzymes. The present work aimed to study the purification of pectinases through aqueous two-phase systems (ATPS). The ATPS systems tested consisted of combinations of polyethylene glycol (PEG) and potassium phosphate and sodium citrate, alcohol (ethanol, n-propanol and isopropanol) and salt (ammonium sulfate, potassium phosphate and sodium citrate).

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2 MATERIALS AND METHODS

2.1 PRODUCTION OF PECTINASES

The enzyme-complex (exo-polygalacturonase – exo-PG, pectin methyl esterase - PME and pectin lyase - PMGL) was produced by the strain *Aspergillus niger* ATCC 9642 by submerged fermentation. Growth medium consisted of citrus pectin (32 g/L), L-asparagine (2 g/L), iron sulfate (1 g/L) and potassium phosphate (0.06 g/L) at 30°C, $pH_{initial}$ 5.5, agitation of 180 rpm and 27 h bioproduction in an orbital incubator (New Ethics), according to the method described by Gomes et al. [19]. After bioproduction, the fermented medium was filtered (Whatman nº1), yielding the crude enzyme extract, which was stored in polyethylene containers, vacuum closed, and held at -20ºC.

2.2 PURIFICATION OF PECTINASES

2.2.1 Aqueous two-phase systems (ATPS)- Alcohol and salt

Different concentrations of alcohol and salt (16/16, 18/20, 20/20 and 24/22 by mass) were tested to evaluate the potential use of alcohol-based ATPS, 20% (by mass) of enzyme exctract and deionized water to complete the total mass 10 g. The stock solutions were prepared with 40% (by mass) of salt. The salts used in this system are ammonium sulfate (Nuclear), potassium phosphate (Vetec) and sodium citrate (Nuclear), and alcohols used were ethanol (Nuclear), n-propanol (Vetec) and isopropanol (Vetec). The systems were weighed into centrifuge tubes (50 mL), homogenized at room temperature during 15 min and for phase separation the samples were centrifuged (centrifuge MPW-351R, Warsaw, Poland) at 4,000 x g for 10 min at 10°C. Control samples were prepared in each assay where instead of the enzyme extract distilled water was placed in the same proportion. The enzyme activity and total protein content of the samples were determined in the precipitate and the supernatant. Results were measured in terms of purification factor (PF), partition coefficient (Ke), volume ratio (V_R) and recovery (R) of exo – PG.

Based on the results of previous tests, further tests were performed using ethanol/citrate, isopropanol/citrate and n-propanol/citrate evaluated for activity of exo-PG, PME and PMGL enzymes. From these results, a factorial design 2^2 was set up by varying the concentration of isopropanol (18 to 22%) and the concentration of citrate salt (20 to 24%). The dependent variables (responses) were: PF, Ke, V_R and R.

2.2.2 Aqueous two-phase systems (ATPS) - PEG/potassium phosphate buffer

The systems were prepared from stock solutions of polyethylene glycol - PEG (Labsynth) 16, 18, 20 and 28% (by mass) wich different molecular weights (1.5; 4.0; 6.0; 8.0 and 10.0 kDa) and

potassium phosphate buffer (Vetec Fine Chemicals) from 8 to 10% (by mass) at pH 6.0 and 7.0 (mixture of monobasic potassium phosphate and dibasic potassium phosphate), sodium chloride (0 to 4.8% by mass) and deionized water to complete the total mass of 30 g (pH 6.0) and 40 g (pH 7.0 and pH 8.0 with only PEG 6.0 kDa). The solutions were stored at 5°C. All systems were prepared in graduated centrifuge tubes. The amount of enzyme was always 8 g, and was the last component added to the system.

For comparison and evaluation of biphasic aqueous purification system, assays were also performed with a commercial enzyme complex, Pectinex Ultra (Novozymes, Danemark), using salt as potassium phosphate and 40% polyethylene glycol. The results were evaluated in terms of PF and R and their standard deviations for exo-PG, PME and PMGL.

2.2.3 Aqueous two-phase systems (ATPS) - PEG/sodium citrate buffer

The systems were prepared from stock solutions of polyethylene glycol - PEG (Labsynth) 5, 10, 20, 25, 40 and 45% (by mass) of different molecular weights (1.5; 4.0; 6.0 and 8.0 kDa) and sodium citrate 5, 6, 10, 20 and 25% (by mass) at pH 5.0 and deionized water to complete the total mass of 40 g. All systems were prepared in graduated centrifuge tubes. The procedure was then followed as described above.

For comparison and evaluation of biphasic aqueous purification system, tests were also performed with a commercial enzyme Pectinex Ultra, using potassium phosphate and 40% polyethylene glycol. The results were evaluated in terms of PF and R and their standard deviations for exo-PG, PME and PMGL respectively.

2.2.4 Purification parameters

The purification factor (PF) was calculated using Eq. 1 [8,20,21]:

$$PF = \frac{A_f}{A_i} \quad (1)$$

where A_f is the specific activity of the enzyme after the purification step (U/mg) and A_i is the initial specific activity of the crude extract (U/mg).

The recovery (R) of the enzyme was calculated by Eq. 2 [8,20,21]:

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$$R = \frac{A_f \times V_f}{A_i \times V_i} \times 100 \quad (2)$$

where A_f is the total activity of enzyme extract phase (U/mL), A_i is the total activity of the crude extract in the diet (U/mL), V_i is the initial volume (mL) of crude extract and V_f is the phase volume (mL).

The volume ratio (V_R) of the system was determined by Eq. 3 [22]:

$$V_R = \frac{V_{top}}{V_{bottom}} \quad (3)$$

where V_{top} is the volume (mL) of the top phase after the purification process and V_{bottom} is the volume (mL) of the bottom phase after the purification process.

The partition coefficient (K_e) was calculated by Eq. 4 [20,22]:

$$K_e = \frac{At_{top}}{At_{bottom}} \quad (4)$$

where At_{top} is the total activity (U/mL) of the enzyme in the top phase after the purification process and At_{bottom} is the total enzyme activity (U/mL) in the bottom phase after the purification process.

2.3 DETERMINATION OF ENZYME ACTIVITY

2.3.1 Exo-polygalacturonase (exo-PG)

The pectinolytic activity of polygalacturonase (exo-PG) was determined by measuring the release of reducing groups using the dinitrosalycilic acid method (DNS), as originally proposed by Miller [23] with some modifications.

The substrate solution consisted of 0.5% (w/v) citrus pectin (Sigma) in sodium acetate buffer pH 5.5. An aliquot of 1,000 µL of this solution was incubated at 37°C during 15 min for temperature equilibration. Then, 500 µL of enzyme extract were added to the substrate solution. After 6 min, 1 mL DNS solution was added to the mixture, and the vial was boiled for 5 min. The mixture was then cooled in an ice bath and, 8.0 mL of 50 mM sodium and potassium tartrate was added for color stabilization. The absorbance was measured at 540 nm (Spectrophotometer, Beckman Coulter,

DU640 model, Germany). For specific activity determination (U/mg), the enzyme activity (U/mL) was divided by protein content (mg/mL), which was quantified by the method of Bradford [24], with bovine serum albumin (Sigma A3294) as standard, and by the fluorimetric method using a commercial Invitrogen (Quanti-it) kit. The sample (10 µL) was added in 190 µL of the reagent with a subsequent digital fluorometer reading (Qbit Fluorometer) used according to the manufacturer's instructions. One unit of exo-PG activity is the amount of enzyme necessary to release 1 µmol of galacturonic acid per minute (U = µmol/min) according to a standard curve (0.1–10 mg/mL) established with D-galacturonic acid (Fluka Chemica) as a reducing sugar.

2.3.2 Pectin methyl esterase (PME)

The pectin methyl esterase activity was determined by the method of Hultin et al. [25]. Inicially was prepared 30 mL of substrate solution (1%, w/v) citrus pectin solution in sodium chloride 0.2 mol/L) with pH adjusted to 7.0 using NaOH 0.01 mol/L in a water bath at 20°C. Then, 1 mL of enzyme extract was added to the substrate and the solution was titrated with sodium hydroxide 0.01 mol/L for 10 min, keeping the reaction mixture pH at 7.0. Specific activity was calculated as described above. One unit of PME was defined as the amount of enzyme able to catalyze the demethylation of pectin corresponding to the consumption of 1 µmol of NaOH/min, under the tested conditions.

2.3.3 Pectin lyase (PL or PMGL*)*

The PMGL activity was determined as described by Ayers et al. [26]. An aliquot of 5 mL of substrate solution (1% citrus pectin solution in Tris-HCl buffer pH 8.5) was mixed with 1 mL of $CaCl_2$ 0.01 mol/L, 1 mL enzyme extract and 3 mL of distilled water. The reaction medium was incubated for 2 h at 30°C. Then 0.6 mL of 9% $ZnSO_4.7H_2O$ and 0.6 mL of NaOH 0.5 mol/L were added to the mixture, centrifuged (centrifuge MPW-351R) at 3,000 x g for 10 min at 5 °C. The supernatant was collected and 3 mL of thiobarbituric acid 0.04 mol/L, 1.5 mL of HCl 0.1 mol/L and 0.5 mL of distilled water were added. The mixture was boiled for 30 min, and then cooled in an ice bath. The absorbance was measured in a spectrophotometer (Beckman Coulter, DU640 model) at 550 nm. Specific activity was calculated as described above. One unit of enzyme activity was defined as the amount of enzyme, which causes a change of 0.01 unit in absorbance at 550 nm under the assay conditions.

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2.4 STATISTICAL TREATMENT

The experimental design results of purification factor and recovery of pectinases were analyzed using the software Statistica 7.0 (Stat Solf Inc., Tulsa, OK, USA).

3 RESULTS AND DISCUSSION

3.1 AQUEOUS TWO-PHASE SYSTEMS (ATPS) WITH ALCOHOLS AND SALTS

The results of purification (Table 1) showed that it was possible to obtain a good recovery of enzyme exo-PG with the aqueous two-phase systems formed with alcohol and salt. However, the runs 3, 6 and 9 yielded purification factors of 3.4, 4.6 and 4.4-fold with sodium citrate/ethanol, sodium citrate/n-propanol and sodium citrate/isopropanol, respectively. For exo-PG enzyme was possible to obtain, in the bottom phase, PF of 4.6 and 3.4-fold with recoveries of 20.2 and 11.2%, using sodium citrate/isopropanol and sodium citrate/ethanol, respectively. Enzyme exo-PG showed higher affinity with bottom phase, rich in salt (20 – 22% by mass).

Table 1. Purification factor and recovery of exo-PG employing salt aqueous two-phase system and alcohol bottom phase.

Run	Biphasic system	Concentration of Alcohol/Salt	PF	Recovery (%)
1	Ethanol/$(NH_4)_2SO_4$	24/22	0	0
2	Ethanol/potassium phosphate	20/20	0	0
3	Ethanol/sodium citrate	24/22	3.4	11.2
4	Isopropanol/$(NH_4)_2SO_4$	18/20	0	0
5	Isopropanol/potassium phosphate	16/16	0	0
6	Isopropanol/sodium citrate	18/20	4.6	20.2
7	n-propanol/$(NH_4)_2SO_4$	18/20	0	0
8	n-propanol/potassium phosphate	16/16	0	0
9	n-propanol/sodium citrate	18/20	4.4	13.5

Based on the recovery results using the system based on sodium citrate/isopropanol, the levels of the concentrations of sodium citrate and isopropanol alcohol were shifted. The results of the experimental design (2^2 factorial design) propanol are presented in Table 2, which presents the real and coded values for independent variables and responses in terms of PF and R of exo-PG, PME and PMGL in the top and bottom phases.

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Table 2. Matrix of 2^2 factorial design encoded (real) values for purification with salt aqueous two-phase system (sodium citrate) and isopropanol alcohol (top and bottom phase) and responses in terms of PF and R of pectinases (exo-PG, PME and PMGL).

Run	Independent variables *			Exo-PG			PME			PMGL		
	X_1	X_2	V_R	Ke	PF	R (%)	Ke	PF	R (%)	Ke	PF	R (%)
Top phase												
1	-1 (18)	-1 (20)	0.42	0.13	0.28	1.4	0	0.92	4.9	0	2.9	15
2	1 (22)	-1 (20)	0.58	0.03	0	1.2	0	0	0	0	0	51.4
3	-1 (18)	1 (24)	0.46	0.12	0.56	1.3	0	1.8	5.8	0	0.71	1.9
4	1 (22)	1 (24)	0.58	0.06	0.12	0.5	0	0	0	0	1.7	4.6
5	0 (20)	0 (22)	0.46	0.01	0.71	1.3	0	1.2	5.8	0	1.5	9.6
6	0 (20)	0 (22)	0.46	0.05	0.62	0.87	0	0	0	0	0	0
7	0 (20)	0 (22)	0.46	0.05	0.96	1.5	0	0	0	0	0	0
Bottom phase												
1	-1 (18)	-1 (20)	0	0	4.7	26.9	0	0	0	0	0	0
2	1 (22)	-1 (20)	0	0	2.9	29.5	0	0	0	0	0	0
3	-1 (18)	1 (24)	0	0	3.7	23.2	0	0	0	0	0	0
4	1 (22)	1 (24)	0	0	4.1	25.9	0	0	0	0	0	0
5	0 (20)	0 (22)	0	0	1.5	25.9	0	0	0	0	0	0
6	0 (20)	0 (22)	0	0	1.6	25.4	0	0	0	0	0	0
7	0 (20)	0 (22)	0	0	1.8	22.9	0	0	0	0	0	0

*Independent variables: X_1= Concentration of alcohol (%); X_2= Concentration of salt (%). Fixed independent variables: enzyme extract 20 % (w/w), ambient temperature, reaction time 15 min

For exo-PG enzyme was possible to obtain, in the bottom phase, PF of 4.7 and recovery of 29.5%, using 20% (by mass) of sodium citrate/18% (by mass) of isopropanol and 20% (by mass) of sodium citrate/22 % (by mass) of isopropanol, respectively. In the bottom phase was not possible to obtain recovery of PME and PMGL showed affinity of these enzymes with top phase.

Results of Table 2 were statistic treated, showing significant effect ($p<0.05$) positive of the interaction of sodium citrate and isopropanol on the purification factor of exo-PG - phase bottom (Fig. 1), indicating that increasing the concentration of either, possible can be enhance the purification factor of exo-PG.

In the top phase (Table 2) the major recovery of PMGL was of 51.4%, using 20% (by mass) of sodium citrate/22% (by mass) of isopropanol. Results of Table 2 were statistic treated showing significant effect ($p<0.05$) negative of the sodium citrate concentration on the recovery of PMGL - top phase (Fig. 2), indicating that increasing the concentration occurs one decrease on the recovery of the enzyme. The effects of studied factors on recovery and purification of the enzyme in the top phase for enzyme exo-PG were not significant at the 95% level (date not presented). The results of purification factor for PME and PMGL enzymes in the top phase has no significant effect (date not presented).

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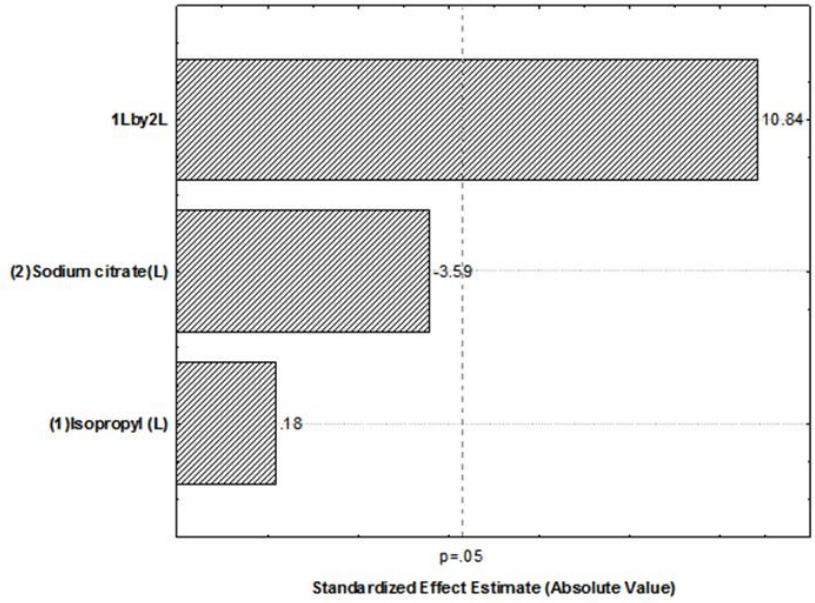
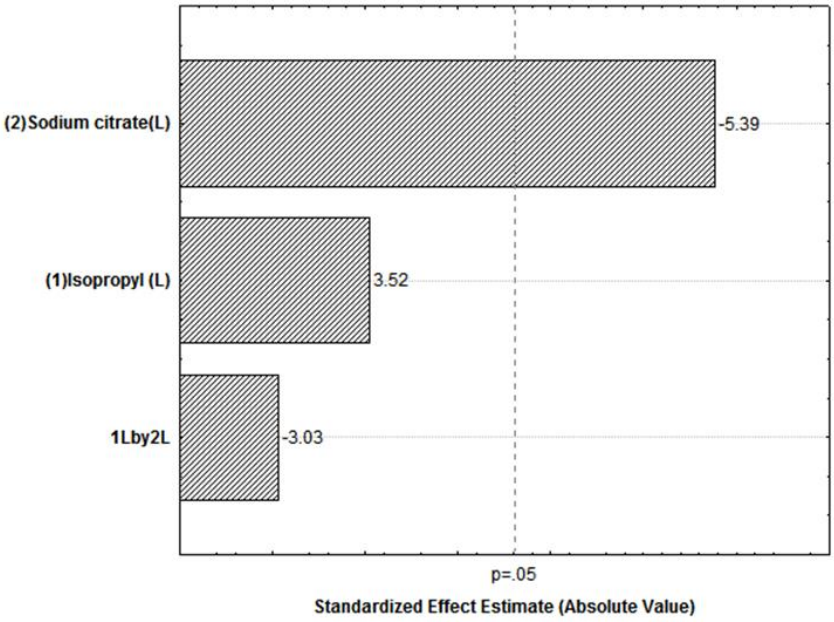
Fig. 1. Pareto chart with the estimated effects (absolute value) of the 2^2 factorial design employing ATPS isopropanol alcohol and sodium citrate in relation to the purification factor of the enzyme exo-PG - bottom phase.



With high concentrations of isopropanol (22% by mass) was observed the inactivation and/or denaturation of the pectinases (Table 2). According to Ooi et al. [8] the aqueous system (alcohol/salt) may inactivate or denature enzymes by incompatibility with the organic phase.

Fig. 2. Pareto chart with the estimated effects (absolute value) of the 2^2 factorial design employing ATPS isopropanol alcohol and sodium citrate in relation to enzyme recovery PMGL - top phase.



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3.1.1 Aqueous two-phase systems (ATPS)– PEG and potassium phosphate buffer

Screening of conditions of PEG/potassium phosphate buffer in the purification of pectinases was tested with different molecular weights PEG at pH 6.0 and pH 7.0. For this study were chosen random points from binodal curve based on results reported by Minami [27]. The results for PEG 1.5, 4.0, 6.0, 8.0 and 10.0 kDa at pH 6.0 are presented in Table 3. The runs with better results for exo-PG were with 16% PEG 4.0 kDa, 8% potassium phosphate and 4.8% NaCl (run 5) and 16% PEG 1.5 kDa, 8 % potassium phosphate and without NaCl (run1), both in the top phase, obtaining 1.4 and 1.2-fold, recovery (R) of 49 and 59% and Ke of 100, respectively. Regarding for PME, the best results were obtained using 18% PEG 6.0 kDa, 10% potassium phosphate and without NaCl (run 9), yielding a purification factor of 4.8-fold and recovery of 478%, and 20% PEG 8.0 kDa, 10% potassium phosphate and without NaCl (run 10) obtaining 2.0-fold, recovery of 220% and Ke of 100. The PMGL showed 4.7-fold and recovery 241% with 18% PEG 10.0 kDa, 10% potassium phosphate and without NaCl (run 13).

Table 3. Purification factors, recovery and partition coefficient using PEG and potassium phosphate buffer at pH 6.0.

Run	PEG (kDa,%)	Salt (%)	NaCl (%)	V_R	Exo-PG			PME			PMGL		
					Ke	PF	R (%)	Ke	PF	R (%)	Ke	PF	R (%)
Top phase													
1	1.5/16	8	0	0	100	1.2	59.3	0	0	0	100	0.64	31.1
2	1.5/16	8	4.8	3.08	100	0.21	7.5	0	0	0	0	0	0
3	1.5/28	8	0	5.63	100	0.01	2.6	100	1.0	396.1	0	0	0
4	4/16	8	0	0	100	0.19	1.2	0	0	0	0	0	0
5	4/16	8	4.8	2.71	100	1.4	48.8	0.02	0.75	26.5	9.7	0.80	28.5
6	4/18	10	0	2.38	100	0	13.0	0	0	0	0	0	0
7	6/16	8	0	3.50	100	0.12	70.3	0	0	0	0.19	0.05	29.6
8	6/16	8	4.8	1.79	100	0.45	27.1	0.98	1.5	88.0	0.03	0.07	6.7
9	6/18	10	0	3.08	100	0.09	9.4	100	4.8	478	0	0	0
10	8/20	10	0	1.84	100	0.11	12.1	0.11	2.0	220	0	0	0
11	10/16	8	0	3.15	100	0.79	65.7	0	0	0	0	0	0
12	10/16	8	4.8	2.00	87.7	1.2	38.6	0	0	0	0	0	0
13	10/18	10	0	1.94	100	0.30	15.4	0	0	0	100	4.7	241
Bottom phase													
1	1.5/16	8	0	0	0	0	0	0	0	0	0	0	0
2	1.5/16	8	4.8	0	0	0	0	0	0	0	0	4.8	13.8
3	1.5/28	8	0	0	0	0	0	0	0	0	0	1.2	85.9
4	4/16	8	0	0	0	0	0	0	0	0	0	0	0
5	4/16	8	4.8	0	0	0	0	0	11.5	538	0	0.45	21.0
6	4/18	10	0	0	0	0	0	0	0	0	0	0	0
7	6/16	8	0	0	0	0	0	0	14.0	201.2	0	0.33	43.9
8	6/16	8	4.8	0	0	0	0	0	1.5	56.2	0	2.3	142.7
9	6/18	10	0	0	0	0	0	0	0	0	0	0	0
10	8/20	10	0	0	0	0	0	0	5.8	394.2	0	0	0
11	10/16	8	0	0	0	0	0	0	21.6	396.1	0	0	0
12	10/16	8	4.8	0	0	0	0	0	4.5	997.7	0	0.37	83.4
13	10/18	10	0	0	0	0	0	0	4.9	509.3	0	0	0

For the exo-PG, on the bottom phase (Table 3) all runs no showed results for PF and R. This is due to the behavior of the protein, where the top phase, rich in PEG, it is more hydrophobic than the salt rich phase [28]. The best results obtained in the bottom phase were to PME with 16% PEG 6.0 and 10.0 kDa, 8% potassium phosphate and without NaCl (runs 11 and 7) with PF 21.60 and 14-fold and recovery 396 and 201%, respectively. For the PMGL best results PF of 4.8 and 2.3-fold and recovery of 13.8 and 142.7% in 16% PEG (1.5 and 6.0 kDa), 8% potassium phosphate and 4.8% NaCl concentration (runs 2 and 8), respectively. The ATPs was efficient in the purification and recovery of the enzyme PME and PMGL.

It is interesting to note that in runs 3, 9 and 10 – Top phase and in runs 5, 7, 10, 11, 12 and 13 – Bottom phase of PME and in run 8 – Bottom phase of PMGL (Table 3) the recovery was higher than 100%. This suggests that the precipitation is removing enzyme inhibitors. This behavior may possibly be attributed to two reasons: i) there is removal of metabolites or secondary metabolites during purification which inhibit the enzyme activity, and ii) a high concentration of salt and/or protein, which helps maintain the protein conformation to the active form [29,30].

In the literature, some studies using aqueous two-phase system also verified that enzymes have affinity by the top phase. Lima et al. [31] achieved the best purification factors in the top phase to the exo-PG and PMGL when used PEG 6.0 and 10.0 kDa and without NaCl.

3.1.2 Aqueous two-phase systems (ATPS) – PEG and sodium citrate buffer

The Table 4 show the results of purification of pectinase using ATPS compound of PEG and sodium citrate buffer.

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Table 4. Purification factors and recovery using PEG and sodium citrate buffer at pH 5.0.

Run	PEG (kDa/%)	Salt (%)	V_R	Exo-PG			PME			PMGL		
				Ke	PF	R (%)	Ke	PF	R (%)	Ke	PF	R (%)
Top phase												
1	1.5/40	10	4.0	10.59	1.0	29.3	4.00	5.9	355.1	0.16	0.54	32.3
2	1.5/25	20	1.37	2.70	0.87	19.5	0	0	0	100	1.1	52.2
3	4/45	10	5.60	3.17	0	37.4	0.90	3.7	724.9	0	0	0
4	4/10	25	0.39	100	1.8	7.7	0.05	4.7	20	100	18.3	78.3
5	6/40	5	16	2.82	2.4	100.2	100	7.8	331.4	0.94	5.7	238.9
6	6/20	10	2.5	0.66	0.3	48.5	0	0	0	0.10	0.57	81.8
7	8/30	6	7.75	0.77	0.7	50.3	0	0	0	0	0	0
8	8/5	20	0.24	0.17	0.9	6.0	0	0	0	0	0	0
Bottom phase												
1	1.5/40	10	0	0	0.01	0.69	0	0.14	22.2	0	0.34	52.0
2	1.5/25	20	0	0	0.30	5.27	0	70.14	2686.4	0	0	0
3	4/45	10	0	0	0.06	2.11	0	3.97	144.2	0	0.09	3.3
4	4/10	25	0	0	0	0	0	8.40	1071.9	0	0	0
5	6/40	5	0	0	0.16	2.22	0	0	0	0	1.1	15.9
6	6/20	10	0	0	0.40	29.3	0	0	0	0	4.6	337.0
7	8/30	6	0	0	0.09	8.5	0	0	0	0	2.7	263.2
8	8/5	20	0	0	0.13	21.5	0	3.57	352.1	0	0	0

The best results obtained in the top phase were to exo-PG with purification factor of 2.4-fold and recorery of 100%, for PMGL with purification factor of 5.7-fold and recorery of 239%, for PME with purification factor of of 7.8-fold and recorery of 331% (run 5). In the bottom phase for PME showed values purification factor of of 70% and recovery of 2,686%, 3.6-fold and 352% (runs 2 and 8), respectively and for PMGL presented purification factor of of 4.6-fold and recorery of 337% (run 6).

4 CONCLUSION

In this study we used pectinases purification strategies in aqueous two-phase systems (ATPS). The best results were when used 40% (by mass) of PEG 5 kDa and 6.0% sodium citrate obtaining purification factor of 2.4, 7.8 and 5.7-fold and recovery of 100, 331 and 239% for exo-PG, PME and PMGL (top phase), respectively.

The efficiency of the aqueous two-phase systems depends on PEG molecular weight, salt concentration and pH that are considered as important parameters for pectinases purification. The ATPS method could be a beneficial, attractive and economical technique for separation and recovery of enzimes. The use of ATPS is a promising method for the pre purification of pectinases from *A. niger* and this partially purified extract is a suitable biocatalyst for a large number of food industrial processes.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare that they do not have conflict of interest.

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