



Integrated strategy for purification of esterase from *Aureobasidium pullulans*

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ABSTRACT

Esterases catalyze the cleavage and formation of ester bonds of a broad range of substrates presenting a wide-spread spectrum of industrial applications. This work aimed to partially purify and characterize an esterase from *Aureobasidium pullulans* LABIOTEC 01 produced in a culture medium containing olive mill wastewater. Esterase purification was evaluated using different strategies, namely the enzyme recovery by PEG-salt aqueous two-phase systems (ATPSs); and the enzyme precipitation with ammonium sulfate, acetone, and ethanol. The best purification factor (18 ± 2) was obtained when the ATPS composed of 20% (w/w) polyethylene glycol (PEG) 6000 and 5.8% (w/w) potassium phosphate buffer (PPB) pH 8.0 was combined with acetone precipitation. The partially purified enzyme presented an optimum pH of 5.0, although it remained active in the pH range of 4.5 to 7.5 ($\geq 50\%$ relative activity). The optimum temperature was found to be 60 °C. Furthermore, the addition of salts such as FeCl₃, CuSO₄ and MnCl₂ promoted an increase in the enzymatic activity (above 100%). The enzyme was found to be stable and showed high activity when exposed to polar solvents such as dimethyl sulfoxide, dimethylformamide, and methanol. The use of an integrated strategy of purification combining simple purification methods such as ATPS and precipitation was herein reported for the first time for esterase. This strategy proved to be an interesting approach to partially purify the esterase produced under submerged fermentation by *A. pullulans*. Furthermore, the enzyme showed potential to be applied in industrial biocatalytic processes using high temperature and different salts or solvents. Also, the production of esterase using olive mill wastewater as substrate demonstrated to be a suitable alternative to reduce and valorize agro-industrial residues.

1. Introduction

The continuous development of bio-sustainable and renewable resource-based technologies is extremely important to reduce the environmental impact of some industries. In this sense, there is a growing interest in developing processes that enable the full use of industrial wastes and residues towards a circular economy [1]. Examples include several agro-food industrial residues such as cheese whey [2], wastes from canning factories [3], potato peel [4], corn steep liquor or sugarcane molasses [5]. Olive mill wastewater, a by-product obtained from the olive oil industry with annual production of approximately 20 million m³, can also be used to produce several bioproducts with commercial interest, such as enzymes [6]. The use of by-products is an interesting strategy not only to reduce the impact of waste management but also to add value to residues that otherwise would be disposed into

the environment.

Enzymes have gained interest to the industry mainly due to their widespread uses under mild conditions. The global market for industrial enzymes was approximately USD 4.2 billion in 2014, and it is expected to reach USD 6.2 billion in 2020 [7]. Esterases (EC 3.1.1.1) are useful enzymes which can be involved in several biocatalytic industrial processes [8]. These enzymes catalyze the cleavage and formation of ester bonds of an extensive range of substrates, preferentially short-chain fatty acids, with no more than 10 carbons [9]. Their broad spectrum of industrial applications includes food and dairy products, ester production, detergents, pharmaceuticals, synthesis of optically pure compounds, degradation of pollutants and production of perfumes [10].

Microbial esterases present several advantages over their counterparts from plant and animal origin since they can properly adjust to several environmental conditions, presenting outstanding

Abbreviations: (ATPS), aqueous two-phase system; (PEG), polyethylene glycol; (DMSO), dimethyl sulfoxide; (DMF), dimethylformamide; (*p*-NPB), *p*-nitrophenyl butyrate; (OMW), olive mill wastewater; (CE), crude extract; (PPB), potassium phosphate buffer; (U), unit of enzyme activity; (PF), purification factor; (R), enzyme recovery; (ASP), Ammonium sulfate precipitation; (AP), Acetone precipitation

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bioconversions at various pH and temperature levels [11].

The application of enzymes in industrial processes can offer recognized advantages over some conventional mechanical and chemical approaches. For example, the use of esterases in the pharmaceutical [8], and the pulp and paper [12], industries lead to significant energy and chemicals saving, thus reducing the environmental impact of those processes. Due to the increasing industrial demand for esterases, the discovery and characterization of novel enzymes with improved performances is of utmost interest. Depending on the microorganism and the purification techniques, yields and enzyme properties can widely vary. Therefore, enzyme characterization is essential to evaluate its potential and applicability in innovative biotechnological processes [13,14].

Enzyme purification generally requires a large number of steps to obtain products with the desired specifications. The design of a purification strategy comprises a series of separation steps, which should lead to a product with a high purification and recovery factor [15]. Overall, the costs related to enzyme recovery and purification can represent up to 80% of the total costs of the process [16].

Several procedures and techniques to purify esterases have been reported [17–19]. However, the majority of the studies use and evaluate a single sequence of purification aiming only at enzymatic characterization and do not consider the peculiarities of those techniques which can generally compromise the efficiency of the recovery process and difficult the scale-up procedure [20,21]. Furthermore, it is necessary to take into account that esterases from different sources can result in different enzymatic extracts with specific compositions (concentration and type of proteins) and contaminants. Hence, it is necessary to develop suitable procedures for each source of the desired enzyme.

This work aimed to partially purify and characterize an esterase produced by *Aureobasidium pullulans* LABIOTEC 01 when cultivated under submerged fermentation using a culture medium containing olive mill wastewater.

2. Materials and methods

The production, partial purification and characterization of esterase from *A. pullulans* LABIOTEC 01 was performed according to the schematic diagram presented in Fig. 1. This figure summarizes the best integrated strategy herein found for esterase purification corresponding

to the design 2: ATPS + acetone precipitation (Section 2.5).

2.1. Materials

Polyethylene glycol (PEG) with molecular weight 4000 (lot number BCBG9026V), 6000 (lot number BCBG3642V) and 8000 (lot number BCBG6496V) Da, and the substrate *p*-nitrophenyl butyrate (*p*-NPB) were purchased from Sigma-Aldrich (Sintra, Portugal). Bradford reagent from Alfa Aesar was supplied by Enzymatic (Santo Antão do Tojal, Portugal). Olive mill wastewater (OMW) was kindly provided by an olive oil mill located in the north of Portugal, which uses a continuous three-phase centrifugation process for olive oil extraction. The residue was stored at 4 °C until use.

2.2. Microorganism

The microorganism *A. pullulans* LABIOTEC 01 was isolated from sugar cane straw and was identified by molecular biology technique. The strain is stocked lyophilized at the Laboratory of Biotechnology, Federal University of Ceará (UFC, Fortaleza, Brazil). After activation the strain was kept at 4 °C in YPD agar containing (in g/L): yeast extract (4.0), peptone (10.0), glucose (20.0) and agar (20.0).

2.3. Inoculum and submerged cultivation

The inoculum was prepared in 250 mL Erlenmeyer flasks with 90 mL culture medium containing (g/L): yeast extract (4.0), peptone (10.0) and glucose (20.0). The pH was adjusted to 5.0 and the sterilization of the medium was performed at 121 °C for 15 min. The microorganism *A. pullulans* LABIOTEC 01 was inoculated and the culture medium was incubated in an orbital shaker (200 rpm) for 24 h at 30 °C. After this period, a homogeneous suspension with an optical density of 0.1 ($\lambda = 620$ nm) was obtained and it was further transferred to the culture medium used for enzyme production.

Esterase production under submerged cultivation was performed in 500 mL Erlenmeyer flask containing 270 mL of medium composed of (g/L): yeast extract (4.0), Tween 80 (4.0), glucose (1.0) and OMW (20.0). The initial pH of the medium was adjusted to 5.5. The culture was incubated in an orbital shaker at 28 °C and 200 rpm.

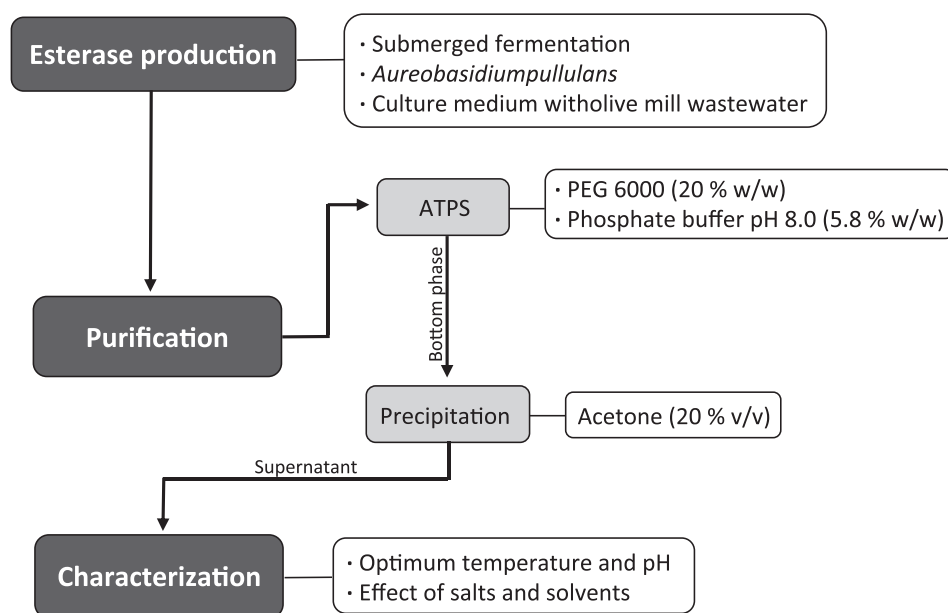


Fig. 1. Schematic diagram of the process used to obtain a partially purified esterase from *Aureobasidium pullulans* LABIOTEC 01 using the design 2 as integrated strategy of purification.

Table 1

Purification factor (PF), esterase recovery (R%) and volume ratio (Vr) for several PEG-potassium phosphate buffer ATPSs with or without NaCl addition.

ATPS	Composition (% w/w)			Esterase purification					
	PEG	PPB ¹	NaCl	Vr	Phase	Activity (U/mL)	Protein (mg/mL)	PF	R%
PEG 4000 + PPB ¹ pH 7.0 ²	14	15	–	0.8 ± 0.0	top	2.5 ± 0.1	5.0 ± 0.2	1.2 ± 0.1	39 ± 1
					bottom	ND ³	–	ND ³	ND ²
PEG 6000 + PPB ¹ pH 8.0 ⁴	13.3	10	3	1.13 ± 0.1	top	6.9 ± 0.2	8.2 ± 0.15	1.4 ± 0.1	73 ± 3
					bottom	7.4 ± 0.16	15.1 ± 0.7	0.8 ± 0.1	89 ± 3
	13.3	10	–	0.82 ± 0.1	top	ND ³	–	ND ²	ND ²
					bottom	15.1 ± 0.4	25.2 ± 0.5	1.6 ± 0.1	148 ± 5
	16	8	4.8	0.74 ± 0.1	top	2.5 ± 0.1	5.1 ± 0.2	0.8 ± 0.1	32 ± 2
					bottom	10.0 ± 0.5	15.4 ± 0.7	1.1 ± 0.1	96 ± 4
	16	8	1.2	0.65 ± 0.1	top	6.6 ± 0.3	5.7 ± 0.4	1.9 ± 0.2	91 ± 3
					bottom	12.8 ± 0.7	16.1 ± 0.9	1.3 ± 0.2	116 ± 4
	16	8	–	0.39 ± 0.0	top	ND ³	–	ND ²	ND ²
					bottom	18.2 ± 1.1	29.7 ± 1.7	1.6 ± 0.2	116 ± 4
	20	5.8	4.8	0.4 ± 0.1	top	4.4 ± 0.4	4.7 ± 0.3	1.5 ± 0.2	71 ± 3
					bottom	11.7 ± 0.35	17.5 ± 0.9	1.09 ± 0.01	81 ± 1
	20	5.8	–	0.13 ± 0.0	top	ND ³	–	ND ²	ND ²
					bottom	83.0 ± 0.8	45.6 ± 2.7	4.7 ± 0.7	203 ± 5
24	12	1.2	0.7 ± 0.0	top	6.6 ± 0.4	5.7 ± 0.2	1.1 ± 0.1	91 ± 3	
				bottom	10.7 ± 0.45	25.6 ± 1.5	0.7 ± 0.03	97 ± 5	
24	12	–	0.57 ± 0.1	top	ND ³	–	ND ²	ND ²	
				bottom	12.3 ± 1.1	30.6 ± 2.2	1.1 ± 0.1	75 ± 3	
PEG 8000 + PPB ¹ pH 7.0 ⁵	1.98	2.3	–	0.2 ± 0.0	top	3.8 ± 0.1	15.8 ± 0.6	0.8 ± 0.1	47 ± 3
					bottom	ND ³	–	ND ²	ND ²

¹ PPB – Potassium phosphate buffer.² ATPS composition obtained from Ref. [14].³ ND – Esterase activity not detected.⁴ ATPS composition obtained from reference [23].⁵ ATPS composition obtained from reference [22].

2.4. Partial purification of esterase

The cell free supernatant obtained at 96 h of cultivation (specific activity 0.35 ± 0.02 U/mg) was divided in aliquots, stored at 4 °C and used as crude extract (CE) in the subsequent purification steps. Before any purification step, the initial enzyme content and activity is determined.

2.4.1. Aqueous Two-Phase Systems (ATPSs)

The ATPSs and the corresponding compositions herein studied (Table 1) were defined according to the literature. These biphasic systems were previously reported for the purification of different enzymes [14,22,23]. The ATPSs (total mass 10 g) were prepared in graduated centrifuge tubes (Eppendorf 5810R) by mixing suitable amounts of PEG (4000, 6000 or 8000 Da, in powder form), a stock solution of potassium phosphate buffer (PPB, pH 7.0 or 8.0, 40% (w/w)) and deionized water. Sodium chloride was also added to some ATPSs to obtain the final compositions presented in Table 1. The tubes containing the ATPS constituents were vortex mixed and maintained at 4 °C. The CE was added at a final concentration of 20% (w/w). Additionally, for each PEG-PPB ATPSs a blank/control was prepared with the same composition but without CE. The biphasic systems were thoroughly mixed and centrifuged for 10 min at 4700g and 4 °C to accelerate phase separation and to prevent enzyme denaturation. Afterward, top and bottom phases were carefully separated. Both phases were assayed for esterase activity and total protein concentration (Section 2.9).

2.4.2. Precipitation with ammonium sulfate, acetone and ethanol

The CE was treated with three precipitating agents, namely ammonium sulfate, acetone or ethanol. Different concentrations were added to the enzymatic extract (20%, 40%, 60% and 80% w/v or v/v, depending on the precipitating agent) and the mixture was left to stand at 4 °C overnight. After precipitation, the sample was centrifuged at 4700g, 4 °C, for 10 min. The precipitate resulting from salt precipitation was resuspended in PPB (50 mM) pH 7.0 and further dialyzed against

this same buffer. The supernatant was also dialyzed. Both precipitate and supernatant obtained from solvent precipitation were treated with a nitrogen flow to enable the total solvent evaporation. The esterase activity and total protein concentration were determined after complete dialysis and solvent evaporation (Section 2.9).

2.5. Design of an integrated purification strategy

The ATPS composed of 20% (w/w) PEG 6000 and 5.8% (w/w) PPB pH 8.0 (Section 2.4.1) was selected and further combined with the best conditions obtained for the precipitation with acetone (AP) and ammonium sulfate (ASP) (20% (v/v) and 80% (w/v), respectively, Section 2.4.2). Therefore, two different integrated purification strategies were evaluated: design 1 (ATPS + ammonium sulfate precipitation) and design 2 (ATPS + acetone precipitation). In both cases, the bottom phase of the biphasic system was collected, dialyzed and further treated with the precipitating agent. The esterase activity and total protein concentration (Section 2.9) were determined for both supernatant and precipitate (after resuspension in a suitable buffer, Section 2.4.2)

2.6. Performance of the purification strategies

The purification strategies were evaluated considering the purification factor, enzymatic activity recovery and volume ratio. The purification factor (PF) [24] was calculated as the ratio between the specific activity (U/mg) obtained after the purification step and the specific activity (U/mg) in the CE or equilibrium phase of the purification step. The specific activity is defined as the ratio between the activity (U/mL) and the total protein concentration (mg/mL). The recovery of the process (R%) was defined as the ratio between the total enzyme activity (U) obtained after the purification step and the total activity (U) fed to the system [25]. The global PF and global R were determined by the ratio between the final and initial specific activity and enzymatic activity, respectively, considering the loss of volume and enzymatic activity in all steps of the purification process. The volume

ratio (Vr) was calculated as the ratio between the volume (mL) of the top phase and the volume (mL) of the bottom phase [26]. The enzymatic activity and total protein concentration was always determined for each aliquot of CE prior to its use in the purification steps and the values obtained were used to calculate the PF and R%.

2.7. Characterization of the partially purified esterase

Several characterization assays were performed for the partially purified enzyme from design 2 using 20% (w/w) PEG 6000 – 5.8% (w/w) PPB pH 8.0 ATPS (bottom phase) and acetone as precipitating agent. The esterase was obtained from the supernatant of the precipitation process after solvent evaporation.

2.7.1. Optimum temperature and pH for esterase activity

The optimum pH was determined at 37 °C using *p*-NPB as substrate (Section 2.9) and 0.1 M Tris–HCl buffer at pH values ranging from 4.5 to 8.5. For the optimum temperature, the enzymatic assay was performed at pH 7.0, varying the temperature from 40 °C up to 80 °C. Esterase activity was expressed as relative enzymatic activity (%), considering that 100% corresponds to the highest activity value obtained for each condition.

2.7.2. Effects of salts and solvents on esterase activity

The effect of different concentrations (1, 5 and 10 mM) of salts (CaCl₂, MgCl₂, NaCl, FeCl₃, KCl, CuSO₄, ZnSO₄, and MnCl₂) on esterase activity was assessed. The partially purified enzyme was pre-incubated with the particular salt solution at 25 °C for 1 h and the enzymatic activity was determined afterward [27] (Section 2.9). A pre-incubation temperature of 25 °C was used as it was considered suitable to preserve esterase stability [28–30]. Additionally, the effect of different solvents (dimethyl sulfoxide (DMSO), dimethylformamide (DMF), methanol, ethanol, n-propanol, butanol, toluene, xylene, n-hexane and isooctane) was also studied. The partially purified enzyme was diluted in the organic solvents at a concentration of 50% (v/v) in the reaction mixtures and further pre-incubated for 1 h at 25 °C [28–30]. After incubation, the enzymatic activity was determined (Section 2.9). A control (without salts or solvents) was incubated under the same conditions and the enzymatic activity achieved was considered as 100%.

2.8. Electrophoresis

The CE (Section 2.4) and the partially purified esterase obtained from design 2 (Section 2.7) were analyzed by electrophoresis. The electrophoresis was performed according to the method reported by Laemmli [31] in a polyacrylamide gel (4% stacking, 12% separating, 0.75 mm thickness) containing sodium dodecyl sulfate. The samples were mixed in a ratio (1:4) with sample buffer containing β-mercaptoethanol, heated at 95 °C for 5 min and applied on the gel. Electrophoresis was performed at 120 V for 100 min (Bio-Rad), and the gel was revealed using the silver nitrate staining [32,33]. A color protein marker II (MB09002, Nzytech) with molecular weight ranging from 11 to 245 kDa was used.

2.9. Enzymatic activity and total protein quantification

The enzymatic activity was determined using *p*-NPB as substrate according to the method described by Gomes, Gonçalves, Garcia-Roman, Teixeira and Belo [34] with some modifications. Briefly, the substrate (*p*-NPB (1.12 mM) in PPB pH 7.0 (50 mM) containing 4% (v/v) of Triton X-100) was incubated with the enzymatic extract at 37 °C during 15 min. Afterwards, the absorbance of the mixture was measured at 410 nm. An extinction molar coefficient of 0.4143 mM⁻¹ was experimentally determined under the assay conditions and was used to calculate the esterase activity. One unit of enzyme activity (U) was defined as the amount of enzyme necessary to yield 1 μmol of *p*-

nitrophenol per min at 37 °C and pH 7.0. Total protein content was determined by the Bradford [35] method using a standard curve prepared with bovine serum albumin (concentration range 5–50 μg/mL, R² = 0.9977). Possible interferences from phase-forming components of the biphasic systems in the Bradford method were minimized using suitable blanks/controls ATPSs, containing the same composition but without the CE. The equilibrium phases of the blank/controls were separated, diluted using the same proportion as the equilibrium phases of the ATPSs containing the CE and were further analyzed for total protein quantification. For the enzymatic assays, the blanks/controls were prepared using the equilibrium phases of the ATPSs containing the CE after thermal denaturation.

2.10. Statistical analysis

Data were subjected to analysis of variance (ANOVA) to detect significant differences among treatments, whereas means were compared by the Tukey's test. Differences were considered significant when *p* < 0.05. All experiments were performed in triplicate.

3. Results and discussion

3.1. Esterase production

The CE used in the purification steps was obtained from submerged cultivation of *A. pullulans* LABIOTEC 01 in a culture medium containing OMW to induce the enzyme production. The maximum esterase activity was found at 120 h of cultivation, reaching values higher than 16.6 U/mL and remaining approximately constant until the end of the fermentation (Fig. 2). However, maximum productivity (the relationship between enzymatic activity and growing time) for esterase from *A. pullulans* was obtained at 96 h (0.15 ± 0.01 U/mL h⁻¹). Afterward, the productivity significantly decreased mainly due to the stabilization of the enzyme production (Fig. 2). For this reason, the CE obtained at 96 h (14.6 ± 0.5 U/mL) was used in subsequent purification steps.

The values of esterase activity obtained in the current study are comparable to those reported in the literature for strains of *A. pullulans* isolated from African tropics (Zimbabwe) and cultivated under submerged fermentation in a culture medium containing olive oil and Tween 80 [36]. However, it is important to highlight that different experimental conditions were used by Kudanga, Mwenje, Mandivenga and Read [36] in the enzymatic assays, namely the substrate (*p*-nitrophenol acetate) and the temperature (30 °C).

3.2. Partial purification of esterase

The esterase was partially purified using different PEG-PPB ATPSs

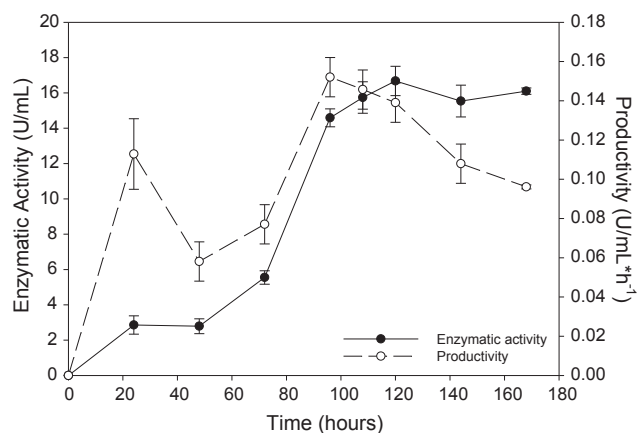


Fig. 2. Esterase activity and productivity obtained for *Aureobasidium pullulans* LABIOTEC 01 using a culture medium containing olive mill wastewater.

described in the literature [14,22,23] (Table 1). PEG-phosphate ATPSs have already shown potential for the recovery of esterase from plant origin [37] and other carboxyl ester hydrolases, such as lipases [22,23]. Furthermore, this type of ATPSs is cheaper (compared with polymer-polymer ATPSs or ATPSs composed of ionic liquids) and suitable to implement the integrated strategy of purification herein proposed. For these reasons, we selected PEG-PPB ATPSs containing PEGs with different molecular weights and potassium phosphate buffer with different pH values. The purification factor and recovery yield found for each biphasic system composed of PEG with different molecular weights and PPB with different pH are presented in Table 1.

For the PEG (4000 or 8000)-PPB pH 7.0 ATPSs only one composition (without the addition of NaCl) was evaluated. It was found that esterase was preferably partitioned to the top phase and no esterase activity was detected in the bottom phase. Similar PF (around 1) and recovery yield (39–47%) were obtained in the top phases of these ATPSs. On the contrary, for the different compositions of PEG 6000-PPB pH 8.0 ATPSs (without the addition of NaCl) evaluated, the esterase was preferably partitioned to the bottom phase, and no esterase activity was determined in the top phase. The addition of NaCl to these ATPSs led, in all cases, to the partitioning of an active form of esterase to both equilibrium phases. It is well known that the presence of additives such as NaCl can alter the protein/enzyme partition in ATPSs by influencing the phase composition of the biphasic systems, the protein hydrophobicity and also the phase potential [38]. The migration of esterase from one equilibrium phase to another can be a consequence of possible electrochemical interactions between the enzyme and the dissociated ions from the additives [39]. For the biphasic system composed of PEG 6000 (16% w/w) and PPB pH 8.0 (8% w/w), the effect of different NaCl concentrations (1.2 and 4.8% w/w) was also studied. It was observed that the increase of NaCl concentration from 1.2 to 4.8% (w/w) favored neither the purification nor the recovery of the esterase to one specific equilibrium phase.

The best results for esterase purification were obtained for the ATPS composed of PEG 6000 (20% w/w) and PPB pH 8.0 (5.8% w/w) with no addition of NaCl, presenting a recovery of 203% and a 4.7-fold purification in the bottom phase. The values of R (%) higher than 100% in the bottom phase (Table 1) can be explained by the removal of potential inhibitors of the enzymatic activity to the top phase and/or by the beneficial presence of salt and PEG, which may help to keep the protein in its active form [40]. This behavior has frequently been reported by other authors who used ATPS to purify enzymes [14,25,40]. Additionally, this fact also strengthens the potential and suitability of ATPSs for the recovery and purification of active forms of enzymes.

Precipitation with ammonium sulfate was also evaluated for the esterase purification from CE. The enzymatic fractionation with ammonium sulfate is based on the increase of the ionic strength which subsequently leads to protein aggregation and precipitation. This is a simple, fast and low cost method widely used for protein purification [41]. Generally, the increase of the ammonium sulfate saturation results in a higher amount of precipitated proteins. It is expected that proteins with few hydrophilic regions precipitate at lower saturation levels, while proteins with more hydrophilic regions are only precipitated at higher saturation levels [42]. The results obtained for each concentration of ammonium sulfate are presented in Table 2. It is important to highlight that the results present in Table 2 correspond to the data obtained for the precipitate since no esterase activity was detected in the supernatant.

A significant improvement ($p < 0.05$) on both the purification factor and esterase recovery was observed when increasing the salt concentration. Saturation with 80% (w/v) of ammonium sulfate provided the best results, which may suggest the presence of hydrophilic regions facing outwards in the esterase structure [42]. The PF was improved from 0.83 (20% w/v ammonium sulfate) to 4.89 (80% w/v ammonium sulfate) while the enzyme recovery increased from 66% (using 20% w/v ammonium sulfate) to 100.0% (using 80% w/v

ammonium sulfate).

The precipitation of crude esterase with different concentrations of chilled ethanol and acetone was also studied, and the results are also presented in Table 2. A concentration of 20% (v/v) of acetone was found to be the best precipitation condition for esterase purification, resulting in a recovery yield of 36% and a 7.1-fold enhancement in the specific activity. In both cases, the increase of solvent concentration to 60% (v/v) led to a higher enzymatic activity recovery, but lower purification factors were achieved. This fact may be explained by the non-selective precipitation of other proteins present in the CE [43,44].

An integrated process can be a suitable alternative to improve the recovery and purification of enzymes since the number of contaminants can be greatly reduced by combining different methodologies [45,46]. Therefore, two design strategies (design 1 and design 2) were herein evaluated for esterase purification. Both strategies include the enzyme recovery through PEG 6000-PPB pH 8.0 ATPS and precipitation. The results obtained for each design are presented in Table 3.

In design 1, the bottom phase of the biphasic system composed of PEG 6000 (20%) and phosphate buffer pH 8.0 (5.8%) was treated with ammonium sulfate (80% w/v). Under these conditions, the precipitate showed a PF value of around 3.1 with $191\% \pm 3$ recovery of enzymatic activity. However, for the design 2 (bottom phase treated with 20% (v/v) acetone), the best results were found in the supernatant fraction, with a purification factor of 7 ± 1 and an enzymatic activity recovery of $35\% \pm 1$.

Overall, the design 2 proved to be the best integrated process to obtain the partially purified esterase, resulting in a global purification factor and enzymatic activity recovery of 18 ± 2 and $90\% \pm 3$, respectively. For design 1, a lower global purification factor (around 8.1) was achieved in the precipitated fraction. However, a higher global activity recovery ($480\% \pm 8$) was obtained. Since the strategy of purification adopted for a specific enzyme is generally dependent on the type of application envisaged, the design 1 can be a suitable approach when esterase application does not need extreme purity, such as in for instance pollutant degradation [47].

To the best of our knowledge, no previous reports have been published on the design of an integrated purification strategy for esterase from *A. pullulans* comprising ATPS and precipitation. Previous studies on the purification of this enzyme are mainly focused on highly selective techniques with the final purpose of enzyme characterization instead of enzyme recovery and industrial application [17–19]. ATPSs are regarded as a suitable approach to recover and concentrate biomolecules in a single extraction step [48]. Furthermore, these biphasic systems can be successfully used in combination with other separation techniques, generally allowing the scale up of the process with excellent reproducibility [48,49]. The few studies reported on esterase purification by ATPS are focused on esterases from plant origin. Jiang, Feng, Liu, Xu, Li and Ji [50] explored the purification of wheat-esterase using ionic liquids-based ATPSs. Under optimum conditions (20% (w/w) of 1-Butyl-3-methylimidazolium tetrafluoroborate and 25% (w/w) of NaH_2PO_4 pH 4.8), the enzyme was obtained with a purification factor of 4.2 and a recovery yield of 89%. Despite the comparable yield, the purification factor is significantly lower than that found in our design 2 (18 ± 2). A different study, described the purification of a plant-esterase by an integrated process involving two successive ATPSs [37]. The first ATPS composed of PEG 1000 (27% w/w) and NaH_2PO_4 pH 5.0 (13% w/w) led to the partitioning of esterase preferably to the top phase (PF = 5.3, R = 85%). Therefore, bottom phase was discarded and NaSO_4 was added to form a new ATPS composed of PEG 1000 (27% w/w) and NaSO_4 pH 5.0 (6% (w/w)). In this case, the esterase partitioned preferably to the bottom phase with a global PF and recovery of 18.5 and 83%, respectively. This approach provided similar PF and a slight lower activity recovery than those obtained from our design 2. However, it is important to consider that a two-step ATPS can represent higher costs and environmental impact when scaling up the process. The discarded bottom (first ATPS) and top (second ATPS) phases will

Table 2

Purification factor (PF) and enzyme recovery (R%) obtained in the precipitate using different precipitating agents (ammonium sulfate, acetone, and ethanol). Results are expressed as mean \pm standard deviation of triplicate independent assays. Values presenting the same letter in a column are not significantly different by Tukey's test ($p < 0.05$).

Precipitating Agent	Concentration % ¹	Activity (U/mL)	Protein (mg/mL)	PF	R%
Ammonium sulfate	20	44.5 \pm 1.4	27.4 \pm 0.9	0.83 \pm 0.15 ^d	66 \pm 2 ^c
	40	52.3 \pm 0.8	13.6 \pm 0.7	1.96 \pm 0.20 ^c	78 \pm 3 ^b
	60	62.7 \pm 1.8	11.4 \pm 0.7	2.79 \pm 0.19 ^b	93 \pm 3 ^a
	80	76.7 \pm 1.1	7.0 \pm 0.3	4.89 \pm 0.21 ^a	100 \pm 2 ^a
Acetone	20	8.6 \pm 0.4	1.5 \pm 0.1	7.1 \pm 0.7 ^a	36 \pm 2 ^c
	40	6.8 \pm 0.2	3.8 \pm 0.3	2.4 \pm 0.4 ^b	29 \pm 1 ^d
	60	21.7 \pm 0.0	18.8 \pm 0.5	1.4 \pm 0.1 ^{bc}	91 \pm 1 ^a
	80	25.8 \pm 0.13	27.9 \pm 1.1	1.2 \pm 0.1 ^c	87 \pm 1 ^b
Ethanol	20	4.8 \pm 0.2	3.0 \pm 0.2	2.1 \pm 0.4 ^{ab}	20 \pm 1 ^d
	40	6.9 \pm 0.3	3.6 \pm 0.5	2.5 \pm 0.2 ^a	29 \pm 1 ^c
	60	17.1 \pm 0.3	12.5 \pm 0.4	1.8 \pm 0.03 ^{bc}	100 \pm 2 ^a
	80	22.73 \pm 1.1	25.2 \pm 0.4	1.2 \pm 0.1 ^c	76 \pm 4 ^b

¹ (w/v) for ammonium sulfate and (v/v) for acetone and ethanol.

Table 3

Purification factor (PF) and enzyme recovery (R%) obtained using design 1 (PEG6000-PPB pH 8.0 ATPS + ammonium sulfate (80% w/v) precipitation) and design 2 (PEG6000-PPB pH 8.0 ATPS + acetone (20% v/v) precipitation). Results are expressed as mean \pm standard deviation of triplicate independent assays.

Design	Phase	Activity (U/mL)	Protein (mg/mL)	PF	R (%)	Global PF	Global R (%)
1 PEG 6000-PPB pH 8.0 ATPS (bottom phase) + ASP ¹	Precipitate	63.4 \pm 1.0	22.2 \pm 1.4	3.1 \pm 0.2	191 \pm 3	8.1 \pm 0.5	480 \pm 8
	Supernatant	8.98 \pm 0.0	7.54 \pm 0.4	1.28 \pm 0.08	26 \pm 1	3.4 \pm 0.2	69 \pm 2
2 PEG 6000-PPB pH 8.0 ATPS (bottom phase) + AP ²	Precipitate	6.5 \pm 0.4	27.3 \pm 0.2	0.25 \pm 0.02	20 \pm 1	0.67 \pm 0.04	50 \pm 3
	Supernatant	11.7 \pm 0.4	1.82 \pm 0.1	7 \pm 1	35 \pm 1	18 \pm 2	90 \pm 3

¹ ASP – Ammonium sulfate precipitation.

² AP – Acetone precipitation.

become part of the process effluents, demanding for a suitable treatment and/or a safe release. The strategy herein used (design 2) generates a lower amount of effluents (only top phase) and allows the easy recycling of the precipitating agent. Using a simple vacuum evaporation, acetone can be recovered and further reused, reducing the environmental impact associated with the release of effluents containing undesirable products [51]. Additionally, acetone may also act as a desalting agent [52]. Therefore, the addition of this solvent to the bottom phase can also contribute to remove some phase-forming salts by co-precipitation [53].

An electrophoresis analysis was performed to evaluate the efficiency of the integrated strategy of purification (Fig. 3). Using this purification strategy, it was possible to eliminate some contaminant proteins present in the CE, while other proteins were concentrated. A common band around 32 kDa, which became reduced in the partially purified extract is clearly visible in the electrophoresis. In addition, the band around 100 kDa in the CE seems to disappear (vestigial) in the partially purified extract. On the other hand, a band around 46 kDa became more evident after the partial purification. The previous works reporting the production of esterases by *A. pullulans* are mainly focused on specific enzymatic activities such as feruloyl esterase (molecular weight of 210 kDa) [54], acetyl esterase (molecular weight not reported) [55] or acetyl xylan esterase (molecular weight not reported) [56]. Nevertheless, esterases from yeast-like organisms (including other *Aureobasidium* sp.) showing predominant enzymatic activity towards butyrate-based substrates, generally present a molecular weight between 40 and 60 kDa [57–59]. In Fig. 3, the common bands observed in this range of molecular weights may correspond to the esterase.

3.3. Esterase characterization

The partially purified esterase obtained from design 2 was characterized regarding its optimum pH and temperature, as well as the effect of different salts and solvents.

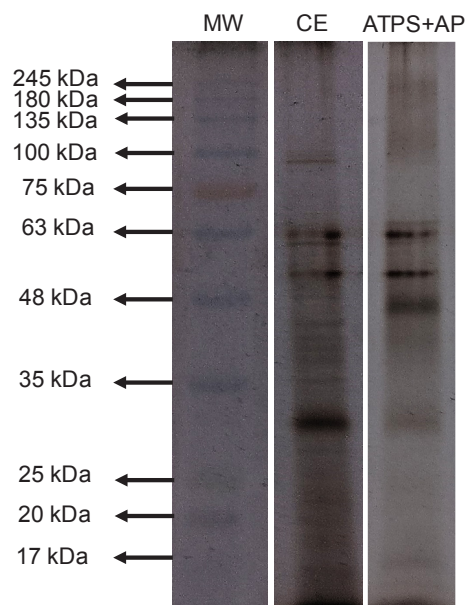


Fig. 3. Electrophoresis analysis of crude extract and partially purified esterase produced by *Aureobasidium pullulans*. MW - Molecular weight marker; CE - crude extract; ATPS + AP - PEG 6000 (20% w/w)-phosphate buffer pH 8.0 (5.8% w/w) ATPS (bottom phase) followed by 20% (V/V) acetone precipitation (supernatant).

3.3.1. Optimum pH and temperature

The enzymatic activity of esterase from *A. pullulans* LABIOTEC 01 was studied for pH values between 4.5 and 8.5. The maximum activity was obtained at pH 5.0 (Fig. 4A). The enzyme remained active in the pH range from 4.5 to 7.5 ($\geq 50\%$ relative activity), but for pH values higher than 7.5 the enzymatic activity significantly decreased. The

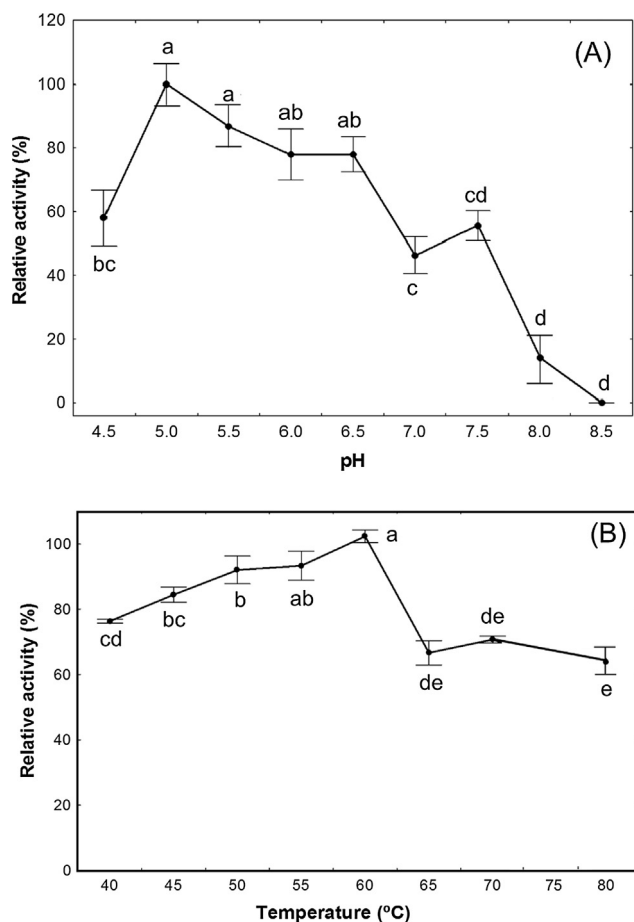


Fig. 4. Optimum pH (A) determined at 37 °C and optimum temperature (B) of the partially purified esterase enzyme performed at pH 7.0. The statistical analysis was conducted by the Tukey's test. The same letters represent no significant differences at the 5% level.

effect of temperature on the enzymatic activity was evaluated in the range 40–80 °C. The enzyme presented a relative activity $\geq 50\%$ in all the temperature range. However, the maximum activity was observed at 60 °C (Fig. 4B). It was possible to observe the increase of the esterase activity up to the maximum, followed by a decrease at higher temperatures (Fig. 4B), thus suggesting some enzyme denaturation. The temperature increase generally causes an increase in the reaction rate, since heat increases the proportion of groups that react and reach the transition state at a given time. However, when the temperature rises above a given point, the heat results in enzyme deactivation. Since most proteins are only marginally stable, denaturation occurs, resulting in a sharp drop in the enzymatic activity [60].

Generally, esterases from yeasts (including *Aureobasidium* spp.) present optimal pH and temperature in the range 7.0–8.0 and 35–60 °C, respectively [36,57,59,61]. Thus, the optimal temperature found for esterase from *A. pullulans* is in agreement with the literature. Nevertheless, the esterase showed a significant decrease in the enzymatic activity for pH > 7.5. The optimal pH found for this esterase (pH 5.0) is closer to the optimal pH reported for some esterases from plant origin and suggests its potential use in more acidic environments [62].

3.3.2. Effect of salts

The influence of different concentrations of NaCl, KCl, CaCl₂, MgCl₂, MnCl₂, ZnSO₄, CuSO₄ and FeCl₃ on the esterase activity was also evaluated by comparison with the control (without the addition of salts). The results obtained are presented in Fig. 5.

The esterase activity was found to be slightly increased ($p < 0.05$)

in the presence of FeCl₃ (1–5 mM), CuSO₄ (5–10 mM) and MnCl₂ (1–10 mM) while it was inhibited by CaCl₂ (83% inhibition for 1 mM), MgCl₂ (55% inhibition for 1 mM), NaCl (26% inhibition for 1–5 mM), KCl (38% inhibition for 10 mM) and ZnSO₄ (35% inhibition for 1–5 mM). These latest salts interfere in the enzymatic catalysis, slowing the enzymatic reaction. On the other hand, increased molarity generally led to a significant rise in the enzymatic activity (Fig. 5). The increase in the enzymatic activity can be due to the ability of some ions to stabilize the binding of the enzyme-substrate complex, maintaining the active conformation of the enzyme and protecting it against thermal denaturation [63,64]. For example, esterase from the fungus *Thermomyces lanuginosus* was notably enhanced either by Ca²⁺ or Ba²⁺ and negatively affected by Fe³⁺ [65]. On the other hand, esterase from the marine fungus *Fusarium* sp. was particularly activated by Mg²⁺ and strongly inhibited by Cr³⁺, Cu²⁺ and Hg²⁺ [21]. Regarding the esterase from *Aureobasidium melanogenum* only Li⁺ presented a positive effect on the enzymatic activity [59].

The study of the esterase behavior in the presence of salts is of utmost relevance since the lack of knowledge of salt interference can compromise the effective application of the enzyme in industrial processes. Additionally, some of these salts may be found in biotechnological processes or even in the raw materials where the enzyme can be used as catalyst.

3.3.3. Effect of solvents

The effect of different organic solvents such as DMSO, DMF, methanol, ethanol, n-propanol, butanol, toluene, xylene, n-hexane, and isooctane on the partially purified esterase was evaluated by comparison with the control (without solvent addition). The results obtained for each solvent are presented in Table 4 together with the log *P*, which is generally used as quantitative measure of the solvent polarity.

High enzymatic stability and activity in organic solvents are essential features for enzyme application in organic synthesis [66]. Additionally, in some cases, the presence of organic solvents can increase the solubility of compounds, favorably shifting the thermodynamic equilibria and eliminating potential microbial contaminations in reaction mixtures [67].

Although different esterases can show different tolerance towards organic solvents, there is a general tendency to be more inactivated in the presence of hydrophilic solvents than in the presence of hydrophobic ones [68]. The esterase deactivation in water miscible organic solvents may be due to the stripping-off of crucial bound-water monolayer from the enzyme which is essential to preserve its conformation and activity [69]. On the other hand, the inactivation in non-polar solvents may be a consequence of solvent interaction with the hydrophobic amino acid residues present near the catalytic site of the enzyme. This interaction may result in a detrimental modification of the esterase conformation and thus can compromise its catalytic action [70].

According to Table 4, esterase showed stability in almost all the organic solvents. Furthermore, the enzyme was activated in the presence of polar solvents such as DMSO, DMF, and methanol. However, the exposure to non-polar hydrophobic solvents such as xylene and isooctane showed a reduction in the relative activity of 31% and 27%, respectively.

Tolerance to more polar solvents is considered a desired property for esterases involved in some asymmetric synthesis since these water miscible solvents can act as a homogeneous co-solvent and enhance the solubility of both the organic substrates and products [61,71]. Therefore, an esterase which preserves or improves its activity in polar solvents assumes special relevance in this context.

4. Conclusions

A sustainable approach, using a culture medium containing OMW, was used to produce esterase from *A. pullulans*, providing an interesting

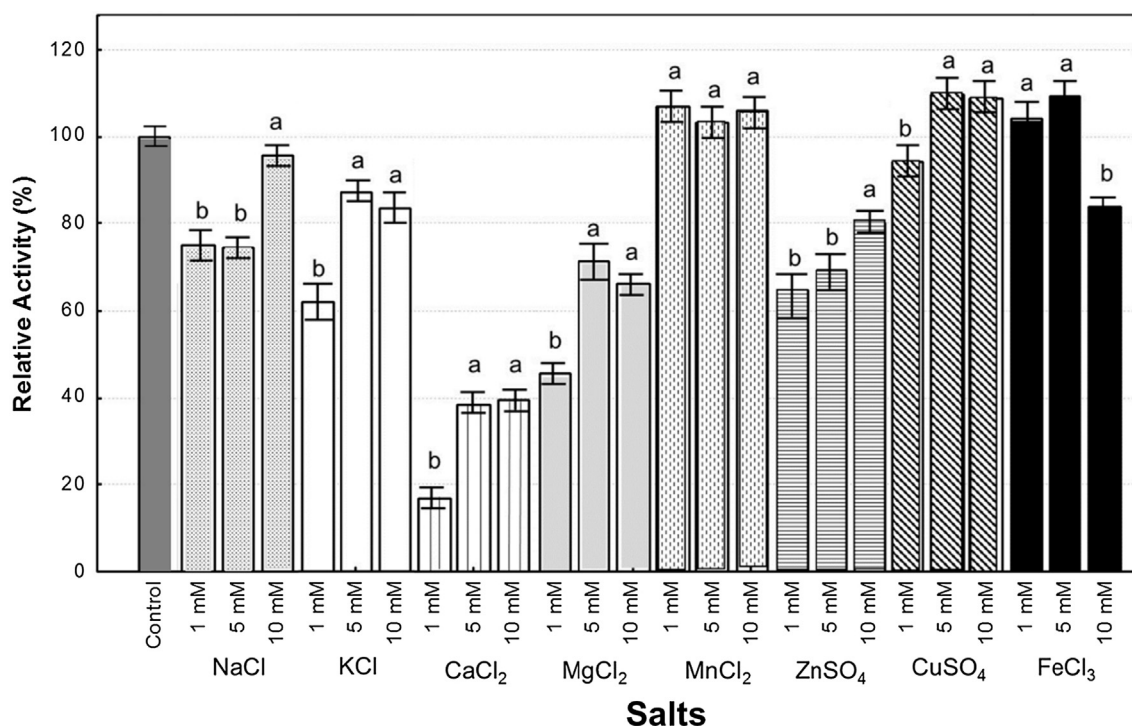


Fig. 5. Effect of different salts at different concentrations (1, 5 and 10 mM) on the activity of the partially purified esterase from *Aureobasidium pullulans* LABIOTEC 01. The statistical analysis was conducted by the Tukey's test. The same letters indicate that no significant differences exist at the 5% level.

Table 4

Effect of different solvents (50% v/v) on the activity of the partially purified esterase enzyme from *Aureobasidium pullulans* pre-incubated for 1 h at 25 °C.

Solvent	Log P^1	Relative activity (%)
Control	–	100 ± 0.2
DMSO	–1.35	116 ± 0.1
DMF	–1.00	115 ± 0.4
Methanol	–0.76	113 ± 0.8
Ethanol	–0.3	99 ± 0.3
Propanol	0.329	93 ± 0.3
Butanol	0.88	102 ± 0.2
Toluene	2.73	102 ± 0.4
Xylene	3.15	69 ± 0.9
n-Hexane	3.5	98 ± 0.3
Isooctane	4.7	73 ± 0.3

¹ P corresponds to the partition coefficient in a standard octanol-water biphasic system [70].

alternative to manage and valorize this waste. A design strategy for the partial esterase purification combining ATPS recovery and acetone precipitation was herein proposed for the first time and resulted in a purification factor of 18 ± 2 and an enzyme recovery around 90%. The enzyme showed optimum temperature and pH at 60 °C and 5.0, respectively. Furthermore, the addition of some salts (FeCl_3 , CuSO_4 , and MnCl_2) promoted an increase of the enzymatic activity. The partially purified enzyme was found to be stable and presented an increased activity in the presence of several organic solvents, namely in polar solvents such as DMSO, DMF, and methanol. Since high temperatures and polar organic solvents are frequently required for the solubilization of both reagents and products, this esterase from *A. pullulans* demonstrated a great potential to be applied in industrial processes, such as asymmetric synthesis.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.seppur.2018.07.062>.

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