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PEG-PHOSPHATE AQUEOUS TWO-PHASE SYSTEM TO EXTRACTION OF PROTEASE FROM ASPERGILLUS FLAVUS

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ABSTRACT

Proteases are enzymes of great interest to biotechnology and their production by filamentous fungi has proven to be an industrially viable alternative. Thus, this research aimed to produce protease obtained by *Aspergillus flavus* UCP 0316 through solid state fermentation. For extraction using the Aqueous Two-Phase System (ATPS) PEG/Phosphate. The experiments were carried out according to a 2^4 factorial design using different PEG molar mass, PEG concentration, concentration of sodium phosphate salt - NaH_2PO_4 and pH as independent variables. The protease partitioned preferentially in the salt-rich phase, the best run was (MPEG = 8000g/mol, 12.5% CPEG, 15% C phosphate (m/m) and pH 6.0) which showed the highest protease activity of 29.8 U/mL, presenting in the system as a whole yield above 100%. ATPS with PEG/Sodium Phosphate proved to be efficient, allowing its use for pre-purification of proteases obtained from *Aspergillus flavus* UCP 0316. Biological evaluation indicated evidence the fibrinolytic activity. The results showed the purity of the sample and its effectiveness of the system in the salt phase, aiming at the potential of the protease obtained to be exploited in industrial applications.

Keywords: *Aspergillus flavus*.; Biotechnology; solid state fermentation; Aqueous two-phase system; purification.

1 INTRODUCTION

Proteases are hydrolytic enzymes responsible for degrading proteins into small peptides and amino acids. Present in all living organisms, they form a notable group of biocatalysts with wide applicability, varying according to their biochemical characteristics and substrate specificity. The protease market is estimated to be worth US$3.54 billion in 2024, and is expected to reach US$4.72 billion by 2029, with a compound annual growth rate (CAGR) of 6% during the forecast period (2024-2029) [1,2].

Due to their wide applicability, proteases exhibit specific activities that are provided in diverse industrial sectors, including meat tenderizing, peptide property, infant formulas, bioremediation, textile, food and pharmaceutical industries. In the pharmaceutical field, fibrinolytic proteases deserve attention due to their ability to degrade fibrin, the main protein involved in blood clotting. Thus, they play a vital role in dissolving blood clots, also known as thrombi, ensuring continuous blood flow through the vessels during normal homeostasis. These fibrinolytic enzymes operate in two distinct ways. They can indirectly promote fibrin lysis by activating circulating plasminogen, converting it into active plasmin that acts on fibrin, a process called indirect fibrinolysis. Or, they can directly degrade pre-existing insoluble fibrin into smaller fragments, called fibrin degradation products, by direct fibrinolysis, thereby restoring typical vascular function. These characteristics make them a promising alternative in the treatment of cardiovascular diseases [3,4].

The purification of proteases is predominantly carried out using traditional techniques, such as prepared with ammonium sulfate, ultrafiltration, successive chromatography and dialysis steps, followed by concentration. To enable the commercial production of enzymes, it is crucial to employ efficient subsequent processing techniques. When applied to biological materials, these processes require rigorous purification steps that are delicate enough to preserve biological activity. It is worth mentioning that the level of purification required is directly linked to the application of the product. The impact of the total cost on the production process is greatly influenced by the type and concentration of the product obtained at the end of the cycle. Therefore, the development and improvement of biocompound degradation and purification techniques have been the subject of increasing interest, driven by growing industry demand [5,6].

An additional highly effective and economically viable method for enzymatic purification is the Aqueous Two-Phase System (ATPS), commonly created by combining two immiscible hydrophilic polymers (natural or synthetic) or by combining a polymer (such as polyethylene glycol, PEG) and a salt (e.g. NaCl, $(NH_4)_2SO_4$) along with buffers (such as phosphate, citrate or phosphate) and water to separate proteins. ATPS offers several advantages in the purification of enzymes and proteins, such as simplicity, low cost and ease of scaling. Enzyme partitioning into ATPS is often influenced by several factors, including the type and concentration of polymers, salts, pH, and temperature. This technique, when applied to the pre-purification of biomolecules, can achieve high levels of purity and recovery, providing a stable medium for the purification of the enzyme, given that the composition of the system is rich in water [7].

2 MATERIAL & METHODS

Microorganism and fermentation process

The strain *Aspergillus flavus* UCP 0316 (Collection 927 – World Culture Collection) was isolated from the Caatinga soil (Northeast Region of Brazil) and obtained from the culture collection of the Catholic University of Pernambuco (UCP) Recife-PE, Brazil, was maintained in solid PDA medium (Potato Dextrose Agar) incubated in an oven at 30°C for 7 days for sporulation and then used for fermentation. Protease production was carried out in solid state fermentation (SSF) using wheat bran as substrate (5g), 10^7 spores/mL, corresponding to a moisture content of 40% 8. SSF were run for 72 h at 30°C. The collected samples were centrifuged to separate the cell mass and obtain a cell-free metabolite liquid for subsequent steps.

Analytical methods

Determination of protein concentration was carried out according to the methods described by Smith [9] using bovine serum albumin (BSA) as a standard substance for the curve. To determine protease activity, the method described by Ginther[10] was used, using 1% (w/v) azocasein. One unit of proteolytic enzyme was defined as the amount of enzyme to produce an increase in absorbance of 0.1 in 1 h at 420nm. To determine fibrinolytic activity, the method described by Wang[11]. One unit of fibrinolytic enzyme was defined as the amount of enzyme to produce an increase in absorbance of 0.1 in 1 h at 275nm.

Extraction of enzymes through the Aqueous Two-Phase System (ATPS)

The ATPS was prepared in 20 15mL graduated tubes containing 50% PEG solutions and 40% sodium phosphate salts according to the conditions determined by 2^4-full factorial design. The following day, 2g of the crude extract was added to all tubes. After adding all system components, the tubes were vortexed for 1 min. After 60 min of rest, the two phases were separated by sedimentation. Then, the upper and lower phases were measured and determined activities.

Definition of ATPS equations

The partition coefficient (K) was defined as the ratio of the protease activity in the upper phase over the lower phase (K=As/Ai) where, A is the protease activity (U/mL) and the indices "s" and "i " represent the upper and lower phases, respectively. The purification factor (PF) was defined as the ratio of the specific activity of the lower phase (AEi) to the specific activity of the crude extract (AEe). Where, specific activity (AE) was defined as the ratio of protease activity (U/mL) to protein concentration (mg/mL). The activity yield (Y) was defined as the ratio of the total activity in the lower phase to the total activity of the crude extract and expressed as a percentage: Y=(Ai.Vi/Ae.Ve).100 where, Ai and Ae are the activity protease in the lower phase and enzymatic extract, respectively. Vi and Ve are the volumes of the lower phase and enzyme extract, respectively. The results were evaluated by analysis of variance (ANOVA) with a significance level of 95%, using Statistica 8.0 Software (Statsoft Inc, 2008).

3 RESULTS & DISCUSSION

The results of the partitioning of proteases produced by *Aspergillus flavus* UCP 0316 are described in Table 1. All tests partitioned for the salt-rich phase, thus verifying that the partition coefficient (K) was less than 1. According to the statistical analysis carried out, it is understood that the salt concentration had a positive effect while the pH had a negative effect, suggesting that the two variables act inversely proportionally, therefore, possibly due to a salting in effect and the isoelectric point of the enzyme favoring the partition to the salt-rich phase. According to statistical analysis, an interaction occurred between the variable molar mass of PEG and salt, strengthening the prospect of salting in effect with molecular exclusion due to the positive effect. In other words, the greater the molar mass of PEG, the more likely the enzyme will partition into the salt-rich phase.

Yield values of 100% above, as obtained in this study, have been reported by works for enzymatic extraction of Protease using the aqueous two-phase system (ATPS), with activity profitability of 145.52% in the PEG system (8000 g/mol), CPEG (12.5 g/mol), 10% phosphate in the purification of serine protease from the microorganism *Paenibacillus* [12] and yield of 157.53% in the extraction of protease from *Aspergillus heteromorphus* using the PEG/ Citrate system [5].

The Peg/sodium phosphate aqueous two-phase system allowed the protease to be partitioned with purification values (PF) greater than 2 in most assays, with the exception of assays (5, 6, 7, and 8) Table 1. The values of the purification factor (Table 1) presented in the present work are higher than the reported works on the purification of protease from *Aspergillus oryzae* CH93 with (PF) values of 1.01 by ammonium sulfate precipitation and 1.32 by column chromatography anion exchange-Q-sepharose [13].

The results obtained for the purification factor, with values above 3 in some tests, and for the yield, with values above 100% in other tests, highlight the effectiveness of the aqueous two-phase system technique. These results indicate no only its extraction capacity, but also its concentration and purification potential.

After analysis of the aqueous two-phase system and evaluation of proteolytic activity, assay 10 was selected to determine fibrinolytic activity. The results revealed a value of 20 U/mL in the salt-rich phase, while a value of 15 U/mL was recorded in the PEG-rich phase. This highlights the potential applicability of this protease under study in the degradation of fibrin thrombi.

Table 1 Extraction of protease by *Aspergillus flavus* UCP 0316 using 2^4 factorial design in the aqueous two-phase system.

Run	PEG MM	C PEG % (m/m)	C phosphate % (m/m)	pH	Protease activity (U/mL)	K	Y% (SALT)	PF (SALT)
1	1500	12,5	10	6	12,53	0,34	111,53	2,30
2	8000	12,5	10	6	15,86	0,39	144,60	2,99
3	1500	17,5	10	6	8,46	0,20	94,97	2,69
4	8000	17,5	10	6	8,2	0,20	107,73	2,88
5	1500	12,5	10	8	23,13	0,48	145,85	1,95
6	8000	12,5	10	8	5,8	0,12	178,65	1,72
7	1500	17,5	10	8	8,93	0,17	120,06	1,83
8	8000	17,5	10	8	4,46	0,09	124,52	1,80
9	1500	12,5	15	6	19,46	0,49	129,78	3,56
10	8000	12,5	15	6	29,8	0,90	100,60	2,92
11	1500	17,5	15	6	26,46	0,71	109,03	3,48
12	8000	17,5	15	6	25,66	0,80	89,62	2,54
13	1500	12,5	15	8	18	0,39	153,65	2,35
14	8000	12,5	15	8	19,66	0,47	137,11	2,30
15	1500	17,5	15	8	23,53	0,50	135,50	2,66
16	8000	17,5	15	8	22,26	0,55	112,47	3,01
17 (c)	4000	15	12,5	7	8,36	0,19	112,62	2,50
18 (c)	4000	15	12,5	7	7,33	0,17	120,65	2,12
19 (c)	4000	15	12,5	7	10,36	0,22	122,21	2,77
20 (c)	4000	15	12,5	7	9,56	0,21	124,40	2,66

C = Central Point; CPEG = PEG concentration; MPEG= PEG molar mass; C phosphate = concentration and sodium phosphate; protease activity; K partition coefficient; Y activity yield; PF purification factor.

4 CONCLUSION

The analysis of the results highlights the potential of ATPS as an innovative approach for the pre-purification of the protease from *Aspergillus flavus* UCP 0316. Its ability to simplify unit operations is remarkable, allowing extraction, concentration and clarification in a single step. This versatility opens doors to future industrial applications, with emphasis on the pharmaceutical area due to its fibrinolytic potential.The main conclusions regarding the research must be presented in this topic.

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