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RECOVERY AND PARTITIONING OF FIBRINOLYTIC PROTEASE FROM *Bacillus* sp. UFPEDA 485 BY AQUEOUS TWO-PHASE SYSTEMS

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ABSTRACT – Fibrinolytic proteases produced by *Bacillus* sp. has attracted interest in the pharmaceutical industry as a promising alternative in thrombolytic therapy due to their effectiveness in degrading fibrin, its production requiring the development of an efficient recovery process. Aqueous two-phase systems (ATPS) have been recognized as an efficient and economical process for recovering enzymes. To optimize the recovery of fibrinolytic protease from the fermentation broth of *Bacillus* sp. UFPEDA 485, a 2^3 full factorial design was used to evaluate the influence of the three independent variables PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}) and sodium sulfate concentration (C_{Na2SO4}) on the partition coefficient (K), purification factor (PF) and yield recovery (Y) of fibrinolytic protease in PEG/Na_2SO_4 aqueous two-phase system. For all ATPS studied, enzymes partitioned to the top phase and the highest extraction was obtained for M_{PEG} 6000 g.mol^{-1}, C_{PEG} 24 % (w/w) and C_{Na2SO4} 11.6 % (w/w) with K = 5.03; PF = 3.30; Y = 91.40% and Fibrinolytic activity in the top phase 821 U.mL^{-1}. Findings reported here show that ATPS composed of PEG/Na_2SO_4 is a valuable strategy for the extraction of fibrinolytic protease and can be considered a promising method for the extraction of enzymes in industrial scale.

1. INTRODUCTION

Fibrinolytic proteases are hydrolytic enzymes that dissolve blood clots. The formation of blood clots is a natural phenomenon of protection of the human body to prevent excessive bleeding from injuries and wounds, but may sometimes block blood flow causing cardiovascular disorders. Thrombolytic therapy using fibrinolytic proteases has shown to be a potential solution to many vascular disorders. The fibrinolytic proteases from *Bacillus* sp. have attracted the interest of researchers and the pharmaceutical industry because of its efficiency as thrombolytic agents in fibrinolysis Raafat et al. (2012).

Currently, there is an industrial need for techniques for the recovery of biomolecules enabling rapid, efficient and low cost processes. Conventional techniques used nowadays for the extraction of products such as ultrafiltration, precipitation and chromatography, are not considered viable for the industry because of the high cost and time consuming process. Therefore, the search for alternative methods has been constant. The application of ATPS in

the recovery of products meets the criteria required by industry. An ATPS is formed when combinations of hydrophilic solutes (two polymers, or a polymer and a salt) in aqueous solution above critical concentrations occur. (Yavari et al. 2013)

The purpose of this study was to analyze the optimal conditions for the extraction of fibrinolytic protease from *Bacillus* sp. UFPEDA 485, using aqueous two-phase system (PEG/Na_2SO_4)

2. MATERIALS AND METHODS

2.1. Microorganism and culture condition

The *Bacillus* sp. UFPEDA 485 strain was obtained from Culture Collection of Department of Antibiotics, at the Federal University of Pernambuco, Brazil. The stock culture was maintained in nutrient broth in cryotubes (10% v/v glycerol) at - 80 ◦C. The soybean medium (MS-2) described by Porto et al. (1996), was used for inoculum growth and for the fibrinolytic proteases production process. The medium composition was: filtered soy flour (2% w/v), K_2HPO_4 (0.435% w/v) and 1% of mineral solution containing: $FeSO_4.7H_2O$ (100 mg); $MnCl_2.4H_2O$ (100 mg), and $ZnSO_4.H_2O$ (100 mg) of distilled water q.s.p 100 mL, NH_4Cl (0.1% w/v); $MgSO_4.7H_2O$ (0.06% w/v), glucose (1% w/v). The process for enzymatic production occurred in shake flasks of 250 mL capacity with 100 mL of production medium at pH 7.8, 150 rpm, at 37 °C for 48 hours.

2.2. Analytical determinations and aqueous two phase systems (ATPS)

Total protein concentration was determined by the Bradford method using as standard bovine serum albumin (BSA) Bradford (1976)

Fibrinolytic activity was determined according to Wang *et al*. (2011). One unit of fibrinolytic protease activity was defined as the amount enzyme required to produce an increase in absorbance equal to 0.01 per minute at 275 nm.

The aqueous two-phase system and the methodology of analysis of the results were in according Medeiros e Silva et al., 2013. The concentrations of Na_2SO_4 and PEG were adjusted according to the planned factorial design (Table 1).

2.4. Statistical analysis and experimental design

A 2^3 full factorial design was used to analyze the influence of the three independent variables: M_{PEG}; C_{PEG} and C_{Na2SO4}. The effects of these three independent variables on enzyme recovery were determined based on response variables enzyme K, PF and Y. The experimental design was composed of 8 runs and 4 repetitions at the central point, needed to calculate the pure error. Statistical significance of the variables was determined at 5% probability level (p<0.05). The analysis of the results was carried with the program Statistic version 8.0

3. RESULTS AND DISCUSSION

Effect of independents variables on the partition coefficient (K), Purification factor (PF) and yield recovery (Y) of fibrinolytic protease

For all the runs, the fibrinolytic protease partition occurred on the top phase rich in PEG, indicating an affinity between the enzyme and the PEG. The best result was obtained at the central point, using M_{PEG} 6000 g.mol^{-1}, C_{PEG} 24% and $C_{Na_2SO_4}$ 11.6%. For this assay, the obtained values at the mean center point were K = 5.03; PF = 3.30; Y = 91.40% and FA = 821 U. mL^{-1}.

Table 1. Matrix of the full factorial design 2^3 with the results of the fibrinolytic protease extraction by ATPS.

Run	Independent variables			Responses		
	[1] M_{PEG} (g.mol^{-1})	[2] C_{PEG} (%)	[3] C_{Na2SO} (%)	[4] K	[5] Y (%)	[6] PF
1	4000	18	10	4.28	87.30	2.71
2	8000	18	10	3.00	66.88	2.05
3	4000	30	10	3.84	80.20	2.97
4	8000	30	10	2.84	68.16	2.58
5	4000	18	13.2	4.05	85.35	2.60
6	8000	18	13.2	2.82	67.23	2.37
7	4000	30	13.2	3.66	72.69	2.99
8	8000	30	13.2	3.16	57.48	2.07
9	6000	24	11.6	4.78	92.56	3.36
10	6000	24	11.6	5.16	90.96	3.30
11	6000	24	11.6	5.30	91.61	3.28
12	6000	24	11.6	4.87	90.47	3.26

(1) PEG molar mass (g mol^{-1}); (2) PEG concentration (%); (3) $_{Na2SO4}$ concentration (%); (4) Partition coefficient (K); (5) yield recovery (Y) and (6) Purification factor (PF).The pure error estimate for K, Y, and PF was 0.06, 0.81 and 0.00, respectively.

The influence of the independent variables, on the responses is described in Table 2.

Table 2. Influence of independent variable on response parameters

Factor	Effect estimate		
	K	Y	PF
(1)M_{PEG}	- 5.79*	- 25.70*	- 17.80*
(2)C_{PEG}	- 0.93	- 11.02*	7.05*
(3)C_{Na2SO4}	- 0.38	- 7.73*	- 2.20
1*2	1.47	4.41*	- 3.31*
1*3	0.79	- 0.34	- 0.83
2*3	0.80	- 6.48*	- 5.66*
1*2*3	0.64	- 2.13	- 7.83*

(1) PEG molar mass (g mol^{-1}); (2) PEG concentration (%); (3) $_{Na2SO4}$ concentration (%); Partition coefficient (K); Purification factor (PF) and yield recovery (Y). * Statistically significant values at the 95% confidence level. The pure error estimate for K, Y, and PF was 0.06, 0.81 and 0.00, respectively

The presented results are corroborated by Kirsch et al, 2012, since in all ATPSs tested, the protease from *Lentinus citrinus* partitioned to the top phase with the best result obtained using M_{PEG} 6000 g.mol^{-1}

The increase of the M_{PEG}, increased the hydrophobicity of the top phase, reducing the free space available for biomolecules and generating an exclusion effect. A similar result was observed by Medeiros e Silva et al, 2013, in the extraction of fibrinolytic protease from

Streptomyces sp. DPUA1576. These authors verified that the decrease on M_{PEG} from 8000 to 1500 ($g.mol^{-1}$) causes an improvement on fibrinolytic protease partition to the top PEG rich phase.

The interaction between M_{PEG} and C_{PEG} favored the increase of the Y and a 91.40% recovery was achieved. These results indicate that none of these independent variable acts alone in the response variables and a synergism occurs. A similar result was observed by Neves et al, 2012, in the extraction of a phytase from *Absidia blakesleeana* URM5604. The reported positive interaction of M_{PEG} with C_{PEG} indicates that an increase in PEG molar mass and PEG concentration enhanced the recovery. The best result for the recovery was 89.67%.

The increase of the C_{PEG} exerted a positive effect and favored the increase in the PF. The best result was 3.30 for the purification factor. Results corroborating this work were obtained by Kirsch et al, 2012.

4. CONCLUSION

According to the presented results, the ATPS composed of PEG/sodium sulfate proved to be a promising method for fibrinolytic protease extraction from the fermentation broth of *Bacillus* sp. UFPEDA 485.

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