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STABILITY OF *Yarrowia lipolytica* IMUFRJ 50682 PROTEASES PARTIALLY PURIFIED BY AQUEOUS TWO-PHASE SYSTEMS

Aparecida S. S. CARVALHO[1]; Rhonyele M. da SILVA[2]; Cristiane N. da SILVA[1]; Filipe S. BUARQUE[2]; Bernardo D. RIBEIRO[2], Ailton C. LEMES[2] & Maria Alice Z. COELHO[1,2*]

[1]*Programa de Pós-Graduação em Ciência de Alimentos, Instituto de Química, Universidade Federal do Rio de Janeiro, Av. Athos da Silveira Ramos, 149. Ilha do Fundão, Rio de Janeiro 21941-909, Brasil*
[2]*Departamento de Engenharia Bioquímica, Escola de Química, Universidade Federal do Rio de Janeiro, Av. Athos da Silveira Ramos, 149. Ilha do Fundão Rio de Janeiro 21941-909, Brasil*
** Corresponding author's email address: alice@eq.ufrj.br*

ABSTRACT

Proteases are the most commercially important enzymes due to their applications in several fields, including food, pharmaceuticals, cosmetics, and so on. Therefore, it is necessary to study stability during storage to establish process conditions to be used in different fields of application. In this study protease produced by *Yarrowia lipolytica* IMUFRJ 50682 via solid-state fermentation was purified using aqueous two-phase system (ATPS). The crude enzyme extract (CEE) and partially purified enzyme (PPE) were stored for 28 days at room, refrigeration, and freezing temperatures to determine enzyme stability. At 7-day intervals, proteolytic activity was measured. The study of the stability of the protease reveals that CEE and PPE can be stored under refrigeration and freezing conditions without decreasing proteolytic activity during 28 days. Furthermore, the results showed that PPE using ATPS with PEG 2000 preserves proteolytic activity for up to 28 days at room temperature, presenting a promising option for future applications.

Keywords: Storage. Polyethylene glycol. Purified protease. Solid-state fermentation.

1 INTRODUCTION

Protease is one of the most important industrial enzymes, representing the second largest class of commercial hydrolytic enzymes, behind only carbohydrases [1]. Proteases are used in many industrial applications, such as the detergent industry, leather, textile, food, pharmaceuticals, and cosmetics industries. Proteases are the most extensively utilized enzymes for dehairing hides and skins [2]. In the food industry, proteases are employed with the function of increasing the texture and flavor, uniformity, and consistency of the dough, as well as controlling the strength of the gluten in the bread [3]. Proteases can also be integrated into detergent formulations to increase cleaning performance [4].

Proteases are obtained from different sources, such as animals, plants, and microorganisms. Microbial proteases are the most feasible alternative, due to their faster production and biochemical diversity [5]. *Yarrowia lipolytica* (*Y. lipolytica*) is a yeast classified as "unconventional" and generally recognized as safe (GRAS) by the Food and Drug Administration (FDA, USA). This yeast has a high potential for producing enzymes, such as proteases, phosphatases, lipases, and esterases [6].

Y. lipolytica can produce proteases through solid-state fermentation using agro-industrial by-products, which decreases the process costs [7]. Nonetheless, other compounds can be secreted into the medium which demands the purification step. Thus, a purification step, such as aqueous two-phase system (ATPS), can be advantageous to obtain a stable enzyme. ATPS are formed by two compounds dissolved in aqueous media, which above given concentrations undergo liquid-liquid demixing. Compared to other alternative protein purification methods, the main advantages of ATPS include the ability to scale up and maintain steady-state operations with low material costs and the rapid attainment of partition equilibrium [8]. Following the purification process, evaluating the stability of the protease is essential for determining its suitability for future applications and commercialization [9]. Thus, the study aims to evaluate the stability of crude (CCE) and partially purified enzyme (PPE) produced by *Y. lipolytica* during 28 days of storage in different temperatures.

2 MATERIAL & METHODS

Y. lipolytica IMUFRJ 50682 isolated from an estuary of Guanabara Bay, Rio de Janeiro, Brazil [10] was used to produce protease. Protease was produced using soybean meal and andiroba oil cake in a 1:1 ratio, 55% moisture, and an inoculum concentration of 0.71 mg of dry biomass/g of substrate [7]. The reactors were placed in a BOD chamber, kept at 28°C, and after a 24-h incubation period, the crude enzyme extract (CEE) was obtained. Posteriorly, the ATPS was prepared using 30 wt% of PEG 2000 + 7 wt% of buffer phosphate (pH 7) to obtain the partially purified enzyme (PPE). The equilibrium curve data for ATPS used in this work were previously reported in the literature by Glyk et al. [11]. The CEE and PPE were stored in Eppendorf microtubes at room temperature (~28°C), refrigeration temperature (10°C), and freezing temperature (-18°C) during 28 days. Protease activity was analyzed weekly for 28 days following the methodology proposed by Charney and Tomarelli [12]. Protease activity (U/mL) was calculated as Carvalho et al. [7]. Equation 1 was used to calculate relative protease activity (%), where initial protease activity refers to protease activity ($U.mL^{-1}$) of day 1. Results were expressed as average values ± standard deviation and were submitted to analysis of variance (ANOVA) one way and comparison of means by Tukey HSD test ($p \leq 0.05$).

$$Relative\ activity\ (\%) = \frac{Protease\ activity\ (U.mL-1) * 100}{Initial\ protease\ activity\ (U.mL-1)} \quad (1)$$

3 RESULTS & DISCUSSION

According to the results, CEE maintained with low reduction in its proteolytic activity during storage at room temperature (1054 $U.L^{-1}$), reducing only 24% after 28 days of storage (Figure 1A). There was no reduction in the proteolytic activity of CEE during storage at refrigeration and freezing temperatures. The PPE decreased its activity after 7 days of storage at room temperature possibly due to denaturation of the protease. However, its activity was retained during the 28 days of storage. The purification process improves enzyme activity, however, it can make the enzymes more susceptible to harmful conditions such as temperature, pH, and salt concentration [13]. Moreover, the PPE retained its proteolytic activity during the 28 days of storage reaching values of 6998, 7723, and 7820 $U.L^{-1}$ at room temperature, refrigeration, and freezing temperatures, respectively. Herrera-Márquez et al. studied the stability of proteases in the presence of detergents at a temperature of 40°C. After 28 days of storage, the maximum relative activity was only 8.3% [14]. This work showed that CEE and PPE have great stability during storage in different temperatures (-18 to 28 ºC). This suggest the feasibility of CEE and PPE application in different processes in food, pharmaceutical, and cosmetic industry.

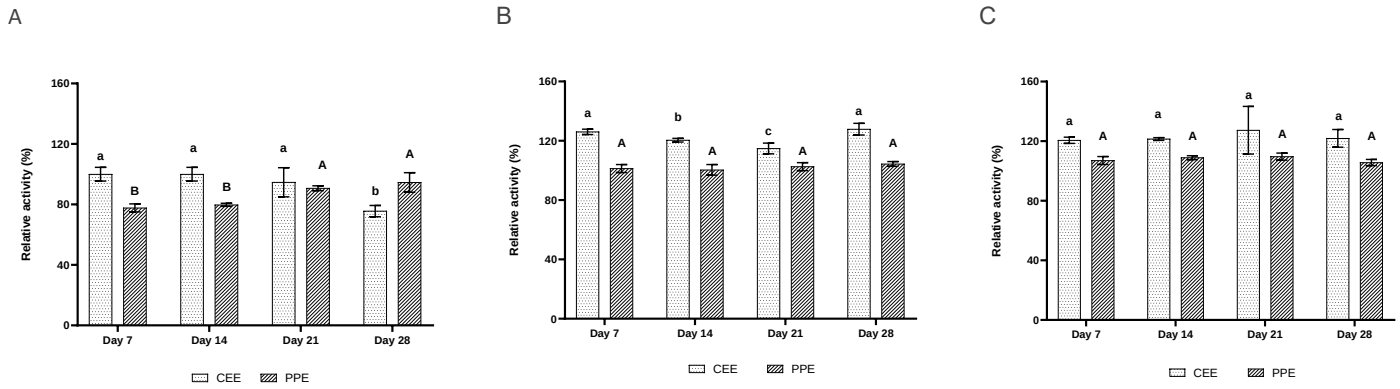

Figure 1 Protease stability for 28 days at room (A), refrigeration (B), and freezing (C) temperature. CEE: crude enzyme extract; PPE: partially purified enzyme. Different upper/lower case letters denote differences (P ≤ 0.05) between the mean values according to Tukey's test.

4 CONCLUSION

The protease from *Y. lipolytica* IMUFRJ 503682 (CEE and PPE by ATPS) showed great stability through 28 days of storage at -18, 10, and 28°C. The PPE retained its activity during the 28 days of storage showing potential to be applied in a wide range of industry processes. Our study showed that *Y. lipolytica* protease produced via solid-state fermentation remains stable in the presence of ATPS salts during storage without requiring an additional purification step, and this reduces process costs.

REFERENCES

[1] ALLIED, A. M. R. Market Research; Global Enzyme market, Opportunity and Forecast, 2018-2024. 2023. Available from: www.alliedmarketresearch.com/enzymes-market.

[2] HASAN, MD J. HAQUE, P., RAHMAN, M. M. 2022. Protease enzyme based cleaner leather processing: A review. Journal of Cleaner Production, Volume 365, 132826, ISSN 0959-6526.

[3] DYER, R. P., WEISS, G. A. 2022. Making the cut with protease engineering, Cell Chemical Biology, Volume 29, Issue 2, Pages 177-190, ISSN 2451-9456.

[4] SINGH, S., MANGLA, J., SINGH, S. 2021. Evaluation of Aspergillus fumigatus NTCC1222 as a source of enzymes for detergent industry. Resources, Environment and Sustainability, 5, 100030. doi:10.1016/j.resenv.2021.100030

[5] CHRISTENSEN, L. F., GARCÍA-BÉJAR, B., BANG-BERTHELSEN, C. H., HANSEN, E. B. 2022. Extracellular microbial proteases with specificity for plant proteins in food fermentation. International Journal of Food Microbiology, Volume 381, 109889, ISSN 0168 1605.

[6] BRÍGIDA, A. I. S., BUARQUE, F.S., NOGUEIRA, V. L. R., MELO, V. M. M., GUISÁN, J. M., RIBEIRO, B. D., GONÇALVES, L. R. B., COELHO, M. A. Z. 2023. Partial purification of crude lipase extract from Yarrowia lipolytica: Precipitation, aqueous two-phase systems (ATPS), and immobilization methods, Cleaner Chemical Engineering, Volume 6,100105.

[7] CARVALHO, A.S.S.; SALES, J.C.S.; NASCIMENTO, F.V.D.; RIBEIRO, B.D.; SOUZA, C.E.C.D.; LEMES, A.C.; COELHO, M.A.Z. 2023. Lipase Production by Yarrowia lipolytica in Solid-State Fermentation Using Amazon Fruit By-Products and Soybean Meal as Substrate. Catalysts, 13, 289.

[8] BASSANI, G., FUCIÑOS, P., PICÓ, G., FARRUGGIA, B. 2010. Candida rugosa lipase Lip1–polyethyleneglycol interaction and the relation with its partition in aqueous two-phase systems. Colloids and Surfaces B: Biointerfaces, 75(2), 532–537. doi:10.1016/j.colsurfb.2009.09.03

[9] SUJITHA, P. SHANTHI, C. 2023. Importance of enzyme specificity and stability for the application of proteases in greener industrial processing- a review, Journal of Cleaner Production, Volume 425, 13891.

[10] HAGLER, A.N.; MENDONÇA-HAGLER, L.C. 1981. Yeasts from Marine and Estuarine Waters with Different Levels of Pollution in the State of Rio de Janeiro, Brazil. Appl Environ Microbiol, 41, 173–178.

[11] GLYK, A., SCHEPER, T., BEUTEL, S. 2014. Influence of Different Phase-Forming Parameters on the Phase Diagram of Several PEG–Salt Aqueous Two-Phase Systems. Journal of Chemical & Engineering Data, 59(3), 850–859.

[12] CHARNEY, J.; TOMARELLI, R.M. 1947. A Colorimetric Method for the Determination of the Proteolytic Activity of Duodenal Juice. J Biol Chem, 171, 501–505.

[13] TANG, S., TAO, S., LI, Y. 2024. Challenges and solutions for the downstream purification of therapeutic proteins, Antibody Therapeutics, Volume 7, Issue 1, Pages 1–12.

[14] HERRERA-MÁRQUEZ, O., FERNÁNDEZ-SERRANO, M., PILAMALA, M., JÁCOME, M. B., LUZÓN, G. 2019. Stability studies of an amylase and a protease for cleaning processes in the food industry. Food and Bioproducts Processing, 117, 64–73.

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