EVALUATION OF BIOCHEMICAL STABILITY OF *Aspergillus oryzae* IPT-301 CELLS IMMOBILIZED IN LOOFA SPONGE

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ABSTRACT

Maintaining the enzymatic activity of microbial cells for an extended period is an industrial challenge, because prolonging the life of biocatalysts is an advantage due to reduction in process costs and consequently, reduction in environmental impact. Thus, the objective of this work was to evaluate the biochemical stability of *Aspergillus oryzae* IPT -301 cells immobilized in loofa sponge, preserved in a refrigerator (4 °C) in stabilizing agents for 45 days. The procedure employed involved submerged culture and simultaneous immobilization in a rotary shaker, at 30 °C, 200 rpm for 28 h. To determine enzymatic activity, the reaction was conducted in a shaking waterbath at 50 °C, 190 rpm for 60 min, then, concentrations of reducing sugar and glucose were quantified. Biocatalysts stored in Tris-Acetate buffer solution and sorbitol 70 %, pH 5.5 (4 °C) showed activities of 1479.09 ± 133.68 U g^{-1} and 1054.86 ± 79.16 U g^{-1}, respectively. It demonstrates the promising use of polyols as a stabilizing agent for stored microbial cells.

Keywords: Polyols. Fructosyltransferase. Fructooligosaccharides. Storage assay.

1 INTRODUCTION

Due to the numerous benefits for human health, the consumption of prebiotic sugar has intensified over the years and fructo-oligosaccharides (FOS) has stood out as a potential ingredient due to its prebiotic function [1,2]. FOS are fructose oligomers produced by a transfructosylation reaction in sucrose molecules catalyzed by extracellular/mycelial enzymes denominated fructosyltransferase (FTases) from diverse fungal strain [1]. However, FTase extracellular reuse is economically unviable, due to the high cost of purified enzymes and low FOS yields [2]. Furthermore, FTases in free form are instable and lost the activity rapidly when are storage, which limits its use in FOS production [3, 4]. A strategy to may severely reduce the process cost is immobilized enzymes in supports, making it possible their reuse, permits the production of biocatalysts that are resistant and robust to the adverse conditions of the reaction medium [3, 5]. Thus, for proper immobilization, the choice of support matrix is an important factor, which the loofa sponge is a potential material for its economically viable, non-toxic, organic and easily accessible [6]. Literature contains studies proving that loofa sponge is an excellent support for immobilization microbial cells[6,7]. However, some factors, such as a temperature, pH and medium composition, may affect the stability of enzymes within microbial cells [8]. So, therefore, the storage step is essential for maintaining the catalytic site and enzyme conformation of FTases in their initial state and extending their lifetime9. Several strategies are adopted for stabilization, such as chemical modifications or enzymatic, and the use of stabilizing agents, such as polyols have attracted attention due to their relatively low cost and easily management [10]. Based on the literature, studies of storage fructosyltransferase using stabilizing agents are scant. This study aimed to evaluate the conservation of *Aspergillus oryzae* IPT-301 cells immobilized in loofa sponge stored under the influence of stabilizing agents and the effectiveness of these products in prolonging the lifespan of the biocatalyst.

2 MATERIAL & METHODS

The submerged cell culture was conducted in a synthetic culture medium with the following composition (in %, m v^{-1}): sucrose 15.0, yeast extract 0.5, $NaNO_3$ 0.5, KH_2PO_4 0.2, $MgSO_4.7\ H_2O$ 0.05, $MnCl_2.4\ H_2O$ 0.03 and $FeSO_4.7\ H_2O$ 0.001. The pH was adjusted to 5,5 [1]. Then, 50 mL of the medium was distributed in 250 mL unbaffled Erlenmeyer flasks and sterilized. Posteriorly, cubes of loofa sponge (*Luffa cylindrica)* with edges of 1.0 cm, previously treated and sterilized, have been added and were inoculated with 0.5 mL of a 10^7 spore. mL $^{-1}$ suspension of *Aspergillus oryzae* IPT- 301 and incubated in a rotary shaker at 30 °C, 200 rpm for 28 h. After that, the content of the flasks was vacuum- filtered and the cells immobilized in organics supports were preserved in a refrigerator (4 °C) in stabilizing agents: sorbitol 70 %, polypropylene glycol (PPG), double-distilled glycerin and 0.2 mol. L^{-1} tris-acetate buffer. The biocatalyst stored in Polyethylene glycol (PEG) was conserved in a dry place under ambient temperature (25 °C), without light incidence, to maintain the same viscosity of the other tested agents, since its freezing range is between the temperatures from 4 to 8 °C. All agents were of analytical grade and had the pH adjusted to 5.5. The transfructosylation activity was monitored along 45 days of storage, in which the samples were collected on the day 0, 5, 15 e 45. For this, 3 cubic particles, with edges of 1.0 cm, of heterogeneous biocatalyst were vacuum-filtered and washed with distilled water, incubated in 3.7 mL of sucrose solution 47% (m v^{-1}) and 1.2 mL of a tris-acetate buffer 0.2 mol L^{-1}, pH 5.5. The stabilizing agents had the pH monitored in these dates. The reaction was conducted in a shaking waterbath at 50 °C, 190 rpm for 60 min and interrupted by the immersion of the reaction medium in boiling water for 10 min and an ice bath for 5 min for enzymatic inactivation [1]. Posterly, the reaction medium was vacuum- filtered and the concentrations of reducing sugar and glucose were quantified by DNS and GOD/PAP ® methods respectively [11]. One unit (1U) of transfructosylation activity was defined as the

amount of FTase that transfers one micromole of fructose per minute under the established experimental conditions [2]. All experiments and analytical procedures were performed in triplicate. The analysis of the means was performed applying Tukey's honest significant difference (HSD) test, with a confidence interval of 95 % using Statistica® software.

3 RESULTS & DISCUSSION

The enzymatic activity was to determine in the days pre-defined during the storage assay as showed at Figure 1. The transfructosylation activity of cells of *A. oryzae* IPT- 301 immobilized in loofa sponge, without agents as a control, in day 0 was 1564.28 ± 138.15 U g^{-1}. This information was used as a reference to establish the percentage of loss of life of the biocatalyst. The decay of transfructosylation activity can be observed over time, as shown in other works [2,4] in which activity decreases linearly, and in extracellular FTase high loss occurs in a few hours of storage.

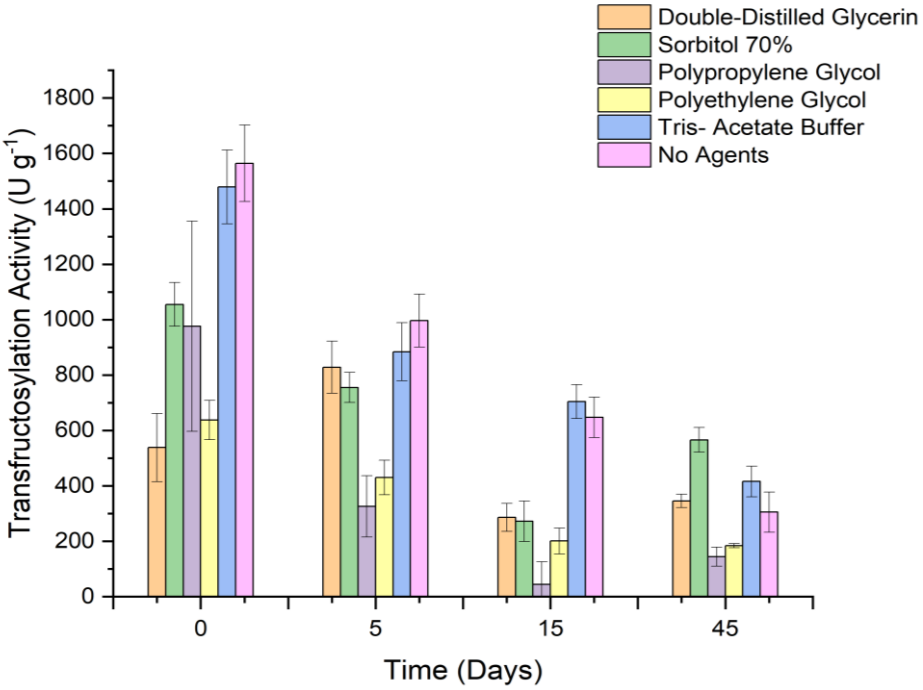

Figure 1 Transfructosylation activity of cells of *Aspergillus oryzae* IPT-301, immobilized in loofa sponge support, storage in refrigerator (4° C) over 45 days. The reaction was carried out for 60 min, at 50 ºC and 190 rpm.

It is noted that heterogeneous biocatalysts stored in Tris-Acetate buffer solution and sorbitol 70 %, pH 5.5 (4 °C) showed greater storage stability over a period of 15 days, without significant difference. The higher activities were observed at the initial, were 1479.09 ± 133.68 U g^{-1} in buffer solution and 1054.79 ± 79.16 U g^{-1} in sorbitol 70 %. At the end of 45 days, the enzymatic activity had a decay of 71.89 % and 46.34 %, respectively. This can be justified by studies that report the presence of sorbitol can reduce the interaction between water molecules and proteins through preferential hydration, improving the thermostability and half- life of enzymes [10, 12]. At the initial of storage assay, the transfructosylation activity of cells preserved in polypropylene glycol and polyethylene glycol showed 717.52 ± 123.07 U g^{-1} and 637.20 ± 70.81 U g^{-1}, respectively. At the end of the test, the activity with these agents shows a reduction in more than 70 %. The presence of polypropylene glycol and polyethylene glycol, may have limited the performance of enzyme active sites, making them unavailable to catalyze sucrose molecules, reducing enzymatic activity, since enzymes can have their stability affected by the composition of the medium and reduce their catalytic efficiency[8,13]. Furthermore, in ambient temperature, FTase extracellular can reduce 50 % of activity in 4 days [3]. Other authors[2] showed, in storage assay data indicated stability to store for 9 days at 4°C, cells of *Aspergillus oryzae* IPT- 301 crosslinked with calcium chloride and entrapped with alginate. However, in the same study, free cells indicated a storage stability of only 5 days. Other studies presented storage results after 5 days with residual activity of 44.07 % ± 8.48 % of fructosyltransferase immobilized in glutaraldehyde-activated polyhydroxybutyrate, maintained in tris-acetate buffer at 0.2 mol L^{-1} and pH 5.5, under refrigeration at 4 °C [14]. In the case of fructosyltransferase extracellular, the extract was stored for a period of 96 h and showed reduction in transfructosylation activity, until it reached approximately 35 % of its initial value, of 13.15 ± 1.47 U mL^{-1} [4]. From these data, it is noticed that the microbial cells when encapsulated/ immobilized have a longer lifespan, under refrigeration conditions. In addition, the use of stabilizing agents, with adjusted pH, can increase this time.

Throughout the experiment, the pH was also monitored for the different agents in 0, 5, 15 and 45 days. Changes in pH can affect the charge distribution of a protein and, consequently, electrostatic interactions between protein groups, between protein and solvent and between the solvent molecules themselves[8]. In addition, the data shows that the pH initial after 45 days, does not change at a 95% significance level, for all stabilizing agents. Immobilized FTase shows higher transfructosylation activities in pH ranges of 5.0 – 6.0. Generally, immobilized enzymes are more robust and resistant to pH changes during reactions [1,2,9,14]. These results suggest that cells of *A. oryzae* IPT -301 immobilized in loofa sponge, can be stored at 15 days under presence of sorbitol 70% and Tris- Acetate buffer solution 0.2 mol L^{-1}, in refrigeration (4 °C) and pH of 5.5, because these agents showed a better efficiency in the conservation of catalytic properties of the enzyme compared to the others tested.

4 CONCLUSION

Therefore, was observed which the use of stabilizing agents in storage, under refrigeration, of microbial cells of *Aspergillus oryzae* IPT- 301, immobilized in loofa sponge, can prolong its lifespan, maintaining the enzymatic activity. The agents that stood out the most were sorbitol 70 % and Tris-acetate buffer, which showed conservation of 95 % of activity in up to 15 days compared to the without any agents under immobilized cell. This work improves the storage data for fructosyltransferase, using polyols, since there are few recent publications on the subject. The mechanism of the process still leaves gaps regarding the interaction of these agents with microbial cells, which brings us as a next step for study.

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