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IMMOBILIZATION OF FRUCTOSYLTRANSFERASE FROM *Aspergillus aculeatus* IN MANAE-AGAROSE WITH AND WITHOUT GLUTARALDEHYDE

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ABSTRACT

Fructosyltransferases are biocatalysts that are capable of hydrolyzing sucrose and transferring fructosyl residues to form fructooligosaccharides (FOS). These compounds are highly beneficial in the food and pharmaceutical industries due to their prebiotic properties. Nevertheless, the industrial application of these enzymes in free form might be restricted by their high production cost, non-reusability, and loss of stability. The objective of this investigation was to evaluate the immobilization of FTase from *Aspergillus aculeatus* on MANAE-agarose support, both with and without glutaraldehyde crosslinking, with the aim to enhance reusability and stability. FTase was produced in a liquid medium, and purified through ion exchange chromatographic columns and sephadex gel filtration. Immobilization on MANAE-agarose with glutaraldehyde demonstrated a higher immobilization efficiency (89.57%) and a greater recovery of enzyme activity (88.83%) compared to the non-crosslinked derivative. The crosslinked FTase exhibited improved reusability, as evidenced by its higher residual activity throughout multiple cycles. FOS production occurred in one hour of reaction by FTase immobilized in MANAE-agarose cross-linked with glutaraldehyde. In contrast, the non-glutaraldehyde cross-linked derivative did not show any detectable FOS production by TLC. These findings highlight the important role of glutaraldehyde for the improved production of FOS and the efficient reuse of FTase immobilized on MANAE-agarose.

Keywords: Fructooligosaccharides. Fructosyltransferase. MANAE-glutaraldehyde. Immobilization. Cross-linking.

1 INTRODUCTION

Fructosyltransferases (FTase; E.C 2.4.1.9) are exceptional biocatalysts that are capable of hydrolyzing sucrose molecules and transferring fructosyl residues to other sucrose molecules, thereby resulting in the formation of fructooligosaccharides (FOS)[1]. These oligosaccharides (FOS) mostly include 1-kestose (GF2), nystose (GF3), and 1F-fructofuranosyl nystose (GF4), where fructosyl units (F) are linked at the β-(2 → 1) bond of fructose molecule[2]. FOS is a class of oligosaccharides that are important to the food and pharmaceutical sectors because of their anti-carcinogenic qualities and effects on nutrient absorption. They are also used as a source of fiber and as an ingredient in low-calorie artificial sweeteners. However, due to their prebiotic qualities, they are commonly utilized in treatments that target the gastrointestinal tract and promote the growth of healthy gut bacteria[3]. *Fusarium, Penicillium*, and *Aspergillus* represent some of the most common FTase producers. Among these, *Aspergillus* is a genus recognized by its large enzymatic production[4].

Although enzymes are an exceptionally useful instrument for accelerating chemical reactions, they are biodegradable, clean, and not consumed[5]. Nevertheless, the majority of soluble enzymes are highly unstable, and the absence of long-term operational stability, recovery, and reuse of the enzyme in biotechnological processes frequently restricts industrial application[6]. In an effort to resolve the problem, a variety of approaches have been investigated; however, the biocatalyst immobilization technique has been particularly effective in enhancing enzyme stability, preserving their separation from the product, and allowing the reuse of the immobilized molecule[7]. The objective of this investigation was to evaluate the immobilization of FTase produced by *Aspergillus aculeatus* on MANAE-agarose support, both with and without glutaraldehyde crosslinking.

2 MATERIAL & METHODS

Cultivation and purification of FTase: The fungus *Aspergillus aculeatus* isolated from the Atlantic forest of western Paraná. was cultivated in Erlenmeyer flasks with 25 mL of Czapek mineral medium supplemented with 1.5% soybean meal and incubated at 28 °C for 6 days to produce FTase. The crude extract was obtained through vacuum filtration and dialyzed for 18 hours. The intracellular extract was loaded onto a DEAE-Sephadex chromatography column and eluted with a linear NaCl gradient for purification. Pure FTase was obtained by loading the concentrated sample onto a Sephadex G-75 column following DEAE.

Enzymatic and protein assays: FTase activity was determined by incubating enzymatic extract with sucrose (60% in 50 mM sodium acetate buffer pH 5.5) incubated at 55°C for 60 min and measured by the DNS method[8] using glucose as a standard. The absorbance was measured at 540 nm. One unit (U) of FTase was defined as the amount of enzyme activity required to produce 1 µmol of reducing sugars. Remaining glucose was also measured using a glucose-oxidase kit at 505 nm. Protein content was estimated using the Bradford method[9] using BSA as a standard.

Immobilization of FTase on MANAE-agarose and reusability: FTase was immobilized on Monoaminoethyl-N-aminoethyl (MANAE-agarose) dissolved in 10 mM phosphate buffer pH 6.5. Cross-linked MANAE-agarose was prepared by adding glutaraldehyde (10% v/v). The immobilization procedure involved the gentle agitation of the enzyme and support with or without glutaraldehyde for 16 hours. Subsequently, the yield and efficiency of FTase immobilization and recovery on MANAE-agarose supports were calculated[11,12,13]. The reusability of the immobilized enzyme was carried out through an enzymatic reaction at 55°C for 1 h per cycle, and after each cycle the derivative was washed with the same buffer as the substrate, and a new cycle of enzymatic activity was repeated.

FOS production and Analysis of carbohydrates by thin layer chromatography (TLC): FOS production was carried out using free purified FTase and immobilized with sucrose substrate (60% in 50 mM sodium acetate buffer pH 5.5) incubated at 55 °C, aliquots were removed at times (1 h, 12 h, 24 h, 48h and 72 h) and boiled for 5 min to stop the reaction. TLC was performed by applying the reaction products to silica plates**.** The mobile phase consisted of butanol:ethanol:water (5:3:2, v/v/v) and the carbohydrates were detected by spraying with 0.2% (w/v) orcinol in methanol:sulphuric acid (9:1, v/v) followed by heating at 100 ºC for a few minutes. Glucose (Glu), fructose (Fru), sucrose (Suc) and FOS were used as standard.

3 RESULTS & DISCUSSION

The FTase of *A. aculeatus* was produced in liquid medium supplemented with 1.5% soybean meal after 6 days of cultivation at 28ºC under stationary conditions. The intracellular extract from the culture after dialysis was used for purification in two chromatographic columns. On the DEAE-Sephadex ion exchange column, the enzyme that remained adhered to the resin was eluted with a NaCl gradient at a concentration between 175 and 300 mM, then purified on the Sephadex G-75 gel filtration column. After all chromatographic steps, FTase was purified 2.18 times and 26% yield, and with a high specific activity of 24,878 U/mg (results not shown). The comparative purification analysis with the two columns in the present study demonstrated similarities with the purification profile of FTase produced by *Aureobasidium pullulans* using a DEAE-cellulose ion exchange column[14].

Immobilization of FTase on MANAE-agarose with or without glutaraldehyde as a crosslinker exhibited a high immobilization yield (Table 1). High enzyme activity was retained, as evidenced by the significant immobilization yield, exceeding 97% in both cases. Furthermore, the immobilization efficiency and recovery of immobilized enzyme activity were significantly higher on MANAE-agarose cross-linked with glutaraldehyde, with values of 89.7% and 88.7%, respectively. Conversely, immobilization of *A. oryzae* FTase on polyhydroxybutyrate (PHB) using glutaraldehyde as a cross-linking agent has been shown to result in a higher yield immobilization, but the activity recovery was 1.4 times lower than that of the non-cross-linked support[15].

Table 1 Comparison of *A. aculeatus* FTase Immobilization on MANAE-agarose supports with and without glutaraldehyde as crosslinker

Support	Immobilization Yield (%)	Immobilization Efficiency (%)	Activity Recovery (%)
MANAE-agarose	97.8	34.5	33.7
MANAE-agarose + glutaraldehyde	99.1	89.6	88.7

The reusability of FTase immobilized on MANAE-agarose without glutaraldehyde was significantly reduced, as evidenced by a significant decrease in residual activity to 64.7% in the second cycle and 16.2% in the third cycle (Fig. 1a). The residual activity of the MANAE-agarose – glutaraldehyde derivative was 100% during the first four cycles, 95% during the fifth cycle, and 74% after the sixth cycle (Fig. 1b). This result suggests that the cross-linking agent (gluraldehyde) is essential for the reusability of FTase that is immobilized on MANAE-agarose.

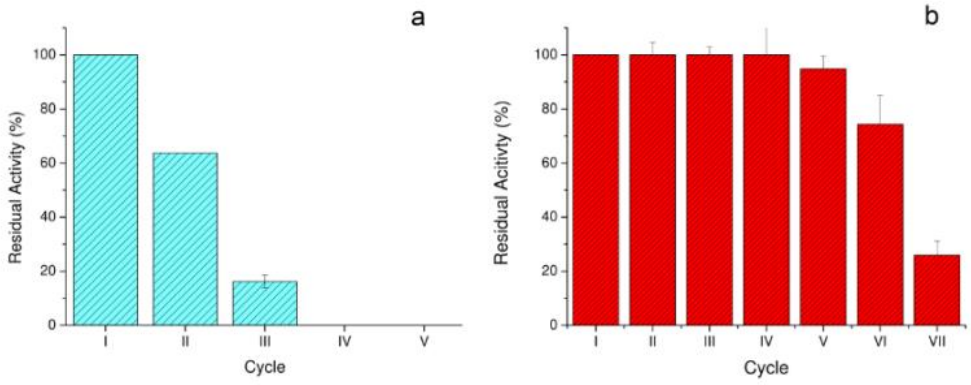

Figure 1 Reusability of FTase immobilized in MANAE-agarose (a) and MANAE-agarose + glutaraldehyde (b).

The production of FOS from sucrose was analyzed by TLC at diferent times (1 h, 12 h, 24 h, 48 h, and 72 h) using free and immobilized FTase (in MANAE-agarose with or without glutaraldehyde) (Fig. 2). The production of FOS was not detectable by TLC within one hour of the reaction, regardless of whether it was immobilized in MANAE-agarose or free FTase. Nevertheless, the production of FOS can be observed within one hour of the reaction when the FTase is immobilized and cross-linked with glutaraldehyde. Some studies have shown that the maximum concentration of FOS molecules can be achieved between 0 and 30 hours, with the highest concentration of GF2 and GF3 occurring before 10 hours of reaction [16]. However, other studies report

that the maximum FOS production can be achieved up to 24 hours and is generally maintained or decreased for longer periods of time.

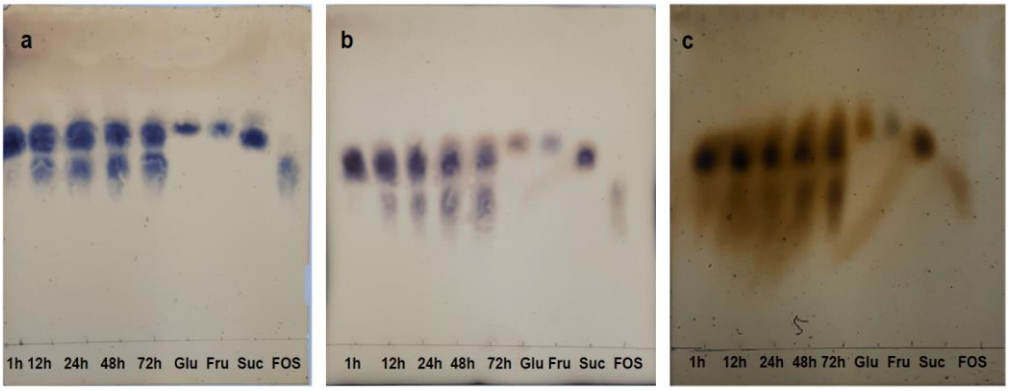

Figure 2 TLC analysis of FOS production from sucrose with FTase from *A. aculeatus* at different times with the free enzyme (a); FTase immobilized only on MANAE-agarose (b); FTase immobilized on MANAE-agarose with glutaraldehyde (c). Standards: Glu- glucose; Fru: fructose; Suc: sucrose and FOS.

CONCLUSION

The comparative study of the immobilization of *A. aculeatus* FTase in MANAE-agarose revealed a low yield, immobilization efficiency, and recovery of the enzymatic activity in the absence of the crosslinker glutaraldehyde. Additionally, the derivative cross-linked with glutaraldehyde revealed enhanced reusability and FOS production. These findings highlight the necessity of the crosslinker glutaraldehyde for the improved production of FOS and the efficient reuse of FTase immobilized on MANAE-agarose.

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