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Investigation of Optimal Conditions for Production, Characterization, and Immobilization of Fructosyltransferase and β-fructofuranosidase by Filamentous Fungi

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Abstract: This work's objective was the extracellular production, partial characterization, and immobilization of the enzymes fructosyltransferase (Ftase) and β-fructofuranosidadase (Ffase) by filamentous fungi. *Aspergillus niger* ATCC 9642 and *Penicillium brasilianum* were evaluated for the production of fructosyltransferase (Ftase) and β-fructofuranosidadase (FASE) enzymes. The *A. niger* presented the highest activity of FTase (24.86 µmol/min.mL) and FFase (28.68 µmol/min.mL) in medium composed of 20% sucrose, 0.5% yeast extract, 1% NaNO3, 0.05% $MgSO_4$.7 H_2O, 0.25% KH_2PO_4, 0.5% NH_4Cl and 0.25% NaCl inoculated using $5x10^7$spores/mL and incubated at 25°C, pH 5.5, 150 rpm for 48 h. Presenting optimum pH and temperature of 2.39 and 60°C. Thermal stability has shown that the enzyme FFase is more thermally stable when compared to FTase. Stability against different pHs showed similar behavior for FTase and FFase; the optimum pH being between 2.0 and 3.0. FTase and FFase showed storage stability in freezing and refrigeration temperature for approximately 400 h. The kinetic parameters, Km and Vmax, for the sucrose substrate were 24.60mM and 104.16 µmol/min.mL for FTase and 3.91mM and 20.24 µmol/min.mL for FFase. The immobilization process displayed a yield of 6744.66% for FFase and 3928.90% for FTase, with enzymatic activities of 364.79 U/g and 220.34 U/g, and 4 and 3 times reuse, respectively.

Keywords: *Aspergillus niger* ATCC 9642; invertases; kinetic parameters; sugar hydrolysis; imobilization.

1. Introduction

Invertases, fructosyltransferases (EC 2.4.1.9) [1] and β-fructofuranosidases (EC 3.2.1.26) [2,3] represent a small fraction of the 75,000 enzyme protein molecules known to date [4]. However, they are extremely relevant since they can be used for inverting sucrose in the preparation of invert sugar and high fructose syrup [5-7] or other compounds such as inulin, cestosis, leads, nystosis, schiosis and raffinose [8]. These enzymes have a wide range of commercial applications, including the production of liquid or soft centers, chocolate and sugarcane molasses fermentation in ethanol. Also, invertase is also used in the manufacture of artificial honey, plasticizers for application in the cosmetics, pharmaceutical, and paper

industries, such as enzymatic electrodes for sucrose detection [6] and fructooligosaccharide (FOS) production [9], which have probiotic action, when included in the diet, by stimulating the consumer's intestinal tract [10,11].

The invertase exists widely in the biosphere, especially in plants [12] and microorganisms such as *Aspergillus niger* [3], *Aspergillus japonicus*, *Aureobasidium pullulans, Saccharomyces cerevisiae, Candida utilis* [13,14], *Zymomonas mobilis* [15], which occur both intracellularly and extracellularly [16-18], with the majority being extracellular [19].

Aspergillus species' enzyme production is sensitive to nutritional and environmental conditions, which indicates significantly different optimal conditions between enzymes, substrates, and/or species/strains used [20]. Therefore, important aspects of enzyme production should be established for each strain, such as sucrose content, agitation, pH, temperature, and other nutrients [21]. Intracellular invertases usually have a wide range of optimal pH, mainly attributed to the presence of groups of carbohydrates in the constitution of proteins, different from extracellular invertases [22].

A limiting factor in the use of the enzyme produced by *Aspergillus* is their high cost. An alternative to the conventional sucrose hydrolysis would be immobilization. It allows for the generation of a product with high purity without generating wastewaters, which is very common in chemical processes [23]. Furthermore, the use of immobilized enzymes may severely reduce the process's cost. It allows for the reuse of the enzyme and eliminates posterior steps after the hydrolysis [24]. High productivity and stability and the low cost of the invertase immobilization process are fundamental requirements for it to compete with the traditional processes [25,26].

Given the above, this work's objective was the extracellular production of the fructosyltransferase (FTase) and β-fructofuranosidadase (FFase) enzymes by the filamentous fungi, with a variation of the production medium composition, spore concentration, time, temperature, and pH. Besides the partial characterization of the enzymes regarding the optimum pH and temperature, kinetic parameters (K_m and V_{max}), thermal stability, different pH and storage, and evaluation of the enzymes' immobilization process and recycle potential.

2. Materials and Methods

Figure 1 presents a flow chart of the present work. Different approaches were performed to evaluate the production and characterization of fructosyltransferase (FTase) and β-fructofuranosidadase (FFase) enzymes.

2.1. Microorganisms.

Aspergillus niger ATCC 9642 was purchased in the lyophilized form from FIOCRUZ (Manguinhos, Brazil), an international distributor of American Type of Culture Collection (ATCC). *Penicillium brasilianum* used in the present study was isolated from tea and previously identified by Zeni *et al*. [27]. The propagation of this culture was done on Potato Dextrose Agar (PDA) and incubated at 30ºC until sporulation (1 week). Harvesting of the slants' spores was done using 20mL of Tween 80–water (0.1%). The spore suspension was collected in a sterile falcon tube and stored at 4ºC until the study began.

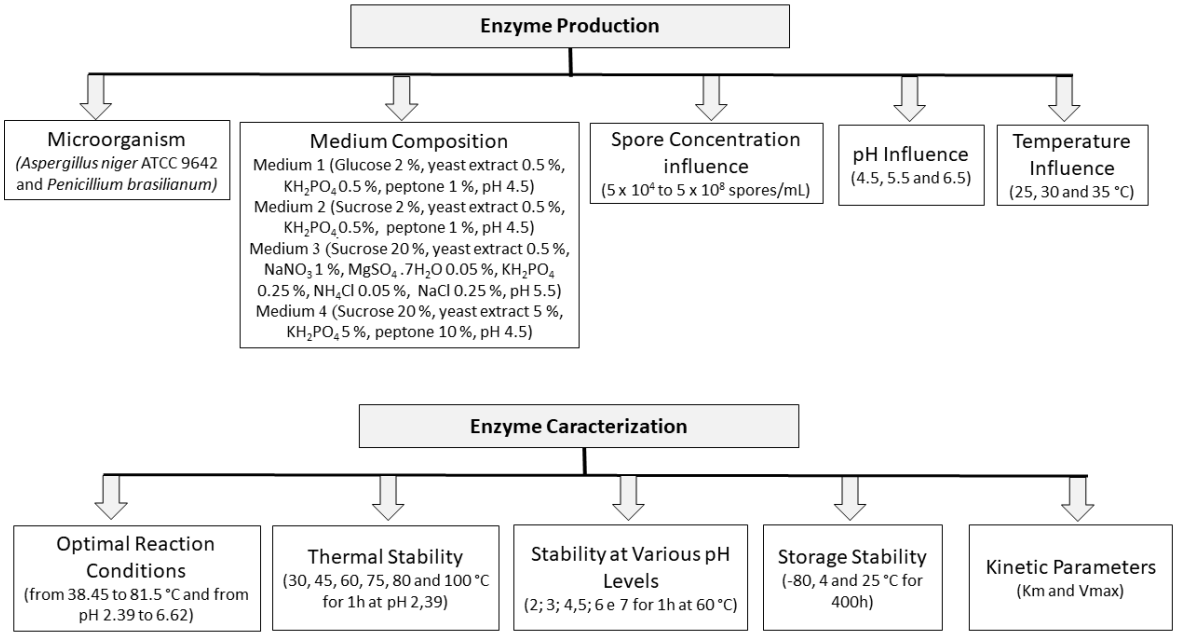

Figure 1. Flow chart of the work.

2.2. Bioproduction of FTASE and FFASE.

In order to study the effects of the microorganism type, the conditions (temperature and pH) and the culture medium on the fructosyltransferase (FTase) and β-frutofuranosidase (FFase) bioproduction, assays were carried out first to evaluate the producing microorganism (*A. niger* and *P. brasilianum*) keeping the medium composition fixed 1. For this, 100 mL of previously sterilized medium, detailed in Table 1, was inoculated with 1 mL of spore suspension ($5x10^6$ spores/mL) of *A. niger* and *P. brasilianum*, respectively.

Subsequently, they were incubated in a shaker at 150 rpm, and 30°C and enzymatic bioproduction kinetics were performed. Periodically, 2 mL aliquots were removed and filtered using filter paper. The obtained crude enzyme extract was used to determine the enzymatic activities of fructosyltransferase (FTase) and β-frutofuranosidase (FFase). With the definition of the best enzyme producing microorganism, new medium formulations (2, 3, and 4) were tested, according to Table 1.

Table 1. Medium composition for the bioproduction of fructosyltransferase (FTase) and β-frutofuranosidase (FFase).

Medium Code	Medium composition	pH	Reference
1	2% glucose, 0.5% yeast extract, 0.5% KH_2PO_4 and 1% peptone	4.5	[28]
2	2%sucrose, 0.5% yeast extract, 0.5% KH_2PO_4 and 1% peptone	4.5	adapted from [28]
3	20% sucrose, 0.5% yeast extract, 1% $NaNO_3$, 0.05% $MgSO_4$.$7H_2O$, 0.25% KH_2PO_4, 0.05% NH_4Cl and 0.25% NaCl	5.5	[29]
4	20% sucrose, 5% yeast extract, 5% KH_2PO_4 and 10% peptone	4.5	adapted from [29]

After selecting the microorganism and culture medium, the influence of the spore concentration ($5x10^4$, $5x10^5$, $5x10^6$, $5x10^7$ e $5x10^8$ spores/mL) on the bioproduction of FFase and FTase was evaluated.

The microorganism and spore concentration selected was evaluated the influence of the pH, the medium (4.5, 5.5, and 6.5), and the temperature (25, 30, and 35ºC) on the bioproduction of FFase and FTase.

2.3. FTASE and FFASE partial characterization.

To determine the optimum values of temperature and pH in terms of enzyme activity, a central composite rotatable design (CCRD) 2^2 was carried out using the enzymatic extract from the microorganism previously selected. The studied range for pH was 2.39 to 6.62 and temperature 38.45 to 81.5°C (Table 3). FTase and FFase activity results were expressed as μmol/min.mL.

The enzyme extract's thermal stability was performed by incubating it for 1 h, with pH adjusted to 2.39 with HCL (0.1 M), in an oven (Brand Quimis, Model Q819V2) of 30, 45, 60, 75, 80, and 100°C. After the incubation period, FTase and FFase activity were determined.

For stability evaluation at different pHs (2, 3, 4.5, 6, and 7), the enzyme extract's pH was adjusted with potassium acetate buffer (0.05 M). The pH-adjusted extracts were incubated in an oven at 60ºC for 1 h and determination of FTase and FFase activity.

In order to determine the storage temperature of the enzyme extract of FTase and FFase, it was subjected to temperatures of -80°C, 4°C, and ambient (± 25°C). The activity was determined periodically under optimized reaction conditions. The results were expressed in terms of the residual percentage of enzymatic activity.

2.4. Determination of the kinetic parameters

The kinetic parameters of hydrolysis of sucrose by FTase and FFase were investigated in potassium acetate buffer (0.05 M) pH 4.5 at 20°C, taking into account that the solubility of sucrose is 1.970 g/L at 20°C [30]. The reaction was initiated by adding 0.5 mL of enzyme extract to 4.5 mL of the substrate at various concentrations (20, 30, 40, 50, 60, 70, and 80% sucrose) and incubated at 60°C, 30 min, and 125 rpm. At time zero and after 30 min of incubation for enzymes, the spectrophotometric assay was performed. For this purpose, 10 μL of each sample was used to quantify the activity of FFase and FTase. The plot, including the initial rate data related to velocity versus substrate concentrations, showed Michaelis-Menten behavior. The double reciprocal (Lineweaver-Burk) plots were used to determine the kinetic parameters (K_m and V_{max}).

2.5. Imobilization in situ of FFase and FTase in a polyurethane matrix.

FTAse and FFase (crude extract) were immobilized in polyurethane (PU) using a proportion of 10 g of poliol for every 2 g of isocyanate, with 1 mL of the enzymatic extract and 0.0060 g of silicone; this composition was determined through previously conducted studies. Initially, the crude enzymatic extract was added to poliol and homogenized. After that, silicone was added to the mix, followed by isocyanate. The resulting foam was kept at 20ºC for 5 min, long enough for polymerization to occur. After that, the polyurethane foam was kept still for 24 h in order to allow it to dry. Following this period, it was then fragmented for posterior evaluation of the enzymatic activity.

2.6. Enzyme assays.

Fructosyltransferase (FTase) and fructofuranosidase (FFase) activity were determined with modifications, according to Ganaie *et al*. [29,31]. When using free enzymes, 4.5 mL of substrate (60% sucrose solution in 0.05 M potassium acetate buffer at pH 4.5) was added to test tubes, then 0.5 mL of crude enzyme extract was added. For immobilized enzymes, 9 mL of substrate (60% sucrose solution in 0.05 M potassium acetate buffer at pH 2.39) was added

to test tubes, followed by 0.2 g of the matrix containing the enzymes. Both enzymes were incubated at 60°C for 30 min in an incubator bath (Nova Ética) at 125 rpm. A control reaction was performed under the same conditions but without sucrose. After the determined time, 10 µL and 2 mL aliquots were removed from the reaction medium to determine glucose concentration using the glucose oxidase kit (Glucose WS, Kovalent) and 2 mL aliquot to quantify the reducing sugars by the Somogyi-Nelson method [32], respectively.

The enzymatic activity calculation is based initially on sucrose's hydrolysis reaction, forming glucose, and fructose; such reaction can be described in Equation 1.

$$RS = G + F \quad (1)$$

Where: RS is reducing sugars/sucrose; G is glucose, and F is fructose.

The enzyme β-fructofuranosidase (FFase) provides the activity known as UF (frutofuranosidase unit), representing the amount of hydrolyzed sucrose per unit time and volume, expressed in µmol/min.mL [33].

To have the value of UF (fructofuranosidase activity unit), one must determine the amount of glucose present in the medium reaction since glucose is one of the products of sucrose hydrolysis. Therefore, fructofuranosidase (FFase) activity was determined according to Equation 2.

$$UF = G \quad (2)$$

Where: UF is fructofuranosidase activity unit, and G is glucose.

The enzyme fructosyltransferase (FTase) is described by UTF (fructosyltransferase activity unit), representing the amount of fructose transferred per unit of time and volume, expressed in µmol/min.mL.

Equation 2 shows that the fructose present in the reaction medium (Fm) is defined according to Equation 3:

$$F_m = RS - G \quad (3)$$

Where: F_m is fructose in the reaction medium, RS is reducing sugars/sucrose, and G is glucose.

Transferred fructose (Ft) is the difference between glucose and medium fructose, Equation 4:

$$F_t = G - F_m \quad (4)$$

Where: F_t is transferred fructose, G is glucose, and F_m is fructose in the reaction medium.

By uniting Equations 3 and 4, we have the final equation representing the amount of fructose transferred, equivalent to the transfructilation activity (Equation 5).

$$UTF = 2G - RS \quad (5)$$

Where 2G indicates the theoretical amount of reducing sugar and RS the actual amount of reducing sugar present in the medium, determined by the Somogyi-Nelson method [34] so that the difference between them indicates the fructose that was transferred.

2.7. Immobilization process efficiency.

The immobilization process's efficiency was determined by the difference between the initial number of enzymatic activity units (U_0) and the number of enzymatic activity units present in the polyurethane matrix (U_f), according to Equation 6.

$$EI\ (\%) = \frac{U_f}{U_0}\ x\ 100 \quad (6)$$

Where: EI (%): efficiency immobilization; U_0: initial enzymatic activity, calculated considering the free enzyme's activity (U/g) and the mass used in the immobilization process

(g); U_f: enzymatic units remaining on the immobilized, calculated by considering the immobilized enzyme's activity (U/g) and the mass used in the immobilization process (g);

2.8. Evaluation of the operational stability of immobilized enzymes.

The immobilized enzymes' operational stability was determined by performing successive reactions, as previously described in the enzymatic activity assay; in this case, however, after each run, the reaction medium was removed with a new medium being added. This process was repeated until the immobilized enzyme's residual activity reached a value of 50% below of the initial activity, determined according to Equation 7.

$$OS\ (\%) = \frac{Lipolytic\ activity\ in\ cycle\ n}{Lipolytic\ activity\ in\ cycle\ 1}\ x\ 100 \qquad (7)$$

Where: OS (%): operational stability; enzymatic activity in cycle *n*: lipolytic activity of each cycle; enzymatic activity in cycle 1*:* initial lipolytic activity.

2.9. Statistical analysis.

The results were statistically processed by analysis of variance (ANOVA) and the differences in average were compared by Tukey test using Statistica software, at 95 % significance level ($p < 0.05$). All experiments were performed in triplicate.

3. Results and Discussion

3.1. Maximization of FTase and FFase bioproduction.

Table 2 presents the results of the production of fructosyltransferase (FTase) and β-frutofuranosidase (FFase) using the filamentous fungi *A. niger* and *P. brasilianum* in submerged fermentation at different times. According to the results, it can be observed that when enzyme production occurred in medium 1 (2% glucose, 0.5% yeast extract, 0.5% KH_2PO_4, 1% peptone at pH 4.5), the filamentous fungi *A. niger* showed the highest activity, 1.85 and 3.01 µmol/min.mL for FFase and FTase at 36 h, respectively. While employing the *P. brasilianum* microorganism, the highest activity was 0.64 and 0.84 µmol/min.mL for FFase and FTase at 72 h, respectively. Thus, the fungi *A. niger* was selected for FPase and FTase bioproduction because of its higher activity. New medium compositions were tested (medium 2, 3, and 4) with this microorganism (Table 2).

In order to verify the influence of sucrose on FFase and FTase production, medium 2 containing 2% sucrose, 0.5% yeast extract, 0.5% KH_2PO_4, 1% peptone at pH 4.5 was tested, observed an increase in enzyme production concerning medium 1 (Table 2).

A new attempt to produce FFase and FTase was evaluated by employing a higher sucrose concentration (20%) in the culture medium, being identified as medium 3. With this, the activity of 6.29 and 7.01 µmol/min.mL for FFase and FTase, respectively. A new formulation, medium 4, in which activity of 8.86 and 0 µmol/min.mL for FFase and FTase, respectively, was also tested. From these results, it was determined that bioproduction medium 3 consisting of 20% sucrose, 0.5% yeast extract, 1% $NaNO_3$, 0.05% $MgSO_4.7H_2O$, 0.25% KH_2PO_4, 0.5% NH_4Cl, and 0.25% NaCl was the most suitable for the bioproduction of β-fructofuranosidases (FFase) and fructosyltransferase (FTase) at 48 h and pH 5.5 using the filamentous fungi *A. niger*.

Table 2. Bioproduction of fructosyltransferase (FTase) and β-fructofuranosidase (FFase) by *A. niger* and *P. brasilianum.*

Microorganisms	Enzymes	Time (h)						
		12	24	36	48	60	72	124
		Activity (µmol/min.mL)						
		Medium 1						
A. niger	FFase	1.37^{ab}±0.46	1.06^{b}±0.26	1.85^{a} ±0.20	0.83^{bc}±0.1	1.11^{b}±0.00	0.44^{c}±0.00	0.60^{c}±0.00
	FTase	2.90^{ab}±0.46	1.68^{bc}±0.66	3.01^{a}±0.33	0.97^{d}±0.20	1.00^{cd}±0.80	-	-
P. brasilianum	FFase	0.32^{cd}±0.11	0.18^{e}±0.05	0.43^{bc}±0.13	0.20^{cd}±0.10	0.21^{d}±0.00	0.64^{a}±0.00	0.55^{b}±0.00
	FTase	-	-	-	-	-	0.82^{a}±0.00	0.67^{a}±0.00
		Medium 2						
A. niger	FFase	-	1.32^{b}± 0.09	1.76^{a}±0.23	2.00^{a}±0.22	-	1.87^{a}±0.00	-
	FTase	-	1.06^{c}±0.04	1.86^{b}±0.08	2.36^{a}±0.24	-	1.27^{c}±0.10	-
		Medium 3						
A. niger	FFase	-	-	-	6.29±1.45	-	-	-
	FTase	-	-	-	7.01±2.22	-	-	-
		Medium 4						
A. niger	FFase	-	-	-	8.86±0.80	-	-	-
	FTase	-	-	-	-	-	-	-

* Means ± standard deviation followed by lower case letters in the lines indicate no significant difference ($p < 0.05$) between production times. (-) corresponds to non-performance of assays to quantify the enzymatic activity at these times.

Ganaie *et al.* [29] evaluated the production of fructosyltransferase from 20 microorganisms for 120 h in a medium containing 20% sucrose, 0.5% yeast extract, 1% $NaNO_3$, 0.05% $MgSO_4.7\ H_2O$, 0.25% KH_2PO_4, 0.5% NH_4Cl, and 0.25% NaCl having a $pH_{initial}$ of 5.5, 200rpm, 28ºC and obtained activities between 31 to 35 U/mL with the filamentous fungi *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, and *Penicillium islandicum*. These values are different from those obtained in this work. They may be associated with the microorganism strain, the production temperature, and the flasks' shaking during cultivation since the production conditions imply different enzymes [20].

Figure 2a and 2b show the results of FTase and FFase bioproduction, respectively, ranging from $5x10^4$ to $5x10^8$ spores/mL. It was observed that the highest activity of FTase, 11 µmol/min.mL, and FFase activity, 10 µmol/min.mL, was obtained in the spore concentration $5x10^7$ spores/mL. Thus, the spore concentration was set at $5x10^7$ spores/mL *A. niger* ATCC 9642 and 48 h for FPase and FTase bioproduction.

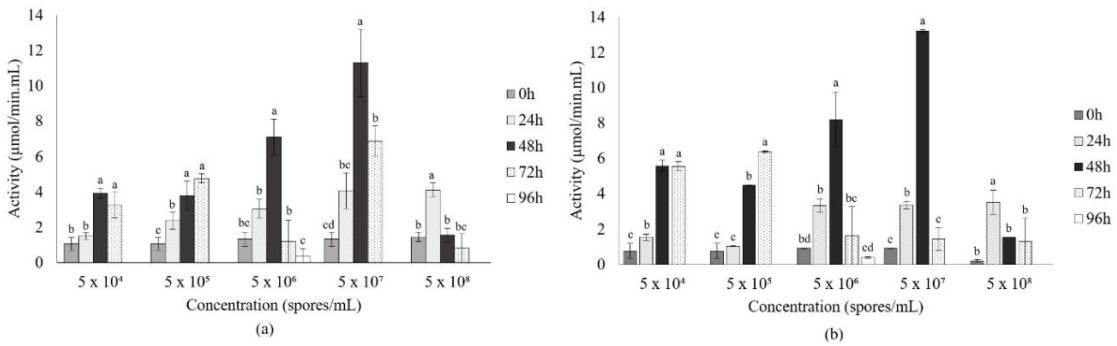

Figure 2. Production of fructosyltransferase (FTase) and β-fructofuranosidase (FFase) as a function of the spore concentration of the filamentous fungi *A. niger* ATCC 9642.

Ganaie *et al.* [31] evaluated the spore concentration in the production of β-frutofuranosidase from the filamentous fungi *Aspergillus flavus*. They obtained the best activities with spore concentrations ranging from $1x10^6$ to $1x10^8$ spores/mL, similar to the present study results.

Figure 3a shows the activity profiles of FTase and FFase using medium composed of 20 % sucrose, 0.5 % yeast extract, 1 % $NaNO_3$, 0.05 % $MgSO_4$.$7H_2O$, 0.25 % KH_2PO_4, 0.05 % NH_4Cl and 0.25 % NaCl, with $5x10^7$ spores/mL, 30°C, 150rpm, 48 h with different pHs (4.5, 5.5 and 6.5). The highest FTase activities were 9.53 and 7.64 µmol/min.mL and 10.62 and 11.67 µmol/min.mL at pH 5.5 and 6.5, respectively, with no statistical difference between them. From these results, it was defined to use for FTase and FFase bioproduction a pH of 5.5.

Figure 3b presents the results of the influence of temperature on FTase and FFase bioproduction, where the highest production of FTase (8.79 µmol/min.mL) and FFase (9.32 µmol/min.mL) was observed at 25°C, differing statistically from the other temperatures studied ($p < 0.05$). Thus, the bioproduction temperature was set at 25ºC.

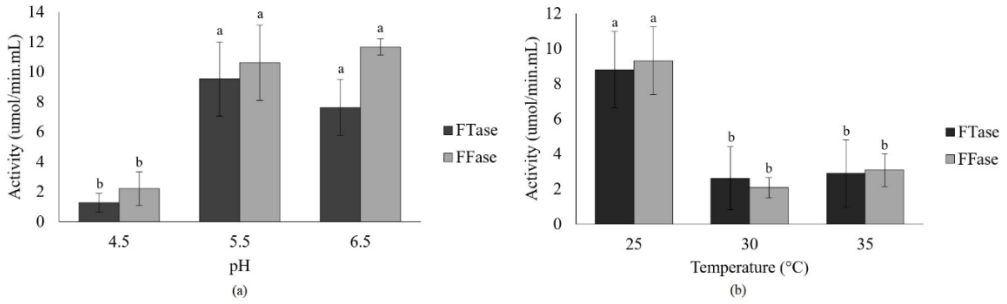

Figure 3. Production of FTase and FFase as a function of pH (a) and temperature (b) of the culture medium of filamentous fungi *A. niger* ATCC 9642.

3.2. Partial characterization of FTASE and FFASE.

The optimal temperature and pH values of the crude enzyme extract produced by *A. niger* ATCC 9642 in submerged cultivation under the maximized conditions (20% sucrose, 0.5% yeast extract, 1% $NaNO_3$, 0.05% $MgSO_4$.$7H_2O$, 0.25% KH_2PO_4, 0.05% NH_4Cl and 0.25% NaCl, with $5x10^7$ spores/mL, 25°C, 150rpm, 48 h, pH 5.5) were defined by complete factorial design 2^2 (3 central points) and are described in Table 3.

Table 3. Matrix of the complete factorial design 2^2 with coded (real) values and responses for FTase and FFase activity as a function of optimal temperature and pH.

Runs	*Independent variables		FTase (µmol/min.mL)	FFase (µmol/min.mL)
	X_1 pH	X_2 Temperature (°C)		
1	-1 (3.0)	-1 (45)	6.98	4.43
2	1 (6.0)	-1 (45)	3.41	4.63
3	-1 (3.0)	1 (75)	27.86	24.86
4	1 (6.0)	1 (75)	3.22	3.74
5	-1.41 (2.39)	0 (60)	27.30	28.68
6	1.41 (6.62)	0 (60)	6.28	7.33
7	0 (4.5)	-1.41 (38.45)	5.02	6.67
8	0 (4.5)	1.41(81.15)	3.28	4.59
9	0 (4.5)	0 (60)	10.72	9.32
10	0 (4.5)	0 (60)	12.97	10.55
11	0 (4.5)	0 (60)	11.00	9.41

*Independent variables: medium (20 % sucrose, 0.5 % yeast extract, 1 % $NaNO_3$, 0.05 % $MgSO_4$.$7H_2O$, 0.25 % KH_2PO_4, 0.5 % NH_4Cl, 0.25% NaCl), $5x10^7$ spores/mL, 150 rpm, 25°C, pH 5.5 and 48 h.

The activities for FTase and FFase were 27.86 µmol/min.mL and 24.86 µmol/min.mL and 27.30 µmol/min.mL and 28.68 µmol/min.mL at pH 3.0 and 2.39 and temperature of 75 and 60°C (run 3 and 5), respectively. Analyzing the variables independently, the decrease in

pH favors the increase of activity (run 3 -pH 3.0 and run 4 -pH 6.0). The temperature increase (run 1 - 45ºC and run 3 -75ºC) promotes this same effect.

It was found that run 5 at pH 2.39 and 60°C showed higher FPase activity (13.86%) than run 3 at pH 3.0 and 75°C. However, it was decided to continue the study with the conditions of run 5 and reduce energy expenditure.

Equations 7 and 9 present the second-order coded models that describe the activities of FTase and FTase as a function of the analyzed variables (temperature and pH) within the studied ranges, respectively. The models were validated by analysis of variance, which obtained correlation coefficients of 0.95 and 0.94 and calculated F of 2.07 and 1.44 times higher than the tabulated F value, which allowed the models' validation. Moreover, the construction of the contour curves presented in Figure 4.

$$FTase = 11.56 - 7.25\ pH + 2.60\ pH^2 + 2.28\ T - 3.75\ T^2 - 5.26\ pH.T \quad (8)$$

$$FFase = 9.77 - 6.40\ pH + 3.53\ pH^2 + 2.08\ T - 2.69\ T^2 - 5.33\ pH.T \quad (9)$$

Where UTF is the fructosyltransferase activity unit (FTase), UF is the β-frutofuranosidase activity unit (FFase), and T is the temperature (° C).

Based on the data obtained in Figure 4, it can be observed that the best pH value for both enzymes is around 2.39, and the temperature is in the range of 60 to 81.15°C. These results are of great importance. They demonstrate that these enzymes tolerate drastic pH and temperature conditions, thus being possible to apply in industrial processes that require these conditions.

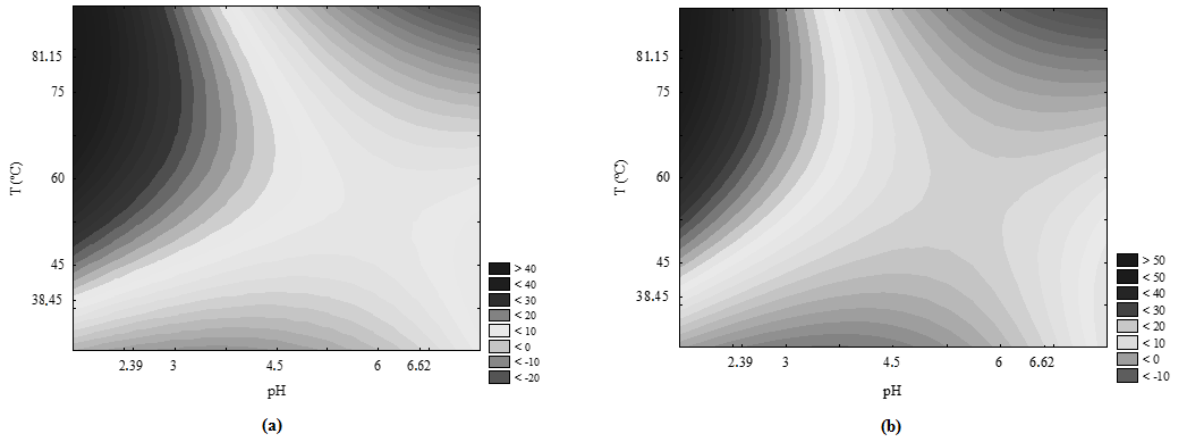

Figure 4. Contour curve for FTase (a) and FFase (b) activity as a function of optimum temperature (°C) and pH.

By correlating the optimal pH and temperature results of the FTase and FFase in the present study with literature data, a considerable variation in values can be observed, which may be related to the environmental and nutritional conditions of production and the strains of the microorganisms used in the study. Production of these enzymes, which may characterize enzymes with distinct characteristics. Milovanović *et al*. [35] evaluated the optimal inverted temperature produced by Saccharomyces cerevisiae observed optimum temperature was 60°C. Rustiguel *et al*. [36] studying *Aspergillus phoenicis* (*Aspergillus saitoi*) production of β-D-frutofuranosidase in submerged fermentation observed optimal temperature for activity at 65°C.

Maiorano *et al*. [37] indicate optimal pH in the range between 5 and 7 for the FTase from *Aspergillus oryzae*. Ganaie *et al*. [38] found that the enzyme, also produced by *Aspegillus oryzae*, showed high transfructylation activities for a pH range from 5.0 to 7.0, with peak activity at pH 6.0. Some studies have shown a more acidic potential, pH between 4.5 and 6.0,

in the invertases from *Lactobacillus reuteri* [39,40]. Industrially applied invertases such as Bioinvertr - Quest International - showed stability in the pH range from 4.0 to 5.0 [41].

Choukade & Kango [42] studied the characterization of fructosyltransferase from *Aspergillus tamarii* NKRC 1229 and obtained maximum activity at pH 7.0 and 20ºC. Rustiguel *et al*. [36] studying *Aspergillus phoenicis* (*Aspergillus saitoi*) production of β-D-frutofuranosidase (invertase) in submerged fermentation observed optimum temperature and pH for activity at 65°C and 4.5, respectively. Almeida *et al*. [43] studied the production of 2 intracellular inverts, where invertase 1 presented maximum activity at pH 3.0 and optimal temperature at 60°C, while invertase 2 presented maximum activity at pH 4.6 and optimal temperature at 55°C.

Figure 5a shows the inverse thermal stability, where FFase is stable at temperatures of 45 to 75°C, with a reduction of approximately 20% of residual activity at temperatures of 80 and 100°C. Already the FTase, when incubated at temperatures of 30 to 100ºC showed a decrease of about 50% of residual enzymatic activity compared to the initial activity (4ºC). The results show that FFase has higher thermal stability than FTase.

Stability at different pHs showed similar behavior for FTase and FFase (Figure 5b). The best pH was 2.00 with residual activity of 139.39 and 119.33% for FTase and FFase, respectively. At pH 3 the enzymes maintained their residual activity. At pHs 4,5, 6, and 7, there is a decrease in residual enzymatic activity of approximately 20 and 40% for FFase and FTase, respectively. The reaction medium's pH is one of the important factors for maintaining protein structure and enzyme activity. It directly interferes with the amino acid side chain ionization state [44]. Consequently, it indicates the possible industrial application of these enzymes.

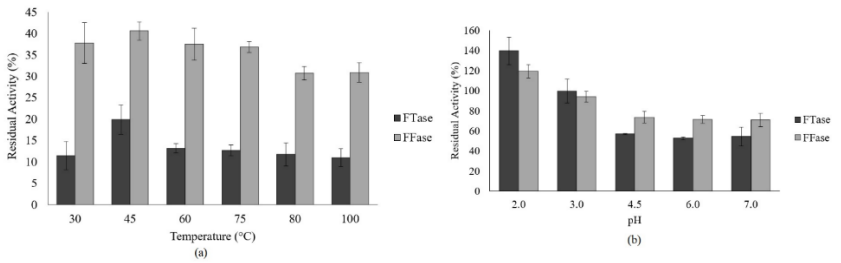

Figure 5. Thermal stability (a) and different pHs (b) of FTase and FFase produced by *A. niger* ATCC 9642.

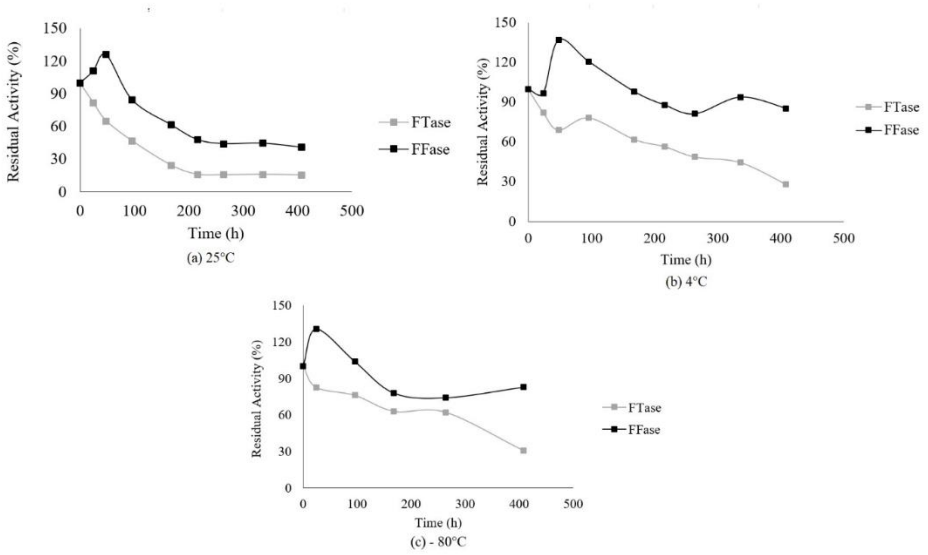

Figure 6. Storage stability of FTase and FFase enzymes at (a) ambient temperature (± 25°C), (b) refrigeration (± 4°C), and (c) freezing temperature (-80°C).

Storage stability results for FTase and FFase at room temperature, refrigeration, and freezing are shown in Figure 6a, b, and c, respectively, where it is observed that at room temperature (Figure 6a), the enzymes FTase and FFase showed a 50% reduction in residual activity at 95 h (4 days) and 200 h (8 days), respectively.

Storage at refrigeration temperature (Figure 6b) caused a 50% decrease in FTase residual activity at 168 h (6 days). In comparison, FFase maintained approximately 60% residual activity during the same period. By storing the enzymes at freezing temperature (Figure 6c), there was a 62% reduction in residual activity of FTase and 74% for FFase in 264 h (11 days), showing to be more stable to storage at a temperature environment and of refrigeration.

3.3. Kinetics analysis of sucrose hydrolysis.

The kinetic parameters K_m and V_{max} of FTase and FFase (Table 4), determined by Lineweaver-Burk graphs, were 24.60 mM and 104.16 μmol/min.mL for FTase and 3.91 mM and 20.24 μmol/min.mL for FFase, respectively. The lower values of K_m are proportional to the higher affinity of the enzymes for the substrate. Thus, the lowest Km of FFase (3.91 mM) for sucrose hydrolysis proved that FTase has a higher affinity for sucrose than FTase (24.60 mM).

Table 4. Kinetic parameters for FTase and FFase during sucrose hydrolysis.

	Equation	R^2	K_m (mM)*	V_{max} (μmol/min.mL)*
FTase	y = 0.2362x + 0.0096	0.99	24.60	104.16
FFase	y = 0.1935x + 0.0494	0.98	3.91	20.24

*Km=Michaelis-Menten constant; Vmax=maximum reaction rate.

Comparing the Km and Vmax data obtained in this study with the values cited in the literature, it is verified that the FFase and FTase present similar values. Lee *et al*. [45] observed that intracellular fructosyltransferase from *Aureobasidium pullulans* presented a K_m of 0.43 mM for sucrose substrate. According to Hirayma *et al*. [46], Fujita *et al*. [47], and Chang *et al*. [48], the purified extracellular β-fructofuranosidases from *Aspergillus niger* ATCC 20611, *Arthrobacter* sp. K-1 and *Aspergillus Oryzae* ATCC 76080 showed, respectively, the following K_m values for sucrose: 0.29 M, 9.1 mM, and 0.53 M. Hayashi *et al*. [49] reported that purified intracellular β-fructofuranosidase from *Aspergillus japonicus* MU-2 showed K_m and V_{max} values for sucrose of 210 mM and 0.65 μmol/ml/min, respectively. Milovanović *et al*. [35], when studying *Saccharomyces cerevisiae* invertase parameters, obtained K_m values of 28.4 mM and V_{max} of 4.5 mM/min.

3.4. Immobilization of enzymes in polyurethane (PU).

The immobilization's yield was defined as the relation between total initial activity, calculated considering the amount of free enzyme (crude enzyme extract) used during immobilization (0.5 mL), and the immobilized enzyme's activity (U_f), determined considering the total activity of the PU matrix containing the enzyme (11.81 g). U_0 and U_f values for free and immobilized enzymes are described in Table 5. It is possible to see that there was an increase in enzymatic activity when they were immobilized in the PU matrix, with yields of 6744.66 % for FFase and 3928.90 % for FTase and enzymatic activities of 364.79 and 220.32 U/g, respectively. These results suggest that the immobilization process improves enzymatic activity by increasing the enzyme's active site exposure due to the expansion of the PU foam

during the *in situ* immobilization process and covalent formation bonds between the enzyme and the PU foam.

Table 5. Effect of the initial enzyme concentration on enzymatic activity (U/g) and yield of the immobilization process (%).

Activity (U/g)	Enzymatic extract weight (g)	Detected activity (U)	Activity (U/g)	PU matrix weight (g)	Detected activity (U)	Yield (%)
Free FFase			**Immobilized FFase**			
62.01	3.09	191.78	364.79	35.46	12934.66	6744.66
Free FTase			**Immobilized FTase**			
64.30	3.09	198.86	220.34	35.46	7812.97	3928.90

According to the studies reported by Ficanha *et al*. [50], from the analysis of the immobilization of *Candida antarctica* B (CAL B) in silica matrix, there was an increase in enzyme activity after the immobilization process, which can be attributed to the better distribution of the enzyme in the support, as well as the greater exposure of the enzyme's active sites, which contribute positively to a later contact between the enzyme and the reaction medium, increasing the enzymatic activity.

3.5. Operational stability (reuse) of the immobilized enzymes.

The immobilized enzyme's operational stability is the key factor affecting the practical applications. The immobilized enzyme with improved stability is more favorable to the practical application com-pared to free enzymes. Figure 7 shows the data regarding the reuses of the immobilized enzymes, considering 50 % of the starting activity. It is possible to observe that achieving this value 4 and 3 cycles for FFase and FTase, respectively, were necessary. This is a considerable advantage since, even with reducing enzymatic activity after each cycle, it is still possible to save time and reduce costs, which is very attractive when considering an industrial application.

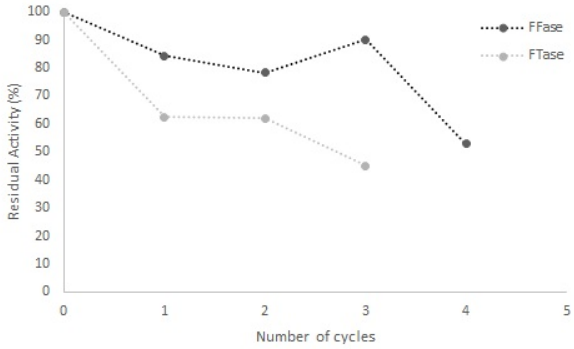

Figure 7. Operational stability (reuse) of the immobilized enzymes.

Immobilization in porous matrices such as calcium alginate or chitosan [51] of FOS producing enzymes has been extensively studied, for example, in the immobilization of the commercial enzyme of *Aspergillus aculeatus* in chitosan granules, where a potential for recyclability of the immobilized up to 8 cycles, when it presented 42.4% of residual activity [23].

The operational stability of *Candida antarctica* B (CAL B) assets in airgel matrix, reported by Ficanha *et al*. [50] ensured a potential for reuse between 8 and 12 cycles for the lipase esterification activity in immobilized form.

4. Conclusions

The data found during this study were positive regarding the extracellular production and immobilization of FTase and FFase produced by *A. niger*, as they possess good thermal stability, are active in acidic pH levels, and when immobilized, may be recycled, allowing their application in industrial processes in food science, chemistry, and medical fields.

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Conflicts of Interest

The authors declare no conflict of interest.

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