SCIENTIA PLENA
www.scientiaplena.org.br
VOL. 20, NUM. 01 2024
doi: 10.14808/sci.plena.2024.011501

Thermoacidophilic β-fructofuranosidase induced with soybean meal produced by a new *Aspergillus flavus* isolated from the Atlantic Forest

β-frutofuranosidase termoacidofílica induzida com farelo de soja produzido por um novo *Aspergillus flavus* isolado da Mata Atlântica

D. Andrades[1]; T. S. Inagaki[1]; L. A. L. Karg[1]; F. A. Y. Minomo[1]; N. S. Brandão[1]; C. Henn[2]; A. Maller[1]; J. L. C. Silva[1]; R. C. G. Simão[1]; M. K. Kadowaki[1]

[1]*Centro de Ciências Médicas e Farmacêuticas – Universidade Estadual do Oeste do Paraná R: Universitária, 2069, 85814-110, Cascavel-Paraná, Brazil*
[2]*Divisão de Reservatório – MARR.CD, Itaipu. Av. Tancredo Neves, 6731,85866-900, Foz do Iguaçu-Paraná, Brazil*

**marinakk@gmail.com*
(Recebido em 01 de março de 2023 aceito em 22 de janeiro de 2024)

High production of β-fructofuranosidase was obtained with the new fungus *Aspergillus flavus* isolated from the Atlantic Forest under solid state fermentation supplemented with soybean meal (53.36 U/ml). This beta-fructofuranosidase obtained after six days of cultivation with soybean meal was partially purified by DEAE-Sephadex ion exchange chromatographic columns, with a final yield of 19%, with an apparent molecular mass of 37 KDa by SDS-PAGE. The optimum pH and temperature for enzyme activity were 5.0 and 60 °C, respectively. The enzyme showed stability with 70% residual activity after 12 hours in the acid pH range (5.0 and 5.5), while at temperatures from 45 ºC to 55 ºC the enzyme showed stability greater than 50%. Therefore, the characteristics of this thermoacidophilic β-fructofuranosidase from *A. flavus* presents potential for use in several biotechnological processes.
Keywords: enzyme, filamentous fungus, purification.

Elevada produção de beta-frutofuranosidase foi obtida com o novo fungo *Aspergillus flavus* isolado da Mata Atlântica em cultivo sólido suplementados com farelo de soja (53,36 U/ml). Esta beta-frutofuranosidase obtida após 6 dias de cultivo com o farelo de soja foi parcialmente purificada por colunas cromatográficas de troca iônica DEAE-Sephadex, com rendimento final de 19%, com massa molecular aparente de 37 KDa por SDS-PAGE. O pH e a temperatura ótima de atividade da enzima foram de 5,0 e 60°C, respectivamente. A enzima exibiu estabilidade ao pH com 70% de atividade residual após 12 horas na faixa de pHs ácido (5,0 e 5,5), enquanto que nas temperaturas de 45ºC a 55ºC a enzima mostrou estabilidade superior a 50%. Portanto, as características dessa invertase termoacidofílica de *A. flavus* apresenta potencial para uso em diversos processos biotecnológicos.
Palavras-chave: enzima, fungo filamentoso, purificação.

1. INTRODUCTION

Beta-D-fructofuranoside fructohydrolase (FFase) (EC 3.2.1.26), commonly called invertase, is an enzyme that irreversibly hydrolyzes the α-1-β-2-glycosidic bonds of sucrose, producing glucose and fructose [1]. The mixture of these monosaccharides is called invert sugar, which has advantages in industrial applications with a sweetness index higher than sucrose, in addition to its products not crystallizing, ensuring a better texture to the food produced [1].

There are numerous isoforms of invertase, classified as acidic, neutral, or alkaline according to their optimal pH [2]. The acids are represented by the GH68 family, which are mostly bacterial, and by the GH32 family, which includes plant and fungal β-fructofuranosidases [1]. However, only alkaline and neutral invertase belong to the GH100 family [3].

Among microorganisms, the yeast *Saccharomyces cerevisiae* is the most extensively studied and is capable of synthesizing two invertases: a glycosylated and periplasmic invertase, and a cytosolic, non-glycosylated invertase that can be secreted or not [4]. There are other yeasts such as *Rhodotorula glutinis* [5] and *Candida utilis* [6] that also produce invertases. However, filamentous fungi also have the potential to produce invertases, such as those produced by species

of *Penicillium aurantiogriseum*, *P. citrinum*, *P. implicatum*, *P. citreonigrum*, *P. glabrum*, *P. islandicum* [7], *A. niger* [8], and *A. fijiensis* [9].

Fungal invertases have been commonly produced by submerged fermentation (SmF) and under solid-state (SSF). However, solid-state fermentation may have some advantages in comparison to SmF, such as: ease of obtaining extracellular enzymes and bioproducts, high volumetric productivity, and lower cultivation costs, due to the use of carbon sources of low commercial value, such as agro-industrial residues or by-products that are generally discarded in agricultural crops [10].

The main sources of carbon used to produce invertase in fungi or yeasts is sucrose, and some unconventional substrates, such as agro-industrial residues [11]. The use of residues from agro-industrial products such as sugarcane bagasse, molasses and food processing residues, fruit peels such as pineapple, pomegranate, and orange, have been used as invertase inducers [11,12,13]. The use of these residues is an attractive alternative since Brazil's economy has a very relevant agricultural base; in addition, the use of soy by-products in the cultivation of microorganisms is interesting since the country is the largest producer and exporter of soybeans in the world, with production of 123 million tons of soybeans in the years 2019–2020 [14]. Consequently, this large agro-industrial production is also responsible for the generation of large amounts of waste and by-products that can cause serious environmental problems [15]. In this context, this study aimed to produce, purify, and characterize a β-fructofuranosidase induced with soybean meal by a new *A. flavus* isolated from the Atlantic Forest of Paraná.

2. MATERIALS AND METHODS

2.1 Isolation and strain identification

The fungus was isolated from soil samples collected at the Bela Vista Biological Refuge, located (25°26'45" S; 54°35'42" W) in Foz do Iguaçu (State of Paraná- Brazil), and this fungus is part of the fungi collection at the State University of Western.

Strain identification was based on the isolation of the genomic DNA of the fungus [16] by analysis of the amplified products of the Internal Transcribed Spacer (ITS) regions of the extracted ribosomal DNA of the fungus [17], and the ITS sequence was deposited in the GenBank database under accession number KJ470626. The phylogenetic tree was built from sequences of regions of *A. flavus* and compared with sequences of other *A. flavus* fungal strains obtained from the NCBI database with the assistance of the Mega X Molecular Evolutionary Genetics Analysis) software [18] applying the Maximum Likelihood method and Jukes-Cantor model [19].

2.2 Cultivation conditions

The liquid medium used for enzyme production was composed of (grams per liter): yeast extract, 5; $(NH_4)_2SO_4$, 2.8; KH_2PO_4, 4; $MgSO_4 \cdot 7H_2O$, 0.9; $CaCl_2 \cdot 2H_2O$, 0.9. Liquid cultivation (under static or agitated conditions) was carried out in 250 ml Erlenmeyer flasks containing 25 ml of sterile medium supplemented with 1% alternative carbon source (banana peel, orange peel, passion fruit peel, walnut peel, pear peel, quinoa bran, soybean meal, rice straw, sorghum straw, and trub, or brewery residue). The agro-industrial residues/by-products were obtained from local agricultural fields and markets. For cultures in solid culture (SSF), 5.0 g of alternative carbon source was used, humidified with sterile mineral medium, and autoclaved at 121 °C. After inoculum of 1 ml of spore solution (10^5 spores/ml), cultures (liquid or solid) were incubated at 28 °C under stationary conditions for six days.

The submerged cultures were filtered after growth by vacuum pump, and the cell-free filtrate obtained was used after dialysis to determine the extracellular enzymatic activity. The crude extract from solid state cultivation was obtained by adding 40 mL of ice-cold distilled water, then stirred in a shaker at 120 rpm for 60 minutes at 4 °C. After this period, the cultures were filtered by vacuum pump, and the filtrate obtained was used after dialysis for enzymatic quantification. Liquid (agitated or stationary) and solid state cultures were carried out in two independent

experiments and the results were presented as the average of the repetitions ± SD. Significant differences between the means of enzymatic activities were analysed with one-way analysis of variance (ANOVA) followed by Tukey's test at the 5% level of significance ($p < 0.05$).

2.3 Enzymatic assay and protein quantification

The β-fructofuranosidase activity was carried out through the determination of reducing sugars with 3.5-dinitrosalicylic acid (DNS) according to Miller's [20] method. The reaction mixture for determining the enzymatic activity was composed of 50 µL of enzymatic extract and 50 µL of 0.2 M sucrose in sodium acetate buffer (50 mM pH 5.0) at 55 ºC for 10 min. This mixture was incubated at 50 °C for 10, 30, and 60 minutes, respectively and was stopped by adding 100 µL of DNS. The estimations of the reducing sugars were at 540 nm in a spectrophotometer, using glucose as a standard. The unit of enzymatic activity was defined as the amount of enzyme capable of releasing 1 µmol of reducing sugar per minute under the test conditions.

The protein concentration was determined using the Bradford method [21] and using bovine serum albumin as a standard.

2.4 Purification

The enzymatic crude extract equilibrated with 20 mM sodium acetate buffer with pH 5.5 was loaded onto the DEAE cellulose column (2 x 20 cm) previously equilibrated with the same buffer and eluted at a flow rate of 1.5 ml/min with a linear NaCl gradient (0.05–1.0 M). Three milliliters of each fraction were collected and subsequently the protein (A_{280}) and enzymatic activity were measured. The fractions that showed invertase activity were pooled, dialyzed in distilled water at 4 °C for 18 h, and loaded onto a second column of DEAE Sephadex equilibrated with the same buffer. The enzyme was eluted with a gradient of 0.05–0.8 M NaCl, and tubes containing β-fructofuranosidase activity were pooled, dialyzed, and lyophilized for analysis by electrophoresis and enzymatic characterization.

2.5 Polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram

The sample obtained after two columns of DEAE was analyzed by SDS-PAGE as described by Laemmli (1970) [22] at 10% acrylamide concentration. Electrophoresis was performed in 0.025 M Tris-HCl, 0.19 M glycine, and 0.1% SDS buffer, pH 8.3, under a current of 40 mA and 120V. After the electrophoretic run by SDS-PAGE, the gel was divided into two parts. A part containing the samples and molecular marker was stained according to the method described by Blum et al. (1987) [23] to verify the protein profile. The following proteins were used as molecular mass markers: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4kDa). The other part of the gel was analyzed for invertase activity according to Liao et al. (2012) [24] with some modifications. The gel was incubated for 30 min in 0.5% (v/v) Triton X-100 at room temperature. After washing in distilled water, the gel was incubated in a 0.3 M sucrose solution dissolved in 50 mM sodium acetate buffer, pH 4.5, for 30 minutes at 40 °C and developed with 0.5% triphenyl tetrazolium chloride dissolved.

2.6 Effect of pH and temperature on enzyme activity

The effect of pH on enzyme activity was determined by incubating the enzyme with different buffers: McIlvaine (pH 2.2–8.0), Tris-HCl (pH 8.5–9.0), and carbonate-bicarbonate (pH 9.5–10.0). The enzyme was pre-incubated in an ice bath in buffer solutions for up to 12 hours. Residual activity was determined using enzyme assay conditions.

To evaluate the optimum temperature of enzyme activity, the assay was conducted at various incubation temperatures ranging from 30 °C to 80 °C. The thermal stability of the enzyme was determined through pre-incubation of the enzyme at different temperatures ranging from 45 ºC to 65 ºC, and aliquots of the incubated enzyme were removed and measured.

2.7 Reproducibility of results

All experiments were performed in triplicate and the results obtained are an average of these three independent experiments.

3. RESULTS AND DISCUSSION

Strain identification

The fungus *A. flavus* isolated from the Atlantic Forest of Western Paraná, Brazil, was taxonomically identified by sequencing the ITS regions of the fungus' ribosomal DNA, and the 529 bp sequence showed 100% similarity with other species of *A. flavus* as shown in the phylogenetic tree of Figure 1. This sequence is deposited and available under accession KJ470626 in the NCBI database.

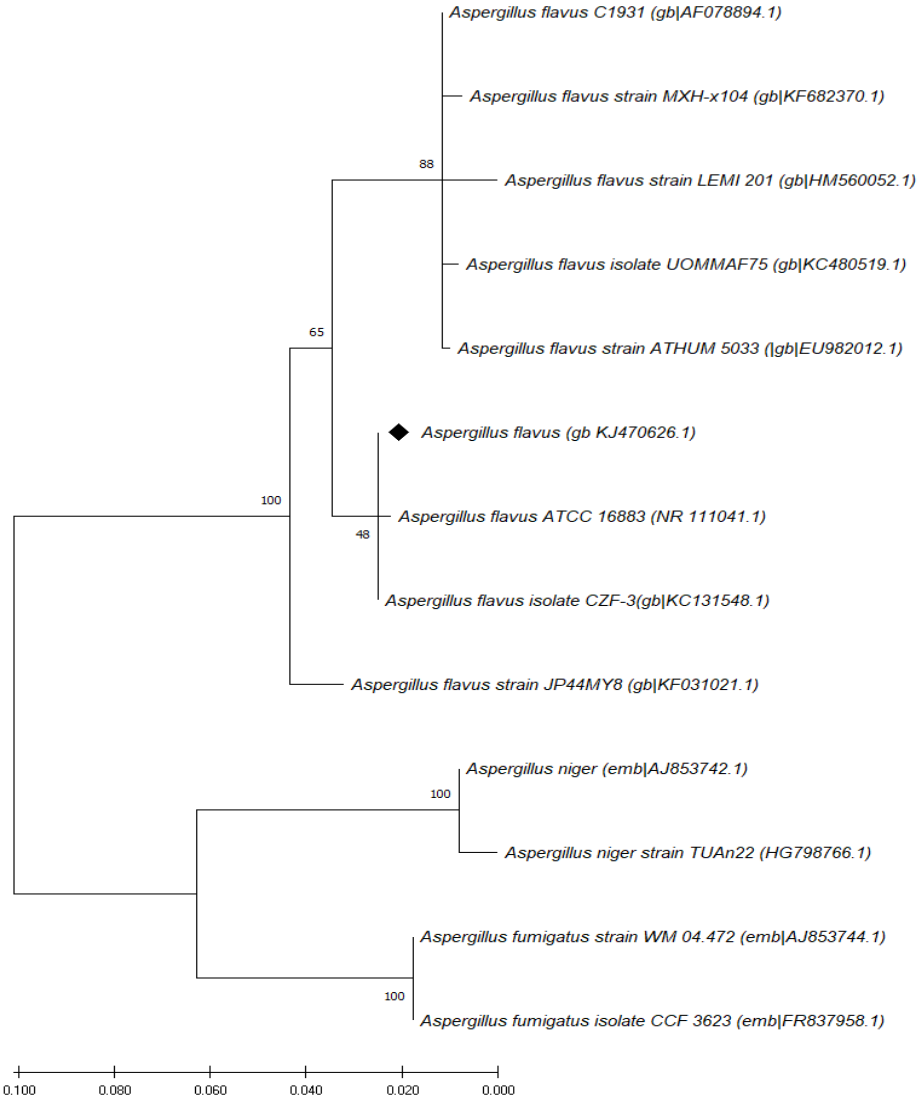

Figure 1. Phylogenetic tree from the sequence of the ITS1, 5.8S, and ITS2 regions of Aspergillus flavus KJ470626.1 in comparison with 12 sequences of others A. flavus strains obtained from the NCBI database.

Influence of carbon sources in liquid and solid cultures for β-fructofuranosidase production

The highest values of β-fructofuranosidase activity were obtained in solid state cultivation compared to liquid cultivations (agitated or stationary) influenced by carbon sources (Table 1). Among all carbon sources, soybean meal was the best inducer of the enzyme regardless of the type of cultivation, with 53.36 U/ml, 12.71 U/mL and 5.39 U/mL under solid, agitated and stationary cultivation, respectively. Soybean meal is an agro-industrial co-product that contains 7.4% soluble sucrose sugar [25], which possibly induced β-fructofuranosidase production by *A. flavus* (table 1). There are still reports of invertases that were produced by the fungi using soy bean (13.7 U/mL) and *Aspergillus carbonarius* with pineapple crown (6.71 U/mL) as inducing sources [26]. Invertase production under solid state fermentation has also been reported with *A. flavus* using fruit peel as a carbon source [27].

Trub also exhibited high activities with (32.04 U/ml in solid culture) and (6.12 U/mL in agitated culture). Fruit peels such as pear and orange were also effective in inducing the enzyme with 16.57 U/mL and 13.05 U/mL, respectively. These results are interesting, considering that most of the carbon sources used for the production of microbial invertase have been sucrose [14,27], thus, the use of agroindustry by products as an alternative source of carbon to replace pure carbohydrates such as sucrose for the production of β-fructofuranosidase makes the fungus *A. flavus* a promising candidate for the production of this enzyme.

Table 1 - Influence of carbon sources on the induction of enzymes of the β-fructofuranosidase by Aspergillus flavus grown under solid-state, stationary and agitated liquid cultivation.

Carbon sources	Enzymatic activity (U/mL)		
	Solid –state cultivation	Stationary – Liquid cultivation	Agitated Liquid cultivation
Orange peel	13.05 ± 0.33^{D}	2.24 ± 0.02^{F}	2.64 ± 0.23^{E}
Banana peel	12.01 ± 0.56^{D}	2.29 ± 0.33^{EF}	2.71 ± 0.23^{E}
Pear peel	16.57 ± 0.66^{C}	0.01 ± 0.01^{G}	1.14 ± 0.01^{F}
Passion fruit peel	12.37 ± 0.55^{D}	3.62 ± 0.09^{CD}	4.21 ± 0.41^{CD}
Quinoa meal	5.52 ± 0.26^{E}	3.19 ± 0.01^{DE}	3.56 ± 0.14^{DE}
Soybean meal	53.36 ± 1.48^{A}	5.39 ± 0.64^{A}	12.71 ± 0.56^{A}
Rice straw	1.63 ± 0.03^{F}	0.98 ± 0.007^{G}	2.45 ± 0.11^{E}
Trub	32.04 ± 1.20^{B}	4.60 ± 0.07^{AB}	6.12 ± 0.50^{B}
Walnut shell	1.87 ± 0.14^{F}	0.42 ± 0.02^{G}	0.59 ± 0.01^{F}
Sorghum straw	8.26 ± 0.28^{E}	4.38 ± 0.05^{BC}	5.13 ± 0.05^{BC}

Means followed by different letters in each column differ significantly between carbon sources using the Tukey test ($p<0.05$).

Purification of β-fructofuranosidase

The crude enzymatic extract of *A. fl*avus was loaded onto two DEAE-cellulose and Sephadex chromatographic columns and the invertase eluted at a concentration of 175 and 225 mM NaCl gradient (Figure 2A and 2B). After purification, invertase had a yield of 19.24% and specific activity of 69.66 U/mg protein (Table 2). The enzyme was partially pure by analysis on 10% SDS-PAGE (Figure 3), with an apparent molecular mass of 45 kDa. This result shows a lower molecular mass than the invertases of *Aspergillus versicolor* with 75 kDa [28] *A. ochraceus* with 79 KDa [29], *Pichia anomala* with 86.5 kDa [30], and *Fusarium solani* with 65 kDa [31]. *Cunninghamella echinulata* had 89.2 kDa, and *A. niveus* invertase had approximately 37 kDa [32, 33].

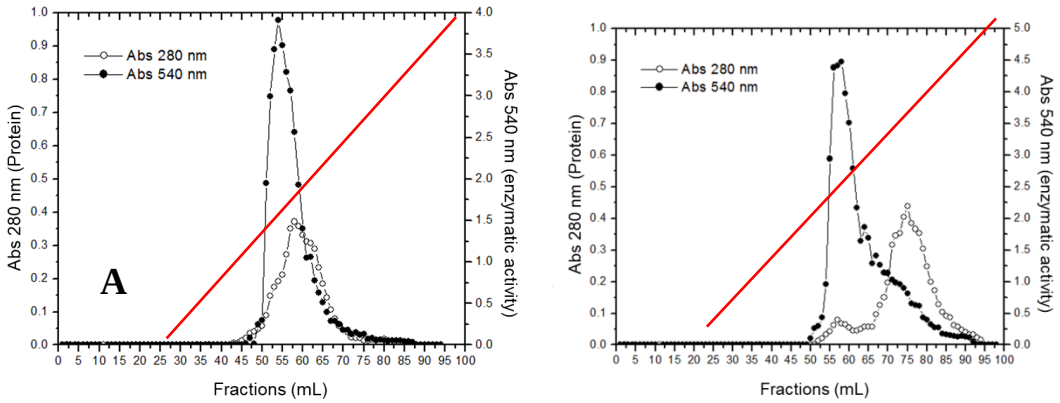

Figure 2. Chromatographic profile of β-fructofuranosidase from Aspergillus flavus in DEAE cellulose (A) and DEAE sephadex (B) equilibrated with 20 mM sodium acetate buffer (pH 5.5), eluted with NaCl (0-1M). Symbols: (○) enzymatic activity; (●) proteins at 280nm.

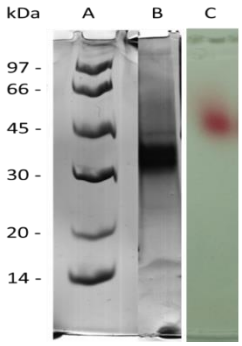

*Figure 3. SDS-PAGE of the β-fructofuranosidase from Aspergillus flavus. **(A)** SDS-PAGE molecular weight standard ladder shown as kDa. Phosphorylase b (97kDa), Albumin (66 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (30 kDa), Trypsin inhibitor (20.1kDa), and α-lactalbumin (14.4 kDa); **(B)** SDS-PAGE fractions eluted from the ion-exchange DEAE-Sephadex chromatography column; **(C)** Zymogram of the β-fructofuranosidase.*

Table 2. Purification of the β-fructofuranosidase from Aspergillus flavus.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (Umg^{-1})	Yield (%)	Purification factor
Crude extract	1934	2011	0.96	100	1.0
DEAE-Cellulose	682	191	3.57	35.26	3.72
DEAE-Sephadex	372.7	5.35	69.66	19.24	72.56

Influence of pH and temperature

The β-fructofuranosidase produced by *A. flavus* exhibited an optimum pH of 5.0 (Figure 4A), demonstrating great similarity to the β-fructofuranosidases produced by *S. cerevisiae* [34], *Aureobasidium pullulans* [35], *Chrysonilia sitophila*, [36], and *Fusarium* sp. [37]. In contrast, strains such as *Saccharomyces cerevisiae* [38] showed an optimum pH 6.0, *A. niger* at pH 7.5 [39].

The enzyme retained its residual activity above 70% for 12 hours at both pH 5.0 and pH 5.5, demonstrating high stability at these pHs (Figure 4B). At pH 4.5 the half-life was approximately 9 hours, while at pH 6.0 and 6.5 the half-life was 3 hours.

The optimal temperature of β-fructofuranosidase was 60 °C (Figure 4C), similar to the invertases of *A. niveus* [40], *A. ochraceus* [29], *A. caespitosus* [41], and *Cunninghamella echinulata* [32], and considerably higher compared to *A. niger*'s invertase [39] of 30 °C. A

characteristic of an invertase with a high optimum temperature is the possibility of using it in industrial processes that demand higher temperatures [33].

Aspergillus flavus β-fructofuranosidase maintained more than 60% of its enzymatic activity after 12 hours of incubation at 45 ºC, while at temperatures of 50 ºC and 55 ºC the stability was approximately 50%. However, at temperatures above 60 ºC the half-life was 2 hours (Figure 4D). This characteristic exhibited by the enzyme is advantageous for industrial applications in which the thermal catalytic stability of approximately 50 °C presents potential for use in the manufacture of cosmetic plasticizing agents, in the production of enzymatic electrodes for sucrose detection, in the paper and pharmaceutical industries [42].

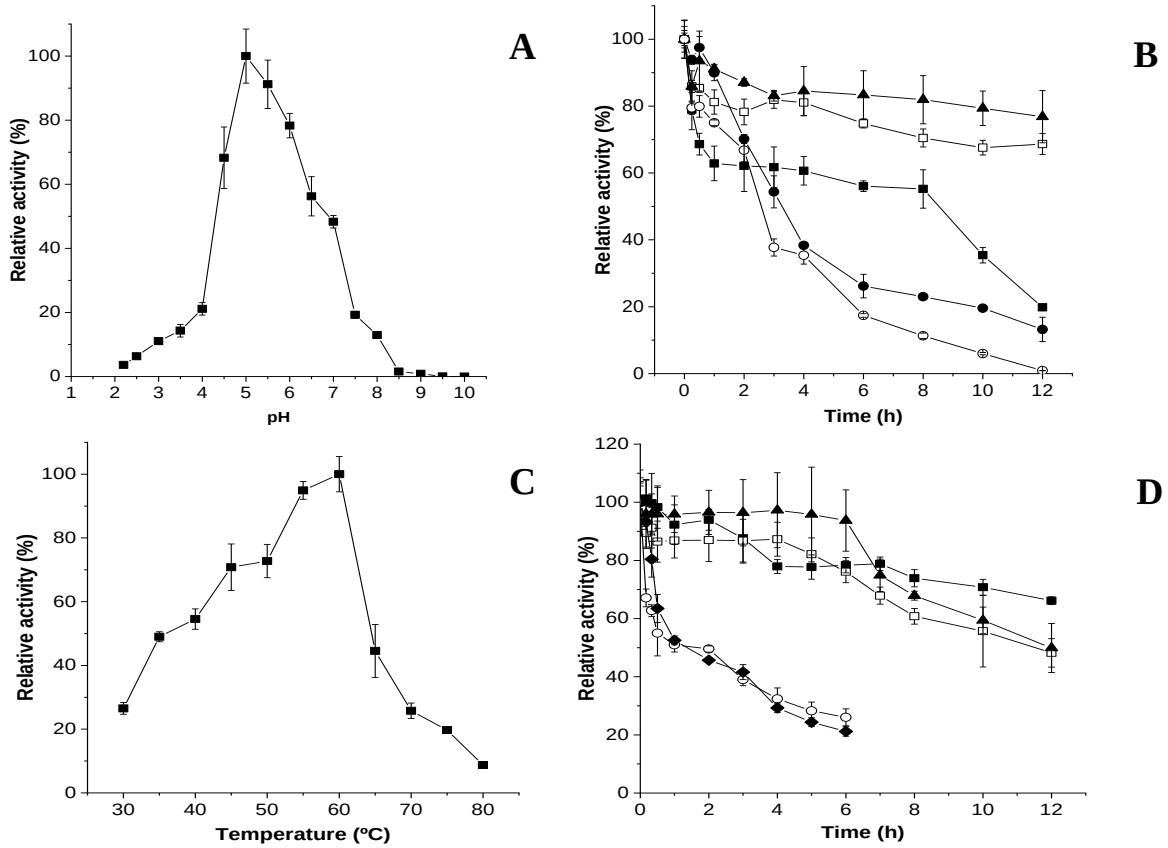

Figure 4. Effect of pH and temperature on β-fructofuranosidase activity and stability. ***(A)*** *The effect of pH on activity, at 55 °C;* ***(B)*** *stability at pH values of 4.5 (-■-), 5.0 (-□-), 5.5 (-▲-), 6.0 (-●-), and 6.5 (-○-) for 12 h, with 100% of residual activity corresponding to the corresponding to the initial time;* ***(C)*** *the effect of temperature on activity was determined with sucrose as the substrate, at pH 5.0;* ***(D)*** *stability at temperatures of 45 °C (-■-), 50 °C (-□-), 55 °C (-▲-), 60 °C (-○-), and 65 °C (-♦-). Residual activity was calculated in relation to the initial enzyme activity. Results were expressed as the average of triplicate assays ± the standard error of the mean.*

4. CONCLUSION

The wild fungus *A. flavus* is a strain isolated from the Atlantic Forest that has shown promise for producing β-fructofuranosidase using soybean meal as inducer an alternative and low-cost carbon source widely produced in Brazil that can be used to produce an enzyme of industrial interest. Thus, this new *A. flavus* isolate proved to be a promising candidate capable of producing thermoacidophilic β-fructofuranosidase with potential use in several biotechnological processes.

5. ACKNOWLEDGEMENTS

D. Andrades and T. S. Inagaki are recipient of fellowship from Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES)-Brazil, F. A. Y. Minomo is recipient of fellowship Fundação Araucária, respectively. The authors acknowledge the financial support and infrastructure from CAPES and Unioeste, respectively.

6. REFERENCES

1. Toledo LET, García DM, Cruz EP, Intriago LMR, Pérez JN, Chanfrau JMP. Fructosyltransferases and invertases: Useful enzymes in the food and feed industries. Enzymes in Food Biotechnology. 2019;451-69. doi: 10.1016/B978-0-12-813280-7.00026-8
2. Lincoln L, More SS. Screening and enhanced production of neutral invertase from *Aspergillus* sp. by utilization of Molasses – A by-product of Sugarcane industry. Adv Biores. 2017;8(4):103-10. doi: 10.15515/abr.0976-4585.8.4.103110
3. Lammens W, Le Roy K, Schroeven L, Van Laere A, Rabijns A, Van Den Ende W. Structural insights into glycoside hydrolase family 32 and 68 enzymes: Functional implications. J Exp Bot. 2009;60(3):727-40. doi: 10.1093/jxb/ern333
4. Kar B, Verma P, den Haan R, Sharma AK. Effect of N-linked glycosylation on the activity and stability of a β-glucosidase from *Putranjiva roxburghii*. Int J Biol Macromol. 2018; 112:490-8. doi: 10.1016/j.ijbiomac.2018.01.201
5. Canli O, Erdal S, Taskin M, Kurbanoglu EB. Effects of extremely low magnetic field on the production of invertase by *Rhodotorula glutinis*. Toxicol Ind Health. 2011;27(1):35-9. doi: 10.1177/0748233710380219
6. Lahiri S, Basu A, Sengupta S, Banerjee S, Dutta T, Soren D, et al. Purification and characterization of a trehalase-invertase enzyme with dual activity from *Candida utilis*. Arch Biochem Biophys. 2012;522(2):90-9. doi: 10.1016/j.abb.2012.03.026
7. Nascimento AKC, Nobre C, Cavalcanti MTH, Teixeira JA, Porto ALF. Screening of fungi from the genus *Penicillium* for production of β- fructofuranosidase and enzymatic synthesis of fructooligosaccharides. J Mol Catal B Enzym. 2016; 134:70-8. doi: 10.1016/j.molcatb.2016.09.005
8. Zhang J, Wang L, Luan C, Liu G, Liu J, Zhong Y. Establishment of a rapid and effective plate chromogenic assay for screening of *Aspergillus* species with high β-fructofuranosidase activity for fructooligosaccharides production. J Microbiol Methods. 2019; 166:105740. doi: 10.1016/j.mimet.2019.105740
9. Coetzee G, Smith JJ, Görgens JF. Influence of codon optimization, promoter, and strain selection on the heterologous production of a β-fructofuranosidase from *Aspergillus fijiensis* ATCC 20611 in Pichia pastoris. Folia Microbiol (Praha). 2022;67(2):339-50. doi: 10.1007/s12223-022-00947-8
10. Guerrero-Urrutia C, Volke-Sepulveda T, Figueroa-Martinez F, Favela-Torres E. Solid-state fermentation enhances inulinase and invertase production by *Aspergillus brasiliensis*. Process Biochem. 2021; 108:169-175. doi: 10.1016/j.procbio.2021.06.014.
11. Veana F, Martínez-Hernández JL, Aguilar CN, Rodríguez-Herrera R, Michelena G. Utilization of molasses and sugar cane bagasse for production of fungal invertase in solid state fermentation using *Aspergillus niger* GH1. Brazilian J Microbiol. 2014;45(2):373-7. doi: 10.1590/S1517-83822014000200002
12. Uma C, Gomathi D, Muthulakshmi C, Gopalakrishnan VK. Production, purification and characterization of invertase by *Aspergillus flavus* using fruit peel waste as substrate. Adv Biol Res (Rennes). 2010;4(1):31-6.
13. Nadeem H, Rashid MH, Siddique MH, Azeem F, Muzammil S, Javed MR, et al. Microbial invertases: A review on kinetics, thermodynamics, physiochemical properties. Process Biochem. 2015 Aug;50(8):1202-10.
14. United States Department of Agriculture (USDA). Oilseeds: World Markets and trade [Internet]; 2020 [cited 2022 Dec 10]. Available from: https://usda.library.cornell.edu/concern/publications/tx31qh68h?locale=en
15. Gonçalves FA, Dos Santos ES, De Macedo GR. Use of cultivars of low cost, agroindustrial and urban waste in the production of cellulosic ethanol in Brazil: A proposal to utilization of microdistillery. Renew Sustain Energy Rev. 2015;50:1287-303. doi: 10.1016/j.rser.2015.05.047
16. Oriente A, Tramontina R, De Andrades D, Henn C, Silva JLC, Simão RCG, et al. Characterization of a novel *Aspergillus niger* beta-glucosidase tolerant to saccharification of lignocellulosic biomass products and fermentation inhibitors. Chem Pap. 2015;69(8):1050-7. doi: 10.1515/chempap-2015-0111

17. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315-322 In: PCR Protocols: A guide to methods and applications. eds. Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White. Academic Press, Inc., New York. (1990). http://dx.doi.org/10.1016/b978-0-12-372180-8.50042-1
18. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018;35(6):1547-9.
19. Jukes TH, Cantor CR. Evolution of Protein Molecules. In: Munro, H.N., Ed., Mammalian Protein Metabolism, Academic Press, New York, 21-132. http://dx.doi.org/10.1016/B978-1-4832-3211-9.50009-7.
20. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 1959;31(3):426-8. doi: 10.1021/ac60147a030
21. Bradford MM. A Rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein- dye binding. Anal Biochem. 1976; 72:248-54. doi: 10.1016/0003-2697(76)90527-3
22. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature. 1970; 227:680-5. doi: 10.1038/227680a0
23. Blum H, Beier H, Gross HJ. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis. 1987;29:93-9. doi: 10.1002/elps.1150080203
24. Liao H, Xu C, Tan S, Wei Z, Ling N, Yu G, et al. Production and characterization of acidophilic xylanolytic enzymes from *Penicillium oxalicum* GZ-2. Bioresour Technol. 2012; 123:117-24. doi: 10.1016/j.biortech.2012.07.051
25. Medic J, Atkinson C, Hurburgh CR. Current knowledge in soybean composition. JAOCS, J Am Oil Chem Soc. 2014;91(3):363-84. doi: 10.1007/s11746-013-2407-9
26. Nascimento GC, Batista RD, Do Amaral Santos CCA, Da Silva EM, De Paula FC, Mendes DB, et al. β-Fructofuranosidase and β -D-Fructosyltransferase from New *Aspergillus carbonarius* PC-4 strain isolated from canned peach syrup: Effect of carbon and nitrogen sources on enzyme production. Sci World J. 2019:1-13. doi: 10.1155/2019/6956202
27. Gopalakrishnan VK, Uma C, Gomathi D, Muthulakshmi C. Production, purification and characterization of invertase by *Aspergillus flavus* using fruit peel waste as substrate. Adv Biol Res (Rennes). 2010;4(1):31-6.
28. Dapper TB, Arfelli VC, Henn C, Regina SM, Dos Santos MF, Torre CL Della, et al. -Fructofuranosidase production by *Aspergillus versicolor* isolated from Atlantic forest and grown on apple pomace. African J Microbiol Res. 2016;10(25):938-48.
29. Guimarães LHS, Terenzi HF, Polizeli MLTM, Jorge JA. Production and characterization of a thermostable extracellular β-d-fructofuranosidase produced by *Aspergillus ochraceus* with agroindustrial residues as carbon sources. Enzyme Microb Technol. 2007;42(1):52-7. doi: 10.1016/j.enzmictec.2007.07.021
30. Rodriguez J, Perez JA, Ruiz T, Rodriguez L. Characterization of the invertase from *Pichia anomala*. Biochem J. 1995;306(1):235-9. doi: 10.1042/bj3060235
31. Bhatti GN, Asgher M, Abbas A, Naax R, Sheikh MA. Studies on kinetics and thermostability of a novel acid invertase from *Fusarium solani*. J Agric Food Chem. 2006;54(13):4617-23. doi: 10.1021/jf053194g
32. Rasbold LM, Heinen PR, Da Conceição Silva JL, De Cássia Garcia Simão R, Kadowaki MK, Maller A. *Cunninghamella echinulata* PA3S12MM invertase: Biochemical characterization of a promiscuous enzyme. J Food Biochem. 2021;45(4):1-9. doi: 10.1111/jfbc.13654
33. Fernandes MLP, Jorge JA, Guimarães LHS. Characterization of an extracellular β-d-fructofuranosidase produced by *Aspergillus niveus* during solid-state fermentation (SSF) of cassava husk. J Food Biochem. 2017;42(1):1-10. doi: 10.1111/jfbc.12443
34. David AE, Wang NS, Yang VC, Yang AJ. Chemically surface modified gel (CSMG): An excellent enzyme-immobilization matrix for industrial processes. J Biotechnol. 2006;125(3):395-407. doi: 10.1016/j.jbiotec.2006.03.019
35. Yoshikawa J, Amachi S, Shinoyama H, Fujii T. Purification and some properties of β-fructofuranosidase I formed by *Aureobasidium pullulans* DSM 2404. J Biosci Bioeng. 2007;103(5):491-3. doi: 10.1263/jbb.103.491
36. Patil PR, Reddy GSN, Sulochana MB. Production, optimization and characterization of β-fructofuranosidase by *Chrysonilia sitophila* PSSF84—A novel source. Indian J Biotechnol. 2011;10:56-64.
37. Bhatti GN, Asgher M, Abbas A, Naax R, Sheikh MA. Studies on kinetics and thermostability of a novel acid invertase from *Fusarium solani*. J Agric Food Chem. 2006;54(13):4617-23.

38. Bhalla TC, Bansuli T, Savitri N. Thakur N. Invertase of *Saccharomyces cerevisiae* SAA-612: Production, Characterization and Application in Synthesis of Fructo-Oligosaccharides. LWT— Food Sci Technol. (2017);77:178-85. doi: 10.1016/j.lwt.2016.11.034
39. Ire FS, Aguguo VJ, Ezebuiro V. Optimization of invertase from *Aspergillus niger* grown on low cost agricultural wastes by response surface methodology (RSM). J Adv Microbiol. 2018;13(4):1-15. doi: 10.9734/JAMB/2018/45480
40. Guimarães LHS, Somera AF, Terenzi HF, Polizeli MLTM, Jorge JA. Production of β-fructofuranosidases by *Aspergillus niveus* using agroindustrial residues as carbon sources: Characterization of an intracellular enzyme accumulated in the presence of glucose. Process Biochem. 2009;44(2):237-41. doi: 10.1016/j.procbio.2008.10.011
41. Alegre ACP, Polizeli MLTM, Terenzi HF, Guimarães LHS. Production of thermostable invertases by *Aspergillus caespitosus* under submerged or solid state fermentation using agroindustrial residues as carbon source. Brazilian J Microbiol. 2009;55(40):612-22. doi: 10.1590/S1517-83822009000300025
42. Kotwal SM, Shankar V. Immobilized invertase. Biotechnol Adv. 2009;27(4):311-22. doi: 10.1016/j.biotechadv.2009.01.009