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

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

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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 45 ºC a 55 ºC 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; (NH₄)₂SO₄, 2.8; KH₂PO₄, 4; MgSO₄·7H₂O, 0.9; CaCl₂·2H₂O, 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 (10⁵ 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 °C 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 (14.4 kDa). 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 °C to 65 °C, 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 other *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>
<thead>
<tr>
<th>Carbon sources</th>
<th>Enzymatic activity (U/mL)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Solid –state cultivation</td>
</tr>
<tr>
<td>Orange peel</td>
<td>13.05±0.33D</td>
</tr>
<tr>
<td>Banana peel</td>
<td>12.01±0.56D</td>
</tr>
<tr>
<td>Pear peel</td>
<td>16.57±0.66C</td>
</tr>
<tr>
<td>Passion fruit peel</td>
<td>12.37±0.55D</td>
</tr>
<tr>
<td>Quinoa meal</td>
<td>5.52±0.26E</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>53.36±1.48A</td>
</tr>
<tr>
<td>Rice straw</td>
<td>1.63±0.03F</td>
</tr>
<tr>
<td>Trub</td>
<td>32.04±1.20B</td>
</tr>
<tr>
<td>Walnut shell</td>
<td>1.87±0.14F</td>
</tr>
<tr>
<td>Sorghum straw</td>
<td>8.26±0.28E</td>
</tr>
</tbody>
</table>

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. flavus 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.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (Umg⁻¹)</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1934</td>
<td>2011</td>
<td>0.96</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>DEAE-Cellulose</td>
<td>682</td>
<td>191</td>
<td>3.57</td>
<td>35.26</td>
<td>3.72</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>372.7</td>
<td>5.35</td>
<td>69.66</td>
<td>19.24</td>
<td>72.56</td>
</tr>
</tbody>
</table>

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 sitophil[a, [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. caesito[osus [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 ºC 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].

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.
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6. REFERENCES


