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Production of β-fructofuranosidase with transfructosylating activity by *Aspergillus tamarii* URM4634 Solid-State Fermentation on agroindustrial by-products

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ABSTRACT

Eight different *Aspergillus* strains were tested for their ability to produce β-fructofuranosidase (FFase) by Solid-State Fermentation. The *Aspergillus tamarii* URM4634 strain was selected as the most performant and tested on six different agroindustrial by-products. Soy, wheat and oat brans, which allowed for the highest hydrolytic (U_H) and transfructosylating (U_{TF}) activities, were tested individually or in mixtures according to a simplex-centroid mixture design in order to investigate their effects on FFase production at different times. The best results in terms of both enzyme activities were obtained with only soy bran. The influence of substrate, moisture and sucrose levels on FFase production was evaluated, and the highest U_H and U_{TF} activities were 229.43 ± 4.88 and 66.93 ± 3.02 U/mL, respectively. The obtained results indicate that *A. tamarii* FFase may be a biocatalyst with great potential for industrial applications such as sugar inversion and fructo-oligosaccharides production.

1. Introduction

β-fructofuranosidase (FFase, EC 3.2.1.26), also named invertase, is an enzyme widely used in food industry that catalyzes the hydrolysis of sucrose β-2,1 glycosidic bonds, thereby producing invert sugar, an equimolar mixture of D-glucose and D-fructose [1]. FFases are included in the GH32 family of glycosyl hydrolases and classified in different isoforms, based on their pH of action, as acid, alkaline and neutral enzymes [2]. The main application of FFases is the production of sugar syrups such as high fructose syrup, high fructose corn syrup and high glucose syrup, which are extensively used in food industry to prepare creams, marshmallows, powder milk for infants, liquefied sugar-containing candies, chocolate covered cherries, digestive aid tablets and artificial honey, as well as in cosmetics as plasticizing agent [3,4]. Glucose obtained by sucrose hydrolysis can be used as natural osmolyte in several applications [5].

At high sucrose concentration, some FFases are able to catalyze the transfer of fructosyl residues to an acceptor compound to produce fructo-oligosaccharides (FOS) by two ways: reverse hydrolysis and transfructosylation [6]. FOS are prebiotic substances consisting of fructose units polymerized to different extent that display several biological and functional properties [7], among which 1-kestose, 1-nystose and 1-fructofuranosyl-nystose are the best known. Some benefits associated with FOS ingestion are stimulation of probiotics growth in the intestinal tract, enhancement of calcium and magnesium absorption, reduction of total cholesterol and prevention of colonic carcinogenesis [8,9].

Most FFases with high transfructosylating activity have been found in fungi belonging the *Aureobasidium* [10], *Penicillium* [2,11] and *Aspergillus* [12] genera. They can be produced by either Submerged (SmF) or Solid-State (SSF) Fermentation. The latter is a process that uses a solid matrix, which is carried out in the absence or a very limited quantity of free water; however, the substrate must possess enough moisture to support microbial growth and metabolism [13]. SSF is considered the most appropriate process when using fungi, because solid substrates resemble their natural habitat, hence improving their growth and secretion of a wide range of extracellular enzymes [14]. An important advantage of SSF is associated with the use of agroindustrial by-products such as seeds, peels, husks and brans as substrates to produce valuable bioactive molecules, which provides economic feasibility and environmental friendliness to the entire process [15,16].

Enzyme production by SSF was extensively studied usually employing only one substrate and techniques of Design of Experiments

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(DOE) to optimize the influencing variables. However, the use of statistical mixture designs to evaluate different substrate formulations for SSF is missing, as far as we are aware, when aiming at the production of FFases or other enzymes to synthesize FOS such as fructosyltransferases or inulinases. Mixture designs are a special class of response surface designs where the factors are the components of a mixture, and the responses are influenced by the proportions variation [17]. Such an approach to evaluate the interactions of different agroindustrial co-substrates in enzyme production by SSF was only reported for glutaminase [18], fructosyltransferase [19], L-asparaginase [20], protease, α-amylase [21] and a multi enzymatic complex composed by lipase, carboxymethyl cellulase, β-glucosidase and α-amylase [22].

Based on this background, the aim of this study was to study β-fructofuranosidase production by *Aspergillus tamarii* URM4634 in different agroindustrial co-substrates by SSF. A simplex centroid mixture design was performed to investigate the occurrence of synergistic or antagonistic effects among different substrates (wheat, soy and oat brans) on FFase production. Moreover, we explored the microbial growth as well as the variables mostly influencing the fermentation process, namely moisture, substrate amount and inducer concentration.

2. Materials and methods

2.1. Microorganisms

Eight fungal strains belonging to the *Aspergillus* genus, provided by "Micoteca-URM" of Mycology Department, Centre of Biosciences of Federal University of Pernambuco (UFPE), Recife, PE, Brazil, were used to investigate FFase production with special focus on the enzyme transfructosylating activity. The strains tested, namely *A. aculeatus* URM4953, *A. heteromorphus* URM269, *A. japonicus* URM5620, *A. niveus* URM5870, *A. phoenicis* URM4924, *A. tamarii* URM4634, *A. terreus* URM4658 and *A. versicolor* URM5701, were preserved in mineral oil, maintained at room temperature (25 ± 1 °C) in Czapek Dox Agar medium and grown in reactivation broth with the following composition (%, *w*/*v*): bacteriological peptone, 1.0; meat extract, 0.3; and glucose, 2.0. After 3 days they were inoculated in Potato Dextrose Agar medium for 7 days at 30 °C.

2.2. FFase production by Solid-State Fermentation

Screening of the best strain for FFase production was performed by Solid-State Fermentation (SSF) using wheat bran as a substrate. The fermentation was performed at 30 °C for 72 h in 125 mL Erlenmeyer flasks containing 5 g of substrate, nutrition solution (10% sucrose and 0.5% yeast extract) and a spore suspension (10^7 spores/mL) corresponding to a 50% moisture content. The FFase crude extract was obtained by addition of 7.5 mL of 0.1 M acetate buffer (pH 5.0) per gram of fermented material and subsequent homogenization in orbital shaker for 90 min at 120 rpm. Solids were removed by centrifugation at 5000 rpm for 15 min at 4 °C, and the crude extract was analyzed and stored at −22 °C.

The *Aspergillus* strain that displayed the highest hydrolytic (U_H) and transfructosylating (U_{TF}) activities was submitted to a screening on different agroindustrial by-products as substrates, namely wheat bran, soy bran, oat bran, corn cobs, orange and lemon peels with granulometry between 0.5 and 2.0 mm. All the by-products were obtained in a local market in the city of Garanhuns, PE, Brazil. Fermentation conditions were the same as those used for strain screening. The activity results were expressed as arithmetic mean ± standard deviation, and the Tukey's test was used to check significant differences ($p < .05$) among samples.

2.3. Statistical mixture design and statistical analysis

To study the effect of fermentation medium composition on FFase production, we used a three-component simplex-centroid mixture design in which wheat, oat and soy brans were selected as the independent variables at four levels, i.e., different proportions of these components, namely 0 (0%), 1/3 (33%), 1/2 (50%) and 1 (100%). For this purpose, both U_H and U_{TF} were determined at different fermentation times (24, 48, 72 and 96 h) and expressed as arithmetic means ± standard deviations. The Tukey' test was used to check significant differences ($p < .05$) among different samples after a given fermentation time.

The following regression models were fitted to the experimental data of FFase activity:

$$Y_i = \sum_{i=1}^{q} \beta_i x_i + \sum \sum_{i<j}^{q} \beta_{ij} x_i x_j + \sum \sum_{i<j<k}^{q} \sum \beta_{ijk} x_i x_j x_k \quad (1)$$

where Y_i is the predicted response (enzyme activity); q is the number of components of the system (3); x_i, x_j and x_k are the coded levels of the independent variables, i.e., wheat, oat and soy bran, respectively; β_i are the coefficients of linear terms, and β_{ij} and β_{ijk} those of binary and ternary interaction terms, respectively.

The statistical analysis of the experimental design was performed using the Statistica 7.0 software package (Statsoft Inc., Tulsa, OK, USA), while the quality of fit of the above models was checked by the analysis of variance (ANOVA), the *F* test and the coefficient of determination (R^2), being considered acceptable $R^2 > 0.90$,

2.4. Full factorial design of experiments of FFase production by SSF

After choosing the best strain and substrate, FFase production experiments were performed according to a 2^3-full factorial design plus three central points, where the independent variables were substrate mass (3, 5 and 7 g), moisture content (40, 50 and 60%) and sucrose concentration (5, 10 and 15% *w*/*v*). The statistical analysis of results obtained according to this experimental design was performed using the same software package mentioned above.

2.5. Hydrolytic and transfructosylating FFase activities

Both U_H and U_{TF} were determined at 55 °C for 1 h after addition of 0.25 mL of enzyme solution to 0.75 mL of 60% (w/v) sucrose (Sigma-Aldrich, St. Louis, MO, USA) solution in 0.1 M acetate buffer (pH 5.0), according to the method described by Sangheetha et al. [23] with some modifications. Briefly, the concentrations of released glucose (*G*) and reducing sugar (*RS*) were determined in medium samples collected at the end of reaction by a commercial glucose oxidase colorimetric kit (Liquiform, Labtest, Lagoa Santa, MG, Brazil) and the 3′5' dinitrosalicylic acid method [24], respectively, from which U_H and U_{TF} were assessed. In particular, the following equations first proposed by Chen and Liu [25] were used to calculate the concentration of transferred fructose (*F*′) and then U_{TF}:

$$F = RS - G \quad (2)$$

$$F' = G - F = 2G - RS \quad (3)$$

where *F*, *RS* and *G* is the concentrations of fructose, glucose and total reducing sugars in the reaction medium, respectively.

One unit of hydrolytic activity was defined as the amount of enzyme required to hydrolyze 1 μmol of sucrose per minute, while one unit of transfructosylating activity as that to transfer 1 μmol of fructose per minute.

2.6. Qualitative aflatoxin detection

Possible aflatoxin production by the three strains that gave the best results in terms of production of FFase with transfructosylating activity was qualitatively checked either in the Coconut Milk Agar Medium according to Lin and Dianese [26] or by the Ammonia Vapor Test [27].

Mycotoxin production was expected to be highlighted, in the former method, by the presence of a fluorescence ring in the agar surrounding colonies under UV-A 365 nm radiation, while, in the latter, by the change from pink to red color in the underside of strain colonies after the addition of 2.0 mL ammonium hydroxide solution (35%). In both cases, after inoculation in Petri-dishes, the strains were grown in triplicate for 7 days at 30 °C.

2.7. Physical-chemical characterization of agroindustrial by-products

The composition of dry agroindustrial by-products in terms of protein, lipid, moisture and ash contents was determined according to the methods of the Association of Official Analytical Chemists (AOAC) [28], with some adaptations, and the carbohydrate content by difference between the total mass and those of the other components as reported by de Castro et al. [14].

The water activity (a_w) was determined by an Aqualab Pre Water Activity Analyzer (Decagon Devices Inc., Pullman, WA, USA), while the Water Absorption Index (WAI) and Critical Humidity Point (CHP) were determined according to Flores-Maltos et al. [29], with some adaptations. Briefly, WAI was determined suspending 1.25 g of co-product in 15 mL of distilled water in 50-mL centrifuge tubes, mixing for 1 min at room temperature (25 ± 1 °C), centrifuging at 8000 *g* for 15 min, discarding the supernatant and weighing the centrifuged pellet. WAI was then expressed as g pellet/g dry weight. To determine CHP, 1.0 g of sample impregnated with water at saturation was placed at 120 °C for 60 min, and then the residual moisture was assessed.

2.8. Determination of fungal biomass in solid state fermentation

The fugal biomass profile was followed under the best conditions for FFase production. Due the impossibility to separate the fungus from substrate in solid-state fermentation, its growth was determined indirectly from the concentration of *N*-acetyl glucosamine released by the acid hydrolysis of chitin present in fungal cell wall, as described by de Castro et al. [14]. Measurements were done after acid hydrolysis of biomass contained in dried fermented samples using concentrated sulfuric acid at 121 °C for 1 h, conditions under which the released glucosamine undergoes successive reductions leading to chromogen that reacts with Ehrlich's reagent (2.67 g of *p*-dimethylaminobenzaldehyde in 1:1 (*v*/v) mixture of analytical reagent grade ethanol and concentrated hydrochloric acid). After that, the absorbance of the reaction mixture was read at 530 nm. Glucosamine concentration was measured at time intervals of 12–24 h of fermentation using glucosamine (Sigma-Aldrich) as a standard. The results were expressed as mg of glucosamine per gram of dry substrate.

3. Results and discussion

3.1. Preliminary screening of fungus and substrate for FFase production

FFase production by Solid-State Fermentation (SSF) was assessed on wheat bran as a substrate using eight different strains belonging to the *Aspergillus* genus. The results of Table 1 show that *A. tamarii* URM 4634, which was already successful in the productions of protease [15] and xylanase [30], ensured the highest hydrolytic (U_H = 55.47 ± 0.18 U/mL) and transfructosylating (U_{TF} = 26.24 ± 3.42 U/mL) activities compared to the other strains. The three best performing strains in terms of both activities, i.e., *A. tamarii* URM4634, *A. aculeatus* URM4953 and *A. terreus* URM4658, were evaluated for their ability to produce aflatoxin in Coconut Milk Agar (CMA) medium and by the Ammonium Vapor Test (AVT). The absence of any fluorescent halo around the colonies of growing mycelia in CMA medium and of any pinkish pigmentation on the reverse of colonies by the AVT indicated no aflatoxin production by all the three strains. These results are consistent with the observations made by da Silva et al. [15] and Yazdani et al. [31] on different strains of *A. tamarii*. Therefore, based on its higher FFase activities as well as the absence of any aflatoxin production, *A. tamarii* URM4634 was selected for further runs to overproduce FFase with high U_{TF}.

Table 1
FFase activities detected after 72 h of wheat bran Solid-State Fermentation by different *Aspergillus* strains.

Microorganism	U_H[1] (U/mL)	U_{TF}[2] (U/mL)
A. aculeatus URM4953	38.37 ± 2.04[b]	18.58 ± 1.81[b]
A. heteromorphus URM0269	14.40 ± 1.14[e]	0.00 ± 0.00[d]
A. japonicus URM5620	19.32 ± 1.04[d]	10.92 ± 0.74[c]
A. niveus URM5870	10.45 ± 1.91[e]	0.00 ± 0.00[d]
A. phoenicis URM4924	19.84 ± 0.54[d]	11.72 ± 0.29[c]
A. tamarii URM4634	55.47 ± 0.18[a]	26.24 ± 3.42[a]
A. terreus URM4658	25.67 ± 0.52[c]	7.81 ± 1.39[c]
A. versicolor URM5701	14.61 ± 0.04[e]	0.00 ± 0.00[d]

Results are the means of triplicates ± standard deviations. Different letters in the same column indicate statistically significant differences among values ($p < .05$).
[1] Hydrolytic activity.
[2] Transfructosylating activity.

Different agroindustrial by-products were then tested as substrates to improve FFase production by SSF, whose results are listed in Table 2. Soy bran ensured the highest values of U_H (135.29 ± 4.26 U/mL) and U_{TF} (42.35 ± 5.89 U/mL), whereas wheat and oat brans allowed for about one-fourth to one-half of the above U_H and about one-fifth to two-thirds of U_{TF}, respectively. On the other hand, lemon and orange peels as well as corn cobs had disappointing performances. Surprisingly, Rustiguel et al. [32] observed a 20% increase in FFase production by *Aspergillus phoenicis*, evaluated only as U_H, when a mixture of soy and wheat brans was used instead of soy bran alone. A mixture of these substrates was successfully used also for the production of FFase with transfructosylating activity by *Penicillium oxalicum* in submerged cultivation ([11].

The centesimal composition and physicochemical parameters of agroindustrial by-products used for FFase production are listed in Table 3. As known, the C:N ratio is one of the most important factors to balance biomass and produce biomolecules of interest, which means that the substrate must have a value of this ratio suitable for the fermentation. FFase production was the highest in substrates with the lowest C:N values (Table 2), i.e., soy (0.77 g/g) and wheat bran (2.60 g/g). These values are close to those detected by de Castro et al. [14] for wheat bran (4.27 g/g), soybean (0.64 g/g) and cottonseed meal (2.15 g/g) used as substrates for protease production by *A. niger*.

Others important parameters are the Water Absorption Index (WAI) and Critical Humidity Point (CHP), which indicate the amounts of water absorbed and intrinsically contained in the support, respectively, the latter not being available to the microorganism for its metabolic functions [29]. Substrates with high WAI and low CHP values are usually preferred in SSF since their moisture content tends to decrease during fermentation. However, despite its intermediate WAI and CHP values

Table 2
FFase activities detected after 72 h of Solid-State Fermentation of different agroindustrial substrates by *Aspergillus tamarii* URM4634.

Substrate	U_H[1] (U/mL)	U_{TF}[2] (U/mL)
Corn cobs	4.90 ± 0.71[d]	2.00 ± 0.87[bc]
Lemon peel	0.27 ± 0.03[e]	0.19 ± 0.05[c]
Oat bran	34.11 ± 0.38[c]	8.66 ± 0.73[bc]
Orange peel	0.19 ± 0.03[e]	0.19 ± 0.05[c]
Soy bran	135.29 ± 4.26[a]	42.35 ± 5.89[a]
Wheat bran	62.82 ± 1.34[b]	26.58 ± 1.60[b]

Results are the means of triplicates ± standard deviations. Different letters in the same column indicate statistically significant differences among values ($p < .05$).
[1] Hydrolytic activity.
[2] Transfructosylating activity.

Table 3
Centesimal composition and physicochemical parameters of dry agroindustrial substrates used to produce FFase with transfructosylating activity by *Aspergillus tamarii* URM4634 by Solid-State Fermentation.

Parameter	Corn cobs	Lemon peel	Oat bran	Orange peel	Soy bran	Wheat bran
Moisture (%)	6.24 ± 0.02^{c}	8.88 ± 0.35^{a}	8.35 ± 0.02^{a}	9.09 ± 0.08^{a}	7.93 ± 0.11^{ab}	6.95 ± 0.74^{bc}
Ash (%)	1.36 ± 0.09^{e}	4.25 ± 0.14^{b}	2.14 ± 0.01^{d}	3.47 ± 0.03^{c}	6.34 ± 0.01^{a}	6.19 ± 0.08^{a}
Protein (%)	1.82 ± 0.29^{e}	6.47 ± 0.00^{d}	15.78 ± 0.29^{c}	7.08 ± 0.00^{d}	56.24 ± 0.00^{a}	18.31 ± 0.43^{b}
Lipids (%)	2.54 ± 0.34^{c,d}	2.14 ± 0.23^{d,e}	6.84 ± 0.13^{a}	1.41 ± 0.24^{e}	3.23 ± 0.30^{c}	4.71 ± 0.57^{b}
Carbohydrates (%)	88.04	78.26	66.89	78.95	26.26	63.84
C:N ratio (g/g)	21.39	6.78	3.16	6.06	0.77	2.60
a_w	0.487 ± 0.006^{d}	0.573 ± 0.001^{a}	0.525 ± 0.001^{c}	0.551 ± 0.001^{b}	0.551 ± 0.001^{b}	0.471 ± 0.003^{e}
WAI[1] (g of water/g of dried substrate)	6.95 ± 0.48^{a}	6.38 ± 0.05^{a,b}	2.03 ± 0.13^{e}	5.72 ± 0.05^{b,c}	4.18 ± 0.04^{d}	5.08 ± 0.33^{c,d}
CHP[2] (%)	14.04 ± 0.60^{c,d}	12.58 ± 0.14^{e}	32.85 ± 2.23^{a}	13.88 ± 0.15^{c,d}	18.23 ± 0.14^{b}	16.70 ± 0.16^{b,c}

Different letters for the same parameter indicate statistically significant differences among values ($p < .05$).
[1] Water Absorption Index.
[2] Critical Humidity Point.

compared to the other substrates, soy bran allowed for the highest FFase production, which suggests that these physicochemical parameters did not play a crucial role in FFase production by *Aspergillus tamarii* URM4634.

3.2. FFase production by Solid-State Fermentation on different agroindustrial substrates

A simplex centroid mixture design was used to investigate the influence of different proportions of the three best substrates (soy, wheat and oat brans) on U_H and U_{TF} at different fermentation times (Table 4). The formulation used in run 1, made up only on soy bran, resulted in a maximum hydrolytic FFase activity as high as 136.26 ± 2.32 U/mL after 48 h of fermentation, 87% of which was preserved even after 96 h. This substrate ensured the highest U_{TF} as well (56.48 ± 1.66 U/mL), but it took twice as long (96 h). Contrary to what expected from the literature, where mixtures of agroindustrial by-products are often pointed out as the best substrates for SSF [32], these results indicate that soy bran alone would be the most suite medium among the tested ones seeking FFase production.

The influence of different formulations on both FFase hydrolytic and transfructosylating activities after 24, 48, 72 and 96 h is illustrated in two-dimensional ternary contour plots (Fig. 1). After 24 h of fermentation, maximum activity zones are located at the side of triangle having mixtures of soy (x_1) and oat (x_3) brans as the vertices, indicating a synergistic effect. Such a binary formulation (run 5) did in fact show about 4.5–5.0-fold increases in U_H and 3.5–4.5-fold increases in U_{TF} compared with the individual substrates; similar synergistic effect was also observed for both enzyme activities with the other binary (runs 4 and 6) and ternary (run 7) mixtures. U_H achieved a maximum value (132.11–136.26 U/mL) after 48–72 h in the medium containing only soy bran as the substrate (run 1), and then decreased by about 10–13% after 96 h. On the other hand, all binary mixtures showed antagonist effects in the same time interval, except the ones made up of soy bran and wheat bran (x_2) (run 4) after 48 and 96 h and of wheat bran and oat bran (run 6) after 96 h.

As regards U_{TF}, the contour plot after 48 h showed the highest value of this response (38.80–40.21 U/mL) in the vertices referring to soy bran (run 1) and wheat bran (run 2) alone, with no statistically significant difference between them ($p > .05$). After 72 h, the highest activity (47.64–51.17 U/mL) was observed in the presence only of soy bran (run 1) and the mixture composed of soy bran and wheat bran (run 4), with no statistically significant difference ($p > .05$). However, after 96 h this activity achieved the maximum value obtained in this study (56.48 ± 1.66 U/mL) in run 1, while all mixtures of substrates showed antagonist effects.

Different models were used to predict the optimum composition of medium for FFase production, taking into consideration either enzyme activity. Among them, the quadratic models based on Eq. (1) allowed by far the best fit ($R^2 > 0.90$) to the experimental data (Table 5), thus proving useful for predictive purposes. In each model, negative and positive terms do refer to antagonistic and synergistic effects, respectively, that reflect the influence of binary and ternary mixtures on enzyme production [21], while their absolute values indicate how strong are these effects. Based on these criteria, the most significant effects on U_H were that of the binary mixture composed by soy bran (x_1) and oat bran

Table 4
Matrix and results along the time of FFase production by *A. tamarii* URM4634 Solid-State Fermentations carried out according to the simplex centroid mixture design.

Run	Independent variables			Fermentation time (h)			
	Soy bran (x_1)	Wheat bran (x_2)	Oat bran (x_3)	24	48	72	96
Hydrolytic activity (U/mL)							
1	1 (5.00)	0 (0.00)	0 (0.00)	13.31 ± 0.20^{d,e}	136.26 ± 2.32^{a}	132.11 ± 3.301^{a}	118.62 ± 1.35^{a}
2	0 (0.00)	1 (5.00)	0 (0.00)	9.58 ± 0.77^{e}	66.05 ± 0.85^{d}	62.82 ± 1.34^{d}	61.23 ± 0.61^{d}
3	0 (0.00)	0 (0.00)	1 (5.00)	11.93 ± 0.04^{d,e}	15.03 ± 0.61^{f}	15.91 ± 0.32^{f}	15.41 ± 0.85^{f}
4	1/2 (2.50)	1/2 (2.50)	0 (0.00)	31.81 ± 1.92^{c}	93.12 ± 1.83^{b}	108.22 ± 0.12^{b}	84.09 ± 2.69^{b}
5	1/2 (2.50)	0 (0.00)	1/2 (2.50)	60.10 ± 0.77^{a}	81.62 ± 0.12^{c}	81.58 ± 1.95^{c}	75.58 ± 0.24^{c}
6	0 (0.00)	1/2 (2.50)	1/2 (2.50)	15.43 ± 1.18^{d}	54.06 ± 1.47^{e}	30.44 ± 3.42^{e}	33.23 ± 0.24^{e}
7	1/3 (1.67)	1/3 (1.67)	1/3 (1.67)	42.42 ± 1.71^{b}	68.42 ± 1.83^{d}	103.31 ± 1.59^{b}	75.48 ± 3.79^{c}
Transfructosylating activity (U/mL)							
1	1 (5.00)	0 (0.00)	0 (0.00)	10.63 ± 0.04^{d}	40.21 ± 0.80^{a}	51.17 ± 0.07^{a}	56.48 ± 1.66^{a}
2	0 (0.00)	1 (5.00)	0 (0.00)	7.19 ± 1.55^{d}	38.80 ± 0.62^{a}	26.58 ± 1.60^{c}	13.83 ± 0.67^{d}
3	0 (0.00)	0 (0.00)	1 (5.00)	8.72 ± 0.10^{d}	10.27 ± 0.41^{c}	22.11 ± 0.28^{d}	12.07 ± 2.80^{d}
4	1/2 (2.50)	1/2 (2.50)	0 (0.00)	20.17 ± 0.34^{c}	21.71 ± 0.68^{b}	47.64 ± 1.39^{a}	30.94 ± 0.48^{b}
5	1/2 (2.50)	0 (0.00)	1/2 (2.50)	37.77 ± 0.27^{a}	26.91 ± 1.87^{b}	26.05 ± 1.91^{c,d}	20.47 ± 0.60^{c}
6	0 (0.00)	1/2 (2.50)	1/2 (2.50)	11.03 ± 1.27^{d}	26.77 ± 0.21^{b}	12.56 ± 3.03^{e}	14.85 ± 1.14^{c,d}
7	1/3 (1.67)	1/3 (1.67)	1/3 (1.67)	28.47 ± 2.70^{b}	36.54 ± 2.87^{a}	39.45 ± 1.72^{b}	35.35 ± 2.76^{b}

Different letters in the same column indicate statistically significant differences among values ($p < .05$).
x_1, x_2, x_3 = coded levels of soy, wheat and oat brans, respectively. Values between brackets are the actual values.

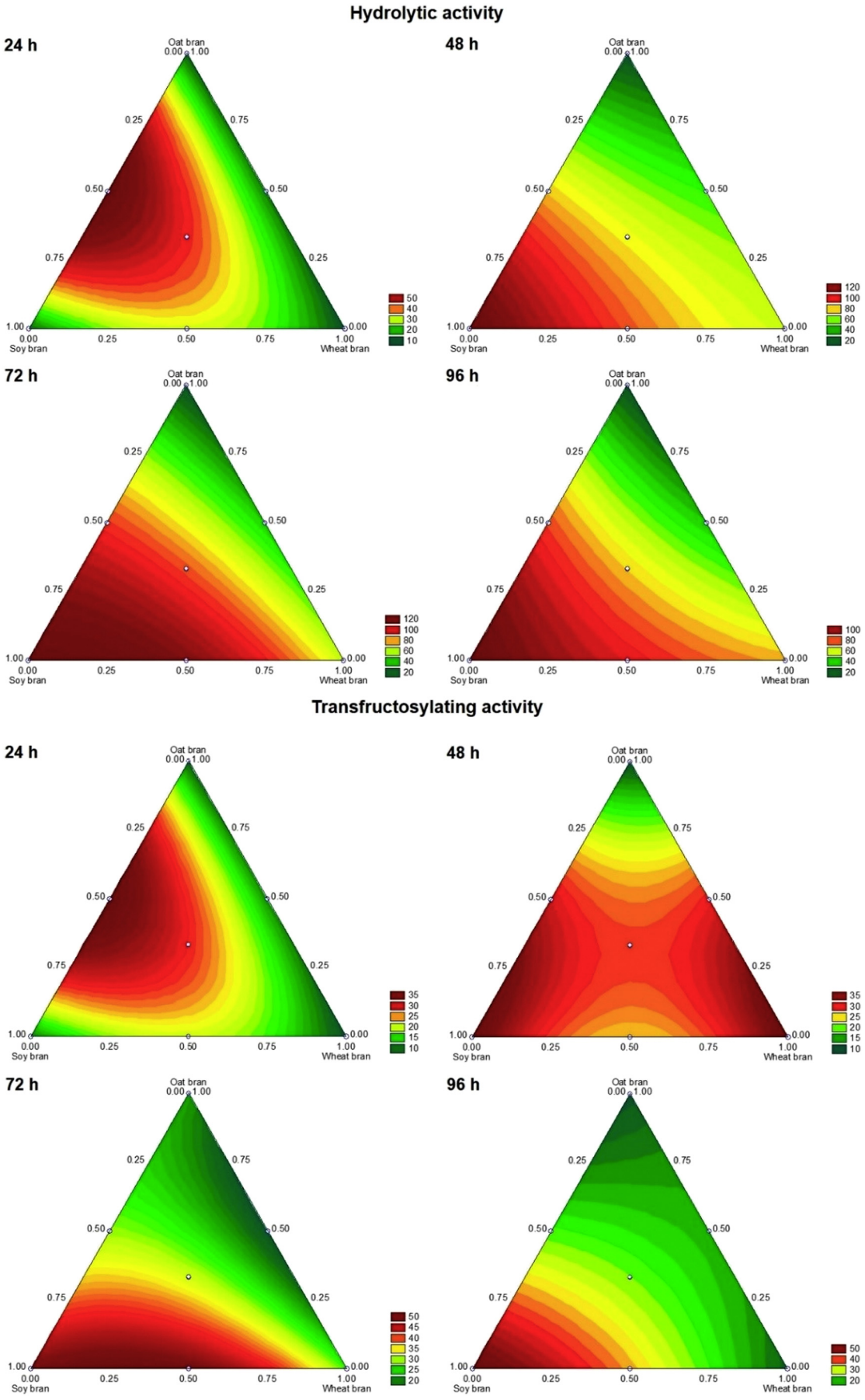

Fig. 1. Mixture contour plots for hydrolytic and transfructosylating activities of *A. tamarii* URM4634 FFase produced by Solid-State Fermentation after 24, 48, 72 and 96 h.

Table 5
Quadratic models describing the effects of substrate mixtures on hydrolytic and transfructosylating activities of FFase produced by *A. tamarii* URM4634 Solid-State Fermentation and related statistical parameters.

Time (h)	$F_{calculated}$	R^2	Equations
Hydrolytic activity			
24	361.75	0.998	$Y = 13.40x_1 + 9.67x_2 + 12.03x_3 + 79.53x_1x_2 + 187.99x_1x_3 + 16.76x_2x_3$
48	32.21	0.994	$Y = 136.88x_1 + 66.67x_2 + 15.65x_3 - 44.56x_1x_2 + 11.47x_1x_3 + 41.67x_2x_3$
72	4.18	0.954	$Y = 130.19x_1 + 61.68x_2 + 13.99x_3 + 79.82x_1x_2 + 68.66x_1x_3 + 8.37x_2x_3$
96	14.08	0.986	$Y = 117.76x_1 + 70.50x_2 + 14.55x_3 - 26.47x_1x_2 + 51.39x_1x_3 - 23.48x_2x_3$
Transfructosylating activity			
24	449.14	0.999	$Y = 10.57x_1 + 7.14x_2 + 8.67x_3 + 46.09x_1x_2 + 113.42x_1x_3 + 13.34x_2x_3$
48	1.85	0.902	$Y = 38.95x_1 + 38.55x_2 + 9.87x_3 - 53.23x_1x_2 + 26.26x_1x_3 + 21.82x_2x_3$
72	2.27	0.919	$Y = 50.30x_1 + 27.28x_2 + 21.26x_3 - 49.21x_1x_2 - 25.09x_1x_3 - 35.42x_2x_3$
96	2.01	0.910	$Y = 55.46x_1 + 12.81x_2 + 11.04x_3 + 3.66x_1x_2 - 34.69x_1x_3 + 28.11x_2x_3$

For all models, p-value < .001 and $F_{tabulated} = 2.90$.
x_1, x_2, x_3 = coded levels of soy, wheat and oat brans, respectively.

(x_3) and that of soy bran alone for long fermentation times (≥ 48 h). Apart from only one exception after 48 h of fermentation, a similar behavior was also observed for U_{TF}.

3.3. Optimization of FFase production by Solid-State Fermentation using soy bran as substrate

After selection of soy bran alone as the best substrate for both enzyme activities, the influence of substrate mass (3, 5 and 7 g), moisture content (40, 50 and 60%) and sucrose concentration (5, 10 and 15% *w*/*v*) on FFase production was investigated using a 2^3-full factorial design, whose conditions and results after 72 h of fermentation are listed in Table 6 in terms of both U_H and U_{TF}. One can see that the maximum U_H (209.11–209.99 U/mL) was obtained in runs 7 and 8 and the maximum U_{TF} (51.08–53.90 U/mL) in runs 4 and 7; therefore, the run 7 carried out using 3 g soy bran, 15% sucrose and 60% moisture was selected as the best compromise to simultaneously optimize them. The former activity was more than thrice that reported for *Aspergillus japonicus* FFase produced on coffee silverskin (65.82 ± 1.46 U/mL) [12], and the latter more than twice those of *A. oryzae* [23] and *Rhizopus stolonifer* [33] FTases using rice bran and cassava waste as substrates, respectively.

The analysis of effects summarized in Table 7 shows that the moisture content exerted a statistically significant positive effect on U_H, i.e., such a response was greatly enhanced by an increase in that independent variable. As known, moisture, which has in general a great importance in any microbial process, is crucial in enzyme production by SSF, because it not only promotes the diffusion of solutes and gases, but also mitigates the osmotic changes brought about by excess metabolites in the vicinity of cells [34]. Moisture contents in the range selected for this study are considered suitable for fungal development; higher values may result in decreased substrate porosity, oxygen limitation and bacterial contamination, whereas lower values may cause poor accessibility of nutrients and then poor microbial growth [13].

On the other hand, U_{TF} was positively influenced only by sucrose concentration. This result is consistent not only with the fact that sucrose is the best carbon source for the production of enzymes with transfructosylating activity [35], but also with the importance of this sugar in the formation of cell constituents as well as its role as an inducer of FFase synthesis. Nonetheless, explaining the statistically significant antagonistic effect of the interaction between substrate concentration and moisture content on this response is a challenge.

As a final effort, we investigated fungal growth during run 7 that ensured the most satisfactory results in terms of both activities (Table 6). For this purpose, due the practical impossibility to discern biomass and substrate masses in SSF, *A. tamarii* URM4634 growth was followed indirectly as the increase in concentration of glucosamine released by cell wall hydrolysis. These results are illustrated in Fig. 2A together with those of FFase production. The glucosamine level progressively increased along the time and reached a maximum value (132.0 ± 2.05 mg/g) after 36 h of cultivation, whereupon a decrease in biomass level took place, likely due to substrate limitation. Doing just a few comparisons, da Silva et al. [15] reported a maximum glucosamine concentration of 119.33 ± 4.8 mg/g after 96 h of SSF using the same *A. tamarii* strain and wheat bran as a substrate, while de Castro et al. [14], cultivating *A. niger* in different inexpensive agroindustrial substrates, achieved a maximum glucosamine level of 83.35 mg/g in soybean meal. FFase production showed peaks of U_H (229.43 ± 4.88 U/mL) and U_{TF} (66.93 ± 3.02 U/mL) after 120 and 96 h, respectively. As far as the FFase productivity is concerned, the best results in terms of hydrolytic (3.48 ± 0.03 U/mL h^{-1}) and transfructosylating (0.70 ± 0.03 U/mL h^{-1}) activities were obtained after 48 and 96 h (Fig. 2B), respectively.

Table 6
2^3-Full factorial design adopted to optimize *Aspergillus tamarii* URM4634 Solid-State Fermentations using soy bran as a substrate. Results are expressed as FFase hydrolytic (U_H) and transfructosylating (U_{TF}) activities after 72 h of fermentation.

Run	Substrate mass (g)	Sucrose concentration (%)	Moisture content (%)	U_H (U/mL)	U_{TF} (U/mL)
1	3	5	40	130.20	23.24
2	7	5	40	130.85	36.86
3	3	15	40	119.13	22.34
4	7	15	40	155.23	51.08
5	3	5	60	175.17	34.06
6	7	5	60	191.67	29.96
7	3	15	60	209.11	53.90
8	7	15	60	209.99	47.67
9 (C)	5	10	50	139.17	32.47
10 (C)	5	10	50	151.09	35.48
11 (C)	5	10	50	159.39	28.99

C = Central point runs.

Table 7
Estimated effects of the independent variables and their interactions on FFase hydrolytic (U_H) and transfructosylating (U_{TF}) activities detected in *Aspergillus tamarii* URM4634 Solid-State Fermentations carried out on soy bran as substrate according to the 2^3-full factorial design summarized in Table 6.

Variable or interaction	U_H	U_{TF}
(1) Substrate mass	2.86	3.48
(2) Sucrose concentration	3.46	5.54*
(3) Moisture content	13.24*	3.49
1 × 2	1.05	1.41
1 × 3	−1.02	−5.73*
2 × 3	2.06	2.64
1 × 2 × 3	−2.70	−1.87

* Values statistically significant at 95% confidence level ($p < .05$).

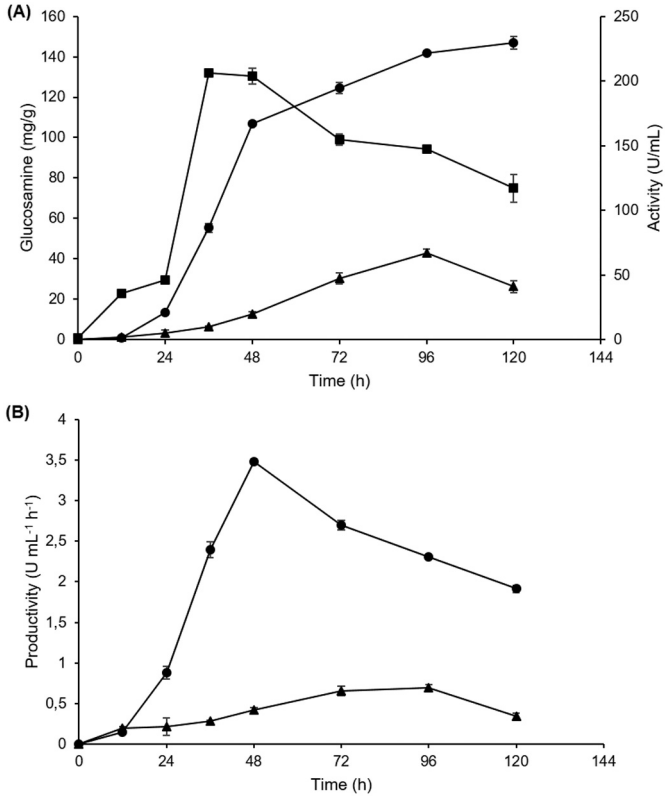

Fig. 2. *A. tamarii* URM4634 Solid-State Fermentation using soy bran as substrate. (A) Biomass growth expressed as glucosamine concentration (■); FFase production expressed as FFase hydrolytic (●) and transfructosylating (▲) activities. (B) FFase productivity in terms of hydrolytic (●) and transfructosylating (▲) activities.

4. Conclusions

Aspergillus tamarii URM4634, selected among different fungal strains as the best FFase producer in Solid-State Fermentation, was tested in different substrates. Among them, soy bran, wheat bran and oat bran showed the high FFase activities; therefore, the influence of each substrate at four levels was evaluated using a three-component simplex-centroid mixture design. The maximum enzyme production in terms of both enzyme activities was obtained using only soy bran as the substrate. The above experimental design provided satisfactory statistical models to predict enzyme production at different fermentation times, thus confirming to be a powerful statistical approach to optimize the performance and find the optimum formulations for SSF. Finally, to maximize FFase production, additional SSF runs were performed according to a 2^3-full factorial design where moisture content, soy bran mass and sucrose concentration were selected as the independent variables. Based on the information obtained from this statistical design, a longer fermentation was carried out under optimal conditions (3 g of substrate, a 15% sucrose concentration and a 60% moisture content), in which the highest hydrolytic (229.43 ± 4.88 U/mL) and transfructosylating (66.93 ± 3.02 U/mL) activities were obtained after 120 and 96 h, respectively. The results obtained in this study, although preliminary, provide a projection of the potential of *A. tamarii* URM4634 FFase in industrial applications such as sugar inversion and FOS production.

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