Optimization of β-Fructofuranosidase Production from Agrowaste by *Aspergillus carbonarius* and Its Application in the Production of Inverted Sugar

Running head: Invertase Production and Application

Ryhára Dias Batista	extsuperscript{1}, Fernanda Guimarães Melo	extsuperscript{1}, Claudia Cristina Auler do Amaral Santos	extsuperscript{1}, Fabrício Coutinho de Paula-Elias	extsuperscript{1}, Rafael Firmani Perna	extsuperscript{2}, Michelle Cunha Abreu Xavier	extsuperscript{3}, Sergio Andres Villalba Morales	extsuperscript{2} and Alex Fernando de Almeida	extsuperscript{1*}

	extsuperscript{1}Graduate Program in Food Science and Technology, Federal University of Tocantins, 109 Norte Av. NS-15, ALCNO-14. Plano Diretor Norte, CEP: 77001-090. Palmas, Tocantins, Brazil

	extsuperscript{2}Federal University of Alfenas (UNIFAL-MG), Institute of Science and Technology, José Aurélio Vilela Road 11999, Km 533, Zip Code 37715-400, Poços de Caldas, MG, Brazil

	extsuperscript{3}Federal University of Tocantins (UFT), Department of Bioprocess Engineering and Biotechnology, Badejos Street 69-72, Jardim Cervilha, Zip Code 77404-970, Gurupi, TO, Brazil

Received: 8 August 2020
Accepted: 14 July 2021

**SUMMARY**

*Research background.* Microbial β-fructofuranosidases are widely employed in food industry to produce inverted sugar or fructooligosaccharides. In this study, a newly isolated *Aspergillus carbonarius* PC-4 strain was used to optimize the β-fructofuranosidase production under a cost-effective process and the sucrose hydrolysis was evaluated to produce inverted sugars.

*Experimental approach.* Optimization of nutritional components of culture medium was carried out using Simplex Lattice mixture design for 72 h and 120 h at 28 °C. One-Factor-at-a-time methodology was used to optimize the physicochemical parameters. Crude enzyme was used for sucrose hydrolysis at different concentrations.
Results and conclusions. The optimized condition of enzyme production was pineapple crown waste (1.3 %, m/V) and yeast extract (0.3 %, m/V) for 72 h (9.4 U/mL), obtaining $R^2$ 91.85 %, $R^2$ adjusted 85.06 %, highest F value (13.52) and low p-value (0.003). One-factor-at-a-time used for optimizing the physicochemical conditions showed optimum temperature (20 °C), pH (5.5), agitation (180 rpm) and time-course (72 h) with an increase of 3.0-folds for enzyme production. The invertase-induced sucrose hydrolysis showed the maximum yield (3,451.7 µmol of reducing sugars) using 10 % of initial sucrose concentration. Higher sucrose concentrations caused inhibition of invertase activity, possibly due to saturation of substrate or formation of sucrose aggregates making it difficult for the enzyme to access sucrose molecules within the created clusters. So, a cost-effective method was developed for the invertase production using agroindustrial waste and the enzyme produced can be used efficiently for inverted sugar production at high sucrose concentration.

Novelty and scientific contribution. This study presents an efficient utilization of pineapple crown wastes to produce invertase by a newly isolated Aspergillus carbonarius PC-4 strain. This enzyme exhibited a good potential for invert sugar production at high initial sucrose concentration, which is interesting for industrial applications.

Key words: invertase, Aspergillus carbonarius PC-4, culture optimization, simplex lattice design, sucrose hydrolysis

INTRODUCTION

Invertases (β-D-fructofuranoside fructohydrolases, E.C. 3.2.1.26) are glycoside hydrolases that catalyze the hydrolysis of sucrose into D-glucose and D-fructose. These enzymes naturally occur in plant, bacteria, yeasts, and filamentous fungi (1, 4). Invertases have drawn the attention of different food and beverage industries due to their feature of generating equimolar mixtures of glucose and fructose, which is the basis to produce high fructose syrups (3). The sucrose hydrolysis by invertases produces a mixture of sugars called invert sugar since sucrose rotates its plane polarized light to the right (dextrorotatory) whereas the hydrolysis products deviate the plane polarized light to the left (levorotatory) (2). Invert syrup with high fructose concentration is used in diverse food industries such as jam and jellied products. Invert syrup minimizes the crystallization rate and maintains softness of sweetmeat, so the products will remain soft throughout their shelf life (9). Therefore, invertases have been associated to the industry of bakery and beverages, reducing 5-15 % of sugar content of soft drinks with the same sweetness. Moreover, invertases can exhibit transfructosylation activity for
production of fructo-oligosaccharides at higher sucrose concentrations with high nutraceutical properties (prebiotics) (4).

A diversity of cultivation strategies based principally in submerged and solid-state fermentation has been used to produce fungal invertases. For industrial microbial cultivation process, medium composition plays a critical role due to its major influences on cell growth and microbial physiology leading to the formation of products (5). Optimization of cultivation medium is one of the critical stages of industrial production processes and must be carried out before to scale up the synthesis of microbial metabolites. Therefore, the optimized production and the variables that affect the enzymatic production should always be investigated, as the optimal conditions vary according to different microbial strains as well as their respective enzyme synthesis (6). For each bioproduct, an increase in productivity reduces the overall cost of the product. Hence, it is one of the most important topics for enzyme research (5,7).

Agroindustrial wastes are utilized in many bioprocesses due to particular interest in renewability, low-cost, and suitable characteristics which allow the production of different value-added metabolites. Tocantins state, Brazil, produces annually 54 million of pineapple fruits. Pineapple manufactured products are classified into two categories of byproducts: pineapple crown postharvest wastes (PCW) and waste from fruit processing in industry. PCW is an important and available source of lignocellulose biomass and this residue disposal can imply significant environmental problems. The pineapple waste appears to be a valuable substrate with great potential if appropriate processes and technologies are applied to transform its different components (2). The aim of this work was to perform the optimization of cultivation parameters for invertase production by Aspergillus carbonarius PC-4 using pineapple crown waste as substrate as well as a Simplex Lattice Design model for nutritional components and OFAT for physicochemical parameters of cultivation; besides studying the biocatalyst application for sucrose hydrolysis at different concentration values in order to synthesize glucose and fructose as reducing sugars.

MATERIALS AND METHODS

Biocatalyst and microbial cultivations

Aspergillus carbonarius PC-4 was isolated from canned peach syrup and it is available in the Laboratory of Biotechnology, Food and Products Analysis, Federal University of Tocantins, Gurupi, Tocantins, Brazil. The identification of this A. carbonarius PC-4 strain was performed by morphological and molecular techniques (13). The gene sequence of A. carbonarius PC-4 was deposited in the NCBI Genbank database and it is available under the accession number AJ876878. Spore
suspension were transferred to Petri plates containing potato dextrose agar (PDA) (Difco, Saint Louis, USA) and incubated at 30 °C for five days. Isolated colonies were inoculated at the center of Petri dishes to ensure the purity of fungi strains. Samples of this strain with a portion of the solid culture media (5 mm x 10 mm) were transferred to sterile glass flasks (6 mL and filled with 4 mL of sterile distilled water, which were identified and hermetically closed with rubber stoppers and aluminium seals (10). Castellani flasks were kept at 4 °C and the viability of strains was verified periodically. The filamentous fungal strain was streaked on PDA slant agar and it was incubated at 28 °C during 72 h for shake flask experiments.

Vogel's medium was used for enzyme production (11). Trace elements solution (solution A) was prepared containing (g/L): citric acid H₂O, 50; ZnSO₄.7H₂O, 50; Fe(NH₄)₂(SO₄)₂.6H₂O, 10; CuSO₄.5H₂O, 2.5; MnSO₄.H₂O, 0.05; H₃BO₃, 0.05; Na₂MoO₄.2H₂O, 0.05. Salts solution (solution B) was prepared containing (g/L): sodium citrate.5H₂O, 150; KH₂PO₄, 250; NH₄NO₃, 100; MgSO₄.7H₂O, 10; CaCl₂.2H₂O, 5. Solution B was also supplemented with 5mL of biotin solution (0.1 mg/mL), 5 mL of solution A and 0.2 mL of chloroform, which were added per liter of solution B. All mentioned solutions were stored at 4 °C. Medium preparation consisted of 50-fold dilution of solution B. Inoculum was obtained from PDA slant agar cultivations by preparing conidial suspensions with distilled water and adjusted to 10⁶ spores/mL. A volume of 1 mL was used to inoculate Erlenmeyers flasks (125 mL) containing 20 mL Vogel's salts medium at pH=6, which was supplemented with pineapple crown as carbon source, besides ammonium chloride and yeast extract as nitrogen sources. Pineapple crown waste was obtained from producers of Miracema do Tocantins, Tocantins, Brazil. Pineapple crown was initially dried and ground to a powder mesh (30 mesh). Then, this powder was washed extensively to remove free soluble sugars. All cultivation media were sterilized at 121 °C for 15 min (Esterilizer Prismatec, São Paulo, Brazil). Shake flasks were incubated at 28 °C and 180 rpm for 72 – 120 h. After cultivations, biomass was separated by vacuum filtration system (Solab, São Paulo, Brazil) and determined gravimetrically, which was expressed as gram per liter of culture medium.

**Physicochemical conditions for enzyme production**

The influence of temperature on invertase production was evaluated by varying the temperature from 15 to 45 °C, with intervals of 5 °C. Culture were grown in medium for 72 h at180 rpm in an orbital shake (Lucadema, São Paulo, Brazil), pH=6.0. The effect of initial pH on invertase production was analyzed from 3.0 to 8.0. The initial medium pH was adjusted by the addition of NaOH 1 mol/L or HCl 1 mol/L. Culture were grown in medium for 72 h, at 180 rpm and 20 °C. The Cultures were grown in medium in an orbital shake at 130, 150, 180 and 210 rpm. Initial pH was adjusted to
5.5 and the incubation temperature was maintained at 20 °C during 120 h. Samples were taken periodically at intervals of 12 h. Cell-free broth was used for enzyme and protein assays.

Invertase activity was determined by adding 0.2 mL crude enzyme to 0.8 mL of a substrate solution containing 2% (m/V) sucrose diluted into 0.1 mol/L citrate-phosphate buffer, pH=5.0. Enzyme assays were performed at 50 °C for 5 min. Samples of 0.2 mL from enzyme reaction were transferred to 0.2 mL of the reagent 3,5-dinitrosalisylic acid and heated at 100 °C during 5 min in a boiled water bath (Cienlab, São Paulo, Brazil), according to Miller (12). A volume of 2 mL of distilled water was added to the reaction tubes, whose samples were submitted to spectrophotometer absorbance analysis at 540 nm (Gehaka, São Paulo, Brazil). Glucose solution (1 g/L m/V) was used for the standard curve. One invertase unit was defined as the amount of enzyme that released 1 µmol of reducing sugar per min under the aforementioned conditions. Invertase production yield was calculated by dividing enzyme units per gram of consumed carbon source, whereas invertase productivity was obtained as the amount of enzyme units per h of cultivation. Protein was determined according Lowry et al. (26), using bovine serum albumin as standard. All experiments were carried out in triplicate.

**Sucrose hydrolysis**

Sucrose solutions of 1, 5, 10, 15, 20 and 30 % (m/V) in 0.1 mol/L citrate-phosphate buffer (pH=5.0) were submitted to enzyme hydrolysis. A volume of 10 mL sucrose solution was maintained for 5 min at 50 °C with subsequent addition of 0.1 mL crude enzyme to the reaction tubes, whose sucrose hydrolysis was performed for 180 min. Samples of 0.4 mL were taken at different intervals and subjected to enzyme inactivation by boiling at 100 °C for 5 min, whose 0.2 mL were added to 3,5-dinitrosalisylic acid to determine the amount of reducing sugars produced from hydrolysis reaction. Results were expressed in µmols of reducing sugar. All experiments were carried out in triplicate.

**Experimental design**

A simplex lattice mixture design was performed for culture medium optimization towards invertase production. The model was fitted to evaluate the influence on invertase production of three factors: ammonium chloride (X₁); yeast extract (X₂); and pineapple crown (X₃). The experimental set considered three levels of concentration for each input variable (0.0, 0.5 and 1.0). The experimental design totalized 12 experiments, which were performed in triplicates. The data factors were chosen after a series of preliminary assays (data not shown). Table 1 shows the experimental points of mixture design with respective real values of variable levels.
Statistical analysis

The statistical analysis of the data was carried out using STATISTICA software 13.4 (27). The significant level was p<0.05. The quadratic regression analysis was performed for all response variables in this work, as follows:

\[ y = \Sigma_{i=1}^{q} \beta_i X_i + \Sigma \Sigma_{i<j}^{q} \beta_{ij} X_i X_j \]  /1/

Where Y is the response variable and corresponds to invertase activity (U/mL), q is the number of ingredients for the design experiment, \( X_i \) and \( X_j \) are proportion variables, \( \beta_i \) is the regression coefficient for linear terms, whereas \( \beta_{ij} \) refers to quadratic terms of the model with binary interaction. Additional experiments were performed to validate the obtained model with optimized values of component variables.

For the analysis of physicochemical conditions to invertase production the data were subjected to analysis of variance (one-way ANOVA) by BIOESTAT 5.0 (28) and Tukey’s test with p\( \leq 0.05 \) was used for the evaluation of statistically significant differences.

RESULTS AND DISCUSSION

Mixture design experiment

Preliminary experiments with A. carbonarius PC-4 showed an invertase activity of 6.7 U/mL with a production yield of 587 U/g of substrate and a productivity of 1.6 U/h (13). Further, the mixture design was chosen to analyse the effect of relative proportions of the ingredients on the composition of the medium, since the main distinction between this experimental design and independent variable experiments is that the input variables are non-negative proportionate amounts in the mixture, which are expressed as fractions of total amount of the mixture. Ammonium chloride was also tested as component of the simplex lattice mixture design besides pineapple crown and yeast extract in the respective levels of the performed experimental design.

Table 1 presents invertase activities, invertase production yield (Yp/s) and enzyme productivity (Pp) for varying compositions of substrates in each experimental run. The invertase activity ranged from 1.0 to 9.7 U/mL for 72 h of cultivation and from 2.0 to 10.2 U/mL for cultivation periods of 120 h, which showed the importance of component variables on fermentative parameters for enzyme production. Analyses based on t test and p-value (< 0.05) for invertase production as the response showed that yeast extract and pineapple crown were significant ingredients of the mixture for cultivation periods of 72 and 120 h, whereas ammonium chloride was significative only after 120 h
cultivation. The effect of evaluated factors on enzyme activity are presented in the pareto chart (Figs. 1a and 1b).

Table 1

<table>
<thead>
<tr>
<th>Fig. 1</th>
</tr>
</thead>
</table>

ANOVA results for quadratic model showed an R² value of 91.85 % and an adjusted R² value of 85.06 % after 72 h cultivation. On the other hand, the analysis of variance for cultivation periods of 120 h revealed an R² value of 89.33 % and an adjusted R² of 80.43 % (Table 2). From these two models, 72 h cultivation was chosen for further experiments, whose substrate components would explain 91.85 % of variability in the response variable, leaving 8.15 % of the variability remaining unexplained. The quadratic model for 72 h cultivation showed a significative F value (13.52) and a low p-value (0.003), which is an interesting result since a shorter period of cultivations with similar enzyme activity values results in better enzyme production yields. The second order models (Equations 2 and 3) obtained expresses the empirical relationship between the invertase production and ingredients of the mixture (ammonium chloride, yeast extract and pineapple crown) for 72 h and 120 h, respectively:

\[
Y = 0.62X_1 + 1.67X_2 + 4.14X_3 + 12.39X_1X_3 + 6.97X_2X_3 \quad /2/
\]

\[
Y = 1.26X_1 + 2.38X_2 + 4.87X_3 \quad /3/
\]

Where Y is invertase activity (U/mL); X₁ is ammonium chloride; X₂ is yeast extract; and X₃ is pineapple crown.

Table 2

The ternary plots show models obtained after 72 h and 120 h cultivation, indicating the existence of optimal region for invertase activity located near to the top of the design triangle for both periods of cultivation (Figs. 1c and 1d). Possibly, higher concentrations of pineapple crown and intermediate concentrations of yeast extract as carbon and nitrogen sources, respectively, have been significatively influenced the predicted values of enzymatic activity (above 9 U/mL). Silva et al. (14) showed that pineapple crown is a potential carbon source, containing xylose and mostly glucose as constituents, and hence an attractive residue for microbial growth. The predicted versus observed values revealed a good co-relation between the model and experimental invertase production. The Simplex Lattice Design methodology promoted an increase of 1.42-fold for enzyme production, when compared to the results obtained by Nascimento et al. (13). In optimized culture conditions, the production parameters were of 723.4 U/g for production yield and 2 U/h of enzyme productivity.
Pineapple crown waste used in this study was chemical characterized and it was constituted of 28.6 % total carbon, 1.8 % total nitrogen, with a C/N ratio of 15.7, 7.7 % humidity, 6.0 % ashes, 6.7 % total lipids, 11.5 % total protein, and 68.1 % total carbohydrates. Therefore, in this study, it was observed an efficient utilization of pineapple crown waste by A. carbonarius PC-4 to produce invertase under submerged culture condition. The invertase production by Aspergillus strains isolated from agro-industrial residues and byproducts has been extensively studied in the last decades. These substrates are rich in nutrients such as carbon and nitrogen sources to attend the microbial nutritional demand and they are also low-cost, which is economically interesting (15,16). Oyedeji et al. (16) observed the increased of invertase production by A. niger IBK1 due to utilization of pineapple peel for growth and hence enzyme production. Pineapple peel contains a considerable number of soluble sugars such as sucrose which makes it suitable for use as a substrate in microbial fermentations. This composition would make the use of pineapple peel for fungal growth attractive, since it is low-cost and rich in carbon and nitrogen and also a source of minerals (17).

Validation of the experimental model

The results predicted by the second order model developed for invertase production by A. carbonarius PC-4 suggested an optimal production with media supplemented with 13.5 g/L of pineapple crown and 3.5 g/L of yeast extract (9.7±0.4 U/mL). The validation experiments corroborate with the model and showed an invertase production of similar value suggested by the second order model (9.4±0.4 U/mL).

Influence of temperature, pH and agitation speed on cell growth and enzyme synthesis

The cultivation temperatures ranging from 15 to 45 °C were used to determine their effects on cell growth and invertase production by A. carbonarius PC-4. Additional culture conditions were previously established and maintained (120 h cultivation, 180 rpm, and initial pH=6.0) (Fig. S1a). The increase of incubation temperature to 20 °C resulted in the highest invertase production (15.5±0.8 U/mL), with yield and productivity values of 783.2 U/g and 2.9 U/h, respectively (Table S1). Invertase production and fermentation parameters remained at high levels until 30 °C, with decreasing values observed above this temperature. At 40 and 45 °C was not observed enzyme production. Other enzymes, e.g. pectinases, amylases, glucosidases, and proteases were produced by A. carbonarius strains using temperatures of cultivation among 28-30 °C (18,19,20,21). The effect of temperature on invertase production by filamentous fungi was widely reported in the literature (3,10,16). Aspergillus spp. strains are generally grown at 30 °C for invertase production. In this work, the temperature of
growth for *A. carbonarius* PC-4 at 20 °C can be explained due to this strain has been isolated from canned peach syrups maintained at low temperature.

The initial pH of cultivation medium is an important physical parameter that affects microbial growth, metabolic activity maintenance and enzyme production by *Aspergillus* spp. strains (22,16,23). Cultivations at different initial pH values were used to evaluate the invertase production by *A. carbonarius* PC-4, maintaining the conditions previously established (120 h, 180 rpm, and 20 °C). The invertase production reached the maximum at pH=5.5 (18.1±0.4 U/mL) (Fig. S1b), with a yield of 1,140.9 U/g and a productivity of 4.3 U/h (Table S1). pH values ranging from 5.0 to 6.5 resulted in invertase production varying from 13.0 to 14.0 U/mL. Similar behaviour was observed for *A. niger* IBK1 in which invertase production was higher in acidic pH range (4.0-6.0), with maximum invertase production occurring at pH=5.0 (16). Dinarvand et al. (22) evaluated the combined effect of pH-temperature and pH-inoculum on invertase and inulinase production by *A. niger* ATCC 20611. Under these conditions, the maximum invertase production occurred at moderately acidic initial pH of 6.5.

The experiments evaluated the effect of agitation speed on *A. carbonarius* PC-4 invertase production, yield and productivity. Four agitation speeds were assayed to evaluate the invertase production by *A. carbonarius* PC-4, maintaining the conditions previously established for 120 h (pH=5.5, 20 °C). The invertase production and additional fermentation parameters increased as the rate of agitation increased up to 180 rpm for 72 h (invertase production of 18.7±0.1 U/mL, yield of 1,184.2 U/g and productivity of 4.4 U/h) (Fig. S1c and Table S1). Low agitation speed (130 rpm) promoted the lowest invertase production reaching the maximum activity of 6.1 U/mL, with a yield of 320.3 U/g and productivity of 0.7 U/h after 48 h.

The influence of agitation speed on enzyme production is an important factor in the fermentation process affecting the successful progress of submerged cultivations in a flask system, since it provides adequate mixing, mass and heat transfer, with consequent improve of dissolved oxygen levels in the cultivation medium (24,25). However, the excessive agitation can produce greater mechanical forces and hydrodynamic shear stress, which implies a variety of effects on microbial cell such as rupture of cell wall and change in filamentous fungi morphology, variation of efficiency and growth rates besides formation of undesired products (25). Al-Hagar et al. (15) reported the increased of invertase production in 1.5-fold under agitation at 30 rpm compared to static condition, reaching the peak of enzyme production at 150 rpm. In this work, invertase production by *A. carbonarius* PC-4 increased 3 -fold when the agitation speed was adjusted from 130 to 180 rpm; with further decreased values of enzyme activity when agitation speed was increased to 210 rpm (13.3 U/mL). Dinarvand et al. (8) related that the increase of agitation speed of shake flask cultivations
was followed by a progressive increase of invertase production and cell growth up to 150 rpm. although higher agitation rates were deleterious for fungal growth, especially due to the formation of hydrogen peroxide, which is prejudicial to the cell.

Inverted sugar syrup production

The hydrolysis of sucrose was carried out in terms of initial concentration of sucrose (5 to 30 %, m/V), at 50 °C, pH=5.0. The reactions were initiated with addition of 0.15 unity of invertase. At low concentration (1 %, m/V) of sucrose, the hydrolysis reactions by invertase from A. carbonarius PC-4 reached the maximum production of reducing sugars of 498 µmol after 90 min of reaction. By Increasing the sucrose concentration up to 10 % (m/V), the maximum reducing sugar production was observed after 150 min of reaction (3,451.7 µmol of reducing sugars) (Fig. 2). Further increase of sucrose concentrations led to decreased hydrolysis rates in the range of 1.9 – 2.4-fold after 180 h.

Fig. 2

Keramat et al. (9) evaluated the effect of sucrose concentration on invertase activity using dynamic light scattering analysis. The authors observed that high sucrose concentrations promoted the intensification of sucrose clusters hindering the enzyme access to the sucrose molecules. Besides this, an increase of sucrose concentration leads to a reduction of water activity due to changes in the distribution of hydrogen bonds between water and sucrose molecules. Therefore, the decrease of hydrolysis rate by increasing the concentration of sucrose also could occur due to folded structures of sucrose molecules and their aggregates that remained non-hydrolysed under invertase catalysis.

The invertase synthesized by A. carbonarius PC-4 presented two important food industry applications: production of invert syrup at low sucrose concentrations the synthesis of fructooligosaccharides at high sucrose concentrations (13). The results observed in this study showed that A. carbonarius PC-4 invertase is suitable to be applied in bioprocess development involving initial sugarcane pressing, juice filtration, sugar hydrolysis in order to obtain high-fructose syrups. Mohd Zain et al. (29) evaluated the application of commercial invertases on sucrose hydrolysis of liquid pineapple waste, reaching high concentration of glucose. Additional applications of invertases are preparation of creams and marshmallows, powder milk for infants, candies containing liquefied sugar center, chocolate-covered cherries, digestive aid tablets, artificial honey besides plasticizing agents for cosmetics (30).

CONCLUSIONS
An efficient utilization of pineapple crown waste in order to produce $\beta$-fructofuranosidase was developed using a newly isolated *A. carbonarius* PC-4 under submerged conditions. Optimized production of $\beta$-fructofuranosidase was achieved using a mixture design (Simplex Lattice Design) and parametric experimental sets leading to 3-fold increase of invertase production from low-cost substrates (pineapple crown and yeast extract). $\beta$-fructofuranosidase produced by *A. carbonarius* PC-4 showed a promising potential for the synthesis of inverted sugars from different initial sucrose concentrations, which is particularly interesting to the food industry, especially the production of sweeteners for confectionery and beverage industries. Therefore, the results obtained in this study revealed *A. carbonarius* PC-4 as a new and adapted microbial strain suitable for the synthesis of invertase from an agro-waste of pineapple industry.

FUNDING

A.F. Almeida thanks the Federal University of Tocantins by Productivity Grant (Edital 10/2018) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Finance Code 001 for the scholarship awarded to R.D. Batista.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

All supplementary material is available at: www.ftb.com.hr.

AUTHOR’S CONTRIBUTION

Ryhára Dias Batista participated in the work conception, designed, and carried out all the experiment work, data analysis and wrote the manuscript. Fernanda Guimarães Melo participated in the experiment work. Claudia Cristina Auler do Amaral Santos was involved in statistical analysis and review of the manuscript. Fabricio Coutinho de Paula-Elias participated of manuscript writing and review. Rafael Firmani Perna was involved in the invertase application, manuscript writing and review. Michelle Cunha Abreu Xavier was involved in statistical analysis and interpretation, manuscript writing and review. Sergio Andres Villalba Morales was involved in data analysis and manuscript writing. Alex Fernando de Almeida conceived, planned, designed, and supervised the entire work, and also contributed to writing and finalizing the manuscript. All authors have read and approved the final manuscript.
ORCID ID
R.D. Batista https://orcid.org/0000-0003-2397-3318
C.C.A.A. Santos https://orcid.org/0000-0001-5512-6119
F.C. de Paula-Elias https://orcid.org/0000-0001-8095-2156
R.F. Perna https://orcid.org/0000-0003-3195-8898
M.C.A. Xavier https://orcid.org/0000-0003-3564-6007
S.A.V. Morales http://orcid.org/0000-0002-2513-8490
A.F. Almeida https://orcid.org/0000-0001-5391-4621

REFERENCES
http://dx.doi.org/10.1590/S0104-66322012000100006

https://doi.org/10.3389/fmicb.2016.02087

https://doi.org/10.1016/j.bjm.2016.10.026

https://doi.org/10.1002/ceat.201400389

http://dx.doi.org/10.1590/S1517-83822014000100007


https://doi.org/10.1021/ac60147a030

https://doi.org/10.1155/2019/6956202

http://dx.doi.org/10.21577/0100-4042.20170281


   http://dx.doi.org/10.4331/wjbc.v6.i3.265

   https://doi.org/10.4172/2161-0517.1000127


   https://docs.tibco.com/pub/stat/13.4.0/doc/pdf/TIB_stat_13.4_quick_ref.pdf?id=1


   http://dx.doi.org/10.1016/j.bej.2010.02.009

   http://dx.doi.org/10.1016/j.procbio.2015.04.015
Table 1. Simplex lattice mixture design arrangement of the actual and coded independent variables for β-fructofuranosidase production by *A. carbonarius* PC-4

<table>
<thead>
<tr>
<th>Run</th>
<th>Ammonium chloride (g/L)</th>
<th>Yeast extract (g/L)</th>
<th>Pineapple crown (g/L)</th>
<th>Invertase (U/mL)</th>
<th>Yp/s (U/g)</th>
<th>Pp (U/h)</th>
<th>Invertase (U/mL)</th>
<th>Yp/s (U/g)</th>
<th>Pp (U/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.0 (1.0)</td>
<td>0.0 (0.0)</td>
<td>1.0 (0.0)</td>
<td>1.4±0.06</td>
<td>1,404.3</td>
<td>0.39</td>
<td>3.0±0.04</td>
<td>2431.0</td>
<td>0.41</td>
</tr>
<tr>
<td>2</td>
<td>0.0 (0.0)</td>
<td>10.0 (1.0)</td>
<td>1.0 (0.0)</td>
<td>3.9±0.01</td>
<td>3,121.6</td>
<td>0.87</td>
<td>5.6±0.06</td>
<td>4,803.8</td>
<td>0.80</td>
</tr>
<tr>
<td>3</td>
<td>0.0 (0.0)</td>
<td>0.0 (0.0)</td>
<td>20.0 (1.0)</td>
<td>9.0±0.18</td>
<td>361.9</td>
<td>2.01</td>
<td>9.2±0.13</td>
<td>346.2</td>
<td>1.15</td>
</tr>
<tr>
<td>4</td>
<td>5.0 (0.5)</td>
<td>5.0 (0.5)</td>
<td>1.0 (0.0)</td>
<td>4.3±0.02</td>
<td>3,884.7</td>
<td>1.08</td>
<td>5.9±0.03</td>
<td>5,345.4</td>
<td>0.89</td>
</tr>
<tr>
<td>5</td>
<td>5.0 (0.5)</td>
<td>0.0 (0.0)</td>
<td>10.0 (0.5)</td>
<td>9.7±0.18</td>
<td>775.3</td>
<td>2.15</td>
<td>9.6±0.13</td>
<td>866.0</td>
<td>1.44</td>
</tr>
<tr>
<td>6</td>
<td>0.0 (0.0)</td>
<td>5.0 (0.5)</td>
<td>10.0 (0.5)</td>
<td>8.9±0.03</td>
<td>669.9</td>
<td>1.86</td>
<td>9.8±0.16</td>
<td>833.6</td>
<td>1.39</td>
</tr>
<tr>
<td>7</td>
<td>10.0 (1.0)</td>
<td>0.0 (0.0)</td>
<td>1.0 (0.0)</td>
<td>1.0±0.13</td>
<td>1,047.4</td>
<td>0.29</td>
<td>2.0±0.09</td>
<td>1,802.5</td>
<td>0.30</td>
</tr>
<tr>
<td>8</td>
<td>0.0 (0.0)</td>
<td>10.0 (1.0)</td>
<td>1.0 (0.0)</td>
<td>2.8±0.19</td>
<td>2,210.0</td>
<td>0.61</td>
<td>3.9±0.11</td>
<td>3,491.1</td>
<td>0.58</td>
</tr>
<tr>
<td>9</td>
<td>0.0 (0.0)</td>
<td>0.0 (0.0)</td>
<td>20.0 (1.0)</td>
<td>7.5±0.19</td>
<td>281.4</td>
<td>1.56</td>
<td>10.0±0.15</td>
<td>357.7</td>
<td>1.19</td>
</tr>
<tr>
<td>10</td>
<td>5.0 (0.5)</td>
<td>5.0 (0.5)</td>
<td>1.0 (0.0)</td>
<td>4.8±0.02</td>
<td>4,079.8</td>
<td>1.13</td>
<td>6.3±0.13</td>
<td>5,676.9</td>
<td>0.95</td>
</tr>
<tr>
<td>11</td>
<td>5.0 (0.5)</td>
<td>0.0 (0.0)</td>
<td>10.0 (0.5)</td>
<td>6.0±0.15</td>
<td>480.6</td>
<td>1.34</td>
<td>6.1±0.31</td>
<td>630.8</td>
<td>1.05</td>
</tr>
<tr>
<td>12</td>
<td>0.0 (0.0)</td>
<td>5.0 (0.5)</td>
<td>10.0 (0.5)</td>
<td>9.6±0.23</td>
<td>723.4</td>
<td>2.01</td>
<td>8.4±0.16</td>
<td>716.2</td>
<td>1.19</td>
</tr>
</tbody>
</table>

YP/s = yield of invertase, Pp = productivity of invertase
Table 2. ANOVA for significance of the regression models

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F</th>
<th>p</th>
<th>Sum of squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>102.09</td>
<td>5</td>
<td>20.42</td>
<td>13.52</td>
<td>0.003</td>
<td>76.28</td>
<td>5</td>
<td>15.25</td>
<td>10.04</td>
<td>0.007</td>
</tr>
<tr>
<td>Total error</td>
<td>9.06</td>
<td>6</td>
<td>1.51</td>
<td></td>
<td></td>
<td>9.11</td>
<td>6</td>
<td>1.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack of fit</td>
<td>0.00</td>
<td>0</td>
<td>0.00</td>
<td></td>
<td></td>
<td>0.00</td>
<td>0</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure error</td>
<td>9.05</td>
<td>6</td>
<td>1.51</td>
<td></td>
<td></td>
<td>9.11</td>
<td>6</td>
<td>1.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total adjusted</td>
<td>111.15</td>
<td>11</td>
<td>10.10</td>
<td></td>
<td></td>
<td>85.39</td>
<td>11</td>
<td>7.76</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Factor</th>
<th>Effect</th>
<th>Error</th>
<th>t</th>
<th>p-value</th>
<th>Factor</th>
<th>Effect</th>
<th>Error</th>
<th>t</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium chloride</td>
<td>1.22</td>
<td>0.87</td>
<td>1.41</td>
<td>0.21</td>
<td>2.52</td>
<td>0.87</td>
<td>2.89</td>
<td>0.03</td>
<td>2.52</td>
</tr>
<tr>
<td>(X₁)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast extract (X₂)</td>
<td>3.33</td>
<td>0.87</td>
<td>3.83</td>
<td>0.01</td>
<td>4.76</td>
<td>0.87</td>
<td>5.47</td>
<td>0.00</td>
<td>4.76</td>
</tr>
<tr>
<td>X₁.X₂</td>
<td>8.27</td>
<td>0.87</td>
<td>9.52</td>
<td>0.00</td>
<td>9.73</td>
<td>0.87</td>
<td>11.16</td>
<td>0.00</td>
<td>9.73</td>
</tr>
<tr>
<td>X₁.X₃</td>
<td>9.12</td>
<td>4.26</td>
<td>2.14</td>
<td>0.07</td>
<td>9.92</td>
<td>4.27</td>
<td>2.32</td>
<td>0.06</td>
<td>9.92</td>
</tr>
<tr>
<td>X₂.X₃</td>
<td>12.40</td>
<td>4.26</td>
<td>2.91</td>
<td>0.03</td>
<td>7.37</td>
<td>4.27</td>
<td>1.72</td>
<td>0.13</td>
<td>7.37</td>
</tr>
<tr>
<td>X₁.X₂</td>
<td>13.94</td>
<td>4.26</td>
<td>3.27</td>
<td>0.02</td>
<td>7.48</td>
<td>4.27</td>
<td>1.75</td>
<td>0.13</td>
<td>7.48</td>
</tr>
</tbody>
</table>

72 h: R²=91.85 %; R² (adjusted)=85.06 %; 120 h: R²=89.33 %; R² (adjusted)=80.43 %
Table S1. Fermentation parameters of β-fructofuranosidase production by \textit{A. carbonarius} PC-4 in different conditions of cultivation

<table>
<thead>
<tr>
<th>Fermentation parameters</th>
<th>Invertase activity</th>
<th>Time (h) *</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature (°C)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>(599.6±3.82)c</td>
<td>(2.23±0.01)c</td>
</tr>
<tr>
<td>20</td>
<td>(783.2±6.77)a</td>
<td>(2.92±0.01)a</td>
</tr>
<tr>
<td>25</td>
<td>(690.0±5.34)b</td>
<td>(2.57±0.01)b</td>
</tr>
<tr>
<td>30</td>
<td>(407.5±1.0)d</td>
<td>(1.52±0.00)d</td>
</tr>
<tr>
<td>35</td>
<td>(131.1±0.9)e</td>
<td>(0.49±0.00)e</td>
</tr>
<tr>
<td>40</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>45</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>(36.82±1.07)i</td>
<td>(0.14±0.00)b</td>
</tr>
<tr>
<td>3.5</td>
<td>(123.2±4.10)h</td>
<td>(0.46±0.00)g</td>
</tr>
<tr>
<td>4.0</td>
<td>(434.0±5.04)f</td>
<td>(1.62±0.01)e</td>
</tr>
<tr>
<td>4.5</td>
<td>(706.9±10.21)c</td>
<td>(2.63±0.01)c</td>
</tr>
<tr>
<td>5.0</td>
<td>(745.8±8.33)c</td>
<td>(2.78±0.01)c</td>
</tr>
<tr>
<td>5.5</td>
<td>(1,149.9±14.28)a</td>
<td>(4.28±0.02)a</td>
</tr>
<tr>
<td>6.0</td>
<td>(934.5±3.13)b</td>
<td>(3.48±0.01)b</td>
</tr>
<tr>
<td>6.5</td>
<td>(906.6±6.08)b</td>
<td>(3.37±0.01)b</td>
</tr>
<tr>
<td>7.0</td>
<td>(639.6±5.12)d</td>
<td>(2.38±0.01)c</td>
</tr>
<tr>
<td>7.5</td>
<td>(573.0±5.06)e</td>
<td>(2.13±0.01)d</td>
</tr>
<tr>
<td>8.0</td>
<td>(307.5±2.04)g</td>
<td>(1.14±0.01)f</td>
</tr>
</tbody>
</table>

Yp/s=yield of invertase, \(Pp=\)productivity of invertase, n.d.=not detected. *Time of the highest values of each fermentation parameters
Fig. 1. Pareto chart and quadratic contour area of ternary plots of the culture media variables on β-fructofuranosidase production by *A. carbonarius* PC-4 after 72 h (a, c) and 120 h (b, d)
Fig. 2. Hydrolysis of sucrose by β-fructofuranosidase from *A. carbonarius* PC-4 produced under submerged culture
Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

a)

![Graph showing temperature (°C) vs. β-fructofuranosidase activity (U/mL)]

b)

![Graph showing time-course (hours) vs. invertase activity (U/mL)]
Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

c)

**Fig. S1.** Effect of temperature (a), pH (b) and agitation speed (c) on β-fructofuranosidase production by *A. carbonarius* PC-4 under submerged conditions. Legend: 130 rpm (■), 150 rpm (●), 180 rpm (▲) and 210 rpm (♦). Culture conditions: effect of temperature and pH experiments were carried out for 72 h; agitation speed experiments were carried out under optimized condition for 120 h of cultivation.