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Screening of fungi from the genus *Penicillium* for production of β-fructofuranosidase and enzymatic synthesis of fructooligosaccharides

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ABSTRACT

Eight new isolated fungi of the genus *Penicillium* were evaluated for β- fructofuranosidase (FFase) production. From these, *Penicillium citreonigrum* was selected for FFase and fructooligosaccharides (FOS) production. The influence of temperature, yeast extract concentration, pH and fermentation time on the FFase activity when using the whole microorganism was evaluated by 2^4 and 2^3 designs. The pH was set at 6.5 and no yeast extract was used in the optimization experiments since both shown low significant effects on FFase activity. After optimization, temperature and fermentation time, were set to 25.5 °C and 67.8 h. Under these conditions, the model predicted a FFase production of 301.84 U/mL. The scaled-up process in a 2 L bioreactor enhanced the enzyme productivity up to 1.5 times (6.11 U/mL h). A concentration of 58.7 g/L of FOS was obtained, where kestose was the main product. Assays performed for enzyme characterization showed that 50 °C and a pH 5.0 are the optimal conditions for FFase activity. FFase showed to be stable at temperatures between 25 and 30 °C and pH 4.0–10.0 and its activity increased in the presence of ions, especially Cu^{4+}. Results obtained in this primary report are a clear indication on the interest of using *P. citreonigrum* as a source of FFase for further FOS production.

1. Introdution

Enzymes named β-fructofuranosidases (FFase) are located in the GH32 family of the glycosil hydrolases and grouped in different isoforms according to their pH of action as acid, alkaline and neutral enzymes [1]. At high sucrose concentration, some FFase are able to catalyze transfructosylation reactions synthesizing fructooligosaccharides (FOS) [2]. Most of these enzymes have been found in fungi such as *Aureobasidium* spp., *Aspergillus* spp. and *Penicillium* spp. [3,4].

FOS are carbohydrates naturally found in small quantities in several plants, though their concentrations are generally too small for produce a significant beneficial effect in humans. For large scale production, enzymes derived from microorganisms such as β-fructofuranosidases (FFase) (EC 3.2.1.26) and fructosyltransferases (FTase) (EC 2.4.1.9) are used [5,6].

Structurally, FOS are non-conventional sugars, namely, kestose (GF_2), nystose (GF_3) and fructofuranosylnystose (GF_4), composed by β(2 → 1)-linked fructose (F) units attached to a terminal glucose (G) moiety by a α(2 → 1) linkage [7]. These oligosaccharides are important prebiotics since their daily intake is related with many health benefits such as: reducing the incidence of intestinal infections, protection against colon cancer, improving mineral absorption, decreasing total cholesterol and lipid blood serum, and an overall improvement of human health [8,9].

For this reason, a rapid market growth on FOS has emerged in the recent years, all over the world. The current largest world market for prebiotic food is the Europe. Asia-Pacific is forecast to emerge as the fastest growing market with a compound annual growth rate (CAGR) of 14% over the analysis period. According to Global Industry Analysts (GIA) report, the global market for prebiotics is projected to surpass $5.9 billion by 2020, driven by expanding applications beyond the food industry and into the animal feed sector [10].

To meet market needs, it is essential keep searching for new microbial strains with potential for FFase production and enzymatic synthesis of FOS. However, the cost of enzyme production and purification is one of the main factors limiting their industrial application. This cost can be reduced by optimizing the fermentation strategies, operational conditions and culture medium composition using statistical tools such as response surface methodology (RSM) to increase enzyme activity.

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The RSM is a powerful technique for testing multiple process variables because fewer experimental trials are needed. Conventional method of optimization involves varying one parameter at a time and keeping others constant. This method is extremely time consuming and often does not bring about the effect of interaction of various parameters [11]. The single dimensional search is laborious and less capable of reaching true optimum due to interactions among variables [12].

Therefore, the aim of this work was the screen and identification of new isolated strains of *Penicillium* fungi with potential for FFase enzyme production. Experimental conditions for FFase production and FOS synthesis were optimized for the strain selected, in shaken flask and bioreactor, using successive experimental designs. The isolated FFase was also characterized regarding its optimal pH, temperature, stability and effect of ions in its enzymatic activity.

2. Experimental

2.1. Chemicals

FOS standards (1-kestose, 1-nystose, and 1-β-fructofuranosylnystose) were purchased in Wako Pure Chemical Industries, Ltd. (Japan Company). Sugars sucrose and fructose were obtained from Merck (USA) and glucose from VWR (Belgium). A glucose–oxidase–peroxidase enzymatic kit was obtained from Labtest (Brazil). Other reagents were purchased from Sigma Chemical (St. Louis, MO, USA), Merck (Darmstadt,Germany), Oxoid (England) or Himedia (India).

2.2. Microorganisms and culture conditions

Eight strains belonging to the *Penicillium* genus were investigated for FFase production. The fungi were obtained from Micoteca of the Federal University of Pernambuco (URM – UFPE). The strains tested, *P. aurantiogriseum* (URM 5139), *P. citrinum* (URM 2725), *P. commune* (URM 4939), *P. aurantiogriseum* (URM 5126), *P. implicatum* (URM 5426), *P. citreonigrum* (URM 4459), *P. glabrum* (URM 4757), *P. islandicum* (URM 5073), were grown and maintained on Czapek Dox Agar medium at 4 °C. A concentrated spore solution wasobtained from a 7 days growing in a Czapek Dox agar medium at 30 °C. Spores were scraped from the plates with a sterile solution of Tween 80 at 0.1% (w/v). The number of spores in the concentrated solution was determined by counting in a microscope, using an improved *Neubauer* chamber. The suspension was finally diluted to a concentration of 10^4 spores/mL.

2.3. Screening of fungi for FFase production

A 35 μL volume of a spore suspension (10^4 spores/mL) was transferred to a 250 mL shaken flask containing 100 mL of fermentation medium with the following composition (% w/v): sucrose, 20.0; yeast extract, 2.75; $NaNO_3$, 0.2; $MgSO_4{\cdot}7H_2O$, 0.05; K_2HPO_4, 0.5; and KCl, 0.05 [13]. The culture medium was previously autoclaved at 121 °C for 15 min and the pH was adjusted to 5.50 before inoculation. Fermentations were carried out at 30 °C under an agitation of 150 rpm. Samples were harvested at 0, 12, 16, 20, 24 and 36 h of fermentation. At the end of fermentation, cultures were firstly vacuum filtered with a Whatman paper number 1, for cell pellets removal. The filtrated solution obtained was used for extracellular FFase activity determination and FOS quantification after re-filtration through a 0.2-μm cut-off filter.

2.4. Determination of FFase transfructosylation activity

The FFase transfructosylation activity was determined according to the method described by Ganaie et al. [14], using sucrose as substrate, at a concentration of 60% (w/v), in citrate buffer, at pH 6.0. The absorbance of glucose released was read at 505 nm on Synergy™ HT Multi-Mode Microplate Reader (BioTek, USA) using an enzymatic glucose determination kit. The units of FFase activity were defined as the amount of enzyme required to release 1 μmol of glucose per minute.

2.5. Sugar analysis

FOS (1-kestose, 1-nystose, and 1-β-fructofuranosylnystose), fructose, glucose and sucrose were analyzed using high-performance liquid chromatography (HPLC) using a LC–10 A equipment (Jasco, Japan) equipped with a Prevail Carbohydrate ES column (5 μm, 250 × 4.6 mm, Alltech). Sugars were detected with an evaporative light scattering detector, Sedex 85 (Sedere). A mixture of acetonitrile and water containing 0.04% ammonium hydroxide (70:30 v/v) was used as mobile phase, at a flow rate of 1.0 mL/min and 25 °C [15,16]. The chromatographic signal was recorded and further integrated using the software LabSolutions (Shimadzu).

2.6. Optimization of FFase production

2.6.1. Complete factorial design on the production of FFase

The enzyme production was optimized for the *Penicillium* strain which FFase activity was higher (Section 2.3: Screening of fungi for FFase production). Two successive full factorial designs (2^4 and 2^3), with three central points, were carriedout to study the effects and interactions of temperature, yeast extract concentration, pH and fermentation time, on the production of FFase. The variables with statistically influence on the FFase activity were selected for further value optimization (Section 2.6.2: Central composite design on the production of FFase). Experimental conditions used in each assay are shown in Tables 1 and 2, respectively.

2.6.2. Central composite design on the production of FFase

A central composite design (2^2) was used to determine the optimal experimental conditions for FFase production. Only the variables found to statistically influence the enzyme production were tested (Section 2.6.1: Complete factorial design on the production of FFase). A 3 levels (−1, 0 and +1) combination was tested, resulting in a set of 10 experiments, with two central points (Table 3).The regression coefficients for linear, quadratic and interactions for each variable were determined and adjusted to a polynomial second order equation (Eq. (1)).

$$Y = \beta 0 + \beta_1 X_1 + \beta_2 X_2 + \beta_1 {X_1}^2 + \beta_{1\beta 2} X_1 X_2 + \beta_2 {X_2}^2 \quad (1)$$

where Y = FFase activity (U/mL), X_1 the temperature, X_2 fermentation time and β_i is the regression coefficient for each factor.

2.7. Statistical analysis

The results were analyzed using Statistica 8.0 [17]. Positive effects were considered significant for *p-values* lower than 0.05 [18].

2.8. Production of FFase in bioreactor

Fermentations were carried out using the *Penicillium* strain that obtained higher FFase activity. A 35 μL volume of a spore suspension (10^4 spores/mL) was transferred to a 250 mL shaken flask containing 100 mL of inoculum medium with the following composition (% w/v): sucrose, 10.0; $NaNO_3$, 0.2; $MgSO_4{\cdot}7H_2O$, 0.05; K_2HPO_4, 0.5; and KCl, 0.05.This inoculum was grown for 3 days at 28 °C and 150 rpm and afterwards transferred to a 2 L stirred tank bioreactor (Autoclavable Benchtop Fermenter Type R'ALF,

Table 1
Experimental conditions assayed and respective β-fructofuranosidase activity values obtained for *Penicillium citreonigrum* according to a 2^4 complete factorial design.

Independent variables (real and (coded) values)					Dependent variable
Run	Fermentation time (h)	Temperature (°C)	pH	Yeast extract (%(w/v))	Activity β-fructofuranosidase (U/mL)
1	36 (−1)	25 (−1)	4 (−1)	0 (−1)	12.03
2	60 (+1)	25 (−1)	4 (−1)	0 (−1)	168.85
3	36 (−1)	35 (+1)	4 (−1)	0 (−1)	17.08
4	60 (+1)	35 (+1)	4 (−1)	0 (−1)	30.66
5	36 (−1)	25 (−1)	6 (+1)	0 (−1)	32.89
6	60 (+1)	25 (−1)	6 (+1)	0 (−1)	200.99
7	36 (−1)	35 (+1)	6 (+1)	0 (−1)	0
8	60 (+1)	35 (+1)	6 (+1)	0 (−1)	1.62
9	36 (−1)	25 (−1)	4 (−1)	2.750 (+1)	30.10
10	60 (+1)	25 (−1)	4 (−1)	2.750 (+1)	195.83
11	36 (−1)	35 (+1)	4 (−1)	2.750 (+1)	1.38
12	60 (+1)	35 (+1)	4 (−1)	2.750 (+1)	1.90
13	36 (−1)	25 (−1)	6 (+1)	2.750 (+1)	60.14
14	60 (+1)	25 (−1)	6 (+1)	2.750 (+1)	287.10
15	36 (−1)	35 (+1)	6 (+1)	2.750 (+1)	0
16	60 (+1)	35 (+1)	6 (+1)	2.750 (+1)	0
17	48 (0)	30 (0)	5 (0)	1.375 (0)	261.09
18	48 (0)	30 (0)	5 (0)	1.375 (0)	263.56
19	48 (0)	30 (0)	5 (0)	1.375 (0)	256.57

Table 2
Experimental conditions assayed and respective β-fructofuranosidase activity values obtained for *Penicillium citreonigrum*, according to a 2^3 complete factorial design.

Independent variables (real and (coded) values)				Dependent variable
Run	Fermentation time (h)	Temperature (°C)	pH	Activity β-fructofuranosidase (U/mL)
1	48 (−1)	20 (−1)	5 (−1)	14.73
2	48 (−1)	30 (+1)	5 (−1)	187.12
3	72 (+1)	20 (−1)	5 (−1)	178.09
4	72 (+1)	30 (+1)	5 (−1)	313.33
5	48 (−1)	20 (−1)	7 (+1)	14.10
6	48 (−1)	30 (+1)	7 (+1)	214.75
7	72 (+1)	20 (−1)	7 (+1)	229.37
8	72 (+1)	30 (+1)	7 (+1)	266.68
9	60 (0)	25 (0)	6 (0)	274.52
10	60 (0)	25 (0)	6 (0)	281.83
11	60 (0)	25 (0)	6 (0)	263.61

Bioengineering AG, Wald, Switzerland), using a working volume of 1L culture medium with the same composition of the inoculum, except for sucrose, which concentration was adjusted for 20% (w/v).Fermentations were performed under temperature and fermentation time conditions previously optimized for the production of FFase, in shaken flask. Samples were collected at 0, 15, 20, 24, 38, 44, 48, 60 and 88 h of fermentation and filtered through a 0.2 μm cellulose acetate membrane for FFase activity and sugars determination. After filtration, the final fermentation broth containing the FFase enzyme produced was also used for FOS enzymatic synthesis.

2.9. Effect of pH and temperature on the enzyme stability and FFase activity

The effect of pH in the FFase activity was studied in a range between 3.0 and 10.0, by variation of one unity of pH per assay. Experiments were carried out at 55 °C, for 60 min, using 20% (w/v) sucrose in a 0.1 M citrate buffer (pH 4.0–6.0), sodium phosphate buffer (7.0 and 8.0) and carbonate bicarbonate buffer (9.0 and 10.0). The effect of temperature on the FFase activity was determined for temperatures between 25 and 80 °C, by increasing five degrees per assay. Tests were carried out for 60 min in 0.1 M citrate buffer (pH 6.0). The enzyme pH stability was estimated by measuring the residual activity after crude extract pre-incubation, at pH 3.0–10.0, for 60 min. Thermal stability of FFase was determined using the value of the residual activity measured after incubation of the crude extract at different temperatures (25–85 °C), for 60 min. The residual activity was measured under standard conditions, as described in Section 2.4.

2.10. Ions effect on FFase activity

The effect of the presence of ions at low (5 mM) and high concentrations (10 mM), on FFase activity was studied. The following salts were tested: $MgSO_4$, NaCl, KCl, $FeCl_3$, $CuSO_4$, $FeSO_4$, $ZnSO_4$, $ZnCl_2$ and $CaCl_2$. The residual activity was determined after incubation at 37 °C, for 60 min, as described in Section 2.4.

2.11. Enzymatic synthesis of FOS using FFase extract

FOS were also synthesized using the extracellular FFase enzymes, produced in bioreactor. The reaction mixture was conducted with 35 mL of FFase extract and 70 mL of a sucrose solution (1:3), in shaken flask (250 mL), at 50 °C and 150 rpm of agitation for 24 h. The pH was set to 5.0 before incubation. At the end of incubation, the reaction was stopped by heating the samples for 10 min at 100 °C. Samples were further diluted, when necessary, and filtered at 0.2 μm for FOS and sucrose analysis by HPLC.

Table 3
Experimental conditions assayed and respective β-fructofuranosidase activity values obtained for *Penicillium citreonigrum* according to a 2^2 central composite design.

Independent variables (real and (coded) values)			Dependent variable
Run	Fermentation time (h)	Temperature (°C)	Activity β-fructofuranosidase (U/mL)
1	43 (−1)	25 (−1)	74.85
2	67 (+1)	25 (−1)	288.17
3	43 (−1)	35 (+1)	19.79
4	67 (+1)	35 (+1)	57.42
5	55 (0)	25 (−1)	241.95
6	55 (0)	35 (+1)	42.62
7	43 (−1)	30 (0)	67.44
8	67 (+1)	30 (0)	252.70
9	55 (0)	30 (0)	241.30
10	55 (0)	30 (0)	239.04

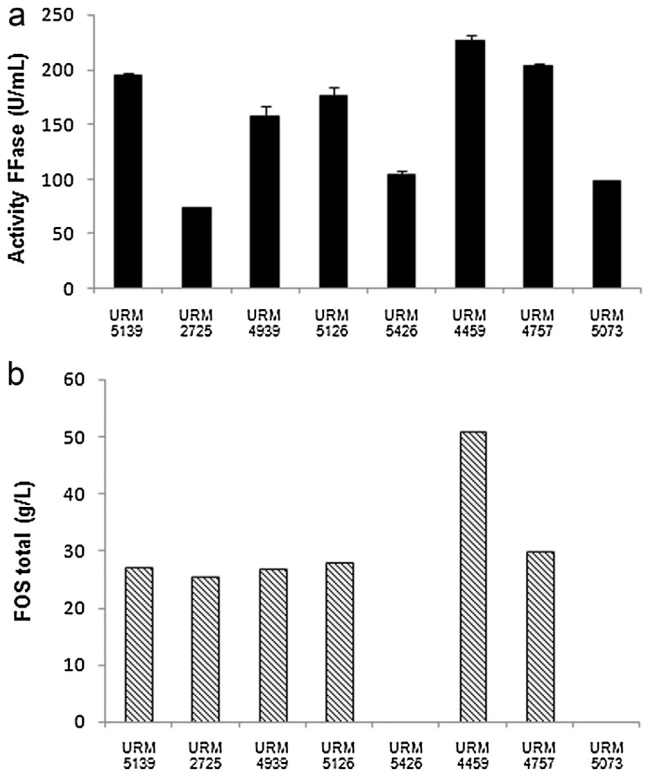

Fig. 1. β-fructofuranosidase (a) and fructooligosaccharides (b) production by the *Penicillium* tested, at 36 h of fermentation. URM 5139 — *P. aurantiogriseum*, URM 2725 — *P. citrinum*, URM 4939 — *P. citrinum*, URM 5126 — *P. aurantiogriseum*, URM 5426 — *P. implicatum*, URM 4459 — *P. citreonigrum*, URM 4757 — *P. glabrum*, URM 5073 — *P. islandicum* (abreviattions: FFase = β-fructofuranosidase, FOS = fructooligosaccharides).

3. Results and discussion

3.1. Screening of fungi for FFase production

All the eight *Penicillium* fungi strains tested showed to have transfructosylation activity after 36 h fermentation (Fig. 1a). The *P. citreonigrum* (UMR 4459) obtained the highest activity of 227.56 ± 4 U/mL, when compared to the other strains.

Mashitah and Hatijah [19] evaluated the production of FFase enzyme of eleven strains of *Penicillium*, *Aspergillus* and *Trichoderma*. Between the strains studied, *Penicillium simplicissimum* obtained the higher extracellular FFase production, confirming the potential of the *Penicillium* genus for FFase production.

To evaluate the sucrose conversion capacity of the FFase enzymes, produced by the eight fungi tested, the amount of sugars contained in the extracellular fermentation mixture were quantified at 36 h fermentation. Results are shown in Fig. 1b as percentage of FOS quantified per total amount of sugars. From Fig. 1 bit is possible to observe that only two strains were not able to convert sucrose into FOS, suggesting that, under the assayed conditions, the extracellular FFase produced by these fungi was limited to sucrose hydrolyze into monosaccharides. Hydrolitic enzymes are usually used for inverted sugar production, at high sucrose concentrations [20]. Other explanation for this phenomenon is the existence of a secondary hydrolysis, wherein the glycosyl hydrolases, belonging to the FFase family, re-hydrolyzes the pre-formed FOS products [21]. In this way, the FOS production becomes limited since an efficient acceptor molecule is necessary for an adequate transglycosilation [22].

P. citreonigrum (URM 4459) produced 50.7 g/L of FOS, representing about 26% more FOS as compared to the following best producer (*P. glabrum* URM 4757). For this reason, the *P. citreonigrum* was selected for further optimization tests of FFase and FOS production.

3.2. Optimization of FFase production

3.2.1. Complete factorial design on the production of FFase

A 2^4 complete factorial design was employed to study the influence of the fermentation time, temperature, pH and amount of yeast extract used in the FFase activity, of the *P. citreonigrum*. The highest FFase activity found (287.10 U/mL) was obtained for the run 14 (Table 1), corresponding to 60 h of fermentation, at 25 °C, pH 6.0 and 2.75% (w/v) of yeast extract.

The Pareto chart, illustrated in Fig. 2, was used for statistical analysis of the variable responses. The figure represents the estimated effects of the independent variables (temperature, fermentation time, pH and yeast extract concentration) and their interactions on the response. Results are shown in a decreasing order of magnitude, where the length of each bar is proportional to the standardised effect. All the observed results at the right of the vertical red line are statistically significant at 95% confidence level.

All the variables studied had a statistically significant effect on the FFase activity (Fig. 2). However, the effect of the yeast extract was very low when compared to the other parameters. The increase of yeast extract concentration, above a given value, was already reported in some studies has having a negative effect on the enzyme and FOS production by strains of *Aureobasidium pullulans* and *Aspergillus oryzae* [16,23]. Thus, the yeast extract was not added in the culture medium used in the further tests. A second full factorial design (2^3) was employed aiming to further improve FFase activity. The ranges of variables temperature (20, 25 and 30 °C), fermentation time (48, 60 and 72 h) and pH (5.0, 6.0 and 7.0) were slightly modified, following the trend of the effects of the first design.

Results obtained with the 2^3 s full factorial design are shown in Table 2. The effects of each independent variable on the FFase activity can be observed in Fig. 3. From the three variables studied, only two showed a statistically significant effect, the temperature and the fermentation time (Fig. 3). Both variables had a positive effect on the production of FFase, the higher the temperature and the fermentation time, the higher the enzyme production obtained. The highest FFase activity found (313.33 U/mL) was obtained for the run 4, corresponding to 72 h of fermentation, at 30 °C and pH 5.0 (Table 2).

A negative effect was observed for the interaction between the independent variables temperature and fermentation time indicat-

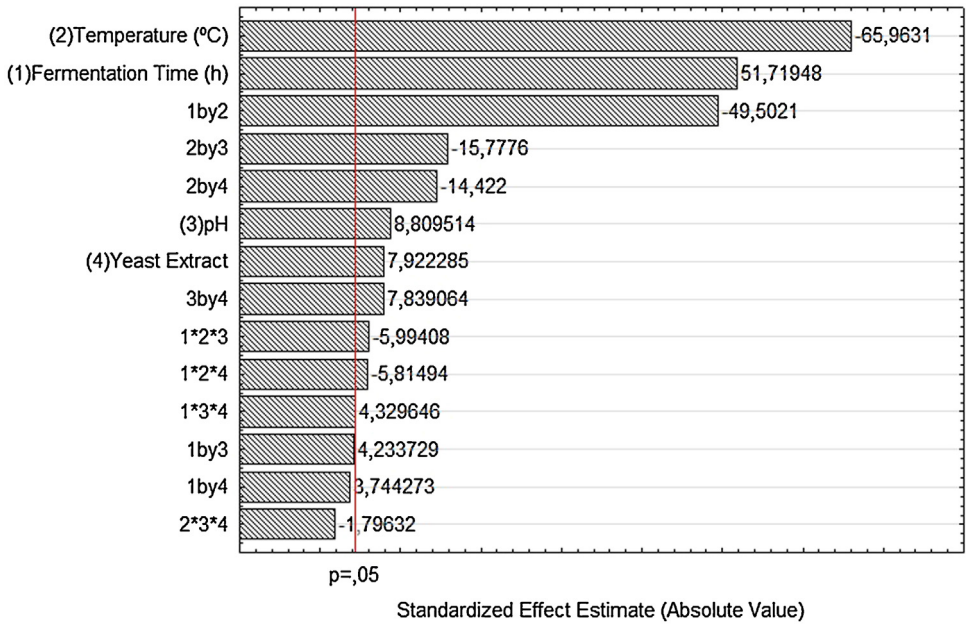

Fig. 2. Pareto chart for the standardised effects of the variables fermentation time, temperature, pH and yeast extract concentration on the production of β-fructofuranosidase by the *Penicillium citreonigrum.*

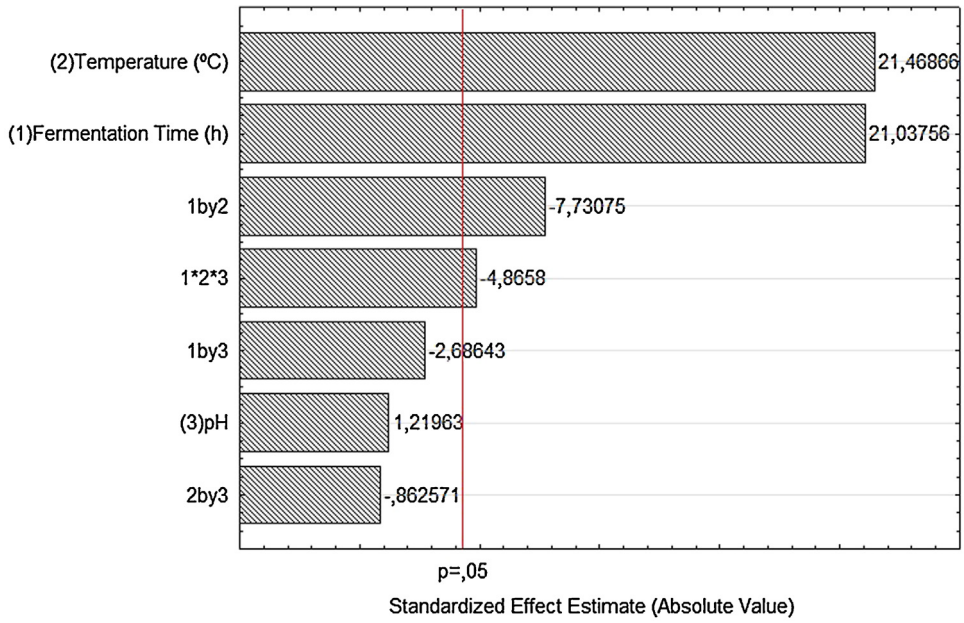

Fig. 3. Pareto chart for the standardised effects of the variables: effects of fermentation time, temperature and pH on the production of β-fructofuranosidase by the *Penicillium citreonigrum.*

ing that, a combination of high temperatures with low fermentation times results in a decreased FFase production (Fig. 3).

Similar results were obtained by Mussatto et al. [24] when studying the effect of temperature on the FFase activity and FOS production by an *Aspergillus japonicus*. While increasing the temperature from 26 to 30 °C an increasing of the FFase production was obtained, although, temperatures above 30 °C showed to have an opposite effect. According to Arumugam et al. [25] high temperatures have generally an adverse effect on the metabolic activity of microorganisms. However, a similar effect might be found for low temperatures. Thus, the temperature used in a fermentation process tends to be a crucial parameter, specially for fungi, since it influences not only the product formation, but also the spore germination and the microorganism growth.

Taking all these considerations into account, the influence of temperature and fermentation time was further optimized using a 2^2 central composite experimental design with three levels (temperature: 25, 30 and 35 °C and fermentation time: 43, 55 and 67 h). Since the pH did not show a significant statistically effect on the enzyme production, it was set as constant for all the further fermentations. The further culture mediums used were prepared with an initial pH of 6.5.

Table 4
Analysis of variance (ANOVA) for the second-order model for level optimization of β-fructofuranosidase production by *Penicillium citreonigrum*.

Factor	Sum of squares	Degrees of freedom	Mean square	F-value	p-Value[a]
(1)Temperature (°C) (L)	39227	1	39227	15392.76	0.005131
Temperature (°C) (Q)	11607	1	11607	4554.79	0.009432
(2)Time (h) (L)	31714	1	31714	12444.69	0.005707
Time (h) (Q)	6491	1	6491	2547.19	0.012612
1L by 2L	7716	1	7716	3027.97	0.011568
Lack of Fit	3562	3	1187	465.91	0.034040
Pure Error	2	1	2		
Total SS	103813	9			

$R^2 = 0.966$ and Adj. $R^2 = 0.923$.
[a] Significant for p-values ≤ 0.05.

3.2.2. Central composite design on the production of FFase

The results obtained with the central composite design (2^2) for producing FFase by *P. citreonigrum* are shown in Table 3. The highest FFase activity found (288.17 U/mL) was obtained for the run 2, corresponding to 67 h of fermentation at 25 °C temperature (Table 3).

An analysis of the variance (ANOVA) of the reduced model was required to confirm both results obtained by graphical methods and the model's adequacy. The results of response surface model of the second order, fitting in the form of ANOVA (Table 4), indicated that the linear and quadratic effects of temperature and fermentation time, and the interaction between variables were significant to the FFase activity response.

The goodness-of-fit of the reduced model was verified by the coefficient of determination R^2 that explains 96.6% of the response variability. Also, the adjusted determination coefficient ($R^2_{adjusted}$) found for the model was 0.923. This value ensured the satisfactory adjustment of the polynomial model to the experimental data. The $R^2_{adjusted}$ corrects the R^2 value for sample size and number of terms in the model. If there are many terms in the model and if the sample size is not very large, the $R^2_{adjusted}$ must be noticeably lower than R^2 [18]. In our case, the $R^2_{adjusted}$ was close to the R^2 value, meaning that the R^2 value does not need much adjustment.

The model obtained is shown by Eq. (2). The coefficients from the model were obtained by a multiple regression analysis on the experimental data using the least squares method, where Y represents the FFase activity, X_1 the temperature and X_2 and fermentation time.

$$Y = 217.9 - 80.86X_1 + 72.70X_2 - 70.53X_1^2 - 43.92X_1X_2 - 52.74X_2^2 \quad (2)$$

All parameters studied, and their interactions were found to have a statistically significant effect on the enzyme production. The effect of the temperature was negative indicating that an increase of the value of this variable above the limits studied would lead to a decrease on the FFase production. On the other hand, the fermentation time had a positive linear effect and a negative quadratic effect, indicating a tendency for maximum production of FFase close to the central point (55 h of fermentation). However, the most important effect was the linear one by increasing the production of FFase with the increasing of the fermentation time, as shown in Fig. 4. The test using minimal temperature (25 °C) and maximal fermentation time (67 h) obtained an increased FFase production that explains the negative interaction between the temperature and fermentation time.

The three-dimensional response surface described by the model (Eq. (2)), for the studied experimental region, is represented in Fig. 4. Increasing the fermentation time and reducing the temperature, within the range studied, a favorable FFase activity might be found (Fig. 4).

Prata et al. [26] evaluated the effect of the fermentation time on the activity of FFase produced from *Penicillium expansum*. The authors found that the highest activity (41 U/mL) was obtained at the end of fermentation (48 h), using a temperature of 25 °C under an agitation of 160 rpm. In the current work, although the optimized fermentation time was higher, the enzymatic activity was seven times higher.

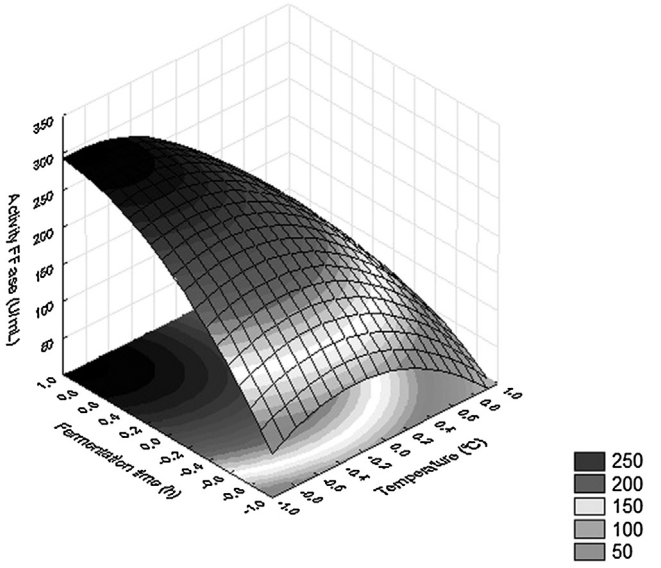

Fig. 4. Response surface obtained in the central composite design for the production of β-fructofuranosidase (FFase) from *Penicillium citreonigrum*.

From the central composite design, the optimal conditions found for FFase production by the *P. citreonigrum,* using the proposed model (Eq. (1)), were 25.5 °C and 67.8 h for temperature and fermentation time, respectively. Under these conditions, the model predicted a FFase activity of 301.84 U/mL.

3.3. Production of FFase in bioreactor

The performance of the FFase production by the *P. citreonigrum* was tested in bioreactor using the optimized conditions found for the shaken flasks experiments. The maximum FFase activity (232.16 ± 1 U/mL) was found for 38 h fermentation (Fig. 5). After this time, the enzymatic activity decreased and a reduction of about 40% of the activity was found for 88 h fermentation.

Although the FFase activity was lower when the fermentations were scaled-up for the bioreactor, the productivity increased. A maximum productivity of 4.30 U/mL h was achieved in the shaken flask experiments while 6.11 U/mL h was achieved when using the bioreactor. The productivity increased 1.5 times when scaling-up the fermentation. This might be explained due to different microorganism growth conditions when using a shaken flask or a bioreactor. For example, for the bioreactor, the pH was controlled during the fermentations and, the agitation type on each reactor is considerably different, influencing the mass and air transference, and consequently, the enzyme production.

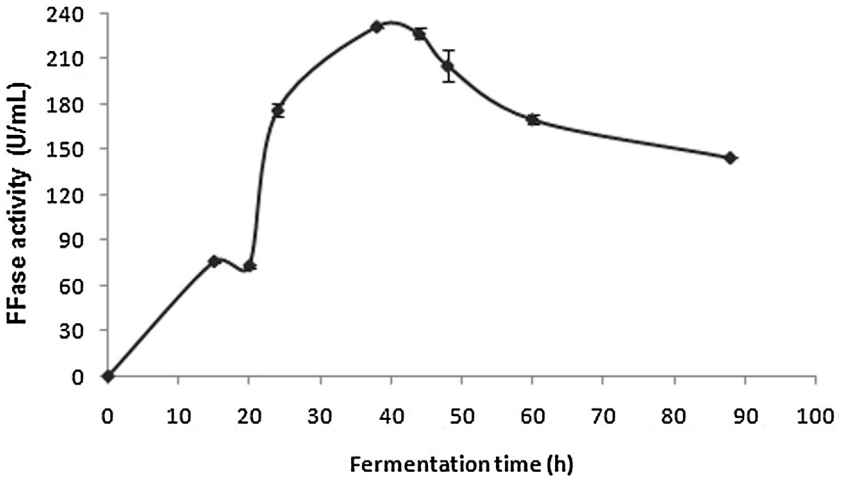

Fig. 5. Production of β-fructofuranosidase (FFase) from *Penicillium citreonigrum* in a 2 L stirred tank bioreactor.

In a work conducted by Driouch et al. [27], using *Aspergillus niger* for the FFase production in a 3L bioreactor (working volume of 2.2 L), under operational conditions of temperature and pH of 37 °C and 5.0, respectively, the authors obtained the higher FFase production of 80 U/mL at 100 h of fermentation (0.8 U/mL h productivity). In the present work, the FFase production was about three times higher (232.16 U/mL) with an eight times higher productivity (6.11 U/mL h).

3.4. Effect of pH and temperature on the activity and stability of the FFase

The pH effect on the FFase activity of a crude extract from *P. citreonigrum* was studied. Results achieved for pH values ranging between 4.0 and 10.0 are shown in Fig. 6a. The highest FFase activity was found for a pH of 5.0. However, for a range between 4.0 and 9.0, the FFase activity did not vary significantly. The FFase was stable over a broad pH range (4.0–10.0), maintaining its activity always above 90% (Fig. 6b). This broad range of pH stability favors its storage and industrial applications [28]. These results are in agreement with previous reports of Xu et al. [28], wherein FFase purified from *Penicillium oxalicum* was also stable over a pH range of 2.0–9.5.

The optimum temperature found for FFase activity was 50 °C (Fig. 6c). The crude FFase showed to be stable for 60 min, at a range of 25–30 °C, maintaining its activity above 95% (Fig. 6d). Similar conditions of temperature and pH were found by Qureshi et al. [29] while using crude extract enzymes from *Mucor geophilus.*

3.5. Effects of different ions on FFase activity

The activity of the crude FFase was increase by 24% in the presence of 5 mM Cu^{4+}, and 29% while using a 10 mM concentration of the same ion (Table 5). The enzymatic activity also increased considerably (more than 23%) in the presence of Mg^{4+}, Zn^{4+} and Zn^{2+} at a concentration of 10 mM. Overall, the FFase activity increased when adding ions to the solution, independently on their nature or concentration. Rustiguel, Jorge & Guimarães [30] reported that metal ions can change the overall charge of proteins, therefore, by affecting their properties, metal ions can interact with the catalytic domains of enzymes reducing or increasing their activities.

3.6. Enzymatic synthesis of FOS

The synthesis of FOS, catalyzed by the extracellular FFase from *P. citreonigrum*, was studied. A concentration of 60% (w/v) sucrose was used as initial carbon source. After 24 h reaction, only 30% (w/w) of the initial sucrose was consumed. The final mixture obtained contained 58.7 g/L of FOS, mainly composed by GF_2 (52.5 g/L) with GF_3 (4.5 g/L) and GF_4 (1.7 g/L). Fig. 7 shows the chromatogram obtain for this mixture These results imply that FFase from *P. citreonigrum* was not able to use the GF_2 synthesized as a fructosyl residue donor to complete the elongation of the glycosidic chain, meaning that almost only sucrose was used as fructosyl donor and acceptor for formation of FOS [31,32]. In the present work, 89.5% of GF_2 in total FOS was obtained at the end of fermentation. GF_2 is known for its higher sweetner power when compared with the longer chain FOS since the increased fructose chain decreases the sweetening power of FOS [33]. Therefore, it would be interesting using the FFase from *P. citreonigrum* for industrial production of GF_2.

Table 5
Influence of ions on β-fructofuranosidase activity from *Penicillium citreonigrum* crude extract.

Compound	Relative activity (%)	
	5 mM	10 mM
No addition	100	100
$MgSO_4$	119.50[a]	123.55[a]
NaCl	116.35[a]	113.20[a]
KCl	113.20[a]	114.55[a]
$FeCl_3$	115.0[a]	96.10
$CuSO_4$	124.0[a]	128.94[a]
$FeSO_4$	123.55[a]	112.75[a]
ZnSO4	119.95[a]	128.49[a]
$ZnCl_2$	117.70[a]	127.14[a]
$CaCl_2$	118.15[a]	116.35[a]

[a] Statistically significant effects (P-value < 0.05).

4. Conclusion

Penicillium citreonigrum was selected as a potential production systems for extracellular FFase. Using RSM it was possible to establish the fermentation conditions (25.5 °C, 67.8 h and pH 6.5) that maximize the FFase production by *P. citreonigrum* in shaken flask, being the maximal FFase activity predicted 301.84 U/mL. The process was scaled-up in a 2 L stirred tank bioreactor applying the optimized conditions found for the shaken flasks experiments and an 1.5 increase on enzyme productivity (6.11 U/mL.h) was achieved. The crude FFase showed stability over a wide range of pH and temperature and had its activity increased in the presence of several ions. *P. citreonigrum* synthesized FOS in a content of 58.7 g/L. The obtained results, although preliminary, are a clear indication on the

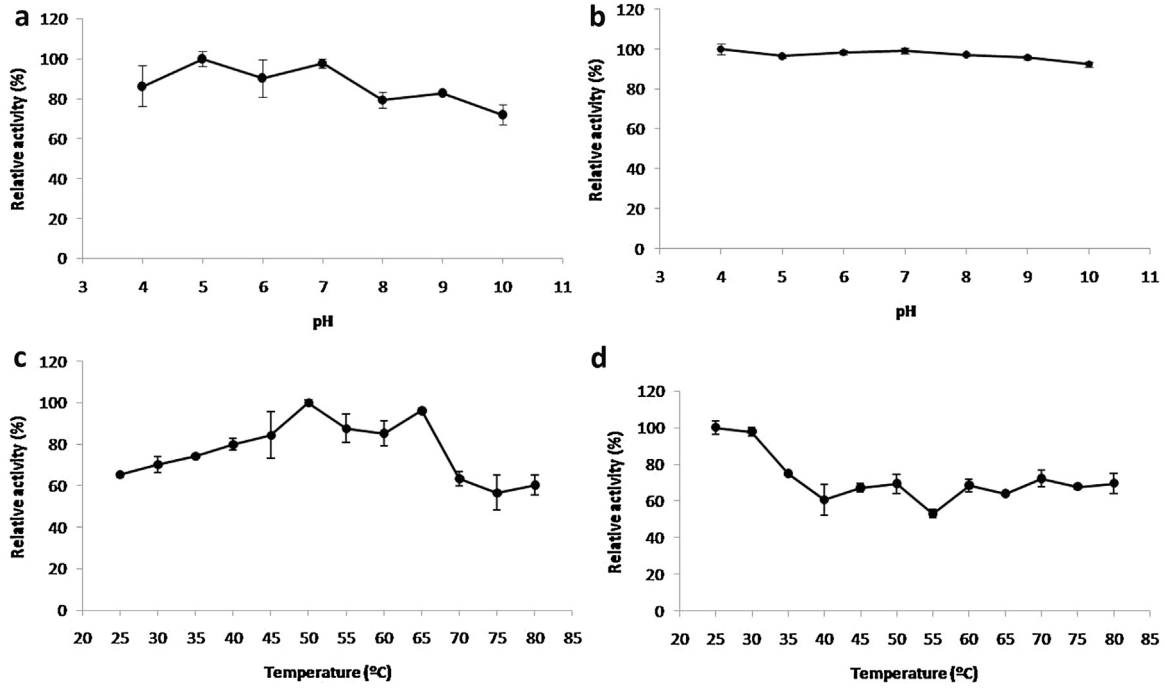

Fig. 6. Optimal pH (a) and temperature (c) and pH (b) and thermal (d) stabilities for the crude β-fructofuranosidase produced by *Penicillium citreonigrum*.

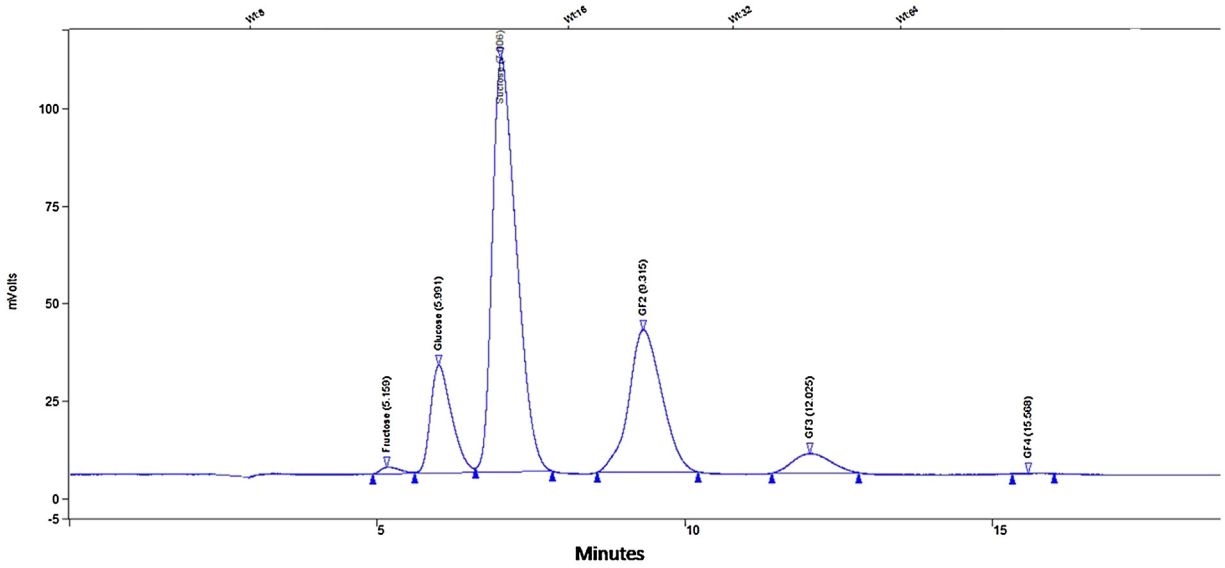

Fig. 7. HPLC chromatogram of the reaction product synthesized by the β-fructofuranosidase crude extract from *Penicillium citreonigrum*, with 60% (w/v) sucrose at 24 h, pH 5.0 and 50 °C. (GF_2:kestose, GF_3:nystose, GF_4: fructofuranosylnystose).

interest of using *P. citreonigrum* as a source of FFase for further use on FOS production.

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