



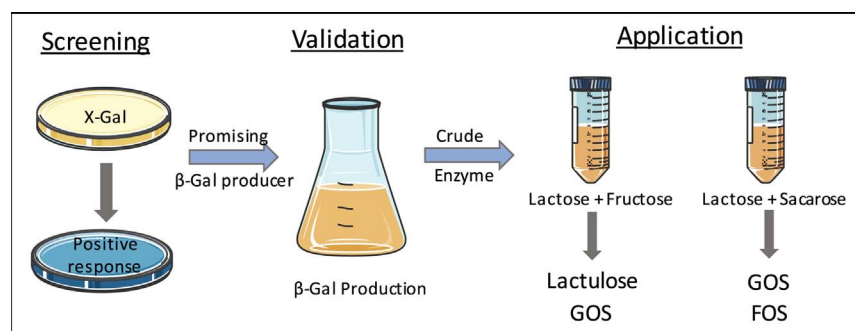
New β -galactosidase producers with potential for prebiotic synthesis

Sara C. Silvério^{a,*}, Eugénia A. Macedo^b, José A. Teixeira^a, Lígia R. Rodrigues^a

^a CEB-Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

^b Laboratory of Separation and Reaction Engineering – Laboratory of Catalysis and Materials (LSRE-LCM), Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

GRAPHICAL ABSTRACT



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ABSTRACT

β -Galactosidases (EC 3.2.1.23) are interesting enzymes with potential application in the pharmaceutical and food industry. In this work, a screening study was carried out to identify new fungal sources of β -galactosidase. A total of 50 fungi were evaluated using a chromogenic test performed in agar plates. The most promising microorganisms were validated as effective β -galactosidase producers under submerged fermentation conditions. The crude β -galactosidases were characterized regarding their optimal pH (3.0–5.5) and temperature (45–65 °C). All enzymes showed ability to synthesize lactose-based prebiotics, namely lactulose (maximal yield 3.3%) and a galacto-oligosaccharide (GOS) (maximal yield 20%). Additionally, some enzymatic extracts with fructosyl-transferase activity allowed to produce other type of prebiotics, namely fructo-oligosaccharides (FOS). This work, reports for the first time the simultaneous synthesis of different mixtures of GOS (2–15% yield and 0.07–0.5 g/L·h⁻¹ productivity) and FOS (4–30% yield and 0.1–1 g/L·h⁻¹ productivity) by crude extracts exhibiting dual enzymatic activity.

1. Introduction

β -Galactosidases (EC 3.2.1.23), frequently known as lactases, are widely used in lactose hydrolysis. This disaccharide is present in mammalian milk in concentrations up to 10% (w/w), depending on the

species (Fox and McSweeney, 1998). The sweetness of lactose corresponds to approximately 20% of that of sucrose and its aqueous solubility is low compared with other sugars (Gänzle et al., 2008). Additionally, lactose is a hygroscopic sugar which frequently suffers undesirable crystallization. The hydrolytic activity of β -galactosidase is

Abbreviations: GOS, galacto-oligosaccharides; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; ONPG, O-nitrophenyl- β -D-galactopyranoside; MUM, Micoteca of University of Minho; DIA-UAC, Food Research Department – Autonomous University of Coahuila; FOS, fructo-oligosaccharides; FFase, β -fructofuranosidase

* Corresponding author.

E-mail address: sarasilverio@deb.uminho.pt (S.C. Silvério).

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Table 1
Results obtained for the 50 fungi in the chromogenic test with X-gal.

Group 1			Group 2			Group 3		
Fungi	Code	Level ^a	Fungi	Code	Level ^a	Fungi	Code	Level ^a
<i>Aspergillus aculeatus</i>	MUM 03.11	1	<i>Penicillium aurantiogriseum</i>	MUM 03.21	2	<i>Alternaria</i> sp.	MUM 00.23	0
<i>Aspergillus bertholletiae</i>	MUM 12.11	0	<i>Penicillium brevicompactum</i>	MUM 02.07	3	<i>Beauveria bassiana</i>	MUM 02.39	2
<i>Aspergillus brasiliensis</i>	MUM 06.179	2	<i>Penicillium cammemberti</i>	MUM 9210	1	<i>Botrytis cinerea</i>	MUM 10.142	2
<i>Aspergillus carbonarius</i>	01UAs130	3	<i>Penicillium chrysogenum</i>	MUM 03.18	2	<i>Byssoschlamys fulva</i>	MUM 9827	3
<i>Aspergillus chevalieri</i>	MUM 00.07	2	<i>Penicillium digitatum</i>	MUM 9811	1	<i>Cladosporium cladosporioides</i>	MUM 9706	2
<i>Aspergillus flavus</i>	MUM 00.06	1	<i>Penicillium expansum</i>	MUM 02.14	1	<i>Curvularia clavata</i>	MUM 9724	1
<i>Aspergillus foetidus</i>	01UAs162	2	<i>Penicillium italicum</i>	MUM 02.25	3	<i>Fusarium culmorum</i>	MUM 9701	0
<i>Aspergillus fumigatus</i>	MUM 9802	2	<i>Penicillium paxilli</i>	MUM 07.03	0	<i>Fusarium solari</i>	MUM 9205	1
<i>Aspergillus ibericus</i>	MUM 03.49	3	<i>Penicillium purpurogenum</i>	DIA-UAC GH2	3	<i>Mucor</i> sp.	DIA-UAC 3P	3
<i>Aspergillus lacticoffeatus</i>	MUM 06.150	2	<i>Penicillium roqueforti</i>	MUM 9838	1	<i>Myceliophthora lutea</i>	MUM 9815	2
<i>Aspergillus minisclerotigenes</i>	MUM 10.229	1	<i>Penicillium spinulosum</i>	MUM 02.34	3	<i>Neurospora crassa</i>	MUM 11.01	3
<i>Aspergillus niger</i>	01UAs83	3	<i>Penicillium verrucosum</i>	MUM 9745	2	<i>Paecilomyces variotii</i>	MUM 9737	3
<i>Aspergillus ochraceus</i>	MUM 9302	0				<i>Phoma violacea</i>	MUM 9708	1
<i>Aspergillus restrictus</i>	MUM 9824	3				<i>Trametes versicolor</i>	MUM 04.100	2
<i>Aspergillus sergii</i>	MUM 10.208	0						
<i>Aspergillus</i> sp.	MUM 10.249	1						
<i>Aspergillus sydowii</i>	MUM 07.14	1						
<i>Aspergillus tamarii</i>	MUM 00.10	0						
<i>Aspergillus transmontanensis</i>	MUM 10.211	2						
<i>Aspergillus tubingensis</i>	MUM 06.152	2						
<i>Aspergillus ustus</i>	MUM 00.12	1						
<i>Aspergillus uvarum</i>	MUM 08.01	2						
<i>Aspergillus versicolor</i>	MUM 00.20	0						
<i>Aspergillus violaceofuscus</i>	MUM 03.02	2						

^a Level of response: (0) no blue color; (1) slight blue color; (2) blue color; and (3) deep blue color.

commonly applied in the food industries aiming to reduce the lactose content of dairy products, preventing lactose crystallization problems and increasing sweetness, flavor and solubility (Gänzle et al., 2008). Furthermore, the hydrolysis of lactose into D-glucose and D-galactose allows the production of lactose-free products suitable for lactose-intolerant people. A significant percentage of the global adult population is affected by lactose intolerance due to β -galactosidase deficiency. This deficiency results in malabsorption of lactose which can cause several undesirable symptoms such as abdominal cramps, bloating, diarrhea or flatulence (Bhatnagar, 2007).

Some β -galactosidases can also catalyze transgalactosylation reactions and have been successfully applied in the synthesis of lactose-based prebiotics, such as galacto-oligosaccharides (GOS) (Torres et al., 2010), lactulose (Silvério et al., 2016) and lactosucrose (Li et al., 2009). These prebiotics are enzymatically obtained through the hydrolysis of lactose followed by the transfer of the galactosyl residue to a suitable acceptor, namely fructose for the disaccharide lactulose; sucrose for the trisaccharide lactosucrose; and lactose for GOS (degree of polymerization from 2 up to 8). The concept of prebiotic was introduced about twenty years ago to define a class of compounds with ability to promote specific changes in the gastrointestinal microflora with benefits upon host well-being and health. The evidence that several beneficial microorganisms at extra-gastrointestinal sites can directly or indirectly be affected by prebiotics stressed the need for an updated concept. Therefore, prebiotic is nowadays considered “a substrate that is selectively utilized by host microorganisms conferring a health benefit” (Gibson et al., 2017). Currently, only few compounds are well-accepted as prebiotics by the scientific community. This restrict group includes fructo-oligosaccharides (FOS), inulin, GOS and lactulose (Gibson et al., 2010; 2017). Besides these compounds, several other non-digestible oligosaccharides have claimed the status of prebiotics, namely lactosucrose, xylo-oligosaccharides (Seesuriyachan et al., 2017), gluco-oligosaccharides (Sharma et al., 2016), pectic oligosaccharides (Babbar et al., 2016) or isomalto-oligosaccharides (Goffin et al., 2011). Prebiotics have gained nutraceutical and pharmaceutical relevance due to their recognized contribute to maintain and restore gastrointestinal microflora, to prevent colitis and constipation, to reduce the incidence

of colon cancer, to promote positive modulation of the immune system or to decrease cholesterol and obesity risk (Patel and Goyal, 2012). The prebiotic market size surpassed USD 3 billion in 2015 and it is expected to reach USD 7.5 billion by 2023 (Global Market Insights, Inc., 2017). To keep up with the growing market trend, efficient production processes ought to be developed, implemented and optimized. The enzymatic synthesis is one of the strategies used at the industrial level for the production of some prebiotics. However, the success of this methodology is in part dependent on the use of effective biocatalysts. In the synthesis of lactose-based prebiotics, such as GOS or lactulose, different microbial sources of β -galactosidase can be used (Torres et al., 2010; Silvério et al., 2016). The search for new and effective biocatalysts represents not only a potential improvement in the production process, but also an opportunity to synthesize novel compounds with enhanced or differentiated biological activity.

In this work, a screening was performed to identify new fungal sources of β -galactosidase with potential for prebiotic synthesis. The most promising microorganisms were validated as effective β -galactosidase producers under submerged fermentation conditions using lactose as carbon source. Finally, the capacity of the crude enzymatic extracts to catalyze the synthesis of several prebiotics was investigated.

2. Materials and methods

2.1. Chemicals

5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal), O-nitrophenyl- β -D-galactopyranoside (ONPG), lactulose and lactosucrose were purchased from Sigma Aldrich (St. Louis, USA). FOS standards (1-kestose (GF2), 1-nystose (GF3) and 1^F-fructofuranosyl-nystose (GF4)) were provided by Wako Pure Chemical Industries, Ltd (Osaka, Japan). A GOS mixture (97% w/w) containing 47% trisaccharides, 42% tetrasaccharides and 8% pentasaccharides was used as GOS standard (Torres et al., 2011). Bradford dye reagent and BSA Standard Ampoules (2 mg/mL) in a solution of 0.9% saline and 0.05% sodium azide were purchased from Alfa Aesar (Karlsruhe, Germany) and Thermo Fisher Scientific (Waltham, USA), respectively.

2.2. Fungal strain

The 50 fungal strains used in the screening test with X-gal were obtained from the collection of cultures of MUM (Micoteca of University of Minho, Portugal) and DIA-UAC (Food Research Department, Autonomous University of Coahuila, Mexico). The strains were divided into three groups: Group 1, containing 24 *Aspergillus* spp.; Group 2, composed by 12 *Penicillium* spp.; and Group 3 which includes 14 fungi from different genera (Table 1). The microorganisms were grown at 25 °C for 7–10 days on Petri plates containing PDA (potato dextrose agar (% w/v): potato extract (0.4), glucose (2) and agar (1.5)) or MEA (malt extract agar (% w/v): malt extract (2), glucose (2), peptone (0.1) and agar (2)). The fungal strains evaluated for β -galactosidase production under submerged fermentation conditions were: *Aspergillus ibericus* (MUM 03.49), *Aspergillus brasiliensis* (MUM 06.179), *Aspergillus restrictus* (MUM 9824), *Aspergillus transmontanensis* (MUM 10.221), *Aspergillus uvarum* (MUM 08.01), *Penicillium brevicompactum* (MUM 02.07), *Penicillium italicum* (MUM 02.25), *Penicillium spinulosum* (MUM 02.34), *Byssoschlamys fulva* (MUM 9827), *Mucor* sp. (DIA-UAC 3P), *Paecilomyces variotii* (MUM 9737) and *Trametes versicolor* (MUM 04.100).

2.3. Chromogenic test

The chromogenic test was performed in Petri plates containing (% w/v): malt extract (2), lactose (2), peptone (0.1) and agar (2). The sterilized medium was supplemented with 0.5% (v/v) of X-gal solution (20 mg/mL in dimethyl sulfoxide). After inoculation, the plates were incubated and protected from light at 25 °C for 7 days. Duplicates were prepared for all the fungi tested. The appearance of blue color in the plates was considered an indication of β -galactosidase production (Manafi, 1996). Four levels of response were defined for the chromogenic test, namely (0) no blue color; (1) slight blue color; (2) blue color; and (3) deep blue color.

2.4. β -Galactosidase production

Spore suspensions for inoculums were prepared in sterile saline solution 0.85% (w/v) NaCl containing 0.01% (w/v) Tween 80. The conidia density was adjusted to 10^6 conidia/mL. Fermentation medium was composed of (% w/v): lactose (2), peptone (0.4), yeast extract (0.4) and salts (KH_2PO_4 (0.2), $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (0.8) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.025) (Nagy et al., 2001). All the fermentations were performed in triplicate during 20 days, at 150 rpm and 28 °C or 37 °C, depending on the microorganism.

2.5. β -Galactosidase activity assay

β -Galactosidase activity was determined by incubating samples (50 μL), at 37 °C for 30 min, with 50 μL of ONPG solution (3 mM) prepared in sodium-citrate buffer (50 mM pH 4.5). The reaction was stopped by the addition of 200 μL of sodium carbonate (0.1 M) (Nagy et al., 2001). The released *O*-nitrophenol was determined spectrophotometrically at 415 nm. One unit (U) of enzyme was defined as the amount of enzyme that liberates 1 μmol of *O*-nitrophenol from ONPG per minute under the assay conditions.

2.6. Biomass wet weight determination

The fermentation broth was filtered and the biomass was conveniently washed with distilled water. In order to remove water excess, the biomass previously filtered was transferred to a Petri plate containing a double paper filter and it was allowed to air dry for 15–20 min at room temperature (Cardoso et al., 2017). Afterwards, the biomass wet weight was determined.

2.7. Protein determination

Protein concentration was determined using the Bradford method. The calibration curve was obtained using stock solutions of bovine serum albumin (BSA) in the concentration range from 1 to 100 $\mu\text{g}/\text{mL}$.

2.8. Sugars quantification

Sugars were quantified by HPLC analysis using a Jasco chromatograph equipped with evaporative light scattering detector (Sedex85, Sedere) and a Prevail Carbohydrate ES column (5 μm , 250 \times 4.6 mm, Alltech) fitted with a pre-column using the same stationary phase. A mixture of acetonitrile:water (75:25 or 70:30, v/v, depending on the sample) pumped at 0.9 mL/min was used as mobile phase. The injection volume was defined as 20 μL . A calibration curve was previously prepared with standards of lactose, lactulose, lactosucrose, GOS and fructooligosaccharides (FOS) in the range 0.1–20 g/L.

2.9. Characterization of the crude enzyme

The fermentation broth was filtered (0.2 μm membrane) and used for further crude enzyme characterization. The effect of temperature in the enzyme activity was evaluated by determining the β -galactosidase activity in 50 mM sodium-citrate buffer (pH 4.5) at different temperatures ranging from 5 to 70 °C using the conditions previously described (section 2.5). The effect of pH on the enzyme activity was evaluated at 37 °C using buffers with different pH ranging from 2.5 to 7.5. The following buffers were used: 50 mM sodium-citrate buffer (pH 2.5–5.5) and 50 mM phosphate-citrate buffer (pH 6.5 and 7.5). For each pH, the enzymatic activity was determined using the conditions previously described (section 2.5). These studies were performed in triplicate.

2.10. Prebiotic production

The prebiotic production was investigated using the crude extract enzyme obtained after a 20-day fermentation, at 28 °C and 150 rpm. First, the biomass was removed by filtration (0.2 μm membrane) and the fermentation broth with β -galactosidase activity was used to study the prebiotic production. The enzymatic synthesis was performed at 37 °C for 30 h by mixing 5 mL of the enzyme crude extract with 5 mL of a sugar solution (200 g/L lactose + 200 g/L fructose for lactulose and GOS production, and 200 g/L lactose + 200 g/L sucrose for lactosucrose and GOS production) prepared in sodium-citrate buffer (50 mM, pH 4.5) (Cardoso et al., 2017). Samples were taken at different time points (1, 3, 5, 10, 20, 25 and 30 h) and were further analyzed by HPLC. All enzymatic synthesis were performed in duplicate.

The substrate conversion and the prebiotic yield were calculated using the following equations, where C_i and C_f (g/L) are the initial and final concentrations of the substrate (lactose or sucrose), respectively, and C_p is the concentration of prebiotics (lactulose, GOS or FOS).

$$\text{Substrate conversion} = \frac{C_i - C_f}{C_i} \times 100 \quad (1)$$

$$\text{Prebiotic yield} = \frac{C_p}{C_i} \times 100 \quad (2)$$

2.11. β -Fructofuranosidase activity assay

β -Fructofuranosidase (FFase) activity was determined by measuring the amount of glucose released from sucrose after 20 min incubation at 30 °C and pH 5.0 (Yoshikawa et al., 2006). Glucose concentration was obtained by HPLC, using the same conditions described above for sugar quantification. One U of the FFase activity was defined as the amount of enzyme that releases 1 μmol of glucose per minute from sucrose.

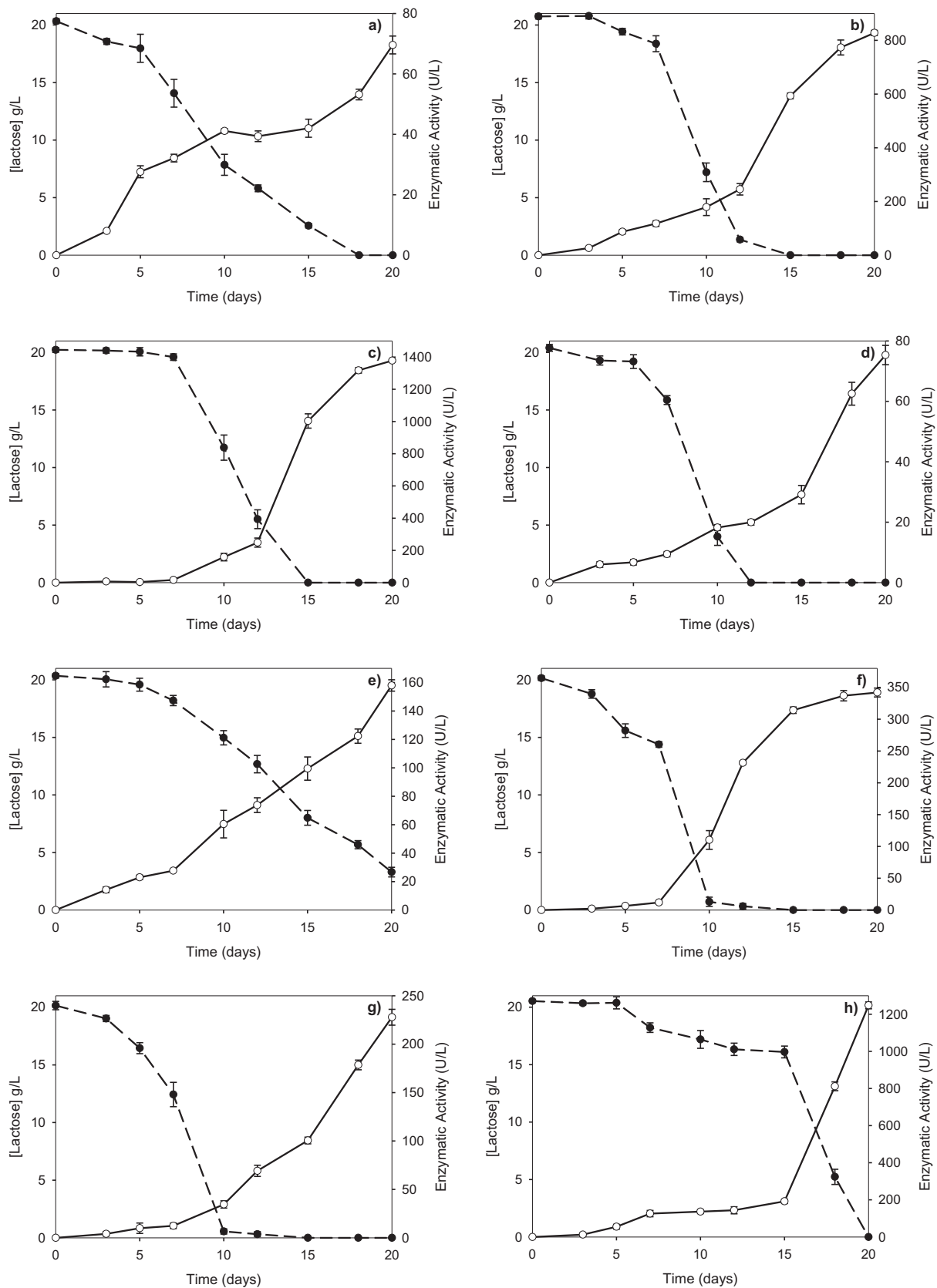


Fig. 1. β -Galactosidase production (empty circle) and lactose consumption (filled circle) under submerged fermentation conditions (150 rpm, 28 °C) for: a) *Aspergillus brasiliensis*; b) *Aspergillus restrictus*; c) *Aspergillus uvarum*; d) *Penicillium brevicompactum*; e) *Penicillium italicum*; f) *Penicillium spinulosum*; g) *Mucor* sp. and h) *Trametes versicolor*. The results are the average of three independent assays \pm standard deviation.

Table 2
Summary of the most relevant results obtained for β -galactosidase production, crude enzyme characterization and prebiotic synthesis.

Fungus	Biomass ^a (g)	β -gal Activity ^a (U/L)	Specific Activity ^a (U/mg)	Optimal pH	Optimal Temp. (°C)	Prebiotics (Lactose + Fructose)		Prebiotics (Lactose + Sucrose)		FPhase Activity ^a (U/L)		
						Lactose conversion ^b (%)	Lactulose ^c (g/L)	Lactose conversion ^b (%)	GOS ^{c,d} (g/L)		Sucrose conversion ^b (%)	FOS ^{c,d} (g/L)
<i>A. brasiliensis</i>	8.0 ± 0.9	70 ± 5.0	1.3 ± 0.3	3.0–5.0 6.5–7.0	55–65	6 ± 1	1.0 ± 0.2 (20 h)	1.6 ± 0.3 (20 h)	2.0 ± 0.5 (30 h)	83 ± 5	5.1 ± 0.2 (10 h)	5560 ± 36
<i>A. restrictus</i>	6.6 ± 0.6	828 ± 9.6	3.6 ± 0.2	3.0–4.5	55–60	44 ± 2	3.3 ± 0.4 (25 h)	17 ± 1 (30 h)	20 ± 0.3 (25 h)	–	–	–
<i>A. uvarum</i>	5.1 ± 0.2	1377 ± 8.1	13 ± 0.7	3.0–4.5	45–60	38 ± 3	3.2 ± 0.2 (30 h)	12 ± 1 (25 h)	15 ± 0.4 (30 h)	33 ± 2	17 ± 0.8 (30 h)	788 ± 11
<i>P. brevicompactum</i>	5.6 ± 0.3	75 ± 3.2	0.52 ± 0.04	4.5–5.5	55	11 ± 2	2.2 ± 0.3 (30 h)	5.8 ± 0.8 (30 h)	5.8 ± 0.2 (30 h)	69 ± 3	30 ± 1.3 ^e (30 h)	10326 ± 88
<i>P. italicum</i>	2.1 ± 0.2	158 ± 4.0	2.8 ± 0.2	3.0–4.5	55	8 ± 2	2.4 ± 0.3(25 h)	4.1 ± 0.3(30 h)	5.0 ± 0.3(25 h)	23 ± 2	4.3 ± 0.4 (30 h)	791 ± 25
<i>P. spinulosum</i>	2.9 ± 0.1	324 ± 2.1	1.5 ± 0.1	3.0–4.0	55–60	14 ± 2	2.6 ± 0.2 (25 h)	5.7 ± 0.4 (30 h)	6.5 ± 0.2 (30 h)	20 ± 2	9.1 ± 0.5 (30 h)	78 ± 8.0
<i>Mucor</i> sp.	8.9 ± 0.4	228 ± 8.2	2.4 ± 0.2	3.0–3.5	45–55	5 ± 1	1.4 ± 0.2 (20 h)	2.0 ± 0.2(20 h)	2.6 ± 0.2 (30 h)	–	–	–
<i>T. versicolor</i>	16.0 ± 0.5	1248 ± 8.4	20 ± 2	3.0–4.0	50–55	19 ± 2	2.8 ± 0.3(20 h)	3.3 ± 0.2 (20 h)	8.6 ± 0.3 (25 h)	88 ± 4	5.9 ± 0.3 (30 h)	4093 ± 97

^a Values obtained after 20 days of fermentation.^b Substrate conversion after 30 h of reaction calculated using Eq. (1).^c Maximal prebiotic concentration and time corresponding to the maximal value.^d Mainly trisaccharides.^e Total FOS concentration of a mixture containing tri-, tetra- and pentasaccharides.

3. Results and discussion

3.1. Chromogenic test

The results obtained for the chromogenic test performed with 50 fungal strains are presented in Table 1, according to the levels of response previously established (section 2.3). Among the 50 microorganisms tested, a total of 42 fungi were identified as potential β -galactosidase producers. In Group 1 (Table 1), 19 *Aspergillus* spp. showed potential to produce β -galactosidase and only 5 strains provided a negative response to the chromogenic test. However, the levels of positive response were different among the potential enzyme producers. For *A. carbonarius*, *A. ibericus*, *A. niger* and *A. restrictus* a deep blue color (level 3) was observed on the bottom of the agar plates and the levels 2 and 1 were obtained for 9 and 6 *Aspergillus* spp., respectively. Among the 12 *Penicillium* spp. studied in Group 2 (Table 1), only *P. paxilli* provided a negative response to the chromogenic test. *P. brevicompactum*, *P. italicum*, *P. purpurogenum* and *P. spinulosum* presented the highest level of response, while *P. aurantiogriseum*, *P. chrysogenum* and *P. verrucosum* presented a level 2 response. A slight blue color (level 1) was observed for 4 *Penicillium* spp. In Group 3 (Table 1), which comprises 14 fungi from different genera, a negative response to the chromogenic test was observed for *Alternaria* sp. and *Fusarium culmorum*. For the 12 fungi identified as potential β -galactosidase producers, *B. fulva*, *Mucor* sp., *N. crassa* and *P. variotti* provided a deep blue color (level 3). The level 2 response was observed for 5 fungi, and 3 of them presented only a slight blue color (level 1).

The chromogenic test with X-gal proved to be a simple and fast approach to evaluate the potential of the 50 fungi for the production of β -galactosidase. However, this test only provides a qualitative assessment and additional studies are needed to confirm the effective production of the enzyme by the fungal strains. According to the results (Table 1), the 12 fungi exhibiting level 3 response were classified as the most promising β -galactosidase producers. Nevertheless, within this level of response, we identified *A. carbonarius*, *A. niger* and *Neurospora crassa* which have already been reported as producers of extracellular and/or intracellular β -galactosidase (> 200 U/L) (El-Gindy, 2003; O'Connell and Walsh, 2008; Comp and Lester, 1971; Tonelotto et al., 2014). Under submerged fermentation conditions and using culture media containing different sugars (lactose, sucrose or arabinose) and also agro-industrial residues (cheese whey or wheat bran), these fungi were able to produce β -galactosidases with catalytic activity towards different substrates such as ONPG, *p*-nitrophenyl- β -D-galactopyranoside or lactose. Therefore, and considering that our main aim is to identify novel β -galactosidase producers, these three fungi will not be included in the following studies. Also, *P. purpurogenum*, a well-known producer of a red pigment (Mendez et al., 2011) was identified in this group. The presence of the pigment could compromise the absorbance measurements in the β -galactosidase activity assay (Mapari et al., 2006) and it also represents an additional contaminant to consider in a possible purification process, thus likewise this fungus was not further used in our work. All the other fungi from level 3 (*A. ibericus*, *A. restrictus*, *P. brevicompactum*, *P. italicum*, *P. spinulosum*, *B. fulva*, *Mucor* sp. and *P. variotti*) were evaluated as β -galactosidase producers under submerged fermentation conditions. Additionally, 4 fungi from level 2, namely *A. brasiliensis*, *A. transmontanensis*, *A. uvarum* and *T. versicolor* were included in the validation study since, as far as we know, their ability to produce β -galactosidase has never been studied.

3.2. β -Galactosidase production

From the submerged fermentations performed for 20 days in a synthetic medium containing lactose it was demonstrated that *B. fulva* and *P. variotti* were not able to produce β -galactosidase, while *A. ibericus* and *A. transmontanensis* presented very low levels of enzymatic activity in the fermentation broth (approximately 28 and 25 U/L,

respectively). These results suggest that changes in type of fermentation, medium composition and temperature may have a negative impact in the β -galactosidase production. Similarly, the use of a different enzymatic assay with a different chromogenic substrate may also affect the detection of β -galactosidase activity (Manafi, 1996). For all the other fungi, the profiles obtained for the lactose consumption and β -galactosidase production are presented in Fig. 1. It was observed that the hydrolysis and consumption of lactose is generally associated with an increase of the extracellular enzymatic activity. All the lactose from the culture medium was exhausted during the fermentation, except for *P. italicum* for which approximately 3 g/L of sugar remained in the fermentation broth after 20 days. Among all the studied fungi, some were better β -galactosidase producers than others according to the following order: *A. uvarum* (1377 U/L) > *T. versicolor* (1248 U/L) > *A. restrictus* (828 U/L) > *P. spinulosum* (324 U/L) > *Mucor* sp. (228 U/L) > *P. italicum* (158 U/L) > *P. brevicompactum* (75 U/L) \approx *A. brasiliensis* (70 U/L). The specific activity (U/mg) at the end of the fermentation was also determined and it is presented in Table 2, together with the final biomass obtained for each fungus. In all cases, no correlation was found between the biomass and β -galactosidase production. The highest specific activities were found for *T. versicolor* and *A. uvarum*. On the other hand, *P. brevicompactum* showed the lowest specific activity value.

3.3. β -Galactosidase characterization

The crude extract obtained after filtration of the fermentation broth was used in the enzyme characterization. The results obtained for the effect of temperature and pH on the enzymatic activity (hydrolytic activity using ONPG as substrate) are shown in Fig. 2 and the optimal values found for each enzyme are described in Table 2. All the fungal β -galactosidases presented optimal pH in the acidic range between 3.0 and 5.5. In general, the enzymatic activity decreased significantly for higher pH values, except for *A. brasiliensis* and *P. brevicompactum*. Regarding *A. brasiliensis*, two ranges of optimal pH were found, namely 3.0–5.0 and 6.5–7.0. This result can possibly be explained by the presence of isoenzymes in the crude extract. *P. brevicompactum* showed an optimal pH in the range 4.5–5.5, nevertheless, its enzymatic activity remained considerable high (> 75%) for pH values between 2.5 and 7.0.

The optimal temperature for all fungi was obtained between 45 °C and 65 °C (Table 2). Generally, the enzymes presented a range of temperatures in which they remain active. However, for *P. brevicompactum* and *P. italicum* the maximal enzymatic activity was observed for a single temperature (55 °C).

The results herein gathered for optimal temperature and pH are similar to those reported in the literature for other β -galactosidases from *Aspergillus* spp. (Gargova et al., 1995; Gonzalez and Monsan, 1991; O'Connell and Walsh, 2008; Tanelotto et al., 2014; Roal et al., 2015), *Penicillium* spp. (Watanabe et al., 1979; Cruz et al., 1999; Nagy et al., 2001) and *Mucor* spp. (Ismail et al., 1997; Shaikh 1999). Generally, fungal β -galactosidases present optimal pH in the acidic range and have relatively high optimal temperature (O'Connell and Walsh, 2008). The optimal conditions obtained for the commercial substrate ONPG, namely the acidic pH, suggest, for instance, the suitability of these enzymes to catalyze lactose hydrolysis in acid cheese whey (Rosolen et al., 2015). Furthermore, these 8 fungal enzymes seem to have potential for the prebiotic synthesis under the reaction conditions generally reported for other fungal β -galactosidases (Torres et al., 2010; Silvério et al., 2016).

3.4. Prebiotic synthesis

To investigate the ability of β -galactosidases to catalyze both the hydrolytic and transfer reactions involved in the production of lactose-based prebiotics, different substrate mixtures were used (Cardoso et al.,

2017). The results obtained for each fungal β -galactosidase are summarized in Table 2. When the crude enzymes were incubated with a mixture of lactose + fructose, two types of prebiotic were formed, namely lactulose and GOS. *A. restrictus* and *A. uvarum* provided the highest lactulose concentration (3.3 and 3.2 g/L, respectively), while the lowest values of lactulose concentration were obtained by *A. brasiliensis* and *Mucor* sp. The GOS concentration presented in Table 2 corresponds mainly to a trisaccharide which probably has the same structure for all the β -galactosidases studied (as suggested by the similar retention time in all the chromatograms using a well-characterized GOS mixture as standard (Torres et al., 2011)). For *A. restrictus* and *A. uvarum* traces of a tetrasaccharide were also detected. This observation is in good agreement with the results presented in Table 2 since the highest concentrations of trisaccharide GOS, the precursor of the tetrasaccharide, were found for these two fungi. Generally, the synthesis of GOS and lactulose was detected 3 h after incubation. However, for *A. brasiliensis*, *P. spinulosum* and *T. versicolor* the production of lactulose was only observed after 5 h. The maximal lactulose and GOS concentration was reached after 25–30 h of reaction for almost all of the fungi, except for *A. brasiliensis*, *Mucor* sp. and *T. versicolor*. For these three fungi, the maximal prebiotic concentration was detected at 20 h of incubation and a decrease was observed afterwards, probably due to some product degradation i.e. secondary hydrolysis of GOS and lactulose (Osman et al., 2014; Sitanggang et al., 2014; Guerrero et al., 2017). On the other hand, the lactose conversion (%) increased over time for all the β -galactosidases studied and the highest percentages of conversion are generally associated with the highest prebiotic concentrations (Table 2).

When the crude enzymes were incubated with a mixture of lactose + sucrose, the only lactose-based prebiotic detected was the trisaccharide GOS (similar retention time in all chromatograms) (Table 2). The maximal GOS concentration was reached after 25–30 h of reaction, being the highest values obtained for *A. restrictus* (20 ± 0.3 g/L), *A. uvarum* (15 ± 0.4 g/L) and *T. versicolor* (8.6 ± 0.3 g/L). Similarly, the highest percentages of lactose conversion after 30 h of reaction were also detected for these three microorganisms (Table 2). The production of lactosucrose was not observed for any studied fungus. Cardoso et al. (2017) also reported the absence of lactosucrose production by β -galactosidase from *Aspergillus lacticoffeatus* under similar reaction conditions. A possible explanation for this fact is the apparent inability of fungal β -galactosidases to catalyze the production of this prebiotic (Cardoso et al., 2017). The enzymatic production of lactosucrose using β -galactosidase as biocatalyst is poorly studied and the few references found in the literature only report the successful use of bacterial β -galactosidase produced by *Bacillus circulans* (Farkas et al., 2003; Li et al., 2009; Duarte et al., 2017). On the other hand, it is important to highlight that lactosucrose production was herein evaluated under unoptimized conditions. It is well-known that reaction parameters such as substrate concentration, substrate molar ratio, enzyme concentration, temperature or reaction time, can significantly affect the amount of lactosucrose and the composition of the final product (Farkas et al., 2003; Li et al., 2009; Duarte et al., 2017). Therefore, different reaction conditions should be carefully studied in the future prior to unequivocally conclude about the inability of fungal β -galactosidase to synthesize this prebiotic.

Although no lactosucrose was produced during the reaction times evaluated, the conversion of sucrose was observed for all the fungal β -galactosidases, except for *A. restrictus* and *Mucor* sp. In general, the conversion was firstly detected at 3–5 h of reaction, but for *A. brasiliensis* and *T. versicolor* it started after 1 h. This observation suggested the presence of FFase activity in the crude extracts. For that reason, an enzymatic assay was performed to determine this catalytic activity (section 2.11). The results obtained are presented in Table 2. As expected, FFase activity was detected in the enzymatic extracts which previously showed sucrose conversion. Furthermore, the highest percentage of sucrose conversion corresponded to the crude extracts with

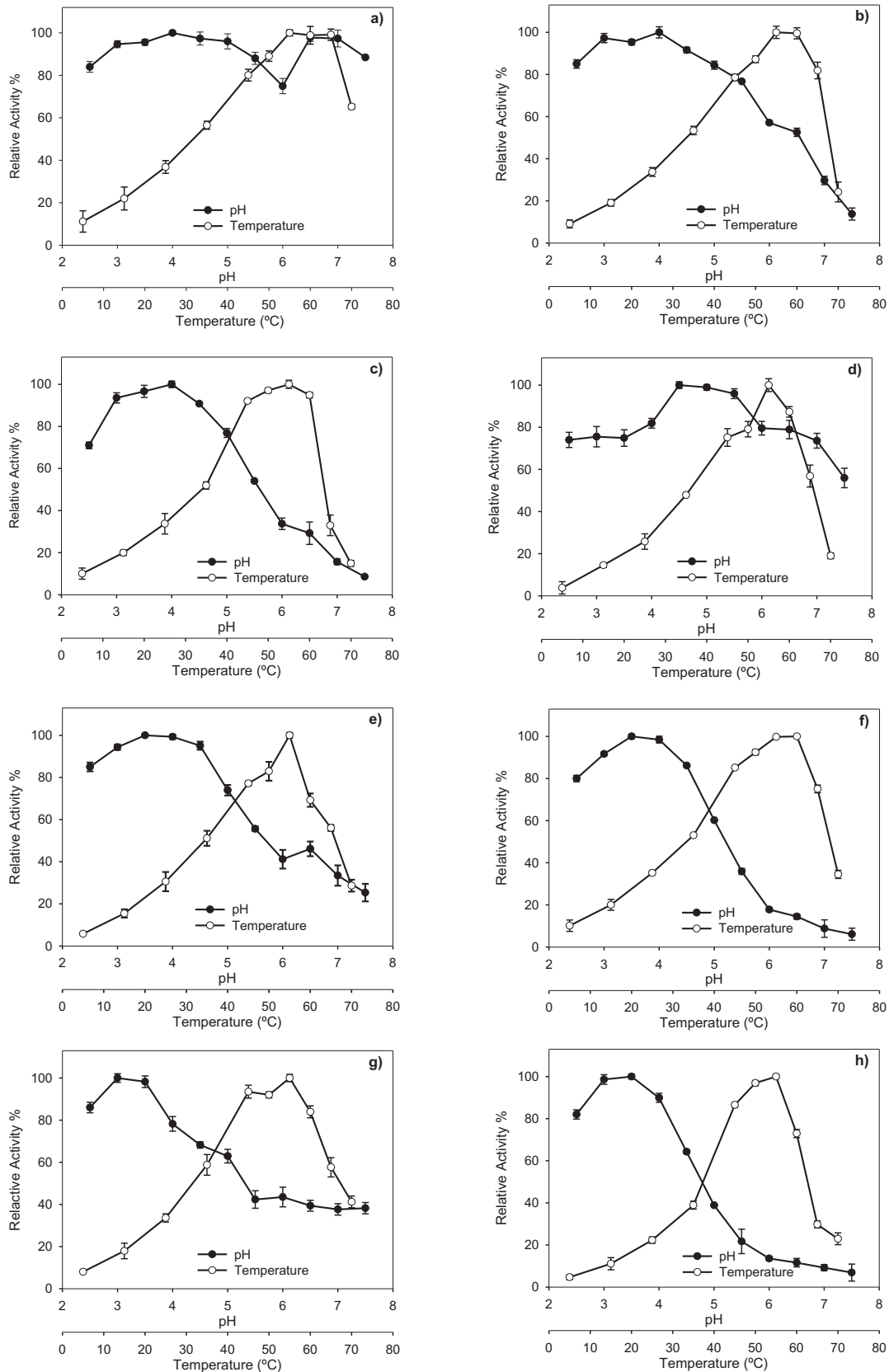


Fig. 2. The effect of pH and temperature on the β -galactosidase activity (hydrolytic activity using ONPG as substrate) of: a) *Aspergillus brasiliensis*; b) *Aspergillus restrictus*; c) *Aspergillus uvarum*; d) *Penicillium brevicompactum*; e) *Penicillium italicum*; f) *Penicillium spinulosum*; g) *Mucor* sp. and h) *Trametes versicolor*. The results are the average of three independent assays \pm standard deviation.

highest FFase activity. The HPLC analysis revealed that sucrose present in the sugar mixtures was not exclusively hydrolyzed into fructose and glucose, it was also used in transfructosylation reactions to produce FOS (Table 2). A similar result was reported by Cardoso et al. (2017) for enzymatic extracts from *A. lacticooffeatus* containing both β -galactosidase and FFase activity.

In general, the enzymatic synthesis of FOS (GF2) was observed after 3–5 h of reaction and their concentration increased during the reaction time. Nevertheless, for *A. brasiliensis* the maximal concentration (5.1 ± 0.2 g/L) was reached at 10 h, followed by a progressive reduction to 3.6 ± 0.3 g/L (30 h) probably due to some product degradation (L'Homme et al., 2003), since no longer structures of FOS were detected by HPLC. In almost all cases, the FOS concentration presented in Table 2 corresponds to a GF2 structure (retention time similar to the standard 1-kestose in all chromatograms). The only exception occurred with the enzymatic extract from *P. brevicompactum* which allowed the synthesis of three different structures of FOS (GF2, GF3 and GF4). After 30 h of reaction, three FOS are detected, together with a GOS structure (trisaccharide). The presence of fructose, glucose and galactose in the mixture was also observed, indicating that considerable amount of both substrates (lactose and sucrose) suffered enzymatic hydrolysis. Fig. 3 illustrates the profile of GOS and FOS production using the crude enzymatic extract from *P. brevicompactum*. The simultaneous synthesis of GF2 and GOS was detected after 1 h (2.7 ± 0.4 and 0.93 ± 0.13 g/L, respectively), while the synthesis of GF3 (3.2 ± 0.4 g/L) and GF4 (3.3 ± 0.3 g/L) was only detected after 3 and 5 h of reaction, respectively. Both GOS and total FOS concentration increased over time. However, after 20 h, the concentration of GF2 (14.2 ± 0.6 g/L) was reduced and the concentrations of GF3 and GF4 were slowly raised. The total FOS concentration obtained at 30 h (approximately 30 g/L) corresponds to 12.4 ± 0.6 g/L of GF2, 11.8 ± 0.4 g/L of GF3 and 5.5 ± 0.3 g/L of GF4. These values represent a FOS yield of around 30% (grams of total FOS per grams of initial sucrose), which is higher than some yields reported in the literature for crude enzymes under optimized conditions. Muñiz-Márquez et al. (2016) obtained around 25% yield using aguamiel, a by-product obtained from maguey, as sucrose source and fructosyltransferase from *Aspergillus oryzae*. A yield of 16% was achieved by Silva et al. (2014) using levansucrase from *Bacillus subtilis*. However, the FOS yield here obtained is far from the maximal theoretical yield (55–60%) generally associated with a two-step process (enzyme production + crude enzyme application) (Sangeetha et al., 2005). The FOS yield is limited by the high amounts of glucose generated during sucrose hydrolysis which will act as enzyme inhibitor. For that reason, several strategies have

been proposed to remove glucose from the reaction medium and increase the yield (Dominguez et al., 2014; Nobre et al., 2016). Glucose inhibition probably exerted a crucial impact in the FOS synthesis under the reaction conditions used in the present work, since the enzymatic hydrolysis of both substrates originated glucose as a reaction product.

The different enzyme concentrations (U/L) used in this study, do not allow a direct comparison of the potential of the several fungi under study for the synthesis of prebiotics. Nevertheless, this simple approach clearly demonstrated the ability of these fungal sources of β -galactosidase to produce lactulose and GOS, and FFase to synthesize FOS. However, it is important to stress the need of additional studies using optimized conditions and/or purified enzymes to better evaluate the potential of each biocatalyst.

The occurrence of hydrolysis and transglycosylation of both lactose and sucrose is a clear evidence of the lack of purity of the obtained crude extracts (Lindamood et al., 1989). Nevertheless, this fact also points out the potential of these enzymatic extracts to simultaneously synthesize two different types of prebiotics, namely lactose-based and sucrose-based oligosaccharides. As far as we know, this strategy of prebiotic production was never explored and could represent an interesting approach to obtain prebiotic mixtures with enhanced biological effect. Actually, the intake of GOS and FOS mixtures can present advantages over the consumption of the individual prebiotics due to the occurrence of possible synergistic effects (Moro et al., 2002). Combinations of GOS and FOS have been described to improve intestinal health (Boehm et al., 2002), to reduce allergic reactions and atopic dermatitis (Vandenplas et al., 2014), to increase bone mineralization (Bryk et al., 2015) or to treat stress-related disorders (Burokas et al., 2017). The optimization of the simultaneous production of FOS and GOS, together with the implementation of strategies for the removal of monosaccharides or unreacted sugars, could significantly contribute to increase the prebiotic yields and should be further investigated. Additionally, the possibility to obtain mixtures of GOS and FOS with different concentrations and compositions offers the opportunity to explore new biological applications for prebiotics.

4. Conclusions

Eight fungal strains were validated as effective β -galactosidase producers. The enzymes presented optimal pH in the acidic range (3.0–5.5) and optimal temperature between 45 and 65 °C. All the β -galactosidases showed potential for the synthesis of GOS and lactulose. Furthermore, some crude extracts exhibiting FFase activity allowed the production of FOS. Therefore, a novel and interesting approach for the simultaneous synthesis of GOS and FOS was proposed based on the application of enzymatic extracts with dual catalytic activity. The diversity of GOS and FOS mixtures originated with this strategy can contribute to obtain new and/or improved prebiotic effects.

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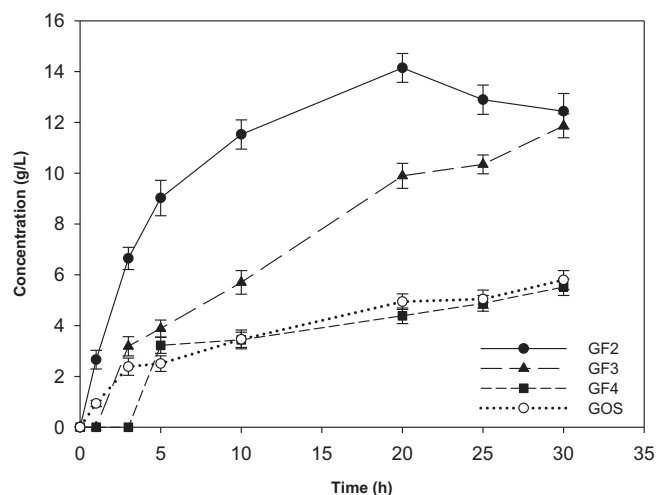


Fig. 3. GOS and FOS production using crude enzymatic extract from *Penicillium brevicompactum*. Enzymatic reaction performed using lactose (100 g/L) and sucrose (100 g/L) as substrates at 37 °C and pH 4.5.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2017.11.045>.

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