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β-galactosidase from *Aspergillus lacticoffeatus*: A promising biocatalyst for the synthesis of novel prebiotics

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ABSTRACT

β-galactosidase (EC 3.2.1.23) are interesting enzymes able to catalyze lactose hydrolysis and transfer reactions to produce lactose-based prebiotics with potential application in the pharmaceutical and food industry. In this work, *Aspergillus lacticoffeatus* is described, for the first time, as an effective β-galactosidase producer. The extracellular enzyme production was evaluated in synthetic and alternative media containing cheese whey and corn steep liquor. Although β-galactosidase production occurred in all media (expect for the one composed solely by cheese whey), the highest enzymatic activity values (460 U/mL) were obtained for the synthetic medium. Ochratoxin A production in synthetic medium was also evaluated and 9 days of fermentation was identified as a suitable fermentation time to obtain a crude extract enzyme with mycotoxin concentration below the legal comparable value established for wine and grape juices (2 ng/mL). The optimal pH and temperature for the crude extract enzyme was found in the range of 3.5–4.5 and 50–60 °C, respectively. The β-galactosidase activity was reduced in the presence of Ba^{2+}, Fe^{2+}, Li^{+}, K^{+} and galactose, while additives (except for ascorbic acid) and detergents exhibited a positive effect on enzymatic activity. This enzyme was able to catalyze the synthesis of prebiotics, namely lactulose (2.5 g/L) and a galacto-oligosaccharide (trisaccharide, 6.3 g/L), either when whole cells or crude enzyme was used as biocatalyst. The lactulose production using fungal whole cells is herein reported for the first time. Additionally, *A. lacticoffeatus* was also found to produce an enzyme with fructosyltransferase activity and other prebiotics, namely fructo-oligosaccharide 1-kestose (2.4 g/L).

1. Introduction

β-galactosidases (EC 3.2.1.23), also known as lactases, are a family of enzymes able to catalyze two different types of reactions, namely hydrolysis and transgalactosylation. The hydrolytic activity is commonly applied in the food industries to reduce the lactose content of dairy products, preventing lactose crystallization problems and increasing sweetness, flavor and solubility (Gänzle et al., 2008). On the other hand, transgalactosylation reactions have been explored in the synthesis of lactose-based prebiotics, such as galacto-oligosaccharides (GOS), lactulose and lactosucrose (Silvério et al., 2015, 2016; Torres et al., 2010), with potential application in the pharmaceutical and food industry. The consumption of these prebiotics is associated with several health benefits such as the maintenance or restoring of a healthy gut microbiota, the reduction of colitis and cancer risk, as well as the increase of the absorption of minerals such as calcium and magnesium (Bruno-Barcena and Azcarate-Peril, 2015; Mao et al., 2014; Seki et al., 2007; Weaver et al., 2011; Zhou et al., 2015). Commercially available lactulose is produced by chemical methods while lactosucrose and GOS are obtained through enzymatic synthesis. However, in the last decade, alternative methods for lactulose production using enzymes such as β-galactosidase has been widely studied, due to the recognized advantages associated with the use of an environment-friendly biocatalyst (Silvério et al., 2016).

The sources of β-galactosidase are extensively distributed in nature, namely in microorganisms, plants and animal organs (Husain, 2010). β-galactosidases from microbial sources exhibit a great industrial relevance mainly due to their easy handling, greater catalytic activity and high production yield (Panesar et al., 2006). However, only a few microbial sources of β-galactosidase are generally recognized as safe (GRAS) and eligible for usage in the pharmaceutical and food industries. Extracellular β-galactosidases from the fungi *Aspergillus niger* and *Aspergillus oryzae* have been classified as GRAS by the Food and Drug Administration (FDA, 2015).

Abbreviations: GOS, galacto-oligosaccharides; **CW**, cheese whe; **CSL**, corn steep liquor; **X-gal**, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; **ONPG**, *O*-nitrophenyl-β-D-galactopyranoside; **OTA**, ochratoxin A; **MUM**, Micoteca da Universidade do Minho; **FOS**, fructo-oligosaccharides; **SDS**, sodium dodecyl sulfate; **PMSF**, phenylmethanesulfonyl fluoride; **EDTA**, ethylenediaminetetraacetic acid; **FFase**, β-fructofuranosidase

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Aspergillus lacticoffeatus was firstly found on coffee beans (*Coffea arabica*), in Venezuela, as well as on beans and soil under *Coffea robusta*, in Indonesia (Samson et al., 2004). This microorganism was included in the *Aspergillus* section *Nigri* which contains some of the most common fungi used for biotechnological purposes, including for enzyme production. Several species of black aspergilli are described as potential causes of food spoilage and deterioration (Samson et al., 2004). However, they are also considered good candidates for genetic manipulation for biotechnological applications given the GRAS status already attributed to *A. niger* (Varga et al., 2007), the most relevant representative of the *Nigri* section. The similarity between *A. lacticoffeatus* and *A. niger* has been reported (Ferracin et al., 2012; Meijer et al., 2011; Varga et al., 2011). Consequently, it is expected that *A. lacticoffeatus* can also produce a properly active β-galactosidase able to catalyze the synthesis of lactose-based prebiotics. These prebiotics are enzymatically produced through the hydrolysis of lactose and further transfer of a galactosyl to a suitable acceptor, namely fructose for the disaccharide lactulose; sucrose for the trisaccharide lactosucrose; and lactose for GOS.

The use of agro-industrial residues as low cost substrates for enzyme production can result in recognized economic and environmental benefits. These residues contain high amounts of organic compounds which can be used to replace the synthetic sources of carbon, nitrogen, and micronutrients (El-Bakry et al., 2015). Cheese whey (CW), the main by-product of the dairy industry, contains considerable amounts of lactose and can be used as a low cost substrate in the culture medium for microorganisms able to metabolize lactose and produce added-value compounds, such as prebiotics (Corzo-Martinez et al., 2015), bioethanol (Koushki et al., 2012) and enzymes (Roal et al., 2015a). Corn steep liquor (CSL), the main by-product of the corn wet-milling industry, is another example of an inexpensive substrate. Due to its high amount of organic nitrogen and vitamins, CSL has been used largely to replace yeast extract and peptone (Nascimento et al., 2009) in the production of biosurfactants (Gudiña et al., 2015), enzymes (Roal et al., 2015a) and food additives (Li et al., 2006).

In this work, the growth and β-galactosidase production by *A. lacticoffeatus* in synthetic and alternative media containing CW and CSL was compared and the ability of the enzyme to catalyze the synthesis of prebiotics was evaluated.

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, lactosucrose and ochratoxin A (OTA) were purchased from Sigma Aldrich (St. Louis, USA). FOS standards (1-kestose and nystose) 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).

2.2. Agro-industrial wastes

Cheese whey (CW) was provided by Queizuar, S.L. (A Coruña, Spain), and corn steep liquor (CSL) was obtained from COPAM: Companhia Portuguesa de Amidos, S.A. (S. João da Talha, Portugal).

2.3. Fungal strain

Aspergillus lacticoffeatus (MUM 06.150) was obtained from MUM (Micoteca da Universidade do Minho, Portugal) collection of cultures. The microorganism was 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)).

2.4. 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. The appearance of blue color in the plates was considered an indication of β-galactosidase production (Manafi, 1996).

2.5. β-galactosidase production

Spore suspensions for inocula 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. Several fermentation media were tested at 28 °C, pH 6.5 and 180 rpm for 6–9 days. Synthetic medium (A) comprised (% *w/v*): lactose (2), peptone (0.4), yeast extract (0.4) and salts (KH_2PO_4 (0.2), $Na_2HPO_4.12H_2O$ (0.8) and $MgSO_4.7H_2O$ (0.025)). CSL was used to replace peptone and yeast extract and 2 media were prepared: medium B – CSL (0.8% *w/v*), and lactose and salts at the same concentrations as in medium A; medium C – CSL (2% *w/v*), and lactose and salts at the same concentrations as in medium A. CW was used as lactose source and after its proper dilution, 4 different media with similar lactose concentration (2% *w/v*, confirmed by HPLC analysis, Section 2.9) were prepared: medium D – CW only; medium E – CW supplemented with salts at the same concentrations as in medium A; medium F – CW supplemented with peptone and yeast extract at the same concentrations as in medium A; medium G – CW supplemented with salts, peptone and yeast extract at the same concentrations as in medium A. All the fermentations were performed in triplicate.

2.6. 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.

2.7. 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 (Balaraman and Mathew, 2006). Afterwards, the biomass wet weight was determined.

2.8. Ochratoxin A (OTA) determination

Fermentation was conducted in medium A for 9 days, at 28 °C and 180 rpm. OTA production at 3, 6 and 9 days was evaluated by high performance liquid chromatography (HPLC) using a Varian Postar 210 pump, a Varian Prostar 410 autosampler, a Jasco FP-920 fluorescence detector (λex = 333 nm and λem = 460 nm) and a reverse phase C18 column YMC-Pack ODS-AQ (250 mm × 4.6 mm, 5 μm) fitted with a pre-column using the same stationary phase. The mobile phase comprising acetonitrile:water:acetic acid (99:99:2, *v/v/v*) was pumped at 1.0 mL/min and the injection volume was defined as 50 μL (Abrunhosa and Venâncio, 2007). A calibration curve was previously prepared with OTA standards in the range 1–50 ng/mL. The OTA concentration was determined as the mean ± SD of triplicate experiments.

2.9. Quantification of sugars

Quantification of sugars was achieved through HPLC analysis using a Jasco chromatograph equipped with evaporative light scattering detector (Sedex85, Sedere) and a Prevail Carbohydrate ES column (5 μm, 250 × 4.6 mm, Alltech) fitted with a pre-column using the same stationary phase. A mixture of acetonitrile: water (75:25, *v*/v) 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 fructo-oligosaccharides (FOS) in the range 0.1–15 mg/mL.

2.10. Characterization of the crude enzyme

The fermentation broth (medium A, 9 days fermentation) was filtered (0.2 μm membrane) and used for further crude enzyme characterization. The effect of temperature on the enzyme activity was evaluated by determining the β-galactosidase activity in 50 mM sodium-citrate buffer (pH 4.5) at different temperatures (15, 25, 35, 40, 45, 50, 55, 60 and 65 °C) using the conditions previously described (Section 2.6). The effect of pH on the enzyme activity was evaluated at 37 °C using buffers with different pH (3.0, 3.5, 4.5, 5.5, 6.5 and 7.5). The following buffers were used: 50 mM sodium-citrate buffer (pH 3.0–5.5) 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 2.6). To study the effect of different metal ions, additives, detergents and sugars, the sodium-citrate buffer (50 mM, pH 4.5) was supplemented with each specific compound. For metal ions, the buffer was mixed with the following ions: KCl, NaCl, LiCl, $BaCl_2$, $MgCl_2$, $ZnSO_4$, $MnCl_2$, $FeCl_2$, $CoCl_2$ and $CuSO_4$. Solutions with final concentration of 10 mM were obtained in each case. For the detergents, different solutions of buffer containing sodium dodecyl sulfate (SDS, 10 mM), Tween-40 (1% *w/v*) or TritonX-100 (1% *w/v*) were prepared. For the additives, buffer was supplemented with phenylmethanesulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) or ascorbic acid to obtain solutions with final concentration of 10 mM. Finally, for the sugars, different concentrations of fructose, glucose and galactose (5, 25, 75 and 100 mM) in sodium-citrate buffer were used. The effect of the metal ions, detergents, additives or sugars was evaluated by incubating 50 μL of crude enzyme extract with 50 μL of ONPG solutions (3 mM) prepared in the several buffer solutions supplemented with each specific compound. In all cases, the enzymatic activity was determined using the β-galactosidase assay described in Section 2.6.

2.11. Prebiotic production

The prebiotic production was investigated using three different approaches:

Method 1 – The fermentation was performed for 6 days, at 28 °C and 180 rpm, using medium A. Afterwards, appropriate volumes of substrate solutions (lactose and fructose for lactulose and GOS production; lactose and sucrose for lactosucrose production) were added to the medium (pH around 5.0) to obtain different substrate concentrations (10:10, 20:20, 30:30 g/L of lactose:fructose or lactose:sucrose) and also different substrate ratios (10:20, 15:15, 20:10 g/L of lactose:fructose or lactose:sucrose). The prebiotic production was evaluated for 3 days using HPLC analysis.

Method 2 – The fermentation was performed for 6 days, at 28 °C or 37 °C and 180 rpm, using medium A. Afterwards, the biomass was collected through sterile vacuum filtration (0.45 μm membrane) and placed in 40 mL substrate solution (150 g/L lactose + 150 g/L fructose for lactulose and GOS production; 150 g/L lactose + 150 g/L sucrose for lactosucrose production) prepared in sodium-citrate buffer (50 mM, pH 4.5). The prebiotic production was evaluated for 3 days using HPLC analysis.

Method 3 – The fermentation was performed for 9 days, at 28 °C and 180 rpm, using the medium A. Afterwards, 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 (300 g/L lactose + 300 g/L fructose for lactulose and GOS production, and 300 g/L lactose + 300 g/L sucrose for lactosucrose production) prepared in sodium-citrate buffer (50 mM, pH 4.5). Samples were taken at 1, 3, 5, 10, 20, 25 and 30 h and further analyzed by HPLC.

The lactose conversion and the prebiotic yield were calculated using the following equations, where Ci and Cf are the initial and final concentrations of lactose, respectively, and Cp is the concentration of prebiotics (lactulose + GOS).

$$lactose\ conversion = \frac{Ci - Cf}{Ci} \times 100 \quad (1)$$

$$prebiotic\ yield = \frac{Cp}{Ci} \times 100 \quad (2)$$

2.12. β-fructofuranosidase activity assay

β-Fructofuranosidase (FFase) activity was determined by measuring the amount of glucose released from sucrose when the enzyme was incubated at 30 °C, in sodium acetate buffer (100 mM, pH 5.0) for 20 min (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 released 1 μmol of glucose per minute from sucrose.

2.13. Statistical analysis

The statistical analysis was performed using SigmaPlot 11.0 software. The *p* values were obtained from *t*-test. $p < 0.05$ was considered to be statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

3. Results and discussion

3.1. β-galactosidase production

Preliminary chromogenic tests performed in agar plates containing X-gal suggested that *A. lacticoffeatus* MUM 06.150 is able to produce β-galactosidase. The appearance of blue color was visible both on the top and bottom of the agar plate (Fig. 1). The insoluble blue product originated from the enzymatic hydrolysis of X-gal and subsequent dimerization of the substituted indole is widely reported as an indicative of β-galactosidase production (Juers et al., 2012). However, the validation of *A. lacticoffeatus* as an effective new producer of the enzyme was achieved under submerged fermentation conditions. The growth of *A. lacticoffeatus*, as well as the production of extracellular β-galactosidase was evaluated in synthetic medium and six alternative media containing CW or CSL. In all cases, the fermentations were conducted for 6 days and were performed in triplicate. The results obtained are presented in Fig. 2. Significant differences were observed for biomass production between the synthetic medium and the alternative media B, F and G (Fig. 2A). Condition F (CW + peptone and yeast extract) provided the highest amount of biomass, while condition B (8 g/L CSL) exhibited the lowest biomass production. The fungus was able to adapt to the different media and grow properly in the form of pellets. However, the size of the pellets was different when it was grown in the CW medium (D) and CW with salts (E). In these particular cases, smaller pellets were observed. This difference in the biomass appearance was observed for the media where no supplementation of nitrogen was used, which suggests the effect of this nutrient in the pellet morphology. In fact, the type and concentration of the nitrogen source have been

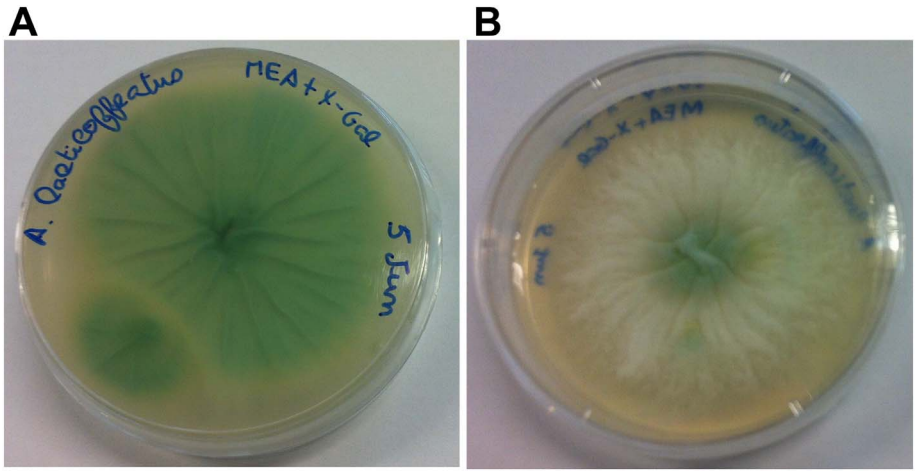

Fig. 1. Chromogenic test with X-gal performed in agar plates protected from light at 25 °C for 7 days: (**A**) bottom view; (**B**) top view. The blue color was considered an indication of β-galactosidase production. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

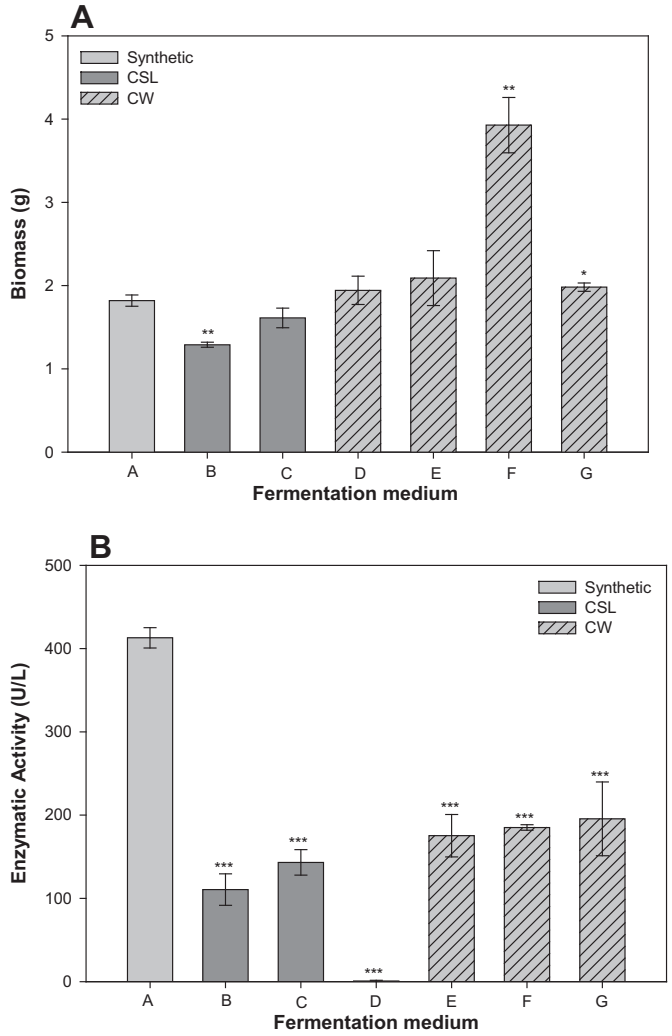

Fig. 2. Biomass production (**A**) and extracellular activity (**B**) after 6 days fermentation in the media: A-Synthetic; B-8 g/L CSL; C-20 g/L CSL; D-CW; *E*-CW + salts; F-CW + peptone and yeast extract; G-CW + salts + peptone and yeast extract. Results correspond to the mean ± SD ($n = 3$). Asterisks indicate a statistically significant difference from medium A (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

reported as an important aspect to the pellet structure with direct action both on the productivity and biomass concentration (Gibbs et al., 2000; Gutiérrez-Sánchez et al., 2013). The results obtained for the extracellular activity (Fig. 2B) showed that medium A provided significantly higher values (413 U/L) than the other 6 media containing the agro-industrial by-products. Furthermore, medium D provided activity values very close to zero, indicating that additional supplementation is needed to produce β-galactosidase.

Comparing the seven fermentation media studied, it seems that the composition of the synthetic medium (A) was the best option to obtain β-galactosidase from *A. lacticoffeatus*. Although the microorganism grew well in all the media studied, the production and secretion of a properly active form of the enzyme was favored in this medium. However, it is important to highlight that the use of CW and CSL as low cost source of lactose and nitrogen, respectively, also provided interesting values of β-galactosidase activity and their application can present recognized economic and environmental advantages (Podleśny et al., 2016; Roal et al., 2015a). For that reason, a further detailed study of β-galactosidase production using these residues (alone or combined in suitable concentrations) could probably improve the results, while significantly reducing the costs of the process.

Since one important application of β-galactosidase is in the production of lactose-based prebiotics with potential application in the pharmaceutical and food industries, it is crucial to have some information on the presence of mycotoxins in the fermentation broth. It is known that mycotoxins are metabolites produced during the secondary metabolism of fungi and several external parameters may regulate their synthesis (Touhami et al., 2016). *A. lacticoffeatus* has been previously reported as an effective OTA producer (Ostry et al., 2013; Samson et al., 2004) and the amount of mycotoxin produced can be dependent on the temperature and incubation time used (Alborch et al., 2011). Consequently, it is important to investigate if this mycotoxin is produced at a harmful level in the fermentation conditions used in this work. The results obtained confirm the presence of OTA with maximal concentration after 6 days (4.9 ng/mL) followed by a considerable reduction to values below the lower limit of detection of the HPLC analysis (≤ 1 ng/mL) after 9 days of fermentation. This reduction could be due to some OTA degradation promoted by light exposure, since several studies have reported that OTA produced by some *Penicillium* and *Aspergillus* spp. are photosensitive toxins (Schmidt-Heydt et al., 2012) and/or by the action of some proteolytic enzymes (Abrunhosa et al., 2010; Abrunhosa and Venâncio, 2007). There is no legal information about the values of OTA concentration accepted in fermentation broths or enzymatic extracts used in the synthesis of prebiotics. Nevertheless, there are already some legal limits established for drink and food products such as: wines and grape juices (2 ng/mL); unprocessed cereals, including rice and buckwheat, and roasted coffee beans (5 ng/g); instant coffees and raisins (10 ng/g); and all food preparations for babies and diet foods for infant medical purposes (0.5 ng/g) (Khoury and Atoui, 2010; Köppen et al., 2010). Considering the legal comparable value established for wine and grape juices, it was demonstrated that at

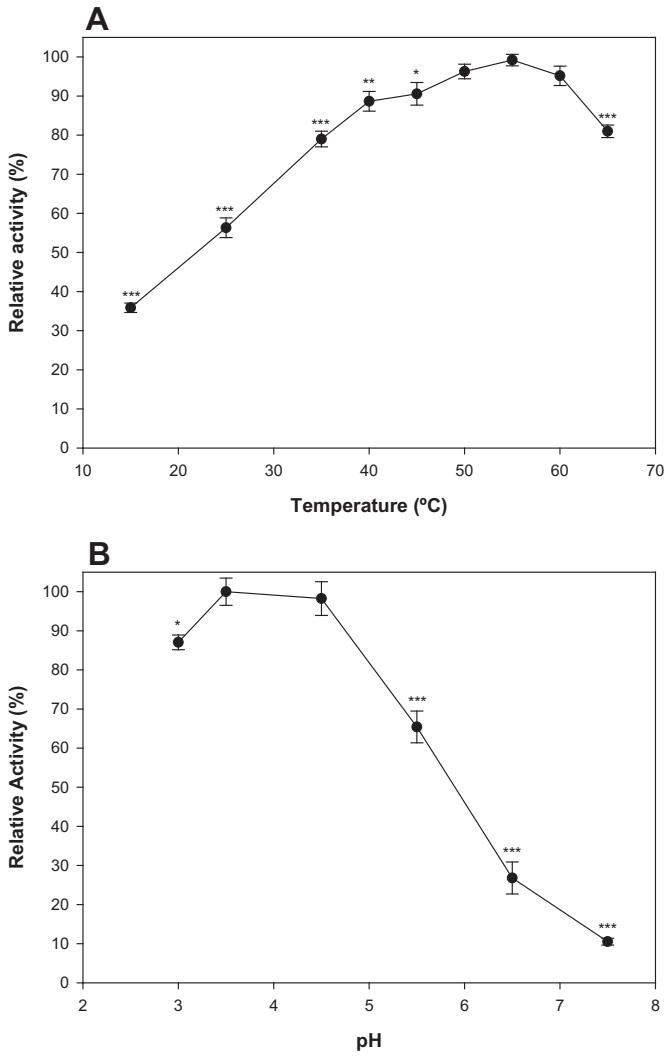

Fig. 3. The effect of temperature (**A**) and pH (**B**) on crude β-galactosidase produced by *A. lacticoffeatus* in medium A after 9 days. Results correspond to the mean ± SD (n = 3). Asterisks indicate a statistically significant difference from 100% relative activity (*p < 0.05, **p < 0.01, ***p < 0.001).

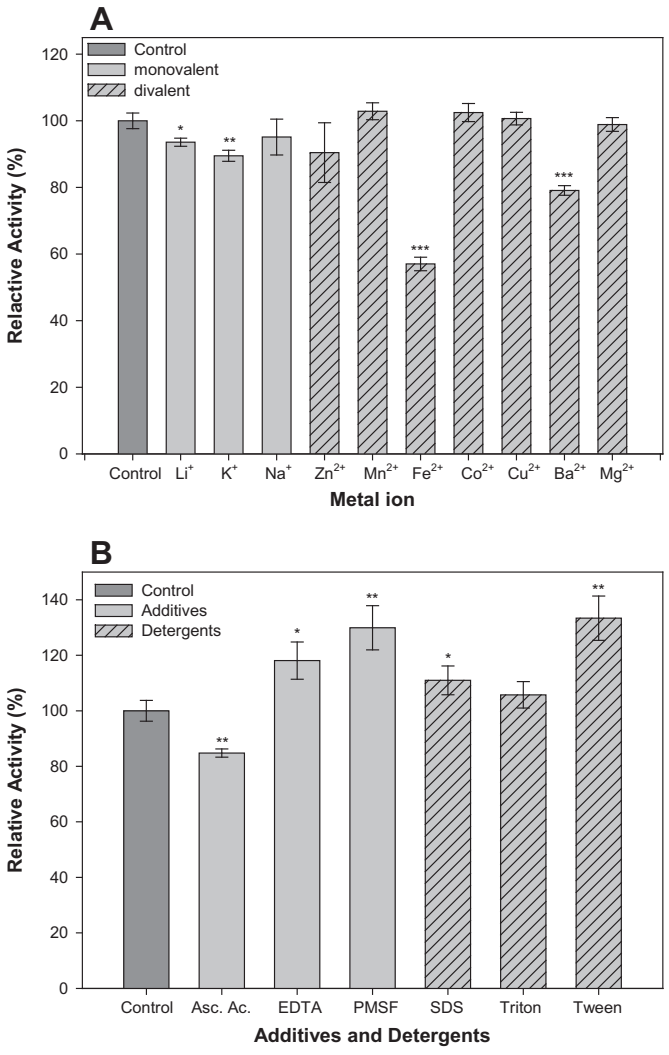

Fig. 4. The effect of mono and divalent metal ions (**A**) and additives and detergents (**B**) on crude β-galactosidase produced by *A. lacticoffeatus* in medium A after 9 days. Sodium citrate buffer (50 mM) pH 4.5 was used as control. Results correspond to the mean ± SD (n = 3). Asterisks indicate a statistically significant difference from control (*p < 0.05, **p < 0.01, ***p < 0.001).

6 days of fermentation OTA concentration is higher than the legal value. However, 9 days can be a suitable period of time to conduct the fermentation, since the enzyme is properly active (460 U/L) and the OTA concentration is ≤ 1 ng/mL.

3.2. β-galactosidase characterization

Crude enzyme characterization was performed using the filtered fermentation broth (9 days). The results obtained for the effect of temperature and pH on the enzymatic activity (hydrolytic activity using ONPG as substrate) are shown in Fig. 3. β-galactosidase from *A. lacticoffeatus* presented an optimal temperature in the range 50–60 °C (Fig. 3A), and remained quite active for temperatures between 35 °C and 65 °C. However, for lower temperatures a considerable reduction of the enzymatic activity was observed. The optimal pH for this β-galactosidase was found in the range 3.5–4.5, and the enzymatic activity decreased significantly for higher pH values (Fig. 3B). These results for 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; Tonelotto et al., 2014). Generally, β-galactosidases produced by fungi present optimal pH in the acidic range and have relatively high optimal temperature (O'Connell and Walsh, 2008). The optimal conditions obtained for the hydrolysis of ONPG by β-galactosidase from *A. lacticoffeatus* (Fig. 3) suggest the suitability of the enzyme for the prebiotic synthesis under the conditions generally used for other fungal enzymes (Torres et al., 2010; Silvério et al., 2016).

The activity of β-galactosidases could also be dependent on the presence of some ions, namely monovalent and divalent cations (Liu et al., 2015). In general, fungal β-galactosidases are described as less dependent on this effect (Mlichová and Rosenberg, 2006), nevertheless some studies have reported the importance of some specific metal ions on the enzymatic activity in *Aspergillus* spp. (Sen et al., 2012). It is known that the way metal ions affect β-galactosidase activity is strongly dependent on the enzyme source. The results obtained for the effect of metal ions are presented in Fig. 4A. Neither the mono nor divalent cations promoted a significant enhancement of the hydrolytic activity. Moreover, β-galactosidase was negatively affected by the presence of Li^+, K^+, Ba^{2+} and Fe^{2+} resulting in a decrease of 6, 11, 21 and 43%, respectively, in the enzymatic activity. The inhibitory effect of some divalent metal ions was also reported for β-galactosidases from *Aspergillus carbonarius* (El-Gindy, 2003), *Aspergillus alliaceus* (Sen et al., 2012) and *Penicillium multicolor* (Takenishi et al., 1983).

The effect of some additives and detergents on β-galactosidase from *A. lacticoffeatus* was also studied (Fig. 4B). It is possible to observe in

Fig. 4B that the enzymatic activity was enhanced or maintained by all the additives and detergents used, except for ascorbic acid which caused a 15% decrease in the β-galactosidase activity. Since ascorbic acid is a reducing agent, its action may be related with the reduction of some amino acid residues (Pal et al., 2013). The negative effect of ascorbic acid was also reported on β-galactosidase from *A. alliaceus* (Sen et al., 2012) and *Enterobacter cloacae* (Ghatak et al., 2010). EDTA, a well-known chelating agent, exhibited a positive effect on the enzymatic activity (an increase around 18%). This increase could be due to the complexing of EDTA with some metal ions present in the fermentation medium which could be negatively affecting the enzyme, for example the K^+, metal ion included in the synthetic medium composition, which promoted a negative effect on the enzymatic activity (Fig. 4A). In addition, the effect of EDTA can suggest that β-galactosidase from *A. lacticoffeatus* does not require metal ions for its hydrolytic activity (El-Gindy, 2003). This fact is corroborated by the results present in Fig. 4A since none of the metal ions studied was found to significantly enhance the enzyme activity. PMSF, a serine protease inhibitor, was also found to promote the enzymatic activity providing an increase of around 30% (Fig. 4B). This effect can probably be related to the inhibition of some proteolytic enzymes possibly present in the crude extract. Furthermore, the results obtained for this additive also suggest that amino acids such as cysteine or serine residues are not associated with the catalytic activity of β-galactosidase from *A. lacticoffeatus* (Badarinath and Halami, 2011). For SDS addition, a negative effect on the β-galactosidase activity was expected, since this anionic detergent has been reported as promoter of protein denaturation (Bhuyan, 2010). However, the results obtained showed an improvement in the enzymatic activity (13% increase) suggesting that probably higher concentrations of SDS are needed to induce the β-galactosidase denaturation. The addition of the nonionic detergents Triton X-100 and Tween-40 to the crude extract contributed to maintain or enhance (33%), respectively, the enzymatic activity.

The effect of glucose and galactose (end products of the lactose hydrolysis), and also fructose on the β-galactosidase activity was investigated, since these monosaccharides can be found in the reaction medium during the enzymatic synthesis of lactose-based prebiotics. According to the results obtained (Fig. 5), no significant effect was detected on the enzymatic activity when fructose was present. On the other hand, glucose had a positive effect in β-galactosidase activity, except for 25 mM, where it became somewhat inhibitory. For galactose, it was possible to verify a strong inhibition of the enzyme in all the concentrations studied. This inhibition became more evident for the highest concentrations (75–100 mM) where only 10% of the enzymatic activity was retained. This result was expected since galactose has been reported as competitive inhibitor of β-galactosidase (van Casteren et al., 2000), acidic enzymes generally being more susceptible to galactose inhibition than to glucose (Boon et al., 2000). Galactose inhibition was also described for other β-galactosidases from the *Aspergillus* spp. (Manzanares et al., 1998; Park et al., 1979; Tonelotto et al., 2014; van Casteren et al., 2000).

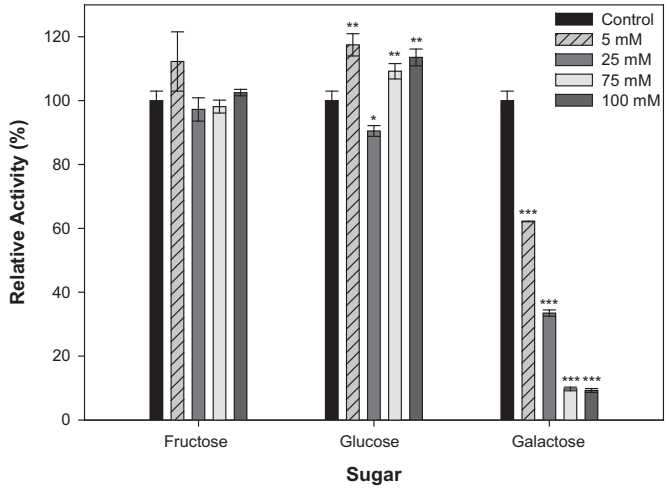

Fig. 5. The effect of different concentrations of fructose, glucose and galactose on crude β-galactosidase produced by *A. lacticoffeatus* in medium A after 9 days. Sodium citrate buffer (50 mM) pH 4.5 was used as control. Results correspond to the mean ± SD ($n = 3$). Asterisks indicate a statistically significant difference from control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.3. β-galactosidase application in the synthesis of prebiotics

To investigate the ability of β-galactosidase from *A. lacticoffeatus* to catalyze both the hydrolytic and transfer reactions involved in the production of lactose-based prebiotics, three approaches were evaluated using different substrate mixtures. In method 1 (addition of different substrate concentrations (10–30 g/L) and different substrate ratios (0.5, 1 and 2) to the ongoing fermentation), the production of prebiotics was not detected for any condition tested. However, lactose and sucrose hydrolysis was observed, as well as the consumption of fructose, galactose and glucose.

For method 2 (biomass transfer to a solution containing 150 g/L of each substrate: lactose + fructose for lactulose and GOS production; lactose + sucrose for lactosucrose production), the production of prebiotics was detected for both temperatures tested (Table 1). When using lactose and fructose as substrates, lactulose and also a trisaccharide GOS were produced. Both compounds were detected after 24 h, their concentration being slightly increased over time to reach a maximum at 72 h (Table 1). The results obtained at 28 °C and 37 °C were similar for lactulose production and for lactose conversion. However, higher concentration of the trisaccharide GOS was detected at 37 °C. Consequently, the prebiotic yield was also superior for the higher temperature studied (Table 1). When lactose and sucrose were used as substrates, no lactosucrose was formed and the only lactose-based prebiotic detected was a trisaccharide GOS. This compound was detected after 24 h and reached a maximal concentration at 72 h. Lactose conversion and GOS concentration was higher at 37 °C. As in method 1, sucrose hydrolysis and subsequent consumption of fructose and glucose occurred, thus suggesting the ability of *A. lacticoffeatus* to also produce an enzyme with sucrase/invertase activity.

In method 3 (crude extract incubated with 150 g/L of each substrate: lactose + fructose for lactulose and GOS production; lactose + sucrose for lactosucrose production), the lactose conversion was lower than in method 2 (Table 1). When lactose and fructose were used as substrates, the formation of lactulose and a trisaccharide GOS was observed. Maximal lactulose concentration (2.5 g/L) was obtained at 25 h and its value was superior to that obtained in method 2. On the other hand, the GOS concentration was similar or lower than that obtained in method 2. Again, no lactosucrose was formed when lactose and sucrose were used as substrates. For this mixture of substrates, the production of a trisaccharide GOS with maximal concentration (6.3 g/L) at 30 h was detected, as well as a trisaccharide FOS identified as 1-kestose. This FOS was observed after 1 h and presented its maximal concentration at 3 h (2.5 g/L). However, it was rapidly degraded and at 5 h its signal was barely detected in the HPLC analysis. The formation of 1-kestose also suggested the presence of an extracellular enzyme with fructosyltransferase activity in the crude extract. An activity test for FFase was conducted to confirm this hypothesis and a value of 16.42 U/mL was found. Therefore, it was proved that *A. lacticoffeatus* is able to produce FFase in the experimental conditions used, being this enzyme the responsible for the hydrolysis of sucrose and subsequent synthesis of 1-kestose. The trisaccharides GOS formed in the several conditions tested have probably the same structure as suggested by their retention time in the HPLC analysis. The absence of lactosucrose formation can possibly be explained by the use of non-optimized reaction conditions or by the inability of fungal sources of β-galactosidase to catalyze the

Table 1
Maximal prebiotic concentration, lactose conversion, and yields obtained for the different approaches evaluated in prebiotic production. The time corresponding to the maximal concentration is also indicated.

Conditions[a]	Prebiotics (g/L)		Lactose conversion[b] (%)	Prebiotic yield[c] (%)
	Lactulose	GOS		
Method 2: lactose + fructose, 28 °C	1.4 (72 h)[d)]	3.8 (72 h)[d]	38	3.5
Method 2: lactose + fructose, 37 °C	1.7 (72 h)[d]	5.8 (72 h)[d]	35	5.0
Method 3: lactose + fructose, 37 °C	2.5 (25 h)[d]	4.0 (30 h)[d]	20	4.3
Method 2: lactose + sucrose, 28 °C	–	4.9 (72 h)[d]	27	3.3
Method 2: lactose + sucrose, 37 °C	–	5.6 (72 h)[d]	34	3.7
Method 3: lactose + sucrose, 37 °C	–	6.3 (30 h)[d]	18	4.2

[a] No prebiotic production was observed for Method 1.
[b] Calculated using Eq. (1).
[c] Calculated using Eq. (2).
[d] Time corresponding to the maximal prebiotic concentration.

production of this trisaccharide. The few works reporting lactosucrose synthesis by β-galactosidase used a bacterial source (*Bacillus circulans*) and found that reaction conditions are determinant for the amount and composition of the transfer products obtained (Farkas et al., 2003; Li et al., 2009; Corzo-Martinez et al., 2015).

Comparing the three methodologies tested for the synthesis of prebiotics, it is possible to conclude that method 1 was ineffective, possible due to the low concentrations of substrates added. On the other hand, the whole cell transfer to a higher substrate concentration (method 2) proved to be more effective for lactulose and GOS production at 37 °C. Method 3 improved lactulose synthesis and the value obtained (2.5 g/L), although low, was comparable to some values reported in the literature for the commercial β-galactosidase from *A. oryzae* (Lee et al., 2004). The GOS concentration obtained when lactose and sucrose were used as substrates also increased in the method 3. Furthermore, 1-kestose was produced in these conditions. Therefore, method 3 seems to be the most efficient for the synthesis of lactose-based prebiotics. Additionally, lactose conversion and prebiotic yield obtained in method 3 can possibly be improved by the use of purified β-galactosidase from *A. lacticoffeatus*, since purification usually leads to an enhancement of the enzymatic activity (Roal et al., 2015b). However, it is important to highlight that the use of the purified enzyme represents additional steps (downstream) and costs in the process of synthesis.

The low prebiotic yields obtained (Torres et al., 2010; Silvério et al., 2016) can be attributed to low values of transgalactosylation activity (Schuster-Wolf-Bühring et al., 2010) and can be improved by decreasing water activity through the use of higher initial substrate concentration and/or organic solvents (Giacomini et al., 2002; Lee et al., 2004). Besides the source of β-galactosidase and the ratio and concentration of substrates, it is known that prebiotic production can also be affected by the reaction temperature, pH and mode of operation. The current work provides important clues on the potential application of the β-galactosidase from *A. lacticoffeatus* in the synthesis of lactose-based prebiotics using reaction conditions similar to those previously reported for other fungal β-galactosidases (Silvério et al., 2016). Therefore, additional studies can be performed to optimize the reaction conditions for β-galactosidase from *A. lacticoffeatus* (pure or crude extract) and to improve the prebiotic production. Nevertheless, the results herein obtained unequivocally show the potential of this enzyme. Furthermore, as far as we know, the use of whole cells from *Aspergillus* sp. is herein reported for the first time in the synthesis of lactulose. Lee et al. (2004) reported the lactulose synthesis using whole cells from *Kluyveromyces lactis* after permeabilization with ethanol. However, in the current work, the whole cells were used without any additional treatment which represents a relevant decrease of the time required for the process of synthesis and the costs involved.

4. Conclusion

A. lacticoffeatus was identified as an effective β-galactosidase producer. The microorganism was able to grow and produce the enzyme using alternative fermentation media containing CW and CSL. However, the best results for enzymatic activity were obtained using the synthetic medium. Crude β-galactosidase has an optimal pH in the acid region (3.5–4.5) and maximal activity in the range 50–60 °C. Furthermore, the divalent cations Ba^{2+} and Fe^{2+}, as well as galactose, had a considerable negative effect in the enzymatic activity. β-galactosidase from *A. lacticoffeatus* was able to catalyze the synthesis of lactulose and a trisaccharide GOS. *A. lacticoffeatus* also produced and secreted an enzyme with fructosyltransferase activity which led to the formation of 1-kestose. The results herein gathered regarding the synthesis of prebiotics are very promising and can be further improved through the optimization of the reaction conditions and/or by using purified β-galactosidase.

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