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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.