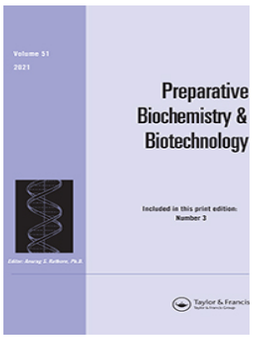

Taylor & Francis
Taylor & Francis Group

Preparative Biochemistry & Biotechnology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/lpbb20

Production and partial purification by PEG/citrate ATPS of a β-galactosidase from the new promising isolate *Cladosporium tenuissimum* URM 7803

Anderson José Paulo, Maria Carolina de Albuquerque Wanderley, Rafael José Vilela de Oliveira, Willie Anderson dos Santos Vieira, Luiz Carlos Alves, Daniela de Araújo Viana Marques, Attilio Converti & Ana Lúcia Figueiredo Porto

To cite this article: Anderson José Paulo, Maria Carolina de Albuquerque Wanderley, Rafael José Vilela de Oliveira, Willie Anderson dos Santos Vieira, Luiz Carlos Alves, Daniela de Araújo Viana Marques, Attilio Converti & Ana Lúcia Figueiredo Porto (2021) Production and partial purification by PEG/citrate ATPS of a β-galactosidase from the new promising isolate *Cladosporium tenuissimum* URM 7803, Preparative Biochemistry & Biotechnology, 51:3, 289-299, DOI: 10.1080/10826068.2020.1815054

To link to this article: https://doi.org/10.1080/10826068.2020.1815054

PREPARATIVE BIOCHEMISTRY & BIOTECHNOLOGY
2021, VOL. 51, NO. 3, 289–299
https://doi.org/10.1080/10826068.2020.1815054

Production and partial purification by PEG/citrate ATPS of a β-galactosidase from the new promising isolate *Cladosporium tenuissimum* URM 7803

Anderson José Paulo[a], Maria Carolina de Albuquerque Wanderley[b], Rafael José Vilela de Oliveira[c], Willie Anderson dos Santos Vieira[d], Luiz Carlos Alves[e], Daniela de Araújo Viana Marques[f], Attilio Converti[g], and Ana Lúcia Figueiredo Porto[h]

[a]Campus Tefé, Federal Institute of Education, Science and Technology of Amazon (IFAM), Tefé, Brazil; [b]Laboratory of Immunopathology Keizo Asami (LIKA), Federal University of Pernambuco, Recife, Brazil; [c]Departament of Micology Prof. Chaves Batista, Federal University of Pernambuco, Recife, Brazil; [d]Department of Agronomy, Federal Rural University of Pernambuco, Recife, Brazil; [e]Institute Aggeu Magalhães-IAM/FIOCRUZ, Federal University of Pernambuco, Recife, Brazil; [f]Laboratory of Biotechnology Applied to Infectious and Parasitic Diseases, Biological Science Institute, University of Pernambuco-ICB/UPE, Santo Amaro, Recife, Brazil; [g]Department of Civil, Chemical and Environmental Engineering, Pole of Chemical Engineering, Genoa, Italy; [h]Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco, Recife, Brazil

ABSTRACT

β-Galactosidase production, partial purification and characterization by a new fungal were investigated. Partial purification was performed by aqueous two-phase system (ATPS) using polyethylene glycol (PEG) molar mass, PEG concentration, citrate concentration and pH as the independent variables. Purification factor (*PF*), partition coefficient (*K*) and yield (*Y*) were the responses. After identification by rDNA sequencing and classification as *Cladosporium tenuissimum* URM 7803, this isolate achieved a maximum cell concentration and β-galactosidase activity of 0.48 g/L and 462.1 U/mL, respectively. β-Galactosidase partitioned preferentially for bottom salt-rich phase likely due to hydrophobicity and volume exclusion effect caused in the top phase by the high PEG concentration and molar mass. The highest value of *PF* (12.94) was obtained using 24% (w/w) PEG 8000 g/mol and 15% (w/w) citrate, while that of *Y* (79.76%) using 20% (w/w) PEG 400 g/mol and 25% (w/w) citrate, both at pH 6. The enzyme exhibited optimum temperature in crude and ATPS extracts in the ranges 35–50 °C and 40–55 °C, respectively, and optimum pH in the range 3.0–4.5, with a fall of enzyme activity under alkaline conditions. Some metal ions and detergents inhibited, while others stimulated enzyme activity. Finally, *C. tenuissimum* URM 7803 β-galactosidase showed a profile suitable for prebiotics production.

KEYWORDS

β-Galactosidase; fungi; phylogenesis; production; aqueous two-phase system

Introduction

β-galactosidases (EC3.2.1.23) are hydrolases capable of hydrolyzing the β-galactopyranosyl terminal residue of lactose (Galβ1-4Glc) to form glucose and galactose as well as the reverse reaction, i.e., sugar condensation to galacto-oligosaccharides (GOS). Therefore, they are able to split β-galactosyl bonds in glycoproteins, polysaccharides, disaccharides and other compounds such as *o*- and *p*-nitrophenyl-β-D-galactosides used to determine β-galactosidase activity. Also known as lactases, they are among the most studied enzymes.[1]

β-Galactosidases are produced by a large number of microorganisms such as filamentous fungi, bacteria and yeasts, but they are also found in vegetables, particularly almonds, peaches, apricots and apples, as well as in animal organs such as intestine, brain and placenta.[2] These enzymes are industrially important because they are used to (a) prevent lactose crystallization in sweetened, condensed and frozen dairy products such as ice cream and condensed milk, (b) solve problems associated with whey utilization and disposal, (c) avoid lactose intolerance complications in lactase-deficient individuals, (d) treat disorders of the gastrointestinal tract through the development of lactose-based supplements; (e) develop biosensors to be used in diagnostic tests.[3,4]

Despite the wide variety of sources, enzymes produced by bacteria, yeasts and filamentous fungi are the most studied and used for various purposes in the biotechnology, food and pharmaceutical industries.[5–7] Even though several filamentous fungi such as *Teratosphaeria acidotherma*,[8] *Aspergillus tubingensis*,[9] *Aspergillus nidulans*,[10] *Aspergillus lacticoffeatus*[11] and *Papiliotrema terrestris*[12] were reported as good producers of these enzymes, studies have been encouraged in recent years on the search for new microbial sources able to produce β-galactosidases with special characteristics.

CONTACT Daniela de Araújo Viana Marques daniela.viana@upe.br Biological Science Institute, University of Pernambuco, R. Arnóbio Marques, 310, 50100-130, Santo Amaro, Recife, PE, Brazil.

Supplemental data for this article can be accessed online at http://dx.doi.org/10.1080/10826068.2020.1815054

Candidates as new β-galactosidase producers, fungi belonging to the genus *Cladosporium* are still little explored biotechnologically. This genus, defined by Link in 1816, is one of the largest, widespread and most heterogeneous genera of Hyphomycetes[13] and comprises more than 189 species. *Cladosporium* spp. are called black yeasts, because they are naturally brownish in color due to the presence of melanic pigment (dihydroxynaphthalene melanin) in their cell wall. Among the species of this genus that have largely been applied biotechnologically, *Cladosporium cladosporioides* is reported as a producer of some enzymes such as pectin methylesterase and polygalacturonase.[14] Moreover, unpublished results obtained with the new isolate of *Cladosporium tenuissimum* (URM 7803) used in this study highlighted its potential both in the hydrolysis of lactose and in the production of prebiotics such as GOS.

The literature reports several techniques for enzymes separation and purification, being ammonium sulfate precipitation, ultrafiltration, successive chromatography steps and dialysis the most commonly used.[15,16] However, these procedures are considered infeasible for large-scale productions, because they are too expensive, time consuming and difficult to scale up.[17,18] It is then necessary to develop alternative low-cost and high-yield methodologies to replace them at least partially.

In this context, Aqueous Two-Phase Systems (ATPS) have emerged as an alternative method to extract and purify enzymes[19–21] because they make use of simple and cheap components. Being among the most promising bioseparation processes, they can be used in the initial stages of the purification process to replace solid-liquid separations, or even in subsequent steps of purification.[22]

During the last few years there has been a renewed interest in new methods in separating biomolecules that allow replacing expensive chromatographies. The literature has already reported the use of ATPS for the separation of several biomolecules such as lipases,[23,24] α-lactalbumin,[25] collagenases,[26–28] ovalbumin,[29] phosvitin and gallic acid,[30] antioxidant peptides,[31] among others. The use of ATPS for β-galactosidase purification was also reported by some authors, all using polyethylene glycol and potassium phosphate.[20,32,33] On the other hand, this is the first study in which a sodium citrate ATPS was used to recover β-galactosidase and in which the enzyme stability was simultaneously checked in the presence of ions, inhibitors, and detergents.

The aim of this work was to investigate β-galactosidase production by the new isolate *Cladosporium tenuissimum* URM 7803, which was identified by rDNA sequencing, for possible application in lactose hydrolysis and production of prebiotics such as GOS. Moreover, we identified the best conditions for its pre-purification using ATPS formed by PEG and sodium citrate and made its partial characterization in terms of influence of temperature, pH and types of ions, inhibitors, and detergents on its activity.

Materials and methods

Materials

Polyethylene glycol (PEG) 400, 3350 and 4000 g/mol was purchased from Sigma (St. Louis, MO, USA), while Tween 80, lactose, peptone, yeast extract and salts were purchased from HIMedia (Mumbai, India). All the other reagents were of analytical grade and acquired from Merck® (Darmstadt, Germany), Micromed® (Brasilia, DF, Brazil) and Sigma®. All the salts used had purity ≥98%.

Microorganism

The *Cladosporium tenuissimum* strain URM 7803 was isolated from culture medium saturated with lactose, deposited at "Micoteca - URM" of Mycology Department of the Federal University of Pernambuco (UFPE), Recife-PE, Brazil, preserved in mineral oil and maintained at 28 °C in Czapek Dox Agar.

DNA extraction, PCR amplification and DNA sequencing

To extract rDNA, cultures grown in test tubes containing malt extract were incubated at 28 °C for six days to obtain the fungal biomass. The material was transferred to 2.0-mL microtubes with screw caps. Acid-washed glass beads (0.5 g) with two different diameters (150–212 µm and 425–600 µm, 1:1; Sigma, Burlington, MA, USA) were added to each tube. The material was crushed by stirring at high speed in a FastPrep homogenizer. The genomic DNA extraction procedure was conducted as previously described by Góes-Neto et al.[34] The mycelium was washed with 24:1 chloroform:isoamyl alcohol and then homogenized in 2% cetyltrimethylammonium bromide buffer. DNA was precipitated in isopropanol, washed with 70% ethanol and resuspended in 50 µL of ultrapure water.

The primer pairs EF1-728F/EF1-986R[35] were used to amplify the translation elongation factor 1-a (TEF1). Polymerase Chain Reaction (PCR) was carried out in 50-µL samples containing 75 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.01% Tween 80, 2.0 mM $MgCl_2$, 200 µM each dNTP, 1.0 µM each primer, and 2 units Taq DNA polymerase (Fermentas, Thermo Fisher, Waltham, MA, USA). Thermal cycling parameters were set as follows: an initial denaturation temperature of 94 °C for 5 min, followed by 40 cycles of denaturation temperature (94 °C) for 45 s, primer annealing at 52 °C for 30 s, primer extension at 72 °C for 90 s and a final extension step at 72 °C for 6 min.

The final amplicons were purified with the kit Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Sequencing was performed by the Laboratory of Molecular Biology and Evolutionary Biology of UFPE, Recife, Brazil. Sequence assembly and editing were performed using the tools called Pregap4 and Gap4, which belong to the Staden package.[36]

Phylogenetic analysis

Forward and reverse sequences were assembled using the Staden package. The sequence generated in the current study was deposited in GenBank (Table 1). The sequence was compared to GenBank sequences using the Basic Local Alignment Search Tool (BLAST) to query the National

Table 1. GenBank and culture collection accession numbers of Cladosporium species employed in phylogeny.

Species (1)	Culture accession number (2)	ef1-α GenBank accession number
*C. angustisporum**	CBS 125983; CPC 12437	HM148236
*C. australiense**	CBS 125984; CPC 13226	HM148240
*C. cladosporioides**	CBS 112388	HM148244
*C. colocasiae**	CBS 386.64; ATCC 200944; MUCL 10084	HM148310
*C. cucumerinum**	CBS 171.52; MUCL 10092	HM148316
*C. feniculosum**	CBS 122129; ATCC 38010; IFO 6537; JCM10683	HM148338
*C. gamsianum**	CBS 125989; CPC 11807	HM148339
*C. grevilleae**	CBS 114271; CPC 2913	JF770472
*C. phaeocomae**	CBS 128769; CPC 18223	JF499875
*C. pseudocladosporioides**	CBS 125993; CPC 14189	HM148402
*C. subuliforme**	CBS 126500; CPC 13735	HM148441
C. tenuissimum	SRRC1616	KT950252
C. tenuissimum	SRRC1634	KT950253
*C. tenuissimum**	CBS 125995; CPC 14253	HM148442
C. tenuissimum	URM 7803	
*C. verrucocladosporioides**	CBS 126363; CPC 12300	HM148472

1: "*" represents holotype isolates. 2: ATCC: American Type Culture Collection, Manassas, VA, USA; CBS: CBS-KNAW Fungal Biodiversity Center, Utrecht, The Netherlands; CPC: Culture Collection of Pedro Crous, housed at CBS; IFO: Institute for Fermentation, Osaka, Japan; MUCL: Mycothèque, Laboratoire de Mycologie Systématique et Appliquée, Université Catholique de Louvain, Louvain-la-Neuve, Belgium; SRRC: Southern Regional Research Center, New Orleans, LA, USA.

Center for Biotechnology Information (NCBI) sequence database for approximate species identification.

Sequences of type and representative strains of *C. cladosporioides* species complex of elongation factor 1-α were downloaded from GenBank and combined with the newly generated sequence. Multiple sequence alignment was performed in MEGA 7.0.14.[37]

The phylogeny was inferred under the maximum likelihood (ML) criterion. ML analyses were done in PhyMl 3,1 aLRT[38] implemented on Phylogeny.fr web server,[39] ML tree searches were performed under the HKY85 substitution model with 1000 pseudoreplicates.

Scanning electron microscopy

Cladosporium tenuissimum URM 7803 spores were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 6.8 (Sigma-Aldrich, São Paulo, SP, Brazil). Then the samples were post-fixed in 1% osmium tetroxide (Sigma-Aldrich) in cacodylate buffer for 1 h in the absence of light at 25 °C. After this step, washing and dehydration were performed in a series of increasing ethanol concentrations for 15 min each (30, 50, 70, 90 and 100%). Samples were subjected to critical point using liquid CO_2, coated with colloidal gold and examined with a scanning electron microscope, model JEOL-5600 LV (Jeol, Tokyo, Japan).

Inoculum preparation and β-galactosidase production

Spore suspensions for inoculum were prepared in sterile saline solution (0.85% NaCl, w/v) containing 0.01% (w/v) Tween 80, and conidia density was adjusted to 10^7 conidia/mL. The fermentation medium was composed of (%, w/v): lactose (2.0), 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)]. Fermentations were carried out at 28 °C, pH 6.5 and 180 rpm for 13 days. Aliquots of the fermented medium (50 mL) were collected every 24 h, and β-galactosidase activity was determined.

β-galactosidase activity

Extracellular β-galactosidase activity was determined by incubating samples (50 µL) at 37 °C for 30 min with 50 µL of 3.0 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG) solution prepared in 50 mM sodium citrate buffer, pH 4.5. The reaction was stopped by the addition of 200 µL of 0.1 M sodium carbonate.[40] The released *o*-nitrophenol was quantified spectrophotometrically at 420 nm. One unit (U) of β-galactosidase was defined as the amount of enzyme that released 1 µmol of *o*-nitrophenol from ONPG per minute under the assay conditions.

Protein determination

Protein content was determined by the bicinchoninic acid method, using the Pierce™ BCA Protein Assay Kit and bovine serum albumin as a standard.[41]

Determination of cell dry mass

In order to determine fungal cell dry mass at specific time intervals, the mycelium was filtered through filter paper (Whatman n° 40), washed once with distilled water and dried up to constant weight at 80° C. The mycelium was placed in desiccator, and then its mass was determined by weighing.[42]

Aqueous two-phase systems

A concentrated solution of sodium citrate (30% w/w) was prepared by mixing at 25 ± 1 °C appropriate amounts of equimolar solutions of dihydrate tri-sodium citrate and monohydrate citric acid so as to obtain different pH values (6.0, 7.0 or 8.0). PEG solutions with different molar masses, specifically 400, 3350 and 8000 g/mol, were dissolved (60% w/w) in phosphate-buffered saline and transferred to 15-mL graduated tubes. Subsequently, water and aliquots of the fermented broth corresponding to 20% (w/w) of the total mass

Table 2. Factor levels of the 2^4-full factorial design used to investigate β-galactosidase extraction and recovery by ATPS.

	Level		
Factor	Low (−1)	Center (0)	High (+1)
PEG molar mass (M_{PEG})	400	3350	8000
PEG concentration (C_{PEG})	20	22	24
Citrate concentration (C_{CIT})	15	17.5	20
pH	6.0	7.0	8.0

(10 g) were added. After 1 min of vortex shaking, the two phases were separated by settling for 120 min. The volume of each phase was then measured, and protein concentration and β-galactosidase activity were determined. To avoid any interference of PEG and citrate salt, all the samples were compared with protein-free standard solutions, with the same phase composition.

The β-galactosidase partition coefficient (K) was determined as the ratio of the β-galactosidase volumetric activity in the top phase (A_T) to that in the bottom phase (A_B):

$$K = \frac{A_T}{A_B} \tag{1}$$

The yield (Y), expressed as a percentage, was defined by the equation:

$$Y = \frac{100}{1 + \frac{1}{R_V K}} \tag{2}$$

where:

$$R_V = \frac{V_T}{V_B} \tag{3}$$

is the volume ratio, *i.e.*, the ratio of the top phase volume (V_T) to that of the bottom phase (V_B).

The purification factor (PF) was calculated as the ratio of the specific activity in the bottom phase ($a_B = A_B/C_B$) to that in the fermented broth ($a_F = A_F/C_F$):

$$PF = \frac{A_B/C_B}{A_F/C_F} \tag{4}$$

where C_B and C_F are the protein concentrations, expressed in mg/mL, in the bottom phase and the fermentation broth, respectively.

Experimental design

A 2^4-full factorial design (Table 2) was utilized to evaluate the influence of four independent variables, namely PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}), citrate concentration (C_{CIT}) and pH, on K, Y, PF and a_B selected as the responses.

For model fitting, the variables were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \tag{5}$$

Where x_i is the coded value corresponding to the X_i actual value, X_0 the average of the two extreme levels and ΔX_i the range of variation of the i-th factor ($1 = M_{PEG}$, $2 = C_{PEG}$, $3 = C_{CIT}$, $4 = pH$).

The full 2^4-design model included a constant term, three main effects, three two-factor interactions and a three-factor interaction. Since no replicates had been carried out at this point, the model was truncated after the two-factor interactions, and the three-factor term was taken as an estimate of the error of an effect. With this assumption, the model was reduced to the equation:

$$\hat{y} = b_0 + \sum b_i x_i + \sum b_y x_i x_j \tag{6}$$

Where $\hat{y}$ is the predicted response, b_0 is the constant term, b_i are the linear coefficients, b_y are the interaction coefficients, while x_i and x_j are the coded values of the independent variables i and j, respectively. The relative significance of all coefficients was assessed from the error estimate based on the third-order term at 95% confidence level.

The goodness of model fitting was evaluated by the coefficient of determination (R^2) and multiple regression; the first-order model equation was determined by the Fischer's test. The experimental and predicted values were compared by the Statistica 8.0 statistical program package (Statistica, Statsoft Inc, 2015, Tulsa, OK, USA) to determine the validity of the developed model.

β-Galactosidase partial characterization

The effect of temperature on enzyme activity was evaluated by determining the β-galactosidase activity at the end of each test, using 50 mM sodium-citrate buffer (pH 4.5) at different temperatures (from 15 to 85 °C) under the conditions described in the previous section. The effect of pH on the enzyme activity was evaluated at 37 °C using two different buffers depending on the pH range under investigation, namely 50 mM sodium-citrate buffer (pH 3.0–5.5) or 50 mM phosphate-citrate buffer (pH 6.5–8.5), under the conditions described in the previous section. To study the effect of different metal ions, additives and detergents, the selected compound was added to 50 mM sodium-citrate buffer (pH 4.5). For metal ions, the buffer was mixed with one of the following salts, KCl, NaCl, $BaCl_2$, $MgCl_2$, $ZnSO_4$, $MnCl_2$, $FeCl_2$, $CoCl_2$ and $CuSO_4$, at a concentration of 10 mM. The same buffer was also used to prepare solutions either of detergents such as 10 mM sodium dodecyl sulfate (SDS), 1% (w/v) Tween-80 or 1% (w/v) Triton X-100 or of additives such as 10 mM phenylmethanesulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) or ascorbic acid.

All these effects were evaluated by incubating 50 µL of enzyme extract with 50 µL of a 3.0 mM ONPG solution prepared in buffer solutions supplemented with each specific compound. In all cases, the enzyme activity was determined using the β-galactosidase activity assay previously described. All characterization tests were carried out in triplicate.

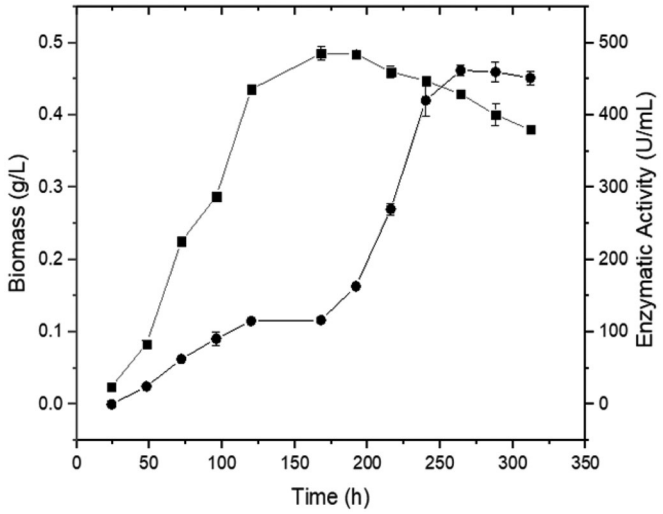

Figure 1. Time behaviors of *β*-galactosidase activity (●) and biomass concentration (■) during *Cladosporium tenuissimum* URM 7803 cultivation on a lactose-based fermentation medium.

Results and discussion

β-Galactosidase production

Cladosporium tenuissimum URM 7803 fermentation was carried out for a period of 312 h, during which samples were taken every 24 h to determine the best conditions for β-galactosidase production. Enzyme activity achieved a maximum value of 462.1 U/mL after 264 h and kept almost constant up to the end of cultivation (451.7 U/mL), while dry cell concentration, after reaching a maximum value of about 0.48 g/L after 168 h, progressively decreased and entered the cell death phase after 212 h (Fig. 1).

Such a partial dissociation of β-galactosidase from the fungus growth suggests that the enzyme preferentially accumulated intracellularly, a characteristic that had already been observed for *Teratosphaeria acidotherma*[8] and *Bacillus safensis,*[43] with a pH-dependent distribution between intracellular and extracellular activities in the former case.

The slow growth of *C. tenuissimum* URM 7803 is typical of fungi belonging to the genus *Cladosporium* that reach maturity usually between 14 and 21 days.[44] Moreover, β-galactosidase produced by this strain had a maximum activity significantly higher than those of other filamentous fungi of the *Aspergillus* genus in submerged fermentation (Supplemental Material, Table S1), hence proving to be a promising novel enzyme with potential for future biotechnological applications.

Morphological and phylogenetic analyses

Members of the genus *Cladosporium* form septate and dark hyphae with lateral and terminal conidiophores of varying size. Their conidia can be produced in chains or even solitary in some species, being able to assume cylindrical, egg-shaped, spindle-like, ellipsoid or spherical forms.[45] In agreement with such a description, macromorphological observation of *C. tenuissimum* URM 7803 revealed dark or brown olive color colonies (Supplemental Material, Fig. S1),

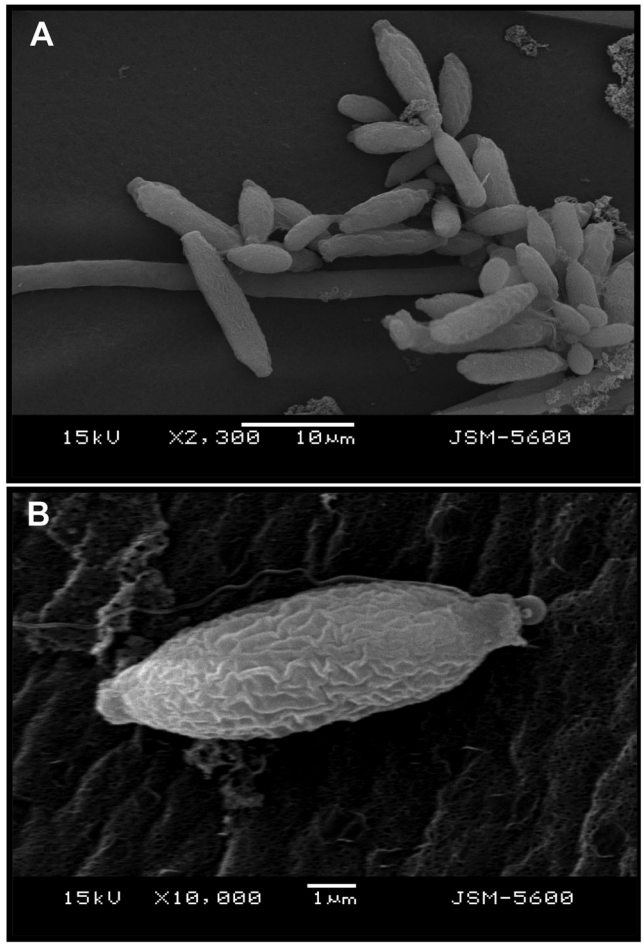

Figure 2. Scanning electron micrographs of *Cladosporium tenuissimum* URM 7803. (A) Ramoconidia and conidia; (B) detail of conidia. Scale bars = 10 µm (A), 1 µm (B).

while the microscopic one conidia with smooth or verrucous surface (Fig. 2).

Phylogenetic analyses showed that ef1-α partial sequence of the β-galactosidase producer was similar to sequences of the *C. cladosporioides* species complex according to the BLAST search. The isolate was placed together with *C. tenuissimum* isolates with very strong support (88%) in the ML tree (Fig. 3) and was confidently assigned to this species.

The genus *Cladosporium* contains species with very significant morphological and genetic similarities, being sequencing of the elongation factor 1-α an important tool to distinguish them.[46] Results of such a sequencing method confirmed that *C. tenuissimum* URM 7803 is a new fungal isolate able to produce β-galactosidase not yet reported in the literature.

Partial β-galactosidase purification by aqueous two-phase systems

Several factors are known to influence partitioning of biomolecules between two phases, among which are the type, concentration and molar mass of the polymer, salt or co-solute salt, pH and temperature. All these factors are in fact able to modulate the characteristics and surface properties of a biomolecule, and then its separation.[47]

Table 3. Factor level combinations and results of the 2^4-full factorial design employed to investigate the extraction and recovery of *Cladosporium tenuissimum* URM 7803 β-galactosidase by PEG/citrate ATPS.

Run	M_{PEG}[a] (g/mol)	C_{PEG}[b] (% w/w)	C_{CIT}[c] (%w/w)	pH	a_B[d] (U/mg)	A_B[e] (U/mL)	K[f]	Y[g] (%)	PF[h]
1	400	20	15	6	–	–	–	–	–
2	**8000**	**20**	**15**	**6**	**3.30**	**261.5**	**0.13**	**56.61**	**12.82**
3	400	24	15	6	–	–	–	–	–
4	**8000**	**24**	**15**	**6**	**3.34**	**238.9**	**0.27**	**51.71**	**12.94**
5	400	20	20	6	0.66	368.5	0.18	79.76	2.57
6	8000	20	20	6	3.83	97.17	1.42	21.03	14.86
7	400	24	20	6	0.49	327.6	0.12	70.91	1.91
8	8000	24	20	6	3.09	101.5	1.11	21.97	11.98
9	400	20	15	8	–	–	–	–	–
10	8000	20	15	8	2.41	198.3	0.20	42.91	9.35
11	400	24	15	8	–	–	–	–	–
12	8000	24	15	8	0.40	34.34	2.47	7.43	1.57
13	400	20	20	8	0.31	276.1	0.14	59.76	1.28
14	8000	20	20	8	1.53	79.13	1.64	17.13	5.95
15	400	24	20	8	0.35	274.3	0.16	59.38	1.35
16	8000	24	20	8	0.90	43.47	3.32	9.41	3.48
17(C)	**3350**	**22**	**17.5**	**7**	**3.68**	**198.7**	**0.29**	**43.01**	**14.24**
18(C)	**3350**	**22**	**17.5**	**7**	**3.25**	**191.9**	**0.34**	**41.55**	**12.60**
19(C)	**3350**	**22**	**17.5**	**7**	**3.56**	**194.8**	**0.37**	**42.16**	**13.80**
20(C)	**3350**	**22**	**17.5**	**7**	**2.76**	**182.2**	**0.39**	**39.43**	**10.71**

[a]PEG molar mass.
[b]PEG concentration.
[c]Citrate concentration.
[d]Specific β-galactosidase activity in the bottom phase.
[e]β-Galactosidase activity in the bottom phase.
[f]Partition coefficient.
[g]Activity yield in the bottom phase.
[h]Purification factor in the bottom phase.
–: no biphasic system. The best results are shown in boldface.

Results of partial purification of β-galactosidase listed in Table 3 show that the runs 1, 3, 9 and 11 carried out at the lowest PEG molecular mass (M_{PEG} = 400 g/mol) did not lead to any biphasic system, likely because the concentrations of components were below or very close to the critical point of the binodal curve.[18] The high concentration of PEG used in this study may have promoted the enzyme partitioning toward the salt-rich, bottom phase, as shown by the very low values of the partition coefficient (K) observed in the runs 5, 7, 13 and 15 (0.12-0.18). A similar effect can be ascribed to the high PEG molar mass. These results taken together suggest that, in general, an increase in polymer size or concentration increased the top phase hydrophobicity, thereby exerting a repulsive volume exclusion effect and then leading to preferential partitioning toward the salt-rich, bottom phase.[19]

The high values of the purification factor observed in the runs 2 (12.82) and 4 (12.94) can be ascribed to a synergistic effect of high M_{PEG} and other factors, which may have promoted the partition of different extract components to the top PEG-rich phase and that of β-galactosidase to the bottom, salt-rich one.[47,48]

The results of statistical analysis (Supplemental Material, Table S2) revealed that most of the effects of the independent variables and their interactions were statistically significant at a 95% confidence level on K and Y, but less on a_B and PF.

All the variables exerted positive linear effects on K, being that of M_{PEG} (x_1) the strongest one, whereas the interaction of C_{PEG} and C_{CIT} (x_2x_3) was negative, which means that enzyme partitioning to the bottom phase would be promoted by an increase in C_{PEG} (x_2) and a simultaneous decrease in C_{CIT} (x_3), or vice versa (Supplemental Table S2). This behavior is also consistent with the dramatic K increase caused by a rise in pH from 6.0 (run 2) to 8.0 (run 16). Likewise, when the pH was raised from 6.0 to 9.0, using a PEG-phosphate ATPS, it was observed an increase from 3 to 9 in K of bromelain.[49]

Only M_{PEG} and pH exerted significant linear positive and negative effects, respectively, as well as a negative interaction effect on PF, which means that β-galactosidase recovery toward the bottom phase would be promoted by an increase in the former independent variable and a decrease in the latter (Supplemental Table S2). This effect, too, may be ascribed to the reduction of space available for β-galactosidase in the top, PEG-rich phase resulting from an increase in PEG molar mass. In previous studies where β-galactosidase was purified by affinity chromatography, Silva and Franco[50] obtained a purification factor of 2.8, while Hemavathi and Raghavarao[32] of only 1.8. Even though the highest PF (14.86) was obtained in the run 6, taking together the results of the three extraction parameters, *i.e.*, K, PF and Y, the conditions of runs 2 (M_{PEG} 8000 g/mol, 20% C_{PEG}, 15% C_{CIT}, pH 6) and 4 (M_{PEG} 8000 g/mol, 24% C_{PEG}, 15% C_{CIT}, pH 6) were selected as the best. On the other hand, M_{PEG} and pH exerted significant antagonist effects on the specific activity in the bottom phase (a_B), in that such a response increased with lowering the pH and increasing M_{PEG}, but, likewise PF, was not statistically significantly influenced by C_{CIT} at 95% confidence level. It is known that pH affects protein partitioning by changing the net charge of the phases; therefore, the hydrophobic and hydrophilic forces existing in the system may be altered, favoring the protein partitioning toward a particular

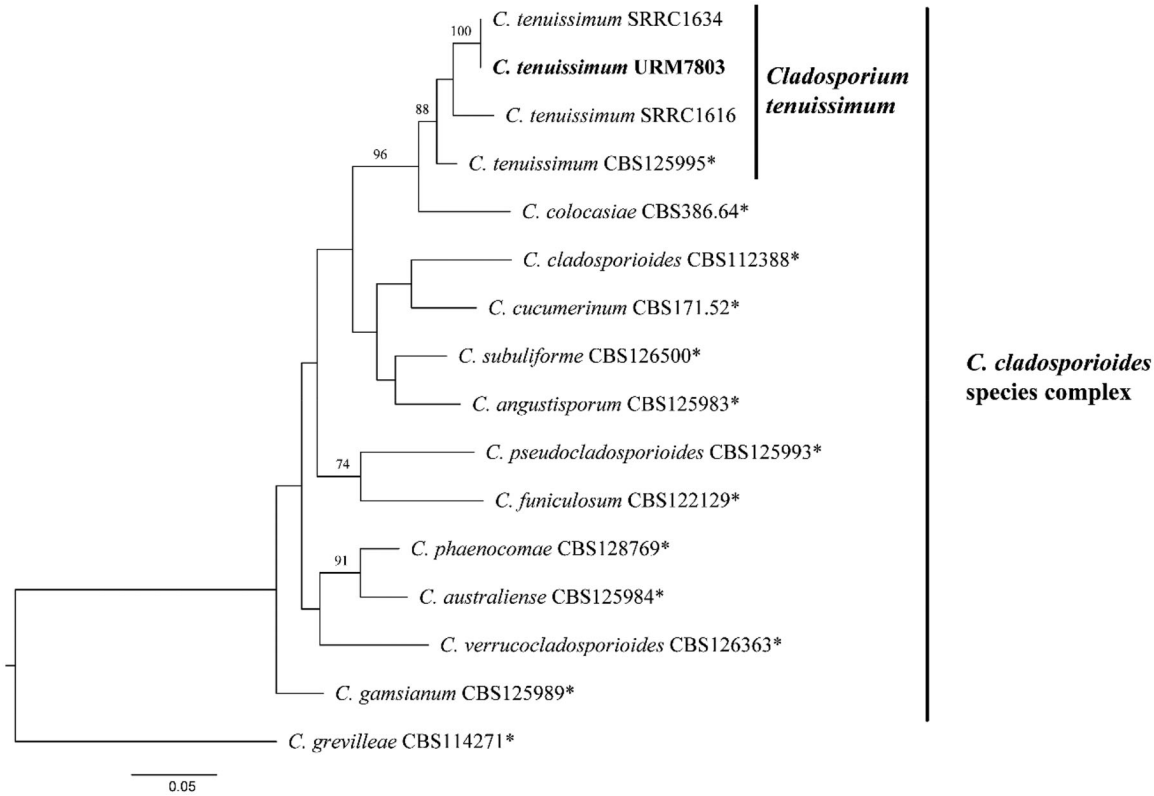

Figure 3 Maximum likelihood tree of *Cladosporium cladosporioides* species inferred from an alignment of ef1-α Supports $\geq$70% are shown above the nodes. Ex-types are marked with "*". Isolate used in the present study is written in bold type. *Cladosporium grevilleae* was used as outgroup taxa. The scale bar indicates the average number of substitutions per site.

phase. However, the change in electrostatic force with pH may vary significantly from one salt to another.[51]

The validity of the regression model was checked by the multiple regression analysis (Supplemental Material, Table S3). For *K*, *PF* and a_B, the *F*-test was significant for all the independent variables, and the model adequate to describe the results, *i.e.*, the calculated *F* values (6.40 for *K*, 4.59 for *PF* and 4.59 for a_B) were higher than the tabulated one (3.06). As far as *Y* is concerned, the *F*-test was not significant at 95% confidence level, due to a too high lack of fit (results not shown). Although the values of the determination coefficient (R^2) obtained by multiple regression analysis for the effects of all independent variables on responses were between 0.550 and 0.630, taking into account that the estimated effects and the corresponding *p*-values were below 0.05, the agreement between experimental data and values predicted by the models can be considered satisfactory.

β-galactosidase partial characterization

The results of *C. tenuissimum* URM 7803 β-galactosidase hydrolytic activity obtained at different temperature and pH values using ONPG as a substrate are illustrated in Figure 4. The enzyme activity reached an optimum at temperature ranging from 35 °C to 50 °C in the crude extract and 40 to 55 °C in the ATPS extract from run 4 (*M*PEG of 8.000 g/mol, *C*CIT of 15%, *C*PEG of 24%, pH 6.0), with an average increase in the relative activity of approximately 31% for the latter. On the other hand, dramatic decreases in enzyme activity were observed in both extracts at lower or higher temperatures. The optimum enzyme pH was found to be between 3.0 and 4.5, and the enzyme activity decreased significantly at higher pH values, especially in the crude extract.

As for other enzymes, β-galactosidase activity may be influenced by the presence of monovalent or divalent cations.[52] However, in general, the type and extent of their effect are quite dependent on the enzyme source, the fungal ones being less sensitive than those from other sources.[53]

As shown in Figure 5, mono and divalent cations exerted a 45–55% increase in the hydrolytic activity of the crude extract, with no statistically significant influence of the cation type and charge number. On the other hand, in the ATPS extract, only Fe^{2+} promoted a 38% increase in such an activity, Zn^{2+}, Cu^{2+} and Co^{2+} reduced it by approximately 25%, while the other ions exerted no effect or only a weak negative effect. The inhibitory effect of some divalent metal ions has also been reported for β-galactosidases from *Aspergillus carbonarius*[54] and *Aspergillus alliaceus*.[55]

The effect of some additives and detergents on the hydrolytic activity of *C. tenuissimum* URM 7803 β-galactosidase was also investigated. It can be seen in Figure 6 that such an activity was improved or not appreciably influenced by all the compounds tested but PMSF, a well-known serine protease inhibitor, which reduced it by around 20 and 12% in the crude extract and the ATPS extract, respectively.

These results suggest a possible inhibition of different proteolytic enzymes present in the crude extract, other than serine proteases, which improved the stability of β-galactosidase.

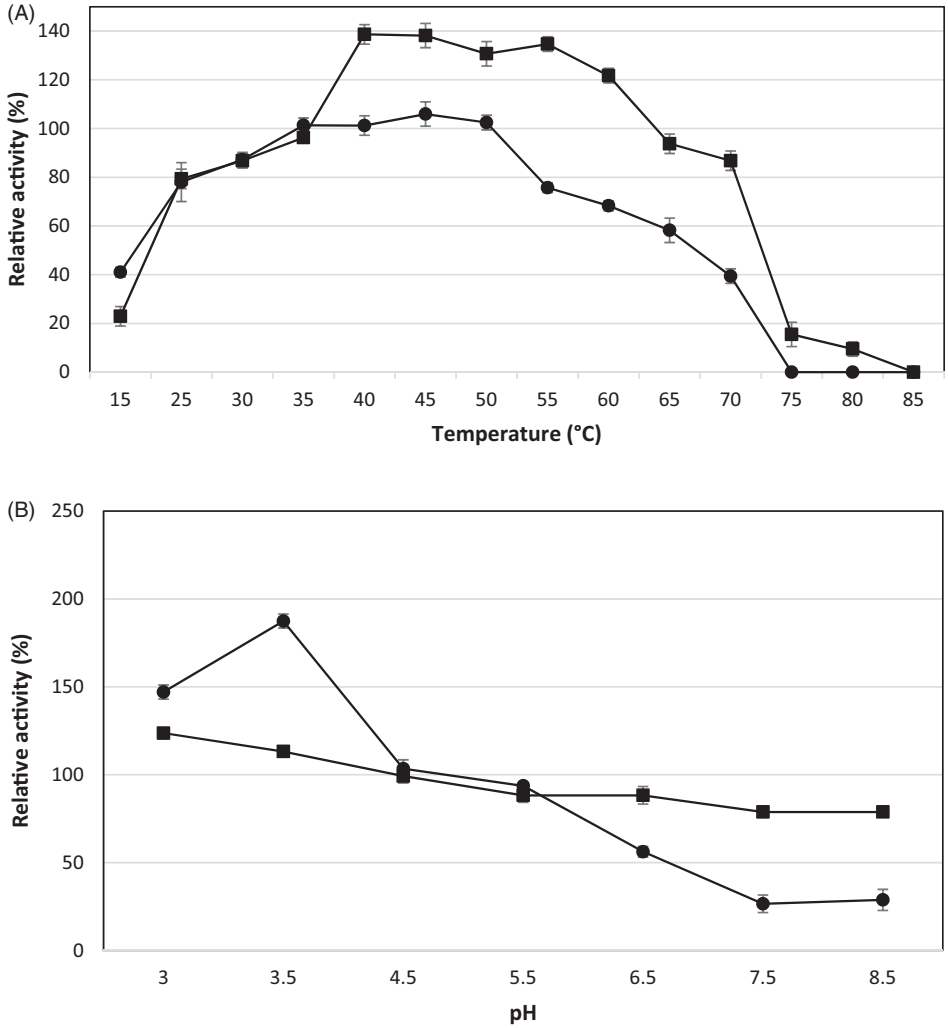

Figure 4. Effect of (A) temperature and (B) pH on the relative hydrolytic activity of β-galactosidase from *Cladosporium tenuissimum* URM 7803 in the crude extract (●) and the extract partially purified by ATPS in run 4 (*M*PEG of 8.000 g/mol, *C*CIT of 15%, *C*PEG of 24%, pH 6.0) (■), expressed as percentage of the maximum activity observed in the crude extract at 37 °C and pH 4.5.

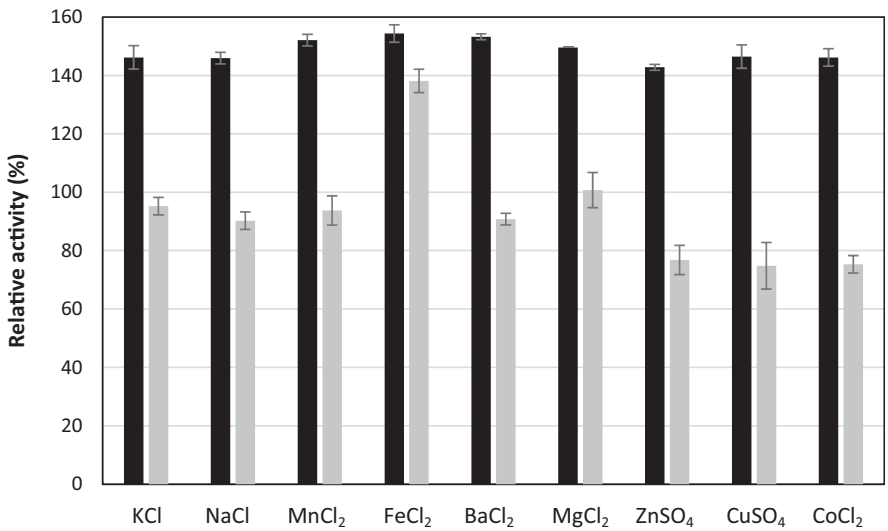

Figure 5. Effect of the addition of different salts on the relative hydrolytic activity of β-galactosidase from *Cladosporium tenuissimum* URM 7803 in the crude extract (■) and the extract partially purified by ATPS (■), expressed as percentage of the maximum activity observed 37 °C and pH 4.5 in the crude extract without any salt.

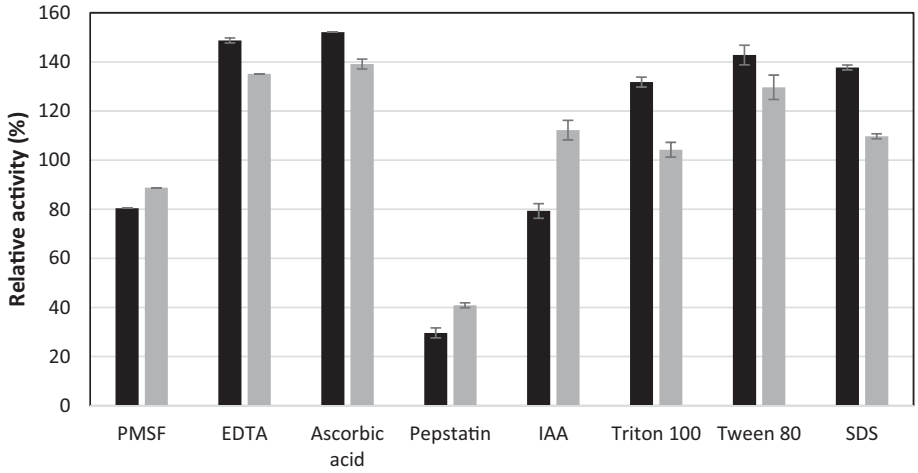

Figure 6. Effect of the addition of different inhibitors or detergents on the relative hydrolytic activity of *β*-galactosidase from *Cladosporium tenuissimum* URM 7803 in the crude extract (■) and the extract partially purified by ATPS (■), expressed as percentage of the maximum activity observed in the crude extract at 37 °C and pH 4.5.

The addition of EDTA, a well-known chelating agent, led to activity increases as high as 48 and 35% in the crude and ATPS extracts, respectively. A combined evaluation of this effect with those of metal ions suggests that *C. tenuissimum* URM 7803 β-galactosidase did not require metal ions for its hydrolytic activity,[54] except for Fe^{2+} that promoted it mainly in the ATPS extract likely due to enzyme complexation/stabilization.

As far as the addition of detergents is concerned, SDS, being a protein denaturing anionic agent,[56] was expected to exert a negative effect on β-galactosidase activity; however, the observed 37% activity increase in the crude extract and 10% increase in the ATPS one indicate that higher concentrations of SDS would be required to induce β-galactosidase denaturation, while some stabilization may have occurred at the low concentration used in this study (10 mM). The addition of the non-anionic detergents Triton X-100 and Tween 80 led to activity increases in the crude extract by no less than 31 and 42%, respectively, but only by 4 and 29% in the ATPS one. This effect may have occurred because nonionic surfactants generally do not cause enzyme inactivation or denaturation.[57]

Conclusions

The *C. tenuissimum* URM 7803 isolate used in this study, which had its species confirmed through the elongation factor 1-α sequencing, proved to be a novel β-galactosidase producer with potential application in the biotech industry. Preliminary extraction and purification of extracellularly produced β-galactosidase was attempted using PEG/citrate aqueous two-phase system (ATPS). Runs carried out using the highest PEG molar mass (8000 g/mol) and lowest pH of citrate solution (6.0) allowed obtaining the best combination of results for the extraction parameters. Under these conditions, the enzyme partitioned to the salt-rich phase, hence making its posterior recovery easier. Partial characterization of β-galactosidase revealed an optimum temperature in the range of 35–50 °C in the fermented broth and 40–55 °C in that submitted to PEG/citrate ATPS, while optimum pH was found to range from 3.0 to 4.5 in both cases, hence behaving as an acidophilic enzyme. Most metallic ions had a positive effect on the enzyme hydrolytic activity in the crude extract, but only Fe^{2+} was able to increase it by 38% in the ATPS extract, whereas Zn^{2+}, Cu^{2+} and Co^{2+} reduced it by around 25%. Surprisingly, the addition of most detergents and well-known inhibitors led to an increase in the relative β-galactosidase activity, suggesting destabilization or inhibition of proteolytic enzymes likely present in the crude extract. The only exception was PMSF that reduced it by 20 and 12% in crude and ATPS extracts, respectively. These results taken together reveal a promising profile for this novel β-galactosidase, which may find a lot of different applications in the food industry sector, among which the synthesis of prebiotics such as galacto-oligosaccharides or the production of lactose-free foods. One of the next efforts will concern the full characterization of this enzyme.

Disclosure statement

The authors are aware of the ethical responsibilities, and the manuscript has no conflict of interest.

Funding

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Process n.: 407814/2013-2), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-Process n.: 88887.163442/2018-00) and Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE-Process n.: BFP-0163-5.01/18).

ORCID

Willie Anderson dos Santos Vieira http://orcid.org/0000-0003-4158-7453
Luiz Carlos Alves http://orcid.org/0000-0002-8384-3008
Ana Lúcia Figueiredo Porto http://orcid.org/0000-0001-5561-5158

References

[1] Awan, M. S.; Khan, S. A.; Rehman, Z. U.; Saleem, A.; Rana, S. M. Influence of Nitrogen Sources on Production of β-Galactosidase by *Aspergillus niger*. *Afr. J. Biotechnol.* **2010**, *9*, 2918–2922. http://www.academicjournals.org/AJB.

[2] Gekas, V.; López-Leiva, M. Hydrolysis of Lactose: A Literature Review. *Process Biochem.* **1985**, *20*, 2–12.

[3] Asraf, S. S.; Gunasekaran, P. Current Trends of β-Galactosidase Research and Application. In *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*. 2nd ed.; Méndez-Vilas, A., Ed.; Formatex Research Center: Bajadoz, Spain, 2010; pp 880–890. https://pdfs.semanticscholar.org/403a/7de56b33d40422cf25186125c8eb2df278e3.pdf.

[4] Panesar, P. S.; Kumari, S.; Panesar, R. Potential Applications of Immobilized β-Galactosidase in Food Processing Industries. *Enzyme Res.* **2010**, *2010*, 473137–473116. DOI: 10.4061/2010/473137.

[5] Hsu, C. A.; Yu, R. C.; Chou, C. C. Production of Beta-galactosidase by Bifidobacteria as Influenced by Various Culture Conditions. *Int. J. Food Microbiol.* **2005**, *104*, 197–206. DOI: 10.1016/j.ijfoodmicro.2005.02.010.

[6] Park, A. R.; Oh, D. K. Galacto-Oligosaccharide Production Using Microbial Beta-Galactosidase: Current State and Perspectives. *Appl. Microbiol. Biotechnol.* **2010**, *85*, 1279–1286. DOI: 10.1007/s00253-009-2356-2.

[7] Oliveira, C.; Guimarães, P. M.; Domingues, L. Recombinant Microbial Systems for Improved β-Galactosidase Production and Biotechnological Applications. *Biotechnol. Adv.* **2011**, *29*, 600–609. DOI: 10.1016/j.biotechadv.2011.03.008.

[8] Isobe, K.; Takahashi, N.; Chiba, S.; Yamashita, M.; Koyama, T. Acidophilic Fungus, *Teratosphaeria Acidotherma* AIU BGA-1, Produces Multiple Forms of Intracellular β-Galactosidase. *J. Biosci. Bioeng.* **2013**, *116*, 171–174. DOI: 10.1016/j.jbiosc.2013.02.018.

[9] Raol, G. G.; Prajapati, V. S.; Raol, B. V. Formulation of Low-Cost, Lactose-Free Production Medium by Response Surface Methodology for the Production of β-Galactosidase Using Halotolerant *Aspergillus Tubengensis* GR-1. *Biocatal. Agric. Biotechnol.* **2014**, *3*, 181–187. DOI: 10.1016/j.bcab.2014.02.007.

[10] Kamran, A.; Bibi, Z.; Aman, A.; Ul Qader, S. A. Hyper Production of β-Galactosidase from Newly Isolated Strain of *Aspergillus nidulans*. *J. Food Process Eng.* **2017**, *40*, e12452. DOI: 10.1111/jfpe.12452.

[11] Cardoso, B. B.; Silvério, S. C.; Abrunhosa, L.; Teixeira, J. A.; Rodrigues, L. R. β-Galactosidase from *Aspergillus Lacticoffeatus*: A Promising Biocatalyst for the Synthesis of Novel Prebiotics. *Int. J. Food Microbiol.* **2017**, *257*, 67–74. DOI: 10.1016/j.ijfoodmicro.2017.06.013.

[12] Ke, Q.; Fulmer, P.; Mizutani, A. Toxicological Evaluation of β-Galactosidase Enzyme Produced by *Papiliotrema Terrestris*. *Regul. Toxicol. Pharmacol.* **2018**, *92*, 213–219. DOI: 10.1016/j.yrtph.2017.12.002.

[13] Dugan, F. M.; Schubert, K.; Braun, U. Check-List of Cladosporium names. *Schlechtendalia* **2004**, *11*, 1–103. https://sundoc.bibliothek.uni-halle.de/diss-online/05/05H208/checklist.pdf.

[14] Al Matar, M.; Makky, E. A. *Cladosporium cladosporioides* from the Perspectives of Medical and Biotechnological Approaches. *3 Biotech* **2016**, *6*, 4. DOI: 10.1007/s13205-015-0323-4.

[15] Li, L.; Tian, H.; Cheng, Y.; Jiang, Z.; Yang, S. Purification and Characterization of a Thermostable Cellulase-Free Xylanase from the Newly Isolated *Paecilomyces Thermophila*. *Enzyme Microb Technol* **2006**, *38*, 780–787. DOI: 10.1016/j.enzmictec.2005.08.007.

[16] Wu, Q.; Li, C.; Li, C.; Chen, H.; Shuliang, L. Purification and Characterization of a Novel Collagenase from *Bacillus pumilus* Col-J. *Appl. Biochem. Biotechnol.* **2010**, *160*, 129–139. DOI: 10.1007/s12010-009-8673-1.

[17] Silva, O. S.; Gomes, M. H. G.; Oliveira, R. L.; Porto, A. L. F.; Converti, A.; Porto, T. S. Partitioning and Extraction Protease from *Aspergillus Tamari* URM4634 Using PEG-Citrate Aqueous Two-Phase Systems. *Biocatal. Agric. Biotechnol.* **2017**, *9*, 168–173. DOI: 10.1016/j.bcab.2016.12.012.

[18] Porto, T. S.; Silva, G. M. M.; Porto, C. S.; Cavalcanti, M. T. H.; Neto, B.B.; Lima-Filho, J.L.; Converti, A.; Porto, A.L.F.; Pessoa Jr, A. Liquid–Liquid Extraction of Proteases from Fermented Broth by PEG/Citrate Aqueous Two-Phase System. *Chem. Eng. Process* **2008**, *47*, 716–721. DOI: 10.1016/j.cep.2006.12.004.

[19] Duarte, A.W.F.; Lopes, A.M.; Molino, J.V.D.; Pessoa, A., Jr; Sette, L.D. Liquid–Liquid Extraction of Lipase Produced by Psychrotrophic Yeast *Leucosporidium Scottii* L117 Using Aqueous Two-Phase Systems. *Sep. Purif. Technol.* **2015**, *156*, 215–225. DOI: 10.1016/j.cep.2006.12.004.

[20] Khayati, G.; Anvari, M.; Shahidi, N. Partitioning of β-Galactosidase in Aqueous Two-Phase Systems Containing Polyethyleneglycol and Phosphate Salts. *Fluid Phase Equilibr.* **2015**, *385*, 147–152. DOI: 10.1016/j.fluid.2014.11.003.

[21] Malhotra, G.; Jeshreena, R.; Chapadgaonkar, S.S. Aqueous Two Phase Purification of Xylanase Obtained from a Bacterial Isolate. *Int. J. Pharma Bio. Sci.* **2016**, *7*, 305–308. DOI: 10.22376/ijpbs.2016.7.4.b305-308.

[22] Rosso, B.U.; Lima, C.D.A.; Porto, T.S.; Nascimento, C.O.; Pessoa, A., Jr, Converti, A.; Carneiro-da-Cunha, M.D.G.; Porto, A.L.F. Partitioning and Extraction of Collagenase from *Penicillium Aurantiogriseum* in Poly(Ethylene Glycol)/Phosphateaqueous Two-Phase System. *Fluid Phase Equilibr.* **2012**, *335*, 20–25. DOI: 10.1016/j.fluid.2012.05.030.

[23] Pimentel, M.C.B.; Araújo, A.I.; Figueiredo, Z.M.B.; Silva, R.A.; Cavalcanti, M.T.H.; Moreira, K.A.; Filho, J.L.L.; Porto, A.L.F. Aqueous Two-Phase System for Citrinin Extraction from Fermentation Broth. *Sep. Purif. Technol.* **2013**, *110*, 158–163. DOI: 10.1016/j.seppur.2013.03.021.

[24] Ramakrishnan, V.; Govea, L.C.; Suralikerimath, N.; Jampani, C.; Halami, P.M.; Narayan, B. Extraction and Purification of Lipase from *Enterococcus faecium* MTCC5695 by PEG/Phosphate Aqueous-Two Phase System (ATPS) and Its Biochemical Characterization. *Biocatal Agric Biotechnol* **2016**, *6*, 19–27. DOI: 10.1016/j.bcab.2016.02.005.

[25] Jiang, B.; Wang, L.; Na, J.; Zhang, X.; Yuan, Y.; Liu, C.; Feng, Z. Environmentally-Friendly Strategy for Separation of α-Lactalbumin from Whey by Aqueous Two Phase Flotation. *Arab J Chem.* **2020**, *13*, 3391–3402. DOI: 10.1016/j.arabjc.2018.11.013.

[26] Shinomiya, K.; Kobayashi, H.; Inokuchi, N.; Nakagomi, K.; Ito, Y. Partition Efficiency of High-Pitch Locular Multilayer Coil for Countercurrent Chromatographic Separation of Proteins Using Small-Scale Cross-Axis Coil Planet Centrifuge and Application to Purification of Various Collagenases with Aqueous-Aqueous Polymer Phase Systems. *J. Liq. Chromatogr. Relat. Technol.* **2011**, *34*, 182–194. DOI: 10.1080/10826076.2011.546151.

[27] Wanderley, M.C.A.; Duarte Neto, J.M.W.; Albuquerque, W.W.C.; Viana Marques, D.A.; Lima, C.A.; Silvério, S.I.C.; Filho, J.L.L.; Teixeira, J.A.C.; Porto, A.L.F. Purification and Characterization of a Collagenase from *Penicillium* sp. UCP 1286 by Polyethylene Glycol-Phosphate Aqueous Two-Phase System. *Protein Expr. Purif.* **2017**, *133*, 8–14. DOI: 10.1016/j.pep.2017.02.010.

[28] Wanderley, M.C.A.; Duarte Neto, J.M.W.; Andrade, A.F.; Melo, R.G.; Viana-Marques, D.A.; Bezerra, R.P.; Porto, A.L.F. First Report on *Chlorella Vulgaris* Collagenase Production and Purification by Aqueous Two-Phase System. *Sustain Chem Pharm.* **2020**, *15*, 100202. DOI: 10.1016/j.scp.2019.100202.

[29] Jiang, B.; Na, J.; Wang, L.; Li, D.; Liu, C.; Feng, Z. Reutilization of Food Waste: one-Step Extraction, Purification and Characterization of Ovalbumin from Salted Egg White by Aqueous Two-Phase Flotation. *Foods* **2019**, *8*, 286. DOI: 10.3390/foods8080286.

[30] Jiang, B.; Wang, X.; Wang, L.; Wu, S.; Li, D.; Liu, C.; Feng, Z. Fabrication and Characterization of a Microemulsion Stabilized by Integrated Phosvitin and Gallic Acid. *J. Agric. Food Chem.* **2020**, *68*, 5437–5447. DOI: 10.1021/acs.jafc.0c00945.

[31] Jiang, B.; Na, J.; Wang, L.; Li, D.; Liu, C.; Feng, Z. and Enrichment of Antioxidant Peptides from Whey Protein Isolate Hydrolysate by Aqueous Two-Phase Extraction and Aqueous Two-Phase Flotation. *Foods* **2019**, *8*, 34. DOI: 10.3390/foods8010034.

[32] Hemavathi, A.B.; Raghavarao, K.S.M.S. Differential Partitioning of β-Galactosidase and β-Glucosidase Using Aqueous Two Phase Extraction. *Process Biochem.* **2011**, *46*, 649–655. DOI: 10.1016/j.procbio.2010.11.008.

[33] Lemes, A.C.; Machado, J.R.; Brites, M.L.; Di Luccio, M.; Kalil, S.J. Design Strategies for Integrated β-Galactosidase Purification Processes. *Chem. Eng. Technol.* **2014**, *37*, 1805–1812. DOI: 10.1002/ceat.201300433.

[34] Góes-Neto, A.; Loguercio-Leite, C.; Guerrero, R. T. DNA extraction from frozen field-collected and dehydrated herbarium fungal basidiomata: performance of SDS and CTAB-based methods. *Biotemas* **2005**, *18*, 19–32. https://pdfs.semanticscholar.org/45a3/7c85a5b607dab54c86dbeb605705877285b1.pdf.

[35] Carbone, I.; Kohn, L.M. A Method for Designing Primer Sets for Speciation Studies in Filamentous Ascomycetes. *Mycologia* **1999**, *91*, 553–556. DOI: 10.1080/00275514.1999.12061051.

[36] Staden, R.; Beal, K.F.; Bonfield, J.K. The Staden Package, 1998. *Methods Mol. Biol.* **2000**, *132*, 115–130. DOI: 10.1385/1-59259-192-2:115.

[37] Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. DOI: 10.1093/molbev/msw054.

[38] Guindon, S.; Gascuel, O. A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. *Syst. Biol.* **2003**, *52*, 696–704. DOI: 10.1080/10635150390235520.

[39] Dereeper, A.; Guignon, V.; Blanc, G.; Audic, S.; Buffet, S.; Chevenet, F.; Dufayard, J.F.; Guindon, S.; Lefort, V.; Lescot, M.; et al. fr: Robust Phylogenetic Analysis for the Non-Specialist. *Nucleic Acids Res.* **2008**, *36*, 465–469. DOI: 10.1093/nar/gkn180.

[40] Nagy, Z.; Kiss, T.; Szentirmai, A.; Biró, S. Beta-Galactosidase of *Penicillium chrysogenum*: Production, Purification, and Characterization of the Enzyme . *Protein Expr. Purif.* **2001**, *21*, 24–29. DOI: 10.1006/prep.2000.1344.

[41] Smith, P.K.; Krohn, R.I.; Hermanson, G.T.; Mallia, A.K.; Gartner, F.H.; Provenzano, M.D.; Fujimoto, E.K.; Goeke, N.M.; Olson, B.J.; Klenk, D.C. Measurement of Protein Using Bicinchoninic Acid. *Anal. Biochem.* **1985**, *150*, 76–85. DOI: 10.1016/0003-2697(85)90442-7.

[42] Vieira, G.R.T.; Liebl, M.; Tavares, L.B.B.; Paulert, R.; Júnior, A.S. Submerged Culture Conditions for the Production of Mycelial Biomass and Antimicrobial Metabolites by *Polyporus Tricholoma* Mont. *Braz. J. Microbiol.* **2008**, *39*, 561–568. DOI: 10.1590/S1517-83822008000300029.

[43] Nath, A.; Datta, S.; Chowdhury, R.; Bhattacharjee, C. Fermentative Production of Intracellular β-Galactosidase by *Bacillus safensis* (JUCHE1) Growing on Lactose and Glucose-Modeling and Experimental. *Biocatal. Agric. Biotechnol.* **2014**, *3*, 246–258. DOI: 10.1016/j.bcab.2014.06.003.

[44] Tamsikar, J.; Naidu, J.; Singh, S.M. *Phaeohyphomycotic Sebaceous* Cyst Due to *Cladosporium Cladosporidioides*: Case Report and Review of Literature. *J. Med. Mycol.* **2006**, *16*, 55–57. DOI: 10.1016/j.mycmed.2005.12.002.

[45] Menezes, C.P.; Pérez, A.L.A.L.; Oliveira, E.L. *Cladosporium* Spp: Morfologia, Infecções e Espécies Patogênicas. *Acta Bra.* **2017**, *1*, 23–27. DOI: 10.22571/Actabra1120176.

[46] Bensch, K.; Braun, U.; Groenewald, J.Z.; Crous, P.W. The Genus *Cladosporium*. *Stud. Mycol.* **2012**, *72*, 1–401. DOI: 10.3114/sim0003.

[47] Lima, C.A.; Freitas Júnior, A.C.V.; Lima Filho, J.L.; Converti, A.; Viana Marques, D.A.; Carneiro-da-Cunha, M.G.; Porto, A.L.F. Two-Phase Partitioning and Partial Characterization of a Collagenase from *Penicillium Aurantiogriseum* URM4622: Application to Collagen Hydrolysis. *Biochem. Eng. J.* **2013**, *75*, 64–71. DOI: 10.1016/j.bej.2013.03.012.

[48] Mayerhoff, Z.D.V.L.; Roberto, C.; Franco, T.T. Purification of Xylose Reductase from *Candida Mogii* in Aqueous Two-Phase Systems. *Biochem. Eng. J.* **2004**, *18*, 217–223. DOI: 10.1016/j.bej.2003.09.003.

[49] Babu, B. R.; Rastogi, N.K.; Raghavarao, K.S.M.S. Liquid-Liquid Extraction of Bromelain and Polyphenol Oxidase Using Aqueous Two-Phase System. *Chem. Eng. Process* **2008**, *47*, 83–89. DOI: 10.1016/j.cep.2007.08.006.

[50] Silva, M.E.; Franco, T.T. Purification of Soybean Peroxidase (Glycine max), by Metal Affinity Partitioning in Aqueous Two-Phase Systems. *J Chromatogr. B* **2000**, *743*, 287–294. DOI: 10.1016/S0378-4347(00)00172-9.

[51] Herculano, P.N.; Porto, T.S.; Maciel, M.H.C.; Moreira, K.A.; Souza-Motta, C.M.; Porto, A.L.F. Partitioning and Purification of the Cellulolytic Complex Produced by *Aspergillus Japonicus* URM5620 Using PEG–Citrate in an Aqueous Two-Phase System. *Fluid Phase Equilib.* **2012**, *335*, 8–13. DOI: 10.1016/j.fluid.2012.08.008.

[52] Liu, Z.; Zhao, C.; Deng, Y.; Huang, Y.; Liu, B. Characterization of a Thermostable Recombinant β-Galactosidase from Thermophilic Anaerobic Bacterial Consortium YTY70. *Biotechnol. Biotechnol. Equip.* **2015**, *29*, 547–554. DOI: 10.1080/13102818.2015.1015244.

[53] Mlichová, Z.; Rosenberg, M. Current Trends of β-Galactosidase Application in Food Technology. *J. Food Nutr. Res.* **2006**, *45*, 47–54.

[54] El-Gindy, A. Production, Partial Purification and Some Properties of Beta-Galactosidase from *Aspergillus Carbonarius*. *Folia Microbiol. (Praha)* **2003**, *48*, 581–584. DOI: 10.1007/BF02993462.

[55] Sen, S.; Ray, L.; Chattopadhyay, P. Production, Purification, Immobilization, and Characterization of a Thermostable β-Galactosidase from *Aspergillus alliaceus*. *Appl. Biochem. Biotechnol.* **2012**, *167*, 1938–1953. DOI: 10.1007/s12010-012-9732-6.

[56] Bhuyan, A.K. On the Mechanism of SDS-Induced Protein Denaturation. *Biopolymers* **2010**, *93*, 186–199. DOI: 10.1002/bip.21318.

[57] Zappone, M.; Kaziska, A.; Bogush, G. *Application of Detergents in Laundering, Handbook of Detergents, Part E: Applications*. CRC Press, Boca Raton, 2009; pp 69–82. DOI: 10.1201/9781420018165-10.