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ORIGINAL PAPER

A novel α-L-rhamnosidase with potential applications in citrus juice industry and in winemaking

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Abstract The production of monoglycosylated flavonoids by α-L-rhamnosidases (EC 3.2.1.40) is an interesting development in biocatalysis. Applications of rhamnosidases in industry include removal of bitterness caused by naringin from citrus juices. In the present work, a psychrotolerant bacterial strain with α-L-rhamnosidase activity was isolated. The α-L-rhamnosidase was found to be able to degrade naringin and was purified and characterized. The α-L-rhamnosidase from *Brevundimonas* sp. Ci19 was able to release both rhamnose and prunin from naringin. The enzyme was partially purified with a performance of 2.7-fold purification. The α-L-rhamnosidase showed an optimum pH between 6.00 and 7.00 with substantial residual activity at pH 5.00 (85.3 %). The optimum temperature was between 20 and 37 °C. The enzyme showed activation in the presence of Ca^{2+} and Cd^{2+} ions and at a high ethanol concentration level (10 % v/v). Activity was found for β-D-glucosidase (EC 3.2.1.21) in the partially purified extract, but it was inactive in the acid pH region. This result indicates the potential for inactivation of β-D-glucosidase along with the high level of α-L-rhamnosidase activity necessary for the production of flavonoid glycosides. The α-L-rhamnosidase from *Brevundimonas* sp. Ci19 showed interesting properties for potential use not only in the citrus juice industry but also in winemaking.

Keywords α-L-rhamnosidase · β-D-glucosidase · *Brevundimonas* sp · Naringin · Purification

This work is dedicated in memoriam to Prof. Carlos M. Abate, colleague and friend.

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Introduction

The production of monoglycosylated flavonoids by rhamnosidases (EC 3.2.1.40) is an interesting application in the field of enzymatic biocatalysis in relation to removal of rhamnose. Recently, application of rhamnosidases for improving the bioavailability of flavonoids has also been described [1]. Flavonoids show a wide range of beneficial effects for human health, including those related to cardiovascular and chronic diseases and certain forms of cancer. They have also been shown to have antimicrobial, antioxidant, antiviral, anti-ischaemic, antitumor, anti-inflammatory, antiallergic, oestrogenic, and radical-scavenging properties [2]. In food processing, rhamnosidases have been applied primarily for debittering of citrus juices. Bitterness in citrus is partly caused by the glycoside flavonoid naringin, which loses its bitter taste upon removal of the rhamnose [3]. This type of research has significant economic relevance in Argentina, where 41 % of the world's lemon production is based as well as 83 % of the industrialized lemon processing in the southern hemisphere [4]. Naringin also has beneficial biological properties such as antioxidant activity, the ability to lower blood lipid levels, anticancer activity, and

inhibition of selected drug-metabolizing cytochromes P450 [5].

In addition to reducing the bitter taste of citrus fruit juices, rhamnosidases are also used for enhancing wine aromas because of their ability to release volatile terpenes bound to a sugar moiety [3, 6]. However, a significant challenge for enzyme technology is finding a biological catalyst that can be used as a process biocatalyst at the industrial level [7]. A number of mammalian tissues, plants, bacteria, and fungi are able to produce α-L-rhamnosidases [8]. However, in terms of the α-L-rhamnosidases produced by bacteria, there has been little research reported to date on psychrotolerant microorganisms with α-L-rhamnosidase activity [6].

Over the course of evolution, microorganisms have developed many strategies to survive and reproduce within a myriad of niches, including under extreme environmental conditions. The isolation of new forms of life allows novel strategies and mechanisms to be identified, providing new insights not only for our basic knowledge of cell biology but also for the use of microorganisms in biotechnological applications. Marine biotechnology has revealed numerous novel biocatalysts with properties including high salt tolerance, hyperthermostability, barophilicity, cold adaptivity, and suitability for large-scale cultivation [9]. The microorganisms isolated from seawater habitats and attracting scientific interest include members of the genus *Brevundimonas* [10], although there is still little known about enzyme production in this taxon.

The present work reports on the isolation, from the Beagle Channel in the Antarctic Sea, of a psychrotolerant bacterium with α-L-rhamnosidase activity, characterized as *Brevundimonas* sp. Ci19. The enzyme produced has shown naringin hydrolysis capability and has been partially purified and characterized. The enzymatic activity of the α-L-rhamnosidase was studied in the presence of metabisulphite, ethanol, and glucose, environmental conditions commonly encountered in winemaking.

Materials and methods

Chemicals

Commercially available rhamnose, 4-nitrophenyl-α-L-rhamnopyranoside (4-NRP), 4-methylumbelliferyl-α-L-rhamnopyranoside, and 4-nitrophenyl-β-D-glucopyranoside (4-NGP) were obtained from Sigma–Aldrich (St. Louis, USA), and naringin, prunin, and naringenin were obtained from Prof. Mirta Daz at the Universidad Nacional de Salta, Argentina. Acrylamide, high molecular weight markers, and supplies for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) were purchased from Bio-Rad (Bio-Rad Laboratories, Hercules, California, USA), and Sephadex G-100 was purchased from GE Healthcare Life Sciences (Pittsburgh, USA). All other chemicals used were of analytical grade.

Microbial source and culture conditions

The microorganisms studied were isolated from seawater samples collected off the coast of Tierra del Fuego near the Argentinean city of Ushuaia. Selection of α-L-rhamnosidase-producing microorganisms was performed on plates with Brunner mineral medium (BMM). Qualitative evaluation of α-L-rhamnosidase activity was performed in Petri dishes supplemented with 4-methylumbelliferyl-α-L-rhamnopyranoside (10 mM), at 4 and 20 °C for 120 and 72 h, respectively. Positive colonies showed fluorogenic characteristics clearly distinguishable by cleavage of the free 4-methylumbelliferone moiety when exposed to long-wave UV radiation. Positive strains were selected for quantitative assaying [11].

The enriched medium contained (g l^{-1}) rhamnose, 5.0; yeast extract, 2.0; milk peptone, 5.0; NaCl, 20.0; and $MgSO_4.7H_2O$, 0.1. The medium for selection and enzyme production contained (g l^{-1}) rhamnose or naringin, 5.0; peptone, 1.0; yeast extract, 0.5; NaCl, 20.0; $MgSO_4.7H_2O$, 0.1; and agar, 15 (pH 6.50). The isolates were cultured in 1.0-l flasks containing 300 ml of the medium in an orbital shaker (250 rpm) at 20 °C for 36 h. All strains with development in the medium for selection were selected for further study.

Characterization and identification of isolates

Total DNA from the selected isolates was prepared according to the method described by Hoffman et al. [12]. Universal primers with specificity to eubacterial 16S rDNA genes (forward primer 27F and reverse primer 1492R) were used to amplify 16S rDNA. Sequences belonging to the same genus or validly published closely related species available in public databases (GenBank and RDP II) were aligned, and phylogenetic and molecular evolutionary analyses based upon 16S rDNA were inferred by the neighbour-joining method [13] using Kimura's evolutionary distance [14]. Phylogenetic trees were constructed using MEGA 5 software, and the bootstrap consensus tree was inferred from 1,000 replicates [15] to represent the evolutionary history of the analysed taxa.

Scanning electron microscope images of *Brevundimonas* sp. Ci19 were prepared by sputter-coating the surface with gold (Balzers SCD 030 metalizer), obtaining a layer thickness from 15 to 20 nm. Microsphere surfaces and morphologies were observed using a Philips 505 SEM at the Centro Integral de Microscopia Electronica, INSIBIO-CONICET, Universidad Nacional de Tucumán.

Enzyme determination

α-L-rhamnosidase activity

Hydrolysis activity of α-L-rhamnosidase on an artificial substrate: 4-NPR For quantification of the α-L-rhamnosidase activity, each reaction was performed using 15 µl of substrate (100 mmol l^{-1} of 4-NPR in dimethylformamide), 900 µl of buffer (100 mmol l^{-1} McIlvaine buffer, pH 5.00), and 100 µl of cell-free extract (enzyme). The reaction was performed for 1 h at 20 °C and was stopped by adding 100 µl of NaOH (100 mmol l^{-1}). One enzyme unit was defined as the amount of enzyme that released 1 µmol of 4-NP in 1 h at the indicated temperature [6].

Hydrolysis activity of the α-L-rhamnosidase on a natural naringin substrate For enzymatic hydrolysis, the intracellular soluble fraction was placed in a Centricon filter unit (50,000 MWCO) and centrifuged, and then 100 µl was incubated with 900 µl of naringin solution (0.5 mg ml^{-1} of naringin solubilized in McIlvaine buffer, pH 4.00 − 6.00). This reaction was performed at 20 °C for time periods from 1 to 12 h. One enzyme unit was defined as the amount of enzyme required to hydrolyse 1 µmol of naringin per ml per hour.

For sample preparation and HPLC analysis, 1 ml of hydrolysed sample was mixed with 1 ml of 6 % TCA. The mixture was centrifuged (10 min at 11,000×*g*), and the supernatant was placed in a 0.45-µm membrane for filtration. The analyses were performed using a high-performance liquid chromatographic system (HPLC, Gilson, Inc.) consisting of a solvent delivery pump (Model 305), a variable-wavelength UV–Vis detector (Model 118), a manual injector with a 100-µl loop (Rheodine 7125), and an integrator (Sykam, Model CR-3A). Separation was performed on a C18 analytical column (Spherisorb, 250 × 4.6 mm I.D., 5 µm particle size). The mobile phase consisted of acetonitrile/water (30:70, v/v). The HPLC system was operated with isocratic elution at a flow rate of 0.8 ml/min at room temperature, and the detector was set at 280 nm. The identification of flavonoid glycosides naringin, prunin, and naringenin was performed by comparing their retention times and UV spectra to those of known standards.

β-Glucosidase activity

The β-glucosidase activity was determined in a vial containing 15 µl of substrate (100 mmol l^{-1} of 4-NGP in dimethylformamide), 900 µl of buffer (100 mmol l^{-1} McIlvaine buffer), and 100 µl of cell-free extract or supernatant (enzyme). The reaction was performed for 1 h at 20 °C and was stopped by adding 100 µl of sodium hydroxide (100 mol l^{-1}). One unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol of 4-nitrophenol per minute under the assayed conditions. All enzymatic measurements were taken in triplicate.

Protein determination

Protein content was measured using Lowry's method [16] with bovine serum albumin (fraction V) as a standard. The specific enzyme activity was determined as U mg^{-1} of protein.

Extraction and partial purification of the α-L-rhamnosidase enzyme

Obtaining the supernatant

Batch cultures of microbial cells were collected by centrifugation (10 min at 12,000×*g*) and then suspended in 5 ml of sterile solution (154 mM NaCl and 100 µM PMSF) followed by cell disruption through a French press at 25,000 psi. This suspension was then centrifuged (10 min at 12,000×*g*) to eliminate cell debris, and α-L-rhamnosidase and β-glucosidase activities were evaluated in the intracellular soluble fraction.

Purification of the α-L-rhamnosidase enzyme

The supernatant obtained after cell disruption was placed in a Centricon filter unit (50,000 MWCO) and centrifuged. The concentrated sample was applied to a Sephadex G-100 column (35.0 cm length, 2.0 cm diameter) previously equilibrated with 50 mM phosphate-buffered saline (PBS, pH 7.00) containing 150 mM sodium chloride. Elution was performed at a flow rate of 0.5 ml min^{-1} (50 mM PBS). Fractions (0.5 ml) corresponding to rhamnosidase activity were collected, and their purity was checked using SDS–PAGE according to Laemmli's method [17]. Protein content was measured using Lowry's method as cited above.

Effect of pH and temperature on enzyme activities

The optimum temperature for α-L-rhamnosidase and β-glucosidase activities was determined by assaying the enzyme at a range of temperatures (8–40 °C), at the optimum pH, and using the techniques described above. The optimum pH was determined by incubating each enzyme preparation with 4-NPR or 4-NGP substrates in McIlvaine buffers at pH 4.00–6.00 and in PBS at pH 7.00–8.72.

The effects of several reagents on α-L-rhamnosidase activity

The effects of several divalent cations (Cu^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+}, and Mg^{2+}) on α-L-rhamnosidase activity

were investigated by their incorporation into the standard assay at 10 mM. The effects of sodium metabisulphite, glucose, and ethanol on α-L-rhamnosidase activity were also investigated, at concentrations ranging from 0 to 250 mg l^{-1}, 0–200 g l^{-1}, and 0–10 % (v/v), respectively. The α-L-rhamnosidase activity was determined under each set of conditions using the enzyme determination methods described above.

Statistical analysis

Statistical analysis was performed using Infostat (2008) and Minitab 1.4 statistical experiment design software. Results are presented as the mean ± standard deviation. Statistical significance values for the means were evaluated using one-way analysis of variance (ANOVA), and subsequent comparisons were performed using Tukey's post hoc test or Fisher's least significant difference (LSD) test. Differences were accepted as significant when $P < 0.05$.

Results and discussion

Isolation, selection, and identification of microorganisms with rhamnosidase activity

Twenty-three microorganisms from seawater samples from sub-Antarctic marine ecosystems were isolated in a liquid medium, using rhamnose as the carbon source. Indigenous colonies belonging to the *Alphaproteobacteria*, *Gammaproteobacteria*, and *Actinobacteria* groups were isolated and labelled as Ci1 to Ci23. Six strains were selected on a solid medium by specific hydrolysis of 4-methylumbelliferyl-α-L-rhamnopyranoside, and intracellular rhamnosidase activity from these strains was quantitatively determined by 4-NPR hydrolysis. Specific activity values from these determinations are shown in Table 1. The 16S rDNA gene sequences of selected wild-type strains allowed determination of the bacterial genus, and these sequences were deposited in GenBank.

Naringin hydrolysis capacity was also evaluated in the selected strains using HPLC, and it was observed that the isolate Ci19 not only showed one of the highest hydrolysis capacities with 4-NPR (9.55 ± 0.11 U mg^{-1} protein) but that it also showed the highest specific activity in naringin hydrolysis showed significant difference with the other strain Ci2 ($p = 0.0074$) (20.20 ± 0.29 U mg^{-1} protein) (Table 1). The α-L-rhamnosidase activity was confirmed via biotransformation of naringin to prunin and naringenin. Hydrolysis activity was evaluated at 0, 15, 30, and 60 min, and the degradation products of naringin were identified by comparison with the retention time of the pure standard compound. Naringin without hydrolysis showed a retention time of 4.067 min, and prunin and naringenin showed retention times of 4.667 min and 13.247 min, respectively (Fig. 1).

Table 1 Strains with α-L-rhamnosidase activity isolated from sub-Antarctic ecosystems

Isolate	Specific hydrolytic activity of *p*-NPR (U mg^{-1})	Specific hydrolytic activity of naringin (U mg^{-1})
Serratia sp. Ci2	8.79 ± 0.09 a	18.20 ± 0.17 a
Devosia sp. Ci5	2.09 ± 0.15 b	Without activity
Microbacterium sp. Ci12	3.43 ± 0.01 c	Without activity
Serratia sp. Ci16	9.73 ± 0.05 d	Without activity
Brevundimonas sp. Ci19	9.55 ± 0.11 d	20.20 ± 0.29 b
Salinibacterium sp. Ci20	10.41 ± 0.18 e	Without activity

Specific hydrolytic activity was determined with *p*-NPR and naringin and is expressed in U mg^{-1}. Data are presented as mean value ± standard deviation calculated from at least three independent experiments. Values with different letters (a–d) are significantly different ($P < 0.05$)

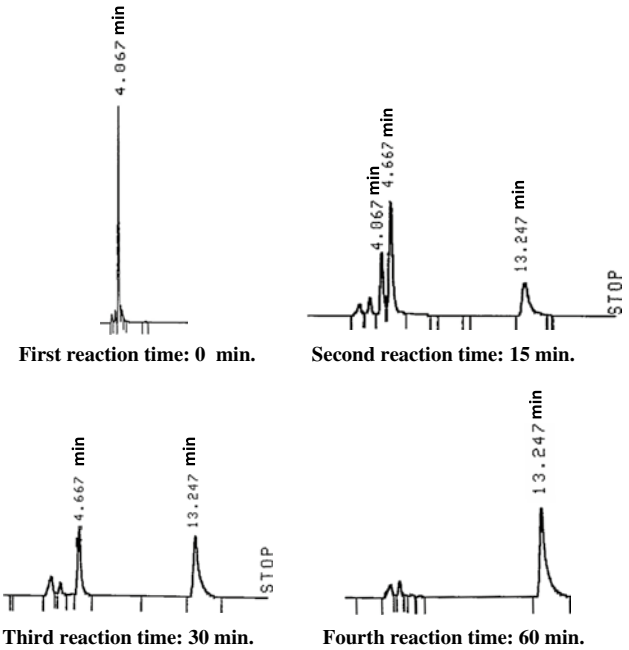

Fig. 1 HPLC of naringin hydrolysis by α-L-rhamnosidase. The flavonoid glycosides prunin and naringenin were evaluated at 0, 15, 30, and 60 min and identified by comparison with the retention time of the pure standard compound. Several peaks were detected: naringin, 4.067 min; prunin, 4.667 min; and naringenin, 13.247 min

The obtainment of flavonoids like prunin indicates the potential industrial application of this α-L-rhamnosidase, since prunin is a very expensive product with antiviral properties [18]. The isolate Ci19 was therefore selected for further study based upon its higher α-L-rhamnosidase activity, especially on the natural naringin substrate.

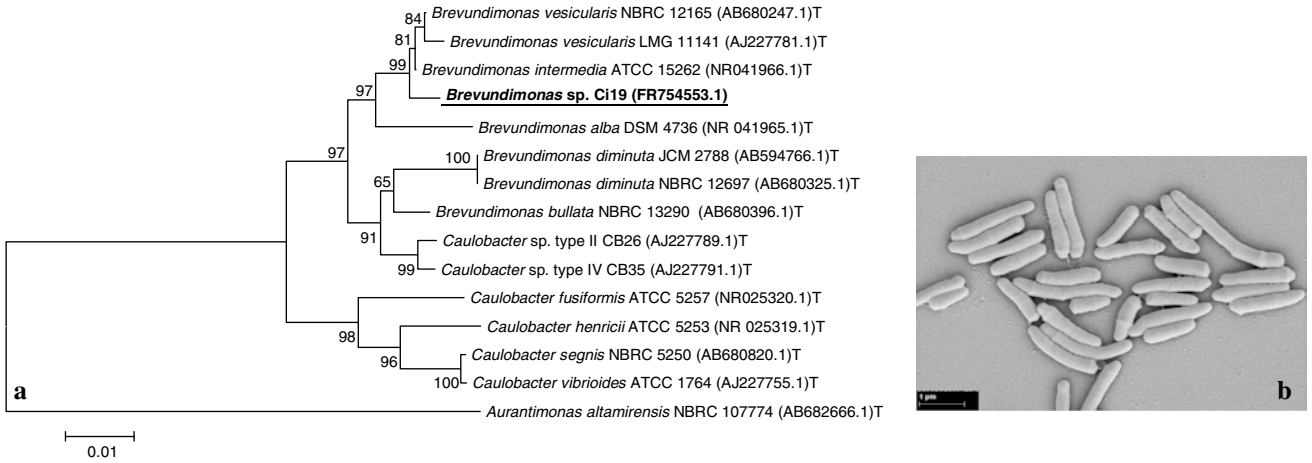

Fig. 2 **a** Phylogenetic tree (Pt) inferred by the neighbour-joining method (Saitou and Nei 1987), using Kimura's evolutionary distance (Kimura 1980) and based upon a comparison of nearly complete 16S rDNA sequences. Accession numbers of 16S rDNA sequences are given in parentheses. **b** Scanning electron microscopic image of *Brevundimonas* sp. Ci19

Characterization of the strain *Brevundimonas* sp. Ci19

The isolate Ci19 was classified within the genus *Brevundimonas*, with a 16S rDNA sequence similarity of 99 % (GenBank accession number FR754553) (Fig. 2a), and the main morphological and physiological characteristics of *Brevundimonas* sp. Ci19 are given in Table 2. The Ci19 strain developed in circular, convex, cream-coloured colonies on agar plates. Microscopically, the microbial cells consisted of flexible, slightly bent rods with a length of 0.8–2.0 μm and a diameter of approximately 300 nm (Fig. 2b). This bacterium was able to grow in saline environments in the range of 10–100 g l^{-1} NaCl, with higher growth rates in the range of 75–100 g l^{-1} NaCl. However, the Ci19 strain was unable to grow in the absence of NaCl. *Brevundimonas* species are characterized by high levels of salt tolerance, but members of the genus show diverse cell morphologies and substrate specificities. Although *Brevundimonas* was at first considered to be a non-marine genus of bacteria, more recent reports on *Brevundimonas* strains isolated from marine habitats have been accumulating [10, 19, 20]. On the other hand, few studies have been reported on α-L-rhamnosidase production by *Brevundimonas* species. The research discussed here is thus significant not only in the isolation of a strain of *Brevundimonas* from Antarctic seawater, but also because of this strain's α-L-rhamnosidase activity on a natural naringin substrate and in view of the industrial application of α-L-rhamnosidase in flavonoid modification [1].

Table 2 Morphological, physiological, and biochemical characteristics of *Brevundimonas* sp. Ci19

Morphology	Rod-shaped cells occurring singly, non-spore-forming, cream-white colour
Gram staining	Negative
Mobility	Positive
Temperature range of growth	0–30 °C
Growth pH	4–8 (optimum at 6)
Growth in NaCl	10–100 g/l (optimum at 75–100)
Hydrolysis of esculin, gelatine, 4-nitrophenyl-β-D-galactopyranoside	Positive

Partial purification of the enzyme

The α-L-rhamnosidase was partially purified by ultrafiltration followed by size exclusion chromatographic techniques, with a resulting increase in enzyme-specific activity of 2.68-fold. A summary of the purification procedures used is presented in Table 3. The increase in specific activity obtained with this purification was lower than those reported for other α-L-rhamnosidase purifications such as from *Aspergillus nidulans* (18-fold) [21].

Effects of pH and temperature on enzyme activity

Typically, a broad optimum pH and temperature range is found for rhamnosidases [22]. In the present study, the behaviour of the α-L-rhamnosidase activity was evaluated at several pH levels and at a range of temperatures (Fig. 3). In terms of pH, the α-L-rhamnosidase activity showed a sharp optimum between pH 6.00 and 7.00 (Fig. 3).

Similar optimum pH values have been reported for *Pseudoalteromonas* 005NJ and *Pichia angusta* α-L-rhamnosidases [6, 23], although different values were obtained for the *A. nidulans a*-L-rhamnosidase [21].

Table 3 Purification of α-L-rhamnosidase from *Brevundimonas* sp. Ci19

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Cell-free extract	0.40	1.34	0.298	100	1.00
Ultrafiltration	0.35	0.556	0.629	87.5	2.11
Sephadex G-100	0.04	0.05	0.80	10.0	2.68

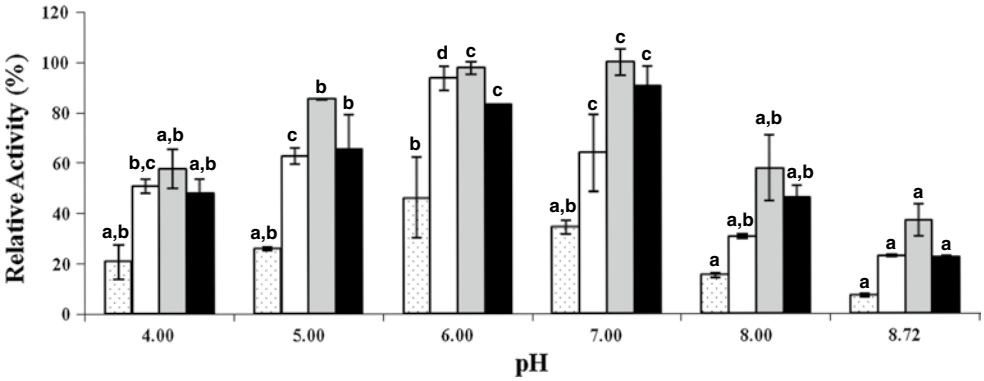

Fig. 3 Effects of pH and temperature (°C) on the activity of partially purified α-L-rhamnosidase from *Brevundimonas* sp. Ci19

The fact that the enzyme in the present study retained from 48 to 58 % of its maximum activity at pH 4.00 and 63–85.3 % of its maximum activity at pH 5.00 is also of interest. On the other hand, in the alkaline pH range, the activity was lower (Fig. 3).

In terms of temperature, at 8 °C the enzyme showed the highest activity between pH 4.00 and 7.00, with no significant differences observed within this pH range ($p = 0.1467$). At 20 °C the highest enzyme activity was observed at pH 6.00, with significantly lower values above and below this pH ($p = 0.0004$). At 30 and 37 °C, the highest α-L-rhamnosidase activity was observed at pH 6.00 and 7.00, with the same behaviour seen at both pH levels ($p = 0.0923$). Thus, in summary, the optimal activity was observed at 20 °C and pH 6.00, or from 30 to 37 °C at pH 6.00 or 7.00, with the observed behaviour of the α-L-rhamnosidase being the same under any of these conditions ($p = 0.0638$). In comparison with other published studies, this optimum temperature range differs from that observed for the α-L-rhamnosidase activity of *Pseudoalteromonas* sp. 005NJ or *A. nidulans*, but is similar to that seen in the *Pseudomonas paucimobilis* FP2001 *a*-L-rhamnosidase [6, 21, 24].

It is known that α-L-rhamnosidase and β-D-glucosidase activities frequently combine to form a naringinase complex, a complex enzyme used in deglycosylation compounds and one with a high potential for use in the food and pharmaceutical industries [1]. In view of this, β-D-glucosidase activity was also evaluated in the supernatant partially purified by ultrafiltration. Activity by β-D-glucosidase was in fact detected, and the optimum pH and temperature were observed at pH 8.00 at 30 and 37 °C, with no significant difference being seen between these two

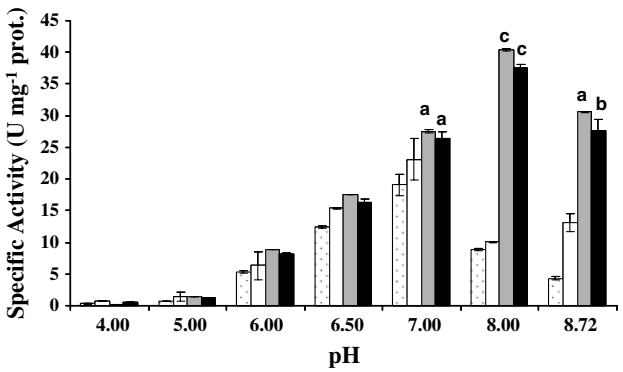

Fig. 4 Effects of pH and temperature (°C) on the activity of β-D-glucosidase from *Brevundimonas* sp. Ci19

temperatures ($p = 0.0616$) (Fig. 4). An important fact to note was that at pH 8.72 the enzyme retained about 75 % of its maximum activity at both temperatures, indicating that β-D-glucosidase is active in the alkaline region (Fig. 4). When producing monoglycosylated flavonoids with naringinase, the expression of β-D-glucosidase activity is not desirable, and this creates the need to apply costly methods for α-L-rhamnosidase purification [1]. Therefore, inactivation of the β-D-glucosidase activity expressed in naringinase while retaining high levels of α-L-rhamnosidase activity is ideal. The differing pH-related behaviour of the *a*-L-rhamnosidase and β-D-glucosidase from *Brevundimonas* sp. Ci19 is thus of interest, given that the *a*-L-rhamnosidase maintains about 85 % of its activity in the acid pH region (pH 5.00) while the β-D-glucosidase was virtually inactive in the acid region while showing its highest activity values in the alkaline region (Fig. 4). This may represent

an important advance towards the development of a simple and inexpensive production method for very expensive flavonoids such as prunin and isoquercetin, starting from naringin and rutin, respectively. Vila-Real et al. [1] also evaluated the effects of temperature and pH on β-D-glucosidase inactivation and achieved selective inactivation of the β-D-glucosidase activity in naringinase while maintaining very high residual activity in the α-L-rhamnosidase.

Effects of divalent cations on the α-L-rhamnosidase activity

Table 4 shows the effects of several divalent cations on the activity of *a*-L-rhamnosidase from *Brevundimonas* sp. Ci19. The enzyme showed activation in the presence of Ca^{2+}, with a 78.36 % increase in activity compared to the control without treatment (which was taken to represent 100 % activity). The activation of α-L-rhamnosidase in the presence of calcium ions was higher than that observed in the *a*-L-rhamnosidase from *A. nidulans*, but only by 14 % [21], and the activation effect observed was similar to the increase in activity seen in *a*-L-rhamnosidase from *Streptomyces avermilitis* [25]. It is known that calcium ions play a key role in L-rhamnose recognition. Fujimoto et al. [26] evaluated the effects of calcium on *a*-L-rhamnosidase, finding that a new domain present in several bacterial families binds L-rhamnose in a calcium-dependent manner and that calcium binding is thus necessary not only for hydrolysis activity but also for sugar binding.

The *a*-L-rhamnosidase activity in the present study showed 14 % more activity than the control (100 %) in the presence of Cd^{2+}. This effect was different than the one observed in *a*-L-rhamnosidase from *Aspergillus terreus*, where Gallego et al. [27] found that Cd^{2+} inhibited the enzyme. The Mg^{2+} and Zn^{2+} ions had little effect on the enzyme activity, with 81.8 % and 91.7 % of the activity remaining in the presence of Mg^{2+} and Zn^{2+}, respectively. The result observed in the presence of Mg^{2+} differs from those observed in *a*-L-rhamnosidase from *A. nidulans*, where the presence of Mg^{2+} produced an inhibition of 63 % [21]. Finally, in the present study the enzyme maintained 64 % of its residual activity in the presence of Co^{2+}, suggesting an inhibition via redox nature caused by this metal [28], and was drastically inhibited in the presence of Cu^{2+}. This latter ion is a sulphydryl oxidant metal, which suggests some importance for a sulphydryl group in the expression of the enzyme activity [23]. Among various ionic compounds, the inhibition effect seemed independent of anions or the charge/size of cations (metal ions) under the conditions of this study (10 mM level).

Table 4 Effects of several divalent cations on the activity of α-L-rhamnosidase from *Brevundimonas* sp. Ci19

Compound (10 mM)	Relative activity (%)
No addition (control)	100 ± 1.47
Mg^{2+}	81.78 ± 2.45
Co^{2+}	64.03 ± 1.44
Cu^{2+}	38.01 ± 22.74
Cd^{2+}	114.06 ± 1.97
Zn^{2+}	91.71 ± 6.91
Ca^{2+}	178.36 ± 5.85

Effects of the sodium metabisulphite, glucose, and ethanol on the α-L-rhamnosidase activity

In wines, *a*-L-rhamnosidases play an important role in the hydrolysis of glycosylated aroma compounds, especially terpenes. Although several authors have demonstrated the oenological importance of fungal rhamnosidases, the information available on bacterial enzymes in this context is still limited [29]. In view of the potential applications for α-L-rhamnosidase activity in wine flavour enhancement, the influence of sodium metabisulphite, glucose, and ethanol was investigated as part of the present study, at concentrations similar to those present during winemaking (Fig. 5). First, at concentrations of sodium metabisulphite between 50 and 100 mg l^{-1}, the α-L-rhamnosidase activity did not differ significantly compared to the control without treatment ($p = 0.1001$), although at 250 mg l^{-1} activity decreased by 35 % (Fig. 5a). With glucose, on the other hand, activity was dramatically inhibited at the concentrations tested (Fig. 5b). This glucose inhibition effect was also observed by Michlmayr et al. [29] for the activity of α-L-rhamnosidase from *Pediococcus acidilactici*. Finally, ethanol concentrations up to 5 % (v/v) did not affect activity, with no significant difference with the untreated control observed ($p = 0.0901$). However, ethanol concentrations of 7.5 and 10.0 % (v/v) showed an activation effect for the α-L-rhamnosidase, with 22 and 43 % increases in activity over that of the control (taken to be 100 %) (Fig. 5c). The enzyme in the present study thus appears to be more stable in the presence of ethanol than the α-L-rhamnosidase from *A. nidulas* or *P. acidilactici* [21, 29]. Ethanol concentrations in wine are around 12 % on average (v/v), and because of this, α-L-rhamnosidase from *Brevundimonas* sp. Ci19 would appear to be an enzyme with potential applications in winemaking.

Conclusion

In food processing, rhamnosidases are used for enhancing wine aromas or debittering of citrus juices. These enzymes

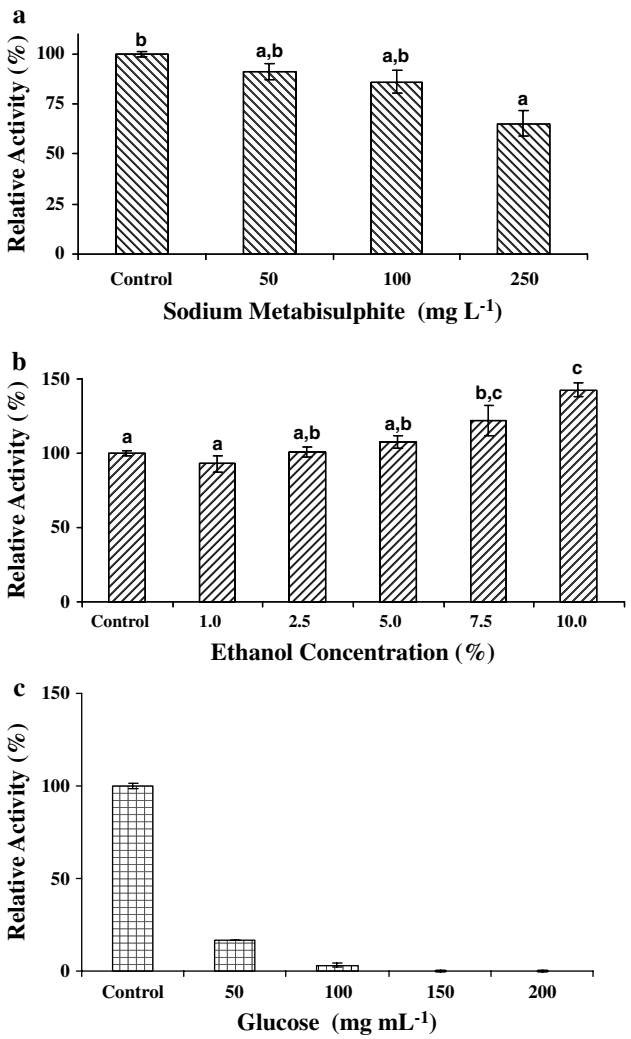

Fig. 5 Effects of several reagents on the activity of partially purified α-L-rhamnosidase from *Brevundimonas* sp. Ci19. **a** Sodium metabisulphite concentrations; **b** glucose concentrations; **c** ethanol concentrations

are largely inactivated at physicochemical conditions of winemaking or in citrus juice production. Microbial rhamnosidases are still a field of great interest for many laboratories around the world. α-L-Rhamnosidase enzyme from the newly isolated, psychrotolerant bacterium *Brevundimonas* sp. Ci19 appears to be an interesting system from an applied point of view, and our results indicate that it could be useful not only in the food processing procedures but also in pharmaceutical industrial. The rhamnosidases are used also for improving the bioavailability of flavonoids with a wide range of beneficial effects for human health. This enzyme was also able to hydrolyse a natural naringin substrate, and this is notable in terms of potential industrial applications since the very expensive product prunin was obtained from enzymatic hydrolysis of naringin. Thus, *Brevundimonas* sp. Ci19 α-L-rhamnosidase described in this study possesses a broad range of properties that may allow its application in a variety of biotechnological processes.

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Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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