Available online at www.sciencedirect.com
ScienceDirect
Enzyme and Microbial Technology 40 (2007) 236–241
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Cold-active α-L-rhamnosidase from psychrotolerant bacteria isolated from a sub-Antarctic ecosystem

A. Gastón Orrillo [a], Pablo Ledesma [a], Osvaldo D. Delgado [a], Giovanni Spagna [b], Javier D. Breccia [a,c,*]

[a] *PROIMI-CONICET (Planta Piloto de Procesos Industriales Microbiológicos), Av. Belgrano y Caseros, 4000 Tucumán, Argentina*
[b] *Gruppo di Biotecnologie Alimentari, Dipartimento di Orto Floro Arboricoltura e Tecnologie Agroalimentari (DOFATA), Facoltà di Agraria, Università di Catania, Via Santa Sofia 98, 95123 Catania, Italy*
[c] *Depto. de Química, Facultad de Ciencias Exactasy Naturales, Universidad Nacional de La Pampa (UNLPAM), Av. Uruguay 151, 6300 Santa Rosa, La Pampa, Argentina*

Received 10 December 2005; received in revised form 31 March 2006; accepted 6 April 2006

Abstract

Psychrotolerant bacteria obtained from sea water and alimentary tracts of benthonic organisms from the sub-Antarctic environment of Tierra del Fuego (Argentina) were screened for cold-active α-L-rhamnosidase production. Only 10 of a total of 140 isolates were positive for α-L-rhamnosidase activity as determined by a qualitative assay; and five of them showed substantial activity at 5 °C. Molecular identification (16S rDNA) of five selected isolates revealed that four of them were closely related to the genus *Pseudoalteromonas* while the other was identified as *Ralstonia pickettii*. The *Pseudoalteromonas* sp. 005NJ strain was selected for further characterization based on the higher relative α-L-rhamnosidase activity at 4 °C. It showed specific growth rates of 0.12–0.19 h^{-1} in the temperature range of −1 to 8 °C, and the α-L-rhamnosidase activity was mainly intracellular. The reaction showed optimal pH and temperature values of 6 and 40 °C respectively and maintained 6% activity at 4 °C. The enzyme was found to be thermo-sensitive, presenting a half-live of 4 min at 50 °C. In presence of 12% (v/v) ethanol the activity decreased by approximately 48%. Cold-active α-L-rhamnosidase might be useful for food processing technologies.

Keywords: Psychrotolerant; α-L-Rhamnosidase; *Pseudoalteromonas*; *Ralstonia*

1. Introduction

Cold-active enzymes have been applied in biotechnology only quite recently compared to their thermophilic counterparts. Enzymatic food processing and bioconversions of thermo-sensitive or volatile organic compounds are rather convenient to perform at low temperatures [1]. In view of such interest, physiological adaptations of psychrophilic microorganisms growing at low temperatures have been reported. For instance they increase the proportion of unsaturated fatty acid, rendering higher membrane fluidity [1]; their enzymes show structural changes that increase its flexibility, resulting in higher catalytic efficiency at low temperatures than those from mesophilic and thermophilic organisms, although thermal-stability is usually reduced [2,3]. Recently, an important contribution for understanding physiological adaptation of microorganisms to cold environments has been reported, showing that chaperonins from psychrotolerant bacteria could play an important role allowing mesophilic bacteria grow at low temperatures [4].

Few studies on bacterial α-L-rhamnosidases [EC 3.2.1.40.] have been reported, and most data have been obtained from eukaryotic organisms [5–10]. α-L-Rhamnosidases, together with α-L-arabinofuranosidase and β-D-glucosidase, might be useful for reducing the acid flavour of fruit juices and for enhancing wine aromas due to the release of volatile terpenes bound to a sugar moiety [10]. On the other hand, biotransformations in organic solvents using flavonoids as sugar donors has been shown to be a very interesting approach [11]. The present work reports on the isolation and characterization of psychrotolerant bacteria producing cold-active α-L-rhamnosidase.

* Corresponding author. Tel.: +54 2954 436787; fax: +54 2954 432535.
E-mail address: javierbreccia@exactas.unlpam.edu.ar (J.D. Breccia).

0141-0229/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2006.04.002

2. Materials and methods

2.1. Microbial sources

Samples of seawater and alimentary tracts of benthonic organisms (*Munida subrugosa*, *M. gregaria*, *Loxechinus albus*, *Patinigera* (Nacella) *deaurata*, *P.* (N.) *magellanica* and *Eurhomalea exalbida*) were collected from the coast of Ushuaia, Tierra del Fuego, Argentina. Samples of 1 g of alimentary tract content of these organisms were suspended in 5 ml of sterile seawater and used for isolation of microbial strains.

2.2. Microorganism isolation and culture conditions

Enrichment medium (g/l): naringin or hesperidin 5; yeast extract 1; milk peptone 1; NaCl 30. Selection medium (g/l): L-rhamnose 10; peptone 1; yeast extract 0.5; NaCl 35; agar 15. Fermentation medium (g/l): milk peptone 10, yeast extract 5; specified carbon source 10 and NaCl 35. For isolation 1 ml of seawater or suspension of alimentary tract content was added to 5 ml of enrichment medium and incubated at 4 °C. After 2 weeks, serial dilutions in 30 g/l NaCl solution were streaked out on the selection medium and the plates were again incubated at 4 °C during 72 h. Isolated colonies were re-streaked in the selection medium for the α-L-rhamnosidase activity qualitative assay [12].

The isolates were then cultured in an orbital shaker (250 rev/min, 25 °C) in 20 ml fermentation medium. After 18 h of cultivation 2 ml L-rhamnose (100 g/l) was added in order to evaluate α-L-rhamnosidase activity and incubation continued until OD_{560} reached 1.1 (approximately 4 h). For intracellular extract samples, the cells were then collected by centrifugation (10 min—12,000 × *g*), suspended in 5 ml of 0.1 mol/l Tris–HCl pH 7.8 buffer, disrupted by passing once through a French press at 25,000 psig and centrifuged for 10 min at 12,000 × *g* to eliminate cell debris. The supernatants were frozen at −20 °C until processing.

2.3. Characterisation and identification of the isolates

DNA extraction and quantification, Intergenic 16S–23S ribosomal internal transcribed spacers (ITS) pattern and 16S rDNA amplification and sequence analysis were carried out as we previously described [13].

2.4. Fermentations

Growth assays were carried out using the fermentation medium with glucose as carbon source. Response to temperature was studied using a 0.2 l capacity jacketed fermentor with a working volume of 0.16 l, operated in the range of −1 to 25 °C. Optical density (OD_{560}) was monitored on-line by using a flow-cell attached to a spectrometer (Ocean optics CHEM2000 UV-Vis, Duiven, The Netherlands). A 2.0 l fermentor (Discovery 210, New Brunswick, USA) was employed for culturing at different pH values (ranging from pH 5.0 to 9.0). It was also used for α-L-rhamnosidase production studies, at an agitation speed of 400 rev/min, aeration of 1 v/v m at 25 °C. The working volume was 1.0 l. Production of α-L-rhamnosidase was performed in fermentation medium, but the carbon source (100 ml of 55 g/l apple pectin) was added after 18 h growth. Aliquots of 20 ml were stroked out and processed as described above for intracellular extract preparation.

2.5. Enzyme assays

Qualitative determination of α-L-rhamnosidase activity was performed in Petri dishes according to Paoni and Arroyo [12], the bacterial colonies growing with L-rhamnose as carbon source were transferred to filter paper and imbibed in 2 mmol/l *p*-nitrophenyl-α-L-rhamnopyranoside (*p*-NPR) in 0.1 mol/l Tris–HCl pH 7.8 and incubated for 2 h at 25 °C. For α-L-rhamnosidase activity quantification, each reaction contained 28.5 μl of substrate (70 mmol/l of *p*-NPR in dimethylformamide), 870 μl of buffer (0.1 mol/l Tris–HCl pH 7.8) and 100 μl of enzyme solution. The reaction was performed during 1 h at 40 °C and stopped by adding 100 μl of 0.1 mol/l NaOH [14]. For activity determination at low temperatures the reaction time was extended. One enzyme unit was defined as the amount of enzyme that released 1 μmol of *p*-NRP in 1 h at the indicated temperature. Protein concentration was determined by the method of Bradford [15], using bovine serum albumin as standard.

3. Results and discussion

3.1. Isolation and identification of cold-active α-L-rhamnosidase producing bacteria

The neritic zone of the sea near the coast of Ushuaia in Argentina has a temperature below 20 °C throughout the year and the sediments consumed by benthonic organisms are mainly lignocellulosic materials that contain flavonoids. These conditions would be an adequate enrichment environment for bacteria that produce α-L-rhamnosidase, an enzyme involved in the deglycosylation of some flavonoids [9]. In total 140 isolates were evaluated for α-L-rhamnosidase activity, 22 of them from sea water samples and approximately 20 isolates/organism studied from the alimentary tracts of benthonic organisms. Ten of these isolates were positive for substrate hydrolysis (*p*-NPR) at 25 °C, and only five showed substantial α-L-rhamnosidase activity at low temperature (5 °C). In order to asses the most cold-active α-L-rhamnosidase among the selected isolates, *p*-NPR hydrolysis at low temperatures was performed (Fig. 1). For all isolates 100% activity was arbitrarily established as the activity shown at 35 °C. At 7 °C the isolate 005NJ retained 19% α-L-rhamnosidase activity while the isolate 003NJ showed no significant differences, the other isolates had activities between 9 and 13%. At 4 °C the activity of the isolate 005NJ decreased to 6%, while all others isolates had less than 2% activity (Fig. 1).

Among prokaryotes, there is considerable variation in the number of *rrn* operons. Intergenic 16S–23S ribosomal internal transcribed spacers (ITS), even when defined as hypervariable in comparison with the adjacent genes, usually only show significant variations at the genus, species or strain level [16]. The sequence alignment for 16S rDNA of the isolates, revealed that all shared high similarities with members of psychrotoler-

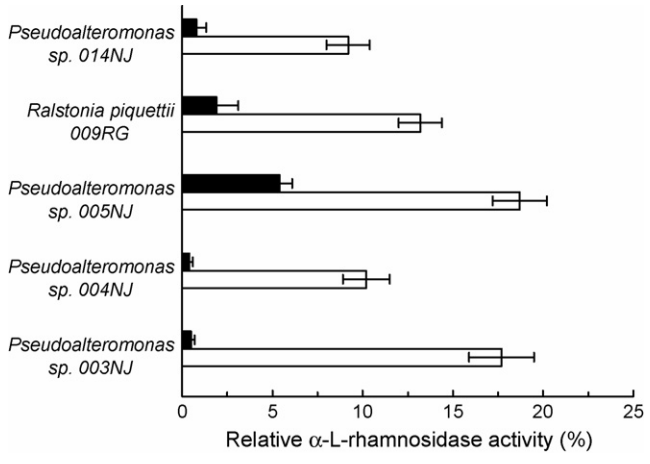

Fig. 1. Relative α-L-rhamnosidase activity of intracellular extracts at (□) 7 °C and (■) 4 °C. The 100% of the activity was arbitrarily taken at 35 °C, corresponding to U/ml: 168, *Pseudoalteromonas* sp. 003NJ; 337, *Pseudoalteromonas* sp. 004NJ; 153, *Pseudoalteromonas* sp. 005NJ; 151, *Ralstonia pickettii* 009RG; 92, *Pseudoalteromonas* sp. 014HJ. The values are presented as the means of duplicate measurements and standard errors.

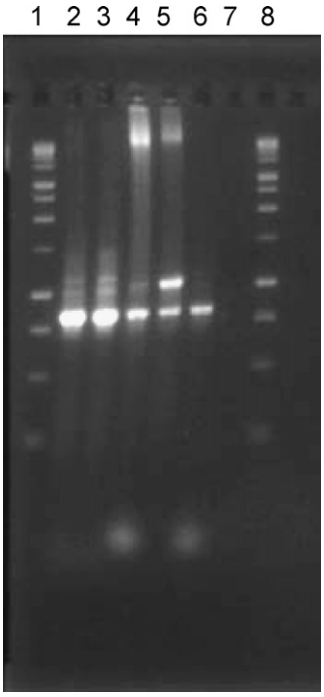

Fig. 2. Agarose gel (2%) of Intergenic 16S–23S ribosomal internal transcribed spacers (ITS-PCR) patterns. Lanes: 1, 100 bp DNA ladder (Promega); 2, *Pseudoalteromonas* sp. 003NJ; 3, *Pseudoalteromonas* sp. 004NJ; 4, *Pseudoalteromonas* sp. 005NJ; 5, *R. pickettii* 009RG; 6, *Pseudoalteromonas* sp. 014HJ.

ant marine bacteria. Four of them 003NJ (DQ311667), 004NJ (DQ311668), 005NJ (AY687990), 014HJ (DQ311669) shared high similarities with *Pseudoalteromonas* spp. and the isolate 009RG (DQ311670) had 100% sequence concordance with *Ralstonia pickettii*. On the other hand, the ITS-PCR fingerprinting of the five strains revealed different patterns, showing typically both large and short intergenic regions (Fig. 2). The amplicon pattern of ITS analysis ranging from 430 bp to about 550 bp suggested that the isolates related to *Pseudoalteromonas* species, are different strains of the genus. These results were confirmed by ARDRA analysis using *Cfo*I and *Hpa*II restriction enzymes (data not shown). The isolate 005NJ, was selected for further studies according to its higher α-L-rhamnosidase activity at 4 °C; it should be classified within *Pseudoalteromonas* genus, being *P. antarctica* (X98336) the closest related species with a 16S rDNA sequence similarity of 99%.

Pseudoalteromonas genus is composed of widespread marine microorganisms requiring a seawater base media for their growth. They are known producers of a wide range of biologically active compounds, such as toxins, antitoxins, anti-tumour and antimicrobial agents, as well as enzymes with a wide spectrum of action [17]. We found *Pseudoalteromonas* strains as well as *R. pickettii* able to grow in presence of flavonoids (hesperidin and naringin), which are recalcitrant compounds and usually inhibitory to microorganisms [18,19], although none of the isolates were able to use the flavonoids as sole carbon source. *Pseudoalteromonas* species were previously isolated from the polynya of Northern Sea Waters and Antarctic ice samples [20,21] and, were usually found in association with higher organisms [17]. We found that all isolates that produced

Table 1
Morphological, physiological, and biochemical characteristics of *Pseudoalteromonas* sp. 005NJ

Morphology	Rod-shaped cells occurring singly, non-spore-forming, white–blue colour with iridescence
Gram staining	Negative
Mobility	Positive
Temperature range of growth	−2 to 30 °C
Growth pH	6–9 (optimum at 7–8)
Growth in NaCl	7.5–100 g/l (optimum at 30–60)
Catalase	Positive
Oxidase	Positive
Hydrolysis of starch, dextrin, gelatine, casein and Tween 80	Positive

α-L-rhamnosidase were derived from seawater samples, while none of the approximately 118 isolates from alimentary tracts of benthonic organisms were α-L-rhamnosidase activity positive. These results were found in the biochemical qualitative assay we used to select positive strains, however, other techniques might be more effective.

3.2. Characterization of the strain Pseudoalteromonas sp. 005NJ

Some morphological and physiological characteristics of *Pseudoalteromonas* sp. 005NJ, are given in Table 1. It formed circular convex colonies coloured white–blue with iridescence. It grew in salty environments between 7.5 and 100 g/l NaCl, with higher growth rates in the range of 30–60 g/l NaCl and was unable to grow in absence of NaCl. A correlation between pigmented strains and the ability to grow at different ionic strengths was pointed out for genus *Pseudoalteromonas* [22]. Pigmented strains (*P. aurantia*, *P. luteoviolacea*, *P. rubra*, *P. denitrificans* and *P. paragorgicola*) required low concentration of NaCl (10–60 g/l), while non-pigmented strains (*P. prydzensis* or *P. issachenkonii*) required up to 150 g/l NaCl for growth, except for *P. atlantica*, which grew in the range between 30 and 60 g/l NaCl. The results obtained with *Pseudoalteromonas* sp. 005NJ were in agreement with the above correlation (Table 1). Remarkably, in contrast to *P. antarctica* that has 99% 16S rDNA sequence similarity, it is non-pigmented and grows optimally in the range 90–125 g/l NaCl.

The usual range of growth temperatures reported for the genus is between 4 and 44 °C [22,23]. *Pseudoalteromonas* sp. 005NJ was unable to grow at 35 °C, but it grew even at subzero temperatures. Fig. 3 shows the specific growth rates of the strain 005NJ in a temperature range from −1 to 25 °C. The growth rate values at low temperatures (−1 to 8 °C) were between 0.12 and 0.18 h^{-1}, which are slightly lower than those reported for another *Pseudoalteromonas* strain isolated from polynya of the North Sea Waters [20].

3.3. α-L-Rhamnosidase production

Several polymers produced by *Pseudoalteromonas* strains, such as exopolysaccharide or *O*-specific polysaccharide, con-

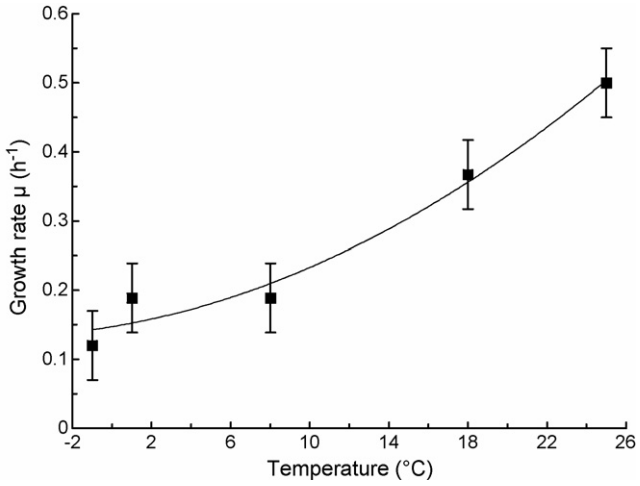

Fig. 3. Effect of temperature on the specific growth rate of *Pseudoalteromonas* sp. 005NJ in submerged cultures using fermentation medium and glucose as carbon source. See details in Section 2.

taining L-rhamnose residues in the repeating units, have been reported [24]. This fact suggests that α-L-rhamnosidase would be an enzyme involved in the turnover metabolism of such compounds. Nevertheless, when rhamnosylated substrates were absent in the medium, the activity α-L-rhamnosidase 005NJ was virtually non-detectable, albeit with normal cell growth. We found intracellular α-L-rhamnosidase in 005NJ only in the presence of rhamnosylated substrates in the culture media (Figs. 4 and 5). Fig. 4 shows the production of α-L-rhamnosidase activity after adding 0.5% (w/v) apple pectin at 18 h growth. The maximum production was found between 3 and 9 h after addition of the polymer. Similar production profiles were exhibited with other rhamnose containing carbon sources such as L-rhamnose,

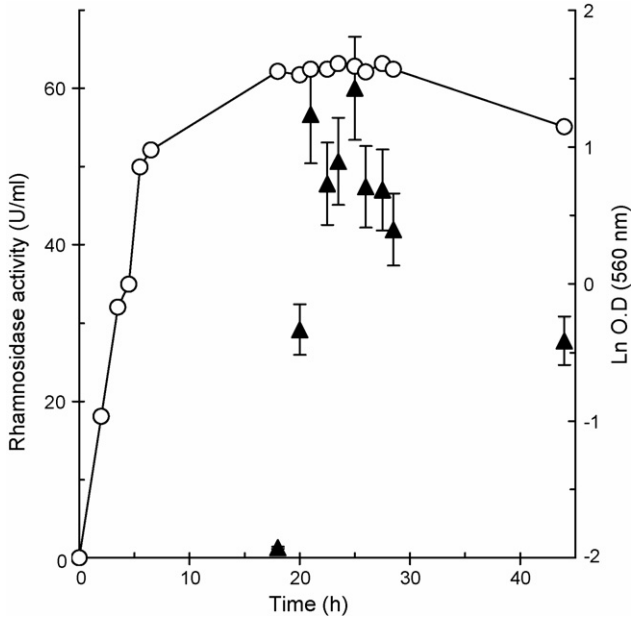

Fig. 4. Production of α-L-rhamnosidase activity by *Pseudoalteromonas* sp. 005NJ in fermentation medium. After 18 h growth, 0.5% (w/v) apple pectin was added as carbon source. (◯) Optical density (560 nm); (▲) α-L-rhamnosidase activity.

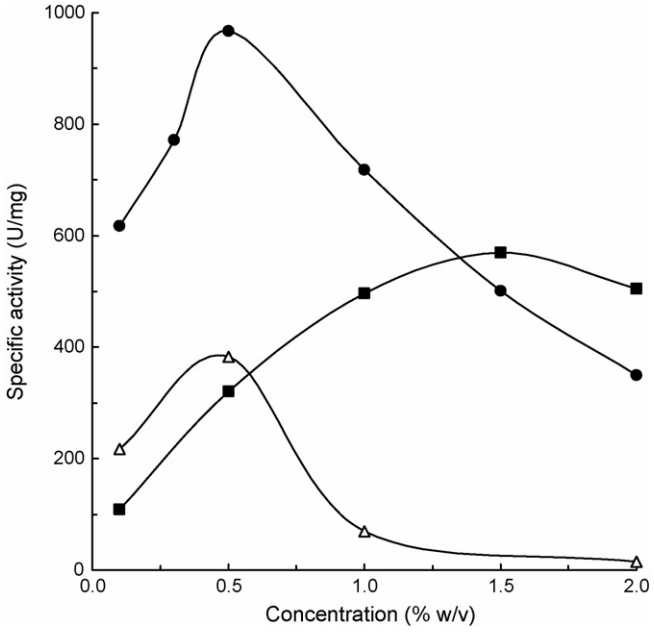

Fig. 5. Effect of carbon source concentration on the specific activity of α-L-rhamnosidase 005NJ. (●) Rhamnose, (■) Arabic gum, (△) Pectin. All samples were dialyzed (10 kDa cut-off membrane) before measuring the activity to avoid underestimation of the activity due to sugar inhibition. The carbon sources were added to the culture medium after 18 h growth at the indicated concentration and after 4 h the specific activity was measured.

naringin and Arabic gum. However, α-L-rhamnosidase production with rhamnosylated compounds gave lower yields (between 35 and 40%) than those obtained with L-rhamnose.

Furthermore, α-L-rhamnosidase specific activity depended on the concentration of the carbon source in the culture media (Fig. 5). The maximum specific activity was obtained at 0.5% (w/v) for L-rhamnose (967 U/mg) and apple pectin (382 U/mg). While for Arabic gum it needed a three times higher carbon source concentration (1.5%, w/v) to reach a maximum of 570 U/mg. Nevertheless, if the relative contents of L-rhamnose in the polymers (5% (w/v) apple pectin [25] and 34% (w/v) Arabic gum [26]) were accounted for, the maximum specific activity values were proportional to the L-rhamnose content in the carbon sources (Fig. 5).

3.4. α-L-Rhamnosidase 005NJ

Usually a broader optimal pH range was found for rhamnosidases [27,28]. α-L-Rhamnosidase 005NJ activity was sensitive to acid pH values, showing a sharp optimum at pH 6 (Fig. 6). The sensitive character to low pH values could be a limiting factor for developing industrial applications of the enzyme in food processing. Regarding the temperature effect on 005NJ α-L-rhamnosidase reaction a maximum of activity at 40 °C was observed (Fig. 6). Similar values have been reported for *Pichia angusta and Absidia* sp. 39 α-L-ramnosidases [7,27]. Unfortunately, evaluations of α-L-rhamnosidase activity at temperatures below 20 °C are scarce in the scientific literature. The lowest temperature assayed for *Absidia* sp. 39 α-L-rhamnosidase was 20 °C with 40% activity [27], while 005NJ enzyme showed

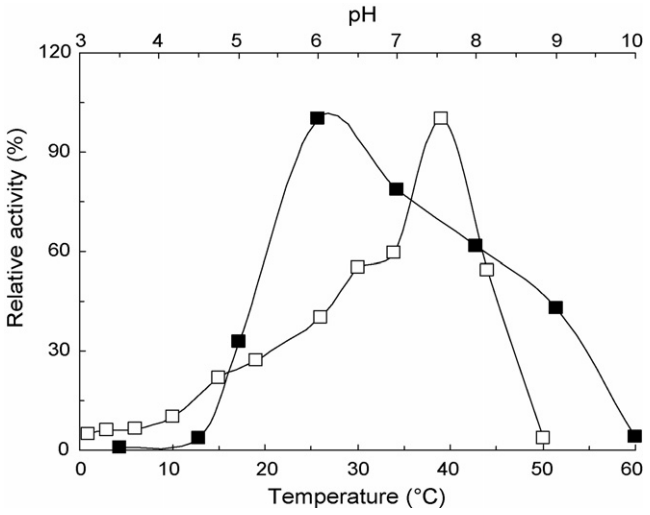

Fig. 6. (■) Effect of the pH and (□) temperature on the α-L-rhamnosidase activity of *Pseudoalteromonas* sp. 005NJ— 50 mmol/l buffers: Tris–acetic acid (pH 4.5–5.0); sodium phosphate (pH 6.0–8.0); Tris–HCl pH 9.0 and solution of 100 mmol/l sodium carbonate pH 10. Hundred percent of the activity correspond to 471 and 316 U/ml for pH and temperature, respectively.

34% activity at this temperature. The thermal stability of 005NJ enzyme was evaluated in the temperature range of 35–50 °C at pH 7.8 (Fig. 7). The enzyme was thermo sensitive, with a half-live of 4 min at 50 °C, in comparison to *P. angusta* α-L-rhamnosidase, which showed a half-live of 30 min at the same temperature [7]. The activation energy (E_a) for α-L-rhamnosidase 005NJ denaturation was 204.0 ± 2.1 kJ/mol, calculated from the slope of the Arrhenius plot of ln k (inactivation rate constant) versus 1/T. Although, 005NJ α-L-rhamnosidase activity was lower in comparison to other reported cold-active enzymes for temperatures between 0 and 10 °C [1], it conserves the thermosensitive character of cold-active enzymes.

In order to asses the potential for using the enzyme in winemaking, the effect of ethanol concentration on the activity was

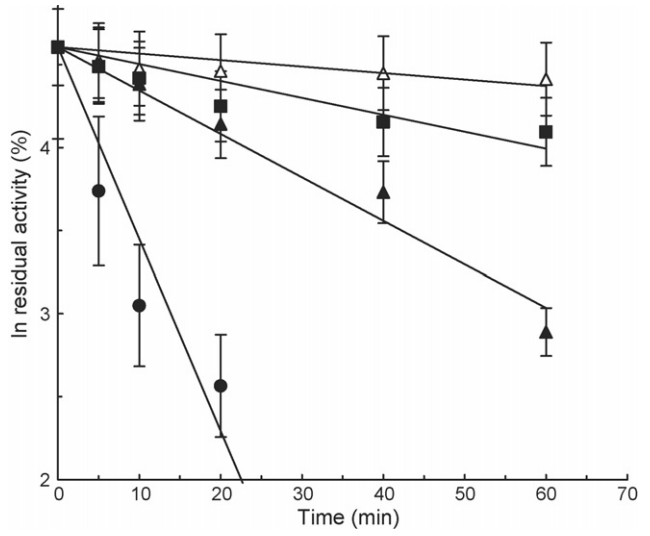

Fig. 7. Thermal inactivation of intracellular α-L-rhamnosidase activity of *Pseudoalteromonas* sp. 005NJ at (△) 35 °C, (■) 40 °C, (▲) 45 °C and (●) 50 °C. The 100% activity corresponds to 351 U/ml.

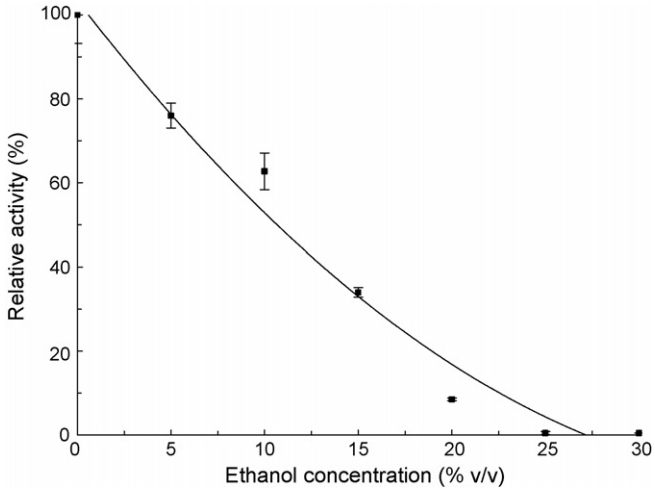

Fig. 8. Effect of ethanol concentration on the α-L-rhamnosidase activity of *Pseudoalteromonas* sp. 005NJ. The 100% activity corresponds to 331 U/ml.

assayed (Fig. 8). When 12% (v/v) ethanol was added to the reaction mixture, α-L-rhamnosidase 005NJ activity decreased, presenting 52% of its original activity in water based solvent. Similar inhibitions have been reported for other α-L-rhamnosidases [6,10], although α-L-rhamnosidases from *Aspergillus terreus* and *P. angusta*, showed a decrease of only 20% in the same ethanol concentration [29,7]. Nevertheless, taking into account the fact that biotransformation of wine does not require the hydrolysis of the whole aroma precursors [30], the susceptibility of the 005NJ α-L-rhamnosidase to ethanol and temperature should not be considered as a disadvantage. The features of 005NJ α-L-rhamnosidase activity at low temperature (5 °C), and its low activity and stability (data not shown) in 12% (v/v) ethanol, could be useful during an enzymatic process since these features may avoid the need for further enzyme removal procedures.

4. Concluding remarks

The aroma and flavour of wine is partly influenced by non-volatile compounds held together by bonds that are broken down by glycosidases. These enzymes are naturally occurring in grapes and yeast but are largely inactive at physico-chemical conditions of winemaking, and are highly variable between and within harvests. Microbial glycosidases are still a field of great interest for many laboratories around the world. These enzymes can improve food quality and several that prove to be efficient are now in use or are being developed. The α-L-rhamnosidase activity of the strain *Pseudoalteromonas* sp. 005NJ appears to be an interesting system from an applied point of view and our results indicate that it could be useful in low temperature food processing procedures.

Acknowledgments

We extend our thanks to Dr. Juan Miquel of Marine Environmental Laboratory, International Atomic Energy Agency,

Monaco, and Elke Noellemeyer, Facultad de Agronomía UNLPAM, Argentina. This work was supported by CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, PIP6205, Sub. 1699/05), Fundación Antorchas and Agregaduría Científica de la Embajada de Italia en Argentina.

References

[1] Russell NJ. Molecular adaptations in psychrophilic bacteria: potential for biotechnological applications. Adv Biochem Eng Biotechnol 1998;61:1–21.
[2] Russell NJ. Toward a molecular understanding of cold activity of enzymes from psychrophiles. Extremophiles 2000;4:83–90.
[3] Gianese G, Argos P, Pascarella S. Structural adaptation of enzymes to low temperatures. Protein Eng 2001;14:141–8.
[4] Ferrer M, Chernikova TN, Yakimov MM, Golyshin PN, Timmis KN. Chaperonins govern growth of *Escherichia coli* at low temperatures. Nat Biotechnol 2003;21:1266–7.
[5] Caldini C, Bonomi F, Pifferi PG, Lanzarini G, Galante Y. Kinetic and inmobilization studies on fungal glycosidases for aroma enhancement in wine. Enzyme Microb Technol 1994;16:286–91.
[6] Orejas M, Ibáñez E, Ramón D. The filamentous fungus produces *Aspergillus nidulans* produces an α-L-rhamnosidase of potential oenological interest. Lett Appl Microbiol 1999;28:383–8.
[7] Yanai T, Sato M. Purification and characterization of an α-L-rhamnosidase from *Pichia angusta* X349. Biosci Biotechnol Biochem 2000;64:2179–85.
[8] Manzanares P, de Graaff H, Visser J. Purification and characterization of an α-L-rhamnosidase from *Aspergillus niger*. FEMS Microbiol Lett 1997;157:279–83.
[9] Manzanares P, van den Broeck HC, de Graaff LH, Visser J. Purification and characterization of two different α-L-rhamnosidases, RhaA and RhaB, from *Aspergillus aculeatus*. Appl Environ Microbiol 2001;67:2230–4.
[10] Spagna G, Barbagallo R, Martino A, Pifferi P. A simple method for purifying glycosidases: α-L-rhamnopyranosidase of *Aspergillus niger* to increase the aroma of Moscato wine. Enzyme Microb Technol 2000;27:522–30.
[11] Sellek GA, Chaudhuri JB. Biocatalysis in organic media using enzymes from extremophiles. Enzyme Microb Technol 1999;25:471–82.
[12] Paoni N, Arroyo R. Improved method for detection of glycosidases in bacterial colonies. Appl Environ Microbiol 1984;47:208–9.
[13] Martinez MA, Delgado OD, Breccia JD, Baigori MD, Siñeriz F. Revision on taxonomic position of the xylanolytic *Bacillus* sp. MIR32 strain reidentified as *Bacillus halodurans* and plasmid mediated transformation of *B. halodurans* species. Extremophiles 2002;6:391–5.
[14] Miake F, Satho T, Takesue H, Yanagida F, Kashige N, Watanabe K. Purification and characterization of intracellular α-L-rhamnosidase from *Pseudomonas paucimobilis* FP2001. Arch Microbiol 2000;173:65–70.
[15] Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of proteins utilizing protein-dye binding. Anal Biochem 1976;72:248–54.
[16] Gürtler V, Stanisich VA. New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. Microbiology 1996;142:3–16.
[17] Holmström C, Kjelleberg S. Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. FEMS Microb Ecol 1999;30:285–93.
[18] Hernandez NE, Tereschuk ML, Abdala LR. Antimicrobial activity of flavonoids in medicinal plants from Tafi del Valle (Tucuman, Argentina). J Ethnopharmacol 2000;73:317–22.
[19] Havsteen VH. The biochemistry and medical significance of the flavonoids. Pharmacol Therapeut 2002;96:67–202.
[20] Middelboe M, Nielsen TG, Bjørnsen PK. Viral and bacterial production in the North Water: in situ measurements, batch-culture experiments and characterization and distribution of virus-host system. Deep Sea Res II 2002;49:5063–79.
[21] Brinkmeyer R, Knittel K, Jurgens J, Weyland H, Amann R, Helmke E. Diversity and structure of bacterial communities in Arctic versus Antarctic pack ice. Appl Environ Microbiol 2003;69:6610–9.
[22] Ivanova EP, Sawabe T, Lysenko A, Gorshkova N, Hayashi K, Zhukova N, et al. *Pseudoalteromonas translucida* sp. nov. and *Pseudoalteromonas paragorgicola* sp. nov., and emended description of the genus. Int J Syst Evol Microbiol 2002;52:1759–66.
[23] Kobayashi T, Imada C, Hiraishi A, Tsujibo H, Miyamoto K, Inamori Y, et al. *Pseudoalteromonas sagamiensis* sp. nov., a marine bacterium that produces protease inhibitors. Int J Syst Evol Microbiol 2003;53:1807–11.
[24] Nazarenko EL, Komandrova NA, Gorshkova RP, Tomshich SV, Zubkov VA, Kilcoyne M, et al. Structures of polysaccharides and oligosaccharides of some Gram-negative marine Proteobacteria. Carbohydr Res 2003;338:2449–57.
[25] Bonnin E, Dolo E, Le Goff A, Thibault JF. Characterization of pectin subunits released by an optimised combination of enzymes. Carbohydr Res 2002;337:1687–96.
[26] Cui W, Mazza G. Physicochemical characteristics of flaxseed gum. Food Res Int 1996;29:397–402.
[27] Hongshan Y, Jinmei G, Chunzhi Z, Fengxie J. Purification and characterization of ginsenoside-α-L-rhamnosidase. Chem Pharmaceut Bull 2002;50:175–8.
[28] Srguleng Q, Hongshan Y, Chunzhi Z, Mingchun L, Hongying W, Fengxie J. Purification and characterization of dioscin-α-L-rhamnosidase from pig liver. Chem Pharmaceut Bull 2005;53:911–4.
[29] Gallego MV, Pinaga F, Ramon D, Valles S. Purification and characterization of an alpha-L-rhamnosidase from *Aspergillus terreus* of interest in winemaking. J Food Sci 2001;66:204–9.
[30] Van Rensburg P, Pretorius IS. Enzymes in winemaking: harnessing natural catalysts for efficient bio-transformations. S Afr J Enol Viticult 2000;21 [special issue].