ISSN 1330-9862
(FTB-2322)

original scientific paper

Selection of Psychrotolerant Microorganisms Producing Cold-Active Pectinases for Biotechnological Processes at Low Temperature

*María S. Cabeza[1,2], Fanny L. Baca[2], Ernesto Muñoz Puntes[2], Flavia Loto[1,3], Mario D. Baigorí[1,3] and Vilma I. Morata[1,2]**

[1]National Council of Scientific and Technical Research (CONICET), Av. Rivadavia 1917, C1033AAJ, Buenos Aires, Argentina

[2]Biotechnology Laboratory, Biology and Food Department, Faculty of Applied Industrial Science, Bernardo de Irigoyen 375, AR-5600, San Rafael, Mendoza, Argentina

[3]Pilot Plant of Industrial and Microbiological Processes (PROIMI), Av. Belgrano and Pje. Caseros 4000, AR-4000 Tucumán, Argentina

Received: July 23, 2009
Accepted: April 28, 2010

Summary

In winemaking, low temperatures are favourable for the production and retention of flavour and colour components, requiring the use of cold-active enzymes. For this reason, 'psychrotolerant' microorganisms have been isolated and selected based on their ability to produce pectinolytic enzymes with satisfactory activity at low temperatures. Different mature grape varieties with designation of origin were sampled from the region of San Rafael (Mendoza, Argentina), and pectinolytic bacterial, fungal and yeast strains were isolated. The pectinolytic activity was measured by cup-plate assay, quantification of released reducing sugars and viscosity reduction of pectin solution. Two bacteria (*Bacillus* sp. SC-G and SC-H) and two yeast strains were selected for their good pectinase activity at low temperatures. Among them, the strain with the highest activity, *Bacillus* sp. SC-H, was selected. According to their 16S rRNA profiles, *Bacillus* sp. SC-G and SC-H can be classified as members of *Bacillus subtilis*. Among the assayed techniques, the rotary evaporation was found to be the most appropriate to obtain enzymatic extracts with highest activity. The optimal conditions for the enzymatic activity were 30 °C and pH=5.0 for the concentrated extract, and 45 °C and pH=6.0 for the filtered supernatant. The concentrated extract presented good activity at 3 °C, confirming that it was a cold-active enzyme. Natural extraction and enzymatic preparation were used to extract pigments and polyphenols from Malbec grapes. Better results were obtained for the enzymatic extract with regard to index, shade, CIELab coordinates, CIELab colour differences and polyphenols (measured using Folin-Ciocalteu).

Key words: pectinase, cold-active enzymes, low temperature, *Bacillus*, pigment and polyphenol extraction

Introduction

Pectinases are used in numerous industrial processes like extraction and clarification of wines and fruit juices, fruit maceration, reduction of juice viscosity, extraction of vegetable oils, and fermentation of coffee and tea (*1*). Pectinolytic enzymes can be classified into two main groups: pectinesterases (PE), able to de-esterify pectins by removal of methoxyl residues, and depolymerases, which readily split the main chain of pectin. The

*Corresponding author; E-mail: vmorata@fcai.uncu.edu.ar

depolymerizing enzymes are divided into polygalacturonases (PG), enzymes that cleave the glycosidic bonds by hydrolysis, and lyases (PL), which break the glycosidic bonds by β-elimination. In addition, the latter two enzymes are classified on the basis of whether they exhibit a preferential hydrolytic power against pectin, pectic acid or oligogalacturonate as the substrate and whether the pattern of action is random (endo-) or terminal (exo-) (*2*).

Pectinolytic enzymes can be used in three steps during vinification: (*i*) during the grape crushing, to increase the volume of free-run juice and to reduce the pressing time; (*ii*) in must before or after its fermentation, to settle out many suspended particles; and (*iii*) to ensure clarification and filtration of wine (*1*). These enzymes facilitate the release and solubility of phenolic compounds from the skin cells. Increased extraction of phenolic compounds, such as tannins, leads to an increased formation of polymeric pigments in aged red wines, resulting in a more intense colour and stability (*3*). Enzymatic degradation of cell wall contributes to the release of the glycosidically-bound terphenols from berries. Hydrolysis of these precursors during fermentation can result in an improved wine aroma (*4*).

It is important to keep low temperatures (10–15 °C) during winemaking, because this increases the production and retention of volatile flavour compounds, improving the aromatic profile of wine (*5–7*). Winemaking under these conditions requires pectinolytic enzymes that can act within this temperature range both for extraction and clarification. Therefore, it is important to find cold-active enzymes (*8,9*).

Within mesophilic microorganisms, some strains are adapted to growth at low temperatures, like psychrophilic microorganisms. They produce molecules with more flexible structures that can flow under this condition. The strains able to grow at or below 7 °C (*i.e.* strains that grow at low temperature) are termed psychrotolerant (psychrotrophic) (*10*). Some studies were done on microorganisms able to produce cold-active pectinolytic enzymes, such as psychrophilic yeasts (*11–13*) and *Mrakia frigida* (*14*), but this alternative would not be useful for the industrial enzyme production due to the need to carry the fermentation at low temperatures, implicating elevated costs.

The present study has been undertaken with the aim to isolate native microorganisms from natural sources in San Rafael, province of Mendoza, Argentina, able to produce pectinases with good activity at 15 °C or below. Microorganism classification was done using morphologic, metabolic and molecular assays. Different methods to isolate the enzyme from the culture supernatant were assayed as well as an activity profile in function of the temperature and pH. Besides, the activity of enzyme during the extraction of grape pigment and polyphenols was studied.

Materials and Methods

Isolation and selection of strains

Isolation of microorganisms that produce pectinolytic enzymes was carried out with grapes from San Rafael, Mendoza, Argentina. Fourteen plates were inoculated as follows: two mature grapes belonging to the same variety were exhaustively washed with 2 mL of peptone water. Yeast extract peptone dextrose (in g/L of distilled water: yeast extract 10, peptone 20, glucose 20, agar 20) agar plates were inoculated with 0.3 mL of the washing fluid and incubated at 30 °C for 5 days. Then 30 colonies were taken from each plate and subcultured on a specially designed medium that contained pectin. The composition (in g/L) was as follows: pectin 2, yeast extract 1, meat peptone 10, soy peptone 10, agar 10, KH_2PO_4 0.2, $CaCl_2$ 0.05, $(NH_4)_2SO_4$ 3, $MnSO_4{\cdot}7H_2O$ 0.05, $FeSO_4{\cdot}7H_2O$ 0.015, $MgSO_4{\cdot}7H_2O$ 0.8 at pH=5. Each sample was cultured on two Petri dishes, one incubated at 15 °C and the other at 30 °C for 5 days. Then the plates were stained with Lugol's solution for detection of a clearance halo formed around the colonies (*15*). The ratio between the halo diameter and the corresponding colony diameter was measured in order to obtain the most productive strain. The reference strain used in this study was *Bacillus pumilus* 29^T, provided by the Spanish Type Culture Collection (CECT, Valencia, Spain).

Microbial taxonomy

Preliminary classification of microorganisms was done using microscopy and Gram staining. Gram-positive, catalase-positive, aerobic or facultatively anaerobic, rod-shaped and endospore-forming bacteria were identified as *Bacillus* strains. Specific morphological and biochemical assays according to Sneath (*16*) were applied in order to identify and determine different characteristics of the species: round spores, swollen sporangium, parasporal crystals, catalase production, anaerobic growth, Voges-Proskauer test (V-P), pH in V-P broth, acid from D-glucose, L-arabinose, D-xylose and D-mannitol, gas from glucose, hydrolysis of casein, gelatin and starch, formation of dihydroxyacetone, growth in nutrient broth at pH=6.8 or 5.7, growth in 2, 5 and 7 % NaCl, growth at 5, 10, 30, 40, 50, 55 or 65 °C.

Bacterial isolates were grown on nutrient agar plates. Surface bacterial growth was resuspended by gentle mechanical agitation in 2 mL of sterile physiological solution. The bacterial suspension was used to inoculate 50 CHB API strips (bioMérieux, Craponne, France). This system enabled the study of the metabolism of 49 carbohydrates and allowed to identify *Bacillus* using API 50 CHB medium in approx. 48 h. The strips were checked at 24 and 48 h and the results were analyzed using the Jacquard coefficient (*17*):

$$s_{ij} = \frac{a}{a+b+c} \qquad /1/$$

where s_{ij} is the similarity coefficient, a is the number of times that both observations are positive (medium acidification), b is the number of times that the observation i is positive and the observation j is negative, c is the number of times that the observation i is negative and the observation j is positive, i is the studied *Bacillus* strain and j is the *Bacillus* strain with which it is compared. This data is available in an identification table (v. 3.0) provided by bioMérieux.

Chromosomal DNA isolation using cetyl trimethylammonium bromide (CTAB)

Saghai-Maroof *et al.* (*18*) protocol was followed, with modifications. A volume of 2 mL of 6-hour cultures was centrifuged for 2 min. The pellet was resuspended in 750 μL of cetyl trimethylammonium bromide extraction buffer and 2 μL of β-mercaptoethanol. It was incubated at room temperature for 30 min and turned over periodically. A volume of 750 μL of chloroform:isoamylic alcohol mixture (24:1) was added. It was centrifuged for 10 min at maximal speed. The supernatant was removed and 0.5 mL of cold isopropanol was added. It was mixed and incubated at 4 °C for 1 h, and turned over periodically. Then, it was centrifuged for 30 min at maximal speed in cold. The supernatant was discarded and the pellet was washed with 0.5 mL of cold ethanol (80 %). After that, it was incubated for 15 min at –20 °C, and then centrifuged for 30 min at maximal speed in cold. The supernatant was discarded and the pellet was dried until ethanol completely evaporated. A volume of 100 μL of double distilled water and 1 mL of RNAse were added. The mixture was conserved at 4 °C.

PCR amplification of the 16S rDNA

PCR reaction was done using a set of universal primers: 27F and 1492R. The reaction volume was 25 μL, from which 20 μL corresponded to the reaction mix (14.4 μL of double distilled sterile water, 5 μL of buffer, 0.2 μL of dNTP, 0.1 μL of primer 27, 0.1 μL of primer 1492 and 0.2 μL of Taq Polymerase) and 5 μL of the template DNA to amplify. A PerkinElmer 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) was used and the amplification program was: 94 °C for 5 min (1 cycle); 94 °C for 1 min, 50 °C for 2 min, 72 °C for 2 min (30 cycles); 72 °C for 7 min, hold at 4 °C.

The electrophoretic runs were done in 0.8 % agarose gels with TAE 1X buffer at 75 V. The dying was made with ethidium bromide for 30 min. A 100-bp DNA ladder (Invitrogen Corporation, Carlsbad, CA, USA) was used as molecular mass marker. DNA was screened using the Molecular Imager Gel Doc XR System (Bio-Rad Laboratories, Hercules, CA, USA).

Sequence analysis

Sequence analysis was made by Macrogen (Seoul, Korea) and edited using Chromas v. 1.55 (Technelysium, Helensvale, Australia) and DNAMAN v. 4.03 (Lynnon Corp, Pointe-Claire, Canada) softwares. The sequences of approx. 1500 bp of the 16S DNAr genes were analyzed for homologies with BLASTN program (*19*) available on NCBI server (National Center of Biotechnology Information, Bethesda, MD, USA, *http://www.ncbi.nlm.nih.gov*), allowing the determination of a relationship with *Bacillus* spp. species.

Enzyme characterization

Pectinolytic activity was assayed by physical (viscosimetry) and chemical methods: cup-plate assay, using 0.03 % (*m*/*V*) ruthenium red for staining; quantification of released reducing sugars, using 3,5-dinitrosalicylic acid (DNS) (*20*) and a pectin solution prepared with 0.25 % pectin from citrus peel (degree of esterification approx. 60 %, Fluka, Buchs, Switzerland) in sodium acetate buffer, pH=5, as substrate. A standard curve was prepared using different concentrations of galacturonic acid (Sigma-Aldrich, St. Louis, MO, USA). The enzymatic reaction mixture consisting of 0.45 mL of substrate and 0.05 mL of enzyme solution was incubated at 15 °C (or 30 °C) for 30 min. The reaction was stopped by adding 0.5 mL of DNS reagent and kept in a boiling water bath for 15 min. After cooling, distilled water (1.5 mL) was added and the absorbance was measured at 530 nm (*21*). Non-enzymatic destruction of the substrate (pectin) and reducing sugars in the enzymatic extract were taken into account by making a blank with identical composition to the enzymatic reaction mixture and without incubating it before the addition of DNS reagent. One unit of pectinase activity was defined as the amount of enzyme required to liberate 1 μmol of reducing sugar per min at 15 °C (or 30 °C). The same treatment was applied to the commercial enzymes (25 mg of solid enzyme in 1 mL of acetate buffer, pH=5): pectinase from *Aspergillus niger* (Fluka BioChemica, Buchs, Switzerland), Inozyme® (Institut Oenologique de Champagne, Epernay, France), Endozym® Éclair (AEB Group, Pascal Biotech®, Paris, France).

Change in viscosity of a pectin solution due to pectinase action was measured using a rotational viscometer, Brookfield HBDV-III®, and an Ultra Low Adapter (ULA) (*22*). First, viscosity of different concentrations of pectin solution (which also contained sucrose and citrate buffer, pH=3.5) was measured to obtain a standard curve. The sample volume was 16 mL and the shear rate 100 rpm. Subsequently, changes in viscosity of different pectin solutions which contained 80 μL of culture supernatant were determined. Viscosity values were transformed into pectin concentrations using the standard curve previously obtained. Enzymatic units were then calculated for each enzyme extract (*Bacillus* sp. SC-G and SC-H) and temperature (15 and 30 °C), using the following equation:

$$U = \frac{c(\mathrm{IPC}) - c(\mathrm{FPC})}{t} \qquad /2/$$

where U is the enzymatic unit (% degraded pectin/min), c(IPC) is the initial pectin concentration, c(FPC) is the final pectin concentration (residual pectin concentration after the action of enzyme extract) and t is the time of enzymatic action (20 min).

Lyase activity was assayed spectrophotometrically by measuring the formation of unsaturated products from polygalacturonic acid (pectate lyase activity) or pectin (pectin lyase activity) at 232 nm (*23*). The standard assay mixture contained 0.2 % substrate (Sigma-Aldrich) in a final volume of 3 mL of 50 mM glycine buffer (pH=10.0) containing 0.5 mM $CaCl_2$. Assay mixtures were incubated for 2.5 min at 50 °C. One unit of enzymatic activity was defined as the amount of enzyme that produces 1 μmol of unsaturated product per min under the assay conditions described. The absorption coefficient of the unsaturated galacturonic acid was 4.600 L/(mol·cm).

Pectin methyl esterase activity was followed by the method presented by Moyo *et al.* (*24*), using a 1 % pectin solution in 0.1 M NaCl, adjusting the pH to 7.5 (using

0.5 M NaOH). To 20 mL of this solution, 10 μL of the enzymatic sample were added, keeping the pH at 7.5 during 30 min with 0.02 M NaOH. One unit of enzymatic activity was defined as the amount of enzyme that releases 1 μmol of carboxylic groups per min under the assay conditions described.

Protein content was quantified by the method of Bradford (*25*), where Coomassie Brilliant Blue G-250 dye binds to proteins. The absorbance was measured at 595 nm. Bovine serum albumin was used as standard, ranging from 0.1 to 1 mg/mL.

The filtrate was concentrated at 45 °C under reduced pressure, using a rotary evaporator. The concentrated extract was stored at –20 °C until use.

Temperature and pH effect on the enzymatic activity

The optimal pH for the enzyme activity was determined by conducting the pectinase assay at various pH values ranging from 3.5 to 11. The optimum temperature for pectinase activity was determined by carrying out the standard assay (optimal pH) at temperatures ranging from 3 to 65 °C. In each case, the substrate was preincubated at the desired temperature for 10 min.

Pigment and polyphenol extraction from grape skin

Assays were performed using skins (without pulp and seeds) from Malbec grapes, from San Rafael region, obtained in 2008 vintage.

The berries were manually pressed and the skins were separated from the pulp and seeds. The skins were cut and opened to better expose their internal side, where the colorant was found and to increase the area contact with the extracting solution. The fragmented skins (1.65 g) were introduced in assay tubes. A volume of 2.5 mL of extracting solution was added, corresponding to a constant volume of concentrated enzymatic extract from *Bacillus* sp. SC-H (150 μL) and sodium acetate buffer (pH=3.5). The blank corresponded to the natural extraction (maceration treatment without enzyme). The skins were macerated and shaken at 130 rpm for 2 h at 15 °C. Then, they were centrifuged at 5000×*g* for 10 min.

Following parameters were calculated using the supernatants: (*i*) colour index according to Glories (*26*): sum of absorbance at 420, 520 and 620 nm; (*ii*) shade according to Sudraud (*27*): ratio of absorbance at 420 nm to that at 520 nm; (*iii*) CIELab coordinates using the MSCV program (*28*): to obtain L* (lightness), C* (chroma), H* (hue), a* (red colour intensity measurement) and b* (yellow colour intensity measurement), using absorbance at 420, 520, 570 and 630 nm; (*iv*) CIELab colour differences (ΔE*):

$$\Delta E^*_{r,s}=[(\Delta L^*_{r,s})^2+(\Delta a^*_{r,s})^2+(\Delta b^*_{r,s})^2]^{1/2} \quad /3/$$

where

$$\Delta L^*_{r,s}=(L^*_r-L^*_s) \quad /4/$$

$$\Delta a^*_{r,s}=(a^*_r-a^*_s) \quad /5/$$

$$\Delta b^*_{r,s}=(b^*_r-b^*_s) \quad /6/$$

r is the natural extraction and s is the studied supernatant; and (*v*) total polyphenolic content (TPC) was done following the Folin-Ciocalteu technique using gallic acid as standard (*29*). All determinations were made in triplicate.

Results and Discussion

Isolation and selection

Strains that presented the largest ratio between clarification haloes (pectinolytic activity) and colony diameters were chosen for further analyses (Fig. 1). Consequently, two bacteria and two yeasts with pectinase activity at 15 °C were selected from about 400 strains.

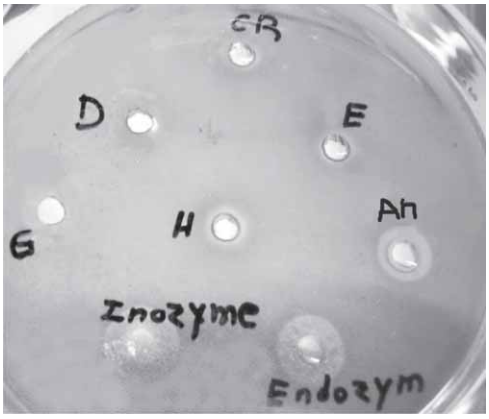

Fig. 1. Hydrolysis haloes after pectinolytic activity in polygalacturonate agar with different extracts and commercial enzymes. Ruthenium red was used for staining. CR – *Bacillus pumilus* CECT 29T, D – yeast SC-D, E – yeast SC-E, G – *Bacillus* sp. SC-G, H – *Bacillus* sp. SC-H, An – pectinase from *Aspergillus niger* (Fluka BioChemica, Buchs, Switzerland), Inozyme (Institut Oenologique de Champagne, Epernay, France), Endozym – Endozym Éclair (AEB Group, Pascal Biotech, Paris, France)

The four selected strains were good cold-active pectinase producers, particularly *Bacillus* sp. SC-H. Yeast strains were stored together with starters for further studies on their enzyme production capacity in the vinification process.

Strain taxonomy

The results of the morphological and metabolic tests employed in classic taxonomy of *Bacillus* sp. SC-G and SC-H were compared with those known for different species of *Bacillus* (*16*). The two selected bacteria presented a great similarity with *Bacillus subtilis*, because all the assayed tests presented results similar to this one. The profile of *B. licheniformis* was much related to *B. subtilis*, the essential difference being the anaerobic growth, which is negative in *B. subtilis* and positive in *B. licheniformis.* The two studied strains did not present an anaerobic growth; consequently, they did not belong to this species.

An API 50CH phenotypic system was applied, using the Jacquard coefficient. As seen in Table 1, it allowed the classification of microorganisms as members of *Bacillus subtilis* cluster, and with greater similarity to *Bacillus amyloliquefaciens* species. Priest *et al.* (*30*) stated that *B. amyloliquefaciens* did not degrade pectin or cellulose, presenting an important difference between the two studied strains that degrade these two substrates.

As it can be seen, *Bacillus* species could not be classified using the API system (low Jacquard coefficients pre-

Table 1. Statistical characterization of *Bacillus* sp. SC-H and SC-G by Jacquard coefficient among the *B. subtilis*

Bacillus subtilis	Jacquard coefficient	
	Bacillus sp. SC-H	*Bacillus* sp. SC-G
B. amyloliquefaciens	**0.77**	**0.58**
B. licheniformis	0.70	0.53
B. subtilis	0.75	0.55
B. pumilus	0.56	0.54
Bacillus sp. SC-H	1.00	0.76
Bacillus sp. SC-G	0.76	1.00

sented in Table 1), because not all *Bacillus* species are included in the data base provided by bioMérieux (Craponne, France).

Almost complete sequences were determined for the 16S rRNA gene of *Bacillus* SC-G (1430 nucleotides) and SC-H (1464 nucleotides). These sequences were included in the GenBank with accession numbers FJ626870 (*31*) and FJ626869 (*32*), respectively. From the homology analysis using BLASTN, the maximal scores corresponded to *Bacillus* species, as seen in Fig. 2. Particularly, *Bacillus* sp. SC-H was more similar to *B. mojavensis*.

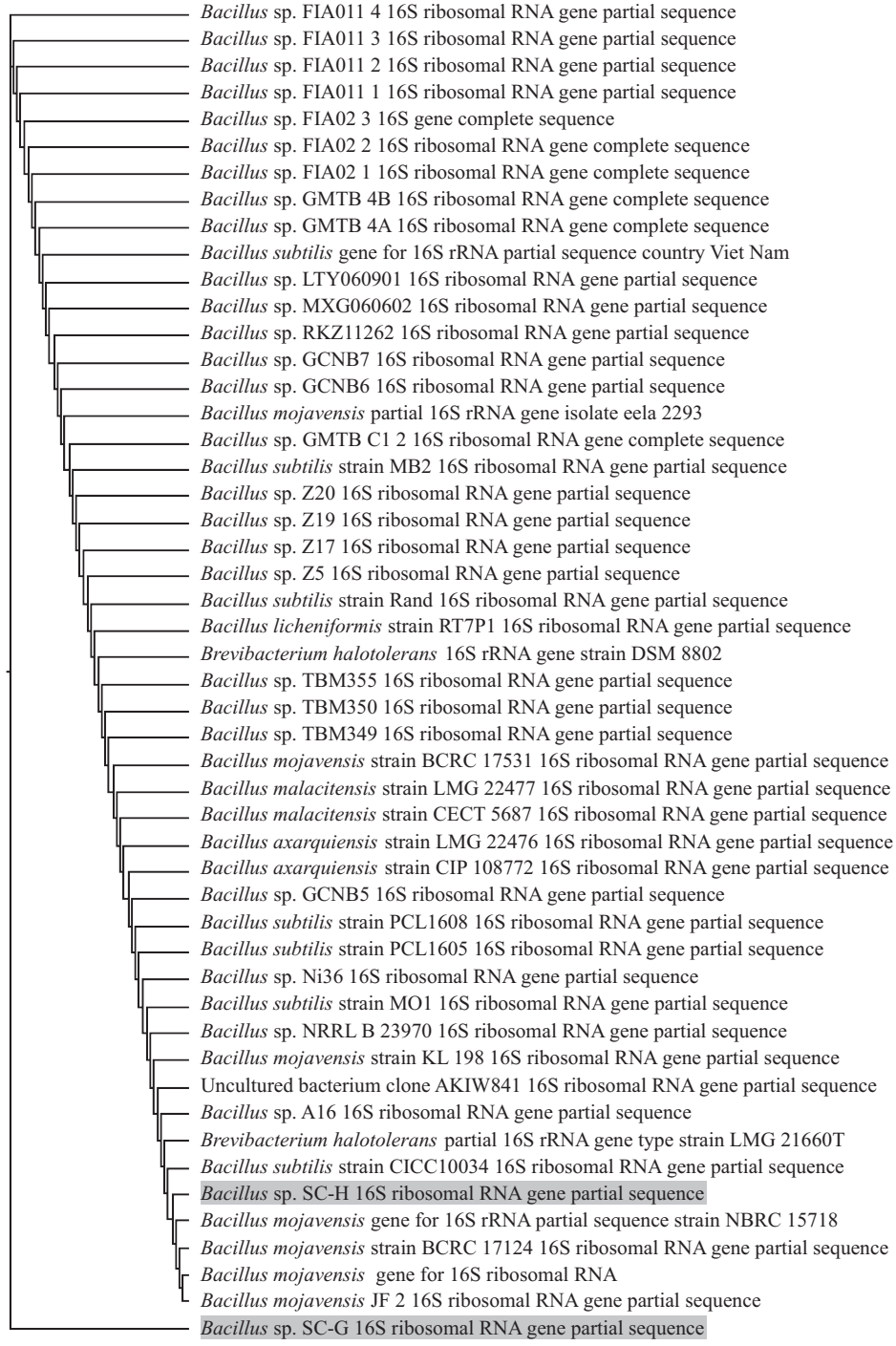

Fig. 2. Neighbour Joining tree (max seq difference: 0.75) based on partial 16S rRNA nucleotide sequences

Pectinolytic activity

Analysis of the enzyme activity was done by quantification of the released reducing sugars with DNS of selected and commercial extracts (Table 2). The evaluation of the activity in the selected strains was done in culture supernatants that were not prepurified. These maximal values for hydrolase activities were comparable to the ones observed by Soriano *et al.* (*33*) for *Bacillus* sp. BP-7 on different substrates (at 40 °C), polygalacturonic acid 0.05 U/mL (pH=5.0) and pectin 0.10 U/mL (pH=7.0).

After the primary selection that resulted in 4 strains, a secondary selection was carried out using the criterion of the highest activity at 15 °C, assessed with the reducing sugar method (DNS) (Table 2). It can be observed that the highest activity levels at 15 and 30 °C corresponded to *Bacillus* sp. SC-H. It can be emphasized that the rotary evaporator concentrate from *Bacillus* sp. SC-H achieved greater activities than commercial extracts in the assayed concentrations, both at 15 and 30 °C. The specific activities also showed great differences in favour of *Bacillus* sp. SC-H extracts. Fig. 1 shows the results of various extracts and commercial enzymes on a polygalacturonate agar plate after staining with ruthenium red, where different halo diameters can be observed.

Determination of polygalacturonase (PG) activity through quantification of reducing sugars can detect both exo- and endo-PG activity. Exo-PG releases small fragments from the polymer and does not reduce viscosity significantly.

Assessment of the activity applying viscosimetry showed that the values at 15 °C (3.4 and 3.3 mU/mL for *Bacillus* sp. SC-G and *Bacillus* sp. SC-H, respectively) were superior to the ones observed at 30 °C (2.7 and 0.9 mU/mL for *Bacillus* sp. SC-G and *Bacillus* sp. SC-H, respectively). Endo-PG activity is characterized by a substantial decrease in viscosity (in general 50 %), while the release of reducing sugars is 1–3 %, as shown in literature (*2*).

The selected enzymatic extracts showed a good endo-pectinase activity at 15 °C. This is favourable for industrial uses since the viscosity decrease is faster by a random mechanism of hydrolysis on the pectin molecule. This efficiency in pectin hydrolysis indicated a greater effect both on must pectin reduction and on the extraction of pigments and polyphenols.

Most comercial pectinase preparations are a mixture of different pectinolytic enzymes. The efficient degradation of polysaccharides in food industry requires a cooperative or sinergic interaction between the enzymes that are responsible for splitting different linkages in pectin structure. Pectin methylesterases (PME) and PG are known to operate in tandem to degrade the methylesterified polyuronides (*34*).

The PME measured in the filtered extract and rotary concentrate from *Bacillus* sp. SC-H was (3.03±0.73) and (17.98±0.19) U/mL, respectively. As it can be seen, the enzymatic activity increased 5.4 times, so this concentration method did not affect the studied enzyme. In other studies similar or lower values than the filtered extract of *Bacillus* sp. SC-H were found, *e.g.* 5.7 U/mL for *Aspergillus japonicus* 586 (*35*), 1 U/mL for *Aspergillus foetidus* and 0.75 U/mL for *Aspergillus niger* (*36*), and 8.1 U/mL for *Botrytis cinerea* Bd90 (*37*).

The lyase activity of the concentrated extract was (2.7±0.4) mU/mL (pectin) and (0.7±0.1) mU/mL (polygalacturonic acid). The enzymatic extract had enzyme(s) that used preferentially pectin as substrate and it was very inferior to that found in literature: 0.02 U/mL of pectate lyase and 0.36 U/mL of pectin lyase in *Bacillus* sp. BP-7 (*33*). It is remarkable that pH assays were very different from those for musts (no activity was detected at pH=3.5).

Enzyme concentration by rotary evaporation

Using this technique, the volume of enzyme preparation was concentrated 5.3-fold (270 to 51 mL). The re-

Table 2. Pectinase activity of selected and reference strains culture supernatants and of commercial enzymes, using the DNS (3,5-dinitrosalicylic acid) method

Plate number (Fig. 1)		Pectinase activity (DNS)* U/mL, Temperature/°C		γ(proteins) mg/mL	Specific activity U/mg, Temperature/°C	
		15	30		15	30
CR	*Bacillus pumilus* CECT 29T	0.11	0.00	0.06	1.98	0.07
D	yeast SC-D	0.01	0.04	0.07	0.13	0.55
E	yeast SC-E	0.04	0.13	0.02	2.05	6.30
G	*Bacillus* sp. SC-G	0.03	0.05	0.04	0.75	1.35
H	*Bacillus* sp. SC-H	0.08	0.18	0.02	3.24	7.04
An	pectinase from *Aspergillus niger***	0.14	0.58	0.18	0.74	3.13
Inozyme	Inozyme**	0.13	0.13	0.14	0.91	0.89
Endozym	Endozym Éclair**	0.06	0.04	0.15	0.38	0.29
–	Rotary evaporator concentrate from *Bacillus* sp. SC-H	0.45	0.93	0.16	2.74	5.73

*amount of enzyme required to release 1 μmol of galacturonic acid per minute under the given assay conditions
**25 mg of solid enzyme in 1 mL of acetate buffer, pH=5

sults are shown in Table 2. The enzymatic activity increased 5.4 times, both at 15 and 30 °C, due to a tiny augmentation in the enzymatic activity. Thus, this concentration technique is more appropriated since the enzyme catalytic capacity was kept intact.

Temperature and pH effect on pectinolytic activity

Fig. 3 shows both extracts (filtered and concentrated), which were active in a wide range of pH, starting their enzymatic activity above pH=3.5. Analyzing the filtrate, it can be seen that two maxima were achieved, one at pH=6.0 (optimum) and a weaker one, at pH=9.0. Analogous to this, extract concentrated using rotary evaporator also presented two maxima, one at pH=5.0 (optimum) and the other at pH=10.0, probably due to changes in the protein during concentration. This is due to the fact that two or more enzymes are present in the extracts. The presence of different maxima was also observed by Mathew *et al.* (*38*), when they optimized the *Penicillium* SPC-F 20 culture, a thermostable polygalacturonase producer. Other studied pectinases showed great diversity in their optima: 8.0 for *Bacillus* sp. DT7 (*39*), 9.1 for *B. subtilis* WSHB04-02 (*40*), 10.5 for *B. pumilus* dcsr1 (*41*), 10 for PL and 8.5 for PME from *B.* KSM-P358 (*42*).

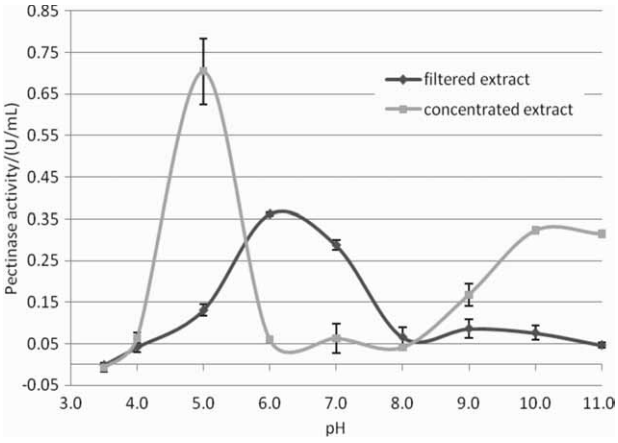

Fig. 3. Optimal pH for pectinase activity from *Bacillus* sp. SC-H. Vertical bars: standard deviation

Fig. 4 shows a series of peaks of enzyme activity at different temperatures. Again, the presence of different pectic enzymes (or isoforms) in the extracts can be observed. The optimal temperatures were 45 and 30 °C for the filtered and the concentrated extract, respectively. It could be observed that the filtered extract obtained from *Bacillus* SC-H had the activity of 0.18 and 0.12 U/mL at 45 and 30 °C, respectively, whereas at 10 °C, the activity was 0.06 U/mL, corresponding to approx. 36 % of the maximum activity. This is in accordance with the objective of this work to achieve good pectinolytic activities at low temperature. The extract concentrated in rotary evaporator showed activity at 3 °C (0.32 U/mL), which corresponded to approx. 34 % of the enzymatic activity observed at 30 °C (0.93 U/mL) for the same sample. This activity at low temperatures was comparable to the polygalacturonase from psychrophilic yeast strain, *Cystofilobasidium capitatum* PPY- 1, found by Nakagawa *et al.* (*11*), whose activity at 5 °C was 50 % of the activity at 20 °C. On the other hand, the pectinolytic activity of the concentrated extract at 15 °C was 0.45 U/mL, which was approx. 48 % of the activity at the optimal temperature, although in the concentration protocol temperature of 45 °C was applied, which indicates a good thermal stability of the enzymatic extract at this temperature.

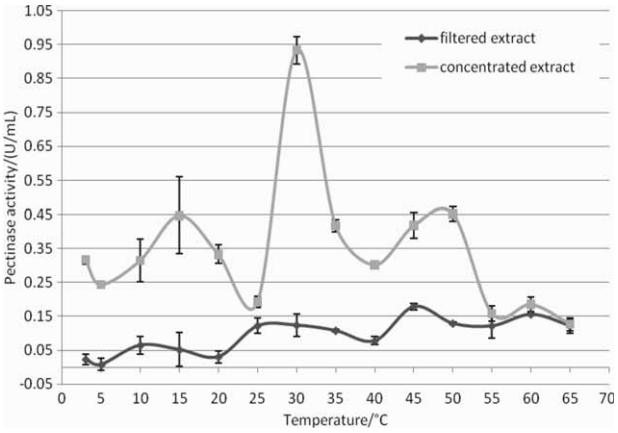

Fig. 4. Optimal temperature for pectinase activity from *Bacillus* sp. SC-H. Vertical bars: standard deviation

Bacillus subtilis SC-H was able to grow at temperatures below 7 °C. Therefore, the microorganism could be considered a 'psychrotolerant' mesophilic species. The fact that the producer of the cold-active enzyme is a mesophilic instead of psychrophilic microorganism is an advantage for industrial production since incubation during the production process is at room temperatures.

Pigment and polyphenol extraction from grape skins

Observing the values shown in Table 3, it can be concluded that colour index and TPC increased notably when *Bacillus* SC-H extract was used. This confirms that the selected enzymatic extract did help in releasing possible cell wall-bound phenolics. Shade did not show statistical differences. The data are in agreement with recent

Table 3. Extraction ability of pigments and polyphenols from Malbec grape skin with extract concentrated from *Bacillus* sp. SC-H in rotary evaporator

	Natural extraction	*Bacillus* sp. SC-H extraction
Colour index	(4.64±0.11)^a	(6.12±0.26)^b
Shade	(0.51±0.05)^a	(0.53±0.02)^a
TPC	(494.00±11.90)^a	(539.90±12.70)^b
L*	(73.80±0.60)^a	(70.20±1.50)^a
C*	(35.57±0.55)^a	(41.43±0.96)^b
H*	(354.70±0.49)^a	(356.80±1.56)^a
a*	(35.43±0.51)^a	(41.36±0.91)^b
b*	(–3.26±0.35)^a	(–2.33±1.18)^a
ΔE*	–	7.0

Different superscript letters in each row mean statistically significant differences ($p<0.05$). ΔE*: CIELab colour difference

results obtained in wine making experiments, where the addition of pectinolytic enzyme preparations at the vinification step also resulted in increased levels of phenolic substances – notably low molecular mass phenols and flavan-3-ol derivatives – in the resulting wines (*43*).

Analyzing the obtained values for the CIELab coordinates, the extract greatly improved the chroma (C*, which includes the contribution of a* and b* in the total colour) and the red colour intensity measurement (a*). Regarding the lightness (L*), hue angle (H*), parameter influenced particularly by yellow pigments that absorb light at about 420 nm, and yellow colour intensity measurement (b*), statistical differences were not observed. The resulting colour conferred a desired characteristic (greater red colour intensity than yellow), since during wine ageing the procyanidin polymerization caused the increase of the yellow component in the colour (*44*) and simultaneously anthocyanidins were either oxidized, which implicated a decrease of the red component of the colour, or combined with procyanidins, stabilizing the red colour (*45*).

Different chromatic characteristics can be detected in a wine glass if the CIELab differences are greater than or equal to 2.7 (*46*). Therefore, enzymatic treatment allowed obtaining a markedly detectable colour difference.

Very good results were achieved with cold maceration, because it improved the extraction of pigments, which increased the colour intensity and/or shade (*47*); tannins (*48*); phenols and polysaccharides, which increased the mouth feel (*49*); and fruit flavour (*50,51*), from grape skins to wine. Cold maceration involved keeping must at low temperatures, typically 0–10 °C during seven to ten days with relatively high SO_2 concentration to allow an aqueous extraction before heating and inoculation for the posterior fermentation (*49*). The cold-active pectinases achieved in this work would be very useful to enhance the extraction of juice and various compounds from grape berry in cold maceration.

Conclusions

The obtained results confirm that *Bacillus* sp. SC-H, a psychrotolerant mesophilic species, is a good producer of cold-active pectinases. It would be possible to add the enzymatic extract during the extraction stage in winemaking and probably to other processes used to extract different compounds from vegetables, especially if low temperatures were used. Methanol levels should be controlled in bottled wines due to the high PME activity. It is important to emphasize that a better extraction of red pigments than yellow ones in grapes was achieved, conferring a very desired and valued characteristic to red wines.

Acknowledgements

This work was financially supported by UNCuyo, No. 06/L036, and CONICET, PEI 6137 and PIP 6472. The authors would also like to thank CECT (Spanish Collection of Type Culture, Valencia) for providing the reference strains.

References

1. D.R. Kashyap, P.K. Vohra, S. Chopra, R. Tewari, Applications of pectinases in the commercial sector: A review, *Bioresour. Technol. 77* (2001) 215–227.
2. P. Blanco, C. Sieiro, T.G. Villa, Production of pectic enzymes in yeasts, *FEMS Microbiol. Lett. 175* (1999) 1–9.
3. A. Ganga, F. Piñaga, A. Querol, S. Vallés, D. Ramón, Cell-wall degrading enzymes in the release of grape aroma precursors, *Food Sci. Technol. Int. 7* (2001) 83–87.
4. B.W. Zoecklein, J.E. Marcy, J.M. Williams, Y. Jasinsky, Effect of native yeasts and selected strains of *Saccharomyces cerevisiae* on glycosyl, glucose potential, volatile terpenes and selected aglycones of white Riesling (*Vitis vinifera*) wines, *J. Food Compos. Anal. 10* (1997) 55–65.
5. E. Killian, C.S. Ough, Fermentation esters-formation and retention as affected by fermentation temperature, *Am. J. Enol. Vitic. 30* (1979) 301–305.
6. R.E. Kunkee, Selection and modification of yeasts and lactic acid bacteria for wine fermentation, *Food Microbiol. 1* (1984) 315–322.
7. A.M. Molina, J.H. Swiegers, C. Varela, I.S. Pretorius, E. Agosin, Influence of wine fermentation temperature on the synthesis of yeast-derived volatile aroma compounds, *Appl. Microbiol. Biotechnol. 77* (2007) 675–687.
8. R. Margesin, F. Schinner, Properties of cold-adapted microorganisms and their potential role in biotechnology, *J. Biotechnol. 33* (1994) 1–14.
9. C. Gerday, M. Aittaleb, M. Bentahir, J.P. Chessa, P. Claverie, T. Collins *et al.*, Cold-adapted enzymes: From fundamentals to biotechnology, *Trends Biotechnol. 18* (2000) 103–107.
10. R.R. Meer, J.W. Baker, F.W. Bodyfelt, M.W. Griffiths, Psychrotrophic *Bacillus* ssp. in fluid milk products: A review, *J. Food Protect. 54* (1991) 969–979.
11. T. Nakagawa, K. Yamada, T. Miyaji, N. Tomizuka, Cold-active pectinolytic activity of psycrophilic–basidiomycetous yeast *Cystofilobasidium capitatum* strain PPY-1, *J. Biosci. Bioeng. 94* (2002) 175–177.
12. T. Nakagawa, T. Nagaoka, S. Taniguchi, T. Miyaji, N. Tomizuka, Isolation and characterization of psychrophilic yeasts producing cold-adapted pectinolytic enzymes, *Lett. Appl. Microbiol. 38* (2004) 383–387.
13. H. Birgisson, O. Delgado, L. García Arroyo, R. Hatti-Kaul, B. Mattiasson, Cold-adapted yeasts as producers of cold-active polygalacturonases, *Extremophiles, 7* (2003) 185–193.
14. R. Margesin, V. Fauster, P.A. Fonteyne, Characterization of cold-active pectate lyases from psychrophilic *Mrakia frigida*, *Lett. Appl. Microbiol. 40* (2005) 453–459.
15. T.M. Fernandes-Salomão, A.C. Rodrigues Amorim, V.M. Chaves-Alves, J.L.C. Coelho, D.O. Silva, E.F. de Araujo, Isolation of pectinase hyperproducing mutants of *Penicillium expansum*, *Rev. Microbiol. 27* (1996) 15–18.
16. P.H.A. Sneath: Endospore-Forming Gram Positive Rods and Cocci. In: *Bergey's Manual of Systematic Bacteriology, Vol. 2*, J.G. Holt (Ed.), William & Wilkins, Baltimore, MD, USA (1986) pp. 1104–1207.
17. R.R. Colwell, B. Austin: Numerical Taxonomy. In: *Manual of Methods for General Bacteriology*, P. Gerhardt (Ed.), American Society for Microbiology, Washington, DC, USA (1981) pp. 441–449.
18. M.A. Saghai-Maroof, K.M. Soliman, R.A. Jorgensen, R.W. Allard, Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics, *Proc. Natl. Acad. Sci. USA, 81* (1984) 8014–8018.
19. Z. Zhang, S. Schwartz, L. Wagner, W. Miller, A greedy algorithm for aligning DNA sequences, *J. Computat. Biol. 7* (2000) 203–214.

20. G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem. 31* (1959) 426–428.

21. F.S. Kittur, A.B. Vishu Kumar, R.N. Tharanathan, Low molecular weight chitosans–preparation by depolymerization with *Aspergillus niger* pectinase, and characterization, *Carbohydr. Res. 338* (2003) 1283–1290.

22. M. Mutlu, K. Sarioğlu, N. Demir, M.T. Ercan, J. Acar, The use of commercial pectinase in fruit juice industry. Part I: Viscosimetric determination of enzyme activity, *J. Food Eng. 41* (1999) 147–150.

23. A. Collmer, J.L. Ried, M.S. Mount, Assay methods for pectic enzymes, *Methods Enzymol. 161* (1988) 329–335.

24. S. Moyo, B.A. Gashe, E.K. Collison, S. Mpuchane, Optimising growth conditions for the pectinolytic activity of *Kluyveromyces wickerhamii* by using response surface methodology, *Int. J. Food Microbiol. 85* (2003) 87–100.

25. M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72* (1976) 248–254.

26. Y. Glories, The colour of red wines. Part one: The balances of anthocyanins and tannins, *Conn. Vigne Vin, 18* (1984) 195–217 (in French).

27. P. Sudraud, Interpretation of red wine absorption curves, *Anal. Technol. Agric. 7* (1958) 203–208 (in French).

28. F. Ayala, J.F. Echávarri, A.I. Negueruela, Simplified Method for Wine Colour Determination, University of Zaragoza, Zaragoza, Spain (2001) (*http://www.unizar.es/negueruela/descargas/MSCVes.zip*) (in Spanish).

29. J.C. Stoclet, A. Kleschyov, E. Andriambeloson, M. Diebolt, R. Andriantsitohaina, Endothelial no release caused by red wine polyphenols, *J. Physiol. Pharmacol. 50* (1999) 535–540.

30. F.G. Priest, M. Goodfellow, L.A. Shute, R.C.W. Berkeley, *Bacillus amyloliquefaciens* sp. nov., nom. rev., *Int. J. Syst. Bacteriol. 37* (1987) 69–71.

31. M.S. Cabeza, F.V. Loto, M.D. Baigori, V.I. Morata: *Bacillus* sp. SC-G 16S ribosomal RNA gene, partial sequence, FJ626870, GenBank, NCBI, USA (2009) (*http://www.ncbi.nlm.nih.gov/nuccore/223470547*).

32. M.S. Cabeza, F.V. Loto, M.D. Baigori, V.I. Morata: *Bacillus* sp. SC-H 16S ribosomal RNA gene, partial sequence, FJ626869, GenBank, NCBI, USA (2009) (*http://www.ncbi.nlm.nih.gov/nuccore/223470546*).

33. M. Soriano, P. Díaz, F.I.J. Pastor, Pectinolytic systems of two aerobic sporogenous bacterial strains with high activity on pectin, *Curr. Microbiol. 50* (2005) 114–118.

34. K. Wakabayashi, J.P. Chun, D.J. Huber, Extensive solubilization and depolymerization of cell wall polysaccharides during avocado (*Persea americana*) ripening involves concerted action of polygalacturonase and pectin methylesterase, *Physiol. Plant 108* (2000) 345–352.

35. M.F.S. Teixeira, J.L.L. Filho, N. Durán, Carbon sources effect on pectinase production from *Aspergillus japonicus* 586, *Braz. J. Microbiol. 31* (2000) 286–290.

36. R. Grebechova, L. Prieto Contreras, Pectinolytic enzyme biosynthesis from moulds *Aspergillus niger* and *Aspergillus foetidus* for food industrial application, *Revista de Investigación, 6* (2006) 153–162 (in Spanish).

37. P. Reignault, M. Mercier, G. Bompeix, M. Boccara, Pectin methylesterase from *Botrytis cinerea*: Physiological, biochemical and immunochemical studies, *Microbiology, 140* (1994) 3249–3255.

38. A. Mathew, A.N. Eldo, A.G. Molly, Optimization of culture conditions for the production of thermostable polygalacturonase by *Penicillium* SPC-F 20, *J. Ind. Microbiol. Biot. 35* (2008) 1001–1005.

39. D.R. Kashyap, S. Chandra, A. Kaul, R. Tewari, Production, purification and characterization of pectinase from a *Bacillus* sp. DT7, *World J. Microbiol. Biotechnol. 16* (2000) 277–282.

40. Q. Wang, X.R. Fan, Z.Z. Hua, J. Chen, Optimizing bioscouring condition of cotton knitted fabrics with an alkaline pectinase from *Bacillus subtilis* WSHB04-02 by using response surface methodology, *Biochem. Eng. J. 34* (2007) 107–113.

41. D.C. Sharma, T. Satyanarayana, A marked enhancement in the production of a highly alkaline and thermostable pectinase by *Bacillus pumilus* dcsr1 in submerged fermentation by using statistical methods, *Bioresour. Technol. 97* (2006) 727–733.

42. T. Kobayashi, K. Sawada, N. Sumitomo, Y. Hatada, H. Hagihara, S. Ito, Bifunctional pectinolytic enzyme with separate pectate lyase and pectin methylesterase domains from an alkaliphilic *Bacillus*, *World J. Microbiol. Biotechnol. 19* (2003) 269–277.

43. I. Revilla, M.L. González-SanJosé, Compositional changes during the storage of red wines treated with pectolytic enzymes: Low molecular-weight phenols and flavan-3-ol derivative levels, *Food Chem. 80* (2003) 205–214.

44. Y. Glories, J.M. Dournel, Role of ethanal in tannin and anthocyanin complexation, *Rapport des Activités de Recherches 1984–1986*, Institut d'Oenologie de Bordeaux, Université de Bordeaux 2, Talence, France (1986) 86–87 (in French).

45. J.M. Dournel, Research on combinations of anthocyanins-flavonols. Influence of these reactions on the colour of red wines, *PhD Thesis*, Faculté d'Oenologie de Bordeaux, Université de Bordeaux 2, Villenave d'Ornon, France (1985) (in French).

46. A. Negueruela, J. Echávarri, F. Ayala, Colour characteristics, *OIV Feuille Verte No. 1102* (2001) (in French).

47. A. Parenti, P. Spugnoli, L. Calamai, S. Ferrari, C. Gori, Effects of cold maceration on red wine quality from Tuscan Sangiovese grape, *Eur. Food Res. Technol. 218* (2004) 360–366.

48. D. Goumy, M. Couasnon, O. Seze, Wine-making process involving cold maceration step, *French patent FR2731228 A1* (1996) (in French).

49. D. Heatherbell, M. Dicey, S. Goldsworthy, L. Vanhanen, Effect of pre-fermentation cold maceration on the composition, color and flavor of Pinot noir wine, *Proceedings of the 4th International Symposium on Cool Climate Viticulture and Enology*, Cornell University Press, Ithaca, NY, USA (1997) pp. 10–17.

50. I. Álvarez, J.L. Aleixandre, M.J. García, V. Lizama, Impact of prefermentative maceration on the phenolic and volatile compounds in Monastrell red wines, *Anal. Chim. Acta, 563* (2006) 109–115.

51. E. Sánchez Palomo, M.S. Pérez-Coello, M.C. Díaz-Maroto, M.A. González Viñas, M.D. Cabezudo, Contribution of free and glycosidically-bound volatile compounds to the aroma of muscat 'a petit grains' wines and effect of skin contact, *Food Chem. 95* (2006) 279–289.