Efficient recovery of whole cell proteins in Oenococcus oeni*—a comparison of different extraction protocols for high-throughput malolactic starter applications*

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Abstract In this study, we compared different total protein extraction protocols to achieve highly efficient isolation and purification of total proteins for the specific protein profiling of *Oenococcus oeni*. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns obtained for the different extraction protocols revealed not only a qualitative similar protein pattern but also quantitative variations with different intensity bands depending on the extraction method used. The selected extraction method added with sonication proved to work extremely well and efficiently and was able to obtain a high-resolution 2-D electrophoresis (2-DE) map. Prominent spots were successfully identified by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry and corresponded to 76 different proteins involved in the main metabolic pathways. The approach allowed to achieve a protein profiling specific for *O. oeni* from Aglianico wine with numerous characterized protein products corresponding to many different *O. oeni* genes and associated with main cellular pathways. Further investigations of the 2-DE protein expression profile will provide useful and interesting information on the molecular mechanisms at the protein level responsible for growth and survival of *O. oeni* in wine.

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Introduction

Oenococcus oeni has long been reported as the main lactic acid bacteria (LAB) species associated with malolactic fermentation (MLF) (conversion of L-malate to L-lactate and carbon dioxide) that leads to the decrease of the acidity and improves the organoleptic qualities and microbiological stability of wines (Sico et al. 2009). From an evolutionary point of view, *O. oeni* may be considered a specialized microorganism, as it has a small genome of approximately 1.8 Mb (Mills et al. 2005) and occupies a very narrow ecological niche. It is one of the naturally occurring LAB in grape must. As a result of natural selection, thanks to its remarkable tolerance for the fluctuating environmental conditions during alcoholic fermentation, *O. oeni* becomes the dominant species among those triggering MLF (Bon et al. 2009). *O. oeni* has been described as a relatively homogeneous species, and a variety of molecular approaches failed to clearly differentiate strains on a molecular level (Sato et al. 2001; Wydau et al. 2006; Zapparoli et al. 2000).

Discrimination was accomplished only recently, using fine techniques, such as differential display PCR, restriction endonuclease analysis-pulsed-field gel electrophoresis (Lechiancole et al. 2006; Vigentini et al. 2009; Gonzalez-Arenzana et al. 2012; Ruiz et al. 2008; Ze-Ze et al. 2000), and multilocus sequence typing (Delaherche et al. 2006; de Las Rivas et al. 2004) that clearly demonstrated an unexpectedly high level of allelic diversity in *O. oeni.* Recently, Marcobal et al. (2008) elucidated these results demonstrating the hypermutable status in the genus, due to the absence of the mismatch repair (MMR) genes *mutS* and *mutL*. As reported for other bacteria, the lack of *mut* genes results in the accumulation of spontaneous errors in DNA replication, or in reduced stringency in recombination, thus generating high levels of polymorphism (Prunier and Leclercq 2005). More interestingly, the lack of MMR also facilitates the generation

of isolates with beneficial mutations, resulting in increased fitness for the environment. This point is of remarkable importance in selecting *O. oeni* starters (Bon et al. 2009). Recently, winery practices tend to use malolactic starters for direct inoculation in wines even if the induction of MLF by inoculation with commercial strains of *O. oeni* is not always successful (Coucheney et al. 2005), and they are not always able to compete well with the house flora so that their use often results in loss of desirable sensory properties. For this reason, appropriate cultures have to be selected from indigenous microorganisms, more competitive, well adapted to the particular product and to the specific production technology, and with high metabolic capacities which can beneficially affect product quality and safety, preserving their typicity (Bonomo et al. 2011). The development of autochthonous starter cultures for food fermentations is a multidisciplinary endeavor requiring not only an ecological study of the spontaneous process but also characterization of useful technological and physiological features of the predominant strains in order to select those with the highest potential for industrial applications (Ruiz et al. 2008). Intraspecific differentiation is therefore a required preliminary step for the selection of strains because technological characteristics, such as survival and resistance to wine conditions, the rate of L-malic acid consumption, the production of enzymatic activities, and the influence on organoleptic quality, are reported to be strain dependent (Zapparoli et al. 2000; Gomez-Alegrìa et al. 2004; Coucheney et al. 2005). Previous works on technological characterization and intraspecific differentiation of various *O. oeni* strains showed differing results due to the different origin of the strains (Lechiancole et al. 2006), and also, several investigations have been conducted on commercial and indigenous strains of *O. oeni* providing evidence of the potential value and the higher activities of autochthonous which are for the enhancement of specific physicochemical conditions of wine (Barbagallo et al. 2004; Sumby et al. 2009). Lately, there has been growing interest in characterizing *O. oeni* strains that are unique to particular geographical wine regions in order to enhance regionality in the wines (Yanagida et al. 2008; Solieri et al. 2010; Ruiz et al. 2010; Capozzi et al. 2010; Vigentini et al. 2009; Canas et al. 2009; Sico et al. 2008; Bartowsky and Borneman 2011). The physiological and molecular responses of *O. oeni* to withstand stress factors that occur in wine have been widely studied (Bourdineaud et al. 2003; Renouf et al. 2008).

As the genome represents a static view of an organism while proteomics (and transcriptomics) a quantitative and dynamic vision of the expressed DNA and, consequently, of protein variations, a combined knowledge of genome features and specific gene expression is required for better understanding the adaptive mechanisms of *O. oeni* in wine and to develop and to select starter cultures for wine fermentation (Cecconi et al. 2009).

The proteomic approach is proved to be a powerful tool for studying the response of bacteria to environment stresses. For example, it allowed to gain new insights into the fundamentals of LAB stress tolerance (Cecconi et al. 2009). Nevertheless, data concerning *O. oeni* are scarce in proteomics, and research groups, working on the analysis of oenococcal protein patterns, often used different analytical procedures and differential expression investigations, frequently due to *O. oeni* inter-strain diversity, with strain-dependent features and response systems (Cecconi et al. 2009; Michlmayr et al. 2010; Vallet et al. 2009; Silveira et al. 2004).

Therefore, in this study, we compared different total protein extraction protocols to achieve highly efficient isolation and purification of total proteins for the specific protein profiling of *O. oeni*. Subsequently, the effectiveness and the performance of the selected protocol were investigated by 2-D electrophoresis (2-DE) and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-ToF MS) for the analysis of *O. oeni* protein expression patterns.

Materials and methods

Bacterial strain and culture conditions

The strain of *O. oeni* S12 (culture collection of the Department of Sciences, University of Basilicata, Potenza, Italy) used in this study was previously isolated, identified, and characterized from Aglianico wines of Basilicata region (Lechiancole et al. 2006; Sico et al. 2008). The strain was maintained as a freeze-dried stock in reconstituted (11 %*w*/*v*) skim milk, containing 0.1 % (*w*/*v*) ascorbic acid, and routinely cultivated in MRS broth containing 20 % (*v*/*v*) tomato juice (MRS-TJ) and adjusted to pH 4.8 at 30 °C for 72 h, before the analyses.

Protein extraction protocols

For *O. oeni* S12 strain, a subculture in MRS-TJ broth was obtained from the active stock culture by 1 % (*v*/*v*) inoculum and incubation for 72 h at 30 °C. The subculture was standardized to a final A_{600}=1 and used to inoculate the medium used for the following analyses.

Late-exponential phase cells were harvested by centrifugation (12,000 rpm, 5 min), washed three times in phosphate buffered saline (PBS), and resuspended in the lysis buffer, complemented with a protease inhibitor cocktail used at 50 μL/g wet biomass. Six different protein extraction protocols were applied and they consisted of a chemical lysis buffer added with a mechanical treatment. The protocols were as follows: (1) 1 % (*w*/*v*) SDS, 20 % (*v*/*v*) glycerol, 20 mmol/L dithiothreitol (DTT), 50 mmol/L Tris–HCl, pH 7.5 added with sonication treatment; (2) 8 mol/L urea, 4 % (*w*/*v*) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

(CHAPS), 65 mmol/L DTT, 50 mmol/L Tris–HCl, pH 7.5 added with sonication treatment; (3) 2 % (*w*/*v*) hexadecyltrimethylammonium bromide (CTAB), 4 mol/L NaCl, 1 mol/L Tris–HCl, pH 8.0 added with sonication treatment; (4) 0.1 mg/mL lysozyme, 50 mmol/L Tris–HCl, pH 7.5 added with sonication treatment; (5) 2 mg/mL lysozyme, 50 mmol/L Tris–HCl, pH 7.5 added with sonication treatment; and (6) 0.1 mg/mL lysozyme, 50 mmol/L Tris–HCl, pH 7.5 added with ultrasonic water bath treatment. For all protocols (except no. 6), protein extracts were obtained by sonication using a Bandelin Sonoplus HD 200 Apparatus, fitted with an UW 200 probe (Bandelin Electronics, Berlin, Germany) for 10 min with the following settings: power MS 73/D, cycle 20 %, and pulse 10 s. During sonication, the tubes containing the cell suspension were immersed in a circulation water bath at −2 °C to maintain suspensions at <10 °C. As lysis buffers 4, 5, and 6, cells were incubated in ultrasonic water bath (P Sonorex Super 10, Bandelin) with lysozyme at 37 °C for 10 min with ultrasound at 10 % power and 10 min without ultrasound for three times (for a total of 60 min), then subjected to direct protein extraction (protocol 6) or followed by sonication treatment (protocols 4 and 5).

Cell debris was removed from all the samples by centrifugation at 12,000*g*, for 10 min, at 4 °C. The total protein extraction was performed in triplicate. Protein concentration was measured by the Bradford (1976) method and the mean values and the standard deviation were calculated from the data obtained with triplicate trials.

Analysis of whole cell proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

An aliquot (100 μL) of each extract was precipitated in ice-cold acetone, dried in speed-vac, resuspended in the sample buffer of Laemmli (1970), and incubated at room temperature for 30 min. After centrifugation at 13,000*g* for 5 min, the supernatant of each preparation was added to separate wells of a 1-mm-thick gel composed of a 13 % T, 2.67 % C running gel and a 4 % T, 2.67 % C stacking gel. Broad range standard proteins (Bio-Rad Laboratories Srl, Segrate, Italy) were used as molecular mass markers.

Electrophoresis was carried out by using 5× running buffer (15 g/L Tris base, 72 g/L glycine, 5 g/L SDS) and 1× loading buffer (Tris–HCl 62.5 mmol/L, pH 6.8, 25 % glycerol, 2 % SDS, 5 % β-mercaptoethanol, bromophenol blue 0.25 g/L) in a Hoefer SE 600 vertical electrophoresis apparatus at 4 °C for 30 min with a voltage of 200 V and then for 5 h at 280 V.

After run, gels was stained with a solution 0.1 % Coomassie brilliant blue G-250 in methanol/water/glacial acetic acid 5:5:2, destained in 10 % glacial acetic acid and 30 % methanol in water, and then digitized as 300 dpi, 16-bit TIFF image using the GelDoc XR apparatus and imported to Diversity Database™ (Bio-Rad Laboratories).

2-D electrophoresis (2-DE) analysis

Aliquots (500 μg of proteins) of the protein extracts were treated according to Wessel and Flügge (1984) and resuspended in 250 μL of rehydration solution containing 7 mol/L urea, 2 mol/L thiourea, 2 % (*w*/*v*) CHAPS, 60 mmol/L DTT, and 0.5 % (*v*/*v*) IPG buffer plus a trace of bromophenol blue.

Isoelectric focusing (IEF) was performed on 13 cm IPG DryStrips (Amersham Biosciences) after rehydration at 20 °C for 10 h. IPG DryStrips IEF was carried out in the linear pH gradient of 3–10 using an IPGphor unit (Amersham Biosciences) at 20 °C for a total of 34.450 Vh. IPG strips were then equilibrated in 1 % (*w*/*v*) DTT containing equilibration buffer (6 mol/L urea, 30 % glycerol, 2 % SDS, 50 mmol/L Tris–HCl, pH 8.8) for 20 min and then in the same solution with 4 % (*w*/*v*) iodoacetamide plus a trace of bromophenol blue for 20 min. 2-D electrophoresis was performed on 13 % polyacrylamide gels using the Hoefer SE 600 vertical electrophoresis system. Runs were carried out at 4 °C for 30 min at 200 V and successively for 5 h at 280 V. Low range standard proteins (Bio-Rad Laboratories) were used as molecular mass markers. Gels were stained with Coomassie blue, destained, and digitized as 300 dpi, 16-bit TIFF images using a ScanMaker 9800 XL-Microtek scanner and imported to the ImageMaster 2D Elite software (Amersham Biosciences).

Protein identification by MALDI-ToF mass spectrometry analysis

Protein spots were excised from the 2-DE gels and digested with trypsin (Promega, Madison, WI, USA) overnight at 37 °C, and the peptide mixtures generated were analyzed by MALDI-ToF MS. The extracted tryptic fragments were mixed with the matrix solution, 1 % (*w*/*v*) α-cyano-4-hydroxy-cinnamic acid solution (CHCA), 50 % (*v*/*v*) acetonitrile, and 0.5 % (*v*/*v*) TFA, and analyzed by MALDI-ToF MS. Mass spectra were acquired in positive reflectron mode at 20 kV using an Ettan MALDI-ToF Pro mass spectrometer (Amersham Biosciences) equipped with a UV nitrogen laser (337 nm) with delayed extraction mode and low mass rejection. For each spectrum, 256 single shots were accumulated. Peptide spectra were internally calibrated using two trypsin peptides ($M+H^+$ 842.509, monoisotopic, and $M+H^+$ 2211.104, monoisotopic). Protein identification was performed by the MASCOT search engine (http://www.matrixscience.com), against the NCBI nr protein and Swiss-Prot/TrEMBL databases using peptide mass fingerprinting (PMF). Proteins were identified by using the probability-based MOWSE score, equal to $-10\mathrm{XLog}(P)$, where P is the probability that the observed match is a random event. Protein scores of >67 were considered statistically significant ($p<0.05$) under the selected variables. The parameters used for database search were as follows: (1) taxonomy

group: *Bacteria*; (2) mass tolerance of 0.2 Da; (3) zero missed tryptic cleavage allowed; (4) carboamidomethylation of cysteine (as a fixed modification); and (5) oxidation of methionine (as a variable modification).

Statistical analysis

Statistical analysis was performed using Systat 10.0 for Windows (SPSS, Chicago, IL, USA). Results were evaluated by analysis of variance (ANOVA) followed by Student's *t* test. Means of the values were considered significantly different when $p<0.05$.

Reagents, culture media, and ingredients

Unless otherwise stated, all reagents were obtained from Sigma-Aldrich (Milan, Italy), while bacteriological media and ingredients were obtained from Oxoid Ltd. (Basingstoke, Hampshire, UK).

Results

The changes in protein patterns obtained in *O. oeni* by different extraction protocols were quantitatively analyzed by SDS-PAGE. Since for each sample the same volume of the extract was applied, the intensity of the bands clearly indicates the quantity of proteins extracted, according to the protein concentration assay (Table 1). Since band intensity in one-dimensional SDS-PAGE might be difficult to estimate because of the limitations of the image analysis technique, only bands with similar intensity in all replicates of each protein extract were considered for the analysis.

In Fig. 1, the SDS-PAGE patterns obtained for the different extraction protocols revealed a qualitative similar protein pattern (except for no. 6) characterized by the presence of 35–38 protein bands with molecular masses (MM) ranging from 100 to 8 kDa. On the contrary, quantitative variations of protein patterns were observed with different intensity bands depending on the extraction method used. Extracts with lysis buffers containing SDS or CHAPS/urea (Fig. 1, lanes 1 and 2) showed a greater intensity of bands than the others with a total protein yield of 258.8 and 252.7 μg/mL, respectively. No differences were found in protein content and band intensity among the extract by CTAB detergent (Fig. 1, lane 3) and those obtained by Tris-lysozyme followed by sonication treatment (Fig. 1, lanes 4 and 5), while the last extract (Fig. 1, lane 6) showed a very low band intensity and a protein content of 35.5 μg/mL, due to the milder mechanical (ultrasonic bath) treatment.

These results highlighted that the extraction method was more efficient to obtain a qualitatively fine protein pattern of *O. oeni* consisting of, in the cell lysis, 50 mmol/L Tris–HCl, pH 7.5, added with 0.1 mg/mL of lysozyme, then followed by mechanical sonication treatment. This selected method is more simple, does not require the aid of reagents which lather or crystallize at a very low temperature required for the sonication step, is very easy to prepare and very cheap, and does not require the use of toxic chemical substances.

Moreover, to investigate the effectiveness and the performance of the selected protein extraction method, total protein extract was separated on 2-DE gel covering pH 3–10 and molecular masses of 14 to 116 kDa ranges. 2-DE analysis was performed to examine the behavior of *O. oeni* cells after treatment with the same lysis buffer but added with the sonication or the ultrasonic bath (panels a and b of Fig. 2, respectively). The extraction method added with sonication proved to work extremely well and efficiently. In fact, as shown in Fig. 2, this method was able to obtain a high-resolution 2-DE map, revealing approximately 228±10 (gel a) compared with the milder mechanical method that detects 194±8 spots (gel b). It was evident on gel (a) that the most abundant protein spots (60 %) were distributed in the p*I* region of 4.0–5.5. Regarding molecular mass, the gel proved four different protein groups including 15, 40, 27, and 18 % of the spots distributed in 10 to 30, 31 to 50, 51 to 70, and 71 to 110 kDa ranges, respectively. A total of 140 prominent spots were excised from gel (a) and subjected to MALDI-ToF analysis for protein identification by PMF. One hundred twenty-nine spots were successfully identified and corresponded to 76 different proteins. A complete list of the identified proteins sampled from the 2-DE gel is reported in Table 2. Most of the proteins were involved in different metabolic pathways, such as protein synthesis, amino acid metabolism, and energy and carbohydrate metabolism. Some

Table 1 Protein recovery from *Oenococcus oeni* cells using different protocols

Protocols[a]	1	2	3	4	5	6
Protein (μg/mL)	258.8±13.5	252.7±9.8	86.2±2.9	84.9±3.5	81.5±4.4	35.5±2.6

Protein content was determined using the Bradford method. Values are reported as mean ± SD of three independent experiments ($n=3$). The mean values are significantly different ($p<0.05$) as analyzed by analysis of variance (ANOVA, Student's *t* test)

[a] Protocols: (1) 1 % (*w*/*v*) SDS, 20 % (*v*/*v*) glycerol, 20 mM DTT, 50 mM Tris–HCl, pH 7.5 added with sonication treatment; (2) 8 M urea, 4 % (*w*/*v*) CHAPS, 65 mM DTT, 50 mM Tris–HCl, pH 7.5 added with sonication treatment; (3) 2 % (*w*/*v*) CTAB, 4 M NaCl, 1 M Tris–HCl, pH 8.0 added with sonication treatment; (4) 0.1 mg/mL lysozyme, 50 mM Tris–HCl, pH 7.5 added with sonication treatment; (5) 2 mg/mL lysozyme, 50 mM Tris–HCl, pH 7.5 added with sonication treatment; (6) 0.1 mg/mL lysozyme, 50 mM Tris–HCl, pH 7.5 added with ultrasonic water bath treatment

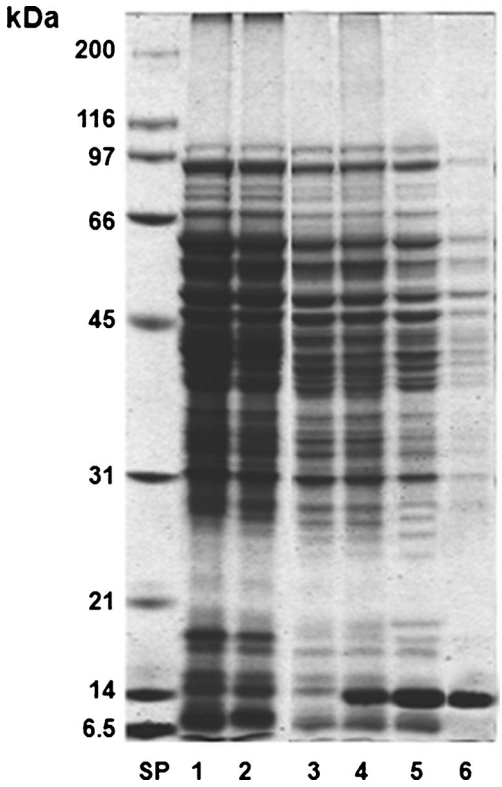

Fig. 1 Whole cell protein patterns obtained by SDS-PAGE (13 % polyacrylamide) for *O. oeni* by using six different protein extraction protocols. *SP*: molecular mass marker; *lane 1*: lysis buffer of 1 % (*w*/*v*) SDS, 20 % (*v*/*v*) glycerol, 20 mM DTT, 50 mM Tris–HCl, pH 7.5 added with sonication treatment; *lane 2*: lysis buffer of 8 M urea, 4 % (*w*/*v*) CHAPS, 65 mM DTT, 50 mM Tris–HCl, pH 7.5 added with sonication treatment; *lane 3*: lysis buffer of 2 % (*w*/*v*) CTAB, 4 M NaCl, 1 M Tris–HCl, pH 8.0 added with sonication treatment; *lane 4*: lysis buffer of 0.1 mg/mL lysozyme, 50 mM Tris–HCl, pH 7.5 added with sonication treatment; *lane 5*: lysis buffer of 2 mg/mL lysozyme, 50 mM Tris–HCl, pH 7.5 added with sonication treatment; *lane 6*: lysis buffer of 0.1 mg/mL lysozyme, 50 mM Tris–HCl, pH 7.5 added with ultrasonic water bath treatment. One hundred microliters of each protein extract was loaded on each lane

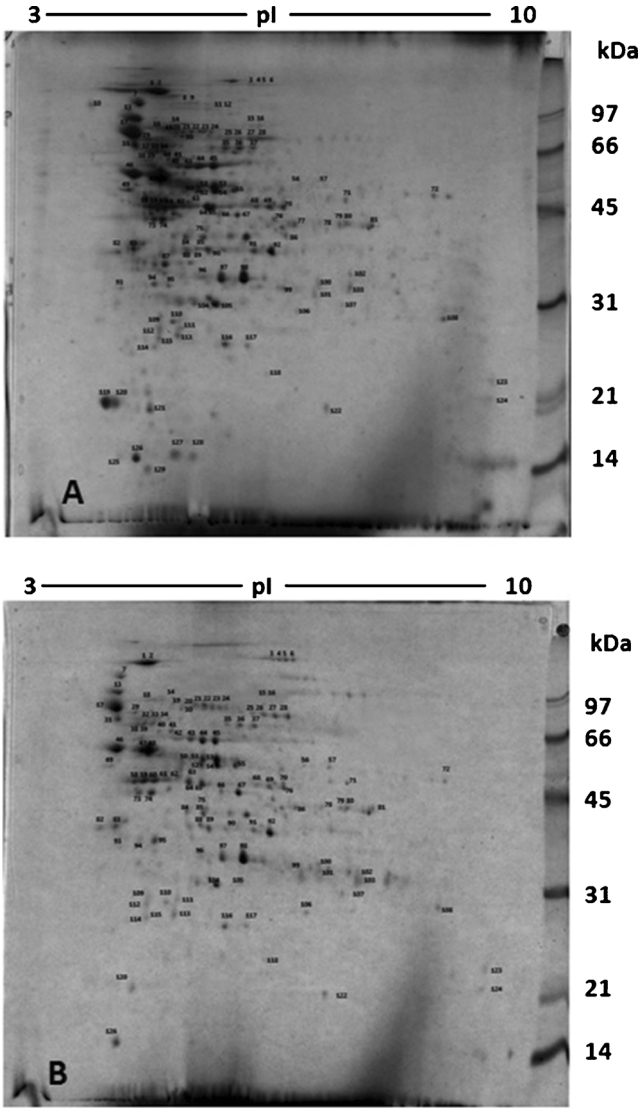

Fig. 2 2-DE analysis of *O. oeni* cells after treatment with the same lysis buffer (0.1 mg/mL lysozyme, 50 mM Tris–HCl, pH 7.5) but added with the sonication (**a**) or the ultrasonic bath (**b**). Five hundred micrograms of the protein extracts were loaded and protein detection was achieved using Coomassie staining. In gel **a**, the proteins identified by MALDI-ToF MS are numbered and listed in Table 2

proteins were present as multiple spots that correspond to multiple isoforms or posttranslational modifications (PTM).

An example of a protein with multiple spots present is shown in Fig. 3; the PMF spectra of the peptides allowed the identification of the stress response membrane GTP-ase as multiple spots (Fig. 2a, spots 8 and 9) due to the posttranslational modifications. As shown in Fig. 3, in the MALDI-ToF mass spectrum (a), corresponding to spot 8 with p*I* of 5.32, a mass signal at *m*/*z* 1,006.58 was detected and attributed to the phosphorylated peptide 444–451 (LHFVAPSR, predicted phosphorylation sites were reported in bold) (http://au.expasy.org/tool/findmod/PHOS.html). In fact, this peak has a mass of 80 Da greater than the calculated mass for peptide 174–192, so this indicates that the peptide was phosphorylated. This signal was absent in mass spectrum (b), corresponding to spot 9 with p*I* of 5.26, in which the nonphosphorylated peptide 444–451 was detected at *m*/*z* 926.58.

Discussion

O. oeni is the main agent of MLF and, therefore, one of the most interesting enological species (Solieri et al. 2010); for this reason, the development of efficient malolactic starters has become one of the main challenges for enological research in recent years (Reguant et al. 2005; Renouf et al. 2008). In enology, biodiversity is closely correlated to habitat and therefore is conditioned from selective factors that inhibit or favor not only the presence of one species over the other but also of a strain or a biotype (Lechiancole et al. 2006). In fact, several reports have shown that the success of starters depends on strain and is influenced by a variety of conditions, including

Table 2 Identification of proteins extracted from *O. oeni* cells

Spot	Protein name	Accession	p*I*	kDa	Function
1, 2	Phosphoketolase	YP-811314	5.08	92.25	Carbohydrate metabolism
3, 6	Bifunctional acetaldehyde-CoA DH	Q04EJ68	6.04	99.15	Energy metabolism
7	Elongation factor G	Q04ED6	4.82	77.86	Protein synthesis
8, 9	Stress response membrane GTP-ase	Q04FP0	5.24	68.23	Stress response
10	Transcription elongation factor	Q04GN3	4.33	52.8	Protein synthesis
11, 12	ATP binding subunit Clp protease	Q04GA6	5.74	78.98	Stress response
13	Molecular chaperone (DNA K)	Q04EE1	4.89	66.2	Stress response
14	Glucosamine-fructose-6P aminotransferase	Q04G45	5.28	66.18	Carbohydrate metabolism
15	mRNA degradation ribonuclease	Q04EK4	5.58	63.06	Nucleotide metabolism
16	Myosin cross-reactive antigen	Q04BD2	5.83	63.93	Stress response
17	Chaperonin GroEL	Q04E64	4.85	57.28	Stress response
18	PEP phosphotransferase	Q04G34	5.11	63.24	Carbohydrate metabolism
19, 20	ATP-synthase	Q04G22	5.26	56.69	Energy metabolism
21, 22	Acetolactate synthase	Q04DC3	5.4	61.29	Amino acid metabolism
23, 27	Pyruvate kinase	Q04F61	5.96	52.30	Energy metabolism
28	Formate-tetrahydrofolate ligase	Q04FS6	6.13	59.45	Nucleotide metabolism
29, 30	F0F1 ATP synthase subunit beta	Q04G20	4.69	50.60	Energy metabolism
31, 32	NAD-dependent aldehyde DH	Q04EF1	5.02	51.53	Energy metabolism
33, 34	Beta-glucosidase	Q04GV7	5.1	55.86	Carbohydrate metabolism
35, 37	Citrate lyase alpha chain	A0NL52	6.05	56.26	Energy metabolism
38, 39	Glucose 6 P-isomerase	Q04G44	4.99	48.66	Carbohydrate metabolism
40, 41	Glutathione reductase	Q04EN7	5.2	48.63	Oxidoreductase activity
42–45	6-Phosphogluconate dehydrogenase	Q04FG3	5.43	53.02	Carbohydrate metabolism
46	Enolase	Q04DH2	4.72	48.45	Energy metabolism
47–48	Elongation factor Tu	Q04FQ4	5.01	43.62	Protein synthesis
49	DNA-direct RNA polymerase alpha subunit	Q04G60	4.73	34.55	Protein synthesis
50–55	Phosphoglycerate kinase	Q04G42	5.49	43.02	Energy metabolism
56	NADPH:quinone reductase	Q04G95	7.03	39.84	Nucleotide metabolism
57	UDP-glucose 6-DH	Q04D91	6.29	43.73	Carbohydrate metabolism
58–60	Alcohol DH	Q04GE6	5.17	40.92	Energy metabolism
61–63	Hypothetical protein OEOE-0344	YP-809892	5.23	41.52	No gene ontology
64–65	Threonine dehydrogenase	Q04BD9	5.48	36.09	Amino acid metabolism
66–67	Phosphoglycerate dehydrogenase	Q04GQ0	5.59	36.55	Energy metabolism
68–70	Cystathionine gamma synthetase	Q04D73	6.02	41.62	Amino acid metabolism
71	*N*-Acetylglucosamine 6-P deacetylase	Q04DA0	6.52	41.71	Carbohydrate metabolism
72	NAD/NADP transhydrogenase alpha-subunit	Q04GT5	8.67	41.02	Oxidoreductase activity
73–74, 88–92	Aldo/keto reductase	Q04HN2	5.15	34.62	Oxidoreductase activity
75	Elongation factor Ts	Q04F87	5.44	31.79	Protein synthesis
76	dTDP-glucose 4,6 dehydratase	Q04E14	5.8	37.46	Dehydratase activity
77, 81	Cystathionine beta synthase	YP-811265	6.98	32.75	Amino acid metabolism
78	Phosphotransacetylase	Q04E26	6.54	35.36	Transferase activity
79–80	Cysteine synthase	ZP-01544102	6.99	32.75	Amino acid metabolism
82	Citrate lyase beta subunit	Q04GP1	4.8	33.31	Lyase activity
83	30S ribosomal protein S2	Q04F88	4.85	28.74	Protein synthesis
84–85	Oxidoreductase	Q04D51	5.32	35.92	Oxidoreductase activity
86	UDP-galactose 4-epimerase	Q04E58			Carbohydrate metabolism
87	Acetoin reductase	Q04FZ3	5.08	27.47	Energy metabolism
93	DNA-binding response regulator	Q04HF8	4.72	25.55	Nucleotide metabolism
94	Phosphate uptake regulator, PhoU	Q04GB6	4.89	25.91	Regulation of phosphate uptake

Table 2 (continued)

Spot	Protein name	Accession	p*I*	kDa	Function
95	DNA-binding response regulator	Q04HF8	4.72	25.55	Nucleotide metabolism
96	Tricorn interacting factor F1	Q04GA3	5.33	34.9	Peptidase
97–98	Short-chain alcohol DH	Q04H46	5.65	27.51	Energy metabolism
99	6-Phosphogluconate DH	Q04FG3	5.43	53.02	Carbohydrate metabolism
100	Glucose-1P-thymidylytransferase	Q04E12	7.74	32.01	Carbohydrate metabolism
101	Uracil phosphoribosyltransferase	Q04DP1	6.6	22.89	Nucleoside/nucleoside synthesis
102–103	Glucose-1P-thymidylytransferase	Q04E12	7.74	32.01	Carbohydrate metabolism
104–105	Phosphoglycerate mutase	Q04HF5	5.5	27.12	Energy metabolism
106	Sigma 54 modulation protein/SSU ribosomal protein S30P	Q0E96	6.2	22.37	Ribosomal protein/binding
107	Dinucleotide binding protein	Q04H44	6.91	22.4	Nucleotide metabolism
108	Redox-sensing transcriptional repressor rex	Q04E62	8.6	24.48	Modulate transcription in response to change in cellular NADH/NAD^+ redox state
109–110	Translation elongation factor P	Q04EK6	5.25	20.5	Protein synthesis
111	Inorganic pirophosphatase	Q04F35	5.22	33.34	Hydrolase/piriphosphatase activity
112	50S ribosomal L11	Q04E42	4.88	20.02	Protein synthesis
113	Alpha-acetolactate decarboxilase	Q04DC2	4.57	26.79	Energy metabolism
114	50S ribosomal L10	Q04E45	5.13	21.17	Protein synthesis
115–116	Flavoprotein	Q04FH9	5.54	20.58	Oxidoreductase activity
117	dTDP-4 dehydrorhamnose 3,5 epimerase	Q04E13	5.73	21.57	Carbohydrate metabolism
118	Universal stress protein	Q04D42	5.85	18.64	Stress response
119–120	DNA binding ferritin like protein (oxidative damage protection)	Q04D79	4.56	18.73	Oxidoreductase activity
121	Heat shock protein (Hsp-20) Lo18 protein	Q04H04 A0NLC2	5.1	16.9	Stress response
122	30 Ribosomal protein S6	Q04HQ7	6.31	16.31	Protein synthesis
123	50S Ribosomal protein L6	Q04G71	9.82	19.26	Protein synthesis
124	50S Ribosomal protein L13	Q04G52	9.95	16.26	Protein synthesis
125, 129	Cellobiose-specific system II B component	Q04GW0	4.8	11.9	Carbohydrate metabolism
126	Co-chaperonin Gro-Es (HSP-10)	Q04E63	4.86	9.72	Stress response
127–128	Methylmalonyl-CoA epimerase	Q04DC1	5.61	16.21	Energy metabolism

Protein spots excised from 2-DE (Fig. 2, gel a) were digested with trypsin and identified by MALDI-ToF MS analysis. Protein identification was performed by the MASCOT (http://www.matrixscience.com), against the NCBI nr protein and Swiss-Prot/TrEMBL databases using peptide mass fingerprinting (PMF)

adaptation to the conditions of production of each wine (Coucheney et al. 2005; Ruiz et al. 2010). Moreover, recent studies of the LAB strain diversity during consecutive vintages have shown the presence of some genotypes in several wineries and suggest the existence of a population of cosmopolitan *O. oeni* strains in a determinate wine-growing region (Izquierdo et al. 2009). Therefore, it is necessary to study previously the representative and best adapted microbiota to the type of wine and winemaking procedures in each elaboration area (Gonzalez-Arenzana et al. 2012).

In a previous work (Lechiancole et al. 2006), the analysis of the expressed genomic fraction of *O. oeni* isolated from Aglianico wines produced in Vulture zone (Basilicata region, Southern Italy) allowed to distinguish and characterize strains from the same wine but made in different wineries, revealing a higher polymorphism related to the origin (winery) of strains and, also, the presence of different biotypes within the same winery. From these outcomes, our study aimed to develop a protein extraction assay to obtain a protein profiling strain specific for *O. oeni* from Aglianico wine.

A rapid, simple, and reproducible protein extraction method was optimized to enhance knowledge of *O. oeni* behavior of a specific winemaking area by an integrated approach based on genotypic observation and proteomic investigation. The most common methods used to prepare whole cell proteins from bacteria are based on the combination of enzymatic or chemical followed by mechanical extraction treatments. We observed the cell reaction to different chemical and mechanical treatments already applied in previous works (Cho et al. 2002; Lehner et al. 2003; Gorg et al. 2004; Arena et al. 2006;

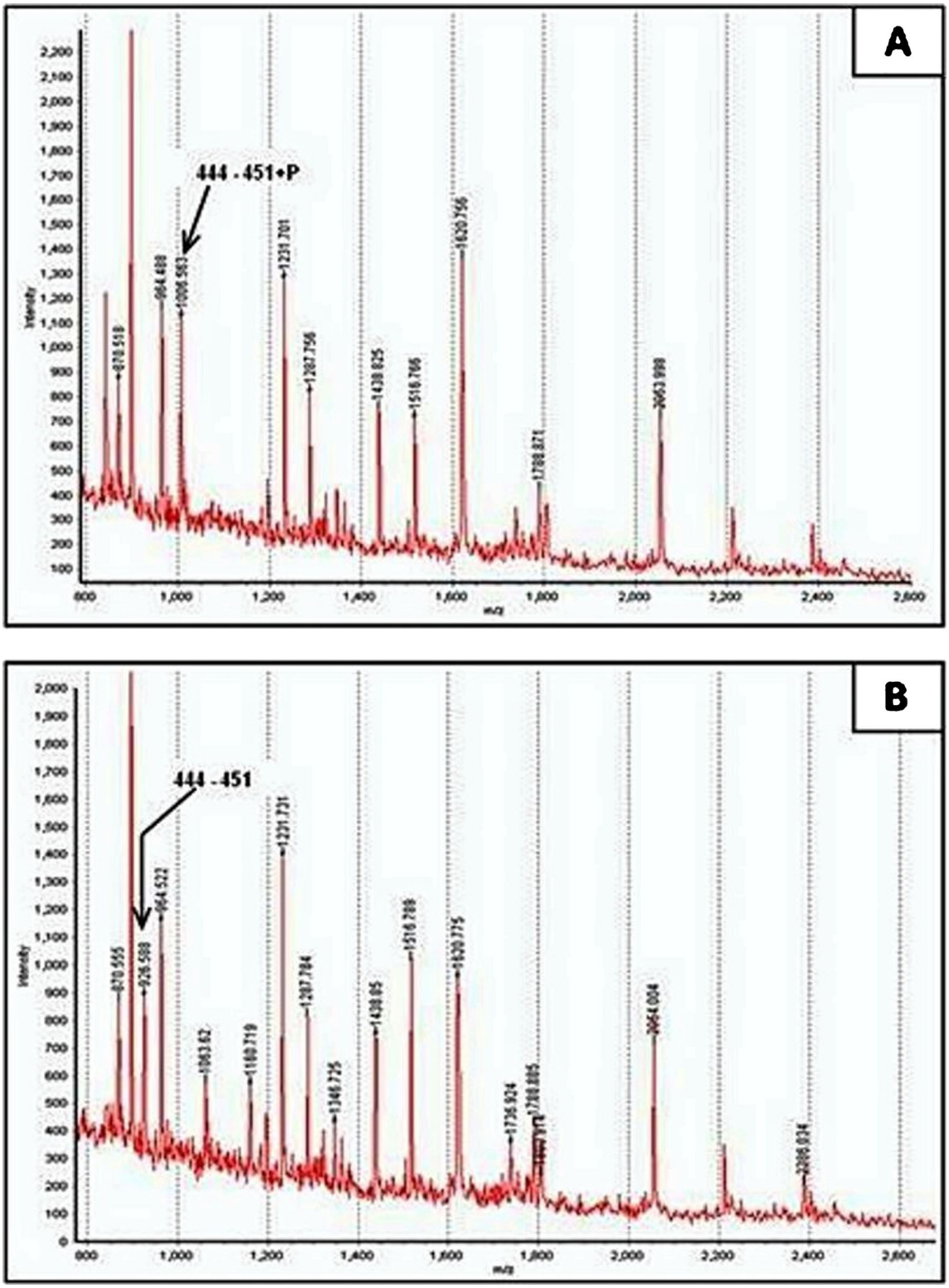

Fig. 3 PMF MALDI-TOF-MS analysis of the tryptic digests from spots 8 and 9 in 2-DE. Mass spectrum **a** corresponds to spot 8 with p*I* of 5.32, in which a mass signal at *m*/*z* 1,006.58 was detected and attributed to the phosphorylated peptide 444–451 (LHFVAP**S**R, predicted phosphorylation sites were reported in bold) (http://au.expasy.org/tool/findmod/PHOS.html). Mass spectrum **b** corresponds to spot 9 with p*I* of 5.26, in which the nonphosphorylated peptide 444–451 was detected at *m*/*z* 926.58. PMF spectra of peptides allowed the identification of the stress response membrane GTP-ase as multiple spots (Fig. 2a, spots 8 and 9) due to the posttranslational modifications

Cecconi et al. 2009; Vallet et al. 2009). As described in these studies, bacterial cells were treated with lysis buffers containing different detergents such as SDS, CHAPS, and CTAB and chaotropic or reducing agents (urea and DTT).

In our study, the comparison of these methods with one of our findings highlighted that the presence of lysozyme in the lysis buffer and the incubation at 37 °C for 60 min in ultrasonic bath before sonication treatment proved to have a good effect on protein extraction, a fine band resolution, and a good quantitative-qualitative correlation. Moreover, the characterization of the protein pattern by a combined strategy (comprising 2-DE and PMF MALDI-ToF MS analysis) allowed to establish that the protein extraction method, developed as specific for *O. oeni* strains of Aglianico del Vulture, works extremely well and efficiently. The use of the enzymatic/chemical treatment followed by mechanical treatment demonstrated that, in our case, the efficiency of protein extraction from *O. oeni* depends on the quality of the cell disruption. The selected extraction method proved to be well performing in terms of good protein yield, concentration, low cost and processing time, and simplicity because of no particular reagents or chemicals are required.

Proteomics offers different opportunities to observe global cellular events by directly visualizing a large part of gene expression products (Arena et al. 2006). In this study, the approach allowed to achieve a protein profiling strain specific for *O. oeni* from Aglianico wine with numerous characterized protein products corresponding to many different *O. oeni* genes and associated with main cellular pathways. Further investigations of the 2-DE protein expression profile will provide useful and interesting information on the molecular mechanisms at the protein level responsible for growth and survival of *O. oeni* in wine.

References

Arena S, D'Ambrosio C, Renzone G, Rullo R, Ledda L, Vitale F, Maglione G, Varcamonti M, Ferrara L, Scaloni A (2006) A study of *Streptococcus thermophilus* proteome by integrated analytical procedures and differential expression investigations. Proteomics 6:181–192

Barbagallo RN, Spagna G, Palmeri R, Torriani S (2004) Assessment of β-glucosidase activity in selected wild strains of *Oenococcus oeni* for malolactic fermentation. Enzym Microb Technol 34:292–296

Bartowsky EJ, Borneman AR (2011) Genomic variations of *Oenococcus oeni* strains and the potential to impact on malolactic fermentation and aroma compounds in wine. Appl Microbiol Biotechnol 92:441–447

Bon E, Delaherche A, Bilhére E, De Daruvar A, Lonvaud-Funel A, Le Marrec C (2009) *Oenococcus oeni* genome plasticity is associated with fitness. Appl Environ Microbiol 75:2079–2090

Bonomo MG, Ricciardi A, Salzano G (2011) Influence of autochthonous starter cultures on microbial dynamics and chemical-physical features of traditional fermented sausages of Basilicata region. World J Microbiol Biotechnol 27:137–146

Bourdineaud JP, Nehmé B, Tesse S, Lonvaud-Funel A (2003) The ftsH gene of the wine bacteria *Oenococcus oeni* is involved in protecting against environmental stress. Appl Environ Microbiol 69:2512–2520

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

Canas PMI, Perez PR, Prieto SS, Herreros MLP (2009) Ecological study of lactic acid microbiota isolated from Tempranillo wines of Castilla-La Mancha. J Biosci Bioeng 108:220–224

Capozzi V, Russo P, Beneduce L, Weidmann S, Grieco F, Guzzo J, Spano G (2010) Technological properties of *Oenococcus oeni* strain isolated from typical southern Italian wines. Lett Appl Microbiol 50:327–334

Cecconi D, Milli A, Rinalducci S, Zolla L, Zapparoli G (2009) Proteomic analysis of *Oenococcus oeni* freeze-dried culture to assess the importance of cell acclimation to conduct malolactic fermentation in wine. Electrophoresis 30:2988–2995

Cho MJ, Jeon BS, Park JW, Jung TS, Song JY, Lee WK (2002) Identifying the major proteome components of *Helicobacter pylori* strain 26695. Electrophoresis 23:1161–1173

Coucheney F, Desroche N, Bou M, Tourdot-Maréchal R, Dulau L, Guzzo J (2005) A new approach for selection of *Oenococcus oeni* strains in order to produce malolactic starters. Int J Food Microbiol 105:463–470

de Las Rivas B, Marcobal A, Munoz R (2004) Allelic diversity and population structure in *Oenococcus oeni* as determined from sequence analysis of housekeeping genes. Appl Environ Microbiol 70:7210–7219

Delaherche A, Bon E, Dupe A, Lucas M, Arveiler B, De Daruvar A, Lonvaud-Funel A (2006) Intraspecific diversity of *Oenococcus oeni* strains determined by sequence analysis of target genes. Appl Microbiol Biotechnol 73:394–403

Gomez-Alegrìa E, Lopez I, Ignacio Ruiz J, Saenz J, Fernandez E, Zarazoga M, Dizy M, Torres C, Ruiz-Larrea F (2004) High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni* strains to lyophilisation and stress environmental conditions of acid pH and ethanol. FEMS Microbiol Lett 230:53–61

Gonzalez-Arenzana L, Lopez R, Santamaria P, Tenorio C, Lopez-Alfaro I (2012) Dynamics of indigenous lactic acid bacteria populations in wine fermentations from la Rioja (Spain) during three vintages. Microb Ecol 63(1):12–19. doi:10.1007/s00248-011-9911-y

Gorg A, Weiss W, Dunn MJ (2004) Current two-dimensional electrophoresis technology for proteomics. Proteomics 4:3665–3685

Izquierdo PM, Ruiz P, Seseña S, Palop ML (2009) Ecological study of lactic acid microbiota isolated from Tempranillo wines of Castilla–La Mancha. J Biosci Bioeng 108:220–224

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lechiancole T, Blaiotta G, Messina D, Fusco V, Villani F, Salzano G (2006) Evaluation of intra-specific diversities in Oenococcus oeni through analysis of genomic and expressed DNA. Syst Appl Microbiol 29:375–381

Lehner I, Niehof M, Borlak J (2003) An optimized method for the isolation and identification of membrane proteins. Electrophoresis 24:1795–1808

Marcobal AM, Sela DA, Wolf YI, Makarova KS, Mills DA (2008) Role of hypermutability in the evolution of the genus *Oenococcus*. J Bacteriol 190:564–570

Michlmayr H, Schümann C, Wurbs P, Barreira Braz da Silva NM, Rogl V, Kulbe KD, del Hierro AM (2010) A β-glucosidase from *Oenococcus oeni* ATCC BAA-1163 with potential for aroma release in wine: cloning and expression in *E. coli*. World J Microbiol Biotechnol 26(7):1281–1289

Mills DA, Rawsthorne H, Parker C, Tamir D, Makarova K (2005) Genomic analysis of *Oenococcus oeni* PSU-1 and its relevance to winemaking. FEMS Microbiol Rev 29:465–475

Prunier AL, Leclercq R (2005) Role of *mutS* and *mutL* genes in hypermutability and recombination in *Staphylococcus aureus*. J Bacteriol 187:3455–3464

Reguant C, Carreté R, Constantí M, Bordons A (2005) Population dynamics of Oenococcus oeni strains in a new winery and the effect of SO_2 and yeast strain. FEMS Microbiol Lett 246:111–117

Renouf V, Delaherche A, Claisse O, Lonvaud-Funel A (2008) Correlation between indigenous *Oenococcus oeni* strain resistance and the presence of genetic markers. J Ind Microbiol Biotechnol 35:27–33

Ruiz P, Izquierdo PM, Sesena S, Palop ML (2008) Intraspecific genetic diversity of lactic acid bacteria from malolactic fermentation of Cencibel wines as derived from combined analysis of RAPD-PCR and PFGE patterns. Food Microbiol 25:942–948

Ruiz P, Izquierdo PM, Sesena S, Palop ML (2010) Selection of autochthonous *Oenococcus oeni* strains according to their oenological properties and vinification results. Int J Food Microbiol 137:230–235

Sato H, Yanagida F, Shinohara T, Suzuki M, Suzuki K, Yokotsuka K (2001) Intraspecific diversity of *Oenococcus oeni* isolated during red wine-making in Japan. FEMS Microbiol Lett 202:109–114

Sico MA, Bonomo MG, Salzano G (2008) Isolation and characterization of *Oenococcus oeni* from Aglianico wines. World J Microbiol Biotechnol 24:1829–1835

Sico MA, Bonomo MG, D'Adamo A, Bochicchio S, Salzano G (2009) Fingerprinting analysis of *Oenococcus oeni* strains under stress conditions. FEMS Microbiol Lett 296:11–17

Silveira MG, Baumgartner M, Rombouts FM, Abee T (2004) Effect of adaptation to ethanol on cytoplasmic and membrane protein profiles of *Oenococcus oeni*. Appl Environ Microbiol 70:2748–2755

Solieri L, Genova F, De Paola M, Giudici P (2010) Characterization and technological properties of Oenococcus oeni strains from wine spontaneous malolactic fermentations: a framework for selection of new starter cultures. J Appl Microbiol 108:285–298

Sumby KM, Matthews AH, Grbin PR, Jiranek V (2009) Cloning and characterization of an intracellular esterase from the wine-associated lactic acid bacterium *Oenococcus oeni*. Appl Environ Microbiol 75:6729–6735

Vallet A, Santarelli X, Lonvaud-Funel A, de Revel G, Cabanne C (2009) Purification of an alcohol dehydrogenase involved in the conversion of methional to methionol in *Oenococcus oeni* IOEB 8406. Appl Microbiol Biotechnol 82:87–94

Vigentini I, Picozzi C, Tirelli A, Giugni A, Foschino R (2009) Survey on indigenous *Oenococcus oeni* strains isolated from red wines of Valtellina, a cold climate wine-growing Italian area. Int J Food Microbiol 136:123–128

Wessel D, Flügge UI (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem 138:141–143

Wydau S, Dervyn R, Anba J, Dusko Ehrlich S, Maguin E (2006) Conservation of key elements of natural competence in *Lactococcus lactis* ssp. FEMS Microbiol Lett 257:32–42

Yanagida F, Srionnual S, Chen YS (2008) Isolation and characteristics of lactic acid bacteria from koshu vineyards in Japan. Lett Appl Microbiol 47(2):134–139

Zapparoli G, Reguant C, Bordons A, Torriani S, Dellaglio F (2000) Genomic DNA fingerprinting of *Oenococcus oeni* strains by pulsed-field gel electrophoresis and randomly amplified polymorphic DNA-PCR. Curr Microbiol 40:351–355

Ze-Ze L, Tenreiro R, Paveia H (2000) The *Oenococcus oeni* genome: physical and genetic mapping of strain GM and comparison with the genome of a "divergent" strain, PSU-1. Microbiology 146:3195–3204