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ORIGINAL ARTICLE

Use of organo-silica immobilized bacteria produced in a pilot scale plant to induce malolactic fermentation in wines that contain lysozyme

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Abstract The exploitation of organo-silica immobilized lactic acid bacteria (LAB) to perform malolactic fermentation (MLF) in wine is described. The immobilization of a large amount of *Oenococcus oeni* cell culture was achieved by a two-step process in an original pilot plant. Cells are entrapped in Ca-alginate microbeads, coated with an organo-silica membrane obtained by two treatments: the first a sol suspension of tetraetoxysilane, the second using methyltriethoxysilane in gas phase. The resulting material improves the physico-chemical features of alginate, avoids cell leakage during fermentation, and protects the cells from antimicrobial compounds. In MLFs carried out at the microvinification scale, the activity of immobilized cells did not differ from that of free cells, and no differences were found in the chemical composition of the wines obtained. The use of immobilized bacteria allowed: (1) simultaneous alcoholic and malolactic fermentations in must inoculated with free yeast and immobilized bacteria; (2) the sequential MLF of three wine lots with the same biomass of immobilized bacteria; (3) the achievement of MLF in a wine with lysozyme added to suppress wild LAB and their potential spoilage.

Keywords Immobilized bacteria · Wine malolactic fermentation · Serial fermentations · Lysozyme · Organo-silica materials

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Introduction

Wine production can appear quite refractory to biotechnology novelties, due to a general suspicion of innovation, in particular in bio-assisted operations. Nonetheless, the use of immobilized microorganisms in winemaking has recently received some attention because it offers many advantages: it improves the fermentation kinetics, allows semi-continuous processes, simplifies cell inoculation and removal, and increases the tolerance of microorganisms to inhibitory substances present in the medium (Maicas 2001). Industrial applications of microbial cells entrapped in organic matrices, attached to the surface of carriers, or confined in membrane reactors have been patented (Dives and Cachon 2005; Kourkoutas et al. 2002). Materials involved in cell immobilization must be safe, biocompatible, cheap, and easy to handle (Groboillot et al. 1994). Among the different organic matrices proposed, Ca-alginate appears particularly suitable for cell encapsulation, due to its easy management and food-grade characteristics; however, its poor mechanical resistance discourages its industrial exploitation in wine fermentations (Maicas 2001).

A suitable solution was recently proposed: a composite material was used to entrap yeast (*Saccharomyces cerevisiae*) or bacteria (*Oenococcus oeni*). The features of Ca-alginate

microbeads were improved through the deposition on their surface of a siliceous membrane made by the conjugation of two different alkoxides: tetraetoxysilane (TEOS), and methyltrietoxysilane (MTES). The silica film obtained covers alginate microbeads uniformly with an average thickness about 10 μm (Callone et al. 2008). The texture that results from the condensation of $[(CH_3)Si]–(OEt)_n$ ($_n$=1,2,3) units of MTES provides narrow porosity and excludes any leakage of immobilized microorganisms while fully preserving chemical exchanges and, therefore, cell bioactivity (Carturan et al. 2004; Callone et al. 2008).

In the present work, organo-silica immobilized *Oenococcus oeni* cells were used to perform malolactic fermentation (MLF) at the microvinification scale. The peculiar features of the immobilization carrier were investigated in order to achieve: (1) simultaneous alcoholic (AF) and malolactic (MLF) fermentation by free yeasts and immobilized bacteria; (2) the use of the same immobilized biomass in serial MLF; (3) the use of immobilized bacteria to perform MLF in the presence of lysozyme, which avoids growth and activity of wild or spoilage lactic acid bacteria (LAB) in wine. This latter application appears the most innovative and requires some explanation. The porosity of the organo-silica membrane is determined by the hydrolysis of Si–OEt groups of MTES and the subsequent condensation of resulting Si-OH, so that $–[(CH_3)SiO_3]–$ (T_3 unit) and $–[(CH_3)Si(OH)_nO_{(3-n)}]–$ (T_{3-n} unit; $_n$=1,2) are the chemical constituents of the membrane. As the T_{3-n}/T_3 ratio increases, the texture of the membrane is loosened, owing to the minor presence of $Si(CH_3)–O–Si(CH_3)$ bounds, and an increase of membrane permeability is expected. The conditions determining the T_{3-n}/T_3 ratio may be adjusted; therefore, the porosity of silica membrane can be tailored a priori to define the molecular bulkiness of crossing species (Carturan et al. 2004). This property was exploited here in order to obtain a membrane with tailored porosity, which excludes contact between the lysozyme added to the wine and LAB immobilized inside silica/alginate microbeads. Lysozyme is an enzyme used in winemaking to avoid wine spoilage due to must or wine native LAB; nevertheless, its non-selective activity affects also LAB cultures added to the wine, and it can inhibit MLF if it is not removed from the fermentation environment (must or wine) with specific treatments (Bartowsky 2003, 2009). The proposed immobilization carrier excludes its contact with the starter LAB, allowing MLF to take place under conditions of complete inhibition of undesirable bacteria that could be responsible of the accumulation of unpleasant or toxic compounds in wine. The present work describes the methods of microbead production in a pilot scale plant, and the behavior of immobilized LAB in microvinification trials.

Materials and methods

Reagents and biological materials

Alginic sodium salt, tetraetoxysilane (TEOS) and methyltrietoxsilane (MTES) were purchased from Sigma-Aldrich (Steinheim, Germany). Other chemicals were reagent-grade products (Sigma-Aldrich, Steinheim, Germany), and used without further purification. A commercial form of lysozyme (Lallzyme©, Lallemand, Canada) was used in experimental fermentations; the concentration in wine was adjusted following the supplier's indications. The *Oenococcus oeni* strain PN4 was selected by Guzzon et al. (2009) for oenological use. It belongs to the Edmund Mach Foundation collection (S. Michele all'Adige, Italy), and is produced in freeze-dried form by Lallemand Inc. (Canada). The *O. oeni* PN4 freeze-dried culture used in the present work had a 2×10^{11} cell/g cell density and was stored at −20°C. When used as a free cell starter, PN4 culture was reactivated at 37°C for 15 min in peptone water (OXOID, Oxford, UK). Before immobilization, freeze-dried cells were rehydrated directly in the sodium alginate solution. Before use, *Saccharomyces cerevisiae* strain ATCC 9763 was stored in slants and cultivated on YM medium to reach a concentration of ca. 1×10^7 CFU/mL.

Basic chemical and microbiological analysis

The chemical composition of musts and wines (sugar, ethanol, titratable acidity, pH, organic acid concentration) was determined using near infra red spectrometry (FT-IR, WineScan 2000, FOSS Instruments, Hillerød, Denmark); total and free SO_2 concentrations were measured with a Crison Compact Titrator (Crison Instruments, Alella, Spain). Plate counts were performed according to the OIV methods (OIV 2009). Epifluorescence microscope observations were carried out with an 80i optical microscope, (Nikon, Tokyo, Japan), equipped by UV lamp at a wavelength of 480 nm. Live/dead cells were differentiated using a Live/Dead BacLight™ Kit (Molecular Probes©, Invitrogen, Carlsbad, CA). Freeze-dried culture, Na-alginate/cells suspension, and Ca-alginate/Silica microbeads were diluted 1:9 w/w with 0.1 M sodium phosphate buffer (pH 7.0), and homogenized in a Stomacher 400 blender (Seward, Worthing, UK) for 120 s to obtain complete dispersion of cells. Viable bacteria were enumerated by plate count as described previously.

Immobilization procedure

Na-alginate 2% w/w solution was prepared by mixing with sterile distilled water. Freeze-dried bacterial culture was dispersed (0.1% w/w) in the Na-alginate solution to

achieve a nominal cell density of 2×10^8 cell/g. The suspension was homogenized with a magnetic stirrer for 30 min at 20°C. Ca-alginate microbeads were obtained using the apparatus described in Fig. 1; 0.1 M $CaCl_2$ solution was used as chelating agent for alginate. Ca-alginate microspheres were immersed for 30 min in an ethanol/water solution (ethanol 508.57 cm^3/L, TEOS 371.46 cm^3/L, HCl 0.01M 119.97 cm^3/L, nominal SiO_2 concentration=100 g/L) previously hydrolyzed for 24h in acid environment (pH 3.0). After this treatment, the microbeads were recovered by filtration through a 0.2 μm membrane, washed with sterile distilled water, and dried at 35°C×15 min. Microbeads were placed in a stainless steel cylinder (Fig. 2) where a vapor mixture of MTES was fluxed across the microspheres by a constant nitrogen flow under continuous agitation (1 L/min; microbeads/MTES ratio 5:1 w/v, 25°C, 15 min). Before use, the microbeads were stored at 5°C in 9.5 g/L sodium chloride solution for at least 10 days.

Cell leakage was evaluated after 24 h of storage in wine, at 20°C, under gentle stirring. Cell counts were obtained by plate count (OIV 2009); results are expressed as a percentage of initial microbeads cell load.

Physical, chemical, and biological characterisation

Dimension, shape, and timing to achieve the 50% of fracture of Ca-alginate microbeads were monitored by optical microscopy observation (80i Optical Microscope, Nikon, Tokyo, Japan) considering 100 beads for each specimen. Micro-structural analysis of microbeads surface was carried out with the aid of a JSM 5500 Scanning Electron Microscope (SEM) (JEOL, Tokyo, Japan) equipped with an EDEX apparatus, and TMP Environmental Scanning Electron Microscope (ESEM) (Philips, the Netherlands), operating at 10 kV (20 kV for Energy Dispersive X-Ray analysis, Oxford Instruments Analytical, UK).

Malolactic fermentation

MLF trials were performed at the microvinification scale (50 L). The musts and wines used in the trials were provided by the winery of the Edmund Mach Foundation (San Michele all'Adige, Italy). Grape must (cv. Chardonnay) had the following composition: reducing sugars 170 g/L; pH 3.20, titratable acidity (as tartaric acid) 7.60 g/L, malic acid 3.60 g/L. Wine (cv. Chardonnay) used in all trials had the following composition: ethanol 12.5%, pH 3.32, total acidity 6.10 g/L, acetic acid 0.23 g/L, malic acid 3.45 g/L. All fermentation was carried out at 20°C. *O. oeni* cells were inoculated in 2×10^6 CFU/mL amounts; in the case of simultaneous AF and MLF, the initial concentration of yeast (*S. cerevisiae* ATCC 9763) was 1×10^6 CFU/mL. In the serial MLF trials, three subsequent fermentations were carried out in 50 L aliquots of wine. Immobilized bacteria, prepared as previously described, were placed in a porous plastic bag. Between two successive MLFs, immobilized bacteria were washed with sterile water and inoculated directly into a new batch of the same wine. When testing the lysozyme effect on immobilized bacteria, two enzyme doses (0.25 and 0.50 g/L) were used, and added to the wine 1 day before the inoculation of bacteria.

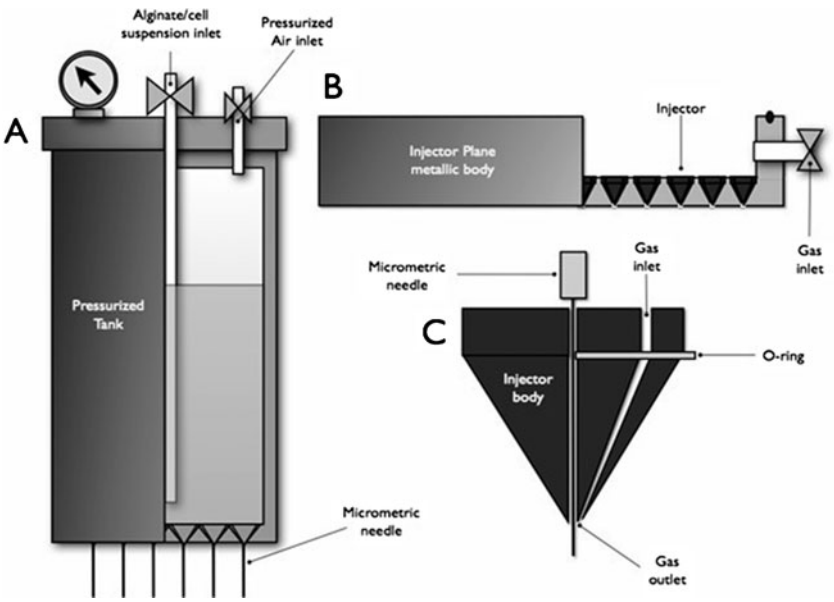

Fig. 1 Diagram of the apparatus for the production of Ca-alginate microbeads holding immobilized *Oenococcus oeni* cells. **A** Pressurized tank; **B** injector plane (ten injectors); **C** injector body with gas outlet and insert for needles

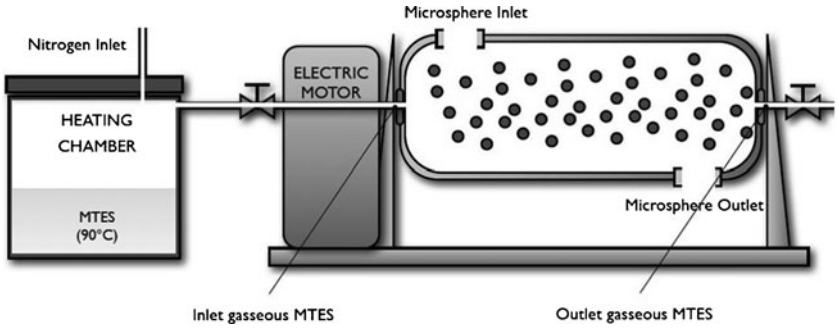

Fig. 2 Diagram of the apparatus for the deposition of gaseous methyltrietoxsilane (MTES) on alginate beads

Results

Design of the apparatus used to immobilize cells

In order to ensure a constant behaviour of entrapped cells, the diameter of encapsulating beads must be set precisely and as constantly as possible (Idris and Suzana 2006). A satisfactory compromise must be reached: smaller dimensions facilitate the migration of nutrients towards the inside of the alginate beads, the chemical exchanges between the cells and the external environment, and consequently cell activity. However, microbeads must be large enough to protect the cells and to facilitate handling during wine processing (Maicas 2001). In order to precisely set the diameter of alginate microbeads, the plant shown in Fig. 1 was built. A pressurized 5L stainless steel tank (Fig. 1A) contains the Na-alginate/cell suspension, which is extruded through ten injectors located on its bottom (Fig. 1B). Inlets for the pressurized air and for the continuous feeding of Na-alginate/cell suspensions, the safety valve, and the manometer are inserted on the tank cover (Fig. 1A). On the bottom, a plane hosts 10 injectors (Fig. 1B, C) through which the Na-alginate is extruded. A 10-L bath located under the tank contains the Ca^{2+} solution where the alginate drops fall. The injector flow rate affects the geometry of Ca-alginate micro drops, the diameter of which was optimized by comparing the behavior of beads of different size. The results are shown in Table 1: beads obtained with an air flow lower than 2 L/min had a mean diameter of >700 μm, and lacked in mechanical stability because they broke in a few days. On the contrary, if the air flux was higher (>2 L/min), the microbeads had higher mechanical strength, but their size distribution was quite broad (mean diameter of <100 μm, relative standard deviation >1.25). The best compromise between mechanical stability and shape uniformity was obtained when the produced microbeads had an average size of 428 μm.

The second immobilization phase provides, in two steps, the deposition of a siliceous membrane on the surface of Ca-alginate microspheres. In the first step, a silica sol is prepared starting from TEOS hydrolysis under acid catalysis in a hydro-alcoholic medium (Avnir et al. 2006). After hydrolysis, ethanol is evaporated under reduced pressure and diluted with water to restore the initial SiO_2 concentration. Ca-alginate microbeads are immersed in this silica sol for 30 min. The second step involves the reaction between gaseous MTES and Ca-alginate beads, already coated with silica sol particles. For this treatment, the equipment shown in Fig. 2 was used. A rotating drum (5 L) has two inlets: one for the feeding of alginate microbeads and one, coaxial to the rotation axis, for the supply of MTES vapor. MTES is evaporated in an external reactor heated at 90°C and connected to the rotor chamber (Fig. 2). A nitrogen gas flux (1 L/min) provides transport of MTES through alginate microbeads, and the immediate removal of ethanol produced during MTES hydrolysis (Carturan et al. 2006). Nitrogen and ethanol are evacuated from the rotor chamber through a specific outlet placed opposite to the gas inlet. The production rate of this apparatus is 500 cm^3/h alginate microbeads. Electronic microscopy observation (Fig. 3A, B) showed a drastic change in the surface morphology of the microspheres after the silica coating: the rough alginate surface became clearly smooth. EDX analysis gives information about the chemical constituents of the surface layers of silica/alginate microbeads (Fig. 3C). The external portion of the microsphere contained mostly silica (85.70±5.40 % w/w) and oxygen (9.16±3.75 % w/w) as expected, while some calcium (4.44±0.80 % w/w) and potassium (0.66±0.25 % w/w) of the underlying calcium alginate structure were also detected. No significant loss of LAB viability was observed: LAB density in the Na-alginate/cell suspension was 3.9×10^8 CFU/mL. In the following steps, the cell concentration inside alginate microbeads did not change, and was always around 2×10^8 CFU/mL (Table 1). The live/dead cell ratio did not change during the whole immobilization process, and the amount of living cells was at least three times higher than that of dead cells. Leakage tests were carried on in wine to verify the effectiveness of cell entrapment. As expected, alginate microbeads that have no outside layers to entrap cells were not able to retain bacteria for long time. After 24 h, an amount equal to the 30% of the initial cell load was released into the medium. In contrast, the deposition of the organo-silica membrane on the alginate microbeads reduced cell leakage to less than 1% of internal cell load (Table 1).

Table 1 Effect of air/alginate mixing on immobilized *Oenococcus oeni* biomass features

Sample	Injector air flow rate (L/min)	Mean diameter (μm)±SD	Cell density (× 10^8 CFU/g microbeads)±SD	Cell leakage after 24 h (% of cell load)	Live/dead cell ratio	50% breaking time (days)[a]
Na-alginate cell/suspension	-	-	3.9±0.1	-	6.14	-
Ca-alginate microbeads	0.0	1,520±220	8.2±0.2	60	4.55	2
	0.5	1,130±250	8.1±0.2	58	5.25	2
	1.0	710±90	8.4±0.2	60	5.66	4
	2.0	428±98	8.4±0.2	57	4.88	> 30
	2.5	340±140	8.6±0.2	55	5.25	> 30
	3.0	60±75	8.6±0.2	55	4.88	> 30
	3.5	50±80	8.7±0.2	56	4.88	> 30
Organo silica coated Ca-alginate microbeads	2.0	390±80	8.2±0.2	< 1	4.26	> 30

[a] Time necessary to observe to the microscopy the fracture of 50% of 100 beads.

Experimental MLF: yeast and bacteria co-fermentation

In the first set of microvinifications, immobilized bacteria (*O. oeni* PN4) and free yeast cells (*S. cerevisiae* ATCC 9763*)* were inoculated simultaneously in three lots of the same must, in which the initial sugar concentrations were adjusted to 170, 200, and 220 g/L (Fig. 4) with the addition of a 1:1 glucose/fructose mixture. The alcoholic fermentation kinetics were not affected, when the fermentations were inoculated with *O. oeni* PN4 strain, free or immobilized, and the final ethanol content was linked stoichiometrically to the initial sugar concentration (Table 2). In the trials inoculated with bacteria, MLF started in less than 2 days, and the full degradation of malic acid took 12 (free cells) or 14 (immobilized cells) days. In the trial without bacterial inoculum, the spontaneous microbiota did not effect any degradation of malic acid (data not shown). When the sugar concentration was increased to 220 g/L, an increase in MLF duration was observed. The complete degradation of malic acid was achieved in 18 (free cells) or 21 (immobilized cells) days. In all cases, the amount of acetic acid after MLF was low, largely below the sensory threshold (Table 2).

Experimental MLF: serial MLFs

Serial MLFs were carried out in three batches of the same wine, in which immobilized cells were inoculated after

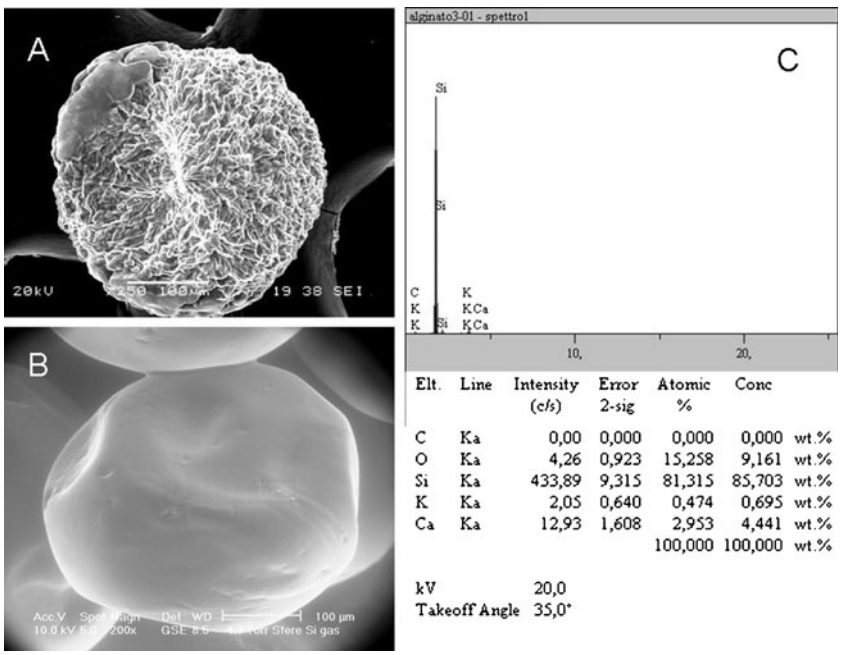

Fig. 3 **A**–**C** Characterization of microbead surface. **A** SEM observation of alginate microbeads before silica coating. **B** ESEM observation of alginate microbeads after silica coating. **C** Spectra of alginate microbeads surface EDX analysis

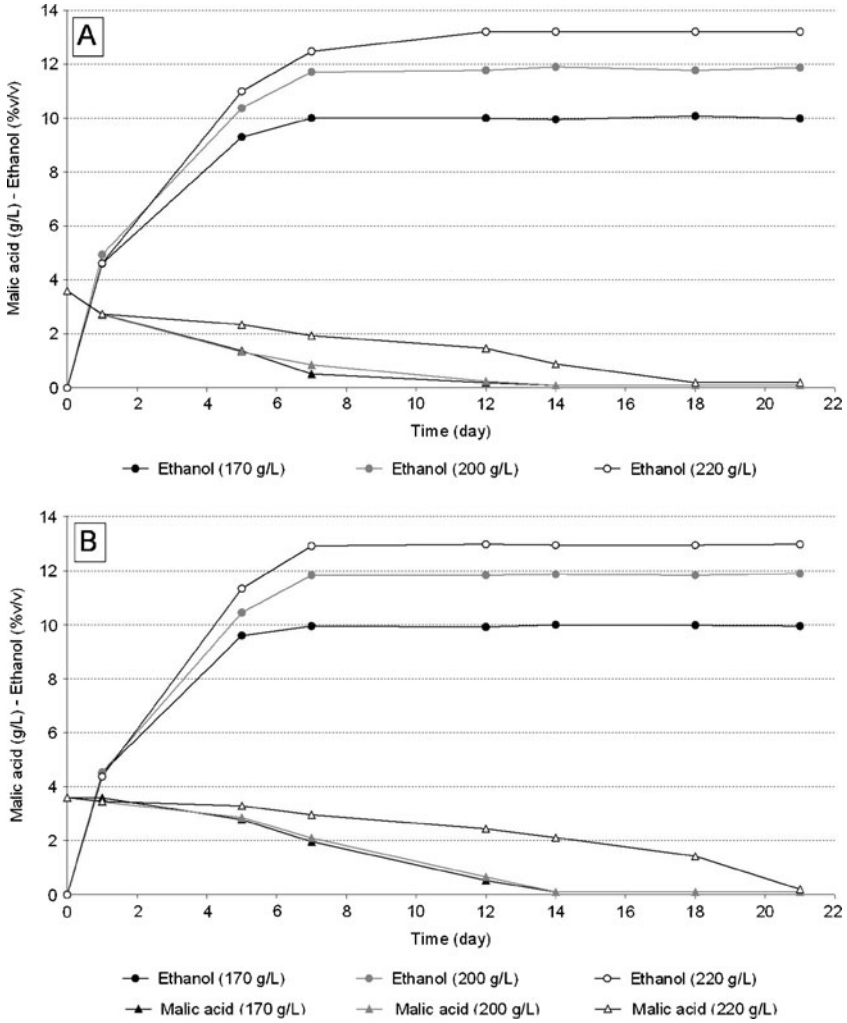

Fig. 4 Simultaneous alcoholic fermentation (AF) and malolactic fermentation (MLF) performed by free yeast and **A** free or **B** immobilized bacteria. Test performed in 50 L of must containing 170, 200, or 220 g/L sugar

being packed in a plastic net bag. After each MLF, the wine was drawn off and replaced with a new lot of the same batch. Immobilized cells were not subjected to any reactivation between the subsequent MLFs, i.e., they worked continuously for 48 days during the course of the trials, and their activity seemed to increase with time, as shown by the quicker starting of MLF and by the increase in fermentation rate (Fig. 5). The delay in the start of the first fermentation was shorter than 3 days, and total consumption of malic acid required 20 days; in the successive two fermentations the malic acid degradation had a shorter latency, and was brought to an end in 14 and 11 days, respectively. The residual malic acid content in all wines was below 0.2 g/L. Initial LAB density in wine was 2.6×10^5 CFU/mL; when the third fermentation was completed, after 48 days, it had not increased significantly (7.9×10^5 CFU/mL), and was still too low to promote MLF (Liu 2001). The malic acid content in the control wine did

Table 2 Wine composition after simultaneous experimental alcoholic fermentation (AF) and malolactic fermentation (MLF)

State of microorganisms	Initial sugar concentration (g/L)	Ethanol (%)	Lactic acid (g/L)	Acetic acid production (g/L)
Free yeast/free bacteria	170	9.99	2.36	0.47
Free yeast/free bacteria	200	11.76	2.34	0.52
Free yeast/free bacteria	220	12.94	2.28	0.56
Free yeast/immobilized bacteria	170	9.99	2.37	0.48
Free yeast/immobilized bacteria	200	11.86	2.30	0.53
Free yeast/immobilized bacteria	220	12.92	2.28	0.48

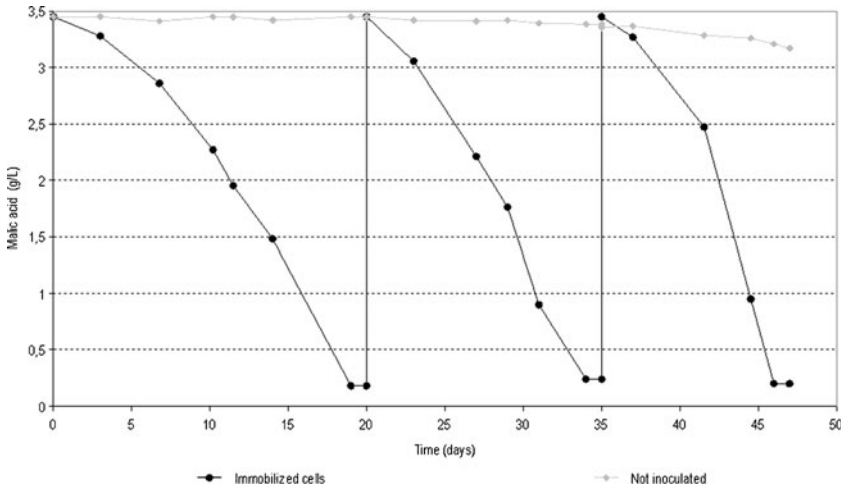

Fig. 5 Serial MLF performed by immobilized *Oenococcus oeni* PN4 strain. Test performed under wine cellar conditions (50L must)

not change in 48 days, despite a slight bacterial growth being observed (LAB final count: 2.3×10^6 CFU/mL), probably indicating a forthcoming, but not yet occurring, MLF start.

Experimental MLF with immobilized cells in the presence of lysozyme

The protective action of the immobilization matrix against lysozyme added to wine was tested in a series of micro-vinification (50 L) trials. Different aliquots of the same wine were added together with 0.25 g/L and 0.50 g/L lysozyme. When no lysozyme was added, the free bacterial biomass performed MLF in 8 days vs. the 15 days required by immobilized cells. The addition of 0.25 g/L lysozyme caused a prompt inhibition of MLF activity of free *O. oeni* cells. As expected, the addition of 0.50 g/L lysozyme confirmed this effect; no malic acid decrease was observed in 30 days when free cells were added (Table 3). On the contrary, the same lysozyme concentrations in wine had no effect against immobilized cells (Fig. 6); MLF occurred regularly with a complete consumption of malic acid and a slight accumulation of acetic acid in wine.

Discussion

Immobilization apparatus and procedure

The extrusion of Na-alginate drops in the presence of an air flux, coaxial to the alginate drops outlet, allows precise definition of microbead size and ensures high dimensional uniformity (Carturan et al. 2006). The pilot plant used to immobilize cells (see Fig. 1) is a scale-up of a laboratory apparatus previously described (Callone et al. 2008). The larger tank volume and the increased number of needles through which alginate/cells suspension are extruded, allowed a fast encapsulation rate, up to 500 cm^3/h, i.e., ten times higher than previously obtained. Moreover, the possibility of continuously feeding the alginate/cell suspension allowed the conversion from a discontinuous to a continuous process, with significant yield improvement.

In this work, all process parameters were re-adjusted in order to better preserve cell viability. The turbulence caused by mixing the Na-alginate/cell suspension and the air flow were minimized by placing the needle tips below the air outlets. The high concentration of live

Table 3 Main kinetic and chemical parameters of experimental MLF performed by free or immobilized microorganisms in wine treated with lysozyme

Description	Latency phase (day)	Day of fermentation	Lactic acid (g/L) after 21 days of inoculation	Acetic acid production (g/L)
Free bacteria 0 g/L lysozyme	2	8	2.17	0.43
Free bacteria 0.25 g/L lysozyme	sf[a]	sf[a]	0.23	sf
Free bacteria 0.50 g/L lysozyme	sf[a]	sf[a]	0.26	sf
Immobilized bacteria 0 g/L lysozyme	2	15	2.27	0.49
Immobilized bacteria 0.25 g/L lysozyme	2	21	2.28	0.45
Immobilized bacteria 0.50 g/L lysozyme	3	21	2.18	0.43

[a] Stuck fermentation

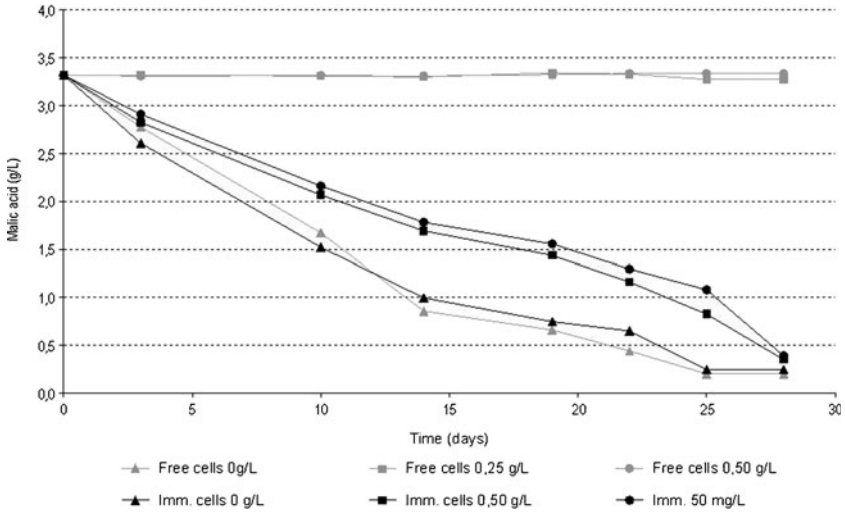

Fig. 6 MLF performed by free or immobilized cells in wine treated with different doses of lysozyme. Test performed under wine cellar conditions (50L must)

cells, and the low amount of dead ones, inside alginate microbeads (>10^8 CFU/g), indicates that the new process did not stress the cells during the inclusion. Moreover, the present results confirm a strict relationship between the alginate microbeads dimension and their physical homogeneity and mechanical resistance. The scale-up of the second silica treatment is more critical in a big volume tank, because it is essential to favor contact between MTES vapor and the microbeads, but at the same time to ensure the immediate elimination of ethanol produced during alkoxide condensation. In the equipment proposed here (Fig. 2) MTES vapor fluxes and saturates the inner volume of the rotating chamber, where the microbeads are kept in continuous agitation. A careful adjustment of the reaction environment is essential to preserve the viability of immobilized cells. In the first step, the ethanol released during TEOS hydrolysis is largely eliminated by evaporation, while the initial addition of water stabilizes the silica solution and keeps the ethanol concentration below values considered toxic to cells (Avnir et al. 2006). In the second step of silica coating, a different approach was used: the ethanol released during MTES condensation was immediately stripped out of the rotating chamber by N_2 gas when the silica coating occurs (Carturan et al. 2001). The proposed apparatus ensures a uniform silica coating of alginate microbeads, as revealed by ESEM observations (Fig. 3); EDX analysis confirmed that the coating layer was composed mainly of silica, indicating that the alginate surface was almost completely covered by the siliceous membrane. The uses of MTES and TEOS in the food industry appears pioneering, no commercial use of these alkoxides in food industry has yet been reported. However, the application of these materials in the biomedical field suggests that they are completely safe towards humans (Carturan et al. 2001, 2004).

Experimental MLF: yeast and bacteria co-fermentation

Low pH, lack of nutrients, and the presence of ethanol and SO_2 are the main causes of stuck or sluggish MLFs, even if LAB cultures are inoculated (Malherbe et al. 2007; Liu 2001). Zapparoli et al. (2009) observed that the simultaneous inoculation of LAB and yeast in must may ensure a progressive and effective adaptation of bacteria to the wine. In the present work, yeasts and bacteria were co-inoculated, and no competition was observed between them. A lower MLF rate was observed when the sugar content was high (220 g/L), probably due to osmotic stress, but it seemed independent from the immobilization. The amount of acetic acid in the wine at the end of fermentation appeared linked to the increasing initial sugar content, which may stimulate bacterial heterofermentative activity (Lonvaud-Funel 1999). However, the PN4 strain confirmed itself to be a low acetic acid producer (Guzzon et al. 2009) if compared to the control AF where MLF did not occur.

Experimental MLF: serial MLFs by immobilized cells

Three subsequent MLFs were carried out using the same immobilized biomass contained in the plastic net sack, and the MLF rate increased in the three trials. This behavior is probably due to adaptation of the cells to the environmental conditions that occur during the prolonged exposure to wine (Garbay and Lonvaud-Funel 1996). Native wine LAB was not able to carry out MLF, as no malic acid degradation occurred in 48 days in wine that was not inoculated. Even though the immobilized biomass was not subjected to any regeneration between three subsequent MLFs, its fermentation activity did not vary. In previous results (Maicas 2001), a periodic regeneration of immobilized biomass involved in MLF was required, due to clogging of immobilization carrier pores, or cell leakage. The preserva-

tion of the bioactivity of silica-covered immobilized cells could be due to the high adaptability of the PN4 strain to wine conditions (Guzzon et al. 2009) and the non-polar character of the siliceous membrane (Callone et al. 2008), which is helpful in avoiding precipitation of organic acid salts on the microbeads surface, which could reduce its permeability.

Experimental MLF with immobilized cells in the presence of lysozyme

Spontaneous MLF can be carried out by different species of wild LAB, and some of these LAB may spoil the wine. Their impact on wine aroma and texture is sometimes negative, and they can produce biogenic amine, due to the degradation of amino acids (Lounvaud-Funel 2001; Bauza et al. 1995). A strict control of wild lactic bacteria growth in wine is therefore mandatory to obtain high quality wines (Bartowsky 2009). Commonly, this is achieved by the addition of sulfur dioxide or lysozyme. The latter is a small single peptide with a muramidase activity, ineffective against eukaryotic cells, that can be added throughout winemaking to inhibit the growth of LAB (Gerbaux et al. 1997; Bartowsky 2009) even in the presence of yeasts. Although lysozyme minimizes wine spoilage due to wild LAB, its addition to wine can also suppress the activity of the inoculated bacteria. The proposed immobilization method overcomes this problem: the MTES siliceous membrane that covers alginate microbeads has a definite and narrow porosity with a cut-off about 30 kDa (Carturan et al. 2004), which is near to the molecular weight of lysozyme. Thus, the siliceous membrane may protect the immobilized cells from the action of lysozyme, preventing direct contact with the enzyme. The inoculation of immobilized bacteria to achieve MLF with the simultaneous addition of lysozyme to wine may allow reliable MLF to be obtained together with the complete inhibition of wild spoilage bacteria. This hypothesis was confirmed: the addition of lysozyme completely inhibited the activity of free cells, while MLF performed by immobilized LAB occurred as expected. The protective action of immobilization carrier against lysozyme was total.

Conclusion

Oenococcus oeni cells were immobilized in a biphasic silica/alginate carrier using a specifically designed pilot scale apparatus for Ca-alginate bead production. The alginate microbeads were coated with an organo-silica membrane, prepared by two successive sol-gel treatments, that improved their mechanical stability and chemical durability. The entrapment of LAB allowed a strict control of their activity during fermentation in must. The effective retention of bacteria by the immobilization carrier and its chemical inertness ensuring a prolonged activity of biomass, and the ability to perform subsequent MLF without affecting alcoholic fermentation when bacteria were inoculated simultaneously with yeast. This allows a reduction of costs and improves the consistency of results. Moreover, the definite porosity of the siliceous membrane constitutes an effective barrier for lysozyme in the medium, enabling the achievement of successful MLF by immobilized LAB, while simultaneously preventing the activity of wine spoilage bacteria.

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