**P-001**

# Cell entrapment by a stereospecific membrane to perform safe wine fermentations.

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## INTRODUCTION AND OBJECTIVES

Wine may be spoiled by lactic bacteria (LAB) coming from grapes or cellar. These microorganisms lead to the production of toxic compounds and off-flavors. The addition of antiseptics, $SO_2$ or lysozyme, can be effective to inhibit spoilage LAB; nevertheless, it also affects starter LAB sticking oenological fermentations. In order to give selectivity to antimicrobial compounds, a physical barrier that entraps useful bacteria, and avoids their contact with antiseptics, was created. Cells were entrapped inside alginate microbeads, coated by an organo-silica membrane, based on tetraethoxysilane (TEOS) and methyltriethoxysilane (MTES). The work elucidated chemical and mechanical features of this material, demonstrating that it allows an effective cells entrapment, while fully preserving their viability and functionality (Callone 2008). Also, a secondary property of siliceous membrane was here exploited: its narrow porosity allows the migration of small molecules, such as nutrients and metabolites, but excludes the contact between cells and high molecular weight agents, such as lysozyme (MW 14000 D, 30×30×50 Å size). Consequently, entrapped bacteria were fully protected against antiseptic action, while wild bacteria were not. We tested the use of LAB immobilized in alginate, covered by a siliceous membrane to perform oenological fermentation, while avoiding wine spoilage bacteria by lysozyme addition in wine.

## MATERIAL AND METHODS

*Microorganism*. *O. oeni* PN4 strain (Guzzon 2009) was used in all trials. LAB were enumerated by plate count on Tomato Juice Agar. Alginate microbeads were dissolved in 0.1 M phosphate buffer, under continuous blending (30 min.).
*Immobilization process*. Cells were dispersed in 2% Na-alginate solution and extruded through needles in presence of an air flow (2 L/min.) in a 0.1 M $CaCl_2$. The 1st coating phase required TEOS hydrolysis in ethanol/water solution, under acid catalysis. After hydrolysis, TEOS was diluted 1:1 with water and evaporated at 30°C, to restore the original SiO2 concentration (100 g/L). Ca-alginate microbeads were treated with TEOS solution for 30 min. The 2nd coating phase was made with MTES evaporated at 90°C and stripped across microspheres for 20 min by a $N_2$ flow (0.1 L/min.).
*Silica/Ca-alginate microbeads characterization*. Optical and electronic microscopy (SEM and ESEM) were used to define size and morphology of specimens. Elemental analysis of Si and Solid State NMR analysis of $^{29}Si$ were performed on dried samples (90°C × 48 h).
*Malolactic fermentation tests*. Malolactic fermentation (MLF) were performed in wine (Ethanol: 12.40%, pH: 3.34, Malic Acid: 2.35 g/L, Tot. $SO_2$: 50 mg/L, Free $SO_2$: 15 mg/L), in 15 L vats. Chemical analysis of wines were performed by FT-IR.

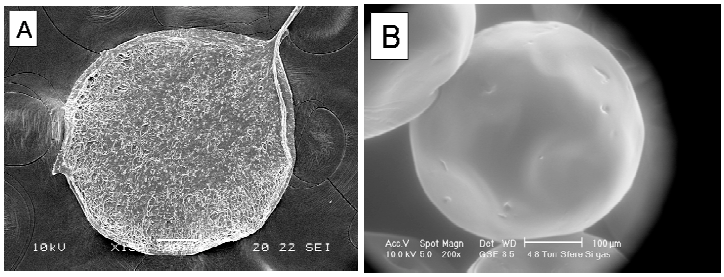


**Figure 1. SEM and ESEM observation of microbeads. A: uncoated; B: after silica coating.**

## RESULTS AND DISCUSSION

***Immobilization process and microbeads characterization.*** Bacteria were immobilized in a biphasic silica-alginate matrix (Callone 2008), alginate/cell suspension viscosity, gas flow, and microbeads aging in $CaCl_2$ solution were defined to get reliable shape and dimension of microspheres: their diameter was 400 $\mu$m and cell concentration reaching the $1.9\pm0.7\times10^8$ CFU/g (Table 1). Silica sol was prepared by TEOS hydrolysis under acid catalysis in hydro-alcoholic medium. To preserve cell viability, ethanol was substituted by water. After the 1st coating silica concentration was 6.20 g/cm$^2$ of microsphere surface. Considering the silica density (1 g/cm$^3$), the thickness of siliceous membrane was calculated for any coating step (Table 1). The deposition of a 2nd siliceous layer was achieved by reaction between MTES in gas phase and the Ca-alginate microbeads, already coated by TEOS. The high reactivity of MTES *vs*. surface exposed Si–OH was exploited in order to stabilize the silica membrane. The use of alkoxides in gas-phase is known to be effective in producing stable coatings on alginate microbeads, without detrimental effects on cell viability (Carturan 2006). Silica layer clearly reduced the surface roughness of uncoated alginate microbeads (Figure 1A): obtained specimens had a very smooth surface (Figure 1B). Elemental analysis indicated that the amount of de-

posited silica was 14.02 g/m$^2$ (Table 1), $^{29}$Si NMR analysis confirmed the presence of high-polymerized TEOS and MTES units on the microbeads surface (Figure 2). Cell concentration inside microbeads did not change after TEOS coating, and increased after MTES treatment (Table 1), due to a remarkable shrinkage of microbeads during the gaseous coating, corresponding to a 21% volume contraction.

After 24 h of incubation an high cell leakage was observed from uncoated and TEOS coated microbeads; indeed, after the MTES coating cell loss was insignificant (Table 1). The siliceous MTES membrane allowed a total cell immobilization. This fact reflects the physical and chemical nature of the *gel* prepared from MTES, which has very narrow porosity. This positive feature is strengthened by cell viability data, which were the highest among all samples. These results confirm the usefulness of this immobilization method as an efficient and promising technology for safe microorganism confinement in heterogeneous phase.

**Table 1. Main features of microbeads before and after the silica coating.**

| Micro beads | Diameter ($\mu$m) | Deposited $SiO_2$ (g/m$^2$) | $SiO_2$ layer thickness ($\mu$m) | Cells density ($\times 10^8$ CFU/cm$^3$) | Cells leakage after 24 h |
|---|---|---|---|---|---|
| Uncoated | 428±93 | - | - | 1.9±0.7 | 57 % |
| Coated by TEOS | 511±91 | 6.2 | 6.2 | 1.7±0.5 | 24 % |
| Coated by TEOS + MTES | 403±67 | 14.0 | 9.2 | 3.7±0.6 | < 1 % |

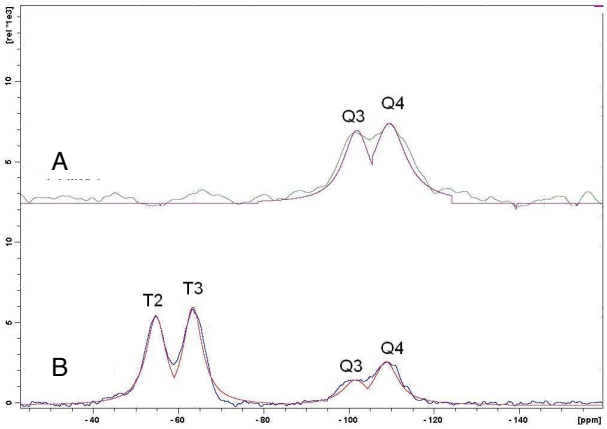


**Figure 2. $^{29}$Si Solid state NMR spectra. A: TEOS coating; B: TEOS+MTES coating. T2: $RSi(OSi)_2OH$; T3:$RSi(OSi)_3$; Q3:$HOSi(OSi)_3$; Q4: $Si(OSi)_4$.**

**MLF *by immobilized cells in the presence of lysozyme.***

Owing to membrane porosity, which excludes high molecular weight macromolecules, the immobilized cells are protected from lysozyme, with the double advantage of killing the potential spoilage LAB and preserving the fermentative activity of LAB starter. The 1 $^{st}$ MLF test was carried out in small volume (1 L wine). The additions of 0.25 and 0.50 g/L of lysozyme caused a complete inhibition of MLF in wine inoculated with free cells: after 30 days the malic acid concentration did not change. Lysozyme did not affect immobilized cells (Figure 3). MLF inoculated with this microorganisms showed a lag phase < 2 days, and took place in 28±1 days. The 2 $^{nd}$ MLF test, carried out in wine cellar (50 L of wine,) confirmed this results. No fermentation occurred in wines inoculated by free cells and treated with 0.25 or 0.50 g/L of lysozyme. On the contrary, immobilized bacteria carried out MLF in 21 days, *i.e.* with 6 days delay, as compared with tests without lysozyme. These results demonstrate that the siliceous membrane effectively protects the cells from the action of lysozyme, preventing their direct contact with the enzyme. This evidence indicates that the MTES membrane has pores < 3 nm in diameter, considering the lysozvme size.

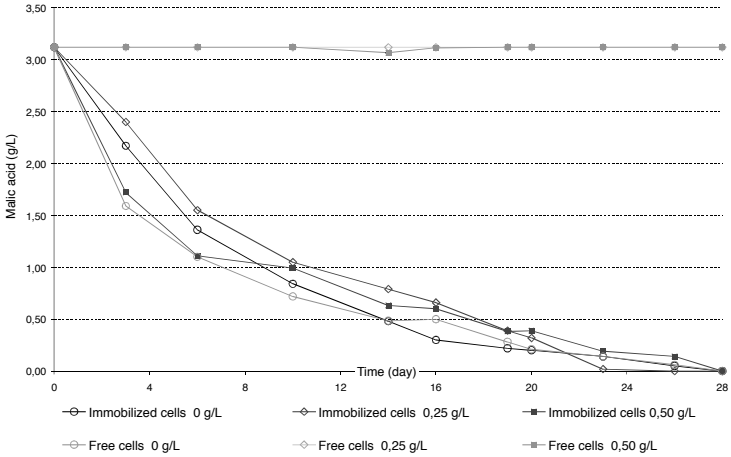


**Figure 3. malolactic fermentation by free or immobilized cells in presence of Lysozyme.**

## CONCLUSION

The production of Ca-alginate beads with a 400 $\mu$m diameter is feasible and effective to immobilize LAB. The Ca-alginate beads are coated with an organo-silica membrane, which improves their mechanical stability and chemical durability. The immobilized biomass showed regular fermentative behavior and was able to fully degrade malic acid in wine. The organo-silica membrane deposed on the bead surface avoids cell leakage and constitutes a barrier against lysozyme added to the wine. These features allow regular MLF and provide secure control of spoilage LAB in wine.

## REFERENCES

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