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Title page

Effect of stressful malolactic fermentation conditions on the operational and chemical stability of silica-alginate encapsulated *Oenococcus oeni*

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Running header: Chemical stability of SiO_2-alginate biocomposites

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Abstract

Oenococcus oeni was encapsulated into inter-penetrated polymer networks of silica-alginate (SiO_2-ALG). Fourier transform infrared spectroscopy analysis proved the presence and the polycondensation of the siliceous material used in SiO_2-ALG capsules. Environmental scanning electron microscopy showed that the structure of SiO_2-ALG biocapsules was rougher than in alginate (ALG) biocapsules. The behaviour of SiO_2-ALG biocapsules was evaluated at pH 3.0-3.6 and alcohol degrees of 12-15%. Repeated-batch malolactic fermentations (MLF) demonstrated that SiO_2-ALG biocapsules can be reused efficiently for five times in either low-pH or high-ethanol wines, while free bacteria only can be used once under the most favourable MLF conditions. The inclusion of siliceous materials into ALG hydrogel improved the stability of the biocapsules, reducing their shrinking and achieving an excellent integrity under winemaking conditions. These results proved the possibility of industrial application of SiO_2-ALG biocapsules in winemaking.

Highlights

- Combination of ALG and SiO_2 enhances chemical stability of biocapsules in wine
- Inclusion of SiO_2 improves the operational stability of ALG-based biocapsules
- SiO_2-ALG biocapsules can be reused efficiently in low-pH or high-ethanol wines

- SiO_2-ALG entrapped bacteria achieve a successful MLF under adverse conditions

Key words: Biomaterial, biocatalysis, immobilization, hydrogel.

1. Introduction

In the last decade, cell encapsulation has been reported as an alternative to free cells for the enhancement of long-term bio-efficiency in fermentative processes (Kourkoutas, Manojlović, & Nedović, 2010; Maicas, 2001). Different materials have been applied as carriers for cell encapsulation, mainly organic polymers (such as ALG, carrageenan, chitosan, agarose, pectin, gelatine, and chitin). Ionotropic ALG hydrogel has been extensively used for this purpose due to being easy and cost-effective to prepare, the encapsulation conditions are mild for cell, and its porous structure allows the free diffusion of substrates and products. However, the low chemical stability and the poor mechanical strength of ALG hydrogel hinder its industrial implementation (Ching, Bansal, & Bhandari, 2015). Different alternatives have been assessed to improve these disadvantages based on coating techniques (Simó, Fernández-Fernández, Vila-Crespo, Ruipérez, & Rodríguez-Nogales, 2017a) as well as on the incorporation of other polymers and/or fillers into ALG gel (Neufeld, & Poncelet, 2004).

Previous researches have been done for the improvement of the stability of ALG capsules using covalent polymeric networks of SiO_2 obtained by the sol−gel process (Thibaud Coradin, Allouche, Boissiere, & Livage, 2006; Kuncová & Trogl, 2010). These inorganic-organic hybrid materials have the advantages of the organic component (ALG) (biocompatibility, elasticity and flexibility) and the inorganic component (SiO_2) (strength, and thermal and chemical stability) (Hwang & Gu, 2013). From a toxicological point of view, SiO_2–ALG capsules are very suitable for food application

since (i) ALG is a natural polymer that has been recognized as a "generally recognized as safe" material (Sosnik, 2014), and (ii) siliceous materials are being used for biomedical applications and hence admitted as safe for humans (Carturan, Dal Toso, Boninsegna, & Dal Monte, 2004).

The classic sol-gel process involves the hydrolysis of alkoxide precursors in the presence of water and catalysts (either acids or bases) to form silanol groups (Si-OH) with the releasing of alcohol molecules. Then, condensation of silanol groups takes place to produce siloxanes (Si-O-Si). Finally, polycondensation of silanol and siloxanes occurs, yielding SiO_2 materials (Niederberger & Pinna, 2009). The use of sol-gel chemistry for cell encapsulation presents some risks for cell viability. On one hand, the released alcohol from the hydrolysis of alkoxide precursors may be toxic to cell. On the other hand, the use of cosolvent and catalysts to increase the low water solubility and the reactivity of alkoxide precursors, respectively, may also cause loss of cell viability. Some solutions have been reported to solve these drawbacks based on either the removal of alcoholic by-products before cell encapsulation or the use of an aqueous sol-gel process with non-alkoxide precursors (Coradin et al., 2006; Kuncová & Trogl, 2010).

A successful strategy was recently proposed by our research team, in which an easy, economic and efficient cell encapsulation procedure based on inter-penetrated polymer networks of SiO_2 and ALG was developed (Simó, Fernández-Fernández, Vila-Crespo, Ruipérez, & Rodríguez-Nogales, 2017b; Simó, Vila-Crespo, Fernández-Fernández, Ruipérez, & Rodríguez-Nogales, 2017). The hybrid composite was obtained by the sol-gel aqueous route using non-alkoxide silicon precursors (colloidal silica and sodium silicate). The resulting SiO_2-ALG biocapsules with entrapped *O. oeni* bacteria were applied in winemaking to develop MLF. SiO_2-ALG biocapsules presented a

100 remarkable increase in mechanical robustness of 328% and 65% in L-malic acid
101 consumption, observing insignificant bacteria release compared to untreated ALG
102 biocapsules (Simó, Vila-Crespo, et al., 2017). Also, SiO_2-ALG encapsulated *O. oeni*
103 showed an enhanced MLF in wines with high concentration of ethanol (13-16%), low
104 pH (3.0-3.3) and at low fermentation temperatures (13-15ºC) compared to ALG
105 capsules (Simó, Fernández-Fernández, et al., 2017b).

106 To the best of our knowledge, only two other works using SiO_2-ALG
107 composites for the encapsulation of *O. oeni* have been reported (Callone, Campostrini,
108 Carturan, Cavazza, & Guzzon, 2008; Guzzon, Carturan, Krieger-Weber, & Cavazza,
109 2012). Unlike our strategy, both works noted a non-aqueous method based on double-
110 layer coating of ALG capsules embedded with *O. oeni* using tetraethoxysilane and
111 methyltriethoxysilane as alkoxide silicon precursors. The coating procedure did not
112 increase the metabolic activity of bacteria, although there was a notable improvement of
113 the hardness of biocapsules.

114 One of the potential main advantages claimed for the use of encapsulated cells
115 includes their easy separation and reuse. Industrial implementation of biocapsules
116 depends on the possibility of their recycling for a long period without compromising
117 their metabolic activity and viability. Moreover, biocapsules must be stable along the
118 fermentative period for the success of alcoholic beverage production (Kourkoutas,
119 Bekatorou, Banat, Marchant, & Koutinas, 2004). A deep knowledge of the effect of the
120 hostile wine environment on biocapsules stability needs to be addressed to obtain a full
121 exploitation of this potential advantage.

122 In this context, the encapsulation of *O. oeni* into inter-penetrated polymer
123 networks of SiO_2 and ALG could involve a great enhancement of the operational and

chemical stability of the biocapsules to the stressful conditions of MLF. For that, the aim of this study was to evaluate the reusability of Si-ALG biocapsules under unfavorable MLF conditions and to test their long-term chemical stability in harsh wine conditions of low pH and high ethanol concentration. The presence of siliceous materials in the Si-ALG biocomposites and their effect on the external and internal structure of the capsules were also verified by Fourier transform infrared spectra (FTIR) and Environmental scanning electron microscopy (ESEM) analysis.

2. Experimental

2.1. Preparation of SiO_2-ALG biocomposites with encapsulated bacteria

Inter-penetrated polymer networks of silica/silicate and ALG (Simó, Vila-Crespo, et al., 2017) were carried out adjusting the pH of a water solution of 1.23 M colloidal silica (Ludox HS40, Sigma-Aldrich, Spain) and 0.06 M sodium silicate (Sigma-Aldrich) to 6.3 by adding 2N HCl. Then, sodium ALG (Panreac, Spain) was mixed with the silica/silicate solution until obtaining a final concentration of 2% (w/v). Freeze-dried bacteria (*O. oeni* strain LALVIN VP 41® MBR, Lallemand, France) were rehydrated in water at 20°C according to the manufacturer's instructions and they were added at a concentration of ~$3 \cdot 10^9$ cfu/g of gel. Population of freeze-dried culture was tested in Tomato Juice Agar (Difco, Sparks, MD, USA) at 22°C. Capsules were made by extrusion of the well-mixed siliceous material-ALG-cell suspension with a 10 mL sterile syringe with a nozzle of 1.78 mm of diameter (BD 166 Plastipak, Spain) into sterile 0.2 M $CaCl_2$ solution under continuous agitation (260 rpm) at 22ºC. Capsules were kept in this solution for 2 h and washed with water at 22ºC. Capsules of ALG were

147 made using the method described for SiO_2-ALG capsules but omitting the addition of
148 siliceous material. All solutions were autoclaved prior to use.

149 *2.2. Environmental scanning electron microscopy (ESEM)*

150 The prepared SiO_2-ALG and ALG biocapsules were dehydrated by freeze-
151 drying at −40ºC**.** The samples were coated with gold and examined in a Quanta 200FEI
152 ESEM (Hillsboro, Oregon, USA) with a backscattered electron detector at a landing
153 energy of 4.0 KeV.

154 *2.3. Fourier transform infrared spectroscopy analysis (FTIR)*

155 Functional groups of freeze-dried SiO_2-ALG and ALG capsules were
156 characterized by FTIR spectrophotometry. The vibrational spectra of the capsules in
157 the 400-4000 cm^{-1} spectral range were measured using a Thermo Scientific Nicolet
158 iS50 FTIR spectrometer (Waltham, MA, USA), equipped with a built-in diamond
159 attenuated total reflection (ATR) system. Spectra of the samples were recorded with a
160 1 cm^{-1} spectral resolution, and 32 scans.

161 *2.4. Operational stability of biocapsules*

162 Reusability of SiO_2-ALG biocapsules was determined using the same inoculum
163 for five consecutive cycles of MLF under different winemaking conditions. Free
164 bacteria were also used as control. Red wine of "Tinta de Toro" variety (3.0 g/L malic
165 acid, pH 3.4 and alcoholic degree of 14.0%, v/v) was partially dealcoholized to obtain a
166 final alcoholic degree of 11% under reduced pressure in a rotary evaporator. A first
167 batch of wine was made adjusting the alcoholic degree of the concentrated wine to
168 12.0%, 13.5% and 15.0% with ethanol and at pH 3.4 using acid or basic concentrated
169 solutions. A second lot of wine was set to an alcoholic degree of 13.0% and pH of 3.0,
170 3.3 and 3.6. MLF was conducted in sterilized red wine (by filtration through a 0.2 μm

pore-size) inoculating either free or SiO_2-ALG encapsulated bacteria at a concentration of ~9 × 10^7 cfu/mL of wine. Rehydrated culture from freeze-dried *O. oeni* strain (LALVIN VP 41® MBR) was used as free inoculum. Bacterial population of free inoculum was tested in Tomato Juice Agar at 22°C (Difco). MLF was carried out in triplicate and its progress was monitored by the determination of L-malic acid concentration (in triplicate) using an enzymatic kit (TDI, Barcelona, Spain). After 120 h of incubation at 22ºC, both free and encapsulated bacteria were removed from wine and introduced into a new wine. Free bacteria were recovered by centrifugation (15 min at 2300 *g*) while encapsulated ones were separated from wine by filtration using a metallic sieve (nominal sieve opening of ~1.5 mm).

2.5. Long-term chemical stability of biocapsules

For capsule stability evaluation, the same two lots of wine developed for the operation stability study were used. Fifteen SiO_2-ALG biocapsules containing bacteria were immersed in 10 mL of red wine at different pH (3.0, 3.3 and 3.6) and alcoholic degrees (12.0%, 13.5% and 15.0%). ALG capsules were also analysed as control for comparison. Mass and diameter of both SiO_2-ALG and ALG biocapsules were determined along the experimental time at 22ºC. The diameter of biocapsules was measured by software IMAGEJ 1.47v (National Institutes of Health, Bethesda, MD, USA). The mass of biocapsules was determined using a semimicro balance with a readability of 0.1 mg (AS 228 220/C/2, Radway, Brancka, Poland). The release of ALG from both capsules was determined by duplicate after 18 days of immersion in wine (fifteen biocapsules in 10 mL of red wine) following the method proposed by Segarra et al. (1995). All experiments were carried out in triplicate.

2.6. Statistical analysis

SPSS v. 17.0 statistical package (SPPS Inc., Chicago, Ill, USA) was used for all statistical analyses. A variance analysis was carried out to determine statistical differences between samples ($p<0.05$). In figures, error bars were calculated as standard error.

3. Results and discussion

3.1. Morphological observations

SiO_2-ALG and ALG biocapsules were nearly spherical and homogeneous in size (Figure 1, images a and b), showing a diameter of 3.24 ± 0.20 and 2.80 ± 0.15 mm, respectively. The sol-gel process using siliceous materials slightly increased the capsule diameter due to the rise of viscosity of ALG solution in presence of colloidal silica/silicate and modified their physical appearance, turning them opaque and white. This behaviour was also observed in alginate-protamine-SiO_2 hybrid capsules where the transparent ALG capsules became opaque and white during the silicification process (Wang et al., 2013).

The surface morphology and the internal structure of ALG and SiO_2-ALG biocapsules containing *O. oeni* were studied by ESEM. Both external and internal structures of ALG biocapsules were relatively smooth (Figure 1, images c and d, respectively). However, the morphology of both structures changed in the presence of silica/silicate and became rough (Figure 1, images e and f). A highly porous network was formed by close-packed silica particles. SEM, thermogravimetric analysis and nitrogen adsorption-desorption experiments performed in SiO_2-ALG biocomposites using the sol-gel route with aqueous precursors revealed that ALG polymer filled the

219 macroporosity of hydrogel rather than being strongly entangled with siliceous material
220 (Coradin & Livage, 2003). Compared to ALG biocapsules, the presence of an inorganic
221 network of SiO_2 notably enhanced the bioactivity of SiO_2-ALG encapsulated *O. oeni*
222 (Simó, Fernández-Fernández, et al., 2017b). This efficiency in L-malic acid
223 consumption could be due (at least partly) to the highly porous structure observed in
224 SiO_2-ALG biocapsules that facilitates the substrate accessibility for encapsulated
225 bacteria. This is consistent with the improvement of diffusion characteristics noted in
226 SiO_2-ALG composites (in comparison with ALG hydrogels), evaluating the diffusion
227 coefficients of reduced nicotinamide adenine dinucleotide, an enzymatic cofactor with a
228 molecular weight of about five-times higher than malic acid (Xu, Lu, Li, Jiang, & Wu,
229 2006).

230

231 *3.2. Chemical characterization of capsules*

232 Chemical composition of ALG and SiO_2-ALG capsules was characterized by FTIR-
233 ATR spectroscopy. The FTIR spectra of the ALG capsules show characteristic bands
234 displayed for alginic acid (Figure 2a). According to literature reports (Gómez-Ordóñez
235 & Rupérez, 2011; Pannier, Soltmann, Soltmann, Altenburger, & Schmitt-Jansen, 2014),
236 the bands at 1612 cm^{-1} and 1412 cm^{-1} are assigned to asymmetric and symmetric
237 stretching vibrations of the COO^- of alginic acid, respectively. A broad peak near 1000
238 cm^{-1} corresponds to vibrational modes of the carbohydrate ring between (1-4)-β-D-
239 mannuronic acid and (1-4)-α-L-guluronic acid of alginic acid (Gómez-Ordóñez &
240 Rupérez, 2011). The FTIR spectra of SiO_2-ALG capsules (Figure 2b) present the bands
241 associated with symmetric and asymmetric stretching vibrations of the COO^- but in very
242 low intensity, suggesting an interaction of the carboxylate groups of ALG with the

siliceous polymer derived from *in situ* gelation of silica/silicate (Xu, Jiang, Lu, Wu, & Yuan, 2006). Under the pH conditions used for bacterial encapsulation (pH 6.3), only hydrogen bonds between hydroxyl groups (–OH) of SiO_2 and carboxyl groups (–COOH) of ALG could take place because the carboxyl groups of ALG are negatively charged and the hydroxyl groups are neutral, whereas SiO_2 presents neutral charge (Coradin & Livage, 2003). A peak at 790 cm^{-1} corresponds to the symmetric stretching of Si-O-Si groups, indicating an extension of SiO_2 polymerization. A peak of high intensity at about 1100 cm^{-1} partially overlapped with the corresponding bands to vibrational modes of the carbohydrate ring of ALG could be assigned to Si-O-Si bending vibration. Finally, the band at 955 cm^{-1} corresponds to Si-OH vibration marking also the presence of silica in this capsule (Xu et al., 2006).

3.3. Reusability of SiO_2-ALG biocapsules

Bacterial reuse reduces production cost, thus the study of the operational stability of SiO_2-ALG biocapsules is essential for their industrial implementation. To examine their reusability, repeated-batch MLF were carried out five times using the same inoculum of SiO_2-ALG encapsulated bacteria. Reusability test of free bacteria was also performed as control for comparison. Operational stability of ALG biocapsules was not studied since malolactic activity of ALG-encapsulated bacteria was notably lower than that observed in SiO_2-ALG biocapsules (Simó, Fernández-Fernández, et al., 2017b).

Figure 3 shows L-malic acid conversion by both SiO_2-ALG biocapsules and free bacteria along five-repeated cyclic operations in red wine at different pH (3.0, 3.3 and 3.6). Wine pH had a marked effect on malolactic activity of both type of inoculum, achieving the best results at pH 3.6. Malolactic conversion by SiO_2-ALG encapsulated

267 bacteria only decreased by 15.7% after five cycles in wine at pH 3.6 (from 98.4% of
268 conversion in run #1 to 83.0% of conversion in run #5). The decrease of malolactic
269 conversion was calculated as relative percentage as

$$\left(1-\frac{C_{MLF5}}{C_{MLF1}}\right) x\ 100$$

270 where C_{MLF1} and C_{MLF5} are the malolactic conversions of the first and fifth MLF,
271 respectively, observed at each pH, alcoholic degree and type of inoculum. The
272 decreases in L-malic acid conversion were slightly higher at pH 3.3 and 3.0 after five-
273 repeated batches, reaching drops of 20.6% (from 97.6% in run #1 to 77.5% in run #5)
274 and 35.3% (from 98.5% in run #1 to 63.7% in run #5), respectively. Malolactic
275 conversion by free bacteria was stable only at pH 3.6 (13.0% of alcoholic degree) after
276 their reuse for five times. This result was similar to that obtained by four successive
277 reinoculations of cultures of *O. oeni* in Monastrell red wine under favourable MLF
278 conditions (pH 3.5 and 11.1% alcoholic degree) (Sergi Maicas, Pardo, & Ferrer, 2000).
279 L-malic acid conversions by free *O. oeni* drastically dropped by 78.4% (from 84.6% in
280 run #1 to 18.3% in run #5) and a 94.9% (from 78.1% in run #1 to 3.97% in run #5) at
281 pH 3.3 and 3.0, respectively.

282 Free and SiO_2-ALG encapsulated *O. oeni* were also tested in five consecutive
283 MLF processes in wines with different alcoholic degrees (12.0%, 13.5% and 15.0%) to
284 determine if there was deactivation of bacteria after repeated use (Figure 3). Alcoholic
285 degree notably affected L-malic acid conversion of both free and encapsulated bacteria
286 along the successive MLF, achieving the best results at the lowest alcoholic degree
287 (12.0%). L-malic acid conversion by SiO_2-ALG encapsulated bacteria was maintained
288 at 90.7% after five cycles in favourable winemaking conditions (12.0%), corresponding
289 with a slight reduction of 7.8% (a high conversion of 98.3% was achieved in the first

290 cycle). Drops of 29.5% (from 99.4% in run #1 to 70.1% in run #5) and 36.9% (from
291 97.6% in run #1 to 61.6% in run #5) in L-malic acid conversion were observed in wines
292 with alcoholic degrees of 13.5% and 15.0%, respectively, after the fifth cycle of use of
293 SiO_2-ALG biocapsules.

294 L-malic acid conversion by free bacteria remained almost unchanged (above
295 98%) in wine with the lowest alcoholic degree (12.0%) after their reuse for five cycles.
296 However, sharp declines of 95.0% (from 85.9% in run #1 to 4.3% in run #5) and 97.0%
297 (from 78.9% in run #1 to 2.3% in run #5) on L-malic conversion were observed at
298 alcoholic degrees of 13.5% and 15.0%, respectively, corroborating the negative impact
299 of ethanol on malolactic activity of *O. oeni* (Bonomo, Di Tomaso, Calabrone, &
300 Salzano, 2018).

301 Growth and metabolism of *O. oeni* in wine depend on a multitude of parameters
302 (Bauer & Dicks, 2004). It is well documented the adverse effect of low-pH wines as
303 well as high-ethanol wines on the ability of *O. oeni* to survive in this unfavourable
304 environment (Bonomo et al., 2018; Sumby, Grbin, & Jiranek, 2014). Ethanol causes
305 breaking of cell membrane structure and membrane fluidity alterations, affecting mainly
306 transport of metabolites, and cell wall and membrane biogenesis (Olguín et al., 2015).
307 Wine pH also plays a crucial role in the beginning of MLF as well as the time required
308 to complete MLF (Knoll et al., 2011). pH values lower than 3.5 negatively affect the
309 growth of *O. oeni* and reduce their ability to metabolize L-malic acid (Betteridge, Grbin,
310 & Jiranek, 2015; Rosi, Fia, & Canuti, 2003).

311 Our results have revealed that the encapsulation into SiO_2-ALG hydrogel
312 notably improved the operational stability of bacteria, remaining high malolactic
313 activity after five successive MLF at the harsh environmental conditions of pH 3.0 and

13% of alcoholic degree, as well as pH 3.4 and 15.0% of alcoholic degree. These results agree satisfactorily with a previous study of our group. (Simó, Fernández-Fernández, et al., 2017b). In this study, we found that the inclusion of colloidal silica and silicates into ALG capsules markedly enhanced the malolactic activity of *O. oeni* at low pH, high ethanol, and low fermentation temperatures. Guzzon et al. (2012) reported successful MLF after three cycles in Chardonnay wine (ethanol 12.5%, pH 3.3, malic acid 3.5 g/L) inoculating *O. oeni* entrapped into ALG capsules coated with an organic-silica membrane. Encapsulated *O. oeni* in polyvinyl alcohol gel could be successfully reused through six cycles of MLF in Tempranillo wine (ethanol 14.2%, pH 3.7, malic acid 1.9 g/L), retaining 75% of efficacy after the sixth batch (Rodríguez-Nogales, Vila-Crespo, & Fernández-Fernández, 2013). Conversely, in Monastrell red wine under gentle winemaking conditions (ethanol 11.0%, pH 3.5, malic acid 3.5 g/L), a drop of about 50% of the initial L-malic acid conversion was observed after six consecutive cycles of MLF inoculating *O. oeni* immobilized on positively-charged cellulose sponge (Sergi Maicas, Pardo, & Ferrer, 2001). *Lactobacillus casei* cells immobilized on delignified cellulosic material showed a high L-malic acid conversion (80%) in the first cycle of MLF under mild winemaking conditions (ethanol 11.2% and pH 3.5), declining gradually up a conversion of 14.7% in the sixth batch (Agouridis, Bekatorou, Nigam, & Kanellaki, 2005). These results highlight that the success in the reusability of immobilized bacteria strongly depends on both the type of the selected matrix and the immobilization method, as well as MLF conditions and bacterial strain. In our study, the differences in malic acid conversion found in the first cycle of MLF between both type of inoculum (free and Si-ALG encapsulated bacteria) may be lower if the fermentative behaviour of the selected strain was higher at low pH and high ethanol concentration.

338 The preservation of malolactic activity of SiO_2-ALG encapsulated bacteria along
339 the operation stability assays could be due to the organic-inorganic matrix, which could
340 provide bacterial protection from the unfavourable winemaking conditions (Kourkoutas
341 et al., 2010). An enhancement of the stability of the hydration layer around the cell due
342 to the presence of non-gelling liquid into ALG hydrogel could increase the cell
343 protection (Sun et al., 2007).

344

345 *3.5. Long-term capsule stability*

346 Together with an adequate operational stability of immobilized bacteria, capsule
347 stability studies also play a decisive role for the industrial implementation of
348 encapsulated bacteria. pH and ethanol concentration in model water solutions markedly
349 affected the stability of ALG-based capsule (Simó, Vila-Crespo, et al., 2017). The
350 weight and swelling of capsules in water increased as pH was higher from 3.0 to 3.9,
351 being more notable this behaviour for ALG capsules than for SiO_2-ALG ones. In
352 contrast, both types of capsules shrank in water-ethanol solutions with more intensity at
353 higher ethanol concentrations (from 10% to 16%) and with less severity in SiO_2-ALG
354 capsules than in ALG ones.

355 In this study, we evaluate the impact of pH and ethanol concentration on SiO_2-
356 ALG capsule stability using samples of wine at different pH (3.0, 3.3 and 3.6) and
357 alcoholic degrees (12.0%, 13.5% and 15.0%). Simultaneously, ALG capsules were also
358 tested as control samples to know the effect of silica/silicate on the capsule stability.
359 Pronounced diameter and weight reductions were found when SiO_2-ALG and ALG
360 capsules were exposed to wine, reducing the free volume in the biocapsules (Figures 4
361 and 5). These results are consistent with the shrinking process observed in SiO_2-ALG

362 and ALG materials in model water-ethanol solutions (Simó, Vila-Crespo, et al., 2017).
363 On the whole, shrinking was decreased as wine pH increased, being always the loss in
364 diameter lower for SiO_2-ALG capsules than for ALG ones (Figure 4). After 27.0 days
365 of immersion of capsules in wine, the differences of weight between both capsules were
366 not so clear as those observed for diameter, testing a less drop in weight for SiO_2-ALG
367 capsules than for ALG ones only at pH 3.0. For instance, depletions of 28.4% and
368 35.9% of diameter and weight of ALG capsules were observed at pH 3.0, respectively,
369 while they were lower for SiO_2-ALG capsules (21.3% and 31.0%, respectively). The
370 differences of shrinking between both capsules can be understood from the reduction of
371 the elasticity and the increase of robustness of capsules due to the inclusion of colloidal
372 silica and silicates into ALG hydrogels (Coradin, Nassif, & Livage, 2003; Simó, Vila-
373 Crespo, et al., 2017). Hydrogels based on ALG tend to shrink when they are exposed to
374 acidic environment (Pasparakis & Bouropoulos, 2006). At a pH value lower than 4.0,
375 the carboxylic groups of mannuronic and guluronic acids of ALG are protonated (pK_a
376 3.38 and 3.36, respectively) and therefore the electrostatic repulsions among these
377 groups decrease and shrinkage is favoured (Wu, Zhu, Chang, Zhang, & Xiao, 2010).

378 In parallel with the increase in the alcoholic degree of the wine, the capsules
379 decreased in diameter and weight (Figure 5). The more the ethanol increased, the more
380 the shrinking increased. To illustrate, reductions of 24.5% and 36.3% were observed at
381 15.0% of alcoholic degree for ALG capsule diameter and weight, respectively, being the
382 loss of capsule diameter and weight less for SiO_2-ALG (21.4% and 26.8%,
383 respectively). SiO_2-ALG and ALG capsules are hydrogels which composition is mainly
384 water and they are dehydrated in the presence of alcohols (Torres, Velasquez, & Brito-
385 Arias, 2011). As it was discussed above, the addition of siliceous material into ALG
386 hydrogel causes more rigidity and robustness (Simó, Vila-Crespo, et al., 2017) and

387 hence the degree of shrinking and dehydration of SiO_2-ALG capsules in alcoholic
388 solutions is lower. The higher capacity of SiO_2-ALG capsules to reduce the water loss
389 creates a more hydrophilic microenvironment (Xu et al., 2006), so improving the
390 fermentative performance of bacteria in an ethanol-rich medium as wine.

391 Finally, to evaluate the capsule stability in wine, the release of ALG polymer
392 from SiO_2-ALG and ALG capsules immersed in wines at different pH and alcoholic
393 degree was evaluated after 18 days (Figure 6). Very small amounts of released ALG
394 (less than 0.3 mg/L) were found in all wines assayed. The release of ALG was slightly
395 higher in ALG capsules than in SiO_2-ALG ones in wines at pH 3.0 and 3.3. Neither the
396 increase of pH nor the alcoholic degree modified the levels of total polysaccharides.
397 Likely, pH-dependent changes in the electrostatic interactions between SiO_2 and ALG
398 polymer as well as ethanol-subordinate variations in capsule shrinking are not involved
399 in the release of ALG polymer. The diffusion of unpolymerized ALG from the inside of
400 the capsules to the wine could explain this behaviour (Strand, Skjåk-Bræk, & Gåserød,
401 2004). These results highlight that rupture and chemical decomposition of capsules did
402 not take place under the alcoholic and acid environment of wine indicating the
403 suitability of SiO_2-ALG biocapsules for the development of MLF.

404

405 **4. Conclusion**

406 Aqueous route using colloidal SiO_2 and sodium silicate as non-alkoxide silicon
407 precursors has been proved as a very suitable strategy to improve the chemical and
408 operational stability of ALG-based biocapsules with entrapped *O. oeni* under hard
409 winemaking conditions. Wine pH and ethanol content play a notable role in the
410 operational stability of both SiO_2-ALG biocapsules and free bacteria being their

411 negative impact most severe for unencapsulated bacteria. SiO_2-ALG biocapsules could
412 be reused for at least five cycles in wines at pH 3.0-3.6 as well as with alcoholic degrees
413 of 12.0%-15.0%. However, unencapsulated bacteria could be successfully reused
414 neither in wines at pH lower than 3.6 nor in wines with alcoholic degree higher than
415 12.0%. Combination of ALG and siliceous material enhanced the long-term chemical
416 stability of biocapsules in wines. These results highlight that the implementation of
417 SiO_2-ALG biocapsules with entrapped *O. oeni* may be a promising technique for highly
418 efficient processes in winemaking.

419

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427

428 **References**

429 Agouridis, N., Bekatorou, A., Nigam, P., & Kanellaki, M. (2005). Malolactic
430 fermentation in wine with *Lactobacillus casei* cells immobilized on delignified
431 cellulosic material. *Journal of Agricultural and Food Chemistry*, *53*(7), 2546–
432 2551. http://doi.org/10.1021/jf048736t

433 Bauer, R., & Dicks, L. M. T. (2004). Control of malolactic fermentation in wine. A

review. *South African Journal of Enology and Viticulture*, *25*(2), 74–88.

Betteridge, A., Grbin, P., & Jiranek, V. (2015). Improving *Oenococcus oeni* to overcome challenges of wine malolactic fermentation. *Trends in Biotechnology*, *33*(9), 547–553. http://doi.org/10.1016/j.tibtech.2015.06.008

Bonomo, M. G., Di Tomaso, K., Calabrone, L., & Salzano, G. (2018). Ethanol stress in *Oenococcus oeni* : transcriptional response and complex physiological mechanisms. *Journal of Applied Microbiology*. *125*(1), 2-15. http://doi.org/10.1111/jam.13711

Callone, E., Campostrini, R., Carturan, G., Cavazza, A., & Guzzon, R. (2008). Immobilization of yeast and bacteria cells in alginate microbeads coated with silica membranes: procedures, physico-chemical features and bioactivity. *Journal of Materials Chemistry*, *18*(40), 4839–4848. http://doi.org/10.1039/b807301e

Carturan, G., Dal Toso, R., Boninsegna, S., & Dal Monte, R. (2004). Encapsulation of functional cells by sol-gel silica: actual progress and perspectives for cell therapy. *Journal of Materials Chemistry*, *14*(14), 2087–2098. http://doi.org/10.1039/b401450b

Ching, S. H., Bansal, N., & Bhandari, B. (2015). Alginate gel particles-a review of production techniques and physical properties. *Critical Reviews in Food Science and Nutrition*, 0. http://doi.org/http://dx.doi.org/10.1080/10408398.2014.965773

Coradin, T., Allouche, J., Boissiere, M., & Livage, J. (2006). Sol-gel biopolymer/silica nanocomposites in biotechnology. *Current Nanoscience*, *2*(3), 219–230. http://doi.org/10.2174/1573413710602030219

Coradin, T., & Livage, J. (2003). Synthesis and characterization of alginate/silica

457 biocomposites. *Journal of Sol-Gel Science and Technology*, *26*(1–3), 1165–1168.
458 http://doi.org/10.1023/A:1020787514512

459 Coradin, T., Nassif, N., & Livage, J. (2003). Silica-alginate composites for
460 microencapsulation. *Applied Microbiology and Biotechnology*, *61*(5–6), 429–434.
461 http://doi.org/10.1007/s00253-003-1308-5

462 Gómez-Ordóñez, E., & Rupérez, P. (2011). FTIR-ATR spectroscopy as a tool for
463 polysaccharide identification in edible brown and red seaweeds. *Food*
464 *Hydrocolloids*, *25*(6), 1514–1520. http://doi.org/10.1016/j.foodhyd.2011.02.009

465 Guzzon, R., Carturan, G., Krieger-Weber, S., & Cavazza, A. (2012). Use of organo-
466 silica immobilized bacteria produced in a pilot scale plant to induce malolactic
467 fermentation in wines that contain lysozyme. *Annals of Microbiology*, *62*(1), 381–
468 390. http://doi.org/10.1007/s13213-011-0272-z

469 Hwang, E. T., & Gu, M. B. (2013). Enzyme stabilization by nano/microsized hybrid
470 materials. *Engineering in Life Sciences*, *13*(1), 49–61.
471 http://doi.org/10.1002/elsc.201100225

472 Knoll, C., Fritsch, S., Schnell, S., Grossmann, M., Rauhut, D., & Du Toit, M. (2011).
473 Influence of pH and ethanol on malolactic fermentation and volatile aroma
474 compound composition in white wines. *LWT - Food Science and Technology*,
475 *44*(10), 2077–2086. http://doi.org/10.1016/j.lwt.2011.05.009

476 Kourkoutas, Y., Bekatorou, A., Banat, I. M., Marchant, R., & Koutinas, A. A. (2004).
477 Immobilization technologies and support materials suitable in alcohol beverages
478 production: A review. *Food Microbiology*, *21*(4), 377–397.
479 http://doi.org/10.1016/j.fm.2003.10.005

480 Kourkoutas, Y., Manojlović, V., & Nedović, V. A. (2010). Immobilization of microbial
481 cells for alcoholic and malolactic fermentation of wine and cider. In N. Zuidam &
482 V. Nedovic (Eds.), *Encapsulation Technologies for Active Food Ingredients and*
483 *Food Processing* (pp. 327–343). New York, USA: Springer.
484 http://doi.org/10.1007/978-1-4419-1008-0_12

485 Kuncová, G., & Trogl, J. (2010). Physiology of microorganisms immobilized into
486 inorganic polymers. In Desiree A. Morrison (Ed.), *Handbook of Inorganic*
487 *Chemistry Research* (pp. 53–101). New York, USA: Nova Science Publishers, Inc.

488 Maicas, S. (2001). The use of alternative technologies to develop malolactic
489 fermentation in wine. *Applied Microbiology and Biotechnology*.
490 http://doi.org/10.1007/s002530100662

491 Maicas, S., Pardo, I., & Ferrer, S. (2000). The effects of freezing and freeze-drying of
492 *Oenococcus oeni* upon induction of malolactic fermentation in red wine.
493 *International Journal of Food Science & Technology*, *35*, 75–79.
494 http://doi.org/10.1046/j.1365-2621.2000.00359.x

495 Maicas, S., Pardo, I., & Ferrer, S. (2001). The potential of positively-charged cellulose
496 sponge for malolactic fermentation of wine, using *Oenococcus oeni*. *Enzyme and*
497 *Microbial Technology*, *28*(4–5), 415–419. http://doi.org/10.1016/S0141-
498 0229(00)00339-2

499 Niederberger, M., & Pinna, N. (2009). Aqueous and nonaqueous sol-gel chemistry. In
500 *Metal Oxide Nanoparticles in Organic Solvents: Synthesis, Formation, Assembly*
501 *and Application* (pp. 7–18). London, UK: Springer London.
502 http://doi.org/10.1007/978-1-84882-671-7_2

503 Olguín, N., Champomier-Vergès, M., Anglade, P., Baraige, F., Cordero-Otero, R.,

504 Bordons, A., Zagorec, M, Reguant, C. (2015). Transcriptomic and proteomic
505 analysis of *Oenococcus oeni* PSU-1 response to ethanol shock. *Food*
506 *Microbiology*, *51*, 87–95. http://doi.org/10.1016/j.fm.2015.05.005

507 Pannier, A., Soltmann, U., Soltmann, B., Altenburger, R., & Schmitt-Jansen, M. (2014).
508 Alginate/silica hybrid materials for immobilization of green microalgae *Chlorella*
509 *vulgaris* for cell-based sensor arrays. *Journal of Materials Chemistry B*, *2*(45),
510 7896–7909. http://doi.org/10.1039/c4tb00944d

511 Pasparakis, G., & Bouropoulos, N. (2006). Swelling studies and in vitro release of
512 verapamil from calcium alginate and calcium alginate-chitosan beads.
513 *International Journal of Pharmaceutics*, *323*(1–2), 34–42.
514 http://doi.org/10.1016/j.ijpharm.2006.05.054

515 Rodríguez-Nogales, J. M., Vila-Crespo, J., & Fernández-Fernández, E. (2013).
516 Immobilization of *Oenococcus oeni* in Lentikats® to develop malolactic
517 fermentation in wines. *Biotechnology Progress*, *29*(1), 60–65.

518 Rosi, I., Fia, G., & Canuti, V. (2003). Influence of different pH values and inoculation
519 time on the growth and malolactic activity of a strain of *Oenococcus oeni*.
520 *Australian Journal of Grape and Wine Research*, *9*(3), 194–199.
521 http://doi.org/10.1111/j.1755-0238.2003.tb00270.x

522 Segarra, I., Lao, C., Lopez, E., & De La Torre, M. C. (1995). Spectrophotometric
523 methods for the analysis of polysaccharide levels in winemaking products.
524 *American Journal of Enology and Viticulture*, *46*(4), 564–570.

525 Simó, G., Fernández-Fernández, E., Vila-Crespo, J., Ruipérez, V., &
526 Rodríguez-Nogales, J. M. (2017a). Research progress in coating techniques of
527 alginate gel polymer for cell encapsulation. *Carbohydrate Polymers*. *170*, 1-14.

528 http://doi.org/10.1016/j.carbpol.2017.04.013

529 Simó, G., Fernández-Fernández, E., Vila-Crespo, J., Ruipérez, V., &
530 Rodríguez-Nogales, J. M. (2017b). Silica-alginate encapsulated bacteria to enhance
531 malolactic fermentation performance in a stressful environment. *Australian*
532 *Journal of Grape and Wine Research*, *23*(3), 342–349.
533 http://doi.org/10.1111/ajgw.12302

534 Simó, G., Vila-Crespo, J., Fernández-Fernández, E., Ruipérez, V., & Rodríguez-
535 Nogales, J. M. (2017). Highly efficient malolactic fermentation of red wine using
536 encapsulated bacteria in a robust biocomposite of silica-alginate. *Journal of*
537 *Agricultural and Food Chemistry*, *65*(25), 5188–5197.
538 http://doi.org/10.1021/acs.jafc.7b01210

539 Sosnik, A. (2014). Alginate particles as platform for drug delivery by the oral route:
540 state-of-the-art. *ISRN Pharmaceutics*, *2014*, 926157.
541 http://doi.org/10.1155/2014/926157

542 Strand, B. L., Skjåk-Bræk, G., & Gåserød, O. (2004). Microcapsule formulation and
543 formation. In V. A. Nedović & R. Willaert (Eds.), *Fundamentals of cell*
544 *immobilisation biotechnology* (pp. 165–183). Dordrecht, Netherlands: Springer.
545 http://doi.org/10.1007/978-94-017-1638-3_9

546 Sumby, K. M., Grbin, P. R., & Jiranek, V. (2014). Implications of new research and
547 technologies for malolactic fermentation in wine. *Applied Microbiology and*
548 *Biotechnology*, *98*(19), 8111–8132. http://doi.org/10.1007/s00253-014-5976-0

549 Sun, Z. J., Lv, G. J., Li, S. Y., Yu, W. T., Wang, W., Xie, Y. B., & Ma, X. (2007).
550 Differential role of microenvironment in microencapsulation for improved cell
551 tolerance to stress. *Applied Microbiology and Biotechnology*, *75*(6), 1419–1427.

552 http://doi.org/10.1007/s00253-007-0960-6

553 Torres, L. G., Velasquez, A., & Brito-Arias, M. A. (2011). Ca-alginate spheres behavior
554 in presence of some solvents and water-solvent mixtures. *Advances in Bioscience*
555 *and Biotechnology*, *2*(1), 8–12. http://doi.org/10.4236/abb.2011.21002

556 Wang, J. Y., Yu, H. R., Xie, R., Ju, X. J., Yu, Y. L., Chu, L. Y., & Zhang, Z. (2013).
557 Alginate/protamine/silica hybrid capsules with ultrathin membranes for laccase
558 immobilization. *AIChE Journal*, *59*(2), 380–389. http://doi.org/10.1002/aic.13834

559 Wu, C., Zhu, Y., Chang, J., Zhang, Y., & Xiao, Y. (2010). Bioactive inorganic-
560 materials/alginate composite microspheres with controllable drug-delivery ability.
561 *Journal of Biomedical Materials Research - Part B Applied Biomaterials*, *94*(1),
562 32–43. http://doi.org/10.1002/jbm.b.31621

563 Xu, S., Jiang, Z., Lu, Y., Wu, H., & Yuan, W.-K. (2006). Preparation and catalytic
564 properties of novel alginate−silica−dehydrogenase hybrid biocomposite beads.
565 *Industrial & Engineering Chemistry Research*, *45*(2), 511–517.
566 http://doi.org/10.1021/ie050940y

567 Xu, S. W., Lu, Y., Li, J., Jiang, Z. Y., & Wu, H. (2006). Efficient conversion of CO_2 to
568 methanol catalyzed by three dehydrogenases co-encapsulated in an alginate-silica
569 (ALG-SiO_2) hybrid gel. *Industrial and Engineering Chemistry Research*, *45*(13),
570 4567–4573. http://doi.org/10.1021/ie051407l

571 Yi, Y., Neufeld, R., & Poncelet, D. (2004). Immobilization of cells in polysaccharide
572 gels. In S. Dumitriu (Ed.), *Polysaccharides: Structural Diversity and Functional*
573 *Versatility* (pp. 867–891). New York, USA: CRC Press.

574

Figure captions

Fig. 1. Optical images of (a) alginate and (b) SiO_2-alginate biocapsules with entrapped *Oenococcus oeni.* (c-f) Environmental scanning electron microscopy (ESEM) of alginate (ALG) and SiO_2-alginate (SiO_2-ALG) biocapsules with entrapped *Oenococcus oeni*. (c,e) External structure of the biocapsules. (d,f) Internal structure of the biocapsules.

Fig. 2. FTIR-ATR spectra of (a) alginate and (b) SiO_2-alginate capsules.

Fig. 3. Conversion of L-malic acid by SiO_2-alginate encapsulated (a-b) and free (c-d) *Oenococcus oeni* in red wines at different pH (3.0, 3.3, and 3.6) and different alcohol degree (12.0, 13.5, and 15.0%) in five subsequent cycles. MLF conditions: red wine (3.0 g/L of L-malic acid), 0.03 g of biocapsules per mL of wine, bacterial load of $\sim 9 \times 10^7$ cfu/mL of wine, temperature of 22°C, and 120 h of incubation. Standard deviations of the assays are represented by error bars (n=3). Different letters in the bars indicate a significant difference between samples ($p<0.05$).

Fig. 4. Evolution of shrinking (expressed as decrease of diameter (in %) and weight (in %)) of alginate (●) and SiO_2-alginate (■) biocapsules loaded with *Oenococcus oeni* submerged in red wines at different pH ((a-b) 3.0, (c-d) 3.3 and (e-f) 3.6). Shrinking was calculated from the average of 20 biocapsules submerged in wine at 22°C. Standard deviations of the assays are represented by error bars. ∗ indicates a statistically significant difference ($p<0.05$) between both capsules.

Fig. 5. Evolution of shrinking (expressed as decrease of diameter (in %) and weight (in %)) of alginate (●) and SiO_2-alginate (■) biocapsules loaded with *Oenococcus oeni* submerged in red wines at different alcohol degree ((a-b) 12.0%, (c-d) 13.5% and (e-f) 15.0%). Shrinking was calculated from the average of 20 biocapsules submerged in wine at 22°C Standard deviations of the assays are represented by error bars. ∗ indicates a statistically significant difference (p<0.05) between both capsules.

Fig. 6. Release of alginate (expressed as total polysaccharides) from alginate (■) and SiO_2-alginate (□) biocapsules loaded with *Oenococcus oeni* submerged in red wines at different pH (3.0, 3.3 and 3.6) and alcohol degree (12.0%, 13.5% and 15.0%) after 18 days. Standard deviations of the assays are represented by error bars. Different letters in the bars indicate a significant difference between samples ($p < 0.05$).

Figure 1
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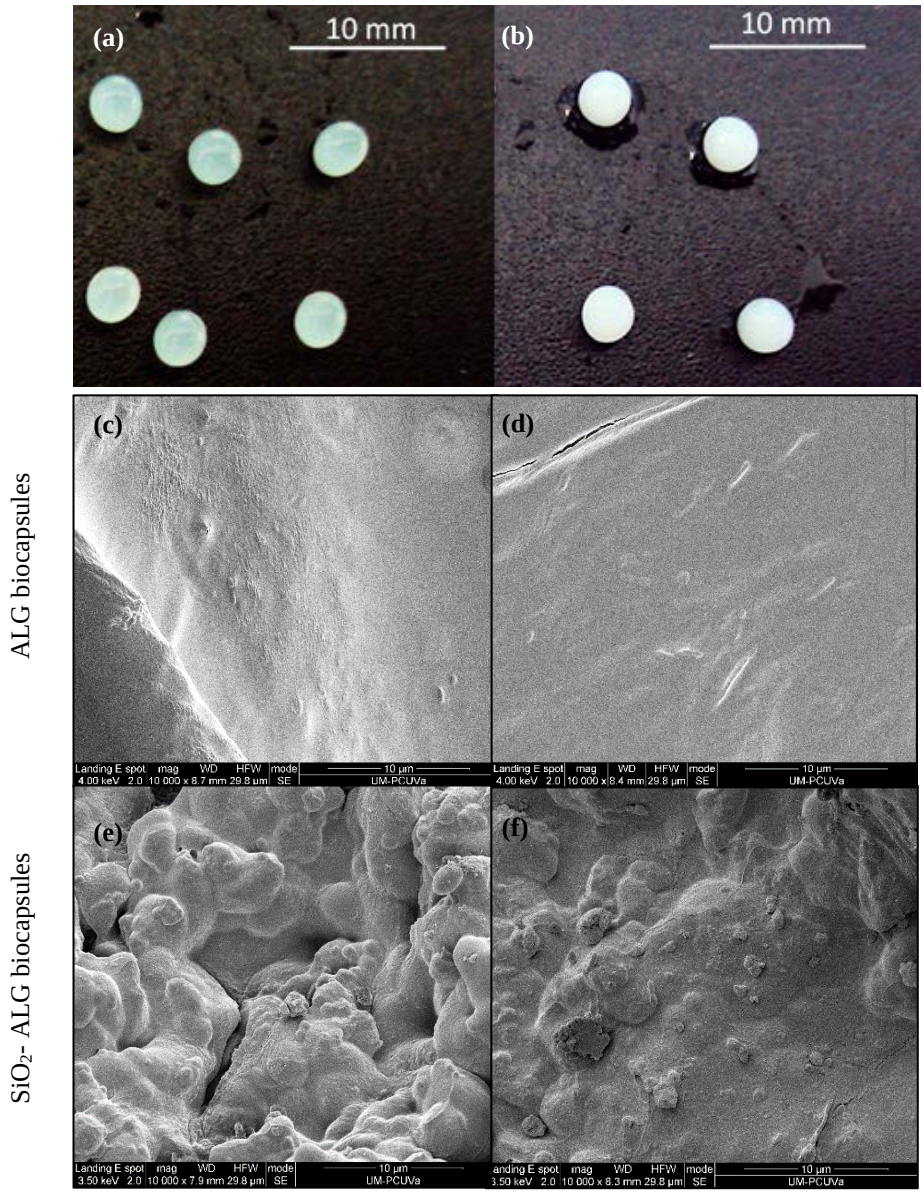

Fig. 1.

Figure 2
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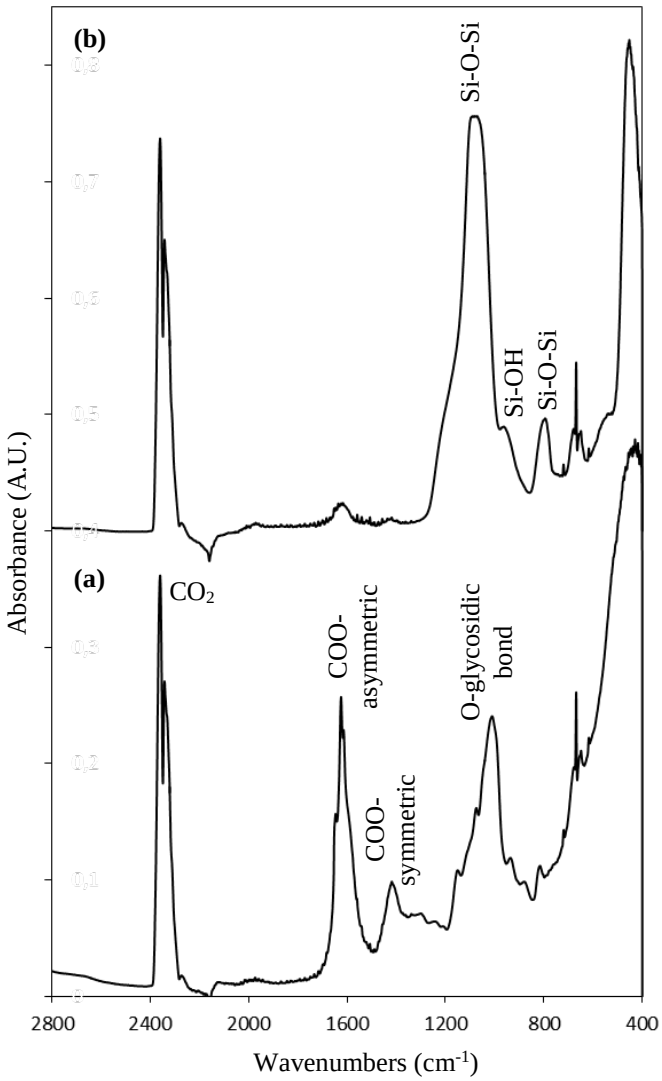

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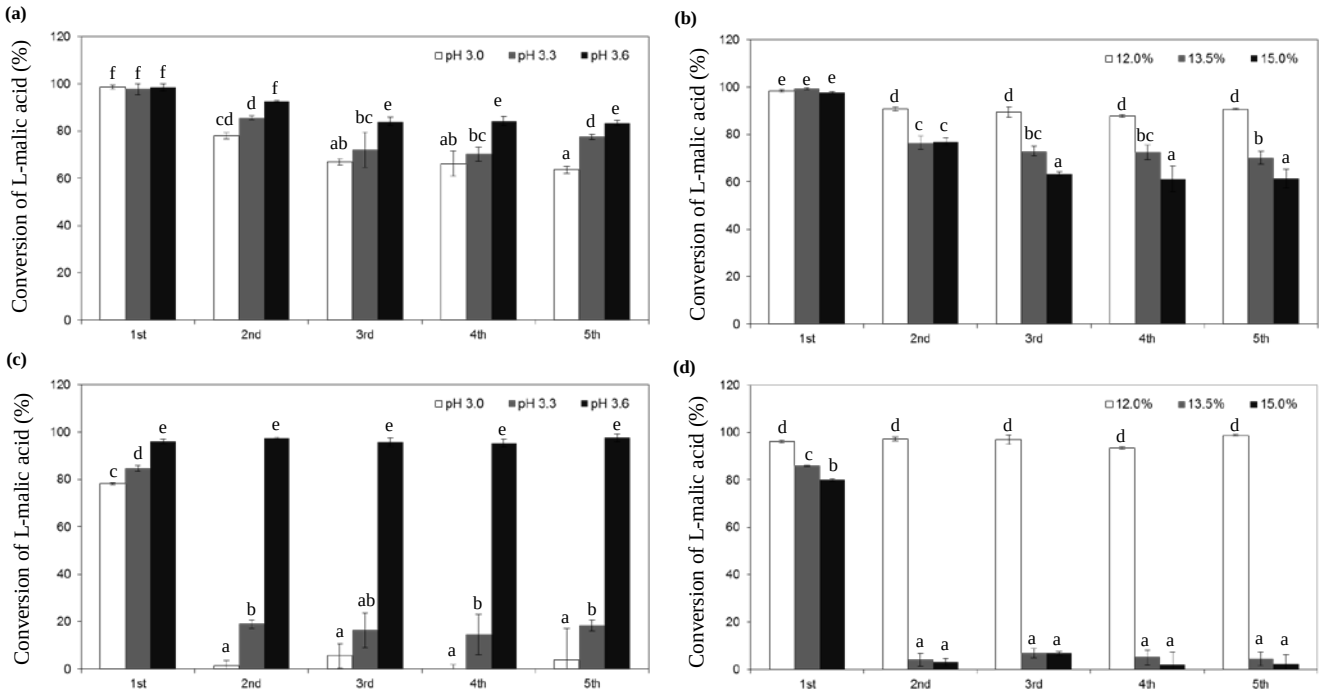

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Figure 4

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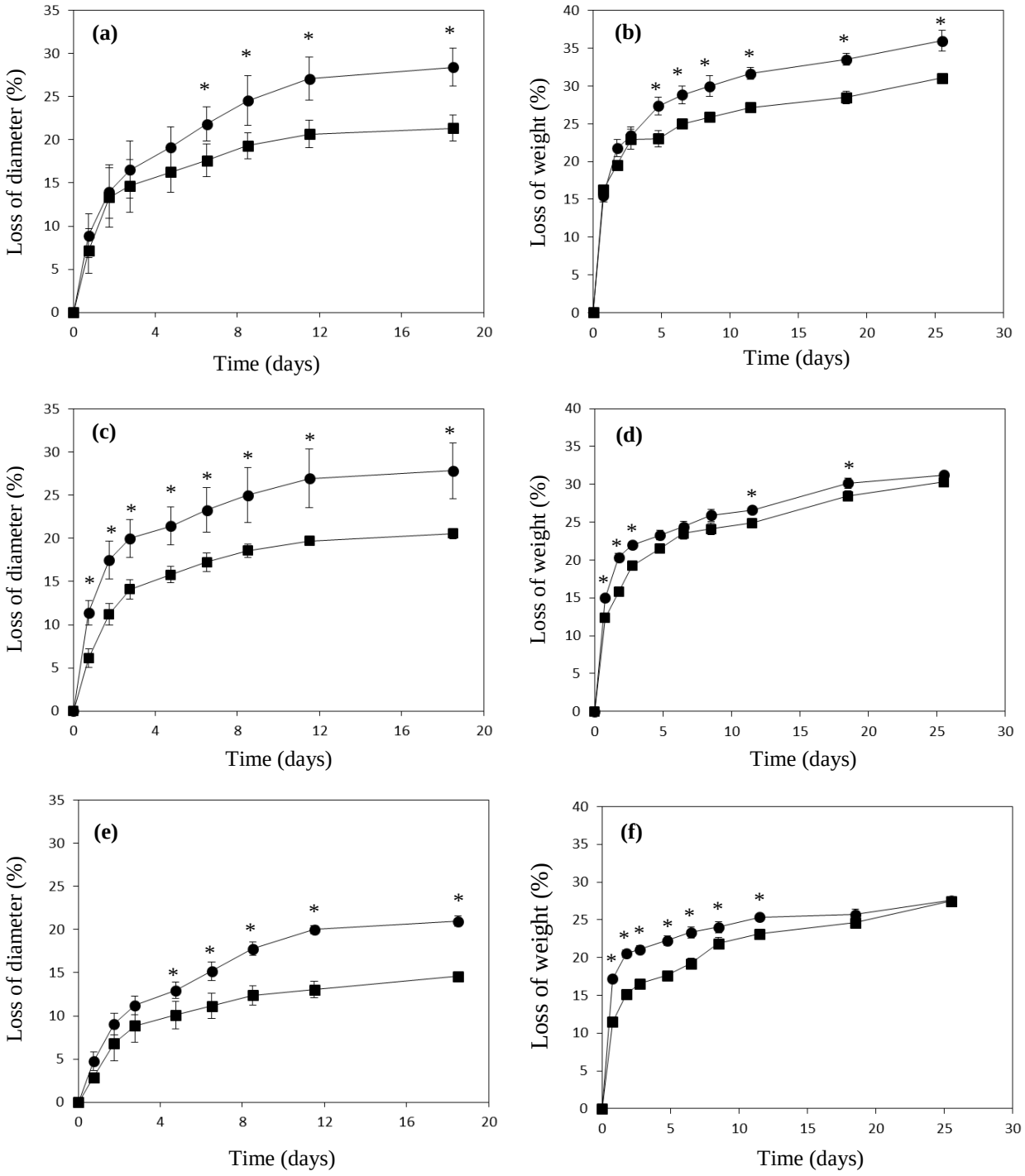

Fig. 4.

Figure 5

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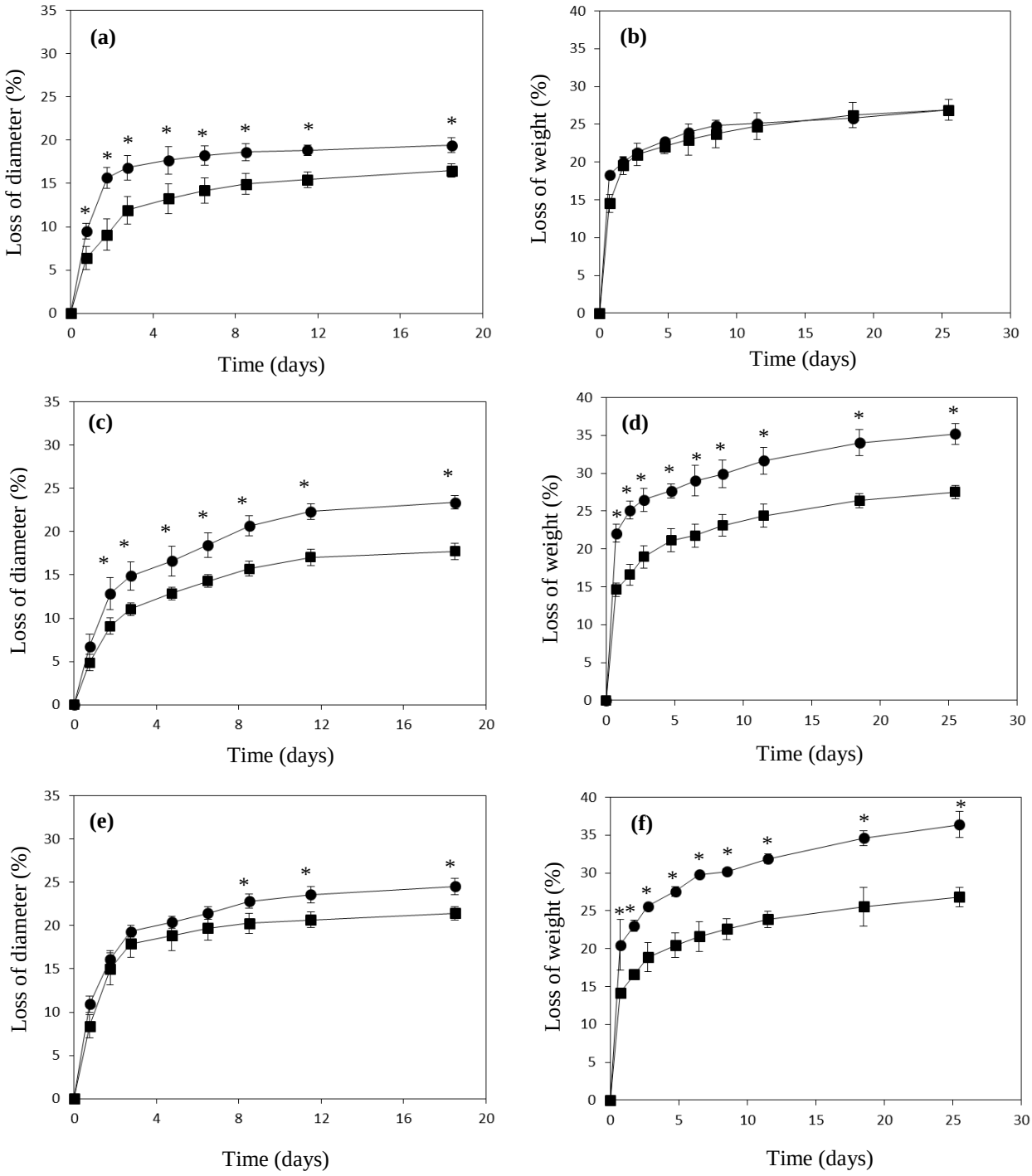

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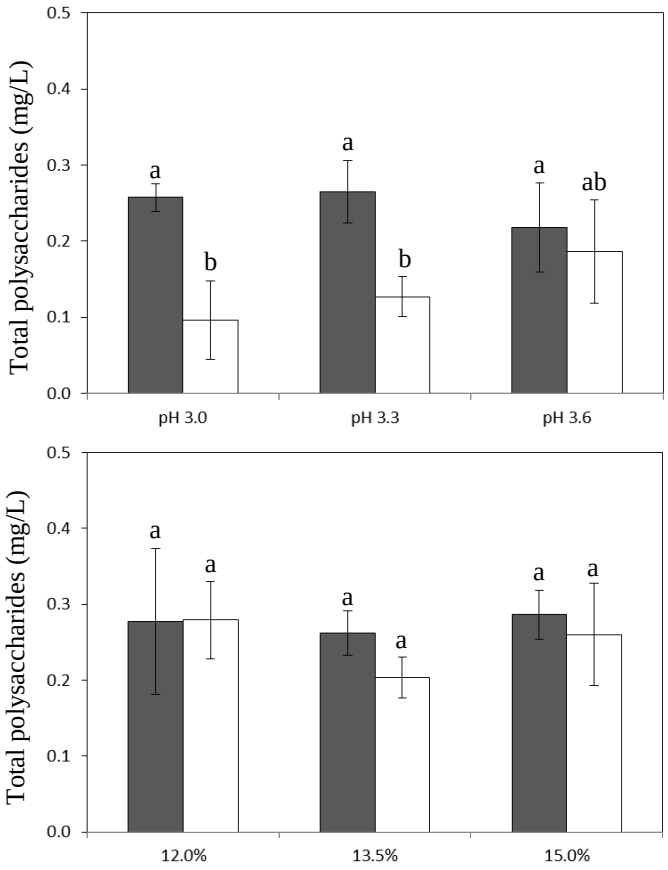

Fig. 6.