Effect of biopolymer matrices on lactose hydrolysis by enzymatically active hydrogel and aerogels loaded with β-galactosidase nanoflowers

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A B S T R A C T

In this work, enzymatically active polysaccharide-based hydrogels and aerogels have been developed. To this end, a thermostable β-galactosidase (TmLac) enzyme from *Thermotoga maritima* embedded in nanoflowers´ format was used to evaluate the capacity of the hydrogel matrices to preserve the hydrolytic activity of the enzyme and the reusability of the hydrogels formed. Commercial agar, unpurified agar and agarose were compared as supporting materials. Although the developed hydrogel capsules can be used at high temperature (75 °C) and reused for the digestion of lactose to a greater extent than the free nanoflowers, loaded hydrogel capsules behaved differently depending on the type of polysaccharide used. Commercial agar was the most promising one since these hydrogel capsules could be reused, maintaining the structural integrity and reaching higher enzymatic activity (after seven cycles at 75 ºC) than the free TmLac-Ca^{2+} nanoflowers.

To facilitate handling and storage, aerogels were developed by freeze-drying the hydrogel capsules. Aerogels of agarose and unpurified agar underwent structural changes during freeze-drying that adversely affected their subsequent use, losing their integrity after being rehydrated. However, commercial agar aerogels were successfully developed and reused thanks to the existing interactions with TmLac-Ca^{2+} nanoflowers (confirmed by FTIR), which resulted in better capsule integrity and enzyme protection. The hydrolytic activity of enzymatically active aerogels based on commercial agar was in the same range of the free TmLac and TmLac-Ca^{2+} nanoflowers, being significantly higher to their counterparts in the hydrated form (hydrogels based on commercial agar).

Keywords:

β-galactosidase – hydrogel capsules -agar - milk products-nanoflowers-aerogels

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1. Introduction

Nowadays, about 75% of the world's adult population is lactose-intolerant (Silanikove, Leitner & Merin, 2015) and people who suffer from lactose intolerance often reduce consumption of bovine milk and other dairy products, which contain high quality proteins.

To overcome these issues, the food industry offers a wide range of lactose-free dairy products, where lactose has been hydrolyzed into glucose and galactose so that also lactose intolerant consumers can enjoy the variety of dairy products in their daily diet. The process of obtaining these lactose-free products at industrial level consists of the direct addition of the fungal enzyme β-galactosidase, generally obtained from *Kluyveromyces lactis* (Zolnere & Ciprovica, 2018; Dekker, Koenders & Bruins, 2019). However, this procedure has a disadvantage since the enzyme remains in the final product (Chen et al., 2008) and is not being able to be reused. A very plausible alternative is to immobilize the enzymes (Liese & Hilterhaus, 2013) which can result in increased shelf-life and stability, allowing their recovery and reuse, thus having a positive impact on production costs.

Furthermore, it is worth mentioning that, in order to reduce the risk of microbial contamination, β-galactosidase enzymes coming from thermophilic microorganism are particularly attractive because they can be easily purified by heat treatments (Marín-Navarro, Talens-Perales, Oude-Vrielink, Cañada, & Polaina, 2014). This explains why, in the last decade, there has been an increased interest in finding microorganisms and enzymes that meet these characteristics. In this regard, *Thermotoga maritima* is a thermophilic bacterium with an optimal growth temperature close to 80 ºC (Huber et al., 1986) is a convenient source for heat-resistant enzymes like β-galactosidase (TmLac) (Kim, Ji, & Oh et al. 2004).

The three-dimensional structure of TmLac has been recently reported by Minguez et al., 2020. Furthermore, its immobilization by using different methodologies and supporting materials has been also reported (Estevinho et al., 2018, Fabra et al., 2019). However, either costs or difficulties

77 in scaling-up immobilization technologies have mainly hampered industrial implementation
78 (Grosová, Rosenberg, & Rebroš, 2008).
79 In recent years, the use of micro- and nanomaterials for enzyme immobilization is gaining attention
80 because they have resulted in improved thermal stability and activity of the immobilized enzymes and
81 can also expand their applicability, and provide manipulation platforms to facilitate their use in
82 different field applications (Ansari & Husain, 2012, Hasanzadeh, Shadjou & de la Guardia., 2018,
83 Hong, Liu, Li, & Chen, 2019, Wang, Mohanty, & Mohanty, 2019). An interesting group of
84 nanomaterials is the so-called nanoflowers (Ge, Lei & Zare, 2012). Since its discovery, different
85 enzymes and other biomolecules have been immobilized with this technique (Liu et al., 2019). In this
86 regard, Talens-Perales, Fabra, Martínez-Argente, Martín-Navarro, & Polaina (2020) have recently
87 compared the efficiency of the immobilization of TmLac in the form of nanoflowers, using different
88 salts of Cu^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+} and Ca^{2+} as inorganic compounds and being TmLac-Ca^{2+} nanoflowers
89 the most promising ones.
90 Even though nanotechnology has opened up a new frontier in the development of organic supports for
91 enzyme immobilization having positive results, one of the concerns is the application at large scale.
92 Furthermore, the separation process of these nanoflowers from the reaction medium at the end of the
93 catalytic process may be difficult and sometimes expensive, thus decreasing applicability (Cipolatti et
94 al., 2016, Cui & Jia, 2017). For this reason, combining physical adsorption of enzymes to
95 nanoflowers with encapsulation of these enzyme-containing nanoflowers within biopolymers can
96 be a good approach to solve some of the previously mentioned issues (Zhao et al., 2017, Bilal &
97 Iqbal, 2019).
98 Among biopolymers, agar is highly attractive for thermostable enzymes since upon solubilization in
99 hot water (~ 90 ºC) and, under specific conditions, agar forms a slightly viscous fluid that can form
100 thermorreversible hydrogels when the temperature is decreased below a certain temperature (i.e.
101 gelling temperature). Agar is a hydrophilic linear polysaccharide derived from red seaweed (*Gelidium*,

102 *Pterocladia*, and *Gracilaria*) and contains alternating β-(1,3)- and α-(1,4)-linked galactose residues
103 with sulphated functional groups (Shankar & Rhim, 2017). It consists of a mixture of agarose and
104 agaropectin. Specifically, agarose is a linear polymer made up of repeating agarobiose units of
105 agarobiose, which is a disaccharide made up of D-galactose and 3,6-anhydro-L-galactopyranose and
106 it is the gelling component of agar. Many factors, such as the agar concentration, the amount and type
107 of impurities and the cooling rate affect the gelation mechanism of agar (Aymard et al., 2001, Lai &
108 Lii, 1997, Lee et al., 2017). At industrial scale, agar extraction process involves the application of
109 alkaline pre-treatments, which is critical for obtaining good quality agar although this treatment
110 significantly decreases the agar extraction yield. In contrast, unpurified agar (without alkali pre-
111 treatment) can be obtained using simpler procedures and with greater yields and the extracts contain
112 bioactive compounds such as proteins and polyphenols. These carbohydrates were selected as an
113 alternative to the widely used alginate for the formation of hydrogel capsules, since they can be
114 manipulated at the optimum temperature of TmLac (~ 75 ºC) in gel form without being melted. The
115 preliminary hypothesis is that the gel strength, influenced by the inner structure of the hydrogel, will
116 be different depending on the material used (Martínez-Sanz et al., 2019) and will probably affect the
117 diffusion of the substrate (lactose) throughout the biopolymer network, thus resulting in different
118 lactose hydrolysis rates and degrees. The physical state of the supporting materials is supposed to also
119 affect the diffusion of substrate. Thus, hydrogel capsules (in which the dispersed phase within the
120 crosslinked biopolymer network is water) and aerogels (a more porous structure in which the water
121 has been replaced by air) will provide different enzymatic activity.
122 Considering these aspects, this work was designed to i) encapsulate TmLac- Ca^{2+} nanoflowers in
123 hydrogel structures based on seaweed-derived hydrocolloids in order to improve their handling
124 properties; ii) to evaluate commercial agar, unpurified agar and agarose (the gelling component of
125 agar) as encapsulation matrices in terms of lactose hydrolyzing capacity of the structures developed
126 thereof; and iii) to evaluate the physical state (hydrogel *vs.* aerogel) of the polysaccharide

encapsulating matrices on the enzymatic activity. Hydrogel capsules were prepared by means of a novel methodology named "oil-induced biphasic hydrogel particle formation" previously described by Alehosseini et al., 2019.

2. Materials and methods

2.1. Chemical and reagents

Salts used for nanoflowers formation ($CaCl_2$) or for buffer preparation (NaCl and $MgCl_2$), p-nitrophenyl ß-D-Galactopyranoside (pNP-Gal) and the glucose assay kit were purchased from Sigma Aldrich. Lactose 1-hydrate was supplied by Panreac Quimica (Spain). Commercial agar, the raw seaweed material (*Gelidium sesquipedale*) and agarose used as encapsulating matrices were kindly donated by Hispanagar S.A. (Burgos, Spain). BlueSafe stain was supplied by Nzytech. Sunflower oil was purchased from a local supermarket.

2.2 Production and purification of TmLac

Procedures used for the production and purification of TmLac from *Thermotoga maritima* are described in Marin-Navarro et al. (2014). Briefly, cultures of *E. coli* Rosetta 2 strain, carrying plasmid pQE-80L in which the TmLac encoding gene had been cloned, were induced to allow the synthesis of histidine-tagged TmLac. The enzyme was purified from crude cell extracts in two stages, first by thermal treatment at 85 ºC, and then by nickel affinity chromatography.

2.3 Production of TmLac-inorganic nanoflowers

Nanoflowers were obtained by the procedure described by Ge et al. (2012) with some modifications as described by Talens-Perales et al (2020). Briefly, a stock solution of TmLac was firstly prepared in phosphate-buffered saline (PBS) at a final concentration of 0.05 mg/mL and,

152 then, 20 µL of 120 mM $CaCl_2$ solution were added to 1500 µL of the enzyme solution and
153 incubated at room temperature (23 ± 2 ºC) for 24 h. After this time, nanoflowers, which appeared
154 as a precipitate sediment, were washed with PBS and resuspended in assay buffer (50 mM
155 phosphate buffer, pH 6.5, 10 mM NaCl, 1 mM $MgCl_2$). The presence and activity of TmLac in
156 the nanoflowers was tested enzymatically by the remaining activity in the supernatant after
157 nanoflower formation, by using 5 mM *p*-nitro phenyl β-D-galactopyranoside (pNP-Gal) as the
158 substrate (Talens-Perales et al., 2020).
159
160 *2.4. Preparation of hydrogel capsules and aerogels*
161 Hydrogel capsules containing TmLac-Ca^{2+} nanoflowers was performed following a protocol
162 adapted from Alehosseini *et al.*, 2019. In brief, an aliquot of 200 µL of nanoflowers resuspended
163 in buffer were mixed with 200 µL stock polysaccharide aqueous solutions (commercial agar,
164 unpurified agar and agarose) (see Supplementary material S1). Stock polysaccharide aqueous
165 solutions were previously prepared by dissolving the required amount of each polysaccharide in
166 hot water at ~90 ºC to have a final concentration of 1% (w/v) of commercial agar or agarose and
167 3.5 % (w/v) of unpurified agar. Then, solutions were cooled until approx. 40 ºC and introduced
168 in a 5 mL plastic syringe with a yellow pipette tip. Hydrogel capsules were formed by dripping
169 the nanoflowers-containing polysaccharide based solutions into a biphasic bath containing an
170 upper sunflower oil layer and a lower cold water layer, for the collection of the hydrogel particles.
171 Control hydrogel capsules (without nanoflowers) were prepared for comparative purposes.
172 Aerogels, with and without nanoflowers, were prepared by freeze-drying the hydrogel capsules
173 using a Genesis 35-EL freeze-dryer (Virtis). To this end, hydrogel samples were frozen in liquid
174 nitrogen (T≤−210 °C) and kept in a freezer at -80 ºC until being lyophilized.
175 Unpurified agar, used as encapsulating matrix, was previously obtained following the procedure
176 described by Martínez-Sanz et al., 2019. Briefly, 50g of seaweed powder were immersed in 500

177 mL of distillated water and heated up to 90 ºC for 2h. The agar-based solution was subsequently
178 separated from the solid residue by filtration with a muslin cloth and the filtrate was allowed to
179 gel and then, it was frozen overnight at - 21 ºC. Afterwards, the material was subjected to two
180 freeze-thaw cycles (-21 ºC/25ºC) to improve the elastic properties of the gels and the obtained
181 material was freeze-dried for further use. The unpurified agar extract was characterized, having
182 392 ± 77 mg/g of carbohydrates (~ 75% galactose), 136 ± 9 mg BSA/g of proteins and 30 ± 2 mg
183 GA/g of polyphenols and 2.8 ± 0.2 % of sulphates (Martínez-Sanz et al., 2019). This explains the
184 higher concentration needed to form gels from the unpurified agar used as encapsulation matrix
185 when compared to that from commercial agar and agarose.
186
187 *2.5. Scanning Electron Microscopy (SEM)*
188 Aerogels were observed in a Hitachi SEM microscope (Hitachi S-4800) at an accelerating
189 voltage of 10 kV and a working distance of 8.0 – 8.5 mm. Samples were mounted on an
190 Aluminium Specimen Mounts, fixed on the support using double-side adhesive tape and coated
191 with a thin layer of gold-palladium sprayed on their surface. Cross-section of aerogels were
192 explored by cryo-fracturing them after immersion in liquid nitrogen, before being mounted on the
193 aluminum support.
194
195 *2.6. Enzymatic activity assays*
196 Enzyme activity determinations were carried out, in triplicate, at the optimum pH (6.5)
197 and temperature (75 ºC) of the enzyme, using lactose as the substrate. Nanoflowers, free or
198 encapsulated, were washed with PBS at room temperature to remove residual unbound enzyme
199 and dispersed in 5% (w/v) lactose in the assay buffer for 30 min. Reactions were finished by
200 heating at 95 ºC for 10 minutes. Lactase activity was measured by quantifying the amount of
201 glucose released, using a glucose assay kit (Sigma).

In order to obtain the kinetics of lactose hydrolysis, the incubations were performed at 75 ºC for 8 h in 5% (w/v) lactose in assay buffer, taking an aliquot after the first 30 min and, then, after every hour. Lactose hydrolysis at each incubation time was measured as already explained.

The possibility of reusing the hydrogel and aerogels capsules was tested by subjecting them to consecutive cycles of lactose hydrolysis at 75 ºC for 3.5 h with gentle agitation. After each incubation time, samples were recovered by centrifugation and washed with assay buffer before being placed again with assay buffer with 5% (w/v) lactose for the following reaction cycle. Remaining lactose in the supernatant of the reaction, after each cycle, was estimated as a function of the amount of glucose measured with a Glocose (GO) Assay Kit.

2.7. Color analysis of immobilized nanoflowers labelled with BlueSafe

To visually observe the immobilized nanoflowers within the hydrogel capsules, capsules were stained with a protein stain called BlueSafe. To this end, loaded and unloaded hydrogel capsules were stained with BlueSafe during 2h and then, capsules were washed overnight with distillated water to remove residual stain. Finally, samples were observed by means of a digital microscope EVO CamII (Vision Engineering, United States).

2.8. Fourier transform infrared (FT-IR) analysis of the materials

The attenuated total reflectance (ATR)-FTIR spectra of the freeze-dried capsules were obtained in a Thermo Nicolet Nexus equipment. The acquisition time was 128 s at 4 cm^{-1} resolution, and the average spectra in the 4000-400 cm^{-1} range are reported.

2.9. Gel strength

The gel strength was measured by using a TA-XTplus Texture Analyser (Stable Micro Systems, Surrey, UK), with a 5 N load cell, using a 2mm diameter cylindrical probe. Hydrogel capsules

were 50% compressed at 1 mm/s deformation rate. Measurements were carried out in ten hydrogel capsules.

2.10 Statistical analysis

Statistical analysis of experimental data was carried out with the IBM SPSS Statistics software (v.23) (IBM Corp., USA) through the analysis of variance (ANOVA). Comparison of the means was done using the Tukey's Honestly Significant Difference (HSD) at 99% confidence level.

3. Results and discussion

3.1 Enzymatically active hydrogel capsules based on commercial agar, unpurified agar and agarose.

Figure 1 shows representative images of the polysaccharide hydrogel capsules prepared with and without TmLac-Ca^{2+} nanoflowers. As clearly observed, all capsules were similar in shape and size (~2 mm diameter), being apparently rougher those prepared with unpurified agar. In order to qualitatively evaluate the presence and distribution of nanoflowers in the loaded capsules, the protein (TmLac) was stained with BlueSafe, which is a highly sensitive single step protein-specific stain. As expected, unloaded agarose hydrogel capsules (without TmLac-Ca^{2+} nanoflowers) were not stained. However, a bluish coloration was observed in commercial agar-based hydrogel capsules, which is indicative of the presence of proteins deriving from the native algae, which had not been eliminated during the extraction process. In fact, it is worth mentioning that *Gelidium sesquipedale* has about 30% protein (Faraj, Lebbar, Debry & Najim, 1987) and, thus, it is possible that a small amount of proteins remained after the industrial purification process with sodium hydroxide, which is performed prior to the agar extraction. In fact, a more intense blue color was observed for the hydrogels prepared from unpurified agar, in agreement

253 with the compositional analysis results of these extracts (136 mg BSA/g of proteins, Martínez-
254 Sanz et al., 2019) and as it was also evidenced by FTIR analysis (as shown below).
255 The incorporation of enzyme-loaded nanoflowers caused a significant increase in the intensity of
256 bluish coloration, which was closely related to the presence of the TmLac and a homogeneous
257 distribution of blue color was observed throughout the hydrogel capsules indicating that
258 nanoflowers were well-distributed within the hydrocolloid matrices. Differences in the bluish hue
259 of loaded hydrogel capsules evidenced a synergetic effect between proteins remaining in the agar
260 matrices and TmLac-Ca^{2+} nanoflowers. Accordingly, the intensity of the bluish coloration in
261 agarose-loaded samples was significantly lower due to the absence of proteins in the unloaded
262 pure agarose matrix (Figure 1E), indicating that the blue color in loaded agarose samples only
263 corresponded to the contribution of TmLac-containing nanoflowers.
264 The enzymatic activity of the immobilized enzyme was assessed by incubating hydrogel capsules
265 in lactose 5% (w/v). For comparative purposes, the free enzyme and TmLac-Ca^{2+} nanoflowers
266 were also incubated at an equivalent concentration. It should be highlighted that direct
267 incorporation of the TmLac within the three hydrogel matrices was also attempted for comparison
268 purposes, but it resulted in leaking of the enzymes to the media and, thus, no remaining
269 hydrolyzing activity of the hydrogel capsules produced, thus requiring the pre-immobilization
270 step in nanoflowers to deliver enzymatically active materials. Table 1 shows the specific activity
271 (measured after 30 min of incubation with lactose) and Figure 2 gathers the kinetics of lactose
272 hydrolysis of the free TmLac, TmLac-Ca^{2+} nanoflowers and hydrogel capsules loaded with
273 TmLac-Ca^{2+} nanoflowers. As observed, TmLac-Ca^{2+} nanoflowers behaved similarly to the free
274 enzyme, in agreement with the results recently reported by Talens-Perales et al., (2020). However,
275 a significant decrease ($p<0.05$) in the enzymatic activity was observed in the loaded hydrogel
276 capsules.

As expected, the immobilized enzyme was slightly less efficient than the unprotected TmLac-Ca^{2+} nanoflowers in the conditions tested (Fig. 2), reaching around 85 % hydrolysis after about 3 h of incubation with lactose. Similarly, Panesar, Kumari, & Panesar 2011 reported 85% of lactose hydrolysis of enzymatically active permeabilized *Kluyveromyces marxianus* cells immobilized into alginate hydrogels and incubated during two hours. Although the differences were small, the presence of impurities interacting with the agar seemed to affect the specific hydrolytic activity of the encapsulated TmLac-Ca^{2+} nanoflowers. This seems to be related to two different factors: (i) the diffusion of substrate through biopolymer matrices, which may be restricted in hydrated systems (hydrogels) and (ii) the biopolymer gel network structure, which will impact the strength of the hydrogel capsules. The catalytic activity of the immobilized enzymes is greatly affected by the porosity, particle size and polarity of the hydrogel matrix, amongst others, as all these factors have an impact on diffusion kinetics (Drozdov, Papadimitriou, Liely & Sanporean, 2016; Nieto et al., 2010) and, thus, the gel structure was expected to affect enzyme accessibility and, consequently, result in slightly decreased enzymatic activity, when compared to that displayed by free enzymes. Additionally, the inherent highly hydrated state of the hydrogel capsules also poses additional substrate (lactose) diffusion restrictions for accessing the active enzyme sites, as water is strongly bonded to form a three-dimensional polymeric network providing mechanical integrity and flow resistance.

To confirm this hypothesis, the mechanical properties of the hydrogel capsules were evaluated by means of penetration tests. Table 2 summarizes the calculated gel fracture strength values for the loaded and unloaded hydrogel capsules. The first thing to highlight is that hydrogel capsules prepared with unpurified agar could not be measured. This can be ascribed to the presence of unpurified compounds (polyphenols, proteins) which greatly affect the gel strength. This hypothesis was reinforced by comparing the agarose capsules with and without nanoflowers, since the incorporation of nanoflowers negatively affected the gel strength presumably because the

presence of nanoflowers could impair the hydrogen-bonding network formation, thus affecting the resulting microstructure. Agar hydrogel capsules are an intermediate between a solid and liquid having both elastic (solid) and flow (liquid) characteristics. The agar gelation process has been widely reported in literature (Guenet & Rochas, 2006, Matsuo, Tanaka, & Ma, 2002, Stanley, 2006). The microstructure of agar hydrogels revealed a porous network made of bundles of agarose helices (Chui, Phillips & McCarthy, 1995, Nordqvist & Vilgis, 2011), in which the agaropectin do not participate in the gel network and obstruct the pores. Thus, TmLac-Ca^{2+} nanoflowers would affect the gelation mechanism of agarose since it would interfere in the formation of hydrogen bonds between agarose chains (the gelation component of the agar). In contrast, this effect was not observed in the commercial agar, where the presence of nanoflowers did not cause significant changes in the gel strength of the hydrogel capsules ($p>0.05$), thus, suggesting that the presence of agaropectin in the commercial agar samples was responsible of this different behavior as it will be later on confirmed by FTIR analysis (see below). Furthermore, these results are also in line with the enzymatic activity results, highlighting that, as suggested, hydrogel network organization, resulting in different gel strength, has an impact on substrate diffusion as, indeed, the loaded agar capsules with greater gel strength were the ones in which a lower enzymatic activity was observed.

The recyclability of the immobilized enzyme after consecutive incubations with 5% (w/v) lactose at 75 ºC was also tested. As shown in Figure 3, the enzymatic activity of the nanoflowers decreased about 65% after four incubation rounds and, a slighter but progressive decay of activity was observed, albeit to a lesser extent, in subsequent incubations. Loaded capsules behaved differently depending on the type of carbohydrate-based encapsulating matrix used. Interestingly, a statistically significant increase in the hydrolysis activity after the first batch was observed in hydrogel capsules formed either with commercial agar or with agarose. This increase was in accordance with that reported for agarose-alginate hydrogel capsules in which bacterial cells

327 loaded with a TmLac were embedded (Fabra et al., 2019). This phenomenon suggests that, after
328 the first incubation at 75 ºC, these hydrogel matrices underwent a structural change, promoting
329 the mobility of molecular chains and leading to the formation of less densely packed structures.
330 This structural relaxation phenomenon was the responsible of the better diffusion of lactose
331 (substrate) through the encapsulation matrix, as it was previously demonstrated for
332 alginate/agarose hydrogel capsules (Fabra et al., 2019). After the second incubation, a progressive
333 decay of activity (in both commercial agar and agarose) was observed, reaching a total loss of
334 ~50 % after seven hydrolysis batches. Nevertheless, the enzymatic activity of the agarose and
335 commercial agar hydrogel capsules was greater for each incubation cycle than for the free
336 nanoflowers, being this enhanced hydrolytic activity attributed to the enzyme protection effect of
337 the hydrogel matrices. Similarly, TmLac-immobilized on the surface of epoxy-coated magnetic
338 beads was able to hydrolyze more than 60 % of the lactose after eight incubations cycles (Marín-
339 Navarro et al., 2014).
340 In contrast, although the enzymatic activity obtained for hydrogel capsules made from unpurified
341 agar showed the same trend as those obtained with commercial agar and agarose, the lactose
342 hydrolysis after each incubation cycle was considerably lower ($p<0.05$) than that of the loaded
343 commercial agar or agarose-based capsules and even from the activity obtained using the free
344 nanoflowers, reaching an enzymatic activity loss around 86%, which was similar to that obtained
345 for free nanoflowers, after the seven batches. As mentioned before, the presence of other
346 compounds (i.e. proteins, polyphenols) in the unpurified agar reduced the gelling capacity of the
347 agar since they interfered in the self-assembling of agarose molecules (via hydrogen bonding)
348 during the gelation process and thus, softer gels with less dense network were formed, as it has
349 been previously confirmed by the mechanical analysis. These less dense structures are
350 hypothesized to offer less protection to the loaded enzymes, thus resulting in similar performance
351 when compared with the TmLac-containing nanoflowers.

352

353 *3.2 Enzymatically-active aerogels based on agar.*

354 In an effort to increase the activity and stability of immobilized enzymes, aerogels were formed
355 by freeze-drying, aiming to increase their handling and applicability as compared to their
356 counterparts prepared in the hydrated form. Furthermore, the substrate (lactose) diffusion through
357 the biopolymer network is also expected to be improved due to the absence of water molecules
358 in the pristine aerogel network. If fact, it is expected that aerogels quickly absorb water by
359 capillarity and thus, the substrate once it will be in contact with the lactose solution. Aerogels are
360 solids that feature very low density, high specific surface area and consist of a coherent open-
361 porous network of loosely-packed chains which are formed by replacement of the liquid in a gel
362 with gas (Ubeyitogullari, Brahma, Rose & Ciftci, 2018). Thus, hydrogel capsules based on
363 commercial agar, unpurified agar and agarose were freeze-dried and the enzymatic activity of the
364 aerogel structures was tested. The visual appearance of the developed aerogels is shown in Figure
365 4, where it is evidenced that whereas those prepared with commercial agar and agarose presented
366 a whitish hue (being more opaque in the case of commercial agar), the aerogel prepared with
367 unpurified agar showed a brownish hue, mostly due to the presence of proteins and polyphenols.
368 The microstructure and porosity of the aerogels formed were also examined by scanning electron
369 microscopy (SEM). Figures 5 and 6 show the surface and cross-section images of the different
370 loaded and unloaded aerogels structures, respectively. Representative micrographs of a detail of
371 some nanoflowers on the surface of the hydrogel capsules covered by different biopolymer
372 matrices are also given in Figure 5. The estimated porous area, which has been obtained from the
373 binary images of SEM micrographs, is also given in Figure 6. As observed, agarose aerogels
374 showed a denser microstructure, in agreement with previous works using other biopolymers such
375 as κ-carrageenan and β-glucan to produce aerogels (Comin, Temelli, & Saldaña, 2012; Manzocco
376 et al., 2017). In contrast, agar-based aerogels showed a less compact network, being the pore size

377 of freeze-dried unpurified agar samples even higher than in the commercial agar aerogels (see
378 Figure 6), as expected from the presence of other components which could interfere in the gelation
379 process of the agarose (gelling component of the agar). Interestingly, the effect that the
380 nanoflowers had on the microstructure of the aerogels depended on the biopolymer used as
381 supporting material. It was clearly observed that nanoflowers were not properly integrated within
382 the agarose matrix (Figure 5C), being not accurately covered by the biopolymer and promoting
383 the formation of a less dense structure with higher pore size than its counterpart prepared without
384 nanoflowers. In contrast, a decrease in the average pore size (as well as in the porous area, see
385 Figure 6) was observed in agar-based aerogels incorporating nanoflowers, providing a denser
386 structure than their unloaded counterparts and being more marked in the case of commercial agar.
387 Therefore, it is reasonable to think that nanoflowers will be better protected by commercial agar-
388 based matrices and, thus, the enzymatic activity will be better preserved. These findings
389 evidenced potential interactions between the agaropectin (the non-gelling fraction of agar) and
390 TmLac-Ca^{2+} nanoflowers, as it will be later on confirmed by FTIR analysis (see below).
391 Agaropectin is a charged polysaccharide, composed of agarose and varying percentages of ester
392 sulphate, D-glucuronic acid and small amounts of pyruvic acid (Labropoulos, Niesz, Danforth, &
393 Kevrekidis, 2002), which interact with the calcium ions arriving from the $CaCl_2$ salt used for
394 nanoflowers´ formation. However, these interactions could be limited in unpurified agar due to
395 the presence of other components.
396 It should be noted that, despite of being properly freeze-dried, neither the unpurified agar samples
397 nor the agarose samples could be used after freeze-drying since they disintegrated after a few
398 minutes of rehydration. This also suggests that some structural and molecular changes occurred
399 in the aerogels depending on the carbohydrate used as supporting material, as it was anticipated
400 by the SEM images and confirmed by FTIR (see below). In fact, similar effects were previously

401 reported for freeze-dried agarose microspheres (Nweke, Turmaine, McCartney, & Bracewell,
402 2017).
403 Thus, in order to further investigate potential molecular interactions between the processed
404 matrices, ATR-FTIR spectra of loaded and unloaded aerogels were recorded. As observed in
405 Figure 7, all the samples presented the most characteristic bands from agar, located at 1260 cm^{-1}
406 and 1371 cm^{-1} assigned to the presence of sulphate ester groups (Volery, Besson, &Schaffer-
407 Lequart, 2004) and at 1150 cm^{-1} ascribed to the vibration mode of ester-sulphate linkages of D-
408 galactose (Chopin, Kerin, & Mazerolle., 1999). The vibrational bands at 1030 and 930 cm^{-1}
409 correspond to the C-O stretching group of the 3, 6-anhydro-D-galactose, while the band at 890
410 cm^{-1} can be assigned to the C-H bending at the anomeric carbon in β-galactopyranosyl residues
411 (Gómez-Ordoñez & Rupérez, 2011, Shankar & Rhim, 2017). It is worth nothing that while the
412 commercial agar and agarose aerogels showed two bands located at 1360 cm^{-1} and 1430 cm^{-1}, the
413 aerogels based on unpurified agar presented a broader band centered at 1400 cm^{-1}. Furthermore,
414 although the amide I band, typically located at 1653 cm^{-1} was masked by the intensity of the band
415 characteristic of physisorbed water (centered at 1630 cm^{-1} approximately), the amide II band
416 (located at 1540 cm^{-1}) was observed in the unpurified agar, evidencing the presence of a
417 significant amount of proteins (as it has been demonstrated by the BlueSafe staining, see Figure
418 1). In fact, the higher intensity of these bands clearly evidenced the higher content of proteins in
419 unpurified agar when compared to that of commercial agar or agarose.
420 All aerogel samples prepared with agar or agarose showed bands in the range of 2840-2950 cm^{-1}
421 , ascribed to the symmetric and asymmetric vibrations of the alkane groups of the agar
422 biopolymer chain (Rhim, Wang, Lee, & Hong, 2014). Interestingly, it was observed that the
423 spectral band at 2936 cm^{-1} split in two when nanoflowers were incorporated into commercial agar
424 aerogels and, the band located at 1371 cm^{-1}, attributed to sulphate groups shifted toward higher
425 wavenumbers, suggesting that there were some interactions between the commercial agar and the

426 TmLac-Ca^{2+} nanoflowers. Specifically, the negative charge of sulphate groups from agaropectins and
427 the positive charge of calcium ions from the nanoflowers could interact, favoring the formation of
428 agaropectin complexes via sulfonate esters. As a result, these molecular interactions, probably fostered
429 by the dehydration process during freeze-drying, could explain the differences observed in aerogels´
430 microstructure (being denser in loaded agar-based aerogels) and their subsequent integrity during
431 enzymatic activity testing. Similarly, interactions between Ca^{2+} and the S=0 stretching groups of the
432 sulphate ester groups from carrageenan have also been reported by Levy-Ontman et al., 2019.
433 The broad absorption band in the region of 2900 and 3800 cm^{-1} corresponds to hydroxyl group
434 vibrational stretching and it is associated to OH bond in water or hydroxyl groups of the agarose,
435 and agar-based samples. Due to the hydrophilic nature of these samples, some water adsorption
436 occurred under environmental conditions and, this was also observed by the above-mentioned
437 bending vibration at 1640 cm^{-1}, characteristic of physisorbed water. As expected, the relative
438 intensity of these bands was higher for unpurified samples due to the presence of other hydrophilic
439 compounds in the matrix, which were not present in the commercial agar. Interestingly, a
440 reduction in the peak intensities of the previously mentioned vibrational bands was observed for
441 loaded commercial agar and unpurified agar-based samples but not in loaded agarose samples, as
442 compared to their unloaded counterparts. This suggests that hydrogen bonding interactions
443 between agaropectin (the non-gelling fraction of agar) and the nanoflowers took place, being
444 more evident in the case of commercial agar where the reduction of the bands' intensity was even
445 higher. As a consequence, these interactions favor the formation of a denser structure in aerogels
446 formed with commercial agar and, the higher porosity observed in unpurified agar and agarose
447 aerogels could be the responsible of the loss of their integrity after being rehydrated.
448 Therefore, taking into account these findings, the enzymatic activity and reusability of the aerogels
449 were carried out with commercial agar aerogels loaded with TmLac-Ca^{2+} nanoflowers. The effect of
450 the freeze-drying process on the specific enzymatic activity (measured after 30 min of incubation with

lactose) of the free enzyme (TmLac) and the corresponding nanoflowers were firstly evaluated and compared with the agar-based aerogels (supplementary material S2). While no differences were observed between fresh and freeze-dried free TmLac, combining nanoflowers with the freeze-drying process resulted in a lower enzymatic activity (around ~ 52%) of the TmLac-Ca^{2+} nanoflowers, as compared to their freshly-prepared counterparts. This can be ascribed to structural changes occurring in nanoflowers during the dehydration process which could negatively affect the catalytic activity of the enzyme. Interestingly, commercial agar aerogels showed a specific activity similar to the free TmLac enzyme indicating that the agar matrix exerted a protective effect on the TmLac-Ca^{2+} nanoflowers likely attributed to the interactions with the encapsulating matrix, as previously observed by FTIR. Besides, it is worth mentioning that the specific activity of the freeze-dried samples was significantly higher ($p<0.05$) than their counterparts in the hydrated form, reaching values in the range of the free TmLac. This confirms that aerogels, structured dry materials with high porosity and large surface area (as previously observed in Figure 6), allowed a faster penetration of the 5% (w/v) lactose (substrate) aqueous solution, thus accelerating the hydrolytic activity.

Once the methodology was proved to successfully increase the hydrolytic activity of the enzymatically active materials, the reaction kinetics of the commercial agar based aerogels was also explored. From Figure 8, a similar pattern was observed between agar-based aerogels and free TmLac or freshly-prepared TmLac-Ca^{2+} nanoflowers, reaching the plateau after 3.5 h incubation. Therefore, this time was chosen to subject the samples to repeated batched of lactose hydrolysis. As shown in Figure 9, agar-based aerogels behaved similar to the hydrated samples (see Figure 3), showing a progressive decrease in the enzymatic activity after each cycle and, resulting in a ~ 33% of hydrolytic activity decay after the third incubation round. Although the hydrated material allowed one more cycle of reuse, reaching a decrease of ~27% in the fourth cycle (see Figure 3), enzymatically active aerogels facilitate the handling and storage of the enzyme with respect to its hydrated counterparts.

4. Conclusion

Three different seaweed-derived matrices (commercial agar, unpurified-agar and agarose) have been used to produce enzymatically active hydrogels and aerogels containing TmLac-Ca^{2+} nanoflowers. The results evidenced that the composition, which conditioned the gel strength of hydrogel capsules, had a strong impact on the properties of the formed hydrogels and on its hydrolytic activity and reusability for the digestion of lactose at high temperature (75 ºC). Although hydrogel capsules prepared with commercial agar were the best performing materials, which could be reused during seven consecutive cycles at 75ºC, keeping a higher enzymatic activity (after seven cycles at 75 ºC) than the non-encapsulated TmLac-Ca^{2+} nanoflowers, its freeze-dried counterpart (commercial agar aerogel) improved handling and storage stability of the developed enzymatically active materials without detrimentally affecting neither its hydrolytic activity nor its reusability at high temperatures. From the results obtained, aerogels based on commercial agar displayed high catalytic activity (i.e. 0.5 g of aerogel capsules hydrolyzed the lactose equivalent of 100 mL of milk in 15 min) and thermal stability.

These results add new insight, to other previous studies, into the potential of this processing method for the development of enzymatically active materials of significant interest in the dairy industry with the additional advantage that they can be easily scaled-up.

Acknowledgment

This research was supported by grants from Spain's 'Secretaría de Estado de Investigación, Desarrollo e Innovación' (AGL2016-75245-R), Agencia Estatal de Investigación (AEI, Grant PCI2018-092886) and cofunded by the European Union's Horizon 2020 research and innovation programme (ERA-Net SUSFOOD2). MJF was supported by a Ramon y Cajal contract (RYC2014-15842) from the Spanish Ministerio de Economia; Industria y Competitividad. The authors thank the Central Support Service

for Experimental Research (SCSIE) of the University of Valencia for the electronic microscopy service.

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651 **Table 1.** Specific hydrolytic activity, measured at 30 min, of the free TmLac, TmLac-Ca^{2+} nanoflowers
652 (TmLac NF) and TmLac-Ca^{2+} nanoflowers encapsulated in commercial agar (AA), unpurified agar (u-
653 AA) and agarose (Ag). Mean value (standard deviation).
654

Material	Activity (μmol Glc · min^{-1} · mg^{-1})
TmLac	23.8 ± 1.0^{a}
TmLac NF	23.5 ± 1.4^{ab}
AA	15.3 ± 1.6^{c}
u-AA	17.2 ± 2.6^{bc}
Ag	16.1 ± 2.7^{bc}

655 Mean value ± standard deviation
656 Different letters in superscripts (a-c) indicate significant differences ($p<0.05$) among the samples.
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667 **Table 2.** Gel strength of commercial agar (AA^-), commercial agar with TmLac-Ca^{2+} nanoflowers
668 (AA), agarose (Ag^-) and agarose with TmLac-Ca^{2+} nanoflowers (Ag) hydrogel capsules. Mean value
669 (standard deviation).
670

Material	F/d (N/mm)
AA -	0.37 ± 0.03^a
AA	0.41 ± 0.02^a
Ag -	0.42 ± 0.03^a
Ag	0.22 ± 0.02^b

671 Mean value ± standard deviation

672 Different letters in superscripts (a-b) indicate significant differences ($p<0.05$) among the samples
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686 **Figure Captions**

687 **Figure 1** Visual appearance of hydrogel capsules with and without nanoflowers: (A) commercial agar,
688 (B) commercial agar with TmLac-Ca^{2+} nanoflowers, (C) unpurified agar, (D) unpurified agar with
689 TmLac-Ca^{2+} nanoflowers, (E) agarose and (F) agarose with TmLac-Ca^{2+} nanoflowers.

690 **Figure 2** Hydrolysis of lactose by free TmLac (TmLac), TmLac-Ca^{2+} nanoflowers (TmLac NF) and
691 enzymatically active hydrogel capsules made of either commercial agar (AA), unpurified agar (u-AA)
692 and agarose (Ag).

693 **Figure 3** Hydrolysis of lactose (5% solution) after serial batches of incubation, at 75 °C for 70 min, of
694 TmLac-Ca^{2+} nanoflowers and hydrogel capsules based on commercial agar (AA), unpurified agar (u-
695 AA) and agarose loaded with TmLac-Ca^{2+} nanoflowers.

696 **Figure 4** Visual appearance of aerogel capsules with and without nanoflowers: (A) commercial agar
697 with TmLac-Ca^{2+} nanoflowers, (B) unpurified agar with TmLac-Ca^{2+} nanoflowers and (C) agarose
698 with TmLac-Ca^{2+} nanoflowers.

699 **Figure 5** Representative SEM images of the aerogel capsules with and without nanoflowers: (A)
700 commercial agar, (B) unpurified agar and (C) agarose (scale markers are 1 mm and 5 μm for left and
701 right micrographs, respectively),

702 **Figure 6** Representative SEM micrographs and binary images of the porosity degree of aerogel
703 capsules with and without nanoflowers: (A) commercial agar, (B) unpurified agar and (C) agarose.
704 (scale marker 10 μm). The porosity area is given in percentage.

705 **Figure 7** ATR-FTIR spectra from loaded and unloaded aerogels based on commercial agar, unpurified
706 agar and agarose.

707 **Figure 8** Hydrolysis of lactose by freeze-dried TmLac (TmLac-FD), freeze-dried TmLac-Ca^{2+}
708 nanoflowers (TmLac NF-FD) and enzymatically active aerogel capsules made of commercial agar
709 (AA-FD).

710 **Figure 9** Hydrolysis of lactose (5% solution) after serial batches of incubation, at 75 °C for 70 min
711 agar-based aerogels (AA-FD).

Figure 1

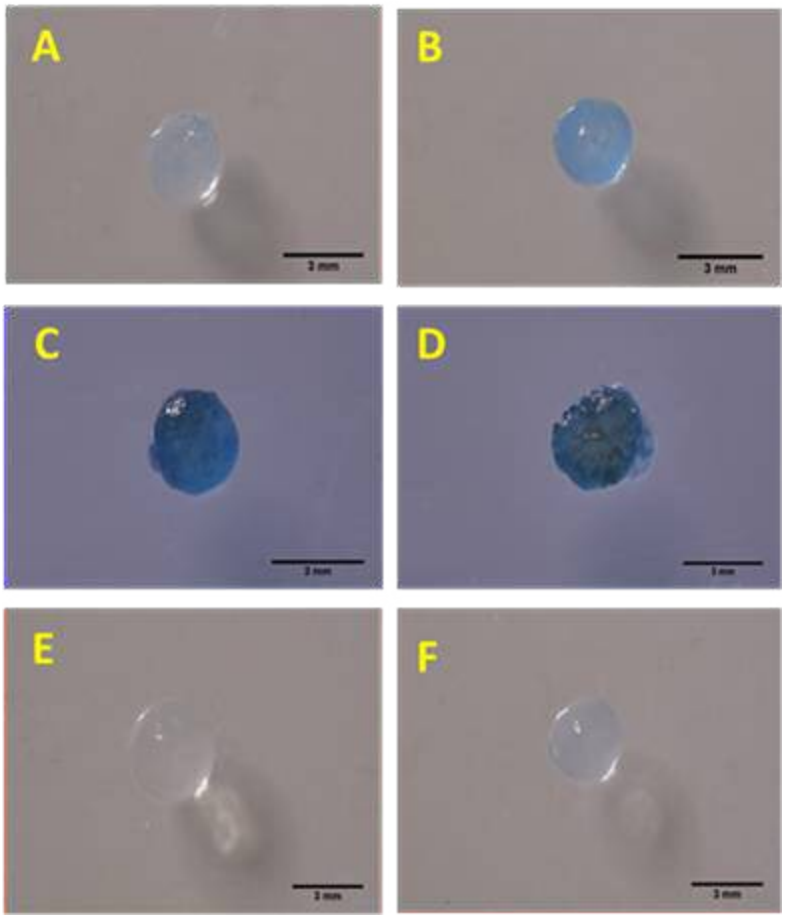


Figure 2.

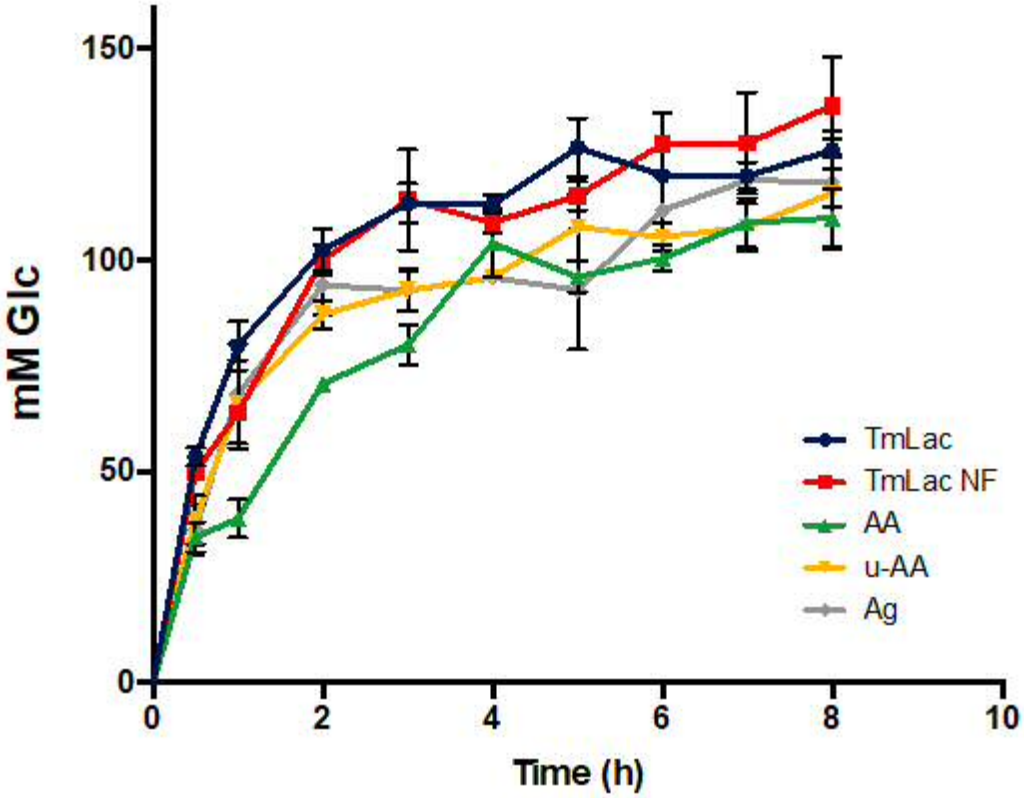


Figure 3

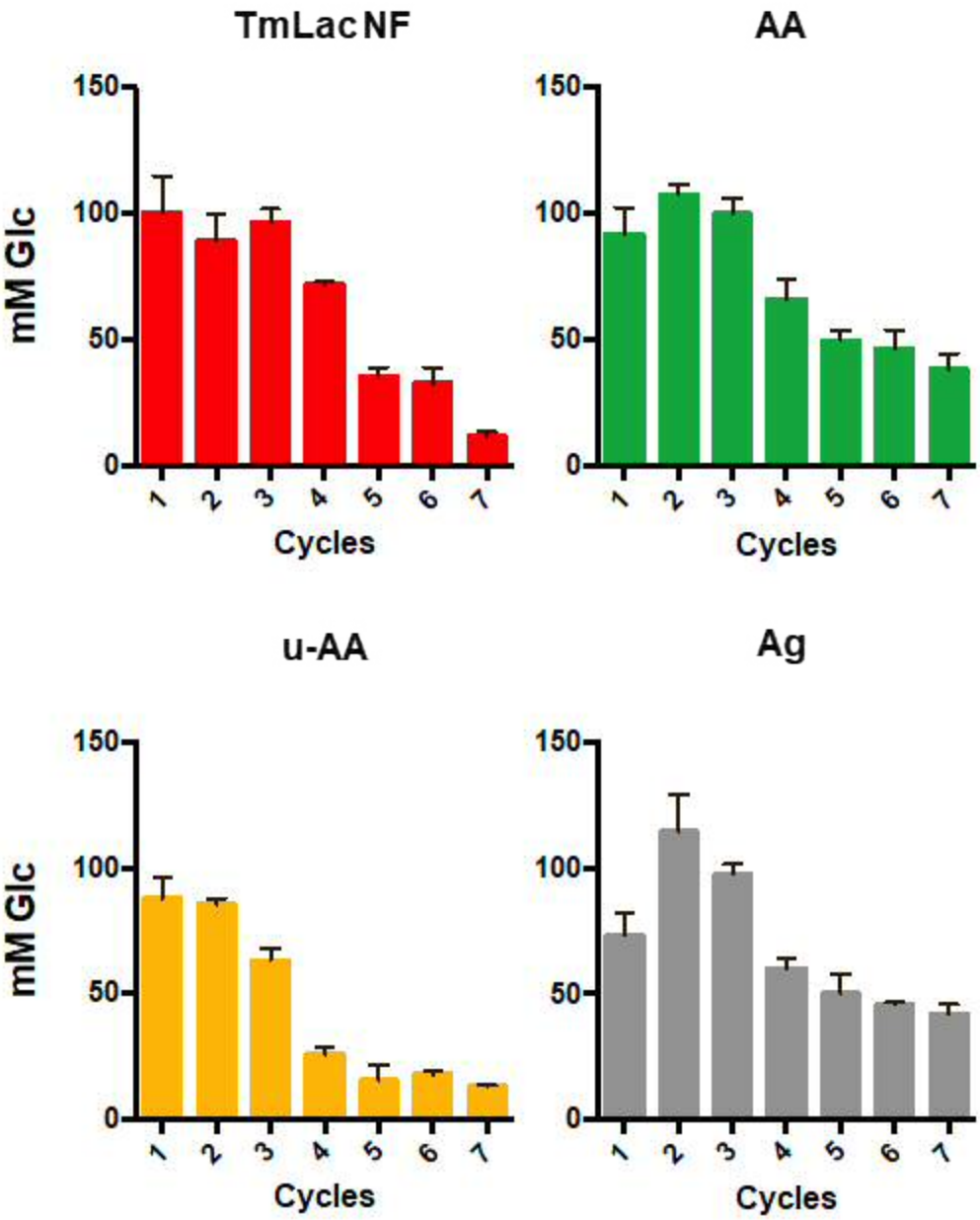


Figure 4

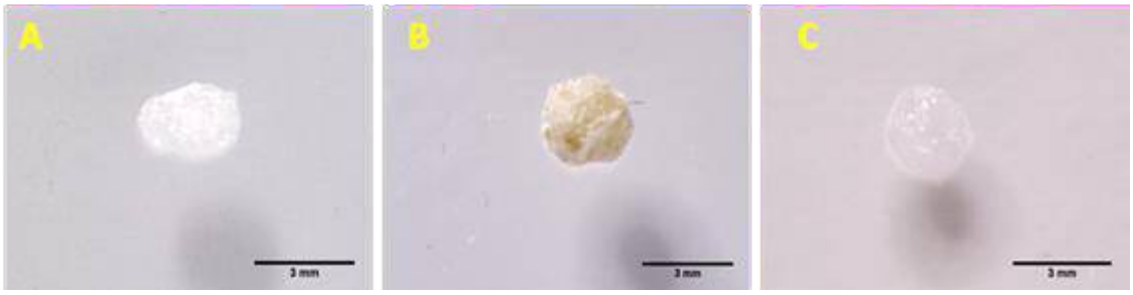


Figure 5.

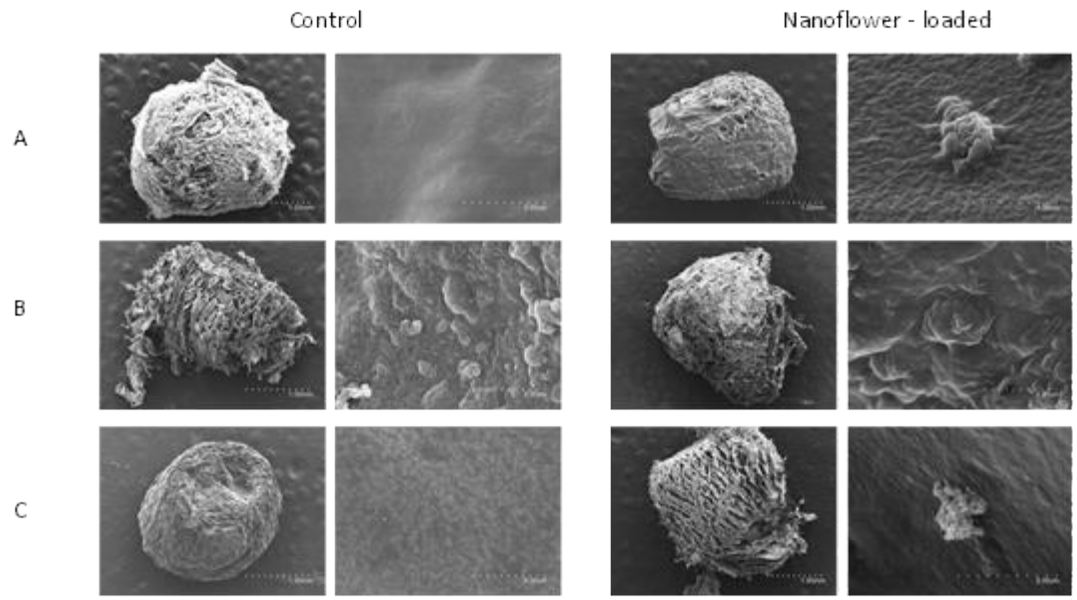
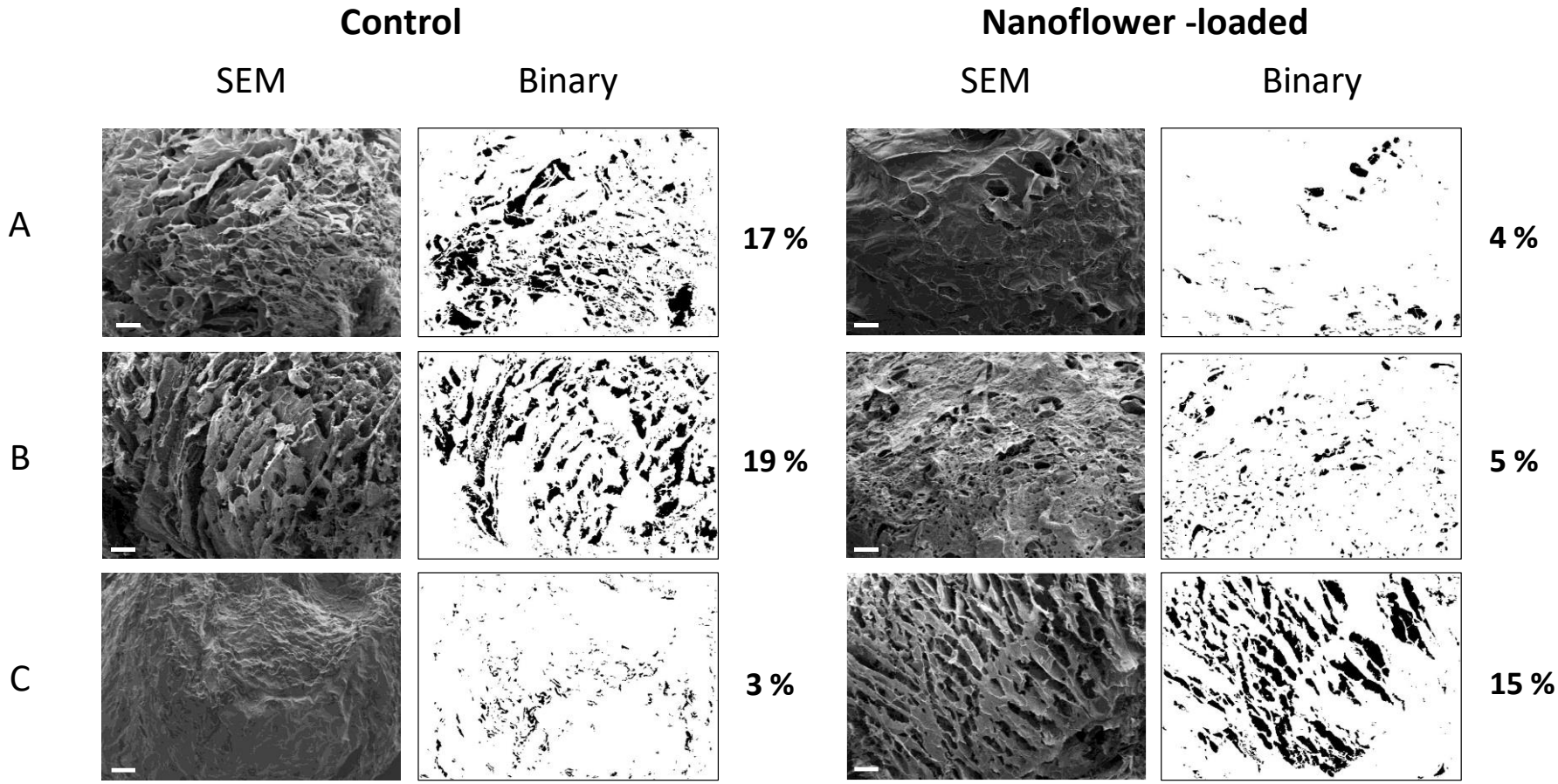


Figure 6


Control
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SEM
Binary
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4 %
5 %
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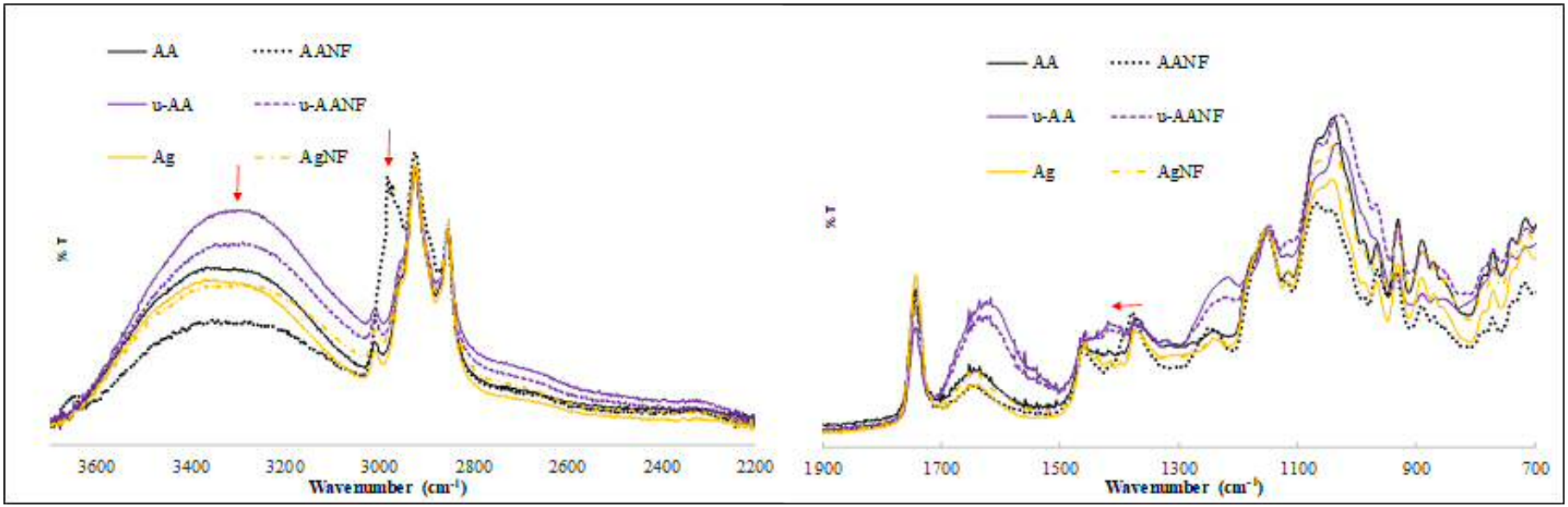
Figure 7


AA
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Wavenumber (cm-1)
AA
AANF
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700
Wavenumber (cm-1)

Figure 8

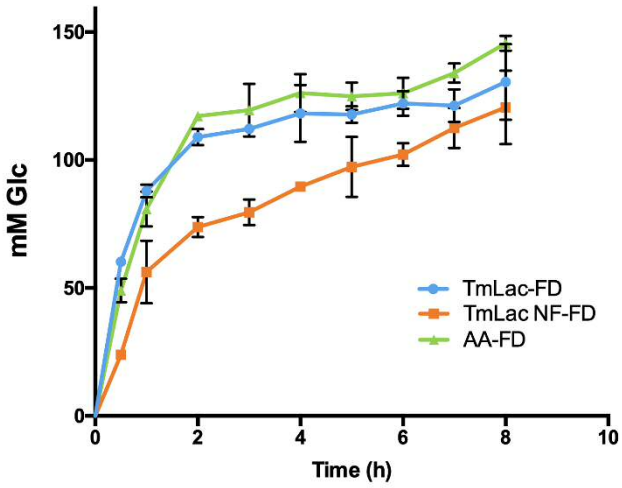
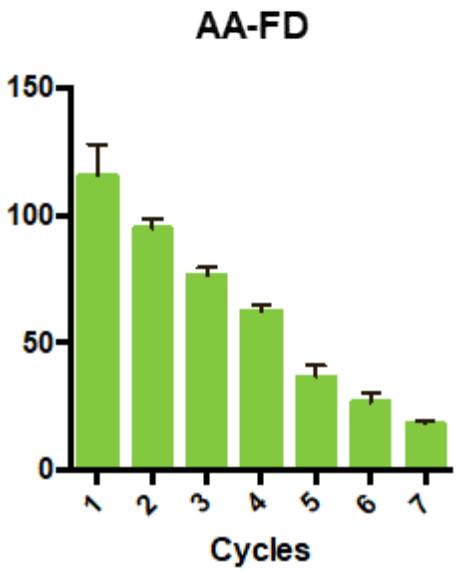


Figure 9



Highlights:

- TmLac-nanoflowers were encapsulated in marine polysaccharide hydrogels and aerogels
- Enzymatic activity was more efficiently preserved in purified agar hydrogels/aerogels
- Active aerogels based on commercial agar showed the highest catalytic activity
- 0.5 g of aerogel beads hydrolyze the lactose equivalent of 100 mL of milk in 15 min