**Matryoshka enzyme encapsulation: development of zymoactive
hydrogel particles with efficient lactose hydrolysis capability.**

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ABSTRACT

This report describes an efficient procedure for enzyme encapsulation and its application for the hydrolysis of lactose. The enzymatic material that has been developed consists of hydrogel particles (ca. 3-4 mm of diameter) composed of either alginate or an alginate-agarose combination, in which bacterial cells loaded with a thermostable β -galactosidase are embedded. The cells were rendered fully permeable to the substrate, either chromogenic *p*-nitrophenyl galactose or lactose, by thermal treatment at 75 °C. Hydrogel particles made of a mixture of alginate and agarose displayed high catalytic activity (i.e. 1 g of beads hydrolyze the lactose equivalent of 100 mL of milk in 15 min) and thermal stability: they could be reused at 75 °C for complete hydrolysis of 5% (w/v) lactose solution at least six consecutive times without significant loss of activity.

Keywords: β -Galactosidase, cell permeabilization, enzyme immobilization, lactose, thermostable enzyme

1. Introduction

β -Galactosidases are ubiquitous enzymes with diverse applications among which stands their widespread use in the food industry for the production of lactose-free milk and milk derivatives. Lactase, usually from fungal source, is directly added to milk, a procedure that involves considerable manipulation and avoids enzyme recovery. Enzyme immobilization in solid supports, a mature technique with multiple applications (Sirisha et al 2016; Sheldon and van Pelt 2013) offers a convenient alternative to the current procedures for lactose-free milk production. Several recent reports describe the immobilization of β -glucosidases in hydrogel capsules and metal-organic frameworks (Chen et al 2018; Nishida et al 2018; Sridhar et al 2018; Traffano-Schiffo et al 2018; Zhang et al 2018). Other strategies of enzyme immobilization offer new possibilities for an even wider range of applications (Altinkaynak et al 2016; Lee et al 2015; Ge et al 2012). Whole cells, usually bacteria or yeasts, can be conceived as a recipient of enzymes and can be a convenient alternative to enzymes as biocatalysts. However, the use of whole cells as catalytic agents required either the attachment of the enzyme onto the cell surface (Casa-Villegas et al 2017) or the permeabilization of the cell to allow the entrance of the substrate to the intracellular space where the enzyme is contained. Permeabilization of microbial cells has been widely used in biochemical or biotechnological studies (Weyler and Henzle 2017; Cánovas et al. 2005; Felix 1982). It is generally accomplished by treating the cells with a chemical agent such as organic solvent or detergent which causes a disruption of the cell membrane which becomes permeable to small molecules while enzymes remain inside the cell. Encapsulation of enzymatically active permeabilized bacterial cells can be a practical procedure for enzyme immobilization. Because of their size, bacteria are much easier to manipulate than purified enzymes. Their size makes them suitable to be encapsulated in a polymeric matrix or retained in a porous membrane. Bacterial cells are also more robust and complex than protein molecules and, thus, they offer more alternatives to be enclosed in a

matrix or attached to different support materials. Encapsulation in hydrogels is particularly attractive for food applications because these semi-solid materials can be prepared using food-grade ingredients (Souza et al., 2018, Zhang et al., 2017, 2016; Estevinho et al 2014). Alginates are unbranched natural copolymers consisting of α -L-guluronic acid and β -D-mannuronic acid residues, being important components of certain algae and bacteria (Draget 2009). These anionic polysaccharides have been widely explored for enzyme encapsulation, given their ability to form hydrogel particles by ionotropic gelation in the presence of divalent cations (Josef et al 2010). Although alginate hydrogels have a number of advantages, related to low cost, biocompatibility, availability and food-grade character, their low mechanical strength, low resistance to physical treatments and the presence of macropores cause enzymatic activity losses during particle formation (Santagapita et al 2012). Agarose, another structural polysaccharide present in different species of red seaweed, is also capable of forming strong hydrogel networks through cold setting and exhibits significant thermal hysteresis (Fujii et al 2000), which provides these hydrogel structures with excellent thermal stability. The formation of double network hydrogels combining both polysaccharides has demonstrated to render materials with improved mechanical properties and stability under different processing conditions (Luo et al 2014), thus constituting an interesting option for cell immobilization.

This communication describes the production of β -galactosidase-active hydrogel particles, made of either alginate or an alginate-agarose mixture, that contain permeabilized *E. coli* cells enriched with this enzymatic activity. The system can be envisioned as a matryoshka doll set assembled in three levels: enzyme-cell-bead. The enzyme used is a thermostable β -galactosidase, from the bacterium *Thermotoga maritima* (Talens-Perales et al 2016; Marin-Navarro et al 2014). This and related β -galactosidase enzymes from Family 2 glycoside hydrolases have industrial applications, the most relevant of which is lactose hydrolysis in milk and milk-related product to avoid lactose intolerance. Other applications are related to the valorization of cheese whey and the synthesis of galactooligosaccharides

(Estevinho et al 2018; Plou et al 2016; Talens-Perales et al 2016; Marin-Navarro et al 2014; Adam et al 2004). In addition to possible practical applications, the choice of β -galactosidase allows very easy determination of the enzyme activity using a chromogenic substrate. This facilitated the development of the methodology reported in this communication that could be implemented for other enzymes with different applications. The fact that *T. maritima* β -galactosidase is thermostable expands the usability of the enzyme. This is a relevant issue from a practical point of view since many possible applications of the most widely used β -galactosidase in industry, the lactase from *Kluyveromyces lactis*, are restricted by the structural liability of this enzyme (Pereira et al 2012; Rico-Diaz et al 2017).

2. Materials and methods

2.1 Materials

Alginate (sodium alginate, medium viscosity), Tween 20, Triton X-100, chloramphenicol (> 98% purity HPLC), ampicillin sodium salt, Ethylenediaminetetraacetic acid (EDTA, > 99% purity), lactic acid (> 85 % purity) and p-nitrophenyl β -D-Galactopyranoside (pNP-Gal) were from Sigma Aldrich. Isopropyl β -D-1-thiogalactopyranoside (IPTG) was from Thermo Fisher Scientific. Agarose (type D1) was kindly supplied by Hispanagar S.A., (Burgos, Spain). Salts used for buffer preparation (NaCl and $MgCl_2$) were from Sigma-Aldrich. Isopropanol (>99.8 % purity) and toluene were from Sigma-Aldrich. Ethanol (96 % v/v extra pure) and acetone were from Scharlab and Panreac, respectively. The protease inhibitor (Complete, EDTA-free) was from Roche.

2.2. Preparation of zymoactive cell suspensions

E. coli transformant cells harboring plasmid TmLac-pQE (Marín-Navarro et al., 2014) were cultured at 37 °C until an OD of 0.6 at 600 nm was reached in 2XYT medium (16 g/L tryptone, mg/mL). Gene induction was carried out with IPTG (1 mM final concentration for 16 hours at 16 °C). Cells were collected by centrifugation at 4000 g for 20 minutes at 4 °C and kept at -20 °C until their use. Aliquots of the collected cells, corresponding to 60 mL of culture, were subjected to different permeabilization treatments. The cells were resuspended in 6 mL of 50 mM phosphate buffer at pH 7.5 to which a protease inhibitor (Complete, EDTA-free, Roche) had previously been added. A total of 10 aliquots of 500 µL were prepared. As a control, one of the samples was sonicated, to break the cells and release the enzyme, so that the activity obtained served as reference value of maximum achievable activity. The other samples were subjected to different treatments with different chemicals: lactic acid, ethanol, acetone, toluene or detergents, known to render *E. coli* cells permeable (Cánovas et al, 2005). The enzyme activity of the cells resulting from each treatment was measured (Table S1 in supplementary material).

2.3 Enzyme assays

Enzyme activity, using 5 mM pNP-Gal as the substrate, in buffer A (50 mM phosphate, pH 6.5, 10 mM NaCl and 1 mM MgCl₂), was determined at 75 °C. Reactions were stopped by adding 0.75 M Na₂CO₃ (final concentration) and monitored spectrophotometrically (400 nm). Reactions using lactose at 5% in buffer A were performed in the same conditions and were stopped at 95 °C for 10 minutes. Lactase activity was determined by the amount of released glucose using a glucose assay kit (Glucose, GO Assay Kit, Sigma).

2.4 Preparation of alginate and agarose solutions

Alginate and agarose stock solutions were prepared as starting material for the synthesis of the alginate and alginate-agarose hydrogel beads. Alginate solution 1.25 % (w/v) in distilled water was prepared under magnetic agitation and mild heating. Similarly, the agarose solution was prepared at 1 % (w/v) by dissolving the powder in distilled water at 90 °C under magnetic agitation. The solution was kept at 60 °C until it was used for the synthesis of the beads.

2.5. Encapsulation of cells in hydrogel beads

Encapsulation of the cells into hydrogel beads was carried out by the gelation technique, using either alginate or an alginate-agarose mixture as biopolymer matrices. In both cases, a cell pellet corresponding to 200 mL of bacterial culture (obtained as described in section 2.2) was dissolved in 4 mL of water. For encapsulation in alginate beads, 2 mL of cells were added to 8 mL of an aqueous alginate (1.25 % w/v) solution in order to reach a final polysaccharide final concentration of 1 % (w/v). For alginate-agarose beads, 2 mL of cells were firstly added to 8 mL of 1.25 % (w/v) alginate and then, 4 mL of this suspension were mixed with 4 mL of 1% (w/v) molten agarose solution cooled down to 40 °C (see previous 2.4 section). Hydrogel beads of ca. 2-3 mm of diameter were prepared by dripping the cell suspensions in either alginate or alginate-agarose into a 50 mM CaCl₂ solution. The beads were allowed to crosslink with Ca²⁺ at room temperature to allow the formation of hydrogels. Then, the beads were collected by filtration and subsequently washed, with distilled water. Encapsulation efficiency was quantified by measuring remaining (unloaded) β-galactosidase activity in the external medium of the beads using 5mM pNP-Gal as substrate.

To test the reusability of the beads, they were incubated in 5 % (w/v) lactose solution prepared in assay buffer, at 75 °C for 1 hour. Then, the sample was centrifuged. The supernatant was heated at 95 °C for 10 minutes to ensure that the hydrolysis reaction was finished, and the

amount of glucose was measured. The beads in the sediment were suspended again in lactose solution and used in a next batch.

2.6. Optical microscopy

In order to evaluate the presence and distribution of bacterial cells within the hydrogel beads, enzyme loaded beads were stained with 1/10 Sybr® Green I Nucleic Acid Stain (Lonza, Barcelona, Spain) solution for 1 min. The beads were then washed twice with water. Surface and cross-sections (aprox. 1-2 mm thick) of the beads were observed by illuminating with a long pass emission B-2E/C fluorescent source (Nikon corporation, Japan). Digital images were taken using an Eclipse 90i Nikon microscope (Nikon corporation, Japan) equipped with 5-megapixels cooled digital colour microphotography camera Nikon Digital Sight DS-5Mc. Acquired images were analysed and processed by using Nis-Elements Br 3.2 Software (Nikon corporation, Japan).

2.7. Cryo-scanning electron microscopy

A JSM-5410 SEM microscope (JEOL, Tokyo, Japan) was used with a Cryo CT-1500C unit (Oxford Instruments, Witney, UK) for the Cryo-SEM observation. Hydrogel beads were placed in the holder and frozen in liquid nitrogen slush ($T \leq -210$ °C). The frozen samples were then transferred to the Cryo unit, fractured, etched for 15min (-90 °C) and gold-coated (2mbar and 2mA). Samples were then transferred to the micro-scope and examined at 10kV, -130 °C, and at a working distance of 15mm.

2.8. Small Angle X-ray scattering (SAXS)

The structure of the capsules at the nanoscale level was investigated by means of small angle X-ray scattering (SAXS). SAXS experiments were carried out in the Non Crystalline Diffraction beamline, BL-11 at ALBA synchrotron light source (www.albasynchrotron.es). The capsule suspension and a blank sample consisting in the CaCl₂ solution (used for background subtraction) were placed in sealed 2 mm quartz capillaries (Hilgenburg GmbH, Germany). The SAXS patterns of the samples were collected before and after two consecutive heating treatments at 70 °C (the samples were designated as “Fresh”, “Batch 1” and “Batch 2”). The energy of the incident photons was 12.4 KeV or equivalently a wavelength, λ , of 1 Å. The SAXS diffraction patterns were collected by means of a 9 CCD detector Quantum ADSC 315r with an active area of 315 x 315 mm², an effective pixel size of 102 x 102 μm^2 and a dynamic range of 16 bits. The sample-to-detector distance was set to 6488 mm, resulting in a q range with a maximum value of $q = 0.2 \text{ Å}^{-1}$. The data reduction was treated by pyFAI python code (ESRF) (Kieffer and Wright, 2013), modified by ALBA beamline staff. The intensity profiles were then represented as a function of q using the IRENA macro suite (Ilavsky & Jemian, 2009) within Igor procedures. The experimental data were fitted using a two-level Beaucage model. This model considers that, for each individual level, the scattering intensity is the sum of a Guinier term and a power-law function (Beaucage, 1995, 1996):

$$I(q) = \sum_{i=1}^N G_i \exp\left(-q^2 \cdot \frac{R_{g,i}^2}{3}\right) + \frac{B_i [\text{erf}(qR_{g,i}/\sqrt{6})]^{3P_i}}{q^{P_i}} + bkg \quad (1)$$

where $G_i = c_i V_i \Delta SLD_i^2$ is the exponential prefactor (where V_i is the volume of the particle and ΔSLD_i is the SLD contrast existing between the i^{th} structural feature and the surrounding solvent), $R_{g,i}$ is the radius of gyration describing the average size of the i^{th} level structural feature, B_i is a q -independent prefactor specific to the type of power-law scattering with power-law exponent, P_i , and bkg is the background.

2.9 Statistical analysis

Experiments were performed in triplicate. Statistical analyses were conducted using IBM SPSS Statistics software (v.23) (IBM Corp., USA) through the analysis of variance (ANOVA). Tukey's Honestly Significant Difference (HSD) was used at the 95% confidence level for multiple comparison tests.

3. Results and discussion

3.1 Preparation of *E. coli* cells as catalytic material

E. coli cells expressing *T. maritima* β -galactosidase, which had not been subjected to any permeabilization treatment, assayed at 75 °C with either pNPGal or lactose as substrates, showed about the same activity that cells that had been disrupted by sonication, or had been treated with permeabilization agents, eg. acetone, isopropanol or toluene (supplementary material, table S1). This showed that the operating conditions at the enzyme optimal temperature assure efficient permeabilization without the need of any other treatment. Incubation of a suspension of the cells at 75 °C for 20 min released no detectable β -galactosidase activity in the supernatant, showing no that there was no enzyme leakage, a necessary condition for using the cells as catalysts. Moreover, Figure 1 shows that the cells fully retained their capability to hydrolyze lactose (5% w/v) after five consecutive cycles. Only a slight decrease was observed at the sixth round.

3.2 Production and assay of zymoactive hydrogel beads.

Handling of free bacteria at industrial scale is cumbersome as it requires centrifugation and filtration steps. Cell encapsulation into beads of suitable size facilitates the use of bacterial

cells as catalytic agent. Initially, encapsulation was carried out in an alginate hydrogel matrix cross-linked with calcium chloride, since it has been previously reported that alginate beads are quite resistant to high temperature (Pravinata et al 2016). However, under the operating conditions performed in this work, the alginate particles were not stable when exposed to the temperature used in the assays. As an alternative to increase the thermal stability, a blend of alginate and agarose was tested. Encapsulation efficiency of bacteria in either alginate or alginate-agarose was virtually 100%, similar to the value reported by Bassani et al. (2019) for microbial cell encapsulation and higher than the efficiency reported for β -galactosidase loaded in polyelectrolyte nanoparticles (Zhang et al., 2018), or in κ -carrageenan hydrogel beads (Zhang et al., 2016), which was around 63%. Figure 2 shows the performance of the two types of hydrogel particles, loaded with zymoactive cells, in the hydrolysis of lactose. The kinetics of lactose hydrolysis was similar in both cases but the alginate capsules lost their integrity after their first use and could not be recycled. Consequently, alginate-agarose particles were chosen and subjected to repeated batches of lactose hydrolysis. Surprisingly, whereas hydrolysis after the first treatment was ca. 70%, similar to that reported for previous experiment, complete hydrolysis (100%) was observed in consecutive batches up to the sixth (Figure 3). Results shown as supplementary material (Table S2) indicated that enzyme leakage from the alginate-agarose particles was negligible. Therefore, the enhancement in the activity seems to represent a sort of activation caused by the incubation of the beads at 75 °C. Since this effect was not observed during the successive batches of hydrolysis with non-encapsulated cells (Figure 1), it is likely due to a change in the network structure of the hydrogel encapsulating material caused by the treatment at 75 °C. In order to test this hypothesis, analysis of the molecular conformation of the polysaccharide matrices by SAXS experiments structural analysis of the hydrogel beads were carried out.

3.3 Molecular conformation and structural analysis of the hydrogel beads.

The molecular conformation of the hydrogel beads was evaluated to ascertain if the increase in the β -galactosidase activity was related to changes in the cohesion of the network formed in the polysaccharide matrices. Figure 4 shows the SAXS patterns of the capsules in their native state and after being subjected to two consecutive heating treatments at 75 °C. As observed, the scattering intensity of the sample decreased noticeably after the first heating cycle. Taking into account that the scattering intensity is proportional to the scattering length density contrast between the sample and the surrounding solvent ($I \propto (SLD_{sample} - SLD_{solvent})^2$) and that the scattering length density from a certain material is proportional to its physical density, the intensity drop may be indicative of a decrease in the packaging density of the molecular chains forming the capsules. The patterns showed the typical behaviour from gel-like structures, which, as seen in Figure 4, can be empirically described by a two-level Beaucage model. The main structural parameters obtained from the fits were the radius of gyration (R_g) and the power-law exponents for the two different structural levels (PL_1 and PL_2) (supplementary material, Table S3). The power-law exponent corresponding to the low q region decreased from 1.6, characteristic from swollen coils, for the fresh capsules, to 1.9, characteristic from Gaussian chains, for the capsules after the first heating cycle. On the other hand, the power-law exponents for the high q region decreased from 4.0 for the native capsules to 3.8 and 3.7 for the capsules after the two consecutive heating cycles, suggesting the transition from a smooth surface to rougher surfaces, at the corresponding length scale. The R_g , which is related to the dimensions of the scattering objects, was gradually reduced after each heating step. In this case, the R_g may be indicative of the distance between adjacent polymeric chains. Therefore, the results suggest that after the first heating treatment the alginate-agarose capsules underwent a structural change at the nanoscale level. It seems that the heating treatment promoted the mobility of the molecular chains, leading to the formation of less densely packed structures, which could favour a better diffusion of the substrate into the hydrogel beads. This transition occurred mainly during the first heating step, with no

significant changes taking place after the second heating cycle, which may explained that the enzymatic activity was not further increased after the second heating step.

Optical and cryo SEM microscopy were also used to analyze the structural properties of the alginate-agarose hydrogel particles. Figure 5 A, B and C, shows the fluorescent microscopy image of an individual hydrogel particle, a detail of its surface and the cross-section morphology, respectively. Sybr® Green I nucleic acid staining revealed a homogeneous distribution of bacteria not only on the bead surface but also within the capsules. A complementary experiment was carried out, in which the enzymatic activity (lactose hydrolysis) of whole beads was compared with that of an equivalent mass of mechanically disrupted beads. The value of enzyme activity was slightly but significantly higher in the disrupted material (Supplementary Material Table S4). This result indicates some restriction to the diffusion of the substrate through the hydrocolloid matrix of the beads. Cryo SEM analysis of the hydrogel particle surface (Figure 6) and cross-sections (Figure 7) was also carried out before and after incubation at 75 °C. A change of the bead surface as a consequence of the thermal treatment was observed. Images C and D in Figure 6, corresponding to the morphological details of the hydrogel capsules after the thermal treatment, revealed the presence of holes or cavities on the bead surface that were not observed in untreated particles (images A and B in Figure 6). In contrast, cross-section images (Figure 7) showed a similar porous aspect in thermally treated or untreated beads. These results provide a likely explanation to the fact that the lactose hydrolyzing activity of the beads increased after the first cycle of utilization. Apparently, the holes caused on the bead surface on the first round of treatment at 75 °C allow a better access of the enzyme substrate (lactose) to the interior of the bead and a more efficient hydrolysis.

4. Conclusions

The methodology described in this communication provides a technically simple and reliable procedure for the encapsulation of whole cells with enzymatic activity in hydrogel particles made of agarose-alginate. The size and rheological properties hydrogel particles facilitate their handling and makes them a convenient material to be used for lactose hydrolysis in different types of containers like, for instance, in a reactor, a column or a simple tea bag for home use. The developed matryoshka-like structures can be operated at high temperature (75 °C) and reused for the digestion of lactose in milk or cheese whey. The results obtained indicate that cell leakage from the alginate-agarose hydrogel particles was negligible and the hydrogel network structure was determinant in the diffusion of the substrate and hydrolysis products, thus explaining the difference in enzymatic activity after different cycles. While the use of *E.coli* cells is not a problem for non-food applications such as the hydrolysis of lactose in whey, it could be objectionable for food applications. However, this limitation could be circumvented by the use of food-grade, probiotic strains such as *E. coli* Nissle 1917, topic which deserves further study.

Acknowledgements

This work was funded by grant AGL2016-75245-R from Spain's 'Secretaría de Estado de Investigación, Desarrollo e Innovación'.MJF was supported by a Ramon y Cajal contract (RYC2014-158) from the Spanish Ministerio de Economía; Industria y Competitividad.

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Figure Legends

Figure 1. Hydrolysis of lactose by recombinant *E. coli* cells containing *T. maritima* β -glucosidase. The cell pellet corresponding to 2 mL of induced culture was added to 0.5 mL of 5% lactose solution and incubated at 75 °C for one hour. After the incubation time, the cells were collected by centrifugation and added to a second batch of the lactose solution. The results of six consecutive batches were analyzed. Lactose hydrolysis was measured as a function of the amount of glucose released in the reaction.

Figure 2. Hydrolysis of lactose by enzymatically active beads made of either alginate (A) or alginate-agarose (AA). The beads were loaded with the cell pellet from 10 mL of an induced *E. coli* culture and incubated with 6 mL of 5% lactose at 75 °C.

Figure 3. Hydrolysis of lactose (5% solution) after serial batches of incubation of agarose-alginate beads at 75 °C for 70 minutes.

Figure 4. SAXS patterns of the native capsules and after two consecutive heating treatments at 75 °C. The dots represent the experimental data and the solid lines correspond to the fits obtained using the two-level Beaucage model.

Figure 5. Micrographs of Sybr® Green I-stained, enzymatically active alginate-agarose beads. **A)** Whole bead. **B)** Detail of the surface of a bead. **C)** Detail of a bead cross-section. The bar length is equivalent to 200µm.

Figure 6. Cryo-SEM of beads surface. Panels **A** and **B** show pictures at different magnification of an untreated bead. Panels **C** and **D** show pictures at different magnification of a bead after 70 minutes of incubation at 75 °C.

Figure 7. Cryo-SEM of beads cross-sections. Panels **A** and **B** show pictures at different magnification of an untreated bead. Panels **C** and **D** show pictures at different magnification of a bead after 70 minutes of incubation at 75 °C.

464 **Figures**

465 **Figure 1**

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467 **Figure 2**

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