"Aerogels of enzymatically oxidized galactomannans from leguminous plants: versatile delivery systems of antimicrobial peptides and enzymes"

Paola Campia^, Erika Ponzini°, Bianca Rossi*, Stefano Farris#, Tiziana Silvetti^, Luca Merlini*, Milena Brasca^, Rita Grandori° and Yves M. Galante*

^Istituto di Scienze delle Produzioni Alimentari, C.N.R.,
Via Celoria2, 20133 Milano, Italy.
paola.campia@ispa.cnr.it
milena.brasca@ispa.cnr.it
tiziana.silvetti@ispa.cnr.it

°Department of Biotechnology and Biosciences
University of Milano-Bicocca (UNIMIB)
Piazza della Scienza 2, 20126, Milano, Italy.
erika.ponzini@unimib.it
rita.grandori@unimib.it

#PackLAB, Unimi Food Packaging Laboratory, *DeFENS*;
Dept. of Food, Environmental and Nutritional Sciences; University of Milan
Via Celoria 2, 20133 Milano, Italy.
stefano.farris@unimi.it

* Istituto di Chimica del Riconoscimento Molecolare, C.N.R.,
Via Mario Bianco 9, 20131 Milano, Italy.
bianca.rossi@polimi.it
luca.merlini@icrm.cnr.it

Correspondence should be addressed to:
Y.M. Galante
Istituto di Chimica del Riconoscimento Molecolare, C.N.R.,
Via Mario Bianco 9, 20131 Milano, Italy.

Telephone number: +39 335 8477648
e-mail address: yves.galante@icrm.cnr.it

Running title: *Aerogels from oxidized leguminous galactomannans as delivery systems*

Abstract

We describe aerogels obtained by laccase/TEMPO-oxidation and lyophilization of galactomannans (GM) from fenugreek, sesbania and guar. Enzymatic oxidation of GM in aqueous solution caused viscosity increase up to fifteen-fold, generating structured, elastic, stable hydrogels, presumably due to establishment of hemiacetalic bonds between newly formed carbonyl groups and free hydroxyl groups. Upon lyophilization, water-insoluble aerogels were obtained, whose mechanical properties and porosity were investigated.

Active principles were absorbed into the aerogels from aqueous solutions and, following rinsing, blotting, re-lyophilization, were released in an appropriate medium. The release of the antibiotic polymyxin B was tested against six different Gram-negative bacterial strains, of the antimicrobial peptide nisin against two Gram-positive and of the muraminidase lysozyme against one anaerobic strain. Protease and lipase release in solution from "enzyme loaded" aerogels was monitored by the increase in enzymatic activity.

These biomaterials could represent new versatile, biocompatible "delivery systems" of actives for biomedical and industrial applications.

Keywords: *galactomannans; polymyxin; nisin; lysozyme; aerogels; delivery systems.*

Abbreviations*: ABTS, 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulphonic acid); CFU, colony forming unit; CNF, cellulose nanofibrillated; DS, delivery system; EOLFG, enzymatically oxidized lyophilized fenugreek gum; EOLGG, enzymatically oxidized lyophilized guar gum; EOLSG, enzymatically oxidized lyophilized sesbania gum; FG, fenugreek gum; GG, guar gum; GO, galactose oxidase; Lcc, laccase; LFG, lyophilized fenugreek gum; LGG, lyophilized guar gum; LMS, laccase mediator system; LSG, lyophilized sesbania gum; MIP, mercury intrusion porosimetry; SG, sesbania gum; TEMPO, 2,2,6,6-Tetramethyl-1-piperidinyloxy radical.*

1. Introduction

Galactomannans (GM) from leguminous plants are widely exploited to generate a considerable range of derivatives with several practical applications. GM are high molecular weight polysaccharides found in the seed endosperms of some *Leguminosae* (belonging to the family *Fabaceae*) where they serve as reserve source for carbon and energy upon germination (Prajapati et al., 2013). They have a branched polymeric structure composed of a backbone of mannose units linked by β-1,4 glycosidic bonds with side units of galactose bound to mannose by α-1,6 glycosidic bonds. The average ratio of galactose to mannose (Gal: Man) is variable, depending on the plant species,and ranges from 1:4.5 in cassia (*Cassia tora*) to 1:1 in fenugreek (*Trigonella foenum-graecum*) (Crescenzi et al., 2004; Daas, Schols & de Jongh, 2000; Daas, Grolle, van Vliet, Schols & de Jongh, 2002; Daniel, Whistler, Voragen & Pilnik, 1994; Liyanage, Abidia, Auldb & Moussa, 2015; McCleary, Clark, Dea & Rees, 1985; Merlini, Boccia, Mendichi & Galante, 2015; Sittikijyothin, Torres & Gonçalves, 2005; Wei et al., 2015). The species-dependent monomers ratio makes them, to various extents, soluble in water at different temperatures, chemically/biochemically quite reactive and flexible in application (Cheng, Prud'homme, Chick & Rau, 2002).

GM and their derivatives are used as rheology modifiers, thickening and suspending agents in food, feed and manufacturing industries (Mathur, 2011). They find application as excipients and co-formulants in the biomedical field, such as in pharmaceutical formulations of tablets and in orally controlled drug delivery systems (ODDS), but also as binders, disintegrants, suspending, thickening, gelling, stabilizing and protective agents, to add cohesiveness to drug powder, as they are susceptible to microbial degradation in the large intestine (Meghwa & Goswami, 2012). GM are also increasingly consumed as dietary fibers with atoxic bioactivities, to lower calories intake and for weight reduction, to control blood glucose, cholesterol and insulin levels, to reduce the risks of heart diseases and colon cancer, as texture modifiers and stabilizers in “specialty” foods (Murthy, Moorthy, Prabhu & Puri, 2010).

In the present paper, we describe the production of aerogels from enzymatically-oxidized, lyophilized (EOL) sesbania and guar gums, following a protocol similar to the one previously reported for fenugreek gum by Rossi et al. (2016). They are referred to as: EOLSG (for sesbania gum), EOLGG (for guar gum) and EOLFG (for fenugreek gum). Because of their biodegradability, biocompatibility, high surface area and porosities with open pore structures, nanostructured aerogels, particularly from polysaccharides (e.g., starch, cellulose, alginates), constitute an emerging platform as “delivery systems” (DS) of active principles, such as drugs, peptides and enzymes (Ulker & Erkey, 2014). However, to our knowledge, GM-based aerogels DS have not yet

been reported by others. We have absorbed into these GM aerogels the following model actives from aqueous solutions: the antibiotic polymyxin B; the antimicrobial peptide nisin; the enzymes lysozyme, protease and lipase, followed in all cases by repeated cycles of water rinsing, to eliminate surface-absorbed compounds, dry blotting and re-lyophilization of the hydrogels to generate "loaded" aerogels. The gradual release of the incorporated actives was monitored either my measuring enzyme activity in solution or in Petri dish on agar culture medium, seeded with different bacterial strains, by evaluating the inhibition halo of cell growth.

Polymyxins, a group of polypeptide antibiotics that consists of 5 chemically different compounds (polymyxins A–E), are secondary metabolites produced by the soil bacterium *Paenibacillus polymyxa*, were discovered in 1947 (Velkov, Thompson, Nation, & Li, 2010), but only polymyxin B (PMB) and polymyxin E (colistin) have been used in clinical practice (Payne, Gwinn, Holmes & Pompliano, 2007; Talbot et al., 2006). They share a common primary sequence of a cationic cyclic decapeptide linked to a fatty acid chain through an alfa-amide linkage (Li, Rayner & Nation, 2007), with a molecular weight of 1,750 Da, the only difference being at position 6,which is occupied by D-Phe in PMB and D-Leu in colistin. Polymyxins have been used against Gram-negative bacteria responsible for nosocomial infections until the early seventies when they were abandoned, because of their human nephro- and neuro-toxicity. More recently, there has been a revival of interest in polymyxins considering the emergence of multidrug resistant (MDR) Gram-negative bacteria to almost all classes of antibiotics. Polymyxin B is active against MDR bacteria, such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumonia*, also called "superbugs" (Falagas, Kasiakou & Saravolatz, 2005; Velkov et al., 2010). Thus, innovative, biocompatible, polysaccharide-derived DS could contribute to a more rational and controlled clinical management of PMB. We have absorbed polymyxin B from an aqueous solution unto plugs carved from wafers of EOLFG, EOLSG and EOLGG, followed by rinsing and re-lyophilization to form "loaded" aerogel plugs and shown its release based on the inhibition halo of cell growth of: *P. aeruginosa*, *Serratia marcescens*, *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Hafnia alvei*, *Enterobacter cloacae*. Controls, i.e., non "loaded" aerogel plugs, had no effect on cell proliferation, while a positive control of free PMB was always present in a separate well of each experimental Petri dish.

Nisin is a natural antimicrobial peptide produced by *Lactococcus lactis*, composed of 34 amino acids, some of which have a D rather than L configuration (de Arauz, Jozala, Mazzola, & Penna, 2009). It inhibits Gram-positive bacteria belonging to genera such as: *Lactococcus*, *Streptococcus*, *Staphylococcus*, *Micrococcus*, *Pediococcus*, *Lactobacillus*, *Listeria* and *Mycobacterium*, and also vegetative cells and spores of *Bacillus* and *Clostridium* (Sahl, Jack, & Bierbaum, 1995). If

combined with a chelating agent (such as EDTA), nisin is able to destabilize the bacterial outer membrane (Vaara, 1992), thus can also be effective against Gram-negative bacteria like *E. coli* and *P. aeruginosa* (Boziaris & Adams, 1999; Fang & Tsai, 2003). In 2006, the European Food Safety Authority approved the use of nisin as an antimicrobial food additive (E234). Currently in the EU, the use of nisin is allowed in: semolina and tapioca puddings, in ripened and processed cheese, in clotted cream, mascarpone and pasteurized liquid egg products, at concentrations ranging from 3 to 12.5 mg/L (2010/69/EU). It is also used in combination with antibiotics to prevent nosocomial infections caused by drug-resistant bacteria (Tong et al., 2014). A control release of nisin into the medium could contribute to its long term stability and effectiveness. Following the same protocol as with PMB, described in details in Methods, release of nisin from the three aerogels was evaluated on culture agar Petri dish from the growth inhibition of the Gram-positive *Enterococcus faecalis* and *Clostridium tyrobutyricum*, the latter under anaerobic conditions.

Finally, a few enzymes were also studied for absorption and release from the three GM aerogels. Indeed, enzymes often show low stability in industrial applications under storage or use conditions, and in free form can lead to sensitization and allergies of operators and users. Three different commercial enzymes of great practical importance were selected: egg lysozyme, a commercial protease and a lipase.

Lysozyme (LSZ, EC 3.2.1.17) is a hydrolytic enzyme obtained from hen's egg white (HEW), composed of 129 amino acids and with muraminidase activity (Silvetti et al., 2010). It has the ability to hydrolyze the β-1,4-glycosidic bond between N-acetyl-muramic acid and N-acetyl-glucosamine in the peptidoglycans of Gram-positive bacteria (Brasca et al., 2013). It is active on bacterial species, such as: *Staphylococcus aureus*, *Micrococcus lysodeikticus*, *Listeria monocytogenes* and *L. innocua*, *Bacillus cereus* and *B. stearothermophilus*, *Clostridium thermosaccarolyticum* and *C. tyrobutyricum*, etc. (Ávila, Gómez-Torres, Hernández & Garde, 2014; Takahashi et al., 2011). Lysozyme is non-toxic to humans and is authorized by EU legislation (EU No. 1129/2011) as a food additive (E1105) at "*quantum satis*" in ripened cheeses and milk products to prevent butyric acid fermentation which causes the "late blowing" of cheese wheels (Brasca et al., 2013).

Proteases constitute a fundamental group of hydrolytic enzymes, mainly employed as detergent additives, but also in several other applications. (Fu et al., 2015; Gupta, Beg & Lorenz, 2002; Karn & Kumar, 2015; Souza et al., 2015). Proteases can be unstable, as they are prone to autolysis (thus they are commercially formulated in high-glycol containing gels and reversibly inhibited with boron salts, whose toxicology is however under scrutiny, see TOXICOLOGICAL PROFILE FOR BORON; U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES; Public Health Service

Agency for Toxic Substances and Disease Registry; November 2010). Alternatives to boron are widely sought to be used as reversible protease inhibitors. Not least, protease dust inhalation can lead to irritation and pulmonary harm in humans, such as workers and users.

Lipases are increasingly used for natural fat degradation, esterification, transesterification and in the production of high value compounds, such as biopolymers, biodiesel, enantiopure pharmaceuticals, flavors and agrochemicals (Borrelli & Trono, 2015; Jaeger & Eggert, 2002; Reetz, 2002;). It would thus be valuable, for both basic studies and applications, to have a readily available, pure and stable lipase entrapped and released from a solid support before use.

Therefore, a great deal of research has been performed on the encapsulation or immobilization of hydrolytic enzymes, e.g.: in gel beads (Verma, Puri & Barrow, 2015) and nanoparticles (Lin, Chen & Liu, 2016; Vaghari et al., 2016;) to improve their long term stability against inactivation.

In this work, we describe the absorption, retention and release in active form of lysozyme, protease and lipase from GM aerogel plugs. Released lysozyme activity was demonstrated microbiologically by actual growth inhibition of *C. tyrobutyricum* (i.e., whose vegetative cells and spores represent its target when added to milk during cheesemaking) under anaerobic conditions. Protease and lipase release from similarly "loaded" and re-lyophilized aerogel plugs was estimated from the increase in their respective enzymatic activity, as a function of time, of solutions in which the plugs were incubated.

To better characterize these materials, compressive tests were also performed and their porosity was determined by Hg absorption under high pressure.

From all of the above, we suggest that aerogels made from enzymatically oxidized and lyophilized GM from leguminous plants, could represent very innovative, versatile, biocompatible delivery systems of various active principles, with potential applications in the biomedical field, as well as in food packaging, feed and industrial products.

2. Materials and Methods

2.1. Materials

Laccase from *Trametes versicolor*, in powder form, was a generous gift of Amano Ltd, UK, and Amano, Japan, with a measured activity of 1600 U/g on ABTS as substrate. It was dissolved with mild stirring in MilliQ water.

The strains of *E. coli (*ATCC 8739) and *S.* Typhimurium (ATCC 14028) were from the American Type Culture Collection (U.S.A.). The other strains were obtained from the bacterial collection of the ISPA-CNR (Institute of Science of Food Production of the Italian National Research Council, Milan,

211 Italy) and included: the Gram-positive *C. tyrobutyricum* (IN15b) and *E. faecalis* (VS485); the Gram-
212 negative *P. aeruginosa* (PS20), *S. marcescens* (S91), *H. alvei* (PS58) and *E. cloacae* (PS25). All
213 these strains were isolated from contaminated dairy samples, identified through partial 16S rRNA
214 sequencing using the universal primer set p8FPL and p806R (McCabe, Zhang, Khan, Mason &
215 McCabe, 1995). The Gram-negative strains were also subjected to *rpo*B sequencing (Sajben,
216 Manczingera, Nagyb, Kredicsa & Vágvölgyia, 2011). Sequences were analyzed by NCBI BLAST
217 search (http://www.ncbi.nlm.nih.gov/BLAST, Altschul, Gish, Miller, Myers & Lipman, 1990).
218 Lysozyme hydrochloride was supplied by Sacco (Cadorago (CO), Italy). Nisin (with a potency of 10^6
219 IU/g) and polymyxin B were purchased from Sigma-Aldrich (St. Louis, MO, USA).
220 The industrial protease (Deterzyme® APY 560 in granular from *Bacillus alcalophicus*) was from
221 Enmex (Tlalnepantla, Mexico). As for the lipase, preliminary experiments were performed with the
222 Type VII enzyme from *Candida rugosa* (Sigma lot 107H1024) in powder form and later with the
223 commercial lipase (Greasex® Ultra L from *Candida lipolitica*) in liquid form (from Novozymes,
224 Bagsvaerd, DK).
225 TEMPO and all other chemicals were from Sigma-Aldrich or Fluka.
226
227 ***2.2. Laccase assay.***
228 Laccase activity was determined using as substrate 2.48 mM ABTS in 100 mM sodium acetate at pH
229 5 (Niku-Paavola, Karhunen, Salola & Raunio et al., 1988).
230 One laccase unit is defined as the amount of enzyme that catalyzes the oxidation of one μmole of
231 ABTS in one min at 25 °C and pH 5.
232
233 ***2.3. Purification and viscosity measurements of GM solutions.***
234 Non purified gum powder from guar (GG), sesbania (SG) and fenugreek (FG), with a Brookfield
235 viscosity at 1% (w/v) in aqueous solution at 20 rpm and 25°C of 5000, 3000 and 1500-2500 mPa*s,
236 respectively, were from a commercial source and kindly supplied by Lamberti S.p.A. Actual GM
237 content of unpurified gum varied between 76 and 80% (w/w), the remaining components being
238 residual aleuronic proteins, seed coat residues, low mol wt sugars, ashes. Before performing
239 oxidation and other experiments, all GM were purified by dispersion (at 10% w/w) in a 3:7 solution
240 of H_2O/ethanol, by stirring at room temperature for 30 min, followed by vacuum filtration. The
241 recovered GM were dispersed (at 10% w/w) in acetone, stirred as before, and finally recovered by
242 vacuum filtration. Before use, they were oven-dried at 60°C overnight. Polysaccharide yield of this
243 procedure was 85-90% (w/w), while the residual 10-15%, composed of proteins and other minor
244 components, was discarded.

"Purified" GG and FG were dissolved in MilliQ water at room temperature at 1200 rpm with an IKA overhead stirrer for 30 min. SG was firstly dispersed using an IKA Ultraturrax in MilliQ water at room temperature for a min and finally at 1200 rpm with an IKA overhead stirrer for 30 min. The solution was kept standing overnight at room temperature without stirring before any further manipulation. Compared to "non purified" GM, viscosity increased by about 10-15% at equal gum concentration in water, indicating a higher polymer amount in the "purified" material. GM solutions for all experiments were prepared the same way.

Viscosity measurements were performed in a volume of 300-400 ml in a beaker at room temperature using a Brookfield DV-I Prime, at 20 rpm, mounted with the appropriate spindle.

2.4. Preparation of the aerogels.

TEMPO-mediated, laccase oxidation of FG, SG and GG was carried out following the procedure previously described by Merlini et al. (2015) with slight modifications. Purified FG, SG and GG were dissolved, as described in § 2.3, at 1.075% (w/w) in 100 ml of MilliQ water at room temperature for 30 min and the solution was kept standing overnight at room temperature. The mediator TEMPO was added to a final concentration of 0.64 mM (10 mg); laccase, 160 U/g $_{(GM)}$ (eq. to about 110 mg), was dissolved in 2 ml of MilliQ water, centrifuged for 4 min at 4000 rpm and the supernatant added to the solution. The reaction was continued for 3 h at 35°C, with constant mechanical stirring at 500 rpm, after which Brookfield viscosity was measured and the mixture was left standing at room temperature. Viscosity was measured again after 6 and 24 h from the start of the reaction.

To obtain the aerogels, whole preparations of enzymatically oxidized FG, SG or GG hydrogels were distributed either into 24 well plates, to obtain 16-20 x 12-16 mm cylindrically shaped samples (see also Rossi et al., 2016), or in Petri dishes (90×12 mm), to give a flat, round aerogel wafer of about 80x5 mm, and frozen for 12 h at -80°C, followed by freeze-drying at -55 °C for 48 h (these materials are referred to as EOLFG, EOLSG and EOLGG, respectively).

2.5. Preparation of actives solutions.

A 1 g/L of polymyxin B stock solution was prepared in sterile distilled water, filter-sterilized and stored at 4°C. A working solution of 300 mg/L was prepared by diluting the stock solution in distilled, sterile water. Nisin working solution (50,000 IU/ml) was prepared by dissolving the appropriate amounts of powder in sterile 0.02 N HCl, sterilized by filtration through a 0.20 µm membrane filter (Corning, USA) and stored at 4 °C. Lysozyme powder was dissolved in distilled, sterile water to give a stock solution of 2 g/L, which was freshly prepared before each set of

experiments and filter-sterilized as before. A lysozyme working solution of 1.25 g/L was prepared by diluting the stock solution with distilled, sterile water.

Before use, 100 mg of protease were dissolved in 1 ml of MilliQ water and centrifuged at 11,200 x g at RT for 5 min, to eliminate insoluble material present in the formulation, the pellet was discarded and the "loading" experiments were carried out with the supernatant.

In the case of lipase, 125 mg of the *C. rugosa* lipase in powder form were likewise dissolved in 1 ml of MilliQ water and centrifuged at 11,200 x g at RT for 5 min, the pellet was discarded and the supernatant used for "loading" experiments. The commercial lipase from *C. lipolitica*, in liquid form, was used as such.

Protein concentration was measured by the Bradford assay (Bradford, 1976) and turned out to be: 33 mg/g for the protease, about 5 mg/g for the *C. rugosa* lipase and 23 mg/ml for the *C. lipolitica* lipase.

2.6. Absorption of the active principles in the aerogels.

Plugs (Ø 8 mm) of EOLFG (from fenugreek gum), EOLSG (from sesbania gum) and EOLGG (from guar gum) were carved with a cork borer as described by Rossi et al. (2016). Polymyxim B, nisin, lysozyme, protease or lipase were absorbed unto the aerogels by immersing one to three plugs for 1 h at room temperature in an Eppendorf tube containing 0.5-1.0 ml of solution of active at a specified concentration. The plugs were then rinsed three times in 1 ml of distilled, sterile water and blotted on UV-sterilized filter paper to remove eventual active solution present on the surface. The "theoretical" amount of active absorbed was calculated from the weight difference of the plug(s) (initial weight: 7-9 mg), measured on each plug before and after immersion in solution, which gave an average weight increase of 13-15-fold.

Protease and lipase protein concentration in the incubation solutions were further checked with the Bradford assay, before and after absorption of the enzymes as explained above, and found to be equal. In all cases, the "loaded" hydrogels were re-lyophilized in order to obtain the corresponding "loaded" aerogels to be tested.

The effect of lyophilization on lipase and protease was evaluated by measuring specific activity of both enzymes after freeze-drying of their respective aqueous solutions, re-dissolution in water and was negligible.

2.7. Bacterial strains and culture conditions

All bacterial strains were routinely grown under standard conditions. Working cultures of *P. aeruginosa*, *S. marcescens*, *E. coli*, *S.* Typhimurium, *H. alvei* and *E. cloacae* were propagated in

313 Brain Heart Infusion broth (Scharlau Microbiology, Barcelona, Spain) and incubated at 30 °C (at 37
314 °C for *E. coli* and *S.* Typhimurium) for 18 h. *C. tyrobutyricum* was cultured anaerobically
315 (Anaerocult A Merck, Darmstadt, Germany) in Reinforced Clostridial Medium (VWR Chemical,
316 Leuven) at 37 °C for 48 h. *E. faecalis* was grown aerobically in M17 broth (Biolife Italiana, Milano,
317 Italy) at 37 °C for 18 h. Before each set of experiments, all enriched cultures were diluted in Ringer
318 solution (Scharlau Microbiology) to reach a concentration of 10^5 CFU/ml. For solid media, 15 g/L
319 agar (Scharlau Microbiology) was added to the broth before sterilization.
320
321 ***2.8. Evaluation of release and activity of active principles***
322 The detection of polymyxin B, nisin and lysozyme released from the aerogels was assessed by a
323 modified method described by Rossi et al. (2016). For anti-bacterial activity, the poured plate method
324 was used. Briefly, each Petri dish was layered with a selective agar medium, seeded with the bacterial
325 inoculum at a concentration of 10^5 CFU/ml. After solidification of the agar, equidistant wells (Ø 8
326 mm) were carved with a sterilized cork borer and filled with: i) different concentrations of the free
327 active; ii) plugs of "loaded" EOLFG, EOLSG or EOLGG containing the appropriate amount of
328 active; iii) a control of "unloaded" EOLFG, EOLSG or EOLGG plug. Inoculated plates were
329 incubated at 30 °C, or 37 °C in the case of *E. coli*, *S.* Typhimurium and *C. tyrobutyricum*, for 24 h.
330 Release of actives from aerogels was evaluated from the inhibition halo zones formed around each
331 well in comparison to the control aerogel and to the diffusion of the free active substance. All manual
332 operations were performed under a vertical laminar flow hood in sterile conditions and experiments
333 were run in duplicate.
334 Release of protease or lipase in water was evaluated by withdrawing aliquots of the incubation
335 solution and determining enzyme activity.
336 Proteolytic activity was calculated from the increase in absorbancy at 440 nm in a 1.0 ml reaction
337 volume composed of 0.25% w/v azocasein, 0.3 mM calcium chloride, 25 mM Trizma base (pH 8) for
338 10 min at 40°C. The reaction was stopped by adding 1.0 ml of 20% trichloroacetic acid, followed by
339 centrifugation at 11,200 x g at RT for 5 min . Protease activity is expressed as Δ O.D. at 440 nm/10
340 min/ml and one Unit of protease activity is the amount of enzyme causing a Δ O.D of 1.0 under those
341 conditions.
342 Lipolytic activity was determined by measuring initial linear absorbance increase at 410 nm produced
343 by p-nitrophenol upon hydrolysis of 1 mM p-nitrophenyl laurate (dissolved in isopropanol) in 10 mM
344 ammonium acetate pH 7.2 and 0.5 % Triton X-100, in 1.0 ml reaction volume (Santambrogio et al.,
345 2013). Lipase activity is expressed as Δ O.D. at 410 nm/min/ml and one Unit of lipase activity is the
346 amount of enzyme causing a Δ O.D of 1.0 under those conditions.

All measurements were performed in triplicate.

2.9 Determination of pore size

Total porosity, average and modal pore size, pore size distribution, and bulk density of the aerogels were measured by mercury intrusion porosimetry (MIP), which consists in forcing mercury into the sample pores at increasing pressures. The output data is the volume of mercury intruded into the sample as a function of the pressure applied, which is inversely proportional to the pores size (Santos et al., 2015). Pascal 140 coupled with Pascal 240 porosimeters (Thermo Fisher Scientific, Waltham, MA) were used for the analysis. Pascal 140 module allows to reach low pressures (up to 400 kPa), thereby measuring only large pores (macropores). Pascal 240 reaches pressures up to 200 MPa, thus allowing measurement of the porosity into the mesopore region. Cylindrical aerogel corks weighing 40 ± 3 mg were used in the analyses. To overcome the potential limitations associated to MIP (Majda et al., 2016) freeze-dried samples were placed in a dessicator containing $CaCl_2$ for 3 weeks to remove residual moisture. The residual moisture content measured with a moisture analyzer HB43–S (Mettler Toledo, Greifensee, Switzerland) was as low as 0.5 ± 0.02 %. During analysis, air inside the samples was removed by three degassing cycles. Mercury intrusion was performed according to the PASCAL (Pressurization with Automatic Speed-up by Continuous Adjustment Logic) system, which minimizes crushes and deformations of the samples. Pore diameter was calculated according to the Washburn equation (Washburn, 1921) assuming a cylindrical shape of the pores (Webb and Orr, 1997), as in (1):

$$P = -2\sigma \cos\frac{\theta}{r} \qquad (1)$$

where P is the applied pressure, r is the radius of the pore, σ is the surface tension of the mercury and θ is the contact angle between mercury and polymer. A surface tension of 480 mN/m and a contact angle of 140° (Kim & Chu, 2000; Joshi et al., 2015) were used for all measurements. SOL.I.D (SOLver of Intrusion Data) software was used for data elaboration.

2.10 Determination of mechanical features.

Cylindrically shaped samples (~20 mm height × 10 mm diameter) of both pristine non-oxidized, lyophilized gums (LFG, LSG, and LGG) and the corresponding laccase-oxidized, lyophilized aerogels (EOLFG, EOLSG, and EOLGG) were evaluated by a compressive test according to the procedure described by Deszczynski, Kasapis & Mitchell (2003) and more recently applied by

Rossi et al. (2016). Briefly, two consecutive cycles of compression were performed with a dynamometer (mod. Z005, Zwick Roell, Ulm, Germany) fitted with a 100 N load cell and connected to two plates (30 mm diameter), placed at a distance of 22 mm apart. Each compression cycle accounted for a maximum deformation of the sample of 2 mm, at a crosshead speed of 2 mm x s^{-1}. Stress–strain and force–time plots allowed for the calculation of compressive modulus, maximum compressive force, cohesiveness, springiness, and resilience using the software TestXpert V10.11 Master (see also Ghafar et al., 2015). All tests were carried out at 23 ± 0.5 °C and 40 ± 2.5 % relative humidity (RH). At least ten replicates were performed with each sample. Statistical differences between mean values were determined by Student's *t*-test, with a significance level (p) < 0.05, using Statgraphic Plus 4.0 software.

3. Results and Discussion

3.1 Release from aerogels and activity of polymyxin B against Gram-negative bacteria.

The properties of EOLFG, EOLSG and EOLGG aerogels as delivery systems were firstly investigated with the antibiotic polymyxin B against six Gram-negative bacterial strains. All the six bacterial species selected are responsible for nosocomial infections. Indeed, *P. aeruginosa* is one of the major multidrug-resistant (MDR) bacterial species and almost all the currently available antibiotics have no effect on it, with the exception of polymyxins (Velkov et al., 2010). Therefore, since the mid nineties, the use of polymyxins has seen a renewed interest, in spite of their suspected nephrotoxicity and neurotoxicity. Thanks to the wide activity spectrum of polymyxin B, we were able to test five other bacterial species of *Enterobacteriaceae*, namely: *S. marcescens*, *E. coli*, *S.* Typhimurium, *H. alvei* and *E. cloacae*; all responsible for nosocomial infections that can lead to life-threatening bacteremia (Alexopoulou et al., 2016; Kim et al., 2015; Steinbach, Töpper, Adam, & Kees, 2015).

The activity of polymyxin B was firstly demonstrated by cell growth inhibition of *P. aeruginosa*. Fig.1a shows the inhibition zone by polymyxin B released from "loaded" plugs of :EOLFG (2), EOLSG (4) and EOLGG (6) on live cells of *P. aeruginosa*.

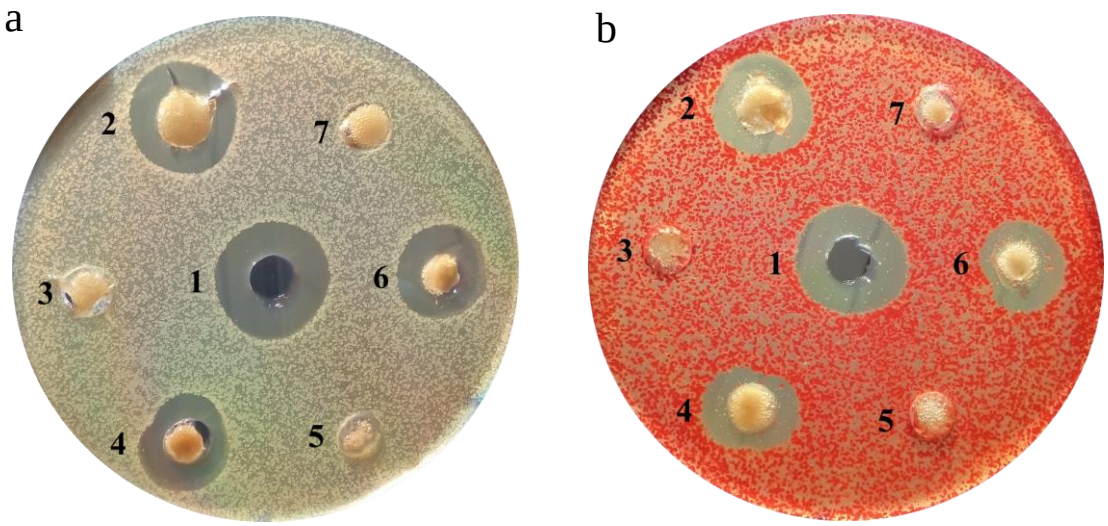


Fig. 1-a,b- Inhibition by polymyxin B released from aerogels of Gram-negative bacterial cell growth. Central well (1), 30 µg (equivalent to 22 nanomoles) of free polymyxin B. Lateral wells: plugs of loaded EOLFG (2), control EOLFG (3); loaded EOLSG (4), control EOLSG (5); loaded EOLGG (6), control EOLGG (7). Fig. 1a: *P. aeruginosa*. Fig. 1b: *S. marcescens*.

The central well (1) was filled with 100 µl of 300 mg/l of polymyxin B (equivalent to 30 µg or 22 nanomoles of active substance). Control plugs of "unloaded": EOLFG (3), EOLSG (5) and EOLGG (7) were fitted in the corresponding wells. The amount of incorporated polymyxin B in aerogel plugs was calculated from the individual average weight increase, performed in triplicate, after 1 h immersion in a polymyxin B solution, rinsing, blotting, and was estimated to be 28.9 ± 8.3 µg for EOLFG, 23.7 ± 2.2 µg for EOLSG and 32.4 ± 6.8 µg for EOLGG**.**

As detailed in § 2.6, "loaded" plugs were rinsed three times in water in order to remove any polymyxin B loosely absorbed on the surface of the aerogels. 100 ul of each rinse water was evaluated for cell growth inhibition on a similarly seeded Petri dish. While the first rinse water showed a very slight inhibition halo, the third one had none (see Fig. 1.S in "Supplementary Material"). Therefore, we conclude that the large halos around the plugs of EOLFG, EOLSG and EOLGG in Fig. 1a are largely or exclusively due to the release of polymyxin B absorbed and retained within the aerogels.

A similar experiment was carried out on a strain of *S. marcescens*, a species recognized to cause human clinical diseases, with a multidrug-resistance profile (Zavascki, Goldani, Li & Nation, 2007). Fig 1b shows the inhibition zone by polymyxin B released from "loaded" plugs of: EOLFG (2), EOLSG (4) and EOLGG (6) of *S. marcescens* cell growth. As for *P. aeruginosa*, the third rinse water of all plugs developed no halos (see Fig 2.S in "Supplementary Material").

Following the same protocol as above, we have evaluated four other Gram-negative bacterial species involved in nosocomial infections, that can be fatal, mostly if infected patients are elderly, young, or people with depressed immune systems (Alexopoulou et al., 2016; Kim et al., 2015; Steinbach et al., 2015). The bacterial strains were the following: *S*. Typhimurium (Fig. 1c), *E. coli* (Fig. 1d), *E. cloacae* (Fig. 1e) and *H. alvei* (Fig. 3.S in Supplementary Material). All strains analyzed were susceptible to polymyxin B cell growth inhibition, which was absorbed and released from the aerogel plugs.

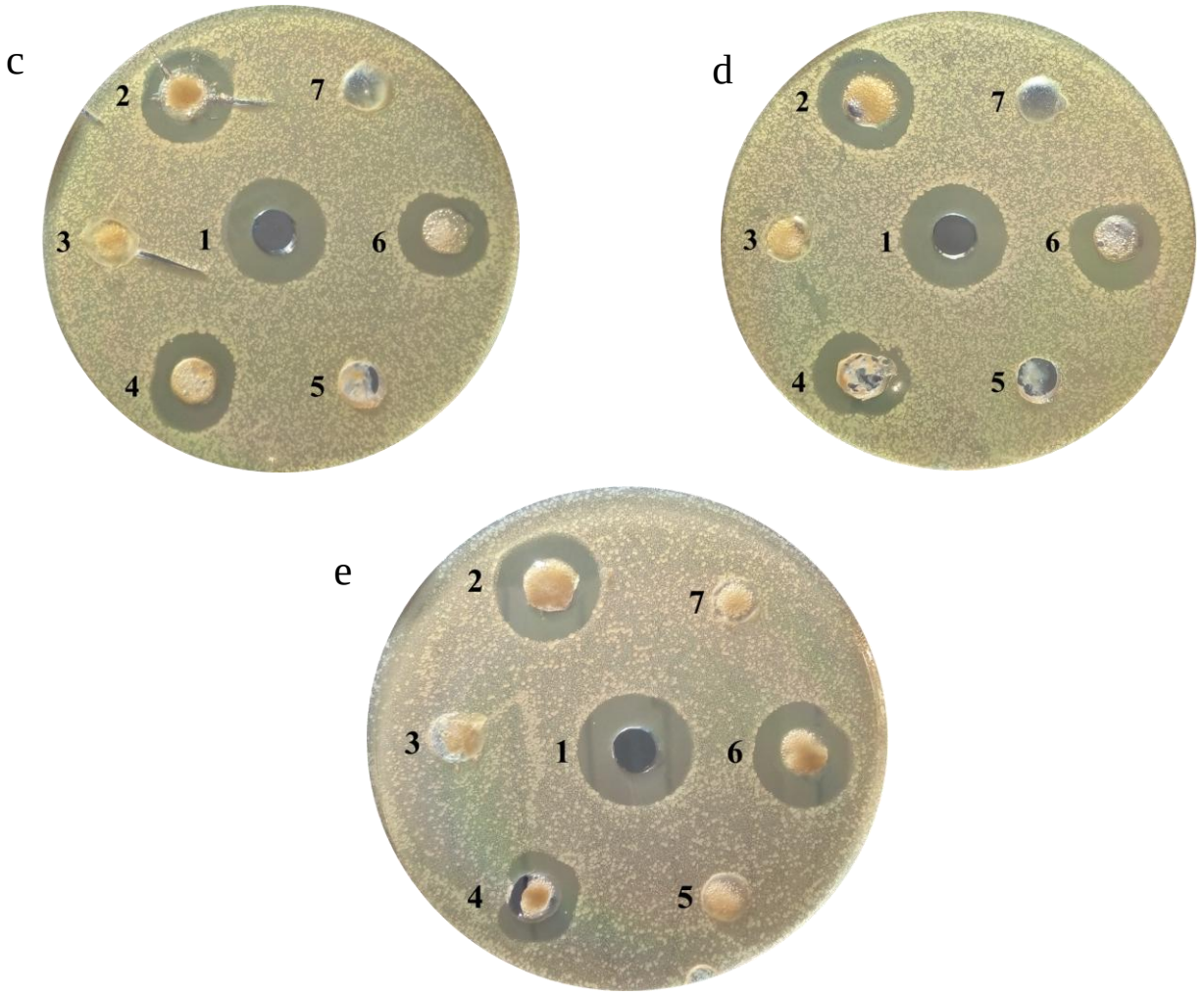


Fig. 1-c-e- Inhibition by polymyxin B released from aerogels of Gram-negative bacterial cell growth. Central well (1), 30 µg (equivalent to 22 nanomoles) of free polymyxin B. Lateral wells: plugs of loaded EOLFG (2), control EOLFG (3); loaded EOLSG (4), control EOLSG (5); loaded EOLGG (6), control EOLGG (7). Fig. 1c: *S.* Typhimurium. Fig. 1d: *E. coli*. Fig. 1e: *E. cloacae.*

It is difficult to make a quantitative evaluation of polymyxin B released from Figs.1, a-e and Fig. 3.S. However, by comparing the diameters of the halos around the "loaded" plugs to the central wells filled with the free active substance, the amounts of polymyxin B diffused appear to be fairly equal.

3.2. Release from aerogels and activity of nisin against Gram-positive bacteria.

The activity of nisin loaded and released from the three GM aerogels was evaluated against Gram-positive bacteria in a similar way as for polymyxin B.

Fig. 2a shows the growth inhibition by nisin released from "loaded" plugs of: EOLFG (2), EOLSG (4) and EOLGG (6) on cells of *E. faecalis* isolated from contaminated dairy samples and Fig. 2b on a strain of *C. tyrobutyricum*, cultured under anaerobic conditions. The amount of incorporated nisin was calculated from the average weight increase of individual aerogel plugs, performed in triplicate, after 1 h of immersion in a nisin solution (50,000 IU/g) and was estimated to be 120.5 ± 34.6 µg for EOLFG, 98.9 ± 9.3 µg for EOLSG and 135.1 ± 28.5µg for EOLGG. All plugs were rinsed three times, as before, in order to remove nisin eventually present on the outer surface. The third rinse water did not develop any halo (see Fig. 4.S in "Supplementary Material").

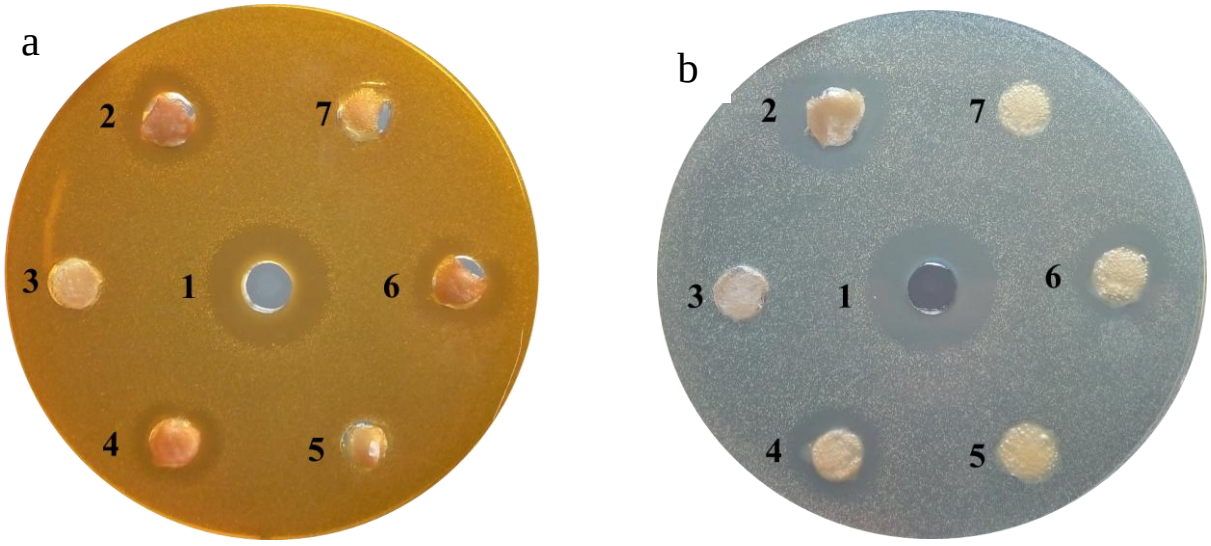


Fig. 2a,b - Inhibition by nisin released from aerogels of Gram-positive bacterial cell growth. Central well (1) 125 µg of free nisin (equivalent to 37 nanomoles). Lateral wells: plugs of loaded EOLFG (2), control EOLFG (3); loaded EOLSG (4), control EOLSG (5); loaded EOLGG (6), control EOLGG(7). Fig. 2a: *E. faecalis*. Fig. 2b: *C. tyrobutirycum.*

3.3. Release and activity of lysozyme against C. tyrobutirycum

The muraminidase lysozyme is used in cheese and milk products to prevent butyric acid fermentation prevalently caused by *Clostridium* species. In a previous work (Rossi et al., 2016) we have described the release of lysozyme from EOLFG aerogel, evaluated by a standard biochemical test, based on the hydrolytic activity of this enzyme on agar plates layered with lyophilized cells of *M. lysodeikticus*, while in the present work a microbiological test was used.

Fig. 5.S shows the inhibition zone by lysozyme released from a "loaded" plug of EOLFG (2) on live cells of *C. tyrobutirycum*, grown under anaerobic conditions. The well on top (1) was filled with 100 µl of 1.25g/L of lysozyme (i.e., 125 µg of protein equivalent to 8.7 nanomoles of active substance). A control plug of EOLFG (3) was fitted in the corresponding well. The amount of incorporated lysozyme was calculated from the average weight increase of individual aerogels, performed in triplicate, after 1 h of immersion in a lysozyme solution and was estimated to be 120.5 ± 34.6 µg of lysozyme for EOLFG. Similar experiments with EOLSG and EOLGG gave somewhat unclear results, as those two aerogels turned out to be too brittle under the anaerobic and temperature conditions used for the test. However, this aspect is being investigated.

Therefore, it appears that with the three selected antimicrobials and under the experimental conditions applied, the release of active was able to control microbial growth *in vitro*, even in the presence of a relatively high cell concentration.

3.4 Uptake and release of protease and lipase from GM aerogels.

The following preliminary controls were performed on the two industrial enzymes tested in order to substantiate our experimental approach:

i. The effect of the lyophilization procedure on the lipase and protease was tested by activity determination of the enzymes, before and after lyophilization from water solution and re-hydration. Results indicated no significant difference caused by the treatment (*data not shown*).

ii. Eventual preferential uptake or exclusion of enzymes by GM gels was tested by measuring protein concentration before and after immersion of the plugs in the solutions of actives. In both cases of protease and lipase, protein concentration was unchanged, which is taken to mean that there is no selective protein exclusion or binding by the hydrogels (*data not shown*).

Enzyme release from "loaded" aerogel plugs in water was monitored by measuring protein concentration and increase in enzyme activity as a function of time.

Fig. 3 shows the kinetics of protease release at RT from the three hydrogels, measured every hour, for 6 h, expressed as percentage of the "theoretical" total protease activity loaded. The mean values and standard deviations are reported in Tab. 1.S (see Supplementary Material).

Protease release appears to be faster in the first 2 h and then gradually reaches a plateau after 6 h.

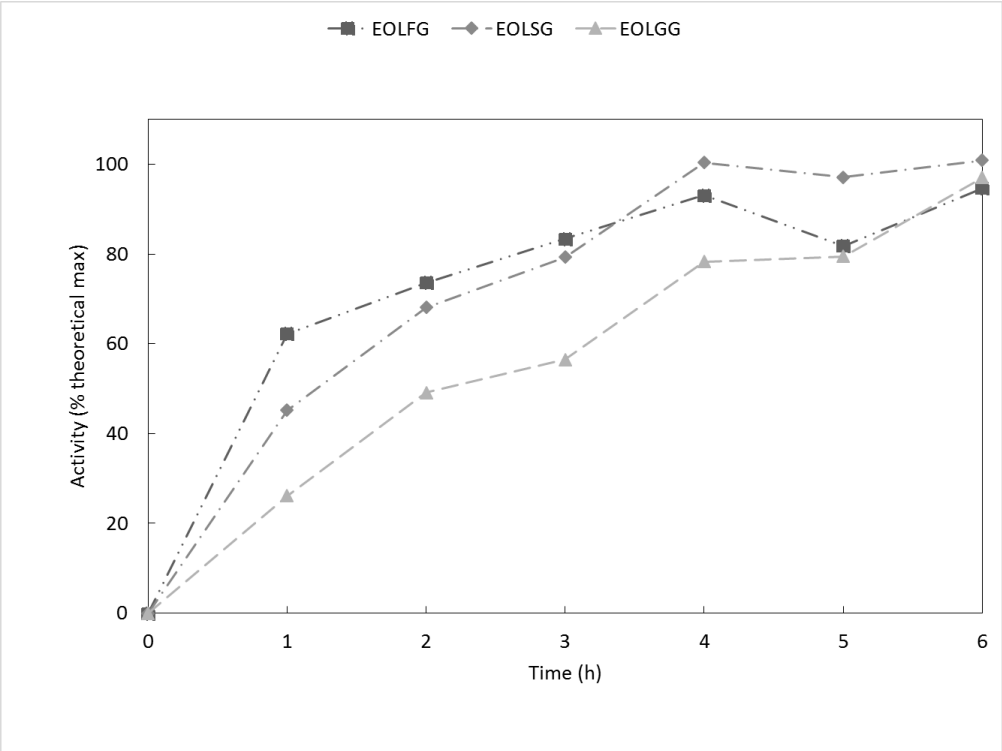


Fig. 3 - Time course of protease release in water solution from "loaded" hydrogel plugs of EOLFG, EOLSG and EOLGG. Each time point was performed in triplicate on separately prepared plugs and is expressed as the average percentage of the "theoretical" total protease activity loaded.

It can be calculated that this represents, in all three cases, an almost complete release of enzyme loaded into the aerogels (Tab. 1.S.).

In the case of lipase, preliminary evaluations were performed with a *C. rugosa* enzyme obtained from Sigma for research purposes, containing over 90% of non protein material, which had to be discarded before further experiments. Loading and release were performed only on EOLFG, as described above, and it was found that almost all the calculated amount of enzyme absorbed was released after 5 h incubation in water at RT (*not shown*). However, in consideration of the little amount of enzyme available, the other two aerogels were not tested.

Therefore, for practical applications (e.g., in detergency), we considered more meaningful to try with an industrial enzyme commercially available, hence the choice of Novozymes' Greasex Ultra L from

C. lipolitica with an activity of 15 U/ml, used as such with no further purification or pre-treatments. In this case, as shown in Fig. 6.S for the three aerogels, the release of lipase in solution was limited to about 30% of the "theoretical" maximum, even after a very long incubation period, as if the stabilizing agents contained in the commercial formulation of this enzyme (e-g., glycols, salts, etc.), prevented or limited its full release. Purification experiments of this lipase are underway to verify the hypothesis, focusing mainly on EOLFG which preserves its structure upon prolonged incubation periods in solution.

The mean values and standard deviations of the experimental data points of Fig. 6.S are reported in Tab. 2.S.

3.5. Determination of aerogels pore size

Data from MIP tests are reported in Tab. 1.

Sample	Bulk density (kg x m^{-3})	Porosity (%)	Pore surface area (m^2 x g^{-1})	Average pore diameter (µm)	Modal pore diameter (µm)
EOLFG	35.9	99.91	11.32	9.83	42.44
EOLSG	32.8	96.19	16.89	6.94	3.59
EOLGG	31.3	93.28	15.54	7.68	102.71

Tab. 1 - MIP parameters of the three GM aerogels

The aerogel from fenugreek (EOLFG) exhibited higher density (35.9 kg x m^{-3}) compared to guar gum (EOLGG) and sesbania (EOLSG) with 31.3 and 32.8 kg x m^{-3}, respectively. Because a number of parameters may affect the bulk density of aerogels (e.g., type of polymer, initial polymer concentration, pH, ionic strength, air inclusion method, and instrumental technique for the measurements), a strict comparison among different materials is relatively hard to make. For example, sodium carboxymethyl cellulose aerogels were reported to have bulk density of 56.8 kg x m^{-3} (Lin, Li, Lu, & Cao, 2015), whereas the bulk density of wheat gluten-based aerogels ranged between 58 and 343 kg m^{-3} (Blomfeldt et al., 2010), therefore a wide range of values is to be expected. On the other hand, previously reported open-cell CNF foams and CNF aerogels had densities of 15 and 7–20 kg x m^{-3}, respectively (Jiang & Hsieh, 2014; Svagan, Samir, & Berglund, 2008), lower than the values we have found, so that different thermal values are to be expected.

From a practical point of view, it is interesting to notice that expanded and extruded polystyrene closed-cell foams, for thermal insulation applications, have densities in the range of 16–45 kg x m^{-3}, whereas those of polyurethane are in the range of 40–55 kg x m^{-3} (Al-Homoud, 2005). Thus, it will be interesting to study the thermal insulation properties of the plant-derived materials we have obtained.

The three aerogels described in this study showed very high open porosity values, with an outstanding 99.9 % measured for the EOLFG sample. This value is in line with a recent work on lightweight foams based on nano-cellulose, which exhibited an open porosity of 99.6% (Wicklein et al., 2015). In our studies, differences in pore size and distribution were observed. EOLFG showed pores of larger size compared to the two other aerogels, as demonstrated by both pore surface area (11.32 m^2 x g^{-1}) and average pore diameter (9.83 μm) (see Tab. 1). EOLSG showed the smallest pores, with pore surface area and average diameter of 16.89 m^2 x g^{-1} and 6.94 μm, respectively. While EOLGG stood halfway in terms of pore size. There was also a difference in pore size distribution according to the nature of the GM aerogel.

As indicated by the modal pore diameter, most of the pores were centered at ~ 3.6 μm diameter for the EOLSG (meaning that the smallest pores are more largely represented), ~ 42 μm for EOLFG, and ~ 103 μm for the EOLGG. This can be seen in the pore size distribution plots of Fig. 7, a-c.S (see Supplementary Material). While the pore diameter frequency distribution of the sample of EOLFG (Fig. 7,a.S) includes several classes of the same height between 10 and 50 μm, the frequency distribution of samples EOLSG (Fig. 7,b.S) and EOLGG (Fig. 7,c.S) is somehow skewed toward high pore diameters. Moreover, with EOLGG and EOLSG the occurrence of a second peak can also be observed, which is broader for EOLSG between 1 and 5 μm. These results indicate that, although with some differences, all the three aerogels tested in this work had more open macropores than mesopores and micropores in their final structure, suggesting a preferential practical application where rapid capillary suction is of utmost importance, e.g., for "fast" delivery systems and in liquid cleaning/removing devices (Blomfeldt et al., 2010).

Finally, Fig. 4 (which is derived from the data of Fig. 7, a-c.S) indicates that in the case of fenugreek there is a wider distribution of pores with intermediate size (20-50 um) than in guar and sesbania, presumably because it is the most extensively cross-linked aerogel.

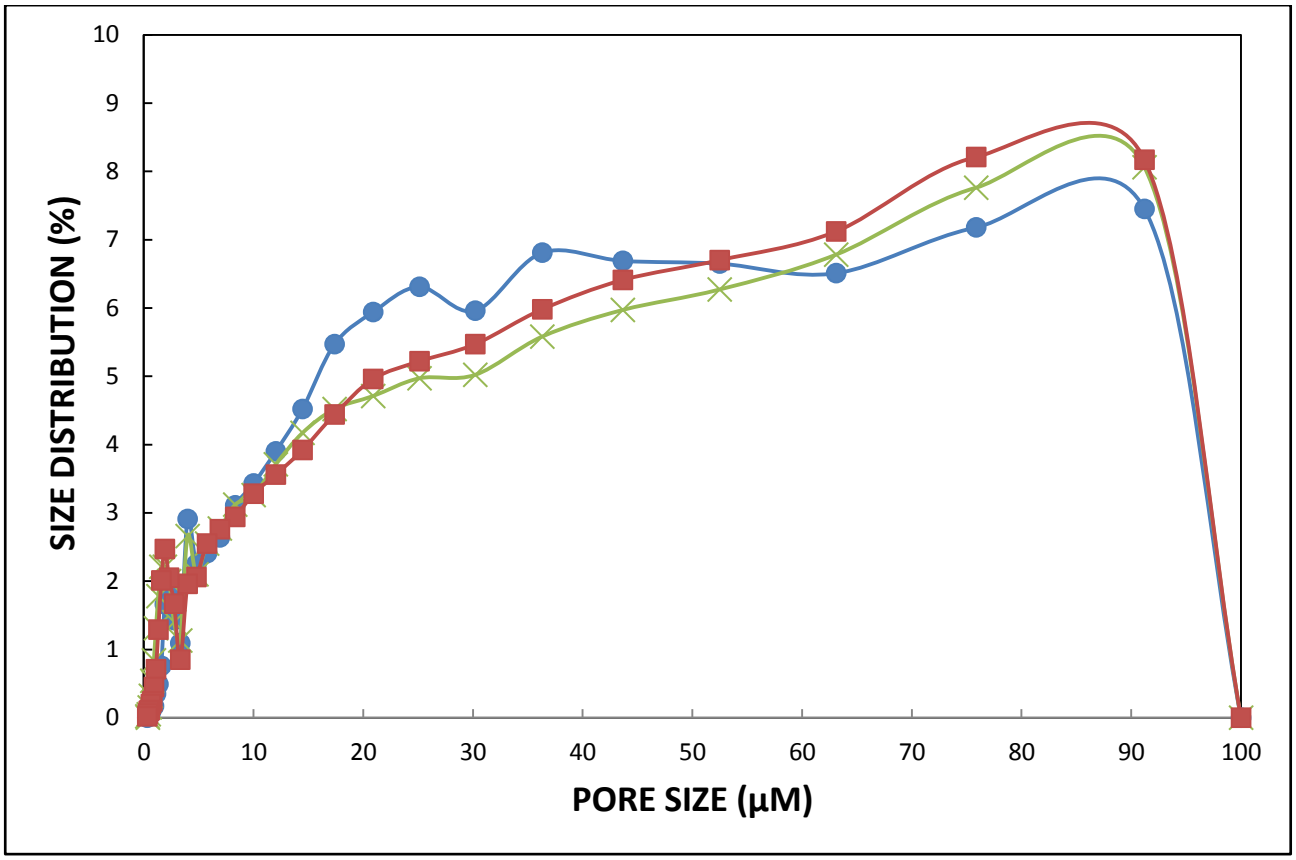


Fig. 4- Pore size distribution in diameter (abscissa) *vs* their % distribution (ordinate) of the three GM aerogels (blu curve (●), EOLFG; green curve (x), EOLSG; red curve (■), EOLGG.

However, if we consider the distribution of larger pores (i.e., between 50-100 µm), guar appears to have the most, closely followed by sesbania.

3.6. Mechanical properties

Large deformation tests allowed to quantify the effect of enzymatic oxidation on the mechanical properties of EOLFG, EOLSG and EOLGG. Tab. 2 reports the mechanical parameters obtained from compressive tests.

Sample	E-mod (kPa)	F_{max} (N)	Cohesiveness	Springiness	Resilience
LFG	0.502 (± 0.085)^{a}	0.081 (± 0.014)^{a}	0.713 (± 0.040)^{a}	0.651 (± 0.042)^{ab}	0.393 (± 0.033)^{ab}
EOLFG	22.775 (± 5.792)^{b}	2.376 (± 0.548)^{b}	0.685 (± 0.034)^{a}	0.611 (± 0.038)^{a}	0.316 (± 0.037)^{c}

LSG	0.249 (± 0.083)^{a}	0.039 (± 0.012)^{a}	0.728 (± 0.033)^{a}	0.665 (± 0.033)^{b}	0.434 (± 0.034)^{b}
EOLSG	12.479 (± 5.165)^{c}	1.332 (± 0.231)^{c}	0.712 (± 0.057)^{a}	0.641 (± 0.063)^{ab}	0.373 (± 0.066)^{a}
LGG	0.433 (± 0.070)^{a}	0.081 (± 0.023)^{a}	0.694 (± 0.043)^{a}	0.632 (± 0.049)^{ab}	0.386 (± 0.048)^{a}
EOLGG	18.577 (± 5.527)^{d}	2.075 (± 0.607)^{b}	0.627 (± 0.049)^{b}	0.542 (± 0.047)^{c}	0.257 (± 0.032)^{d}

Table 2 -Mechanical parameters from compressive tests on pristine, lyophilized and laccase-oxidized, lyophilized fenugreek (LFG, EOLFG), sesbania (LSG, EOLSG) and guar gum (GG, EOLGG) aerogels.

Results are expressed as mean values and standard deviation (in brackets). Different superscripts within a group (i.e., within each parameter) refers to a statistically significant difference ($p < 0.05$).

The three non-oxidized, lyophilized GM aerogels (i.e., LFG, LSG, LGG) did not exhibit appreciable differences in terms of F_{max} and E-mod. However, after laccase oxidation it was possible to observe statistically significant differences between samples. In absolute terms, EOLFG aerogel was stiffer and harder than EOLSG and EOLGG, with EOLSG showing the lowest mean values of both F_{max} and E-mod. Nevertheless, if we consider the relative increase of the above parameters, the largest occurred for EOLSG (F_{max} and E-mod increased ~ 50 and 34 times, respectively). This observation seems to indicate that the oxidation, and resulting internal crosslinking, was somewhat more "efficient" in the case of SG than FG and GG, though SG ultimately yielded a relatively weaker aerogel.

Concerning the other mechanical parameters, cohesiveness represents the ability of the sample to stand a second deformation in relation with the sample behavior during the first deformation cycle. Springiness indicates the capability of the sample to spring back after the first compression. Resilience is a measure of the ability of the sample to recover its original shape. After oxidation, cohesiveness and springiness decreased significantly only for the GG samples. Resilience decreased significantly upon oxidation for all the three samples, consistent with the inverse relationship between this parameter on one hand, and the compressive modulus and maximum force on the other (Ghafar et al., 2015).

Although the results of the mechanical tests unambiguously demonstrate positive effect of enzymatic oxidation on gel structuring, it is clear that the crosslinking efficiency depends on the specific polysaccharide and the formation of hemiacetal bonds at inter– and intra– molecular level.

4. Conclusions

There is an increasing interest in the development of new biomaterials from renewable resources, possibly by means of sustainable biochemical reactions. Indeed, plant polysaccharides represent a valuable and sustainable alternative to traditional synthetic polymers produced from monomers of fossil, non-renewable origin and are increasingly applied in a growing number of industrial fields, either in their natural or chemically/biochemically modified forms. Enzyme modifications of polysaccharides is still an open field of investigation and product development. However, while glycohydrolases (e.g., amylases, cellulases, mannanases, etc.) are currently widely used enzymes for depolymerization, the family of redox enzymes (e-g., dehydrogenases and oxidases) have so far been relatively less studied and applied to generate "functional" polymers and new biomaterials (see also Karaki, Aljawish, Hueau, Muniglia & Jasniewski, 2016). Galactomannans are employed to produce a considerable range of derivatives with numerous applications in food, feed and industrial fields. These are versatile and quite reactive polysaccharides, that, from an agronomic point of view, offer the advantage of being reserve polysaccharides of leguminous plants grown in sub-tropical and semi-arid areas of the world, requiring little or no fertilizing and limited artificial watering, compared to starch-containing cereals. In the present work, we have applied to three different GM the laccase-mediator oxidation system previously described (Lavazza et al, 2011), followed by lyophilization, and have shown that oxidation is a key factor in the generation of aerogels with enhanced overall features and performance. However, differences among the oxidized GM suggest that the chemical composition of the polysaccharides (i.e., the galactose : mannose ratio) appears to play an important role. Fenugreek exhibited the best performance in terms of mechanical properties over guar and sesbania gums, which is likely due to its highest amount of galactose along the mannose backbone (Gal : Man = 1:1).

We suggest that the findings reported might be useful in the designing of novel nanostructured biomaterials with tailored functional properties as biocompatible, flexible delivery systems of various actives (for a comprehensive review, see Ulker & Erkey, 2014), as well as for food packaging or insulating devices.

More studies are needed to establish their real potential and applicability. Few lines of investigation are ongoing to clarify various aspects of these systems, such as: freeze-drying protocols to influence pore and size formation, range of actives that can be uploaded and released, kinetics of release, role

of temperature and humidity on aerogels performance, long term activity preservation, stability in order to ensure long-term antimicrobial protection.

Acknowledgements

We wish to thank Dr. Sergio Riva for constant support, suggestions and criticism throughout this project, Dr. Lucia Galimberti for performing the MIP and Prof Alessandra Polissi for suggesting the testing and the gift of a polymyxin B sample.

The supply of GM gums from guar, sesbania and fenugreek by Lamberti SpA is gratefully acknowleged.

This project was supported by the program **"Suschem Lombardia: prodotti e processi chimici sostenibili per l'industria lombarda"**. Accordo Quadro Regione Lombardia-CNR, 16/07/2012 (protocol no. 18096/RCC), by **Cariplo Foundation** (grant 2014-0478).

673 ***References***

674

675 Alexopoulou, A., Vasilieva, L., Agiasotelli, D., Siranidi, K., Pouriki, S., Tsiriga, A., Toutousa, M. &
676 Dourakis, S. P. (2016). Extensively drug-resistant bacteria are an independent predictive factor of
677 mortality in 130 patients with spontaneous bacterial peritonitis or spontaneous bacteremia. *World*
678 *Journal of Gastroenterology, 22 (15)*, 4049.
679 Al-Homoud, M. (2005). Performance characteristics and practical applications of common building
680 thermal insulation materials. *Build. Environ., 40*, 353-366.
681 Altschul, S.F., Gish, W., Miller, W., Myers, E. W. & Lipman D.J. (1990). Basic local alignment
682 search tool. *J. Mol. Biol., 215*, 403-410.
683 Ávila, M., Gómez-Torres, N., Hernández, M., & Garde, S. (2014). Inhibitory activity of reuterin,
684 nisin, lysozyme and nitrite against vegetative cells and spores of dairy-related Clostridium species.
685 *Intl. J. Food Microb., 172*, 70-75.
686 Blomfeldt, T. O. J., Olsson, R. T. Menon, M., Plackett, D., Johansson, E. & Hedenqvist, M. S.
687 (2010). *Macromol. Mater. Eng., 295*, 796-864.
688 Borrelli, G. M. & Trono, D. (2015). Recombinant lipases and phospholipases and their use as
689 biocatalysts for industrial applications. *Int. J. Mol. Sci., 16 (9)*, 20774-20840.
690 Boziaris, I. S., & Adams, M. R. (1999). Effect of chelators and nisin produced in situ on inhibition
691 and inactivation of Gram negatives. *International Journal of Food Microbiology, 53 (2)*, 105-113.
692 Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities
693 of proteins utilizing the principle of protein-dye binding. *Analitycal Biochemistry, 72*, 248-254.
694 Brasca, M., Morandi, S., Silvetti, T., Rosi, V., Cattaneo, S., & Pellegrino, L. (2013). Different
695 analytical approaches in assessing antibacterial activity and the purity of commercial lysozyme
696 preparations for dairy application. *Molecules, 18*, 6008-6020.
697 Cheng, Y., Prud'homme, R. K., Chick, J., & Rau, D. C. (2002). Measurement of Forces between
698 Galactomannan Polymer Chains: Effect of Hydrogen Bonding. *Macromol., 35*, 10155-10161.
699 Commission Directive 2010/69/EU of 22 October 2010. (2010). *Official Journal of the European*
700 *Union*, 1-31.
701 Commission Regulation (EU) No 1129/2011 of 12 November 2011. (2011). *Official Journal of the*
702 *European Union*, 1-177.
703 Crescenzi, V., Dentini, M., Risica, D., Spadoni, S., Skjak-Braek, G., Capitani, D., Mannina, L., &
704 Viel, S. (2004). C(6)-Oxidation Followed by C(5)- Epimerization of Guar Gum Studied by High
705 Field NMR. *Biomacromol., 5*, 537-546.

706 Daas, P. J. H., Schols, H. A., & de Jongh, H. H. J. (2000). On the galactosyl distribution of
707 commercial galactomannans. *Carboydr. Res., 329*, 609-619.
708 Daas, P. J. H., Grolle, K., van Vliet, T., Schols, H. A., & de Jongh, H. H. J. (2002). Toward the
709 Recognition of Structure-Function Relationships in Galactomannans. *J. Agric. Food Chem., 50*,
710 4282-4289.
711 Daniel, J. R., Whistler, R. L., Voragen, A. G. J., & Pilnik, W. (1994). Starch and other
712 Polysaccharides. In B. Elvers, S. Hawkins & W. Russey, *Ulmann's Encyclopedia of Industrial*
713 *Chemistry, Vol. A25* (pp. 1-62), Weinheim, Germany: VCH.
714 de Arauz, L. J., Jozala, A. F., Mazzola, P. G., & Penna, T. C. V. (2009). Nisin biotechnological
715 production and application: a review. *Trends in Food Science & Technology, 20 (3),* 146-154.
716 Deszczynski, M., Kasapis, S., & Mitchell, J. R. (2003). Rheological investigation of the structural
717 properties and aging effects in the agarose/co-solute mixture. *Carbohydrate Polymers, 53*, 85-93.
718 European Food Safety Authority (EFSA) (2006). Opinion of the Scientific Panel on food additives,
719 flavourings, processing aids and materials in contact with food (AFC) related to the safety in use of
720 nisin as a food additive in an additional category of liquid eggs. *EFSA Journal, 4 (12),* 1-8.
721 Falagas, M. E., Kasiakou, S. K., & Saravolatz, L. D. (2005). Colistin: the revival of polymyxins for
722 the management of multidrug-resistant gram-negative bacterial infections. *Clinical Infectious*
723 *Diseases, 40 (9),* 1333-1341.
724 Fang, T. J., & Tsai, H. C. (2003). Growth patterns of *Escherichia coli* O157: H7 in ground beef
725 treated with nisin, chelators, organic acids and their combinations immobilized in calcium alginate
726 gels. *Food Microbiology, 20 (2),* 243-253.
727 Fu, J., Su, J., Wang, P., Yu, Y., Wang, Q. & Cavaco-Paulo, A. (2015). Enzymatic processing of
728 protein-based fibers. *Appl. Microbiol. Biotechnol.,99 (24),* 10387-10397.
729 Ghafar, A., Parikka, K., Sontag-Strohm, T., Osterberg, M., Tenkanen, M., & Mikkonen, K. S.
730 (2015). Strengthening effect of nanofibrillated cellulose is dependent on enzymatically oxidized
731 polysaccharide gel matrices. *European Polymer J., 71*, 171–184.
732 Gupta, R., Beg, Q. K. & Lorenz, P. (2002). Bacterial alkaline proteases: molecular approaches and
733 industrial applications. *Appl. Microbiol. Biotechnol., 59 (1),* 15-32.
734 Jaeger K. E. & Eggert T. (2002). Lipases for biotechnology. *Curr. Opin. Biotechnol., 13 (4),* 390-
735 397.
736 Jiang, F. & Hsieh, Y. L. (2014). Super water absorbing and shape memory nanocellulose aerogels
737 from TEMPO-oxidized cellulose nanofibrils via cyclic freezing - thawing. *J. Mater. Chem. A, 2*,
738 350-359.

739 Joshi, M. K., Pant, H. R., Tiwari, A. P., Kim, H. J., Park, C. H. & Kim, C. S. (2015). Multi-layered
740 macroporous three-dimensional nanofibrous scaffold via a novel gas foaming technique. *Chem.*
741 *Eng. J., 275*, 79-88.
742 Karaki, N., Aljawish, A., Humeau, C., Muniglia, L. & Jasniewski, J. (2016). Enzymatic
743 modification of polysaccharides: Mechanisms, properties, and potential applications: A review.
744 *Enzyme and Microbial Technol. 90*, 1-18.
745 Karn, S. K. & Kumar, A. (2015). Hydrolytic enzyme protease in sludge: recovery and its
746 application. *Biotechnology and Bioprocess Engineering, 20 (4)*, 652-661.
747 Kim, S. H. & Chu, C. C. (2000). Pore structure analysis of swollen dextran-methacrylate hydrogels
748 by SEM and mercury intrusion porosimetry. *J. Biomed. Mater. Res., 53*, 258-266.
749 Kim, Y. J., Oh, D. H., Song, B. R., Heo, E. J., Lim, J. S., Moon, J. S., Park, H. J., Wee, S. H. & Sung,
750 K. (2015). Molecular characterization, antibiotic resistance, and virulence factors of methicillin-
751 resistant *Staphylococcus aureus* strains isolated from imported and domestic meat in Korea.
752 *Foodborne Pathogens and Disease, 12 (5)*, 390-398.
753 Lavazza, M., Formantici, C., Langella, V., Monti, D., Pfeiffer, U., & Galante, Y. M. (2011).
754 Oxidation of galactomannan by laccase plus TEMPO yields an elastic gel. *J. Biotechnol. 156*, 108-
755 116.
756 Li, J.; Rayner, C. R. & Nation, R. L. (2005). Colistin-associated acute renal failure: revisited. *South.*
757 *Med. J., 98*, 1229-1230.
758 Lin, R. Li, A., Lu, L. & Cao, Y. (2015). Preparation of bulk sodium carboxymethyl cellulose
759 aerogels with tunable morphology. *Carbohydrate Polymers, 118*, 126-132
760 Lin, Y., Chen, Z. & Liu, X.Y. (2016). Using inorganic nanomaterials to endow biocatalytic systems
761 with unique features. *Trends in Biotechnol., 34 (4)*, 303-315.
762 Liyanage, S., Abidia, N., Auldb, D., & Moussa, H. (2015). Chemical and physical characterization
763 of galactomannan extracted from guar cultivars (*Cyamopsis tetragonolobus L.*). *Industr. Crops &*
764 *Products, 74*, 388–396.
765 Mathur, M. K. (2011). *Industrial Galactomannan polysaccharides*. Boca Raton, Florida: CRC
766 Press.
767 Majda, D., Zimowska, M., Tarach, K., Góra-Marek, K., Napruszewska, B. D. & Michalik-Zym, A.
768 (2016). Water thermoporosimetry as a tool of characterization of the textural parameters of
769 mesoporous materials. *J. Therm. Anal. Calorim., in press*. DOI: 10.1007/s10973-016-5400-3.
770 McCabe K. M., Zhang Y. H., Khan G., Mason E. O. & McCabe E. R. B. (1995). Amplification of
771 bacterial DNA using highly conserved sequences: automated analysis and potential for molecular
772 triage of sepsis. *Pediatrics, 95*, 165-169.

McCleary, B. V., Clark, A. H., Dea, I. C. M. &, Rees, D. A. (1985). The fine structures of carob and guar galactomannans. *Carbohydr. Res., 139*, 237–260.

Meghwal, M., Goswami, T. K. (2012). A review on the functional properties, nutritional content, medicinal utilization and potential application of fenugreek. *J Food Process. Technol., 3*, 181.

Merlini, L., Boccia, A. C., Mendichi, R., & Galante, Y. M. (2015). Enzymatic and chemical oxidation of polygalactomannas from the seeds of a few species of leguminous plants and characterization of the oxidized products. *J. Biotechnol., 198*, 31-43.

Murthy, P. S., Moorthy, R., Prabhu, K. M., & Puri D. (2010). Anti-diabetic and cholesterol lowering preparation from fenugreek seeds. Patent US7815946 B1.

Niku-Paavola, M. L., Karhunen, E., Salola, P., & Raunio, V. (1988). Ligninolytic enzymes of the white-rot fungus *Phlebiaradiata*. *Biochem. J., 254*, 877-884.

Payne, D. J., Gwynn, M. N., Holmes, D. J. & Pompliano, D. L. (2007). Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nat. Rev. Drug Discovery, 6*, 29-40.

Prajapati, V. D., Jani, G. K., Moradiya, N. G., Randeria, N. P., Nagar, B. J., Naikwadi, N. N., & Variya, B. C. (2013). Galactomannan: A versatile biodegradable seed polysaccharide. *Intl. J. Biol. Macromol., 60*, 83- 92.

Reetz M. T (2002). Lipases as practical biocatalysts. *Curr. Opin. Biotechnol., 6 (2)*, 145-150.

Rossi, B., Campia, P., Merlini, L., Brasca, M., Pastori, N., Farris, S., Melone, L., Punta., C. & Galante, Y. M. (2016). An aerogel obtained from a chemo-enzymatically oxidized fenugreek galactomannans as a versatile delivery system. *Carbohydr. Polym., 144*, 353–361.

Sahl, H. G., Jack, R. W., & Bierbaum, G. (1995). Biosynthesis and biological activities of lantibiotics with unique post-translational modifications. *European J. Biochem., 230*, 827-853.

Sajben E., Manczingera L., Nagyb A., Kredicsa L. & Vágvölgyia C. (2011). Characterization of *Pseudomonas* isolated from decaying sporocarps of oyster mushroom. *Microbiol. Res., 166*, 255-267.

Santambrogio, C., Sasso, F., Natalello, A., Brocca, S., Grandori, R., Doglia, S. M. & Lotti, M. (2013). Effects of methanol on a methanol-tolerant bacterial lipase. *Appl. Microbiol. Biotechnol, 97 (19)*, 8609-8618.

Santos, S. M., Carbajo, J. M., Quintana, E., Ibarra, D., Gomez, N., Ladero, M., Eugenio, M. E. & Villar, J. C. (2015). Characterization of purified bacterial cellulose focused on its use on paper restoration. *Carbohydrate Polymers, 116*, 173-181.

Silvetti, T., Brasca, M., Lodi, R., Vanoni, L., Chiolerio, F., de Groot, M. & Bravi, A. (2010). Effects of lysozyme on the microbiological stability and organoleptic properties of unpasteurized beer. *J. Inst. Brewing, 116*, 33-40.

807 Sittikijyothin, W., Torres, D., & Gonçalves, M. P. (2005), Modelling the rheological behaviour of
808 galactomannan aqueous solutions. *Carbohydrate Polymers, 59,* 339-350.
809 Souza, P. M., Assis Bittencourt, M. L., Caprara, C. C., Freitas, M., Almeida, R. P. C., Silveira, D.,
810 Fonseca, Y. M., Filho, E. X. F., Pessoa Junior, A & Magalhães, P.O. (2015). A biotechnology
811 perspective of fungal proteases. *Brazilian J. Microb., 46 (2),* 337-346.
812 Steinbach, C. L., Töpper, C., Adam, T., & Kees, M. G. (2015). Spectrum adequacy of antibiotic
813 regimens for secondary peritonitis: a retrospective analysis in intermediate and intensive care unit
814 patients. *Annals of Clinical Microb. & Antimicrobials, 14 (1),* 1.
815 Svagan, A. J., Samir, M. A. S. A. & Berglund L. A. (2008). Biomimetic foams of high mechanical
816 performance based on nanostructured cell walls reinforced by native cellulose nanofibrils. *Adv.*
817 *Mater., 20,* 1263-1269.
818 Takahashi, H., Kuramoto, S., Miya, S., Koiso, H., Kuda, T., & Kimura, B. (2011). Use of
819 commercially available antimicrobial compounds for prevention of *Listeria monocytogenes* growth
820 in ready-to-eat minced tuna and salmon roe during shelf life. *J. Food Protection, 74(6*), 994-998.
821 Talbot G. H., Bradley, J., Edwards, J. E., Gilbert, D., Scheld, M. & BartletT, J. G. (2006). Bad bugs
822 need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force
823 of the Infectious Diseases Society of America. *Clin. Infect. Dis., 42,* 657-668.
824 Tong, Z., Zhang, Y., Ling, J., Ma, J., Huang, L., & Zhang, L. (2014). An in vitro study on the
825 effects of nisin on the antibacterial activities of 18 antibiotics against *Enterococcus faecalis*. *PloS*
826 *one, 9(2).*
827 Ulker, Z. & Erkey, C. (2014) An emerging platform for drug delivery: Aerogel based systems. *J.*
828 *Controlled Release, 177*, 51-63.
829 Vaara, M. (1992). Agents that increase the permeability of the outer membrane. *Microbiological*
830 *reviews, 56 (3),* 395-411.
831 Vaghari, H., Jafarizadeh-Malmiri, H., Mohammadlou, M., Berenjian, A., Anarjan, N., Jafari, N. &
832 Nasiri, S. (2016). Application of magnetic nanoparticles in smart enzyme immobilization.
833 *Biotechnol. Lett., 38 (2),* 223-233.
834 Velkov, T., Thompson, P. E., Nation, R. L., & Li, J. (2009). Structure− activity relationships of
835 polymyxin antibiotics. *Journal of Medicinal Chemistry, 53 (5),* 1898-1916.
836 Verma, M.L., Puri, M. & Barrow, C.J. (2016). Recent trends in nanomaterials immobilised enzymes
837 for biofuel production. *Crit. Rev. Biotechnol., 36 (1),* 108-119.
838 Washburn, E. W. (1921). Note on a method of determining the distribution of pore sizes in a porous
839 material. *Proc. Natl. Acad. Sci.,* 7, 115-116.

Webb, P. A. & Orr, C. (1997). *Analytical methods in fine particle technology*. Norcross: Micromeritics Publishers.

Wei, Y., Lin, Y., Xie, R., Xu, Y., Yao, J., & Zhang, J. (2015). The flow behavior, thixotropy and dynamical viscoelasticity of fenugreek gum. *J. Food Eng., 166*, 21-28.

Wicklein, B., Kocjan, A., Salazar-Alvarez, G., Carosio, F., Camino, G., Antonietti, M. & Bergström, L. (2015). Thermally insulating and fire-retardant lightweight anisotropic foams based on nanocellulose and graphene oxide. *Nat. Nanotechnol., 10*, 277-283.

Zavascki, A. P., Goldani, L. Z., Li, J., & Nation, R. L. (2007). Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. *J. Antimicrobial Chemotherapy, 60 (6)*, 1206-1215.

Table(s)

Table 1 - MIP parameters of the three GM aerogels

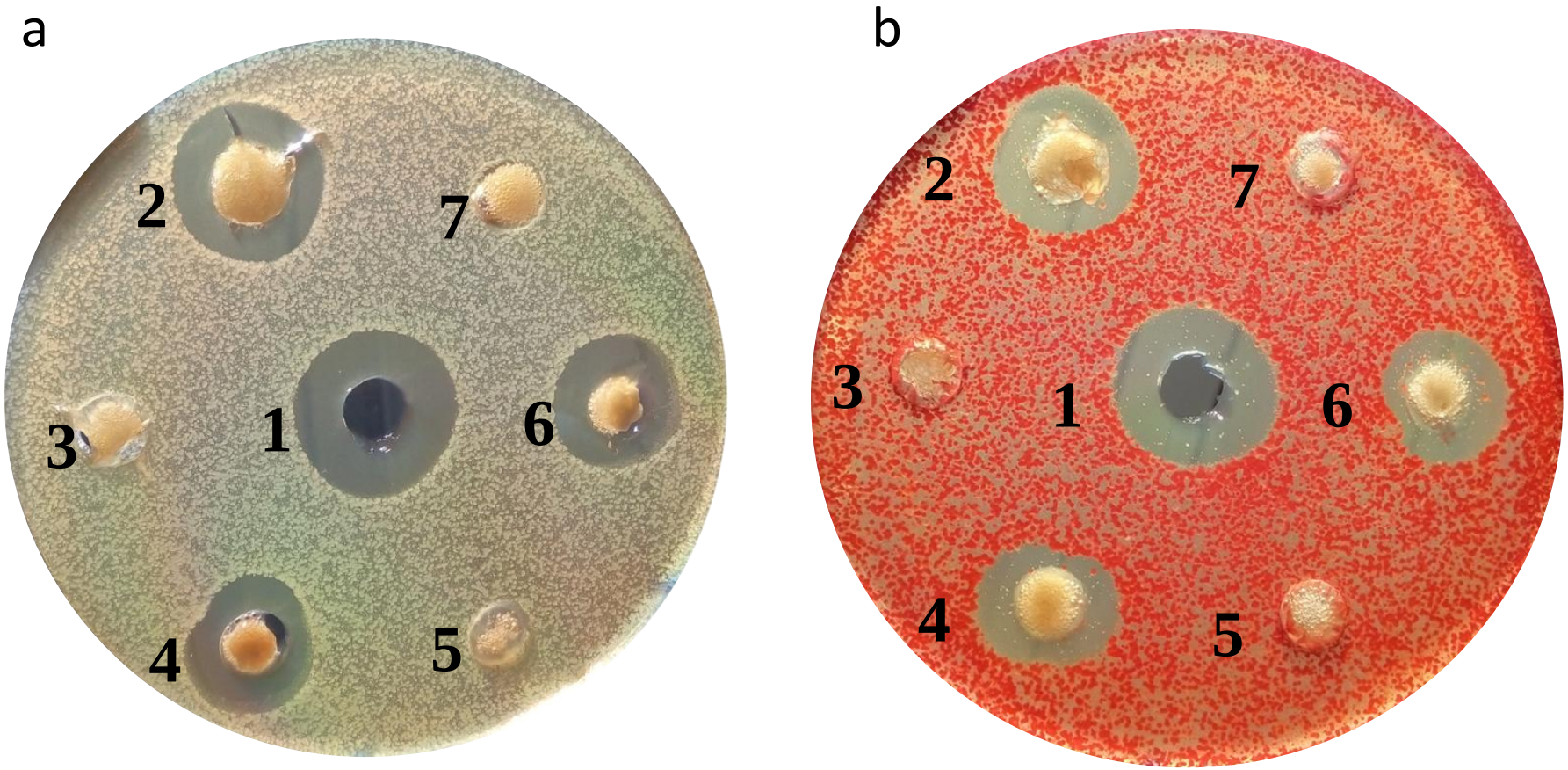
Sample	Bulk density ($kg \times m^{-3}$)	Porosity (%)	Pore surface area ($m^2 \times g^{-1}$)	Average pore diameter (µm)	Modal pore diameter (µm)
EOLFG	35.9	99.91	11.32	9.83	42.44
EOLSG	32.8	96.19	16.89	6.94	3.59
EOLGG	31.3	93.28	15.54	7.68	102.71

Table 2 - Mechanical parameters from compressive tests on pristine, lyophilized and laccase-oxidized, lyophilized fenugreek (LFG, EOLFG), sesbania (LSG, EOLSG) and guar gum (GG, EOLGG) aerogels.

Sample	E-mod (kPa)	F_{max} (N)	Cohesiveness	Springiness	Resilience
LFG	0.502 (± 0.085)^{a}	0.081 (± 0.014)^{a}	0.713 (± 0.040)^{a}	0.651 (± 0.042)^{ab}	0.393 (± 0.033)^{ab}
EOLFG	22.775 (± 5.792)^{b}	2.376 (± 0.548)^{b}	0.685 (± 0.034)^{a}	0.611 (± 0.038)^{a}	0.316 (± 0.037)^{c}
LSG	0.249 (± 0.083)^{a}	0.039 (± 0.012)^{a}	0.728 (± 0.033)^{a}	0.665 (± 0.033)^{b}	0.434 (± 0.034)^{b}
EOLSG	12.479 (± 5.165)^{c}	1.332 (± 0.231)^{c}	0.712 (± 0.057)^{a}	0.641 (± 0.063)^{ab}	0.373 (± 0.066)^{a}
LGG	0.433 (± 0.070)^{a}	0.081 (± 0.023)^{a}	0.694 (± 0.043)^{a}	0.632 (± 0.049)^{ab}	0.386 (± 0.048)^{a}
EOLGG	18.577 (± 5.527)^{d}	2.075 (± 0.607)^{b}	0.627 (± 0.049)^{b}	0.542 (± 0.047)^{c}	0.257 (± 0.032)^{d}

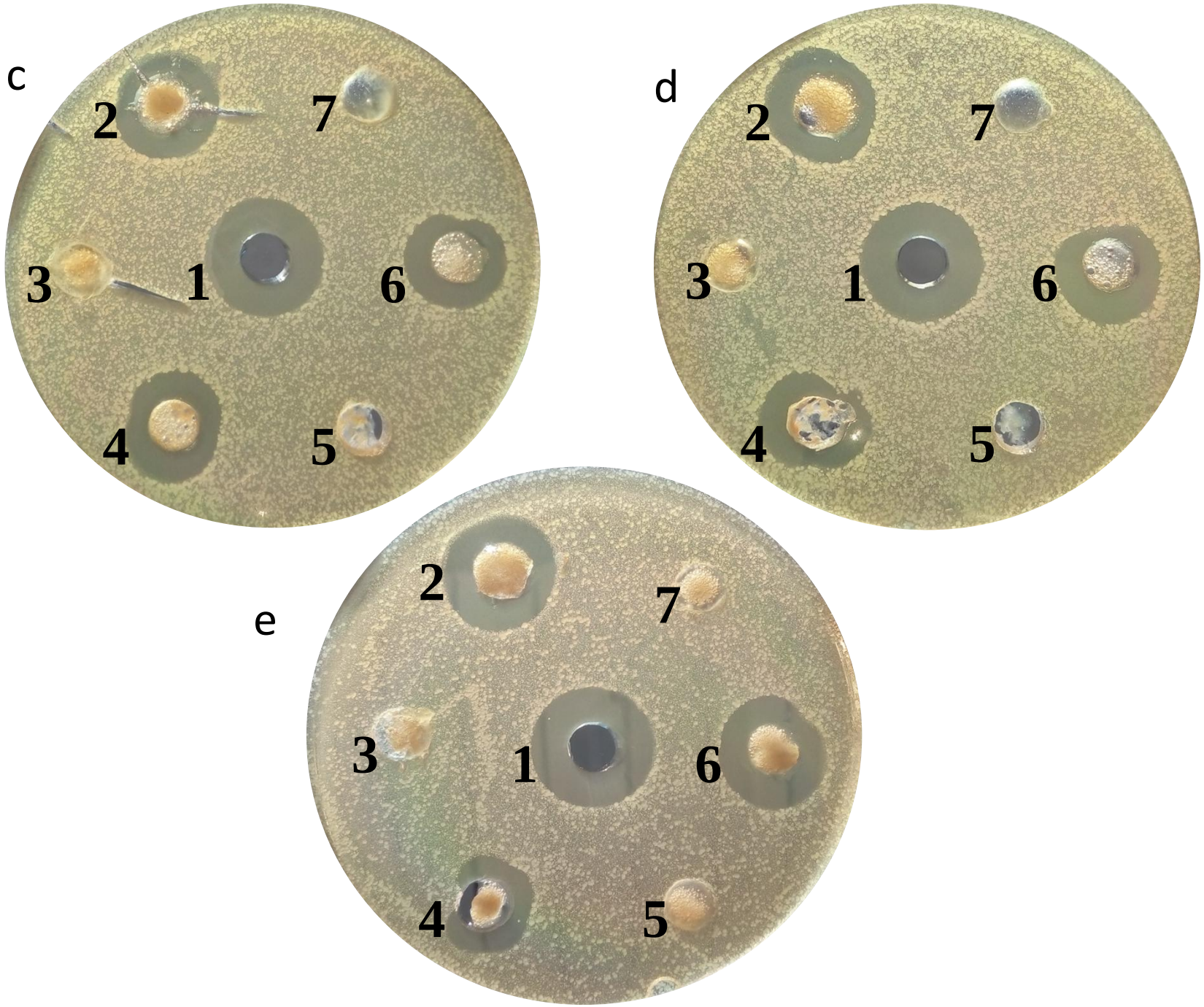
Figure(s)

Fig. 1-a,b



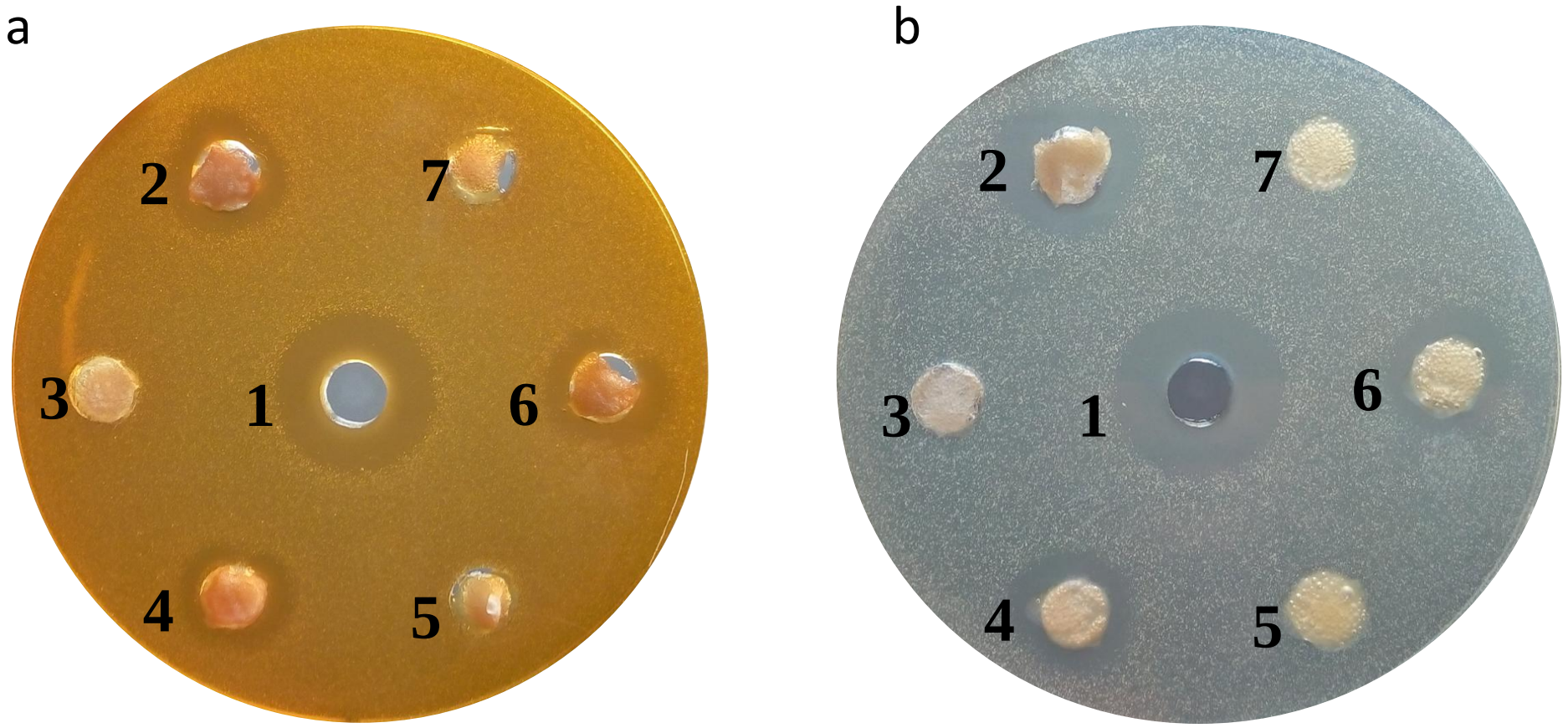
Figure(s)

Fig. 1-c,d,e



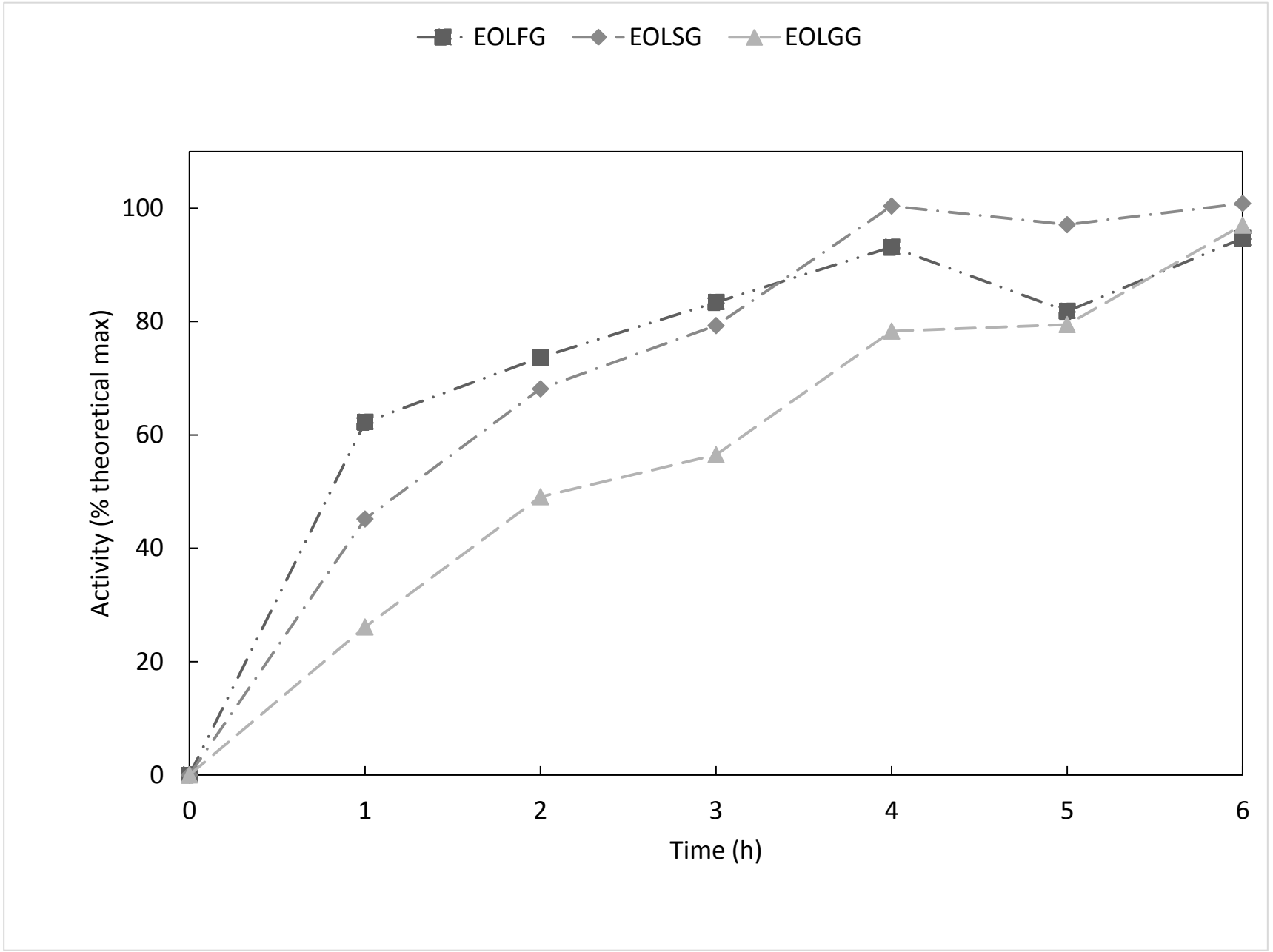
Figure(s)

Fig. 2-a,b



Figure(s)

Fig. 3



Figure(s)

Figure 4

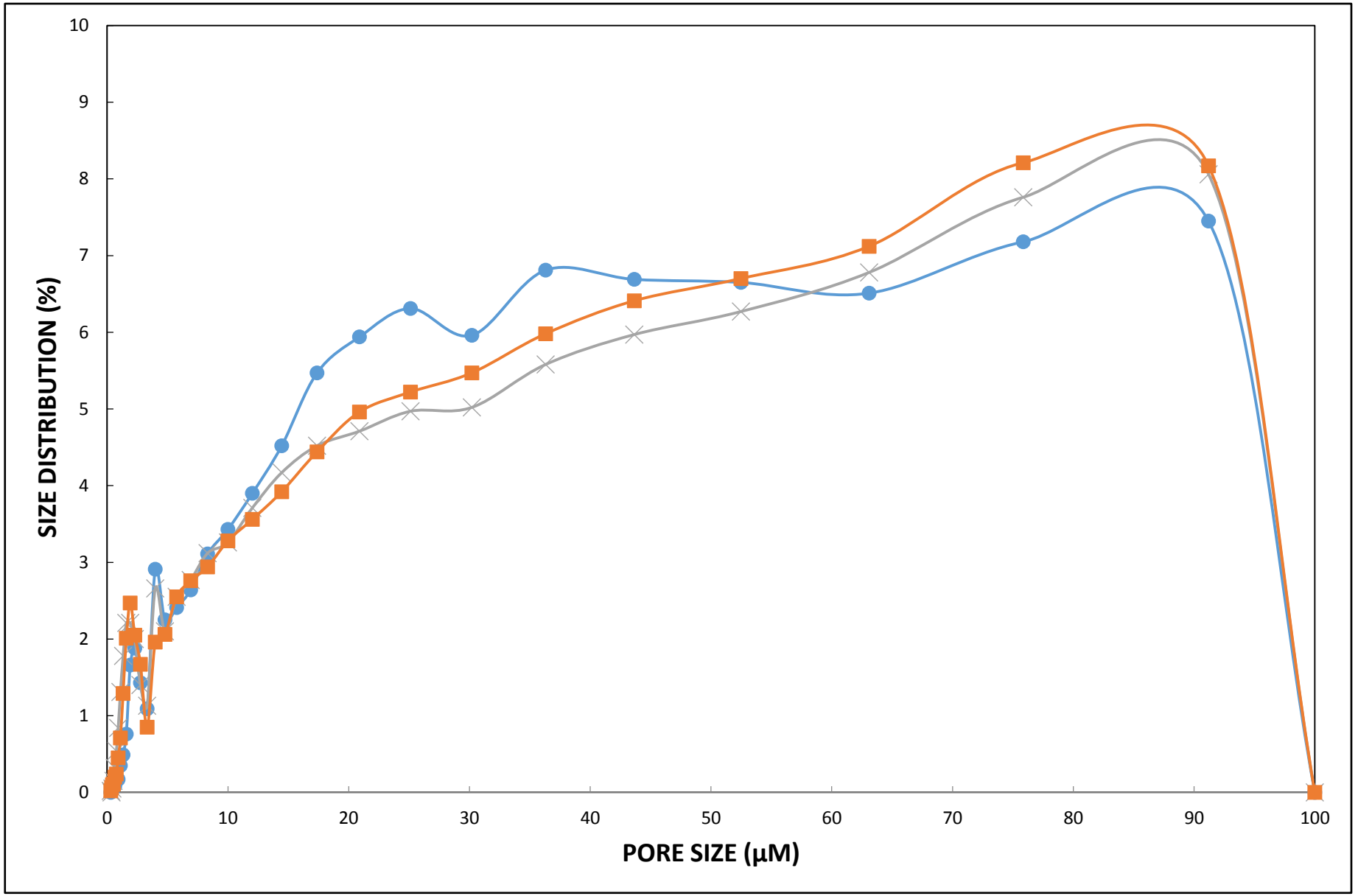


Figure legends

Fig. 1- Inhibition by polymyxin B released from aerogels of Gram-negative bacterial cell growth. Central well (1), 30 µg (equivalent to 22 nanomoles) of free polymyxin B. Lateral wells: plugs of loaded EOLFG (2), control EOLFG (3); loaded EOLSG (4), control EOLSG (5); loaded EOLGG (6), control EOLGG (7). Fig. 1a: *P. aeruginosa*. Fig. 1b: *S. marcescens*. Fig. 1c: *S.* Typhimurium. Fig. 1d: *E. coli*. Fig. 1e: *E. cloacae.*

Fig. 2 - Inhibition by nisin released from aerogels of Gram-positive bacterial cell growth. Central well (1) 125 µg of free nisin (equivalent to 37 nanomoles). Lateral wells: plugs of loaded EOLFG (2), control EOLFG (3); loaded EOLSG (4), control EOLSG (5); loaded EOLGG (6), control EOLGG(7). Fig. 2a: *E. faecalis*. Fig. 2b: *C. tyrobutirycum.*

Fig. 3 - Time course of protease release in water solution from "loaded" hydrogel plugs of EOLFG, EOLSG and EOLGG. Each time point was performed in triplicate on separately prepared plugs and is expressed as the average percentage of the "theoretical" total protease activity loaded.

Fig. 4- Pore size distribution in diameter (abscissa) *vs* their % distribution (ordinate) of the three GM aerogels (blue curve (●), EOLFG; green curve (x), EOLSG; red curve (■), EOLGG.

Fig. 1.S

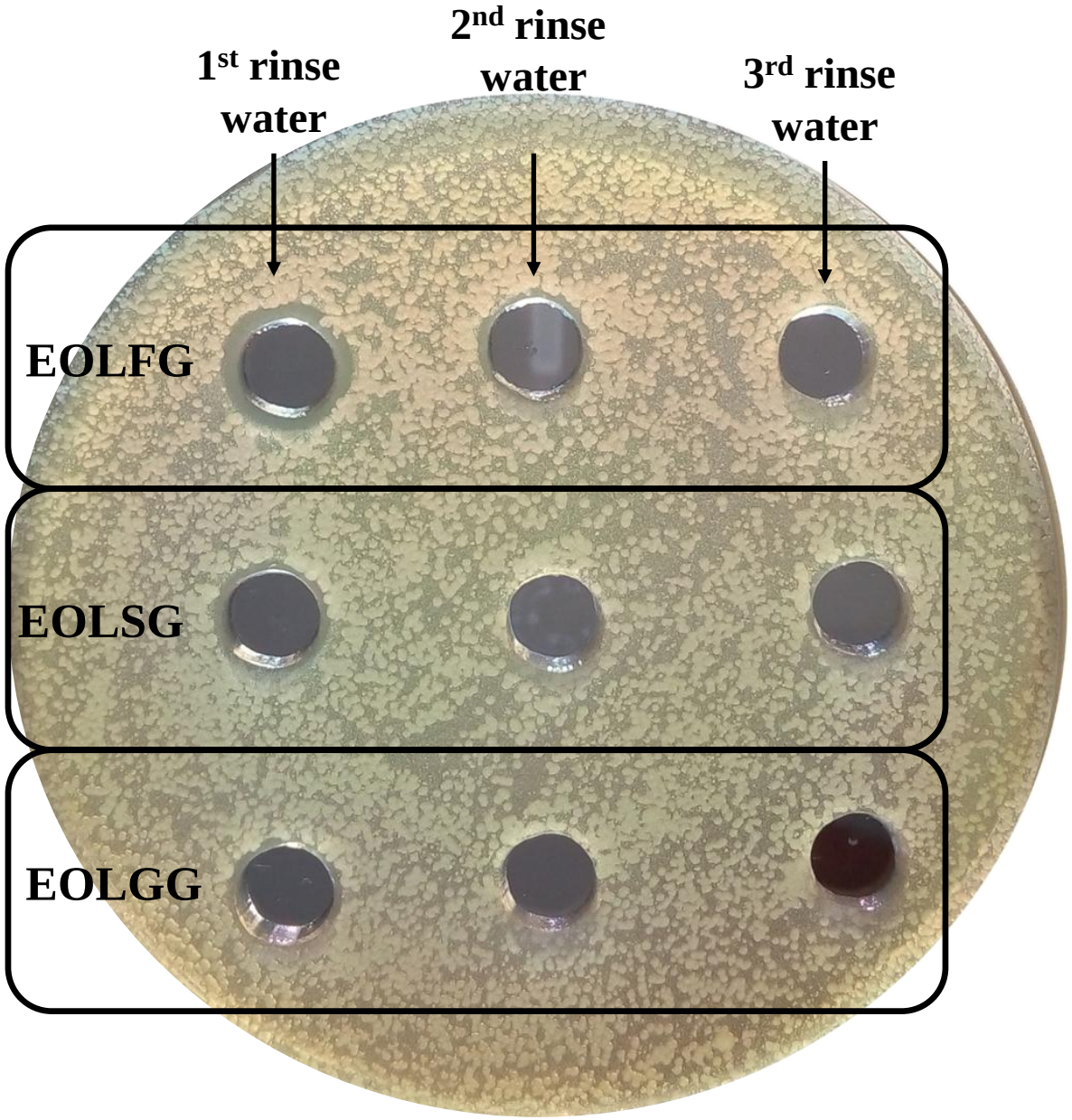

1st rinse water
2nd rinse water
3rd rinse water
EOLFG
EOLSG
EOLGG

Fig. 2.S

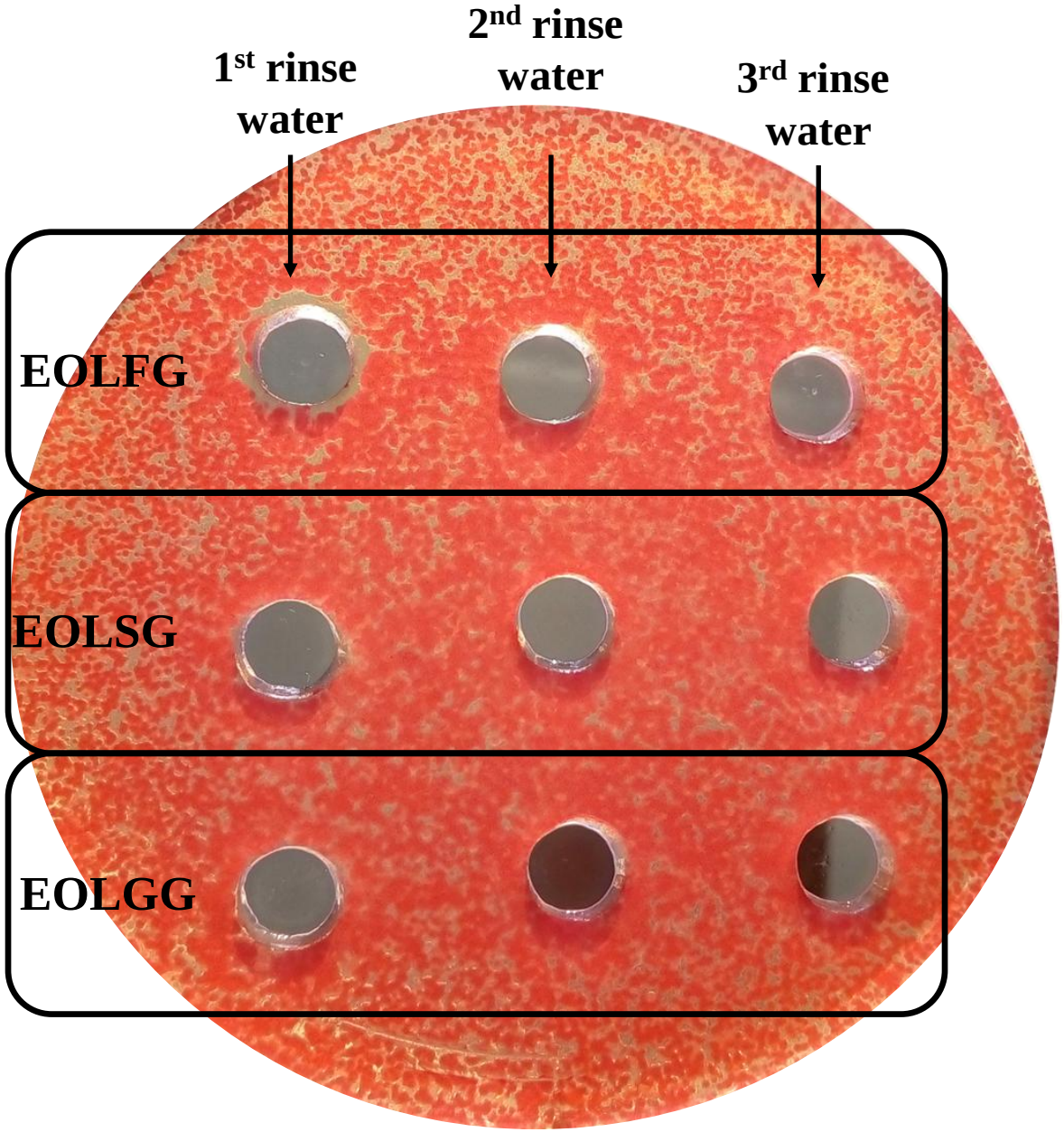

1st rinse water
2nd rinse water
3rd rinse water
EOLFG
EOLSG
EOLGG

Fig. 3.S

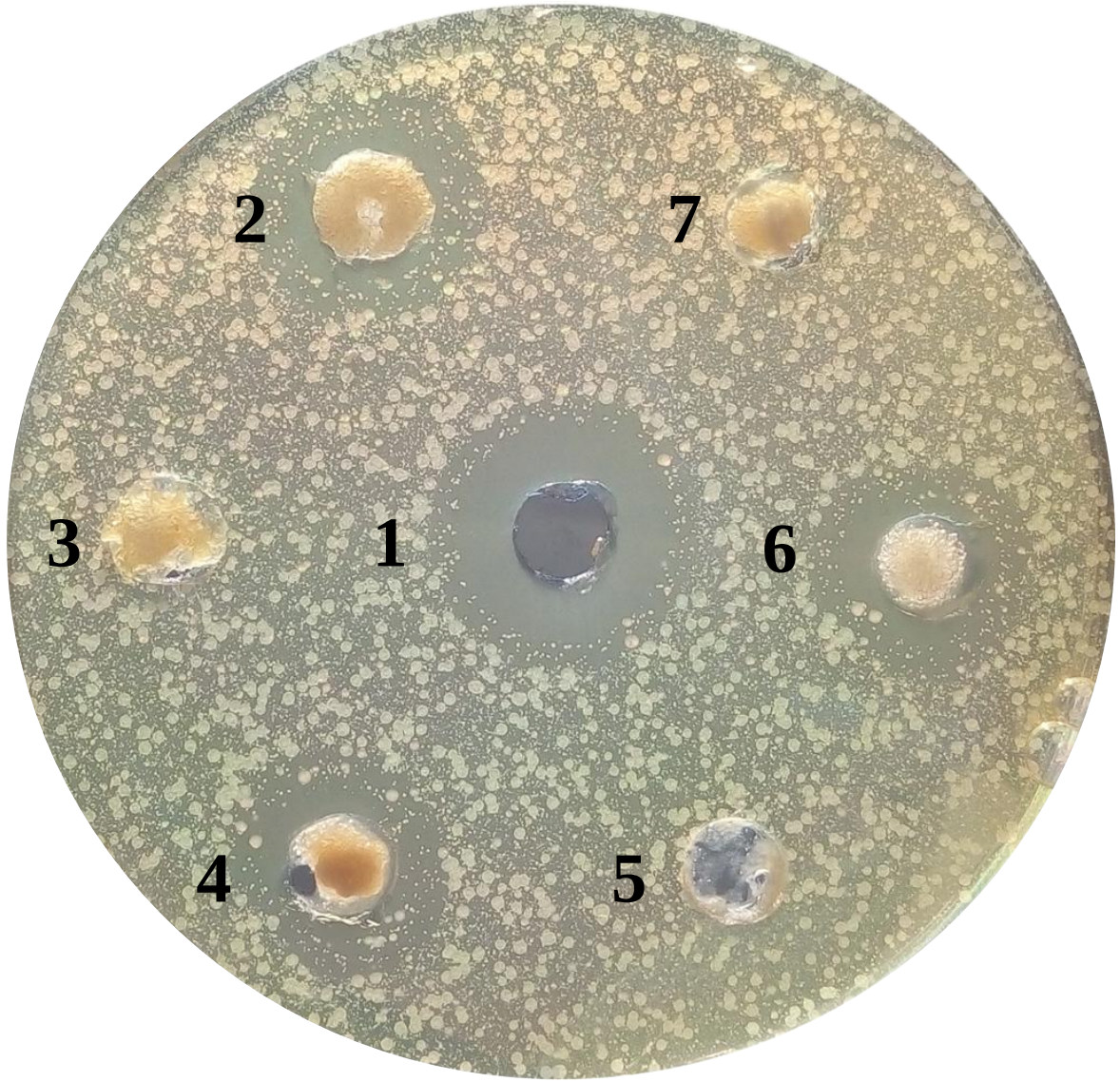


Fig. 4.S

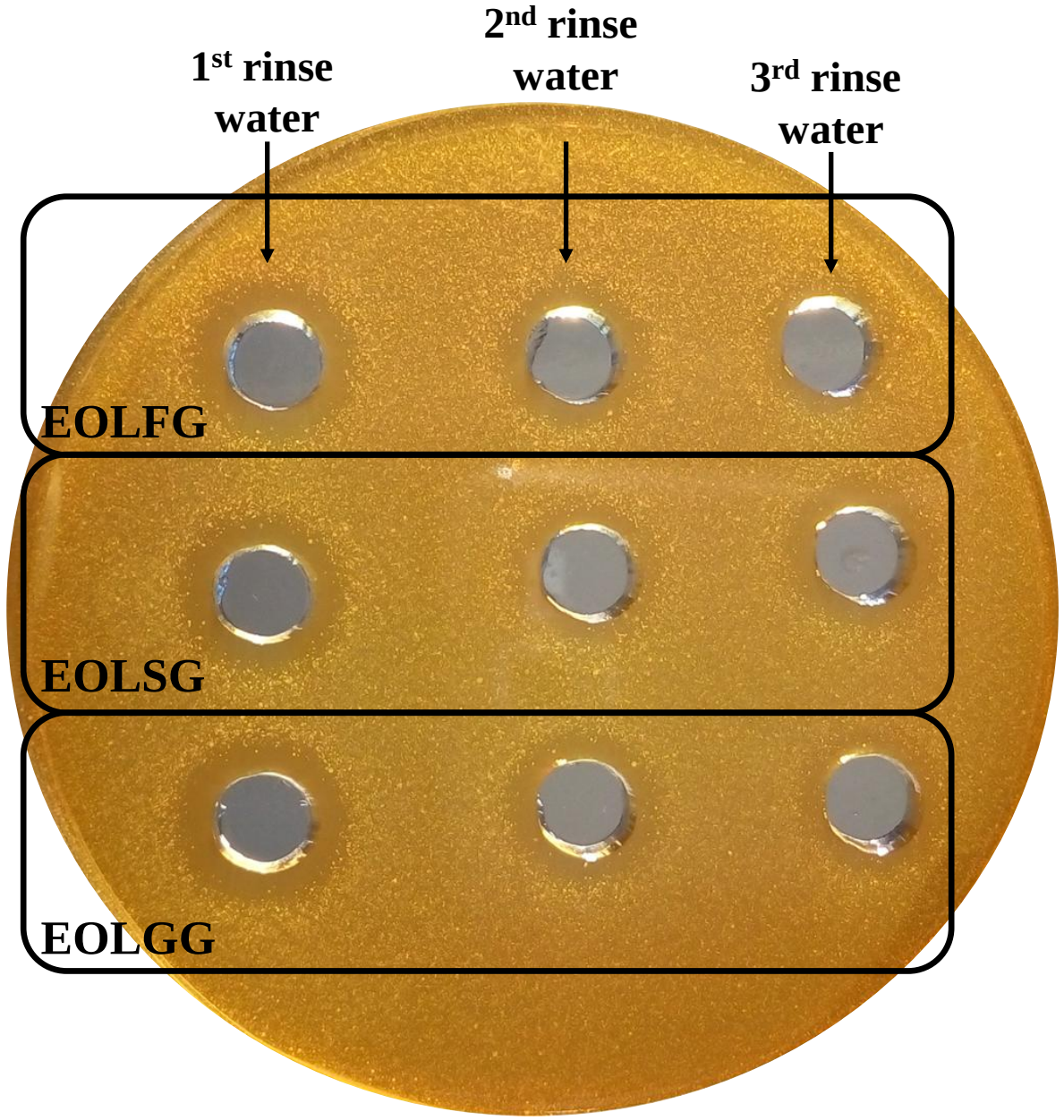

1st rinse water
2nd rinse water
3rd rinse water
EOLFG
EOLSG
EOLGG

Fig. 5.S

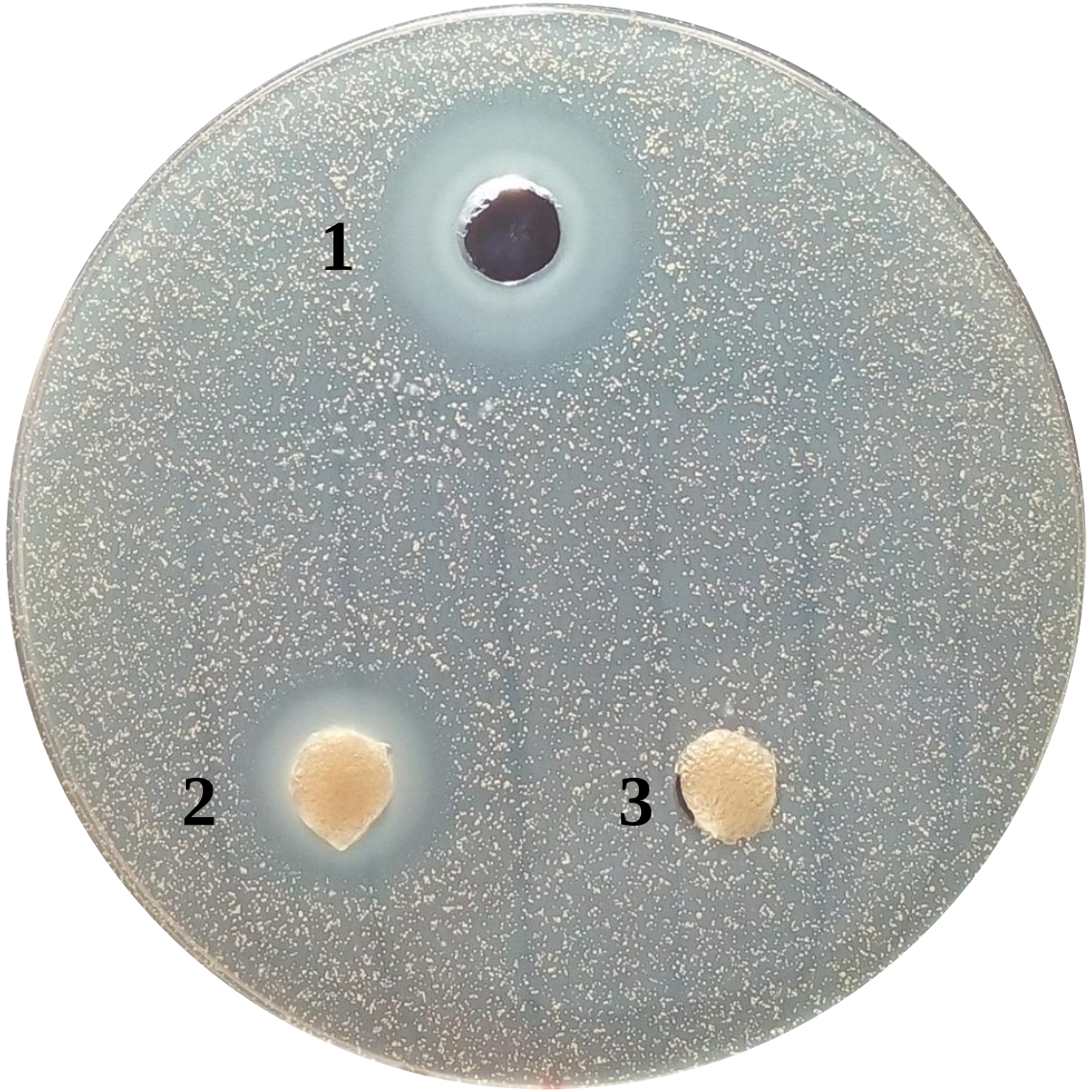


Fig. 6.S

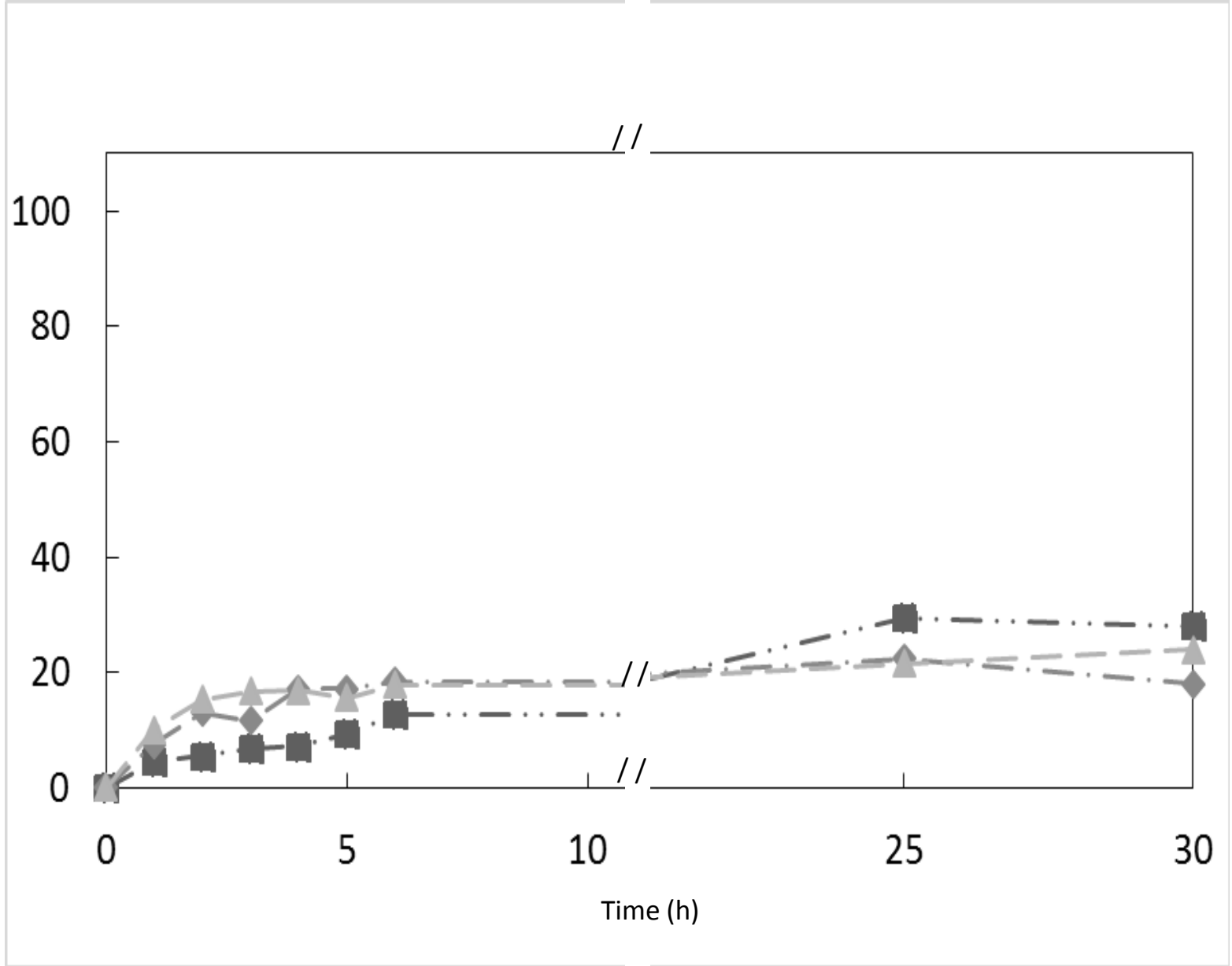
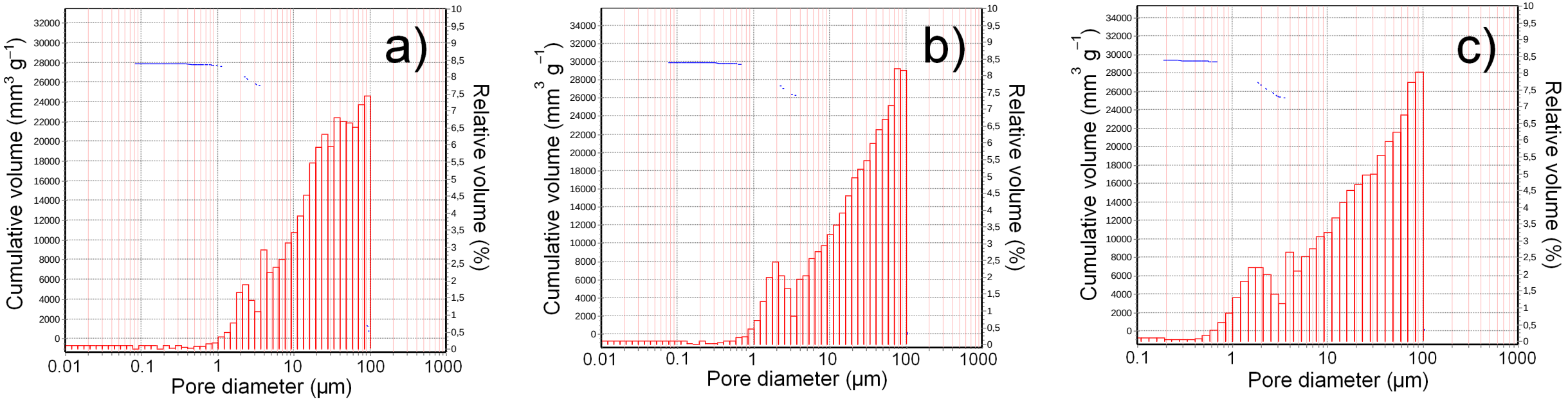


Figure 7.S


a)
b)
c)
Cumulative volume (mm^3 g^{-1})
Relative volume (%)
Pore diameter (µm)
0.01
0.1
1
10
100
1000

SUPPLEMENTARY MATERIALS

Legends to Figures

Fig. 1.S - Rinse water of EOLFG, EOLSG, EOLGG plugs loaded with polymyxin B (from Fig. 1.a) and checked for cell growth inhibition of *P. aeruginosa*. 100 ult of (from left to right): 1st, 2nd, 3rd rinse water were added to each well.

Fig. 2.S - Rinse water of EOLFG, EOLSG, EOLGG plugs loaded with polymyxin B (from Fig. 1.b) and checked for cell growth inhibition of *S. marcescens*. 100 ult of (from left to right): 1st, 2nd, 3rd rinse water were added to each well.

Fig. 3.S- Inhibition by polymyxin B released from aerogels of Gram-negative *Hafnia alvei* cell growth. Central well (1), 30 µg (equivalent to 22 nanomoles) of free polymyxin B. Lateral wells: plugs of loaded EOLFG (2), control EOLFG (3); loaded EOLSG (4), control EOLSG (5); loaded EOLGG (6), control EOLGG (7).

Fig. 4.S - Rinse water of EOLFG, EOLSG, EOLGG plugs loaded with nisin (from Fig. 2.a) and checked for cell growth inhibition of *E. faecalis*. 100 ult of (from left to right): 1st, 2nd, 3rd rinse water were added to each well.

Fig. 5.S - Inhibition of *C. tyrobutirycum* growth by lysozyme released from EOLFG aerogel: (1) 125 µg (equivalent to 8.7 nanomoles) of free lysozyme. Plugs of: (2) loaded EOLFG, (3) control EOLFG.

Fig. 6.S - Time course of commercial *C. lipolitica* rrelease in water solution from "loaded" hydrogel plugs of EOLFG, EOLSG and EOLGG. Each time point was performed in triplicate on separately prepared plugs and is expressed as the average percentage of the "theoretical" total lipase activity loaded.

Fig. 7.S - Pore size distribution plots of EOLFG (a), EOLSG (b), EOLGG (c)

Tab. 1.S – Time course of protease release in water from "loaded" hydrogel plugs of EOLFG, EOLSG and EOLGG. Experiments were performed in triplicate on independently prepared plugs.
Average activity values and percentage of the "theoretical" maximum activity loaded into the aerogels are reported for each time point, with standard deviations. Protease activity is expressed as Δ O.D. at 440 nm/10 min/ml.

	EOLFG		EOLSG		EOLGG	
Time, h	Protease activity	Theoretical max, %	Protease activity	Theoretical max, %	Protease activity	Theoretical max, %
1	23.8±1.2	54.3±6.0	17.5±13.1	45.1±25.8	11.2±4.0	26.1±9.4
2	26.7±2.8	60.5±3.2	25.2±8.7	68.1±8.7	21.0±4.7	49.1±11.0
3	34.0±4.7	76.9±1.6	29.5±12.0	79.3±15.2	24.2±6.4	56.5±14.9
4	35.8±4.5	81.2±2.8	28.2±9.7	76.3±9.4	33.5±7.3	78.3±17.1
5	39.7±0.9	90.6±12.3	35.0±4.7	97.1±8.8	34.0±4.7	79.5±11.0
6	43.2±5.4	97.8±3.3	36.8±9.2	100.8±2.6	41.5±6.4	97.0±14.9

Tab. 2.S – Time course of *C. lipolitica* release in water from "loaded" hydrogel plugs of EOLFG, EOLSG and EOLGG. Experiments were performed in triplicate on independently prepared plugs.
Average activity values and percentage of the "theoretical" maximum activity loaded into the aerogels are reported for each time point, with standard deviations. Lipase activity is expressed as Δ O.D. at 410 nm/min/ml.

	EOLFG		EOLSG		EOLGG	
Time, h	Lipolytic activity	Theoretical max, %	Lipolytic activity	Theoretical max, %	Lipolytic activity	Theoretical max, %
1	0.7±0.1	4.5±2.4	1.4±0.4	7.5±1.9	2.2±1.2	10.1±5.5
2	0.8±0.4	5.5±3.1	2.4±1.8	13.0±10.0	3.3±1.8	15.3±8.6
3	1.0±0.5	6.8±4.1	2.2±0.7	11.6±4.2	3.6±1.8	16.7±7.8
4	1.1±0.6	7.3±4.7	3.2±0.8	17.2±5.6	3.6±1.6	16.9±6.5
5	1.4±0.8	9.4±5.8	3.2±1.0	17.1±6.2	3.3±0.9	15.6±4.2
6	1.9±1.0	12.8±7.8	3.4±1.3	18.5±7.6	3.8±0.9	17.9±3.3
25	4.8±0.7	29.5±8.0	4.3±0.9	22.4±2.3	4.4±0.6	21.5±4.9
30	4.3±0.1	28.8±11.6	3.4±0.7	18.0±2.0	4.9±0.7	24.0±4.7