Polysacharide-based Materials as Support for *Ideonella Sakaiensis* with Applications in Wastewater Remediation

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Abstract. The accumulation of microplastics (MPs) in surface water is a growing environmental challenge due to their persistence and the health risks they pose to both aquatic organisms and humans. The biological treatment in wastewater plants employs activated sludge microorganisms to decompose organic compounds in the effluent and plays a key role in reducing the spread of microplastics into the environment. This paper presents the preparation and characterization of different polysaccharide-based scaffolds acting as a carrier for bacteria specialized in MP degradation. The bacterial-loaded sodium alginate scaffold was prepared by the ionotropic gelation method, using calcium chloride as a cross-linker. The performance of bacterial immobilization is determined by the material properties, such as porosity and structural resistance, which are strongly influenced by the concentration of polysaccharides, crosslinking method, and the composition of the polysaccharide mixture. Bacterial cell viability was assessed using fluorescence microscopy, which indicated that alginate crosslinked in $CaCl_2$ solution is the most versatile material for this application.

1 Introduction

Plastic production has grown exponentially since the 1950s and this trend is driven mainly by the indiscriminate consumption of products with short lifespans, such as packaging, as well as by the use of essential single-use plastics such as medical devices, personal protective equipment, syringes, sterile packaging for instruments, etc. [1]. In recent years, scientists and environmental non-governmental organizations (NGOs) have

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increasingly drawn attention to the hazardous environmental effects of plastic waste, providing evidence of its negative impact on both wildlife and human health [2]. Significant stocks of plastics have accumulated in terrestrial and aquatic environments, resulting from inadequate collection systems, improper disposal practices, and insufficient recycling efforts.

Another aspect of plastic pollution refers to the presence of small fragments, less than 5 mm in size, known as "microplastics". They result from the breakdown and degradation of bigger plastic pieces, under the mechanical forces, biological or chemical process [3] or are originating from daily care products, such as shower gels, nail polish, toothpaste etc. [4]. Water collected from domestic or industrial sources and rainwater arrive in wastewater treatment plants (WWTPs) were only 60-80% of the MPs is removed [5, 6], while the rest can be found on the effluent mostly in the form of microfibres and microbeads [7]. Moreover, from the total amount of MPs removed in WWTPs, more than 65% accumulates in the sewage sludge [6], and this is problematic, especially when the sludge is further utilized as fertilizer in agriculture [8]. In WWTPs, microplastics are removed in the highest proportion by flotation, sedimentation, and screening, during primary treatments. The effectiveness of the biological treatment is consistently lower [9]; usually less than 15% of the MPs is retained from the water flux during this step, as the time is insufficient for the MPs degradation [6]. Bioremediation of wastewater using bacteria, fungi, and algae has been intensively explored to remove different pollutants from wastewater, such as heavy metals, pesticides, and organic pollutants [10]. Pressed by the urgent need to find solutions against plastic pollution, the scientific community was committed to finding reliable and scalable solutions for the bioremediation of the contaminated environment [11]. In this context, *Ideonella sakaiensis* emerged as a promising candidate for the degradation of polyethylene terephthalate (PET), a significant component of the plastic litter that ends up in landfills or aquatic ecosystems. This aerobic and gram-negative bacterial strain can produce PETase and MHETase enzymes that catalyze the hydrolysis of PET into terephthalic acid (TPA) and ethylene glycol (EG) [12].

In the biotechnological process involving the use of bacteria, the immobilization of the microorganisms on a support is often encountered in order to stimulate microbial growth and protect the microorganisms from inhibitory compounds [13]. Alginate is a polysaccharide naturally found in brown algae and the cell wall of certain bacteria, suitable for microorganism immobilization, due to the mild crosslinking reaction, which takes place at room temperature and at neutral pH. Ionic gelation of alginate is explained by the formation of new bonds between the carboxyl ($–COO^-$) and hydroxyl (OH^-) and divalent (M^{2+}) or trivalent (M^{3+}) metals. In this study, the bacterial-loaded sodium alginate scaffold was prepared by the ionotropic gelation method, using calcium chloride as a cross-linker. The poor mechanical strength of sodium alginate-based hydrogels represents a major constraint for their use in industrial applications. Chitosan is a readily accessible and cost-effective polysaccharide with excellent biodegradability, nontoxicity, biocompatibility, and antibacterial properties. The hydroxyl and amino groups ($-NH_2$) on the chitosan molecular chain can react with various reagents, leading to materials with tunable properties. The formation of a polymer network between chitosan and alginate chains can be achieved through electrostatic interactions, involving physical cross-

linking between the protonated amino group (-NH^{3+}) of chitosan and carboxylate groups (-COO^{-}) of alginate. Combining chitosan with alginate and gelatin led to materials with enhanced biological or mechanical properties, suitable for a large palette of applications, including wound dressing membranes [14], soil conditioner [15], hydrogels involving stimulus response acting as promising biomedical devices [16] or intelligent actuators [17]. Encapsulation of different active substances (such as carvacrol, *Tabacum Nicotiana* extract, etc.), essential oils, etc., in gelatin/chitosan-based composites has been recently reported [18-20]. All the above-mentioned polymers, i.e., alginate, chitosan, and gelatin, are promising candidates for developing sustainable gel networks for contaminants removal. In this paper, we report the preparation of three types of hydrogels, i.e., alginate, chitosan/alginate, and chitosan/gelatin, and the hydrogel exhibiting the most favorable physico-chemical properties was embedded with live bacteria through a simple and effective process.

2 Experimental

2.1 Materials

Sodium alginate (MW = 120.000-190.000 g/mol), chitosan medium molecular weight, calcium carbonate solution (CaCO3, Sigma-Aldrich), and gelatin from porcine skin were purchased from Sigma-Aldrich. L-glutamic acid and calcium chloride (CaCl2) were obtained from Roth and VWR Chemicals, respectively.

2.2 Methods

2.2.1. Cell immobilization in calcium alginate gels

A 1% (w/v) solution was prepared by dissolving the required quantity of sodium alginate (in water, at room temperature, under strong stirring. Similarly, the $CaCO_3$ solution 0.8 %, 0.16 % solution of L-glutamic acid, calcium chloride 0.1 M solution. All solutions were prepared using ultrapure water (obtained from a Millipore Simplicity UV system) to ensure high purity. The solutions were further autoclaved at 120°C for 15 minutes and cooled down to room temperature before mixing them with the cell suspension. The following steps of the experiments were performed in a sterile environment, in a microbiological hood. The *Ideonella sakaiensis* strain was grown at 37°C as a pure culture in nutrient broth supplemented with 1% glucose, at pH 7 (MLT, Arad, Romania) until the absorbance at 600 nm (O.D.600) reached 1.100. The cells were centrifuged at 3500 rpm for 5 min, and the cell pellet was separated from the supernatant and redispersed in culture medium to obtain the desired cell density in the inoculum, which was determined by spectrophotometry (Biowave 2, WPA, USA). Cell immobilization in the polymeric medium was performed at room temperature, at physiological pH between 7.35 - 7.45, by dropping the cell suspension into the alginate solution, under slight magnetic stirring. Subsequently, 10 mL of $CaCO_3$ solution was added, under slight stirring (400 rpm), followed by the addition of 10 mL of glutamic acid solution. The suspension became opaque and slightly viscous, indicating the beginning of the internal gelation process. The

suspension was transferred into a Petri dish containing $CaCl_2$, where it underwent ionic crosslinking to form a compact hydrogel.

2.2.2. Preparation of chitosan-alginate hydrogels

Aqueous solution of alginate, 4% (w/v), was prepared by dissolving the corresponding sodium alginate quantity in hot water (85°C) for 2 h. After chitosan of 2% (w/v) was dissolved in 1% acetic acid, the sodium alginate solution was added at a ratio of 1:1 (v/v). The mixture was mixed gently for 10 min, and poured into a $CaCl_2$ solution of 0.1 M. After 50 min, the soft hydrogel was cross-linked with glutaraldehyde 2% (v/v) and left overnight. The hydrogel was successively washed with deionized water and lyophilized for 90 h. The sample was denoted CA1.

2.2.3. Preparation of chitosan-gelatine hydrogel

The chitosan-gelatine hydrogel was prepared by using a similar method, with a few modifications. Gelatine of 4% (w/v) was first dissolved in deionized water, at 50°C, for 2 h, under continuous stirring. The gelatine solution was further mixed with chitosan 2% (w/v) solution, prepared as above, at a ratio of 2:1 (v/v) gelatine:chitosan. The mixture was gently mixed for 10 min and crosslinked with glutaraldehyde 2%. After 20 h, the hydrogel was carefully washed with deionized water and lyophilized for 90 h. The sample was denoted as CG2.

2.3 Characterization

2.3.1. Scanning Electron Microscopy (SEM)

The lyophilized hydrogels were characterized by using a Hitachi SU-70 scanning electron microscope (SEM). To enhance the sample conductivity, an AuPd conductive layer was applied by using the SC7620 Mini Sputter Coater/Glow Discharge System.

2.3.2. Infrared Spectroscopy in Attenuated Total Reflection mode (FTIR-ATR)

The FTIR spectra were recorded using a Bruker Tensor 27 equipped with a diamond crystal ATR module (Bruker Optik GmbH, Ettlingen, Germany), in the spectral range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}, and applying 32 scans. OPUS software version 7.2 (Bruker Optics, Germany) was used for spectra acquisition. The spectra were processed using Origin 8.5 software.

2.2.3. Gel cytotoxicity on the I. sakaiensis line

The stock culture of *Ideonella sakaiensis* was obtained by inoculating the *I. sakaiensis* strain (DSM 112585, Austria) in liquid nutrient medium with 1% glucose (MLT, Romania) and incubated at 37° C for 8 hours under shaking (200 rpm), then evaluated by reading the absorbance at 600 nm (O.D. 600). Gels containing 0.5 x 10^8 cells/mL of *I.*

sakaiensis were removed, rinsed in three sterile water baths and then in one bath of liquid Amies medium (Rmbio, Missoula, USA). After rinsing, gel samples were crushed between glass slides and then treated with 5 µL of SYTO 9 fluorescent dye (LIVE/DEAD BacLight, Thermofisher, USA), incubated in the dark for 15 min, and fixed with oil mounting.

The samples were examined under an inverted fluorescence microscope (Olympus IX71) with λex = 490 nm and λem = 515 nm, at 4 h and 24 h for gels incubated at 4°C and 37°C.

3 Results and discussion

1.1 FTIR characterization

The key functional groups of the obtained hydrogels were identified by analysing the vibrational patterns associated with specific polymer bonds in the FTIR spectra, and the resulted spectra are presented in Figure 1. The broad band between 3500 and 3290 cm^{-1} corresponds to the overlapping symmetric and asymmetric stretching vibrations of -OH groups present in both chitosan and alginate (Figure 1a), as well as -NH groups in chitosan [21]. In the CA1 sample, the absorption band at 2925 cm^{-1}, which belongs to the stretching vibrations of the $-CH_2$ group from alginate [22], overlaps with the band at 2873 cm^{-1}, attributed to the asymmetric stretching of C-H bonds in methyl groups ($-CH_3$) of chitosan.

This combined band shift to 2930 cm^{-1} and gets wider in the CA1 sample, due to hydrogen bonding between acetylated amines of chitosan and the -OH and -COOH groups of alginate [21, 23]. The FTIR spectra of alginate hydrogel show the characteristic peaks at 1600 cm^{-1} and 1400 cm^{-1} assigned to asymmetric and symmetric stretching vibration of the carboxylate salt ion (COO^-), and the peak at 1028 cm^{-1} belongs to the stretching vibrations of the -C-OH group of the polysaccharide structure [24]. In the chitosan spectrum, the amide bands appeared at 1650 cm^{-1} (C=O stretching of amide I), 1575 cm^{-1} (N-H bending of amide II) and 1319 cm^{-1} (C-N stretching of amide III) [23]. FTIR analysis confirmed the formation of an imine group (C=N) through covalent bonding between chitosan's amino groups and glutaraldehyde's aldehyde groups.

Gelatine exhibited characteristic absorption bands at 1627 cm^{-1}, 1530 cm^{-1}, 1447 cm^{-1}, and 1239 cm^{-1}, corresponding to the presence of amide functional groups (Figure 1b) [25, 26]. The small signals at 1333 cm^{-1} and 1028 cm^{-1} were assigned to methylene groups and the C=O bond, respectively. The peak intensity of the main gelatine's functional groups changed in the CG2 sample, which indicates the formation of new hydrogen bonding between the amide groups (-NH and -CO) in gelatine and amino ($-NH_2$) and hydroxyl (-OH) groups in chitosan. In addition, the reduction of the sharp spectral peak of around 3273 cm^{-1} indicates the involvement of –OH and –NH groups in hydrogen bonding, leading to a more compact and crosslinked network structure.

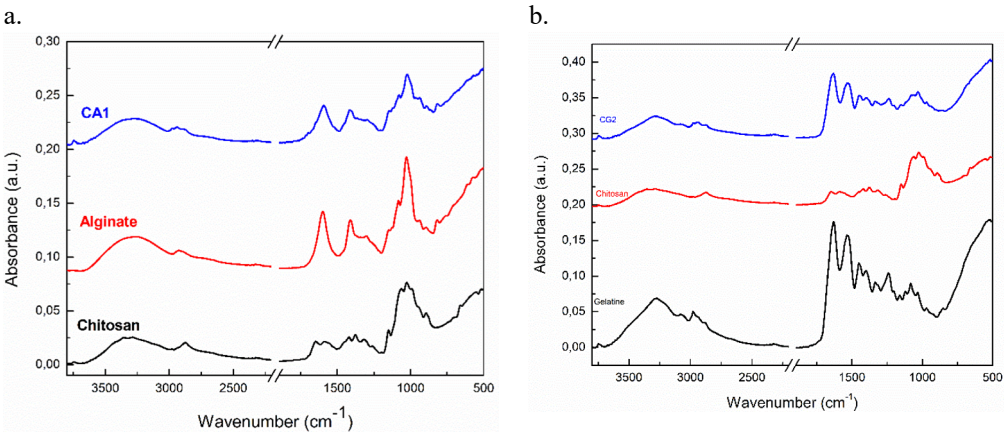

Fig. 1. FTIR spectra of alginate, CA1, and CG2 hydrogels

1.2 SEM analysis

The pore size of the hydrogels and their distribution are critical parameters that influence both physico-chemical and biological performance. SEM micrographs (Figures 2-4) reveal significant differences between the samples, resulting from the composition of the polymer solution and the crosslinking method used.

The cross-sectional area of pure alginate showed the formation of an irregular structure, comprising highly dense and smooth areas, interrupted by macroscopic cavities of different sizes (Figure 2). This can be explained by the successive steps during the crosslinking process. In the first step, internal gelation occurred under the influence of Ca^{2+} ions resulted from the dissociation of the insoluble salt $CaCO_3$, a process initiated by the addition of glutamic acid. In the second step, external gelation occurred when the viscous alginate/ cells suspension, which was transferred into a Petri dish containing $CaCl_2$. The suspension underwent rapid gelation from the exterior toward the interior, entrapping the bacterial cells within the polymeric network.

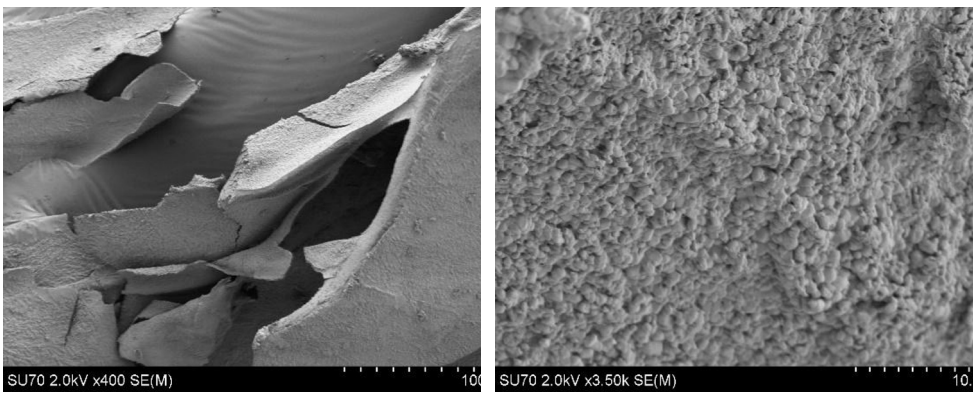

Fig. 2. SEM images of the alginate sample

The addition of the chitosan altered the morphological profile of the alginate hydrogel. As shown in Figure 3, the CA1 sample exhibited a distinct layered structure, with numerous pores of different sizes, many of which appeared clogged or partially obstructed, most probably explained by a phase separation during the crosslinking process.

SEM micrographs of the CG2 sample show a smooth surface, with few open and large pores (Figure 4). From the SEM image taken at higher magnification, gelatine particles can be formed during the crosslinking method. .

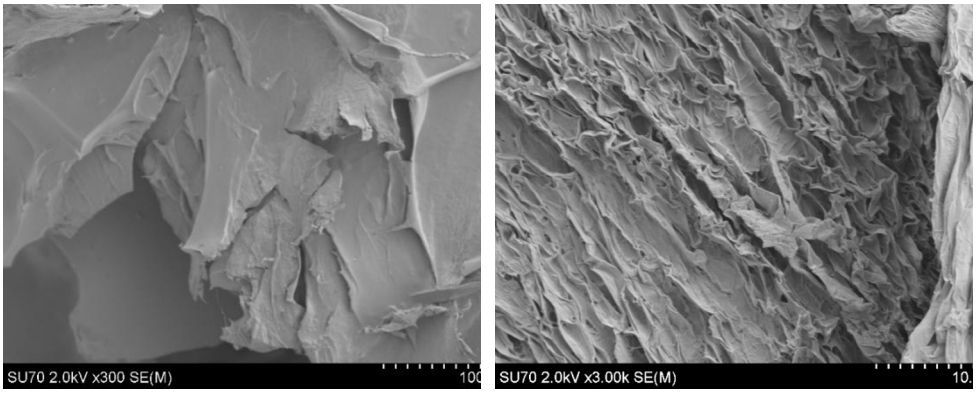

Fig. 3. SEM images of CA1 sample

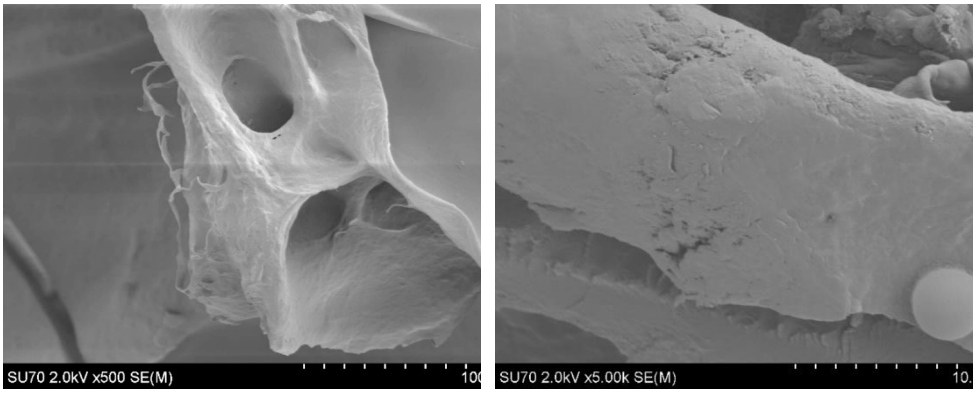

Fig. 4. SEM images of CG2 sample

1.3 Hydrogel cytotoxicity on *I. sakaiensis* line

The immobilization of bacterial cells in the alginate structure is expected to provide a suitable environment, considering the good biocompatibility and water retention capacity of the polymer matrix. Therefore, the polymeric substrate could be considered an excellent carrier for bacterial colonies, protecting cells from environmental stress (pH, toxic substances, etc.), which finally extends their viability. When the substrate meets appropriate homogeneity and porosity, it facilitates efficient solute exchange and nutrient diffusion inside the polymer network, while promoting the effective removal of toxic metabolic waste toward the hydrogel boundaries. *I. sakaiensis* emerged as a promising

candidate for wastewater treatment technologies, due to its ability to metabolize different contaminants derived from plastic waste, utilizing them as a carbon source [27].

After 24 hours of incubation, all hydrogels retained viable bacterial cells; however, the samples incubated at 4°C exhibited a lower bacterial density compared to those maintained at 37°C (Figure 5). Bacterial growth was quantitatively assessed by measuring the optical density at 600 nm (OD_{600}), with a recorded value of 0.414, which corresponds to a cell density of 2.07×10^8 cells/mL within the alginate hydrogel.

Despite the initial retention, the large pores of the alginate gels facilitated the cells' release during the 48 h experiment of cell viability. Their diffusion from alginate hydrogel can be attributed to the small size of *I. sakaiensis* cells, i.e., 0.6 to 0.8 μm in width and 1.2-1.5 μm in length [12], which is considerably smaller than the average pore size of the alginate hydrogel.

In the case of CA1 and CG2, the cells were mostly adsorbed on the surface of the hydrogels and, to a lesser extent, were able to penetrate the polymer network, most likely due to the lower pore density (data not shown).

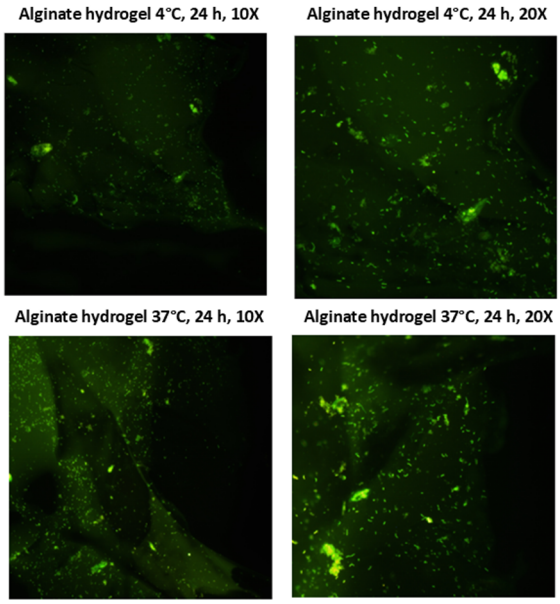

Fig. 5. Embedded bacterial cells in alginate hydrogel

2 Conclusions

The efficiency of MPs removal in WWTPs is still reduced due to the lack of standardised methods and available technologies. Therefore, consistent work needs to be done to design advanced materials for water purification, such as membrane filtration, particularly membrane bioreactors, and to improve the existing biological wastewater treatment technologies.

Addressing the systemic failure in plastic waste management, enhancing public awareness to reduce plastic consumption, and developing new technologies for

macroplastic recycling and microplastic collection in WWTPs are essential to prevent further environmental degradation.

Against the measures already implemented to reduce plastic pollution, such as new recycling approaches or the growing use of biodegradable plastics, certain environmental concerns remain unsolved, and, in some cases, are merely replaced by new challenges. For instance, in recent years, biodegradable plastics have become very popular and highly promoted as an alternative to non-degradable plastics, but generate unpredictable and unknown effects that are not completely understood at present. Therefore, the effective removal of the plastic particles in WWTPs remains a critical challenge, and microbial degradation may offer a promising solution to solve this issue.

The materials presented in this study were developed as a substrate for the immobilization of *I. sakaiensis* cells. The obtained system proved to be reliable, especially in the case of alginate hydrogel, but further research is needed in order to optimise the composition of the polymer matrix. Ideally, the polymer substrate should allow the refilling with cell suspension for several cycles, with little degradation of the hydrogel and reduced loss of cell viability during the operation. The optimization of the polymer substrate/cells system should take into account the morphology of the polymer network, pore size and interconnectivity, biodegradability, biocompatibility, cost effectiveness, and scalability of the material.

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