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Article

Encapsulation of *Acidithiobacillus thiooxidans* in Sulfur-Loaded Hydrogel Films for Increased Nutrient Uptake by Plants

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ABSTRACT: Designing smart carrier materials for inoculants used in agriculture is essential to ensuring proper shelf life and cell survival in soil. Herein, we propose to encapsulate *Acidithiobacillus thiooxidans* and elemental sulfur (ES) particles in a single fertilizer material based on biodegradable hydrogel (HG) films made of carboxymethylcellulose cross-linked by citric acid (CA). The goal was to study the direct availability of ES to the bacterium for improved cell survival and sulfate delivery to plants. Film swelling was evaluated as a method for bacterium inoculation. HG films cross-linked with 5 wt % CA displayed a superior swelling ratio than with 10 wt % CA (129 and 10 $g \cdot g^{-1}$, respectively) and were less affected by the acid pH used for *A. thiooxidans* growth. The encapsulated bacterium proved to be viable, achieving up to 62% of ES oxidation after reactivation in culture medium. Soybean plants fertilized with the HG films reached 36% of sulfur use efficiency, comparable to the performance of the soluble positive control, thus confirming its effectiveness as a sulfur biofertilizer.

KEYWORDS: *A. thiooxidans, carboxymethyl cellulose, inoculation, fertilizer, hydrogel, sulfur*

1. INTRODUCTION

Sulfur (S) is essential for plant development, but its availability in agricultural soils has been increasingly low, impacting plant growth and its resistance to abiotic and biotic stresses.[1−3] Elemental sulfur (ES) is an abundant waste that can be used as a greener S-fertilizer.[4−6] Once in soil, it needs to be oxidized into sulfate by soil microorganisms in order to become available for plant uptake.[6−8] In this context, the coapplication of S-oxidizing microorganisms is an interesting strategy to optimize ES use efficiency.

While many heterotrophic microorganisms indirectly participate in S transformation in soils, their S-oxidation rate is still limited, and their activity depends on the availability of organic carbon.[9−11] The chemolithotrophic bacterium *Acidithiobacillus thiooxidans* is the most effective microorganism in S oxidation, deriving its energy specifically from oxidizing reduced S forms.[2,7,8,12] S deficiency directly impacts *A. thiooxidans* availability in agricultural soils, and consequently, incorporating the bacterium in a biofertilizer could have a beneficial role of restoring its soil population.[2,9,13−15]

Microbial inoculants have been vastly studied to enhance agronomic efficiency.[16] Microbial survival in soil is a major challenge, as cell viability can be highly affected by environment conditions such as soil salinity, pH, and water content.[17] Additionally, the inoculant must compete with the local microbiota and adapt to the nutrients that are accessible. Choosing an appropriate material to protect the bacterium during storage and once it is applied in soil is therefore a crucial aspect of inoculant formulation. Solid matrices have the advantage of being easier to handle and store in addition to reducing transportation costs. Ideally, the material should keep moisture to preserve the cells and promote their controlled release.[16,18] Carboxymethylcellulose (CMC) is a highly available, low-cost, nontoxic, hydrophilic, and biodegradable biopolymer, characteristics that support its use as a matrix for inoculants and fertilizers.[19,20] Moreover, CMC can be chemically cross-linked to form a hydrogel (HG) structure, with a 3D network that absorbs and retains large volumes of water.[21]

The aim of this work was to develop HG films based on CMC cross-linked with citric acid for the simultaneous encapsulation of ES and *A. thiooxidans*. This study also proposes a simple inoculation method based on HG swelling in an activated culture medium. We propose that ES particles within the matrix could provide nutrient support for the initial microbial survival and boost sulfate production, improving its delivery to plants. The CMC matrix can have an agronomic role as a soil conditioner and by controlling nutrient release.[21−23] Additionally, CMC could be used as a source of organic carbon by heterotrophic microorganisms from soil and further contribute to sulfur oxidation. Therefore, we hypothesize that (1) in the inoculation method, *A. thiooxidans* would be incorporated into the films as a result of the HG swelling, penetrating the structure, and (2) the ES-containing HG films would protect the bacterium for application as a sulfur fertilizer.

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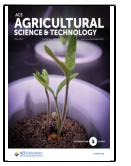

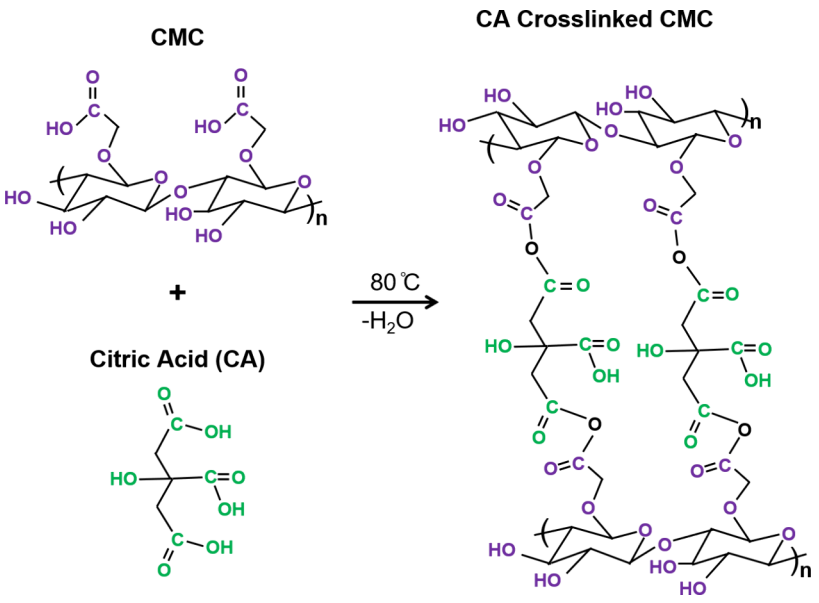

Figure 1. Chemical scheme of the cross-linking reaction between CMC and CA.

2. MATERIALS AND METHODS

2.1. Materials. Hydrogel films were prepared using sodium carboxymethyl cellulose (Synth, Brazil), citric acid (Sigma-Aldrich, USA), and elemental sulfur 98% (Synth, Brazil).

2.2. Preparation of CMC/CA Hydrogel Films. Carboxymethyl cellulose (CMC) hydrogel (HG) films were prepared using citric acid (CA) as a cross-linker with and without elemental sulfur (ES) incorporation. Figure 1 shows the chemical scheme of the cross-linking reaction between CMC and CA.

First, CMC and CA were slowly dissolved in distilled water to obtain a 2 wt % polymer concentration at room temperature and constant mixing with an overhead stirrer. CA was added either at 5 or 10 wt %, relative to the polymer mass (HG-5 and HG-10, respectively). Films containing ES (<125 μm) were prepared with 20 wt % ES, relative to the total mass of the films (forming HG-5-ES and HG-10-ES, with 5 and 10 wt % CA, respectively). After a homogeneous solution was formed, it was poured into plastic Petri dishes and dried in an oven at 80 °C for approximately 24 h, allowing cross-linking formation. Figure 2 illustrates the scheme of hydrogel film preparation as well as the following steps of *A. thiooxidans* inoculation, which will be discussed below.

2.3. Characterizations. Morphology of the films was investigated with scanning electron microscopy (SEM) using a secondary electron detector (JEOL, JSM 6510). All samples were coated with gold before analysis in an ionization chamber (BALTEC Med. 020). The chemical structures of the materials were elucidated with Fourier transform infrared (FTIR) spectroscopy (Bruker, VERTEX 70).

2.4. Swelling Ratio Estimation. The swelling ratio (SR) of hydrogels indicates their ability to absorb water based on the weight difference compared to the dry material. The SR of the films was estimated with distilled water, both in neutral (pH = 5.3) and acid (pH = 3.2) conditions, with the latter pH being corrected with diluted H_2SO_4 solution. The optimum pH for *A. thiooxidans* growth is between 2.0 and 3.5;[7] however, hydrogel swelling can be significantly reduced in low pH. Therefore, we were interested in estimating the film swelling degree in a pH low enough to favor bacterium survival without substantially interfering with the hydrogel swelling behavior.

Pieces of the dry films with an average of 12 mg and similar sizes were immersed in 20 mL of water or acidified medium in triplicates. The films were weighted in consecutive time intervals after gently drying their surface. The swelling ratio was calculated according to the following eq 1:

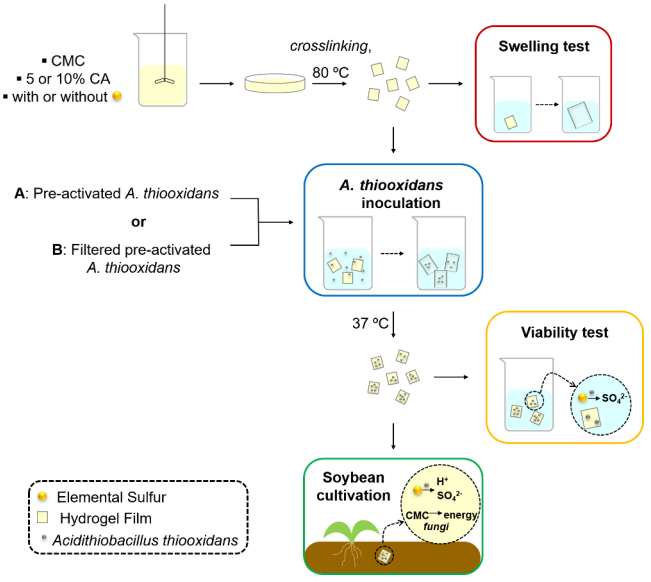

Figure 2. Scheme of hydrogel films preparation and subsequent *A. thiooxidans* inoculation, with tests for film swelling, cell viability, and soybean cultivation in a greenhouse.

$$SR = \frac{ms}{m0} \quad (1)$$

where m_0 is the dry film mass and m_s is the swollen HG film mass.

2.5. Bacterium Inoculation in Films. Based on the estimated swelling degrees, only the films with 5 wt % CA were selected for bacterium inoculation, i.e., HG-5 and HG-5-ES. Before inoculation, the films were hand cut into small squares with around 4 cm length and rinsed with distilled water to remove residual CA and sodium.

The preactivation of *A. thiooxidans* (FG01 strain)[24,25] was conducted in an adapted 9K culture medium containing 3 g/L $(NH_4)_2SO_4$, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.1 g/L KCl, and 1% (m/v) of elemental sulfur.[26] The medium pH was corrected to 2.8 with diluted H_2SO_4. The bacterium was activated by incubating 10% (v/v) of stock culture in an Erlenmeyer flask containing the 9K medium, which was maintained in an orbital shaker incubator at 30 °C and 150 rpm for 10 days. Cell concentration was estimated turbidimetrically with a McFarland 0.5 standard in a UV−vis

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spectrophotometer (FEMTO, 700 Plus, Brazil) at a 625 nm wavelength.

Bacterium inoculation in the films was conducted by two different methods. In Method A, HG-5 (0.2 g) and HG-5-ES (0.5 g) were both tested to compare the matrix effect on cell inoculation and survival. The masses were calculated to standardize the amount of culture medium that the films could absorb based on their SR, allowing a similar amount of bacterium inoculation. The films were immersed in 20 mL of preactivated bacterium medium for 30 min, maintained on an orbital shaker at 150 rpm. Prior to this, the bacterium pH was adjusted to approximately 3.2 to allow film swelling and, consequently, bacterium incorporation. The films were then collected and dried in an oven at 37 °C for approximately 5 h. Based on the results from experiments with films prepared by Method A, some modifications to this method were proposed in Method B. Since our interest was to obtain a sulfur-loaded fertilizer, only HG-5-ES films were tested. The same general procedure from Method A was used, but a filtration step was included prior to pH correction by vacuum filtration with Whatman paper no 1. The aim was to improve film swelling and bacterium incorporation as sulfur particles and other solids from the culture medium could interfere in those processes.

2.6. Cell Viability Study in Culture Medium. Bacterium survival after inoculation in the films was evaluated indirectly, based on sulfur oxidation estimations, calculated by eq 2:

$$S\ \text{oxidation}(\%) = \frac{S\ \text{final}}{S\text{applied}} \times 100 \quad (2)$$

where S refers to the sulfur mass in the form of sulfate (in the nutrient medium) at the end of the incubation and the applied S consists of the initial sulfur mass from the films that was added to the nutrient medium.

2.6.1. Cell Viability from Films Inoculated by Method A. Inoculated films from Method A (HG-5/A and HG-5-ES/A) were investigated after the drying step to test the bacterium survival and activation from the solid matrices. The main goal was to observe the dynamics involved in bacterium release from the different matrices (with and without ES) and sulfur oxidation from the different systems, as an initial indication of bacterium survival in the proposed carriers. In the case of HG-5/A, the previously weighed and inoculated films were added to 50 mL of 9K medium containing 1% (m/v) of elemental sulfur. A control with no film and 10% (v/v) of preactivated bacterium was also prepared to observe the bacterium activity in standard conditions. For HG-5-ES/A, the inoculated films were added to 50 mL of 9K medium without elemental sulfur addition in order to evaluate sulfur oxidation from the ES provided by the film. A control with no film and 10% (v/v) of preactivated bacterium was also analyzed, with the addition of 0.2% (m/v) of elemental sulfur (the same ES mass contained in the films). Triplicates of the films and the controls were kept in an orbital shaker incubator at 30 °C and 150 rpm for 10 days.

Following the incubation period, the samples were filtered and sulfate concentration was estimated by the turbidimetric method with $BaCl_2$ using an UV−vis spectrophotometer at 420 nm (FEMTO, 700 Plus, Brazil).[27,28] Sulfate from the 9K medium was also quantified in order to exclude its contribution from the final sulfate concentrations, avoiding an overestimation of sulfur oxidation. Additionally, pH measurements were conducted using a pH meter (Gehaka, Brazil), and cell concentration was estimated turbidimetrically, as described in the previous section.

2.6.2. Cell Viability from Films Inoculated by Method B. Films prepared by Method B (HG-5-ES/B) were studied to verify whether the proposed modifications in the cell inoculation procedure could improve cell survival in the matrix. Additionally, the films were investigated after being stored for 1 month, aiming to observe the storage time effect on bacterium survival. Again, the films (immediately after the drying step and after 1 month of storage) were added to 50 mL of 9K medium without elemental sulfur addition. A control with no film and 10% (v/v) of filtered preactivated bacterium was prepared, with 0.2% (m/v) of elemental sulfur. Triplicates of the films and the controls were kept in an orbital shaker incubator at 30 °C and 150 rpm for 10 days. After incubation, the samples were filtered and measurements of the pH and sulfate concentration were conducted, as described in the previous section.

2.7. Greenhouse Experiment. The agronomic efficiency of HG-5-ES/B was investigated in a pot experiment with soybean (*Glycine max* L.) at a greenhouse in Embrapa Instrumentation, Brazil, during a period of 39 days from October to November 2022. The plants received 12 h of light daily, provided both naturally and also with artificial light. The greenhouse had an average room temperature of 25 °C, and the soil moisture was kept at 70% WHC.

An oxisol soil from São Carlos, Brazil, was selected for this experiment, collected from the top layer (0−20 cm). Prior to the experiment, the soil was prepared by drying for 24 h in an oven at 40 °C, followed by sieving (<2.0 mm), and limestone powder addition (3:1 wt %) for acidity correction.[29] Soil analysis results can be found in Table S1, including cation-exchange capacity, pH ($CaCl_2$), soil organic matter, sum of bases, soil base saturation, soil acidity (H + Al), and nutrient concentration (P, S, K, Ca, and Mg).[29]

The fertilization effects of combining ES and *A. thiooxidans* in a CMC hydrogel matrix were compared to other treatments: a control with no sulfur fertilization (No−S), a positive reference of potassium sulfate (K_2SO_4, Synth, Brazil), and ES in the form of commercial pellets (ES-pellet). A fixed dose of 50 mg of S/kg of soil was supplied in the form of K_2SO_4, ES-pellet, and HG-5-ES/B. Monoammonium phosphate (MAP, Yara, Brazil) was added to all treatments to complete the doses of phosphorus (P, 200 mg/kg) and nitrogen (N, 300 mg/kg). Potassium (K) was supplemented with KCl (Sytnh, Brazil) to No−S, HG-5-ES/B, and the ES-pellet, equalizing the dose supplied by the positive control with K_2SO_4 (134 mg/kg). A micronutrient solution was prepared with H_3BO_3, $CuSO_4$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ to supplement micronutrient doses of borate (B, 0.8 mg/kg), copper (Cu, 1.5 mg/kg), manganese (Mn, 4 mg/kg), zinc (Zn, 5 mg/kg), and molybdenum (Mo, 0.15 mg/kg).

Prior to the experiment, the designated S sources, MAP, and KCl were mixed with 1 kg of soil in a plastic bag, allowing for complete homogenization of the fertilizers in the soil. Following this, pots were filled with the fertilized soil (1 kg) and four soybean seeds (*G. max* L.) were incorporated at a distance of approximately 4 cm from the soil surface. The micronutrient solution was then added to each pot, and water was supplied to complete 70% WHC. After 1 week, two seedlings were removed from each pot, keeping two plants per pot in all treatments. Four replicates of each treatment were used in the experiment, and the pots were kept in randomized positions, altered once a week.

Plant harvest was conducted 39 days after seed sowing. The final plant height was measured prior to the harvest. Following this, plant shoots were kept in paper bags and dried in a forced air oven at 60 °C until constant weight for shoot dry biomass measurements. Soil samples were dried and sieved to remove the roots, after which they were further homogenized and dried in a forced air oven at 60 °C until constant weight. Nutrient contents from both soil and shoot samples were estimated to elucidate nutrient absorption by plants under the different treatments and nutrient release from the fertilizers. Prior to this, the shoots were ground to <1 mm with a Wiley mill (Marconi, Brazil).

Available sulfate in soil was extracted with acid ammonium acetate solution, and sulfate concentration was then estimated turbidimetrically using an UV−vis spectrophotometer (FEMTO, 700 Plus, Brazil) at 420 nm.[28] Sulfate from the shoots was extracted by digesting approximately 500 mg of the biomass in 10 mL of 65% nitric acid in a digestion block at 150 °C for 3 h, followed by filtering and completing the volume to 50 mL with distilled water. Sulfate determination was then conducted with an inductively coupled plasma optical emission spectrometer (ICP OES 5110, Agilent Technologies, Australia) equipped with a Mira Mist nebulizer and a cyclonic mist chamber at 180.7 wavelength. Sulfur uptake (mg/pot) and sulfur use efficiency (SUE, %) were then estimated by the following equations:

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$$S\ \text{uptake}\left(\frac{\text{mg}}{\text{pot}}\right) = \text{shoot biomass} \times S\text{shoot} \quad (3)$$

$$\text{SUE}(\%) = \frac{S\text{uptake(fertilized)} - S\text{uptake(control)}}{S\text{applied}} \times 100 \quad (4)$$

2.8. Statistical Analysis. Statistical analysis of the results was performed with the OriginPro 9.0 software. The data from the greenhouse experiment were submitted to Levene's test to assess homogeneity of variance and Shapiro–Wilk test to assess normality. After the first exploratory analysis, the data that met the assumptions were submitted to variance analysis (ANOVA) by the F-test. When the F-test was significant, differences among treatments were compared by Duncan's test at a significance level of 0.05.

3. RESULTS

Carboxymethyl cellulose (CMC) hydrogel (HG) films were studied as strategic carrier materials for *A. thiooxidans* inoculation. Different citric acid (CA) ratios for CMC cross-linking were compared, as well as the incorporation of elemental sulfur (ES) particles. Table 1 displays the nomenclature of the HG films prepared with different CA and ES contents.

Table 1. Hydrogel (HG) Film Nomenclature

nomenclature	CA cross-linker (wt % polymer)	ES(wt % film)	*A. thiooxidans* inoculation method
HG-5	5%	-	-
HG-10	10%	-	-
HG-5-ES	5%	20%	-
HG-10-ES	10%	20%	-
HG-5/A	5%	-	A
HG-5-ES/A	5%	20%	A
HG-5-ES/B	5%	20%	B

Before bacterium incorporation, FTIR spectroscopy was used to characterize the materials and confirm cross-linking formation (Figure 3a). CMC spectra displayed the following characteristic peaks: a wide band related to O–H stretching in the range of 3666–2980 cm^{-1}; C–H vibration peaks between 2980 and 2775 cm^{-1}; asymmetric (1587 cm^{-1}) and symmetric (1414 and 1323 cm^{-1}) carboxylate (COO^-) stretching modes; primary and secondary alcohol C–O vibrations at 1100, 1051, 1022, and 995 cm^{-1}; and the β-1,4-glycosidic band at 897 cm^{-1}.[19,20,23,30] Bands related to the acidic CMC form were not observed in the powder. In the prepared HG films, a new band at 1715 cm^{-1} appeared, indicating ester bond formation. In carboxylic acids such as citric acid, this C=O stretching band usually occurs in lower frequencies, which was not present in the films' spectra.[20] Additionally, a small band was identified in HG-5-ES and HG-10-ES at around 465 cm^{-1}, corresponding to the S–S stretching from the ES particles.

The swelling ratio (SR) of the hydrogel films was then investigated to elucidate how the different cross-linking degrees (i.e., the amount of chemical bonds formed by CA connecting the CMC chains) and film compositions could affect the swelling behavior. Figure 3b shows that film swelling was significantly reduced with an increased CA incorporation. In neutral conditions, HG-5 achieved almost 7 times the SR of HG-10 with 10 min of immersion. After 24 h (1440 min), HG-5 mass increased up to 129 times, while HG-10 swelling was not significantly improved, reaching only 10 times the initial mass. The presence of ES particles significantly reduced the SR

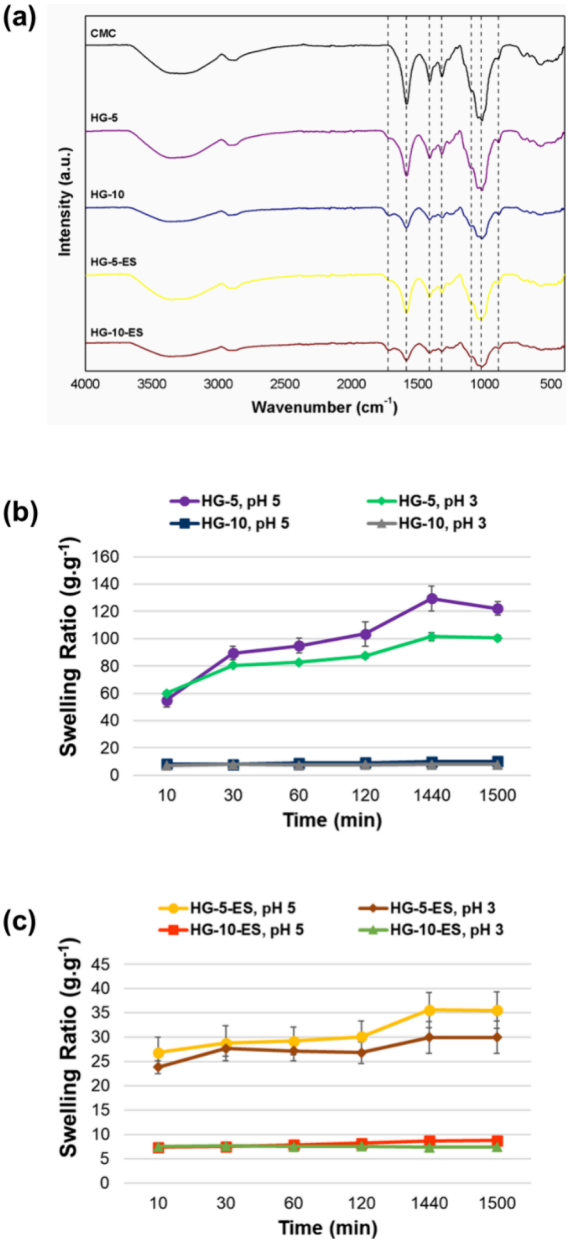

Figure 3. (a) FTIR spectra of CMC, HG-5, HG-10, HG-5-ES, and HG-10-ES. Swelling ratios at pH 5 and 3 of hydrogel films: (b) HG-5 and HG-10; (c) HG-5-ES and HG-10-ES.

of 5 wt % CA hydrogels, with HG-5-ES featuring only half of HG-5 SR within the first 10 min (Figure 3c). After reaching equilibrium in 24 h, the final SR value of HG-5-ES was 36 g·g^{-1}, against 122 g·g^{-1} from HG-5. The SR of 10 wt % CA films was not as affected by ES incorporation. At the end of the experiment, a small difference was observed, with 10 and 9 g·g^{-1} in HG-10 and HG-10-ES, respectively.

Comparing the behavior of HG films under acidic conditions (Figure 3b,c), HG-5 achieved the highest SR (100 g·g^{-1}). Still, the swelling of HG-5 in acid pH was inferior than that observed in neutral conditions, with a decrease of 18% of the SR at the end, although the performances of films with 10 wt % CA and ES incorporation were not significantly influenced by pH acidification. The lowest SR values in 25 h were obtained by HG-10 and HG-10-ES, respectively, at 8.1 and 7.4 g·g^{-1}.

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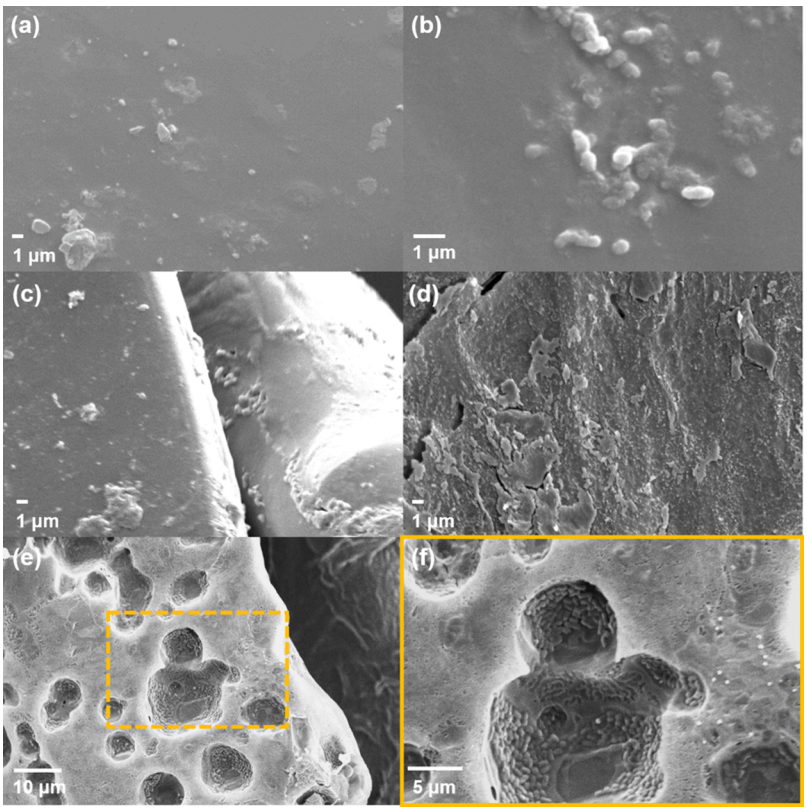

Figure 4. SEM images of films after preparation with Method A: (a) HG-5/A and (b) HG-5-ES/A; SEM images of the films after incubation in 9K medium: (c) HG-5/A and (d) HG-5-ES/A and (e) an ES particle from HG-5-ES/A after incubation, with (f) a magnified section.

Based on the superior SR of films with 5 wt % CA, HG-5 and HG-5-ES were selected for *A. thiooxidans* incorporation. The SEM images of the films before and after bacterium inoculation by Method A can be seen in Figure S1 and Figure 4a,b, respectively. HG-5 (Figure S1a) presents a smooth and continuous surface with no detectable defects. In contrast, HG-5-ES (Figure S1b) presents two distinct phases, with an irregular morphology attributed to ES particles either dispersed on the surface or underneath it. *A. thiooxidans* cells can be identified in HG-5/A and HG-5-ES/A (Figure 4a,b), suggesting the inoculation step was successful. Moreover, it is possible to notice solid particles adhered on the surface of HG-5/A (Figure 4a), indicating that precipitates or ES particles from the culture medium may form deposits on the film.

Bacterium survival and activation from both solid matrices were evaluated by incubating the films in a nutrient medium. HG-5/A films served as a reference for bacterium release to an S-containing medium. Figure 4c,f shows the SEM images of the materials after incubation. HG-5/A displays *A. thiooxidans* cells still attached to the surface (Figure 4c), which could indicate an insufficient release of the bacteria to the medium. Most of the HG-5-ES/A films disappeared at the end of the incubation, but Figure 4d shows one of the remaining films covered by bacterium cells. While HG-5/A kept a similar morphology from before the test (Figure 4a), with a uniform and smooth matrix surface, HG-5-ES/A displays a rough film surface and is more heterogeneous, which is consistent with a more significant degradation process. *A. thiooxidans* cells were clearly identified on ES particles released from HG-5-ES/A (Figure 4e,f) after film decomposition, vastly colonizing the ES surface. It is worth mentioning that, at the end of the incubation, fungi spores were observed in the culture medium of some replicates in the presence of both HG-5/A and HG-5-ES/A (see Figure S2). Nevertheless, HG-5/A films were not similarly decomposed, even when in the presence of fungi.

Cell concentration and sulfur oxidation results confirmed that *A. thiooxidans* was viable after incorporation in both hydrogel matrices (Table 2), with no indication of any toxic effects from the hydrogel film on the bacterium. Few studies have evaluated *A. thiooxidans* survival and viability after encapsulation in a solid matrix, and the existing data is usually based on cell count or measurements of cell respiratory activity.[31−33] We argue that it is essential to analyze *A. thiooxidans* viability with sulfur oxidation experiments as a

Table 2. Cell Viability Results from Films Prepared by Method A and Method B of Bacterial Inoculation, Regarding Final Cell Concentration, pH, and S Oxidation

films inoculated by Method A			
treatment	**bacterium**(cells/mL)	**pH**	**S oxidation (%)**
control (1% S)	$5.0 \times 10^8 \pm 0.1$	0.9 ± 0.1	65.3 ± 7.9
HG-5/A	$2.0 \times 10^8 \pm 0.0$	1.4 ± 0.1	21.9 ± 12.8
control (0.2% S)	$2.1 \times 10^8 \pm 0.0$	1.2 ± 0.0	112.9 ± 10.2
HG-5-ES/A	$1.2 \times 10^8 \pm 0.1$	2.3 ± 0.4	41.2 ± 14.4
films Inoculated by Method B			
treatment	**Bacterium** (cells/mL)	**pH**	**S oxidation** (%)
control (0.2% S)	$1.9 \times 10^8 \pm 0.1$	1.7 ± 0.0	106.5 ± 1.3
HG-5-ES/B - 0 days	$1.8 \times 10^8 \pm 0.1$	2.5 ± 0.3	61.8 ± 10.8
HG-5-ES/B - 30 days	$3.8 \times 10^7 \pm 0.0$	3.8 ± 0.1	27.9 ± 3.5

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direct indication of the bacterium activation efficiency and potential use.

The preactivated bacterium was diluted to 3.7×10^7 cells/mL in the studied controls, and at the end of the experiment, cell concentration significantly increased (>10^8 cells/mL). Comparing the controls, it is possible to see that bacterium growth was favored by a higher ES supply (i.e., 1% S); still, S-oxidation was more efficient in the presence of 0.2% S, reaching full conversion into sulfate, which means that S was sufficiently provided at this rate to support bacterium activity. This is expected as cells require more time to convert a higher amount of S and thus present a faster oxidation rate for lower S concentrations.

As expected, the encapsulated *A. thiooxidans* achieved cell concentrations and oxidation rates lower than those of the controls (Table 2). Despite this, the hydrogels displayed a satisfying performance, with 22% and 41% of S-oxidation for HG-5/A and HG-5-ES/A, respectively. It is important to highlight that, regardless of the observed fungi spores, the bacterium cell count and high S oxidation in all film replicates suggest *A. thiooxidans* was able to grow and show bioactivity.

Bacterium inoculation in HG-5-ES was also tested using filtered preactivated bacterium (Method B), aiming to improve the inoculation efficiency. The produced HG-5-ES/B film morphology features a smoother surface compared with HG-5-ES/A, and the cells appear to be more evenly dispersed (Figure 5a). S oxidation from HG-5-ES/B was significantly higher than achieved by HG-5-ES/A (Table 2), with 61% of oxidation (50% higher), confirming that film efficiency can be improved by the filtration step. After these results, the effect of storage on cell viability was studied with HG-5-ES/B films stored over 1 month. Cells were less evident in the stored films (Figures 5b,c). Although HG-5-ES/B performance suffered a decline with storage time, reaching approximately half of the S oxidation and reduced cell growth, results indicate that the CMC matrix was still able to protect a sufficient number of cells for activation after application.

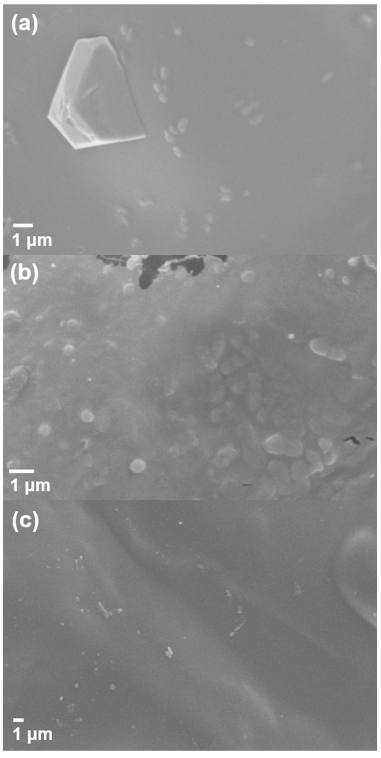

Figure 5. SEM images of the HG-5-ES/B film (a) as-prepared, (b) after incubation in 9K medium without prior storage time, and (c) stored over 30 days.

The combination of ES and *A. thiooxidans* in the CMC hydrogel matrix was studied for the cultivation of soybean (*G. max* L.), a crop with high demand of S fertilization.[34,35] Figure 6 shows plants grown under no sulfur (No−S), K_2SO_4 (the positive control), HG-5-ES/B, and a commercial ES-pellet after 39 days. Flowering was observed at the end of the experiment in plants from the positive control and HG-5-ES/B, suggesting they entered the reproductive stage earlier than with the other treatments. Over the last days, the yellowing of older leaves was noticed in all treatments, but these nutrient deficiency symptoms were more significantly evident in plants grown under the ES-pellet (Figure 6). Despite this, Table 3 shows that ES-pellet fertilization achieved similar plant height and production of shoot biomass compared to those of the other treatments.

After harvest, HG-5-ES/B displayed the highest concentration of sulfur in the shoot biomass with almost 5 g/kg (Table 3). This confirms that sulfate was efficiently provided to soybean from the hydrogels containing ES and *A. thiooxidans*, achieving a sulfur nutrition statistically equivalent to that of the positive control (4.3 g/kg). ES-pellet showed an intermediate result with around 3 g/kg, being statistically comparable to the other fertilized treatments but also to the No−S control, which had the lowest biomass sulfur concentration. It should be noted that the ES pellets were still somewhat intact after the experiment (Figure S3), while HG-5-ES/B was fully biodegraded. Based on the produced shoot biomass of each treatment, the calculated average sulfur uptake from HG-5-ES/B was around 25 mg/pot against 23 mg/pot from K_2SO_4, 18 mg/pot from ES-pellet, and 7 mg/pot from No−S (Table 3). Sulfur use efficiency (SUE) from the HG films was the highest, although not statistically different from the other fertilizers, with 36% SUE from HG-5-ES/B and 22% from the ES-pellet.

4. DISCUSSION

Hydrogel films were prepared to be tested as biodegradable matrices for *A. thiooxidans* incorporation and, simultaneously, as carriers for ES in a single fertilizer material. This strategy was proposed to optimize bacterial survival after application in soil. *A. thiooxidans* use in agriculture is still understudied and mostly done with direct liquid inoculum application. Solid matrices can reduce costs and are easier to handle in field; however, since *A. thiooxidans* is highly sensitive to osmotic stress and usually unable to grow on organic carbon,[7] developing a dry inoculant material is a major challenge. For this reason, there are very few reports in the literature of *A. thiooxidans* inoculation in solid matrices. Núñez-Ramírez et al. notably encapsulated the bacterium in a biopolymer by spray drying for biomining.[31] Herein, the cells were encapsulated with ES, and to the best of our knowledge, this is the first time that this bacterium has been inoculated in CMC hydrogel films for crop fertilization.

The influence of HG structural differences and ES incorporation on film swelling behavior was investigated to indicate the capacity of the films to absorb and retain *A. thiooxidans*, particularly at the bacterium cultivation pH (0.5 < pH < 3.5).[7,36] The hypothesis is that cells could be incorporated within the HG pores and structure as a result

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Figure 6. (a) Representative image of soybean plants after 39 days of cultivation under, respectively (from left to right), No−S, K_2SO_4, HG-5-ES/B, and ES-pellet treatments.

Table 3. Average Results from the Greenhouse Experiment with Soybeans after 39 days of Cultivation[a]

treatment	plant height (cm)	shoot biomass(g/pot)	S-shoot(g/kg)	S-uptake(mg/pot)	SUE (%)
No−S	43.8 ± 4.0 a	5.2 ± 0.1 a	1.4 ± 2.0 b	7.3 ± 9.0 b	-
K_2SO_4	47.2 ± 6.5 a	5.4 ± 0.4 a	4.3 ± 0.4 a	23.4 ± 0.6 a	31.8 ± 1.2 a
HG-5-ES/B	46.2 ± 4.9 a	5.3 ± 0.2 a	4.8 ± 1.3 a	25.3 ± 5.5 a	35.7 ± 11.1 a
ES-pellet	43.3 ± 1.9 a	5.7 ± 0.4 a	3.3 ± 0.6 ab	18.4 ± 2.7 ab	21.9 ± 5.4 a

[a]Indexes a and b signal the statistical differences for each measurement based on Duncan's test ($p < 0.05$).

of film swelling in the activated culture medium. This is proposed as a simpler inoculation method with lower energy and chemical consumption compared to other techniques such as spray drying or chemical reticulation.

CMC was successfully cross-linked by CA, either at 5 or 10 wt %. HG films with 5 wt % CA featured a satisfactory SR, superior to similar works in the literature, with up to 120 $g \cdot g^{-1}$ in water and 100 $g \cdot g^{-1}$ in acid pH (Figure 3b). In Lima et al., for example, CMC hydrogels with 3 wt % CA achieved around 40 $g \cdot g^{-1}$ in water and 10 $g \cdot g^{-1}$ in pH 4.[19] In Bauli et al., the swelling in pH 6 and 2 corresponded to 2.36 and 0.83 $g \cdot g^{-1}$, respectively.[23] On the other hand, the SR was significantly reduced by increasing the CA ratio (i.e., 10 wt %), probably due to the effect of a higher cross-linking density on network rigidity, restricting CMC chain movement and, thus, the film capacity to swell.[20]

ES dispersion within the polymer clearly reduced the SR of 5 wt % CA hydrogels but did not affect films with 10 wt % (Figure 3b,c). Although ES chemical interaction with the CMC matrix is low, it probably hindered CMC active functional groups, reducing water and intramolecular interactions that promote the swelling.[37] ES particles could also have blocked the HG pores,[38] and as hydrophobic fillers, they might limit water penetration.[38] In Bauli et al., 1 wt % vermiculite in CMC-HG decreased the swelling due to inhomogeneous filler dispersion and agglomeration.[23] Despite this, HG-5-ES performance was still adequate and far superior to that observed for films with 10 wt % CA, thus being a viable option. HG-5 swelling was also reduced by the acidic medium (Figure 3b,c). Higher H^+ concentration in solution leads to the protonation of the carboxylic groups from CMC, thus reducing the electrostatic repulsion involved in HG swelling.[19] HG-5-ES performance in acid was not particularly affected, as ES particles already hindered the carboxylic and hydroxyl groups from CMC, which is interesting for inoculating *A. thiooxidans*.

HG films cross-linked with 5 wt % CA (i.e., HG-5 and HG-5-ES) were selected as candidates for *A. thiooxidans* inoculation, based on their superior SR. Cell survival and activation from HG films were first studied by incubating HG-5/A and HG-5-ES/A in a 9K medium. We were interested in understanding if ES incorporation would be favorable to *A. thiooxidans* growth and activity as an easily accessible nutrient or if it would be detrimental to the inoculation process, since ES reduces HG water retention capacity. For this, HG-5/A films were used as a reference for cell release dynamics in a S-containing medium, as their higher SR could implicate in greater cell adsorption capacity.

Results from incubation indicated that the HG successfully protected *A. thiooxidans*. While cell concentration and oxidation rates were lower than the controls (Table 2), this was expected since the encapsulated bacteria are not as readily active or directly exposed to nutrients, requiring some time to be reactivated. In the HG-5/A case, cells also need to be released to the nutrient medium to access ES, which was not fully achieved based on SEM images (Figure 4c). It should be noticed that the initial cell concentration from the films could be lower than the controls: part of the bacterium does not attach or get adsorbed into the films during the inoculation process, and most importantly, some cells do not survive the drying conditions. Despite this, inoculated cells were viable to

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grow in the 9K medium, achieving around 1×10^8 cells/mL (Table 2). This concentration is in line with results for some biofertilizers and liquid inoculants found in the literature.[39−41] In Núñez-Ramírez et al., *A. thiooxidans* cells achieved 90% survival rate (around 10^9 cells/mL) after encapsulation in biopolymers by spray drying.[31] In Long et al., *A. ferrooxidans* immobilization by PVA-boric acid reached a 2×10^7 cell count in 100 mL batches.[42]

The amount of ES incorporated into the films proved to be adequate to support bacterium growth, leading to up to 41% of S oxidation. Most of the HG-5-ES/A films were completely decomposed after the incubation test. Although fungi spores were observed in some of the replicates and could have favored the process,[9,43] film degradation was observed in all of the replicates. Film decomposition could be explained as a result of embedded ES particles being locally oxidized by the bacterium producing sulfuric acid, which can enhance CMC degradation by acid hydrolysis.[44] Most importantly, CMC is a biodegradable material, being depolymerized under microbial enzymatic attack, including by some bacteria.[45−47] Enzymatic and acid degradation were probably more effective in HG-5-ES/A, as cells from HG-5/A needed to be released to the liquid medium to oxidize ES.

It is important to highlight that some fungi cannot oxidize sulfur or can only achieve that at very low oxidation rates, especially in such harsh conditions; thus, it is most likely that the fungi contaminant did not influence S oxidation results.[9] This suggests that *A. thiooxidans* can have a non-competitive interaction with heterotrophic fungi in this system,[2,9] which could be important once the films are applied in soil and in contact with the local microbiota. Overall, results showed that even when fungi were present, the bacterium was activated and grew, based on the cell count and high sulfur oxidation.

Films prepared by Method A of inoculation featured small particles adhered on the surface (Figure 4a), probably from precipitate formation during bacterium preactivation.[48] Since these particles can reduce film swelling and thus its capacity for cell uptake,[23,38] a filtering step was then included in Method B. This proved to be an important addition to the inoculation process, leading to a more homogeneous dispersion of cells in the films (Figure 5a) and notably improving the sulfur oxidation efficiency (Table 2). Cell activity was affected by the storage time but still showed a considerable sulfate production, proving that the CMC matrix can protect a considerable percentage of the inoculated bacterium before reactivation.

The encapsulated *A. thiooxidans* in sulfur-loaded hydrogel films was then tested as a fertilizer. Although HGs are frequently used in agriculture for their controlled-release capacity,[49] their application with *A. thiooxidans* is novel. Results from the greenhouse experiment confirmed that HG-5-ES/B can adequately provide sulfate to soybean plants, achieving values superior to those of the No−S treatment and a performance similar to that of the positive control of readily available sulfate (Table 3). Fertilization with the HG films produced the highest sulfur concentration in shoot biomass and the highest SUE, although statistically the results were similar among the fertilized treatments. For example, SUE from HG-5-ES/B was higher than the one achieved by Klaic et al.[50] In a similar approach, the authors studied starch-based fertilizers containing ES and *A. niger* fungi, featuring 16% SUE for Italian ryegrass cultivation, a result that was statistically comparable to that of commercial ES pellets.

Although the greenhouse results were not conclusive in indicating if the HG films could be more efficient than other fertilizers, the films still offer other advantages. Highly soluble fertilizers such as K_2SO_4 tend to lixiviate, for instance, which not only reduces their efficiency overtime but can also cause environmental pollution. Moreover, soluble fertilizers may increase soil salinity, while HG can regulate the ionic concentration in soil due to their ion exchange capacity, thus improving the overall soil fertility. Compared to the HG-5-ES/B fertilization, plants grown under commercial ES-pellets did not reach their reproductive stage and featured a more extensive yellowing of leaves (Figure 6). More importantly, S-shoot biomass from the ES-pellet was statistically comparable to the No−S control, contrary to HG-5-ES/B (Table 3), and the pellets were recovered after the harvest (Figure S2), suggesting a very slow oxidation rate. This could lead to insufficient sulfate availability in subsequent soybean growth stages, which can restrict grain yields and affect the quality of the soybeans in terms of its protein content. Although sulfur fertilization did not seem to significantly influence soybean growth under the studied conditions, the proposed fertilizers were highly efficient to sustainably provide sulfur nutrition to plants.

In conclusion, the sulfur-oxidizing bacterium *A. thiooxidans* was successfully inoculated in a solid matrix of CMC for agricultural use, providing a safer and more practical option for handling and storage than commonly used liquid inoculants. The ES-containing hydrogels achieved efficient S supply while protecting the bacterium with directly accessible sulfur particles. The films acted as multifunctional matrices, adsorbing the bacterium with their swelling mechanism in a simple and mild inoculation method, in addition to keeping cell viability and allowing cell release to the medium for access to free sulfur particles. The agronomic efficiency in S supply was confirmed with soybean cultivation, displaying a comparable biomass production and S uptake to the soluble positive control.

Overall, the study offered new insights into *A. thiooxidans* inoculation in a solid carbohydrate-based material, which is still underexplored in the literature. Most importantly, results showed that CMC hydrogel films are viable matrix candidates to encapsulate both ES and *A. thiooxidans* in an alternative environmentally friendly sulfur fertilizer.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsagscitech.5c00025.

Soil characterization, SEM images of the HG films as prepared, and SEM images of the ES-pellets after cultivation (PDF)

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Author Contributions

S.F.V. conceived and designed the experiments, collected the data, performed the analysis, and wrote the paper − original draft. A.S.G. designed the experiments and wrote the paper − review and editing. G.G.F.G. designed the experiments, performed the analysis, and wrote the paper − review and editing. C.R. conceived the experiments, supervised the project, and wrote the paper − review and editing.

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Notes

The authors declare no competing financial interest.

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